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

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Thank you.

Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

(Signature)

Declarations

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Publications

1. Watt, G and Karl, T. In vivo Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease (2017) *Frontiers in Pharmacology*, *8*.

2. Watt, G*., Shang, K*., Zieba, J., Olaya, J., Li, Henry., Garner, B., Karl, T. Chronic treatment with 50 mg/kg cannabidiol (CBD) improves cognition and moderately reduces $A\beta_{40}$ levels in 12-month-old *$A\beta PP_{swe}$/PS1Δ E9* transgenic mice (2020) *Journal of Alzheimer's Disease.* Accepted – 29/1/2020.
   ** contributed equally, i.e. shared first (GW & KS) authors*

3. Watt, G., Przybyla, M., Zak, V., van Eersel, J., Ittner, A., Ittner L., Karl, T. Novel behavioural characteristics of male human *P301* mutant tau transgenic mice – a model for tauopathy (2020) *Neuroscience*. Accepted – 31/1/2020.

Manuscripts Currently Submitted

4. Watt, G., Chesworth, R., Przybyla, M., Garner, B., Ittner, A., Ittner M., Karl, T. Evaluation of the behavioural effects of chronic cannabidiol (CBD) on 4-month-old male TAU58/2 transgenic mice. *Submitted to Pharmacology Biochemistry and Behavior*

5. Watt, G., Olaya, J., Garner, B., Karl, T. Therapeutic effects of chronic 100 mg/kg cannabidiol (CBD) treatment in male double transgenic *$A\beta PP_{swe}$/PS1Δ E9 (APPxPS1)* transgenic mice. *Submitted to International Journals of Neuropsychopharmacology*

Additional Publications and Manuscripts

Publications

1. Sharman, M., Verdile, G., Kirubakaran, S., Parenti, C., Singh, A., Watt, G., Karl, T., Chang, D., Li, C., Münch, G. Targeting Inflammatory Pathways in Alzheimer's Disease: A Focus on Natural Products and Phytomedicines (2019) *CNS Drugs, 33*.

Book Chapters

2. Chesworth, R., Watt, G., Karl, T. Cannabinoid Modulation of Object Recognition and Location Memory—A Preclinical Assessment (2018) In *Handbook of Behavioral Neuroscience, 27.*

Manuscripts Currently Submitted

3. Kreilaus, F., Masanetz, R., Watt, G., Przybyla, M., Ittner, A., Ittner, L., Karl, T. The behavioural phenotype of 14-month-old female TAU58/2 transgenic mice. *Submitted to Behavioural Brain Research*

Conference Presentations – Oral Presentations

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Health Beyond Research and Innovation Showcase, 2018

The therapeutic effect of cannabidiol (CBD) on male APPxPS1 and Tau58/2 transgenic mice [oral presentation]

International Behavioural Neuroscience Society Annual Meeting, 2018

The therapeutic effect of cannabidiol (CBD) on male APPxPS1 and Tau58/2 transgenic mice [oral presentation]

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Kioloa Neuroscience Colloquium, 2017

The therapeutic effect of cannabidiol (CBD) in the Tau58/2 transgenic mouse model of Alzheimer's disease [poster presentation]

Brain Sciences UNSW Conference, 2017

The therapeutic effect of cannabidiol (CBD) in the J20 transgenic mouse model of Alzheimer's disease [poster presentation]

Australian Dementia Forum , 2018

The therapeutic effect of cannabidiol (CBD) on male APPxPS1 and Tau58/2 transgenic mice [poster presentation]

Macquarie Neurodegeneration meeting , 2018

The therapeutic effect of cannabidiol (CBD) on male APPxPS1 and Tau58/2 transgenic mice [poster presentation]

Health Beyond Research and Innovation Showcase, 2019

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The therapeutic effect of cannabidiol (CBD) on male APPxPS1 transgenic mice [poster presentation]

Abstract

Background: Alzheimer's disease (AD) is characterised by progressive cognitive decline and by the accumulation of amyloid-β (Aβ) and tau hyperphosphorylation in the brain causing neurodegeneration and neuroinflammation. Current AD treatments do not stop or reverse the disease progression, highlighting the need for more effective therapeutics. The phytocannabinoid cannabidiol (CBD) has demonstrated antioxidant, anti-inflammatory and neuroprotective properties making it an interesting therapeutic candidate for AD therapy. Previous studies in male *APP_{swe}/PS1ΔE9* (*APPxPS1*) transgenic mice revealed that the beneficial cognitive effects of CBD are only associated with subtle changes in neuropathological measures. Importantly, CBD has been shown to work in a dose-dependent manner and cannabinoids are known to have biphasic effects. Traditionally, transgenic mouse models for AD have focused on Aβ pathology, however, recently a number of tauopathy transgenic mouse models have been developed, including the TAU58/2 transgenic model. Despite the important role of tau in AD, there have be no *in vivo* studies which have investigated the effect of CBD in a tauopathy model of AD to this date.

Methods: The aims of my thesis were to investigate the therapeutic potential of 50 and 100 mg/kg CBD in male *APPxPS1* transgenic mice, to conduct a comprehensive behavioural analysis of a novel tauopathy mouse model of AD, TAU58/2 transgenic mice, and to investigate the therapeutic potential of 50 mg/kg CBD in the latter mouse model. The effect of CBD on reversing AD-relevant phenotypes was assessed using a chronic CBD treatment strategy including daily intraperitoneal injections for 3 weeks prior to any testing, which then continued throughout behavioural testing. After the initial 3-week-treatment period, comprehensive tests for AD-relevant behaviours were conducted and at the conclusion of testing several neuropathological markers were analysed in *APPxPS1* males.

Results: Chronic CBD treatment (50 and 100 mg/kg) reversed social recognition memory deficits in 7.5-month-old male *APPxPS1* mice and both social recognition memory and reversal spatial learning deficits in 12-month-old male *APPxPS1* mice respectively. Chronic CBD treatment at a dose of 50 mg/kg also tended to reduce insoluble $A\beta_{40}$ pathology in the hippocampus. Furthermore, I found an age-dependent expression of markers of neuroinflammation and microglial activation in AD transgenic mice. 7.5-month-old *APPxPS1* males demonstrated reduced levels of neuroinflammatory cytokines (i.e. tumor necrosis factor [TNF]-α and interleukin [IL]-1β) in the hippocampus whereas markers of microglial activation (i.e. ionized calcium binding protein [IBA1]) in the cortex were not affected. In addition, these *APPxPS1* males exhibited elevated cortical brain derived neurotrophic factor (BDNF) levels. 12-month-old *APPxPS1* males did not show altered cortical levels of TNF-α and IL-1β, although their cortical IBA1 levels were increased. Cortical BDNF was not altered at this age and peroxisome proliferator-activation receptor γ (PPARγ) levels in the same brain region were not affected at either age tested. Neither dose of chronic CBD affected markers of neuroinflammation or PPARγ levels. Cortical BDNF was increased in only WT controls by 100 mg/kg CBD and neither genotype was affected by 50 mg/kg CBD. In a second part of this thesis, I determined that 4-month-old TAU58/2 males demonstrated a number of novel behavioural impairments, including reduced sociability, impaired sensorimotor gating and motor impairments, all of which can be symptoms of AD patients, highlighting the relevance of this model as a novel tauopathy mouse model of AD. Despite the beneficial effects of CBD in male *APPxPS1* mice, CBD was effective in reversing motor deficits in 4-month-old male TAU58/2 transgenic mice.

Conclusion: Chronic CBD treatment (50 and 100 mg/kg) reversed cognitive deficits in *APPxPS1* transgenic mice. In addition, chronic CBD treatment at a dose of 50 mg/kg tended to reduce

insoluble $A\beta_{40}$ pathology in the hippocampus. However, chronic CBD treatment did not affect markers of neuroinflammation and neurodegeneration at the ages and doses tested. 4-month-old TAU58/2 males demonstrated a number of AD-relevant behavioural impairments, highlighting its relevance as a tauopathy mouse model of AD. However, chronic CBD treatment did not reverse motor impairments in 4-month-old TAU58/2 males. The findings in this thesis indicate that CBD demonstrates therapeutic benefits on cognition, emphasizing the clinical relevance of CBD treatment in AD. However, the underlying mechanisms involved and the effectiveness of CBD on tauopathy mouse models requires further investigation.

List of Abbreviations

2-AG	–	2-arachiodonoylglycerol
5-HT1A	–	serotonergic receptor 1A
5-HT2A	–	serotonergic receptor 2A
Aβ	–	amyloid-β
A_2	–	adenosine receptor 2
ABCA7	–	ATP-binding cassette transporter A7
ACE1	–	angiotensin converting enzyme 1
ACh	–	acetylcholine
AChE	–	acetylcholinesterase
AD	–	Alzheimer's disease
AEA	–	anandamide
AMPA	–	2-amino3-(-4-butyl-3-hydroxyisoxazol-5-yl)propionic acid
ANOVA	–	analysis of variance
APH1A	–	anterior pharynx-defective 1A
APOE	–	apolipoprotein E
APP	–	amyloid precursor protein
APPxPS1	–	APP_{swe}/PS1ΔE9 double transgenic mice
ASR	–	acoustic startle response
BDNF	–	brain derived neurotrophic factor
BIN1	–	bridging integrator 1
BW	–	body weight
CAMK2A	–	calcium/calmodulin dependent protein kinase II α
CB	–	cheeseboard task
CB1	–	cannabinoid receptor 1
CB2	–	cannabinoid receptor 2
CBD	–	cannabidiol
CDK5	–	cyclin dependent kinase 5
CLU	–	clusterin
CR1	–	complement receptor 1
CSF	–	cerebral spinal fluid

DAGL	–	diacylglycerol
DYRK1A	–	dual specificity tyrosine phosphorylation regulated kinase 1A
eCBS	–	endocannabinoid system
ECE1	–	endothelin converting enzyme 1
ELISA	–	enzyme-linked immunosorbent assay
EPHA1	–	ephrin type A receptor 1
EPM	–	elevated plus maze
FAAH	–	fatty acid amide hydrolase
FC	–	fear conditioning
FDA	–	Food and Drug Administration, USA
FTD	–	frontotemporal dementia
GABA	–	gamma-aminobutyric acid
GFAP	–	glial fibrillary acidic protein
GMSC	–	mesenchymal stem cells derived from gingiva
GPR18	–	G-protein coupled receptor 18
GPR55	–	G-protein coupled receptor 55
GSK-3β	–	glycogen synthase kinase 3β
IBA1	–	ionized calcium binding protein
IDE	–	insulin degrading enzyme
IFN-γ	–	interferon γ
IL-1β	–	interleukin-1β
IL-6	–	interleukin-6
iNOS	–	inducible nitric oxide synthase
i.p.	–	intraperitoneal
ITI	–	inter-trial interval
MAGL	–	monoglyceride lipase
MAPK1	–	mitogen-activated protein kinase 1
MAPK12	–	mitogen-activated protein kinase 12
MAPK14	–	mitogen-activated protein kinase 14
MAPT	–	microtubule associated protein tau
MCI	–	mild cognitive impairment
mRNA	–	messenger ribonucleic acid

MS4A	–	membrane-spanning 4A family
NAPE-PLD	–	*N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D
NCSTN	–	nicastrin
NF-κB	–	nuclear factor-κB
NFTs	–	neurofibrillary tangles
NMDA	–	N-methyl-D-aspartate
NO	–	nitric oxide
NORT	–	novel object recognition task
NSAID	–	non-steroidal anti-inflammatory drugs
OF	–	open field
p-GSK-3β	–	phosphorylated glycogen synthase kinase 3β
p38 MAPK	–	p38 mitogen-activated protein kinase pathway
PHFs	–	paired helical filaments
PICALM	–	phosphatidylinositol binding clathrin assembly protein
PPARα	–	peroxisome proliferator-activated receptor α
PPARγ	–	peroxisome proliferator-activated receptor γ
PPI	–	prepulse inhibition
PS1	–	presenilin 1
PS2	–	presenilin 2
PSENEN	–	presenilin enhancer
RI	–	Resident-Intruder
RM	–	repeated measures
ROS	–	reactive oxygen species
S100B	–	S100 calcium binding protein B
SEM	–	standard error of means
SI	–	social interaction
SORL1	–	sortilin-related receptor L
SPT	–	social preference test
THC	–	Δ^9-tetrahydrocannabinol
TNF-α	–	tumour necrosis factor α
TREM2	–	triggering receptor expressed on myeloid cells 2

TRPV1	–	vanilloid receptor 1
VEH	–	vehicle
WT	–	wild-type like mouse

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Chapter 1: Introduction

Based on Publication 1

Watt, G and Karl, T. In vivo Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease (2017) *Frontiers in Pharmacology*, *8*.

Declaration

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Georgia Watt

1.1 Alzheimer's Disease

Dementia refers to the general loss of cognitive ability and encompasses a number of distinct diseases (Duong et al. 2017). It is the second leading cause of death in Australia, accounting for 5.4% of deaths in males and 10.6% of deaths in females each year (Brown et al. 2017). Alzheimer's disease (AD) is the most common form of dementia and the most common neurodegenerative disease in the world. It is responsible for over 60% of dementia cases and affects over 33 million people worldwide (Barnes and Yaffe 2011). Unfortunately, as a result of the ageing population, this number is expected to reach 115 million by the year 2050 (Wisniewski and Goni 2014). In Australia, the total cost of dementia was $14.25 billion in 2016 and is predicted to increase to $36.8 billion in 2056 (Brown et al. 2017). AD is characterised by progressive cognitive decline and specific neuropathological features including the accumulation of the amyloid-β (Aβ) and tau proteins (Chapman et al. 2001). It has been shown that the pathological changes seen in AD occur decades before symptoms start to show. To account for this, AD has been classified into three stages which define the varying stages of pathophysiology and cognitive decline: preclinical AD, mild cognitive impairment (MCI) due to AD and dementia due to AD (Alzheimer's Association 2018). The preclinical stage of AD refers to pathophysiological changes in the brain which precede cognitive and behavioural changes, this may begin over 20 years before symptoms appear (Alzheimer's Association 2018). A study in people carrying a genetic mutation for familial AD predicted their age of onset of preclinical neuropathological changes based on the age at which their parents developed symptoms (Bateman et al. 2012). They found that 25 years prior to symptom onset $A\beta_{42}$ levels were reduced in the cerebral spinal fluid (CSF). While 15 years prior to symptom onset, Aβ deposition in plasma was increased, tau levels in CSF was increased and brain atrophy began. In addition, they noted that 10 years prior to onset, glucose metabolism was

reduced (Bateman et al. 2012). Towards the end of the preclinical stage, impairments in episodic and semantic memory develop and are followed by slight deficits in executive function as the transition into MCI due to AD occurs. In the MCI due to AD stage, more cognitive domains become affected, however, initially these deficits are hard to detect as they do not dramatically impair everyday life (Alzheimer's Association 2018). As the MCI stage progresses, impairments in verbal recall, attention and visuospatial memory become evident with a general decline in memory (Webster et al. 2014). In the dementia due to AD stage, there is a global disruption of cognitive ability, with severe impairments in speech and facial recognition, all of which eventually render the patients in need of 24-hour care (Alzheimer's Association 2018; Webster et al. 2014). Alongside these cognitive deficits, AD patients also experience motor dysfunction including impaired gait and posture, increased rigidity, bradykinesia and tremors (Wirths and Bayer 2008), as well as an increased susceptibility to other illnesses (Alzheimer's Association 2018). The rate of AD progression varies greatly across patients, but on average people diagnosed with AD over 65 live for 4 to 8 years post diagnosis, although some survive for up to 20 years with the disease (Alzheimer's Association 2018; Tom et al. 2015). Reasons for varying disease progression are linked to early-onset or late-onset AD and which genetic mutation is carried by the patient (for familial AD), with late-onset and *presenilin 1* (*PS1*) mutations being associated with faster disease progression (Armstrong 2014; Ryan and Rossor 2010).

1.1.1 Genetics: Familial vs sporadic AD

AD is classified into two types, sporadic AD (>95% of cases) and familial AD (<5% of cases). A major distinction between the two types of AD is the age of onset. Familial AD is predominantly early-onset, where patients are <65 years old, while sporadic AD is more commonly late-onset.

However, there have been cases of early-onset sporadic AD and vice versa for familial AD. Although sporadic AD is the most common form, it is less understood than familial AD. Familial AD results from autosomal dominant mutations in the *amyloid precursor protein* (*APP*) gene or in the *presenilin 1* and *2* (*PS1* and *PS2*) genes. *APP* is the precursor molecule that is cleaved into the Aβ peptide, while *PS1* and *PS2* encode the γ-secretase and β-secretase complexes that mediate pathogenic *APP* splicing (as seen in Figure 1) (Sisodia and St George-Hyslop 2002). Mutations which occur in the *PS1* gene have been found to be the most common and result in a more aggressive form of familial AD (Vilatela et al. 2012). The cause of sporadic AD is less clear and yet to be defined, however, studies indicate that it may result from a complex interaction between several environmental factors and various susceptible genes (Kamboh 2004; Karch and Goate 2015). In recent years, numerous genes have been reported as susceptible genes for sporadic AD with many having roles in the inflammatory response as well as cholesterol metabolism and endocytosis (genes listed in Table 1) (Karch and Goate 2015). Genes of particular interest in regard to AD include *triggering receptor expressed on myeloid cells 2 (TREM2), ATP-binding protein cassette transporter A7 (ABCA7)* and *apolipoprotein E (APOE)*. TREM2 is highly expressed on microglia and when mutated causes impaired lipid signalling which may induce microglia activation and promote neuroinflammation (Dorszewska et al. 2016). Decreased ABCA7 expression in the brain has also been associated with an increased risk of AD. It is expressed in the brain and has been detected macrophages and microglia. It is thought to regulate phagocytosis, modulate APP processing and cholesterol metabolism (Kim et al. 2013) and has been associated with increased Aβ deposition and brain atrophy (De Roeck et al. 2019). APOE plays a significant role in cholesterol trafficking as well as neuroplasticity and neuroinflammation. Importantly it binds to Aβ and thus can impact clearance of Aβ oligomers and aggregation. Furthermore, the ε4

allele dramatically increases AD risk and reduces age of onset (Dorszewska et al. 2016). It is commonly assumed that familial AD has a faster, more aggressive disease progression, however recent research suggests that the disease progression of familial and sporadic AD is similar, and the major difference between the two types is their initial cause and age of onset (Armstrong 2014; Day et al. 2016; Ryan and Rossor 2010). Both forms of AD exhibit a neurodegenerative cascade that appears to be instigated by the accumulation of Aβ and hyperphosphorylated tau proteins (Chapman et al. 2001). The cascade induces neuroinflammation and oxidative stress, which creates a neurotoxic environment that potentiates neurodegeneration and eventually leads to cognitive decline (Ahmed et al. 2015; Hardy and Selkoe 2002).

Modifiable and non-modifiable risk factors of AD

Risk Factor	Role in AD	References
Genetic risk factors for familial AD		
APP	Precursor molecule which is cleaved into the Aβ peptide	Chartier-Harlin et al. 1991
PS1	Encodes the γ-secretase which mediates APP splicing	Rogaev et al. 1995
PS2	Encodes the β-secretase which mediates APP splicing	Sherrington et al. 1996
Genetic risk factors for sporadic AD		
CR1, CD33, MS4A family, TREM2, ABCA7, EPHA1, CLU	Inflammatory response	Harold et al. 2009; Naj et al. 2011; Guerreiro et al. 2013; Lambert et al. 2009; Hollingworth et al. 2011
APOE, CLU, ABCA7, SORL1	Cholesterol metabolism	Harold et al. 2009; Hollingworth et al. 2011; Sando et al. 2008b; Rogaeva et al. 2007
BIN1, PICALM, CD2AP, EPHA1, ABCA7, SORL1	Endocytosis	Harold et al. 2009; Naj et al. 2011; Hollingworth et al. 2011; Kauwe et al. 2011; Rogaeva et al., 2007

Other non-modifiable risk factors for AD		
Age	AD risk increases with age	Herbert et al. 2013
Gender	AD is more common in females than males	Carter et al. 2012
Family History	People with first-degree relative with AD have an increased risk	Green et al. 2002; Loy et al. 2014
Modifiable risk factors for AD		
Cardiovascular disease risk factors	Mid-life obesity, hypertension, prehypertension and high cholesterol have been associated with increased AD risk. Diabetes and smoking are also associated with increased AD risk	Rönnemaa et al. 2011; Launer et al. 2000; Gottesman et al. 2017; Solomon et al. 2009
Diet and exercise	Healthy diet, in particular the Mediterranean diet, and regular physical activity are associated with reduced AD risk	Morris et al. 2015; Rolland et al. 2008
Education	Increased years of formal education, mentally stimulating jobs and other mentally stimulating activities are associated with a lower risk of AD	Sando et al. 2008a
Traumatic brain injury (TBI)	Moderate and severe TBI's increase AD risk. Risk increases for those who experience repeated TBI's (e.g. boxers and football players)	Van Den Heuvel et al. 2007
Social interaction	Social and mental activity promotes brain health and is linked with reduced AD risk	Wang et al. 2002; Saczynski et al. 2006
Sleep	Sleep disturbances have been associated with increased AD risk	Lim et al. 2013; Ju et al. 2014

Table 1. Modifiable and non-modifiable risk factors of AD. *Alzheimer's disease, AD; amyloid precursor protein, APP; amyloid-β, Aβ; presenilin, PS; complement receptor, CR; membrane-spanning 4A family, MS4A; triggering receptor expressed on myeloid cells 2, TREM2; ATP-binding protein cassette transporter A7, ABCA7; ephrin type A receptor 1, EPHA1; clusterin, CLU; apolipoprotein E, APOE; sortilin-related receptor L, SORL1; bridging integrator 1, BIN1; phosphatidylinositol binding clathrin assembly protein, PICALM; CD2-associated protein, CD2AP; traumatic brain injury (TBI).*

1.1.2 Non-modifiable and modifiable risk factors

In addition to genetic risk factors, other non-modifiable risk factors for AD have been identified (Alzheimer's Association 2018). AD is often referred to as an age-related disease as risk increases dramatically with age, with 32% of people over the age of 85 in the US being diagnosed with AD (Hebert et al. 2013). AD is also more common in females (Carter et al. 2012), the underlying mechanisms for the gender-differences are not clearly understood although the fact that women live longer is thought to play a role (Carter et al. 2012). Furthermore, studies have found that the disease progresses differently in men and women, with women being relatively protected in the preclinical stage but then experiencing greater cognitive decline and brain atrophy in later stages of the disease (Ferretti et al. 2018). Risk also increases in people who have a first-degree relative with AD, even if the family member does not have familial AD (Green et al. 2002; Loy et al. 2014). A number of modifiable risk factors have also been identified, although the strength of evidence supporting these as risk factors for AD varies (as seen in Figure 1).

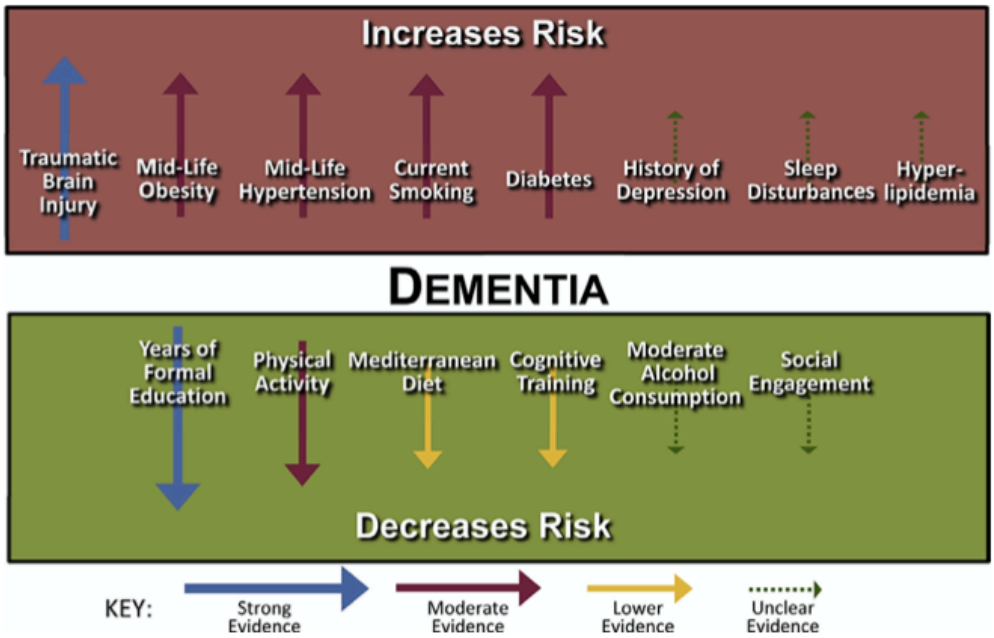

Figure 1: The strength of evidence for non-modifiable risk factors of AD (Baugmart et al., 2015).

These are primarily associated with cardiovascular health, including midlife obesity (Rönnemaa et al. 2011), hypertension (Launer et al. 2000), prehypertension (Gottesman et al. 2017) and high cholesterol (Solomon et al. 2009). Other modifiable risk factors include poor diet, less physical activity, lower education, low levels of social interaction, sleep disturbances and traumatic brain injury (Alzheimer's Association 2018; Baumgart et al. 2015) (as seen in Table 1).

1.1.3 Pathophysiology of AD

1.1.3.1 Role of Aβ protein in AD

AD is characterised neuropathologically by the accumulation of extracellular Aβ and intracellular tau hyperphosphorylation (Haass and Selkoe 2007). Pathological Aβ is produced from the cleavage of APP by protease and secretase activity, including the α–, β– and γ-secretases (Sisodia and St George-Hyslop 2002). The non-amyloidogenic pathway is initiated by α-secretases cleaving APP inside the Aβ domain, thus preventing the formation of Aβ and releasing APPsα (Reddy and Beal 2008). The amyloidogenic pathway is initiated by β-secretases which cleave APP and the by product is subsequently cleaved by γ-secretases to generate toxic Aβ (Sisodia and St George-Hyslop 2002) (as seen in Figure 2). The two main forms of pathogenic Aβ which arise from APP splicing are $A\beta_{42}$ and $A\beta_{40}$. $A\beta_{42}$ is thought to be the more toxic form of the protein as it aggregates and forms oligomers and fibrils more readily than $A\beta_{40}$ (Chapman et al. 2001). Aβ accumulation, to an extent, is part of healthy ageing. Nevertheless, when the Aβ plaque load and the ratio of $A\beta_{42}$:$A\beta_{40}$ are substantially elevated, neurodegeneration begins to occur (Mucke et al. 2000; Rowe et al. 2007). Insoluble Aβ plaques consist of aggregated Aβ and have been found to cause synaptic loss and neuritic dystrophy (Mancuso et al. 2011; Mucke and Selkoe 2012). The

neurodegenerative cascade and associated neurotoxicity commonly seen in AD is thought to occur downstream to the accumulation of Aβ (Imbimbo et al. 2005). Importantly, studies have implicated that soluble Aβ oligomers are more toxic than the insoluble Aβ plaques (Haass and Selkoe 2007).

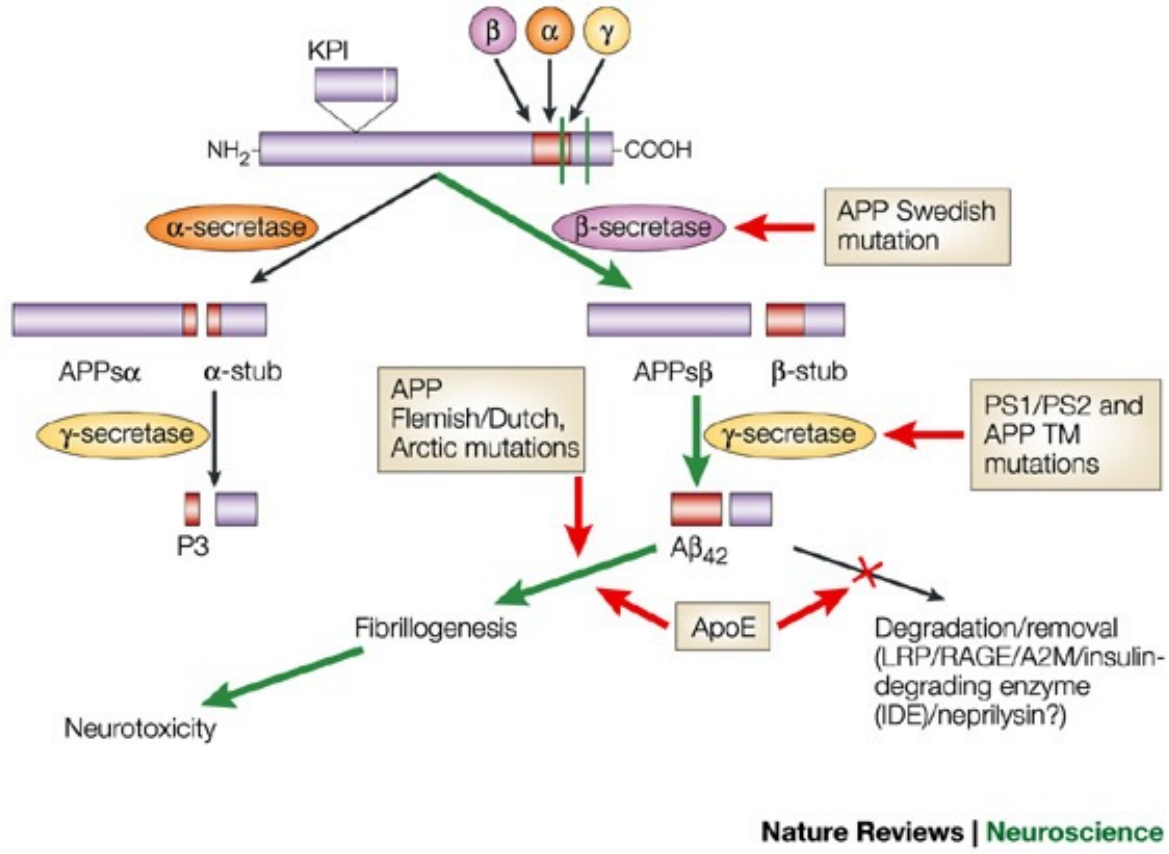

Figure 2: Schematic of the proteolytic events and cleavage products that are generated during the processing of APP (Sisodia and St George-Hyslop 2002).

In vitro studies suggest long-term potentiation is effectively blocked by soluble oligomers but not Aβ plaques unless they are treated with acid to release the oligomers inside (Shankar et al. 2008). Biochemical analyses indicated that soluble Aβ oligomer levels are more closely correlated with synaptic loss and cognitive deficits than Aβ plaques (Kayed et al. 2003; Shankar et al. 2008). This supports the hypothesis that the soluble oligomers are more neurotoxic than the insoluble forms accumulating in plaques, which may act as reservoirs of the soluble oligomers (Mucke and Selkoe

2012). It is important to note that although the soluble Aβ oligomers are considered more detrimental, insoluble Aβ plaques also cause neurodegeneration and microglia have been reported to surround the plaques and induce neuroinflammation (Marchalant et al. 2008). Furthermore, various neurotransmitter systems have been found to be disrupted in response to Aβ accumulation. Most importantly, it correlates with the elevation of glutamate levels in the CSF of AD patients (Pomara et al. 1992) and the loss of cholinergic neurons in brain areas relevant for memory processing, which is accompanied by a decrease in acetylcholine (ACh) (Schliebs and Arendt 2011). Aβ has also been shown to alter N-methyl-D-aspartate (NMDA) receptor activity, which triggers neuroinflammation (Marchalant et al. 2012) and oxidative stress for example, increasing the generation of reactive oxygen species (ROS) (Iuvone et al. 2004).

1.1.3.2. Role of tau protein in AD

The normal tau protein is a component of microtubules, which are found in the axons of cells. It is essential for microtubule stabilisation, which is crucial for cytoskeleton scaffolds that allow for cellular trafficking (Pîrscoveanu et al. 2017) and therefore, the intracellular transport of organelles (e.g. mitochondria) (Medina and Avila 2014). Under pathological conditions, tau becomes hyperphosphorylated and detaches from microtubules. Intracellular hyperphosphorylated tau then aggregates to form paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) (TurabNaqvi et al. 2020) (as seen in Figure 3). NFTs replace microtubules, which impairs microtubule stabilisation, therefore impairing cellular communication (Mudher et al. 2004). Impaired cellular communication potentiates neurodegeneration by inducing apoptosis, synapse dysfunction and degeneration and neuritic atrophy (Massoud and Gauthier 2010; Octave and Pierrot 2008; Pîrscoveanu et al. 2017). When hyperphosphorylated, tau results in mitochondrial

dysfunction as well as the generation of ROS and nitrogen species, further contributing to the neurodegeneration (Patterson et al. 2011; Rapoport 2003).

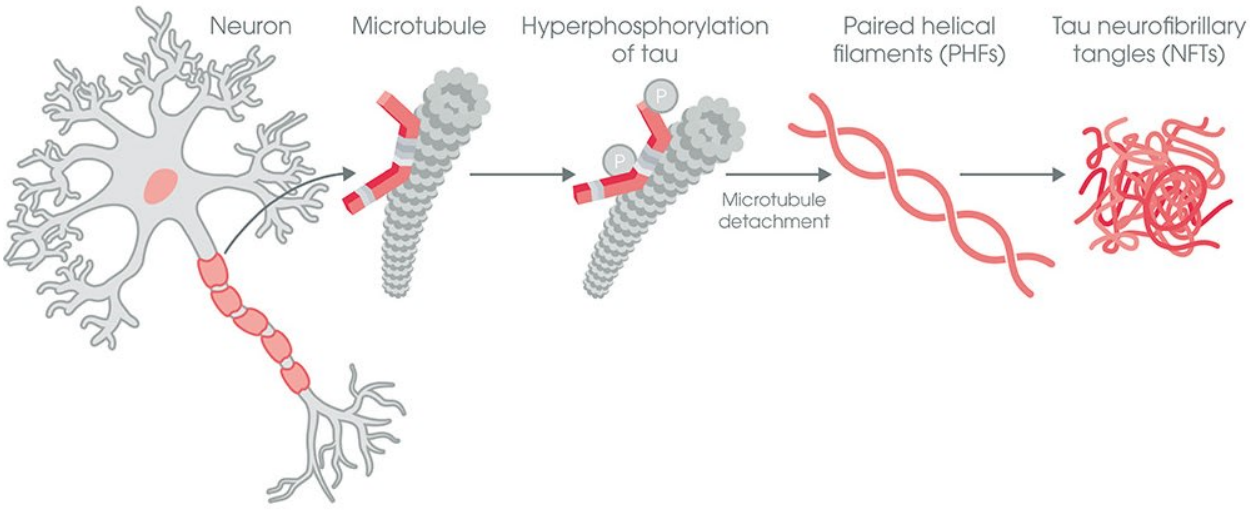

Figure 3: Progression of tau in pathological conditions to form NFTs (Abcam 2020).

Importantly, tau pathology develops in a distinct spatial distribution, beginning in the entorhinal cortex and then spreading to the hippocampus and cortical areas (Pedersen and Sigurdsson 2015). This spatial distribution correlates strongly with cognitive decline in AD, arguably to a greater extent than Aβ (Pedersen and Sigurdsson 2015). Cellular and animal models have provided evidence that tau plays a central role in neurodegeneration and AD transgenic mouse models have demonstrated that Aβ-toxicity is tau-dependent and can be inhibited by the reduction of tau levels (Ittner and Götz 2011). Genetic variants of the *microtubule associated protein tau* (*MAPT*) have been connected with familial frontotemporal dementia (FTD), however, there is conflicting literature surround whether *MAPT* mutations are associated with AD (Kauwe et al. 2008). A number of studies have found that polymorphisms in *MAPT* are associated with AD risk (Laws et al. 2007; Liu et al. 2013; Myers et al. 2005; Myers et al. 2007), while others have reported no association (Abraham et al. 2009; Mukherjee et al. 2007; Setó-Salvia et al. 2011). A major

limitation in these studies has been low statistical power and small sample sizes which may account for these discrepancies (Zhou and Wang 2017). It is evident that further research into the association of *MAPT* mutations and AD risk is necessary.

1.1.3.3 Role of neuroinflammation in AD

Neuroinflammation and the immune response also play a crucial role in AD pathogenesis. They have been found to exist alongside neurodegeneration early in the disease progression (Centonze et al. 2007). Furthermore, the role of neuroinflammation in AD is emphasised as microglia (i.e. primary immune cells in the brain) are activated in response to the presence of Aβ (Bilkei-Gorzo 2012) and are known to aggregate around Aβ plaques (Marchalant et al. 2008). In addition, the identification of genetic risk factors for sporadic AD, which are largely associated with the immune system, further emphasises the role of neuroinflammation in AD (Minter et al. 2016). Initially, the involvement of the immune response is thought to be beneficial as microglia clear Aβ plaques through phagocytosis and limit disease progression. However, eventually the levels of Aβ oligomers and plaques becomes too large for sufficient clearance and results in the constant activation of glial cells (i.e. microglia and astrocytes) (Heneka and O'Banion 2007). Activated microglia may assume two states i) a pro-inflammatory state, where they release numerous pro-inflammatory mediators including cytokines (including interleukin [IL] 1, tumor necrosis factor α [TNF-α], IL-6 and interferon γ [IFN-γ]), ROS and reactive nitrogen species or ii) an anti-inflammatory state, where they release anti-inflammatory cytokines and growth factors (including transforming growth factor-β, IL-10, nerve growth factor and IL-1 receptor antagonist) to promote healing (Kim and Joh 2006; Schwab and McGeer 2008). Importantly, microglial phagocytosis is enhanced in the anti-inflammatory state. However, it has been found that in AD, that microglia

assume the pro-inflammatory state (Schwab and McGeer 2008). The prolonged activation of pro-inflammatory microglia and subsequent release of pro-inflammatory mediators is thought to accelerate neurodegeneration and the development of AD (Heneka and O'Banion 2007). Neuroinflammation can directly promote AD pathogenesis through inducing neurotoxic effects via pro-inflammatory mediators or indirectly, where inflammation enhances Aβ levels by inducing pathogenic APP processing (Heneka and O'Banion 2007). Furthermore, once this has occurred it's hypothesised that the removal of the stimulus (e.g. Aβ) is unable to resolve the inflammatory cascade: a possible explanation for the ineffectiveness of anti-Aβ treatments in late AD (Minter et al. 2016). The inflammatory response can also lead to the release of prostaglandins, glutamate and the inhibition of glutamate reuptake, which all enhances excitotoxicity and oxidative damage (Marchalant et al. 2008). Interestingly, neuroinflammation and neurodegeneration in AD patients correlate more closely than either Aβ or tau proteins (DiPatre and Gelman 1997; Terry et al. 1991), emphasising their role in AD.

1.1.3.4 Role of oxidative stress in AD

Oxidative stress is thought to occur early in AD pathogenesis, prior to the development of learning and memory deficits and is proposed as an early indicator of developing AD (Bilkei-Gorzo 2012; Dalle-Donne et al. 2006; Praticò et al. 2002). Oxidative stress results in cell death and neurodegeneration when the brain's antioxidant defence systems cannot cope with the elevated levels of ROS produced (Smith et al. 2000). Various aspects of AD pathogenesis contribute to establishing an oxidative environment. Aβ is one of the central features initiating this process, as it induces neuroinflammation causing activated microglia to release free radicals and nitric oxide (NO) (Behl and Moosmann 2002; Smith et al. 2000) as well as increasing the generation of ROS

(Sayre et al. 1997). Hyperphosphorylated tau also contributes to oxidative stress by increasing ROS production as a result of mitochondrial dysfunction (Smith et al. 2000).

1.2 Mouse Models of AD

Mouse models of AD are used to understand behavioural and pathological changes that occur in the disease. Mouse models are a beneficial model of AD research as they have similar genetic codes (85 percent identical) and there is a high degree of phylogenetic conservation, in particular in the hippocampus and entorhinal cortex circuits which are vulnerable in AD, to humans (Hall and Roberson 2012). Despite these genetic similarities, mouse models also provide a simplified system that facilitates the isolation of individual components of AD (e.g. Aβ and tau) and enables experimental manipulation (Hall and Roberson 2012). Mouse models of AD are developed in a number of ways, including pharmacological, lesion and transgenic models. These models can mimic behavioural and cognitive impairments which are present in AD patients, for example memory loss, social withdrawal and aggression (Hall and Roberson 2012). They also replicate AD-relevant pathological changes including Aβ accumulation and aggregation, tau hyperphosphorylation and NFT formation, neurotoxicity, neuroinflammation, oxidative stress, gliosis and neurodegeneration (Hall and Roberson 2012). Different models express particular phenotypes to varying degrees; no existing model recapitulates all features of AD (Hall and Roberson 2012). The combination of behavioural and pathological changes allows us to relate behavioural changes with underlying pathogenesis, allowing for better understanding of the disease as well as effectiveness of pharmacological intervention. Therefore, mouse models play a crucial role in the preclinical assessment of novel therapeutics. However, it must be noted that many drug targets for AD which have demonstrated therapeutic benefits in preclinical research

have not translated to the clinical setting, either due to a lack of efficacy or causing severe adverse effects in humans (Franco and Cedazo-Minguez 2014; Laurijssens et al. 2013).

1.2.1 Pharmacological rodent models of AD

Pharmacological rodent models of AD are predominantly developed by the injection of Aβ into the brain by intrahippocampal or intracerebroventricular injections (Puzzo et al. 2015). They result in AD-relevant pathology, for example, the intrahippocampal injection of Aβ results in neuronal loss in the CA1, CA2 and CA3 regions, which is accompanied by apoptosis and gliosis markers (Van der Stelt et al. 2006). Inoculation with $A\beta_{42}$ results in elevated neuroinflammatory markers (Esposito et al. 2011). It is also associated with spatial memory deficits (Martín-Moreno et al. 2011). Although these models replicate aspects of AD pathogenesis, they are acute and do not demonstrate the gradual rise in Aβ which can be more closely modelled in transgenic rodent models of AD.

1.2.2 Transgenic mouse models of AD

Transgenic mouse models of AD are developed by the introduction of genetic mutations related to AD. The most commonly used gene mutations are those responsible for familial AD (e.g. *APP*, *PS1* and *PS2*). However, more recent models have been generated based on other genetic mutations related to AD, including *APOE* (Hall and Roberson 2012). Although no genetic mutations related to tau in AD patients have been identified, it is necessary to model tau pathology to understand its contribution to AD pathogenesis. Tauopathy models are often developed by expressing transgenic human tau mutations which are associated with FTD (e.g. *MAPT* mutations) (Hall and Roberson 2012). When investigating novel treatment interventions, it's important to determine if they will

have an effect on Aβ and tau pathology (Ittner and Götz 2011; Ittner et al. 2010). In this thesis I will use established mouse models for both Aβ- and tau-pathology which will be discussed in depth below.

1.2.2.1 APPxPS1 double transgenic mouse model

The double transgenic *APPswe/PS1ΔE9* (*APPxPS1*) mouse model is one of the most commonly used transgenic mouse models of AD and the Aβ-based transgenic mouse model used in this thesis. This model expresses chimeric mouse/human *APP* (Mo/HuAPP695$_{swe}$/Swedish mutations K595N/M596l) and mutant human *PS1 (PS1/ΔE9*) (Jankowsky et al. 2004) and is maintained as hemizygotes on the congenic C57BL/6J x C3H/HeJ background (as described by Borchelt et al., 1997). Importantly, there is an *APPxPS1* mouse model which is on a pure C57Bl/6 background (Pugh et al. 2007). The co-expression of *APP* and *PS1* genes causes more accelerated amyloid pathology than single transgene models. Amyloid plaques begin to develop at 4 months and increase with age (Wang et al. 2003), although it is important to note that plaques do not necessarily correlate with cognitive decline (Sloane et al. 1997). This model also expresses neuroinflammatory markers (e.g. increased inducible nitric oxide synthase [iNOS], TNF-α and IL-1β levels) (Cheng et al. 2014c; Kalifa et al. 2011) and impaired neurogenesis and oxidative stress (Hamilton and Holscher 2012). The *APPxPS1* mouse model exhibits a number of behavioural and cognitive changes, including social recognition memory deficits (Cheng et al. 2013; Cheng et al. 2014a; Cheng et al. 2014c) and spatial memory deficits in the Barnes maze (O'Leary and Brown 2009; Reiserer et al. 2007), cued and non-cued versions of the cheeseboard task (Cheng et al. 2014b; Kulkarni et al. 2008; Pillay et al. 2008), Morris water maze (Butovsky et al. 2006; Cao et al. 2014; Donkin et al. 2010; Gallagher et al. 2013; Jardanhazi-Kurutz et al. 2010) and spatial

reward test (Phillips et al. 2011). This model has also been reported to have object recognition deficits in the novel object recognition task in males (Cheng et al. 2013) and females (Donkin et al. 2010) and impaired contextual fear associated memory (Bonardi et al. 2011; Kilgore et al. 2010). This model effectively mimics AD with pathological changes due to Aβ accumulation and aggregation as well as wide-ranging behavioural and cognitive changes. Importantly, these behavioural changes are gender- and age specific which may be at least partially responsible for inter-study differences (summarised in Table 2).

Behavioural phenotype of *APPswe/PS1ΔE9* double transgenic mice

Tests	Gender	Age	Deficit	Reference
Anxiety and locomotion				
Elevated plus maze	Males and Females	7 months	Anxiolytic-like phenotype (on day 1, but not on day 2)	Lalonde et al. 2004
	Males and Females	7 months	Anxiogenic-like phenotype	Reiserer et al. 2007
	Males	7 months	Increased anxiety levels	Cheng et al. 2013a
	Males	7 months	No change in anxiety	Cheng et al. 2014a
Light Dark	Males and Females	7 months	No anxiety change	Reiserer et al. 2007
	Males	7 months	Increased locomotive activity but normal anxiety levels	Cheng et al. 2013a
	Females	9 months	Decreased anxiety levels and increased locomotor activity	Cheng et al. 2013b

Social recognition memory				
Social preference test	Males	7 months	Impaired social recognition memory	Cheng et al. 2013a
	Males	7 months	Impaired social recognition memory	Cheng et al. 2014a
	Males	10 months	Impaired social recognition memory	Cheng et al. 2014b
Spatial learning and retention memory				
Cheeseboard (cued version)	Not mentioned	2-3 months	Impaired spatial learning	Pillay et al. 2008
	Not mentioned	24 months	Impaired spatial learning	Kulkarni et al. 2008
Cheeseboard (not cued)	Females	9 months	Impaired spatial memory in reversal cheeseboard task	Cheng et al. 2013b
Morris water maze	Females	4/6/12 months	Impaired learning at all ages, impaired retention memory at 12 months	Jardanhazi-Kurutz et al. 2010
	Males	8 months	Intact learning, impaired retention memory	Cao et al. 2007
	Males and Females	9 months	Impaired reversal learning in females. Intact spatial learning and retention memory in males	Gallagher et al. 2013
	Not mentioned	9 - 10 months	Impaired learning in initial and reversal stages	Butovsky et al. 2006
	Females	10 months	Impaired spatial learning	Donkin et al. 2010
Barnes maze	Males and Females	7 months	Impaired learning (only when hidden version of	Reiserer et al. 2007

			task follows the cued version)	
	Males and Females	16 months	Impaired learning and modest impairment in retention memory (stronger in males than females)	O'Leary and Brown, 2009
Spatial reward test	Not mentioned	6-9, 11, 13, 15, 18 months	Impaired learning at 6 months. Reduced re-learning/memory from 9 months	Phillips et al. 2011
Novel object recognition memory				
Novel object recognition task	Males	7 months	Impaired object recognition memory	Cheng et al. 2014a
	Females	9 months	No object recognition memory deficit	Cheng et al. 2013b
	Females	10 months	Impaired object recognition memory	Donkin et al. 2010
	Females	12 months	Intact object recognition memory, but reduced compared to WT controls	Jardanhazi-Kurutz et al. 2010
Fear associated memory				
Contextual fear conditioning	Females	4 months	Impaired context extinction	Bonardi et al. 2011
	Male and Females	4 and 6 months	Impaired fear associated contextual memory at 6 months (when conducted 24 h after conditioning, but not 1 h)	Kilgore et al. 2010
Contextual and cue fear conditioning	Males	7 months	No changes in fear associated memory	Cheng et al. 2014a
Conditioned taste preference				

Conditioned taste preference	Males and Females	2/5 months	Impaired conditioned taste aversion	Pistell et al. 2008

Table 2. Summary of behavioural phenotypes reported in *APP$_{swe}$/PS1ΔE9* double transgenic mice.

1.2.2.2 TAU58/2 transgenic mouse model

The TAU58/2 transgenic mouse model expresses the human *04NR* tau isoform with the neuronal *P301S* mutation under the control of the mouse Thy1.2 promoter on a C75BL/6J background, as previously described (van Ersel et al. 2015). The expression of human *04NR* tau causes tau and NFT pathology. Tau pathology is present from 3 months of age onwards in the cortex, hippocampus, brainstem and amygdala in both male and female mice and progresses with age, although disease progression is slower in females (Przybyla et al. 2016; van Ersel et al. 2015). NFT pathology is present in the amygdala at 6 months of age and in the cortex, hippocampus and brainstem at 10 months of age, suggesting that the amygdala is the primary site of pathological tau deposition (Przybyla et al. 2016; van Ersel et al. 2015). Furthermore, glial fibrillary acidic protein (GFAP) expression was increased in the brainstem at 3 and 10 months of age but not in the cortex (van Ersel et al. 2015). Another study in TAU58-2/B mice (genetically similar to TAU58/2 transgenic mouse model, but a different breeding colony maintained at a different institute) found subtle tau hyperphosphorylation in the hippocampus and cortex of 8-month-old mice, which increased with age and spread to the brainstem and cerebellum by 12 months of age (Van Der Jeugd et al. 2016). In the TAU58 models, this neuropathology is accompanied by impaired gait and motor impairments in pole test and challenge beam from 2 months of age and rotarod from 6 months of age in males and 12 months of age in females (Van der Jeugd et al. 2013; van Ersel et al. 2015) . The TAU58 models also demonstrate reduced body weight and reduced spatio-temporal anxiety in the elevated plus maze (EPM) and light-dark test from 2-3 months of age onwards

(Przybyla et al. 2016; Van Der Jeugd et al. 2016; van Ersel et al. 2015). The reduced spatio-temporal anxiety has been reported as a disinhibitory phenotype, however, this has not been further investigated in behavioural tests to assess disinhibition more directly.

Furthermore, the cognitive phenotype of this model has not yet been widely investigated. In the TAU58-2/B model, mice demonstrated spatial working memory impairments in the y-maze and impaired instrumental short-term memory in the puzzle box task, thus indicating impaired executive function. Furthermore, reduced sociability was reported from 6 months of age (Van Der Jeugd et al. 2016). It is necessary to further investigate the cognitive and social impairments of this model as these relate to the cognitive deficits and social withdrawal symptoms of AD which would support its use as an AD transgenic mouse model of tauopathy (summarised in Table 3).

Behavioural phenotype of TAU58 transgenic mice					
Tests	**Model**	**Gender**	**Age**	**Deficit**	**Reference**
Anxiety/disinhibition and locomotion					
Elevated plus maze	TAU58/2	Males	3, 6 and 10 months	Anxiolytic-like / disinhibitory phenotype at all ages	Przybyla et al. 2016
Open field	TAU58/2	Males	3, 6 and 10 months	Increased locomotion in 10-month-old TAU58/2. No difference in anxiety at any age	Przybyla et al. 2016
	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	No difference in locomotion or anxiety at any age in both genders	Van der Jeugd et al. 2015
Light dark	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Anxiolytic-like/ disinhibitory phenotype at all ages in both genders	Van der Jeugd et al. 2015
Body weight and motor impairments					

Body weight	TAU58/2	Males and females	1, 2, 4 and 6 months	Reduced body weight at 2, 4 and 6 months in both genders	Van Eersel et al. 2015
	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Reduced body weight at all ages (gender mixed)	Van der Jeugd et al. 2015
Pole Test	TAU58/2	Males and females	2 and 6 months	Impaired motor function at both ages in both genders	Van Eersel et al. 2015
Challenge beam	TAU58/2	Males and females	2 and 6 months	Impaired motor function at both ages in both genders	Van Eersel et al. 2015
Rotarod	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Motor impairments in 6-7 and 10-11-month-old mice (gender mixed)	Van der Jeugd et al. 2015
Grip strength	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Motor impairments in 10-11 month old mice (gender mixed)	Van der Jeugd et al. 2015
Sociability					
Social interaction	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Decreased social exploration in 6-7 and 10-11-month-old males and females	Van der Jeugd et al. 2015
Cognition					
Spontaneous alternation paradigm	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Impaired spatial working memory in 6-7- and 10-11-month-old males and females	Van der Jeugd et al. 2015
Puzzle box	TAU58-2B	Males and females	2-3, 6-7 and 10-11 months	Impairment in dig trials on day 1 in 10-11-month-old mice, and impairment in plug trial on day 2 in 6-7- and 10-11-month-old mice	Van der Jeugd et al. 2015

Table 3. Summary of behavioural phenotypes reported in TAU58 transgenic mice

1.3 Current Treatments and Clinical Trials

Despite the growing prevalence of AD and the increase in our understanding of the disease progression and diagnostics, effective drug therapies are missing. However, various novel therapeutic targets have been identified for the treatment of AD based on preclinical research and have shown promise to reduce AD neuropathology and even restore memory impairments in AD patients. In the following sections I will outline the approved treatments for AD and a number of promising strategies currently under investigation in clinical trials.

1.3.1 Currently Approved AD Medication - Acetylcholinesterase (AChE) inhibitors and NMDA receptor antagonists

Current approved treatments provide only symptomatic relief early in the disease progression and possess therefore only limited therapeutic benefits (Mangialasche et al. 2010). There are only four approved drugs on the market for AD; three AChE inhibitors (rivastigmine, donepezil and galantamine) and one NMDA receptor antagonist (memantine) (Mangialasche et al. 2010). In addition to limited therapeutic benefits, they are also associated with adverse effects. AChE inhibitors can cause nausea, vomiting, diarrhoea and weight loss (Kaduszkiewicz et al. 2005), while memantine is known to cause hallucinations, dizziness and fatigue (Herrmann et al. 2011). Most importantly, these treatments do not stop or reverse the disease progression, highlighting the need for novel therapeutics (Salomone et al. 2012).

1.3.2 Clinical trials

Since the discovery of AD, hundreds of therapeutics have been tested in clinical trials and only the four drugs mentioned above have made it onto the market. More specifically, in the last 10 years

over 50 drugs have passed phase II trials but none have progressed past phase III trials (Bachurin et al. 2017) and in February 2019, there were 132 therapeutic agents in current clinical trials (Cummings et al. 2019). The majority of these therapeutics have focused on targeting the Aβ protein, however other target areas include tau aggregation, the cholinergic system, and neuroinflammation (Hung and Fu 2017). Anti-Aβ therapies are mostly classified into anti-Aβ immunotherapies, β and γ-secretase inhibitors and anti-aggregation therapeutics (Bachurin et al. 2017). Of these, the most successful have been the immunotherapies. A number of these have made it to phase III clinical trials after demonstrating beneficial effects on cognition and AD-relevant brain pathology (e.g. solanezumab, aducanumab, bapineuzumab). Unfortunately, over the years solanezumab and bapineuzumab were discontinued due to failure to have beneficial effects on cognitive ability (Doody et al. 2014; Salloway et al. 2014), while aducanumab was discontinued after a phase III futility analysis determined it would not achieve its goal (Howard and Liu 2019). Interestingly, most recently, aducanumab may be reinvestigated as the developing company, Biogen Inc., found through reanalysis of the data that it was effective in people who received a high dose with a long time exposure and are seeking to re-investigate its therapeutic potential in 2020 (Howard and Liu 2019; Kaplon et al. 2019). β- and γ-secretase inhibitors have also been investigated as potential treatment options. However, β-secretases are difficult to target, and γ-secretases affect a wide range of functions thus when inhibited result in a number of adverse effects, including impaired cognition and functionality, gastrointestinal toxicity and increased incidence of skin cancer in AD patients (Imbimbo and Giardina 2011; Schenk et al. 2012).

The majority of anti-tau therapies that have progressed to clinical trials are also immunotherapies with preclinical trials having demonstrated their ability to reduce NFTs and tau hyperphosphorylation (Jadhav et al. 2019; McGeer et al. 2006; Mullane and Williams 2013;

Schenk et al. 2012). The only results from tau immunotherapy clinical trials are in primary proteinopathies (e.g. progressive supranuclear palsy), as no results from AD clinical trials have been released to date. These clinical trials were stopped prematurely due to failure to reach endpoints. Despite the limited therapeutic benefits and further clinical trials in primary proteinopathies being discontinued, two of these tau immunotherapies (ABBV-8E12 and gosuranemab) will be evaluated in phase II clinical trials *(http://www.ClinicalTrials.gov* identifiers NCT03712787 and NCT03352557*)*. Other anti-tau therapies include selective inhibitors of tau protein aggregation, for example LMT-X Methylene Blue, which have advanced to phase III clinical trials. One clinical trial for LMT-X Methylene Blue failed to demonstrate significant therapeutic benefits (Gauthier et al. 2016), while other clinical trials are currently in progress with results pending (*http://www.ClinicalTrials.gov* identifiers NCT03446001 and NCT03539380).

Epidemiological data have shown that non-steroidal anti-inflammatory drugs (NSAIDs) are associated with reduced risk of AD (McGeer et al. 2006). Likewise, animal studies indicated that NSAID treatments can attenuate AD pathogenesis, suggesting that treating neuroinflammation may slow the progression of the disease (Maccioni et al. 2009). However, NSAID treatments have also been associated with severe long-term adverse effects (e.g. gastrointestinal problems) and have shown limited efficacy in reducing or preventing clinical symptoms of AD in humans (McGeer et al. 2006; Rojo et al. 2008).

Importantly, it is unlikely that any drugs acting on a single pathway or molecular target will mitigate the complex pathoetiological cascade leading to AD. Instead, a multifunctional drug approach targeting a number of AD pathologies simultaneously may provide better, wider-ranging benefits than current therapeutic approaches (Bedse et al. 2015; Van der Schyf and Geldenhuys 2011). The endocannabinoid system (eCBS) is now being investigated as a novel therapeutic target

for AD as it can regulate various systems which contribute to the disease pathogenesis, including learning and memory, brain plasticity and inflammation (Aizpurua-Olaizola et al. 2017).

1.4 The Endocannabinoid System (eCBS) and AD

1.4.1 The eCBS

The eCBS is comprised of cannabinoid receptors (the best described being cannabinoid receptors 1 and 2 [CB1 and CB2]), endocannabinoids (anandamide [AEA] and 2-arachiodonoylglycerol [2-AG]), and the enzymes required for their synthesis and degradation (fatty acid amide hydrolase [FAAH], monoglyceride lipase [MAGL], diacylglycerol [DAGL] and *N*-acyl-phosphatidylethanolamines-hydrolysing phospholipase D [NAPE-PLD]) (D'Addario et al. 2012; Di Marzo et al. 2015) (as seen in Figure 4). The eCBS plays a role in regulating a number of different systems in the brain, including neuroplasticity, learning and memory, neuronal development, nociception, inflammation, metabolism, sleep-wake cycle and regulation of stress and emotion, making it an ideal target for a wide range of potential therapies (Aizpurua-Olaizola et al. 2017). CB1 and CB2 receptors are differentially distributed throughout the brain, in line with their distinct functions (Benito et al. 2003).

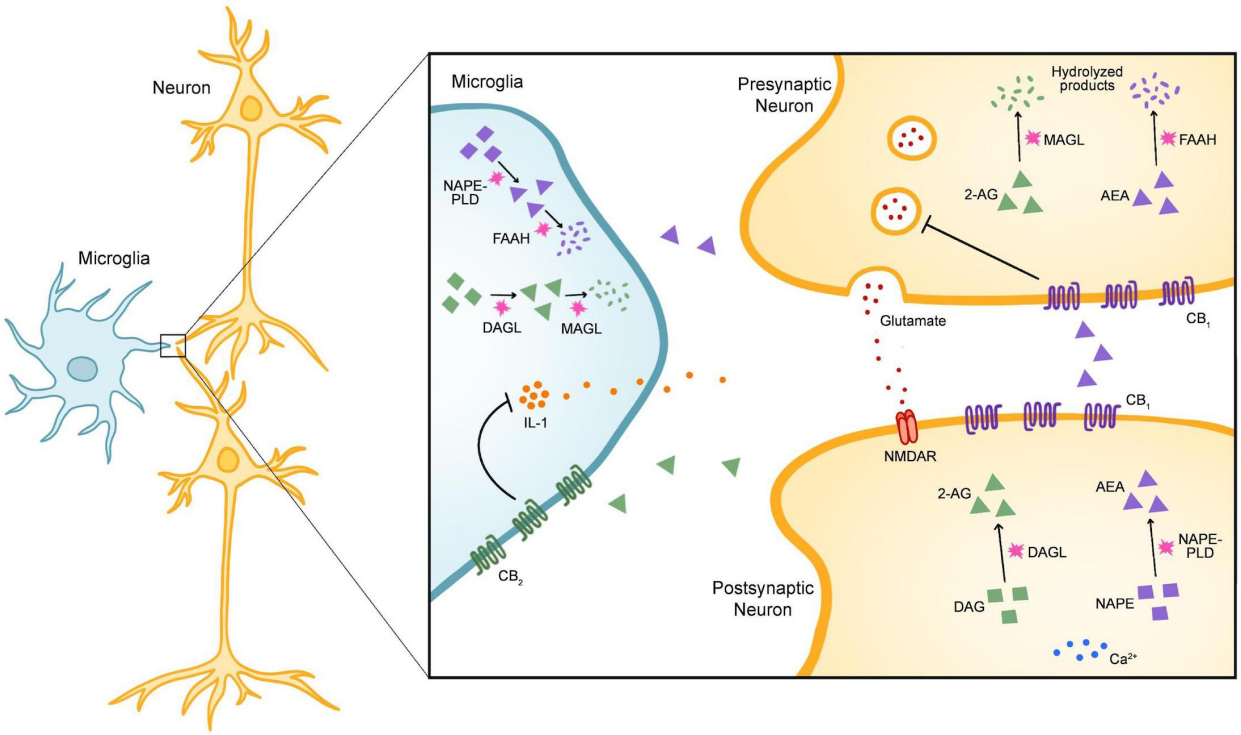

Figure 4: Components of the eCBS and their interactions in microglia and neurons (Araujo et al. 2019).

CB1 receptors are some of the most abundant and widely distributed G-protein coupled receptors (GPR) in the brain. They are expressed in the hippocampus, amygdala, cerebellum, substantia nigra, basal ganglia, striatum and cerebral cortex. Thus, CB1 receptors are involved in a broad range of processes including learning and memory processes, control of movement, regulation of emotion and complex cognitive function (Di Iorio et al. 2013). They also play a role in neuroprotection by protecting the central nervous system from neurotransmitter overstimulation or over inhibition (Di Iorio et al. 2013). In contrast, CB2 receptors are expressed on immune cells (i.e. microglia) and therefore play a role in immunomodulation (Buckley et al. 2000). CB1 and CB2 are not the only receptors involved in the eCBS. Cannabinoids interact with a number of other receptors as well, including other cannabinoid receptors (e.g. orphan receptor GPR55 and GPR18), the vanilloid receptor 1 (TRPV1), the peroxisome proliferator-activated receptors (PPAR) α and

γ, and various neurotransmitter receptors, for example noradrenaline, glutamate, opioids, gamma-aminobutyric acid (GABA), ACh and serotonin (5-HT) (Pertwee et al. 2010).

1.4.2 The role of the eCBS in AD

The eCBS regulates a number of age-related processes, all of which are impaired in AD. For example, it is known to modulate mitochondrial activity through reducing the production of ROS (Athanasiou et al. 2007), reduce oxidative stress via its antioxidant properties (Marsicano et al. 2002), modulate the immune system through microglial activation (Germain et al. 2002) and promote the clearance of damaged macromolecules (Bilkei-Gorzo 2012). Thus, highlighting the therapeutic potential of targeting the eCBS for AD therapy. Further support for this theory is the ability of endocannabinoids to reduce the hallmark features of AD (i.e. Aβ generation and tau hyperphosphorylation) (Esposito et al. 2006a; Eubanks et al. 2006), promote Aβ clearance (Tolón et al. 2009), modulate neuroinflammation (Ehrhart et al. 2005) and potentiate ACh homeostasis (Eubanks et al. 2006). Importantly, post-mortem analyses have found that several of the components of the eCBS are altered in composition and signalling in AD brain tissue, for example CB2 and FAAH upregulation in microglia surrounding Aβ plaques (Benito et al. 2003; Ramírez et al. 2005; Solas et al. 2013), decreased AEA levels in midfrontal and temporal cortices (Jung et al. 2012) and impaired MAGL recruitment and increased DAGL expression which result in altered 2-AG signalling (Mulder et al. 2011).

Importantly, CB1 receptors are known to be associated with cognitive and psychoactive adverse effects. Furthermore, their expression has been found to be either reduced or unaltered in AD, while CB2 receptors are not associated with these side effects and are upregulated in AD. In particular, CB2 expression has been found to correlate with $A\beta_{42}$ levels and plaque deposition

(Benito et al. 2003; Ramírez et al. 2005). Therefore, in order to avoid possible side effects due to CB1 activation (i.e. psychoactivity (Onaivi et al. 1995) and potential detrimental effects on cognitive domains (Mallet and Beninger 1998)), research has focused on targeting CB2 receptors, manipulating endocannabinoid levels or using low doses of CB1/CB2 receptor agonists (Marchalant et al. 2012). Importantly, due to the immunomodulatory role of CB2 receptors, agonists targeting this receptor are able to modulate the activation of microglia thereby reducing the release of pro-inflammatory cytokines (i.e. TNF-α and NO) (Ehrhart et al. 2005; Ramírez et al. 2005) and also promote Aβ phagocytosis (Tolón et al. 2009).

Recently, phytocannabinoids (cannabinoids present in *Cannabis sativa*) have been investigated for their potential as therapeutics in a number of neurological disorders (Karl et al. 2016). The two main active constituents of the *Cannabis sativa* plant are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). However, as THC is an agonist for the CB1 receptor, it is associated with negative cognitive and psychoactive effects when used in isolation, indicating that it may not be an ideal treatment (Iuvone et al. 2004). Interestingly, when used in combination with CBD as Sativex® (GW pharmaceuticals, Salisbury, United Kingdom) to treat inflammation and spasms in multiple sclerosis it has been found to be well tolerated with limited adverse effects in humans (Iuvone et al. 2009; Thomas et al. 2007). Importantly, CBD is not an agonist at the CB1 receptors and therefore is not associated with the negative side effects seen with THC (Fadda et al. 2004). Thus, CBD alone has been investigated as a novel therapeutic for AD.

1.5 Cannabidiol (CBD)

CBD has recently gained a lot of attention for its therapeutic potential to treat a range of neurodegenerative and inflammatory disorders, due to its neuroprotective, anti-inflammatory and antioxidant properties (Iuvone et al. 2009). The antioxidant and neuroprotective properties of CBD

were demonstrated *in vitro* in a study which demonstrated it was more protective than the well-known antioxidant α-tocopherol in a model of glutamate neurotoxicity (Hampson et al. 1998). These properties were further supported by an *in vitro* study in lipopolysaccharide-macrophages which demonstrated that CBD treatment decreased protein levels of TNF-α, iNOS and NF-kB (Rajan et al. 2016) and in another study in lipopolysaccharide-microglia where CBD treatment inhibited the release of cytokines [i.e. TNF-α and IL-1β] and glutamate (dos-Santos-Pereira et al. 2019). Another *in vitro* study in a model of allergic contact dermatitis highlighted CBD's anti-inflammatory effects as it dose-dependently inhibited the release of IL-6, IL-8, TNF-α and monocyte chemotactic protein-2 (Petrosino et al. 2018). A study in a rodent model of ethanol-induced neurotoxicity demonstrated that CBD protected against hippocampal and entorhinal cortical neurodegeneration (Hamelink et al. 2005). In a rodent model of arthritis, CBD was able to reduce cytokine production and release (i.e. interferon [IFN]-γ and TNF-α), decrease the inflammatory response by inhibiting lymphocyte proliferation and reduce oxidative stress via ROS production (Malfait et al. 2000). CBD's antioxidant and anti-inflammatory properties were demonstrated in a mouse model of hepatic ischemia, as it suppressed reactive gliosis by reducing TNF-α expression and neurotrophin S100 calcium binding protein B (S100B) in lipopolysaccharide-mice (De Filippis et al. 2011). Furthermore, these properties were evident in a study in male swiss mice which demonstrated that CBD was able to prevent a haloperidol-induced increase in microglial activation and release of inflammatory mediators [i.e. IL-1β, IL-6 and TNF-α], as well as prevent haloperidol-induced lipid peroxidation (Sonego et al. 2018).

1.5.1 Pharmacology of CBD

1.5.1.1 CBD in the eCBS

CBD has a complex interactive link with the eCBS. It has demonstrated low displacement at the CB1 and CB2 receptors compared to other cannabinoids, such as THC (Thomas et al. 1998). CBD has also been shown to have low affinity for both cannabinoid receptors (Petitet et al. 1998) and has antagonistic properties against the synthetic cannabinoid CP 55,940, which is a potent agonist at both CB1 and CB2 receptors. Interestingly, CBD antagonizes CP 55,940 at a much lower concentration than it binds to the cannabinoid receptors, suggesting it may act at a prejunctional site which is not the cannabinoid receptors (Pertwee et al. 2002). CBD acts as an inverse agonist at the CB1 and CB2 receptors, which may explain some of its anti-inflammatory properties as inverse agonists at CB2 receptors are able to inhibit the migration of immune cells (Lunn et al. 2006). More recently, research has indicated that CBD may act as a negative allosteric modulator at CB1 receptors, which could account for how CBD attenuates negative effects of THC (Chung et al. 2019). CBD has also been found to act as an antagonist at the cannabinoid receptors GPR55 and GPR18 (McHugh et al. 2010; Ryberg et al. 2007), as well as to activate the putative abnormal CBD receptor (Pertwee 2008) (summarised in Figure 4).

1.5.1.2 CBD in other areas

CBD has been reported to interact with a number of non-cannabinoid receptors. It acts as an agonist at the PPARγ receptors, where it is thought to mediate, at least partially, its anti-inflammatory effects (Esposito et al. 2011). It has shown to activate TRPV1 and is thought to also demonstrate some of its anti-inflammatory effects via this activation (Bisogno et al. 2001). In addition, a study in gingival mesenchymal stem cells (GMSCs) found that CBD stimulation at TRPV1 inhibited glycogen synthase kinase 3β (GSK-3β) and subsequently reduced Aβ generation and tau hyperphosphorylation (Diomede et al. 2017). It has also been shown to inhibit the adenosine

transporter and subsequently increase adenosine receptor 2 (A_2) signalling, which has been reported to mediate some of CBD's anti-inflammatory and neuroprotective effects (Carrier et al. 2006; Castillo et al. 2010; Sagredo et al. 2007). Furthermore, CBD interacts with a number of neurotransmitter systems, including the glutamate receptors (i.e. NMDA receptors, 2-amino-3-(-4-butyl-3-hydroxyisoxazol-5-yl)propionic acid [AMPA] receptors and kainite receptors), serotonergic receptors 1A (5-HT1A) and 5-HT2A and nicotinic ACh receptor (Mahgoub et al. 2013; Russo et al. 2005) (summarised in Figure 5).

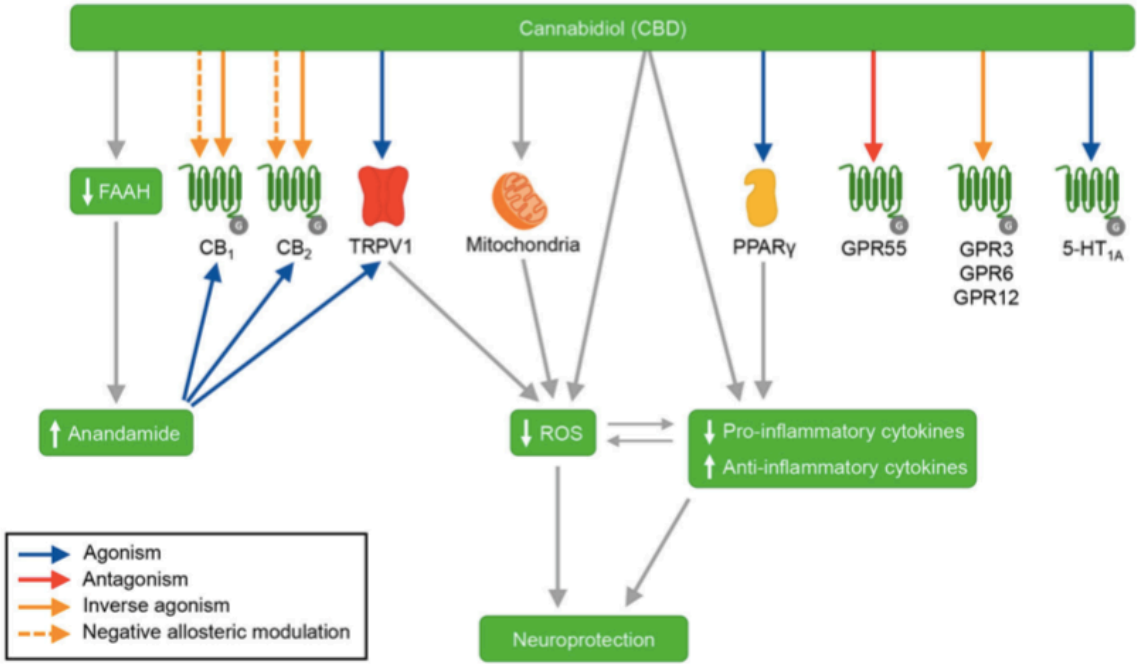

Figure 5: Summary of CBDs mechanisms of action (Peres et al. 2016).

1.5.2 In vitro effects of CBD in AD

In PC12 neuronal cells, CBD protected against Aβ-induced neurotoxicity and oxidative stress by promoting neuronal survival and reducing ROS, lipid peroxidation, caspase-3, DNA fragmentation and intracellular calcium levels (Iuvone et al. 2004). Caspase-3 and DNA fragmentation are

hallmark features of apoptosis (Gschwind and Huber 1995; Nicholson and Thornberry 1997), while elevated intracellular calcium levels are thought to contribute to Aβ-induced toxicity (Mattson 2002), therefore indicating the neuroprotective and anti-apoptotic properties of CBD. Additional studies in Aβ-induced PC12 neuronal cells demonstrated that CBD was able to inhibit tau hyperphosphorylation (Esposito et al. 2006a) as well as the transcription of the proinflammatory gene, iNOS and the subsequent release of NO (Esposito et al. 2006b). The inhibition of tau hyperphosphorylation was associated with a decrease in phosphorylated GSK-3β (p-GSK-3β) (Esposito et al. 2006a). p-GSK-3β is the active form of GSK-3β, also known as tau protein kinase, and, in its active form, is responsible for tau hyperphosphorylation and NFT formation (Esposito et al. 2006a). Oxidative stress can promote the phosphorylation of GSK-3β into its active form and therefore, CBD's ability to reduce p-GSK-3β is thought to be at least partially mediated by its antioxidant properties (Chen et al. 2004; Esposito et al. 2006a). Additionally, a study in GMSCs pre-treated with CBD demonstrated that CBD downregulated genes coding for kinases involved in aberrant tau production and secretases involved in Aβ production. CBD pre-treatment also upregulated genes for enzymes involved in Aβ degradation and increased mRNA expression of genes involved in ubiquitination (Diomede et al. 2017) (genes presented in Table 4).

Genes regulated by CBD pre-treatment in GMSCs	
Effect of CBD	**Genes**
Genes coding for kinases involved in aberrant tau production	
Downregulated	*GSK-3β, CDK5, DYRK1A, CAMK2A, MAPK1, MAPK12, MAPK14*
Genes coding for secretases involved in Aβ production	

Downregulated	*PS1, PS2, NCSTN, PSENEN, APH1A*
Genes for enzymes involved in Aβ degradation	
Upregulated	*ACE1, ECE1, IDE*

Table 4. Summarising genes regulated by CBD pre-treatment in GMSCs as seen in Diomede et al. 2017. *Glycogen synthase kinase 3β, GSK-3β; dual specificity tyrosine phosphorylation regulated kinase 1A, DYRK1A; calcium/calmodulin dependent protein kinase II α, CAMK2A; mitogen-activated protein kinase, MAPK; presenilin, PS; nicastrin, NCSTN; presenilin enhancer, PSENEN; anterior pharynx-defective 1A, APHA1A; angiotensin converting enzyme 1, ACE1; endothelin converting enzyme 1, ECE1; insulin degrading enzyme, IDE.*

CBD was also able to reduce p38 MAPK, which is a stress-activated kinase that activates the transcription factor nuclear factor-κB (NF-κB) (Esposito et al. 2006b). NF-κB regulates the expression of genes that are involved in an array of different processes including cell differentiation, proliferation, apoptosis and the oxidative, inflammatory and immune responses (Moncada et al. 1991). Importantly, NF-κB is involved in the expression of iNOS and therefore, CBD's ability to inhibit p38 MAPK and NF-κB emphasises its antioxidant properties, through the downstream inhibition of NO production (Esposito et al. 2006b).

Another study in N13 microglial cells reported that CBD was able to increase microglial migration, demonstrating its ability to modulate immune cells (Martín-Moreno et al. 2011). It was hypothesized that CBD exerts these effects through the cannabinoid or A_{2A} receptors, as CB1 and CB2 antagonists blocked the beneficial effects of CBD and an A_{2A} receptor agonist exhibited a similar response (Martín-Moreno et al. 2011). A study in PC12 and SHSY5Y cells further emphasised CBD's antioxidant effects as it improved cell viability in response to tert-butyl-hydroperoxide treatment (Harvey et al. 2012). Another study in SHSY5Y cells demonstrated that CBD was able to directly protect against Aβ mediated neurotoxicity as well as prevent microglial-activated neurotoxicity (Janefjord et al. 2014). Additionally, a study in $SHSY5Y^{APP+}$ cells

demonstrated that CBD induced APP ubiquitination and therefore reduced subsequent Aβ production (Scuderi et al. 2014). Furthermore, CBD was found to increase cell survival by reducing the apoptotic rate. These effects were mediated at least partially through the PPARγ (Scuderi et al. 2014). Furthermore, an *in vitro* study in hippocampal slices from C57/Bl6 mice found that pre-treatment with CBD was able to reverse $A\beta_{42}$-dependent long-term potentiation impairments (Hughes and Herron 2019). This study found that these neuroprotective effects of CBD were prevented when a PPARγ antagonist was present, but not in the presence of 5-HT_{1A}, A_{2A} and CB1 antagonists. Therefore, indicating that CBD also protects synaptic plasticity via PPARγ (Hughes and Herron 2019) (summarised in Table 5).

Effect of Cannabidiol on AD-like Pathology

Model	Effect	References
***In vitro* studies using CBD**		
PC12 Neuronal Cells	Protected against Aβ neurotoxicity and oxidative stress, increased cell survival, decreased ROS production and lipid peroxidation	Iuvone et al. 2004
	Inhibited tau hyperphosphorylation	Esposito et al. 2006a
	Prevented transcription of pro-inflammatory genes	Esposito et al. 2006b
Primary Rat Microglia	Increased microglial migration and prevented ATP-induced intracellular calcium increase	Martin-Moreno et al. 2011
PC12 and SHSY5Y Cells	Improved cell viability after treatment with *tert*-butyl hydroperoxide treatment	Harvey et al. 2012

SHSY5Y Cells	Protected against Aβ neurotoxicity and microglial-activated neurotoxicity	Janefjord et al. 2014
SHSY5Y^{APP+} Cells	Induced APP ubiquitination and subsequent Aβ production and increased cell survival by reducing apoptotic rate	Scuderi et al. 2014
$A\beta_{42}$ treated hippocampal slices from C57BL/6 mice	Pre-treatment with CBD prevented development $A\beta_{42}$ mediated deficits in long term potentiation	Hughes and Herron, 2018
Mesenchymal Stem Cells	Pre-treatment with CBD caused downregulation of genes coding for kinases linked to tau phosphorylation and secretases involved in Aβ generation	Diomede et al. 2017
***In vivo* studies using CBD**		
Mice inoculated with human $A\beta_{42}$ peptide	Attenuated Aβ induced neuroinflammatory responses by decreasing expression of pro-inflammatory gene and mediators	Esposito et al. 2007
	Dose-dependently decreased Aβ-induced neuroinflammation and promoted hippocampal neurogenesis. Effects exerted though PPARγ receptors	Esposito et al. 2011
Mice intraventricularly injected with fibrillar Aβ	Decreased microglial activation and reversed a spatial reference memory deficit in the Morris water maze	Martin-Moreno et al. 2011
APPxPS1 transgenic mice (mixed background)	Reversed social and object recognition memory deficits in the cheeseboard task and novel object recognition task, respectively	Cheng et al. 2014a
	Prevented development of social recognition memory deficits. No effect on Aβ load but	Cheng et al. 2014b

	subtle effects on inflammatory markers, cholesterol and dietary phytosterol retention	
Young *APPxPS1* transgenic mice (mixed background)	Improved memory deficits in two-object recognition task but not active avoidance task. Reduced astrogliosis around Aβ plaques but did not affect Aβ levels	Aso et al. 2014
***In vivo* studies using CBD-THC**		
Young *APPxPS1* transgenic mice (mixed background)	Improved memory deficits in the two-object recognition task and the active avoidance task. Decreased soluble $A\beta_{42}$ levels, changed plaque composition, reduced astrogliosis, microgliosis and inflammatory related molecules	Aso et al. 2014
Aged *APPxPS1* transgenic mice (mixed background)	Restored cognition in the two-object recognition task but had no effects on Aβ load or related glial reactivity	Aso et al. 2016
Transgenic tauopathy mouse model ($PK^{-/-}$ /Tau^{VLW} mice)	Reduced Aβ and tau deposition in the hippocampus and cerebral cortex, increased autophagy, decreased gliosis, increased the ratio of reduced/oxidized glutathione and reduced levels of iNOS	Casarejos et al. 2013

Table 5. Summary of the effects of CBD and CBD-THC combinations on AD models. *Amyloid-β, Aβ; reactive oxygen species, ROS; amyloid precursor protein, APP; cannabidiol, CBD; peroxisome proliferator-activated receptor, PPAR; Δ^9-tetrahydrocannabinol, THC; inducible nitric oxide synthase, iNOS.*

1.5.3 In vivo effects of CBD in AD

1.5.3.1 Pharmacological mouse models of AD

The *in vivo* therapeutic potential of CBD was initially reported in pharmacological models of AD (e.g. inoculation with fibrillar Aβ). These studies described the anti-inflammatory and

neuroprotective effects of CBD. The *in vivo* anti-inflammatory effects of CBD were confirmed in a pharmacological mouse model of AD where the mice were intrahippocampally injected with human $A\beta_{42}$ and then treated daily with intraperitoneal (i.p.) injections of CBD (2.5 or 10 mg/kg) for 7 days (Esposito et al. 2007). The results from this study demonstrated that CBD dose-dependently inhibits GFAP mRNA and protein expression. GFAP is the best known marker of activated astrocytes and thought to be one of the main features of reactive gliosis (Esposito et al. 2007). Therefore, these results imply that CBD reduces Aβ-induced reactive gliosis. In addition, CBD reduced both iNOS and IL-1β protein expression and the related NO and IL-1β release (Esposito et al. 2007). NO and IL-1β are a few of the many active substances released by Aβ-stimulated microglia and therefore have been identified as potential modulators of neuronal damage. NO is a free radical and important in neuroinflammatory and neurodegenerative conditions, which include accelerating protein nitration and increasing tau hyperphosphorylation (Esposito et al. 2007). IL-1β is involved in the cytokine cycle responsible for neurodegeneration, the synthesis and processing of APP, the activation of astrocytes and the overexpression of iNOS and overproduction of NO (Esposito et al. 2007). Data from *in vitro* studies suggest that CBD may be able to reduce iNOS protein expression and NO release as a result of its ability to rescue the Wnt/β-catenin pathway by reducing GSK-3β activation, which plays a role in tau hyperphosphorylation (Esposito et al. 2006a). Furthermore, the ability of CBD to attenuate reactive gliosis may result from its ability to act as an inverse agonist at the CB2 receptors, which is thought to be involved in reactive gliosis (Thomas et al. 2007; Walter and Stella 2004).

The anti-inflammatory and neuroprotective effects of CBD were further investigated in a rat model of AD-related neuroinflammation. This study evaluated the involvement of the PPAR receptors in the therapeutic effects of CBD, as PPARγ receptors are increased in AD patients (Esposito et al.

2011). Adult, male rats were inoculated with human $A\beta_{42}$ in the hippocampus and then treated with 10 mg/kg CBD either in the presence or absence of a PPARγ or PPARα receptor antagonist for 15 days. CBD dose-dependently decreased Aβ-induced expression of iNOS, GFAP, S100B and p50 and p56 antibodies in rat astrocytes (Esposito et al. 2011). iNOS and GFAP, as mentioned previously, are key elements in reactive gliosis and therefore their reduction demonstrates CBD's anti-inflammatory properties. CBD's ability to reduce reactive gliosis is further emphasised by the inhibition of S100B. S100B is an astroglia-derived neurotrophin that plays a crucial role in the pro-inflammatory cycle and the promotion of APP to $A\beta_{42}$. It is also responsible for inducing tau hyperphosphorylation by disrupting the Wnt/β catenin pathway (Esposito et al. 2011). Furthermore, the reduction of p50 and p56 expression indicates CBD's ability to inhibit NF-κB and therefore emphasises the responsibility of both PPARγ and NF-κB in CBD's anti-inflammatory properties (Esposito et al. 2011). Importantly, the therapeutic benefits of CBD were blocked when co-administered with the PPARγ antagonist (but not the PPARα antagonist), suggesting that CBD-induced anti-inflammatory properties are mediated, at least partially, through the PPARγ receptors (Esposito et al. 2011). Finally, the study found that CBD restored CA1 pyramidal neurons to a similar integrity to that of the control rats. CBD also down-regulated gliosis and repaired neurogenesis in the dentate gyrus (Esposito et al. 2011).

One study investigated the effects of CBD on cognition in a pharmacological model of AD (intraventricularly injected with 2.5 μg of fibrillar Aβ). They were then treated with 20 mg/kg CBD via daily i.p. injections for one week and then three times per week for the following two weeks. The spatial learning of the mice was assessed in the Morris water maze (Martín-Moreno et al. 2011). CBD treatment reversed the cognitive deficits of Aβ-treated mice. Interestingly, selective CB2 agonists did not prevent the cognitive deficit, indicating that CBD exerted this

therapeutic effect via other mechanisms (Martín-Moreno et al. 2011). CBD treatment also prevented Aβ-induced IL-6 gene expression. This suggests that the behavioural benefits documented may be mediated by glial cell modulation, as IL-6 both promotes astrogliosis and activates microglia (Rubio-Perez and Morillas-Ruiz 2012). However, CBD did not influence TNF-α gene expression (Martín-Moreno et al. 2011). *In vitro* results from this study supported this finding as CBD treatment promoted microglial activation in cultured microglia (Martín-Moreno et al. 2011) (summarised in Table 5).

1.5.3.2 Transgenic Aβ mouse models of AD

Although pharmacological models of AD are useful in producing AD-like symptoms, transgenic mouse models are a more ideal model of AD as they result from gene mutations related to the disease and pharmacological intervention will be interacting with the long-term accumulation of AD-related pathology. Initially, two studies were conducted in our laboratory to elucidate the remedial and preventative potential of chronic CBD treatment in AD transgenic mice. To assess the remedial effects of CBD, adult male *APPxPS1* mice were treated for 3 weeks with CBD (20 mg/kg CBD, daily i.p. injections) post onset of cognitive deficits and AD pathology (Cheng et al. 2014a). CBD treatment was able to reverse cognitive deficits in object recognition memory and social recognition memory without influencing anxiety parameters (Cheng et al. 2014a).

In the preventative treatment study, male *APPxPS1* mice at the age of 2.5 months were treated for 8 months with either 20 mg/kg CBD or vehicle pellets using a daily voluntary oral administration protocol (Cheng et al. 2014c). This assessed the long-term effect of CBD prior to onset of AD-like symptoms. Long-term CBD treatment was able to prevent the development of social recognition memory deficits without affecting anxiety domains in AD transgenic mice (Cheng et al. 2014c). These beneficial effects were not associated with a reduction in oxidative damage. There was also

no difference in hippocampal or cortical soluble and insoluble levels of $A\beta_{40}$ and $A\beta_{42}$ in the AD transgenic mice regardless of treatment. Furthermore, cortical lipid oxidation levels were not altered by CBD treatment. However, the study did report a complex interaction between CBD treatment, AD genotype and cholesterol and phytosterol levels, suggesting cholesterol and phytosterol may be involved in the mechanisms behind the beneficial effects of CBD. There was also a subtle impact of CBD on inflammatory markers of the brain (Cheng et al. 2014c).

A later study carried out by international colleagues compared the effect of CBD, THC and a CBD-THC combination treatment in *APPxPS1* mice. Mice were treated with 0.75 mg/kg CBD, THC or CBD-THC for 5 weeks via daily i.p. injections in the early symptomatic phase of AD (~6 months). The study found that CBD treatment reduced memory impairments in the two-object recognition task but not in the active avoidance task. It was also found that CBD could reduce astrogliosis around Aβ plaques (Aso et al. 2014). A follow-up study by Aso et al., (2016) investigated the effect of CBD-THC combination treatment on memory and brain pathology in aged male *APPxPS1* mice and littermate controls (12 months of age) as well as non-aged controls (3 months of age) (Aso et al. 2016). Compared to non-aged controls, vehicle treated aged mice demonstrated impaired cognition in the two-object recognition task. Interestingly, CBD-THC combination restored the memory deficit of *APPxPS1* mice but not in wild-type like (WT) control mice (Aso et al. 2016). In contrast to their previous study testing young *APPxPS1* mice, this study found that CBD-THC combination treatment did not influence the Aβ load or the related glial reactivity in aged AD transgenic mice (Aso et al. 2016) (summarised in Table 5).

1.5.3.3 Transgenic tau mouse models of AD

To date, no *in vivo* studies have investigated the effect of purified CBD in tauopathy models of AD. One study conducted by Casarejos et al., (2013) investigated the effect of Sativex (CBD-THC combination therapy, 1:1 ratio, 1.5 mg/kg of each compound) in a mouse model of tauopathy, the parkin-null, human tau overexpressing ($PK^{-/-}$/Tau VLW) mice. This mouse model was foremost a model of FTD, parkinsonism and lower motor neuron disease. However, Sativex decreased gliosis, increased the ratio of reduced/oxidized glutathione and reduced levels of iNOS (Casarejos et al. 2013), thereby demonstrating neuroprotective and antioxidant properties. Importantly, the study reported that Sativex reduced Aβ and tau deposition in the hippocampus and cerebral cortex as well as increased autophagy (Casarejos et al. 2013). Although the mouse model is not directly related to AD, the therapeutic benefits of Sativex in this study are relevant to AD neuropathology, suggesting these findings are highly translatable to AD (summarised in Table 5).

1.5.4 Clinical Trials with CBD

There are no clinical trials to date that have assessed the potential of CBD as an AD therapeutic intervention and limited clinical trials have assessed CBD's effect on cognition. Although, a study in schizophrenia patients treated with 600 mg CBD per day found no improvement in cognition (Boggs et al. 2018). Importantly, an open-label, 10 week trial assessing a high CBD/low THC sublingual solution on treating behavioural symptoms in mild-moderate AD patients is soon to commence (*http://www.ClinicalTrials.gov* identifier NCT04075435).

Importantly, CBD has been approved by the U.S. Food and Drug Administration (FDA) in the form of Epidiolex (highly purified CBD) for the treatment of seizures caused by Dravet syndrome and Lennox-Gastaut syndrome. Clinical trials demonstrated that Epidiolex was generally well tolerated, although there were some adverse effects including, somnolence and diarrhoea (Laux et

al. 2019). It is important to note these clinical trials were targeted at children, so these adverse effects could be absent or exaggerated in the aged individuals and should be considered in future clinical trials.

Other clinical trials evaluating CBD therapy for other human diseases have reported that CBD is well tolerated with limited adverse effects when taken chronically, for example trials in schizophrenia (600 mg per day for 6 weeks) (Boggs et al. 2018) and Huntington disease (10 mg/kg per day for 6 weeks) (Consroe et al. 1991). In addition, recent research has indicated that CBD can protect against the detrimental effects caused by THC-induced activation of the CB_1 receptors (e.g. psychoactivity and impaired cognition) and may actually provide greater therapeutic benefits than either phytocannabinoid alone. Nevertheless, Sativex, a combination therapy of CBD and THC is approved as an anti-inflammatory drug treatment against spasms in multiple sclerosis and does not appear to be associated with any adverse THC effects, suggesting that CBD effectively blocks those in the dose / ratio chosen (Collin et al. 2010; Novotna et al. 2011).

As CBD has previously been investigated in clinical trials, the translation of preclinical research of CBD as a novel AD therapy could be realised relatively quickly. To date, only a few studies have investigated the effect of CBD in Aβ-dependent transgenic mouse models of AD and at quite low doses, even though research has demonstrated that CBD works in a dose-dependent manner. Importantly, no studies have investigated the effect of CBD in tauopathy models of AD despite tau playing a crucial role in the disease pathogenesis. Thus, this thesis will investigate the effect of varying doses of CBD in two mouse models of AD, the *APPxPS1* double transgenic mice and the TAU58/2 transgenic mice.

1.6 Summary and aims of study

1.6.1 Study rationale

AD is characterised by progressive cognitive decline which is accompanied by the accumulation of Aβ plaques and tau hyperphosphorylation. These neuropathological features induce various changes in the brain, inducing a neurodegenerative cascade. A main feature of the neurodegenerative cascade is neuroinflammation which contributes to the neurotoxic environment and exacerbates the AD condition. Unfortunately, current AD treatments are unable to stop or reverse the disease progression, highlighting the need for novel therapeutics. One potential therapeutic is the phytocannabinoid CBD, a constituent of *Cannabis sativa*. It has been shown to reduce AD pathology by interacting with the eCBS and various neurotransmitter and neuroinflammatory systems to counteract neurodegeneration, neuroinflammation and oxidative stress (as reviewed in (Watt and Karl 2017)). Furthermore, *in vitro* studies have demonstrated that CBD can increase Aβ clearance and reduce Aβ-induced neuroinflammation, neurodegeneration and tau hyperphosphorylation. It has also been shown to promote hippocampal neurogenesis. Importantly, pharmacological and transgenic rodent models of AD have demonstrated that CBD can restore and prevent cognitive deficits, which have been associated with reduced Aβ pathology and neuroinflammation. In addition, CBD is preferential to other cannabinoids, in particular THC, as it devoid of the psychoactive and cognitive side effects, making it a more attractive therapeutic option. Therefore, I hypothesise that CBD has therapeutic benefits in AD.

Previous studies in Aβ mouse models of AD have revealed that the beneficial cognitive effects of CBD are only associated with subtle changes in neuropathological measures. However, CBD has been shown to work in a dose-dependent manner and cannabinoids are known to have biphasic effects. In addition, despite the important role of tau in AD, there have be no *in vivo* studies which have investigated the effect of CBD in a tauopathy model of AD. Therefore, to investigate the

therapeutic benefits of CBD in AD, I firstly investigated the therapeutic potential of 50 and 100 mg/kg CBD in the *APPxPS1* double transgenic mouse model. I then conducted a comprehensive analysis of a novel tauopathy mouse model of AD and finally investigated the therapeutic potential of 50 mg/kg CBD in the TAU58/2 mouse model.

The ability of CBD to reverse cognitive and behavioural deficits and AD-relevant neuropathological changes in transgenic mouse models of AD was assessed by chronic CBD treatment for 3 weeks after the onset of AD symptoms (in cognition and neuropathology). After 3 weeks of treatment, comprehensive tests for behaviour (anxiety and locomotor activity), motor function and cognition (social and object recognition memory, spatial learning and memory and fear associated memory) were conducted. Biochemical analyses of amyloid pathology, microglia activation, neuroinflammation, neurodegeneration and CBD-relevant receptors were conducted using enzyme-linked immunosorbent assay (ELISA) and western blotting techniques. In these studies, I will assess the ability of CBD to reverse AD-relevant cognitive and behavioural symptoms as well as AD-relevant neuropathology. This will establish CBD's therapeutic potential for AD.

1.6.2 Major aims

Aim 1: Determine if 50 mg/kg CBD reverses cognitive and neuropathological impairments of *APPxPS1* transgenic mice (Chapter 2)

Aim 2: Determine if 100 mg/kg CBD reverses cognitive and neuropathological impairments of *APPxPS1* transgenic mice (Chapter 3)

Aim 3: Characterise TAU58/2 mice in novel behavioural paradigms to determine its use as a tauopathy mouse model of AD (Chapter 4)

Aim 4: Determine if 50 mg/kg CBD reverses behavioural impairments of TAU58/2 transgenic mice (Chapter 5)

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Chapter 2: Effect of 50 mg/kg CBD on AD-relevant phenotypes of *APPxPS1* transgenic mice

Chronic treatment with 50 mg/kg cannabidiol (CBD) improves cognition and moderately reduces Aβ$_{40}$ levels in 12-month-old male *APPswe/PS1ΔE9* transgenic mice

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Declaration

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Georgia Watt

2.1 Abstract

Alzheimer's disease (AD) is characterised by progressive cognitive decline and pathologically by the accumulation of amyloid-β (Aβ) and tau hyperphosphorylation causing neurodegeneration and neuroinflammation. Current AD treatments don't stop or reverse the disease progression, highlighting the need for more effective therapeutics. The phytocannabinoid cannabidiol (CBD) has demonstrated antioxidant, anti-inflammatory and neuroprotective properties. Furthermore, chronic CBD treatment (20 mg/kg) reverses social and object recognition memory deficits in the *APPxPS1* transgenic mouse model with only limited effects on AD-relevant brain pathology. Importantly, studies have indicated that CBD works in a dose-dependent manner. Thus, this study determined the chronic effects of 50 mg/kg CBD in male *APPxPS1* mice. 12-month-old mice were treated with 50 mg/kg CBD or vehicle via daily intraperitoneal injections for 3 weeks prior to behavioural testing. A variety of cognitive domains including object and social recognition, spatial and fear-associated memory were evaluated. Pathological brain analyses for AD-relevant markers were conducted using ELISA and western blot. Vehicle-treated male *APPxPS1* mice demonstrated impaired social recognition memory and reversal spatial learning. These deficits were restored after CBD treatment. Chronic CBD tended to reduce insoluble $A\beta_{40}$ levels in the hippocampus of *APPxPS1* mice but had no effect on neuroinflammation, neurodegeneration or PPARγ markers in the cortex. This study demonstrates that therapeutic-like effects of 50 mg/kg CBD on social recognition memory and spatial learning deficits in *APPxPS1* mice are accompanied by moderate brain region-specific reductions in insoluble $A\beta_{40}$ levels. The findings emphasise the clinical relevance of CBD treatment in AD, however, the underlying mechanisms involved require further investigation.

Keywords: Alzheimer's disease, cannabidiol (CBD), transgenic *APP_{swe}/PS1*ΔE9 mice, cognition, amyloid-β, neuroinflammation, IBA1, BDNF, PPARγ

2.2 Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterised by progressive cognitive decline. AD initially manifests as short-term memory loss but progresses to include a wide range of cognitive and behavioural symptoms including impaired facial recognition, social withdrawal, impaired motor function and impaired spatial orientation (Association 2016). The two hallmark features of AD are the accumulation of amyloid-β (Aβ) into senile plaques and hyperphosphorylation of tau protein leading to neurofibrillary tangles (NFTs). These two proteins are thought to be central to the neurodegenerative cascade characteristic of AD (Chapman et al. 2001). This cascade includes neuroinflammation and oxidative stress, which exacerbates the excitotoxic environment resulting in atrophy of the brain. Atrophy begins in brain regions important for memory (i.e. hippocampus and amygdala), but eventually progresses to cause global disruptions in the brain (Ahmed et al. 2015; Hardy and Selkoe 2002).

Recently, the importance of neuroinflammation in AD has received increasing interest. Studies have demonstrated that microglia are activated in response to Aβ accumulation to i) phagocytose and clear Aβ plaques and ii) to cause a neuroinflammatory response and release an array of inflammatory cytokines [e.g. tumour necrosis factor (TNF)-α, interleukin-1β (IL-1β) and IL-6] (Mandrekar-Colucci and Landreth 2010). The inflammatory response is part of the body's defence mechanisms, however, persistent activation and release of inflammatory cytokines contribute to the cytotoxic environment in the brain and promote neurodegeneration (Heneka and O'Banion 2007).

Current AD treatments do not stop or reverse the disease progression and only provide limited therapeutic benefits, highlighting the need for new therapeutic candidates. Cannabidiol (CBD) is a non-toxic phytocannabinoid from the *Cannabis sativa* plant with neuroprotective, anti-

inflammatory and anti-apoptotic properties. Furthermore, it has been shown to increase Aβ clearance and reduce tau hyperphosphorylation *in vitro* (reviewed in (Karl et al. 2016; Watt and Karl 2017)).

In a previous study, CBD reduced a spatial reference memory deficit in a pharmacological mouse model of AD (Martín-Moreno et al. 2011). Our laboratory followed up on this initial finding and tested CBD in the double transgenic *APP_{Swe}/PS1ΔE9* (*APPxPS1*) mouse model for the familial/early-onset type of AD (Bettens et al. 2013; Götz and Ittner 2008). Chronic intraperitoneal (i.p.) treatment with 20 mg/kg CBD reversed social and object recognition memory deficits in 8-month-old male *APPxPS1* mice (Cheng et al. 2014a). This study did not test the remedial effects of CBD on AD-relevant brain pathology. CBD also prevented the development of a social recognition memory deficit, however only induced subtle beneficial effects on neuroinflammatory markers (e.g. TNF-α and IL-1β) and had no effect on Aβ pathology when given orally for 8 months starting prior to the development of AD symptoms (Cheng et al. 2014c).

It is important to note that cannabinoids are known to have biphasic effects, for example in regards to anxiety where low doses have anxiolytic effects and high doses have anxiogenic effects (Viveros et al. 2005). Furthermore, CBD works in a dose-dependent manner and has shown to demonstrate stronger anti-inflammatory effects at higher doses (Esposito et al. 2007). In addition, CBD has not been associated with adverse effects even at high doses of up to 480 mg/kg in mouse models (Zuardi et al. 1991). Thus, in the present study, we evaluated the remedial dose of CBD to 50 mg/kg in 12-month-old male *APPxPS1* transgenic mice starting treatment 3 weeks before conducting a battery of cognitive tests: the social preference test (SPT), the novel object recognition task (NORT), the cheeseboard task (CB) and the fear conditioning paradigm (FC). At the conclusion of behavioural testing, brain tissue was collected and analysed for Aβ levels and

markers for neuroinflammation (i.e. TNF-α and IL-1β), neurogenesis (i.e. brain-derived neurotrophic factor: BDNF), microglia activation (i.e. ionized calcium binding adaptor molecule 1: IBA1), as well as the peroxisome proliferator-activated receptor gamma (PPARγ). These targets were chosen as CBD reduces microglia activity (i.e. production of cytokines such as TNF-α and IL-1β) but increases microglia migration to promote phagocytosis of Aβ plaques (Martín-Moreno et al. 2011). Furthermore, PPARγ is dysregulated in AD and appears to be involved in the therapeutic effects of CBD in a pharmacological AD model (Esposito et al. 2011; Scuderi et al. 2014).

2.3 Experimental Procedures

2.3.1 Animals

Double transgenic mice expressing chimeric mouse/human *APP* (*Mo/HuAPP695$_{swe}$/Swedish* mutations *K595N/M596l*) and mutant human *PS1* (*PS1/ΔE9*) were maintained as hemizygotes on the congenic C57BL/6J x C3H/Hej background. Male double transgenic mice (*APPxPS1*) and their non-transgenic wild type-like control littermates (WT) were bred and group housed in independently ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Australian BioResources (Moss vale, Australia). Mice were transported to Neuroscience Research Australia (NeuRA) at ~10 weeks of age. At NeuRA they were group housed in filter top cages (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (PuraCob premium: Able Scientific, Perth Australia) and some tissues for nesting (Kimwipes®, Kimberley-Clark, Australia) (as described previously in (Cheng et al. 2014a)). Mice were kept in 12:12 h light:dark schedule [light phase: white light (illumination:210 lx); lights on 0700-1900h]. Environmental temperature and relative humidity were automatically regulated at 21±1°C and 40-60% respectively. Food and water were provided ad libitum, except where specified (i.e. food deprivation in cheeseboard). Adult, male A/J mice from Animal Resources centre (Canning Vale, Australia) were used in the SPT as social opponents. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3.2 Drug preparation and administration

Powdered cannabidiol (CBD: provided by GW Pharmaceuticals, Salisbury, United Kingdom) was dissolved in equal parts Tween80 (Sigma-Aldrich Co, St Louis, USA) and 100% ethanol and

diluted with 0.9% sodium chloride to the appropriate concentration to a final ratio of 1:1:18. Ethanol and Tween80 each made up 5% of the volume. A vehicle control solution (VEH) was made up similarly but without the addition of CBD. Vehicle and CBD treatments were administered at a dose of 50 mg/kg body weight (BW) via i.p. injections using an injection volume of 10 ml/kg BW. Mice of both genotypes were either treated with vehicle or CBD daily for 3 weeks prior to the start of behavioural tests (test age: 12 months ± 3 weeks, as seen in Table 1) (WT-VEH $n = 10$; *APPxPS1*-VEH $n = 10$; WT-CBD $n = 11$; *APPxPS1*-CBD $n = 8$). Treatment was continued throughout behavioural testing (total treatment duration 7 weeks) but given in the afternoon (between 1400-1600 h) after test completion, to avoid any potential acute effects of CBD becoming a test confounder. This treatment design was identical to our previously published studies (Cheng et al. 2014a). BW of mice was recorded at least weekly.

Treatment	Vehicle		CBD	
Genotype	WT	*APPxPS1*	WT	*APPxPS1*
Age at start of treatment	52±1	50±1	52±1	51±1
Social preference test	55±1	53±1	55±1	54±1
Novel object recognition task	55±1	54±1	55±1	54±1
Cheeseboard task	56±1	54±1	55±1	55±1
Fear conditioning paradigm	59±1	57±1	58±1	58±1
Tissue collection	59±1	58±1	59±1	59±1

Table 1 – *Test biography.* Treatment schedule and test order of *APPxPS1* and WT mice: Age [weeks] of non-transgenic WT control littermates and double transgenic *APPxPS1* mice at the start of treatment, throughout behavioural testing and at the conclusion of treatment. *Cannabidiol, CBD; wild type-like mice, WT; APP_{swe}/PS1 E9 double transgenic mice, APPxPS1*

2.3.3 Behavioural testing

At 12 months of age, mice were tested in a battery of behavioural tests (for test biography and test age, see Table 1) with an inter-test interval of at least 48 h to minimize the effect of repeated testing. Tests were conducted within the first 5 h of the light phase (between 0900-1400h) and all equipment was cleaned with 70% ethanol between trials.

2.3.3.1 Social preference test (SPT)

The SPT was used to test sociability and social recognition memory (Moy et al. 2004). The apparatus consisted of three chambers: a central chamber (9 cm x 18 cm x 20 cm) and two outer chambers (6 cm x 18 cm x 20 cm). All walls were made from clear Plexiglas, with openings (4 cm x 4 cm) in the dividing walls leading to the outer chambers. In each of the outer chambers there was a circular cage (mouse enclosure: 15 cm high, 7 cm in diameter and bars spaces 0.5 cm apart to allow contact between mice but prevent fighting). The chambers and enclosures were cleaned with 70% ethanol between trials and fresh corn cob bedding was added before each trial.

SPT was conducted within one day (after 21 days of treatment). Test animals were isolated for 1h prior to the start of testing. In the habituation trial, test animals were placed in the centre chamber and allowed to freely explore all three chambers for 5 min. In the sociability trial, an unfamiliar, male A/J was placed into one of the mouse enclosures (allocation of outer chamber was quasi-randomized). Then the test mouse was placed in the centre chamber and allowed to explore all three chambers for 10 min. In the social preference trial, an unfamiliar, male A/J was placed in the empty mouse enclosure and the familial A/J mouse placed back into the same chamber as during the sociability trial. Then the test mouse was placed in the centre chamber and allowed to explore all three chambers for 10 min. The inter-trial interval (ITI) was 10 min. Test design was chosen

based on previous studies conducted within our laboratory (Cheng et al. 2014a). ANY-maze^{TM} (Stoeting, Wood Dale, USA) tracking software was used to record time spent, number of entries and distance travelled in each chamber and time spent *sniffing* (nose within 1 cm of mouse enclosure) mouse enclosures.

2.3.3.2 Novel object recognition task (NORT)

The NORT uses the innate preference of mice for novelty to distinguish between familiar and unfamiliar objects and to assess object recognition memory (Dere et al. 2007). The apparatus consisted of a single arena (35 cm x 35 cm x 30 cm) made of Perspex. NORT was conducted over 3 days (after 23 days of treatment) with 2 x 10 min trials a day with an ITI of 1 h. On day 1, both trials were habituation trials where the test mouse was placed in the empty NORT arena and allowed to explore freely. On day 2, the first trial was another arena habituation trial. In the second trial, two identical objects were placed in the arena for the test mouse to freely investigate (object habituation trial). On day 3, in the first trial, two identical objects (distinct from day 2) were placed in the arena and in the second trial, one of the objects was changed to an unfamiliar object. Protocol was chosen based on previous literature (Cheng et al. 2014a). ANY-maze^{TM} tracking software was used to record time spent near each object (nose within 1 cm of object). In addition, manual scoring was used to record exploratory behaviours towards the objects (*nosing* and *rearing*).

2.3.3.3 Cheeseboard task (CB)

The CB assesses spatial reference memory acquisition and retention (Lopez et al. 2010). The apparatus consists of a circular board (1.1 m in diameter, elevated 60 cm from the ground) with one flat side and one side with 32 wells evenly distributed into 8 radial zones (4 wells in each zone,

5 cm apart – the last well is 10 cm from the edge). Visual cues are placed on the floor and the walls surrounding the board. CB was conducted over 14 days (after 28 days of treatment). The test consisted of a 3-day habituation where the test mouse was placed on the flat side of the board for 3 x 2 min trials with an ITI of 10 min. In the 5-day training phase, a food reward was placed in one of wells and the mice were trained to learn the location of the food reward. The test was concluded after 2 min if the mouse did not find the food reward and mouse was placed next to the food reward. There were 3 x 2 min trials per day with an ITI of 10 min and location of food reward was kept constant. On the following day (day 9), a probe trial was conducted where mice were placed on the board for 2 min with no food reward. Time spent in the target zone was recorded using ANY-maze^{TM} tracking software. Following this, reversal training (days 10-13) and reversal probe trials (day 14) were conducted (in similar fashion except the food reward was moved to the opposite well) to assess the ability of the mice to learn a new location of the food reward. Mice were food deprived and kept at 85-90% of their initial BW (BW was monitored daily) during testing. They were fed for 1-2 h post completion of daily testing. Test design was chosen based on previous studies conducted in our laboratory (Cheng et al. 2013).

2.3.3.4 Fear conditioning (FC)

Fear conditioning (FC) assesses hippocampus and amygdala dependent fear associated memory (Phillips and LeDoux 1992). In this test, a previously neutral stimuli (e.g. tone and context, conditioned stimuli: CS) were paired with an aversive stimulus (e.g. foot shock, unconditioned stimulus: US). FC was conducted over 3 days (after 48 days of treatment). On conditioning day, test mice were placed in the test chamber. After 2 min, an 80 dB conditioned stimulus (tone) was presented for 30 s with a co-terminating 2 s 0.4 mA foot shock, this CS-US pairing was repeated

after a 2 min ITI. The test concluded 2 min after the delivery of the second foot shock. On the following day (context test), the test mice were returned to the test chamber for 7 min. 24 h later (cue test), the test mice were placed in a distinct context. After 2 min, the conditioned tone stimulus was presented for 5 min. The test concluded 2 min after the cue presentation ended. Protocol design was based on previous studies in our laboratory (Cheng et al. 2014a). Time spent *freezing* was measured on all three experimental days using ANY-Maze™ tracking software.

2.3.4 Biochemical Analyses

2.3.4.1 Brain tissue preparation

Four days after the conclusion of behavioural tests, mice were anaesthetized and were perfused with phosphate buffered saline (PBS) transcardially. The brain was divided sagittally and the left hemisphere was fixed in 4% paraformaldehyde for 72 h then stored in 30% sucrose solution. The right hemisphere was dissected, and cortex and hippocampal tissue were snap frozen in liquid nitrogen and stored in -80°C. Final CBD/Vehicle treatment was given 24 h before euthanasia.

Frozen hippocampal (10-27 mg) and cortical tissue (39-67 mg) was homogenized in 12 volumes of TBS extraction buffer (140 mM NaCl, 3 mM KCl, 25 mM Tris1M pH7.4, 2 mM 1,10 phenanthroline stock, containing 1% Igepal CA630 and Sigma protease inhibitor cocktail and 10 µM phenylmethylsulfonyl fluoride [PMSF]) using a Precelleys 24 homogenizer (2 x 30s, 6000 x g). Homogenates were centrifuged at 16,100 g for 30 min at 4°C. The TBS-soluble supernatant was collected and stored at -80°C until used. The pellet was homogenized with 10 volumes of 6.25 mM guanidine HCL (gHCL) in 50 mM Tris, pH8 in Precelleys 24 homogenizer (2 x 30s, 6000 g) and left in the rotary at 4°C overnight. Homogenates were centrifuged at 16,100 g for 30 min at 4°C. The gHCL-soluble supernatant (TBS-insoluble) were collected and stored at -80°C. Protein

was quantified using the bicinchoninic acid method (BCA assay). Sample numbers for ELISA and western blotting: n = 10 for WT-VEH, n = 11 for WT-CBD, n = 10 for *APPxPS1*-VEH, n = 8 for *APPxPS1*-CBD.

2.3.4.2 Enzyme-linked immunosorbent assay (ELISA)

The concentration of $A\beta_{40}$ and $A\beta_{42}$ proteins in TBS-soluble and gHCL-soluble hippocampal fractions of *APPxPS1* mice only were quantified using BetaMark™ β-Amyloid x-40 and x-42 ELISA Kit (TMB) (Australian Biosearch [842301 and 842401]). The concentration of TNF-α and IL-1β proteins in TBS-soluble cortical fractions of *APPxPS1* mice and WT controls were quantified using Invitrogen TNF-α Mouse ELISA kit (ThermoFisher Scientific) and Invitrogen IL-1β ELISA kit (ThermoFisher Scientific).

2.3.4.3 Western blotting

TBS-soluble cortex fractions were analysed by SDS-PAGE Western Blotting using antibodies: IBA1 (5 µg of protein per lane, 1:1000, Novachem [019-19741]), BDNF (20 µg of protein per lane, 1:1000, Abcam [ab108319]), PPARγ (40 µg of protein per lane, 1:250, Cell Signalling [C26H12]) and housekeeper antibody: actin (1:1000, Sigma-Aldrich [A2066]). Goat anti-rabbit IgG HRP-conjugated secondary antibody (1:2000, Millipore [AP132P]) and enhanced chemiluminescence were used to detect signals. Signals were quantified using image J software. Data were normalized to actin levels and expressed as relative values.

2.3.5 Statistical analysis

Two-way analysis of variance (ANOVA)s were utilized to analyse behavioural and molecular (except Aβ levels) parameters for main effects of 'CBD', 'genotype' and potential interactions. Three-way repeated measures (RM) ANOVAs were also used to analyse effects of 'chamber' (SPT), 'object' (NORT), 'days' (CB) and 'min' (FC). Single sample *t*-tests determined if 'time spent in chamber' (SPT), 'time exploring novel object' (NORT) and 'time spent in target zone' (CB) were greater than chance levels (i.e. 50% for SPT and NORT, 12.5% for CB). One-way ANOVA was used to evaluate the effect of CBD treatment on $A\beta_{40}$ and $A\beta_{42}$ levels in *APPxPS1* mice. Data are presented as means ± standard error of means (SEM) and differences were regarded as significant if $p < 0.05$. Genotype effects are shown as '*' (where $^{*}\, p < 0.05$, $^{**}\, p < 0.01$ and $^{***}\, p < 0.001$), treatment effects as '$^{\#}$' (where $^{\#}\, p < 0.05$, $^{\#\#}\, p < 0.01$ and $^{\#\#\#}\, p < 0.001$), RM results are shown as '$^{\wedge}$' (where $^{\wedge}\, p < 0.05$, $^{\wedge\wedge}p < 0.01$ and $^{\wedge\wedge\wedge}\, p < 0.001$) and *t*-test and one-way ANOVA results are shown as '$^{+}$' (where $^{+}\, p < 0.05$, $^{++}\, p < 0.01$ and $^{+++}\, p < 0.001$). F-values and degrees of freedom are presented for ANOVA. Analyses were conducted using SPSS 25.0 for Mac.

2.4 Results

2.4.1 Behaviour

2.4.1.1 Sociability and social recognition memory

Sociability: Single sample *t*-test for percentage time spent in the mouse chamber confirmed this preference [WT-VEH t(9) = 2.6, $p = 0.028$; *APPxPS1*-VEH t(9) = 4.7, $p = 0.001$; WT-CBD t(10) = 3.7, $p = 0.004$; trend in *APPxPS1*-CBD t(7) = 2.3, $p = 0.055$] (Figure 1A). In line with this, three-way RM ANOVA revealed a significant effect of 'chamber' [$F(1,35) = 39.0$, $p < 0.0001$], where all mice demonstrated a preference for the mouse chamber over the empty chamber regardless of genotype or CBD treatment (no interactions, i.e. all p's > 0.05) (data not shown).

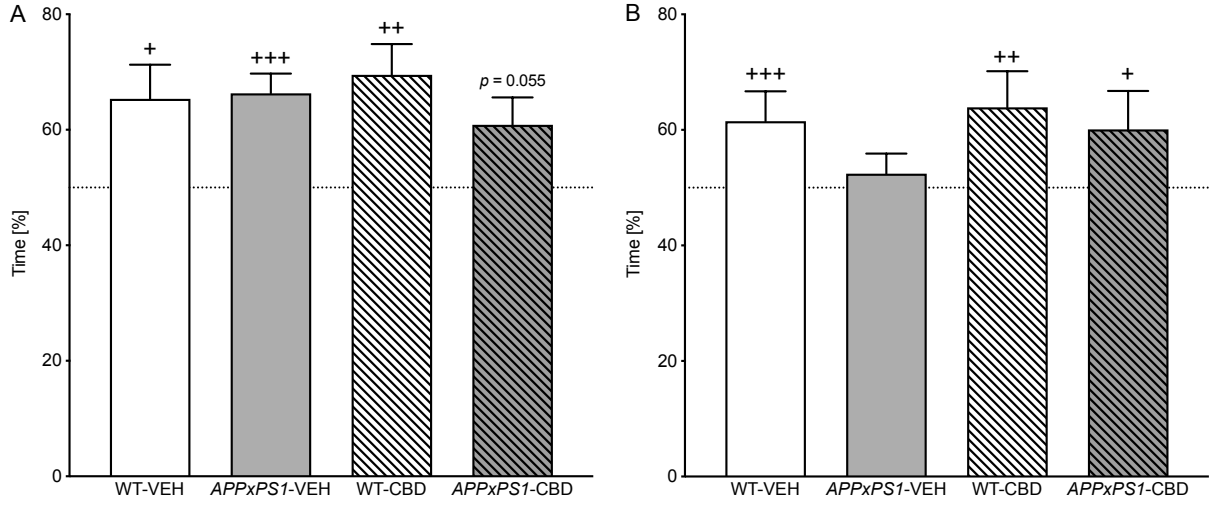

Figure 1A-B: ***Sociability and social recognition in the SPT.*** **A)** Time spent [%] in the mouse chamber and **B)** time spent [%] in the novel mouse chamber for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. *t*-test results for zone times above chance level (12.5%) are presented by '+' ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$).

Social recognition memory*:* In the social recognition trial, single sample *t*-tests demonstrated that all groups, except the *APPxPS1*-VEH group, had a preference for the novel mouse chamber [WT-VEH t(8) = 5.2, $p = 0.001$; *APPxPS1*-VEH t(9) = 0.7, $p = 0.5$; WT-CBD t(9) = 3.8, $p = 0.004$;

APPxPS1-CBD t(6) = 3.5, $p = 0.013$] indicating impaired social recognition memory in *APPxPS1*-VEH mice, which was restored in *APPxPS1*-CBD mice (Figure 1B). In line with this, three-way RM ANOVA revealed an effect of 'chamber' [F(1,32) = 39.1, $p < 0.0001$] and strong trends for interactions between 'chamber' and 'genotype' [F(1,32) = 4.0, $p = 0.053$] and 'chamber' and 'CBD' [F(1,32) = 4.1, $p = 0.051$]. When split by the corresponding factors, there was a 'chamber x treatment' interaction in *APPxPS1* mice [F(1,15) = 5.820, $p = 0.029$] and a 'chamber x genotype' interaction in VEH treated mice [F(1,17) = 7.952, $p = 0.012$] (data not shown).

2.4.1.2 Spatial learning and memory

Spatial learning: Three-way RM ANOVA for the latency to find the location of a food reward averaged across trials per day revealed a main effect of 'days' for both the training [F(4,140) = 54.007, $p < 0.0001$] and the reversal training periods [F(3,105) = 46.82, $p < 0.0001$] indicating that all mice learnt the location of the food reward (Figure 2A-B). For the initial training, no interaction effects of 'treatment' or 'genotype' with 'days' were found [all p's > .05] (Figure 2A). However, in the reversal training, we detected a significant 'days' by 'genotype' by 'treatment' interaction [F(3,105) = 2.8, $p = 0.044$], where *APPxPS1*-VEH mice were slower to learn the location of the food reward than WT mice and this learning deficit was absent in CBD-treated mice (Figure 2B).

Spatial memory: Two-way ANOVA for time spent in the target zone (%) for the initial probe trial revealed no main effect of 'genotype' [F(1,35) = 0.64, $p = 0.429$] but a main effect of 'CBD' [F(1,35) = 5.522, $p = 0.025$], with CBD treatment increasing time spent in the target zone across genotypes (Figure 2C). There was also a moderate trend for a 'genotype x treatment' interaction

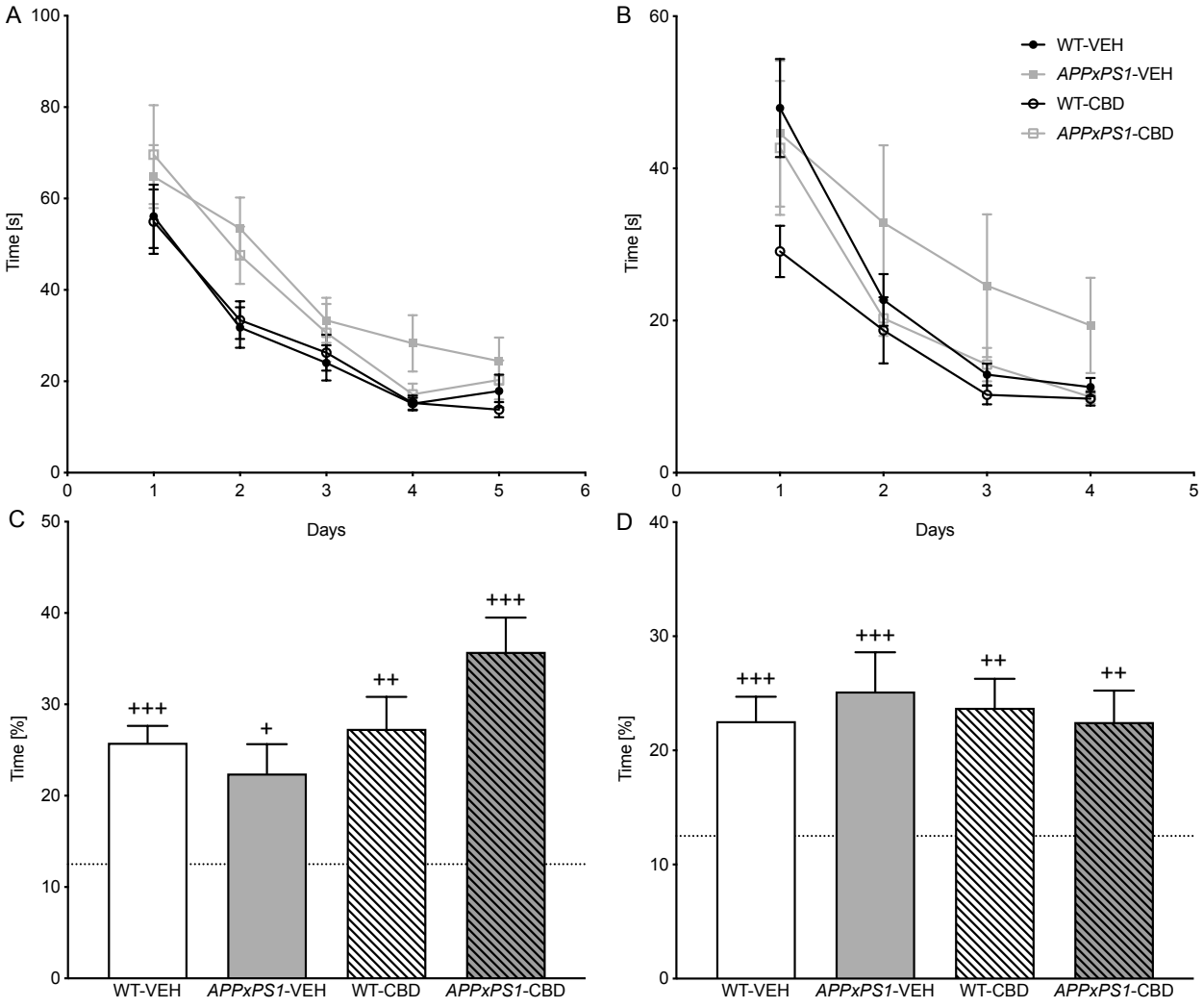

Figure 2A-D: ***Spatial learning and recognition memory in the CB task.*** **A)** Latency to find the food reward over 5 days in the initial training [s], **B)** latency to find the food reward over 4 days in the reversal training [s], **C)** time spent in the target zone in the initial probe trial [%] (as a percentage of total time minus time spent in the centre) and **D)** time spent in the target zone in the reversal probe trial [%] (as a percentage of total time minus time spent in the centre) for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. RM ANOVA found a 'time x genotype x treatment' interaction effect ($p = 0.04$) in reversal training. *t*-test results for zone times above chance level (12.5%) are presented by '+' ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$).

[$F(1,34) = 3.477$, $p = 0.071$] with *APPxPS1* mice displaying a stronger response to CBD treatment.

Single sample *t*-tests confirmed that all groups demonstrated a preference for the target zone above

chance levels [WT-VEH t(9) = 7.2, $p < 0.0001$; *APPxPS1*-VEH t(9) = 3.1, $p = 0.013$; WT-CBD t(10) = 4.2, $p = 0.002$; *APPxPS1*-CBD t(7) = 6.2, $p < 0.0001$] (Figure 2C).In the reversal probe trial, there were no main effects of 'genotype' or 'treatment' (all p's > 0.05). Single sample *t*-tests revealed that all groups demonstrated a preference for the target zone [WT-VEH t(9) = 4.7, $p = 0.001$; *APPxPS1*-VEH t(9) = 3.7, $p = 0.005$; WT-CBD t(10) = 4.4, $p = 0.001$; *APPxPS1*-CBD t(7) = 3.6, $p = 0.008$] (Figure 2D).

2.4.1.3 Novel object recognition memory

Three-way RM ANOVA for time spent *nosing* the objects revealed a significant 'object' effect [F(1,33) = 26.113, $p < 0.0001$]. More importantly, there was also a significant 'object' by 'genotype' interaction [F(1,33) = 5.190, $p = 0.029$] with only WT mice showing a clear preference

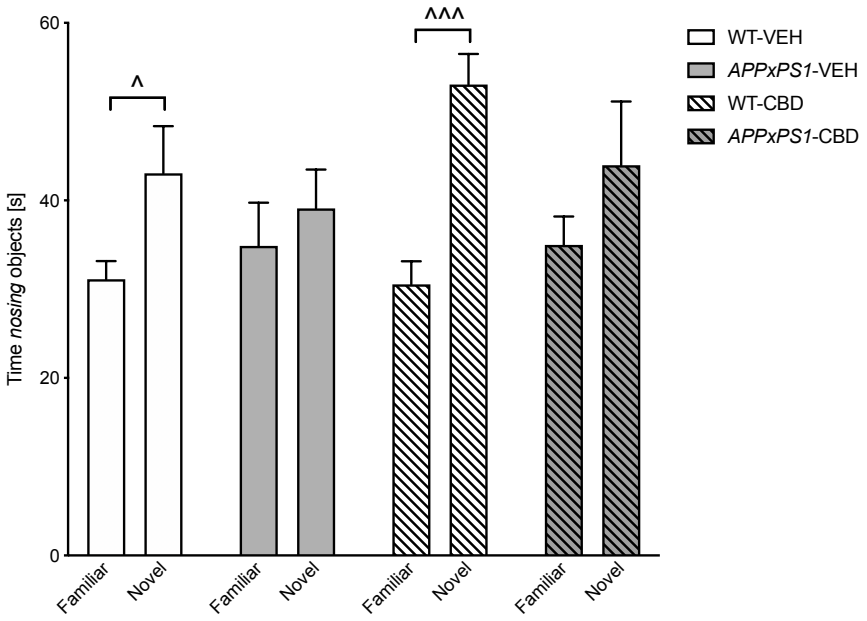

Figure 3: ***Novel object recognition memory in NORT.*** **A)** Time spent *nosing* [s] the novel object and familiar object for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. RM ANOVA found an 'object x genotype' effect ($p = 0.028$). RM ANOVA effects are shown as '^' (where ^$p < 0.05$ and ^^^$p <$ 0.0001).

for the novel object. Indeed, when split by 'genotype', an effect of 'object' was only evident in WT [$F(1,18) = 37.915$, p, < 0.0001] but not *APPxPS1* transgenic mice [$F(1,16) = 2.189$, $p = 0.158$] (Figure 3). These findings were supported by single sample *t*-tests: *APPxPS1* mice of both treatment groups did not show a preference for the novel object, while WT-CBD and WT-VEH (trend) developed such a preference (WT-VEH $t(8) = 2$, $p = 0.077$; *APPxPS1*-VEH $t(9) = 0.9$, $p = 0.380$; WT-CBD $t(10) = 8.4$, $p < 0.0001$; *APPxPS1*-CBD $t(6) = 1.2$, $p = 0.293$) (data not shown).

2.4.1.4 Fear-associated memory

Two-way ANOVA revealed all groups demonstrated the same amount of *freezing* in the first 2 min of conditioning (no interaction effects, all p's > 0.05) (data not shown). Three-way RM ANOVA for *freezing* over time in the context trial revealed an effect of 'time' [$F(6,210) = 14.092$, $p < 0.0001$] but no main effects of 'genotype', 'treatment' or interactions with 'time' (Figure 4A).

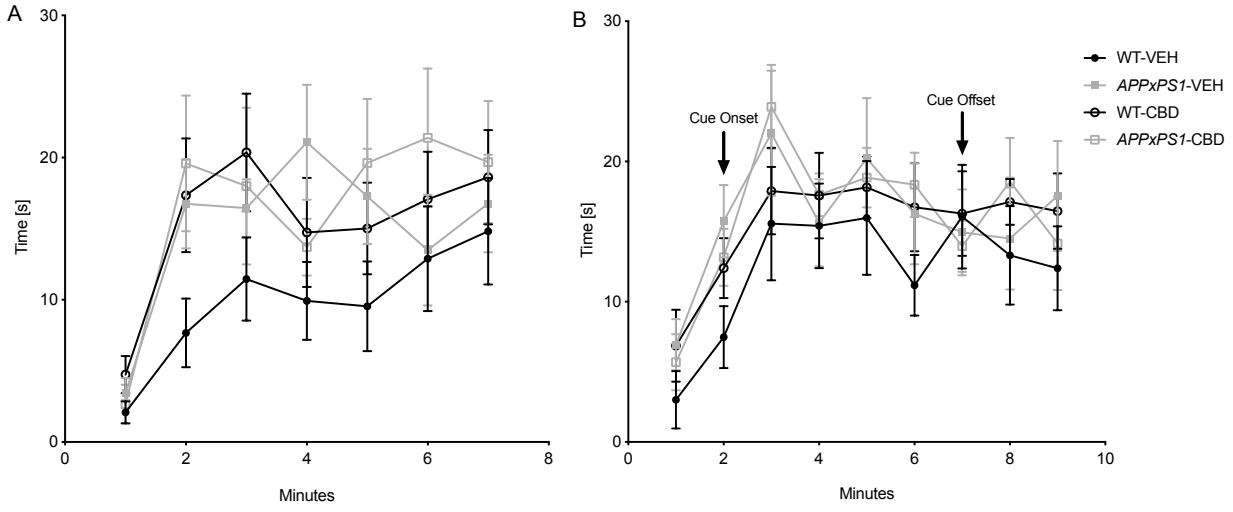

Figure 4A-B: ***Fear associated memory in the FC paradigm.*** Time spent freezing [s] across 1-min blocks in **A)** the context trial and **B)** the cue trial for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM.

In the cue version of the FC task, three-way RM ANOVA revealed an effect of 'time' [$F(8,280) = 14.125$, $p < 0.0001$], but no effect of 'genotype', 'treatment' or interactions (all p's > 0.05) (Figure 4B). Three-way RM ANOVA analysing percentage time spent f*reezing* prior to *versus* during cue presentation indicated that all groups associated the cue with the US ['cue': $F(1,35) = 61.450$, $p < 0.0001$] regardless of genotype or CBD treatment (no interaction effects, all p's > 0.05) (data not shown).

2.4.2 Brain Pathology

One-way ANOVA revealed a moderate trend for insoluble $A\beta_{40}$ to be reduced in the hippocampus of CBD-treated *APPxPS1* mice compared to *APPxPS1*-VEH mice [$F(1,17) = 3.339$, $p = 0.086$] (Figure 5A). One-way ANOVA revealed no other CBD-driven change in insoluble $A\beta_{42}$, soluble $A\beta_{40}$ or soluble $A\beta_{42}$ in *APPxPS1* mice (all p's > 0.05) (Figure 5B-D).

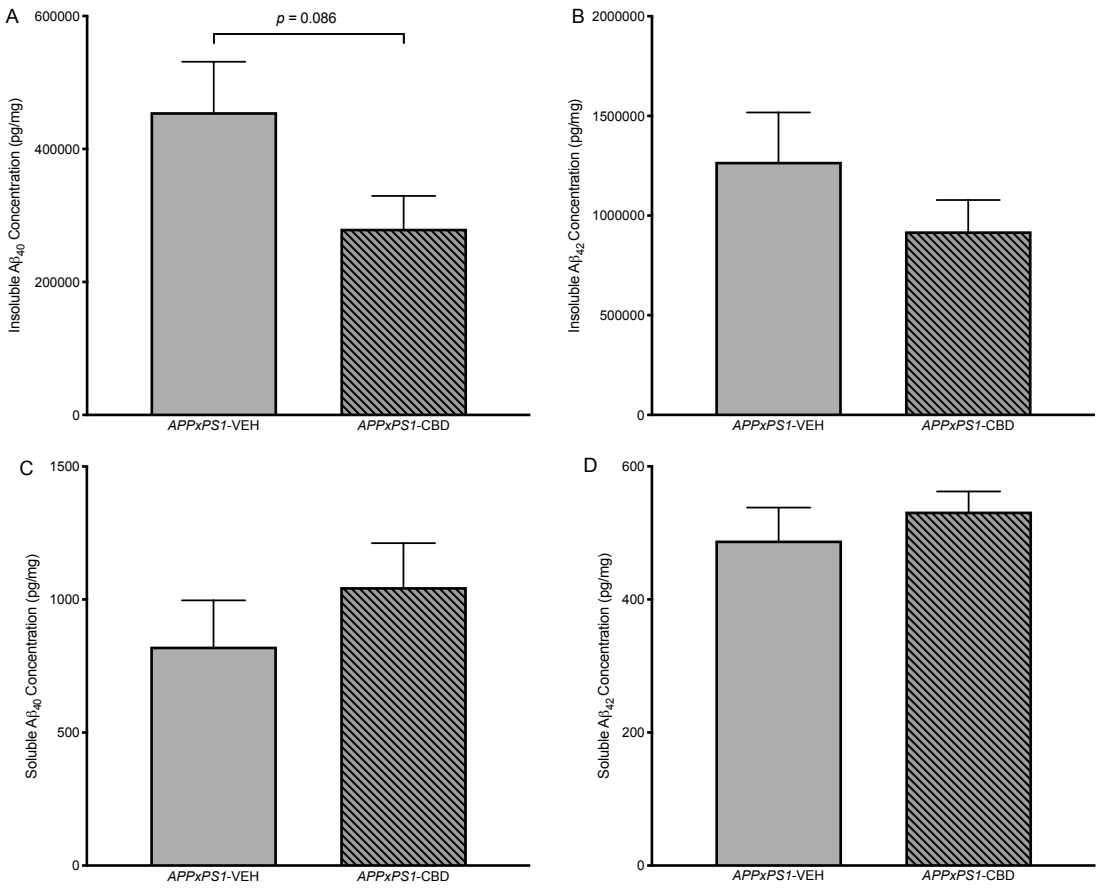

Figure 5A-D: ***ELISA results for insoluble and soluble Aβ40 and Aβ42 levels in the hippocampal tissue of APPxPS1 mice*****. A)** Insoluble $A\beta_{40}$ levels, **B)** insoluble $A\beta_{42}$ levels, **C)** soluble $A\beta_{40}$ and **D)** soluble $A\beta_{42}$ for *APPxPS1* transgenic mice treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM.

Two-way ANOVA for IBA1 levels revealed a main effect of 'genotype' [$F(1,35) = 6.813$, $p = 0.013$], where IBA1 levels were higher in the cortex of *APPxPS1* mice compared to WT controls. There were no main effects of 'treatment' or 'genotype x treatment' interactions (both p's > 0.05) (Figure 6A). Cortical levels of BDNF, PPARγ, TNF-α and IL-1β were not affected by 'genotype', 'treatment', or interactions thereof (all p's > 0.05) (Figure 6B-E).

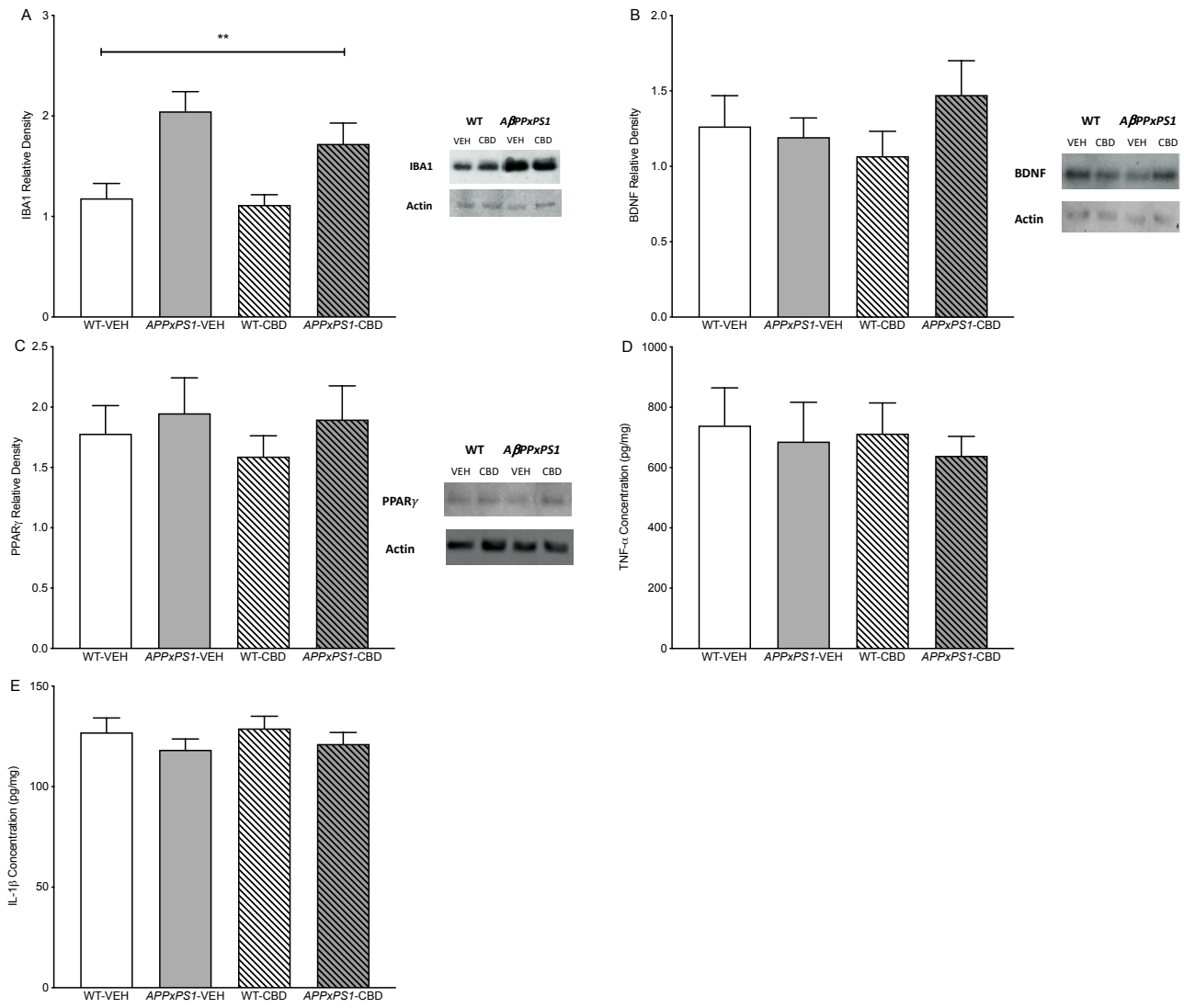

Figure 6A-E: ***ELISA and western blot results for neuroinflammatory levels in the cortical tissue of APPxPS1 mice and WT controls.*** Western blot results for **A)** IBA1 levels, **B)** BDNF levels, and **C)** PPARγ and ELISA results for **D)** TNF-α levels, and **E)** IL-1β levels for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM.

2.5 Discussion

This study demonstrated that chronic 50 mg/kg CBD reversed behavioural deficits in social recognition memory and reversal spatial learning of around 13.5-month-old male double transgenic *APPxPS1* mice. CBD treatment had a moderate effect on $A\beta_{40}$ levels in the hippocampus of *APPxPS1* mice, which exhibited increased cortical IBA1 levels compared to control mice. Cortical levels of BDNF, PPARγ, TNF-α and IL-1β were not affected by genotype or CBD treatment.

In line with previous studies, vehicle-treated *APPxPS1* mice exhibited impaired social recognition memory (Cheng et al. 2013; Cheng et al. 2014a; Cheng et al. 2014c). This cognitive deficit was not affected by changes to general social behaviours, as the sociability version of the test revealed an intact preference of AD transgenic males to explore a mouse over an empty chamber. The memory deficit was reversed after chronic 50 mg/kg CBD treatment. These findings support and extend our previous report on the effectiveness of chronic 20 mg/kg i.p. CBD treatment to restore social recognition memory deficits of 8-month-old male *APPxPS1* mice (Cheng et al. 2014a). In this context, it is also relevant to note that long-term CBD treatment has been found to prevent the development of those deficits in 10.5-month-old AD transgenic mice when given orally for 8 months (Cheng et al. 2014c). These results are clinically relevant as social withdrawal and loss of facial recognition are symptoms of AD (Association 2016).

In the CB task, while initial spatial learning was not affected in any mice, the reversal spatial learning was impaired in vehicle-treated AD transgenic mice. Studies have previously reported that reversal spatial learning in rodents measures cognitive flexibility (Boulougouris and Robbins 2010; Izquierdo et al. 2017). This suggests that although the spatial learning of *APPxPS1* mice was intact, they exhibit impaired cognitive flexibility. Importantly, CBD was able to restore the

reversal learning deficit. In addition, although there was no retention memory deficit in the *APPxPS1* mice, it is interesting to note that CBD had an overall beneficial effect on spatial retention memory across genotypes.

Only one other study has looked at the effect of CBD on spatial learning in mouse models of AD to date. The research team reported that 20 mg/kg CBD was able to reverse a spatial learning deficit in a pharmacological model (i.e. Aβ was injected into the hippocampus). Reversal learning was not assessed in that study (Martín-Moreno et al. 2011). Interestingly, damage to the hippocampus or disruptions in hippocampal function have been found to affect reversal learning more than initial spatial learning (Cirulli et al. 2000; Cirulli et al. 2004; Pouzet et al. 1999; Walsh et al. 2011), which is highly relevant to our finding, as the functionality of this brain region is severely compromised in AD (Bruen et al. 2008; Liang et al. 2008) and in the *APPxPS1* mouse model (Zhang et al. 2011). Importantly, spatial memory deficits of *APPxPS1* males may be dependent on age, genetic background or gender of the test animals. Previously, our laboratory has found no spatial learning deficits in 7-month-old males (Cheng et al. 2013) but impaired reversal spatial retention memory in 9-month-old *APPxPS1* female mice (Cheng et al. 2014b). Furthermore, another study in *APPxPS1* males on a pure C57BL/6J background demonstrated impaired spatial learning and retention memory from 9-15 months of age (Yoshiike et al. 2008).

In NORT, *APPxPS1* mice exhibited deficient object recognition memory. This deficit was not restored with CBD treatment. Previously, our laboratory found that 3 weeks of CBD treatment at 20 mg/kg was able to reverse this deficit in 8-month-old *APPxPS1* males. It is possible that at 12 months of age AD-relevant pathology is already too advanced for CBD to be able to still be beneficial. Similar to our previous study, treatment with CBD or a CBD+THC combination restored object recognition deficit in younger 6-month-old *APPxPS1* males (on C3HeJ x C57Bl/6J

F3 x C57Bl/6Jn1 background); unfortunately, older age groups were not assessed in that study (Aso et al. 2014).

In the fear conditioning paradigm, there was no significant difference between the WT and *APPxPS1* mice in the conditioning, context or cue phases of the test. This in agreement with our previous finding, where 6-month-old *APPxPS1* males did not show a deficit in fear associated memory (Cheng et al. 2014a), suggesting that even at an older age, i.e. later in the disease progression, *APPxPS1* males do not develop fear-associated memory deficits.

Important for future therapeutic applications is the fact that CBD did not affect behavioural test performances when mice were healthy (i.e. WT controls). This finding is in agreement with previous literature indicating that CBD has no or very limited effects in healthy animals (Aso et al. 2014; Cheng et al. 2014a; Cheng et al. 2014c; Martín-Moreno et al. 2011). In addition, controlled clinical trials have found CBD to be well tolerated and have limited side effects (Boggs et al. 2018; Naftali et al. 2017).

The hippocampus and cortex are two key regions affected in AD. Therefore, we measured Aβ levels in the hippocampus, as Aβ deposition is thought to start here in the *AβPP/PS1* mouse model (Borchelt et al. 1997; Jankowsky et al. 2004). We measured all other markers in the cortex as previous studies in AD mouse models have reported altered expression of TNF-α, IL-1β, IBA1 and BDNF predominantly in this region (Babcock et al. 2015; Cheng et al. 2014c; Peng et al. 2009; Venneti et al. 2009). The beneficial effect of CBD on the behavioural performance of *APPxPS1* mice was not accompanied by major changes in the brain markers chosen including for Aβ pathology and neuroinflammation. Our analyses detected a moderate effect of CBD on hippocampal Aβ load, i.e. CBD-treated *APPxPS1* mice tended to have reduced insoluble $A\beta_{40}$ levels. A previous study in our laboratory using 20 mg/kg CBD in a preventative treatment setting

could not detect any therapeutic effect on Aβ load (Cheng et al. 2014c), probably due to the fact that CBD works in a dose-dependent manner (Esposito et al. 2007) and that the ages differed between studies (Cheng et al. 2014c). Importantly, the effect of CBD on Aβ levels has not been widely investigated. *In vitro*, CBD has been found to induce APP ubiquitination and to reduce $A\beta_{40}$ production in $SHSY5Y^{APP+}$ cells (Scuderi et al. 2009).

Currently, there is some evidence suggesting that particular CBD-THC combination treatments may have some therapeutic-like effects beyond pure CBD treatment designs. For example, Aso and colleagues (2014) found that 0.75 mg/kg CBD had no effect on soluble or insoluble $A\beta_{40}$ or $A\beta_{42}$ in 6-month-old *APPxPS1* males but when combined with THC, CBD reduced soluble $A\beta_{42}$ levels in the cortex (Aso et al. 2014). Importantly, the same laboratory found no effect of CBD-THC combination treatment on Aβ levels in the cortex or hippocampus in 12-month-old *APPxPS1* transgenic males (Aso et al. 2016) in line with our previous statement that therapeutic effects of cannabinoid medication in AD transgenic mice might be dependent on the disease stage of the mouse model tested. In addition, in a tauopathy mouse model for frontotemporal dementia, parkinsonism and lower motor neuron disease, Sativex® (CBD-THC, ratio of 1:1, 1.5 mg/kg of each CBD and THC) reduced Aβ plaques and aggregation in the cortex and hippocampus (Casarejos et al. 2013). These discrepancies across studies highlight the need to further investigate the effect of CBD on Aβ pathology and to also consider particular CBD-THC combination treatments.

We also evaluated the effects of CBD on other AD-relevant brain processes. To investigate neuroinflammation and microglia activation, we looked at TNF-α and IL-1β levels as well as IBA1, the latter being of interest as it is expressed only in microglia and macrophages and is upregulated when microglia are activated (Sasaki et al. 2001). Indeed, microglia expression and activation is

increased in response to Aβ and microglia are known to surround Aβ plaques (Martín-Moreno et al. 2011). Cortical IBA1 levels were elevated in *APPxPS1* mice compared to WT controls but TNF-α and IL-1β protein levels were not affected. In a previous study, we had detected a moderate increase in mRNA levels of TNF-α and IL-1β in 8-month-old *APPxPS1* males (Cheng et al. 2014c). However, similar to our studies, 9-15-month-old *APPxPS1* mice showed elevated mRNA expression of TNF-α and IL-1β without accompanying changes in protein expression (Babcock et al. 2015). In addition, a study in 27-28-month-old *APPxPS1* mice also did not detect any changes in TNF-α or IL-1β protein levels (Pihlgren et al. 2009) suggesting that the neuroinflammatory profile of *APPxPS1* is highly age-dependent. We found no effect of CBD on neuroinflammation nor microglia activation in the cortex of *APPxPS1* mice, which could be related to the lack of neuroinflammation in the AD transgenic mice. In this context it is interesting to note that CBD did not reduce elevated TNF-α mRNA levels in a pharmacological mouse model of AD (Martín-Moreno et al. 2011). The lack of effect of CBD on microglia activation is in contrast to previous *in vitro* findings which have reported that CBD reduces microglia activation (Martín-Moreno et al. 2011).

We also investigated cortical BDNF levels as a marker of neurodegeneration and as decreased BDNF levels have been found in AD patients (Siegel and Chauhan 2000) and appear linked to impaired cognition (Bathina and Das 2015). BDNF levels were not altered in *APPxPS1* transgenic males at 12 months of age. Previous studies in the same *APPxPS1* model have reported reduced BDNF levels in the hippocampus and cortex in 8-11-month-old males (Hou et al. 2010; Li et al. 2014) whereas $A\beta PP^{SWE}/PS\text{-}1^{M146V}$ transgenic mice did not demonstrate altered BDNF expression in the cortex at 12 and 14 months of age (Peng et al. 2009). In our study, CBD did not affect BDNF levels. A previous study in male Swiss mice found that acute CBD (10 mg/kg) elevated BDNF

levels in the hippocampus and prefrontal cortex. Interestingly, this effect was not sustained when measured 7 days after CBD treatment (Sales et al. 2019).

Finally, we measured cortical PPARγ levels as studies have indicated that CBD activates the PPARγ receptors and PPARγ antagonists prevent CBD's beneficial effects in pharmacological rodent models of AD (e.g. inoculated with $A\beta_{42}$ in hippocampus) (Esposito et al. 2011). Interestingly, PPARγ agonists have become a target for AD therapy (Heneka et al. 2007; Landreth et al. 2008; Shie et al. 2009). We found that cortical PPARγ protein levels were not altered in *APPxPS1* mice and not affected by CBD treatment either. This is the first study looking at PPARγ biology in the *APPxPS1* mouse model and future studies should expand on our early research considering other brain regions and age groups.

In conclusion, this study is the first to date to investigate the effect of 50 mg/kg CBD on cognition and AD-relevant brain pathology in 12-month-old male *APPxPS1* mice. We found that chronic CBD treatment was able to reverse social recognition memory deficits and an impairment in reversal spatial learning. In addition, CBD treatment resulted in a moderate reduction of insoluble $A\beta_{40}$ levels in the hippocampus of *APPxPS1* transgenic mice, which were also characterised by increased cortical IBA1 levels. The study emphasises the clinical relevance of CBD treatment for AD-relevant behaviours although the mechanisms involved need further investigation.

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Chapter 3: Effect of 100 mg/kg CBD on AD-relevant phenotypes of *APP$_{Swe}$/PS1ΔE9* double transgenic mice

Therapeutic effects of chronic 100 mg/kg cannabidiol (CBD) treatment in male double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) mice

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3.1 Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterised by cognitive decline as well as accumulation of amyloid-β (Aβ), tau hyperphosphorylation, neurodegeneration and neuroinflammation in the brain. There are no current AD treatments which reverse or prevent the progression of the disease, highlighting the need for novel therapeutics. The phytocannabinoid (CBD) has demonstrated antioxidant, anti-inflammatory and neuroprotective properties. Studies have found that chronic CBD treatment (20 mg/kg and 50 mg/kg) reverses social recognition memory deficits of *APPxPS1* transgenic mice without having pronounced effects on AD-relevant brain pathology. Thus, this study determined the therapeutic-like effects of chronic high dose CBD treatment (i.e. 100 mg/kg body weight intraperitoneally) in 7.5-month-old male *APPxPS1* mice. Mice were assessed for anxiety, two forms of recognition memory, and social behaviours including territorial aggression. Neuropathological analyses for a number of AD-relevant markers were also conducted using ELISA and western blot. Vehicle-treated *APPxPS1* transgenic males demonstrated reduced *wrestling* behaviour and increased sociopositive behaviours as well as impaired social recognition memory. The deficits in recognition memory were restored by CBD. *APPxPS1* mice also exhibited reduced hippocampal levels of TNF-α and IL-1β and elevated cortical levels of BDNF regardless of treatment condition. CBD increased proBDNF levels in WT controls. PPARγ and IBA1 protein levels were not affected by genotypes or CBD treatment. This study demonstrates that high dose CBD restores social recognition memory, thus emphasising the clinical relevance of CBD in AD. The mechanisms involved in CBD's therapeutic effects require further investigations.

Keywords: Alzheimer's disease, cannabidiol (CBD), transgenic *APP$_{swe}$/PS1* E9 mice, cognition, amyloid-β, TNF-α, IL-1β

3.2 Introduction

Alzheimer's disease (AD) is the second leading cause of death in Australia (Alzheimer's Association 2018). It is a debilitating neurodegenerative disease characterised by cognitive decline. AD initially presents in patients as short-term memory loss, but eventually progresses to cause severe memory problems, for example inability to recognise familiar faces and spatial disorientation (Alzheimer's Association 2018). Pathologically, AD is characterised by the accumulation of amyloid-β (Aβ) into plaques and the hyperphosphorylation of tau proteins into neurofibrillary tangles (NFTs) (Chapman et al. 2001). These features are thought to be central to the neurodegenerative cascade associated with AD, which includes neurodegeneration, neuroinflammation and oxidative damage (Ahmed et al. 2015). This initially occurs in areas of the brain crucial for memory (i.e. hippocampus and amygdala), but eventually spreads across all regions of the brain (Ahmed et al. 2015; Hardy and Selkoe 2002). This results in a number of non-cognitive related symptoms, including social withdrawal and aggression (Alzheimer's Association 2018). Despite the increase in research for AD, no treatments are currently available which prevent disease progression or work at later disease stages. Current AD treatments are only symptomatic and provide limited therapeutic benefits in early stages of the disease (Alzheimer's Association 2018).

In recent years, cannabidiol (CBD) has gained increasing attention as a potential novel therapeutic due to its neuroprotective, anti-inflammatory and anti-apoptotic properties (Iuvone et al. 2004). Furthermore, *in vitro* CBD has been able to elevate Aβ clearance and reduce tau hyperphosphorylation (reviewed in (Karl et al. 2016; Watt and Karl 2017)). In a pharmacological model of AD, CBD (20 mg/kg) restored a spatial reference memory deficit (Martín-Moreno et al. 2011). Our own studies in a transgenic mouse models of familial AD, the double transgenic

APPswe/PS1Δ9 (*APPxPS1*) model revealed that 20 mg/kg CBD reverses deficits in social recognition memory and object recognition memory (Cheng et al. 2014a) and 50 mg/kg CBD reverses deficits in social recognition memory and spatial learning with an accompanying moderate reduction in insoluble hippocampal $A\beta_{40}$ levels (Watt et al. 2020). In a preventative study, CBD was also able to prevent the development of social recognition memory deficits in these mice and tended to reduce neuroinflammatory markers in the cortex (i.e. tumour necrosis factor [TNF]-α, interleukin [IL]-1β and IL-6) (Cheng et al. 2014c). Importantly, *in vitro* studies have demonstrated that CBD, and cannabinoids in general, work in a dose-dependent manner (Esposito et al. 2007). In the present study, we evaluated the effects of chronic high-dose CBD treatment on reducing AD-relevant behavioural symptoms as well as brain pathology. Treatment of 7-month-old male *APPxPS1* transgenic mice with 100 mg/kg CBD started 3 weeks before conducting a battery of behavioural tests including the elevated plus maze (EPM), the novel object recognition task (NORT), the social preference test (SPT), and the Resident-Intruder paradigm. At the conclusion of behavioural testing, hippocampal and cortical tissue were collected and analysed for Aβ levels, neuroinflammation (i.e. TNF-α and IL-1β), neurogenesis (i.e. brain-derived neurotrophic factor [BDNF]), microglia activation (i.e. ionized calcium binding adaptor molecule 1 [IBA1]) and the peroxisome proliferator-activation receptor γ (PPARγ). These targets were chosen due to CBD's ability to reduce microglial activity (i.e. TNF-α and IL-1β production) and promotion of Aβ phagocytosis by increasing microglia migration (Martín-Moreno et al. 2011). In addition, PPARγ is both dysregulated in AD and appears to be involved in the therapeutic effects of CBD in AD mouse models (Esposito et al. 2011; Scuderi et al. 2014).

3.3 Experimental Procedures

3.3.1 Animals

Double transgenic male mice expressing chimeric mouse/human *APP* (*Mo/HuAPP695swe/Swedish mutations K595N/M596L*) and mutant human *PS1* (PS1/ΔE9) were obtained from Jackson Laboratory (Bar Harbor, USA; stock no. 004462, line 85) and maintained as double hemizygotes on a mixed C57BL/6J x C3H/HeJ background as described previously (Borchelt et al. 1997; Jankowsky et al. 2004). Male $APP_{Swe}/PS1\Delta E9$ transgenic mice (*APPxPS1*; $n = 22$) and non-transgenic littermates (wild type-like [WT]; $n = 29$) were bred and group-housed in independently ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at the Australian BioResources (Moss Vale, Australia). Mice were transported to our mouse holding and test facilities at Western Sydney University (WSU) at approximately 10 weeks of age. At WSU they were group-housed in filter top cages (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (Tecniplast Australia, Rydalmere, Australia), crinkle cut (Cink-l'Nest, Kraft) and tissues for nesting (as described previously in (Cheng et al. 2014a)). Mice were kept in a 12:12 h light:dark schedule [light phase: white light (illumination: 124 lx), dark phase: red light (illumination: < 2 lx]. Food (Rat & Mouse Pellets, Gordon's Specialty Stockfeeds Pty Ltd., Yanderra, Australia) and water were provided *ad libitum*. Adult, male A/J mice from Animal Resources Centre (Canning Vale, Australia) were used in the SPT as standard opponents. Research and animal care were approved by WSU Animal Care and Ethics Committee (#11335 and #12905) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes

3.3.2 Drug preparation and administration

Powdered cannabidiol (CBD: GW Pharmaceuticals, Salisbury, United Kingdom) was dissolved in equal parts Tween 80 (Sigma-Aldrich Co, St Louis, USA) and 100% ethanol. It was diluted with 0.9% sodium chloride to the appropriate concentration to a final ratio of 1:1:18. Ethanol and Tween 80 made up 10% of the volume. A vehicle control (VEH) was made up similarly but without the addition of CBD. VEH and CBD treatments were administered at a dose of 100 mg/kg body weight via intraperitoneal (i.p.) injections using an injection volume of 10 ml/kg body weight. Male *APPxPS1* and WT controls were either treated with VEH or CBD daily starting 3 weeks prior to the commencement of behavioural testing (test age: 7 months +/- 1 week) (WT-VEH n = 14; *APPxPS1*-VEH n = 11; WT-CBD n = 15; *APPxPS1*-CBD n = 11). Treatment continued throughout behavioural testing (total treatment duration: 6 weeks) and was given in the afternoon (between 1400 – 1600) after test completion, to avoid any acute effects of CBD confounding test outcomes from experiments run in the first hours of the light phase (i.e. before 1400). This treatment design was modelled on previous published studies (Cheng et al. 2014a). The body weight of mice was recorded on a weekly basis.

3.3.3 Behavioural testing

Mice were tested in several tests to detect behavioural and cognitive deficits in *APPxPS1* males (Cheng et al. 2013; Cheng et al. 2014a) (for test age and test biography see Table 1). All tests were conducted in the beginning of the light phase between 0930 and 1400. An inter-test interval of at least 48 h was used to minimise effect of repeated testing. Between trials equipment and apparatus were cleaned with 80% ethanol, except when specified.

Treatment	Vehicle		CBD	
Genotype	WT	*APPxPS1*	WT	*APPxPS1*
Age at start of treatment	194 ± 16	197 ± 22	196 ± 19	198 ± 23
Elevated plus maze	219 ± 20	223 ± 27	220 ± 22	228 ± 32
Novel object recognition task	223 ± 22	226 ± 28	223 ± 23	231 ± 33
Social preference test	228 ± 16	231 ± 20	229 ± 16	236 ± 25
Holeboard	230 ± 17	234 ± 22	231 ± 16	239 ± 26
Resident-Intruder paradigm	231 ± 18	235 ± 23	232 ± 17	240 ± 25
Tissue Collection	235 ± 16	239 ± 22	236 ± 17	244 ± 27

Table 1 - *Test biography.* Age [days] of male *APPxPS1* and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle throughout behavioural testing. Ages are presented as mean ± age range.

3.3.3.1 Elevated plus maze (EPM)

The EPM was used to utilize the natural conflict of mice to explore a novel environment and to avoid brightly lit, elevated open areas to assess anxiety (Lister, 1987). The maze consists of two open arms (30.5 cm x 6.5 cm, no sidewalls) and two closed arms (30.5 cm x 6.5 cm, sidewall height 18.5 cm). The arms are connected with a central platform (6 cm x 6 cm) and is elevated 40 cm above the ground. Test mice were placed in the centre platform, facing an enclosed arm and allowed to explore for 5 min. ANY-maze^{TM} tracking software (Stoelting, Wood Dale, USA) was used to record time spent and distance travelled in open and enclosed arms.

3.3.3.2 Novel object recognition task (NORT)

The NORT was used to assess object recognition memory by utilising the innate preference of rodents for novelty (Dere et al. 2007). The test consists of a single Perspex chamber (35 x 35 x 30 cm) and two distinct objects (toy giraffe and toy elephant [LEGO®DUPLO®, Billund, Denmark]). The test was conducted over two days. On the first day, mice were habituated to the empty arena in a 10 min trial. On the second day 2 x 10 min were conducted with an inter-trial interval (ITI) of 15 min. In the first trial, two identical objects were placed in the arena and the test mouse was allowed to explore. In the second trial, one of the objects was changed to a novel object and the test mouse was allowed to explore. The objects and their locations were counterbalanced across genotypes. Time spent *nosing* and *rearing* the objects were recorded using ANY-maze™ tracking software.

3.3.3.3 Social preference test (SPT)

The SPT was used to assess sociability and social recognition memory (Moy et al., 2004). The apparatus consists of a three-chamber box, where two outer chambers (16 cm x 18 cm x 20 cm) are joined by a central chamber (9 cm x 18 cm x 20 cm). In each outer chamber there is a mouse enclosure (diameter: 7cm; height: 15 cm; bars spaced: 0.5 cm). Test mice were isolated for 1 h prior to the start of testing. In the habituation trial, mice were allowed to freely explore the apparatus for 5 min. In the sociability trial, an unfamiliar age- and gender-matched standard opponent (male A/J mouse) was placed in one of the mouse enclosures in the outer chambers. The test mouse was allowed to explore for 10 min. In the social novelty trial, a second unfamiliar standard opponent was placed in the previously empty mouse enclosure and the standard opponent from the sociability trial was kept. The test mouse was again allowed to explore for 10 min. The

ITI was 3 min and between trials the test mouse was kept in the centre chamber. Chambers and enclosures were cleaned with 80% ethanol and fresh corn cob bedding was added to the chambers prior to each new test mouse. Time spent, entries and distance travelled in each chamber and time spent *nosing* mouse enclosures were recorded by ANY-maze^{TM} tracking software.

3.3.3.4 Resident-Intruder paradigm (RI)

The RI was used to assess territorial aggression (Brain and Poole, 1974). Prior to the start of testing, test mice were isolated in their home cage for 30 min. At the start of testing, an age- and gender-matched standard opponent (male A/J mouse) and the test mouse were placed simultaneously in opposite corners of the home cage of the test mouse. The mice were then allowed to freely interact for 10 min. The duration and frequency of agnostic behaviours (i.e. *tail rattling, aggressive grooming,* and *wresting*) and socio-positive behaviours (i.e. *anogenital sniffing, sniffing,* and *following*) were recorded by ANY-maze^{TM} tracking software. Tests were stopped when fighting escalated (defined as >10 bites or continuous fighting for >10 s) to avoid injuries (only evident in *APPxPS1*-VEH $n = 1$ and *APPxPS1*-CBD $n = 1$).

3.3.4 Biochemical Analyses

3.3.4.1 Brain tissue preparation

Four days after the conclusion of behavioural tests (age range: 238 ± 16 days), mice were anaesthetized and perfused with phosphate buffered saline (PBS) transcardially. The brain was divided sagittally and the left hemisphere was fixed in 4% paraformaldehyde for 24 h and then stored in 30% sucrose solution. The right hemisphere was dissected, and cortex and hippocampal

tissue were snap frozen in liquid nitrogen and stored in -80˚C. Final CBD/vehicle treatment was given 24 h before euthanasia.

Frozen hippocampal tissue (12 – 26 mg) and cortical tissue (10 – 24 mg) was homogenized in 12 volumes of TBS extraction buffer (140 mM NaCl, 3 mM KCl, 25 mM Tris1M pH7.4, 2 mM 1,10 phenanthroline stock, containing 1% Igepal CA630 and Sigma protease inhibitor cocktail and 10 µM phenylmethylsulfonyl fluoride [PMSF]) using a Precelleys 24 homogenizer (2 x 30s, 6000 x g). Homogenates were centrifuged at 16,100 g for 30 min at 4°C. The TBS-soluble supernatant was collected and stored at -80°C until used. The pellet was homogenized with 10 volumes of 6.25 mM guanidine HCL (gHCL) in 50 mM Tris, pH8 in Precelleys 24 homogenizer (2 x 30s, 6000 g) and left in the rotary at 4°C overnight. Homogenates were centrifuged at 16,100 g for 30 min at 4°C. The gHCL-soluble supernatant (TBS-insoluble) were collected and stored at -80°C. Protein was quantified using Qubit protein assay kit.

3.3.4.2 Enzyme-linked immunosorbent assay (ELISA)

In our analysis of Aβ pathology, we only investigated $A\beta_{42}$ levels as it has been reported to be the more pathogenic and harmful form of the protein (Chapman et al. 2001). The concentration of $A\beta_{42}$ protein in TBS-soluble and gHCL-soluble hippocampal fractions of *APPxPS1* mice was quantified using BetaMark™ β-Amyloid x-42 ELISA Kit (TMB) (Australian Biosearch). The concentration of TNF-α and IL-1β protein in TBS-soluble hippocampal fractions of *APPxPS1* mice and WT controls were quantified using Invitrogen TNF-α Mouse ELISA Kit (ThermoFisher Scientific) and Invitrogen IL-1β ELISA Kit (ThermoFisher Scientific). Outliers were defined as ± 2 standard deviations from the mean (soluble Aβ: CBD $n = 1$; insoluble Aβ: VEH $n = 1$, CBD $n = 1$).

3.3.4.3 Western blotting

Both proBDNF and mature BDNF were analysed with cleavage of proBDNF forming mature BDNF, the biologically active compound (Bathina and Das 2015). In addition, we analysed two isoforms of PPARγ, PPARγ1 and PPARγ2, as they have distinct depositions in the brain, with PPARγ2 being predominantly expressed in adipose tissue and PPARγ1 more ubiquitously expressed (Heikkinen et al. 2007). TBS-soluble cortical fractions were analysed by SDS-PAGE Western Blotting using antibodies: IBA1 (5 µg of protein per lane, 1:1000, Novachem [019-19741], band size: ~14 kDa), BDNF (20 µg of proteins per lane, 1:1000, Abcam [ab108319], band 1 [proBDNF] size: ~37 kDa, band 2 [mature BDNF] size: ~14 kDa), PPARγ (2 µg of protein per lane, 1:2000 Millipore [ABN1445], band 1 [PPARγ2 isoform] size: ~60 kDa, band 2 [PPARγ1 isoform] size: ~55 kDa) and housekeeper antibody: actin (1:1000, Sigma-Aldrich [A2066]). Goat anti-rabbit IgG HRP-conjugated secondary antibody (1:2000, Millipore [AP132P] and enhanced chemiluminescence was used to detect signals. Signals were quantified using image J software. Data were normalised to actin levels and internal control and expressed as relative values.

3.3.5 Statistical analysis

Behavioural data from the EPM and the RI test as well as molecular data (except for $A\beta_{42}$ protein levels) were analysed using two-way analysis of variance (ANOVA) for the main effects of 'genotype', 'treatment' as well as 'genotype' by 'treatment' interactions. Performance in NORT and SPT was assessed using single sample *t*-tests to investigate if time spent *nosing* a novel object (NORT) and time spent in chamber and *nosing* a mouse (SPT) were greater than chance (50%). Single sample *t*-tests were used to evaluate the effect of CBD treatment on $A\beta_{42}$ levels in *APPxPS1* mice. Data are shown as means ± standard error of means (SEM). Differences were regarded as

statistically significant if $p < 0.05$. F-values and degrees of freedom are presented for ANOVAs. Significant genotype effects are shown by '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), treatment effects are indicated by '#' ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) and significant 'genotype x treatment' interactions are reported by '+' ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). Significant *t*-test results are shown by '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$). Statistical analyses were conducted using SPSS 25 for Mac.

3.4 Results

3.4.1 Anxiety/Locomotion

3.4.1.1 Elevated plus maze (EPM)

Two-way ANOVA for total distance travelled in the EPM revealed no difference between *APPxPS1* mice and WT controls and CBD treatment did not affect locomotion either [no main effects, all p's > 0.05] (Table 2). Similarly, there were no main effects of 'genotype', 'treatment' or 'genotype' x 'treatment' interactions on the anxiety-related parameters percentage time spent and percentage distance travelled on the open arms (all p's > 0.05) (Table 2). When evaluating the first and second halves of the open arms, with the second half of the open arm being more aversive to explore (as it is further away from the more secure centre zone and enclosed arms), two-way ANOVA confirmed that there were no effects of genotype or CBD treatment on time spent in either half of the open arms (all p's > 0.05) (Table 2).

Treatment	Vehicle		CBD	
Genotype	WT	*APPxPS1*	WT	*APPxPS1*
Total distance travelled [m]	7 ± 1	7 ± 0	7 ± 0	7 ± 1
Distance travelled in open arms [%]	17 ± 3	20 ± 5	15 ± 3	19 ± 3
Time spent in open arms [%]	31 ± 6	31 ± 7	26 ± 5	33 ± 5
Time spent in first half of open arms [%]	23 ± 4	19 ± 4	21 ± 4	24 ± 4
Time spent in second half of open arms [%]	5 ± 2	9 ± 3	3 ± 1	7 ± 2

Table 2 – *Elevated plus maze behaviours*. Locomotion and anxiety-related behaviours in the EPM for male *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle. Data are presented as mean ± SEM.

3.4.2 Cognition

3.4.2.1 Novel object recognition task (NORT)

Single sample *t*-tests indicated that no experimental group demonstrated a preference for the novel object above chance levels when assessing the full 10 min test period (all *p*'s > 0.05; Figure 1A).

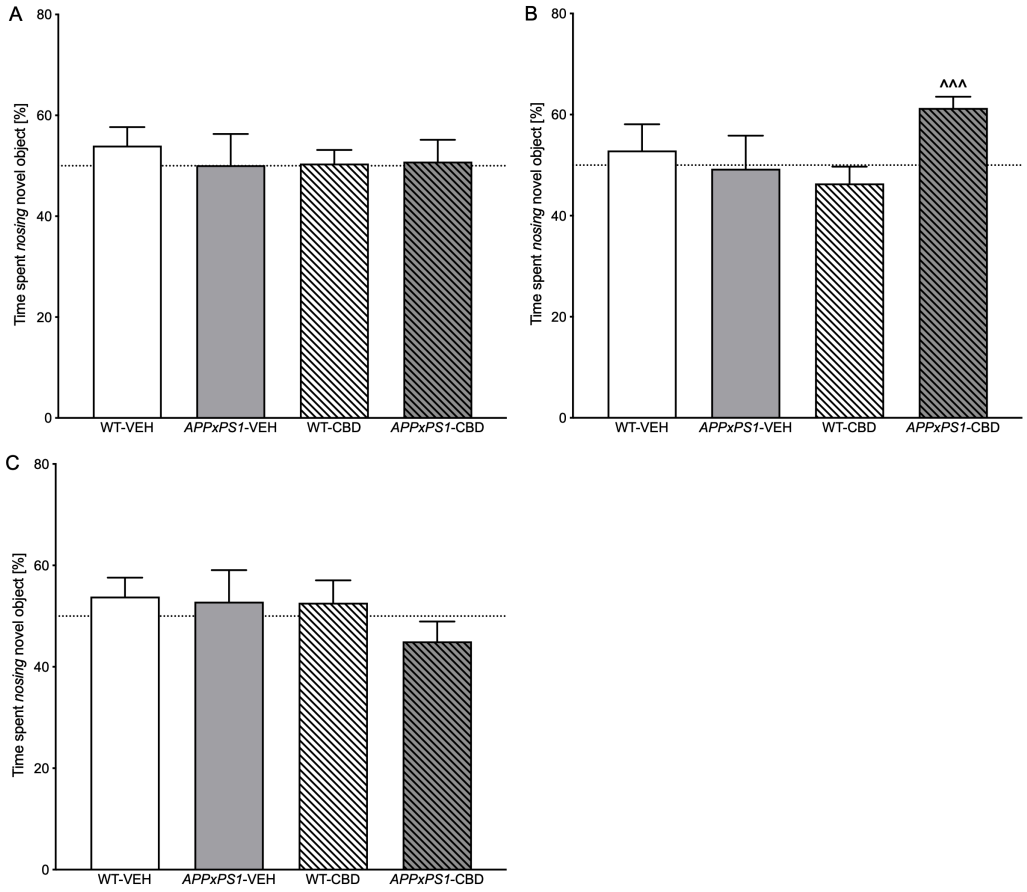

Figure 1A-C: ***Novel object recognition memory.*** Time spent [%] **A)** *nosing* novel object, **B)** *nosing* novel object in first 5 minutes of test and **C)** *nosing* novel object in last 5 minutes of test for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. *t*-test results are presented as '^' (^^^$p < 0.001$).

However, when split into 5-min bins, *APPxPS1*-CBD mice exhibited a preference for the novel object in the first 5 min of testing whereas all other experimental groups failed to develop this

preference [WT-VEH $p = 0.586$; *APPxPS1*-VEH $p = 0.915$; WT-CBD $p = 0.294$; *APPxPS1*-CBD $p < 0.0001$] (Figure 1B). No group demonstrated a preference for the novel object in the second 5-min bin (all p's > 0.05; Figure 1C).

3.4.2.2 Sociability and social recognition memory

Sociability: Single sample *t*-tests revealed that all groups demonstrated an above chance preference for the mouse chamber [WT-VEH $p < 0.0001$; *APPxPS1*-VEH $p < 0.0001$; WT-CBD $p < 0.0001$; *APPxPS1*-CBD $p < 0.0001$] (Figure 2A). This was supported by a three-way ANOVA for time spent in mouse vs empty chamber which revealed a 'chamber' effect [$F(1,47) = 140.5$, $p < 0.0001$], where all groups demonstrated a preference for the mouse chamber (data not shown). Interestingly, it also revealed a 'chamber' by 'treatment' effect [$F(1,47) = 4.607$, $p = 0.037$], where CBD treated mice spent more time in the empty chamber (data not shown). This finding was also evident in the two-way ANOVA for time spent in the mouse chamber, which revealed a main effect of 'treatment' [$F(1,47) = 4.242$, $p = 0.045$], where CBD treatment reduced time spent in the mouse chamber (data not shown).

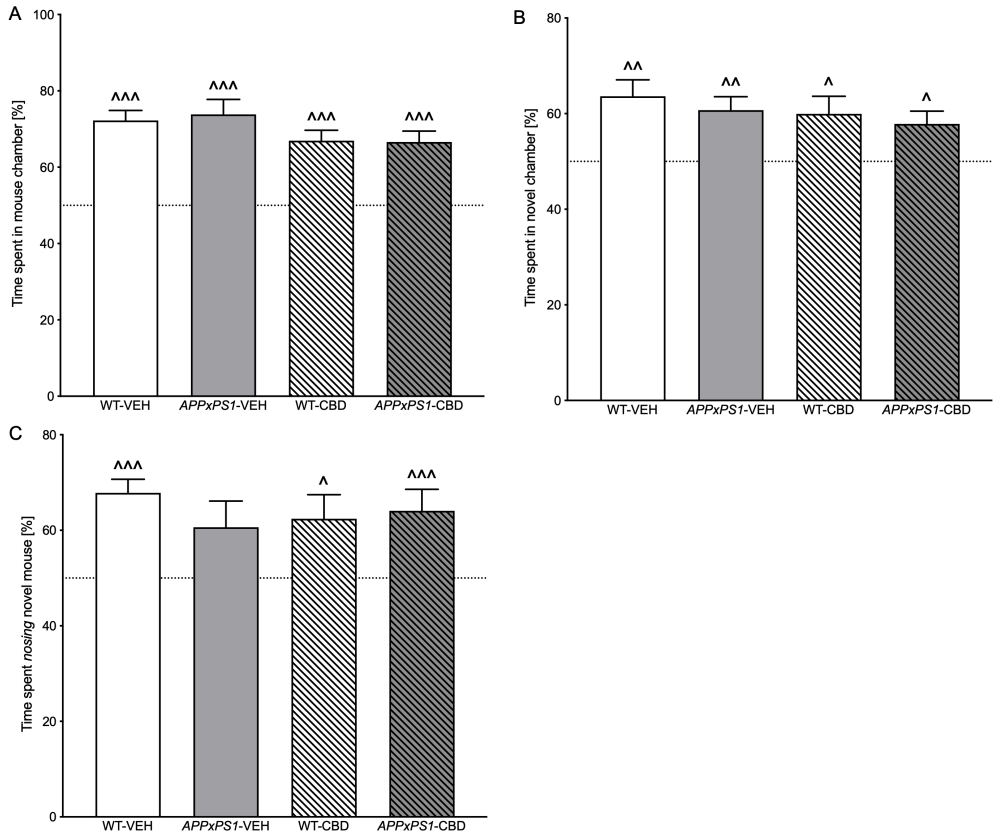

Figure 2A-C: ***Sociability and social recognition in the social preference test.*** Time spent [%] **A)** in mouse chamber, **B)** in novel mouse chamber and **C)** *nosing* the novel mouse enclosure for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. *t*-test results are presented as '^' (^$p < 0.05$, ^^$p < 0.01$ and ^^^$p < 0.001$).

Social recognition memory: Single sample *t*-tests indicated a preference for the novel chamber in all experimental groups when assessing time spent in chamber [WT-VEH $p = 0.002$; APPxPS1-VEH $p = 0.004$; WT-CBD $p = 0.016$; APPxPS1-CBD $p = 0.016$] (Figure 2B). This was supported by a three-way ANOVA for time spent in novel vs familiar mouse chamber, as all groups demonstrated a preference for the novel mouse chamber [$F(1,47) = 35.08$, $p < 0.0001$]. There were no 'genotype', 'treatment' or 'genotype x treatment' interactions [all p's > 0.05] (data not shown). This was supported by a two-way ANOVA for time spent in the novel mouse chamber which

revealed no main effects of 'genotype', 'treatment' or 'genotype x treatment' interactions [all p's > 0.05] (data not shown).

However, we also analysed time spent *nosing* the familiar and novel mouse as this is a more accurate measure to test for social recognition memory. *APPxPS1*-VEH mice failed to demonstrate a preference for the novel mouse [*APPxPS1*-VEH: t(10) = 1.939, $p > 0.05$] whereas this preference was intact in all other experimental groups [WT-VEH: t(13) = 6.276, $p < 0.0001$; WT-CBD: t(14) = 2.469, $p = 0.027$; *APPxPS1*-CBD: t(10) = 3.129, $p = 0.01$] (Figure 2C). Interestingly, this was not supported by a three-way ANOVA for time spent *nosing* the novel vs familiar mouse nor a two-way ANOVA for time spent *nosing* the novel mouse, as neither revealed 'genotype', 'treatment' or 'genotype x treatment' interactions [all p's > 0.05] (data not shown).

3.4.3 Resident-Intruder paradigm behaviours

Two-way ANOVA for total time spent interacting with a standard opponent mouse (i.e. across all socio-positive and aggressive behaviours shown) revealed no main effects of 'genotype', 'treatment' or 'genotype' x 'treatment' interactions (all p's > 0.05; Figure 3A). Interestingly, the total time spent on only socio-positive behaviours (i.e. *sniffing*, *anogenital sniffing*, and *following*) was elevated in *APPxPS1* transgenic males compared to WT controls [genotype: F(1,44) = 12.163, $p = 0.001$] regardless of CBD treatment (no significant 'genotype' x 'treatment' interaction, $p > 0.05$) (Figure 3B). In line with this, *APPxPS1* males spent significantly more time on *sniffing* [F(1,44) = 14.536, $p < 0.0001$] and this phenomenon was not affected by CBD treatment (i.e. no 'genotype' x 'treatment' interaction; $p > 0.05$). The time spent on *anogenital sniffing* tended to be different between genotypes [F(1,44) = 3.770, $p = 0.059$] as well but this finding was affected by CBD treatment ['genotype' x 'treatment' interaction: F(1,44) = 4.341, $p = 0.043$]. When split by treatment, it became obvious that *APPxPS1*-VEH mice spent significantly longer on this behaviour

compared to corresponding control mice [F(1,22) = 8.515, $p = 0.008$], whereas this genotype effect was absent in CBD-treated animals [F(1,22) = 3.741, $p = 0.923$]. There was also a trend for a CBD effect in the *APPxPS1* males with CBD decreasing the duration of *anogenital sniffing* in these mice [F(1,17) = 3.664, $p = 0.073$ – absent in WT controls: F(1,27) = 0.727, $p = 0.402$]. *APPxPS1* mice also exhibited a higher frequency of *anogenital sniffing* [F(1,44) = 4.126, $p = 0.048$] compared to WT mice regardless of treatment (no genotype' x 'treatment' interaction, $p > 0.05$; Table 3). There was no effect of genotype or CBD treatment on *following* or *rearing* on social opponents [all p's > 0.05] (Table 3).

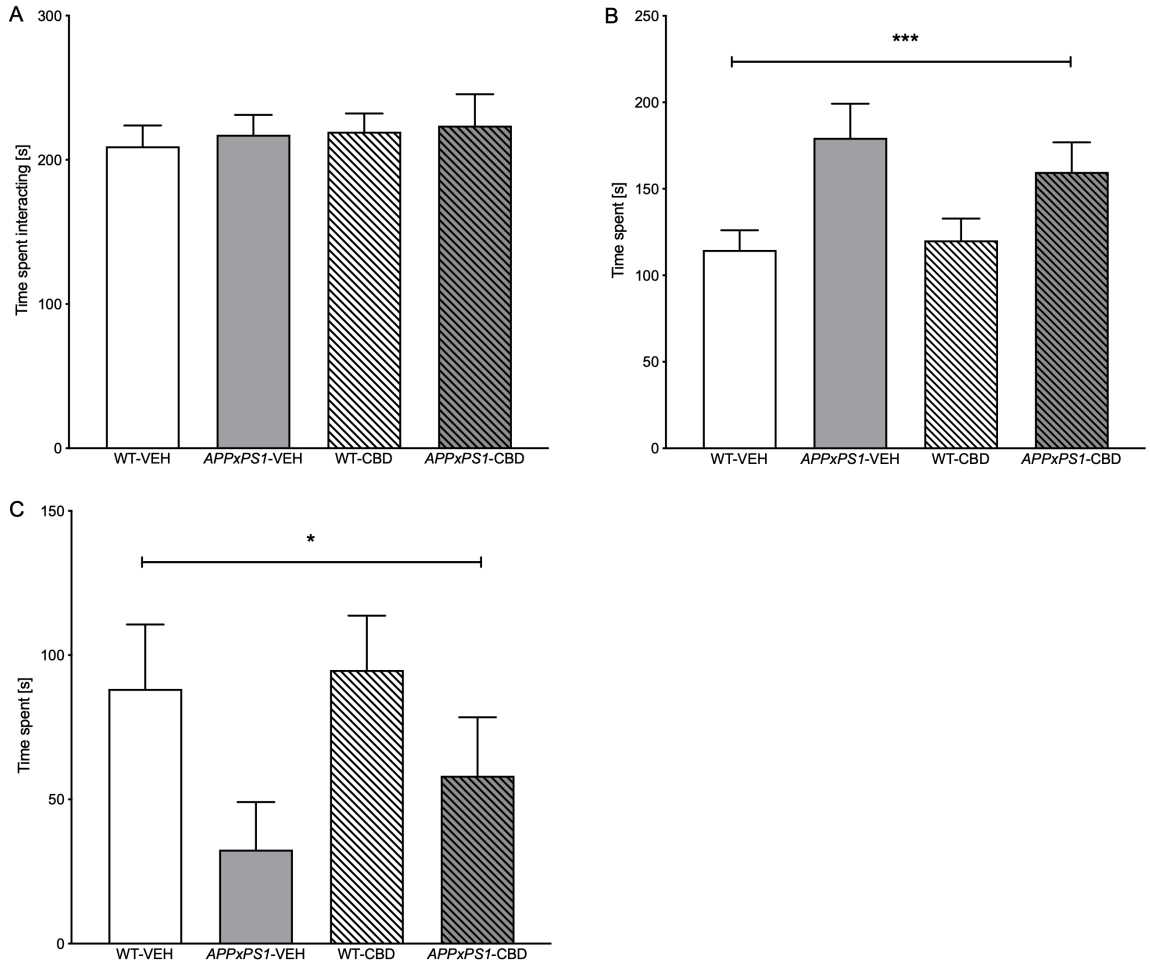

Figure 3A-C: ***Aggression in Resident-Intruder paradigm.*** **A)** Total active interaction time [s], total time showing **B)** socio-positive behaviours and **C)** aggressive behaviours for *APPxPS1* transgenic mice and non-

transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. 'Genotype' effects are presented as '*' (*$p < 0.05$ and ***$p < 0.001$).

Treatment	Vehicle		CBD	
Genotype	WT	*APPxPS1*	WT	*APPxPS1*
Time spent exhibiting [s]				
Sniffing	84 ± 9	125 ± 16	80 ± 7	124 ± 14
Anogenital Sniffing +	29 ± 4	52 ± 8	35 ± 6	34 ± 5
Following	2 ± 1	3 ± 1	5 ± 2	2 ± 1
Wrestling	60 ± 18	20 ± 13	65 ± 13	32 ± 13
Tail Rattling	3 ± 1	1 ± 1	5 ± 1	5 ± 2
Aggressive Grooming	25 ± 7	11 ± 6	25 ± 7	21 ± 9
Rearing	6 ± 1	5 ± 2	5 ± 1	6 ± 2
Frequency exhibiting [n]				
Sniffing	41 ± 3	43 ± 4	37 ± 3	46 ± 3
Anogenital Sniffing	15 ± 2	21 ± 3	14 ± 2	18 ± 2
Following	1 ± 0	2 ± 0	2 ± 1	2 ± 1
Wrestling	20 ± 6	7 ± 5	19 ± 5	10 ± 4
Tail Rattling	4 ± 1	2 ± 1	6 ± 1	5 ± 2
Aggressive Grooming	10 ± 2	4 ± 2	8 ± 3	7 ± 2
Rearing	5 ± 1	3 ± 1	3 ± 0	4 ± 1

Table 3 - *Resident-Intruder behaviours.* Aggressive and socio-positive behaviours in the Resident-Intruder task for male *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle. Data are presented as mean ± SEM. The 'genotype x treatment' interaction is presented as '+' ($^{+}p < 0.05$).

Interestingly, the total time spent on aggressive behaviours (i.e. *wrestling, tail rattling*, and *aggressive grooming*) was lower in *APPxPS1* mice compared to WT controls [genotype: $F(1,44) = 4.908$, $p = 0.032$) (Figure 3C). This was predominantly due to AD transgenic mice exhibiting significantly less *wrestling* behaviour (in regards to both time spent and frequency) than WT

controls [time: $F(1,44) = 5.470$, $p = 0.024$; frequency: $F(1,44) = 4.620$, $p = 0.037$ - no 'genotype' effects for *tail rattling* and *aggressive grooming*; all p's > 0.05; Table 3). Interestingly, CBD-treated mice spent more time *tail rattling* than vehicle-treated mice ['treatment' effect: $F(1,44) = 4.877$, $p = 0.032$; Table 3] regardless of genotype (no 'genotype' x 'treatment' interaction, $p > 0.05$).

3.4.4 Brain pathology

3.4.4.1 Amyloid β

Single sample *t*-test for soluble $A\beta_{42}$ hippocampal levels in *APPxPS1* mice revealed that there was no significant difference between treatment groups [$t(10) = 1.4$, $p = 0.18$; Table 4]. Similarly, insoluble $A\beta_{42}$ hippocampal levels were not different between vehicle- and CBD-treated *APPxPS1* mice [$t(8) = 0.69$, $p = 0.51$] (Table 4).

Treatment	Vehicle	CBD
Soluble $A\beta_{42}$	11 ± 3	23 ± 7
Insoluble $A\beta_{42}$	13 ± 6	7 ± 3

Table 4 - *ELISA results for soluble and insoluble $A\beta_{42}$ levels in the hippocampal tissue of APPxPS1.* Soluble $A\beta_{42}$ and insoluble $A\beta_{42}$ for male *APPxPS1* transgenic mice treated with 100 mg/kg cannabidiol (CBD) or vehicle. Data are presented as means ± SEM.

3.4.4.2 Neuroinflammation

Two-way ANOVA for hippocampal TNF-α levels revealed a main effect of 'genotype' [$F(1,32) = 6.329$, $p = 0.017$], where *APPxPS1* mice had a lower concentration of hippocampal TNF-α compared to WT controls. There was no 'genotype' x 'treatment' interaction ($p > 0.05$) and no

significant effect of CBD on TNF-α [F(1,32) = 3.034, p = 0.094] (Figure 4A). Similarly, hippocampal IL-1β concentration was lower in *APPxPS1* mice compared to control WT mice as well [F(1,36) = 5.753, p = 0.022] with CBD having no effect on this marker [both p's > 0.05] (Figure 4B). Testing for cortical IBA1 protein levels revealed no significant differences regardless of genotype or CBD treatment (all p's > 0.05) (Figure 5A).

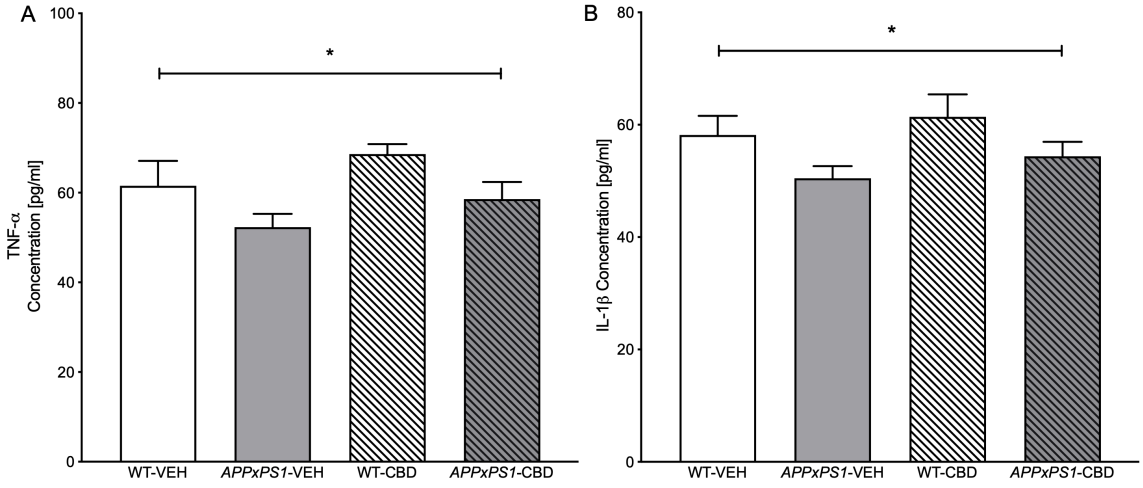

Figure 4A-B: ***ELISA results for TNF-α and IL-1β in the hippocampal tissue of APPxPS1 mice and WT controls.*** ELISA results for **A)** TNF-α levels and **B)** IL-1β levels for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. 'Genotype' effects are presented as '*' (*p < 0.05).

3.4.4.3 Neurodegeneration

Two-way ANOVA for cortical proBDNF (37 kDa) revealed a strong trend for an overall 'genotype' effect [F(1,40) = 3.860, p = 0.056] and more importantly, a main 'genotype' x 'treatment' interaction was detected [F(1,40) = 5.580, p = 0.02]. Split by 'treatment', vehicle-treated *APPxPS1* mice showed increased proBDNF levels compared to respective control males [F(1,20) = 10.158, p = 0.005; p > 0.05 for CBD-treated mice] (Figure 5B). When splitting by 'genotype', CBD increased proBDNF levels in WT mice [F(1, 23) = 4.964, p = 0.036], while no

such effect was seen in *APPxPS1* mice ($p > 0.05$).

Two-way ANOVA for cortical mature BDNF (14 kDa) revealed only a moderate trend for a

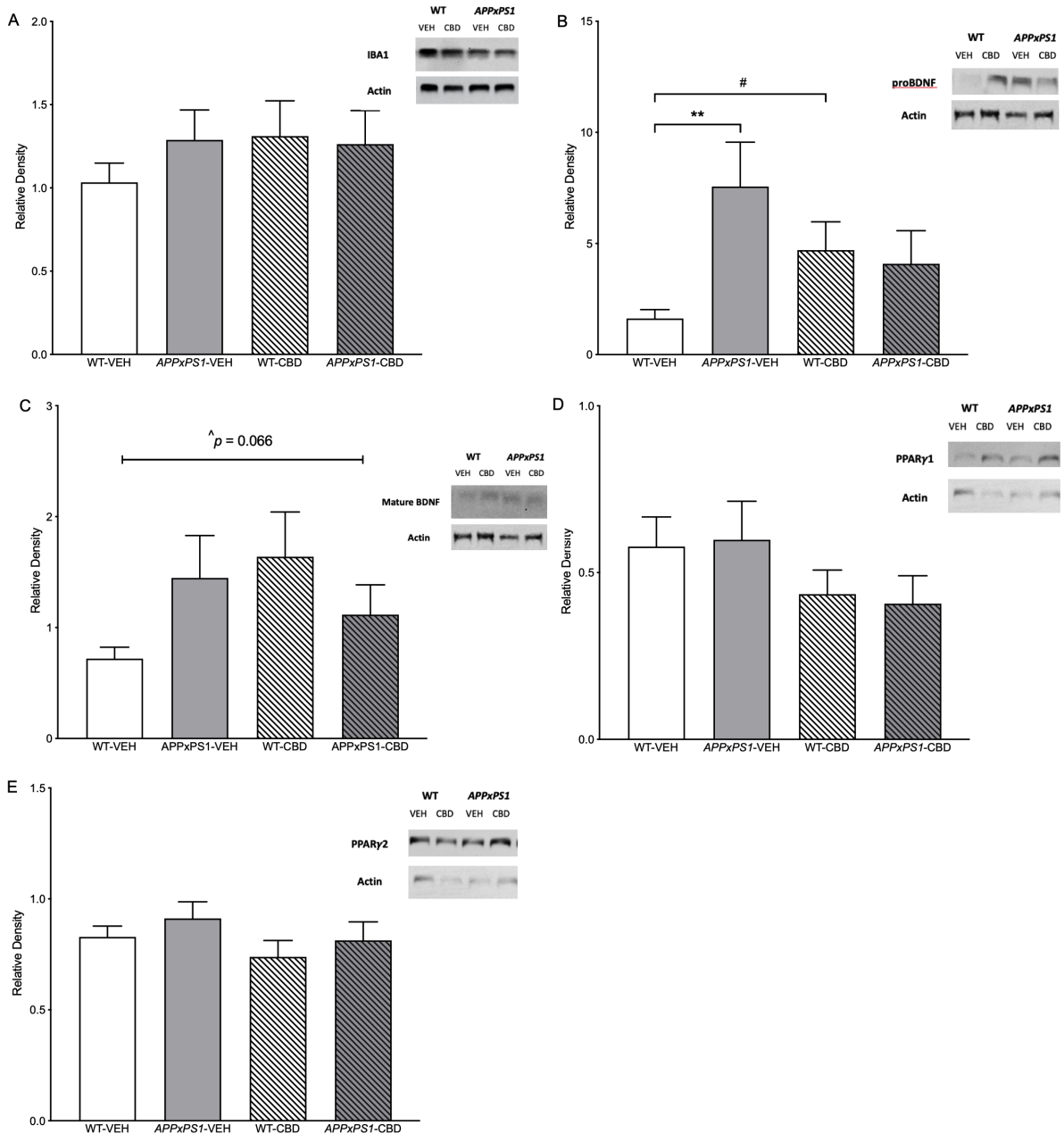

Figure 5A-E: ***Western blot results for neuroinflammation and neurodegeneration markers in the cortical tissue of APPxPS1 mice and WT controls.*** Western blot results for **A)** IBA1, **B)** proBDNF, **C)** mature BDNF, **D)** PPARγ1 and **E)** PPARγ2 levels for *APPxPS1* transgenic mice and non-transgenic WT control littermates treated with 100 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as

means ± SEM. ‘Genotype x treatment’ effects are presented as ‘^’ (^$p < 0.05$), ‘treatment’ effects as ‘$^{\#}$’ ($^{\#}p < 0.05$) and ‘genotype’ effects as ‘*’ (*$p < 0.05$, **$p < 0.01$).

‘genotype’ x ‘treatment’ interaction [$F(1,38) = 3.595$, $p = 0.066$]. No significant main effects of ‘genotype’ or ‘treatment’ were detected (Figure 5C).

3.4.4.4 PPARγ levels

Two-way ANOVA for cortical PPARγ1 isoform revealed no significant effect of CBD treatment [$F(1,43) = 3.358$, $p = 0.074$]. There was no main effect of ‘genotype’ and no significant ‘genotype’ x ‘treatment’ interaction (both p’s > 0.05) (Figure 5D). There were no statistically significant differences between experimental groups when analysing PPARγ2 protein levels (all p’s > 0.05) (Figure 5E).

3.5 Discussion

This study demonstrated that chronic treatment with 100 mg/kg CBD reversed a social recognition memory deficit in 7.5-month-old male double transgenic *APPxPS1* mice. *APPxPS1* mice also exhibited reduced hippocampal levels of TNF-α and IL-1β regardless of treatment as well as a moderate increase in protein levels of proBDNF (but not mature BDNF) in the cortex. CBD did not affect TNF-α or IL-1β levels of mice nor did it affect BDNF levels in *APPxPS1* transgenic mice. However, CBD did increase proBDNF in the WT controls. CBD did not affect $A\beta_{42}$ levels in AD transgenic mice and PPARγ1, PPARγ2 and IBA1 protein levels were similar across genotypes and treatment groups.

This study found that anxiety was unaltered between *APPxPS1* males and WT controls in the EPM. Interestingly, our previous baseline study in 7-month-old *APPxPS1* males reported increased anxiety in the EPM (Cheng et al. 2013). However, when followed up in 8-month-old *APPxPS1* males treated with 20 mg/kg CBD, we found no difference in anxiety between vehicle-treated *APPxPS1* males and WT controls (Cheng et al. 2014a). However, potential stress associated with daily injections and handling (Baek et al. 2015) and/or the 5% ethanol included in the vehicle treatment (Acevedo et al. 2014) may account for these differences in anxiety-relevant phenotypes between studies. Interestingly, a study in male *APPxPS1* mice on a pure C57BL/6J background demonstrated an anxiolytic-like phenotype at 10 months of age, but not at 2 and 5 months of age (Pugh et al. 2007). This finding suggests that the baseline anxiety phenotype of *APPxPS1* transgenic mouse models is highly dependent on age and the genetic background. Chronic CBD treatment had no effect on EPM anxiety parameters, in line with our earlier study using 20 mg/kg CBD treatment (Cheng et al. 2014a). Although CBD has been found to have anxiolytic-like effects,

these have been predominantly reported when utilising acute CBD administrations (Campos and Guimarães 2008; Gomes et al. 2011).

In the NORT, WT mice failed to develop a preference for the novel object although we have used similar NORT protocols successfully in the past (Cheng et al. 2014a; Watt et al. 2020). It is interesting to note that vehicle-treated *APPxPS1* mice did not demonstrate a preference for the novel object in the first 5 min of the recognition trial and that this deficit was restored in the chronic CBD treatment group, similar to what has been reported previously when using 20 mg/kg CBD (Cheng et al. 2014a) as well as CBD-enriched extract (0.75 mg/kg) and a CBD+THC combination treatment (1:1 ratio, 0.75:0.75 mg/kg). A number of studies have utilised a 5 min trial protocol in *APPxPS1* mice to detect impaired object recognition memory in the past (Yan et al. 2013; Zhang et al. 2012).

In the SPT, vehicle-treated *APPxPS1* males demonstrated impaired social recognition memory (Cheng et al. 2013; Cheng et al. 2014a; Cheng et al. 2014c), which was restored after chronic treatment with 100 mg/kg CBD. Previously we had reported that lower doses of CBD are also effective in restoring deficits in social recognition memory of 8- and 12-month-old *APPxPS1* males (Cheng et al. 2014a; Watt et al. 2020). A loss of facial recognition memory and social withdrawal are commonly seen in AD patients suggesting clinical relevance of CBD's effect on mouse behaviour (Alzheimer's Association 2018). Importantly, all mice showed intact sociability confirming that the cognitive deficits were not affected by changes in social behaviour.

Although unpaired t-tests revealed that vehicle-treated *APPxPS1* males demonstrated impaired social recognition memory in SPT, this was not reflected when two- and three-way ANOVAs were conducted. This discrepancy suggests that only a subtle social recognition memory impairment was detected. Previous studies have reported that *APPxPS1* males begin to show cognitive deficits

from 7-8 months and that the disease progresses with age (Cheng et al. 2013). However, it is possible that at 7.5 months of age (as was tested in this experiment) male *APPxPS1* mice are only demonstrating mild cognitive impairments. Future experiments should be conducted at an older age to ensure a significant social recognition memory impairment.

Increased aggression is commonly seen in AD patients (Silveri 2007). Our findings indicate that territorial aggression was reduced in our *APPxPS1* model. Other studies have found increased levels of aggression in *APPxPS1* males, but these animals were on the pure C57BL/6J background and there were also differences in the aggression test protocol utilised (i.e. isolation-induced territorial aggression had been assessed and the social opponents were on C57BL/6J background) (Minkeviciene et al. 2004; Pugh et al. 2007). In addition, a study in *APPxPS1* mice on a mixed background (i.e. C57BL/6 x C3H/HeN) found that transgenic males demonstrated less aggression even after isolation (Olesen et al. 2016). This is a different background subline to our *APPxPS1* model, however, another study has suggested that they are the same subline (Metaxas et al. 2018). These distinct differences in methodologies have been found to account for variations in aggressive phenotypes (Olivier and Young 2002; Takahashi and Miczek 2013). Chronic CBD treatment increased *tail rattling* but had no effect on any other aggressive behaviours measured. Interestingly, cannabinoids have been investigated as a potential treatment for agitation and aggression associated with AD. However, it has been suggested that these therapeutic effects are largely mediated via the cannabinoids receptors, of which CBD is not a major agonist (Liu et al. 2015). We also recorded socio-positive behaviours during RI testing and found some were elevated in *APPxPS1* males. Furthermore, CBD decreased *anogenital sniffing* of AD transgenic mice. A number of studies outline that lower dose CBD can restore social interaction deficits (Gururajan

et al. 2012; Kaplan et al. 2017; Osborne et al. 2017) but at the 100 mg/kg CBD dose, the phytocannabinoid did not affect overall social interaction time (Long et al. 2012).

The findings from this study highlight potential limitations of AD mouse models as a number of common symptoms of AD were not found in the *APPxPS1* males. In particular, *APPxPS1* males did not demonstrate aggression or recognition memory impairments, which are well known symptoms seen in AD patients (Silveri 2007; Webster et al. 2014). However, it is important to note that mouse models often don't recapitulate all aspects of the disease they model (Hall and Roberson 2012) and this mouse model in particular only models the Aβ-dependent pathology associated with AD.

$A\beta_{42}$ levels were measured in the hippocampus as Aβ deposition is thought to start in this region in *APPxPS1* mice (Borchelt et al. 1997; Jankowsky et al. 2004). CBD treatment did not affect soluble or insoluble $A\beta_{42}$ levels in *APPxPS1* males. In previous work, we found that CBD treatment (50 mg/kg) moderately reduced hippocampal insoluble $A\beta_{40}$ levels but did not affect $A\beta_{42}$ levels in 12-month-old *APPxPS1* mice (Watt et al. 2020). Similarly, CBD-enriched extract (0.75 mg/kg) had no effect on soluble $A\beta_{42}$ in the cortex of 6-month-old *APPxPS1* males suggesting that CBD effects on Aβ levels are age- and dose-dependent (Aso et al. 2014). It is of interest to mention here that combining CBD and THC within one treatment effectively reduced $A\beta_{42}$ in 6-month-old *APPxPS1* males, supporting recent evidence that CBD-THC combination treatments might be therapeutically relevant (Pamplona et al. 2018; Russo 2011; Russo 2019).

TNF-α and IL-1β have been found to be elevated in AD mouse models (Babcock et al. 2015) as well as in AD patients (Swardfager et al. 2010). Furthermore, microglial expression and activation are increased in response to Aβ (Martín-Moreno et al. 2011; Sasaki et al. 2001). In the current study, hippocampal TNF-α and IL-1β were reduced while cortical IBA1 levels were unaffected.

We previously found cortical mRNA expression of TNF-α and IL-1β tended to be increased in vehicle-treated 8-month-old *APPxPS1* males (Cheng et al. 2014c) and increased IBA1 protein expression in 12-month-old *APPxPS1* males regardless of CBD treatment (Watt et al. 2020), suggesting changes in microglia function may only commence at a later disease stage. The distinct findings in TNF-α and IL-1β expression may be at least partially due to different methods (i.e. protein *versus* mRNA expression). Supporting this, 9-15-month-old *APPxPS1* mice exhibited increased mRNA expression of TNF-α and IL-1β, which was not confirmed when analysing protein levels (Babcock et al. 2015).

Reduced levels of BDNF, a marker for neurogenesis, have been reported in AD patients (Siegel and Chauhan 2000) and appear associated with reduced cognition (Bathina and Das 2015) and increasing BDNF levels reversed cognitive deficits in mouse models of AD (Blurton-Jones et al. 2009; Shin et al. 2014). Interestingly, *APPxPS1* mice expressed higher cortical levels of proBDNF but not the mature form of BDNF and CBD increased proBDNF levels in WT controls but not transgenic mice. Previous studies found reduced BDNF levels in the hippocampus and cortex of 8-11-month-old *APPxPS1* transgenic males (Hou et al. 2010; Li et al. 2014), whereas our own work in 12-month-old *APPxPS1* males found no changes to mature BDNF (Watt et al. 2020). Colleagues have reported a CBD-induced increase in hippocampal BDNF levels although this finding was only evident at 5 and 10 mg/kg (Magen et al. 2009; Sales et al. 2019) but not at 30 mg/kg (Zanelati et al. 2010), suggesting dose-dependent effects.

PPARγ agonists have been found to improve memory, reduce Aβ levels and microglia activation in AD mouse models (Heneka et al. 2005; Yan et al. 2003) and have a beneficial effect on memory in mild to moderate AD cases (Geldmacher 2006; Watson et al. 2005). Furthermore, CBD appears to act as an agonist at PPARγ receptors and elicit some of its anti-inflammatory and neuroprotective

effects via these receptors (Esposito et al. 2011). Nonetheless, PPARγ1 and PPARγ2, were not affected in AD transgenic mice at the age tested and CBD had no effect on those levels, in line with our previous study analysing treatment effects of 50 mg/kg CBD (Watt et al. 2020).

In conclusion, this is the first study evaluating the effect of high dose CBD on behavioural impairments and AD-relevant neuropathology in 7.5-month-old *APPxPS1* male mice. CBD restored a social recognition memory deficit and *APPxPS1* males demonstrated lower hippocampal levels of TNF-α and IL-1β and elevated cortical levels of proBDNF (the latter being decreased by CBD in WT controls). This study demonstrates the therapeutic potential of CBD to reverse cognitive deficits in 7.5-month-old *APPxPS1* males. However, the underlying mechanisms for this effect remain elusive and need further investigations.

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Chapter 4: Behavioural characterisation of TAU58/2 transgenic males

Novel behavioural characteristics of male human P301S mutant tau transgenic mice – a model for tauopathy

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Georgia Watt

4.1 Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterised by progressive cognitive decline and the accumulation of two hallmark proteins, amyloid-β (Aβ) and tau. Traditionally, transgenic mouse models of AD have generally focused on Aβ pathology, however, in recent years a number of tauopathy transgenic mouse models have been developed, including the TAU58/2 mouse model. These mice develop tau pathology and neurofibrillary tangles from 2 months of age and show motor impairments and alterations in the behavioural response to elevated plus maze testing. The cognitive and social phenotype of this model has not yet been assessed comprehensively. Furthermore, the behavioural changes seen in the elevated plus maze have previously been linked to both anxiety and disinhibitory phenotypes. Thus, this study assessed 4-month-old TAU58/2 males comprehensively for disinhibitory and social behaviours, social recognition memory, and sensorimotor gating. TAU58/2 males demonstrated reduced exploration and anxiety-like behaviours but no changes to disinhibitory behaviours, reduced sociability in the social preference test (SPT) and impaired acoustic startle and prepulse inhibition. Aggressive and socio-positive behaviours were not affected. Our study identified new phenotypic characteristics of young adult male TAU58/2 transgenic mice and clarified the nature of changes detected in the behavioural response of these mice to elevated plus maze testing. Social withdrawal and inappropriate social behaviours are common symptoms in both AD and frontotemporal dementia (FTD) patients and impaired sensorimotor gating is seen in moderate-late stage AD, emphasising the relevance of the TAU58/2 model to these diseases.

Keywords: TAU58/2 transgenic mouse; animal model; Alzheimer's disease; behaviour; tau pathology

4.2 Introduction

Alzheimer's disease (AD) is the most common form of dementia. It affects over 35 million people worldwide and this number is expected to reach over 115 million by the year 2050 (Alzheimer's Association 2018). AD is most commonly associated with progressive cognitive decline, which initially starts with short-term memory loss that progresses to cause a global disruption of cognitive ability (Alzheimer's Association 2018). The disease also causes an array of other behavioural symptoms including social withdrawal, aggression, mood disruptions, agitation and motor impairments (Alzheimer's Association 2018; Buchman and Bennett 2011). Pathologically, there are two hallmark features central to AD, these are the accumulation of the amyloid-β (Aβ) protein into senile plaques and the hyperphosphorylation of the tau protein into neurofibrillary tangles (NFTs). These two features are thought to be central to the neurodegenerative cascade associated with AD (Chapman et al. 2001).

In the last couple of decades, the role of tau in AD has been emphasised as tau pathology appears to correlate more closely with cognitive decline than Aβ pathology (Bejanin et al. 2017). The tau protein binds to microtubules and regulates microtubule assembly and stabilisation as well as intracellular transport of organelles (e.g. mitochondria) (Medina and Avila 2014). In AD, the hyperphosphorylation of tau into NFTs causes microtubules to destabilise and disrupts cellular transport, eventually causing cell death (Medina and Avila 2014). The discovery of the association between the *microtubule associated protein tau* (*MAPT)* gene and frontotemporal dementia (FTD) lead to the development of a number of transgenic tau mouse models (Götz et al. 2007), one of which is the TAU58/2 transgenic mouse model.

The TAU58/2 mouse model has previously been reported as a model for FTD as the *MAPT* gene is a risk factor for this disease (Przybyla et al. 2016; Van Der Jeugd et al. 2016; van Ersel et al.

2015). However, recent studies have reported that polymorphisms in *MAPT* are risk factors for AD as well (Zhou and Wang 2017). TAU58/2 transgenic mice express neuronal *P301S* mutant tau under the control of the mouse Thy1.2 promotor (previously described by (van Ersel et al. 2015)) and develop tau and NFT pathology from 2 months of age. This pathology is evident in the hippocampus, cortex, brain stem and amygdala and progresses with age (Przybyla et al. 2016; Van Der Jeugd et al. 2016), similar to the progression of tau pathology in AD (Chong et al. 2018; Maruyama et al. 2013). Previous studies have reported that these mice demonstrate behavioural changes including motor impairments, increased time spent in the open arms of the elevated plus maze (EPM) and increased distance travelled and time spent in light zone in aged TAU58/2 mice in light-dark test, which could indicate reduced anxiety-like behaviours or a disinhibitory-like phenotype (Przybyla et al. 2016; Van Der Jeugd et al. 2016; van Ersel et al. 2015). The EPM findings have been linked to a potential disinhibitory phenotype, but paradigms more directly testing disinhibitory behaviours had not been considered. Disinhibition is often used as an umbrella term referring to various behaviours of inhibitory dysfunction or behavioural undercontrol, including impulsivity, risk taking behaviour, poor risk assessment and disregard for social norms (Reynolds et al. 2013). Importantly, it is seen in both AD and FTD patients (Silveri 2007). In addition, the cognitive performance of these mice as well as their social and aggressive behaviours have not been assessed in any detail. Thus, the present study characterised two test cohorts of 4-month-old male TAU58/2 transgenic mice and their wild type-like littermates in elevated plus maze, cliff avoidance, step-down passive avoidance, social preference test (SPT), social interaction, Resident-Intruder, social dominance and prepulse inhibition. This study will extend the functional evaluation of the TAU58/2 mouse model to novel behavioural domains and increase its validity for research into FTD and AD.

4.3 Experimental Procedures

4.3.1 Animals

Male TAU58/2 transgenic mice expressing the human *0N4R* tau isoform with the *P301S* mutation under the control of the mouse Thy1.2 promoter on a C57BL/6J background, as previously described (Przybyla et al. 2016; van Ersel et al. 2015), and their wild type-like control littermates (WT) were bred and group housed in independently ventilated cages (Airlaw, Smithfield, Australia) at the Australian BioResources (ABR: Moss Vale, Australia). Test mice were transported to the animal facilities of the School of Medicine, Western Sydney University, at around 10 weeks of age, where they were group-housed in independently ventilated cages (GM500 Tecniplast Australia, NSW, Australia) with corn cob bedding (Tecniplast Australia, NSW, Australia), crinkle cut (Crink-l'Nest, Kraft) and tissues for nesting. Mice were kept in a 12:12 h light:dark schedule [light phase = white light (illumination: 124 lx) - dark phase = red light (illumination: < 2 lx)]. Food (Rat & Mouse Pellets, Gordon's Specialty Stockfeeds Pty Ltd., NSW, Australia) and water were provided *ad libitum*. When handling TAU58/2 mice it became apparent that transgenic mice did not habituate to the experimenter and at times attempted to jump off the researchers' hand whereas WT control mice were calm and easy to handle after an initial handling period. Adult, male A/JArc mice were purchased from the Animal Resources Centre (Canning Vale, Australia) and were used as standard opponents in the social interaction test, Resident-Intruder paradigm and SPT. Adult, male C57BL/6JAbr mice from ABR were used as standard opponents in the social dominance tube task. All research and animal care procedures were approved by the Western Sydney University Animal Care and Ethics Committee (A11335) and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.3.2 Behavioural testing

Four-month-old male TAU58*/2* and WT mice (total number of mice: N = 54) were tested comprehensively in disease-relevant behavioural domains. Two test cohorts were used (Cohort 1: WT n = 14; TAU58/2 = 20, Cohort 2: WT n = 14; TAU58/2 n = 16) to reduce the effect of repeated testing (for test order and age see Table 1). An inter-test interval of at least 48 h was guaranteed between any two test paradigms. All tests were conducted during the first 5 h of the light phase to minimise circadian rhythm effects on test performance. Equipment and apparatus were cleaned between trials using 80% ethanol.

	Age [days]
Test Cohort 1	
Elevated plus maze	120 ± 1
Social interaction	121 ± 1
Social dominance tube task	122 ± 1
Cliff avoidance	123 ± 1
Step-down passive avoidance (2 s shock duration)	124 ± 1
Resident-Intruder paradigm	128 ± 1
Test Cohort 2	
Social preference test	127 ± 1
Step-down passive avoidance (1 s shock duration)	130 ± 1
Prepulse inhibition	134 ± 2

Table 1 – *Test biography and test age.* Age [days] of male TAU58/2 transgenic mice and non-transgenic WT control littermates throughout behavioural testing (cohort 1: WT n = 14; TAU58/2 n = 20, cohort 2: WT n = 14; TAU58/2 n = 16). Ages are presented as mean ± SEM.

4.3.2.1 Elevated plus maze (EPM)

The EPM was used to investigate anxiety by utilising the natural conflict of mice to explore a novel environment and avoid brightly lit, elevated open areas (Lister 1987). The EPM apparatus consisted of a grey PVC '+' maze that consisted of two open arms **(**30.5 cm x 6.5 cm, no sidewalls) and two closed arms (30.5 cm x 6.5 cm, sidewall height 18.5 cm) with a central platform connecting the arms (6 cm x 6 cm). It was elevated 40 cm above the floor. Mice were placed individually in the centre of the EPM, facing an enclosed arm and allowed to explore freely for 5 min. ANY-maze™ (Stoelting, Wood Dale, USA) tracking software was used to record time spent and distance travelled in open and enclosed arms.

4.3.2.2 Social interaction (SI)

The SI was used to test for social interaction and social withdrawal (File and Seth 2003; Rung et al. 2005). Individual test mice and age-matched male A/J standard opponents were placed in opposite corners of a grey Perspex™ arena (35 x 35 x 30 cm) and allowed to interact freely for 10 min. The duration and frequency of socio-positive behaviours *nosing, anogenital sniffing, allogrooming rearing, crawling* and *following* and duration of time spent interacting (all behaviours combined) were recorded manually using ANY-maze™.

4.3.2.3 Social dominance tube test

The custom-made social dominance test was used to assess non-territorial dominance (Lijam et al. 1997). One test mouse and an aged-matched C57BL/6J control mouse were placed in opposite ends of a clear Plexiglas™ tube (diameter: 4 cm; length: 30.5 cm) with a metal divider in the middle for a maximum of 60 s. When both mice had reached the centre facing one another, the

divider was removed. The test was carried out until one mouse forced the other mouse to back out of the test tube, in which case the former was defined as the dominant mouse and the latter the subordinate mouse. The percentage of dominant mice in each genotype was calculated and the latency for a mouse to force the opponent out of the tube was recorded with a stopwatch. Test mice that were subordinate were given a latency of 60 s.

4.3.2.4 Social preference test (SPT)

The custom-made SPT was used to assess sociability and social recognition memory (Moy et al. 2004). The SPT apparatus consisted of a clear plastic three-chamber apparatus containing corn cob bedding, with a centre chamber (9 cm x 18 cm x 20 cm) and two outer chambers (16 cm x 18 cm x 20 cm). In each outer chamber was a mouse enclosure (diameter: 7 cm; height: 15 cm; bars spaced: 0.5 cm). Prior to the start of testing, test mice were isolated for 1 h. During the habituation trial, test mice were allowed to freely explore the apparatus for 5 min. In the sociability trial, an unfamiliar standard opponent (adult, male A/J mouse) was placed in one of the mouse enclosures in the outer chambers and the test mouse was allowed to explore all three chambers and the enclosures (one containing the A/J mouse) for 10 min. In the social novelty trial, a second unfamiliar standard opponent was placed in the previously empty mouse enclosure and the standard opponent from the sociability trial was kept. The test mouse was returned to the apparatus and allowed to explore all three chambers and the two enclosures containing A/J mice (novel and familial) for 10 min. The inter-trial interval (ITI) was 5 min and chambers and enclosures were cleaned with 80% ethanol between trials. Fresh corn cob bedding was added to the chambers prior to each new test mouse. Time spent in chambers and time spent *nosing* the enclosures were recorded by ANY-maze™ tracking software.

4.3.2.5 Resident-Intruder paradigm (RI)

The RI was used to assess territorial aggression (Brain and Poole 1976). The test mouse was isolated in their home cage for 30 min prior to the start of testing. An age-matched A/J mouse was used as a standard opponent. At the start of the test, test mouse and standard opponent were placed simultaneously in opposite corners of the home cage. The mice were then allowed to freely interact for one 10 min trial. The duration and frequency of agonistic behaviours including *tail rattling, aggressive grooming* and *fighting* and socio-positive behaviours like *anogenital sniffing, sniffing* and *following* and duration of time spent interacting (all behaviours combined) were recorded manually using ANY-maze™ tracking software. In cases where mice did not show a behaviour, a latency of 600 s was given (i.e. cut-off time for testing). Testing was stopped when escalated fighting occurred (>10 bites or fighting for longer than >10s) to avoid injuries (excluded mice: WT $n = 1$; TAU58/2 $n = 1$).

4.3.2.6 Cliff avoidance

The cliff avoidance paradigm was used to assess disinhibitory behaviour (Matsuoka et al. 2005). The custom-made apparatus consisted of a clear plastic cylinder with a flat top (diameter: 16 cm; height: 25 cm). Bedding surrounded the base. The test mouse was placed in the centre of the cylinder and latency to jump down [s] was recorded. The number of *head-dips* and *forepaws off the edge* [n] (where mice hold on with hind paws and reach forepaws off the edge towards the ground) were also recorded manually. The test was stopped after 7 min.

4.3.2.7 Step-down passive avoidance test

The step-down passive avoidance assesses disinhibitory behaviours (Prado et al. 2006). The apparatus consisted of a fear conditioning chamber (Med Associates, St. Albans, VT, USA) (29.5 cm x 24.5 cm x 21 cm) which contained a custom-made Perspex^{TM} elevated platform (7 cm x 7 cm x 6 cm). The test consisted of three 5 min trials. In trial 1, the test mouse was placed on the platform and the latency to step down was recorded [s], with 'stepping down' defined as all four paws off the platform. Once the mouse had stepped down, it immediately received a 0.4 mA foot shock. This test was run twice, once in each test cohort, using two different durations for the electric foot shock (2 s duration for the first cohort and 1 s duration for the second cohort). Only data from cohort 2 will be discussed in detail, as a ceiling effect was seen in the first cohort (i.e. no mouse stepped down on trial 2). After shock delivery, the mouse was removed from the chamber. Trial 2 occurred after a 1.5 h ITI: the mouse was placed on the platform again and the latency to step down was recorded [s]. A cut-off time of 5 min was used in cases where the mouse did not step down. The number of *head-dips* was recorded manually, and *freezing* was recorded for trial 2 and trial 3 using Video Freeze ®, Med Associates Inc. software (freezing threshold = 15; detection method = linear; minimum freezing duration = 30 frames). Trial 3 occurred 24 h after trial 1 and was identical to trial 2. One WT mouse was excluded from the test because it jumped off the platform as soon as it was placed into the apparatus.

4.3.2.8 Prepulse inhibition (PPI)

PPI was used to assess the acoustic startle response (ASR) and sensorimotor gating of test mice (Wang et al. 2012). The apparatus consisted of Plexiglas^{TM} mouse enclosures in startle chambers (SR-Lab, San Diego Instruments, San Diego, USA). The test consisted of three days of habituation,

where mice were placed into the enclosures before being transferred into the apparatus for 5 min with a constant background noise (70 dB). On the test day, one 30 min trial was run, which consisted of a 5 min acclimatisation period with a 70 dB background noise, followed by 97 trials in a pseudorandomised order: 5 x 70 dB trials (background); 5 x 80 dB trials; 5 x 100 dB trials; 15 x 120 dB trials (startle) and 3 sets of prepulse trials using either 74, 82 or 86 dB prepulses presented either 32, 64, 128 or 256 ms [variable interstimulus (prepulse-pulse) interval; ISI] prior to a startle pulse of 120 dB (PPI response). The ITI between individual PPI trials varied randomly from 10 - 20 s. The startle response to each trial was calculated as the average mean amplitude detected by the accelerometer and was calculated as the mean amplitude to all 120dB startle trials (ASR). We also analysed Tmax, defined as the mean latency to maximum startle response, (i.e. latency until mice show their maximum startle response post stimulus presentation). Percentage PPI (% PPI) was calculated as [(mean startle response (120 dB) - PPI response)/mean startle response (120 dB)] x 100. % PPI was averaged across ISI's to produce a mean % PPI for each prepulse intensity.

4.3.3 Statistical analysis

Data of the two test cohorts were analysed using one-way analysis of variance (ANOVA) to investigate the main effects of 'genotype' or two-way repeated measures (RM) ANOVA to include effects of 'chamber' (SPT), 'trial' (step-down passive avoidance), 'startle pulse intensity' and 'prepulse intensity' (both PPI). Performance in SPT was also assessed using a one sample *t*-test to investigate if time spent in the chambers was greater than chance levels (50%). Chi square test was used to assess 'step downs' (cliff avoidance test) and 'wins' (social dominance tube task). Differences were regarded as significant if $p < 0.05$. F-values and degrees of freedom are presented for ANOVAs and significant RM ANOVA effects are reported by '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^p

< 0.001). Significant genotype effects are shown in figures and tables by '*' (*p < 0.05, **p < 0.01; ***p < 0.001). Data are shown as means ± standard error of means (SEM) and analyses were conducted using SPSS 25 for Mac.

4.4 Results

4.4.1 Disinhibition/Anxiety Behaviours

4.4.1.1 Elevated plus maze (EPM)

One-way ANOVA for total distance travelled in the EPM revealed no difference between TAU58/2 mice and WT controls [$F(1,32) = 0.642$, $p = 0.429$] (Table 2). However, TAU58/2 mice explored the open arms significantly more compared to WT control littermates as percentage time spent and percentage distance travelled in open arms [time: $F(1,32) = 19.517$, $p < 0.0001$; distance: $F(1,32) = 13.539$, $p = 0.001$] were elevated in transgenic mice (Table 2).

	WT	TAU58/2
Total distance travelled [m]	10 ± 0	9 ± 1
Distance travelled in open arms [%]	10 ± 4	33 ± 4 ***
Time spent in open arms [%]	16 ± 4	46 ± 5 ***

Table 2 – *Locomotion in the EPM.* Locomotion and anxiety-related behaviours in the EPM for male TAU58/2 transgenic and non-transgenic WT control littermates (WT $n = 14$; TAU58/2 $n = 20$). Data are presented as mean ± SEM. 'Genotype' effects are indicated with '*' (***$p < 0.0001$).

4.4.1.2 Cliff avoidance

One-way ANOVA for latency to jump [$F(1,32) = 0.567$, $p = 0.457$] and Chi-square test for the percentage of mice that jumped [i.e. 80% of TAU58/2 males and 57% of WT mice: $\chi^2 = 1.44$, n = 34, d.f. = 2, $p = 0.150$] revealed no differences between TAU58/2 males and WT (Table 3). Interestingly, TAU58/2 mice exhibited significantly less *head-dipping* [$F(1,32) = 15.168$, $p < 0.0001$] and less *forepaws off the edge* compared to WT controls [$F(1,32) = 4.890$, $p = 0.034$] (Table 3).

	WT	TAU58/2
Cliff avoidance		
Latency to jump [s]	257 ± 47	209 ± 33
Number of h*ead-dips* [n]	55 ± 9	22 ± 3 ***
Number of *forepaws off the edge* [n]	1 ± 0	0 ± 0 *
Step down passive avoidance – 2 s shock duration (experiment 1)		
Latency to step-down in trial 1 [s]	116 ± 23	184 ± 31
Latency to step-down in trial 2 [s]	300	271 ± 20
Latency to step-down in trial 3 [s]	300	300
Number of *head-dips* in trial 1 [n]	20 ± 2	21 ± 3
Number of *head-dips* in trial 2 [n]	10 ± 2	8 ± 1
Number of *head-dips* in trial 3 [n]	3 ± 1	4 ± 1
Time spent *freezing* in trial 2 [s]	167 ± 12	114 ± 10**
Time spent *freezing* in trial 3 [s]	204 ± 7	167 ± 11*
Step down passive avoidance – 1 s shock duration (experiment 2)		
Latency to step-down in trial 1 [s]	59 ± 13	35 ± 11
Latency to step-down in trial 2 [s]	140 ± 34	139 ± 32
Latency to step-down in trial 3 [s]	188 ± 38	158 ± 34
Number of *head-dips* in trial 1 [n]	16 ± 3	9 ± 2*
Number of *head-dips* in trial 2 [n]	9 ± 2	9 ± 2
Number of *head-dips* in trial 3 [n]	9 ± 2	10 ± 3

Time spent *freezing* in trial 2 [s]	81 ± 21	71 ± 22
Time spent *freezing* in trial 3 [s]	108 ± 25	82 ± 25

Table 3 – *Cliff avoidance and step-down passive avoidance behaviours.* Disinhibitory behaviours in cliff avoidance and step-down passive avoidance (Experiment 1 and 2, foot shocks of 2 s and 1 s duration, respectively) as well as and *freezing* behaviour (step-down passive avoidance only) for male TAU58/2 transgenic and non-transgenic WT control littermates (cliff avoidance: WT $n = 14$; TAU58/2 $n = 20$ - step-down passive avoidance, experiment 1: WT $n = 14$; TAU58/2 $n = 20$; experiment 2: WT $n = 14$; TAU58/2 $n = 16$). Data are presented as mean ± SEM. Effects of 'genotype' are indicated by '*' (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$).

4.4.1.3 Step-down passive avoidance

In experiment 1 (using a 2 s shock duration), there was no genotype difference in latency to step down in trials 1 and 2 [$F(1,32) = 2.628$, $p = 0.115$; trial 2: $F(1,32) = 1.462$, $p = 0.235$]. Furthermore, no animals stepped of the platform in the third trial (Table 3). There was no difference between genotypes in *head-dipping* in any trial either [all p's > 0.05] (Table 3). Interestingly, TAU58/2 males exhibited significantly less *freezing* in trials 2 and 3 compared to WT controls [trial 2: $F(1,32) = 12.155$ $p = 0.001$; trial 3: $F(1,32) = 6.201$, $p = 0.018$] (Table 3). In experiment 2 (using a 1 s shock duration), the latency to step down was similar between genotypes in each of the three trials [trial 1: $F(1,28) = 2.113$, $p = 0.157$; trial 2: $F(1,28) = 0$, $p = 0.992$; trial 3: $F(1,28) = 0.342$, $p = 0.563$] (Table 3). In line with cliff avoidance testing, TAU58/2 were less explorative (i.e. frequency of *head-dipping*) compared to control males in the first trial of the second experiment [$F(1,28) = 4.459$, $p = 0.04$]. There was no difference between genotypes in time spent *freezing* in either trial [all p's > 0.05] (Table 3).

4.4.2 Social Behaviours

4.4.2.1 Social interaction (SI)

One-way ANOVA for total time spent interacting with the standard opponent (i.e. active social interaction time) revealed no difference between the TAU58/2 and WT control mice [$F(1,31) = 0.137$, $p = 0.713$] (Figure 1A).

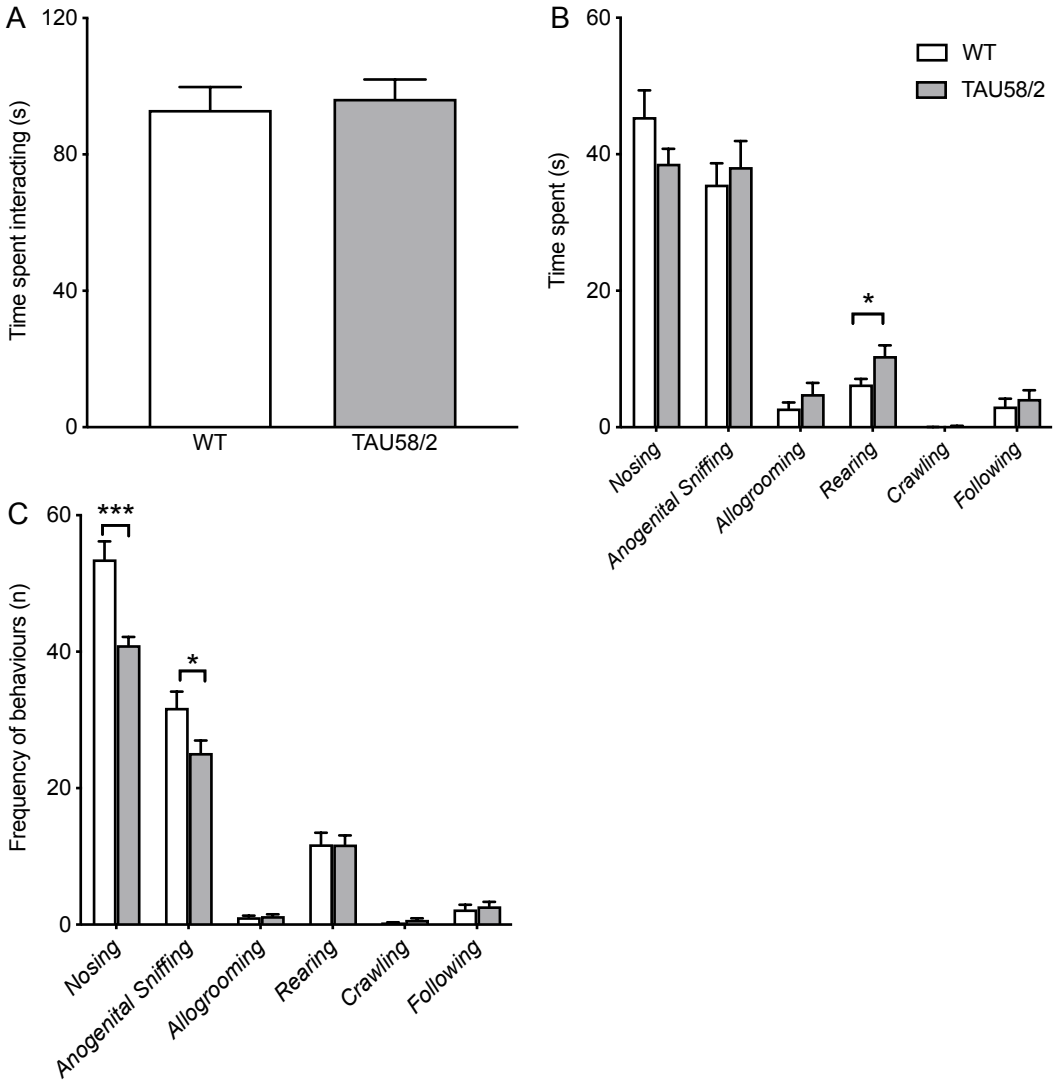

Figure 1A-C: *Sociability in SI.* A) Total active social interaction time [s], **B)** time spent [s] and **C)** frequency [n] of *nosing, anogenital sniffing, allogrooming, rearing, crawling* and *following* for TAU58/2 transgenic mice and non-transgenic WT control littermates. Data are presented as means ± SEM. 'Genotype' effects are presented as '*' (*$p < 0.05$ and ***$p < 0.0001$).

However, more detailed quantitative analysis of individual behaviours revealed that TAU58/2 mice spent significantly more time *rearing* on the A/J mouse compared to WT controls [$F(1,31) = 4.241$, $p = 0.048$] (Figure 1B) whereas the frequencies of *nosing* [$F(1,31) = 22.942$, $p < 0.0001$] and *anogenital sniffing* [$F(1,31) = 4.969$, $p = 0.033$] were reduced (Figure 1C). There were no other differences in regard to duration or frequency of any other behaviours recorded (all p's > 0.05; Figures 1B-C).

4.4.2.2 Social dominance

The Chi square test for percentage 'wins' revealed no difference between TAU58/2 (65%) and WT controls (57%) [$\chi^2 = 1.022$, n = 34, $p = 0.3067$]. One-way ANOVA for latency to force the opponent mouse out of the tube confirmed that there was no difference between genotypes [WT = 35 ± 6; TAU58/2 = 37 ± 6 - $F(1,31) = 33.025$, $p = 0.818$], indicating TAU58/2 males reach control-equivalent levels of social dominance in pairwise encounters.

4.4.2.3 Social preference test (SPT)

Sociability*:* One sample *t*-tests for percentage time in the mouse chamber to clarify preference levels for a mouse over an empty chamber revealed that only WT mice showed an above chance preference for the mouse chamber (WT: $t(14) = 3.714$, $p = 0.002$; TAU58/2: $t(5) = 1.471$, $p = 0.162$) (Figure 2A). Similarly, one sample *t*-tests for the percentage time spent *nosing* the mouse enclosure revealed that only WT mice showed an above chance preference for *nosing* the mouse enclosure (WT: $t(12) = 2.794$, $p = 0.014$) whereas TAU58/2 transgenic mice failed to develop a preference for the mouse enclosure over the empty enclosure (TAU58/2: $t(15) = 1.009$, $p = 0.329$)

(Figure 2B). In line with this we also found a trend for a 'chamber' by 'genotype' interaction when using a two-way RM ANOVA [$F(1,29) = 3.6$, $p < 0.07$] (data not shown).

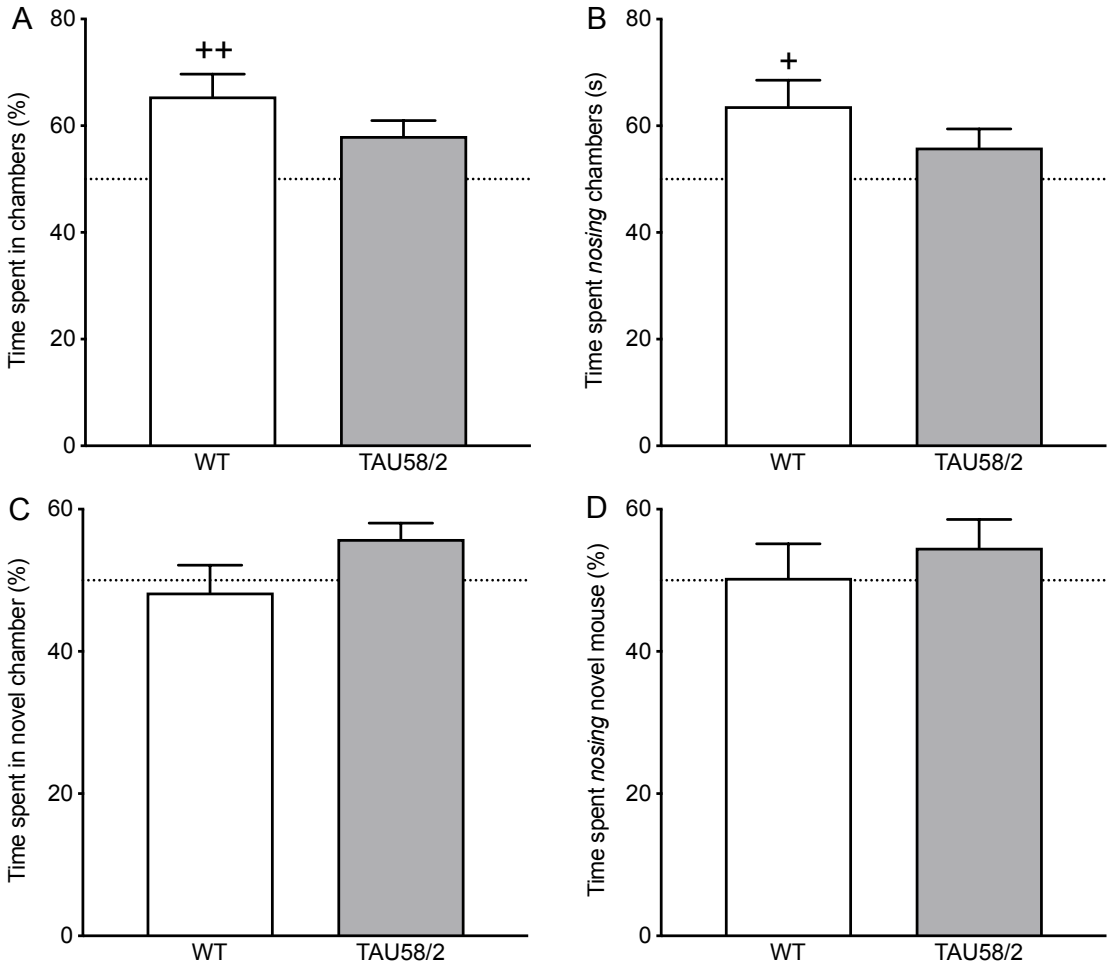

Figure 2A-D: ***Sociability and social recognition in the SPT.*** Time spent [%] **A)** in the mouse chamber, **B)** *nosing* the mouse enclosure, **C)** in the novel mouse chamber and **D)** *nosing* the novel mouse enclosure for TAU58/2 transgenic mice and non-transgenic WT control littermates. Data are presented as means ± SEM. *t*-test results are presented as '+' ($^{+}p < 0.05$ and $^{++}p < 0.01$).

Social recognition memory: One sample *t*-tests for the percentage time spent in the novel chamber (i.e. containing the novel mouse) and percentage time *nosing* the novel chamber to clarify

preference levels for the novel mouse over a familiar mouse revealed neither group showed a preference for the novel mouse above chance levels (all p's > 0.05; Figure 2C-D).

4.4.2.4 Territorial aggression (Resident-Intruder paradigm)

One-way ANOVA for time spent interacting with the standard opponent mouse (i.e. sum of all social behaviours shown) revealed a trend for a 'genotype' effect [$F(1,30) = 3.021$, $p = 0.092$], where TAU58/2 mice trended to interact less (Table 4).

	WT	TAU58/2
Time spent exhibiting behaviour		
Interacting [s]	113 ± 16	88 ± 6$^{\#}$
Wrestling [s]	6 ± 4	4 ± 2
Tail rattling [s]	2 ± 1	2 ± 1
Aggressive grooming [s]	7 ± 4	2 ± 1
Frequency exhibiting behaviour		
Wrestling [n]	4 ± 3	3 ± 1
Tail rattling [n]	2 ± 1	2 ± 1
Aggressive grooming [n]	4 ± 3	2 ± 1

Table 4 – *Territorial aggression in the RI.* Territorial aggression in the Resident-Intruder test for male TAU58/2 transgenic and non-transgenic WT control littermates (WT $n = 14$; TAU58/2 $n = 20$). Data are presented as mean ± SEM. $^{\#}p = 0.092$ indicates a trend for a 'genotype' effect.

In line with the social interaction findings, time of sociopositive behaviours such as *nosing* and *following* were decreased in TAU58/2 mice compared to WT controls (data not shown). There

was no difference in time spent or frequency of aggressive behaviours such as *wrestling*, *tail rattling* and *aggressive grooming* [all p's > 0.05 – Table 4].

4.4.3 Sensorimotor gating

4.4.3.1 Acoustic startle response (ASR)

RM ANOVA for acoustic startle response revealed a significant effect of 'startle pulse intensity' [$F(2,58) = 29.229$, $p < 0.0001$] on ASR in all mice with 120 dB generating the highest startle response. Importantly, there was an interaction between 'startle pulse intensity' and 'genotype', where TAU58/2 startled significantly less than WT controls with increasing startle pulse intensities [$F(2,58) = 13.417$, $p < 0.0001$] (Figure 3A). One-way ANOVA split by 'startle pulse intensity' confirmed an effect of 'genotype' at 100 dB and 120 dB [100 dB: $F(1,29) = 32.865$, $p < 0.0001$; 120 dB: $F(1,29) = 18.742$, $p < 0.0001$] but not at 70 dB [i.e. background noise: $F(1,29) = 1.811$, $p = 0.189$]. Furthermore, one-way ANOVA for Tmax revealed a main effect of 'genotype' at 100 dB and 120 dB [100dB: $F(1,29) = 40.443$, $p > 0.0001$ – 120 dB: $F(1,29) = 154.201$, $p = 0.0001$], where Tmax was longer in TAU58/2 males than control animals (Figure 3B).

Prepulse inhibition (PPI)

RM ANOVA for percentage prepulse inhibition revealed an effect of 'prepulse intensity' [$F(2,58) = 51.355$, $p < 0.0001$], where all mice startled more with increasing prepulse intensities. This effect was not affected by genotype [no 'prepulse intensity' x 'genotype' interaction: $F(2,58) = 0.039$, $p = 0.962$]. Importantly, there was an overall 'genotype' effect [$F(1,29) = 18.458$, $p < 0.0001$] with TAU58/2 males showing less prepulse inhibition than WT controls. One-way ANOVAs split by prepulse intensities revealed a 'genotype' effect at all prepulse intensities [74 dB: $F(1,29) = 10.663$,

$p = 0.003$; 82 dB: $F(1,29) = 19.382$, $p < 0.0001$; 86 dB: $F(1,29) = 20.366$, $p < 0.0001$], indicating impaired sensorimotor gating in the TAU58/2 mice compared to WT controls (Figure 3C).

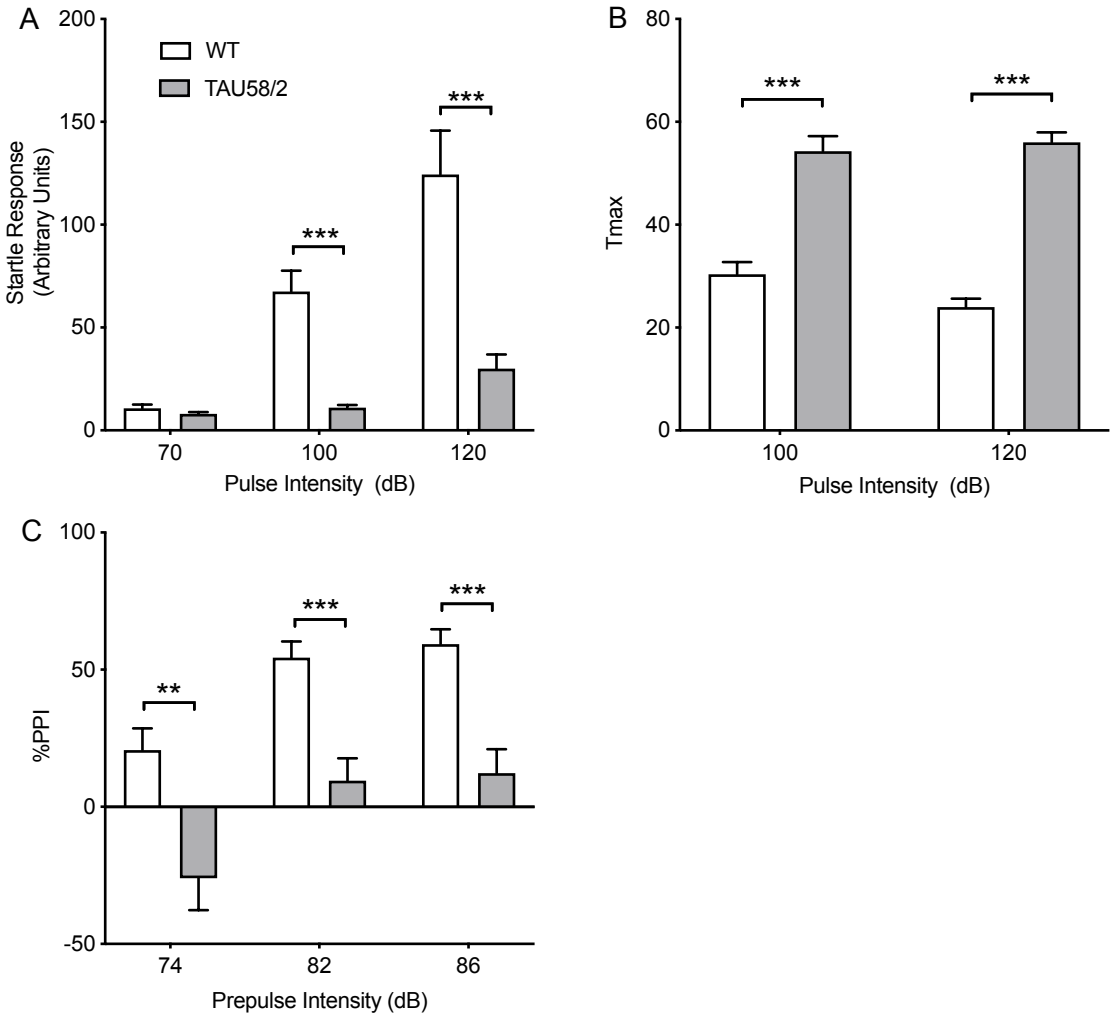

Figure 3A-C: ***ASR and PPI*****. A)** Average startle response to 70, 100 and 120 dB acoustic startle stimuli [arbitrary units], **B)** average Tmax during acoustic startle response trial to 100 and 120 dB acoustic startle stimuli and **C)** percentage prepulse inhibition [%] to 74, 82, 86 dB (averaged across ISIs) for TAU58/2 transgenic mice and non-transgenic WT control littermates. Data are presented as means ± SEM. Genotype effects per startle pulse intensity are presented as '*' (**$p < 0.01$ and ***$p < 0.001$).

4.5 Discussion

This study demonstrated that 4-month-old male TAU58/2 transgenic mice display an anxiolytic-like rather than a disinhibitory phenotype, exhibit reduced sociability, and show impaired acoustic startle response and prepulse inhibition. Social dominance and territorial aggression were intact in this mouse model for tauopathy.

The study found that TAU58/2 males demonstrated reduced anxiety in the EPM as they spent more time and travelled further in the open arms compared to control mice. This supports a previous study which have shown that TAU58/2 males spend significantly more time in the open arms compared to WT controls (Przybyla et al. 2016). Previously, this finding was interpreted as a potential disinhibitory phenotype (Przybyla et al. 2016), similar to what other studies on various tauopathy transgenic mouse models (e.g. for mutations such as *P301S* and *P301L*) have discussed when assessing EPM or open field (OF) phenotypes. These studies reported that tauopathy transgenic models spent more time in and were faster to enter the aversive zones of these test paradigms compared to WT controls – importantly, these changes to anxiety-related behaviours were not seen at all ages investigated (Dumont et al. 2011; Pennanen et al. 2006; Van Der Jeugd et al. 2016). However, the EPM (and to a degree also the OF) is most commonly used to assess anxiety in rodent models (Lister 1987; Sachs et al. 2013; Van Gaalen and Steckler 2000) . Thus, we assessed potential disinhibition behaviours in TAU58/2 mice further, using more specific paradigms including cliff avoidance and step-down passive avoidance (Sachs et al. 2013). Both tests revealed no differences between TAU58/2 males and WT controls. Thus, studies assessing disinhibition behaviours using EPM and OF should be followed up using disinhibition-specific test paradigms to confirm their initial findings.

Interestingly, TAU58/2 males exhibited significantly less *head-dips* and less *forepaws off the edge* than WT controls in the cliff avoidance test. TAU58/2 also showed reduced levels of *head-dipping* in the first trial of the step-down passive avoidance task. This suggests that TAU58/2 males may display reduced explorative tendencies. Interestingly, lower exploratory drive appears to be specific to spatio-temporal paradigms as TAU58/2 transgenic mice spent more time *rearing* compared to WT controls in social test settings like the social interaction and Resident-Intruder tests.

The step-down passive avoidance paradigm also assesses fear-associated memory (measured by assessing *freezing* behaviour across trials). In experiment 1 where a longer foot shock duration was utilised, TAU58/2 males *froze* significantly less 2 h and 24 h post shock delivery compared to control littermates. Interestingly, this reduction in *freezing* was not seen when presenting a 1 s shock. This could suggest that fear-associated memory is impaired in the TAU58/2 when presented with a more pronounced unconditioned stimulus. It could also suggest that fear extinction in the TAU58/2 males is stronger, however, this was not specifically tested in this step-down test paradigm.

TAU58/2 transgenic males displayed reduced sociability compared to WT controls. Similarly, and although overall active social interaction time was not affected by genotype, TAU58/2 males displayed less bouts of *sniffing* and *anogenital sniffing* compared to WT controls in the social interaction test, however, time spent exhibiting these behaviours was not different between genotypes. This was also confirmed by data from the Resident-Intruder test, where TAU58/2 mice spent significantly less time *sniffing* the other mouse and also tended to spend less time following the standard opponent compared to WT controls. This supports previous findings where male and female Tau58-2/B transgenic mice demonstrated reduced social interaction from 6 months of age

(Van Der Jeugd et al. 2016). Social withdrawal is commonly seen in both AD and FTD, highlighting the clinical relevance of these findings (Alzheimer's Association 2018).

Social dominance and territorial aggression were not affected in male TAU58/2 mice. Few studies have looked at aggression in tauopathy mouse models, and our study is the first to look at aggression in the TAU58/2 model. One study in THY-Tau22 transgenic mice reported increased aggression in these mice (Van der Jeugd et al. 2013). Interestingly, aggression is seen more commonly in AD patients than FTD patients (Barber et al. 1995; Silveri 2007).

Disinhibition often presents in AD patients as increased aggression, hyperactivity and socially intrusive behaviour (Lesser and Hughes 2006). Our results indicate that these symptoms are not present in the TAU58/2 males at the age tested. However, disinhibition is more commonly seen in FTD than in AD (Snowden 2001; Zamboni et al. 2008). Furthermore, some of the phenotypic alterations we detected in the TAU58/2 transgenic mice are in line with AD, as social withdrawal is commonly seen in AD patients (Alzheimer's Association 2018) and loss of empathy and inappropriate social behaviours are commonly seen in FTD patients (Snowden 2001).

Testing recognition memory, neither control nor transgenic mice demonstrated a preference for the novel mouse over the familiar mouse in the social novelty preference trial (i.e. social recognition memory) although we have used the same test protocol successfully in the past for other AD transgenic mouse models (Cheng et al. 2014a; Cheng et al. 2014c). Thus, we cannot make any conclusions about potential social recognition memory deficits in TAU58/2 mice. Importantly, previous studies have reported that tau pathology and NFTs accumulate in the amygdala first and in a greater load compared to the cortex and hippocampus (Przybyla et al. 2016). Thus, cognitive deficits including social recognition memory may present at an older age in TAU58/2 males whereas the changes detected in anxiety-related behaviours are likely to be

mediated by the amygdala and therefore already evident at 4 months of age (i.e. the age at which our test mice were assessed). Future studies will be necessary to evaluate if such cognitive deficits do develop at an older age as hippocampal-dependent cognitive decline is commonly seen in AD and less prevalent in FTD (Silveri 2007).

Interestingly, TAU58/2 males demonstrated deficits in both acoustic ASR and PPI. They startled significantly less than the WT controls at 100 dB and 120 dB suggesting a hearing impairment in these mice. However, TAU58/2 males did startle more in response to the 120 dB pulse intensity compared to 100 dB, indicating that the mice were not completely hearing impaired. In addition, we reported that TAU58/2 males demonstrated a significantly longer latency for their maximum startle response compared to WT controls. TAU58/2 males also demonstrated lower %PPI compared to WT controls, indicating impaired sensorimotor gating. Sensorimotor processes are modulated by a number of brain regions relevant to AD, including the hippocampus (Lipska et al. 1995; Miller et al. 2010; Swerdlow et al. 2004), amygdala (Takeuchi et al. 2011) and prefrontal cortex (Swerdlow et al. 2001). In addition, the cholinergic system has been reported to be directly involved in sensorimotor gating (Hejl et al. 2004), as anti-cholinergic compounds have been shown to impair PPI, while cholinergic agonists enhance it (Kumari and Gray 1999). Patients with moderate to advanced stages of AD show impaired sensorimotor gating (Ally et al. 2007; Takeuchi et al. 2011; Ueki et al. 2006). Thus, our finding of impaired sensorimotor gating is in line with partial validity of this mouse model for AD.

It is important to note that differences in the baseline ASR may influence the %PPI read out used for assessing sensorimotor gating (Csomor et al. 2008; Yee et al. 2005). However, studies have also reported that when ASRs are distinct between groups and analysed as a covariate, the %PPI remained significant, suggesting that %PPI differences are not solely dependent on the ASR

(Csomor et al. 2008). Furthermore, an impaired ASR may rather reflect an alternative impairment outside of the sensorimotor gating system (O'Leary et al. 2017). Indeed, impaired ASR and %PPI have been detected in another mouse model for AD, i.e. the 5xFAD transgenic mouse model. These AD transgenic mice present with ASR deficiencies at 3-4 months of age but show no accompanying deficits in %PPI or peripheral hearing – the latter develops only from 7 months of age onwards suggesting that deficits in ASR and PPI can develop separately (O'Leary et al. 2017). Studies have also reported that impaired motor function (Brooks and Dunnett 2009) and low anxiety levels (Plappert and Pilz 2002) can reduce the ASR of mice, which are all evident in TAU58/2 transgenic mice. However, these impairments are also evident in the 5xFAD phenotype, but develop later (i.e. at 6-9 months of age) when PPI deficits are already present (O'Leary et al. 2017).

It is possible that the potential hearing impairment found in PPI could have influenced the findings in the sociability tests (i.e. SPT and Resident-Intruder). A number of studies have highlighted the crucial role ultrasonic voaclisations play in communication between mice (as reviewed in Portfors and Perkel 2014). Importantly, it has been demonstrated that male mice use ultrasonic vocalisations to determine social hierarchies as well as territorial boundaries (Chabout et al. 2012). Therefore, the inability to detect this communication in TAU58/2 males may confound our findings in social interaction and aggression tests. The potential hearing impairment in the TAU58/2 males was an unexpected finding and hearing, specifically, was not investigated in this study. Therefore, future research should be conducted to determine the extent of the hearing impairment in TAU58/2 males and the role that it plays in the reduced sociability phenotype identified in this study.

In conclusion, this study describes for the first time that 4-month-old TAU58/2 transgenic males show reduced sociability as well as impaired acoustic startle response and sensorimotor gating.

The experiments also clarify that this mouse model exhibits an anxiolytic-like rather than disinhibitory-like phenotype. Our results suggest that TAU58/2 males demonstrate dementia-relevant symptoms, including social withdrawal and impaired sensorimotor gating, however, further testing is necessary to evaluate cognitive deficits.

4.6 References

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Chapter 5: Effect of 50 mg/kg CBD on AD-relevant phenotypes of TAU58/2 transgenic mice

Evaluation of the behavioural effects of chronic cannabidiol (CBD) on 4-month-old male TAU58/2 transgenic mice

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5.1 Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterised by progressive cognitive decline, motor impairments, and accumulation of hallmark proteins, amyloid-beta (Aβ) and tau. Traditionally, transgenic mouse models for AD have focused on Aβ pathology, however, recently a number of tauopathy transgenic models have been developed, including the TAU58/2 transgenic model. Cannabidiol (CBD), a non-toxic constituent of the Cannabis *sativa* plant, has been shown to prevent and reverse cognitive deficits in Aβ transgenic mouse models of AD. Importantly, the therapeutic properties of CBD on the behavioural phenotype of tauopathy mouse models have not been investigated. We assessed the impact of chronic CBD treatment (i.e. 50 mg/kg CBD, intraperitoneal administration starting 3 weeks prior to behavioural assessments) on disease-relevant behaviours of 4-month-old TAU58/2 transgenic males in paradigms for anxiety, motor functions, and cognition. TAU58/2 transgenic males demonstrated reduced body weight, anxiety and impaired motor functions. Furthermore, they demonstrated increased *freezing* in fear conditioning compared to control animals. Interestingly, both sociability and social recognition memory were intact in AD transgenic mice. Chronic CBD treatment did not affect behavioural changes in transgenic males. In summary, 4-month-old TAU58/2 transgenic males exhibited no deficits in social recognition memory, suggesting that motor deficits and changes in anxiety at this age do not impact on social domains. The moderate increase in fear-associated memory needs further investigation but could be related to differences in fear extinction. Future investigations will need to clarify CBD's therapeutic potential for reversing motor deficits in TAU58/2 transgenic mice by considering alternative CBD treatment designs.

Keywords: Alzheimer's disease; cannabidiol; TAU58/2 transgenic mice; motor function; social recognition memory; fear-associated memory

5.2 Introduction

Alzheimer's disease (AD) is characterised by progressive cognitive decline which initially manifests as short-term memory loss. In the later stages of the disease there is a global disruption of cognitive ability as well as impairments in motor function and increased aggression and agitation (Alzheimer's Association 2018; Buchman and Bennett 2011). Neuropathologically, AD is characterised by two hallmark proteins, amyloid-β (Aβ) and tau. Aβ accumulates extracellularly into amyloid plaques and tau hyperphosphorylates intracellularly to form neurofibrillary tangles (NFTs) (Chong et al. 2018). Plaques and tangles are thought to be central to the neurodegenerative cascade which causes neuroinflammation and oxidative stress and contributes to neurodegeneration (Chapman et al. 2001). In the last couple of decades, the role of tau in AD has been emphasised as tau pathology appears to correlate more closely with cognitive decline than Aβ pathology (Bejanin et al. 2017).

The phytocannabinoid cannabidiol (CBD) is a non-toxic and non-psychotomimetic constituent of the *Cannabis sativa* plant and has been shown to be neuroprotective, anti-inflammatory and anti-oxidant (Iuvone et al. 2009). Importantly, CBD has been reported to reduce Aβ-induced neurotoxicity and tau hyperphosphorylation as well as promote hippocampal neurogenesis *in vitro* (Esposito et al. 2006a; Esposito et al. 2011; Iuvone et al. 2004). *In vivo*, the therapeutic effectiveness of purified CBD has only been established for AD transgenic mouse models for Aβ pathology. Our own studies discovered that chronic CBD treatment (oral or intraperitoneal (i.p.) administration, at a dose of 20 mg/kg) reversed and prevented the development of cognitive deficits in a transgenic mouse model for mutant *amyloid precursor protein* (*APP*) and *presenilin 1* (*PS1*) (Cheng et al. 2014a; Cheng et al. 2014c). A follow up study using the same mouse model confirmed that chronic administration of 50 mg/kg CBD reversed the development of cognitive

deficits and was associated with a moderate reduction of insoluble $A\beta_{40}$ (Watt et al., 2020 - accepted 29/1/2020). In addition, a study in this same model demonstrated that CBD-rich cannabis extract (0.75 mg/kg) reversed an object recognition memory deficit and reduced astrogliosis (Aso et al. 2014).

Importantly, the therapeutic effects of purified CBD have not yet been assessed in transgenic mouse models targeting tauopathy. TAU58/2 transgenic mice carry a mutation in the *microtubule associated protein tau* (*MAPT*) and polymorphisms in *MAPT* are a risk factor for AD (Zhou and Wang 2017). These mice have also been described as a model for frontotemporal dementia (FTD) (Przybyla et al. 2016; van Ersel et al. 2015). TAU58/2 transgenic mice express the human *04NR* tau isoform with a neuronal *P301S* mutation and present with tau and NFT pathology from 2 months of age onwards which progresses with age (Przybyla et al. 2016; van Ersel et al. 2015). TAU58/2 as well as TAU58-2/B transgenic mice (the latter being genetically similar to the TAU58/2 model but originate from an independent breeding colony at another institute) develop motor impairments and reduced spatio-temporal anxiety from 3 months of age onwards (Przybyla et al. 2016; Van Der Jeugd et al. 2016; van Ersel et al. 2015). The TAU58-2/B model shows impaired spatial memory in the Y-maze from 6 months of age (more pronounced in male mice) and deficient instrumental short-term memory in the puzzle box task. Furthermore, reduced sociability in a simple social interaction task has been detected (Van Der Jeugd et al. 2016). Social and cognitive domains have not yet been assessed in any detail in the TAU58/2 transgenic mouse model.

Social withdrawal and loss of facial recognition are common symptoms of AD (Alzheimer's Association 2018). Furthermore, AD patients have demonstrated motor impairments, including impaired gait and posture, bradykinesia and tremors (Wirths and Bayer 2008). We aimed to

investigate early stage AD as previous literature has suggested that early therapeutic intervention is more effective (Cummings et al. 2007) and TAU58/2 show behavioural changes from 2-3 months of age onwards. Thus, we tested novel behavioural domains including sociability, social recognition memory, as well as fear-associated memory in 4-month-old TAU58/2 transgenic males and evaluated the effects of chronic treatment with 50 mg/kg CBD on these domains as well as the established motor deficits and anxiolytic-like phenotype.

5.3 Experimental Procedures

5.3.1 Animals

Transgenic TAU58/2 mice express the human *0N4R* tau isoform with the *P301S* mutation under the control of the murine Thy1.2 promoter on a C57BL/6J background backcrossed over 10 generations as previously described (van Ersel et al. 2015). Male TAU58/2 mice (n = 26) and non-transgenic littermates [wild type-like (WT): n = 28] were bred and group housed in independently ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Australian BioResources (Moss Vale, Australia). Test mice were transported to the School of Medicine, Western Sydney University, at around 10 weeks of age, where they were group-housed in independently ventilated cages (GM500 Tecniplast Australia, NSW, Australia**)** with corn cob bedding (Tecniplast Australia, NSW, Australia), crinkle cut bedding (Crink-l'Nest, Kraft) and tissues for nesting. Mice were kept under a 12:12 h light/dark schedule [light phase between 0900 and 2100 hours with white light (illumination 124 lx) and dark phase with red light (illumination < 2 lx)]. Food (Rat & Mouse Pellets, Gordon's Specialty Stockfeeds Pty Ltd., NSW, Australia**)** and water were provided ad libitum. Adult, male A/JArc mice from Animal Resources Centre (Canning Vale, Australia) were used as standard opponents in the social preference test (SPT). Research and animal care procedures were approved by Western Sydney University Animal Care and Ethics Committee (#A11335) and are in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.3.2 Drug preparation and administration

Powdered CBD (CAS: 13956-29-1; THC Pharma GmbH, Frankfurt/Main, Germany) was dissolved in Tween80 and 100% ethanol and diluted with 0.9% sodium chloride as published

previously (Cheng et al. 2014a). Ethanol and Tween80 were added in equal parts to comprise 10% of the total volume. The vehicle control was made similarly without the addition of CBD. 50 mg/kg CBD and vehicle were administered by i.p. injections at a volume of 10 ml/kg. This dose was chosen based on previous studies conducted within our laboratory which found that 50 mg/kg CBD was effective in reversing behavioural deficits in *APPxPS1* males and had moderate effects in reducing Aβ levels (Watt et al. 2020).

At 3.5 months (± 1 week**)** of age, mice were treated with either CBD or vehicle daily for 3 weeks prior to the start of behavioural testing. WT and TAU58/2 transgenic mice were assigned to the two treatment groups in a quasi-randomized manner (WT-VEH n = 14; TAU58/2-VEH n = 12; WT-CBD n = 14; TAU58/2-CBD n = 14). Treatment was continued throughout behavioural testing until test completion (i.e. total treatment duration was around 5 weeks). This treatment regimen was chosen based on our previous studies evaluating CBD as a therapeutic target in AD transgenic mouse models for Aβ pathology (Cheng et al. 2014a). Injections were administered in the afternoon, between 1400 and 1600 hours, to avoid potential acute effects of CBD confounding behavioural outcomes during the behavioural testing (Cheng et al. 2014a). Treatment and testing schedule are presented in Table 1.

5.3.3 Behavioural testing

A limited number of behavioural tests were conducted in TAU58/2 transgenic mice to assess anxiety, motor function and social as well as cognitive functions (for test order see Table 1). All tests were conducted in the light phase between 0930 and 1400 hours (i.e. in the first half of the light phase), with an inter-test interval of at least 24 h to minimize the effect of repeated testing.

Between trials equipment and apparatus were cleaned using 80% ethanol. Body weight of all test mice was recorded weekly.

Treatment	Vehicle		Cannabidiol (CBD)	
Genotype	WT (*n* = 14)	TAU58/2 (*n* = 12)	WT (*n* = 14)	TAU58/2 (*n* = 14)
Age at start of treatment	99 ± 1	100 ± 1	99 ± 1	101 ± 1
Elevated plus maze	120 ± 1	121 ± 1	120 ± 1	122 ± 1
Pole test	121 ± 1	122 ± 1	121 ± 1	123 ± 1
Accelerod	122 ± 1	123 ± 1	122 ± 1	124 ± 1
Social preference test	123 ± 1	124 ± 1	123 ± 1	125 ± 1
Beam walking test	125 ± 1	126 ± 1	125 ± 1	127 ± 1
Fear conditioning	126 ± 1	127 ± 1	126 ± 1	128 ± 1

Table 1 - ***Test biography.*** Test order, sample size, and test age [days] for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg CBD or vehicle throughout behavioural testing. Ages are presented as mean ± SEM.

5.3.3.1 Elevated plus maze (EPM)

The EPM was used to investigate the anxiolytic effects of CBD treatment by utilising the natural conflict of mice to explore a novel environment and avoid brightly lit, elevated open areas (Lister 1987). The EPM apparatus consisted of a grey PVC '+' maze with two open arms (30.5 cm x 6.5 cm, no sidewalls, illumination 70 lx) and two closed arms (30.5 cm x 6.5 cm, sidewall height 18.5 cm, illumination 10 lx) with a central platform connecting the arms (6 cm x 6 cm). It is elevated 40 cm above the floor. Mice were placed in the centre of the EPM, facing an enclosed arm and

allowed to explore for 5 min. ANY-maze™ (Stoelting, Wood Dale, USA) tracking software was used to record time spent and distance travelled in open and enclosed arms.

5.3.3.2 Pole test

The pole test was used to measure motor coordination and grip strength (van Ersel et al. 2015). The pole test apparatus consists of an aluminium pole (diameter: 1 cm; length 50 cm) which is installed vertically on a base platform. The pole is wrapped in masking tape to provide grip. Mice were placed on the pole with head facing upwards. Latency to turn around and to reach the platform at the bottom were recorded using a stopwatch (cut off time: 60 s). Mice were given 60 s if they fell off or slid down the pole without displaying any attempt to climb down the pole. Each mouse was given 3 trials with an inter-trial interval (ITI) of 30 min. Apparatus was cleaned with 80% ethanol between trials.

5.3.3.3 Accelerod

The accelerod was used to assess motor coordination, balance and ataxia (Buitrago 2004). The accelerod apparatus consists of a five-lane rotating beam (Rota-rod, Med Associates Inc., St. Albans, USA). During training, mice were given 3 x 2 min trials with an ITI of 30 min. Mice were placed on the accelerod at a constant rotation speed (12 rpm) for 2 min. If mice fell during training, they were instantly replaced on the accelerod. In the test phase (24 h after training), mice were given 3 x 5 min trials with an ITI of 30 min. Mice were placed on the accelerod at an accelerating speed (4 - 40 rpm within 4.5 min). The latency to fall off the accelerod was recorded automatically. The apparatus was cleaned with 80% ethanol between trials.

5.3.3.4 Beam walking test

The beam walking test was used to assess motor coordination and balance (Pothakos et al. 2009). The test set up consisted of plastic beams (length: 70 cm) with either a 2 cm or 1 cm square cross-section. The beams were placed horizontally, 80 cm above the ground with one end with an enclosed box into which the mouse can escape. Padding was placed on the floor in case mice fell off beam to avoid injuries. During training (day 1), the mouse was placed on the beam and trained over 3 trials to traverse the beam into the enclosed box. On day 2 (test day), the mouse was placed first on the larger beam for 2 test trials, which were then followed by another 2 trials on the smaller beam with an ITI of 30 min. The latency to reach the enclosed platform and number of hindlimb slips were recorded manually. Test cut off time was 60 s.

5.3.3.5 Social preference test (SPT)

The SPT was used to assess sociability and social recognition memory (Moy et al., 2004). The SPT apparatus consists of a three-chamber apparatus, with a centre chamber (9 cm x 18 cm x 20 cm) and two outer chambers (16 cm x 18 cm x 20 cm). In each outer chamber is a mouse enclosure (diameter: 7 cm; height: 15 cm; bars spaced: 0.5 cm). Prior to the start of testing, mice were isolated for 1 h. During the habituation trial, mice were allowed to freely explore the apparatus for 5 min. In the sociability trial, an unfamiliar standard opponent (male, adult A/J mouse) was placed in one of the mouse enclosures in the outer chambers and the test mouse was allowed to explore for 10 min. In the social novelty trial, a second unfamiliar A/J standard opponent was placed in the previously empty mouse enclosure and the standard opponent from the sociability trial was kept. The test mouse was returned to the apparatus and allowed to explore for 10 min. The ITI was 5 min and chambers and enclosures were cleaned with 80% ethanol between trials. Fresh corn cob

bedding was added to the chambers prior to each new test mouse. Time spent, entries and distance travelled in each chamber were recorded and time spent *nosing* mouse enclosures were manually scored using ANY-maze^{TM} tracking software. Mice were excluded if they climbed out of the apparatus during testing.

5.3.3.6 Fear conditioning paradigm (FC)

The FC was used to assess fear associated learning and memory where a neutral stimulus is paired with an aversive stimulus to then elicit a fear response. The apparatus consisted of a fear conditioning chamber (Med Associates Inc.) (29.5 cm x 24.5 cm x 21 cm) with a gridded floor. On day 1 (conditioning), mice were placed into the test chamber for a 7 min trial. After 2 min an 80 dB conditioned stimulus (tone) was presented for 30 s with a 2 s co-terminating 0.4 mA foot shock twice with an ITI of 2 min. The test concluded 2 min later. On day 2 (context trial), mice were returned to the apparatus for 7 min. On the final day (Day 3: cue trial), mice were placed into a distinct context for 9 min. After 2 min, the tone was presented for 5 min. The test concluded after another 2 min. Time spent *freezing* was recorded in 1 min bins on all days using *Video Freeze*® (Med Associates Inc. - software setting: freezing threshold = 15; detection method = linear; minimum freezing duration = 30 frames) and presented as *freezing* over time and percentage of time spent *freezing* in each trial. Response to cue presentation in the cue trial was also analysed by comparing percentage of time spent *freezing* in the 2 min prior (i.e. no cue presentation) and the 5 min post cue onset (i.e. during cue presentation).

5.3.4 Statistical analysis

Two-way analysis of variance (ANOVA) was used to analyse behavioural parameters for main effects of 'CBD', 'genotype' and 'CBD' by 'genotype' interactions. Repeated measures (RM) three-way ANOVA was used when analysing the effects of 'week' (body weight), 'arm' (EPM), 'chamber' (SPT), 'min' and 'cue' (both FC) across the experimental groups. Data are presented as mean ± standard error of mean (SEM) and differences were regarded as significant if $p < 0.05$. F-values and degrees of freedom are presented for ANOVA. Significant ANOVA results for 'genotype' are indicated by '*' (i.e. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$), 'treatment' by '#' (i.e. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$) and 'time' x 'genotype' interactions by '+' (i.e. $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$). Significant RM results are indicated by '^' (i.e. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, and $^{\wedge\wedge\wedge}p < 0.001$). Analyses were conducted using SPSS 23.0 for windows.

5.4 Results

5.4.1 Body weight development

Three-way RM ANOVA revealed a main effect of 'week' [$F(3,150) = 16.6$. $p < 0.0001$] with all mice increasing body weight over time (Figure 1). The body weight development was not affected by genotype or CBD treatment (no interactions, all *p's* > 0.05). We also detected a main effect of 'genotype' [$F(1,50) = 81.3$, $p < 0.0001$] but not 'CBD' [$F(1,50) = 0.2$, $p = 0.6$] with TAU58/2 transgenic mice having a lower body weight compared to WT controls across the recording period regardless of treatment condition.

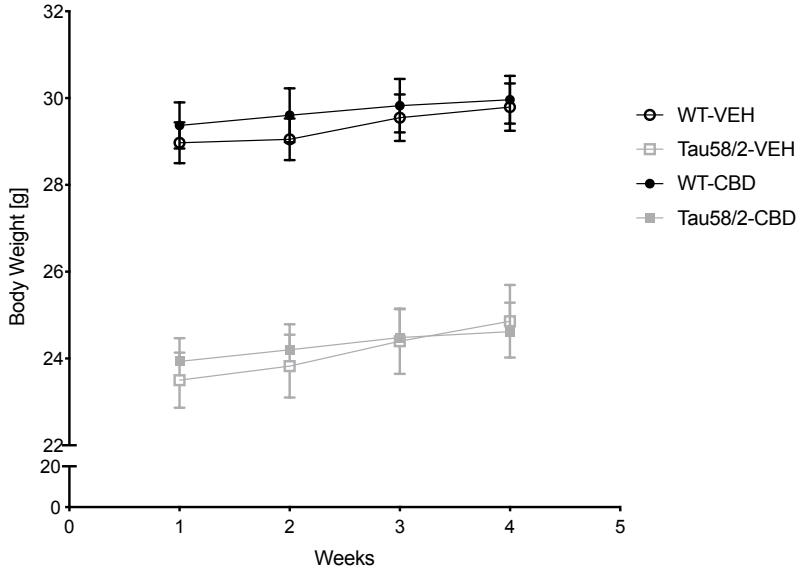

Figure 1: ***Body weight development.*** Body weight [g] per week for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. A main effect of 'genotype' is presented by '*' (***$p < 0.0001$).

5.4.2 Locomotion and anxiety-related EPM behaviours

Two-way ANOVA for total distance travelled in the EPM revealed no main effects of 'genotype', 'CBD' or interaction effects suggesting that all mice showed similar levels of locomotion (all *p's* > 0.05; Table 2).

Treatment	Vehicle		Cannabidiol (CBD)	
Genotype	WT	TAU58/2	WT	TAU58/2
Elevated plus maze				
Total distance travelled [m]	9.2 ± 0	9.1 ± 1	9.4 ± 1	8.8 ± 1
Beam walking test				
Frequency of hindlimb slips on large beam [n] ***, #	0.1 ± 0	0.5 ± 0.1*	0.2 ± 0.1#	0.9 ± 0.3
Frequency of hindlimb slips on small beam [n] ***, #	0.6 ± 0.2	1.8 ± 0.3*	0.8 ± 0.2#	2.6 ± 0.3

Table 2 - *Locomotion in EPM and motor coordination in the beam walking test*. Hindlimb slips for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg CBD or vehicle. Data are presented as mean ± SEM. Main 'genotype' effects are presented as '*' (***$p < 0.0001$) and main effects of CBD treatment as '#' ($^{\#}p < 0.05$).

Importantly, percentage distance travelled in the open arms revealed a main effect of 'genotype' [$F(1,50) = 64.6$, $p < 0.0001$] with TAU58/2 transgenic mice travelling further in the open arms compared to WT controls regardless of treatment condition (no interaction with 'CBD': $p > 0.05$; Figure 2A). Similarly, percentage time spent in open arms was significantly increased in TAU58/2 mice across treatment conditions [$F(1,50) = 77.6$, $p < 0.0001$; no interaction with 'CBD': $p > 0.05$; Figure 2B]. Chronic CBD had no effect on locomotion or anxiety-related parameters (no overall 'CBD' effects: p's > 0.05).

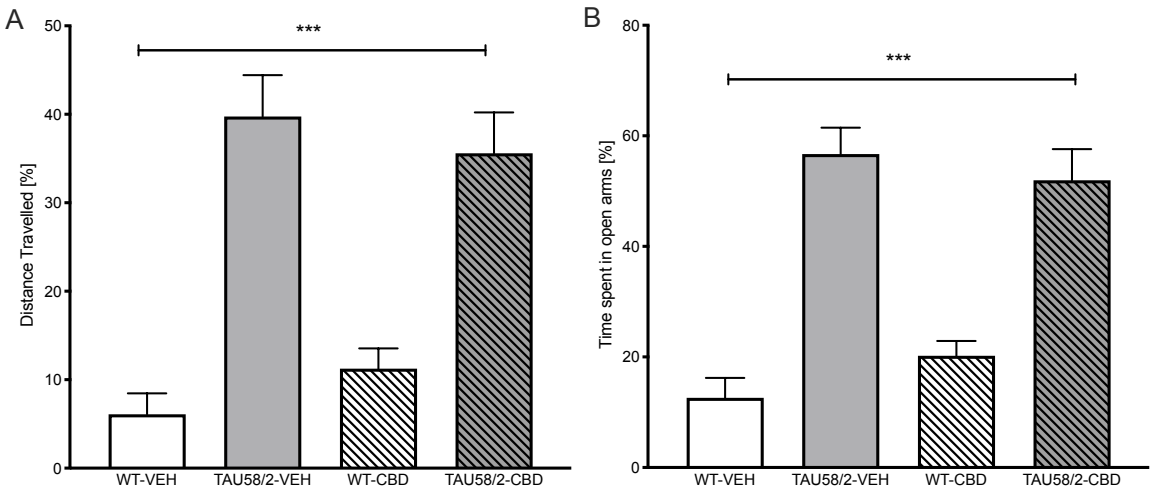

Figure 2A-B: ***Anxiety-related behaviours in elevated plus maze.*** **A)** Percentage distance travelled in open arms [%], and **B)** percentage time spent in open arms [%] for TAU58/2 transgenic mice and non-transgenic WT control littermates with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. Main 'genotype' effects are presented by '*' (***$p < 0.001$).

5.4.3 Motor Function

In the pole test, two-way ANOVA for the latency to reach the platform revealed a main effect of 'genotype' [$F(1,50) = 16.1$, $p < 0.0001$], where TAU58/2 mice took significantly longer to climb down the pole. There we no main effects of CBD treatment or interaction effects (all p's > 0.05; Figure 3A). In the accelerod test, TAU58/2 transgenic males fell off the accelerating rods significantly earlier compared to WT controls [$F(1,50) = 95.0$, $p < 0.0001$]. The accelerod performance and the TAU58/2-related motor deficit were not affected by CBD treatment (no 'CBD' and no 'CBD' by 'genotype' interaction: all p's > 0.05; Fig 3B). Finally, in the beam walking test paradigm, two-way ANOVA for latency to cross the larger and smaller beams revealed a main effect of 'genotype' [large beam: $F(1,50) = 29.9$, $p < 0.0001$; small beam: $F(1,50) = 55.6$, $p < 0.0001$] with TAU58/2 mice taking significantly longer to cross the beams compared to WT control animals. Similar to before, there were no 'CBD' and no 'CBD' by 'genotype' interaction effects (all p's > 0.05) indicating that CBD did not reverse this motor deficit (Figure 3C).

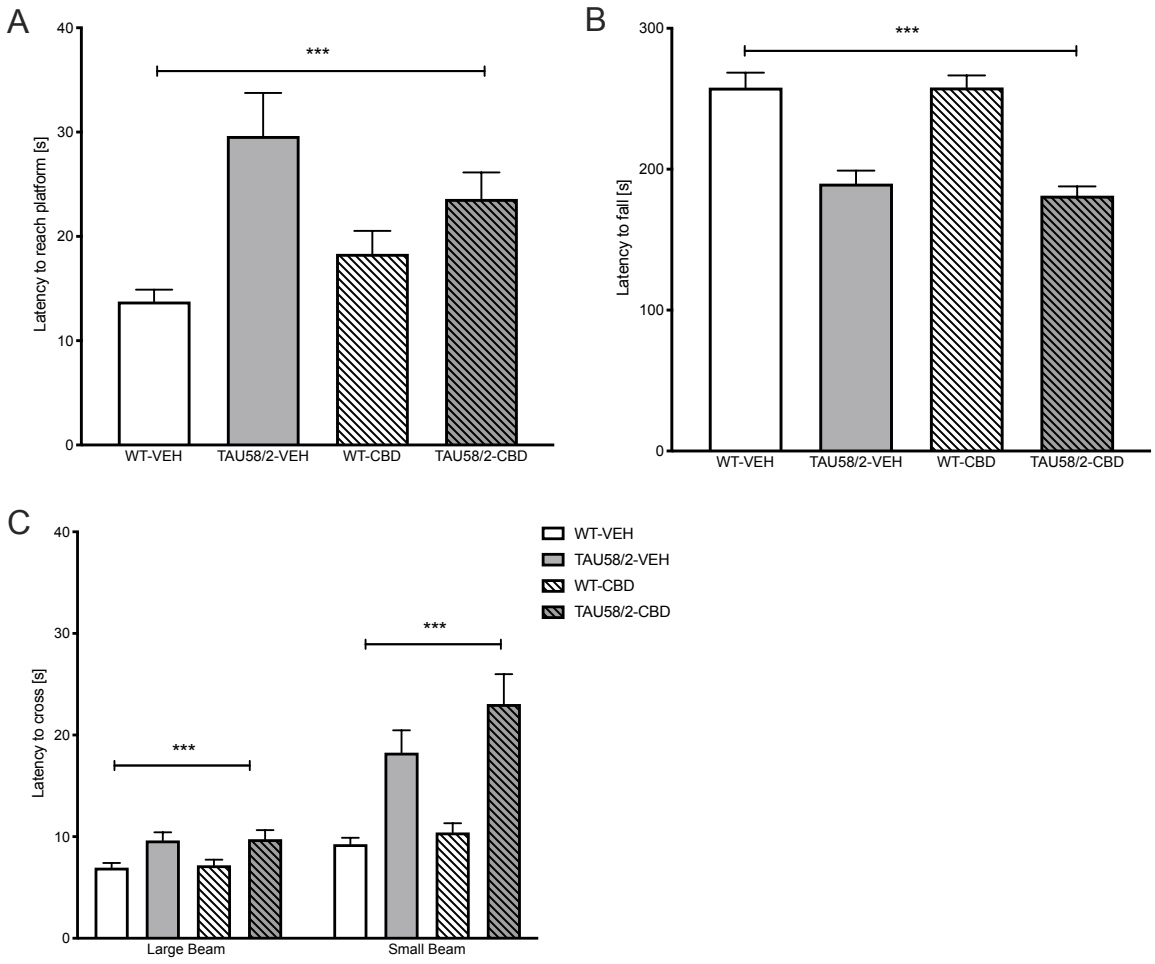

Figure 3A-C: ***Motor function and coordination in the pole test, accelerod and beam walking paradigm.*** Latency [s] to **A)** reach the platform in the pole test, **B)** fall off accelerod and **c)** cross large and small beams for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. Main genotype effects are presented by '*' (***$p < 0.001$).

In line with this, the frequency of hindlimb slips on the beams was also significantly increased in TAU58/2 mice compared to control mice [large beam: $F(1,50) = 18.7$, $p < 0.0001$; small beam: $F(1,50) = 32.\ 1$, $p < 0.0001$]. Interestingly, there was also a main effect of 'CBD' [large beam: $F(1,50) = 4.2$, $p < 0.05$; small beam: $F(1,50) = 4.1$, $p < 0.05$] with CBD-treated mice slipping significantly more often compared to VEH-treated animals (Table 2).

5.4.4 Cognition

5.4.4.1 Social preference test (SPT)

Sociability: Three-way RM ANOVA revealed a main effect of 'chamber' for time spent *nosing* the A/J mouse [$F(1,49) = 256.1$, $p < 0.0001$]. All mice regardless of genotype or CBD treatment showed intact sociability in the SPT (i.e. preference for the chamber containing a mouse) as there were no significant interactions of 'chamber' with 'genotype' or 'treatment' (all p's > 0.05; Figure 4A).

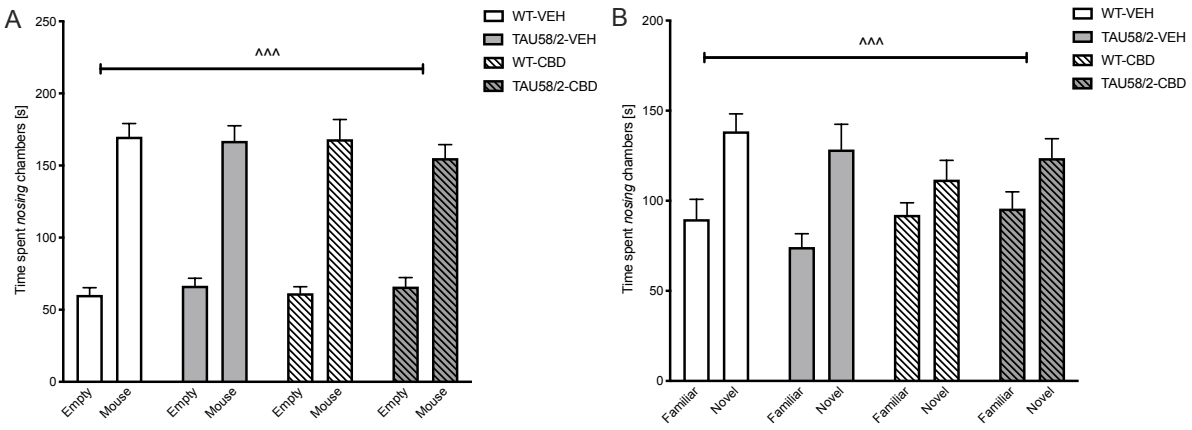

Figure 4A-B: ***Sociability and recognition memory in the SPT.*** Total time spent *nosing* [s] **A)** empty and mouse enclosures (i.e. sociability test) and **B)** familiar and novel mouse enclosures (i.e. social recognition memory test) for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. RM ANOVA results for 'chamber' are presented by '^' (^^^$p < 0.001$).

Social recognition memory*:* In the social recognition trial, all mice exhibited a preference for the novel mouse over the familiar mouse [$F(1,46) = 26.6$, $p < 0.0001$]. There was also a trend for a 'chamber' by 'treatment' interaction [$F(1,46) = 3.6$, $p = 0.06$], where CBD-treated mice showed a moderately reduced preference for the novel mouse (Figure 4B). No 'chamber' by 'genotype' by 'CBD' interaction was found ($p > 0.05$).

5.4.4.2 Fear associated memory

All mice responded to the electric foot shocks delivered during the conditioning trial as determined by vocalization of the test mouse. In line with this, three-way RM ANOVA confirmed a main effect of 'time' [$F(6,300) = 35.0$, $p < 0.0001$], where *freezing* increased over time during conditioning.

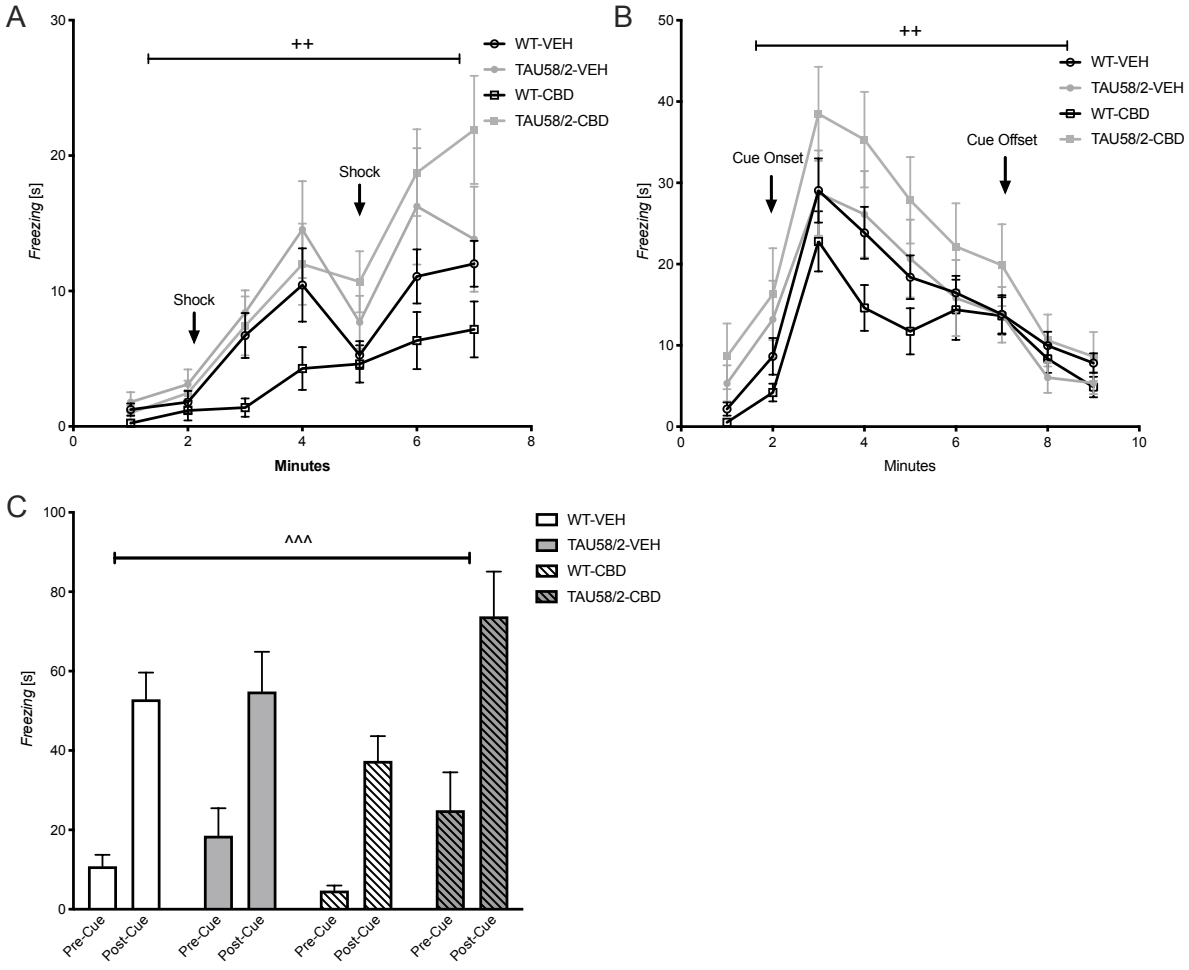

Figure 5A-C: ***Fear associated memory in the FC.*** Time spent *freezing* [s] over 1 min blocks during **A)** conditioning and **B)** cue trials, as well as **C)** time spent *freezing* [%] averaged for the 2 min before and 2 min after cue onset during the cue trial for TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg cannabidiol (CBD) or vehicle (VEH). Data are presented as means ± SEM. RM ANOVA results for 'time' are presented by '^' ($^{\wedge\wedge\wedge}p < 0.001$) and 'time' x 'genotype' interactions are presented by '+' ($^{++}p < 0.01$).

Interestingly, we also detected an interaction of 'time' and 'genotype' [F(6,300) = 3.5, $p = 0.003$] with TAU58/2 mice *freezing* more across time than WT controls (Figure 5A). However, in the first two mins of conditioning, where baseline *freezing* is evaluated prior to any shock delivery, time spent *freezing* was similar between genotypes and no main effect of 'CBD' or 'genotype' by 'CBD' interaction were detected either (all *p's* > 0.05; Table 3).

Treatment	Vehicle		Cannabidiol (CBD)	
Genotype	WT	TAU58/2	WT	TAU58/2
Freezing first 2 min of conditioning [s]	3 ± 1	5 ± 1	1 ± 1	3 ± 1
Total context *freezing* trial [%] *	26 ± 4	28 ± 5	18 ± 3	36 ± 6
Total cue *freezing* [%] $^{*p = 0.05}$	24 ± 3	23 ± 5	18 ± 3	35 ± 7

Table 3 – *Fear associated memory.* Fear associated memory in FC for male TAU58/2 transgenic mice and non-transgenic WT control littermates treated with 50 mg/kg CBD or vehicle. Data are presented as mean ± SEM. Main genotype effects are presented as '*' (*$p < 0.05$; trend value also listed).

Two-way ANOVA for total *freezing* during the context trial revealed a 'genotype' effect [F(1,50) = 6.9, $p = 0.012$] with increasing context *freezing* being evident in TAU58/2 transgenic mice compared to their control littermates. CBD had no effect on contextual *freezing* or the genotype effect found (all *p's* > 0.05) (Table 3). In the cue test, a main effect of 'time' [F(8,368) = 52.3, $p < 0.0001$] and a significant 'time' by 'genotype' interaction [F(8,368) = 3.0, $p = 0.003$] were detected, with TAU58/2 transgenic mice *freezing* more across time than WT controls regardless of treatment (no 'time' by 'genotype' by 'CBD' interaction: $p > 0.05$; Figure 5B). Analysing *freezing* per 1 min block during cue presentation (i.e. 3rd minute to 7th minute) revealed a 'time' by 'genotype' interaction [F(4,184) = 2.556, $p = 0.04$] which appeared to be mostly driven by the CBD treatment

group with TAU58/2 transgenic mice showing more *freezing* (Figure 5B). Two-way ANOVA for total cue *freezing* (i.e. time spent *freezing* during cue presentation) revealed a trend for a similar 'genotype' effect [$F(1,46) = 3.9$, $p = 0.05$] (Table 3).

Importantly, all groups responded to the cue at onset (i.e. comparing percentage *freezing* in the first 2 min of cue session with the percentage *freezing* during the 5 min of cue presentation) as there was a main effect of 'cue' [$F(1,46) = 58.920$, $p < 0.0001$]. There were no main effects of 'genotype' or 'CBD' and no interactions for the *freezing* response at cue onset (all p's > 0.05; Figure 5C).

5.5 Discussion

This study is the first to investigate the therapeutic potential of chronic CBD treatment in a tauopathy-specific transgenic mouse model of AD. The results confirmed that TAU58/2 males exhibit lower body weight, show decreased anxiety in the EPM, and have impaired motor functions across test paradigms. The research also established that sociability and social recognition memory are intact in AD transgenic mice at the age tested and that the *freezing* responses to conditioned context and a cue are increased compared to control mice. The chosen CBD treatment regime had no significant beneficial effects on the behavioural changes detected in 4-month-old TAU58/2 males.

It is important to note that the test age of the TAU58/2 males was chosen based on the previously described motor impairments which begin to develop at 2 months. We wanted to determine the behavioural profile of this model before their motor impairments became too advanced and thereby representing a potential confound in the cognitive paradigms chosen. TAU58/2 males demonstrated lower initial body weight compared to their WT littermates, however, their body weight increased over weeks similar to WT controls. This supports previous studies which have reported lower body weight in this model as well (Przybyla et al. 2016). Chronic CBD treatment did not affect body weight. This is in line with previous studies which have reported that 4-week Sativex® treatment in $PK^{-/-}/Tau^{VLW}$ mice had no effect on body weight (Casarejos et al. 2013). Furthermore, pure CBD treatment (at doses 1, 10 and 20 mg/kg) in a mouse model of type 1 diabetes found that 4 and 11 weeks of treatment also had no effect on body weight (Rajesh et al. 2010).

In the EPM, TAU58/2 males demonstrated reduced anxiety as they spent more time and travelled further in the open arms. This confirms previous yet unpublished findings within our laboratory as

well as other laboratories (Przybyla et al. 2016; Van Der Jeugd et al. 2016). As expected, chronic CBD treatment did not affect anxiety levels of any experimental test group. This is the first study to look at CBD effects in tauopathy transgenic mice, however, treating *APPxPS1* transgenic mice, a mouse model for Aβ pathology, chronically with CBD doses (20 mg/kg) revealed a similar phenomenon, i.e. anxiety levels being unaffected by CBD treatment (Cheng et al. 2014a). Acute CBD has been reported to reduce anxiety behaviours in rats (at a dose of 15-60 nmol) (Campos and Guimarães 2008; Gomes et al. 2011) and humans (at a dose of 400 – 600 mg) (Bergamaschi et al. 2011; Crippa et al. 2011). In addition, chronic CBD (50 mg/kg) treatment has been reported to moderately reduce anxiety in mice (Long et al. 2010). Thus, the CBD treatment design (50 mg/kg) of our study and the established anxiolytic-like phenotype of TAU58/2 mice made it highly unlikely that CBD would have restored EPM behaviours.

TAU58/2 males demonstrated motor impairments in the pole test, accelerod and beam walking test. Motor deficits are a common symptom seen in several other transgenic tauopathy mouse models (Ittner et al. 2008; Leroy et al. 2007; Lewis et al. 2000), the TAU58/2 mouse model of the current study, as well as the TAU58/2-B model (Van Der Jeugd et al. 2016; van Ersel et al. 2015). The chronic CBD treatment did not restore motor impairments in 4-month-old TAU58/2 males. It is noteworthy to mention here that 4-week treatment with Sativex®, a CBD-THC combination treatment (cannabinoid ratio of 1:1), did not affect impaired motor functions of the $PK^{-/-}$ / Tau transgenic mouse model either (Casarejos et al. 2013). A few studies have reported that CBD (5 mg/kg) treatment could prevent motor impairments induced by bile-duct ligation and reserpine, which was measured by locomotion in the open field test and oral movements, respectively (Magen et al. 2010; Peres et al. 2016). Furthermore, acute CBD (1 mg/kg) after hypoxia-ischemia in newborn rats was able to reverse motor impairments in accelerod (Pazos et al. 2012). There have

been no reports of CBD worsening motor function. The moderate negative effect of CBD on hindlimb slips should be investigated further using more advanced motor function test equipment such as DigiGait or other devices.

Previous *in vitro* studies demonstrated that pre-treatment with CBD (10^{-9}, 10^{-8}, 10^{-7} M) can reduced tau hyperphosphorylation in a dose-dependent manner (Esposito et al. 2011) by reducing phosphorylated glycogen synthase kinase 3β (pGSK-3β), which is responsible for tau hyperphosphorylation and NFT formation (Esposito et al. 2006a). In addition, a study in mesenchymal stem cells derived from gingiva (GMSCs) found that pre-treatment with CBD downregulated genes encoding kinases involved in aberrant tau (Diomede et al. 2017). Therefore, suggesting that CBD treatment may be able to reverse motor impairments which result directly from tau pathology. However, a study in another hSYN mice crossed onto $tau^{-/-}$, $tau^{-/+}$ and $tau^{+/+}$ backgrounds found that reducing tau levels (determined by background crossing) did not restore motor function deficits in accelerod, challenge beam, gait and hindlimb clasping. This suggests that tau pathology may not be the only mediating factor behind motor impairments (Morris et al. 2011), although it should be mentioned here that Le Corre and coworkers found a direct link between the reduction of tau phosphorylation and improving motor functions in tau transgenic mice (Le Corre et al. 2006). It is possible that the CBD treatment design utilised in the current study was started too late, the treatment period was too short, or that the CBD dose chosen was too low to be able to affect the motor impairments of TAU58/2 transgenic mice. Future studies should look at the preventative effects of CBD in this model to determine if it could prevent the development of motor impairments. In amyotrophic lateral sclerosis, i.p. treatment with other cannabinoids (e.g. THC [20 mg/kg] and AM1241 [1 mg/kg]) after the onset of disease symptoms has been found to be beneficial in delaying the onset of motor impairments and prolonging survival

in the G93A-SOD1 mouse model (Kim et al. 2006; Raman et al. 2004), but CBD has not been evaluated yet.

In the SPT, all mice demonstrated intact sociability regardless of genotype and treatment. Interestingly, TAU58/2-B male transgenic mice develop a social interaction deficit at 6 months of age (not evident at 2-3 months of age) (Van Der Jeugd et al. 2016), suggesting that tauopathy-related social deficits of mice may develop at a later stage. In the social recognition trial, all mice also demonstrated intact social recognition memory, which has not been investigated previously in either TAU58 mouse model. Another study reported a social recognition deficit in Tau22 transgenic mice at 9-10 months of age (Lo et al. 2013) and 3xTg-AD mice ($PS1_{M146V}$, APP_{SWE} and tau_{P301L}) demonstrated reduced sociability and impaired recognition memory at 20 months of age (Arsenault et al. 2013).

We assessed fear associated memory as it is mediated through brain regions severely impacted in AD (e.g. amygdala and hippocampus) (Hall et al. 2001). TAU58/2 males demonstrated intact fear-associated memory in both context and cue trials. In fact, the results indicate that TAU58/2 males froze more than WT littermates in all trials, a phenomenon, which appeared to be mostly driven by the CBD-treated groups despite no significant interactions being present between genotype and treatment condition. Although TAU58/2 transgenic males demonstrated an anxiolytic-like phenotype in the EPM, the increased *freezing* in the fear conditioning paradigm (and also in the first 2 min of the conditioning period prior to shock exposure) is likely driven by different brain regions as spatio-temporal anxiety is measured in EPM whereas fear-associated memory is assessed in FC. This is supported by a study in interleukin-1 null mice which also exhibited reduced anxiety in EPM but enhanced *freezing* in fear conditioning (Koo and Duman 2009). This increased *freezing* response could be an indication of an improved fear-associated memory.

However, a study in *SOD1* transgenic mice suggested increased *freezing* to be associated with impaired fear extinction rather than facilitation of fear associated memory (Sgobio et al. 2008). Our study did not assess fear extinction as the initial aim was to establish first fear-associated memory in these mice using protocols which had been established for AD transgenic mice (Cheng et al. 2013). Indeed, AD mouse models have previously been found to develop impairments in FC (Comery et al. 2005; Corcoran et al. 2002); however, future studies should run in depth baseline analyses into the fear extinction phenotype of the TAU58/2 transgenic mouse model.

In summary, this was the first study to evaluate the effects of chronic CBD treatment (50 mg/kg) on behavioural deficits of 4-month-old TAU58/2 males. TAU58/2 transgenic males exhibited no deficits in social recognition memory function, suggesting that motor deficits and changes in anxiety behaviour evident at this age do not impact on social domains. The moderate increase in fear-associated memory needs further investigation but could be related to differences in fear extinction. Future investigations will need to clarify CBD's therapeutic potential for reversing the motor deficits of the TAU58/2 mouse model by considering alternative CBD treatment.

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Chapter 6: General Discussion

6.1 Summary of findings

The studies in this thesis indicate that CBD is able to reverse cognitive deficits in male *APPxPS1* mice at early and later stages of the disease (i.e. 7.5 and 12 months of age). Chronic CBD treatment reversed a social recognition memory deficit in 7.5-month-old *APPxPS1* males and both social recognition memory and reversal spatial learning deficits in 12-month-old male *APPxPS1* mice. In addition, chronic CBD treatment at a dose of 50 mg/kg tended to reduce insoluble $A\beta_{40}$ pathology in the hippocampus. However, CBD did not affect markers of neuroinflammation and neurodegeneration at the ages and doses tested. These findings suggest therapeutic benefits of CBD treatment in AD. Importantly, these findings are highly relevant to the clinical situation as loss of facial recognition and spatial disorientation are common cognitive deficits seen in AD patients (Alzheimer's Association 2018). This thesis also indicated that 4-month-old TAU58/2 males demonstrate dementia-relevant symptoms, including social withdrawal, impaired sensorimotor gating and motor impairments. Despite the beneficial effects of CBD in *APPxPS1* transgenic mice, the thesis work did not find that CBD was effective in reversing behavioural deficits detected in young male TAU58/2 transgenic mice. In the following sections I will discuss my findings on the therapeutic benefits of CBD treatment on *APPxPS1* mice, outline why CBD may have been ineffective in TAU58/2 transgenic mice, and outline CBD's potential for human AD therapy including necessary future research strategies to further evaluate CBD for the clinic.

6.1.1 Dose-dependent effects of CBD in APPxPS1 mice

Initially, our laboratory found that chronic 20 mg/kg CBD treatment reversed and prevented cognitive deficits in *APPxPS1* males (Cheng et al. 2014a; Cheng et al. 2014c). In the preventative

arm of this research, long-term CBD treatment only subtly reduced neuroinflammatory markers and had no effect on Aβ levels in 10-month-old *APPxPS1* males (Cheng et al. 2014c). Importantly, *in vitro* studies have demonstrated that CBD works in a dose-dependent manner (Esposito et al. 2007; Esposito et al. 2011) and in addition, cannabinoids have been found to have biphasic effects (Guimarães et al. 1990). Therefore, in this thesis I investigated if higher CBD doses would still be effective in reversing cognitive deficits of male *APPxPS1* transgenic mice, while having a greater effect on reducing their AD-relevant brain pathology. My work found that increasing the dose of CBD to 50 and 100 mg/kg improved recognition memory, and importantly without inducing psychological or physiological adverse effects (as determined by daily monitoring, body weight recording, and testing baseline behaviours such as anxiety). Increasing the dose to 50 mg/kg CBD also tended to reduce hippocampal levels of insoluble $A\beta_{40}$ but not $A\beta_{42}$. In the 100 mg/kg CBD study, only $A\beta_{42}$ levels were assessed as this is thought to be the more pathogenic form of the protein (Chapman et al. 2001) but hippocampal levels of soluble or insoluble $A\beta_{42}$ were not affected by CBD treatment. It is important to note that the *APPxPS1* males tested in the 50 mg/kg CBD study were 12 months of age, while those used in the 100 mg/kg CBD study were 8 months of age. This was due to wanting to investigate the effect of CBD in reversing the AD phenotype in late and early stages of the disease. However, as the phenotype in *APPxPS1* mice is age-dependent, this means that some of the cognitive deficits seen in 12-month-old *APPxPS1* males have not yet developed in the 8-month-old mice and similarly, neuropathology is likely to be less prominent in younger *APPxPS1* mice.

In addition to determining the effect of CBD on Aβ pathology, this thesis also investigated the effect of CBD on markers for neuroinflammation and microglial activation (i.e. TNF-α, IL-1β and IBA1). Previously, 10-month-old *APPxPS1* males tended to show elevated TNF-α and IL-1β

levels which appeared to be reduced by long-term, preventative, low dose CBD treatment via oral administration (Cheng et al. 2014c). Here, I found that 12-month-old *APPxPS1* male mice did not demonstrate altered levels of these markers when being treated with CBD via the i.p. route and post onset of AD-relevant symptoms. This discrepancy may be due to the previous study being a preventative study, in which treatment began before the onset of symptoms and lasted for 8 months with oral administration of low dose CBD (Cheng et al. 2014c). In addition, the previous study measured mRNA levels of TNF-α and IL-1β while this thesis looked at protein levels by ELISA. A similar discrepancy was found in 9-15-month-old *APPxPS1* mice where mRNA expression of TNF-α and IL-1β were altered in AD transgenic mice, but these changes were not reflected in the respective protein expression (Babcock et al. 2015). Interestingly, in 8-month-old male *APPxPS1* mice, I found that TNF-α and IL-1β levels were reduced compared to WT controls and 100 mg/kg CBD had no effect on these levels. As CBD is known to have anti-inflammatory effects, the fact that CBD did not increase neuroinflammatory markers is not surprising. However, the finding that TNF-α and IL-1β were lower in AD transgenic mice than WT controls is interesting and conflicts previous literature in *APPxPS1* mice, which reported that levels are either elevated (Couturier et al. 2012; Minogue et al. 2014) or no different to WT controls (Babcock et al. 2015; Pihlgren et al. 2009). These findings could be due to the profile of cytokine protein expression being age-dependent in the *APPxPS1* mouse model. Previous studies have demonstrated such an effect, for example a study in female *APPxPS1* mice found that TNF-α and IL-1β protein levels in the hippocampus were elevated at 12 and 18 months, but not 6 months and cortical levels were only elevated in 18-month-old female *APPxPS1* mice (Couturier et al. 2012). In addition, another study in *APPxPS1* males and females demonstrated that TNF-α and IL-1β levels increased with age in 12- and 24-month-old mice (Minogue et al. 2014).

It would be interesting to investigate mRNA and protein expression of neuroinflammatory markers in parallel and at different ages to further understand these distinct findings, but this was beyond the scope of the current PhD project. However, it is interesting to note that 12-month-old male *APPxPS1* mice exhibited elevated IBA1 levels, a marker for microglial activation, however, this was not evident in 8-month-old *APPxPS1* males suggesting an age-dependent development of brain inflammation in these mice. Neither dose of CBD had an effect on IBA1 levels. Although, as 100 mg/kg CBD was used at an age where neuroinflammation is potentially not yet very pronounced, this dose should be further investigated in older *APPxPS1* mice when altered pathology is present.

In this thesis, I found little difference in the effects of 50 and 100 mg/kg CBD on the *APPxPS1* phenotype, which conflicts previous *in vitro* and *in vivo* studies which have shown that CBD and other cannabinoids work in a dose-dependent manner, with increasing the dose having a more marked effect on various aspects of neuropathology relevant to AD, including TNF-α, IL-1β, iNOS, NO, GFAP and S100B (Esposito et al. 2007; Esposito et al. 2011). Importantly, studies have also demonstrated that cannabinoids, including CBD, have biphasic effects, suggesting that their effects at high and low doses may be opposite. For example, low doses of CBD have been shown to have an anxiolytic-like effect, which is not seen at higher doses in male Wistar rats (Guimarães et al. 1990), although one study found that 10 mg/kg CBD induced anxiogenic effects in Lister-hooded rats (ElBatsh et al. 2012). In addition, in a murine collagen-induced arthritis model, CBD demonstrated a bell-shaped dose-dependent curve for its immunosuppressive and anti-inflammatory effects, where 5 mg/kg CBD demonstrated optimal effects through reducing serum TNF, reactive oxygen species and lymphocyte proliferation (Malfait et al. 2000). The bell-shaped neuroprotective effects of CBD were also demonstrated in a mouse model of middle

cerebral artery occlusion and a gerbil model of cerebral ischemia, where the mouse study found that i.p. injections of 1 and 3 mg/kg of CBD prevented cerebral infarction whereas 0.1 and 10 mg/kg were not effective (Mishima et al. 2005). In the gerbil study a range of CBD doses (1.25, 2.5, 5, 10 and 20 mg/kg) was evaluated and 5 mg/kg CBD elicited the greatest neuroprotective effect (Braida et al. 2003). Although these studies are not specifically related to AD, the underlying pharmacokinetics are relevant to how CBD dosing affects the phytocannabinoids ability to reduce AD pathology. Future research should evaluate the potential biphasic effects of CBD on AD pathology in more detail.

The dose-dependent effects of CBD on memory have not yet been investigated, although they have been in other cannabinoids, i.e. THC. Low doses of THC (0.3 and 1 mg/kg) do not affect memory, but higher doses (3 or 10 mg/kg) impair memory performance (Puighermanal et al. 2009). However, it is important to note that beneficial effects of THC at a dose of 3 mg/kg has been demonstrated on memory of aged mice (Bilkei-Gorzo et al. 2017), suggesting that the dose-dependent effects of cannabinoids differ in ageing organisms. From our studies and previous studies in the literature low (0.75 and 20 mg/kg) and high doses (50 and 100 mg/kg) of CBD appear to be beneficial in reversing memory deficits in pharmacological and transgenic mouse models of AD (Aso et al. 2014; Cheng et al. 2014a; Martín-Moreno et al. 2011). However, as these have all been individual studies, a large scale dose comparison study would be beneficial to determine the most effective dose of CBD on reversing memory deficits as well as correlating these beneficial effects with neuropathological markers.

6.1.2 Differences in CBD effects between APPxPS1 and TAU58/2 mice

The studies in this thesis investigated the effect of chronic CBD treatment in transgenic mouse models for the two major hallmarks of AD, i.e. Aβ pathology and tau hyperphosphorylation, using *APPxPS1* and TAU58/2 mouse models of AD. CBD treatment was effective in reversing cognitive deficits in the *APPxPS1* mouse model and had subtle effects on Aβ pathology but did not restore any of the behavioural deficits detected in 4-month-old TAU58/2 males.

This thesis demonstrated that TAU58/2 males exhibited AD-relevant behavioural impairments, including reduced sociability, impaired sensorimotor gating and motor impairments. However, at the age tested TAU58/2 males did not demonstrate cognitive deficits in social recognition and fear-associated memory. It is possible that cognitive deficits do not develop in this mouse model until a later age. Supporting this idea, a study in TAU58/2-B mice indicated that they demonstrated impairments in spatial working memory and instrumental short-term memory in the y-maze and puzzle box paradigm from 6 months of age, but not at 2-3 months of age (Van Der Jeugd et al. 2016). In addition, unpublished data from our laboratory found that at 15 months of age, TAU58/2 females demonstrated impaired spatial learning in the cheeseboard task. Furthermore, in the Tau22 transgenic mouse model social recognition memory deficits were reported in 9-10-month-old mice (Lo et al. 2013).

TAU58/2 males also demonstrated an anxiolytic-like phenotype in the elevated plus maze and elevated *freezing* in the context and cue trials of the fear conditioning paradigm (FC). Elevated *freezing* in FC may indicate improved fear-associated memory. However, a study in *SOD1* transgenic mice suggested increased *freezing* in the context trial can also be associated with impaired fear extinction rather than facilitation of fear-associated memory (Sgobio et al. 2008).

The protocol used in our study did not assess fear extinction, and thus this finding could not be clarified but would be an interesting follow up study.

Although *in* vitro studies provided evidence that CBD would be effective in reducing tau pathology and corresponding behavioural deficits associated with it, chronic CBD (50 mg/kg) did not restore the behavioural deficits in 4-month-old TAU58/2 transgenic males. Importantly, and as mentioned above, the TAU58/2 males did not demonstrate cognitive deficits at the age tested and therefore, the studies in this thesis were unable to determine the role of CBD in restoring cognitive deficits in 4-month-old TAU58/2 males. The anxiolytic-like phenotype of the TAU58/2 males was also unaffected by CBD treatment. However, this was not surprising as chronic CBD treatment has been shown to have no effect on anxiety (Cheng et al. 2014a), while acute CBD treatment has been associated with an anxiolytic-like effect (Campos and Guimarães 2008; Gomes et al. 2011). Therefore, if CBD were to have an effect it would likely accentuate the anxiolytic-like phenotype rather than reverse it to the level of WT controls. Importantly, in this thesis chronic CBD treatment (50 mg/kg) did not reverse motor deficits in 4-month-old TAU58/2 males.

There are distinct differences between the two mouse models used in this thesis which are related to the AD pathology they are representing, i.e. the *APPxPS1* mouse model is an Aβ-dependent model of AD while the TAU58/2 mouse model is a tauopathy mouse model of AD. Not surprisingly, the behavioural deficits identified are distinct as well. The major phenotype of the *APPxPS1* mouse model is that they develop cognitive deficits while the most prominent phenotype in the TAU58/2 mouse model at this age is that they develop motor impairments and an anxiolytic-like phenotype. Previous literature and unpublished data from colleagues in the Karl laboratory have demonstrated that at an older age TAU58/2 mice do develop cognitive deficits (Van Der Jeugd et al. 2016) Therefore, the discrepancy of CBD's effects in the *APPxPS1* mouse model

compared to the TAU58/2 model may be due to CBD being more effective against Aβ pathology than tau pathology or it may be due to CBD being effective against cognitive deficits but not motor impairments. It is possible that these differences are also due to the models exhibiting different levels of neuroinflammation at the ages tested. In the *APPxPS1* mouse model, altered neuroinflammation expression in the cortex and hippocampus appears to occur from 8 months of age (Babcock et al. 2015; Cheng et al. 2014c). It is important to note that although the results from this thesis did not find elevated neuroinflammation in *APPxPS1* males that the studies which have reported this have measured mRNA expression, while this thesis looked at protein expression. However, in the TAU58/2 mice, microgliosis in the cortex has only been reported at 10 months of age (van Ersel et al. 2015). Thus, suggesting that CBD's anti-inflammatory effects may not yet be evident in the TAU58/2 mice at the age tested in this thesis.

An *in vitro* study reported that CBD inhibited tau hyperphosphorylation in PC12 neuronal cells treated with Aβ (Esposito et al. 2006a). However, it is important to note that in this study, Aβ was administered inducing AD-like pathology and subsequently tau hyperphosphorylation. This brings to question whether in this study CBD is inhibiting tau pathology through a direct pathway, or if it rather prevents Aβ pathology with follow-on effects on the production of tau. Interestingly, GSK-3β is one of the enzymes involved in tau production (Vallée et al. 2017) and at least one study suggested that CBD impacts on tau pathology through the reduction of GSK-3β (Esposito et al. 2006a).

However, it is also possible that CBD may be indirectly reducing tau pathology in the study by Esposito and colleagues by decreasing Aβ levels which results in reduced GSK-3β expression as well (Vallée et al. 2017). In addition, as Esposito and coworkers administered CBD at the same time as Aβ (Esposito et al. 2006a), it is also possible that CBD actually prevented the development

of tau pathology rather than reversing it. Importantly, a study in gingival mesenchymal stem cells (GMSC)s found that CBD pre-treatment resulted in the downregulation of genes which code for kinases involved in the production of aberrant tau as well as secretases involved in Aβ generation (Diomede et al. 2017). These findings suggest that CBD does have the ability to alter tau pathology directly.

Importantly, no *in vivo* studies have investigated the effect of CBD on a tauopathy mouse model to this date. However, a study in a mouse model for frontotemporal dementia and parkinsonism found that Sativex® (CBD-THC combination) reduced soluble and phosphorylated tau pathology as well as neurofibrillary tangles. Sativex® treatment also reduced stress, aggression, and stereotypy but not motor impairments in this model (Casarejos et al. 2013). This suggests that the motor impairments in tauopathy mouse models may not be driven by tau pathology, as the reduction of tau did not have beneficial effects on motor deficits (Casarejos et al. 2013).

Furthermore, it is possible that the discrepancy of CBD's effects in the *APPxPS1* mouse model compared to the TAU58/2 model may be due to CBD being able to reverse cognitive deficits, but not motor impairments. There have been limited studies investigating the ability of CBD to reverse motor impairments. However, the results from this thesis found that chronic CBD treatment (50 mg/kg) was unable to reverse motor impairments in various tasks, including accelerod, pole test and challenge beam. In addition, the study above found that chronic CBD-THC treatment was also unable to reverse motor impairments in a different tauopathy mouse model (Casarejos et al. 2013). Importantly, it appears that CBD can be effective when administered in a preventative regimen. Acute CBD treatment (1 mg/kg) was able to prevent hypoxia-ischemia induced motor function impairments in accelerod when given 10 min after hypoxia-ischemia and tested 30 days later (Pazos et al. 2012). Another study where CBD was given before and 3 h after ischemic injury

reported that 3 mg/kg CBD partially prevented the development of motor impairments on the accelerod (Hayakawa et al., 2008). Therefore, suggesting a preventative treatment strategy may be more effective in treating motor impairments in TAU58/2 mice. It is evident that there is a need for further research into the degree of CBD interacting with tau pathology and whether this interaction is pronounced enough to have therapeutic-like effects, i.e. reversal of AD-relevant behavioural deficits, or whether a preventative treatment strategy is necessary.

6.2 Limitations and future research

6.2.1 Sex specificity

One of the limitations of this study is that all experiments were conducted in male mice. It is important to note that the mouse models tested develop sex-specific phenotypes, for example, a notable difference is that in the *APPxPS1* mouse model female mice do not show a social recognition memory deficit but rather spatial memory deficits (Cheng et al. 2013; Cheng et al. 2014b). *APPxPS1* females were also found to develop Aβ pathology earlier and more extensively than male *APPxPS1* mice (Wang et al. 2003). TAU58/2 females were found to develop motor deficits in accelerod later than males, but not in other motor or behavioural tests. Furthermore, they have been found to develop tau pathology later than males (van Ersel et al. 2015). As discussed in Section 1.1.2, AD is more common in women and studies have demonstrated that there are differences in disease progression between women and men, with a later onset of clinical symptoms in women but acceleration of cognitive decline once symptoms appear (Ferretti et al. 2018). Therefore, the gender differences seen in the mouse models are highly relevant to the clinical situation. Investigating the effect of CBD in female *APPxPS1* and TAU58/2 transgenic

mice was outside the scope of this thesis. However, the therapeutic benefits of CBD should be determined in female mice in future studies.

6.2.2 Remedy versus prevention strategy

This thesis focused on remedial treatment strategies, i.e. investigating the effect of CBD treatment on behavioural and neuropathological deficits of AD transgenic mice after the onset of AD-relevant symptoms. Research articles suggest that early therapeutics may be more effective in AD patients and may even prevent the development of the disease (Iuvone et al. 2009; Riedel 2014). Furthermore, previous studies in our laboratory found that a preventative study of 20 mg/kg CBD administered orally for 8 months prevented the development of social recognition memory deficits in *APPxPS1* males, with moderate effects on neuroinflammatory cytokines (i.e. TNF-α and IL-1β) (Cheng et al. 2014c). Thus, it would be highly relevant for future studies to investigate the preventative effect of higher doses of CBD, to determine whether a higher dose in a preventative treatment regimen would have a pronounced effect on the neuropathology of AD transgenic mice. As mention previously, CBD treatment commencing immediately after insults appears to prevent the development of motor impairments (Magen et al. 2009; Peres et al. 2016). Therefore, it would be interesting to conduct preventative studies on the effect of various doses of CBD in both the *APPxPS1* and the TAU58/2 mouse models to determine if an earlier treatment paradigm could prevent the development of motor and behavioural impairments. This is in line with current research indicating that in order for AD therapeutics to be effective they must begin early on in the disease progression (Iuvone et al. 2009; Riedel 2014). However, in order for this to be translated into the clinic early markers of AD will need to be identified.

6.2.3 CBD-THC combination treatment/ entourage effect of cannabinoids

There is increasing evidence demonstrating that CBD-THC combination treatments are more effective than either cannabinoid alone. Although THC as a therapy can be problematic because of its association with negative psychoactive and cognitive effects, studies have demonstrated that CBD can block this activity (Fadda et al. 2004; Chung et al. 2019). The underlying mechanisms of how CBD is able to attenuate the negative side effects of THC is not entirely clear. Some studies have suggested that it may be through CBD acting as a negative allosteric modulator at the CB1 receptors (Chung et al. 2019; Laprairie et al. 2015). In contrast, other research indicates that it may be through indirectly antagonising THC via mechanisms independent of CB1 for example via the TRPV1, A_{2A} and 5-HT1A receptors (as reviewed in McPartland et al. 2015). In addition, recently THC has been found to even have beneficial effects on cognition in aged mice (Bilkei-Gorzo et al. 2017). A study in *APPxPS1* mice found that when administered separately, CBD and THC were both able to reverse an object recognition memory deficit. However, only when given together did they reverse learning deficits in the active avoidance test as well and only the combination treatment had a beneficial effect on Aβ levels (Aso et al. 2014). In addition, in the aforementioned mouse model of frontotemporal dementia and parkinsonism, the combination of CBD and THC was able to reduce Aβ and tau pathology, oxidative stress, and reverse behavioural abnormalities including stress, aggression, and stereotypy (Casarejos et al., 2014). This phenomenon of combination treatments being potentially more effective than purified CBD is thought to be due to a possible 'entourage effect' of cannabinoids, which refers to the tendency of cannabinoids to provide greater therapeutic potential when administered together as they target different signalling pathways (Nahler et al. 2019). Further research should investigate if CBD-THC combination

treatments or CBD-enriched cannabis extracts would have additional effects on AD-relevant behavioural impairments and brain pathology.

6.3 Significance of outcomes

The results from this thesis indicate that chronic CBD treatment is able to reverse social recognition memory and reversal spatial learning deficits in male *APPxPS1* transgenic mice at early and late stages of the disease (i.e. 7.5 and 12 months of age). In addition, in 12-month-old *APPxPS1* males 50 mg/kg CBD tended to reduce insoluble $A\beta_{40}$ levels in the hippocampus. The ability of CBD to reverse these cognitive deficits is clinically relevant as the loss of facial recognition and spatial disorientation are common symptoms of AD, emphasising the translatability of CBD to a potential AD therapeutic. In addition, the studies in this thesis indicate that chronic treatment with 50 and 100 mg/kg CBD was well tolerated in AD transgenic mice and did not result in adverse effects. This finding is in line with previous studies which have indicated that CBD is well tolerated not only in AD transgenic mouse models (Aso et al. 2014; Cheng et al. 2014a; Cheng et al. 2014c) but also in clinical trials (Boggs et al. 2018; Laux et al. 2019). Importantly, CBD has been approved as Epidiolex for the treatment of seizures caused by Dravet syndrome and Lennox-Gastaut syndrome and has been approved in combination with THC as Sativex® for the treatment of spasms in multiple sclerosis (Laux et al. 2019). As CBD has been investigated in previous clinical trials, the translation of preclinical research of CBD as a novel AD therapeutic could be realised relatively quickly. Importantly, based on the initial research carried out in this thesis, chronic CBD treatment did not appear to reverse behavioural deficits in the TAU58/2 transgenic mouse model. This highlights the need for further research into

determining cognitive deficits in the TAU58/2 mouse model and the evaluation of varying doses of CBD and preventative treatment regimens to understand the role of CBD on tauopathy.

Chapter 7. Major Conclusions

In conclusion, the studies from this thesis found that male *APPxPS1* mice demonstrate social recognition memory deficits at 7.5 months of age and reversal spatial learning deficits at 12 months of age. Chronic CBD treatment at doses of 50 and 100 mg/kg were able to reverse these deficits. In addition, chronic CBD treatment at a dose of 50 mg/kg tended to reduce insoluble $A\beta_{40}$ pathology in the hippocampus. However, chronic CBD treatment did not affect markers of neuroinflammation and neurodegeneration at the ages and doses tested. These findings highlight the therapeutic benefits of CBD treatment in AD, but the mechanisms involved in these effects of CBD require further investigation. The outcomes of this study are highly relevant to the clinical setting as loss of facial recognition and spatial disorientation are common cognitive deficits seen in AD patients. Importantly, both doses of CBD were well tolerated by the mice and devoid of adverse effects, further emphasising the translatability of CBD into the clinic. This thesis also found that 4-month-old TAU58/2 males demonstrated a number of behavioural impairments, including reduced sociability, impaired sensorimotor gating and motor impairments, all of which are symptoms in AD patients, supporting its relevance as a novel tauopathy mouse model of AD. Although, at 4 months of age TAU58/2 males did not demonstrate cognitive deficits in the domains tested. Despite providing evidence for the beneficial effects of CBD in male *APPxPS1* mice, this thesis did not find that CBD was effective in reversing behavioural deficits in 4-month-old TAU58/2 transgenic mice. Aβ and tau pathology are both crucial in AD pathogenesis, thus there is a need for further research to investigate if CBD is effective in treating behavioural deficits in tauopathy mouse models, in particular its effects on cognitive deficits. In summary, the results from this thesis indicate that CBD demonstrates therapeutic benefits on cognition, yet the underlying mechanisms involved and the role of CBD on tauopathy require further investigation.

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