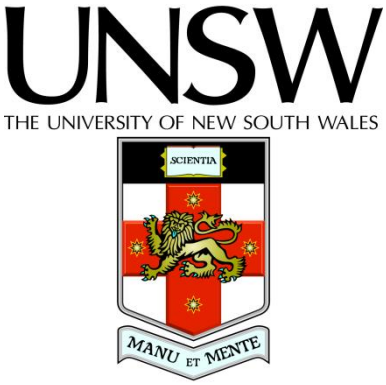
The therapeutic potential of the phytocannabinoid cannabidiol for Alzheimer's disease

David Cheng

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy



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Conclusions: APPxPS1 transgenic mice demonstrated novel cognitive deficits relevant for AD. These deficits could be prevented and ameliorated by CBD treatment potentially via its anti-inflammatory actions and its impact on dietary phytosterols. CBD possesses therapeutic potential for the treatment of AD symptomatology and should be evaluated further in clinical trials.

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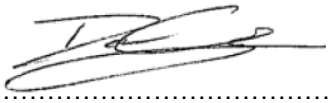

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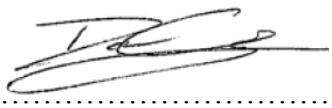
Signed  ……

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I hereby certify that all co-authors of the published or submitted papers agree to David Cheng submitting those papers as part of his Doctoral Thesis.

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Publications

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III. Cheng, D., Low, J.K., Logge, W., Garner, B., Karl, T. 2014. Chronic cannabidiol treatment improves social and object recognition in double transgenic APP_{Swe}/PS1ΔE9 mice. *Psychopharmacology (Berl)*. 231(15): 3009-17

IV. Cheng, D., Spiro, A.S., Jenner, A.M., Garner, B., Karl, T. 2014. Long-term cannabidiol treatment prevents the development of social recognition memory deficits in Alzheimer's disease transgenic mice. *Journal of Alzheimer's Disease*. DOI: 10.3233/JAD-140921

Additional Publications

I. Karl, T., Cheng, D., Garner, B., Arnold, J.C. 2012. The therapeutic potential of the endocannabinoid system for Alzheimer's disease. *Expert Opin. Ther. Targets*. 16(4): 407-20

II. Karl, T., Bhatia, S., Cheng, D., Kim, W.S., Garner, B. 2013. Cognitive phenotyping of amyloid precursor protein transgenic J20 mice. *Behav Brain Res*. 228(2): 392-7

III. Logge, W*., Cheng, D.*, Chesworth, R., Bhatia, S., Garner, B., Kim, W.J., Karl, T. 2012. Role of Abca7 in mouse behaviours relevant to neurodegenerative diseases. *PLoS One*. 7(9): e45959

*Equal contribution

IV. Kim, W.J., Li, H.Y., Ruberu, K., Chan, S., Elliott, D., Low, J.K., Cheng, D., Karl, T., Garner, B. 2013. Deletion of Abca7 increases cerebral amyloid-β deposition in the J20 mouse model of Alzheimer's disease. *J. Neuroscience*. 33(10): 4387-4394

Conference Presentations

2nd National Cannabis Conference 2012, Brisbane, QLD, Australia

Preliminary *in vitro* and *in vivo* evidence for the therapeutic potential of cannabidiol in Alzheimer's disease [Oral Presentation]

33rd Australian Neuroscience Society 2013, Melbourne, VIC, Australia

In vitro and *in vivo* evidence for the therapeutic potential of cannabidiol in Alzheimer's disease [Poster Presentation]

Brain Sciences UNSW Colloquium 2013, Sydney, NSW, Australia

Therapeutic potential of cannabidiol for Alzheimer's disease [Poster Presentation]

41st Annual Tow Meeting 2013, Sydney, NSW, Australia

Targeting Alzheimer's disease social recognition and cognitive deficits with cannabidiol [Oral Presentation]

Abstract

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List of tables and figures

Tables

Figures

List of abbreviations

2-AG	-	2-arachidonoylglycerol
2-LG	-	2-linoleoylglycerol
5-HT_{1A}	-	5-hydroxytryptamine1A
AA	-	arachidonic acid
abn-CBD	-	Abnormal-cannabidiol
ACh	-	acetylcholine
AChE	-	acetylcholinesterase
AD	-	Alzheimer's disease
AEA	-	N-arachidonoylethanolamide/anandamide
AICD	-	APP-intracellular C-terminal domain
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANCOVA	-	analysis of covariance
ANOVA	-	analysis of variance
APOE	-	apolipoprotein E
APP	-	amyloid precursor protein
APPxPS1	-	APP_{Swe}/PS1ΔE9
ASR	-	acoustic startle response
ATP	-	adenosine triphosphate
Aβ	-	amyloid-β
BBB	-	blood brain barrier
BSTFA	-	N,O-bis(trimethylsilyl)trifluoroacetamide
Ca^{2+}	-	calcium ions
CB	-	cheeseboard
CB_1	-	cannabinoid receptor 1
CB_2	-	cannabinoid receptor 2
CBD	-	cannabidiol
CNS	-	central nervous system
COP	-	cholesterol oxidation products
COX	-	cyclooxygenase
CTF	-	C-terminal fragment of APP
Cu^{2+}	-	copper
DAGL	-	diacylglycerol lipase
ECS	-	endocannabinoid system
ELISA	-	enzyme-linked immunosorbent assay
EPM	-	elevated plus maze
FAAH	-	Fatty acid amide hydrolase
FC	-	fear conditioning
GC-MS	-	gas-chromatography mass-spectrometry
GFAP	-	glial fibrillary acidic protein
gHCL	-	guanidine hydrochloride

GPR55	-	G-protein-coupled receptor 55
i.p.	-	intraperitoneal
IFN-γ	-	interferon-γ
IL-1β	-	interleukin-1β
IL-6	-	interleukin-6
iNOS	-	nitric oxide synthase
ISI	-	interstimulus interval
ITI	-	inter-trial interval
LD	-	light-dark test
LOD	-	Limits of detection
LTP	-	long-term potentiation
MAPT	-	microtubule-associated protein tau
MGL	-	Monoacylglycerol lipase
MPAC	-	Metal-protein attenuating compounds
MWM	-	Morris water maze
NAPE-PLD	-	N-acyl phosphatidylethanolamine phospholipase D
NFT	-	neurofibrillary tangles
NMDA	-	N-methyl-D-aspartate
NO	-	nitric oxide
NORT	-	novel object recognition task
NSAID	-	Non-steroidal anti-inflammatory drug
NTF	-	N-terminal fragment of APP
OF	-	Open field
PEA	-	palmitoylethanolamide
PPAR	-	peroxisome proliferator-activated receptor
PPI	-	prepulse inhibition
PS1	-	presenilin 1
PS2	-	presenilin 2
qPCR	-	quantitative polymerase chain reaction
RM	-	repeated measures
ROS	-	reactive oxygen species
sAPP	-	soluble APP
SEM	-	standard error of means
SPT	-	social preference test
THC	-	Δ^9-tetrahydrocannabinol
TMCS	-	Trimethylchlorosilane
TNF-α	-	tumour necrosis factor-α
TRP	-	transient receptor potential
VEH	-	vehicle
Zn^{2+}	-	zinc

Table of Contents

Chapter 1: Introduction

As the life expectancy of the world's population grows, many ageing individuals face an increasing risk of developing dementia. Dementia is the severe loss of cognitive abilities that is not part of the normal ageing process. There are over 330,000 Australians living with dementia and Alzheimer's disease (AD) is the most prominent form, accounting for up to 70% of all people with dementia. AD is predicted to affect 1 in 85 people globally by the year 2050. The healthcare cost for dementia in Australia in the years 2009-2010 amounted to at least $4.9 billion and is predicted to be the third greatest source of health and residential aged care spending within the next two decades. AD is classified into three progressive clinical stages: mild, moderate and severe (Zandi et al. 2002). During the mild stage, AD patients experience short-term memory loss, subtle difficulties in learning and communication as well as spatial disorientation. Memory decline in the moderate stage (e.g. noticeable lapses in short-term memory and loss of reading and writing ability) begins to affect everyday tasks resulting in increased frustration and loss of emotional control. The severe stage of AD is characterised by a disruption of cognitive abilities including severely impaired learning, inability to recognise familiar people and loss of control over bodily functions. The decline in health and cognition may last up to 17 years after the first diagnosis of AD, depending on the severity of the disease at first diagnosis.

Two distinct types of lesions are observed in post-mortem examinations of AD-affected brains, representing the major hallmarks of the disease: 1) extracellular deposits comprised of small amyloid-β (Aβ) peptides that form amyloid or senile plaques and 2) intracellular accumulation of microtubule-associated protein tau (MAPT) leading to the formation of neurofibrillary tangles (NFT) (Selkoe 1996) (for more detail see section 1.1).

AD is characterised as either sporadic (late-onset) or familial (early onset). Sporadic AD is the most common and least understood form of AD, accounting for up to 95% of reported AD cases (Gotz and Ittner 2008). The age of onset for sporadic AD is usually around the age of 65. The cause of sporadic AD remains to be elucidated, but is believed to result from a complex interaction of various environmental risk factors and multiple susceptibility genes (Kamboh 2004). For example, the apolipoprotein E (*APOE*) genotype is associated with altered risk for sporadic AD. The importance of *APOE* for AD has been confirmed by genome-wide association studies, which have also identified several other risk factors that are related to lipid homeostasis (e.g. *GAB2*) (Belbin et al. 2011). Familial AD is autosomal dominant and accounts for the minority of cases, with an earlier age of onset than the sporadic form, often occurring at 40-50 years of age. Familial AD is linked to mutations in three genes encoding for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2). As of 2012, studies have identified over 30 mutations for *APP*, more than 180 mutations for *PS1* and roughly 10 mutations for *PS2*, with mutations in the *PS1* gene accounting for the majority (50-70%) of all familial AD cases (Cruts et al. 2012). In AD, the disease-causing mutations affect the metabolism or stability of Aβ generated through the amyloidogenic pathway leading to their accumulation, as evidenced by extracellular deposits in the AD brain: *APP* is responsible for the generation of Aβ, while *presenilins* (either PS1 or PS2) are the active components of the γ-secretase complex, one of the enzymes responsible for the cleavage of Aβ from APP (Figure 1). Although *PS1* and *PS2* share structural similarities, their physiological functions are not likely to overlap. In addition to mutation frequency differences between the two genes, only *PS1* has been found to be functionally important in the embryonic development of mice (Herreman et al. 1999; Shen et al. 1997), suggesting different biological roles for the two genes.

Figure 1 provides a simple representation of the enzymatic cleavage of APP to either several soluble APP fragments (non-amyloidogenic pathway) or the release of plaque-forming Aβ peptides (amyloidogenic pathway).

Figure 1 has been removed due to Copyright restrictions

Figure 1: Non-amyloidogenic and amyloidogenic pathways of APP cleavage. NTF, N-terminal fragment of APP; CTF, C-terminal fragment of APP; sAPP, soluble APP; AICD, APP-intracellular C-terminal domain (Zhang and Saunders 2007)

Current AD treatments (to be outlined in section 1.2) provide limited relief for cognitive and functional decline in patients and are ineffective against disease progression. Furthermore, these treatments are accompanied by a range of adverse side effects (e.g. nausea, vomiting, abdominal pain, headache, depression, dizziness, and on rare occasions, seizures, heart failure and bradycardia) (Benito et al. 2007; Marchalant et al. 2008; Micale et al. 2007). Therefore, it is absolutely necessary to explore new therapeutic avenues.

1.1. Biology of Alzheimer's disease

1.1.1. The role of β-amyloid in AD

Amyloid precursor protein (APP) is cleaved and processed by the enzymes α-, β- and γ-secretases via two pathways, a non-amyloidogenic and an amyloidogenic pathway (Figure 1) (LaFerla et al. 2007). The non-amyloidogenic pathway accounts for the majority of APP processing in the healthy brain: APP is cleaved by α-secretase to generate soluble sAPP α, which has neuroprotective properties (Kojro and Fahrenholz 2005). A minority of APP is processed by the amyloidogenic pathway, leading to the generation of Aβ. Following the latter pathway, APP is processed into Aβ in two steps: first, β-secretase cleaves APP resulting in soluble APP and a cell-membrane bound fragment; second, γ-secretase cleaves this fragment further, producing Aβ and the APP intracellular domain (AICD) which regulates gene transcription. Aβ peptides can be between 36-43 amino acids in length (Marsden et al. 2011), but the majority of Aβ produced by the amyloidogenic pathway are 40 residues in length ($A\beta_{40}$), whereas approximately 10% form the 42 residue-length variant ($A\beta_{42}$) (Benilova et al. 2012; Citron et al. 1996). $A\beta_{42}$ is the longer, hydrophobic isoform that is more prone to fibril formation. It is found predominantly in cerebral plaques. Mutations in *APP*, APP-processing enzymes or *PS1* appear to influence the overproduction of $A\beta_{42}$ and other Aβ peptides in AD (such as the $A\beta_{43}$ variant which also possesses potent amyloidogenic and neurotoxic properties (Dolev et al. 2013; LaFerla et al. 2007; Saito et al. 2011).

Aβ peptides are found in human brains and may have functional roles in several processes, including the regulation of cholesterol transport, antioxidant and anti-microbial properties (Baruch-Suchodolsky and Fischer 2009; Soscia et al. 2010; Umeda et al. 2010). They possess high turnover rates and are associated with synaptic vesicle release, implying a role in neurotransmission (Marchesi 2011). In AD, deposits of Aβ

occur in the entorhinal and frontal cortices, and are found predominantly in the superior frontal cortex and the hippocampal formation (Bouras et al. 1994). The presence of elevated Aβ in the brain is strongly correlated with both cognitive decline in patients diagnosed with early dementia (Naslund et al. 2000), and impaired episodic memory in some non-demented individuals (Marchesi 2011; Pike et al. 2007). In addition to plaque formation and cell death, Aβ accumulation in the brains of AD patients may induce subtle changes to dendritic structures over time, causing neuronal dysfunctions that remain undetected for years and eventually leading to the damage and destruction of synapses that are essential for cognition (Marchesi 2011).

A number of hypotheses have been put forward to explain the role that Aβ might play in AD pathophysiology. The most prominent hypothesis is the *amyloid cascade hypothesis* which suggests the deposits of Aβ are responsible for causing tau phosphorylation into NFTs, with both processes leading to neuronal death and ultimately dementia. This view is based on studies of familial AD that have found mutations in *APP* suggesting that Aβ deposition is the pivotal process of AD pathology (Hardy and Allsop 1991; Selkoe 1996). In some cases, Aβ deposition has been observed to occur before any signs of tau pathology, and can exacerbate the hyperphosphorylation of tau (Gotz et al. 2001; Lewis et al. 2001; Oddo et al. 2004).

However, AD is a complex disease that includes various other pathological impairments resulting from Aβ accumulation, including a neuroinflammatory component (Barger and Basile 2001). The *amyloid cascade-neuroinflammation hypothesis* suggests resting microglia become activated in response to the presence of Aβ and cluster at sites of amyloid deposition in the brain, initiating inflammatory processes and the release of neurotoxic factors, such as proinflammatory cytokines and reactive oxygen and nitrogen species (Streit 2004). This series of pathological events results in the manifestation of

several characteristic AD-pathologies such as neurodegeneration, neuroinflammation, neurotoxicity and oxidative damage.

1.1.2. The role of tau proteins in AD

The *microtubule-associated protein tau* (*MAPT*) gene is responsible for tau expression, for which six isoforms exist and are generated through alternate splicing of *MAPT* (Goedert et al. 1989). Tau proteins are mainly found in neuronal axons. Under normal physiological conditions, tau is a highly soluble and unfolded protein that promotes the assembly of microtubules from tubulin (Weingarten et al. 1975). Thereafter, it maintains the stability of those microtubules. Tau also functions to promote microtubule-dependent axonal transport of organelles and biomolecules by modulating the motor proteins, dynein and kinesin (Dixit et al. 2008). Phosphorylation of tau may be crucial for the prevention of acute apoptotic cell death by stabilising β-catenin (Li et al. 2007), a phosphoprotein involved in Wnt signalling (a physiological process implicated in the regulation of adult hippocampal neurogenesis). Interestingly, defects in axonal transport are thought to play a role in early AD pathogenesis (Stokin et al. 2005).

In the healthy brain, 2-3 amino acid residues on tau are phosphorylated. In AD, tau proteins are hyperphosphorylated (average of 9 phosphates per molecule) leading to lowered tau affinity for microtubules, increased tau resistance to calcium-activated neutral proteases and finally the aggregation and formation of NFTs (Lie et al. 2005). Thus, tau protein is a major component of NFTs. NFTs are found and thought to develop initially in the entorhinal cortex before spreading to the CA1 region of the hippocampus, the subiculum, inferior temporal cortex and eventually to other neocortical regions (Bouras et al. 1994). It has been suggested that the formation of

NFTs (known as the tau fibrillisation pathway) consists of at least two key steps: 1) neutralisation of the tau-binding function allowing for the intracellular accumulation of tau proteins; 2) self-association of tau molecules to their microtubule-binding ability to form β-sheet enriched filaments, resulting in NFTs (Kuret et al. 2005). Tau filaments associated with AD consist primarily of paired helical filaments that appear as twisted structures (less commonly with straight morphology) (Perry et al. 1987). The significance of tau filament morphological differences remains speculative. Paired helical filaments are considered to be the primary structure distinct to AD (Wischik et al. 1985), while straight filaments are abundant in neuropil threads (unmyelinated axons, dendrites and glial cells) and may be precursors of the paired helical filaments, twisting together to form the helical structures (King et al. 2001).

Importantly, accumulation of tau and the associated NFTs induce cognitive deficits which correlate with neurodegeneration. For example, transgenic animal models expressing tau mutations demonstrate cognitive deficits (Barten et al. 2012; Beharry et al. 2013; Levenga et al. 2013). Extensive tau pathology is generally associated with the later stages of AD, but changes in tau biology could potentially occur much earlier (Kuret et al. 2005). The link between tau and Aβ remains unknown, with tau processes possibly occurring downstream of Aβ processes. Studies suggest the regulation of tau does not halt the ongoing amyloid pathogenesis (Oddo et al. 2007; Roberson et al. 2007), but may mediate Aβ toxicity and the resultant memory deficits through the reduction of tau or by targeting tau-dependent mechanisms of AD (Ittner et al. 2010; Roberson et al. 2007). Currently, no tau mutations have been linked to AD (Andorfer et al. 2003; Armstrong 2013; Wolfe 2009). Transgenic animal models expressing tau mutations do not develop Aβ pathology, which suggests that tau pathology in AD may be induced by Aβ but not *vice versa*, lending support to the *amyloid cascade hypothesis*.

1.1.3. Neuroinflammation, neurotoxicity, neurodegeneration and oxidative stress in AD

AD is considered to have an inflammatory component with the observation that microglia cluster at Aβ plaques. Microglia are the resident immune cells of the central nervous system (CNS) and have macrophage-like properties. They originate from myeloid tissue and migrate into the developing CNS. These cells remain in the CNS and become quiescent, a resting state that lasts as long as the CNS remains healthy. Microglia are found throughout the brain and spinal cord but predominantly in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Lawson et al. 1990; Morales et al. 2014; Venneti et al. 2009). They become activated within hours of CNS injury, carrying out various roles including pro- and anti-inflammatory functions and antigen presentation to immune cells (Ashton and Glass 2007). Not surprisingly, they are prominent around areas of neurodegeneration (Yong 2010). Under normal conditions, the presence of Aβ activates microglia which secrete pro-inflammatory enzymes for the phagocytosis and removal of Aβ. In AD, the clusters of activated microglia seem to be incapable of completely removing amyloid, most likely due to the impaired phagocytic or clearance ability of microglia (Fiala et al. 2005). Subsequently, Aβ plaques develop along with the build-up of inflammatory cytokines that contribute to the pronounced inflammation and neurotoxicity seen in AD (Streit 2004).

Microglia are responsible for neuroinflammatory processes which may function as a double-edged sword. On one hand, the activation of microglia may be beneficial for the brain by conferring properties such as neuroprotection, axonal regeneration, neurogenesis, and remylination (Yong 2010). On the other, the prolonged activation of microglia can result in extensive neuroinflammation which is detrimental for conditions such as AD. Indeed, evidence suggests neuroinflammation may be a driving force of AD pathology (Wyss-Coray 2006). Activated microglia induce neuroinflammation

through the release of inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). IFN-γ is also able to induce neuronal dysfunction by enhancing neurotoxic effects through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Mizuno et al. 2008). These cytokines also elicit neurotoxic effects by inducing the release of glutamate, an excitatory amino acid, which consequently triggers excito-neurotoxicity (Barger and Basile 2001; Takeuchi 2010). Neurotoxicity and neuroinflammation in AD are therefore consequences of prolonged microglial activation or rather, its impaired phagocytic ability (Fiala et al. 2005; Yong 2010).

Long-term disruptions to various neurotransmitter systems including glutamatergic and acetylcholine (ACh) neurotransmission (Schaeffer and Gattaz 2008; Schliebs and Arendt 2011). Under normal physiological conditions, glutamate activates N-methyl-D-aspartate (NMDA) receptors, allowing calcium ions (Ca^{2+}) to flow into the post-synaptic neuron, triggering a signalling cascade that produces synaptic plasticity such as long-term potentiation (LTP), thereby facilitating higher order processes such as learning and memory (Parsons et al. 2013). In AD, NMDA receptors are overstimulated by the presence of excess glutamate, leading to sustained Ca^{2+} influx. This prolonged Ca^{2+} overload increases the production of nitric oxide, inhibiting mitochondrial activity and depletes intracellular adenosine triphosphate (ATP) levels (Takeuchi 2010). The loss of energy results in impaired dendritic and axonal transport, and neuronal function, generating an excitotoxic state and eventually neurodegeneration.

Neurodegeneration also leads to the loss of cholinergic neurons and a reduction in ACh receptor density (Schliebs and Arendt 2011). ACh is an essential neurotransmitter distributed throughout the CNS and plays a role in cortical development and activity, control of circadian rhythm, cerebral blood flow and importantly, the modulation of

cognition, learning and memory. The weakened ACh signalling contributes to cognitive and memory impairment in AD. Furthermore, a number of cholinergic neuronal receptors (e.g. nicotinic receptor ion channels and muscarinic M1 receptors) are also decreased through degeneration occurring in the AD brain (Parsons et al. 2013).

Studies in post-mortem brain tissue of AD patients have also found prominent reactive oxygen species (ROS)-mediated injuries (i.e. oxidative damage), especially in regions containing high plaque and NFT load (Mattson 2004; Pratico and Sung 2004). Importantly, oxidative stress is absent from areas that are not affected in AD such as the cerebellum (Hensley et al. 1995). ROS are highly unstable and are mediated by efficient antioxidant systems, thus their levels are relatively low under normal conditions. However, in situations where the production of ROS exceeds the limits of those systems, oxidative homeostasis is altered resulting in increased oxidative stress. Importantly, sources of oxidative stress include Aβ peptides, activated microglia and altered mitochondrial function (Colton et al. 1996; Reddy and Beal 2005). For example, microglia produce nitric oxide (NO), peroxynitrite, superoxide and hydrogen peroxide (Colton et al. 1996). The ROS produced by microglia include both extracellular and intracellular ROS. Extracellular ROS are highly neurotoxic thereby inducing oxidative damage (Reynolds et al. 2007), while intracellular ROS are crucial for proinflammatory function and cellular homeostasis by acting as second messengers (Takeuchi 2010). Myelin breakdown, also a consequence of oxidative stress, is an early and largely unrecognised feature of AD that causes decreased neurotransmission, which may also contribute to the onset of AD (Bartzokis 2011).

In conclusion, AD is characterised by additional Aβ-driven pathological changes including neuroinflammation, neurotoxicity, neurodegeneration and oxidative stress, providing various targets for AD treatment options.

1.2. Treatment options for Alzheimer's disease

The currently approved treatment options for AD patients either target NMDA receptor hyperactivity or inhibit acetylcholinesterase (AChE). However, both strategies offer limited efficacy. Various other treatment options that have been suggested to confer protection, prevent the onset of AD and even improve memory in AD patients are currently under investigation. Such potential treatment options are at various preclinical and clinical trial stages and have so far returned mixed results. The current and proposed treatments will be discussed briefly in the following sections.

1.2.1. NMDA receptor antagonists

Only one (non-competitive) NMDA receptor antagonist, memantine, has been approved for treating AD and is used to reduce further cell death by decreasing Ca^{2+} influx induced by the overstimulation of the glutamatergic system. The administration of memantine reduces the decline in quality of daily living (Reisberg et al. 2003), allowing patients to retain their ability to perform daily tasks such as dressing themselves for longer compared to placebo group. However, the beneficial effects appear to be confined to early stages of AD (with limited efficacy after a few years), as the drug was not effective in patients with moderate to severe cases of AD (Modrego et al. 2010). Furthermore, a meta-analysis could not confirm the effectiveness of memantine for mild AD (Schneider et al. 2011) and side effects such as hallucinations, dizziness, and fatigue have been reported by AD patients (Herrmann et al. 2011).

1.2.2. AChE inhibitors

AChE inhibitors such as donepezil, rivastigmine and galantamine are used to improve the cholinergic tone in AD patients by preventing further ACh loss from degenerating cholinergic neurons in the amygdala, hippocampus and frontal cortex (areas that are relevant for memory). Studies have shown significant improvements in cognitive performance as well as activities of daily living but this effect was absent from more severe stages of AD (Mancuso et al. 2011). Donepezil and galantamine have also been documented to inhibit NO-induced cytotoxicity (caused by the production of free radicals and mitochondrial dysfunction) and counteract neuronal cell death (Takada-Takatori et al. 2009). However, a variety of side effects accompany the use of AChE inhibitors, including diarrhoea, vomiting and nausea, insomnia, fatigue and dizziness.

1.2.3. Anti-inflammatory treatments

Non-steroidal anti-inflammatory drugs (NSAIDs) target the neuroinflammatory component of AD via the inhibition of cyclooxygenase (COX) enzymes, and at higher dosages, interleukin-1β (IL-1β) and interleukin-6 (IL-6). At least one study has found that treatment with ibuprofen attenuated plaque density and expression of inflammatory cytokines (such as IL-1β) in a transgenic mouse model of AD (Lim et al. 2000). Suppression of COX-2 (one of two COX enzyme isoforms) may provide therapeutic benefits for patients with AD as the isoform is increased in many areas of the AD brain, correlating with elevated Aβ protein levels (Zandi and Breitner 2001). Epidemiological studies and meta-analyses support the use of NSAIDs as an alternative AD treatment, citing less cognitive decline and reduced risk of AD, possibly through inhibition of the immune response (Zandi and Breitner 2001). However, the general consensus is that NSAIDs can only offer protection up until a certain point prior to the diagnosis of

dementia. More importantly, their prolonged use results in serious side effects including gastrointestinal ulcerations, renal and cardiovascular complications (Zandi and Breitner 2001).

1.2.4. Antioxidant treatments

The use of antioxidant treatments (including vitamin E and vitamin C) may have the capacity to prevent further tissue damage caused by ROS and improve neuronal survival in AD (Zandi et al. 2002). A recent clinical trial has shown that vitamin E is able to benefit patients with mild to moderate AD (where patients were also prescribed AChE inhibitors as part of their regular treatment), as the vitamin supplementation slowed functional decline and reduced caregiver burden (Dysken et al. 2014). However, a study from 2005 suggests that the use of vitamin E supplements should be closely monitored as daily high doses may increase the risk of morbidity and mortality (Boothby and Doering 2005).

1.2.5. Anti-Aβ therapies

The reduction of Aβ has previously been postulated to provide a beneficial effect for AD, as it is thought to be the main culprit in triggering the cascade of events that lead to a neurotoxic state, a loss of functional synapses and neurodegeneration. Treatments such as active immunisation with Aβ prevented amyloid plaque formation, neuritic dystrophy and astrogliosis in a transgenic mouse model of AD (Schenk et al. 1999), and slowed cognitive decline in AD patients (Hock et al. 2003). Despite these initial findings, clinical trials were halted after several patients began experiencing secondary effects, including clinical symptoms consistent with inflammation of the CNS (i.e. meningoencephalitis) (Check 2002; Nicoll et al. 2003).

1.2.6. Metal 'attenuation' trials

Zinc (Zn^{2+}) and copper (Cu^{2+}) are normally found in high concentrations near excitatory NMDA synapses and required for transition-metal homeostasis (Bush 2013). In AD, essential metals in the brain such as Zn^{2+} and Cu^{2+} interact with Aβ to exacerbate amyloid pathology. Furthermore, transition-metal homeostasis is severely disrupted in AD leading to the *metal hypothesis* of AD (Bush 2013). Metal-protein attenuating compounds (MPAC) are low-affinity-metal-complexing compounds that are designed to inhibit Zn^{2+}- and Cu^{2+}-induced Aβ oligomers without interrupting essential metal homeostasis (Lannfelt et al. 2008). A phase II clinical trial of PBT2 (a quinoline MPAC), orally administered for 12 weeks to patients with mild AD reduced amyloid aggregation and toxicity, and acted as an ionophore thereby increasing the availability of Cu^{2+} and Zn^{2+}, and promoting neuronal function (Lannfelt et al. 2008). However, cognitive efficacy was restricted to two measures of executive function and patients reported various adverse effects including headache, dizziness, somnolence, nasopharyngitis, and fatigue (Lannfelt et al. 2008). Furthermore, the short duration of the trial also means that the safety and efficacy of MPACs has yet to be determined in large-scale clinical trials.

In conclusion, current treatments do not stop or reverse the progression of AD and no novel treatments have been approved for AD since memantine over a decade ago. Furthermore, a plethora of side effects accompany current treatments including nausea, vomiting, headaches, cataracts, constipation or diarrhoea, and weight loss. A theory has emerged, supported by the lack of efficacy of current treatments that suggests existing interventions may be administered too late to have any lasting beneficial effects as the extent of the damage caused by AD pathology might already be too severe (Hampel

2012; Karl et al. 2012b; Riedel 2014). Thus, new interventions need to adopt a preventative and multimodal approach that is able to simultaneously target various pathological processes involved in AD (e.g. neurotoxicity, neuroinflammation, oxidative damage). Such a treatment strategy may provide increased therapeutic benefit for patients (Hsiung and Feldman 2008; Tariot et al. 2004). Interestingly, one clinical study using a combination of an NMDA receptor antagonist with an AChE inhibitor (memantine and donepezil) has shown greater treatment efficacy in improving cognitive functions in patients than receiving AChE inhibitor alone (Tariot et al. 2004). Importantly, studies support the use of memantine and AChE inhibitors in combination to target different mechanisms (i.e. multimodal) in treating AD (Parsons et al. 2013). The use of an intervention that can be applied early and even prior to the manifestation of AD (i.e. prevention) with few or no severe side effects would be ideal as current treatment interventions may be administered too late into the progression of the disease. In particular, the endocannabinoid system has been proposed to provide therapeutic potential for patients and will be discussed in Section 1.4.

1.3. Rodent models of Alzheimer's disease

Animal models of AD, including pharmacological, lesion and transgenic models are used to investigate and better understand disease pathogenesis. They demonstrate various AD-relevant changes in pathology including Aβ aggregation and toxicity, NFT formation, neuronal damage, neuroinflammation and oxidative stress. Importantly, animal models of AD demonstrate a number of behavioural and cognitive impairments which are relevant for the clinical symptoms of AD patients. Behavioural and cognitive tests are used to evaluate AD models, while the analyses of pathological neural changes in these animal models provide valuable insight into the mechanisms involved in AD.

Typically, behavioural testing of AD mouse models includes cognitive tests for associative learning (e.g. fear conditioning) (Bonardi et al. 2011; Kilgore et al. 2010), short-term recognition memory (e.g. novel object recognition task and social preference test) (Faizi et al. 2012; Jardanhazi-Kurutz et al. 2010), long-term spatial memory (e.g. Morris water maze and cheeseboard task) (Faizi et al. 2012; Gallagher et al. 2013; Pillay et al. 2008), and sensorimotor gating (i.e. prepulse inhibition) (Wang et al. 2012). The integrity of the hippocampus is crucial for performance in spatial memory tests, but other brain regions may also influence spatial memory including the hippocampal connections (i.e. entorhinal and perirhinal cortices), the amygdala, cerebellum, anterior cingulate cortex, striatum and the prefrontal cortex (Puzzo et al. 2014). Learning, short-term and long-term memory are evaluated in these paradigms thereby considering hippocampal as well as amygdala function. Other behaviours such as anxiety, locomotion and exploration, as well as social behaviours are also assessed (Faizi et al. 2012; Reiserer et al. 2007). In the following section, a brief description of AD rodent models currently available will be given.

1.3.1. Pharmacological and lesion models of AD

Pharmacological models are generated based on Aβ pathology (Streit 2004). The injection of Aβ peptide into the hippocampus of rats or mice induces significant neuronal loss in the CA1, CA2 and CA3 regions, followed by the appearance of typical markers of apoptosis and gliosis. No other signs of neurodegeneration have been found suggesting the effects are likely to be caused by reversible neuronal dysfunction (van der Stelt et al. 2006). Spatial memory deficits have been attributed to pharmacological AD models (Martin-Moreno et al. 2011). Lesion models are used to study the effects of structural deficits in areas that are relevant for AD such as the cholinergic nucleus

basalis magnocellularis (Murray and Fibiger 1985). These models exhibit deficits in spatial (i.e. long-term) memory and are useful for determining the function of specific brain regions. However, they are not representative of AD pathology.

1.3.2. Transgenic mouse models

The generation of transgenic mouse models of AD was made possible through three key events: 1) the isolation and sequencing of the Aβ peptide in 1984; 2) the cloning of APP and discovery of its role in Aβ generation, and; 3) the discovery of the first *APP* mutation in autosomal-dominant AD (Ashe and Zahs 2010). Transgenic mouse models are generated based on human mutations found in the genes responsible for familial AD (i.e. *APP*, *PS1* and *PS2*) (McGowan et al. 2006). Mutations in the *MAPT* gene are also used to generate animal models expressing tau pathology. Although no tau-associated mutations have been found in AD to date, tau-based transgenic mouse models are used to better understand tau pathogenesis and its interactions with amyloid pathology (Ittner and Gotz 2011; Ittner et al. 2010).

In patients with familial AD, mutations in *APP* or one of the presenilins (*PS1* or *PS2*) are sufficient to cause progressive cognitive impairment, the formation of plaques and tangles and neuron loss in the brain. The expression of *APP* gene mutations in the mouse brain results in the accumulation of amyloid plaques and memory deficits without noticeable tau pathology, while the expression of presenilin mutations induce memory impairments (Hwang et al. 2002) without the typical AD-relevant neuropathological changes (i.e. amyloid pathology) in transgenic mice (Ashe and Zahs 2010). Varied neuronal loss can be observed in some transgenic animal models (e.g. PS1 or MAPT transgenic mice) (Shen et al. 1997; Yoshiyama et al. 2007) similar to neurodegeneration occurring in AD brains. Microglial activation has also been shown to

induce neurodegeneration in transgenic mice (Ramirez et al. 2005). Transgenic mouse models also develop various behavioural and cognitive symptoms some that are akin to and highly relevant for AD (Ashe and Zahs 2010). Therefore, existing transgenic mouse models provide a great advantage over pharmacologically-induced AD and lesion models, offering valuable insight into specific mechanisms of AD pathology that may be linked to various behavioural and cognitive deficits. Mechanisms prior to and post onset of disease can be studied in great detail and potential therapeutic options can be assessed in validated transgenic mouse models. This is of importance for AD as subtle pathological and/or behavioural changes occur several years prior to its clinical diagnosis.

The co-expression of more than one mutation in transgenic mice allows the possibility for a greater understanding of the specific roles of AD mutations and pathological interactions that can occur. In particular, the co-expression of mutant *PS1* with *APP* exacerbates and accelerates Aβ pathology compared to single transgene mice (Borchelt et al. 1997; Howlett et al. 2004). In addition to impaired spatial memory and other cognitive deficits (e.g. recognition memory, associative learning), different lines of APPxPS1 transgenic mice (varying in *APP* and *PS1* mutations) also display a range of other behavioural deficits including hyperlocomotion and alterations in anxiety. Memory deficits are age-dependent and evident as early as 2-3 months of age, alongside Aβ deposition. By investigating double transgenic mice co-expressing *APP* and *PS1*, it may be possible to gain a better understanding of AD symptomatology and pathogenesis.

Table 1: Mouse models of Alzheimer's Disease				
Gene(s) involved	**Model**	**Mutation**	**Phenotype**	**Reference**
Single transgene				
APP	PDAPP	V717F (Indiana)	Robust plaque pathology. Synapse loss but no overt cell loss and no NFT pathology	(Games et al. 1995)
	Tg2576	K670N, M671L (Swedish)	Cognitive deficits but no cell loss or NFT pathology	(Hsiao et al. 1996)
	APP23	K670N, M671L (Swedish)	Hippocampal neuronal loss associated with amyloid plaque formation	(Sturchler-Pierrat et al. 1997)
	APP_{Dutch}	APP751	Severe cerebral amyloid angiopathy, smooth muscle cell degeneration, haemorrhages and neuroinflammation.	(Herzig et al. 2004)
PS1	$PS1_{M146v}$ or $PS1_{M146L}$	M146v or M146L	No abnormal pathology but altered mitochondrial activity and dysregulation of calcium homeostasis	(Duff et al. 1996)
MAPT	JNPL3	P301L	Mice show marked tangle pathology, cell loss, age-dependent motor impairments and motor neuron loss in spinal cord	(Lewis et al. 2000)
	Tau_{P301S}	P301S	Lower limb paralysis and widespread neurofibrillary pathology in brain and spinal cord and neuronal loss in the spinal cord	(Yoshiyama et al. 2007)
	Tau_{V337M}	V337M	Development of neurofibrillary pathology	(Tanemura et al. 2002)
	Tau_{R406W}	R406W	Impaired associative memory	(Tatebayashi et al. 2002)
	rTg4510	P301L	Abnormal *MAPT* pathology, progressive NFT pathology, cell loss, cognitive deficits evident from 2.5 months of age. Mice possess an inducible *MAPT* and turning off the transgene improves cognitive performance, but NFT pathology worsens	(Santacruz et al. 2005)
Double transgene				
APP, PS1	PSAPP	Tg2576 with M146L	Co-expression of *APP* and *PS1* mutation causes accelerated development of plaques compared to single transgenic mice. *PS1*-driven elevation of Aβ	(Holcomb et al. 1998)
APP	TgCRND8	K670N/ M671L with V717F	Rapid extracellular plaque development coinciding with cognitive deficits.	(Chishti et al. 2001)
APP	J20	K670N/ M671L with V717F	Age-dependent plaque formation, impaired spatial learning and memory, and poor long-term object recognition memory	(Mucke et al. 2000)
MAPT	Htau	Human tau and disrupted exon one of tau	Hyperphosphorylated *MAPT* from 6 months of age and develop NFT by 15months	(Andorfer et al. 2003)
Triple transgene				
APP, PS1, MAPT	3xTgAD	Swedish, M146V and P301L	Retention/retrieval deficits occurring prior to plaques and tangles. Progressive neuropathology with plaques appearing first before evidence of tangles at 6 months	(Oddo et al. 2003)

Adapted from (McGowan et al. 2006).
Additional information can be found at http://www.alzforum.org/research-models/

1.3.3. APP_{Swe}/PS1ΔE9 double transgenic mouse

The double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) mouse model that was the focus of this thesis was a double transgenic mouse model generated by the co-injection of a chimeric human murine *APP* construct bearing the Swedish mutation (APP_{Swe}) and the exon-9-deleted *PS1* (PS1/ΔE9) mutation (Jankowsky et al. 2004a; Jankowsky et al. 2004b). As mutation, background strain and gender can impact experimental outcomes (Fairless et al. 2008; Moy et al. 2008), care must be taken when describing the exact model investigated. The double transgenic mice used in this thesis express chimeric mouse/human APP (Mo/HuAPP695Swe/Swedish mutations K595N/M596L) and mutant human PS1 (PS1/ΔE9), and were maintained as hemizygotes on the congenic C57BL/6JxC3H/HeJ (B6C3; i.e. mixed) background as originally described (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b; Jankowsky et al. 2001). As a result of the co-expression of *APP* and *PS1* mutant genes, the model exhibits accelerated amyloid pathology in comparison to single transgene mouse models (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b; Machova et al. 2010). Amyloid plaques appear in the brain as early as 4 months of age (Wang et al. 2003a) and spatial memory deficits were more pronounced with age, correlating with increased amyloid deposition (Hooijmans et al. 2009; Savonenko et al. 2005; Zhang et al. 2011). APPxPS1 mice also demonstrate neuroinflammation (i.e. increased NOS and TNF-α levels) (Kalifa et al. 2011), impaired neurogenesis and evidence of age-dependent oxidative damage (Hamilton and Holscher 2012).

Studies have investigated and reported various behavioural and cognitive alterations displayed by this APPxPS1 double transgenic mouse model, including deficits in spatial memory, object recognition, altered associative memory, sensorimotor gating, and changes in anxiety and locomotor activity (Table 2). A number of inter-study

discrepancies have been marked with an asterisk ('*') in the table for various tests which are likely to be due to differences in age and possible sex-specific effects.

Studies utilising the Morris water maze (MWM) report spatial learning and memory deficits in APPxPS1 mice at various ages. One study has suggested that female APPxPS1 mice have learning deficits (i.e. acquisition) in the MWM as early as 4 months of age (Jardanhazi-Kurutz et al. 2010). Indeed, the majority of studies consistently report learning deficits in 8-10 month old APPxPS1 mice (Butovsky et al. 2006; Donkin et al. 2010; Gallagher et al. 2013). Based on the existing literature, reference memory (i.e. performance in the probe trial) becomes impaired as female mice age to 12 months old (Jardanhazi-Kurutz et al. 2010). However, sex specificity may influence the outcomes as 9-month old female APPxPS1 mice were reported to demonstrate learning impairments while age-matched male transgenic mice did not show any deficits (Gallagher et al. 2013). Furthermore, one study has reported that 8-month old male APPxPS1 mice demonstrate spatial memory deficits in the probe trial, absent deficits in acquisition in contrast to other findings (Cao et al. 2007).

Other tests of spatial memory, such as the Barnes maze and the cued-cheeseboard (CB) task (dry-land variations of the MWM), revealed similar impairments in task acquisition in APPxPS1 mice for all ages tested (Table 2) (Pillay et al. 2008; Reiserer et al. 2007), although all studies combined males and females. Slight inter-study differences were found in the Barnes maze between APPxPS1 mice possibly due to differences in age (i.e. 7 and 16 months) (O'Leary and Brown 2009; Reiserer et al. 2007). The cued-CB task, which relies on positive reinforcement, has been suggested to be more sensitive for the detection of cognitive impairment in APPxPS1 mice compared to the MWM, allowing deficits to be observed at 2-3 months age (Pillay et al. 2008) and also later at 24 months of age (Kulkarni et al. 2008).

Table 2: Behavioural and cognitive phenotypes of APP_{Swe}/PS1ΔE9 double transgenic mice				
Tests	**Sex**	**Age**	**Deficit**	**Study**
Barnes maze	Males and Females	7 months	Impaired acquisition in the Barnes maze but only when hidden version was tested post cued version (not the other way around)	(Reiserer et al. 2007)
	Males and Females	16 months	Acquisition and retention modestly impaired (more pronounced in males) - no phenotype in reversal trials	(O'Leary and Brown 2009)
Cheeseboard (cued version)	Not mentioned	2-3 months	Impaired task acquisition (i.e. to located/approach flagged reward well)	(Pillay et al. 2008)
	Not mentioned	24 months	Impaired acquisition	(Kulkarni et al. 2008)
Conditioned taste preference	Males and Females	2/5 months	Fail to exhibit conditioned taste aversion to a saccharin and lithium chloride solution	(Pistell et al. 2008)
Contextual fear conditioning	Females	4 months	Retention not impaired. Deficit in context extinction	(Bonardi et al. 2011)
	Males and Females	4 and 6 months	Impaired retention in 6-month old transgenic mice (when context test was conducted 24 h and 14 days after conditioning, but not after 1 h)	(Kilgore et al. 2010)
Elevated plus maze*	Males and females	7 months	Anxiolytic-like phenotype on day 1 but not on day 2 of EPM	(Lalonde et al. 2004)
	Males and females	7 months	No anxiolytic phenotype, although transgenic mice spent less time in the closed arms	(Reiserer et al. 2007)
Morris water maze*	Males	8 months	Intact acquisition. Less platform crossings in probe trial	(Cao et al. 2007)
	Males and females	9 months	Females impaired in reverse MWM acquisition, but no spatial memory deficit reported for males	(Gallagher et al. 2013)
	Not mentioned	9-10 months	Impaired acquisition in reference task and reverse MWM	(Butovsky et al. 2006)
	Females	10 months	Impaired acquisition	(Donkin et al. 2010)
	Females	4/6/12 months	Impaired acquisition at all ages investigated and retention deficit in 12-month old mice	(Jardanhazi-Kurutz et al. 2010)
Novel object recognition task*	Females	10 months	Impaired novel object recognition (4 hour ITI)	(Donkin et al. 2010)
	Females	12 months	Intact novelty preference in transgenic mice but preference for novel object is reduced compared to control mice	(Jardanhazi-Kurutz et al. 2010)
Olfactory test	Not mentioned	6-9, 11, 13, 15, 18 months	No differences in olfactory ability	(Phillips et al. 2011)
Open field	Males	15 months	Hyper-locomotion in OF	(Hooijmans et al. 2009)
Spatial reward test	Not mentioned	6-9, 11, 13, 15, 18 months	Impaired learning at 6 months and reduced re-learning/memory from 9 months onwards	(Phillips et al. 2011)

Inter-study inconsistencies denoted by an asterisk '*'

Female APPxPS1 mice also demonstrate impairments in object recognition at 10 months of age in the novel object recognition task (NORT) (Donkin et al. 2010). However, another study has shown that object recognition was not impaired in 12-month old female APPxPS1 mice (Jardanhazi-Kurutz et al. 2010). Male APPxPS1 mice have not been investigated in the NORT.

Deficits in sensorimotor gating have been observed for female APPxPS1 mice at 7 months of age (Wang et al. 2012), while male APPxPS1 mice have not yet been assessed in these test paradigms. Other reported characteristics of the model include increased locomotor activity (hyperlocomotion) and decreased anxiety (Hooijmans et al. 2009; Lalonde et al. 2004). Finally, the anxiolytic phenotype of APPxPS1 mice (Lalonde et al. 2004) was not observed in APPxPS1 mice in a separate study (Reiserer et al. 2007).

The inconsistencies between studies may be related to the fact that some studies consider sex-specific effects (Gallagher et al. 2013; Pistell et al. 2008; Wang et al. 2003a) whereas others use both male and female mice within one test cohort (Lalonde et al. 2004; O'Leary and Brown 2009; Reiserer et al. 2007). As summarised in Table 2, it is very likely that the reported behavioural and cognitive phenotypes are sex-dependent. For example, female APPxPS1 mice exhibit AD-like symptomatology (i.e. spatial memory deficits and Aβ pathology) earlier than age-matched male APPxPS1 mice (Gallagher et al. 2013). Furthermore, differences in testing conditions (e.g. level of test illumination, circadian rhythm) can impact on the behaviour of mice (Post et al. 2011). For example, the time of testing was not reported for the two studies in which discrepancies were found for anxiety in APPxPS1 mice, thus limiting the comparability of the individual findings (Lalonde et al. 2004; Reiserer et al. 2007). In another example, discrepancies in object recognition protocols (inter-trial interval of 1 h

compared to 4 h) are likely reasons for different findings in this task between studies (Donkin et al. 2010; Jardanhazi-Kurutz et al. 2010).

In order to preserve the comparability of future findings, greater care must be taken to ensure that the mutation and background of the model, gender and age of test animals as well as housing conditions of test animals, test protocol, and test conditions are sufficiently described to allow for inter-study comparisons.

1.4. The Endocannabinoid system and Alzheimer's disease

The endocannabinoid system (ECS) has emerged as a potential therapeutic target for patients with AD and appears to be a neuroprotective system that responds to neurotoxic insults including Aβ deposition (Fowler et al. 2010). It is an intercellular signalling system comprised of G-protein coupled cannabinoid receptors 1 (CB_1) and 2 (CB_2), endogenous ligands and their homologues and metabolic enzymes (see Table 3). The ECS is involved in a variety of physiological processes including appetite, pain sensation, mood and cognition.

CB_1 receptors were first characterised in the late 1980s and subsequently cloned in 1990 (Pazos et al. 2004). Autoradiographic studies have found that CB_1 receptors are highly expressed throughout the brain by many different classes of neurons and also at lower levels by glial cells and many peripheral cell types. They are found in abundance in the basal ganglia, cerebellum, and more importantly the parahippocampal and entorhinal cortices, and the hippocampus, suggesting an involvement of CB_1 receptors in learning and memory (Pazos et al. 2004). CB_1 receptors are also expressed in peripheral tissues such as adipocytes, liver, pancreas and skeletal muscle possibly accounting for metabolic-related cannabinoid effects (i.e. CB_1 receptor antagonists induce weight loss). CB_1 receptors may also be involved in cannabinoid-mediated modulation of immune

functions (Cabral et al. 2008), however its activation also leads to psychoactive effects (Ramirez et al. 2005).

Table 3: Components of the endocannabinoid system

Type	Component	Function	Reference
Endocannabinoids	2-arachidonoylglycerol (2-AG)	Stimulation of chemotactic response of microglial cells through CB_2 receptors	(Cabral et al. 2008)
	N-arachidonoylethanolamide (AEA, or anandamide)	Produced by immune cells and is selective for CB_1 receptors	(Cabral et al. 2008; Tanasescu and Constantinescu 2010)
Exocannabinoids	Phytocannabinoids	Various constituents of Cannabis sativa including the main psychoactive Δ^9-tetrahydrocannabinol (THC) and non-psychoactive cannabidiol (CBD)	(Pertwee 2008; Tanasescu and Constantinescu 2010)
	Cannabimimetics	Synthetic cannabinoids that mimic actions of phytocannabinoids	(Tanasescu and Constantinescu 2010)
Homologues of endocannabinoids	2-linoleoylglycerol (2-LG)	Potentiates activity of other endocannabinoids including 2-AG	(Ben-Shabat et al. 1998)
	palmitoylethanolamide (PEA)	Pain reduction and anti-inflammatory effects	(Hansen 2010)
Metabolic enzymes	N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)	Synthesis of AEA and PEA	(Fowler et al. 2010; Watkins et al. 2010)
	diacylglycerol lipases (DAGL) α and β	Synthesis of 2-AG	(Fowler et al. 2010; Watkins et al. 2010)
	Monoacylglycerol lipase (MGL)	Degradation of 2-AG	(Cravatt et al. 2001)
	Fatty acid amide hydrolase (FAAH)	Degradation of AEA and 2-AG, regulation of endocannabinoids	(Cravatt et al. 2001)
Cannabinoid receptors	Cannabinoid type 1 (CB_1)	Involved in learning and memory, immune functions and psychosis	(Cabral et al. 2008; D'Souza 2007)
	Cannabinoid type 2 (CB_2)	Mediation of neuroinflammatory responses in CNS	(Ramirez et al. 2005; Walter et al. 2003)
	Abnormal-cannabidiol (abn-CBD) or GPR18	Microglial-neuronal communication, neuronal migration and proliferation	(McHugh 2012)
	GPR55	Potential cannabinoid receptor, but classification is complex due to several molecular differences from CB_1 and CB_2 receptors	(Brown 2007; Henstridge 2012)
	GPR119	Potential cannabinoid receptor, implicated in regulation of energy balance and body weight	(Brown 2007)

CB_2 receptors were classified and cloned a few years later (Munro et al. 1993). In the periphery, they are expressed on a variety of immune cells including B lymphocytes, natural killer cells, monocytes/macrophages, polymorphonuclear neutrophils and T cells (Koppel and Davies 2008) suggesting a role in immunomodulation. Importantly, CB_2 receptors are also densely expressed on activated microglia cells and do not possess psychoactive properties (Ramirez et al. 2005; Walter et al. 2003). Their expression on microglia suggests a possible role in mediating neuroinflammatory responses in the CNS. CB_2 receptors are up-regulated in microglia in response to neuroinflammation. Stimulation of CB_2 receptors in microglia not only drives the proliferation and migration of microglia, but can also block their differentiation to a neurotoxic phenotype (Stella 2010).

Other cannabinoid receptors have been postulated to exist as cannabinoid ligands exert effects in CB_1 and CB_2 receptor-deficient mice (Jarai et al. 1999). For example, palmitoylethanolamide (PEA) is able to interact with the ECS to reduce pain associated with inflammation but does not have any direct effect on CB_1 or CB_2 receptors (Jarai et al. 1999).

There are two main groups of cannabinoids that interact with the receptors of the ECS, namely endogenous and exogenous cannabinoids (see Table 3). The primary endocannabinoids *N*-arachidonoylethanolamide (AEA; also known as anandamide) and 2-arachidonoylglycerol (2-AG) are synthesised on demand from arachidonic acid by *N*-acyl phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) or diacylglycerol lipases (DAGL) α and β respectively (Fowler et al. 2010; Watkins et al. 2010). AEA is CB_1 receptor specific (Tanasescu and Constantinescu 2010) and 2-AG is selective for CB_2 receptors (Cabral et al. 2008). Both are metabolised by fatty acid amide hydrolase (FAAH) which is essential for the regulation of endocannabinoid

levels (Cravatt et al. 2001). Exogenous cannabinoids include various phytocannabinoids derived from the marijuana plant, *Cannabis sativa* and synthetic cannabinoids (i.e. cannabimimetics such as CP55,940 and WIN55,212-2) (Tanasescu and Constantinescu 2010). Over 70 different phytocannabinoids have been identified and derived from the cannabis plant (Elsohly and Slade 2005). Among these, the main psychoactive component is Δ^9-tetrahydrocannabinol (THC) and the main non-psychoactive constituent is cannabidiol (CBD). Phytocannabinoids exert various effects by engaging the receptors of the ECS, especially CB_1 and CB_2 receptors. Importantly for AD, the ECS appears to be altered and presents a novel target for therapeutic compounds.

1.4.1. Therapeutic value of the ECS for AD

Post-mortem analyses have found up-regulated cannabinoid expression (i.e. CB_2 and FAAH) in the AD brain, possibly to exert a protective function (Benito et al. 2003; van der Stelt et al. 2006). In AD, Aβ deposition induces dramatic changes in the phenotype of glial cells, including the up-regulation of some components of the ECS, decreased CB_1 receptor binding and inefficient signal transduction in the hippocampus and basal ganglia (Benito et al. 2007), overexpression of CB_2 receptors in microglia cells and increased FAAH in astrocytes (Benito et al. 2007; Benito et al. 2003; Walter et al. 2003). Furthermore, *in vitro* studies have found the stimulation of CB_2 receptors increases Aβ phagocytosis (Ehrhart et al. 2005), antagonised Aβ-induced microglial activation (Ramirez et al. 2005) and induced the removal of Aβ by human macrophages (Tolon et al. 2009). Therefore, the manipulation of the ECS provides a novel and potential treatment avenue for patients with AD.

Pharmacological interventions targeting CB_1 receptors such as THC have been investigated. Few studies suggested a beneficial effect of THC for AD, where it induced

a positive effect on appetite, increased body weight, and improved several clinical symptoms in AD patients, including nocturnal motor activity and agitation (Volicer et al. 1997; Walther et al. 2006). However, selective CB_1 activation negatively impacts ACh levels, resulting in altered LTP, a mechanism that is crucial for learning and memory, especially in AD (Hasselmo and Barkai 1995; Steffens et al. 2003). Working memory performance is decreased following THC administration, correlating with a reduction in ACh release (Egashira et al. 2008; Nava et al. 2000). Furthermore, THC induces memory loss, sedation, motor impairment and psychotropic effects that ultimately lead to psychosis (Englund et al. 2013), thus rendering THC and CB_1 activation unsuitable as a therapeutic option for AD.

CB_2 receptors may provide another route for AD therapy. In AD patients, overexpression of CB_2 receptors is a phenomenon directly correlated with increased Aβ deposition and is hypothesised to be an anti-inflammatory response of the CNS to protect neurons from degeneration (Benito et al. 2003; Ramirez et al. 2005; van der Stelt et al. 2006) suggesting a role in AD pathology. CB_2 receptors are expressed in activated microglia and their activation increases microglial cell proliferation and migration, and reduces the release of inflammatory cytokines such as TNF-α and free radicals that induce oxidative stress, relevant for the pathogenesis of AD (Stella 2010). One study has shown that CB2 receptor agonists may promote Aβ clearance, reduced cytokine production and Aβ-induced spatial memory deficits in a pharmacological animal model of AD (i.e. Aβ-injected rats) (Wu et al. 2013). The deletion of CB_2 receptors also impaired short- and long-term memory in mice suggesting a role in cognition (Ortega-Alvaro et al. 2011). However, the effect of CB_2 receptor agonists has not been assessed in humans, and thus the safety of its use for AD is unclear.

On the other hand, the non-psychoactive phytocannabinoid, CBD has been investigated in both preclinical and clinical settings and does not induce adverse psychotropic properties. Further, it exerts beneficial effects which appear to be highly relevant and beneficial for the treatment of AD. The therapeutic potential of CBD for AD will be discussed in more detail in the following section.

1.5. Cannabidiol (CBD)

CBD is a non-psychotropic phytocannabinoid that interacts with the ECS. Its non-psychoactivity is likely due to the lack of intrinsic effect on CB_1 receptors (Felder et al. 1995; Showalter et al. 1996). CBD also has low selectivity for CB_2 receptors. However, studies have shown CBD interacts with these receptors by antagonising the actions of CB_1 and CB_2 agonists and possibly by acting as an inverse agonist for these receptors (Iuvone et al. 2009; Thomas et al. 2007). It has been postulated that CBD may interact with the abnormal-cannabidiol-sensitive receptor (abn-CBD) or AEA receptors (Jarai et al. 1999), based on the observation that CBD exerts similar effects on mice lacking CB_1 and CB_2 receptors as it does on their wild type-like littermates. CBD also indirectly increases AEA production perhaps to affect lipid metabolism (Rimmerman et al. 2011). However, the molecular mechanisms in which CBD exerts its effects are still under debate with evidence suggesting that its actions are not confined to the receptors of the ECS. CBD may also activate various non-cannabinoid receptors including several TRP channels and 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) (e.g. CBD exerts anti-depressive effects and attenuates catalepsy, which may be mediated through 5-HT_{1A} activation) (Booz 2011; Gomes et al. 2013). It interacts with several transient receptor potential (TRP) channels and as an antagonist of G-protein-coupled receptor 55 (GPR55) suggesting various potential mechanisms to exert its effects, but further investigation is required to

understand its pharmacological relevance (Booz 2011). Recent evidence suggests that CBD also interacts with a family of nuclear receptors known as peroxisome proliferator-activated receptors (PPAR), providing an avenue of interest for the pharmacological effects of CBD (O'Sullivan et al. 2009). The importance of PPARs will be revisited later (section 1.5.1).

Importantly, CBD exhibits various properties including neuroprotection, anti-inflammatory and antioxidant effects (Booz 2011; Iuvone et al. 2009; Krishnan et al. 2009; Scuderi et al. 2011; Zuardi 2008), which counter a number of AD-relevant pathological symptoms. Studies have shown that the administration of CBD modulates the function of the immune system. Studies show CBD is able to attenuate inflammatory cell migration and induce neuroprotection following ischemic damage (Hampson et al. 1998) or after ethanol-induced brain injury in rodents (Booz 2011; Garcia-Arencibia et al. 2007; Hamelink et al. 2005). Several mechanisms might explain this neuroprotective effect of CBD, including the blockade of excitotoxicity, the reduction of calcium influx, antioxidant properties or enhanced tropic factor support. CBD is able to modulate the levels of inflammatory cytokines such as TNF-α, IFN-γ and IL-1. Specifically, CBD decreases cytokine generation, proinflammatory mediators and nitric oxide, demonstrating its neuroprotective and anti-inflammatory properties (Malfait et al. 2000).

The chemical structure of CBD contains two hydroxyl groups which suggests it also has potent antioxidant properties (i.e. potentially act as reductants; Figure 2). Oxidative damage in neuronal cultures has also been prevented by CBD (Hampson et al. 1998). CBD substantially limited neuronal damage to the hippocampal and entorhinal cortical brain regions, enhanced neuronal replacement and prevented neuronal cell death after

ethanol-induced lesioning, perhaps mediated by its ability to reduce oxidative stress (Hamelink et al. 2005).

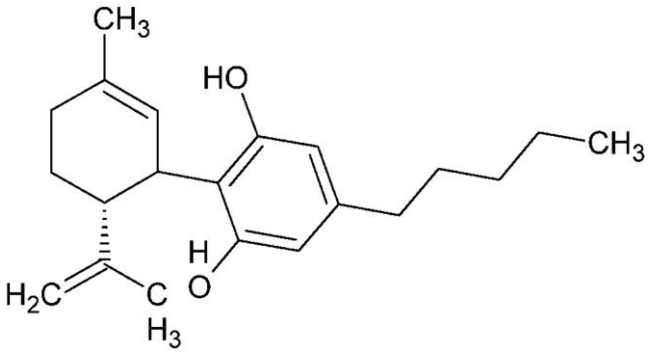

Figure 2: Chemical structure of CBD. Two hydroxyl groups on the benzene ring suggest potent antioxidant properties. Reprinted from Booz (2011) with permission from Elsevier

In addition to its non-psychoactive properties, the acute administration of CBD has been found to exert anxiolytic and antipsychotic effects (Zuardi 2008). On the other hand, the chronic administration of CBD does not seem to induce any adverse effects on anxiety in mice (Long et al. 2010). Importantly, CBD and CBD-rich cannabis extracts do not appear to affect spatial learning in animals, possibly owing to the THC-antagonising effects of CBD in the latter (Lichtman et al. 1995). Indeed, chronic CBD treatment does not impair spatial learning and working memory in the MWM (Wolf et al. 2010), but instead promotes hippocampal neurogenesis in mice (Campos et al. 2013; Wolf et al. 2010); properties that are highly applicable to the AD condition.

1.5.1. CBD and AD

The mounting evidence of the beneficial properties of CBD suggests that treatment with CBD may counteract AD-relevant pathologies. Preliminary *in vitro* studies provide support for the notion that CBD is a potentially useful treatment for AD. Table 4 provides a brief summary of the existing findings on the mechanisms of CBD. Aβ-induced neurotoxicity, ROS production and lipid peroxidation were prevented by the application of CBD in rat PC12 cells *in vitro* (Iuvone et al. 2004). Aβ-induced NO production was also attenuated by CBD through antioxidant and neuroprotective (anti-

apoptotic) mechanisms (Esposito et al. 2006b). These findings are consistent with previous studies that show cannabidiol reduced glutamate, NMDA- and kainite-induced excitotoxicity in rat cortical neurones (Hampson et al. 1998), and is especially relevant for the neurotoxic component of AD (i.e. inflammatory cytokine and glutamate release by activated microglia). Interestingly, Hallak and colleagues demonstrated that CBD interacts with the glutamatergic system by augmenting the effects of the NMDA receptor antagonist, ketamine, in humans (Hallak et al. 2011). Furthermore, CBD also inhibited Aβ-induced tau protein hyperphosphorylation (which would normally lead to the formation of neurofibrillary tangles) in differentiated PC12 cells *in vitro* (Esposito et al. 2006a).

Table 4: Mechanisms and effects of cannabidiol

Model	Effect of CBD treatment	Mechanisms	References
In vitro			
PC12 cells exposed to Aβ	Increased cell survival, decreased ROS production and lipid peroxidation	Inhibition of caspase 3 involved in the signalling pathway for CBD	(Iuvone et al. 2004)
	Inhibits nitrite production and iNOS protein expression	Inhibition of phosphorylated p38 MAP kinase and activation of nuclear factor-kB	(Esposito et al. 2006b)
	Rescues Aβ-induced toxicity and inhibits tau protein hyperphosphorylation	Rescue of Wnt/β-catenin pathway	(Esposito et al. 2006a)
Primary cortical neurons	Reduction of glutamate-induced toxicity	NMDA, AMPA and kainate receptors	(Hampson et al. 1998)
APP-expressing human neuroblastoma cells	Induced ubiquitination of APP and decreased Aβ production	Selective activation of PPAR-γ	(Scuderi et al. 2013)
In vivo			
Aβ-injected mice	Reduction in IL-1β, iNOS expression and subsequent NO release	Interaction with glial pathways	(Esposito et al. 2007)
	Suppression of IL-6 and prevented spatial memory deficits	Induced microglial migration	(Martin-Moreno et al. 2011)
CB_1 receptor deficient mice	Increased adult neurogenesis with no effect on cognition in WT mice	Indirect activation of CB_1 receptor	(Wolf et al. 2010)
Aβ-injected rats	Induced hippocampal neurogenesis and reduced reactive gliosis	Activation of PPAR-γ	(Esposito et al. 2011)
WT mice	Antagonises CB_2 receptor agonists	Inverse CB_2 receptor agonism	(Thomas et al. 2007)

Limited *in vivo* studies have been conducted to investigate the possible benefits of CBD in pharmacological animal models of AD (Table 4). The neuroinflammatory response caused by the injection of Aβ into the hippocampus of mice and rats was attenuated by the administration of CBD (Esposito et al. 2007; Esposito et al. 2011). Furthermore, reactive gliosis and the subsequent neuroinflammation caused by Aβ injury were significantly reduced by CBD. In another study, the injection of Aβ into the hippocampus of mice induced spatial memory deficits in the MWM as well as increased levels of glial fibrillary acidic protein (GFAP), nitric oxide synthase (iNOS), nitrite and IL-1β generation, which were attenuated by the administration of CBD (Martin-Moreno et al. 2011). As mentioned earlier, CBD interacts with PPARs, a family of nuclear hormone receptors that are generally regulated by steroids and lipid metabolites, to control the expression of genes related to lipid and glucose homeostasis and inflammatory responses (Esposito et al. 2011; O'Sullivan et al. 2009). In particular, the pharmacological actions of CBD may be mediated by the activation of the ligand-activated PPAR-γ receptor (O'Sullivan et al. 2009), whose biological functions are to regulate lipid and glucose metabolism and suppress inflammatory gene expression. PPAR-γ can be found on microglia and astrocytes, and their activation reduces APP overexpression and consequently Aβ overproduction in human neuroblastoma cells overexpressing APP (Scuderi et al. 2013). Importantly, PPAR-γ appears to be selectively involved in the anti-inflammatory and neuroprotective effects of CBD. In Aβ-injected mice, CBD treatment (for 15 days) resulted in a reduction of NO, TNF-α and IL-1β release in the hippocampus compared to Aβ mice not receiving CBD, but the blockade of PPAR-γ significantly blunted the effects of CBD on reactive gliosis and subsequently neuronal damage (Esposito et al. 2011). CBD-induced activation of PPAR-γ also promoted hippocampal neurogenesis, leading to a reduction in reactive

gliosis, decreased neurodegeneration and increase in cell proliferation in Aβ-injected rats (Esposito et al. 2011; Scuderi et al. 2013). Thus CBD may also target progressive neuronal loss and deficient maturation of new neurons characteristic of AD (Lazarov and Marr 2010; Li et al. 2008).

1.5.2. Clinical applications of CBD

The use of CBD for the treatment of AD patients has yet to be investigated, highlighting the importance of preclinical studies. However, studies have previously evaluated the use of CBD in clinical settings. These studies have found that CBD is well tolerated with no side effects when administered chronically to humans (Consroe et al. 1991; Cunha et al. 1980), including patients with schizophrenia (Zuardi et al. 2006). When administered on its own, CBD does not produce psychological or physiological effects in humans (Perez-Reyes et al. 1973), and was also reported to improve sleep duration in insomniac humans (Carlini and Cunha 1981). Higher dosages of CBD (600 mg) may produce a sedative effect, which was accompanied by decreased plasma cortisol levels (a hormone responsible for stress responses) (Zuardi et al. 1993). CBD appears to protect against the deleterious effects of THC on cognition, including recognition memory and performance on verbal memory tasks (Morgan et al. 2012; Morgan et al. 2010; Pertwee 2008). CBD and THC are combined in nabiximols (Sativex), an oromucosal spray used to treat muscle spasticity caused by multiple sclerosis, which has been approved for use in various countries including Australia (listed for use by the Therapeutic Goods Administration as of November 2012; but is not currently available to patients). Clinical studies have shown that nabiximols, when used at the prescribed therapeutic doses, do not induce psychoactive or cognition-impairing effects but may still carry significant abuse potential due to the presence of THC (Aragona et al. 2009).

As CBD has been investigated in clinical trials, further assessment of its potential as an AD treatment in preclinical studies will ensure a quick transition to clinical trials for AD patients. Research so far has focused on elucidating the *in vitro* effects of CBD, with only a few studies determining the effects of CBD on pharmacological animal models of AD. Importantly, no studies have been conducted on CBD and transgenic mouse models of AD although they are commonly used in evaluating new therapeutic targets, calling for a greater focus on preclinical research (Iuvone et al. 2009; Karl et al. 2012b). Thus, this thesis will examine the effects of CBD treatment for the first time in an established and validated transgenic mouse model of AD, APPxPS1 double transgenic mice.

1.6. Summary and aims of study

1.6.1. Methodological considerations

In order to gain a better understanding of AD mouse models, recent phenotyping studies have evaluated transgenic mouse models in alternate spatial memory paradigms to avoid various issues associated with the MWM, including stress, floating behaviour, thigmotaxis, hypothermia and physical fatigue (Gerlai 2001; Iivonen et al. 2003; Mizunoya et al. 2004; Wolfer et al. 1998). In the following experiments, I employed the cheeseboard task (Karl et al. 2012a), a dry-land alternative to the MWM, which focuses on positive reinforcement (Llano Lopez et al. 2010) by training the mice to find a hidden food reward and assessing their memory for the location. More importantly, the CB has been determined to be a reliable test of spatial learning and memory in another mouse model of AD (J20 amyloidogenic mouse line expressing Swedish $670/671_{KM->NL}$ and Indiana $717_{V->F}$ *hAPP* mutations) (Karl et al. 2012a; Kim et al. 2013). Furthermore, novel behavioural domains including social recognition memory (Faizi et al. 2012) and

sensorimotor gating (Wang et al. 2012) have been considered in more recent studies. The social preference test allows the assessment of sociability and social novelty preference (i.e. social recognition) in mice (Moy et al. 2004; Moy et al. 2008). AD mice have previously been reported to demonstrate social recognition memory deficits (i.e. failure to distinguish between a novel and familiar social opponent) by studies investigating the Thy1-hAPP(Lond/Swe+) (Faizi et al. 2012) and APPxPS1 mice maintained on pure C57BL/6J background (Filali et al. 2011). Importantly, AD patients have difficulties in recognising familiar faces (Reisberg et al. 1982).

Female APPxPS1 mice have been reported to demonstrate a sensorimotor gating deficit at 7 months of age (Wang et al. 2012). However, sex specific effects are likely to impact the experimental outcomes (Gallagher et al. 2013; Gogos et al. 2009; Ison and Allen 2007; Karl et al. 2011; Wang et al. 2003a), thus I also investigated male APPxPS1 mice. Interestingly, suppression of the P50 event-related potential of sensorimotor gating has been reported for patients with AD (Jessen et al. 2001).

Anxiety was also measured as some studies suggest acute CBD treatment may induce anxiolytic effects (Campos and Guimaraes 2008; Campos et al. 2013; Guimaraes et al. 1990; Long et al. 2010; Moreira et al. 2006; Onaivi et al. 1990). On the other hand, sub-chronic (i.e. 2-week) CBD treatment did not produce noticeable anxiolytic effects as detected by the EPM (Campos et al. 2013). The effect of long-term (8 months) CBD treatment on anxiety has not been investigated previously and no other study uses an oral treatment regime similar to that described in this thesis. The dosage and treatment duration used in the upcoming studies in this thesis were chosen based on a previous study investigating CBD treatment in a pharmacological model of AD (Martin-Moreno et al. 2011). Thus, a dosage of 20 mg/kg was selected and administered intraperitoneally (i.p.) for 3 weeks prior to the start of behavioural and cognitive testing.

Thus, in a first step, I assessed both male and female APPxPS1 transgenic mouse model beginning at 7 months of age in a test battery comprised of the CB task, the novel paradigms (social preference test and prepulse inhibition) as well as associative learning and anxiety behaviour. Following this, I investigated the effect of CBD treatment on male APPxPS1 transgenic mice in the selected behavioural and cognitive paradigms as well as pathology. Mice were either treated with CBD or vehicle (control) for 3 weeks (intraperitoneal administration) after the development of AD-relevant symptoms or for 5 months prior to AD onset (oral treatment) before behavioural and cognitive assessment. In order to minimise the impact of daily intraperitoneal injections, I developed an oral treatment strategy that allowed the possibility of investigating the long-term effects of CBD treatment in APPxPS1 mice. The technique was adapted from existing studies (Zhang 2011) and involved lacing a highly palatable and sweetened gel pellet with CBD and allows for accurate dosing based on the body weight of the mice. Details regarding CBD treatment, experimental procedures and analyses will be described in Chapters 3 and 4.

1.6.2. Rationale of study

The deposition of Aβ induces a multitude of changes in the brain, and the impaired clearance ability of microglia allows Aβ to aggregate, contributing to the pathology of AD. Furthermore, activated microglia incurs the build-up of inflammatory cytokines, neurotoxic chemicals (e.g. glutamate) and ROS such as NO, resulting in pronounced neuroinflammation, neurotoxicity and oxidative damage that exacerbate the condition of AD. One potentially therapeutic cannabinoid, CBD, interacts with the ECS and a number of other systems (e.g. glutamatergic system and PPAR-γ) to generate neuroprotective, anti-inflammatory, antioxidant effects and promotion of neurogenesis,

countering the changes occurring in AD pathology. A limited number of mostly *in vitro* studies support this claim by showing that CBD reduces Aβ-induced neuroinflammation, neurotoxicity, tau hyperphosphorylation and promotes hippocampal neurogenesis. CBD can also modulate APP processing, resulting in a decrease in Aβ production. Importantly, it has been shown to be beneficial for cognition in pharmacological rodent models of AD and is advantageous over other cannabinoids such as THC as it is also devoid of cognition-impairing characteristics. The lack of psychoactive properties makes CBD an attractive candidate for a cannabinoid-based therapeutic strategy for AD. Therefore I hypothesised that CBD has therapeutic potential for AD. The upcoming studies included in this thesis, are the first to investigate various novel behavioural and cognitive domains in the APPxPS1 transgenic mouse model and more importantly, the effect of CBD in this transgenic animal model of AD. The effectiveness of CBD in preventing and reversing a variety of behavioural and cognitive impairments and AD-relevant pathological changes in APPxPS1 mice was assessed by administering CBD chronically prior to (from an early age, long term for 8 months) and after (for 3 weeks) the onset of AD pathogenesis in the transgenic mice. Comprehensive assessments of their behaviour (sensorimotor gating, locomotor activity and anxiety) and cognition (social and object recognition memory, spatial learning and memory, and associative learning and memory) were conducted. Biochemical analyses of amyloid pathology, oxidative damage, cholesterol and neuroinflammation were performed using enzyme-linked immunosorbent assay (ELISA), gas-chromatography mass-spectrometry (GC-MS) and quantitative polymerase chain reaction (qPCR). The studies conducted in the following chapters are both novel and insightful for the phenotypic repertoire of the APPxPS1 mice and the effect of CBD treatment in a transgenic mouse model of AD. In these very first studies,

I assessed the efficacy of CBD to prevent/reverse AD-relevant cognitive and behavioural symptoms as well as pathophysiology in order to evaluate CBD's therapeutic potential for AD.

1.6.3. Major Aims

Aim 1: Characterise APPxPS1 mice in novel behavioural and cognitive paradigms to clarify the inconsistencies of earlier reports (Chapter 2)

Aim 2: Determine if CBD reverses cognitive impairments of APPxPS1 transgenic mice (Chapter 3)

Aim 3: (a) Assess the potential of long-term CBD treatment to prevent the development of cognitive deficits in APPxPS1 mice (b) Assess the potential of long-term CBD treatment to prevent the development of AD-relevant brain pathophysiology (amyloid load, oxidative damage, cholesterol and inflammation) (Chapter 4)

Chapter 2: Behavioural and cognitive characterisation of APP_{Swe}/PS1ΔE9 double transgenic mice (males and females)

2.1. Novel behavioural characteristics of the APPSwe/PS1ΔE9 transgenic mouse model of Alzheimer's disease

Publication I

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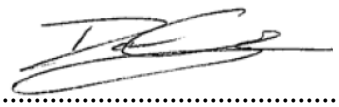
Declaration

I certify that this publication was a direct result of my research towards this PhD, and that reproduction in this thesis does not breach copyright regulations.



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David Cheng

Novel Behavioural Characteristics of the *APP_{Swe}/PS1ΔE9* Transgenic Mouse Model of Alzheimer's Disease

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Highlights

- There are inconsistencies in the behavioural phenotype of APPxPS1 transgenic mice
- Male APPxPS1 mice showed task-dependent hyperlocomotion and increased anxiety
- Transgenic mice exhibited normal spatial cognition and fear conditioning
- APPxPS1 mice displayed impaired social recognition memory
- Sensorimotor gating of APPxPS1 males was unaffected

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Abstract

In order to better understand animal models of Alzheimer's disease, novel phenotyping strategies have been established for transgenic mouse models. In line with this, the current study characterised male APPxPS1 transgenic mice on mixed C57BL/6JxC3H/HeJ background for the first time for social recognition memory, sensorimotor gating, and spatial memory using the cheeseboard test as an alternative to the Morris water maze. Furthermore, locomotion, anxiety, and fear conditioning were evaluated in transgenic and wild type-like animals. APPxPS1 males displayed task-dependent hyperlocomotion and anxiety behaviours and exhibited social recognition memory impairments compared to wild type-like littermates. Spatial learning and memory, fear conditioning, and sensorimotor gating were unaffected in APPxPS1 transgenic mice. In conclusion, this study describes for the first time social recognition memory deficits in male APPxPS1 mice and suggests that spatial learning and memory deficits reported in earlier studies are dependent on the sex and genetic background of the APPxPS1 mouse line used. Furthermore, particular test conditions of anxiety and spatial memory paradigms appear to impact on the behavioural response of this transgenic mouse model for Alzheimer's disease.

Keywords: Alzheimer's disease; transgenic APP_{Swe}/PS1ΔE9 mice; behaviour; social recognition memory; sensorimotor gating; cheeseboard;

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, predicted to affect 1 in 85 people globally in 2050. Disease progression from mild to severe stages encompasses impaired learning and communication, spatial disorientation, and memory loss. Two major post-mortem histological diagnostic features describe AD: 1) cleavage of the amyloid precursor protein (APP) produces amyloid beta (Aβ) depositions, which form senile plaques, and 2) hyper-phosphorylation of tau protein causes intracellular neurofibrillary tangles [1-2]. Importantly, elevated levels of Aβ in post-mortem brain tissue correlated with AD-typical memory decline in patients diagnosed with dementia [3]. Familial AD (FAD) is the hereditary form of AD (early onset, autosomal dominant) and accounts for <10% of AD cases (the remaining are classified as sporadic forms of AD) [1]. A number of mutations in genes encoding the amyloid precursor protein (*APP*), and presenilins, a family of enzymes responsible for the processing of APP, have been identified for FAD. Presenilin 1 and 2 (*PSEN1*, *PSEN2*) are responsible for the activity of γ-secretase, one of the enzymes responsible for the cleavage of APP into Aβ isoforms [1-2].

Murine models are most commonly used to investigate the pathology of AD. The mice used in this study were generated by the co-injection of a chimeric human/murine *APP* construct bearing the Swedish double mutation (APP_{Swe}) and the exon-9-deleted *PSEN1* mutation (*PSEN1/*Δ*E9*) [4-5]. $APP_{Swe}/PS1\Delta E9$ (APPxPS1) double transgenic mice exhibit increased levels of Aβ at 4 months of age and develop accelerated plaque pathology, which is correlated with age [4-6]. Furthermore, impairments in cholinergic and muscarinic transmission develop alongside Aβ accumulation in the brain of APPxPS1 mice at 5-7 months of age, reminiscent of AD pathology [7-8].

Various behavioural and cognitive deficits have been documented for this transgenic AD mouse model. Most notable are spatial memory impairments in the Barnes maze and Morris

water maze (MWM), with the earliest deficits appearing at 7 and 8 months respectively [9-10]. These cognitive deficits were more pronounced with age and correlated with increasing plaque deposition [11-13], which is sex-specific [6]. Other behavioural characteristics reported for APPxPS1 mice include decreased anxiety and increased locomotor activity [14]. However, some of the reported behavioural characteristics were inconsistent across laboratories. For example, Reiserer and colleagues could not replicate the anxiolytic phenotype reported earlier [10, 14]. More importantly, spatial memory deficits in the reversal task of the MWM were detected in 9-10-month-old mice [15] whereas another study reported no deficits in the reversal task in 12-month-old mice [16]. Furthermore, some studies combined both male and female mice within one test cohort [10, 14, 17], even though other studies revealed sex-specific differences in APPxPS1 mice [6, 18].

In order to better understand animal models of AD, recent phenotyping studies in transgenic mouse models of AD have considered alternative spatial memory paradigms (i.e. cheeseboard; [19]) and also evaluated transgenic mice in novel behavioural domains such as social recognition memory [20] and sensorimotor gating [21]. In the present study we tested the APPxPS1 transgenic mouse model in these novel paradigms to determine the behavioural phenotype of this mouse model in more detail.

2. Materials and methods

2.1 Animals

Double transgenic mice expressing chimeric mouse/human APP (Mo/HuAPP695swe / Swedish mutations K595N/M596L) and mutant human PSEN1 (PS1/ΔE9) mice were obtained from Jackson Laboratory [Bar Harbor, USA; strain name: B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax; stock no. 004462] and maintained as hemizygotes on the congenic C57BL/6JxC3H/HeJ background as described previously [4-5, 22-23]. Male double transgenic mice (APPxPS1: n = 12) and their non-transgenic littermates (WT: n = 17) were bred and group-housed in independently ventilated cages (Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). Test mice were transported to Neuroscience Research Australia (NeuRA) at around 10 weeks of age, where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) equipped with some tissues for nesting. Mice were kept under a 12: 12 h light: dark schedule [light phase: white light (illumination: 124 lx) – dark phase: red light (illumination: < 2 lx)]. Food and water were provided *ad libitum*, except where specified. Adult, male A/J mice from Animal Resources Centre (Canning Vale, Australia) were placed in the animal enclosures of the social preference test.

Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Behavioural Phenotyping

Starting at 7 months of age, mice were tested in a battery of behavioural tests (for test order see Table 1; for test details see below) with an inter-test interval of at least 48h. All tests were

conducted during the first 5 h of the light phase to minimise effects of the circadian rhythm on the performance of test mice.

2.2.1 Light-dark test (LD): In the LD, the distance travelled and time spent in a brightly illuminated, aversive test arena compared to a dark area are indicators of anxiety in rodents [24-25]. The apparatus (for details see [26]) was an infrared photobeam-controlled open-field activity test chamber (MED Associates Inc., St Albans, USA) containing a dark box insert that covered half the chamber and was opaque to visible light. Mice were placed at the opening (faced towards the dark compartment) at the start of the experiment. The time spent as well as the distance travelled in the two chambers was recorded for 10 min.

2.2.2 Elevated plus maze (EPM): The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated and open area [27-28]. The grey plus maze was "+" shaped (for details of apparatus see [29]). Mice were placed at the centre of the "+" (faced towards an enclosed arm) and were allowed to explore the maze for 5 min. The time spent and distance travelled in the open and enclosed arms were recorded using AnyMaze^{TM} (Stoelting, Wood Dale, USA) tracking software.

2.2.3 Social preference test (SPT): The SPT was used to assess sociability and social novelty preference (i.e. social recognition memory) in test mice [30-31]. The apparatus consisted of 3 chambers, a central chamber (length: 9 cm, width: 18 cm, depth: 20 cm) and two outer chambers (16 cm x 18 cm x 20 cm). The dividing walls were made of clear Plexiglas, with square passages, 4 cm high and 4 cm wide. One circular cage (i.e. mouse enclosure) was placed into each outer chamber. The mouse enclosures were 15 cm in height with a diameter of 7 cm and bars spaced 0.5 cm apart to allow nose contact between mice (i.e. test mouse and A/J mouse) but prevent fighting. The chambers and enclosures were cleaned with 30% ethanol in-between trials (inter-trial interval of 5 min) and fresh corn cob bedding was added prior to each test trial.

Test animals were isolated for an hour prior to the start of testing. During the habituation trial, WT and APPxPS1 mice were placed individually in the central chamber and allowed to freely explore the apparatus and the two empty enclosures for 5 min. For the sociability test an unfamiliar adult male A/J mouse was placed in one of the two enclosures (i.e. opponent chamber) in a quasi-randomised fashion. Then the test mouse was returned to the apparatus and allowed to explore all three chambers for 10 min. Finally, test animals were observed in a 10 min social recognition test. For this, a second, unfamiliar A/J mouse was placed in the previously empty chamber so that the test mouse had the choice to explore either the familiar A/J mouse (from the previous trial) or the novel, unfamiliar mouse. AnyMaze^{TM} tracking software was used to determine the time spent in the different chambers, number of entries and distance travelled by the test mice in each trial. Time spent *sniffing* the opponent (i.e. A/J mouse) was recorded manually (i.e. snout of test mouse within the enclosure containing the opponent mouse or < 5 mm away from enclosure).

2.2.4 Fear Conditioning: Fear conditioning assesses associative learning whereby a previously neutral stimulus elicits a fear response after it has been paired with an aversive stimulus. On conditioning day, mice were placed into the test chamber (Model H10-11R-TC, Coulbourn Instruments, USA) for 2 min. Then an 80 dB conditioned stimulus (CS) was presented for 30 seconds with a co-terminating 0.4 mA 2 second foot shock (unconditioned stimulus; US) twice with an inter-pairing interval of 2 min). The test concluded 2 min later. The next day (context test), mice were returned to the apparatus for 7 min. On day 3 (cue test), animals were placed in an altered context for 9 min. After 2 min (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 2 min with the absence of the CS. Time spent *freezing* was measured using Any-Maze^{TM} software [32-33]. To avoid any influence of foot shock exposure on further testing, an inter-test interval of

several months was chosen and all following tests were carried out in test rooms other than the fear conditioning test.

2.2.5 Cheeseboard (CB): The CB was used as a less stressful dry-land alternative of the MWM [19]. Mice at 10-11 months of age were trained to find a food reward on a wooden board over a number of days (for specifics of test apparatus see [34]). A total of 32 bottle caps were evenly distributed across the CB and external cues were located around the board. One cap contained a food reward (100 µl sweetened condensed milk; diluted 1:4 with water) although all caps were brushed lightly with diluted sweetened condensed milk to eliminate the chance that mice use odour cues to find the target cap. For this, all mice were food-deprived and kept at 85-90% of their pre-test body weight throughout testing (mice were fed for 1-2 h per day). A camera was mounted above the CB to measure latency to find the reward and time spent in the different CB zones (i.e. board was separated into 8 equal zones) using Any-Maze^{TM} software.

During habituation, (3 days to the blank side on the inverted platform of the CB) three 2 min trials were conducted each day for three days with a 10 min intertrial interval (ITI). For spatial reference memory acquisition, mice were trained over 9 days (three trials per day with a 10 min ITI) to locate the food reward. The location of the target well was kept constant for each mouse between trials and across days but quasi-randomised and counterbalanced across genotypes. If the target well was not located within 2 min, mice were placed next to the target well and allowed to consume the food reward. To test for spatial reference memory, a probe trial was conducted on day 10, where no wells were baited and mice were given 2 min to explore the board freely. The time the mice spent in the different zones of the CB (i.e. % exploration time) was recorded (as previously described [32]).

To test reversal learning (start of training 24h post probe trial), the location of the food reward was moved to the opposite side of the CB. Mice completed 4 days of reversal training (three trials per day with a 10 min ITI) before a reversal probe trial was carried out.

2.2.6 Sensorimotor gating (i.e prepulse inhibition: PPI): PPI was used to test for sensorimotor gating deficits as it has been demonstrated by others that sensorimotor gating can be impaired in AD mouse models and can be directly correlated with amyloid burden [21, 35]. Test mice were placed in Plexiglas mouse enclosures of the startle chambers (SR-Lab, San Diego Instruments, San Diego, USA) and allowed to habituate to the enclosure and test apparatus for 5 min over 3 consecutive days prior to PPI testing with a consistent background noise of 70 dB. The 30 min PPI test session consisted of a 5 min acclimation period to 70 dB background noise, followed by 97 trials presented in a pseudorandom order: 5 x 70 dB trials; 5 x 100 dB trials; 15 x 120 dB trials to measure the acoustic startle response (ASR) and 15 sets of 5 trials comprising of a prepulse of either 74, 82 or 86 dB presented 32, 64, 128, or 256 ms (variable interstimulus interval; ISI) prior to a startle pulse of 120 dB to measure the PPI response. The intertrial interval (ITI) varied randomly from 10 – 20 seconds. Responses to each trial were calculated as the average mean amplitude detected by the accelerometer [36-37]. ASR was calculated as the mean amplitude to all startle trials and percentage PPI (%PPI) was calculated as [(mean startle response (120 dB) – PPI response)/mean startle response (120 dB)] x 100. %PPI was averaged across ISIs to produce a mean %PPI for each prepulse intensity. For ASR habituation, blocks of ASR to 120 dB were averaged at the beginning, middle and end of the PPI protocol (5 trials per block).

2.2.7 Olfactory test (i.e. cookie test): Olfactory abilities play a crucial role in social interaction between mice [38]. A simple test [24, 30] was performed to assess the gross olfactory abilities of male WT and APPxPS1 transgenic mice. Test mice were familiarised with a high carbohydrate food (Froot Loops: Kellogg Pty. Ltd., Strawberry Hills, Australia)

in their home cages, 24 h prior to the test. Consumption was observed by the experimenter to ensure the novel food was palatable for the mice. On the test day, test mice were habituated for 5 min to a large opaque cage (47 x 18 x 13 cm) containing 2 cm deep bedding. The animal was removed from the cage thereafter, and one Froot Loop was buried randomly in the cage bedding. The animal was then returned to the cage and given 10 min to locate the buried food. The latency to find the Froot Loop was recorded as a measure of olfactory abilities.

2.3 Statistical Analysis

Analysis of the behavioural parameters was performed using one-way analysis of variance (ANOVA) to investigate main effects of 'genotype' or repeated measures (RM) ANOVAs for effects of 'chamber' (SPT), 'time' (CB) '1 min block' (FC), 'startle block' and 'prepulse intensity' (both PPI) as published previously [34]. Performance in the CB probe trials and social preference test were also assessed using one sample t-tests to investigate whether the percentage of time spent in the target zone or novel chamber were greater than chance (12.5% and 50% respectively). Differences were regarded as significant if $p < .05$. F-values and degrees of freedom are presented for ANOVAs and significant genotype effects (ANOVA) are shown in figures and tables as '*' ($^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$). RM ANOVA effects for chamber are presented by '#' ($^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$). Data are shown as means ± standard error of means (SEM). Analyses were conducted using SPSS 20.0 for Windows.

3. Results

3.1 Anxiety

One-way ANOVA for total distance travelled in the LD revealed an effect of 'genotype' [$F(1,29) = 11.7$, $p < .01$; Table 2], suggesting that APPxPS1 transgenic mice exhibit a hyperlocomotor phenotype. Importantly, no effects of APPxPS1 were detected on the anxiety-related parameters time in light chamber [$F(1,29) = 0.001$, $p = .9$] and percentage distance travelled in the same zone [$F(1,29) = 0.07$, $p = .8$] (Table 2). Nevertheless, APPxPS1 mice demonstrated increased levels of anxiety in the EPM. Both, the percentage of time spent in the open arms [$F(1,28) = 4.6$, $p < .05$] as well as the percentage open arm entries [$F(1,28) = 4.5$, $p < .05$] were significantly lower in transgenic mice when compared with their WT counterparts (Table 2). Furthermore, there was a strong trend for an increase in total time spent on the open arms [$F(1,28) = 4.0$, $p = .05$] (Table 2). No other significant differences were found for any of the parameters investigated [$p > .05$ for all parameters], including the total time spent in enclosed arms and the total distance travelled in enclosed arms (data not shown).

3.2 Cognition

3.2.1 Social Preference Test: All mice demonstrated sociability in the 3-chamber social preference test. RM ANOVA detected a significant effect of test chamber for all mice for total time spent in chamber [$F(1,29) = 50.1$, $p < .001$; 'genotype' x 'chamber' interaction: $F(1,29) = 4.4$, $p < .05$] (Fig. 1A). One-way ANOVA for total time spent in opponent chamber revealed that transgenic mice spent a less time in the mouse chamber than WT control mice [$F(1,29) = 4.7$, $p < .05$]. However, one sample t-test confirmed that both WT and transgenic mice developed a preference for the opponent chamber (containing a stranger/unfamiliar mouse) [WT: $t(11) = 10.8$, $p < .001$; APPxPS1: $t(18) = 3.4$, $p < .01$].

In the social recognition test, RM ANOVA revealed a significant effect of 'chamber' for all mice for total time spent in test chambers [$F(1,29) = 4.4$, $p < .05$] (Fig. 1B) and time spent *sniffing* [$F(1,29) = 7.8$, $p < .01$] (Fig. 1C). Importantly, only WT mice demonstrated a preference for the chamber containing the novel mouse [time spent in chamber: $F(1,11) = 5.9$, $p < .05$ – time spent *sniffing*: $F(1,11) = 8.9$, $p = .01$] while transgenic mice spent an equal amount of time with the familiar and the novel mouse [time spent in chamber: $F(1,18) = 0.1$, $p = .7$ – time spent *sniffing*: $F(1,18) = 0.9$, $p = .4$]. T-tests for percentage time spent with novel mouse and percentage time *sniffing* the novel mouse confirmed that WT [$t(11) = 2.2$, $p < .05$ – $t(11) = 3.6$, $p < .01$] but not APPxPS1 transgenic mice [$t(18) = .3$, $p = .7$ – $t(18) = 1.0$, $p = .3$] developed a clear preference for the chamber containing the novel mouse (data not shown). One-way ANOVA revealed no significant genotype differences for percentage of time spent in the novel chamber [$F(1,29) = 2.3$, $p = .1$] and percentage of time *sniffing* the novel opponent [$F(1,29) = 2.2$, $p = .2$]. Locomotion of WT and APPxPS1 mice was identical in both the familiar [$F(1,29) = 1.3$, $p = 0.3$] and the novel chamber [$F(1,29) = 0$, $p = 1.0$] (Table 2).

3.2.3 Fear Conditioning: All mice responded to the electric foot shocks delivered during the conditioning phase (i.e. vocalisation). Furthermore, the baseline *freezing* prior to conditioning was similar across genotypes [$F(1,29) = .3$, $p = .5$; Table 3]. Contextual fear conditioning (i.e. total time spent *freezing* during context test) of APPxPS1 mice was WT-like [$F(1,29) = 2.9$, $p = .1$]. In the cue test, all mice demonstrated the ability to associate the CS with the US as evidenced by a significant increase in *freezing* behaviour in response to the presentation of the cue [RM ANOVA for '1 min block': $F(1,29) = 9.3$, $p < .01$ - no '1 min block' by 'genotype' interactions; Table 3].

3.2.4 Cheeseboard: Mice of both genotypes showed normal task acquisition as indicated by RM ANOVA for 'time' [$F(8,208) = 38.8$, $p < .001$ – no interaction with 'genotype'; Fig. 2A],

demonstrating a significant decrease in latency to find and consume the reward across days. In the probe trial, all mice demonstrated a preference for the target zone [WT: $t(11) = 3.1$, $p < .01$; APPxPS1: $t(15) = 3.7$, $p < .01$], as they spent significantly more time than chance (i.e. 12.5%) in the target zone, indicating successful recall of the reward location (Fig. 2B). Furthermore, one-way ANOVA for percentage time in target zone revealed the performance of transgenic mice did not differ significantly from that of WT mice [$F(1,26) = .3$, $p = .6$].

During reversal learning, all test animals adapted to the change in reward location and exhibited decreased latencies to find the food reward over days [RM ANOVA for 'time': $F(3,78) = 4.7$, $p < .001$ – no interaction with 'genotype'; Fig. 2C]. Finally, all mice developed a preference for the new target zone in the reversal probe trial as they spent significantly more time than chance in the designated zone [WT: $t(11) = 3.0$, $p < .05$; APPxPS1: $t(15) = 2.9$, $p < .05$; Fig. 2D]. Transgenic mice did not differ significantly in their preference to explore the target zone [$F(1,26) = .2$, $p = .6$].

3.3 Sensorimotor gating

3.3.1 Acoustic startle response (ASR) and ASR habituation: RM ANOVA revealed a significant effect of 'pulse intensity' [$F(2,50) = 25.3$, $p < .001$] on the ASR of all mice with 120 dB pulses generating the highest startle responses (Fig 3A). A trend was found for the effect of 'genotype' [$F(1,25) = 3.9$, $p = .06$], suggesting that ASR was generally higher in transgenic APPxPS1 mice compared to WT mice. However, one-way ANOVAs for the different startle pulses revealed no significant differences between WT and APPxPS1 mice [$p > .05$ for all startle pulses].

Statistical analysis suggested that mice did not habituate significantly to the 120 dB pulse [RM ANOVA for 'startle block': $F(2,50) = 2.6$, $p = .08$], although this appeared to be due to a failure of APPxPS1 rather than WT mice to habituate to a 120 dB startle stimulus [trend for

'startle block' x 'genotype' interaction: $F(2,50) = 2.7$, $p = .08$] (Fig. 3B). Indeed, when data were split by 'genotype', it was found that WT mice demonstrated significant habituation towards the 120 dB pulse [WT: $F(2,20) = 6.8$, $p < .01$], while transgenic mice exhibited no reduction in ASR across trials [APPxPS1: $F(2,30) = .002$, $p = 1.0$] (Fig. 3B].

3.3.2 Prepulse inhibition: Prepulse intensities had a significant effect on %PPI as increasing prepulse intensities resulted in more pronounced prepulse inhibition [RM ANOVA: $F(2,50) = 28.4$, $p < .001$] (Fig. 3C). Importantly, sensorimotor gating was not altered in transgenic mice as no effects of 'genotype' were found at any prepulse intensity [$p > .05$ for all parameters investigated; Fig. 3C].

3.4 Olfaction (Cookie test)

All mice found and consumed the buried food reward within the allotted time as measured in seconds (WT: 300.6 ± 61.6 - APPxPS1: 227.8 ± 46.4). The performance of transgenic mice in the olfactory test was comparable to WT mice [latency to find buried food: $F(1,27) = .9$, $p = .3$], suggesting WT-like olfactory abilities of transgenic AD mice.

4. Discussion

This is the first report that APPxPS1 males develop social recognition memory impairments. Furthermore, transgenic males displayed task-dependent hyperlocomotion and anxiety behaviours. Spatial learning and memory in the CB paradigm as well as sensorimotor gating and fear conditioning were all unaffected in 10-month-old APPxPS1 mice.

Agitation and increased motor activity (restlessness) is one characteristic of AD patients [39]. Measuring the locomotor activity of APPxPS1 mice revealed that transgenic animals developed a hyperlocomotive phenotype in the LD test at the age of 7 months. This finding is in line with a study testing 8-month old APPxPS1 male in the open field [11] although other studies reported wild type-like locomotion of APPxPS1 [10, 14, 40]. Importantly, a detailed comparison of all these studies suggests that the characteristics of the particular APPxPS1 mouse model tested (i.e. number of backcrosses onto C57BL/6J background), the sex of test animals and the methodology used to analyse locomotion (e.g. test duration and the level of stress caused by test apparatus) may account for inconsistent behavioural responses across studies. Methodological differences might also explain why hyperlocomotion of APPxPS1 males of the current study was detected in the LD test but not the EPM.

Male APPxPS1 mice displayed wild type-like anxiety levels in the LD test, which is consistent with earlier reports [10]. However, transgenic males were more anxious in the EPM compared to control animals. This task-specific anxiety phenotype may be related to the human clinical setting as there are AD patients who experience symptoms of anxiety [41]. In contrast, Lalonde and co-workers detected decreased anxiety levels in APPxPS1 mice (males and females were tested together) and interpreted this phenotype as a loss of behavioural inhibition, akin to dis-inhibitory tendencies observed in AD patients [14].

While control mice exhibited a clear preference for the novel opponent as expected [30], APPxPS1 males did not differentiate between the novel and familiar opponent mouse

suggesting deficits in social recognition memory (as measured by time spent in chamber and time spent *sniffing* opponent). This effect was not confounded by the hyperactive phenotype of APPxPS1 mice observed in the light-dark test as locomotion was identical in both chambers across genotypes. All test mice were also characterised in the cookie test, as the test performance is dependent on olfactory abilities and as AD patients and some mouse models of AD exhibit impaired olfaction [42-43]. All test animals showed normal olfactory abilities in the cookie test. In addition, Rey and colleagues showed that the olfactory discrimination under baseline conditions (using a 5 min delay between first and second exposure to novel/familiar odours) was identical for control and APPxPS1 mice [44]. Interestingly, transgenic mice of that study exhibited impaired odour retention with a 15 min delay. However, as the inter-trial interval of the social preference test in our study was 5 min, it is unlikely that the social recognition memory deficit of APPxPS1 mice was influenced by a reduced ability of transgenic mice to recall odours they had encountered earlier. Furthermore, control and APPxPS1 males displayed normal sociability (i.e. preference to investigate a mouse over an empty chamber) although this preference was more pronounced in WT mice. Importantly, the task-dependent anxiety phenotype of transgenic mice in the elevated plus maze (but not the light-dark test) did not impact on the natural drive of mice to explore another mouse. Both WT and APPxPS1 mice exhibited a clear preference to investigate the social stimulus presented during the sociability test. However, the intact yet reduced levels (compared to control mice) of social interaction/investigation of another mouse observed in APPxPS1 mice may be influenced by the anxiety phenotype detected in the elevated plus maze.

In support of an impaired social recognition memory in AD mice is a recent study reporting impaired social recognition in the Thy1-hAPP(Lond/Swe+) transgenic mouse model [20]. In this context, it is interesting to note that AD patients have difficulties to recognise familiar

faces [45]. Brain regions responsible for recognition memory are the perirhinal cortex and hippocampus [46], both regions are compromised in AD patients [47]. Furthermore, the amygdala, which is associated with social behaviours, undergoes atrophy in AD patients [48]. Thus, impairments in social recognition memory may be caused by pathological changes in these brain regions in APPxPS1 mice. Further research will have to address potential histological differences in these regions between WT and APPxPS1 mice.

The deficit in recognition memory was specific as fear conditioning (i.e. associative learning) was intact in 7-month-old transgenic mice, which is similar to what had been reported in 4-month-old APPxPS1 females [49]. Furthermore, task acquisition and retention of spatial memory of APPxPS1 males were not impaired in the hidden version of the CB paradigm. APPxPS1 mice have been described to develop spatial learning and memory deficits, which are most often evaluated in the MWM. In females, deficits in spatial learning were evident in 9-10-month-old APPxPS1 mice [15, 50] and retention deficits were detected in 12-month-old transgenic animals [51]. However, only one study has investigated male APPxPS1 mice on C57BL/6JxC3H/HeJ background to date. Cao and co-workers reported intact task acquisition but impaired spatial memory retention for 8-month-old transgenic males [9], whereas APPxPS1 males backcrossed to C57BL/6J developed spatial learning and memory deficits at the age of 9-15 months. This suggests an influence of the genetic background of APPxPS1 males on the development of cognitive deficits [52-53]. Importantly, the CB paradigm used in current study is classified as the dry version of the MWM [54] and has been validated as an alternative spatial memory test to detect cognitive impairments in AD transgenic mice [19]. Nonetheless, comparing results between MWM and CB testing requires caution as the MWM can impact severely on the stress response of mice (for this and other issues relevant to MWM testing of mice see [54-58]). Thus, the anxiety phenotype of male APPxPS1 mice may explain the differences between the cognitive performance of transgenic animals in the

MWM [9] and the CB of the current study. Two previous studies found deficits in spatial learning and memory of APPxPS1 males using the CB paradigm. However, one study used WT and transgenic mice at the age of 24 months [59] and both studies detected cognitive impairments in the cued (but not the hidden) version of the CB only [21, 59].

A meta-analysis has found social withdrawal is among the first symptoms displayed by AD patients, occurring up to 33 months on average prior to the diagnosis of AD [39]. In line with this, 10-month-old APPxPS1 males appear to demonstrate social recognition memory impairments in the absence of any other cognitive deficits. Thus, testing APPxPS1 males on a mixed background, which are significantly older than the cohort tested in the current study, might result in spatial memory deficits in the hidden versions of both CB and MWM.

Studies have identified suppression of the P50 event-related potential of sensorimotor gating in AD patients [60]. The present study found that sensorimotor gating as measured by prepulse inhibition was unaltered in 10-month-old APPxPS1 males. This is supported by a previous study that found no PPI deficits in a mixed cohort of 12-month-old male and female mice of another APPxPS1 line [61]. However, a more recent study showed that female APPxPS1 of the same line developed sensorimotor gating deficits at the age of 7 months [35]. However, sex and PPI protocol-specific effects are likely [6, 37, 62-63].

In conclusion, this investigation describes for the first time social recognition memory deficits in male APPxPS1 mice. Furthermore, this deficit manifests at least 3 months prior to any evidence of other cognitive deficits such as spatial learning and memory impairments. The deficits in social recognition could be linked to possible impairments of the prefrontal cortex and hippocampus caused either by the deposition of Aβ or other underlying pathological symptoms. The observed anxiety phenotype and the absence of any spatial deficits in 10-month-old male APPxPS1 mice on a mixed background emphasise the

necessity to consider sex and genetic background effects in AD mouse models and to pay attention to details of the cognitive paradigms undertaken.

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7. Figure Legends

Fig. 1A-B: Sociability (A) and social recognition memory (B and C) in the social preference test: A) Total time spent in test chambers containing either an unfamiliar mouse (i.e. opponent) or an empty mouse enclosure (i.e. empty) [s]; **B)** Time spent in a test chamber containing either a familiar or an unfamiliar (i.e. novel) mouse [s]. **C)** Time spent *sniffing* a familiar or an unfamiliar (i.e. novel) opponent (i.e. A/J mouse) [s]. Data for non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) males are shown as means + SEM. Significant genotype effects of ANOVA are indicated with '*' ($^{*}p < .05$) whereas RM ANOVA for chamber effects are presented by '#' ($^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$).

Fig. 2A-D: Spatial learning and memory in the cheeseboard (CB): A) Latency [s] to find the food reward (averaged across 3 daily trails) during training; **B)** Percentage time [%] spent in the target zone of the CB (i.e. in close proximity to the reward well) during the 2 min probe trial; **C)** Latency [s] to find the food reward (averaged across 3 daily trials) during reversal training; **D)** Percentage time [%] spent in the target zone of the CB during the 2 min reversal probe trial. Data for non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) males are shown as means + SEM.

Fig. 3A-C: Sensorimotor gating: A) Acoustic startle response (ASR: startle amplitude in arbitrary units) to different startle pulses (i.e. 70 dB = background noise, 100 dB, 120 dB); **B)** Habituation of the ASR to a 120 dB startle pulse over blocks of trials; **C)** Percentage prepulse inhibition (%PPI) averaged over trials for different prepulse intensities (72/74/78 dB). Data for non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) males are shown as means + SEM. Significant genotype effects (ANOVA) are indicated by '*' ($^{*}p < .05$).

8. Tables and Figures

Test age [d]	Behavioural paradigm
194 ± 12	Light-dark test (LD)
196 ± 12	Elevated plus maze (EPM)
207 ± 12	Social preference test (SPT)
214 ± 12	Contextual and cued fear conditioning (FC)
308 ± 6	Cheeseboard (CB)
321 ± 6	Reversal cheeseboard (rCB)
340 ± 12	Sensorimotor gating (Prepulse inhibition: PPI)
379 ± 12	Olfaction (Cookie test)

Table 1: Test age [d] and test biography of non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) male mice are shown.

	WT	**APPxPS1**
LD		
Total distance travelled [cm]	1315.3 ± 114.5	2256.0 ± 204.8 **
Distance travelled in the light chamber [%]	35.0 ± 4.4	33.8 ± 1.9
Time spent in the light chamber [%]	33.6 ± 4.3	33.7 ± 2.1
Time spent in the light chamber [s]	191.9 ± 24.3	194.6 ± 12.3
EPM		
Time spent on open arms [%]	21.3 ± 5.6	9.2 ± 2.8 *
Time spent on open arms [s]	29.9 ± 8.7	13.0 ± 3.8 $^{+}$
Entries into open arms [%]	28.0 ± 4.6	16.2 ± 3.4 *
SPT		
Familiar mouse chamber Total distance travelled [cm]	321.4 ± 29.5	397.9 ± 49.1
Novel mouse chamber Total distance travelled [cm]	416.7 ± 54.5	416.2 ± 43.1

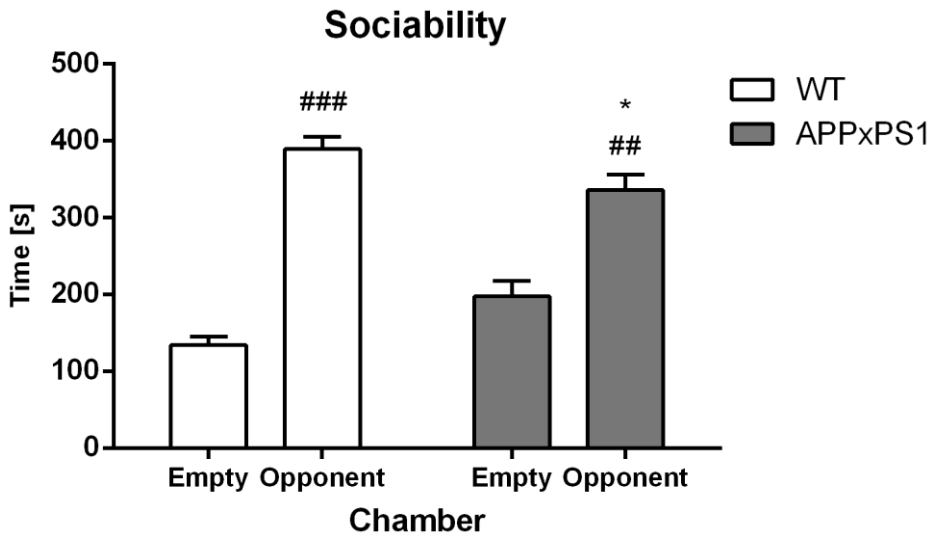
Table 2: Locomotion (total distance travelled) and anxiety behaviours (percentage locomotion and time spent in aversive zones) in the light-dark test (LD), the elevated plus maze (EPM) and the social preference test (SPT: total distance travelled in social recognition test only) of non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) male mice are shown as mean ± SEM. Significant effects of 'genotype' are indicated with '*' ($^{*}p < .05$ and $^{**}p < .01$) whereas trends of 'genotype' are shown with '+' ($^{+}p = .05$).

Fear conditioning	**WT**	**APPxPS1**
Conditioning		
Baseline *freezing* [s]	9.4 ± 4.5	6.9 ± 2.3
Context		
Total time spent *freezing* [s]	61.2 ± 13.3	100.4 ± 16.4
Freezing - first 2 min [s]	17.2 ± 5.5	21.7 ± 5.7
Cue		
Time spent *freezing* 1 min prior to cue onset [s]	6.7 ± 2.8	9.9 ± 2.2
Time spent *freezing* 1 min post cue onset [s]	10.2 ± 2.0	16.3 ± 3.4
Freezing – first 2 min [s]	10.2 ± 4.4	12.4 ± 3.0

Table 3: Fear-associated memory: Time spent *freezing* [s] at baseline (first 2 min of conditioning trial), during the context test, and 1 min prior to and post tone presentation in the cue version of the fear conditioning paradigm is shown for non-transgenic control (WT) and double transgenic APP_{Swe}/PS1ΔE9 (APPxPS1) male mice. Data are presented as mean ± SEM.

Figure 1

A


Sociability
500
400
300
200
100
0
Time [s]
###
*
##
WT
APPxPS1
Empty
Opponent
Empty
Opponent
Chamber

B

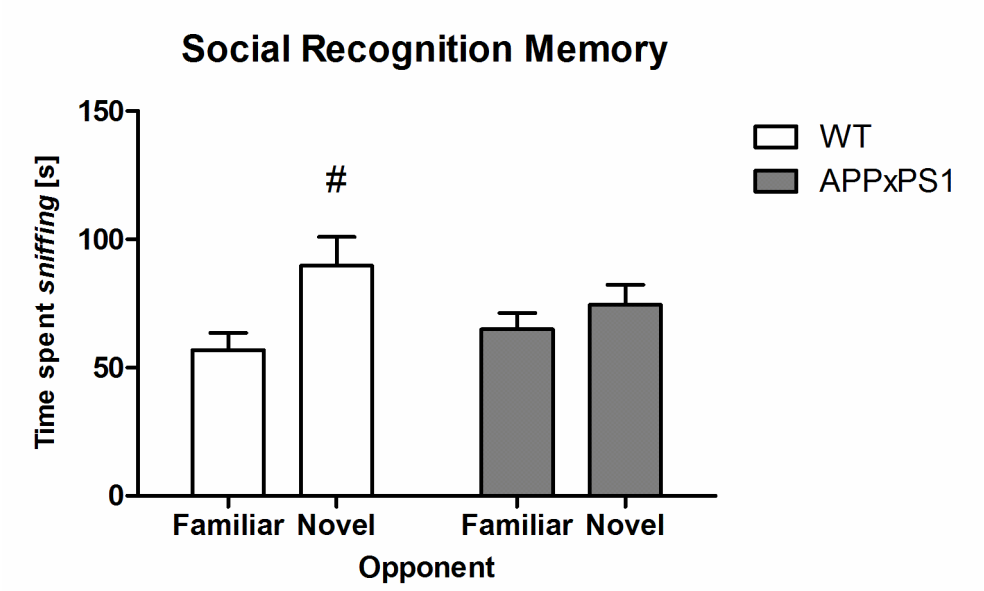

Social Recognition Memory
150
100
50
0
Time spent sniffing [s]
#
WT
APPxPS1
Familiar
Novel
Familiar
Novel
Opponent

Figure 1
C

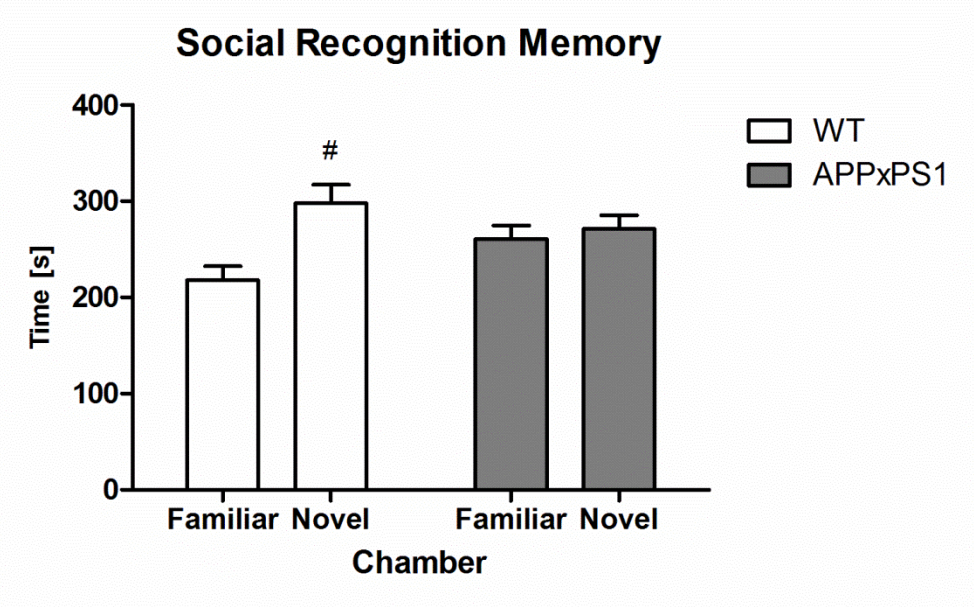
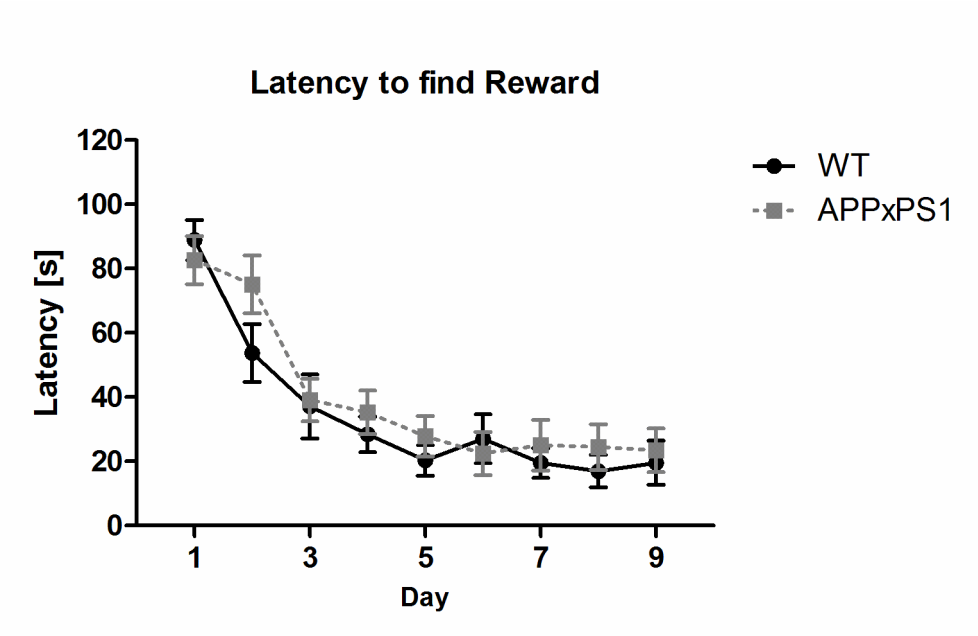

Social Recognition Memory
400
300
200
100
0
Time [s]
#
WT
APPxPS1
Familiar
Novel
Familiar
Novel
Chamber

Figure 2

A


Latency to find Reward
Latency [s]
120
100
80
60
40
20
0
1
3
5
7
9
Day
WT
APPxPS1

B

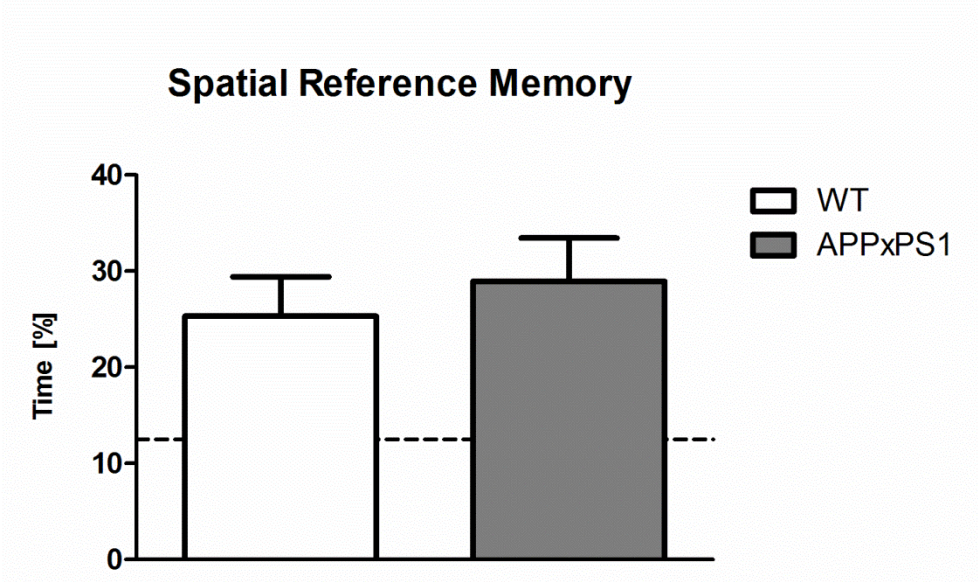
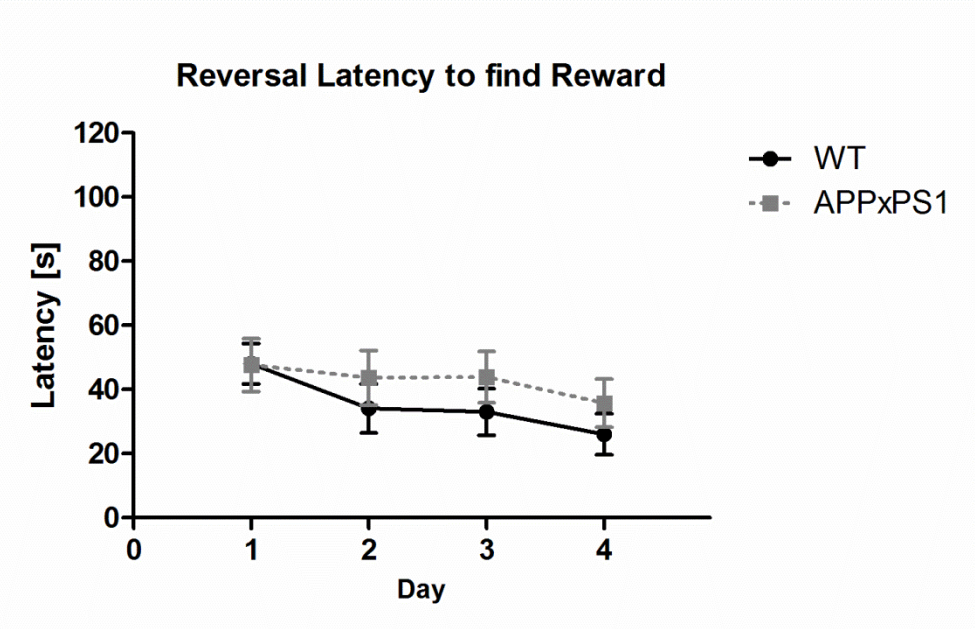

Spatial Reference Memory
Time [%]
40
30
20
10
0
WT
APPxPS1

Figure 2

C


Reversal Latency to find Reward
Latency [s]
120
100
80
60
40
20
0
0
1
2
3
4
Day
WT
APPxPS1

D

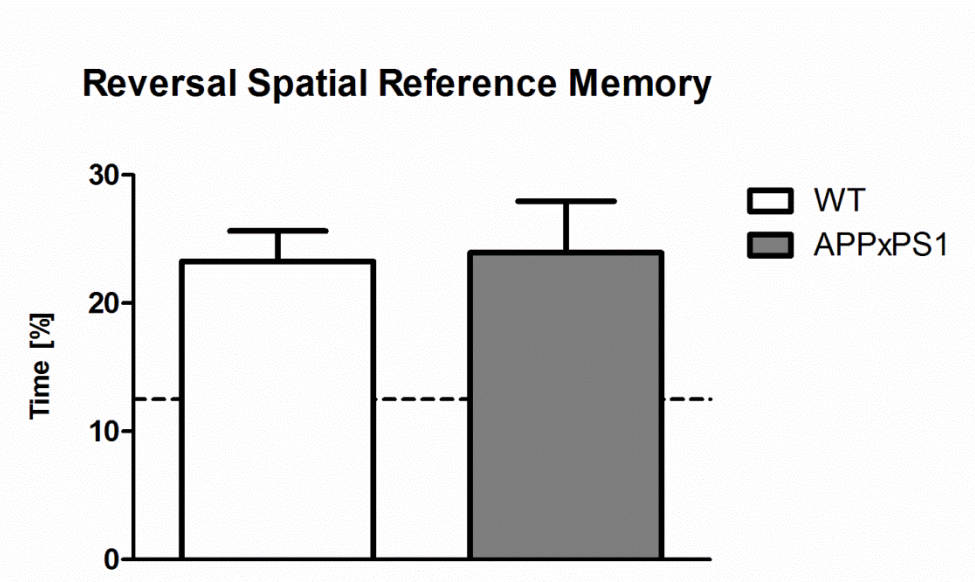
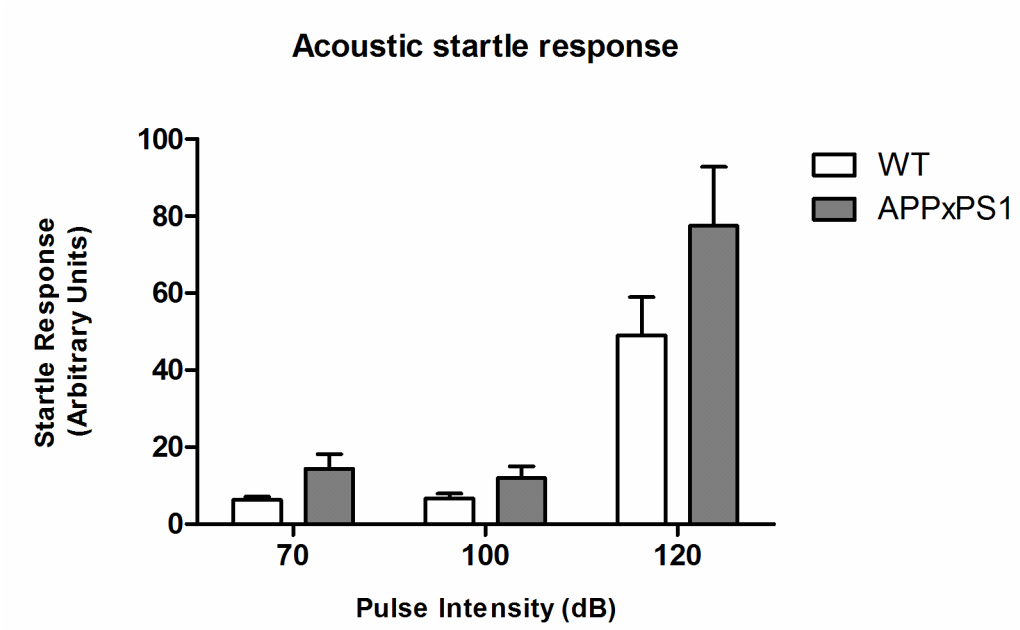

Reversal Spatial Reference Memory
Time [%]
30
20
10
0
WT
APPxPS1

Figure 3

A


Acoustic startle response
Startle Response (Arbitrary Units)
100
80
60
40
20
0
70
100
120
Pulse Intensity (dB)
WT
APPxPS1

B

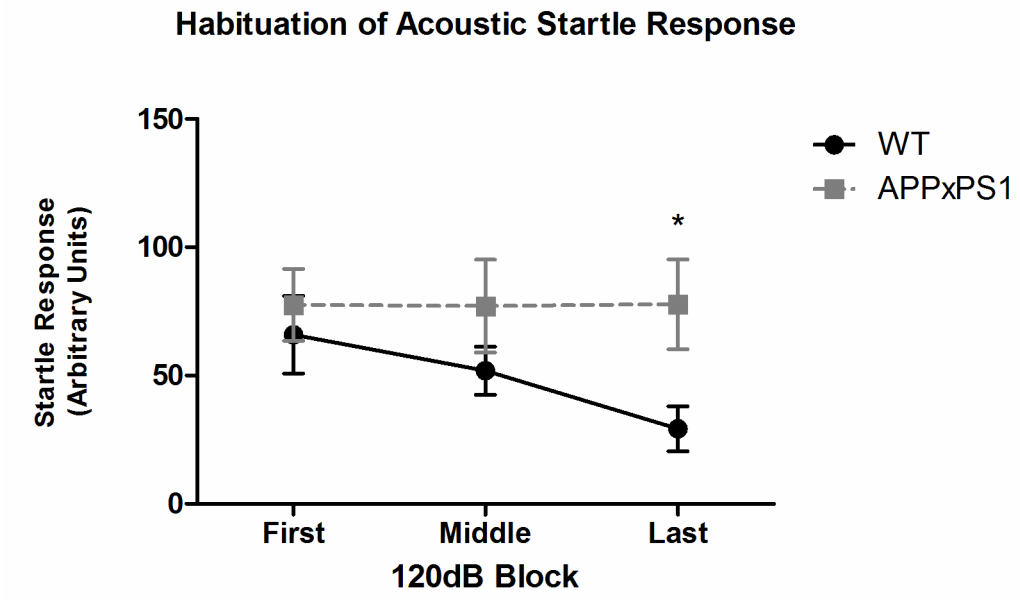
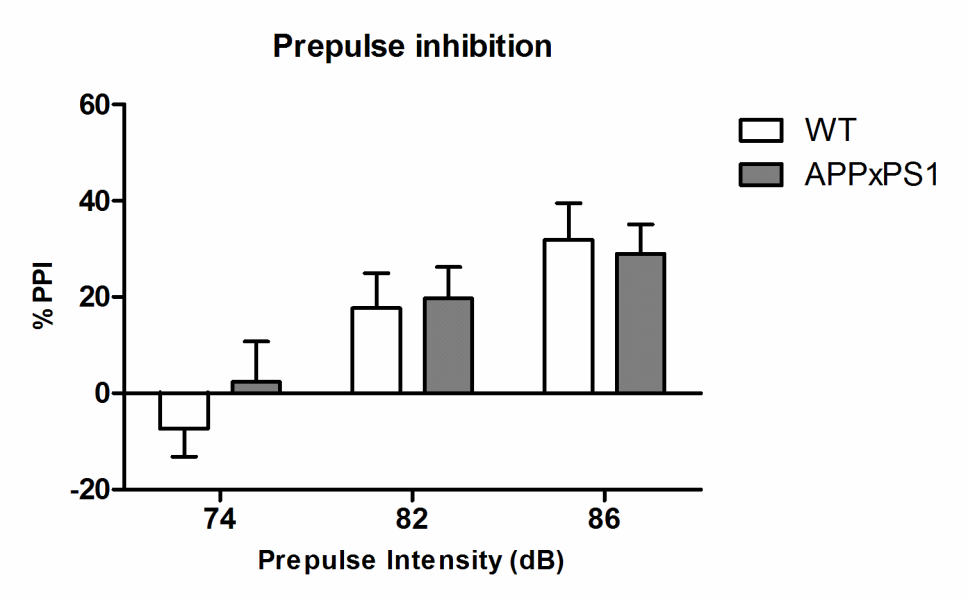

Habituation of Acoustic Startle Response
Startle Response (Arbitrary Units)
150
100
50
0
*
First
Middle
Last
120dB Block
WT
APPxPS1

Figure 3
C


Prepulse inhibition
60
40
20
0
-20
% PPI
74
82
86
Prepulse Intensity (dB)
WT
APPxPS1

2.2. Novel behavioural characteristics of female APP_{Swe}/PS1ΔE9 double transgenic mice

Publication II

Cheng, D., Low, J.K., Logge, W., Garner, B. and Karl, T. Novel behavioural characteristics of female APP_{Swe}/PS1ΔE9 double transgenic mice. *Behavioural Brain Research*. 2014; 260: 111-118

Declaration

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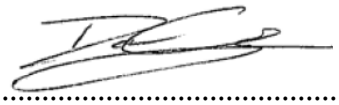

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David Cheng

Novel behavioural characteristics of female *APP$_{Swe}$/PS1ΔE9* double transgenic mice

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Highlights

- Characterisation of novel behaviours of APPxPS1 transgenic female mice
- APPxPS1 female mice demonstrate spatial memory deficit in cheeseboard task
- APPxPS1 females show task-dependent hyperlocomotor and anxiolytic-like behaviour
- Unaltered sensorimotor gating and associative learning and memory of APPxPS1 mice

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Abstract

Murine models are commonly used to evaluate progression of Alzheimer's disease. APP_{swe}/PS1ΔE9 (APPxPS1) mice have previously been reported to demonstrate impaired learning and memory in the Morris water maze test. However, this paradigm introduces a variety of behaviours that may confound performance of the mice, thus an alternative was sought. A battery of behavioural tests (light-dark test, elevated plus maze, novel object recognition task, social recognition test, cheeseboard task and prepulse inhibition) was used to investigate various behavioural and cognitive domains with relevance to Alzheimer's disease. We found 9-month old female APPxPS1 mice exhibited impaired spatial memory in the reversal cheeseboard task. In addition, task-dependent hyperlocomotion and anxiolytic-like behaviours were observed in the light-dark test. Female APPxPS1 demonstrated intact object recognition memory. Sensorimotor gating was not significantly decreased compared to control mice except for one particular interstimulus interval. The social recognition test failed to detect preference for social novelty in control females. In conclusion, this is the first study to describe a memory deficit in female APPxPS1 mice in the hidden cheeseboard task. Transgenic females also exhibited task-dependent reduction in anxiety behaviours and hyperlocomotion. These novel findings enhance our understanding of the behavioural phenotype of APPxPS1 females and present the cheeseboard as a valid alternative to other established spatial memory tests. Furthermore, the task-dependency of some of these findings suggests that behavioural profiling of APPxPS1 transgenic mice should be assessed using a variety of behavioural paradigms.

Keywords: Alzheimer's disease; transgenic *APP_{Swe}/PS1ΔE9* mice; behaviour; social recognition memory; sensorimotor gating; cheeseboard

1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia. Post-mortem brain tissue of AD patients is characterised by amyloid-β peptide (Aβ) aggregation causing plaques and tau protein hyperphosphorylation, the latter being associated with neurofibrillary tangle formation (reviewed in [1]). AD patients exhibit behavioural and cognitive symptoms such as social withdrawal, deficits in language comprehension, and severe cognitive decline of short-term and long-term memory including the inability to recognise friends and relatives [2, 3].

AD is classified as two subtypes: 1) sporadic AD (late onset) is the most common form of AD and results from a complex interaction of various environmental risk factors and susceptibility genes (e.g. *APOE*) [4]; and 2) familial AD (early onset, autosomal dominant), which accounts for <10% of all AD cases [5] and is caused by mutations in one of three genes: amyloid precursor protein (*APP*), presenilin 1 (*PS1*) or presenilin 2 (*PS2*) [6]. Transgenic mouse models of familial AD are commonly used to investigate progression of AD. Co-expression of mutant *APP* and *PS1* in double transgenic mice such as the $APP_{Swe}/PS1\Delta E9$ transgenic mice (APPxPS1) [7-11] accelerates the rate of amyloid brain pathology compared to mouse models targeting only one risk gene for AD [12].

We recently determined new behavioural characteristics of male APPxPS1 transgenic mice including impaired social recognition memory, task-dependent hyperlocomotion and anxiety, but no disruptions to sensorimotor gating [13]. Importantly, female APPxPS1 mice have previously been found to exhibit more extensive amyloid pathology compared to male transgenic mice [9] and increased Aβ accumulation has been shown to be correlated with deficits in spatial memory [14]. Female mice also develop cognitive impairments but the nature of those deficits reported varies across studies. For example, some studies detected learning deficits in the Morris water maze (MWM) at 8 and 10 months of age [15, 16] whereas others described memory retention deficits at the age of 10 and 12 months [17, 18]

or no impaired memory retention at all [19]. Mice are not natural swimmers and inconsistencies in cognitive deficits of transgenic mice across studies could, for example, be due to differential levels of stress induced by variations in MWM protocols [20]. This would be in line with the observation that APPxPS1 females appear less anxious than control mice in some but not other laboratories [21, 22].

An alternative to the MWM is the cheeseboard task (CB), which has been proposed to be a less stressful variant of the MWM as it focuses on positive reinforcement [23]. This is important when the mouse model in question exhibits an anxiety-related phenotype. The CB also avoids some of the issues surrounding MWM testing (e.g. *floating* behaviour, hypothermia, thigmotaxis and physical fatigue [23-28]. Importantly, we have recently established the CB to reliably detect cognitive deficits [29, 30]. Thus, in the current study, we aimed to further characterise female APPxPS1 double transgenic mice in novel behavioural paradigms with relevance to AD. We employed the CB as a novel test for spatial memory assessment and determined social behaviours in APPxPS1 females for the first time. We also analysed social and object recognition memory, fear-associated memory, sensorimotor gating as well as anxiety behaviour in these mice. Female APPxPS1 mice were tested at an age, where previous studies had reported relevant behavioural deficits and AD-relevant brain pathology [9, 14, 21, 22, 31].

2. Materials and Methods

2.1 Animals

Double transgenic mice expressing chimeric mouse/human *APP* (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and mutant human *PS1* (PS1/ΔE9) mice (APPxPS1) were obtained from Jackson Laboratory (Bar Harbor, USA; stock no. 004462, line 85) and maintained as double hemizygotes on C57BL/6JxC3H/HeJ background as described previously [7, 8, 12, 32]. Female transgenic mice (APPxPS1; N = 9) and their non-transgenic littermates (WT; N = 12) were bred and group-housed in independently ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). Test mice were transported to Neuroscience Research Australia (NeuRA) at around 10 weeks of age, where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) with corn cob bedding (Bed-O'Cobs: Able Scientific, Perth, Australia) and some tissues for nesting. Mice were kept under a 12: 12 h light: dark schedule [light phase: white light (illumination: 124 lx) – dark phase: red light (illumination: < 2 lx)]. Environmental temperature was automatically regulated at 21 ± 1 °C and relative humidity was 40-60%. Food (Rat and Mouse Breeder Diet: Gordon's Specialty Stockfeeds, Yanderra, Australia) and water were provided *ad libitum*, except where specified. Adult, female A/JArc mice from the Animal Resources Centre (Canning Vale, Australia) were used as standard opponents in the social preference test. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Behavioural Phenotyping

Starting at 7 months of age, mice were tested in a number of behavioural tests (Table 1) with an inter-test interval of at least 48 h as described earlier [13]. All tests were conducted during the first 5 h of the light phase to minimise effects of the circadian rhythm on the performance of test mice [33].

2.2.1 Light-dark test (LD): The apparatus was an infrared photobeam-controlled open-field activity test chamber (MED Associates Inc., St Albans, USA) containing a dark box insert that covered half the chamber and was opaque to visible light. Light (illumination: 20 lx) and dark (illumination: <2 lx) compartments were connected by an opening located at the centre of the partition (for details see [34]). Mice were placed at the opening (faced towards the dark compartment) at the start of the experiment. The time spent and distance travelled in the two chambers were recorded for 10 min. Time spent as well as distance ratio (distance travelled in light chamber / total distance travelled) in the more aversive light chamber were selected as anxiety parameters.

2.2.2 Elevated plus maze (EPM): The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated and open area [35, 36]. The '+' apparatus consisted of two alternate open arms (illumination: 70 lx) and two alternate enclosed arms (illumination: 10 lx) connected by a central platform, and was elevated 70 cm above the floor. Mice were placed at the centre of the '+' of the grey PVC plus maze (for further details of apparatus see [37]) facing towards an enclosed arm and were allowed to explore the maze for 5 min. The time spent and distance travelled in the open and enclosed arms were recorded using AnyMaze^{TM} (Stoelting, Wood Dale, USA) tracking software.

2.2.3 Novel object recognition task (NORT): Object recognition memory in the NORT is demonstrated by the animal's ability to distinguish between familiar and unfamiliar objects (rodents have an innate preference towards novelty [38]). The NORT was conducted over 3

days (as described previously [39]): two trials (10 min per trial) were conducted per day with a 1 h inter-trial interval (ITI). On day 1, mice were habituated to the empty arena during both trials. On day 2, mice were habituated to the empty arena during trial 1 and to two identical objects during trial 2. On the test day, mice were exposed to two identical objects in the sample trial (training trial / trial 1; objects distinct from day 2), and then one familiar and one novel object in the test trial (test trial / trial 2). The objects and their locations were counterbalanced across genotypes. Time spent *nosing* and *rearing* on the objects were recorded using AnyMaze^{TM} tracking software (and confirmed by manual scoring). The percentage of time spent *nosing* towards the novel object indicated short-term object recognition memory (% novel object recognition) and was calculated using [(novel object *nosing* time / novel + familiar object *nosing* time) × 100].

2.2.3 Social preference test (SPT): The SPT was used to assess sociability and social recognition memory [40]. The apparatus consisted of 3 chambers (as described in [13]). One circular cage (i.e. mouse enclosure) was placed into each outer chamber. The mouse enclosures allowed nose contact between mice (i.e. test mouse and A/JArc standard opponent mouse) but prevented fighting. The chambers and enclosures were cleaned with 30% ethanol in-between trials and fresh corn cob bedding was added to the chambers prior to each test trial.

Test animals were isolated for an hour prior to the start of testing. During the habituation trial, mice were allowed to freely explore the apparatus for 5 min. For the sociability test an unfamiliar standard opponent was placed in one of the two enclosures (i.e. opponent chamber) in a quasi-randomised fashion. Then the test mouse was returned to the apparatus and allowed to explore all three chambers for 10 min. Finally, test animals were observed in a 10 min social recognition test. For this, a second, unfamiliar standard opponent was placed in the previously empty chamber so that the test mouse had the choice to explore either the

familiar mouse (from the previous trial) or the novel, unfamiliar mouse. AnyMaze™ tracking software was used to determine the time spent in the different chambers, *sniffing* behaviour, number of entries and distance travelled by the test mice in each trial.

2.2.4 Fear Conditioning test (FC): Fear conditioning assesses associative learning whereby a previously neutral stimulus elicits a fear response after it has been paired with an aversive stimulus. On conditioning day, mice were placed into the test chamber (Model H10-11R-TC, Coulbourn Instruments, USA) for 2 min. An 80 dB conditioned stimulus (CS) was then presented for 30 seconds with a co-terminating 0.4 mA 2 second foot shock (unconditioned stimulus; US) twice with an inter-pairing interval of 2 min). The test concluded 2 min later. The next day (context test), mice were returned to the apparatus for 7 min. On day 3 (cue test), animals were placed in an altered context for 9 min. After 2 min (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 2 min with the absence of the CS (for more details see [41, 42]). Time spent *freezing* was measured using Any-Maze™ software.

2.2.5 Cheeseboard (CB): The CB was used to test mice for spatial learning and memory deficits. Mice at 8-9 months of age were trained to find a food reward (100 μl sweetened condensed milk; diluted 1:4 with water) on a wooden board over a number of days (for details of test apparatus see [13, 39]). All mice were food-deprived and kept at 85-90% of their pre-test body weight throughout testing. The latency and distance travelled to find the reward and time spent in the different CB zones (i.e. board was separated into 8 equal zones) was measured using Any-Maze™ software.

During habituation (three days to the blank side of the CB), three 2 min trials were conducted each day for three days with a 10 min inter-trial interval (ITI). For spatial reference memory acquisition, mice were trained over nine days (three trials per day with a 10 min ITI) to locate the food reward. If the target well was not located within 2 min, mice were placed next to the

target well and allowed to consume the food reward. To test for spatial reference memory, a probe trial was conducted on day 10, where no wells were baited and mice were given 2 min to explore the board freely. The time the mice spent in the different zones of the CB (i.e. % exploration time) was recorded (as previously described [41]).

To test reversal learning (start of training 24 h post probe trial), the location of the food reward was moved to the opposite side of the CB. Mice completed four days of reversal training (three trials per day with a 10 min ITI) before the reversal probe trial (24h post training).

2.2.6 Sensorimotor gating (i.e prepulse inhibition: PPI): PPI was used to test for sensorimotor gating deficits, which can be impaired in AD mouse models and can be directly correlated with amyloid burden [31, 43]. Test mice were placed in Plexiglas mouse enclosures of the startle chambers (SR-Lab, San Diego Instruments, San Diego, USA) and allowed to habituate to the enclosure and test apparatus for 5 min over 3 consecutive days prior to PPI testing with a consistent background noise of 70 dB. The 30 min PPI test session consisted of a 5 min acclimation period to 70 dB background noise, followed by 97 trials presented in a pseudorandom order: 5 x 70 dB trials; 5 x 100 dB trials; 15 x 120 dB trials to measure the acoustic startle response (ASR) and 15 sets of 5 trials comprising of a prepulse of either 74, 82 or 86 dB presented 32, 64, 128, or 256 ms (variable interstimulus interval; ISI) prior to a startle pulse of 120 dB to measure the PPI response. The inter-trial interval (ITI) varied randomly from 10 – 20 seconds. Responses to each trial were calculated as the average mean amplitude detected by the accelerometer [29, 44]. ASR was calculated as the mean amplitude to all startle trials. For ASR habituation, blocks (i.e. averaged across 5 trials) of the ASR to 120 dB startle pulses presented at the beginning, in the middle and at the end of the PPI protocol were used to determine the effect of 'startle block'. Percentage PPI (%PPI) was calculated as [(mean startle response (120 dB) – PPI response)/mean startle response

(120 dB)] x 100. %PPI was averaged across ISIs to produce a mean %PPI for each prepulse intensity. We analysed both the %PPI for the mean startle response across all 120 dB startle trials (i.e. 15 trials) as well as %PPI for the mean startle response across only the middle 120 dB startle trials (i.e. 5 trials).

2.3 Statistical Analysis

One-way analysis of variance (ANOVA) and of covariance (ANCOVA) were used to analyse behavioural parameters for main effects of 'genotype' and repeated measures (RM) ANOVAs for effects of 'chamber' (SPT), 'time' (CB) '1 min block' (FC), 'startle block', 'startle pulse', 'ISI' and 'prepulse intensity' (all PPI) as published previously [39]. Performance in the cheeseboard probe trials and social preference test were also assessed using one sample t-tests to clarify whether the percentage of time spent in the target zone (CB), with the novel object (NORT) or in the opponent/novel chamber (SPT) were greater than chance (12.5% and 50% respectively). Differences were regarded as significant if $p < .05$. Data are shown as means ± standard error of means (SEM). F-values and degrees of freedom are presented for ANOVAs and significant genotype effects are shown in figures and tables as '*' ($^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$). Analyses were conducted using SPSS 20.0 for Windows.

3. Results

3.1 Anxiety and locomotion

APPxPS1 females exhibited decreased anxiety levels in the LD test as confirmed by one-way ANOVA [time spent in the light chamber: $F(1,19) = 5.5$, $p < .05$ - light chamber distance ratio: $F(1,19) = 7.7$, $p < .05$] (Table 2). Transgenic females also exhibited increased locomotor activity [total distance travelled: $F(1,19) = 13.2$, $p < .01$] (Table 2). ANCOVA revealed that spending more time in the light chamber was correlated with increased overall levels of locomotion but only in control females ['genotype' by 'total distance travelled': $F(1,17) = 8.7$, $p < .009$; followed by regression analyses: WT: $p = .02$ $R^2 = .413$ - APPxPS1: $p = .1$ $R^2 = .317$; data not shown]. These findings were task-specific as APPxPS1 females showed wild type-like anxiety and locomotion behaviour in the EPM (all $p > .05$; Table 2).

3.2 Cognition

3.2.1 Novel Object Recognition Task: There was no effect of 'genotype' on overall object recognition (i.e. *nosing* time) during the training trial (data not shown). In the test trial, both WT and APPxPS1 mice displayed significantly more *nosing* towards the novel object over the familiar one (Percentage *nosing*: WT: 65.8 ± 5.4; APPxPS1: 62.4 ± 5.1). One sample t-test confirmed that all mice preferred *nosing* the novel object, demonstrating intact object recognition [WT: $t(11) = 2.9$, $p < .05$; APPxPS1: $t(8) = 2.4$, $p < .05$].

3.2.2 Social Preference Test: All mice demonstrated intact sociability. RM ANOVA revealed that both WT and transgenic females spent significantly more time in the opponent chamber compared to the empty chamber ['chamber': $F(1,19) = 43.5$, $p < .001$; 'genotype': $F(1,19) = .6$, $p = .4$; 'genotype' × 'chamber': $F(1,19) = 1.2$, $p = .3$] (Supplementary Fig. 1A). One sample t-test confirmed that all mice had a significant preference for the mouse over the empty chamber [WT: $t(11) = 3.7$, $p < .01$; APPxPS1: $t(8) = 6.6$, $p < .001$]. However,

regardless of genotype, females failed to show a preference for the novel mouse in the social recognition test [RM ANOVA for 'chamber': $F(1,19) = .9$, $p = .4$; one sample t-test: $p > .05$ for both genotypes] (Supplementary Fig. 1B).

3.2.3 Fear Conditioning: All mice responded to the electric foot shocks (i.e. vocalisation) delivered during the conditioning phase. Baseline *freezing* prior to the conditioning phase was similar for both genotypes as was the total time spent *freezing* in the context test (all $p = .5$; Table 3). In the cue test, all mice regardless of genotype demonstrated the ability to associate the CS with the US as evidenced by a significant increase in *freezing* behaviour in response to the presentation of the cue [RM ANOVA for '1 min block': $F(1,19) = 13.5$, $p < .01$] (Table 3). The time spent *freezing* during cue presentation was not affected by 'genotype' [RM ANOVA for '1 min block': $F(1,19) = .1$, $p = .8$ (Fig. 1)].

3.2.4 Cheeseboard: Mice of both genotypes showed normal task acquisition as indicated by a reduced latency to find the food reward over time (averaged across trials) [RM ANOVA for 'time': $F(8,136) = 21.6$, $p < .001$; no interactions] (Fig. 2A), which was accompanied by a significant decrease in distance travelled to reach the reward [$F(8,152) = 11.0$, $p < .001$] (Fig. 2B). In the probe trial, all mice demonstrated a preference (i.e. time spent > 12.5%) for the target zone indicating successful recall of the reward location [WT: $t(10) = 4.2$, $p < .01$; APPxPS1: $t(8) = 3.9$, $p < .01$] (Fig. 2C).

In the reversal task, all mice learned to find the new (i.e. reversed) reward location over days [latency to find the reward: $F(3,51) = 16.0$, $p < .001$ - distance travelled to reach the reward: $F(3,57) = 21.6$, $p < .001$] (Fig. 2D-E). However, APPxPS1 females demonstrated no preference for the new target zone in the reversal probe trial [WT: $t(10) = 2.9$, $p < .05$; APPxPS1: $t(8) = 1.8$, $p = .1$]. The time the animals spent in the opposite zone (i.e. the previous location of the food reward) was not different (i.e. no preference) between genotypes [WT: $t(10) = -1.0$, $p = .4$; APPxPS1: $t(8) = .6$, $p = .6$] (Fig. 2F).

3.3 Sensorimotor gating

3.3.1 Acoustic startle response (ASR) and ASR habituation: The startle response of all mice to a 120 dB startle stimulus averaged across trials (WT: 72.0 ± 16.2; APPxPS1: 88.7 ± 25.7) was similar [$F(1,19) = .3$, $p = .6$]. RM ANOVA revealed a significant effect of startle pulse intensity (i.e. 70 dB versus 100 dB versus 120 dB) ['startle pulse': $F(2,38) = 24.5$, $p < .001$] on the ASR of all mice. Furthermore, all mice habituated to the 120 dB pulse across the PPI test session regardless of 'genotype' [RM ANOVA for 'startle block': $F(2,38) = 6.3$, $p < .01$ – no 'startle block' by 'genotype' interaction] (Fig. 3A).

3.3.2 Prepulse inhibition: Sensorimotor gating increased with increasing prepulse intensities [RM ANOVA: $F(2,38) = 56.0$, $p < .001$] (Fig. 3B). %PPI was not significantly different between APPxPS1 and control females for any prepulse intensity investigated (averaged across ISIs) ['genotype': all $p > .05$] although APPxPS1 generally exhibited lower %PPI (Fig. 3B). Data were also analysed using RM ANOVA for 'prepulse intensity', 'ISI', and 'genotype'. The analysis detected a main effect of 'prepulse intensity' [$F(2,38) = 56.0$, $p < .001$] and an interaction between 'prepulse intensity' and 'ISI' [$F(6,114) = 2.4$, $p = .03$] (all other p's $> .05$). These findings were evident for %PPI calculated for the 120 dB startle response averaged across all 120 dB trials as well as %PPI calculated for the 120 dB startle response of the middle 120 dB startle block (data not shown for the latter).

4. Discussion

Our study characterised new behavioural domains of APPxPS1 females and clarified some of the inconsistencies found in earlier studies. Female APPxPS1 transgenic mice exhibited a deficit in reversal spatial memory in the CB paradigm. Transgenic females also demonstrated task-dependent hyperlocomotion and a reduction in anxiety behaviours. No genotype effects were found in sociability, sensorimotor gating, object recognition memory or fear-associated memory.

Anxiety and stress are factors which may affect the cognitive performance of animals [26]. Thus, we assessed APPxPS1 females in the light-dark test and the elevated plus maze. 7-month old transgenic mice were hyper-locomotive and less anxious than control littermates in the LD test. This is the first study to detect hyperlocomotion in APPxPS1 female mice, which is in line with earlier studies using male APPxPS1 mice [13, 45]. Importantly, agitation and increased motor activity (restlessness) is a reported characteristic of AD patients [3]. The anxiolytic-like phenotype of APPxPS1 females is consistent with two earlier studies in 7-month old APPxPS1 transgenic mice, although males and females were assessed together in those studies and decreased anxiety levels were found in the EPM but not the LD test [21, 22]. These data suggest that the anxiety phenotype of APPxPS1 mice needs to be assessed carefully as task-specific responses are likely. Furthermore, differences in test protocols can have a significant impact on test outcomes [46], which demands the detailed reporting of protocol specifics (e.g. illumination levels were not described in the previous studies investigating the anxiety response of APPxPS1 mice [21, 22]).

Female APPxPS1 transgenic mice exhibited a deficit in spatial memory during the reversal phase of the hidden version of the cheeseboard whereas task acquisition and spatial memory during initial CB testing were unaffected. Importantly, APPxPS1 mice in the cued version of the CB paradigm displayed impaired performance in the probe and reversal probe trials at 24

months of age [47] but not at 2–3 months of age [31]. The earliest MWM–related memory deficits in female APPxPS1 mice were described for 10–12 month old mice [17, 18], although acquisition deficits have been reported for 8-9 month old female mice [14]. Thus, the CB paradigm appears more sensitive to detect spatial memory deficits of APPxPS1 females than the MWM. Aside from differences in stress arousal and impact on mouse physiology [23, 25-28], CB and MWM recruit different behavioural strategies and test motivators (i.e. foraging versus survival) and also require very different motor skills. These and other factors could be responsible for the differences seen in the cognitive performance of APPxPS1 transgenics in CB and MWM. For example, CB performance might be linked to the prelimbic-infralimbic regions of the prefrontal cortex [48], while the MWM might be more dependent on amygdala function [49-51]. Importantly, it is a common phenomenon when testing APPxPS1 mice in spatial memory tasks that transgenic mice develop either impairments in task acquisition [15, 16] or memory retention [17, 18]. Future research should address this in more detail. Overall, these data suggest that age and test design must be carefully considered when investigating and comparing between the spatial learning and memory deficits of APPxPS1 transgenic females.

Female APPxPS1 mice exhibited intact object recognition memory when using an ITI of 1 hour. This confirms findings of earlier studies investigating female APPxPS1 transgenic mouse models, which reported impaired object recognition memory after an ITI of 4 h [16] but not 1 h [17, 52] (however, Bonardi and colleagues do not specify the mutation of their model [52]). In addition, our female APPxPS1 mice demonstrated intact contextual and cued fear conditioning. Other studies have found deficits in contextual FC, however, these transgenic mice (males and females pooled together) carried a different mutation in the APP_{Swe} gene (K670N/M671L) [53].

Female APPxPS1 mice and their WT counterparts displayed normal sociability. However, none of the female mice demonstrated a preference for social novelty, although the same SPT protocol was effective in male APPxPS1 mice tested in an earlier study [13]. This phenomenon might be related to the fact that male WT mice generally demonstrate enhanced olfactory discrimination of social olfactory cues (e.g. urine) compared to female mice [54], which might allow males to better distinguish between different social opponents. This capacity to distinguish between different mice is essential for the SPT. Although male and female mice have successfully established a preference for social novelty using similar protocols in previous studies [55], these mice were on a pure C57BL/6J background. It is known that the background strain impacts on social behaviour and therefore on the effectiveness of test protocols [56, 57].

Patients with AD have been shown to exhibit suppression of the P50 event-related potential of sensorimotor gating [58]. Therefore, we tested APPxPS1 females for sensorimotor gating deficits. Prepulse inhibition of 11-month old APPxPS1 females was not significantly altered compared to WT mice. Importantly, another study reported that APPxPS1 transgenic females develop sensorimotor gating deficits at the age of 7 months when employing an ISI of 100 ms only [43]. As APPxPS1 females of our study showed non-significant lower levels of PPI, additional animals were tested (total N = 17–19 per genotype) for sensorimotor gating. Increasing the sample size resulted in decreased prepulse inhibition in APPxPS1 females, but only at an ISI of 128 ms (Supplementary Figure 2). Thus, differences between the current study and the report by Wang *et al.* suggest that PPI of APPxPS1 females is highly protocol-dependent, similar to what has been described for other mutant mouse models [59].

In conclusion, this study described hyperlocomotion, reduced anxiety, and impairment in spatial memory in the cheeseboard task in female APPxPS1 mice. These phenotypic features were task-specific, which suggests that behavioural profiling of AD transgenic mice needs to

be assessed using a variety of behavioural paradigms to avoid false positive or negative results.

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7. Figure Legends

Fig. 1: Fear-associated learning in fear conditioning (FC): Time spent *freezing* during the cue test for each '1 min bin'. Data for non-transgenic control (WT) and double transgenic $APP_{Swe}/PS1\Delta E9$ (APPxPS1) females are shown as means + SEM.

Fig. 2A-F: Spatial learning and memory in the cheeseboard (CB): A) Latency [s] and **B)** distance travelled [m] to find the food reward averaged across 3 daily trials; **C)** Percentage time [%] spent in the target zone of the CB (i.e. in close proximity to the reward well) during the 2 min probe trial; **D)** Latency [s] and **E)** distance travelled [m] to find the food reward averaged across 3 daily trials; **F)** Percentage time [%] spent in the target zone of the CB during the 2 min reversal probe trial. Data for non-transgenic control (WT) and double transgenic $APP_{Swe}/PS1\Delta E9$ (APPxPS1) females are shown as means + SEM. Significant preference for target zone (as analysed by t-test) is indicated by '#' ($^{\#}p < .05$ and $^{\#\#}p < .01$).

Fig. 3A-B: Sensorimotor gating: A) Habituation of the ASR to a 120 dB startle pulse over blocks of 5 trials; **B)** Percentage prepulse inhibition (%PPI) averaged over all 120 dB startle trials and ISIs for different prepulse intensities (72/82/86 dB). Data for non-transgenic control (WT) and double transgenic $APP_{Swe}/PS1\Delta E9$ (APPxPS1) females are shown as means + SEM.

Supplementary Fig. 1A-B: Sociability (A) and social recognition memory (B) in the social preference test: A) Total time spent in test chambers containing either an unfamiliar mouse (i.e. opponent) or an empty mouse enclosure (i.e. empty) [s]; **B)** Time spent in a test chamber containing either a familiar or an unfamiliar (i.e. novel) mouse [s]. Data for non-transgenic control (WT) and double transgenic $APP_{Swe}/PS1\Delta E9$ (APPxPS1) females are shown as means + SEM. Significant RM ANOVA for 'chamber' are presented by '#' ($^{\#\#}p < .01$ and $^{\#\#\#}p < .001$).

Supplementary Fig. 2A-B: Sensorimotor gating: A) Percentage prepulse inhibition (%PPI) averaged over all 120 dB startle trials and ISIs for different prepulse intensities (72/82/86 dB) (*N* = 17-19 per genotype). **B)** %PPI for different prepulse intensities (72/82/86 dB) at an interstimulus interval (ISI) of 128 ms. RM ANOVA revealed a main effect of 'prepulse intensity' [$F(2,68) = 57.9$, $p < .001$], 'ISI' [$F(3,102) = 3.6$, $p < .02$] and an interaction between 'prepulse intensity' and 'ISI' [$F(6,204) = 4.6$, $p < .001$] (all other p's > .05). Data for non-transgenic control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) females are shown as means + SEM. Significant effects of 'genotype' (as analysed by one-way ANOVA) were indicated by '*' (i.e. *p < .05).

8. Tables and Figures

Test age [weeks]	Behavioural paradigm
30 ± 1	Light-dark test (LD)
30 ± 1	Elevated plus maze (EPM)
30 ± 1	Novel object recognition task (NORT)
31 ± 1	Social preference test (SPT)
34 ± 1	Contextual and cued fear conditioning (FC)
36 ± 1	Cheeseboard (CB)
38 ± 1	Reversal cheeseboard (rCB)
46 ± 1	Sensorimotor gating (Prepulse inhibition: PPI)

Table 1: Test age [weeks] and test biography of wild type-like control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) female mice are shown.

	WT	**APPxPS1**
LD		
Time spent in the light chamber [s]	136.1 ± 18.4	239.7 ± 40.4 *
Distance ratio in the light chamber [%]	23.2 ± 3.3	40.1 ± 6.8 *
Total distance travelled [m]	14.2 ± 1.5	24.1 ± 2.4 **
EPM		
Time spent on open arms [s]	3.3 ± 1.2	5.9 ± 1.6
Entries onto open arms [%]	13.1 ± 3.4	16.2 ± 4.3
Total distance travelled [m]	8.3 ± 1.0	10.9 ± 1.2

Table 2: Anxiety [i.e. distance ratio (LD), entries onto open arms (EPM) and time spent (LD and EPM)] and locomotion (total distance travelled) in the light-dark test (LD) and the elevated plus maze (EPM) of wild type-like control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) female mice are shown as mean ± SEM. Significant ANOVA effects of 'genotype' are indicated with '*' (*p <.05 and **p < .01).

	WT	**APPxPS1**
Conditioning: Baseline *freezing* [s]	7.8 ± 2.7	5.0 ± 2.6
Total time spent *freezing* in context [s]	114.9 ± 24.3	144.7 ± 33.7
Time spent *freezing* 1 min prior to cue [s]	17.25 ± 3.8	19.8 ± 3.5
Time spent *freezing* 1 min post cue [s]	27.2 ± 4.3 $^{\#}$	28.7 ± 3.2 $^{\#}$

Table 3: Fear-associated memory: Time spent *freezing* [s] at baseline (first 2 min of conditioning trial), during the context test, and 1 min prior to and post tone presentation in the cue version of the fear conditioning paradigm is shown for wild type-like control (WT) and double transgenic *APP$_{Swe}$/PS1ΔE9* (APPxPS1) female mice. Data are presented as mean ± SEM. Significant RM ANOVA for '1 min block' (i.e. 1 min prior to cue *versus* 1 min post cue) are presented by '#' ($^{\#}p < .05$).

Figure 1

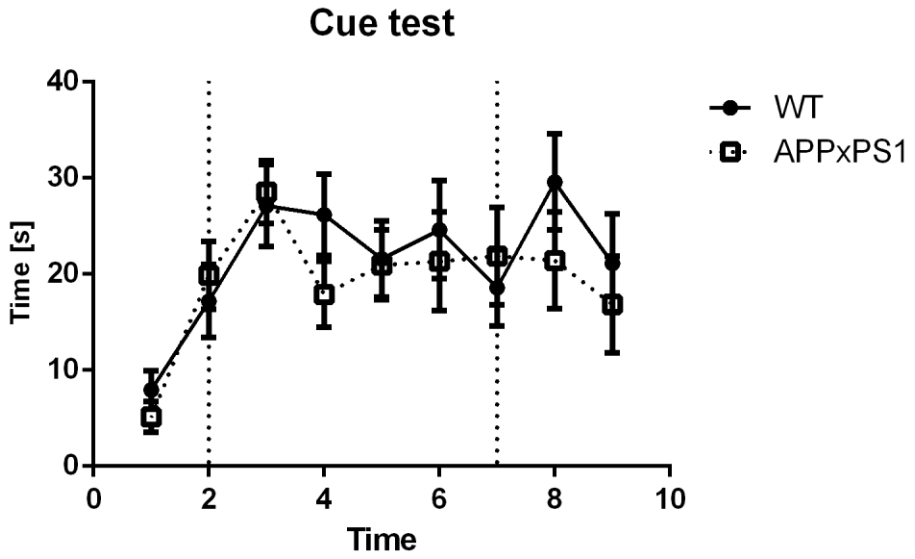
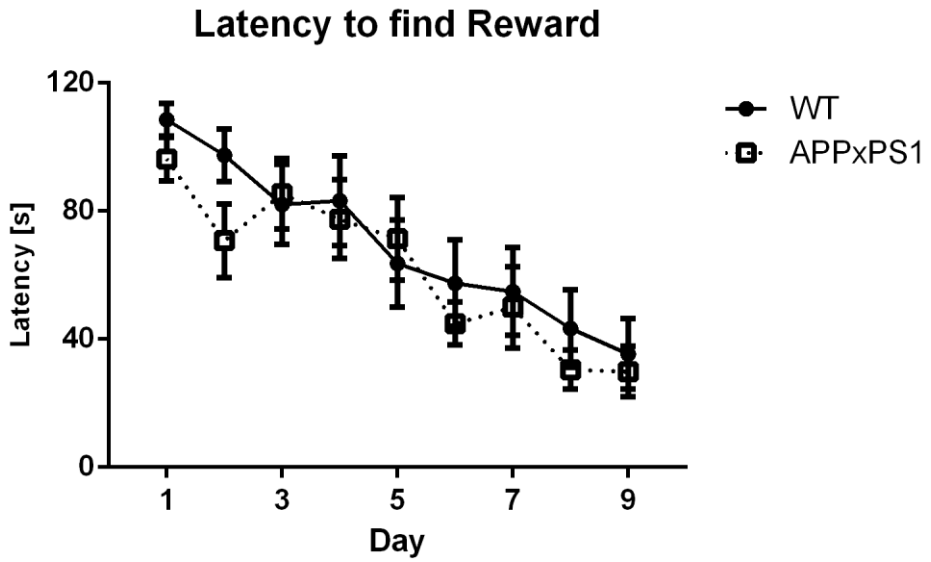


Figure 2

A


Latency to find Reward
120
80
40
0
Latency [s]
1
3
5
7
9
Day
WT
APPxPS1

B

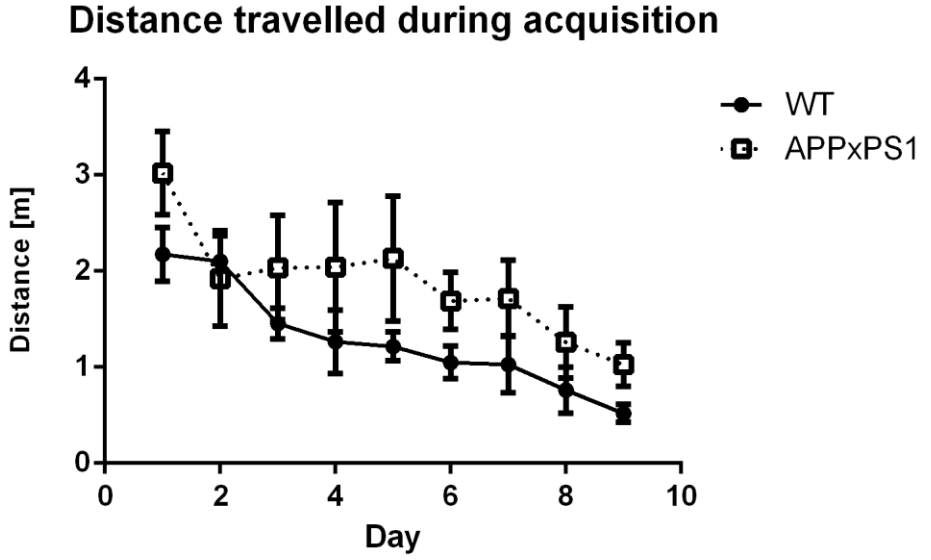
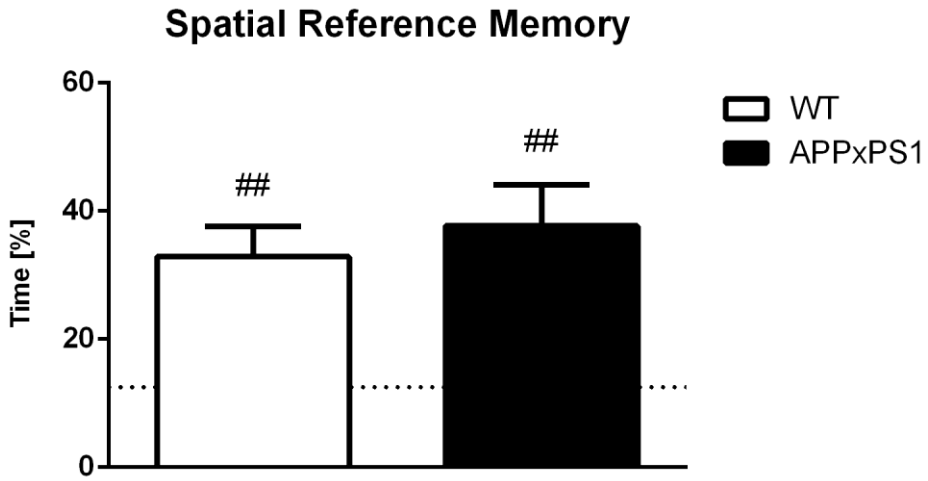

Distance travelled during acquisition
4
3
2
1
0
Distance [m]
0
2
4
6
8
10
Day
WT
APPxPS1

Figure 2

C


Spatial Reference Memory
Time [%]
60
40
20
0
##
##
WT
APPxPS1

D

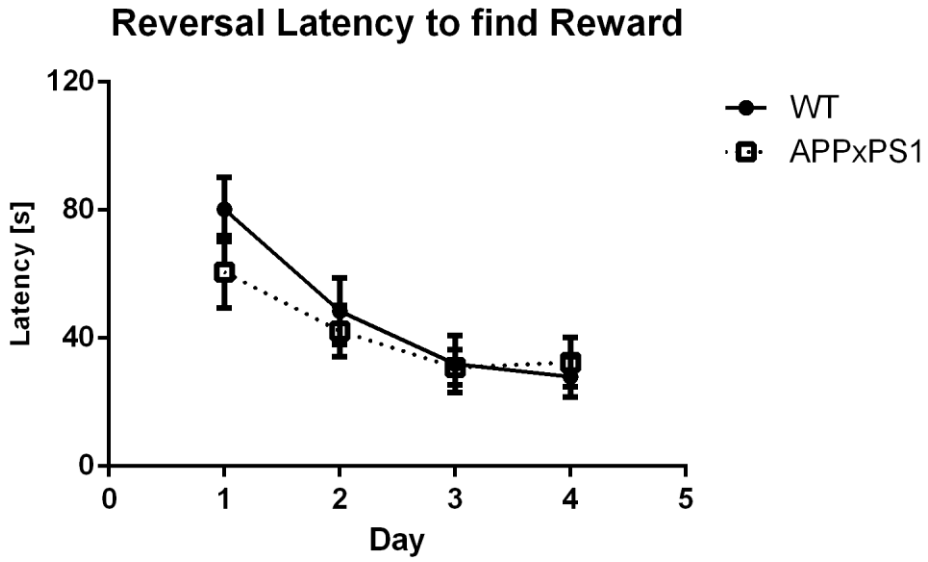
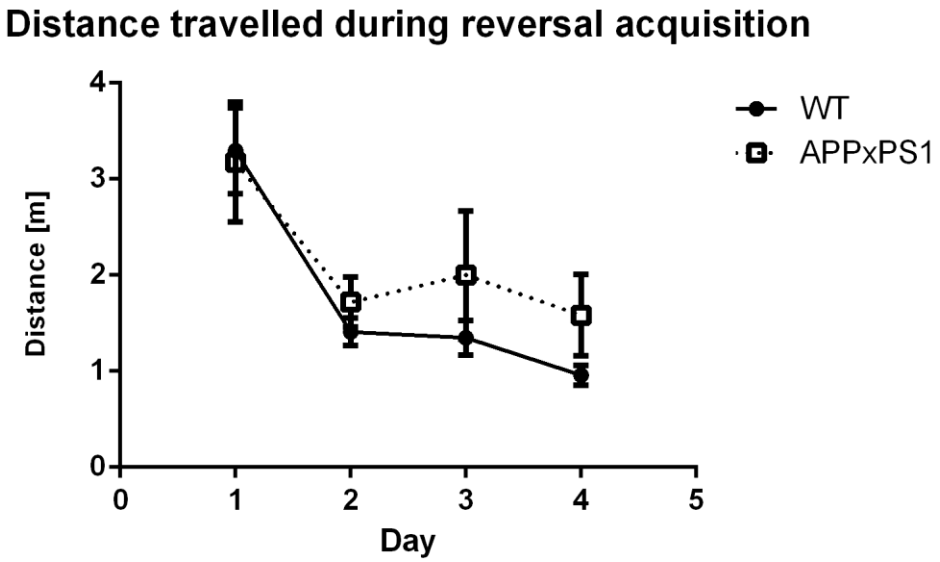

Reversal Latency to find Reward
Latency [s]
120
80
40
0
0
1
2
3
4
5
Day
WT
APPxPS1

Figure 2

E


Distance travelled during reversal acquisition
Distance [m]
Day
WT
APPxPS1
0
1
2
3
4
5

F

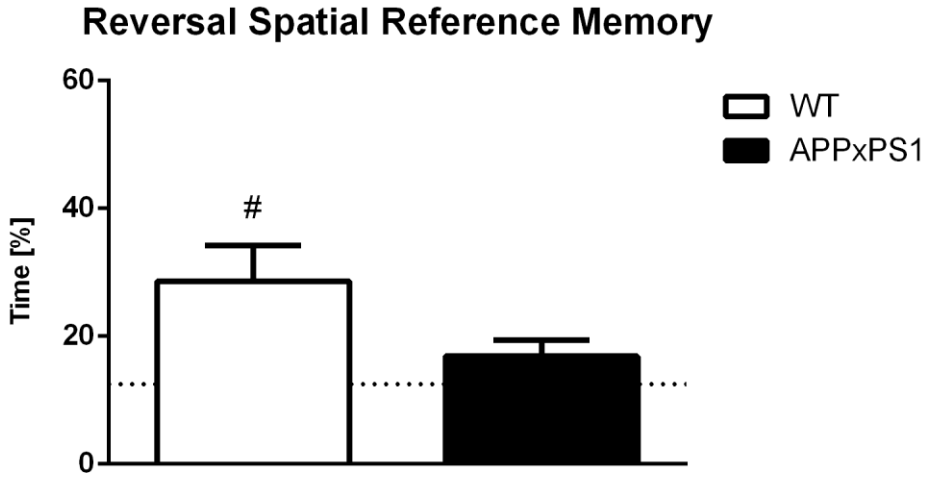
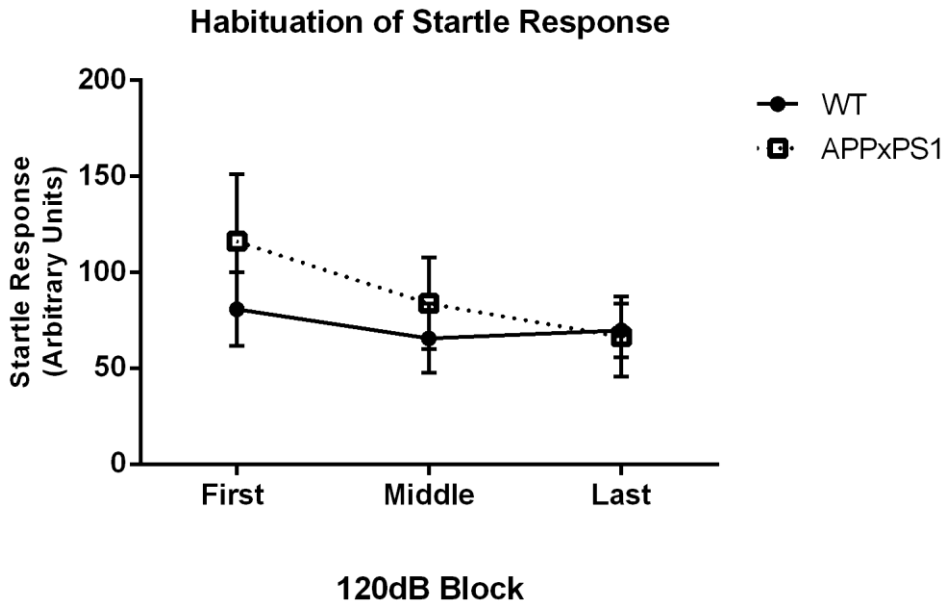
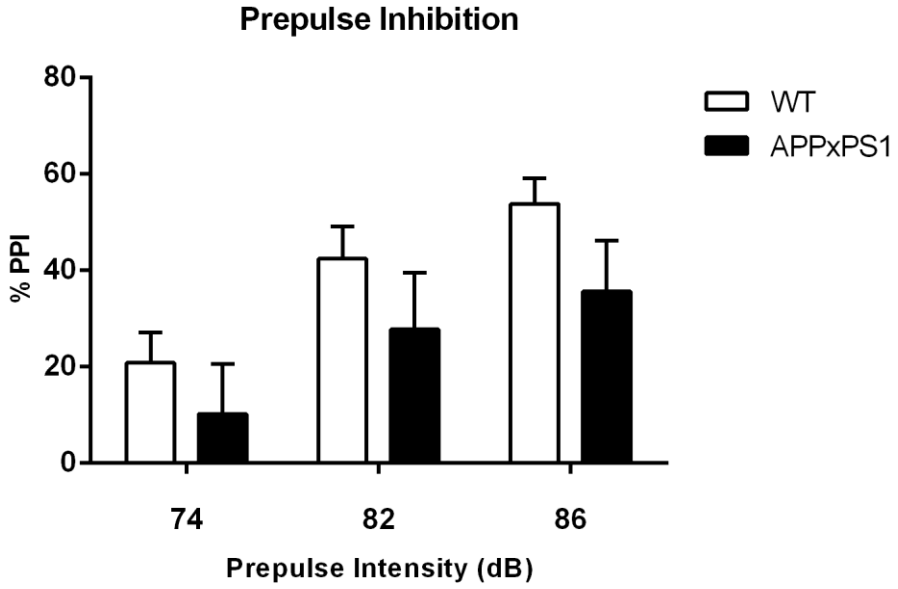

Reversal Spatial Reference Memory
Time [%]
#
WT
APPxPS1
0
20
40
60

Figure 3

A


Habituation of Startle Response
Startle Response (Arbitrary Units)
200
150
100
50
0
First
Middle
Last
120dB Block
WT
APPxPS1

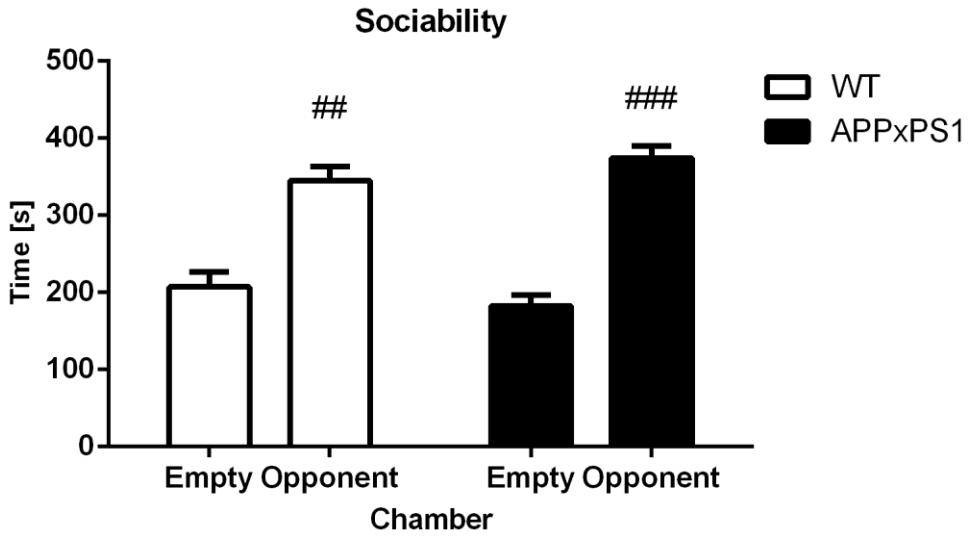
B


Prepulse Inhibition
% PPI
80
60
40
20
0
74
82
86
Prepulse Intensity (dB)
WT
APPxPS1

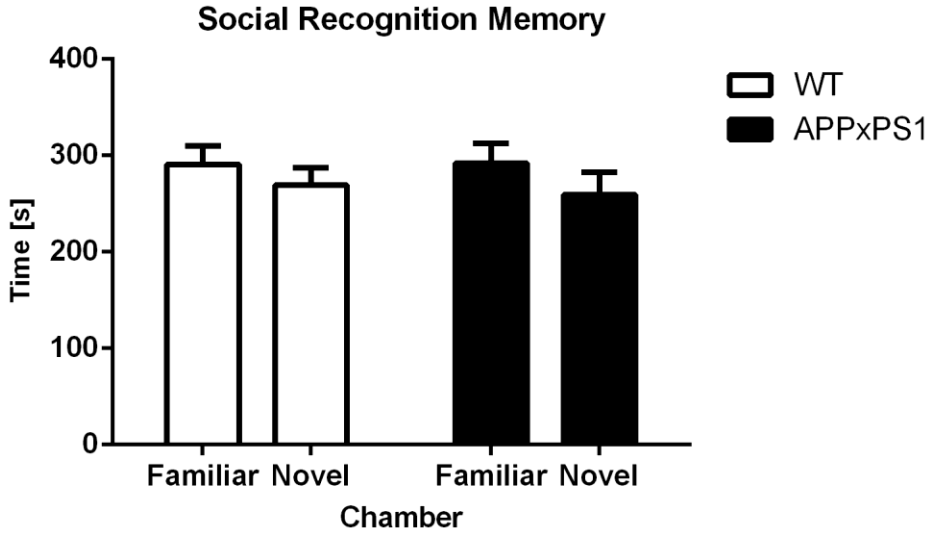
9. Supplementary Data

Supplementary Fig. I.

A



B

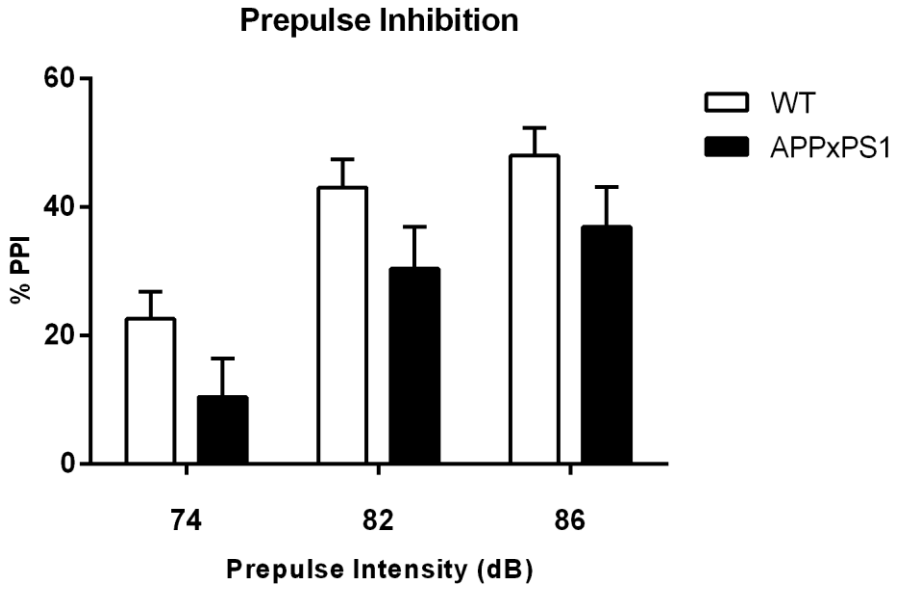


(A and B) Sociability (A) and social recognition memory (B) in the social preference test: (A) total time spent in test chambers containing either an unfamiliar mouse (i.e. opponent) or an empty mouse enclosure (i.e. empty) (s); (B) time spent in a test chamber containing either a

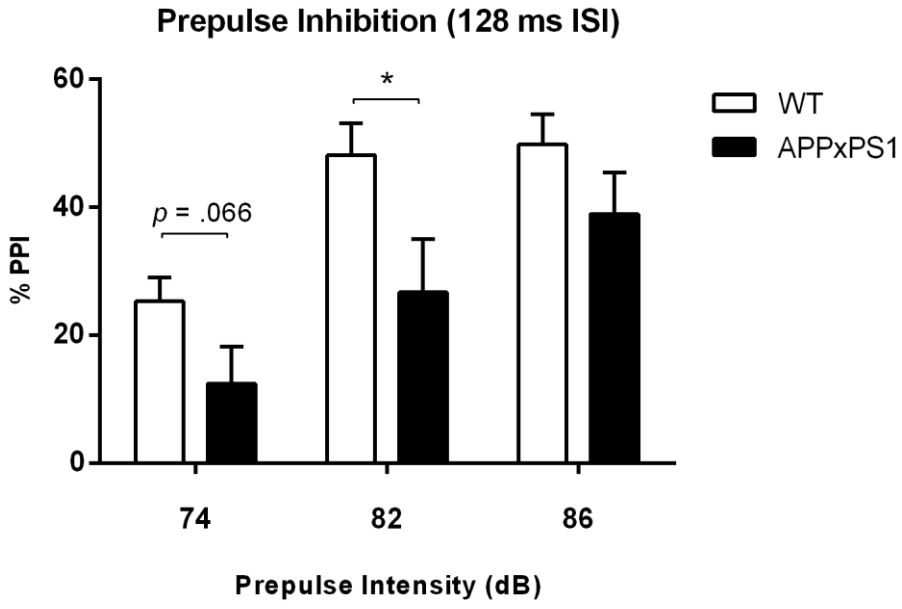
familiar or an unfamiliar (i.e. novel) mouse (s). Data for non-transgenic control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) females are shown as means + SEM. Significant RM ANOVA for 'chamber' are presented by '#' ($^{\#\#}p < .01$ and $^{\#\#\#}p < .001$).

Supplementary Fig. II.

A



B



(A and B) Sensorimotor gating: (A) Percentage prepulse inhibition (%PPI) averaged over all 120 dB startle trials and ISIs for different prepulse intensities (72/82/86 dB) (N = 17–19 per genotype). (B) %PPI for different prepulse intensities (72/82/86 dB) at an interstimulus

interval (ISI) of 128 ms. RM ANOVA revealed a main effect of 'prepulse intensity' [$F(2,68) = 57.9$, $p < .001$], 'ISI' [$F(3,102) = 3.6$, $p < .02$] and an interaction between 'prepulse intensity' and 'ISI' [$F(6,204) = 4.6$, $p < .001$] (all other p's > .05). Data for non-transgenic control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) females are shown as means + SEM. Significant effects of 'genotype' (as analysed by one-way ANOVA) were indicated by '*' (i.e. *$p < .05$).

Chapter 3: Reversal of AD-relevant phenotypes of APP_{Swe}/PS1ΔE9 double transgenic mice

3.1. Chronic cannabidiol treatment improves social and object recognition in double transgenic APP_{Swe}/PS1ΔE9 mice

Publication III

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Declaration

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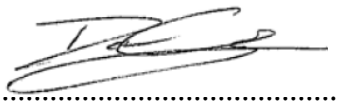

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David Cheng

Chronic cannabidiol treatment improves social and object recognition in double transgenic APP_{swe}/PS1ΔE9 mice

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Abstract

Rationale Patients suffering from Alzheimer's disease (AD) exhibit a decline in cognitive abilities including an inability to recognise familiar faces. Hallmark pathological changes in AD include the aggregation of amyloid-β (Aβ), tau protein hyperphosphorylation as well as pronounced neurodegeneration, neuroinflammation, neurotoxicity and oxidative damage. *Objectives* The non-psychoactive phytocannabinoid cannabidiol (CBD) exerts neuroprotective, antioxidant, and anti-inflammatory effects and promotes neurogenesis. CBD also reverses Aβ-induced spatial memory deficits in rodents. *Materials and methods* Thus we determined the therapeutic-like effects of chronic CBD treatment (20 mg/kg, daily intraperitoneal injections for 3 weeks) on the APP_{swe}/PS1ΔE9 (APPxPS1) transgenic mouse model for AD in a number of cognitive tests, including the social preference test, the novel object recognition task and the fear conditioning paradigm. We also analysed the impact of CBD on anxiety behaviours in the elevated plus maze. *Results* Vehicle-treated APPxPS1 mice demonstrated impairments in social recognition and novel object recognition compared to wild type-like mice. Chronic CBD treatment reversed these cognitive deficits in APPxPS1 mice without affecting anxiety-related behaviours. *Conclusions* This is the first study to investigate the effect of chronic CBD treatment on cognition in an AD transgenic mouse model. Our findings suggest that CBD may have therapeutic potential for specific cognitive impairments associated with AD.

Keywords: Alzheimer's disease, novel therapeutic, cannabidiol, transgenic APP_{swe}/PS1ΔE9 mice, cognition, behaviour, social recognition memory, object recognition memory

Introduction

Alzheimer's disease (AD) is the most prominent form of dementia. Patients suffering from AD demonstrate a decline in general cognitive ability including mild to severe memory loss, social withdrawal, an inability to recognise familiar faces and increased incidences of wandering (Chung and Cummings 2000; Reisberg et al. 1982). AD is characterised by two pathological hallmarks: 1) the aggregation of amyloid-β (Aβ) protein forming plaque deposits; and 2) tau protein hyperphosphorylation, resulting in neurofibrillary tangle formation (Gotz and Ittner 2008). Neurodegeneration, neuroinflammation, neurotoxicity and oxidative damage are also prominent in post-mortem brain tissue of AD patients (Barger and Basile 2001; Koppel and Davies 2008; Marchalant et al. 2008; Pomara et al. 1992; Pratico and Sung 2004; Williams et al. 2006).

Existing treatments for AD such as acetylcholinesterase inhibitors and *N*-methyl-*D*-aspartate (NMDA) receptor antagonists provide short-term relief for cognitive and functional decline but are generally ineffective against disease progression (Benito et al. 2007; Marchalant et al. 2008; Micale et al. 2007). Using compounds which target different aspects of AD pathology simultaneously (i.e. multimodal drug approach) may provide increased therapeutic benefits for patients compared to the more traditional interventions (Farlow et al. 2010; Tariot et al. 2004). Targeting the endocannabinoid (EC) system might be such an approach. The EC system has recently emerged as a possible therapeutic target for patients with AD, as it appears to be a neuroprotective system that responds to neurotoxic insult, including Aβ deposition [for review, see (Karl et al. 2012b)]. In particular, the phytocannabinoid cannabidiol (CBD), a non-psychoactive component of *cannabis sativa*, promises potential for the multimodal treatment of AD due to its neuroprotective, anti-inflammatory and antioxidant properties (Booz 2011; Iuvone et al. 2009; Krishnan et al. 2009; Scuderi et al. 2011; Zuardi 2008). Based on these properties, CBD may be able to counter many pathological symptoms

of AD, and indeed a number of *in vitro* studies have shown that CBD treatment attenuates Aβ-induced neurotoxicity and cell death (Iuvone et al. 2004), tau protein-induced hyperphosphorylation (Esposito et al. 2006), and promotes hippocampal and adult neurogenesis (Esposito et al. 2011; Wolf et al. 2010).

Importantly, only a few studies have investigated the therapeutic potential of CBD *in vivo* and only in pharmacological rodent models for AD (i.e. intraventricular injection of Aβ). Treatment with CBD reduced Aβ-induced neuroinflammation (Esposito et al. 2007; Esposito et al. 2011), rescued spatial memory deficits in the Morris water maze (MWM) and promoted microglial migration, a cellular mechanism that may enable the removal of Aβ deposits (Martin-Moreno et al. 2011).

Thus the multimodal nature of CBD suggests that CBD might be effective in treating a range of AD-relevant behaviours and brain pathologies. In the current study, we investigated for the first time the effectiveness of chronic CBD treatment to diminish behavioural deficits of an established transgenic mouse model of familial AD. Familial AD (early onset, autosomal dominant) is caused by mutations in one of three genes: amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) (Gotz and Ittner 2008). The double transgenic APP_{swe}/PS1ΔE9 (APPxPS1) mouse model we utilised exhibits accelerated amyloid pathology due to the co-expression of APP and PS1 mutant genes (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b; Machova et al. 2010). We have previously reported that male APPxPS1 mice demonstrated a social recognition deficit and increased anxiety-like behaviour by the age of 6 – 7 months (Cheng et al. 2013), while other studies report impairment in object recognition in female APPxPS1 mice (Donkin et al. 2010; Jardanhazi-Kurutz et al. 2010). In the present study, we hypothesised that chronic treatment with CBD will reverse the behavioural and cognitive deficits of male APPxPS1 transgenic mice. To investigate this, we placed APPxPS1 transgenic males on a 3-week CBD treatment schedule

before assessing them in a battery of behavioural and cognitive tests: the social preference test (SPT), the elevated plus maze (EPM), the novel object recognition task (NORT) and the fear conditioning paradigm (FC).

Materials and methods

Animals

Double transgenic mice expressing chimeric mouse/human *APP* (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and mutant human *PS1* (PS1/ΔE9) mice (APPxPS1) were obtained from Jackson Laboratory (Bar Harbor, USA; stock no. 004462, line 85) and maintained as double hemizygotes on C57BL/6JxC3H/HeJ background as described previously (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b; Jankowsky et al. 2001). Male transgenic mice (APPxPS1; N = 23) and their non-transgenic littermates [wild type-like (WT); N = 22] were bred and group-housed in independently ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). Test mice were transported to Neuroscience Research Australia (NeuRA) at around 10 weeks of age, where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) with corn cob bedding (Bed-O'Cobs: Able Scientific, Perth, Australia), a red transparent, polycarbonate igloo (certified mouse igloo from Bio-Serv, Frenchtown, NJ, USA), and some tissues for nesting. Mice were kept under a 12: 12-h light/ dark schedule [light phase between 0830 – 2030 h: white light (illumination: 124 lx) – dark phase: red light (illumination: < 2 lx)]. Environmental temperature was automatically regulated at 21 ± 1 °C and relative humidity was 40-60%. Food (Rat and Mouse Maintenance Pellets: Gordon's Specialty Stockfeeds, Yanderra, Australia) and water were provided *ad libitum*, except where specified. Adult, Male A/JArc mice from the Animal Resources Centre (Canning Vale, Australia) were used as standard opponents in the social preference test. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Drug preparation and administration

Powdered cannabidiol (CAS: 13956-29-1 THC Pharm GmbH, Frankfurt/Main, Germany) was dissolved in equal amounts of Tween 80 (Sigma-Aldrich Co., St Louis, USA) and 100% ethanol to the appropriate concentration and diluted with 0.9% sodium chloride as published previously (Long et al. 2010; Long et al. 2012). Ethanol and tween 80 comprised 10% of the total volume. A vehicle (control) treatment was set up similarly without the addition of CBD. Vehicle and CBD (20mg/kg body weight) were administered by intraperitoneal (i.p.) injection (injection volume of 10ml/kg body weight). The dose was chosen based on a previous study in a pharmacologically induced Aβ mouse model (Martin-Moreno et al. 2011).

Mice were assigned to either vehicle or CBD groups and treated daily for 3 weeks prior to the start of the behavioural assessment, beginning at 24 weeks of age (± 1 week). This treatment design was selected as the only other study evaluating the therapeutic potential of chronic CBD treatment for AD also used a 3-week treatment design and found that CBD could reverse Aβ-induced spatial memory deficits in a pharmacological model of AD (Martin-Moreno et al. 2011). Furthermore, therapeutic-like effects of CBD in mouse models of schizophrenia have been found after 3 weeks of CBD treatment (Long et al. 2010; Long et al. 2012). Body weight of animals was monitored weekly. Treatment continued until the end of behavioural testing when mice were 32 weeks of age (± 2 weeks). The total duration of treatment was 8 weeks. Injections occurred post-testing in the afternoon (between 1400 – 1600 h) to avoid acute effects of CBD administration modifying the behavioural performance of APPxPS1 and WT mice (Deiana et al. 2012) (for treatment details see Table 1).

Behavioural test battery

Mice were tested in a battery of tests sensitive to detect behavioural and cognitive deficits in APPxPS1 mice (Cheng et al. 2013; Karl et al. 2012a; Logge et al. 2012) (for test details see Table 1). All tests were conducted early in the light phase between 0930 – 1400 h except for the elevated plus maze, which was conducted late in the dark phase of the light cycle (0500 - 0830 h). An inter-test interval of at least 48 h was used to minimise the effect of repeated testing. Equipment and apparatus were cleaned between trials using 70% ethanol except where specified.

Social preference test (SPT): The SPT was used to assess sociability and social recognition memory (Moy et al. 2004) and performed as described in an earlier study assessing baseline behaviours of APPxPS1 males (Cheng et al. 2013). Test animals were isolated for 1 h prior to the start of testing. During the habituation trial, mice were allowed to explore a three-chamber apparatus, consisting of a centre chamber (length: 9 cm; width: 18 cm; depth: 20 cm) and two outer chambers (16 cm × 18 cm × 20 cm), freely for 5 min. For the sociability test an unfamiliar standard opponent (male A/J mouse) was placed in one of two animal enclosures (i.e. opponent chamber) in a quasi-randomised fashion (mouse enclosures allowed nose contact between mice but prevented fighting). The test mouse was returned to the apparatus and allowed to explore all three chambers and the animal enclosures for 10 min. Finally, test animals were observed in a 10 min social recognition test. For this, a second, unfamiliar standard opponent was placed in the previously empty chamber so that the test mouse had the choice to explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse. The inter-trial interval (ITI) was 5 min. The chambers and enclosures were cleaned with 30% ethanol in-between trials and fresh corn cob bedding was added to the chambers prior to each test trial. ANY-maze^{TM} (Stoelting, Wood Dale, USA) tracking software was used to determine the time spent in the different chambers, number of entries and distance travelled by the test mice in each trial. Animals were excluded as non-

performers if they climbed on top of the enclosures for more than 30 s during testing. A total of 7 animals (*N*) were excluded (3 WT-VEH, 2 APPxPS1-VEH, 1 WT-CBD, 1 APPxPS1-CBD) leaving *N* = 8-11 animals per test condition.

Elevated plus maze (EPM): The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated and open area (Montgomery 1955; Montgomery and Monkman 1955) and was employed to investigate potential anxiolytic-like effects of chronic CBD treatment. Indeed, CBD may exert acute anxiolytic effects that are detectable by the EPM (Guimaraes et al. 1990; Onaivi et al. 1990). The '+' apparatus consisted of two alternate open arms (35 cm x 6 cm; without side walls; highly illuminated: 70 lx) and two alternate enclosed arms (35 cm x 6 cm; height of enclosing walls 28 cm; dimly illuminated: 10 lx) connected by a central platform (6 cm x 6 cm), and was elevated 70 cm above the floor. Mice were placed at the centre of the '+' of the grey PVC plus maze [for further details of apparatus see (Karl et al. 2008)] facing an enclosed arm and were allowed to explore the maze for 5 min. The time spent and distance travelled in the open and enclosed arms were recorded using ANY-maze™ tracking software.

Novel object recognition test (NORT): Object recognition memory in the NORT is demonstrated by the animals ability to distinguish between familiar and unfamiliar objects [as rodents have an innate preference towards novelty (Dere et al. 2007)]. The NORT was conducted over 3 days [as described previously (Logge et al. 2012)]: two trials (10 min per trial) were conducted per day with a 1 h inter-trial interval (ITI). On day 1, mice were habituated to the empty arena during both trials. On day 2, mice were habituated to the empty arena during trial 1 and to two identical objects during trial 2. On the test day, mice were exposed to two identical objects in the sample trial (trial 1; objects distinct from day 2), and a familiar and a novel object in the test trial (trial 2). The objects and their locations were counterbalanced across genotypes. Time spent *nosing* and *rearing* on the objects were

recorded using ANY-maze™ tracking software. The percentage of exploration time (time spent *nosing* + *rearing* objects) for the novel object (% novel exploration) was calculated using [(novel object exploration time / novel + familiar object exploration time) × 100] and indicated short-term object recognition memory. Animals were excluded if they showed low levels of activity (i.e. <15 s of total object exploration time).

Fear conditioning (FC): Fear conditioning assesses hippocampus- and amygdala-dependent associative learning whereby a previously neutral stimulus elicits a fear response after it has been paired with an aversive stimulus. On conditioning day, mice were placed into the test chamber (Model H10-11R-TC, Coulbourn Instruments, USA) for 2 min. Then an 80 dB conditioned stimulus (CS) was presented for 30 seconds with a co-terminating 0.4 mA 2 second foot shock (unconditioned stimulus; US) twice with an inter-pairing interval of 2 min). The test concluded 2 min later. The next day (context test), mice were returned to the apparatus for 7 min. On day 3 (cue test), animals were placed in an altered context for 9 min. After 2 min (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 2 min with the absence of the CS [for more details see (Chesworth et al. 2012; Duffy et al. 2010)]. Time spent *freezing* was measured for all three experimental days using Any-Maze™ software.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to analyse behavioural parameters for main effects of 'genotype' and 'treatment' in all tests. Repeated measures (RM) two-way ANOVA was used to evaluate effects of 'chamber' (SPT) and '1 min block' (FC) as published previously (Logge et al. 2012). Performance in the SPT was also assessed using one sample t-tests to clarify whether the percentage of time spent in the opponent/novel chamber was above chance (50%). Differences were regarded as significant if $p < .05$. Data

are shown as means ± standard error of means (SEM). F-values and degrees of freedom are presented for ANOVAs and significant genotype and treatment effects are shown in figures and tables as '*' ($^{*}p < .05$) and '#' ($^{\#}p < .05$) respectively whereas t-test results for social novelty preference are presented by '+' ($^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$). Analyses were conducted using SPSS 20.0 for Windows.

Results

Cognition

Sociability and social recognition: All mice across treatments demonstrated a preference for sociability in the 3-chamber social preference test. RM ANOVA revealed a significant effect of 'chamber' for time spent in both chamber [$F(1,41) = 74.2$, $p < .001$], where all mice spent significantly more time in a chamber with an opponent over an empty chamber. T-test for percentage of time spent with the novel mouse confirmed that all mice demonstrated significant levels of sociability above chance [WT-VEH: $t(10) = 7.2$, $p < .001$; APPxPS1-VEH: $t(10) = 5.4$, $p < .001$; WT-CBD: $t(10) = 2.4$, $p < .05$; APPxPS1-CBD: $t(11) = 5.8$, $p < .001$] (Fig. 1a).

In the social preference test, RM ANOVA revealed a significant effect of 'chamber', with test mice spending more time with the novel mouse than the familiar mouse [chamber: $F(1,34) = 26.7$, $p < .001$]. Importantly, an effect of 'genotype' for time spent in the chamber with the novel mouse was only found for vehicle-treated, but not CBD-treated, mice with APPxPS1-VEH mice showing reduced time spent with the novel mouse compared to WT-VEH mice [$F(1,34) = 6.6$, $p < .05$] (Fig. 1b). Indeed, one sample T-test revealed that all groups, except APPxPS1-VEH animals, spent a significantly greater percentage of time with the novel mouse than the familiar mouse [WT-VEH: $t(7) = 3.7$, $p < .01$; APPxPS1-VEH: $t(8) = .8$, $p = .4$; WT-CBD: $t(9) = 2.8$, $p < .05$; APPxPS1-CBD: $t(10) = 3.4$, $p < .01$].

Novel object recognition: Two-way ANOVA revealed no overall effects of 'genotype' or 'treatment' [all $p > .05$], but an interaction between 'genotype' and 'treatment' was found for novel object recognition [$F(1,35) = 4.7$, $p < .05$]. To investigate this interaction, data were split by 'treatment' which revealed a deficit in vehicle-treated APPxPS1 mice in novel object recognition compared to WT-VEH mice [$F(1, 19) = 4.5$, $p < .05$; Fig. 2]. No genotype differences were found between CBD-treated mice [$p > .05$]. Furthermore, an effect of

'treatment' was found between APPxPS1-VEH and APPxPS1-CBD groups [$F(1,17) = 4.7$, $p < .05$], giving evidence that CBD treatment restored object recognition in APPxPS1 transgenic mice.

Fear conditioning: All mice responded to the electric foot shocks delivered during the conditioning phase as determined by their vocalisation. Two-way ANOVA found that all mice demonstrated similar amounts of *freezing* behaviour at baseline and during the context test [all $p > .05$] (Table 2). Further, all mice responded with an increase in *freezing* post CS onset [RM ANOVA: $F(1,41) = 13.7$, $p < .001$] regardless of 'genotype' or 'treatment' (Table 2).

Anxiety

EPM testing found no effects of 'genotype' or 'treatment' on anxiety-like measures such as percentage of time spent in the open arms, percentage of distance travelled in the open arms and percentage of open arms entries (all $p > .05$) (Table 3).

Discussion

Our study is the first to investigate the treatment effects of CBD in an established transgenic mouse model of AD. Daily treatment with CBD for 3 weeks rescued social recognition memory and improved object recognition deficits of male APPxPS1 transgenic mice. These effects were specific for recognition memory as CBD had no impact on fear-associated memory or anxiety measures.

The SPT measures both sociability and social recognition in the APPxPS1 transgenic mouse model. Sociability was intact in APPxPS1 mice whereas AD transgenic mice exhibited a clear social recognition memory deficit. This failure to distinguish between novel and familiar social opponents confirms our previous report for untreated male APPxPS1 mice of a similar age (Cheng et al. 2013). Importantly, animals of that study were also tested for their olfactory ability and no differences between transgenic and control mice were detected. The current study shows that chronic CBD treatment reversed the social recognition deficit observed in APPxPS1 mice, which regained a WT-like preference for the novel opponent post-treatment. AD mice have previously been reported to develop impaired social recognition memory, including Thy1-hAPP(Lond/Swe+) transgenic mice (Faizi et al. 2012) and APPxPS1 mice maintained on pure C57BL/6J background (Filali et al. 2011), while AD patients have difficulties recognising familiar faces (Reisberg et al. 1982). Thus CBD's ability to rescue social recognition memory is worthy of further investigation as this therapeutic-like potential might have relevance in clinical settings.

Male APPxPS1 mice also demonstrated deficits in object recognition. Again, CBD treatment improved object recognition memory of APPxPS1 mice, as transgenic mice showed a wild type-like preference for a novel object post CBD administration. Importantly, male APPxPS1 mice have not been assessed for object recognition memory previously, but impaired object recognition has been found in female APPxPS1 mice (Donkin et al. 2010; Jardanhazi-Kurutz

et al. 2010). Furthermore, in support of our findings, male APPxPS1 mice bred on the C57BL/6J background also displayed deficits in object recognition (Yoshiike et al. 2008) as did other double transgenic AD mouse models including the APP(swe)/PS1(L166P) and APP(NLh)/PS1(P264L) lines (Duszczyk et al. 2012; Webster et al. 2013). Impairments in object recognition have been linked to dysregulation of the glutamatergic system (Nilsson et al. 2007) as well as hippocampal and parahippocampal dysfunction (Fernandez et al. 2007). Interestingly, Hallak and colleagues found that CBD interacts with the glutamatergic system (Hallak et al. 2011); the phytocannabinoid augmented the effects of an N-methyl-D-aspartate (NMDA) receptor antagonist in a human study. CBD was also shown to be protective against glutamate neurotoxicity (Hampson et al. 1998). Finally, memantine, another NMDA receptor antagonist improved object recognition in another transgenic AD mouse model (Scholtzova et al. 2008) suggesting that CBD may improve recognition memory via the glutamatergic pathway. It is interesting to note here that facial recognition is not linked to deficits in NMDA-receptor activity (Rammsayer 2001), demonstrating the potential multimodality of CBD.

APPxPS1 transgenic mice did not develop an impairment in fear-associated memory which is line with what we previously observed in male APPxPS1 mice (Cheng et al. 2013). Furthermore, CBD did not impact on this behavioural domain in WT or APPxPS1 transgenic mice. One other study found an effect of subchronic CBD treatment (i.e. 2-week treatment with 10 mg/kg CBD) on *freezing* behaviour in the conditioned emotional response paradigm (ElBatsh et al. 2012). CBD increased the *freezing* response of drug-treated Lister-hooded rats 24 h post conditioning. This phenomenon could be related to an impact of CBD on anxiety (potentially anxiogenic but no baseline *freezing* was evaluated in those rats) or cognitive behaviours (potentially improving contextual fear conditioning). Importantly, ElBatsh and co-workers tested rats not mice and applied ten foot shock-context pairings during the

conditioning phase, which presents a more stressful test protocol than the one utilised in our study. In this context, it is important to mention that chronic CBD treatment did not modify the anxiety response of APPxPS1 mice in the elevated plus maze in the current study and we also could not find an effect of genotype in this paradigm. Previously, we reported increased anxiety in male APPxPS1 mice when we employed a light-phase EPM test (Cheng et al. 2013). Thus, the differences between the earlier study and the current investigation is likely due to the circadian rhythm (animals were tested in the EPM during the dark phase in the current study) and the necessary daily injection procedure for all mice tested. Importantly, the earlier study did not reveal any differences in anxiety-like behaviour in the light-dark test (Cheng et al. 2013), suggesting that the anxiety phenotype of APPxPS1 mice is not only circadian rhythm but also task-dependent. Finally, previous studies reporting an anxiety phenotype in APPxPS1 mice did not specify test conditions (e.g. illumination, test time) thereby making a direct comparison of study outcomes impossible (Lalonde et al. 2004; Reiserer et al. 2007). CBD has previously been found to induce anxiolytic-like properties (Campos and Guimaraes 2008; Campos et al. 2013; Guimaraes et al. 1990; Long et al. 2010; Long et al. 2012; Moreira et al. 2006; Onaivi et al. 1990). However, these effects were predominantly evident after acute treatment regimes (Campos and Guimaraes 2008; Campos et al. 2013; Guimaraes et al. 1990; Long et al. 2010; Moreira et al. 2006; Onaivi et al. 1990) whereas our behavioural testing commenced only after 3-week CBD treatment. Therefore, it is not that surprising that we could not detect an anxiolytic-like effect of CBD treatment on EPM behaviours. Our results are in line with Campos and colleagues, who recently showed that subchronic (i.e. 2-week) CBD treatment (30mg/kg) failed to produce anxiolytic-like effects in the EPM or the novelty suppressed feeding paradigm in control mice (although CBD reversed the anxiogenic effects of 14 days of unpredictable stress (Campos et al. 2013).

In this study we demonstrate for the first time a beneficial effect of chronic CBD treatment on recognition memory in a transgenic mouse model for AD. These findings are novel and expand on earlier work describing the effectiveness of CBD to rescue Aβ-induced spatial memory deficits in wild type-like mice (Martin-Moreno et al. 2011). The same study described a profound effect of CBD on Aβ-induced neuroinflammation (i.e. reduction of interleukin-6, a biomarker for inflammation) and attenuation of microglial activation (Martin-Moreno et al. 2011). Furthermore, other studies reported similar anti-inflammatory effects of CBD in rats and mice (Esposito et al. 2007; Esposito et al. 2011). Importantly, APPxPS1 mice reportedly show elevated levels of neuroinflammation (i.e. increased nitric oxide species and TNF-α) in the hippocampus (Kalifa et al. 2011) and neuroinflammatory processes are thought to be linked to neurodegeneration and cognitive impairment in humans (Johnson et al. 2013). Thus CBD might have improved the cognitive performance of APPxPS1 mice via the inhibition of neuroinflammation in transgenic mice. CBD's anti-inflammatory properties may also be strengthened indirectly by the phytocannabinoid's interaction with the endocannabinoid system (Thomas et al. 2007). Further, a previous study reported that APPxPS1 mice demonstrate impaired neurogenesis from the age of 10 months (Hamilton and Holscher 2012), which may contribute to the impaired cognitive abilities of transgenic mice compared to WT mice. Importantly, CBD has been shown to promote neurogenesis (Campos et al. 2013; Wolf et al. 2010) suggesting that the phytocannabinoid may also affect the cognitive performance of APPxPS1 mice via increasing neurogenesis in these mice. Other currently discussed mechanisms of action for CBD include the enhancement of endocannabinoid-mediated actions, by inhibiting the inactivation of the endocannabinoid anandamide, and the modulation of intracellular Ca^{2+} concentration (for more details see Campos et al. 2012; Hill et al. 2012; Izzo et al. 2009). Future studies are needed to clarify the

mechanisms behind CBD's therapeutic-like effects in general and on AD-relevant pathological brain processes in particular.

In conclusion, our study provides the first evidence of CBD's therapeutic-like potential in a transgenic mouse model for AD. Chronic CBD treatment reversed deficits in social recognition and object recognition, without affecting fear-associated memory or anxiety behaviour. Thus CBD may have therapeutic potential as a treatment for AD patients.

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Figure Legends

Fig. 1 Sociability and social recognition in the social preference test. **(a)** Percentage of time spent [%] in test chambers containing an unfamiliar mouse (i.e. opponent) compared to the total time spent with both the opponent and the empty mouse enclosure (WT-VEH: *N* = 11;APPxPS1-VEH: *N* = 11 - WT-CBD: *N* = 11 - APPxPS1-CBD: *N* = 12); **(b)** Percentage of time spent [%] in test chamber containing an unfamiliar (i.e. novel) mouse compared to the total time spent with both the novel and the familiar mouse (WT-VEH: *N* = 8 - APPxPS1-VEH: *N* = 9 - WT-CBD: *N* = 10 - APPxPS1-CBD: *N* = 11). Data for non-transgenic WT control and double transgenic APPxPS1 male mice in vehicle and CBD groups are shown as means + SEM. One-sample t-test results for novelty preference are presented by '$^{+}$' ($^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$). Abbreviations: APPxPS1, male APP_{Swe}/PS1ΔE9 transgenic mice; CBD, cannabidiol; WT, wild type-like mice

Fig. 2 Novel object recognition task. Time spent *nosing* towards the novel object over the familiar object expressed as a percentage of total time spent interacting with both objects [%]. Data for non-transgenic WT-like control and double transgenic APPxPS1 male mice in vehicle and CBD groups are shown as means + SEM (WT-VEH: *N* = 11 - APPxPS1-VEH: *N* = 11 - WT-CBD: *N* = 11 - APPxPS1-CBD: *N* = 12). Significant 'genotype' effects are indicated with '*' ($*p < .05$), and 'treatment' effects are indicated with '$^{\#}$' ($^{\#}p < .05$). Abbreviations: APPxPS1, male APP_{Swe}/PS1ΔE9 transgenic mice; CBD, cannabidiol; WT, wild type-like mice

Tables and Figures

Table 1: Treatment schedule and test order of APPxPS1 and WT mice

Treatment	Vehicle		CBD	
Genotype	WT *(N = 11)*	APPxPS1 *(N = 11)*	WT *(N = 11)*	APPxPS1 *(N = 12)*
Age at start of treatment	25 ± 1	25 ± 1	24 ± 1	25 ± 1
Mice received daily i.p. injections of 20 mg/kg CBD for 3 weeks prior to behavioural testing				
Social Preference Test	28 ± 1	28 ± 1	27 ± 1	28 ± 1
EPM	29 ± 1	29 ± 1	28 ± 1	29 ± 1
NORT	30 ± 1	30 ± 1	29 ± 1	30 ± 1
Fear conditioning	32 ± 2	33 ± 3	32 ± 2	33 ± 2

Age [weeks] of APPxPS1 mice and their WT counterparts at the start of treatment, throughout behavioural tests and at the end of treatment (total duration of CBD treatment was eight weeks)

APPxPS1, male APP_{Swe}/PS1ΔE9 transgenic mice; CBD, cannabidiol; WT, wild type-like mice; EPM, elevated plus maze; NORT, novel object recognition task;

Table 2: Fear-associated memory

	Vehicle		CBD	
	WT	APPxPS1	WT	APPxPS1
Baseline (first 2 min)				
Conditioning *freezing* [s]	9.7 ± 3.7	4.3 ± 1.4	4.7 ± 1.5	7.7 ± 2.2
Context *freezing* [s]	26.1 ± 5.5	23.3 ± 6.5	23.9 ± 7.2	35.9 ± 8.0
Cue *freezing* [s]	20.9 ± 3.2	21.5 ± 4.8	21.8 ± 3.8	34.3 ± 6.2
Context				
Total time spent *freezing* [s]	87.9 ± 17.4	92.4 ± 22.3	89.8 ± 19.8	117.7 ± 25.0
Cue				
Time spent *freezing* 1 min prior to cue onset [s]	13.1 ± 1.7	16.3 ± 3.7	15.2 ± 2.4	21.7 ± 3.3
Time spent *freezing* 1 min post cue onset [s]	21.5 ± 3.8	20.8 ± 4.9	23.4 ± 4.3	26.4 ± 3.9

Time spent *freezing* [s] during baseline, context test and the cue test is shown for non-transgenic WT control and double transgenic APPxPS1 male mice treated with either vehicle or CBD. Data are presented as mean ± SEM (WT-VEH: $N = 11$ - APPxPS1-VEH: $N = 11$ - WT-CBD: $N = 11$ - APPxPS1-CBD: $N = 12$)

APPxPS1, APP_{Swe}/PS1ΔE9 transgenic mice; CBD, cannabidiol; WT, wild type-like mice

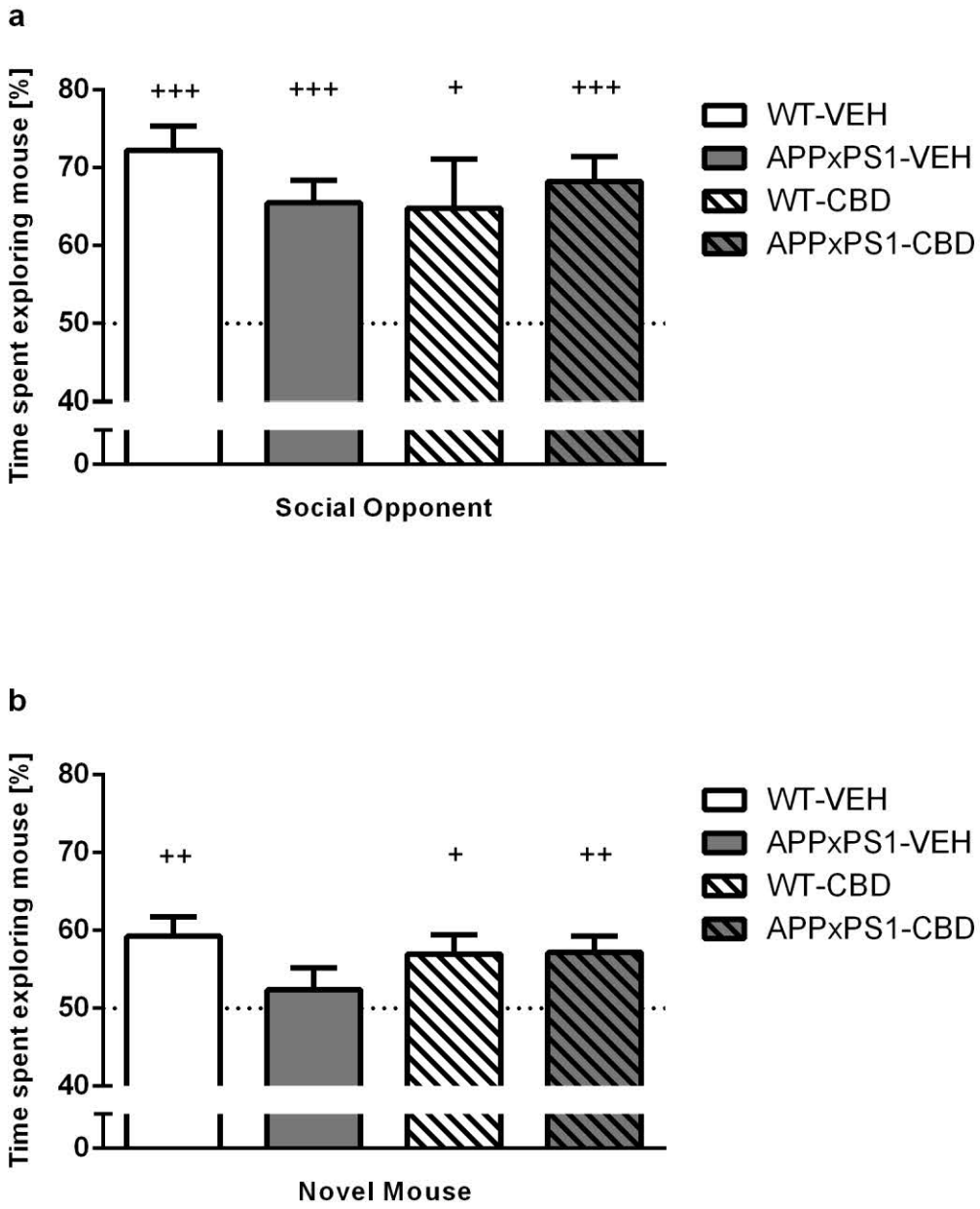
Table 3: Anxiety-like behaviours

	Vehicle		CBD	
	WT	APPxPS1	WT	APPxPS1
Time spent on open arms [%]	5.7 ± 2.7	4.1 ± 1.0	4.7 ± 2.3	7.2 ± 2.1
Time spent on open arms [s]	11.0 ± 5.2	7.9 ± 1.9	8.1 ± 3.3	12.3 ± 3.5
Entries into open arms [%]	15.5 ± 3.5	16.4 ± 2.0	15.5 ± 4.0	20.2 ± 2.5
Distance travelled on open arms [%]	4.1 ± 1.8	3.7 ± 1.0	3.5 ± 1.3	5.7 ± 3.1
Total distance travelled [m]	7.1 ± .6	8.3 ± .6	7.2 ± .5	8.0 ± .6

Anxiety-like behaviours in the EPM of non-transgenic WT control and double transgenic APPxPS1 male mice treated with either vehicle or CBD. Data are presented as mean ± SEM (WT-VEH: $N = 11$ - APPxPS1-VEH: $N = 11$ - WT-CBD: $N = 11$ - APPxPS1-CBD: $N = 12$)

APPxPS1, APP_{Swe}/PS1ΔE9 transgenic mice; CBD, cannabidiol; EPM, elevated plus maze; WT, wild type-like mice

Figure 1


a
Time spent exploring mouse [%]
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Social Opponent
WT-VEH
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WT-CBD
APPxPS1-CBD
b
Time spent exploring mouse [%]
80
70
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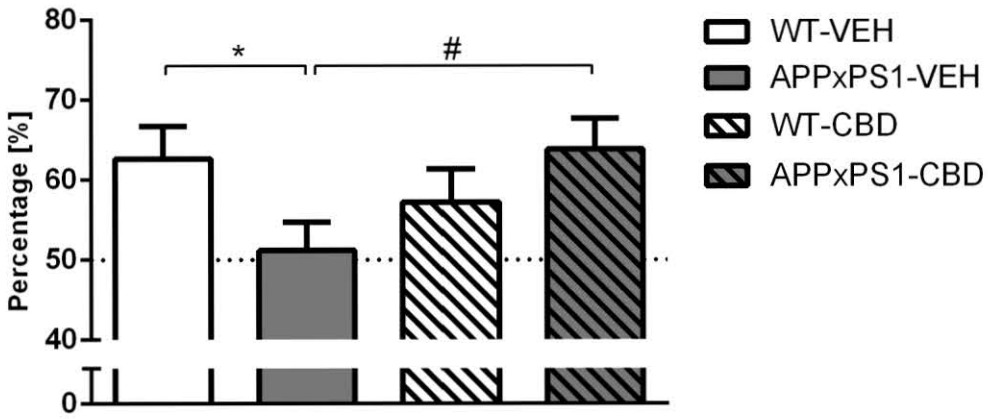
Novel Mouse
WT-VEH
APPxPS1-VEH
WT-CBD
APPxPS1-CBD

Figure 2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APPxPS1-CBD

Chapter 4: Preventing the development of AD-relevant phenotypes of APP_{Swe}/PS1ΔE9 double transgenic mice

4.1. Long-term cannabidiol treatment prevents social recognition deficit in APP_{Swe}/PS1ΔE9 transgenic mice

Publication IV

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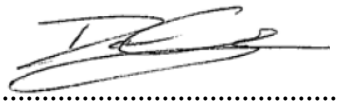

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David Cheng

Running title: Protective effects of cannabidiol

Long-term cannabidiol treatment prevents the development of social recognition memory deficits in Alzheimer's disease transgenic mice.

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Abstract

Impairments in cognitive ability and widespread pathophysiological changes caused by neurotoxicity, neuroinflammation, oxidative damage and altered cholesterol homeostasis are associated with Alzheimer's disease (AD). Cannabidiol (CBD) has been shown to reverse cognitive deficits of AD transgenic mice and to exert neuroprotective, antioxidative and anti-inflammatory properties *in vitro* and *in vivo*. Here we evaluate the preventative properties of long-term CBD treatment in male *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) mice, a transgenic model of AD. Control and AD transgenic mice were treated orally from 2.5 months of age with CBD (20 mg/kg) daily for 8 months. Mice were then assessed in the social preference test (SPT), elevated plus maze (EPM) and fear conditioning (FC) paradigms, before cortical and hippocampal tissues were analysed for amyloid load, oxidative damage, cholesterol, phytosterols and inflammation. We found that AβPPxPS1 mice developed a social recognition deficit, which was prevented by CBD treatment. CBD had no impact on anxiety or associative learning. The prevention of the social recognition deficit was not associated with any changes in amyloid load or oxidative damage. However, the study revealed a subtle impact of CBD on neuroinflammation, cholesterol and dietary phytosterol retention, which deserves further investigation. This study is the first to demonstrate CBD's ability to prevent the development of a social recognition deficit in AD transgenic mice. Our findings provide the first evidence that CBD may have potential as a preventative treatment for AD with a particular relevance for symptoms of social withdrawal and facial recognition.

Keywords: Alzheimer's disease, transgenic *AβPP$_{Swe}$/PS1ΔE9* mice, cannabidiol, social recognition memory, behaviour, amyloid load, oxidative stress, cholesterol, neuroinflammation, phytosterol

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, which is associated with progressive memory loss. Other behavioural and cognitive symptoms include social withdrawal, poor facial recognition ability, increased motor agitation and likelihood of wandering [1, 2]. AD is characterised by two main post-mortem pathological hallmarks; amyloid-β (Aβ) protein aggregation forming plaque deposits and tau protein hyperphosphorylation resulting in neurofibrillary tangles. Microglia, the resident immune cells of the central nervous system, are activated for the phagocytosis of Aβ [3-5], but impaired clearance or reuptake of Aβ results in the release of inflammatory cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and chemokines, that cause neuroinflammation. Brain tissue damage is further exacerbated by the release of glutamate and reactive oxygen and nitrogen species, resulting in neurotoxicity and oxidative damage respectively [6]. Increased oxidative stress may be an early indication of AD risk [7, 8]. Disturbances in brain cholesterol metabolism are associated with the major pathological features of AD (including Aβ and tau pathology). In particular, decreased cholesterol synthesis correlates with the severity of neurodegeneration and dementia [9, 10], while late-stage AD patients also show decreased cholesterol circulation [11, 12]. Interestingly, dietary phytosterols (or plant sterols) found naturally in many foods (such as vegetable oils, nuts, grains and grain-derived products) [13] can either interfere with critical functional processes in AD or decrease amyloidogenic processing [14]. Some phytosterols may even be relevant additional biomarkers for AD [15].

Current treatments available to AD patients do not slow the progression of the disease and only offer limited benefits for the cognitive abilities of patients (reviewed in [16]). Thus, it is important to explore novel alternative treatment strategies. The phytocannbinoid cannabidiol (CBD) may be a potential new candidate for AD therapy (for review see [17]). CBD is derived from the *cannabis sativa* plant and is devoid of psychoactive properties. It has neuroprotective, anti-inflammatory, and antioxidative properties [18-22], thereby countering a number of AD-relevant pathological

symptoms. *In vitro* studies have found that CBD prevents Aβ-induced tau protein hyperphosphorylation [23], neurotoxicity [23, 24], attenuates cell death, and promotes neurogenesis in mouse hippocampal cells [25, 26]. These biological functions of CBD promise therapeutic value for the neurodegenerative and neurotoxic components of AD. Indeed, *in vivo* studies reported that CBD reduced Aβ-induced neuroinflammation in rats and mice [25, 27] and rescued learning deficits in the Morris water maze in a pharmacological mouse model of AD [28]. The memory restoring properties of CBD were linked to a reduction in microglial activation and pro-inflammatory cytokines (i.e. decreased IL-6) [28].

Current research suggests existing interventions may be administered too late in the disease process when the damage caused by AD pathology is already too severe [17, 29, 30]. Thus, in the current study, we evaluated for the very first time the effectiveness of long-term oral CBD treatment to prevent the development of cognitive deficits and AD-relevant brain pathophysiology in an established transgenic mouse model of familial AD [31]. The double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) mouse model co-expresses mutant amyloid-β precursor protein (AβPP) and presenilin 1 (PS1) genes [31-34]. Amyloid plaques are found as early as at 4 months of age in these AD transgenic mice [35]. Our past research established that male AβPPxPS1 mice demonstrate social recognition deficits, increased anxiety, and task-specific hyperlocomotion whereas sensorimotor gating and spatial memory were intact at 10-12 months of age [36]. Importantly, we also demonstrated recently that 3-weeks of CBD treatment effectively reversed the social and object recognition memory deficits of AβPPxPS1 males [37]. In the present study male AβPPxPS1 mice were treated with CBD (20 mg/kg) or vehicle using a daily voluntary oral administration scheme for 8 months beginning at 2.5 months of age when AD-like pathophysiology is still sparse (i.e. no Aβ burden reported for 4-month old AβPPxPS1 mice: [35]). Following this, mice were assessed in social recognition memory, associative memory (i.e. fear conditioning) and anxiety, before brain samples were analysed for amyloid load, oxidative damage (i.e. markers of cerebral lipid oxidation), cholesterol levels as well as dietary phytosterols, and neuroinflammation markers. We

selected the cytokines TNF-α and IL-1β as both have been most strongly implicated in the promotion of AD pathology in humans and in AD transgenic mouse models [38, 39]. Furthermore, inflammation driven by these cytokines is attenuated by CBD [27, 28].

2. Methods

2.1 Animals

Double transgenic mice expressing chimeric mouse/human AβPP (Mo/HuAβPP695swe/Swedish mutations K595N/M596L) and mutant human PS1 (PS1/ΔE9) were obtained from Jackson Laboratory [Bar Harbor, USA; strain name: B6C3-Tg(*AβPP$_{Swe}$/PS1ΔE9*)85Dbo/Mmjax; stock no. 004462] and maintained as hemizygotes on the congenic C57BL/6JxC3H/HeJ background as described previously [31-33, 40]. Male double transgenic mice (AβPPxPS1) and their non-transgenic littermates (WT) were bred and group-housed in independently ventilated cages (Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). Test mice were transported to Neuroscience Research Australia (NeuRA) at around 10 weeks of age, where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) with corn cob bedding (PuraCob Premium: Able Scientific, Perth, Australia) and some tissues for nesting. Mice were kept under a 12:12 h light:dark schedule [light phase: white light (illumination: 210 lx); lights on 0700 – 1900 h]. Environmental temperature was automatically regulated at 21 ± 1 °C and relative humidity was 40-60%. Food (Gordon's Rat and Mouse Maintenance Pellets: Gordon's Specialty Stockfeeds, Yanderra, Australia) and water were provided *ad libitum*, except where specified. Adult, male A/J mice from Animal Resources Centre (Canning Vale, Australia) were placed in the animal enclosures as standard opponents for the social preference test. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Drug treatment

Powdered cannabidiol (CAS: 13956-29-1, THC Pharm GmbH, Frankfurt/Main, Germany) was used at a dose of 20 mg/kg body weight, based on previous studies evaluating the behavioural properties of different doses of CBD [41] and the effectiveness of 20 mg/kg CBD to reverse spatial memory

deficits of a pharmacological mouse model of AD [42]. Although chronic administration of CBD appears to be well tolerated by transgenic mice [37, 43], the stress of chronic injections may impact behavioural, cognitive and/or physiological results. Thus, the gel pellet preparation and the oral administration regime were adapted from Zhang and colleagues [44, 45]: CBD or vehicle were dissolved in a highly palatable, sweetened and chocolate flavoured gel pellet, and administered at a volume of 8 ml/kg body weight. Due to the insolubility of CBD in water, CBD was first dissolved in 100% ethanol and an equal amount of Tween 80 (Sigma-Aldrich Co., St Louis, USA), then vortexed vigorously. CBD was dissolved in gel pellets with a final composition of 2.0% ethanol, 2.0% Tween 80, 15.2% Splenda (Splenda Low Calorie Sweetener: Johnson & Johnson Pacific Pty, Broadway, Australia), 8.7% gelatine (Davis Gelatine: GELITA Australia Pty, Josephville, Australia), 20.1% chocolate flavouring (Queen Flavouring Essence Imitation Chocolate: Queen Fine Foods Pty, Alderley, Australia) and 52.0% water for irrigation. Vehicle gel pellets were identical but contained no CBD. Mice were initially habituated to vehicle gel pellets in their home cages for seven days. Following this, the mice were isolated within their home cages for the treatment by placing a plastic divider in the home cage. Then animals were given either a vehicle or a CBD gel pellet (treatments were quasi-randomised), which they consumed within 2-5 mins. The plastic divider was removed once mice had consumed the gel pellets. Mice were treated daily, late in the afternoon, to avoid potential acute effects of CBD confounding test outcomes (Table 1).

2.3 Behavioural phenotyping

Starting at 10 months of age, mice were tested in a number of behavioural tests (Table 1), with an inter-test interval of at least 48 h as described earlier (N = 8-14 mice per genotype/treatment) [36, 46, 47]. All tests were conducted during the first 5 h of the light phase to minimise effects of circadian rhythm.

2.3.1 Social preference test (SPT)

The SPT was used to assess sociability and social recognition memory [48] and performed as described previously [36, 37]. Test animals were isolated for an hour prior to the start of testing. During the habituation trial, mice were allowed to freely explore a three-chamber apparatus, consisting of a centre chamber (length: 9 cm; width: 18 cm; depth: 20 cm) and two outer chambers (16 cm × 18 cm × 20 cm), freely for 5 min. For the sociability test an unfamiliar standard opponent (male A/J mouse) was placed in one of two animal enclosures (i.e. opponent chamber) in a quasi-randomised fashion (mouse enclosures allowed nose contact between mice but prevented fighting). The test mouse was returned to the apparatus and allowed to explore all three chambers and the animal enclosures for 10 min. Finally, test animals were observed in a 10 min social recognition test. For this, a second, unfamiliar standard opponent was placed in the previously empty chamber so that the test mouse had the choice to explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse. The inter-trial interval (ITI) was 5 min. The chambers and enclosures were cleaned with 30% ethanol in-between trials and fresh corn cob bedding was added to the chambers prior to each test trial. AnyMaze^{TM} (Stoelting, Wood Dale, USA) tracking software was used to determine the time spent in the different chambers, number of entries and distance travelled by the test mice in each trial. Two mice (1 WT-VEH and 1 WT-CBD) were excluded from the sociability test due to recording issues.

2.3.2 Elevated plus maze (EPM)

The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated and open area [49, 50] and was employed to determine potential effects of chronic CBD treatment on anxiety behaviour. The '+' apparatus consisted of two alternate open arms (35 cm x 6 cm; without side walls) and two alternate enclosed arms (35 cm x 6 cm; height of enclosing walls 28 cm) connected by a central platform (6 cm x 6 cm), elevated 70 cm above the floor. Mice were placed at the centre of the '+' of the grey PVC plus maze (for further details of apparatus see [51]) facing an enclosed arm and were allowed to explore the maze for 5 min. The time spent on open arms, the percentage of entries onto open arms over total arm entries

(open arm entries) and the distance travelled on the open and enclosed arms were recorded using AnyMaze™ tracking software. One mouse was excluded (AβPPxPS1-CBD group) for falling off the apparatus.

2.3.3 Fear Conditioning (FC)

FC assesses hippocampus- and amygdala-dependent associative learning whereby a previously neutral stimulus elicits a fear response after it has been paired with an aversive stimulus. On conditioning day, mice were placed into the test chamber (Model H10-11R-TC, Coulbourn Instruments, USA) for 2 min. An 80 dB conditioned stimulus (CS) was presented twice for 30 seconds with a co-terminating 0.4 mA 2 second foot shock (unconditioned stimulus; US) with an inter-pairing interval of 2 min. The test concluded 2 min later. The next day (context test), mice were returned to the apparatus for 7 min. On day 3 (cue test), animals were placed in an altered context for 9 min. After 2 min (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 2 min, absent the CS (for more details see [52, 53]). Time spent *freezing* was measured on all three experimental days using Any-Maze™ software.

2.4 Biochemical analyses

Mice were anaesthetised and blood was collected through cardiac puncture. Blood samples were centrifuged (5000 rpm, 5 min, 4 °C) in a microcentrifuge (Model No. 5415 R, Eppendorf, Hamburg, Germany), and the plasma fraction was collected and stored at -80 °C. Euthanised mice were perfused with phosphate buffered saline (PBS) transcardially as described previously [54]. Brains were sagittally divided and the right hemisphere was snap frozen in liquid nitrogen before being stored at -80 °C. Cortex and hippocampal samples were dissected and weighed on dry ice prior to biochemical analyses [Sample numbers for ELISA and GC-MS were: $N = 8$ for WT-vehicle, $N = 10$ for AβPPxPS1-vehicle, $N = 10$ for WT-CBD and $N = 10$ for AβPPxPS1-CBD].

2.4.1 Aβ enzyme-linked immunosorbent assay (ELISA) for Aβ pathology

Frozen cortex (20-30mg) and hippocampal samples (~5mg) were homogenised and prepared as TBS soluble, and guanidine HCl (gHCl) soluble (TBS insoluble) fractions and stored at -80 °C as described previously [54]. Both TBS-soluble and gHCl-soluble fractions were used in enzyme-linked immunosorbent assay (ELISA) to investigate the effect of CBD on Aβ levels in transgenic mice. Protein was quantified using the bicinchoninic (BCA assay) method.

$A\beta_{40}$ and $A\beta_{42}$ protein in TBS-soluble and gHCl-soluble fractions of brain homogenates were quantified using Beta Amyloid x-40 and x-42 ELISA kits (Cat No. SIG-38954 and SIG-38956 respectively, Covance, Emeryville, USA) as described previously [54, 55].

2.4.2 Gas chromatography-mass spectroscopy (GC-MS) for cholesterol, oxidative damage and CBD plasma levels

An Agilent 7000B triple quadrupole mass selective detector interfaced with an Agilent 7890A GC system gas chromatograph, equipped with an automatic sampler and computer workstation (Agilent Technologies, Santa Clara, USA) was used to analyse markers of oxidative damage in the cortical samples and CBD presence in plasma samples. GC-MS triple quadrupole provided very high analytical sensitivity for all analytes measured. Limits of detection (LOD: 0.05 ng/ml) were significantly less (at least 10-fold) than the levels of each analyte measured in plasma and brain. 150 µl of plasma were used for the analysis of CBD. The concentration obtained from the GC-MS was therefore multiplied by a factor of 6.67 to give the total amount of CBD per ml of plasma as shown in the Results section. The injection port and GC-MS interface were kept at 270 °C and separations were carried out on a fused silica capillary column (20 m x 0.18 mm i.d. x 0.18 m film thickness, Restek Rxi-5 ms). Helium was the carrier gas with a flow rate of 0.8 ml/min (average velocity = 59 cm/s).

2.4.3 F_2-isoprostanes, oxidised sterols (oxysterols) and cholesterol

Frozen cortex samples (10-20 mg) were homogenised and hydrolysed overnight for GC-MS analysis as described previously [56]. Samples were loaded into solid phase extraction columns (UCT CUQAX223 3 ml; United Chemical Technologies, Bristol, USA). Sterols and oxysterols,

arachidonic acid, DHA and F_2-isoprostanes were eluted from the SPE column separately. The sterol/oxysterol fractions were derivatised in 20 µl acetonitrile and 20 µl Selectra-SIL BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] containing 1% TMCS (trimethylchlorosilane; United Chemical Technologies, Bristol, USA) prior to GC-MS analysis. Quantification of cholesterol oxidation products (COP) was as previously described [57]. Cholesterol was quantified using lathosterol-d_6 heavy isotope standard in a separate (0.6 µl split ratio 25:1). Relative molar response factors of all analytes were calculated from calibration curves constructed from different concentrations in triplicate. The F_2-isoprostane and fatty acid fractions were prepared and analysed by GC-MS as described previously [56]. Quantification of F_2-isoprostanes and fatty acids were calculated by comparison of specific SRM transitions with their corresponding heavy isotope internal standards.

2.4.4 Quantification of CBD in plasma

Concentration of cannabidiol in plasma was quantified using the GC-MS as previously described with slight modifications [58, 59]. Plasma samples (150µl) were treated using sodium acetate buffer pH 4.0, with MTBE and Hexane (1:1 v/v), rotated for 30 min and centrifuged at 1500 rpm for 2 min at 4 °C, dried down, derivatised in 20 µl BSTFA and 20 µl 1% TMCS and incubated at 70 °C for 30 min. Derivatised samples were dried down, reconstituted in 40 µl of toluene and analysed using the GC-MS (1 µl splitless). MRM was performed using EI mode similar to sterol analysis. Column temperature was held for 1 min and increased 40 °C/min to 210 °C, then 20 °C/min to 300 °C and held for 4 min. Quantification of CBD was calculated by comparison with specific MRM transitions corresponding with its heavy isotope internal standard (CBD-d3, Lipomed, Arlesheim, Switzerland).

2.4.5 Inflammatory markers (quantitative polymerase chain reaction)

RNA extraction: Frozen cortex samples (10-20mg) were homogenised in Tri-reagent (TRIzol Reagent, cat no. 15596-018, Life Technologies, Mulgrave, VIC, Australia) as described previously [57]. RNA levels were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher

Scientific, Scoresby, Australia), and diluted in RNase-free water to obtain a concentration of 0.5µg/µl. cDNA was synthesised using a Tetro cDNA Synthesis kit (Bioline, Alexandria, Australia), according to manufacturer instructions. The SensiFAST SYBR No-ROX kit (Bioline, Alexandria, Australia) was used to determine levels of inflammatory markers. Template concentration was 100ng (1:10 dilution; 1µl cDNA). Forward and reverse primers for interleukin-1β (IL-1β; forward, 5′-CAACCAACAAGTGATATTCTCCATG-3; reverse, 5′-GATCCACACTCTCCAGCTGCA-3′), and tumour necrosis factor-α (TNF-α; forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′; reverse: 5′-TGGGAGTAGACAAGGTACAACCC-3′) were used as biomarkers for the quantification of inflammation in transgenic mice, with β-actin as a housekeeping gene. Polymerase chain reaction (PCR) assays were reacted (3-step cycling; IL-1β: 45 cycles; TNF-α: 50 cycles) and analysed using Roche LightCycler 480 (Roche Diagnostics, Castle Hill, Australia). Three mice were excluded (1x WT-CBD, 1x AβPPxPS1-vehicle and 1x AβPPxPS1-CBD) as outliers (2 standard deviations away from mean).

2.5. Statistical analyses

One-way ANOVA was used to analyse effect of 'treatment' on Aβ levels in AβPPxPS1 mice. Two-way analysis of variance (ANOVA) was used to analyse behavioural parameters and biochemical data obtained for oxidative damage, CBD levels and quantification of inflammation for main effects of 'genotype' and 'treatment' in all tests. Repeated measures (RM) ANOVA was used to evaluate the effects of 'chamber' (SPT) and '1 min block' (FC) as published previously [36, 47]. Performance in the SPT was also assessed using one sample *t*-tests to clarify whether the percentage of time spent in the opponent/novel chamber was greater than chance (50%). Differences were regarded as significant if $p < .05$. Data are shown as means ± standard error of means (SEM). F-values and degrees of freedom are presented for ANOVAs and significant 'genotype' and 'treatment' effects are shown in figures and tables as '*' ($^{*}p < .05$, $^{**}p < .01$) and '#' ($^{\#}p < .05$)

respectively whereas RM-ANOVA results for social novelty preference are presented by '$^{+}$' ($^{+}p <$ .05, $^{++}p < .01$, $^{+++}p < .001$). Analyses were conducted using SPSS 20.0 for Windows.

3. Results

3.1 Behaviour

3.1.1 Sociability and social recognition

RM ANOVA revealed an effect of 'chamber' [$F(1,39) = 197.9$, $p < .001$] (Fig. 1A). All mice spent more time investigating the social opponent over the empty chamber, indicating intact sociability for all mice regardless of genotype and treatment. *T*-tests for percentage of time spent with the novel mouse confirmed that all mice demonstrated significant levels of sociability above chance [WT-VEH: $t(6) = 7.4$, $p < .001$; AβPPxPS1-VEH: $t(13) = 6.2$, $p < .001$; WT-CBD: $t(8) = 12.3$, $p < .001$; AβPPxPS1-CBD: $t(12) = 8.6$, $p < .001$] (data not shown).

In the SPT, RM ANOVA revealed a significant effect of 'chamber' for time spent investigating the novel over the familiar mouse [$F(1,41) = 23.6$, $p < .001$]. Importantly, a significant interaction between 'genotype' and 'treatment' was found [$F(1,41) = 4.8$, $p < .05$], where only vehicle-treated AD transgenic mice did not develop a preference for the novel mouse (Fig.1B). Two-way ANOVA also revealed a trend towards an effect of CBD treatment [$F(1,41) = 3.1$, $p = .09$]. Indeed, ANOVA split by 'genotype' revealed that CBD increased the time AD transgenic mice spent with the novel mouse [$F(1,25) = 5.0$, $p < .05$], with no such effect observed in WT mice [$F(1,16) = .2$, $p = .7$] (Fig. 1B) showing that CBD had a beneficial effect on social recognition memory. *T*-tests confirmed that all animals, except vehicle-treated AβPPxPS1 mice, spent a significantly greater percentage of time with the novel mouse than the familiar mouse [WT-VEH: $t(7) = 2.5$, $p < .05$; AβPPxPS1-VEH: $t(13) = .3$, $p = .8$; WT-CBD: $t(9) = 3.3$, $p < .01$; AβPPxPS1-CBD: $t(12) = 3.7$, $p < .01$] (data not shown).

3.1.2 Anxiety

AβPPxPS1 transgenic mice demonstrated WT-like locomotion and anxiety ($p > .05$ for total distance travelled, time spent on open arms and open arm entries). Chronic treatment with CBD had no effect on EPM behaviours (all $p > .05$; Table 2).

3.1.3 Associative learning

All mice responded to the electric foot shocks during conditioning (i.e. vocalisation detected in all mice). Two-way ANOVA found transgenic mice demonstrated increased amounts of *freezing* at baseline (i.e. first 2 mins pre-conditioning) regardless of treatment [$F(1,41) = 4.5$, $p < .05$]. However, *freezing* duration during the first 2 min of the context and cue trials was similar for all mice across test conditions (all $p > .05$; Table 3) and all mice exhibited intact context memory regardless of treatment [$p > .05$; Fig. 2A]. Furthermore, memory of the cue was intact as all animals showed increased *freezing* post cue presentation [RM ANOVA: $F(1,41) = 52.9$, $p < .001$], regardless of 'genotype' or 'treatment' (Fig. 2B and Table 3).

3.2. Brain pathophysiology

3.2.1 Amyloid load

One-way ANOVA revealed that CBD had no effect on soluble and insoluble $A\beta_{40}$ or $A\beta_{42}$ in the cortex of AβPPxPS1 mice, although insoluble $A\beta_{42}$ was slightly higher after CBD treatment ['treatment': Soluble $A\beta_{40}$: $F(1,18) = .3$, $p = .6$; Insoluble $A\beta_{40}$: $F(1,18) = 2.4$, $p = .1$; Soluble $A\beta_{42}$: $F(1,18) = .1$, $p = .7$; Insoluble $A\beta_{42}$: $F(1,18) = 3.5$, $p = .08$] (Table 4). Similarly, Aβ levels remained unchanged after CBD treatment in the hippocampus ['treatment': Soluble $A\beta_{40}$: $F(1,17) = .4$, $p = .6$; Insoluble $A\beta_{40}$: $F(1,18) = 1.1$, $p = .3$; Soluble $A\beta_{42}$: $F(1,15) = .3$, $p = .6$; Insoluble $A\beta_{42}$: $F(1,18) = .1$, $p = .7$] (Table 4).

3.2.2 Oxidative damage

Total F_2-isoprostanes (free and esterified corrected for arachidonic acid; AA) were not significantly altered in AβPPxPS1 mice when compared to their WT littermates, regardless of 'treatment' (all $p > .05$) (Table 5). We also measured the levels of oxysterols in the cortex. For enzymatically oxidised sterols, AβPPxPS1 mice demonstrated significantly decreased overall levels of 24-hydroxycholesterol compared to WT littermates ['genotype': $F(1,34) = 4.9$, $p < .05$], whereas 'treatment' had no effect on sterols [$F(1,34) = .07$, $p = .8$] and no 'genotype' by 'treatment' interactions were found. No differences were found across all four groups for 27-

hydroxycholesterol, and the reactive species oxidised sterols, 7β-hydroxycholesterol and 7-ketocholesterol (all $p > .05$) (Table 5).

3.2.3 Cholesterol

Cholesterol was increased in cortical tissues of AβPPxPS1 mice compared to WT animals [$F(1,34) = 12.1$, $p < .01$] and CBD increased cholesterol levels [$F(1,34) = 11.0$, $p = .01$]. Further one-way ANOVA revealed that cholesterol was significantly higher in vehicle-treated AD transgenic mice [$F(1,16) = 7.7$, $p < .05$] compared to control mice, while CBD increased the cholesterol levels in WT mice [$F(1,16) = 25.1$, $p < .001$] but not AβPPxPS1 mice [$F(1,18) = 1.3$, $p = .3$] (Table 5).

Two-way ANOVA revealed a significant 'genotype' by 'treatment' interaction for the cortical levels of the dietary phytosterol, brassicasterol [$F(1,34) = 6.1$, $p < .05$], which was caused by CBD increasing brassicasterol levels in AβPPxPS1 mice only [WT: $F(1,16) = .5$, $p = .5$ AβPPxPS1: $F(1,18) = 6.9$, $p < .05$; Table 5]. Furthermore, the analysis detected a 'genotype' effect in CBD-treated mice [vehicle: $F(1,16) = .2$, $p = .7$, CBD: $F(1,18) = 9.9$, $p < .01$; Table 5]. The dietary phytosterol, campesterol was also increased in AβPPxPS1 mice [$F(1,34) = 4.4$, $p < .05$]. More specifically, cortical campesterol was elevated in CBD-treated AβPPxPS1 mice [vehicle: $F(1,16) = .2$, $p = .6$, CBD: $F(1,18) = 9.0$, $p < .01$]. There was also a trend for CBD to increase the cortical levels of this phytosterol in transgenic mice [WT: $F(1,16) = .0$, $p = 1.0$, AβPPxPS1: $F(1,18) = 3.4$, $p = .08$; Table 5]

3.2.4 Inflammatory markers

Two-way ANOVA revealed no significant differences in the levels of mRNA for two inflammatory cytokine markers across test conditions ['genotype': IL-1β: $F(1,39) = 1.0$, $p = .3$ - 'TNF-α': $F(1,39) = 1.1$, $p = .3$] (Fig. 3A-B). There was no significant effect of 'treatment' on these cytokines [IL-1β: $F(1,39) = 1.3$, $p = .3$ - TNF-α: $F(1,39) = 2.5$, $p = .1$] either, although cytokine levels of CBD-treated AβPPxPS1 mice appeared closer to corresponding WT levels than levels of vehicle-treated AD transgenic mice (Fig. 3A-B).

3.2.5 CBD plasma levels

Two-way ANOVA revealed that all mice treated with CBD demonstrated significantly increased levels of plasma CBD (ng/ml) [WT-CBD: 2.1 ± 0.6; AβPPxPS1-CBD: 1.9 ± 0.4 - 'treatment': $F(1,30) = 21.3$, $p < .001$]. No significant 'genotype' differences or interactions were found (all $p > .05$). CBD could not be detected in mice that were treated with vehicle (values < 0.05 ng/ml).

4. Discussion

Our study demonstrates for the first time the effects of long-term oral CBD treatment on the social recognition memory and pathophysiology of a double transgenic AβPPxPS1 mouse model for AD. We provide first evidence of a possible impact of CBD on dietary phytosterols, which can exert beneficial effects on cognition. We also suggest that the therapeutic effect of CBD may be linked to neuroinflammatory processes or changes in cholesterol but further research using additional CBD doses will be necessary to clarify this.

The SPT determined that vehicle-treated AβPPxPS1 mice exhibit a social recognition memory deficit, confirming our earlier findings [36, 37]. Importantly, long-term CBD treatment prevented this social recognition deficit from occurring in AβPPxPS1 mice. We previously found that intraperitoneal administration of CBD for three weeks reversed this cognitive deficit in the same AD mouse model [37]. Other recent studies also report social recognition deficits in AD transgenic mouse models, providing evidence for the increasing relevance of social recognition memory testing for AD research [60, 61]. Anxiety can confound the performance of mice in cognitive tests [62] and acute CBD has been found to modify anxiety-related behaviours [41, 63-67]. However, the AβPPxPS1 transgene did not influence anxiety parameters nor did CBD treatment.

The beneficial effect of CBD on social recognition memory was not associated with a direct effect on Aβ levels. Insoluble and soluble levels of $A\beta_{40}$ and $A\beta_{42}$ were no different between vehicle and CBD-treated AβPPxPS1 mice in cortex and hippocampus. Similarly, another study described improvements in spatial memory in *$A\beta PP_{Swe}/PS1\Delta E9$* mice on a C57BL/6J background, which was not accompanied by changes in Aβ levels [68]. The same study also found that levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in the parietal cortex did not correlate with cognitive deficits [68]. Nonetheless, various *in vitro* studies show CBD can attenuate Aβ-induced processes [23-26], reverse Aβ-induced memory impairments in rodents [28] and reduce Aβ formation [69].

The *in vivo* formation of isoprostanes is a marker for cerebral lipid oxidation and directly correlated with an increase in oxidative stress [7, 70-72]. Patients with AD are also known to have increased

concentrations of F_2-isoprostanes in CSF even prior to disease diagnosis [7, 8, 71]. Levels of oxidation were not significantly altered in AβPPxPS1 mice in comparison to their age-matched WT littermates, nor did we detect changes in the level of lipid oxidation in the cortex of CBD-treated animals, despite its known antioxidant properties [73, 74]. These findings may be due to age as nucleic acid oxidation is significantly higher in 3 and 5-month old AβPPxPS1 mice compared to age-matched control mice. Importantly, this phenomenon is not evident in 10 and 15 month old mice, which is the age when brain tissue was collected for the current study [75].

Cholesterol was increased in AβPPxPS1 mice compared to WT mice, while CBD treatment increased cholesterol levels in WT mice. Our finding of increased cholesterol in AβPPxPS1 mice could indicate either an impaired reuptake process, or a compensatory mechanism for protection against neurodegeneration in AD mice. Maintenance of sufficient cholesterol is important to help combat synapse loss and neurodegeneration [76] and such a response is consistent with the reduced levels of 24-OH cholesterol detected in the AβPPxPS1 mice compared to WT. Formation of 24-OH cholesterol is the major pathway of cholesterol removal from the brain [77]. Insufficient amounts of cholesterol may interrupt essential processes such as myelin formation, synaptic transmission and cognitive ability in mice [78, 79], while a reduction in oxysterols has been shown to correlate with the severity of dementia and brain atrophy [9, 10, 80]. Interestingly, 8-month old *AβPP$_{Swe}$/PS1ΔE9* mice on a pure C57BL/6J background did not demonstrate significantly different levels of cholesterol, while 15-month old transgenic mice had significantly lower cholesterol levels compared to control mice [81]. It is noteworthy that decreased levels of cholesterol in cerebral spinal fluid and plasma have been found in patients with AD [11, 12].

Phytosterols are present naturally in a variety of different foods, including grains (e.g. sorghum and bran) found in mouse food pellets. CBD increased the levels of brassicasterol and campesterol in AβPPxPS1 mice. The accumulation and long-term consumption of dietary phytosterols do not interfere with memory [82, 83]. On the other hand, dietary supplementation of a fish oil-rich diet with phytosterols reduced insoluble $Aβ_{42}$ in *AβPP$_{Swe}$/PS1ΔE9* mice on a C57BL/6J background

[68], while the phytosterol stigmasterol attenuated scopolamine-induced spatial memory deficits of mice [84]. These findings suggest a potentially beneficial effect of increased phytosterol levels for cognitive symptoms in the AD brain. It is possible that CBD interacted with AD pathophysiology by increasing the retention of specific phytosterols. Further research needs to be conducted in order to understand the effect of increased phytosterol levels in AD brains and how CBD might be involved in these processes.

Daily long-term administration of 20 mg/kg CBD did not result in a statistically significant effect. However, AβPPxPS1 mice have previously been shown to exhibit elevated levels of neuroinflammation (increased nitric oxide species and TNF-α) in the hippocampus [85] and inflammatory changes have been linked to impaired spatial memory of AβPPxPS1 mice [86]. Furthermore, Martin-Moreno and colleagues have demonstrated that Aβ-induced neuroinflammation was decreased after CBD treatment [28]. Thus, we suggest that CBD might be able to combat increased inflammation in AβPPxPS1 mice thereby impacting on the cognitive performance of these mice. Future research should consider additional CBD doses to determine the effects of long-term CBD treatment on neuroinflammation in AD mouse models. Furthermore, an escalating CBD dosage regime could be used as the dosage of AD-approved treatments is often increased over time as the condition of patients deteriorates [87].

In conclusion, our study is the first to demonstrate that long-term CBD treatment can prevent the development of a social recognition deficit in AβPPxPS1 mice. The findings suggest the mechanism involved in this prevention may be linked to CBD-induced retention of dietary phytosterols or neuroinflammatory processes in the brain of AD mice. We provide the first evidence that CBD has potential to be used as a long-term preventative treatment option for AD and may be especially relevant for symptoms of social withdrawal and facial recognition. The behavioural inertness of CBD and the fact that CBD is well tolerated in humans [88, 89] suggests that preclinical research findings could easily be followed up in clinical trials. Future studies using cytokine arrays or an 'omics' approach may reveal which biochemical/genetic pathways contribute

to the beneficial effects of CBD. It will also be important to clarify what receptors mediate the therapeutic-like effects of CBD: the peroxisome proliferator-activated receptor-γ [25, 69], N-methyl-D-aspartate receptors [90, 91], and receptors of the endocannabinoid system [92] are promising targets.

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7. Figure Legends

Figure 1A-B: Sociability and social recognition were measured using the social preference test. Graphs show total time spent [s] in test chambers by the test mice containing A) either an unfamiliar mouse (i.e. opponent) or an empty mouse enclosure (i.e. empty); or B) either a familiar or an unfamiliar (i.e. novel) mouse. Data for non-transgenic wild type-like control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) male mice after vehicle or cannabidiol (CBD) treatment are shown as means + SEM. Significant 'treatment' effects are indicated with '#' (#$p < .01$). RM ANOVA for novelty preference are presented by '$^{+}$' ($^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$).

Figure 2A-B: Fear-associated learning was assessed in the fear conditioning test. Time spent *freezing* during A) the context test and B) the cue test for each is shown per '1 min bin'. Data for non-transgenic control wild type-like (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) mice after vehicle or cannabidiol (CBD) treatment are shown as means ± SEM.

Figure 3A-B: Neuroinflammation markers in cortical tissue. Quantitative PCR was used to measure the concentration of A) interleukin-1β and B) TNF-α derived from the cortex of control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) male mice after vehicle or cannabidiol (CBD) treatment. Concentrations [ng/µl] are presented as mean ± SEM.

8. Tables and Figures

Treatment	Vehicle		CBD	
Genotype	WT	AβPPxPS1	WT	AβPPxPS1
Age at start of treatment	91.5 ± 11.5	97.1 ± 18.3	89.0 ± 8.2	95.9 ± 12.5
Number of days treated prior to start of testing	*228.1 ± 38.5*	*234.8 ± 31.3*	*226.1 ± 33.0*	*237.8 ± 35.5*
Social Preference Test	319.6 ± 34.5	331.9 ± 41.5	315.1 ± 30.8	333.8 ± 38.6
Elevated plus maze	324.1 ± 34.8	336.0 ± 41.7	319.3 ± 31.3	338.1 ± 38.9
Y-Maze	326.9 ± 34.5	338.4 ± 41.5	321.8 ± 31.2	340.6 ± 38.6
Fear conditioning	329.9 ± 34.5	341.4 ± 41.5	324.8 ± 31.2	343.6 ± 38.6
Tissue collection	333.1 ± 34.8	345.0 ± 41.7	328.3 ± 31.3	347.1 ± 38.9
Total Days of treatment	*241.6 ± 38.9*	*247.9 ± 31.6*	*239.3 ±33.4*	*251.2 ± 35.8*

Table 1: Age of *$A\beta PP_{Swe}/PS1\Delta E9$* (AβPPxPS1) mice and their WT counterparts (in days ± SEM) at the start of treatment, throughout behavioural testing and at the end of treatment.

	Vehicle		CBD	
	WT	AβPPxPS1	WT	AβPPxPS1
Time spent on open arms [s]	7.2 ± 2.7	6.0 ± 2.3	6.7 ± 1.6	7.3 ± 2.7
Entries onto open arms [%]	13.3 ± 4.0	7.8 ± 3.1	11.6 ± 2.1	11.2 ± 2.7
Total distance travelled [m]	7.1 ± 0.9	8.0 ± 1.4	7.2 ± 0.9	7.9 ± 1.0

Table 2: Anxiety-related behaviours (i.e. time spent on and entries onto open arms) and locomotion (total distance travelled) in the elevated plus maze (EPM). Parameters for wild type-like control mice (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPPxPS1) mice after vehicle or cannabidiol (CBD) treatment are shown as mean ± SEM.

	Vehicle		CBD	
	WT	AβPPxPS1	WT	AβPPxPS1
Baseline (first 2 min)				
Conditioning *freezing* [s]	2.3 ± 1.1	5.8 ± 1.7	4.7 ± 1.5	8.5 ± 1.9
Context *freezing* [s]	32.0 ± 7.8	47.1 ± 7.4	37.0 ± 6.0	36.7 ± 7.0
Context				
Total time spent *freezing* [s]	132.2 ± 29.6	152.3 ± 28.1	131.6 ± 15.4	150.7 ± 25.8
Cue				
Time spent *freezing* 2 min prior to cue onset [s]	18.0 ± 5.9	29.1 ± 5.7	25.5 ± 5.2	28.6 ± 4.4
Time spent *freezing* 2 min post cue onset [s]	$40.4 \pm 7.3^{+++}$	$37.4 \pm 5.8^{+}$	$38.7 \pm 6.7^{+}$	$40.4 \pm 5.2^{+}$

Table 3: Fear-associated memory in the fear conditioning paradigm. *Freezing* (i.e. time spent *freezing* [s]) at baseline, and during context test and cue test for non-transgenic wild type-like control (WT) and double transgenic *$A\beta PP_{Swe}$/PS1ΔE9* (AβPPxPS1) male mice after vehicle or cannabidiol (CBD) treatment are presented as mean ± SEM. Significant effects of cue presentation on *freezing* response are indicated by '$^{+}$' ($^{+}p < .05$ and $^{+++}p < .001$).

AβPPxPS1	Vehicle	CBD
Cortex		
Soluble $A\beta_{40}$ [pg/mg]	1033.2 ± 211.2	904.6 ± 118.8
Soluble $A\beta_{42}$ [pg/mg]	654.3 ± 102.6	613.4 ± 65.8
Insoluble $A\beta_{40}$ [pg/mg]	8184.6 ± 701.0	9758.3 ± 751.6
Insoluble $A\beta_{42}$ [pg/mg]	25601.4 ± 2138.6	30897.8 ± 1847.6
Hippocampus		
Soluble $A\beta_{40}$ [pg/mg]	464.4 ± 99.3	390.8 ± 69.6
Soluble $A\beta_{42}$ [pg/mg]	155.7 ± 196.8	196.8 ± 54.1
Insoluble $A\beta_{40}$ [pg/mg]	9854.5 ± 2217.7	12776.1 ± 1738.7
Insoluble $A\beta_{42}$ [pg/mg]	22295.1 ± 7937.3	26370.0 ± 8467.1

Table 4: Amyloid-β. Soluble and insoluble amyloid load in double transgenic *$A\beta PP_{Swe}$/PS1ΔE9* (AβPPxPS1) male mice after vehicle or cannabidiol (CBD) treatment are shown as means ± SEM.

	Vehicle		CBD	
	WT	AβPPxPS1	WT	AβPPxPS1
Oxidised sterols				
Reactive species oxidised				
7β-hydroxycholesterol [ng/mg]	0.29 ± 0.02	0.31 ± 0.05	0.30 ± 0.02	0.32 ± 0.02
7-ketocholesterol [ng/mg]	0.53 ± 0.04	0.50 ± 0.04	0.47 ± 0.03	0.57 ± 0.05
Enzymatically oxidised				
24-hydroxycholesterol [ng/mg]	38.5 ± 2.8	34.2 ± 3.0	39.4 ± 2.7	31.9 ± 1.8 *
27-hydroxycholesterol [pg/mg]	48.3 ± 5.3	38.9 ± 5.7	46.1 ± 2.5	43.4 ± 4.6
F_2-isoprostanes (normalised for arachidonic acid)				
Total [pg/ng]	10.0 ± 1.0	8.1 ± 0.5	9.1 ± 0.4	8.3 ± 0.5
Cholesterol				
Total cholesterol [ng/mg]	20.5 ± 1.0	29.6 ± 2.8 *	29.3 ± 1.4 $^{\#\#\#}$	33.1 ± 1.4
Dietary phytosterol				
Brassicasterol [pg/mg]	51.2 ± 3.8	48.3 ± 6.1	46.7 ± 4.9	70.4 ± 5.8 $^{**\ \#}$
Campesterol [ng/mg]	15.1 ± 1.8	16.5 ± 2.3	15.0 ± 1.7	21.2 ± 1.1 *

Table 5: Oxysterols, F_2-isoprostanes, cholesterol and phytosterol levels. Oxidative damage and total cholesterol in the cortex of non-transgenic wild type-like control (WT) and double transgenic *$A\beta PP_{Swe}$/PS1ΔE9* (AβPPxPS1) male mice after vehicle or cannabidiol (CBD) treatment. Concentrations (in ng and pg) are presented as mean ± SEM. Significant effects of 'genotype' are indicated by '*' ($^{*}p < .05$ and $^{**}p < .01$) and effects of 'treatment' by '#' ($^{\#}p < .05$ and $^{\#\#\#}p < .001$)

Figure 1A

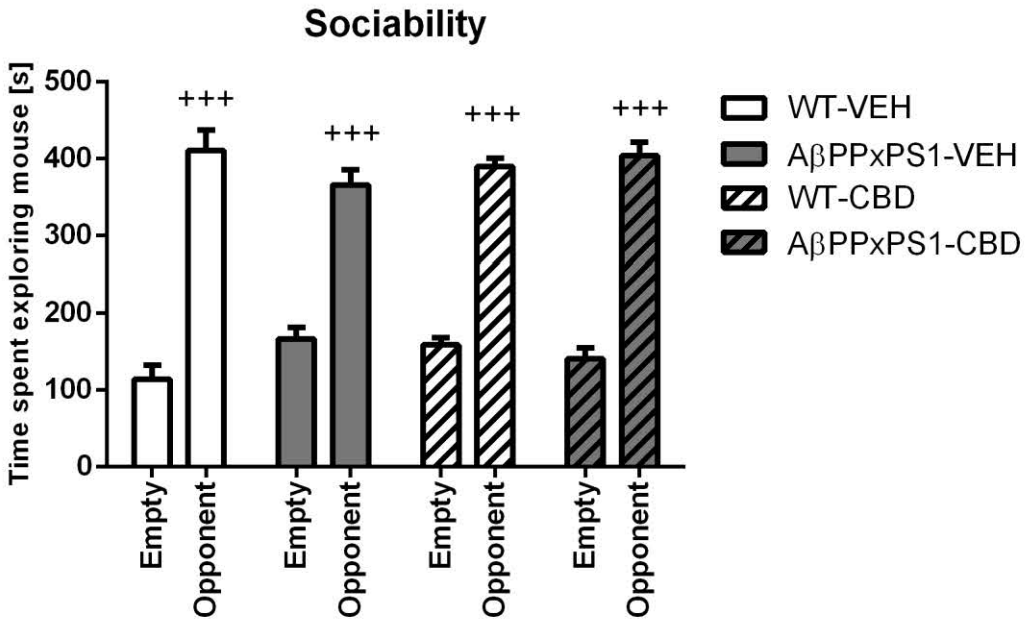


Figure 1B

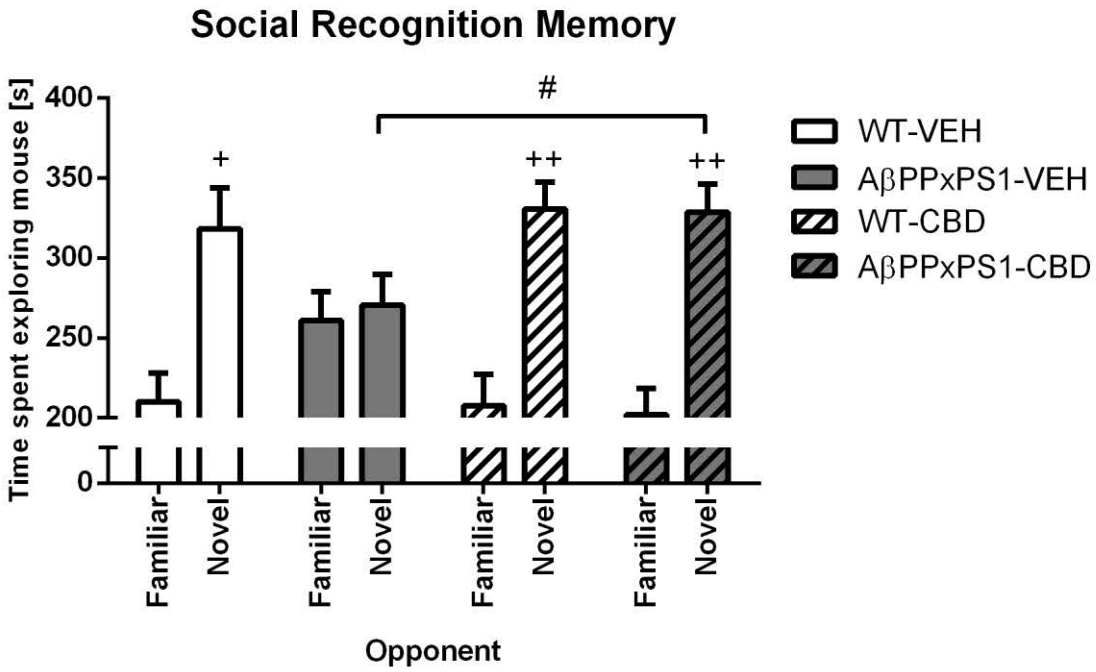


Figure 2A

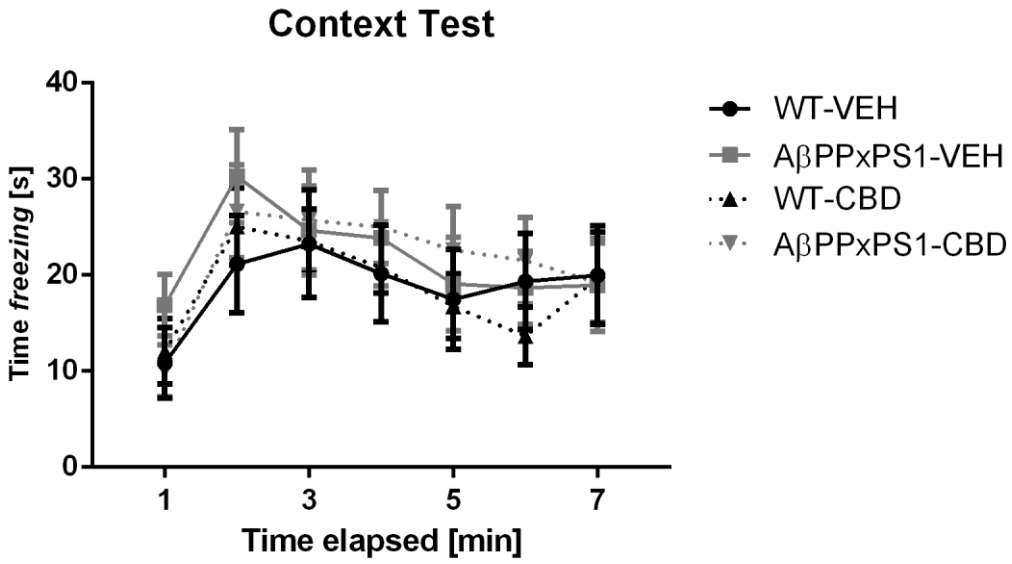


Figure 2B

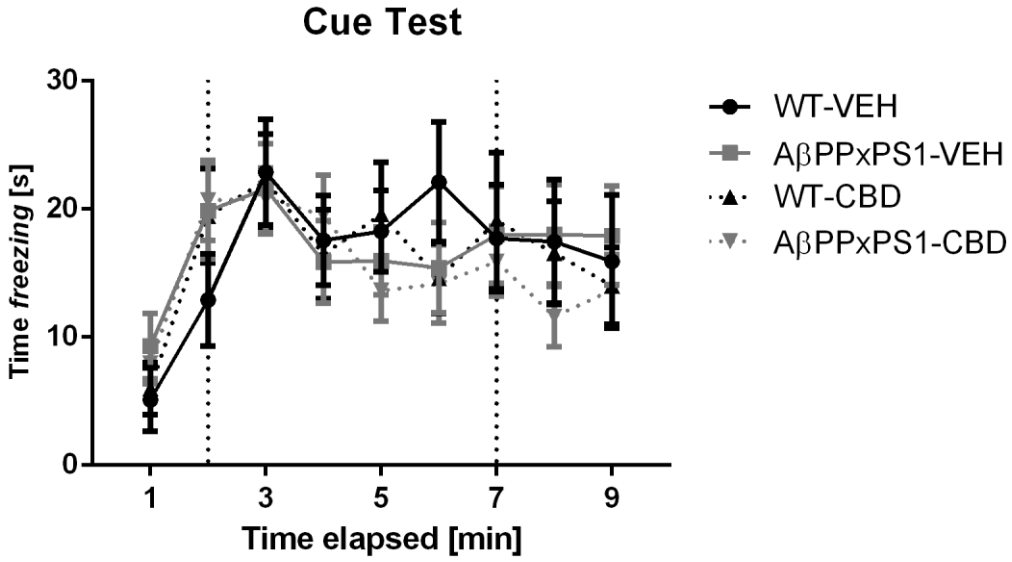


Figure 3A

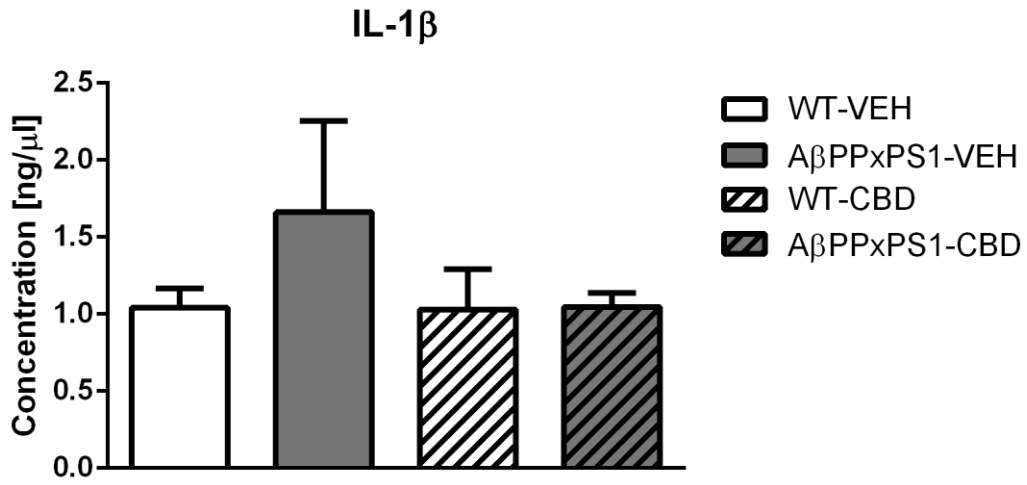
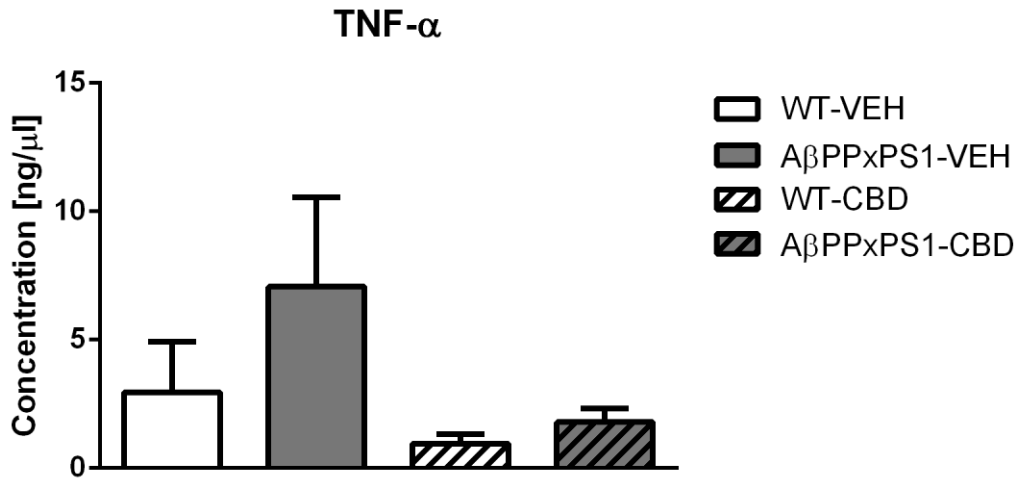


Figure 3B



Chapter 5: General discussion

The research included in this thesis has determined a number of novel findings in relation to the behavioural and cognitive phenotypes of transgenic APPxPS1 mice and the effects that cannabidiol (CBD) treatment exerts on these phenotypes and the AD-relevant pathophysiology of these animals. After carefully reviewing the literature on APPxPS1 transgenic mice, a number of inconsistencies in the behavioural and cognitive phenotypes of these AD transgenic mice were discovered: for example, different ages of onset for spatial memory deficits in male APPxPS1 mice and different types of memory impaired in the Morris water maze (e.g. acquisition *versus* reference memory) (Cao et al. 2007; Gallagher et al. 2013)]. Thus, in a first step, APPxPS1 transgenic mice were evaluated in behavioural tests relevant for AD. As expected, APPxPS1 mice were found to demonstrate AD-relevant cognitive impairments (Section 5.1). Based on a limited number of studies, which have been reviewed by us and others (Iuvone et al. 2009; Karl et al. 2012b; Krishnan et al. 2009), I hypothesised that CBD might present a novel therapeutic candidate for AD, and thus would produce beneficial effects in transgenic mouse models for AD. Indeed, treatment with CBD was able to reverse (section 5.2) and prevent (section 5.3.1) some of the cognitive impairments established in our laboratory. Furthermore, CBD appeared to exert multimodal effects on AD-relevant pathophysiology, including the potential reduction in neuroinflammatory processes (section 5.3.2).

5.1. Summary of findings

5.1.1. Baseline phenotypes of APPxPS1 mice

A number of behavioural and cognitive tests were used to determine the phenotypic repertoire of the APPxPS1 transgenic mouse model. Mice were assessed in social recognition memory (social preference test: SPT), object recognition (novel object recognition task: NORT), spatial memory (cheeseboard task: CB), fear-associated learning (fear conditioning: FC), anxiety (light-dark test and the elevated plus maze: LD, EPM respectively) and sensorimotor gating (prepulse inhibition: PPI). Studies have previously shown that APPxPS1 mice demonstrate disrupted spatial memory (Morris water maze and Barnes maze) and alterations in anxiety-like behaviours, while other AD mouse models have been described to show social recognition deficits (as discussed in Section 1.3). These behavioural characteristics may be relevant for the clinical situation as AD patients experience symptoms of anxiety (Echavarri et al. 2013), disruptions in sensorimotor gating (Jessen et al. 2001) and social withdrawal (Chung and Cummings 2000). Importantly, patients demonstrate cognitive deficits including poor object and facial recognition (Kivisaari et al. 2013; Reisberg et al. 1982), spatial disorientation (Vlcek and Laczo 2014), impaired associative learning and memory, long-term memory and declarative memory (Kivisaari et al. 2013; Woodruff-Pak 2001). Male and female mice were tested separately in this thesis. I will now provide a summary of the major findings obtained from my investigation of the APPxPS1 double transgenic mouse model.

Social recognition memory and performance in the SPT is influenced by sociability of mice and their olfactory abilities (Moy et al. 2004; Moy et al. 2008). The latter are impaired in some AD patients and mouse models of AD (Mesholam et al. 1998; Wesson et al. 2011). Therefore, it was necessary to assess sociability and olfactory ability of

APPxPS1 test mice. I discovered that both male and female APPxPS1 mice demonstrated intact sociability at 7 months of age. Transgenic mice also demonstrated WT-like olfactory abilities, which is in line with another study that reported unaltered olfactory abilities in APPxPS1 mutant mice (Rey et al. 2012). However, I found that male APPxPS1 mice were impaired in social recognition memory while the SPT protocol used was not sensitive enough to assess social recognition in females. Indeed, a previous study had shown that male mice have enhanced olfactory discrimination of social olfactory cues (e.g. urine) in comparison to female mice (Wesson et al. 2006), thus allowing male mice to better distinguish between different social opponents. This could explain why the SPT only worked in male mice. The relevance of an impaired social recognition memory of male AD transgenic mice for AD research has been confirmed in other AD mouse models (Faizi et al. 2012) including APPxPS1 mice on a C57BL/6 background (Filali et al. 2011). Interestingly, this deficit in social recognition memory may mimic the AD condition as patients do not recognise familiar faces (Reisberg et al. 1982), suggesting the AD mouse model possesses face validity (i.e. degree to which the symptomatology of the animal model is similar to the human disease).

I found that 7-month old female transgenic mice demonstrated intact object recognition following an ITI of 1 h in the NORT paradigm. Previous studies found that 10-month old APPxPS1 female mice display object recognition deficits but only when using an ITI of 4 h (Donkin et al. 2010), but not 1 h (Jardanhazi-Kurutz et al. 2010). Male mice were also investigated, but this data was not included in the main findings as the test was unsuccessful for control mice (Appendix I).

Both male and female transgenic mice demonstrated intact fear-associative learning and memory for both the auditory cue and the context at 7 months of age. Two other studies

have found deficits in APPxPS1 mice using a fear conditioning-related paradigm: the first reported a deficit in contextual fear conditioning in 6-month old APPxPS1 mice (but combined both male and female mice within one test cohort) (Kilgore et al. 2010), and the second found a deficit in the extinction of contextual fear in 4-month old female APPxPS1 mice (Bonardi et al. 2011). The differences in the reported behavioural outcomes between those studies and my investigations are likely due to variations in the FC test protocol. For example, the number of foot-shocks administered during the acquisition of context fear (i.e. 6 shocks) (Bonardi et al. 2011) and the shock intensity (0.75 mA) (Kilgore et al. 2010) were much higher in those studies than in the current study.

Male APPxPS1 mice did not differ from their WT counterparts in the CB task, demonstrating intact spatial learning and memory at 10 months of age. Indeed, males have been reported to demonstrate intact spatial memory in the MWM at the same age (Gallagher et al. 2013). The findings in males suggest that the development of impaired recognition memory and spatial memory are dissociated, as social recognition deficits manifested at an age where spatial memory deficits could not be detected. This is interesting, as AD patients experience social withdrawal years prior to the clinical diagnosis of AD (Chung and Cummings 2000), emphasising the face validity of the APPxPS1 mouse model.

Female APPxPS1 transgenic mice exhibited a spatial memory deficit during the reversal phase of the hidden CB task at 8-9 months of age. It is important to note that APPxPS1 mice of earlier studies displayed impaired spatial memory at 24 (Kulkarni et al. 2008) but not 2–3 months of age (Pillay et al. 2008) in the cued version of the CB paradigm. Unfortunately, the sex of the mice investigated was not disclosed in those studies.

Many research groups assess spatial memory in APPxPS1 mice using the MWM. The CB task was selected over the MWM due to various stressors introduced by MWM testing (e.g. mice are not natural swimmers, thigmotaxis, hypothermia) as outlined previously (Chapters 1 and 2). Importantly, different results between CB and MWM testing are to be expected due to differences in test-evoked stress arousal, the recruitment of different test motivators and behavioural strategies (i.e. food foraging versus survival), and the necessity to use different motor skills. Furthermore, although both the CB and MWM represent tests of spatial memory, the two tests are dependent on different brain regions. Performance in the CB task appears to be linked to activity in the prelimbic-infralimbic regions of the prefrontal cortex (Ragozzino et al. 1999), while the MWM is more dependent on amygdala function (Bannerman et al. 1999; Kim et al. 2001; Morris et al. 1982). Thus, comparisons between deficits detected by the CB and the MWM must be evaluated very cautiously. The earliest MWM–related spatial memory (i.e. retention) deficits in APPxPS1 mice have been described for 12-month old female mice (Jardanhazi-Kurutz et al. 2010) and 8-month old male mice (Cao et al. 2007). A recent study reported sex-specific task acquisition deficits in the MWM in 8-9 month old female but not male APPxPS1 mice (Gallagher et al. 2013). Thus it is possible that similar sex-specific effects (and potentially mechanisms) may impact the findings in the hidden version of the CB task used for my study.

I also investigated sensorimotor gating and found that 12-month old male and 10-11 month old female APPxPS1 transgenic mice exhibited intact sensorimotor gating (i.e. PPI). Male transgenic mice had not been assessed previously, while a recent study found that female APPxPS1 transgenic mice developed a sensorimotor gating deficit at 7 months of age (Wang et al. 2012). However, upon closer examination of the PPI protocol used, the researchers had employed a constant interstimulus interval (ISI) of

100 ms (Wang et al. 2012), which is in contrast to the variable ISI protocol used in my study (i.e. ISI of 32, 64, 128, 256 ms). Previous studies have confirmed the importance of PPI protocol characteristics (such as ISI, duration and intensity of the prepulse and startle stimuli, and protocol duration) for PPI test outcomes (Swerdlow et al. 2000; Wang et al. 2003b). As female APPxPS1 mice exhibited a trend towards reduced PPI, I included an additional test cohort of female mice, thereby increasing the total cohort size from N = 9-12 to N = 17-19. Doing so resulted in a significantly decreased prepulse inhibition in female APPxPS1 mice compared to control WT mice, but only when the ISI was 128 ms (Chapter 2.2. supplementary data). This is in line with the previous study utilising an ISI of 100 ms (Wang et al. 2012) suggesting that PPI deficits in APPxPS1 mice are highly protocol dependent, similar to what our team has reported for a mutant mouse model of schizophrenia (Karl et al. 2011). Interestingly, the alpha-7 subunit of the cholinergic nicotinic receptor is involved in sensory gating and the integrity of the cholinergic system is disturbed in AD patients. Consequently, patients exhibit reduced suppression of the P50 event-related potential (following the second click of the double-click paradigm) in comparison to control subjects indicating disturbed sensorimotor gating (Jessen et al. 2001).

Overall, my investigations revealed various novel behavioural and cognitive deficits in the APPxPS1 transgenic mouse model, including deficits in social recognition memory and impaired spatial memory relevant for the clinical condition. Table 5 provides a brief overview of these behavioural findings for APPxPS1 transgenic mice, linking them to various AD-relevant symptoms. Importantly, factors such as sex, age and test design must be carefully considered when investigating learning and memory deficits of APPxPS1 transgenic mice across different studies to ensure comparisons between studies are accurate.

Table 5: Summary of baseline phenotypes determined by the current study for APPxPS1 mice				
Behavioural Test	**Findings for APPxPS1 mice**		**AD symptoms**	**References**
	Male	*Female*		
Light/Dark test	No genotype differences in LD-related anxiety parameters; Hyperlocomotion	Decreased anxiety; Hyperlocomotion	Loss of behavioural inhibition and dis-inhibitory tendencies	(Chung and Cummings 2000)
Elevated plus maze	Increased anxiety	No genotype differences	Increased anxiety	(Echavarri et al. 2013)
Novel object recognition task	Test did not work for WT mice	No genotype differences	Impaired declarative memory, poor recognition of and confusion of objects	(Kivisaari et al. 2013)
Social preference test	Intact sociability; Impaired social recognition	Test did not work for WT mice	Social withdrawal and poor/impaired facial recognition	(Reisberg et al. 1982)
Contextual/ Cued Fear conditioning	No genotype differences	No genotype differences	Associative learning and memory (eyeblink classical conditioning) impaired before disruptions to declarative learning and memory	(Woodruff-Pak 2001)
Cheeseboard/ Reversal Cheeseboard	No genotype differences	Spatial memory deficit in reversal probe trial	Disorientation, impaired long-term memory	(Vlcek and Laczo 2014)
Prepulse inhibition	No genotype differences	Protocol-dependent impairment in sensorimotor gating	P50 event-related sensorimotor gating deficit	(Jessen et al. 2001)
Cookie Test	No genotype differences	Not assessed	Impaired olfactory ability	(Stamps et al. 2013)

5.1.2. Treating AD-relevant phenotypes of APPxPS1 mice with CBD

Male AD transgenic mice were treated daily with CBD (20 mg/kg, i.p.) for 3 weeks before being tested in behavioural and cognitive tests. It was found that CBD treatment had beneficial effects in AD transgenic mice, restoring cognitive abilities in social and object recognition memory. This is the first time a cognition-rescuing effect of CBD has been described for an AD transgenic mouse model.

Previously, it had been found that acute CBD treatment induces anxiolytic-like effects in rodents (Campos and Guimaraes 2008; Campos et al. 2013; Long et al. 2010; Moreira et al. 2006; Onaivi et al. 1990). As anxiety may impact the performance of mice in various cognitive tests (Lipp and Wolfer 1998), I evaluated anxiety behaviours

in vehicle and CBD-treated mice. In my assessment of the APPxPS1 mice, there was no difference in performance between transgenic and WT control mice in the EPM. Furthermore, chronic CBD treatment did not affect any anxiety parameters. These findings are in line with reports by Campos and colleagues demonstrating that subchronic treatment (i.e. 2-week) with CBD (30 mg/kg) does not result in anxiolytic effects (Campos et al. 2013). CBD treatment restored the ability of APPxPS1 mice to remember the social opponent and familiar object, suggesting that it may possess cognition-improving effects for AD-relevant social and object recognition memory deficits. These therapeutic-like properties of CBD are highly relevant for the clinical situation, as patients are known to experience symptoms of social withdrawal (Chung and Cummings 2000), have difficulties recognising familiar faces (Reisberg et al. 1982) and poor object recognition (Kivisaari et al. 2013).

Recent research and opinion papers suggest that an early therapeutic intervention may provide greater symptomatic improvements for AD patients over existing treatments or may even prevent disease manifestation (Hampel 2012; Iuvone et al. 2009; Karl et al. 2012b; Riedel 2014). Thus I investigated the preventative potential of long-term CBD treatment in APPxPS1 transgenic mice (i.e. daily diet supplementation for 8 months). For this, a novel voluntary oral administration technique was selected as it has only minimal impact on the well-being of test mice (i.e. compared to alternatives of oral gavage or repeated osmotic minipump implantation) and also represented a true model of the clinical situation. I discovered that long-term early CBD treatment prevents the development of social recognition deficits characteristic for vehicle-treated APPxPS1 mice. This effect was long-lasting as the initial deficit had been established in 7-month old APPxPS1 transgenic mice whereas here 11-month old mice were tested. My findings suggest that CBD has the potential to be highly efficacious and long-lasting in

AD therapy. Importantly, CBD did not disrupt anxiety behaviour or induce major changes in home cage behaviour (as judged during the daily treatment procedure) demonstrating the tolerability and behavioural inertness of long-term CBD treatment.

Assessment of the effect of CBD on spatial memory in male APPxPS1 mice was not carried out due to the finding that male mice did not demonstrate deficits at 10 months of age. However, my research found that 8-9 month old female APPxPS1 mice have spatial memory deficits. I have generated preliminary evidence that suggests CBD treatment (i.p.) in female APPxPS1 mice may alleviate spatial memory deficits or improve spatial memory performance (oral treatment) as measured by the CB task (See Appendix II and III respectively). Female APPxPS1 mice were treated with CBD for 3 weeks (i.p.) post onset, and for 9 months (oral) prior to the onset of AD-like symptomatology. In both cases, CBD improved spatial memory performance in female APPxPS1 mice compared to vehicle-treated AD transgenic mice. Follow-up studies (due to insufficient *N* for i.p. and inconsistent replication for oral CBD studies) on the spatial memory deficits of APPxPS1 females will be necessary to strengthen these findings.

In order to determine the effects of long-term CBD treatment on AD-relevant pathophysiology, I analysed amyloid load, inflammation, oxidative damage and cholesterol levels to define potential mechanisms involved in the therapeutic potential of CBD. The protection of social recognition memory by CBD treatment did not appear to be linked to changes to amyloid levels as $A\beta_{40}$ and $A\beta_{42}$ expression in both the cortex and hippocampus were unaltered in CBD-treated transgenic mice. This finding might be unexpected, however, another study has shown that pharmacologically-induced improvements in the spatial memory of APP_{Swe}/PS1ΔE9 mice on a C57BL/6J background were not accompanied by reduced cortical Aβ levels either (Koivisto et al.

2014): levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in the parietal cortex did not correlate with cognitive deficits of AD transgenic mice (Koivisto et al. 2014). Koivisto and colleagues concluded that cognitive impairments were alleviated in AD mice via multiple mechanisms, including increased levels of brain phospholipids and dendritic spine plasticity, and potentially effects on Aβ production (although the latter was not observed) (Koivisto et al. 2014). Nonetheless, previous studies have shown that CBD can reverse Aβ-induced spatial memory impairments in rodents (Martin-Moreno et al. 2011), reduce Aβ formation *in vitro* (Scuderi et al. 2013) and attenuate various Aβ-induced processes such as tau hyperphosphorylation, neurotoxicity and neuroinflammation (Esposito et al. 2006a; Esposito et al. 2011; Iuvone et al. 2004). Thus, the beneficial effect of CBD on Aβ pathology should not be ruled out yet.

Brain tissue damage is exacerbated by the release of glutamate and ROS leading to increased neurotoxicity and oxidative damage in AD. Importantly, the *in vivo* formation of isoprostanes directly correlates with increased oxidative stress in AD (Dalle-Donne et al. 2006; Morrow 2005; Pratico et al. 2001; Pratico et al. 2004), and patients are known to have increased concentrations of F_2-isoprostanes in cerebrospinal fluid prior to disease diagnosis (Dalle-Donne et al. 2006; Pratico et al. 2002; Pratico et al. 2001). Thus, I analysed the levels of cortical lipid oxidation in APPxPS1 mice, which were unaffected by genotype and CBD treatment despite the known antioxidant properties of CBD (Hamelink et al. 2005; Rajesh et al. 2010). Importantly, age may be a determining factor in the level of oxidative damage observed as previous studies have reported increased nucleic acid oxidation in 3 and 5-month old APPxPS1 mice whereas 10 and 15-month old APPxPS1 mice demonstrated similar levels of oxidation as age-matched WT mice (Hamilton and Holscher 2012). This could explain why 11-month old APPxPS1 mice of the current study did not exhibited increased cortical oxidative

damage. Indeed, oxidative stress may be an early event in the AD condition (Iida et al. 2002; Lovell and Markesbery 2007), and is also a prominent feature of the normal ageing process (Dalle-Donne et al. 2006).

I also analysed cortical levels of cholesterol and their metabolites (oxygenated derivatives of cholesterol, oxysterols, such as 24-OH cholesterol and 27-OH cholesterol) as alterations in brain cholesterol metabolism have been linked to the two major pathological features of AD, Aβ and tau pathology. Furthermore, decreased cholesterol synthesis correlates with the severity of neurodegeneration and dementia (Papassotiropoulos et al. 2000; Solomon et al. 2009), and late stage AD patients have decreased cholesterol circulation (Kolsch et al. 2010; Mielke et al. 2005). Finally, insufficient amounts of cholesterol may interrupt essential processes such as myelin formation, synaptic transmission and cognitive ability in mice (Haque and Mozaffar 1992; Suzuki et al. 2013), while a reduction in oxysterols [normally associated with a diverse number of biological functions including lipid metabolism (Schroepfer 2000)] has been shown to correlate with the severity of dementia and brain atrophy (Leoni and Caccia 2011; Papassotiropoulos et al. 2000; Solomon et al. 2009). APPxPS1 mice demonstrated high levels of cortical cholesterol regardless of treatment, while CBD increased cholesterol levels in WT mice. Increased cholesterol in APPxPS1 mice could indicate either an impaired cholesterol reuptake process or a compensatory protective mechanism against neurodegeneration as maintenance of sufficient cholesterol is necessary to combat synapse loss and neurodegeneration (Koudinov and Koudinova 2001). Such a response is consistent with the reduced levels of 24-OH cholesterol detected in APPxPS1 mice compared to WT mice as the formation of 24-OH cholesterol is the major pathway for the removal of cholesterol from the brain

(Lutjohann et al. 1996). Indeed, cholesterol has been observed to accumulate around Aβ plaques and presynaptic terminals in the AD brain (Gylys et al. 2007).

The diet of the mice consisted of maintenance food pellets, made up of ingredients such as grains (e.g. sorghum and bran), providing a natural source of phytosterols. Dietary phytosterols (also known as plant sterols) can readily cross the blood brain barrier (BBB) (Marangoni and Poli 2010) and can either interfere with critical functional processes in AD or have a beneficial effect by decreasing amyloidogenic processing (Burg et al. 2013). They may even provide relevant additional biomarkers for AD (Vanmierlo et al. 2011). Thus, I analysed phytosterol levels (i.e. brassicasterol and campesterol) in APPxPS1 mice. There were no differences in these levels between WT and vehicle-treated APPxPS1 mice. However, CBD-treated APPxPS1 mice demonstrated elevated levels of brassicasterol and campesterol compared to the other experimental groups. Since diets were the same for all mice, food pellets do not provide an explanation of this intriguing finding. On the other hand, there have been no studies that have identified phytosterols in CBD specifically; but, cannabis contains phytosterols such as campesterol, stigmasterol and β-sitosterol (Foote and Jones 1974). Thus, perhaps CBD also contains various phytosterols and may provide one explanation for the increase in cortical phytosterols observed in APPxPS1 mice treated with CBD. Importantly, AD mice including APPxPS1 (C57BL/6J background) mice have been found to exhibit a compromised BBB (resulting in increased permeability) which might explain why only CBD-treated AD but not WT mice showed increased phytosterol levels (Minogue et al. 2014; Ujiie et al. 2003). Importantly, previous studies show dietary supplementation of phytosterols reduced the levels of insoluble $A\beta_{42}$ in APPxPS1 mice on a C57BL/6J background (Koivisto et al. 2014), while the phytosterol stigmasterol attenuates scopolamine (muscarinic M1 receptor antagonist)-induced

spatial memory deficits in mice (Park et al. 2012). As scopolamine induces hypocholinergic neurotransmission, the increased phytosterol levels may benefit cognition by enhancing cholinergic neurotransmission, which is highly relevant for the AD brain (Park et al. 2012). Further research needs to be conducted to fully elucidate the role of dietary phytosterol in the AD brain and how CBD might affect this role.

Activated microglia induce the release of inflammatory cytokines, such as IL-1β and TNF-α, and chemokines that cause neuroinflammation in the AD brain. Although not statistically significant, the analysis of the cortical levels of IL-1β and TNF-α (Chapter 4) suggest that neuroinflammation might be moderately increased in the cortex of APPxPS1 mice and that CBD might be able to attenuate increased inflammation. Previously, 10-12 month old APPxPS1 mice were reported to have significantly elevated levels of neuroinflammation (i.e. increased nitric oxide species and TNF-α) in the hippocampus (Kalifa et al. 2011), and neuroinflammation has been linked to deficits in spatial memory in APPxPS1 mice (Gallagher et al. 2013). Importantly, Aβ-induced neuroinflammation and spatial memory deficits were decreased by CBD treatment in a pharmacological model of AD (Martin-Moreno et al. 2011) and the potent anti-inflammatory nature of CBD is supported by various *in vitro* studies (Esposito et al. 2006a; Iuvone et al. 2004).

The beneficial effects of CBD on the behavioural deficits in APPxPS1 mice might also be related to its interaction with other potential biological pathways specific for CBD actions, which have not been investigated in the current project.

5.1.3. Additional targets not yet investigated

CBD is known to interact with the nuclear hormone receptor PPAR-γ (responsible for regulating glucose and lipid metabolism and suppression of inflammatory responses), as

mentioned in Section 1.5. Indeed, the ability of CBD to reverse the object recognition deficits of APPxPS1 transgenic mice is similar to the reported actions of rosiglitazone, a potent PPAR-γ agonist (Escribano et al. 2009). Escribano and colleagues reported that the PPAR-γ agonist ameliorated the object recognition deficit of another AD transgenic mouse model ($hAPP_{Swe-Ind}$). In another study rosiglitazone was able to reverse fear-associative memory deficits in the Tg2576 mouse model for AD (Denner et al. 2012). Interestingly, the reversal of Aβ-induced spatial memory deficits following subchronic CBD treatment was accompanied by a reduction in IL-6 production, a biomarker for inflammation (Martin-Moreno et al. 2011). PPAR-γ also appears to be selectively involved in the anti-inflammatory and neuroprotective effects of CBD in Aβ-injected mice, reducing nitric oxide, TNF-α and IL-1β release in the hippocampus, and the promotion of hippocampal neurogenesis (Esposito et al. 2011; Scuderi et al. 2013). Preclinical studies have shown PPAR-γ agonists are able to prevent and reverse cognitive deficits in AD transgenic mouse models (Denner et al. 2012; Escribano et al. 2009). Furthermore, clinical trials show PPAR-γ agonists improved delayed recall (Watson et al. 2005) and enhanced cognitive performance on a number of AD-relevant memory tests (e.g. Mini-Mental State Exam, Wechsler Memory Scale-Revised) in patients with mild AD (Sato et al. 2011). In line with these findings, the effect of CBD on the transgenic mice used in my study points in the direction of a possible prevention of elevated cortical inflammatory cytokines mentioned earlier. The interaction of CBD with PPAR-γ provides a promising new avenue to understand the mechanism behind the beneficial effects of CBD on cognitive domains of APPxPS1 mice.

The glutamatergic pathway may also be involved in the beneficial properties of CBD in APPxPS1 mutant mice. This is based on findings showing that CBD interacts with the selective NMDA receptor antagonist ketamine (Hallak et al. 2011) and protects against

glutamate neurotoxicity (Hampson et al. 1998). Furthermore, impairments in object recognition are strongly linked to dysregulation of the glutamatergic system and dysfunctional hippocampal and parahippocampal areas (Fernandez et al. 2007; Nilsson et al. 2007). The beneficial effect of CBD on object recognition in my studies may be similar to that of the NMDA receptor antagonist memantine, which has been shown to improve object recognition in another transgenic mouse model of AD (Scholtzova et al. 2008). Interestingly, memantine has been found to impair rather than improve object recognition in healthy humans without affecting facial recognition memory (Rammsayer 2001), suggesting CBD may be efficacious for a wider range of behavioural and cognitive deficits than memantine.

In summary, I have investigated various AD-relevant pathophysiological pathways that CBD may exert its therapeutic effects on cognition. In particular, the elevation of cortical phytosterol levels (brassicasterol and campesterol) or a possible prevention of inflammatory cytokine release may mediate the prevention of social recognition deficits in AD mice. Based on these findings and those of others, CBD may exert its effects by interacting with various systems including the glutamatergic pathway and PPAR-γ.

5.2. Limitations and future research

One limitation of this study was the absence of dose-response experiments. The studies included in this thesis investigated one dosage of CBD only. This dosage was based on an initial (and only) study demonstrating the amelioration of spatial memory deficits and reduction in inflammatory cytokines by an identical dose of CBD in a pharmacological mouse model of AD (Martin-Moreno et al. 2011). It would be worthwhile testing higher dosages of CBD, which may have a more prominent effect on disease progression and pathophysiology of APPxPS1 mice. In addition, an escalating

dose regime could be used, as AD patients commonly receive higher dosages of AD medication as their condition worsens (Tariot et al. 2012). Importantly, previous studies of our laboratory have shown that CBD is behaviourally inert in rodents at much higher dosages [e.g. 50 mg/kg (Long et al. 2010)] than what was used in my investigations.

5.3. Significance of outcomes

APPxPS1 mice demonstrate a number of AD-relevant behavioural deficits, including social recognition memory and spatial memory deficits that have not been assessed previously, adding further to the face validity of the model. Based on the limited number of *in vitro* and *in vivo* studies on CBD effects in AD, I investigated if CBD can reverse or prevent behavioural deficits characteristic for APPxPS1 mice. Indeed, CBD reversed the social recognition memory and object recognition memory deficits of 7-month old male APPxPS1 mice and long-term CBD treatment prevented the manifestation of social recognition memory deficits in 10-11 month old male APPxPS1 mice. The ability of CBD to rescue or prevent social and object recognition memory in AD mice suggest a significant therapeutic-like potential for clinical settings. Importantly, CBD has been found to be tolerable in mice (Long et al. 2012; Martin-Moreno et al. 2011) and people (Consroe et al. 1991; Cunha et al. 1980) including schizophrenia patients (Zuardi et al. 2006) and does not produce psychological or physiological effects (Perez-Reyes et al. 1973). CBD has been reported to improve sleep duration in humans suffering from insomnia (Carlini and Cunha 1981), and higher dosages of CBD (600 mg) may produce a sedative effect, which was accompanied by decreased stress responses (Zuardi et al. 1993). It is highly relevant for the translational value of my study that the clinically tested nabiximol (Sativex), a combination of CBD and Δ^9-tetrahydrocannabinol (THC) in the form of an oromucosal spray, has already

been approved for the treatment of muscle spasticity in patients with multiple sclerosis. Studies have shown therapeutic doses of nabiximols do not induce psychopathology or result in cognition-impairing effects (Aragona et al. 2009). Furthermore, CBD attenuates the psychotic-like effects of cannabis in recreational users (Morgan et al. 2012), attenuates the deleterious effects of THC on cognition (Pertwee 2008), and protects against THC-induced recognition memory impairments and verbal memory deficits in humans (Morgan et al. 2010; Pertwee 2008). Importantly, my study demonstrates the CBD does not need to be administered in conjunction with THC to improve cognitive symptoms of AD.

The fact that CBD can be administered long-term on a daily basis without negative impacts on the behaviour and health of mice suggests that CBD might have potential as a preventative supplement. This may allow protection against the development of cognitive deficits in at-risk patients. More research must be conducted in order to fully elucidate the viability of CBD as a mode of prevention for AD symptomatology and perhaps pathophysiology (at higher dosages). Furthermore, the current lack of definite early AD-diagnosing tests provides a challenge for early intervention programs. Nonetheless, a multitude of possible biomarkers and genetic risk factors that may contribute to AD are currently discussed in the field (Belbin et al. 2011; Ray et al. 2007). The fact that CBD has already been tested in clinical settings suggests that with greater preclinical research, its transition to clinical AD trials may not be too far away in the future.

Chapter 6: Major Conclusions

In conclusion, this thesis has generated the following findings:

1. Male APPxPS1 mice demonstrate a social recognition deficit at 7 months of age prior to any detectable spatial memory deficits
2. Female APPxPS1 mice demonstrate a spatial memory deficit at 8-9 months of age in the newly established reverse cheeseboard task
3. 3-week treatment with CBD (20 mg/kg) ameliorated deficits in social and object recognition of 7-month old male APPxPS1 mice
4. Long-term oral CBD (20 mg/kg) treatment prevented the development of social recognition deficits in 10-11 month old male APPxPS1 mice
5. The beneficial effect of long-term oral CBD on social recognition was not mediated by changes in amyloid load. However, the preventative effect of CBD might be linked to its anti-inflammatory properties and its effects on phytosterol retention

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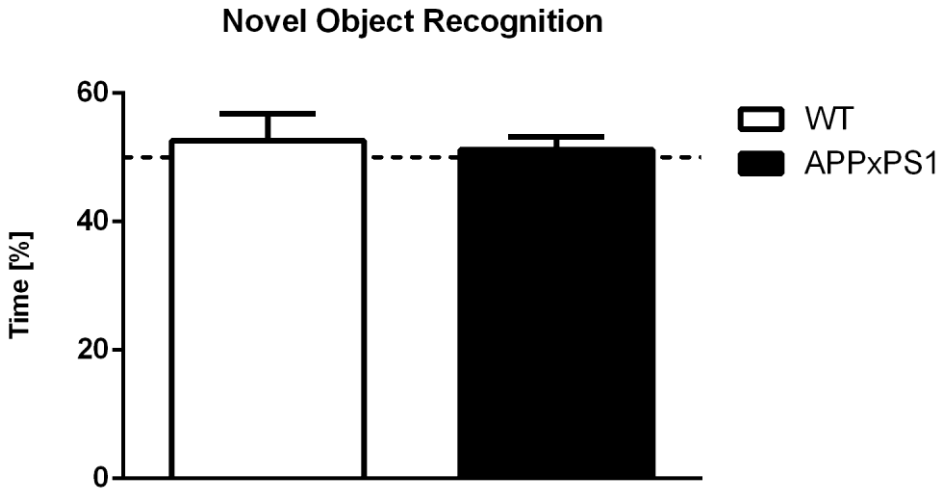
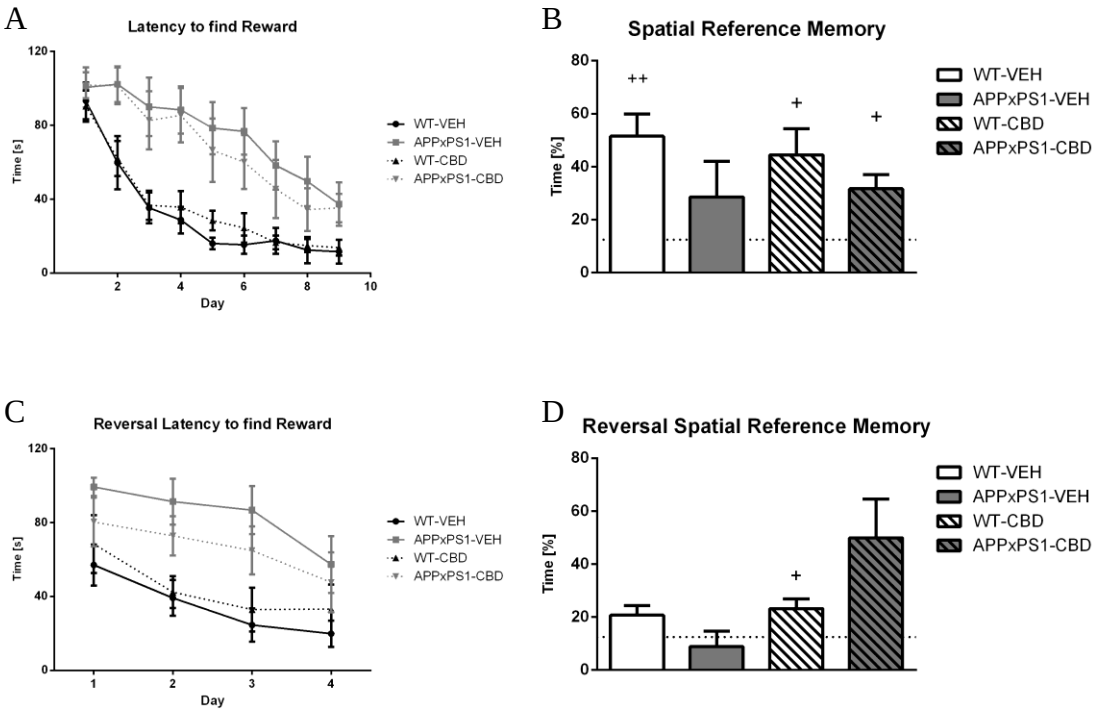
Appendix I: Novel object recognition task



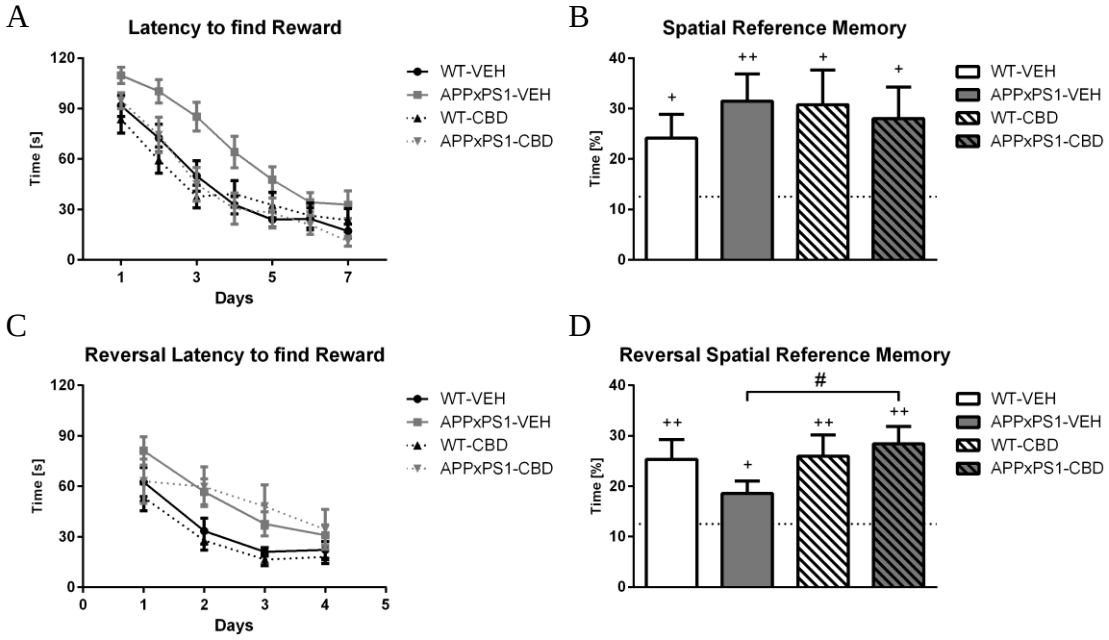
Novel object recognition memory for male mice. Baseline data for time spent *nosing* towards the novel object over the familiar object expressed as a percentage of total time spent interacting with both objects [%]. Data for non-transgenic WT control and double transgenic $APP_{Swe}/PS1\Delta E9$ (APPxPS1) male mice are shown as means + SEM [WT: N = 11; APPxPS1: N = 18].

Appendix II: Preliminary findings for effects of CBD treatment (i.p.) on spatial learning and memory



Preliminary data for spatial learning and memory during the cheeseboard (A-D) for female mice treated with either vehicle or CBD (20 mg/kg; daily i.p. for 3 weeks prior to 11 months of age): (A) latency [s] to find the food reward averaged across 3 daily trials; (B) percentage time [%] spent in the target zone of the CB (i.e. in close proximity to the reward well) during the 2 min probe trial; (C) latency [s] to find the food reward averaged across 3 daily trials; (D) percentage time [%] spent in the target zone of the CB during the 2 min reversal probe trial. Data for non-transgenic control (WT) and double transgenic *APP_{Swe}/PS1ΔE9* (APPxPS1) females are shown as means + SEM [WT-VEH: $N = 8$; APPxPS1-VEH: $N = 7$; WT-CBD: $N = 8$; APPxPS1-CBD: $N = 5$]. Significant preference for the target zone (as analysed by the t-test) is indicated by '$^{+}$' ($^{+}p < .05$ and $^{++}p < .01$).

Appendix III: Effect of long term (oral) CBD treatment on spatial learning and memory



Spatial learning and memory in the cheeseboard (A-D) for female mice treated with either vehicle or CBD (20 mg/kg; daily oral treatment from 2.5 months of age until 11-12 months of age): (A) latency [s] to find the food reward averaged across 3 daily trials; (B) percentage time [%] spent in the target zone of the CB (i.e. in close proximity to the reward well) during the 2 min probe trial; (C) latency [s] to find the food reward averaged across 3 daily trials; (D) percentage time [%] spent in the target zone of the CB during the 2 min reversal probe trial. Data for non-transgenic control (WT) and double transgenic *APP$_{Swe}$/PS1ΔE9* (APPxPS1) females are shown as means + SEM [WT-VEH: N = 13; APPxPS1-VEH: N = 15; WT-CBD: N = 13; APPxPS1-CBD: N = 9]. Effect of treatment indicates significant improvement in spatial memory by CBD in APPxPS1 mice, but not a prevention of an existing spatial memory deficit. Significant preference for the target zone (as analysed by the t-test) is indicated by '$^{+}$' ($^{+}p < .05$ and $^{++}p < .01$), and significant effect of treatment is indicated by '$^{\#}$' ($^{\#}p < .05$).