Investigating the Effects of FTY720 on Spatial Memory and Neuroinflammation in a Rodent Model of Alzheimer's Disease

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List of Abbreviations

AC	Acid ceramidase
AChE	Acetlycholinesterase
ACM	Acid sphingomyelinase
AD	Alzheimer's disease
ADAS-COG	Alzheimer's disease assessment scale-cognitive
ADL	Activities of daily living
APoE4	Apolipoprotein E4
APP	Amyloid Precursor Protein
AQP4	Aquaporin 4
Aβ	Amyloid β
BACE1	APP cleaving enzyme-1
BBB	Blood-Brain Barrier
BIN1	Bridging integrator 1
BPSD	Behavioural and psychological symptoms of dementia
CAMKIIα	Calcium-calmodulin kinase IIα
CAMKIV	Calcium-calmodulin kinase IV
cAMP	cyclic Adenosine monophosphate
CaN	Calcineurin
CDK5	Cyclin-dependent kinase 5
chAT	Choline acetlytransferase
CREB	cAMP response element binding protein
DAMP	Damage-associated molecular pattern
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular-signal-related-kinase
fAD	familial Alzheimer's Disease
FTLD	Frontotemporal Lobar Dementia
GFAP	Glial Fibrillary Acidic Protein
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3-β
HDAC	Histone deacetylase inhibitor
Iba1	Ionized calcium-binding adaptor molecule 1
iNOS	inducible nitric oxide synthase
LPC	Lysophosphatidylcholine
LTD	Long-term depression
LTP	Long-term potentiation
MAC	Membrane attack complex
MAPK	Mitogen-associated protein kinase
MAPT	Microtubule associated protein tau
MBP	Myelin Basic Protein
MCI	Mild cognitive impairment
MHCII	Major Histocompatibility Complex II
MOG	Myelin oligodendrocyte glycoprotein
MWM	Morris Water Maze

NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NOD	Nucleotide oligomerization domain
NOL	Novel Object Localization
NSAID	Non-steroidal anti-inflammatory drugs
nSMase	neutral sphingomyelinase
OF	Open Field
OPC	Oligodendrocyte precursor cell
PET	Positron Emission Tomography
PHF	Paired helical filaments
PLP	Myelin proteolipid protein
PSEN1/2	Presenilin 1/2
RAGE	Receptor for advanced glycation end-products
RRMS	Relapsing-remitting multiple sclerosis
RT-qPCR	Real-time quantitative polymerase chain reaction
S1PR	Sphingosine 1-phosphate receptor
sAD	sporadic Alzheimer's Disease
SSRI	Selective serotonin reuptake inhibitor
TNFα	Tumour necrosis factor α
TRAF2	TNF receptor associated factor 2
TREM 2	Triggering receptor expressed on myeloid cells 2
VDCC	Voltage-dependent calcium channel
WT	Wild-type

Scientific Abstract

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disorder characterized by memory loss and cognitive decline, with loss of global functioning over time eventually leading to death. At the molecular level, it is recognised that the aberrant aggregation and accumulation of both β-amyloid 1-42 into fibrillar plaques, and hyperphosphorylated tau into neurofibrillary tangles are the two major contributors to the axonal degeneration, synapse loss and eventual neuronal cell death that are characteristic of disease pathology. Current therapies for AD are symptomatic and seek to redress the neurotransmitter imbalances that contribute to memory-cognitive deficits, but cannot stop or reverse disease progression. More recently, CNS neuroinflammation in AD has been recognised as a third major branch of pathology contributing to neuronal cell death. Chronic activation of glial cells confers a pro-inflammatory phenotype in the CNS, propagating the release of pro-inflammatory cytokines and further glial activation. Therefore, immune modulation in the CNS constitutes a promising therapeutic target for the amelioration of the inflammatory profile in AD. FTY720, an immunomodulatory pro-drug used for relapsing-remitting multiple sclerosis, is a potent sphingosine 1-phosphate receptor (S1PR) agonist. S1PRs have been found to be expressed on all major glial cell types, constituting a prime target for FTY720-mediated modulation of the activity of these cell types.

We utilised the McGill-R-Thy1-APP rat model of AD to assess the effects of FTY720 on spatial memory and the neuroinflammatory profile of AD. At 6 months of age, rats were orally treated with 1mg/kg FTY720 for 6 months, before subjection to Morris Water Maze (MWM), Novel Object Localization (NOL) and Open Field (OF) behavioural assessments, with sacrifice at 12 months. FTY720 was found to improve spatial memory in AD animals in the MWM but not the NOL task, and no changes in locomotion or anxiety-like behaviour were observed in the OF. Amyloid precursor protein (APP) expression was found to be significantly increased in both the hippocampus and cerebellum, which was attenuated by FTY720. Western blot analysis of glial fibrillary acidic protein (GFAP), vimentin and ionized calcium-binding adaptor molecule 1 (Iba1) astrocyte and microglial markers in the hippocampus showed no significant difference in the expression levels of these proteins between wild-type (WT) and APP animals, and FTY720 also had no significant effect on their expression. Expression levels of myelin basic protein were found to be increased in WT animals with FTY720 treatment, but not in transgenics, while there was found to be no effect of genotype or treatment in myelin oligodendrocyte glycoprotein expression. Taken together, these results suggest that FTY720 can ameliorate behavioural aspects of AD *in vivo* but its effect on molecular indicators of reactive gliosis is inconclusive.

Lay Abstract

Alzheimer's disease (AD) is a progressive, age-related disorder of the brain which is characterized primarily by memory loss and a decline in a person's cognitive ability i.e. ability to understand and process information. The exact cause or combination of causes of the disease are not yet fully understood, but it is thought that the incorrect processing of a protein known as the amyloid precursor protein (APP) leads to the production of a smaller protein known as amyloid β (Aβ) 1-42. Aβ 1-42 is very sticky and accumulates in the brain into structures known as plaques, causing the death of the primary brain cell type, the neuron, in areas important for learning and memory such as the hippocampus and cortex. Another protein known as tau is also thought to contribute to neuronal cell death in AD, and activating this protein (hyperphosphorylation) is thought to lead to the destruction of the processes emitted from neurons which allow them to communicate, known as the synapse, or nerve terminal. Current therapies are only useful for treatment of disease symptoms but cannot stop the progression of the disease. More recently, brain inflammation as a result of the activation of two types of brain cell collectively known as 'glia', but individually known as astrocytes and microglia, has been thought to worsen disease progression. In the short-term, activation of these cells in response to Aβ is beneficial, as these cells can 'eat' the Aβ, clearing it from the brain. However, eventually these cells reach an Aβ-digesting plateau where they can no longer consume any more Aβ, but they are still activated, so it is after this chronic stage of activation that they begin to release a number of small proteins known as cytokines and chemokines, that message to other glial cells to become active, or to undergo programmed cell death (apoptosis). We hypothesised that an immune-response altering drug known as FTY720 that activates a group of cell-surface receptors known as the sphingosine 1-phosphate receptors could reduce the overactivation of these glial cells, and as a result, improve spatial memory and protect against the inflammation-mediated loss of the protective membrane covering neuronal processes, known as the myelin sheath, in genetically modified AD rats. We found that spatial memory in the Morris Water Maze memory task was improved in FTY720-treated AD animals, but not in the Novel Object Localization test. We also found that FTY720 can reduce the expression of APP in both the hippocampus and cerebellum, but does not affect inflammatory markers of astrocytes and microglia. Finally we found that FTY720 increases expression of the myelin protein myelin basic protein in non-AD animals, but not AD animals. Taken together, these results suggest that FTY720 can reduce the Aβ burden and improve spatial memory, but cannot reduce inflammatory glial activation.

Aims and Hypothesis

We hypothesize that FTY720 can improve spatial memory in APP transgenic rats, while attenuating neuroinflammatory markers of reactive gliosis and de-myelination in the hippocampus and cerebellum.

Based on this hypothesis, the aims of the study can be classified as such:

- To investigate the effects of FTY720 treatment on animal behaviour, namely spatial memory, locomotor activity and anxiety-like behaviour as tested by the Morris Water Maze (MWM) Novel Object Localization (NOL) and Open Field (OF) analyses respectively.
- To identify the effect of FTY720 on amyloid precursor protein (APP) expression, as well as on markers of neuroinflammation in the hippocampus i.e. astrocyte and microglial activation, as well as demyelination via western blot.
- To examine the effect of FTY720 on gene expression of APP and neuroinflammatory/myelination markers in the cerebellum via real-time quantitative polymerase chain reaction (RT-qPCR).

Value of Research

Alzheimer's disease (AD) has a high prevalence worldwide and accounts for about 60-80% of dementia cases, so of approximately 45-50 million people globally living with dementia, around 60-80% have Alzheimer's dementia. It is estimated that without a cure, the number of people afflicted by AD could increase to around 150 million worldwide by the year 2050, which highlights both the scope of the problem, and the requirement for the development of interventional therapy. Current AD therapies attempt to redress neurotransmitter imbalances such as those of acetylcholine and glutamate attributed to the loss of specific neuronal subpopulations, but these therapies only focus on symptomatic AD and cannot halt or slow clinical course of the disease. AD patients experience significant progressive negative impact on activities of daily living, with near total loss of declarative memory and loss of global functioning rendering patients in need of full-time care, unable to carry out a wide range of activities that were once effortless independently of assistance.

The aetiology of AD is the subject of much debate; 95%+ of AD presentations are comprised of sporadic AD (sAD) cases, with the major risk alleles for sAD including the microglial TREM2 mutation or astrocytic APoE4, but it is accepted that Aβ is a key player in both familial and sporadic AD. More recently, CNS neuroinflammation has been identified as another significant branch of pathology in AD. Given that S1PRs are expressed on astrocytes and microglia, the key perpetuators of inflammation in AD, we believe that targeting these receptors with the immunomodulatory S1PR agonist/functional antagonist S1PR1 constitutes a viable therapeutic approach toward the amelioration of neuroinflammation in AD. Though there are some studies highlighting the beneficial effects of FTY720 on neuroinflammation through activation of CNS resident glial S1PR1s, there has yet to be any research done on the effect of FTY720 on CNS myelination state in AD, and there has yet to be any research carried out with the single transgenic McGill APP rat model of AD, which is one of the best models available for recapitulating human AD phenotype without producing the excessively severe phenotype observed in the triple-transgenic or 5xFAD mouse models of the disease. This study aims to identify whether long-term FTY720 treatment can improve spatial memory, reduce markers of inflammation and positively affect markers of myelination in the CNS in aged AD rats.

Outputs

Papers

Bechet, S.**,** **Davison, L.,** Dev, K.K. Systematic Review and meta-analysis of the efficacy of Sphingosine 1-Phosphate (S1P) receptor agonist FTY720 (fingolimod) in Animal Models of Alzheimer's disease (in preparation).

Introduction

1. Characterization of Alzheimer's Disease

1.1 Alzheimer's Disease Aetiology.

Alzheimer's disease (AD), first characterized by Alois Alzheimer in 1907, is a chronic, progressive neurodegenerative disorder that contributes to around 60-70% of the incidence of dementia, an age-related disorder of memory and cognition (Winblad et al. 2016).The exact cause of AD is poorly understood, but its incidence is believed to be multi-factorial with a number of common risk alleles for the disease already identified in tandem with environmental factors, but disease aetiology is largely unknown for the more common late-onset form of the disease. The aggregation and accumulation of a 42-amino acid protein known as Amyloid-β (Aβ) in areas of the brain important for memory and cognition such as the hippocampus and cortex is thought to be the triggering event in AD, and is described by the 'Amyloid Hypothesis'(Hardy and Selkoe 2002). However, it is also accepted that intracellular hyperphosphorylation of the microtubule-associated protein Tau into Neurofibrillary Tangles (NFTs) is an important disease contributor in terms of axonal degeneration and synapse loss, which are thought to precede cognitive decline (Gong and Iqbal 2008). More recently, neuroinflammation caused by glial overactivation in the AD brain has been considered a third major contributor to neuronal cell death (Heneka et al. 2015). It is estimated that without a cure, the number of those affected by AD could increase to about 150 million globally by 2050, underpinning the urgency with which an effective novel therapeutic is required (Anand, Gill and Mahdi 2014).

It is recognised that AD may be divided into two separate classes depending on the age of onset of cognitive symptoms. The early-onset form, which is known as familial AD (fAD), accounts for only 5% of all AD cases and occurs as the result of mutations in genes designated for the production of proteins involved in the cleavage of the amyloid precursor protein (APP), which subsequently drives the production of the aggregation-prone Amyloid-β42 (Aβ) isoform over the non-amyloidogenic Aβ40 isoform (Zhu et al. 2015). These proteins are known as the presenilins (PSEN1 and 2) and form the catalytic subunit of the γ-secretase enzyme (Thinakaran 1999). fAD is understood to present in affected individuals below the age of 65, but may develop as early as 40 years old, particularly in individuals with Trisomy 21 (Down's Syndrome), who near-universally develop fAD due to the presence of a third chromosome 21 and by extension, a third copy of the *app* gene, conferring a high risk of over-expression of APP (Rossor et al. 2010, Geller and Potter 1999).

Late-onset or 'sporadic' AD (sAD) is far more common than fAD, accounting for 95% of all cases of AD. The incidence of sAD increases with increasing age, as only 15% of people aged 65-74 develop the disease compared to 44% at 75-84 years in the USA, with women affected twice as much as men (Association 2016). Though genes are not believed to play a leading role in sAD, they do confer an increased risk of a particular individual acquiring the disease, with common risk alleles for AD including the ε4 form of the Apolipoprotein E (APoE4) lipid carrier (increasing risk of AD by 3-5 times), bridging integrator 1 (BIN1), and identified most recently, the triggering receptor expressed on myeloid cells 2 (TREM2), as its R47H variant is reported to increase risk of late-onset AD by 2-3 times (Guerreiro et al. 2013, Zhang et al.

2018). Other risk factors for sAD include head trauma, depression and hypertension (Schwarzbold et al. 2008).

1.2 Clinical Presentation and Diagnosis of Alzheimer's Disease.

While a number of cognitive and memory-related symptoms are characteristic of patients presenting with AD, these symptoms may first appear in an individual prior to the onset of AD in a milder form, known as Mild Cognitive Impairment (MCI). MCI is thought to constitute prodromal AD in the presence of raised Aβ levels, with increased hippocampal neuronal activity as a result of Aβ deposition being predictive of AD in MCI patients (Huijbers et al. 2015). MCI is a condition in which affected persons experience a mild but noticeable (measurable) change in cognitive function with little impact on activities of daily living (ADL). Approximately 15-20% of over 65s have MCI (Roberts and Knopman 2013). MCI patients have been shown to be more likely to develop AD or other dementias than those without, highlighting MCI as an important indicator for the potential development of late-onset AD (Kantarci et al. 2009). A systematic review of 32 MCI studies found that 32% of MCI individuals presented with AD within 5 years of MCI diagnosis (Ward et al. 2013).

Clinical symptoms of AD vary from patient to patient and are dependent on a patient's disease stage, (i.e. early or late) as well as patient's genotype with respect to risk alleles. However, there are six main clinical symptoms that are common to the majority of individuals affected by AD, namely; memory deficits, language impairment, difficulties in visuospatial and executive functioning as well as altered attention and affect (Gold and Budson 2008). Of these six, memory impairment is recognised as the most likely indicator of a potential incidence of AD. Decline or impairment in episodic memory i.e. the explicit, declarative recall of events or facts about one's life or life experience, where these events occurred and when, is one of the earliest symptoms of AD (Sperling et al. 2011). Coupled with episodic memory loss is difficulty in creating new short-term episodic memories e.g. difficulty in remembering recent conversations. Behavioural symptoms such as apathy and co-morbid depression are also common early symptoms of AD (Association 2016). As the disease progresses, patients experience increased symptoms of confusion, irritability, aggression, language difficulty, long-term memory loss, dysarthria, dysphagia and global motor deficits (Waldemar et al. 2007). Less than 3% of AD sufferers survive more than 14 years post-diagnosis (Mölsä, Marttila and Rinne 1995).

Diagnosis of AD can be divided into two main streams – diagnosis based on the profile of clinical symptoms that an individual presents with, or diagnosis via measurement of clinical biomarkers of AD. The first set of guidelines for diagnosis of AD based on clinical symptoms were laid-out in the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), followed by guidelines published by the National Institute for Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) in 1984, which stated that a patient had 'probable AD' if they presented with short- and long-term memory impairment and one of; aphasia, apraxia, agnosia or executive functioning disturbance (Cohen 2013). The NINCDS-ADRDA guidelines recognized that ultimate

diagnosis of AD was dependent on neuropathology identified at autopsy, as a number of other dementias present with similar subsets of symptoms.

Early diagnosis of AD through analysis of disease-specific biomarkers has been recognised as vitally important for the improvement of AD prognosis, given the progressive and irreversible nature of the disease. Positron Emission Tomography (PET) scanning is used to identify pathological glucose metabolism (hypometabolism – flurodeoxyglucose - PET) in the brain, which has been shown to be the most accurate discriminator between AD individuals and those with other forms of dementia (Bloudek et al. 2011). PET can also be used to detect the presence of Aβ fibrils through radio-ligand binding of Aβ40 and 42 isoforms, the extent of which can be used as a measure of the extent of pathology in AD patients (Marcus, Mena and Subramaniam 2014). Obtaining CSF for the analysis of AD biomarkers is a more invasive method of AD diagnosis but can be used to detect levels of Aβ42 and phosphorylated tau in the cerebral lymphatic system. While the amount of Aβ in the CSF does not correlate well with the incidence of AD, it has been found that Aβ42 is decreased in the CSF of AD patients, which is thought to be due to the deposition of Aβ in plaques, reducing the levels of circulating Aβ (Motter et al. 1995). Tau protein (total tau), has been shown to increase in AD patient CSF and correlates with neurodegeneration in AD but cannot separate AD from other tauopathic dementias (Johnson-Wood et al. 1997, Vandermeeren et al. 1993, Formichi et al. 2006). More recently an initiative known as the Alzheimer's Disease Sequencing Project has sought to perform whole-genome and whole-exome sequencing on 1400 families with histories of late-onset AD to identify the expression of risk alleles before the onset of disease, and holds great promise for earlier diagnosis of AD in the future (Beecham et al. 2017).

1.3 Mechanisms of Neuropathology in Alzheimer's Disease.

It has been well-characterized that there are a number of pathological hallmarks of AD that contribute to disease progression, but the most commonly associated pathological indicator of the disease is the overproduction and deposition of Aβ42 protein into extracellular neuritic plaques in the CNS, principally in areas of the brain associated with learning and memory, as well as executive functions – the hippocampus, cortex and sub-cortical regions. Aβ is a 4kDa protein produced by proteolytic cleavage of a ~100kDa neuronal cell-surface glycoprotein known as the amyloid precursor protein (APP)(Guo et al. 2012).The precise function of APP has not been fully elucidated, but a number of *in vivo* studies have suggested that APP may have a number of trophic functions for neurons, including cell growth, motility, cell survival and neurite out-growth, which may be mediated either by the full-length protein, or by the cleaved, soluble ectodomain (Young-Pearse et al. 2007, Oh et al. 2009).

APP is processed by a family of proteolytic enzymes known as the secretases, which are responsible for driving the production of the two most common forms of Aβ, Aβ40 and Aβ42, via sequential cleavage of APP through the α- and β secretase pathways respectively followed by γ-secretase-mediated cleavage (**Figure 1.1**). Aβ protein, when processed under normal physiological conditions, is responsible for cell survival (predominantly Aβ40), however, in AD, an imbalance between production and removal leads to a pathological accumulation of the protein, and generation of β-sheet fibrillar plaques (Plant et al. 2003). While the presence of plaque pathology has long been thought to be the major Aβ-related toxic insult in AD, more recent evidence has suggested that the

soluble, oligomeric intermediates produced prior to mature fibrils may be the toxic species mediating neuronal cell death (Lambert et al. 1998). It is thought that oligomeric Aβ-mediated cellular neurotoxicity is caused by interactions with glutamatergic receptors, voltage-gated calcium channels and GM1 gangliosides on neuronal membranes, producing reduced synaptic excitatory neurotransmission and loss of dendritic spines, calcium homeostasis disturbance and fibrillization of oligomeric Aβ species respectively (Shankar et al. 2008, Demuro et al. 2005, Hong et al. 2014). Aβ oligomers have also been shown to potently inhibit hippocampal long-term potentiation (LTP) through the removal of cell-surface AMPA receptors leading to synaptic depression and LTP inhibition (Hsieh et al. 2006).

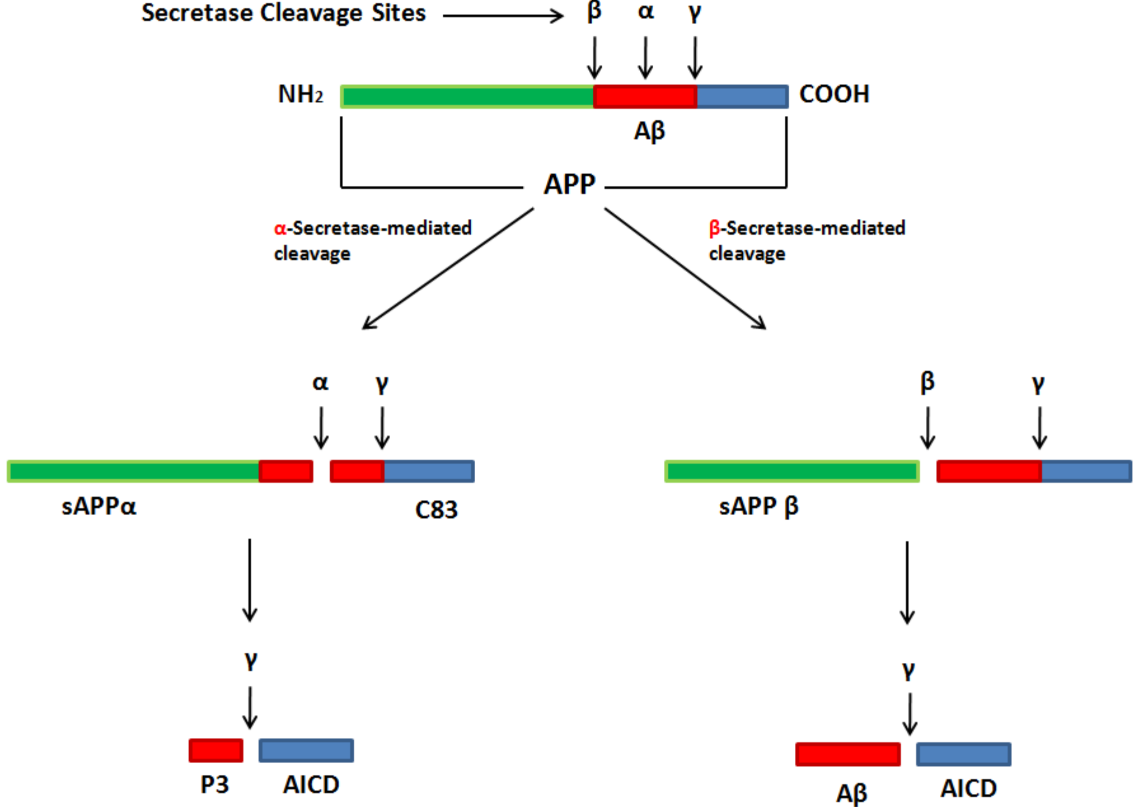


Figure 1.1 Processing of APP via β-secretase-mediated cleavage produces amyloidogenic product. APP cleavage is mediated by either the α-secretase or β-secretase initially, followed by γ-secretase mediated cleavage. Processing of APP along the β-secretase pathway produces Aβ protein with 42 amino-acid residues, which has a high capacity to aggregate, compared to the non-amyloidogenic 40 amino-acid protein that is produced as a result of α-secretase-mediated cleavage (Zhu et al. 2015).

It has also been shown that cholinergic neurons in the basal forebrain are particularly vulnerable to Aβ oligomer neurotoxicity, and that the degeneration of cholinergic synapses as a result of the presence of Aβ oligomers correlates with cognitive decline in AD patients (Wong et al. 1999, Terry et al. 1991). In conjunction with this, the remaining cholinergic neurons exhibit severely reduced choline acetlytransferase (ChAT) transcription, reducing the production of acetylcholine and therefore reducing forebrain cholinergic neurotransmission, which has been shown to be involved in memory and learning (Boccia et al. 2003, Fine et al. 1997). The interaction of Acetlycholine-esterases (AChE) with developing Aβ oligomers has also been identified as a source of Aβ-mediated cholinergic dysfunction, with a specific motif of AChE being capable of promoting amyloid formation and the synthesis of mature fibrils (De Ferrari et al. 2001).

The second major pathological hallmark of AD is the presence of intracellular neurofibrillary tangles (NFTS) which are composed of insoluble aggregates of the microtubule-associated protein tau (MAPT). Tau binds to neuronal microtubules and is responsible for cytoskeletal stabilization by promoting tubulin assembly (Duan et al. 2017). Tau is closely associated with microtubules, cytoskeletal structures that are responsible for providing a framework for the ATP-dependent activities of kinesin and dynein, axonal motor proteins designated for the transport of sub-cellular cargo such as mitochondria, endocytic vesicles and lysosomes to and from synapses respectively. Tau acts as an obstacle to physiological kinesin- and dynein-mediated cargo transport when aggregated (Chaudhary et al. 2018).

Ultrastructural studies on AD brain specimens have shown that NFTs are composed of paired-helical filaments (PHFs), pathological aggregates of tau oligomers that contribute to axonal degeneration and synapse loss in AD (Grundke-Iqbal et al. 1986). Hyperphosphorylation of tau by glycogen synthase kinase 3-β (GSK3β) is thought to mediate its detachment from microtubules, impairing axonal transport, thereby causing synaptic atrophy and neuronal cell death (Lucas et al. 2001). GSK3β has also been shown to reduce acetylcholine synthesis in the basal forebrain and mediate apoptosis, highlighting its potential role in the cholinergic deficit associated with memory loss in AD, and the apoptosis of cholinergic neurons (Hoshi et al. 1996, Turenne and Price 2001). As well as hyperphosphorylation, a number of post-translational modifications of tau protein have been shown to contribute to its potential to aggregate and become neurotoxic. C-terminal truncation of tau at aspartic acid residue D421 mediated by cleaved-caspase 3 in the hippocampus has been shown to produce a more aggregation-prone tau sequence that contributes to neuronal cell death and subsequent cognitive decline (Chung et al. 2001). Interestingly, a certain degree of crosstalk has been observed between hyperphosphorylated tau and Aβ, with cross-breeding of human APP transgenic mice and tau transgenic mice producing enhanced aggregation of tau, increased dendritic spine loss and accelerated cognitive decline compared to tau-only transgenics, highlighting a synergistic effect between the two proteins (Chabrier et al. 2014).

Neuroinflammation has recently emerged as a third major contributor to AD pathogenesis, with glial over-activation in response to Aβ and NFT deposition conferring a pro-inflammatory phenotype in the CNS. CNS inflammation in acute-phase response has been shown to be neuroprotective, while chronic inflammation has been observed to be detrimental to brain health. A number of studies initially showed that there was a sustained inflammatory response

evident in the brains of AD patients (Akama and Van Eldik 2000, Combs et al. 2000, Griffin et al. 1995). Early studies involving the use of non-steroidal anti-inflammatory drugs (NSAIDs) suggested that these drugs may have a protective effect in AD, further consolidating the theory that inflammation plays a significant role in AD pathology (Beard et al. 1998, Rich et al. 1995). Inflammation is now routinely observed in post-mortem tissues of AD patient brain samples (Gomez-Nicola and Boche 2015, Hopperton et al. 2018). Interestingly, post-mortem examination of head-trauma patients has revealed an increase in cerebral Aβ deposits, which has been shown to be caused by production of the pro-inflammatory cytokine IL-1, which increases APP production for the generation of amyloidogenic Aβ42 (Goldgaber et al. 1989, Plassman et al. 2000). Elevated levels of the pro-inflammatory cytokine IL-1β has been shown to increase the production of other pro-inflammatory cytokines such as IL-6, which leads to activation of downstream kinases such as cyclin-dependent kinase 5 (CDK5), which is thought to be a contributor to the hyperphosphorylation of tau and NFT production (Quintanilla et al. 2004). It is evident from these studies that pro-inflammatory protein mediators are capable of driving both Aβ deposition and tau hyperphosphorylation, implicating chronic inflammation as a key driver of worsening AD pathology severity.

Blood-brain barrier leakiness has also been identified as a pathological by-product of Aβ-oligomer-mediated neurovascular unit disruption, reducing the expression of tight junction proteins ZO-1, claudin-5 and occludin and permitting the permeation of peripheral immune cells into the CNS where they contribute to further parenchymal inflammation (Wan et al. 2015). Both microglia and astrocytes are pathologically activated and accumulate around senile AD plaques, where they attempt to phagocytose the mature fibrillar Aβ that these plaques contain (Medeiros and LaFerla 2013). This leads to the activation of intracellular signalling pathways and the release of a battery of pro-inflammatory cytokines, chemokines and reactive oxygen species such as IL-6, Tumour Necrosis Factor α (TNFα) and inducible nitric oxide synthase (iNOS) that contribute to increased recruitment and activation of glial cells, thus propagating neuroinflammation. Complement is also a potent contributor to CNS neuroinflammation in AD. The Blood-Brain Barrier has been observed to increase in its permeability, becoming ‘leaky’ with the onset of neurodegeneration, and this increase in permeability permits the entry both of peripheral macrophages and a strongly pro-inflammatory factor known as complement, into the CNS. The cytotoxic effector of complement is known as membrane attack complex (MAC), which creates pathological transmembrane channels that disrupt cell membrane structure causing cell lysis, and the propagation of inflammation (Morgan 2018) (**Figure 1.2**).

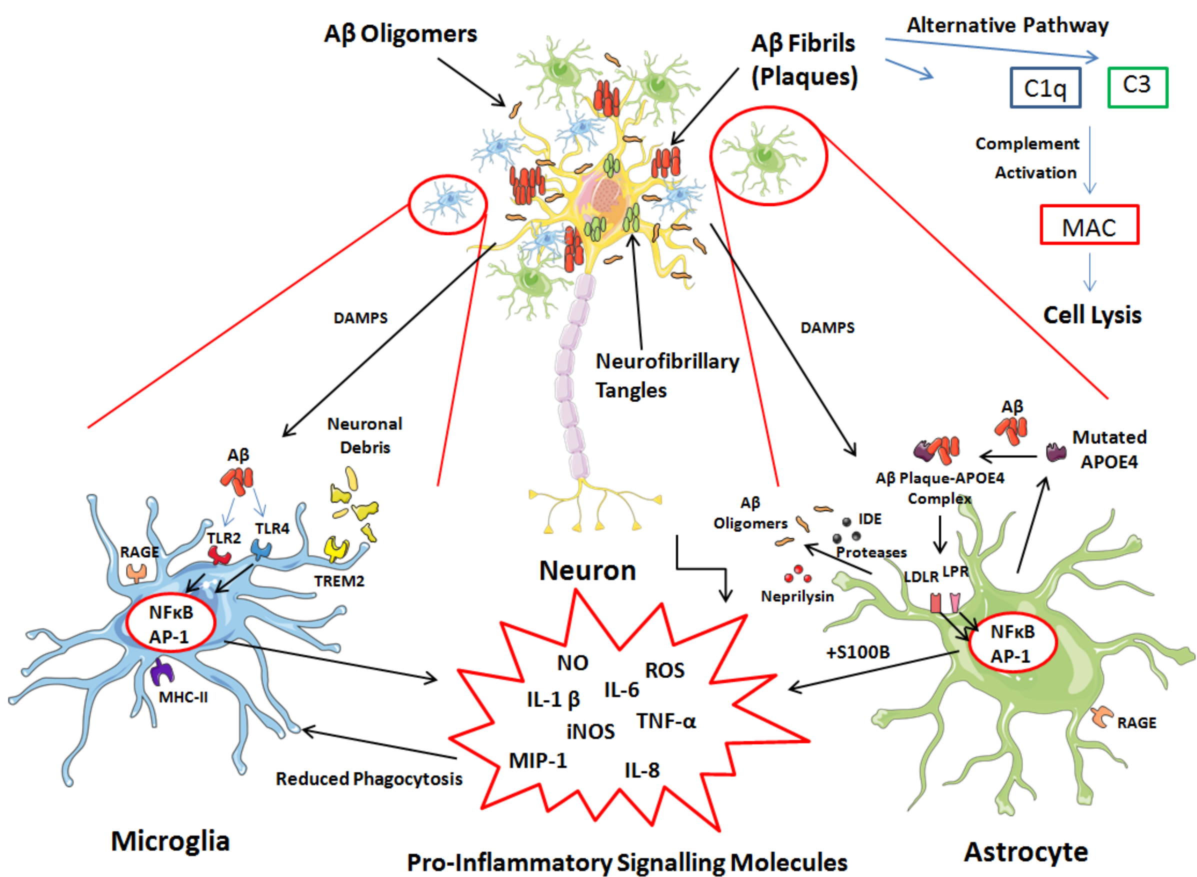


Figure 1.2 The pro-inflammatory reactive glial response to Aβ-deposition and Tau hyperphosphorylation in the CNS. The presence of p-Tau, soluble oligomeric Aβ and mature fibrillar Aβ deposited in plaques is highly neurotoxic and leads to the release of neuronal DAMPs, which bind to astrocytic and microglial receptors, causing their activation, chemotaxis to the site of damage, and phagocytosis of Aβ. A battery of pro-inflammatory cytokines is concurrently released, which encourage further glial activation, but ultimately lead to reduced phagocytosis of toxic aggregated protein. Neuronal cell death may also occur via the alternate complement pathway which leads to complement activation and subsequent production of the membrane attack complex (MAC), ultimately lysing cells (Heneka et al. 2015).

1.4 Alzheimer's Disease Therapeutics.

Given the high incidence of AD and its potential to increase significantly over the next 30 years in terms of economic and societal burdens, there is a need to develop novel therapeutics and methods by which identification of affected persons can occur either in early-stage disease, or optimally, before the presentation of clinical symptoms (Jia et al. 2018, Mancuso et al. 2011). Current therapeutics on the market are palliative, with modest efficacy and a number of side effects. These drugs focus primarily on restoring the basal levels of neurotransmitters involved in cognitive function, memory and learning that are reduced in AD, but cannot reverse the clinical course of AD.

The current standard of care is the oral administration of acetylcholinesterase (AChE) inhibitors, whose function is to increase the amount of available acetylcholine at cholinergic synapses, which are largely depleted by cholinergic cell death in the Nucleus Basalis of Meynert in the basal forebrain in AD (Ferreira-Vieira et al. 2016). This neurotransmitter imbalance is attributed to deficits in cognitive functioning and memory in AD. Tacrine - first developed as a partial antagonist to morphine to alleviate withdrawal symptoms - was the first AChE inhibitor approved for AD in 1993, however it was removed from the market due to significant hepatotoxicity and gastrointestinal side effects (BRODIE, WAY and LOWENHAUPT 1952, Alfirevic et al. 2007).

The three primary AChE inhibitors in clinical use are rivastigmine (Exelon®, Novartis, Basel, Switzerland) donepezil (Aricept®, Pfizer, New York, USA) and galantamine (Razadyne®, Jansen, Beerse, Belgium) (Farlow 2002). A number of double blind, randomized, placebo controlled trials of these three drugs showed improvements in cognitive and global functioning of patients with mild to moderate AD, without significant differences in efficacy between the three (Birks 2006). This was evaluated by improvements in AD Assessment Scale – Cognitive (ADAS-cog), behaviour and global clinical rating over 6-12 month periods. Improved symptoms included attention, thinking, language, communication and memory (Qaseem et al. 2008). Early administration of AChE inhibitor therapy has shown to improve cognitive profile when compared with delayed administration, as a 52 week study of rivastigmine efficacy in mild-to-moderate severity patients who began treatment 6 months after diagnosis versus immediately after diagnosis highlighted lower cognitive performance in the group whose treatment was delayed (Farlow et al. 2000).

For moderate to severe AD, the N-methyl-D-aspartate (NMDA) receptor antagonist memantine is the first-choice option, either as a monotherapy or in combination with AChE inhibitors, such as donepezil, which has been shown to be efficacious in the treatment of moderate-to-severe AD but not mild-to-moderate AD (Farlow et al. 2010, Howard et al. 2012). Memantine is a non-competitive, low-to-moderate affinity NMDA receptor antagonist that binds to NMDA-operated calcium channels (Kishi and Iwata 2013). More recently, it has been identified that memantine more potently inhibits extra-synaptic NMDARs over synaptic ones in the hippocampus (Xia et al. 2010). This purported mechanism of action is based on a hypothesis suggesting that synaptic NMDAR activation promotes cell survival, where extra-synaptic NMDAR activation promotes cell-death. This is likely to have implications for neurodegenerative disorders involving dysfunction in NMDAR signalling, with pro-survival vs apoptotic signalling not purely dependent on overall NMDAR activity, but via the degree of

synaptic vs. extra-synaptic activation (Hardingham and Bading 2010). In the Huntington's disease YAC128 model mouse for example, inhibition of extra-synaptic NMDARs via memantine was found to reduce striatal extra-synaptic NMDAR expression without affecting synaptic NMDA-mediated transmission, as well as inhibiting pro-apoptotic p38 MAPK phosphorylation (Dau et al. 2014). Extra-synaptic NMDAR antagonism is also highly relevant to the use of FTY720 as a neuroprotective therapeutic for AD, as a 2017 study has shown that FTY720 induces upregulation of GLUN2A-containing neuroprotective NMDARs on dendritic spines in cultured hippocampal neurons. FTY720 was also shown to induce the relocation of extra-synaptic GLUN2B-containing NMDARs to the synapse, altering extra-synaptic/synaptic NMDAR ratio and thus rendering hippocampal neurons less sensitive to Aβ-42 – mediated calcium homeostatic disruption (Joshi et al. 2017). This effect is likely mediated through S1PRs, particularly 1 and 3, as antagonism of these receptors was also shown to attenuate the neuroprotective effects of FTY720 from Aβ-42-mediated neurotoxicity. From a meta-analysis of nine studies of memantine monotherapy involving 2433 patients who met the inclusion criteria, it was found that cognitive function, behavioural disturbance, activities of daily living, global function assessment and stage of dementia scores were significantly improved compared to placebo in moderate to severe AD, and memantine is FDA approved for this indication (Matsunaga, Kishi and Iwata 2015).

While memantine and AChE inhibitors have proven to be efficacious therapeutics for treatment of symptomatic AD, they do not address the underlying pathological accumulation of aggregated or misfolded proteins in the form of oligomeric Aβ or hyperphosphorylated tau aggregates in AD. Similarly, therapeutics targeting the behavioural and psychological symptoms of dementia (BPSD), such as selective serotonin reuptake inhibitors (SSRIs) for co-morbid depression and atypical antipsychotics such as olanzapine and quetiapine for agitation/aggression, attempt to control co-morbidities but are not curative (Zec and Burkett 2008, Ballard and Corbett 2010). Disease-modifying approaches such as Aβ and tau immunization emerged around twenty years ago as a means by which pathological aggregates of these proteins may be targeted by antibodies and phagocytosed by glial cells in the brains of patients affected by mild-to-moderate AD. Both active immunization (vaccination) and passive immunization (monoclonal antibodies) are under investigation. After promising *in vivo* animal studies, the first active vaccination trial was undertaken in humans in 2002, using injections of Aβ1-42 (AN-1792) in conjunction with a T helper cell adjuvant (QS-21) (https://clinicaltrials.gov/ct2/show/NCT00021723). However, phase II of the trial was discontinued due to the occurrence of a rare side effect known as meningoencephalitis in 6% of patients receiving AN-1792. This was thought to be due to T-cell mediated autoimmunity (Gilman et al. 2005). It was also found that only 19.7% of patients receiving AN-1792 developed the desired antibody response.

Given the instance of rare side effects and variable antibody response in patients, passive immunization against specific amino acid residues of the Aβ1-42 protein was considered. The monoclonal mouse antibody 3D6 (bapineuzumab), recognizing Aβ1-5, entered phase III clinical trials in 2007, but in phase II trials was not found to meet the targets of efficacy established prior to the trial, and also produced vasogenic edema in a number of patients in the treatment group (Johnson-Wood et al. 1997). Efficacy was limited to ApoE4 non-carriers with mild-to-moderate AD, and bapineuzumab entered phase III clinical trials for this indication. Poor

correlation between the extensive amyloid clearance identified at autopsy, and the minimal improvement in clinical cognitive scores in mild-to-moderate AD as a result of Aβ immunization has called into question the legitimacy of the 'amyloid hypothesis' (Wisniewski and Konietzko 2008). Refer to **Table 1.1** for a summary of AD therapeutics.

Name	Target	Structure	Approval Status	Company	Reference
Donepezil	Acetylcholinesterase Inhibition		Approved	Eisai Inc., Pfizer, Inc.	(Graham, Bonito-Oliva and Sakmar 2017)
Galantamine	Acetylcholinesterase Inhibition		Approved	Johnson and Johnson Pharmaceutical Research & Development L.L.C.	(Graham, Bonito-Oliva and Sakmar 2017)
Rivastigmine	Acetylcholinesterase Inhibition		Approved	Novartis Pharmaceutical	(Graham, Bonito-Oliva and Sakmar 2017)
Memantine	NMDA Receptors Inhibition		Approved	H. Lundbeck A/S	(Graham, Bonito-Oliva and Sakmar 2017)
Riluzole	NMDA Receptors Inhibition		Phase II Clinical Trial	Icahn School of Medicine, Mount Sinai	(Graham, Bonito-Oliva and Sakmar 2017)
Semagacestat	Y-Secretase Inhibition		Phase III Clinical Trial **Discontinued**	Eli Lilly & Co.	(Graham, Bonito-Oliva and Sakmar 2017)
Verubecestat	BACE Inhibition		Phase III Clinical Trial	Merck & Co. Inc.	(Graham, Bonito-Oliva and Sakmar 2017)
AN-1792	Aβ Immunization	42-amino acid Aβ peptide	Phase II Clinical Trial **Discontinued**	Janssen Pharmaceuticals	(Graham, Bonito-Oliva and Sakmar 2017)
Bapineuzumab	Aβ Immunization		Phase III Clinical Trial **Discontinued**	Pfizer Inc.	(Graham, Bonito-Oliva and Sakmar 2017)

Table 1.1 Summary of first-line AD therapeutics and promising therapeutics that have been discontinued in the clinical phase.

1.5 Animal Models of Alzheimer's Disease.

In order to elucidate the molecular mechanisms of AD pathology and identify potential therapeutic drug candidates pre-clinic, the development of human AD-relevant *in vivo* animal models has been an area of great interest. There does not currently exist an animal model that can recapitulate all aspects of human AD, but there are many models, mainly rodent – murine and rat – that can mimic the amyloid and tau pathologies observed in human AD, with concomitant neuronal degeneration and memory/cognitive deficits, to varying degrees. The mouse is the most commonly used animal model due to the relatively low cost of maintenance, ease of breeding and genetic manipulation. Though the rat is not as commonly used as mouse for modelling of AD, it is genetically, physiologically and morphologically closer to humans than mice, has a larger brain size for ease of processing and analysis, and is more co-operative to handling than mice (Jacob and Kwitek 2002). However, as genetic manipulation of rats is more difficult than mice due to rat one-cell embryos having less visible pronuclei making transgene injection significantly more difficult, coupled with the fact that rat embryo survival rate is low following transfection, mice are the more commonly used organism (Charreau et al. 1996). Historically, other vertebrates used to model AD have included sea lamprey, zebrafish, sheep, pigs and dolphins, while invertebrates such as the fruit-fly and roundworm have also proven to be useful models (Götz and Ittner 2008).

Mouse models of AD can be broadly classified as being either transgenic, homology-directed recombination dependent (HDR - i.e. knock-in or knock-out of AD-relevant genes), and non-genetic, i.e. involving stereotaxic injection of synthetic Aβ1-42 aggregates into AD-relevant areas of the brain (Götz, Bodea and Goedert 2018). Wild-type mice do not develop Aβ plaques during the course of aging as humans do, and this is thought to be due to differences in 3 amino acids in the Aβ sequence (Dyrks et al. 1993). The generation of transgenic AD mouse models is based on the random integration of gene variants associated with human fAD – *APP, PSEN1* and *PSEN2* genes - for mimicking the APP overexpression and preferential generation of the Aβ1-42 isoform from γ-secretase-mediated cleavage of APP associated with amyloid pathology in AD. The first of the Aβ-plaque-generating transgenic AD mouse models expressed the familial V717F (Indiana) *APP* gene and the now commonly studied Swedish *APP* double mutation APP^{swe} (K670N/M671L) (Games et al. 1995) Single transgenic mice exhibiting tau pathology were first generated by expressing a mutated form of the gene for familial frontotemporal lobar dementia (FTLD) known as microtubule-associated protein tau (*MAPT*) gene (P301L mutation), which confers an overexpression of tau protein for NFT formation and associated frontotemporal cognitive deficits (Lewis et al. 2000).

Following the advent of single transgenic models, a number of multi-transgenic models for amyloid pathology were developed, the most common being the *APP*/*PS1* model involving co-injection of the APP^{swe} mutant of the *APP* gene and the ΔE9 mutant of the *PS1* gene, and the 5xFAD model, expressing 3 *APP* mutations and 2 *PSEN1* mutations conferring strong amyloid pathology (Jankowsky et al. 2001, Oakley et al. 2006). These models are capable of recapitulating key pathological hallmarks of human Aβ pathology, namely Aβ-plaque formation, reactive gliosis, neuronal cell death, synapse loss and cognitive decline in an age-dependent manner, however tau pathology is lacking. The 3xTg model is a combination triple-transgenic model which expresses mutations in *APP* (Swedish) and *MAPT* (P301L) in *PS1* (M146V) knock-in mouse oocytes and is one of the most widely used AD mouse models,

utilised for the study of the effects of Aβ-plaque deposition with concomitant frontotemporal tauopathy (though much later on in the disease process) on memory and cognition (Oddo et al. 2003). See **Table 1.2** for a summary of the most commonly-used mouse models of AD.

As previously eluded to, rats have a number of disadvantages compared to mice in terms of generating AD models, however, rats are physiologically and genetically more relevant to humans and display a wider and more complex array of behavioural phenotypes. The first of the transgenic rat models were developed in 2004 and similarly to early mouse models, they expressed either the human Swedish (K670N/M671L) and Indiana (V717F) *APP* mutations, or these two mutations together with the human Finnish (M146L) *PS1* mutation. These first models were found to have both hippocampal and cortical intracellular Aβ inclusions, causing aberrant tau phosphorylation, extracellular-signal-related kinase (ERK) 2 activation and learning/memory dysfunction (Echeverria et al. 2004). The McGill-R-Thy1-APP model is another rat model expressing human APP_{751} with the Swedish and Indiana mutations, with intraneuronal Aβ1-42 detectable as early as post-natal day 7, and plaque deposition evident from 6 months. Astrocyte density is increased in the hippocampus by 6 months, as is microglia density in the subiculum, with active amoeboid microglia evident in the hippocampus and cortex by 18 months (Leon et al. 2010, Hanzel et al. 2014) **(Table 1.4)**. The TgF344 model expressing the Swedish *APP* and the PS1ΔE9 transgene is capable of producing cerebral amyloid pathology that precedes cognitive dysfunction, as well as gliosis, neuronal cell death and tauopathy (Cohen et al. 2013). Interestingly, rats are far less utilised for the study of tau pathology than they are for Aβ due to the widespread belief that rats only express three 4R (repeat) tau isoforms, while humans express four 4R tau isoforms. However, it has since been shown that rats do contain a total of six tau isoforms similar to humans, but the ratio of 3R to 4R sequences differs (Hanes et al. 2009). Therefore there is a distinct possibility that rats may be increasingly utilised for the study of tauopathy in the future. See **Table 1.3** for a summary of commonly used rodent transgenic models for AD.

Transgenic Mouse	Transgene(s)	Promoter	Strain	Pathogenesis
Tg2576	APP KM670/671NL (Swedish)	PrP	C57BL/6	Parenchymal plaque formation at 11-13 months with some vascular amyloidosis (Hsiao et al. 1996)
PDAPP	APP V717F (Indiana)	PDGF	C57B6 x DBA2	Parenchymal Aβ plaque deposition at 6-9 months (Games et al. 1995)
APP23	APP KM670/671NL (Swedish)	Thy-1	C57BL/6	Appearance of parenchymal Aβ plaques by 6 months and significant vascular amyloidosis (Sturchler-Pierrat et al. 1997)
TgCRND8	APP KM670/671NL (Swedish), APP V717F (Indiana)	PrP	C3H/He × C57BL/6	Strong parenchymal plaque deposition as early as 3 months (Chishti et al. 2001)
5xFAD	APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V	Thy-1	(C57BL/6 x SJL)F1	Severe Amyloid pathology – intraneuronal Aβ present at 1.5 months, plaque deposition at 2 months – neuron loss and spatial learning deficits at 4 months (Oakley et al. 2006)
PS1M146V	PSEN1 M146V	PDGF	Swiss Webster x B6D2F1	Increased Aβ42 in the absence of plaque pathology (Duff et al. 1996)
APP22	APP751 (Swedish/London)	Thy-1	C57BL/6	Double APP expression vs. WT. Plaque pathology at 18 months of age, dystrophic neurites, tau phosphorylation (Sturchler-Pierrat et al. 1997)
APP/PS1	APP KM670/671NL (Swedish), PSEN1 L166P	PS1M146V x Tg2576	C3H/HeJ × C57BL/6J	Earlier-onset and more extensive plaque pathology than APP alone (Radde et al. 2006)
3xTg	APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V	Thy-1.2	C7BL/6;129X1/SvJ;129S 1/Sv	Aβ plaque pathology at 6 months and NFTs at 12 months (Oddo et al. 2003)

Table 1.2 Commonly-used transgenic mouse models of AD.

Transgenic Rat	Transgene(s)	Promoter	Strain	Pathogenesis
APP21	APP KM670/671NL (Swedish), APP V717F (Indiana)	Ubiquitin-C	Fischer 344	No amyloid plaque pathology. Neuronal death in female hippocampi at 19 months. Resilient to endogenous amyloid pathology but can host exogenous amyloidogenic seeds (Agca et al. 2008)
McGill-R-Thy1-APP	APP KM670/671NL (Swedish), APP V717F (Indiana)	Thy-1.2	HsdBrl:WH Wistar	Intraneuronal Aβ at 1 week, plaque pathology at 6 months subiculum, by 13 months in hippocampus and cortex. Synapse and neuron loss, gliosis all present at 18 months (Leon et al. 2010)
APP/PS1	APP KM670/671NL (Swedish), APP V717F (Indiana), PSEN1 L166P	Ubiquitin-C	Fischer 344	Amyloid plaques and cerebral amyloid angiopathy seen at 19 months – Aβ42 serum levels increased 6X at 19 months (Agca et al. 2016)
TgF344-AD	APP KM670/671NL (Swedish), PSEN1: deltaE9	PrP	Fischer 344	Age-dependent accumulation of Aβ plaques in hippocampus and cortex (6 to 26 months). Cerebral amyloid angiopathy and dystrophic neurites reported. Gliosis apparent at 6 months. Tau pathology also present at 16 months. (Cohen et al. 2013)

Table 1.3 Commonly-used transgenic rat models of AD.

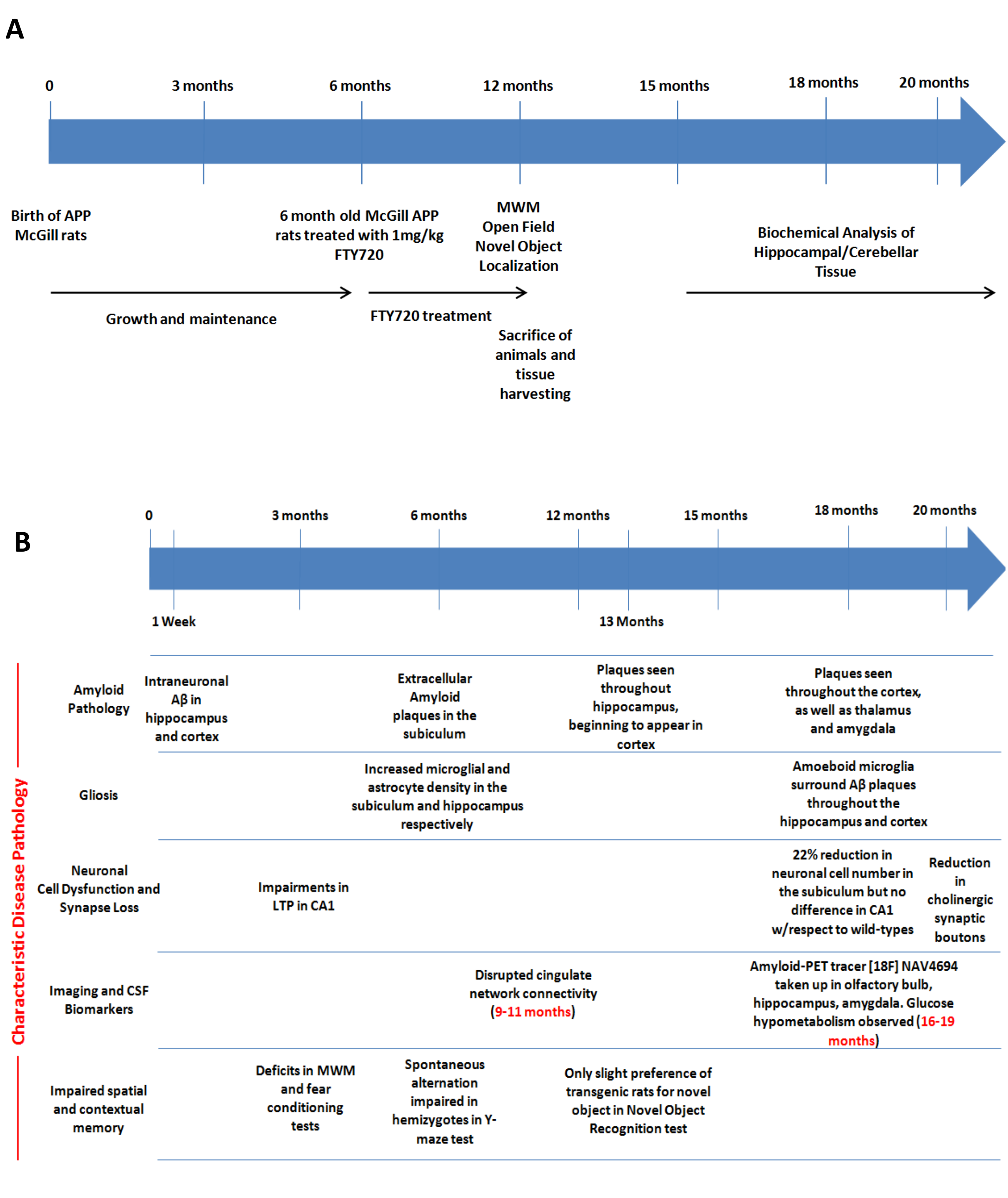


Table 1.4 Experimental Timeline and Characteristic McGill APP pathology milestones.
A) Timeline of treatment and experiments conducted in this study on the McGill APP rat model. **B)** Timeline of characteristic disease milestones reported in the APP McGill rat.

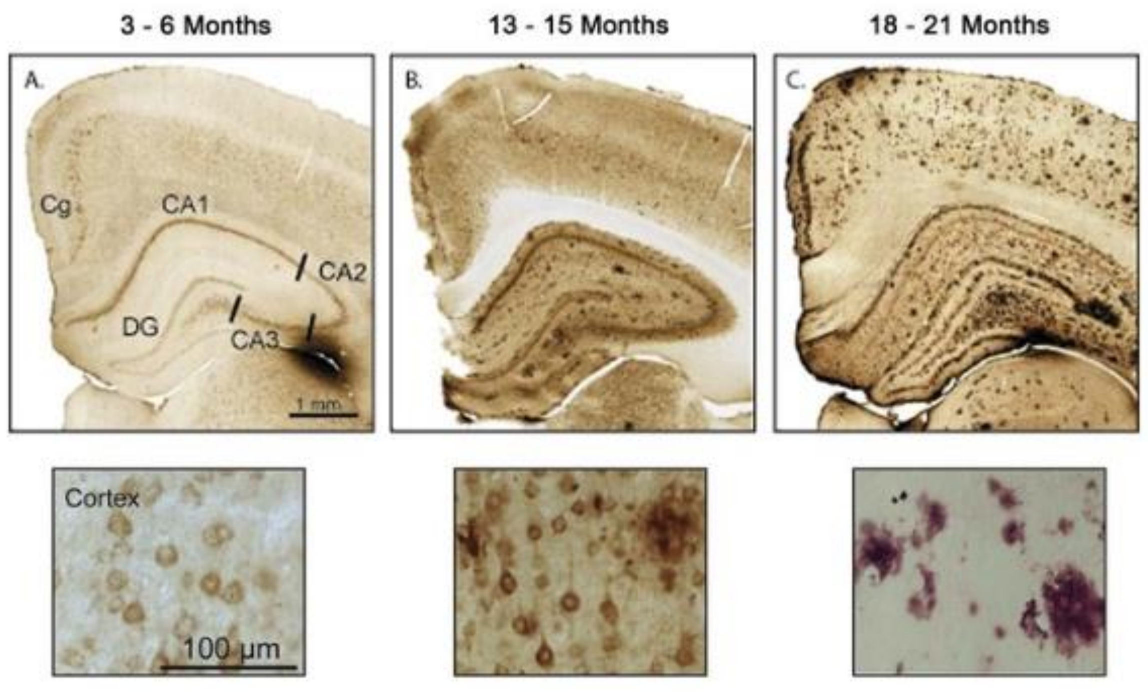


Fig 1.3 Histological sections showing Aβ-deposition in McGill-R-Thy1-APP transgenic rats over time – cortex and hippocampus. (Iulita et al. 2017)

2. Affected Cells Types in Alzheimer's Disease

2.1 The Role of Neurons in Alzheimer's Disease; Primary Targets of Degeneration.

Neurons are a class of electrically excitable cells that receive, process and transmit electrical and chemical signals. Neurons make up the basic functional unit of the brain and are responsible for transmission of chemical signals across an anatomical region of space known as the synaptic cleft, which may terminate on another neuron (CNS) or a muscle cell (PNS). It is estimated that there are roughly 70-85 billion neurons in the brain, the ratio of neurons to glia a subject of debate, with recent evidence suggesting a 1:1 ratio compared to the previously held idea of a 10:1 glia-neuron ratio, but ratios vary depending on brain region (von Bartheld, Bahney and Herculano-Houzel 2016). Neurons have been recognized to be the primary brain cell type affected by AD-related cytotoxicity, with the pathological accumulation of Aβ and NFTs producing synapse degeneration through loss of dendritic spines and dysfunction of axonal transport, with dystrophic neurites representing a common feature of Aβ-plaque-associated neurons (Sadleir et al. 2016). Neurotoxicity in AD occurs most commonly in the basal forebrain, cortex and hippocampus.

Cholinergic neurons are associated with the release of acetylcholine, a neurotransmitter that activates nicotinic and muscarinic cholinergic receptors in the CNS and PNS, controlling a number of autonomic functions. Cholinergic neurons in the CNS are part of either local circuits i.e. interneurons, or groups of projection neurons, connecting functionally relevant areas of the brain. Of the projection cholinergic neurons, the cholinergic neuronal cluster found in the basal forebrain, particularly the nucleus basalis of Meynert, is vulnerable to severe degeneration in AD (Arendt et al. 1983). These neurons project to the cortex, amygdala and hippocampus, and loss of these connections has severe implications in AD, particularly in cortical and hippocampal innervations (Richardson and DeLong 1988). Disruption of cortical cholinergic input can cause deficits in attention and decision-making in response to ongoing events (Muir et al. 1992). It has also been shown that selective blocking of CA3 cholinergic receptors in the hippocampus can impair the encoding of memories and storage of information (Rogers and Kesner 2004).

Dysregulation of calcium homeostasis has also been shown to be implicated in the neurodegenerative process in AD. The calcium ion (Ca^{2+}) is critical for a host of neuronal functions, including second messenger activity, exocytosis of synaptic vesicles, synaptic plasticity and cognitive function (Bezprozvanny and Mattson 2008, Ferreiro, Oliveira and Pereira 2004). Neurons in close proximity to Aβ plaques have been shown to exhibit increased intracellular Ca^{2+} levels released from the endoplasmic reticulum, with subsequent dysfunction of neuronal signalling networks, particularly in synaptic integration (Kuchibhotla et al. 2008). This rise in hippocampal intracellular Ca^{2+} concentration has been shown to negatively affect synaptic LTP through increased activation of voltage-dependent calcium channels (VDCCs) and release from intracellular stores, with concurrent decrease of influx via NMDA receptors (Foster 2007). Dysregulation of calcineurin (CaN), calcium-calmodulin kinase IIα (CAMKIIα) and calcium-calmodulin kinase IV (CAMKIV) as a result of the presence of Aβ has also been shown to potentiate long-term depression (LTD), phosphorylate tau proteins and inhibit synaptic vesicle trafficking along axons respectively (Mulkey et al. 1994, Yamamoto et al. 2005, Park et

al. 2017). Hyper-phosphorylated Tau accumulation in the hippocampus has been shown to produce marked de-phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) in neurons in an extracellular signal-related kinase (ERK)-mediated fashion, the significance of which lies in phosphorylated CREB's basal functioning as a potentiator of synaptic LTP and memory formation through transcription of specific genes involved in memory formation (Teich et al. 2015).

A number of studies have shown that oxidative stress is another contributor to neuronal degeneration and in AD, oxidative damage is shown to surpass levels seen in elderly controls, suggesting a mechanism outside of the normal aging process (Wang et al. 2014). Lipid peroxidation, protein and DNA oxidation and reduction in antioxidant levels all contribute to the oxidative stress profile in AD. Lipid peroxidation products 4-hydroxynonal, malondialdehyde and acrolein levels are all elevated in AD hippocampus (Markesbery and Lovell 1998, Lovell et al. 1995, Lovell, Xie and Markesbery 2001). The levels of specific antioxidant species are seen to decrease in AD, including vitamins A, C and E and while the expression levels of antioxidant enzymes increases, their functioning is seen to decrease, and this is true of the superoxide dismutase, catalase and glutathione peroxidise enzymes (Kim et al. 2006, Omar et al. 1999). Mitochondrial stress is recognized as an early and prominent feature of AD in neurons, with reduced glucose metabolism as a result of the reduced neuronal expression of genes relevant to the synthesis of electron transport chain enzymes in mitochondria, including cytochrome oxidase, an important outcome of this stress (Chandrasekaran et al. 1994).

2.2 The Role of Astrocytes in Alzheimer's Disease.

Astrocytes are a class of 'star-shaped' glial cells present in the CNS and the spinal cord which have a host of supportive and homeostatic functions in their interactions with neurons, endothelial cells and other glia. Glial cell numbers in the human brain have been reported in the range of 40-130 billion cells, with astrocytes comprising 20-40% of that number (von Bartheld et al. 2016). Astrocytes express a number of sub-cellular markers that allow them to be distinguished from neurons and other glia, particularly the glial fibrillary acidic protein (GFAP), calcium-binding protein S100B and glutamine synthetase, among others (Sofroniew and Vinters 2010). Astrocytes provide structural support to the blood brain barrier (BBB) through the interactions of astrocytic endfeet with pericytes and endothelial cells to form the neurovascular unit, actively providing a link between the cerebral vasculature and neurons, a process known as 'neurovascular coupling' (Alvarez, Katayama and Prat 2013). Astrocytes also regulate the movement of water at the BBB through the transmembrane aquaporin 4 (AQP4) water channel (Nielsen et al. 1997).

Critical to the functioning of neurons in the CNS, astrocytes provide support in a number of ways. At the tripartite synapse they take up excess neurotransmitter such as glutamate through the relevant transporters and recycle them back to the pre-synaptic neuron. They release gliotransmitters in response to Ca^{2+} influx in a exocytotic manner which includes the release of glutamate, ATP, D-serine and GABA to modulate glial-glial and glial-neuron signalling (Volterra and Meldolesi 2005). Relevant to the study of AD pathology, astrocytes

have been shown to play an important role in synaptic plasticity and LTP in the hippocampus through the regulation of glutamatergic neurotransmission via postsynaptic AMPA receptors in an ATP-dependent manner (Ota, Zanetti and Hallock 2013). Astrocytes are also the largest producers of the apolipoprotein cholesterol carrier in the CNS, which is responsible for the transport of cholesterol to neurons for maintaining membrane fluidity and synthesising myelin for axonal insulation, but mutations in the ε4 allele have been shown to confer a higher risk of the development of sAD (Zhang et al. 2018, Saher et al. 2005).

In AD, astrocytes are present in a pathologically active state commonly referred to as 'reactive gliosis.' Reactive gliosis is characterised by increased expression levels of the astrocytic markers GFAP and and S100B which are seen to be routinely elevated in post-mortem AD brains (Griffin et al. 1989, Meda, Baron and Scarlato 2001). The degree of reactive gliosis has been recognized to correlate well with measures of cognitive decline; however, the same cannot be said of the degree of reactive gliosis and the severity of Aβ plaque pathology (Simpson et al. 2010). Calcium dysregulation, glutamatergic dysfunction (excitotoxicity) and altered phagocytosis are three important effects of Aβ-deposition mediated astrocytic activation. Astrocytes in the vicinity of senile Aβ plaques function to internalize and breakdown Aβ, which requires their activation, but if astrocyte activation in AD crosses the boundary from acute to chronic, it may progress from being neuroprotective activation to pathological activation that contributes to neuroinflammation (Ries and Sastre 2016).

Astrocytes have been identified as being directly responsible for mediating Aβ-induced tau phosphorylation and cleavage, with the production and release of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 occurring as a result of this (Garwood et al. 2011).

As with macrophages, which classically have been described as having two polarization states, M1 and M2 (but now are recognised as being capable of existing in more than two states), astrocytes exhibit a shift from so-called 'resting' astrocytes to either A1 or A2 reactive astrocytes, depending on the harmful stimulus, either neuroinflammation or ischemia respectively (Martinez and Gordon 2014). The release of IL-1β, TNF-α and IL-6 from activated microglia can induce phenotypic changes in astrocytes, producing A1 reactive astrocytes. While A2 reactive astrocytes are seen to upregulate neurotrophic factors, and are thus considered protective, induction of the A1 reactive astrocyte phenotype causes these cells to lose their ability to promote neuronal growth, survival and synaptogenesis, leading to neuronal and oligodendrocyte cell death in AD and a number of other neurodegenerative diseases (Liddelow et al. 2017).

Calcium dysregulation is one such downstream effect of prolonged reactive gliosis. Fibrillar Aβ in culture and the plaques around which astrocytes are associated *in vivo* causes spontaneous calcium signals and oscillations of intracellular calcium concentration (Lee, Kosuri and Arancio 2014). Abnormal intracellular astrocytic calcium levels due to the presence of extracellular Aβ is partially mediated by increased release from intracellular ER stores, and partially from Aβ-mediated activation of astrocyte cell-surface receptors that lead to calcium entry, such as the metabotrophic glutamate receptor mGluR5 (Toivari et al. 2011, Grolla et al. 2013). Pathologically elevated Ca^{2+} concentrations intracellularly in astrocytes affects the release of the gliotransmitter NMDA-R co-agonist D-serine, which in turn affects the activation of

NMDAR's on post-synaptic synapses, and Ca^{2+} influx through NMDARs is one of the primary mechanisms by which synaptic LTP is maintained (Henneberger, Bard and Rusakov 2012). Aβ can also induce the release of the gliotransmitter glutamate, which activates extrasynaptic NMDA receptors (Talantova et al. 2013). In ex vivo astrocyte studies, it has been shown that Aβ1-42 decreases the expression of the glutamate transporters GLT-1 and GLAST, increasing the concentration of astrocyte-released glutamate in the synapse, contributing to excitatory overactivation of NMDA receptors (excitotoxicity)(González-Reyes et al. 2017).

2.3 The Role of Microglia in Alzheimer's Disease.

Microglia are the resident innate, phagocytic immune cells of the CNS, originating from myeloid progenitor cells in the embryonic yolk sac, from which they migrate, proliferate and become ramified (i.e. express a large number of highly branched processes) throughout the brain parenchyma (Ginhoux et al. 2013). In the adult brain, microglia constitute approximately 0.5-16% of all cells in the brain and spinal cord (Mittelbronn et al. 2001).The main function of microglia is to dynamically survey their CNS microenvironment through extension and retraction of their processes in order to regulate tissue maintenance and respond to CNS cellular injury and pathogen infiltration (Colonna and Butovsky 2017). Microglia are also active during the formation of neural circuits during development, participating in the phagocytosis and removal of unnecessary neurons and synapses in a process known as 'synaptic pruning' (Schafer et al. 2012).

Microglial activation in acute CNS injury is a highly regulated process, with a host of distinct receptors expressed on the microglial cell surface to aid the removal of pathogenic species in an efficient manner e.g. Toll-like receptors, CD36, major histocompatibility complex II (MHC II) and complement receptors (Neumann, Kotter and Franklin 2009, Lue et al. 2010, Liu and Hong 2003). The protective effect of microglia in acute CNS injury, however, can eventually become deleterious if it is maintained for a long period of time, such as in progressive neurodegenerative disorders like AD. The physiological process of aging can itself cause changes in microglial morphology and activation, as is indicated by increased expression of microglial activation markers MHCII and the receptor for advanced glycation end-products (RAGE), increased pro-inflammatory cytokine release (TNF, IL-6, IL-1β) and hypersensitivity of microglia to inflammatory stimuli (Perry, Matyszak and Fearn 1993, Sierra et al. 2007). Chronic microglial activation in AD as a result of excessive, sustained neuronal damage and inflammatory stimuli is a potent contributor to neurodegeneration and there are a number of mechanistic pathways through which microglial-mediated neuroinflammation contributes to the worsening of AD phenotype.

In AD, microglia become activated through the recognition of damage-indicating factors known as damage-associated molecular patterns (DAMPs), which activate pattern recognition receptors (PRRs) (Kigerl et al. 2014). DAMPs are produced by damaged cells and trigger a microglial response to brain injury. Activated microglia undergo a morphological change in response to damage signals, switching from the 'resting' ramified morphology to the 'active' amoeboid morphology, and this involves the retraction of cellular processes to facilitate

phagocytosis and increase cellular mobility (Kreutzberg 1996). PRRs such as the nucleotide oligomerization domain (NOD)-like receptors (NLRs), particularly the NLRP3 contributes to microglial activation and inflammasome assembly in AD (Halle et al. 2008). ATP released by damaged neurons in the hippocampus is also capable of activating microglia via purinergic receptors on the microglial cell surface, while chemokines such as fractalkine (CXCL3) bind to microglial CX3CR1 receptors to promote microglial chemotaxis to the site of damage (Koizumi et al. 2007, Harrison et al. 1998). AD patient studies have shown that the microglia-secreted cytokines IL-1β, IL-6, TNF-α, IL-8, TGFβ and macrophage inflammatory protein 1α (MIP1α) are all altered in their expression profile compared to controls (Sastre, Klockgether and Heneka 2006).

Both Aβ and NFTs are capable of producing an inflammatory response in microglia. Knockdown of the *app* gene delays and decreases microglial activation, and Aβ load is proportional to the extent of microglial activation (DeGiorgio et al. 2002, Solito and Sastre 2012). Aβ can stimulate the mitogen-associated protein kinase pathway (MAPK) and downstream transcription factor NFκB, driving the transcription of pro-inflammatory cytokines such as TNF-α (Combs et al. 2001). In turn, microglial-mediated inflammation can stimulate transcription of the β-secretase (BACE1), increasing production of amyloidogenic Aβ peptide (Sastre, Walter and Gentleman 2008). Aβ can activate microglia through a number of mechanistic pathways, primarily, through TLR2 and TLR4 binding, RAGE, scavenger receptors CD36 and CD40, formyl peptide and complement receptor activation, which all contribute to increased phagocytosis and inflammatory phenotype in the AD brain (Solito and Sastre 2012). Models of tauopathy such as the P301S mouse model exhibit increased microglial activation around neurons with dystrophic neurites caused by tau hyperphosphorylation (Yoshiyama et al. 2007). More recently, a TREM2 mutation has been identified as a significant risk factor for sAD. TREM2 functions to promote microglial activation and survival via the Wnt/β-catenin pathway under physiologically normal conditions, and *Trem2* haplodeficiency mouse models of AD exhibit reduced microglial accumulation around Aβ plaques, highlighting the role of TREM2 in promoting the microglial response to aggregated Aβ (Zheng et al. 2017, Wang et al. 2015). Overall, there is sufficient evidence to suggest that microglial activation is necessary for Aβ phagocytosis, but sustained activation of microglia in response to Aβ, NFTs and the battery of pro-inflammatory cytokines released by damaged neurons, astrocytes and other microglia contribute to a strongly pro-inflammatory CNS phenotype that serves to compound the neuronal degeneration and synaptic impairment that is characteristic of AD.

2.4 The Role of Oligodendrocytes in Alzheimer's Disease.

Oligodendrocytes are the third major subclass of glial cell in the CNS, whose primary responsibility concerns the proper myelination of axons. Axonal myelination in the CNS is crucial to the efficient transmission of electrical signals from neuron to neuron, increasing conduction velocity by up to fifteen times when compared with unmyelinated axons (Giuliodori and DiCarlo 2004). Oligodendrocytes provide crucial support to neurons through the generation of a layer of fatty insulation known as the myelin sheath (Bradl and Lassmann 2010). Oligodendrocytes wrap myelin around axons to prevent the leakage of ions along the length of the axon while maintaining membrane potential, thus promoting fast and efficient

nerve impulse transmission. Myelin is composed of 80% lipid, and 20% protein, the latter of which consists of the mature myelin markers myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and myelin proteolipid protein (PLP), among others (Pietrucha-Dutczak et al. 2006). Oligodendrocytes can myelinate large numbers of CNS axons concurrently, usually up to around 50 axons per oligodendrocyte(Cai and Xiao 2016).

The breakdown of myelin and alterations in oligodendrocyte activity is understood to be one of the earliest potential signs of the onset of AD, preceding Aβ deposition and tau hyper-phosphorylation in a number of *in vivo* models of AD (Desai et al. 2009). It is thought the decline in the robustness of myelin with increased age leaves it vulnerable to stressors and pathologically aggregated protein species such as Aβ and NFTs causing cognitive decline (Bartzokis 2004). Studies suggest that age-related myelin degeneration can promote the formation of oligomeric Aβ into mature plaques in a ferritin-iron dependent manner, which in turn destroys more myelin in a feed-forward mechanism (Bartzokis, Lu and Mintz 2007). A number of mechanisms of Aβ-mediated alteration in oligodendrocyte and myelination profile have been identified, including through *PS1* mutation-mediated alterations in oligodendrocyte precursor cell (OPC) differentiation and MBP subcellular mislocation. Corpus callosum white matter damage and inflammatory demyelination as a result of direct Aβ deposition also contribute to an demyelinating AD phenotype as well as reduction in MBP, PLP and cholesterol levels as a result of the presence of Aβ in CNS white matter, which negatively affects the synthesis and viability of myelin and axonal health (Desai et al. 2011, Jantaratnotai et al. 2003, Roher et al. 2002).

In early-stage AD, Aβ-mediated axonal demyelination and neuroinflammation drives increased proliferation of OPCs and differentiation to mature oligodendrocytes, as well as increased transcription of genes linked to remyelination and the clearance of myelin debris (Behrendt et al. 2013, Glezer, Lapointe and Rivest 2006). However, oligomeric Aβ species in white matter can prevent OPC remyelination (Horiuchi et al. 2012). It has been shown that Aβ can also induce oligodendrocytic apoptosis via the activation of the neutral sphingomyelinase (nSMase) pathway, which drives the production of ceramide, an inducer of apoptosis. Oxidative stress as a result of Aβ-plaque deposition is another strong contributor to oligodendrocyte damage, as the content of reduced glutathione (GSH) is low in these cells and thus affects their ability to scavenge oxygen radicals from the high levels of iron they contain (Back et al. 1998). Low GSH also leaves oligodendrocytes more susceptible to Aβ-mediated activation of the nSMase pathway, and supplementation of oligodendrocytes with GSH precursors *in vitro* prevents nSMase-mediated cell death, whereas depletion of GSH increased Aβ-mediated cell death (Lee et al. 2004). Aβ also increases cleaved-caspase 3 expression, which leads to the apoptosis of mouse oligodendrocyte precursor cells *in vivo* (Desai et al. 2010).

NFT formation has also been shown to affect oligodendrocyte function and myelination profile in AD. Expression of G272V tau mutation in mice leads to the formation of tau filaments in oligodendrocytes which subsequently exhibit fibrillary lesions akin to those seen in human tauopathies (Götz et al. 2001). Oligodendrocytes are also highly sensitive to DAMPs in their CNS microenvironment, leaving them susceptible to excessive ATP and glutamate receptor activation and thus excitotoxicity in AD (Matute et al. 2007). Oligodendrocytes express a wide variety of cell-surface receptors such as ionotropic glutamate and purinergic receptors. High

levels of ATP released from damaged neurons in AD leads to sustained activation of p2x7 purinergic receptors on oligodendrocytes which causes caspase-3 mediated apoptosis due to increased Ca^{2+} influx. This prolonged activation eventually leads to oligodendrocyte cell death and the destruction/impaired synthesis of myelin (Nasrabady et al. 2018).

3. S1PRs and Alzheimer's Disease

3.1 The Sphingosine 1-Phosphate Receptor Axis – Structure and Function.

Sphingosine 1-phosphate (S1P) is a bioactive lysosphingolipid that has a role in a number of important cellular responses such as proliferation, migration, adherence, cytoskeletal organization and immune cell trafficking (Hla 2004). S1P is present at nanomolar concentrations in the blood (200-900nM), where it is plasma bound (Murata et al. 2000). There is a significant body of evidence to suggest that S1P has a role as an intracellular signalling molecule alongside its canonical role as an extracellular signalling molecule. S1P signals through histone deacetylases (HDACs), TNF receptor associated factor 2 (TRAF2) and the β-site amyloid precursor protein (APP) cleaving enzyme-1 (BACE1) among others, before being broken down by an S1P lyase (Maceyka et al. 2012). The homeostatic levels of S1P are maintained by two sphingosine kinases; SphK1 an SphK2, two S1P phosphatases and one S1P lyase (Le Stunff et al. 2002). SphK1 is phosphorylated at serine 225 in an ERK-dependent manner in response to signals such as growth factors, hormones and cytokines, which promotes its migration from the cytosol to the plasma membrane where it acts on its substrate, sphingosine, to produce S1P (Pitson 2011). SphK2 however, is present in a number of intracellular compartments and associates with the nucleus to control gene transcription of S1P, which is itself a HDAC inhibitor (Hait et al. 2009).

S1P binds in the order of nanomolar affinity with a family of five G-protein coupled receptors (GPCRs) known as the S1PR family, of which there are 5 subtypes, S1PR1-5 (Brinkmann 2007). The S1PR1-3 subtypes are widely expressed in the cardiovascular system, but more pertinently, S1PR1 is present on lymph nodes and regulates the egress of B and T cells from the lymphatic organs they reside in (Matloubian et al. 2004). S1PR4 is expressed at low levels in the lymphoid system while the S1PR5 is expressed predominantly in the white matter tracts of the CNS, as well as on natural killer cells (Im et al. 2000, Gräler, Bernhardt and Lipp 1998). S1PR activation has pleiotropic effects, ranging from immune cell trafficking and cellular communication to vascular homeostasis, neurogenesis and angiogenesis (Brinkmann 2007). G-protein coupling of S1PRs and their downstream effector pathways are summarised in **Figure 1.3**.

In terms of identifying CNS expression of S1PRs, mRNA-based analysis of cell-specific receptor subtype expression has been carried out via PCR and in-situ hybridization, but more recently the development of antibodies against these receptors has facilitated the use of immunohistochemistry and western blot for analysis of their expression profiles (Nishimura et al. 2010). All five S1PRs are found in the developing as well as the mature brain (Dubin, Herr and Chun 2010). Constitutive knockout of S1PR1 produces a schizophrenia-like behavioural phenotype in mice (Contos et al. 2002). S1PR1 is expressed on neuronal precursor cells and

mature neurons, affecting neurogenesis, neuronal migration and brain-derived neurotrophic factor (BDNF)-mediated neurite outgrowth (Martin 2014). S1PRs are also widely expressed on astrocytes, making them prime targets for modulation due to their prominent role in CNS inflammation in degenerative disease. S1PR1, 2, 3 and 5 are all expressed on astrocytes, however S1PR1 and 3 have been shown to have higher expression levels than either S1PR2 or 5 (Rao et al. 2003). When activated by PAMPs or DAMPs, S1PR1 and 3 are upregulated in activated astrocytes, implicating these receptors in the neuroinflammatory cascade (Van Doorn et al. 2010). Microglial S1PR expression profile is dynamically dependent on activation state, with down-regulation of S1PR1 and 3 and upregulation of S1PR2 evident upon activation (Tham et al. 2003). In oligodendrocytes, S1PR expression depends on cell maturation, with S1P5 more expressed on mature cells than the other S1PRs, but prior to maturation, all S1PR expression levels are the same (Choi and Chun 2013).

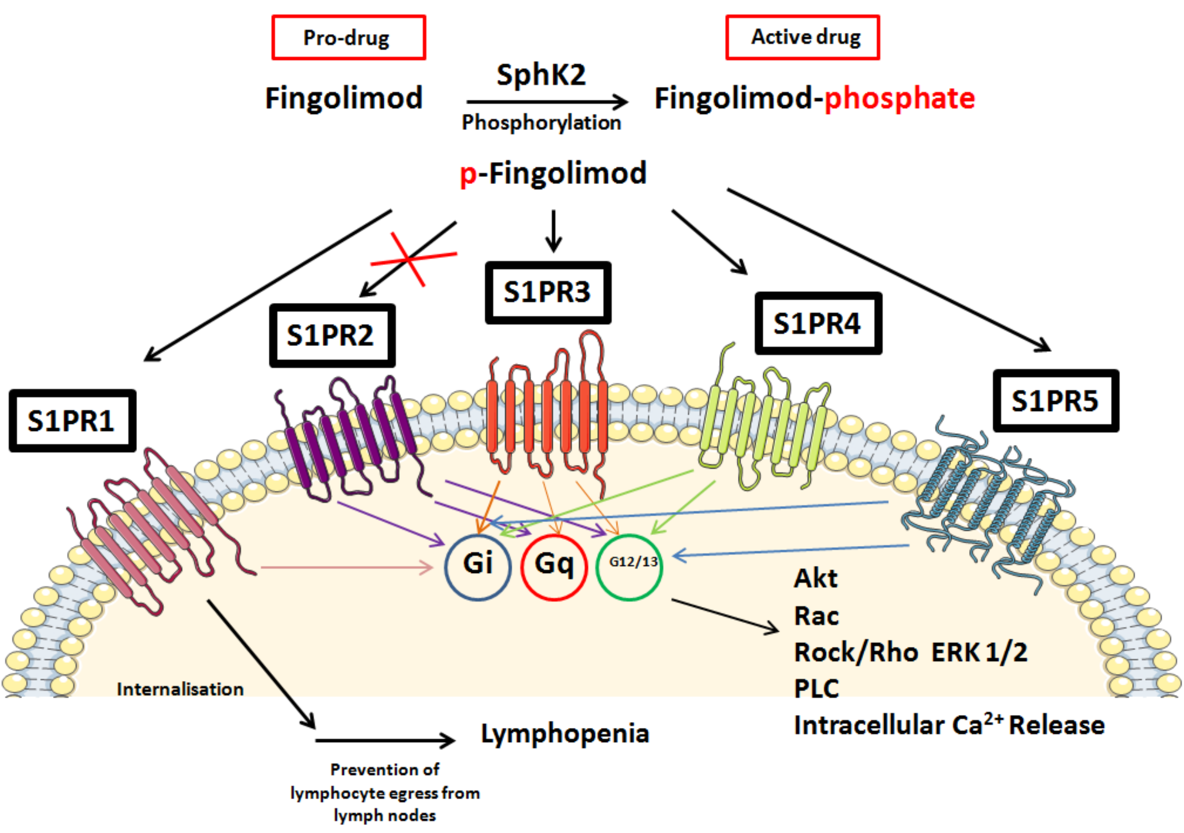


Figure 1.4 p-FTY720 is an agonist for S1PR1 and S1PR3-5 but not S1PR2. The S1PR system is a G-protein coupled receptor system, which, when activated by an S1PR agonist such as p-FTY720, causes their internalization, preventing B and T cell egress from lymph nodes, resulting in lymphopenia. Activation of these receptors leads to the activation of downstream intracellular signalling cascades, which in the case of S1PR1 leads to neurogenesis, neurite outgrowth, oligodendrocyte survival and astrocyte migration (Dev et al. 2008).

3.2 Sphingosine 1-Phosphate Receptor Pharmacology – S1PR Agonists.

A number of S1P receptor modulators are currently undergoing clinical trials for efficacy in the treatment of a variety of disorders, but all are being tested for various forms of multiple sclerosis (MS). FTY720 (Gilenya®/fingolimod) is an orally bioavailable structural analogue of sphingosine 1-phosphate. For relapsing-remitting multiple sclerosis (RRMS), efficacy of FTY720 in experimental autoimmune encephalomyelitis (EAE) has prompted phase II and III clinical trials (Kappos et al. 2006). The phase III study of FTY720 had superior efficacy to the standard-of-care intramuscular interferon- β1a (IFN- β1a) in terms of relapse rate and inflammatory lesion number (Chiba and Adachi 2012). Fingolimod is currently approved for second-line treatment of MS in the EU and is FDA-approved as a first-line treatment in the USA. FTY720 was first derived via chemical modification of a natural product known as myriocin, a potent immunosuppressant isolated from a broth culture of *Isaria sinclairii* (Fujita et al. 1994).

Due to its similarity to S1P, FTY720 is rapidly phosphorylated *in vivo* by the SphK2 enzyme, to produce p-FTY720 which binds to all of the S1PRs with high affinity (0.3-3.1nM), apart from S1PR2 (Billich et al. 2003, Chun and Hartung 2010). Activation of the S1PR1 on B- and T-lymphocytes as well as lymph nodes (LNs) by FTY720 prevents the egress of these lymphocytes from the LNs, thus down-regulating the trafficking of these immune cells in the periphery, and this is mediated by the receptor-internalising effects of FTY720 (Matloubian et al. 2004, Kabashima et al. 2006). This produces significant lymphopenia and immunosupression. This immunosuppressive effect of FTY720 has been shown to be capable of treating corneal graft rejection as a result of T-cell mediated autoimmunity in animal models of transplantation (Liu et al. 2015). Most notably, FTY720 has been shown to be efficacious for the treatment of RRMS, but is not recommended as a first –line therapeutic for mild RRMS (Ayzenberg, Hoepner and Kleiter 2016). S1PR1 modulation by FTY720 has also been implicated in neurogenesis, neurite outgrowth, oligodendrocyte survival and astrocyte migration as further examples of its neuroprotective effects (Dev et al. 2008).

A number of other S1PR modulators, particularly S1PR1 agonists, are currently undergoing clinical trials for a range of diseases. KRP-203 is an S1PR1 agonist with a similar structure to FTY720, and like FTY720, KRP-203 has potent immunosuppressant activity and is involved in the regulation of lymphocyte mobilization (Shimizu et al. 2005). Its mechanism of action is also analogous to FTY720, involving the phosphorylation and functional antagonism of the S1PR1, but also the S1PR5 (Lukas et al. 2014). Pre-clinical data suggests that KRP-203 may have potential clinical application for organ transplantation as it has been shown to prolong graft survival and reduce rejection of rat skin and heart allografts (Shimizu et al. 2005). Siponimod, (also known as BAF312) like KRP-203, is a selective S1PR1/5 agonist (Gergely et al. 2012). Akin to FTY720, siponimod prevents lymphocyte egress from lymph nodes via phosphorylation of its receptor targets, producing profound lymphopenia (Fryer et al. 2012). Siponimod has been shown to completely suppress experimental autoimmune encephalomyelitis (EAE), inhibit lysophosphatidylcholine (LPC)-induced demyelination in organotypic slice culture and attenuate both LPS and TNF-α/IL-17-induced IL-6 production in both astrocytes and microglia (Gergely et al. 2012, O'Sullivan et al. 2016).

CS-0777 is a selective S1PR1 modulator that, like the other major S1PR1 modulators, is phosphorylated *in vivo*, produces lymphopenia and suppresses EAE (Nishi et al. 2011).

Ponesimod (ACT-128800) is an orally bioavailable selective S1PR1 agonist that produces lymphopenia, but a significant difference between ponesimod and the other major S1PR modulators is that ponesimod is eliminated from the body much more quickly (1 week after discontinuation) and its lymphocyte-modulating effects are rapidly reversible. This is in direct contrast to a drug like FTY720, which is eliminated much more slowly and has a much longer half-life ($t_{1/2}$ = 7 days with FTY720, vs. 21-33 hours with ponesimod) (Kovarik et al. 2004, D'Ambrosio, Freedman and Prinz 2016). Ozanimod (RPC1063), ceralifimod (ONO-4641) and GSK2018682 are all selective dual S1PR1/5 agonists that prevent lymphocyte egress from secondary lymphoid tissues, and all are undergoing clinical trials for efficacy and safety in the treatment of RRMS (Park and Im 2017) For a summary of the S1PR modulators currently undergoing clinical trials see **Table 1.5**.

Name	S1PR Target	Indication(s)	Stage	Company
Fingolimod (FTY720)	S1PR1/3/4/5	Relapsing-remitting multiple sclerosis (RRMS) Neurodegeneration Schizophrenia Rett Syndrome Stroke Amyotrophic lateral sclerosis Primary progressive multiple sclerosis (PPMS)	Approved (2010) Phase IV Phase II Phase III	Novartis Pharmaceuticals
Siponimod (BAF312)	S1PR1/5	Secondary progressive multiple sclerosis (SPMS) RRMS	Phase III Phase II	Novartis Pharmaceuticals
MT-1303	S1PR1	Crohn's Disease Systemic lupus erythematosus RRMS Inflammatory bowel disease	Phase II Phase 1 Phase II Phase1	Mitsubishi Tanabe Pharma Corporation
Ozanimod (RPC1063)	S1PR1/5	Multiple Sclerosis Ulcerative colitis Crohn's Disease	Phase III Phase III Phase II	Celgene
Ponesimod (ACT-128800)	S1PR1	RRMS vs. teriflunomide in RRMS Psoriasis	Phase II Phase III Phase II	Actelion
Cerafilimod (ONO-4641)	S1PR1/5	RRMS	Phase II	ONO Pharma USA Inc.
KRP-203	S1PR1	Subacute cutaneous lupus erythematosus Hematological Malignancies	Phase II Phase I	Novartis Pharmaceuticals
CS-0777	S1PR1	Multiple Sclerosis	Phase I	Daiichi Sankyo Inc.
GSK2018682	S1PR1/5	RRMS	Phase I	GlaxoSmithKline

Table 1.5 Summary of the S1PR modulators currently undergoing clinical trials.
Information gathered from clinicaltrials.gov

3.3 Relevance of Sphingosine 1-Phosphate Receptors to Alzheimer's Disease.

Deregulation of lipid metabolism in the CNS has recently been implicated in the pathogenesis of AD, with post-mortem AD brains exhibiting decreases in total phospholipid and sulfatide contents, along with increases in ceramide and cholesterol levels(Asle-Rousta et al. 2013a). Elevated expression of the acid sphingomyelinase (ACM) and acid ceramidase (AC) enzymes in the brain has been recorded, leading to increased ceramide production and a decrease in sphingomyelin (Filippov et al. 2012). Accumulation of ceramide in the AD brain leads to inhibition of glycolysis, increased inducible-nitric oxide synthase and oxidative stress, increased apoptosis and stabilizing of the amyloidogenic APP-cleaving enzyme-1 (BACE1), promoting Aβ production and subsequent CNS deposition (Asle-Rousta et al. 2013a). Dysregulation of sphingolipid metabolism as evidenced by an increase in sphingosine coupled with a reduction in S1P (which is associated with the loss of SphK1 and enhancement of S1P-lyase activity) in the hippocampus and inferior temporal cortex of post-mortem AD brains has also been implicated in Aβ accumulation in these areas (Ceccom et al. 2014). This suggests that the loss of the numerous protective effects of S1P – inhibition of apoptosis, stimulation of proliferation, differentiation and migration of various cell types in the brain – coupled with the damaging effects of ceramide accumulation, may constitute a potential therapeutic target to redress the imbalance between the synthesis and degradation of these two lipid species (He et al. 2010).

Mimetics of S1P have emerged as promising therapeutic agents for the restoration of this balance of ceramide-to-S1P ratio. A number of animal models of AD have been utilised to study the potential neuroprotective and neurorestorative effects of S1PR modulation in AD. In one study, bilateral intrahippocampal injection of Aβ1-42 was used to mimic AD, and chronic daily administration of 1mg/kg of FTY720 for 14 days attenuated spatial learning and memory impairment in the MWM test, as well as preventing hippocampal neuronal damage and pro-apoptotic caspase-3 activation (Asle-Rousta et al. 2013a). Another model involving bilateral injection of Aβ1-42 into the frontal cortex of Sprague-Dawley rats compared the effect of intraperitoneal FTY720 administration against treatment with the NMDA receptor antagonist, Memantine. AD animals receiving FTY720 were shown to have a comparable restoration in memory to Memantine following the passive avoidance task. Treatment with both drugs significantly attenuated Aβ-induced neuron loss and reduced gene expression of MAPK genes and other pro-inflammatory markers in the hippocampus (Hemmati et al. 2013).

Another study utilising bilateral injection of Aβ1-42 into the hippocampus of Wistar rats, used the sphingosine kinase inhibitor N,N-Dimethylsphingosine (DMS) and the S1PR1 blocker W123 to inhibit phosphorylation of FTY720 and block S1PR1 receptor activation respectively, and it was found that the protective effects of FTY720 for AD are likely mediated via S1PR1 modulation on CNS resident cells as a result of these inhibitors' ability to suppress the neuroprotective effects of FTY720 (Asle-Rousta et al. 2014). In addition, a study investigating the effects of FTY720 pre-treatment on infection-induced enhancement of Aβ accumulation in APP/PS1 mice found that FTY720 was capable of attenuating the astrocytic activation and increased BBB permeability exhibited by *B. Pertussis*-infected AD mice. Observed increases in soluble and plaque Aβ as a result of infection were also attenuated by FTY720. This study

appears to indicate that FTY720 promotes an increase in Aβ-phagocytosis by astrocytes and is useful in attenuating neuroinflammatory glial activation in AD but only in the presence of infection (McManus et al. 2017). Finally, a study involving chronic administration of the S1PR1 selective agonist SEW2871 for two weeks found that the reduction in S1PR1 expression produced by bilateral intrahippocampal administration of Aβ1-42 into rats was attenuated by SEW2871 treatment (Asle-Rousta et al. 2013b). The drug was also capable of ameliorating spatial memory impairments in the MWM task, highlighting the importance of deregulated S1PR1 signalling in AD, and highlighting SEW2871 as a potential alternative S1PR1 agonist for the treatment of AD. Taken together, these studies provide a significant body of evidence to suggest that S1PR signalling is deregulated in AD and contributes to Aβ42 production, neuroinflammation and oxidative stress, which can be attenuated by FTY720 treatment.

Materials and Methods

1. Materials

All primary and secondary antibodies utilized for western blot are summarised in **Table 2.1**. Primary antibodies used: chicken anti-glial fibrillary acid protein (GFAP, Abcam, ab7260), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, Wako, 019-19741), rabbit anti-β-Actin (Abcam, ab8227), mouse anti-myelin oligodendrocyte glycoprotein (MOG, Millipore, MAB5680), rabbit anti-myelin basic protein (MBP, Abcam, ab40390), mouse anti-vimentin (Santa Cruz, sc-6260) and mouse anti β-amyloid 1-16, clone 6E10 (BioLegend, SIG39320). Secondary antibodies used: donkey horseradish peroxidise (HRP)-conjugated anti-rabbit (GE Healthcare, GENA934-1ML), goat HRP-conjugated anti-chicken (Sigma, SAB3700199-2MG), goat HRP-conjugated anti-mouse (Sigma, A8924-5ML).

All TaqMan Fast Gene Expression Assays primers are listed in **Table 2.2.** Gene targets: *gapdh, gfap aif-1, app, S1pr1, mbp, mog, vim* (Thermofisher).

2. Animals

All experiments were carried out in accordance with the guidelines laid out by the EU in Directive 2010/63/EU on the protection of animals used for scientific purposes and with the protocols approved by the Trinity College Dublin ethics committee. The project was authorised by the Health Products Regulatory Authority (HPRA). Rats were kindly donated by Prof. Michael Rowan of the School of Medicine in Trinity College Dublin for use in this project. 16 of the animals carried a single *APP* transgene with two human mutations, the Swedish (APP KM670/671NL) and Indiana (APP V717F) mutations, conferring an overexpression of the human APP751 protein under the control of the murine Thy1.2 promoter, on a Wistar (HsdBrl:WH) background. As a result, these animals produce severe amyloid-like pathology, with homozygotes exhibiting the accumulation of intracellular and extracellular amyloid plaques (Aβ42), pathological glial reactivity, cholinergic synapse loss and cognitive impairment in an age-dependent manner (Leon 2010). 7 of these animals were H_2O (vehicle) treated, while 9 were treated with FTY720. The remaining 11 animals were wild-type (WT) Wistar rats treated with either H_2O (6) or FTY720 (5). As FTY720 is an orally bioavailable compound, the drug was administered through the drinking water of the animals at a concentration of 1mg/kg/day, beginning at 6 months of age, up until sacrifice at 12 months (6 months administration). Animals were classified by genotype (APP transgenic vs. WT) and by treatment (H_2O or FTY720) producing four separate groups – WT H_2O, WT FTY720, APP H_2O and APP FTY720. Animals were housed socially in a fixed-temperature room under artificial illumination with a 12h light/dark cycle. All animals were given *ad libitum* access to food and water. The dose of FTY720 was selected based on other studies in rodents and is analogous to doses that have been tested in clinical trials of FTY720 for multiple sclerosis (Asle-Rousta et al. 2014, Fukumoto et al. 2014, Montalban et al. 2015).

3. Behavioural Testing

APP rats and their healthy age-matched controls were subjected to both the novel object localisation (NOL) and Open Field (OF) behavioural tests just prior to sacrifice at 12 months to analyse the effect of FTY720 on spatial memory and recognition in the animals. The NOL task was carried out at 12 months of age in a circular test arena with spatial cues on the wall of the

arena. The 3 objects chosen for the test were similar in size and were washed in 70% ethanol between each trial to remove olfactory cues. Animal cages were transported to the behavioural testing suite at least 30 minutes prior to the start of behavioural testing in order to acclimatise to the novel environment. On the first day, rats were habituated to the test arena which contained spatial cues but no objects during a single 10 minute trial. The next day, rats underwent 3 x 2 minute acquisition trials (10 minute inter-trial interval) where they were allowed to freely explore the test arena, including the objects. This was followed by a single 2 minute test 10 minutes after the last training trial and the time spent exploring each object was recorded. Exploration was defined as touching the object but not climbing, or the animals' snout was directed at the object at a distance of less than 2 cm. The discrimination index was calculated as the time spent exploring the displaced object (Od) over the average time exploring the stationary objects (Os) plus time exploring displaced object multiplied by 100, (Od/(Os+Od))*100, and was used to measure spatial memory.

The OF behavioural test was also carried out at 12 months, just prior to sacrifice. Testing was carried out in a circular testing arena measuring 100cm in width by 50cm in height. Animals were placed in the testing arena in the same position and allowed to move freely for 5 minutes before removal from the arena. Using ANY-maze 6.1 software, the circular arena was divided into three concentric circles, thus dividing the arena into 'inner', 'middle' and 'outer' zones **(Figure 1)**. The total path length (distance travelled) was recorded by the software, as was the amount of time spent in both the inner and outer circles, as well as the number of entries to these circles. This has been shown to be useful as a marker of anxiety-like behaviour in other studies utilising the APP McGill rat model, where increased time and number of entries into the outer zone is indicative of this anxiety-like behaviour (Galeano et al. 2014).

Finally, the Morris Water Maze test for spatial memory was also conducted at 12 months of age. Animals were trained for 5 days (D1-5) with 3 x 2 minute trials, with a 20 minute inter-trial interval. On D6, animals were tested with a single 2 minute trial. The time spent and the number of entries into both the platform area and the platform quadrant were recorded. All behavioural testing was carried out and video recorded by Dr. Steven Fagan.

4. Tissue Preparation/Harvesting

At 12 months of age the animals were sacrificed by CO_2 asphyxiation and transcardially perfused with ice-cold PBS. The brain was dissected out and one hemisphere was post-fixed in 4% paraformaldehyde for 24h before being dehydrated in 30% sucrose solution and snap frozen in isopentane. From the other hemisphere, the cortex and hippocampus were isolated and snap frozen in liquid nitrogen. The spleen, kidneys and cerebellum were also removed and snap frozen in liquid nitrogen. All tissues were stored at -80^{0}C until further use.

5. Western Immunoblot

Hippocampi from all animals were manually homogenised in RIPA buffer (10%w/v) containing a protease/phosphatase inhibitor cocktail (Cell-Signaling Technology, 5872). The homogenate was then briefly sonicated and subsequently centrifuged at 12,000 x g for 20 minutes at 4^0C. A Pierce™ BCA protein assay (Thermo Scientific, 23225) was carried out on the tissue homogenates from each animal to determine total protein concentration. Each sample was equalised to 4 mg/ml with RIPA buffer and then diluted 1:1 with 2x Laemmli buffer (Bio-Rad, 1610737) containing a 1:20 dilution of the reducing agent 2-mercaptoethanol, producing a final concentration of 2mg/ml protein. Samples were then boiled at 90^0C for 10 minutes for the purpose of protein denaturation. 20µg (10µl) of protein per sample were loaded for separation onto 10-15% polyacrylamide gels (depending on protein size) using the Bio-Rad Mini-PROTEAN© Tetra System. Gels were then transferred onto methanol-activated Immobilon-P polvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010). Membranes were blocked in 5% Marvel (milk powder) in 0.05% PBS-Tween (PBS-T) overnight at 4^0C. Membranes were then incubated in primary antibody diluted in 5% Marvel in 0.05% PBS-T overnight at 4^0C in a 50ml Falcon Tube on a roller. Membranes were washed 3 x 10 minutes in 0.05% PBS-T and then incubated with the appropriate secondary antibodies in 5% Marvel in 0.05% PBS-T at room temperature for 1.5 hours. Membranes were then washed 3 more times in PBS-T before application of enhanced chemiluminescent substrate (Thermo Scientific, 32106) to the membrane for 90 seconds followed by imaging using a Li-Cor C-Digit Western Blot Scanner. All membranes were also probed for β-Actin (43kDa) as a loading control.

6. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Whole rat cerebellums were removed from -80^0C, and were allowed to thaw for 10 minutes. A ~30mg section of cerebellum was removed from each brain and placed in Ra1 buffer containing β-mercaptoethanol. Brains were homogenised using blue arrow-head homogenisers fitted to a Pellet pestle (Sigma) in Ra1 buffer. The homogenate was then filtered through a Nucleospin® filter (violet) and centrifuged for 1 min at 11,000 x g. The filter was discarded and 350µl of 70% ethanol was added to the lysate. The lysate was then passed through a blue Nucleospin® RNA Column and centrifuged. 350µl of membrane desalting buffer was added to the column before another centrifugation step. 95µl of a 1:10 dilution of reconstituted DNAse reaction mixture was added to the centre of the column and left to incubate for 15 minutes. 200µl of RAW2 buffer was then added to inactivate the enzyme, before two washes with RA3 buffer were carried out, in-between centrifugation steps. The RNA was then eluted into a sterile 1.5ml Eppendorf® tube with 60µl RNAse-free H_2O and stored at -80^0C. All buffers, filters and collection columns were obtained from the Macherey-Nagel™ Nucleospin™ RNA isolation kit. RNA was quantified using a Nanodrop Lite® spectrophotometer (Thermo Scientific). RNA samples from all groups were equalised to the group with the lowest concentration using RNAse-free H_2O.

cDNA was transcribed from RNA using 10xRT buffer (Thermofisher), 25x dNTP mix (100mM, 4367381, Thermofisher) 10xRT random primers (Thermofisher), MultiScribe™ Reverse Transcriptase (Thermo Scientific) and RNAse-free water. 10µl of the Reverse Transcriptase

Master Mix was then mixed with 10µl of the equalised RNA. The solution was then run through a PCR thermal cycler (BioRad) for 10 minutes at 25^{0}C, 120 minutes at 37^{0}C and five seconds at 85^{0}C.

cDNA was then diluted 1:4 in RNAse-free water, and 4µl of each sample was added to the wells of a MicroAmp® Fast 96-well Reaction Plate (ThermoFisher). 5µl of TaqMan© Fast Master Mix (ThermoFisher) was added to 0.5µl of GAPDH housekeeping TaqMan© Gene Expression Assay (ThermoFisher)and 0.5µl of the target TaqMan© Gene Expression Assay (ThermoFisher). 6µl of the TaqMan©-primer mix was added to each cDNA sample. The plate was sealed with MicroAmp® Optical Adhesive Film (ThermoFisher) and the plate was spun in a centrifuge (ref) for 2 mins at 2000 x g. The plate was then loaded into the Applied Biosystems StepOnePlus Real-Time qPCR System© and run according to: 2 minutes at 50^{0}C, 20 seconds at 95^{0}C, 40 cycles of 15 seconds at 95^{0}C and 1 minute at 60^{0}C. Target genes were quantified using the comparative Ct method, and the average ΔCt of all of the WT H_2O animals was taken as control.

7. Statistical Methods

All experimental data obtained was grouped, analysed and graphically represented using GraphPad Prism 5 software (GraphPAD Software, Inc.). Western blot images captured with the Licor C-Digit were processed through Image Studio Lite 5.2 software, which was used to determine the pixel density of each band as a measure of protein expression. To distinguish between genotype and treatment, a Two-Way ANOVA was used to analyse Western Blot data, with Tukey's post-hoc test utilised for multiple comparisons, expressed as mean ± SEM, taking into account the separate observations of two independent observers, with * = $P<0.05$, ** = $P<0.01$. Open Field analysis was carried out using ANY-maze® software and Open Field, Novel Object Localisation and Morris Water Maze tests were analysed via Two-way ANOVA, expressed as mean ± SEM. Behavioural data are displayed as the observations of three independent observers carrying out the analysis – the candidate, Mr. Luke Davison, a post-doctoral researcher, Dr. Steven Fagan, and an undergraduate student, Mr. Adam Walmsley. The scoring method was mutually agreed upon among the three observers. Only the undergraduate student was fully-blinded to the treatment groups. Animals were given numbered codes, which were not memorised by any of the observers, so while full-blinding did not occur for the former two observers, neither had side-by-side treatment-to-test-animal keys available when scoring behavioural videos, and the animal numbers with associated behavioural scores were decoded after behavioural observation. RT-RT-qPCR data was collected and analysed using StepOne® software, and presented as RQ (relative quantitation), or fold increase of the target gene in each sample when compared to WT H_2O control. Data were analysed using Two-Way ANOVA and expressed as mean ± SEM with * = $P<0.05$, ** = $P <0.01$.

Primary Antibodies						
Target	**Mol Weight.**	**Host Species**	**Supplier**	**Cat. Number**	**Dilution**	**Storage Temp.**
GFAP	50kDa	Chicken	Abcam	ab7260	1:5000	-20^0C
Iba1	17kDa	Rabbit	Wako	019-19741	1:1000	-20^0C
B-Actin	43 kDa	Rabbit	Abcam	ab8227	1:2500	-20^0C
MOG	27kDa	Mouse	Millipore	MAB5680	1:1000	-20^0C
MBP	20.5 kDa 18 kDa 17.5 kDa 14 kDa	Rabbit	Abcam	ab40390	1:1000	-20^0C
Vimentin	54 kDa	Mouse	Santa Cruz	sc-6260	1:1000	4^0C
APP Clone 6E10	100kDA	Mouse	BioLegend	SIG-39320	1:1000	4^0C

Secondary Antibodies					
Target	**Host Species**	**Supplier**	**Catalogue No.**	**Dilution**	**Storage Temp.**
HRP-Conjugated Anti-Rabbit	Donkey	GE Healthcare	GENA934-1ML	1:5000	4^0C
HRP-Conjugated Anti-Chicken	Goat	Sigma	SAB3700199-2MG	1:10000	-20^0C
HRP-Conjugated Anti-Mouse	Goat	Sigma	A8924-5ML	1:2000	-20^0C

Table 1.1: Primary and Secondary Antibody List

TaqMan® Gene Expression Assays					
Gene Target	**Species**	**Supplier**	**Catalogue No.**	**Dye**	**Application**
gapdh	Rat	Thermofisher	4453320	VIC-MGB	PCR
gfap	Rat	Thermofisher	4448892	FAM-MGB	PCR
aif-1	Rat	Thermofisher	4448892	FAM-MGB	PCR
app	Human	Thermofisher	4453320	FAM-MGB	PCR
S1pr1	Rat	Thermofisher	4453320	FAM-MGB	PCR
mbp	Rat	Thermofisher	4453320	FAM-MGB	PCR
mog	Rat	Thermofisher	4453320	FAM-MGB	PCR
vim	Rat	Thermofisher	4448892	FAM-MGB	PCR

Table 1.2: List of TaqMan® Gene Expression Assays.

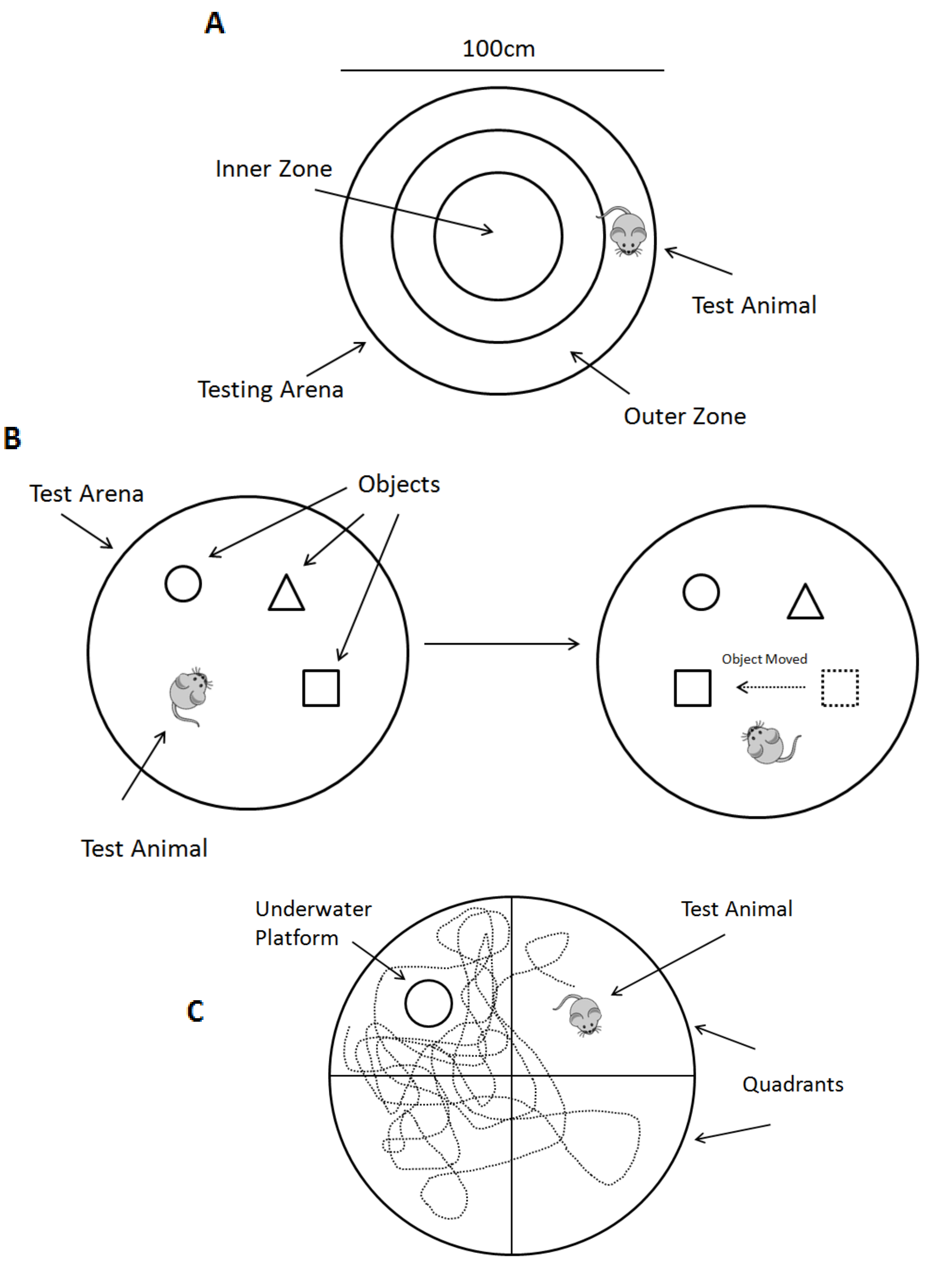


Figure 1: Behavioural Analysis Paradigms. A) Open Field experimental setup. The 100cm diameter circular test arena was divided up into three concentric circles with a width of 16.66cm (radius of the centre zone was taken – 16.66cm). These divisions mark the 'zones' – outer, middle and inner. **B)** Novel Object Localization task involves free exploration of 3 different objects in an arena 3 times, followed by one test trial, with one of the objects being displaced in the test trial as is indicated by the dotted line. **C)** Morris Water Maze task setup. Animals were given 5 days of 3 x 2 minute acquisition trials and a single two-minute test trial, with number of entrances into both the platform area and platform quadrant, as well as amount of time spent in these zones, being recorded. For full details of behavioural analysis, see **Section 3. Behavioural Testing.**

Results

1. Novel Object Localization shows no effect of FTY720 in rAPPtg model.

The Novel Object Localization (NOL) test was carried out to assess the impact of APP over-expression on spatial memory and recognition in transgenic rats with respect to their age-matched WT controls. In tandem, this behavioural experiment was performed to assess whether oral FTY720 treatment could attenuate the deficits in spatial memory that were expected to be exhibited by the APP transgenic animals. Finally, this experiment was conducted to corroborate the findings of another study which used a similar behavioural test, the Novel Object Recognition test, in the same rat APP transgenic model (Galeano et al. 2014). Here, we identified no significant reduction in discrimination index of APP transgenic animals with respect to their WT controls, and no significant increase in discrimination index with FTY720 treatment was observed (**Figure 1**).

Details of the habituation, training and testing procedure are outlined in **3. Behavioural Testing**. The discrimination index of each group was compared to every other group and it was found that there was no significant difference between either differing genotypic groups i.e. WT vs. APP transgenic, or differing treatment groups i.e. H_2O vs. FTY720. Data was analysed using Two-Way ANOVA to account for the effects of two independent variables, genotype and treatment. Tukey's post-hoc test was conducted to compare all pairs of means. This confirms the finding made by Galeano et al. which showed that spatial memory and object recognition capabilities are not inhibited by APP over-expression in the McGill APP transgenic rat model. Taken together, this data indicates that in this particular model of AD, at this particular time-point (12 months), spatial memory and object recognition are not affected by the over-expression of APP.

2. Open Field test suggests FTY720 does not alter locomotion or anxiety-like behaviour in rAPPtg model.

The Open Field (OF) test was conducted to assess the effect, if any, of APP over-expression on locomotor activity of rats compared to their WT controls. In conjunction with this, the experiment was also conducted to assess whether, given that APP over-expression did have an effect on locomotor activity, normal locomotor behaviour could be restored to levels exhibited by WT controls following treatment with FTY720. A previous study assessing locomotion in 4-7 month old APP McGill rats did not find any abnormalities in locomotion with respect to age-matched controls (Petrasek et al. 2018)_The OF test was also utilized to identify the effect of APP transgenesis and FTY720 treatment on anxiety-like behaviour, as measured by an animal's aversion to being located in the centre of the testing arena._The parameter measured to assess locomotor behaviour was path length i.e. the total distance travelled by the animal in the test arena. Details of the experimental setup for the OF task are outlined in **3. Behavioural Testing.** No differences in path length were observed between differing genotypic groups or differing treatment groups (H_2O vs. FTY720) (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 2A**). No significant differences between any of the groups were observed in terms of total time spent in the outer zone, total time spent in the inner zone, number of entries into the outer zone and number of entries into the inner zone (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 2B-E**). Data collected on anxiety behaviours in this model from other studies appears to be inconsistent, with some observing clear aversion to the central area in APP transgenics

only, while others observe this behaviour in wild-type groups also (Petrasek et al. 2018, Galeano et al. 2014, Martino Adami et al. 2017). Taken together, this data indicates that locomotor ability is not affected either by APP over-expression or FTY720 treatment, and neither of these factors affects anxiety in these animals either, with aversion to the centre circle being observed across all groups.

3. Morris water maze displays FTY720 treatment improves spatial memory in rAPPtg.

In order to identify whether the APP transgenic rats in this study exhibited the expected deficits in spatial memory and to see if FTY720 treatment could ameliorate this spatial learning deficit, 12 month old APP McGill rats and their WT littermates were subjected to the MWM behavioural test. There was found to be a significant increase in the amount of time spent in the platform-containing quadrant in APP transgenic rats treated with FTY720 compared to vehicle-treated transgenics, with no significant difference in time spent in the quadrant observed between either vehicle or FTY720-treated WT groups (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 3A**). Next, there was found to be no significant differences in the time spent in the platform zone between WT and APP vehicle-treated groups and WT and APP FTY720-treated groups, however there was a trend toward increased time spent in the platform zone in both the WT and APP FTY720-treated groups with respect to vehicle-treated animals of both groups (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 3B**). There was found to be no significant difference in the frequency/number of entries of animals between all 4 treatment groups into the platform-containing quadrant (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 3C**). Finally, the frequency of entry of the animals into the platform-containing quadrant was observed, and there were found to be no significant differences in the frequency of entry ito the platofrm-containing quadrant among any of the groups (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 3D**). Taken together, these results suggest that FTY720 can improve spatial memory in APP transgenic rats, as is evident by increased time spent in the platform-containing quadrant in this treatment group with respect to vehicle-treated APP transgenic rats. A trend towards increase time in the platform zone of ~50% is also observed in both WT and APP transgenic FTY720-treated groups, further consolidating the idea that FTY720 has a beneficial effect on spatial memory.

4. FTY720 attenuates APP expression in rAPPtg model.

In order to confirm that the single-transgenic McGill APP rat model of AD did indeed over-express APP, and thus is a useful model of the disease, western blot analysis of hippocampal homogenate for the 100kDa APP protein was carried out. As well as this, both in order to confirm that all animals had been genotyped correctly and to identify the extent of *app* expression outside of the hippocampus, in this case, in the cerebellum, RT-qPCR analysis of cerebellar RNA was conducted. Finally, in order to identify whether FTY720 treatment affected cerebellar gene expression of the protein receptor target of the drug, S1PR1, RT-qPCR analysis of RNA from cerebellar tissue was carried out. It was found that vehicle-treated APP transgenic rats did indeed express APP, and as expected, no protein expression was identified in either the WT vehicle-treated or WT FTY720-treated rats, as rats do not normally express APP with

natural aging (Benedikz, Kloskowska and Winblad 2009) (**Figure 4A,B**). Interestingly, there was found to be a significant decrease in APP expression with FTY720 treatment (Two-Way ANOVA, post-hoc Tukey's test *=p<0.05) (**Figure 4A, B**). RT-qPCR analysis of cerebellar RNA reflected the western blot result, highlighting a ~600-fold increase on average of *app* gene expression in the vehicle-treated APP transgenic rats (Two-Way ANOVA, post-hoc Tukey's test **=p<0.01) (**Figure 4C**). RT-qPCR analysis on cerebellar *S1pr1* gene expression identified no significant differences between either groups of differing genotype (WT vs. APP) or treatment (H_2O vs. FTY720) (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 4D**). Taken together, these results suggest that FTY720 may be useful as a therapeutic agent for the reduction of amyloid load, and therefore may constitute a disease-modifying therapy. Given that FTY720 affects APP expression at the gene level, this suggests that it may affect an intracellular signalling cascade involved in gene transcription. These results also indicate that though FTY720 is an S1PR1 functional antagonist, it does not affect expression of the receptor at the level of RNA.

5. FTY720 treatment does not alter expression of astrocyte markers.

Given that reactive astrocytosis has been shown to be a key feature of CNS neuroinflammation in AD, particularly in the vicinity of Aβ-plaques, and that GFAP and vimentin are considered standard markers of reactive astrocytes, both of these proteins were analysed via western blot from hippocampal homogenate in the APP McGill rat and WT controls (Sofroniew 2009, Janeczko 1993, Jain, Wadhwa and Jadhav 2015). In conjunction with this, RT-qPCR analysis was conducted on cerebellar RNA of both of these proteins, as it would be expected that if GFAP and vimentin proteins are upregulated in reactive astrocytes, then gene expression should be similarly increased. Finally, these experiments were conducted in order to identify whether FTY720 could reverse or even attenuate long-term astrocyte reactivity as illustrated by reduced protein and gene expression of GFAP and vimentin. Western blot analysis of GFAP revealed no significant increase or decrease in GFAP expression with either APP transgenesis or FTY720 treatment in respect to vehicle-treated WT animals (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 5A, B**). RT-qPCR analysis of the *gfap* gene in cerebellar tissue similarly showed no significant effect of genotype or treatment on the expression level of this gene (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 5C**). Vimentin western blot and RT-qPCR analyses on hippocampal and cerebellar tissue respectively yielded similar results to GFAP, with no significant differences between any of the 4 groups evident from the data (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 5D-F**). Taken together, these results appear to suggest that in this model of AD, APP expression is not inducing reactive astrocytosis, and FTY720 also has no effect on this phenotype, which is contrary to much of what has been published on astrocytic activation in this model (Leon et al. 2010).

6. Levels of myelin protein are not affected by APP genotype or FTY720 treatment.

Given that myelin breakdown and alterations in oligodendrocyte function are some of the earliest potential signs of AD (in a triple-transgenic rodent model of AD), even preceding Aβ deposition, protein markers of myelination, namely MOG and MBP, were analysed via western blot to identify whether the expression of a single APP transgene in the McGill rats negatively

affected myelination state by reducing the expression or causing the breakdown/degradation of these proteins (Desai et al. 2009). These experiments were also conducted to evaluate the potential for FTY720 to either prevent demyelination in these animals (should it occur), or promote remyelination in APP transgenic animals. Western blot analysis of MOG from hippocampal homogenate showed no significant effect of APP transgenesis on the expression of this protein with respect to WT controls, and no significant effect of FTY720 on WT or transgenic animals was observed either (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 6A, B**). Western blot analysis of MBP from hippocampal homogenate showed a significant increase of ~75% in the WT FTY720-treated group of the 21.5kDa isoform of the protein, and a trend towards increase in the same treatment group across the other three MBP isoforms. No significant difference in MBP expression was observed between vehicle-treated WT and APP transgenic animals, with FTY720 having no significant effect on MBP expression in APP transgenics (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 6C-G**). Taken together, these results suggest that APP expression does not affect the expression levels of the myelin proteins MOG and MBP, indicating that demyelination is not a knock-on effect of AD phenotype in this model. However, this data suggests that FTY720 treatment increases baseline levels of MBP expression in WT animals, highlighting the potential of FTY720 treatment as a myelin protein-protective agent in normal ageing.

7. Levels of myelin genes are not affected by APP genotype or FTY720 treatment.

Given that myelin breakdown and alterations in oligodendrocyte function are some of the earliest potential signs of AD (in a triple-transgenic rodent model of AD), even preceding Aβ deposition, the genes of protein markers of myelination, namely MOG and MBP, were analysed via RT-qPCR to identify whether the expression of a single APP transgene in the McGill rats negatively affected myelination state by reducing the expression levels of the genes responsible for the coding of these myelin proteins (Desai et al. 2009). These experiments were also conducted to evaluate the potential for FTY720 to either prevent demyelination in these animals (should it occur), or promote remyelination in APP transgenic animals at the level of the gene. RT-qPCR analysis of RNA from cerebellar tissue showed no significant difference in either *mog* or *mbp* expression between groups of differing genotype or treatment (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 7A, B**). Taken together, these results suggest that neither APP expression nor FTY720 treatment affect the expression of genes for myelin proteins in the cerebellum.

8. Microglial activation is not affected in rAPPtg or FTY720 treatment.

Microglia, as with astrocytes, have been shown to be directly implicated in contributing to CNS neuroinflammation in AD, converting from a 'resting' ramified morphology to an 'active' amoeboid morphology to facilitate phagocytosis of Aβ when DAMPs from degenerating neurons bind to microglial receptors, thus facilitating the release of pro-inflammatory cytokines to propagate inflammatory glial activation (Kreutzberg 1996, Sastre et al. 2006). Based on this knowledge, western blot analysis on hippocampal homogenate of the microglial marker Iba1 was carried out to assess whether APP expression correlated with increased expression of this marker and thus activation of microglia. In tandem with this, RT-qPCR

analysis of *aif-1* gene expression in cerebellar tissue was conducted to assess whether APP expression in the cerebellum would upregulate the expression level of this gene, indicating an increase in the expression of Iba1 protein with microglial activation in response to Aβ. Finally, these experiments were conducted to assess whether FTY720 could reduce the expected increase in Iba-1 protein and gene expression as a result of Aβ deposition in both the hippocampus and cerebellum, thus attenuating harmful chronic microglial activation. Western blot analysis conducted by two independent observers did not reveal any significant differences in Iba1 expression between WT and transgenic animals (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 8A, B**). RT-qPCR analysis of the *aif-1* gene similarly did not produce any significant difference in expression levels of the gene between transgenic and WT animals (Two-Way ANOVA, post-hoc Tukey's test) (**Figure 8C**). Taken together, these results appear to suggest that microglia are either not activated by the presence of APP in the hippocampus, or that WT animals have abnormally high levels of microglial activation, and that FTY720 did not have any effect on the expression levels of the protein. The RT-qPCR data also suggests that APP expression in the cerebellum does not lead to upregulation of the Iba1 gene, suggesting that Iba1 protein levels, and therefore levels of microglial activation, are not increased in either WTs or transgenics.

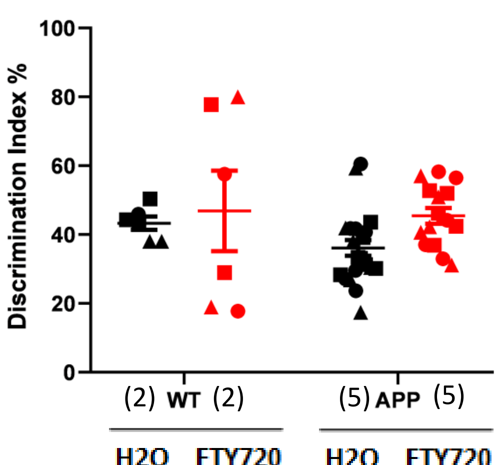


Figure 1. Novel Object Localization shows no effect of FTY720 in rAPPtg model.
Result of the Novel Object Localization behavioural test presented as % discrimination index. Discrimination index is calculated as time spent exploring the displaced object divided by the sum of the time spent exploring the displaced object and the two non-displaced objects, multiplied by 100. No significant difference in the propensity of the animals to spend more time exploring the displaced object over the stationary objects was observed either as a result of genotype or treatment. Data presented as mean ± SEM, Two-Way ANOVA and post-hoc Tukey's test (n=2-5). Observations were made by three independent researchers and depicted as ■ , ● and ▲ for each person, with the black versions of these shapes representing H2O-treated groups and the red versions representing FTY720-treated groups. Animal numbers are indicated in the parentheses.

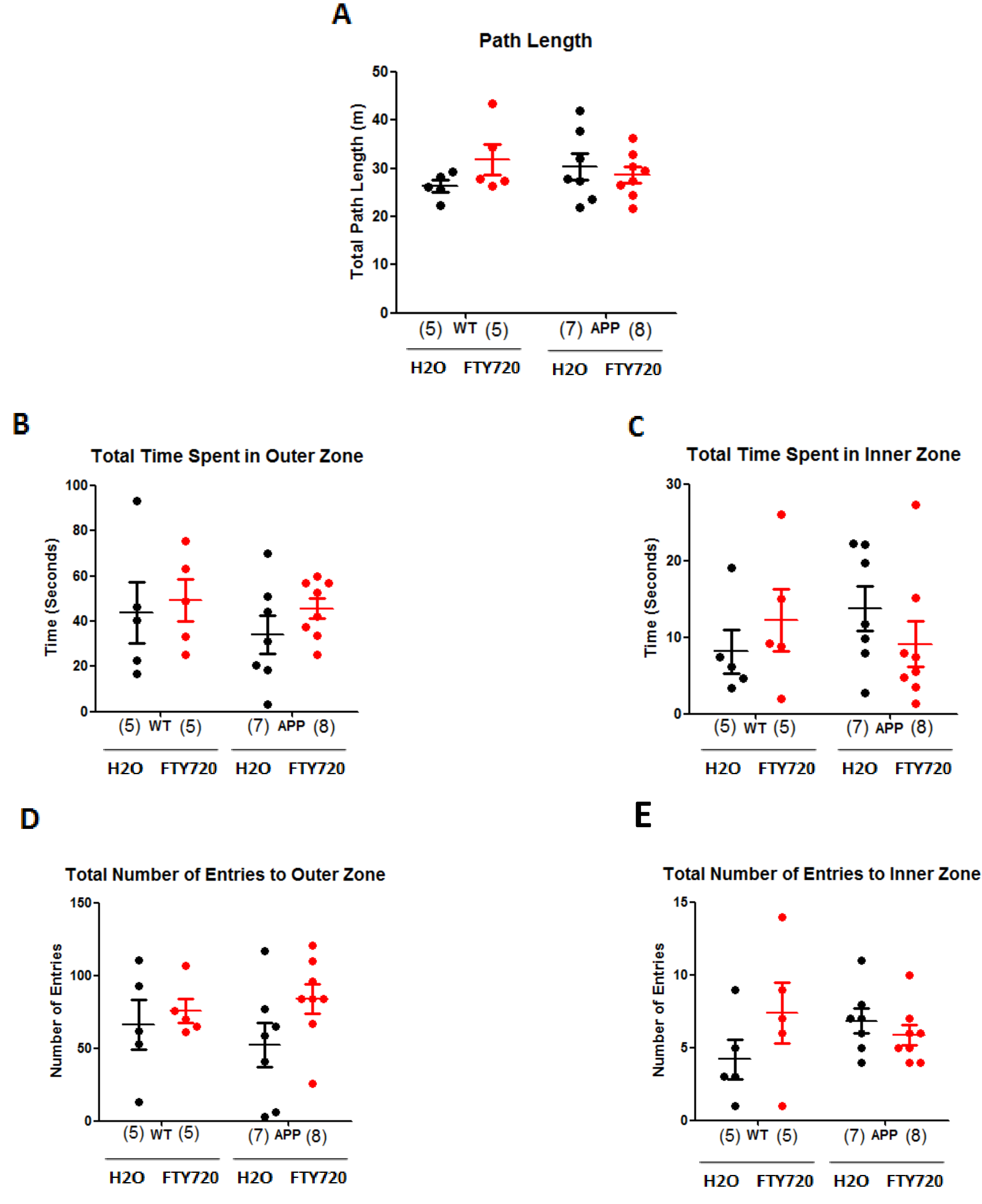


Figure 2. Open Field test suggests FTY720 does not alter locomotion or anxiety-like behaviour in rAPPtg model. A) Total distance covered by the animals in metres (m). **B)** Total time (in seconds) spent by each animal in the outer third of the test arena. **C)** Total time (in seconds) spent in the inner circle of the test arena. **D)** Total number of entries of each animal into the outer zone of the test arena. **E)** Total number of entries of each animal into the inner circle of the test arena. None of the above metrics of locomotion and/or anxiety-like behaviour were seen to have significant differences between animals either of differing genotype, or differing treatment. Data presented as mean ± SEM, Two-Way ANOVA and post-hoc Tukey's test (n=5-9). Animal numbers are indicated in the parentheses.

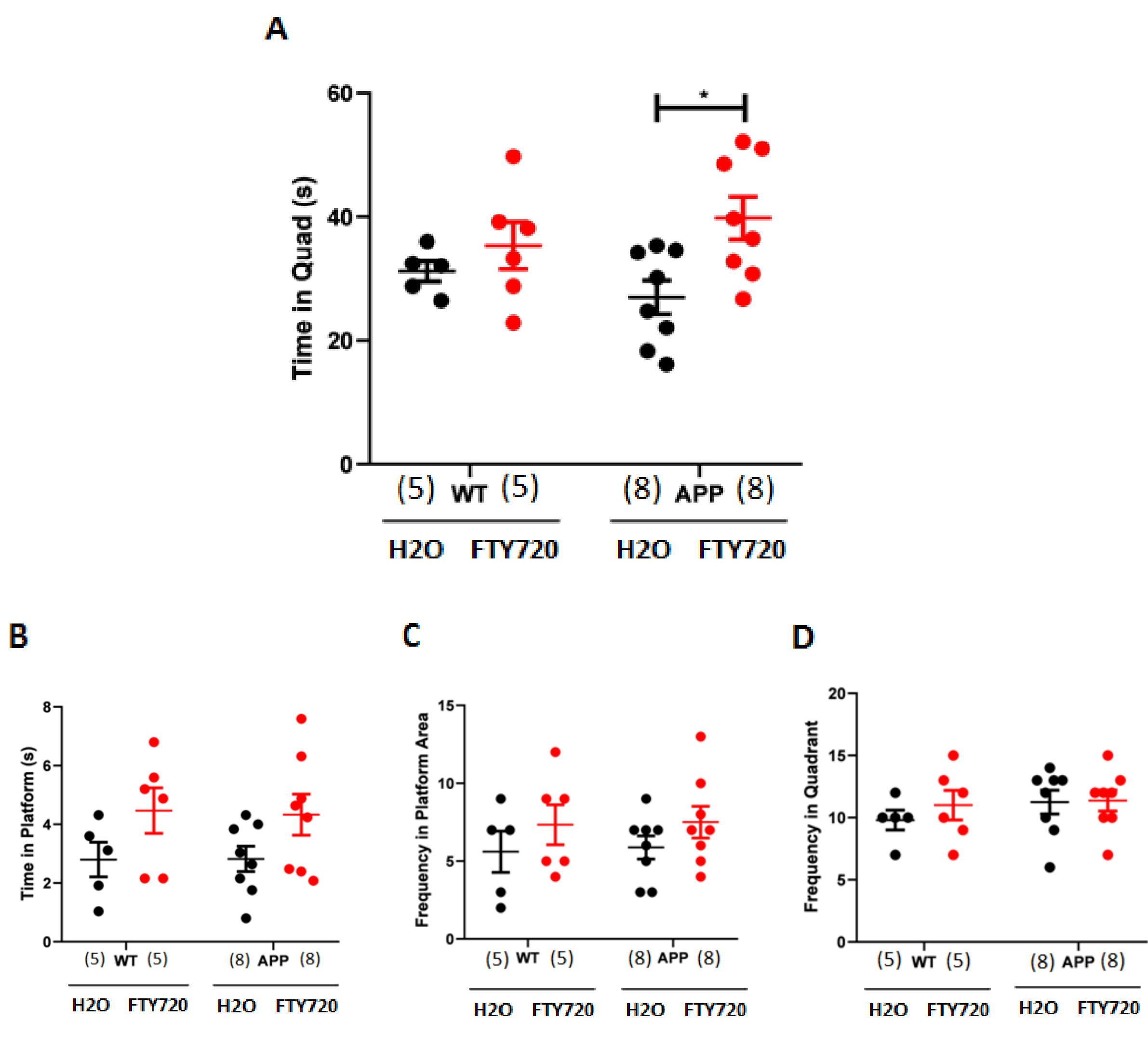


Figure 3. Morris water maze displays FTY720 treatment improves spatial memory in rAPPtg. A) Time spent in seconds in the platform-containing quadrant of the arena. A significant increase in time spent in the platform-containing quadrant was observed in FTY720-treated APP animals. **B)** Time in seconds spent by each animal in the MWM platform zone. A trend toward increased time in the platform zone was observed for both WT and APP FTY720-treated groups, but this effect was not found to be significant. **C)** No significant effect of genotype or FTY720 treatment was observed for the frequency of entry of the animals into the platform area. **D)** No significant effect of genotype or FTY720 treatment was observed for the frequency of entry of the animals into the platform-containing quadrant of the arena. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=5-8). *=P<0.05. Animal numbers are indicated in the parentheses.

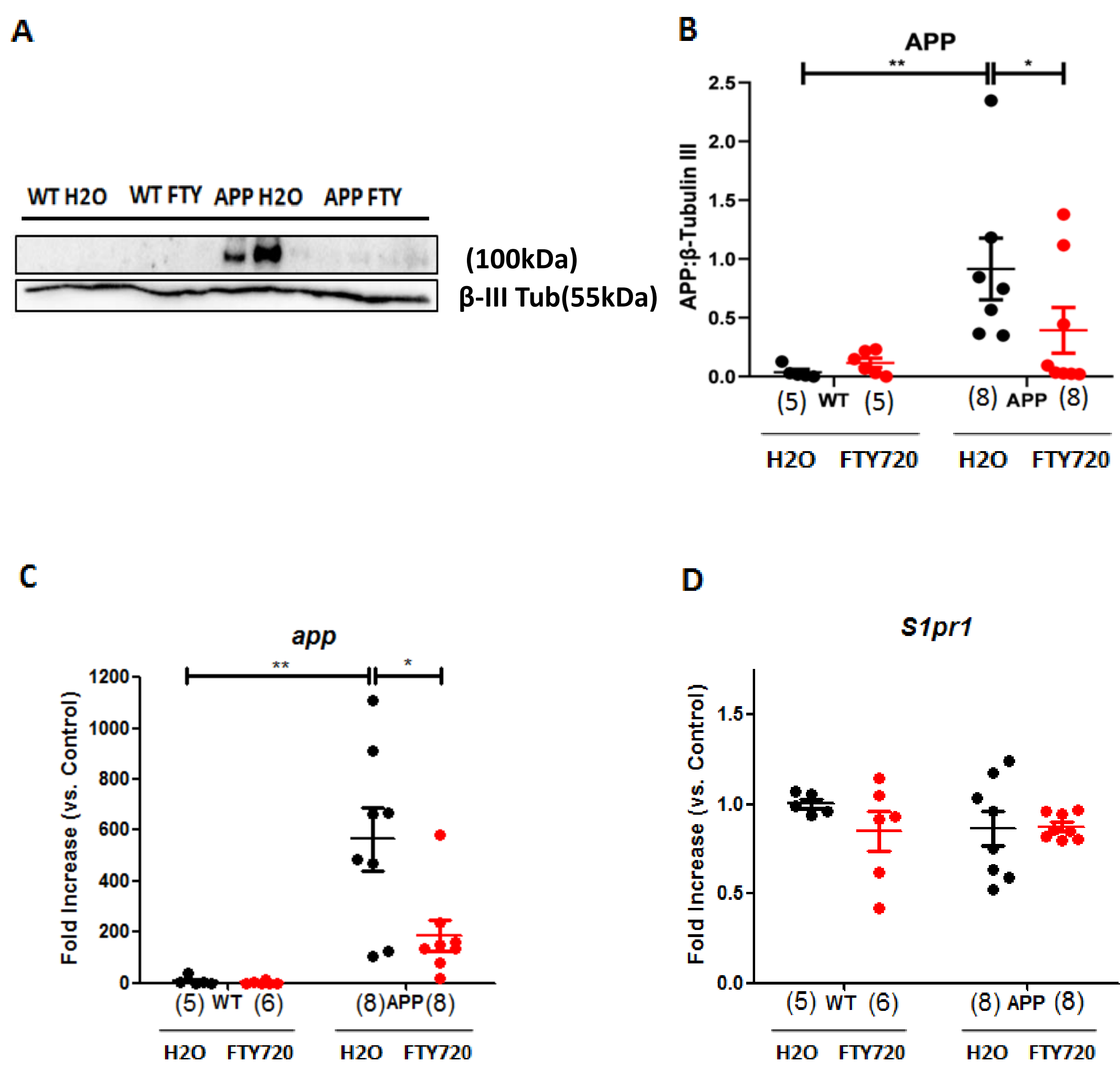


Figure 4. FTY720 attenuates APP expression in rAPPtg model. A) Representative western blot of APP expression with β-III Tubulin as housekeeping control. **B) C)** RT-qPCR data indicating relative quantitation of *app* to *gapdh* in the cerebellum, with the WT H_2O-treated group taken as control. *app* gene expression is significantly increased in APP transgenic animals, and this expression at the gene level is attenuated by FTY720 treatment. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=5-8). *=P<0.05, **=P<0.01. Animal numbers are indicated in the parentheses.

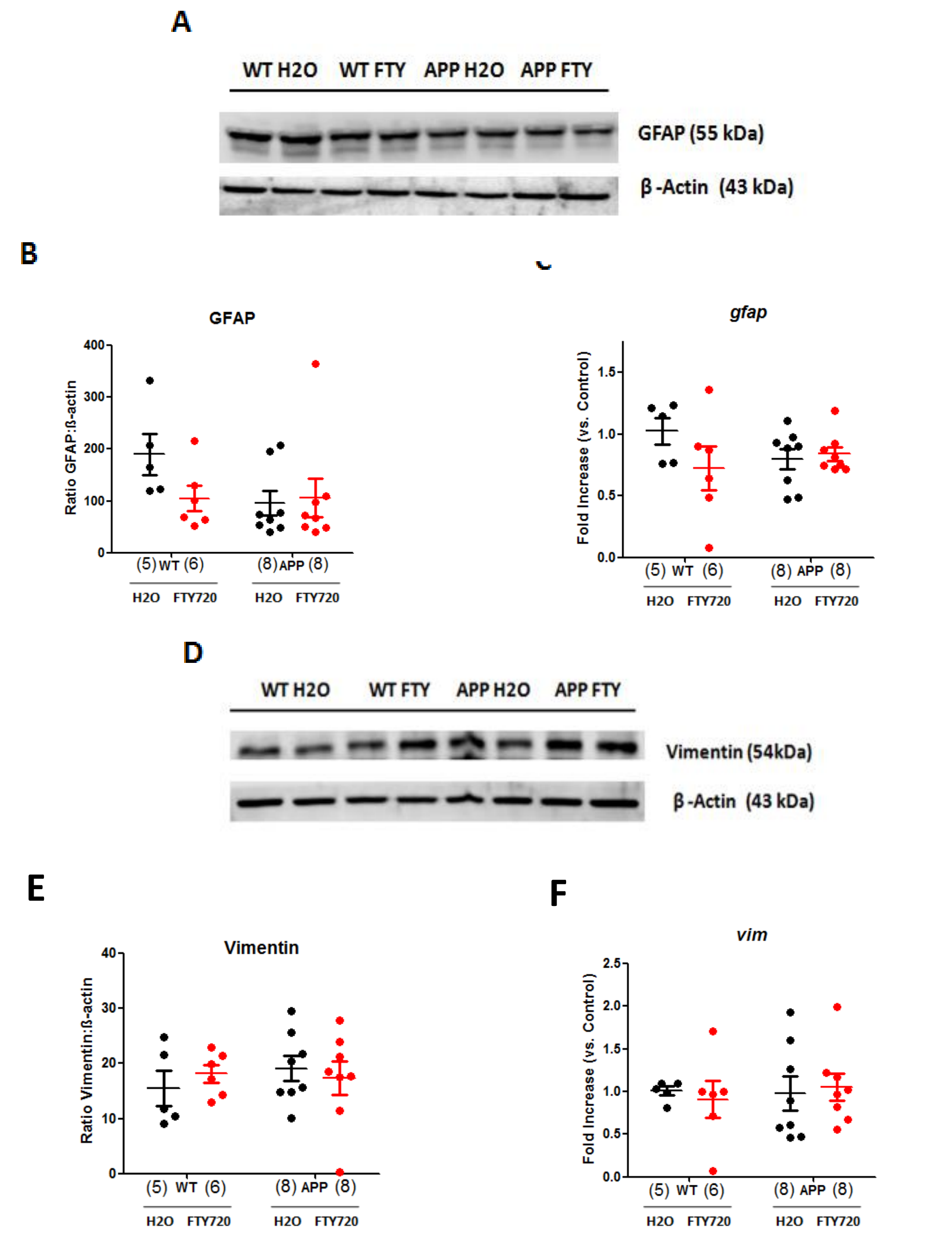


Figure 5. FTY720 treatment does not alter expression of astrocyte markers. A, D) Representative western blot images of the astrocyte protein markers, GFAP and Vimentin respectively with β-Actin as housekeeping control. **B, E)** Graphs of the ratio of GFAP:β-Actin and Vimentin:β-Actin respectively. No significant difference was observed between animals of differing genotype or animals of differing treatment for either protein. **C, F)** RT-qPCR data indicating relative quantitation of *gfap* and *vim* respectively to *gapdh* in the cerebellum, with the WT H_2O-treated group taken as control. There was no significant difference observed between groups of differing genotype or treatment. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=5-8). Animal numbers are indicated in the parentheses.

H2O FTY720 H2O FTY720

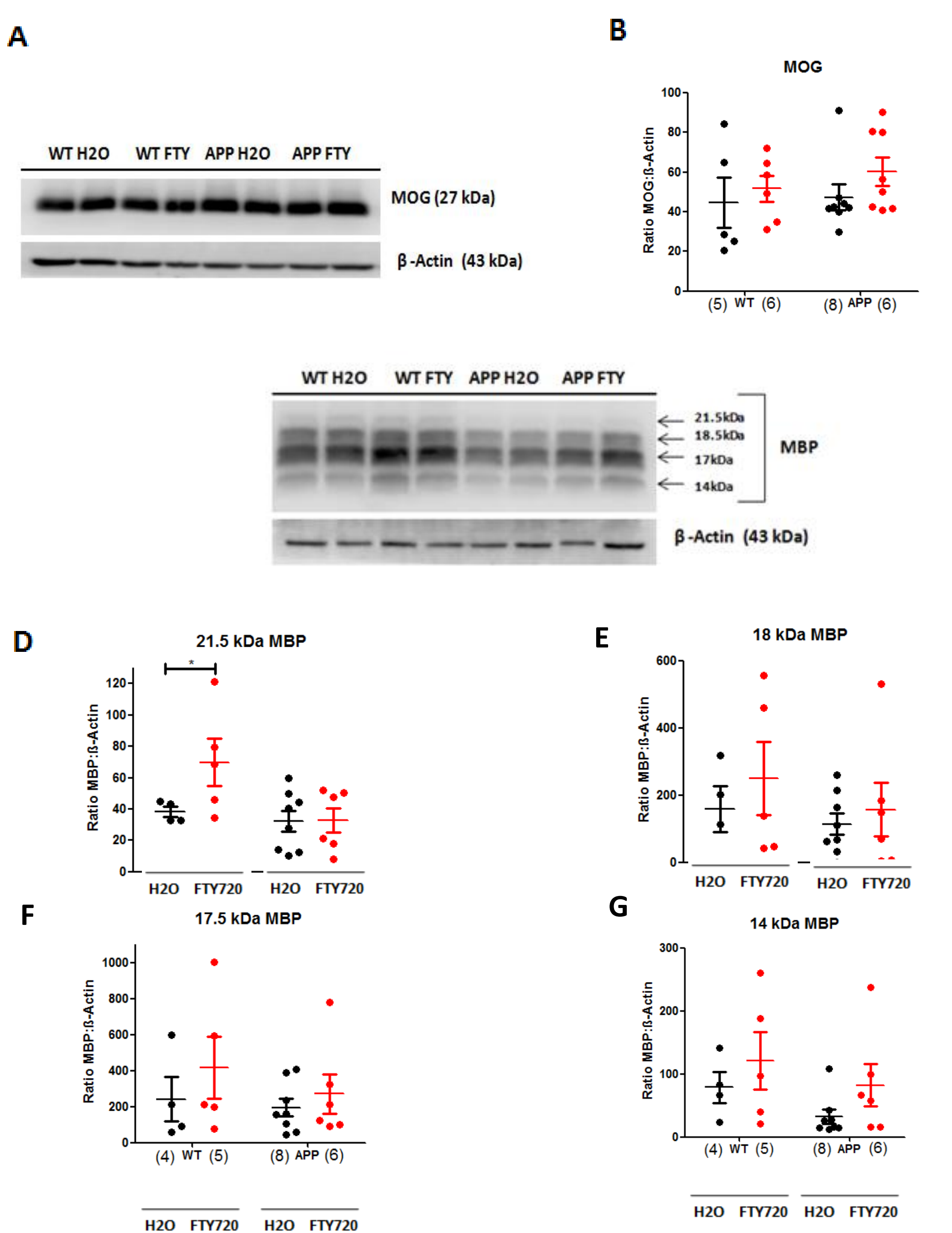
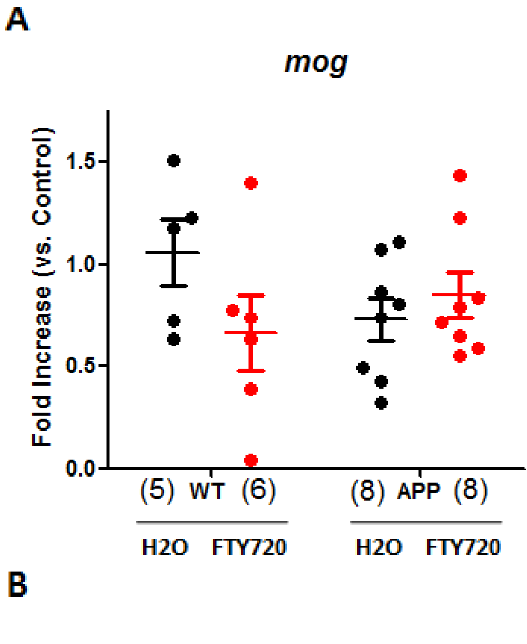


Figure 6. Levels of myelin protein are not affected by APP genotype or FTY720 treatment. A) Representative western blot image of MOG with β-Actin as housekeeping control. **B)** Graph of the ratio of MOG:β-Actin for each group, which showed no significant difference in MOG expression between groups. **C)** Representative western blot image of MBP, with β-Actin as housekeeping control. **D-G)** Graphs of the ratio of MBP isoforms of molecular weight 21.5kDa, 18kDA, 17.5kDA and 14kDA respectively indicate no significant effect of genotype or treatment on MBP expression. However, a trend toward increased basal expression of MBP as a result of FTY720 treatment in WT animals was observed. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=4-8). *=P<0.05. Animal numbers are indicated in the parentheses.



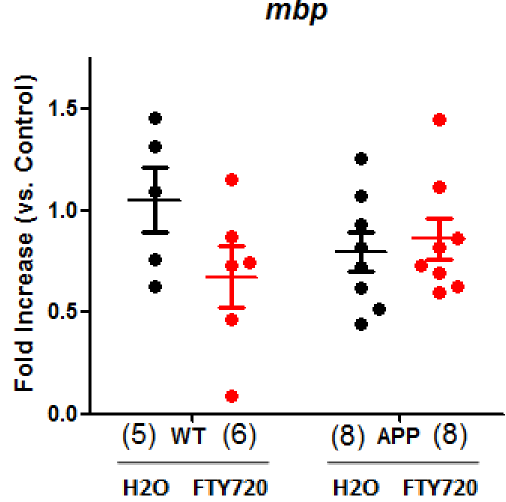
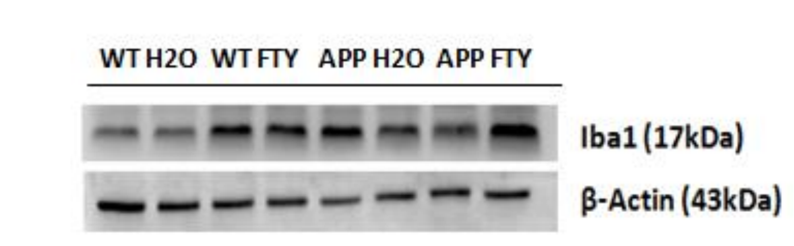
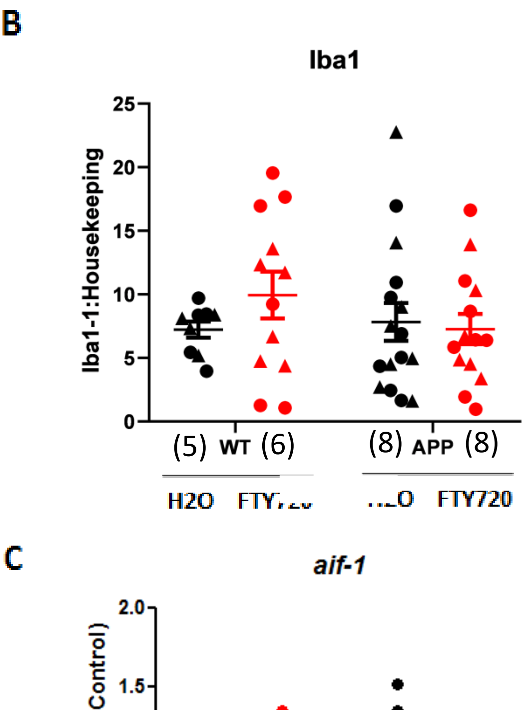


Figure 7. Levels of myelin genes are not affected by APP genotype or FTY720 treatment. A-B) RT-qPCR data indicating relative quantitation of *mog* and *mbp* respectively to *gapdh* in the cerebellum, with the WT H_2O-treated group taken as control. Neither genotype nor FTY720 group affected expression levels of either of these genes. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=5-8). Animal numbers are indicated in the parentheses.

A



B



C

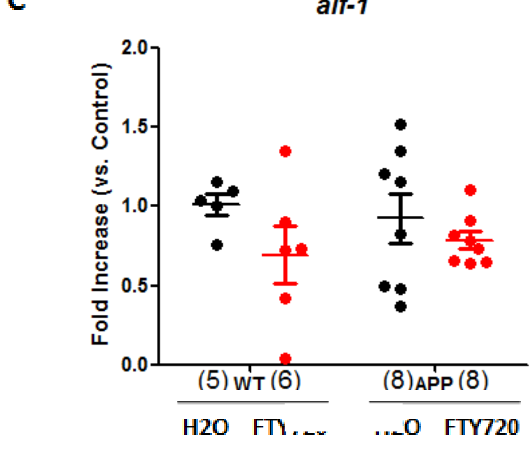


Figure 8. Microglial activation is not affected by APP transgenesis or FTY720 treatment. A) Representative western blot of Iba1 expression with β-Actin as housekeeping control. **B)** Graph of the ratio of Iba1:β-Actin. No significant differences in Iba1 expression were observed between either WT vs. transgenic animals and H_2O vs. FTY720-treated animals. Western blotting was repeated by two independent researchers (Adam, Luke). **C)** RT-qPCR data indicating relative quantitation of *aif-1* to *gapdh* in the cerebellum, with the WT H_2O-treated group taken as control. Expression levels of this gene were not affected by APP transgenesis or FTY720 treatment. Western blot data is the result of observations from two independent researchers, depicted as ● and ▲ for H2O-treated animals, and the same shapes in red for FTY720-treated animals. Data presented as mean ± SEM, Two-Way ANOVA, post-hoc Tukey's test (n=5-8). Animal numbers are indicated in the parentheses.

Discussion

1. Summary of findings

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disorder characterized by memory loss and cognitive decline, with loss of global functioning over time eventually leading to death. Neuroinflammation has been implicated in AD pathogenesis in addition to Aβ and tau-mediated neuronal cell death (Heneka et al. 2015) Furthermore, a number of studies have demonstrated the therapeutic efficacy of FTY720 in ameliorating spatial memory deficits, as well as reducing Aβ-mediated neuronal loss and chronic inflammatory glial activation *in vivo* (Asle-Rousta et al. 2013a, Hemmati et al. 2013, McManus et al. 2017). In the present study, we examined the effect of FTY720 on spatial memory in McGill APP rats through the MWM and Novel Object Localization (NOL) tests, as well as locomotion and anxiety-like behaviour through the Open Field (OF), which have all previously been studied in the McGill APP rat (Leon et al. 2010, Petrasek et al. 2018). FTY720 was not found to affect spatial memory in the NOL test, and McGill APP rats were found to have a similar preference for the relocated object as was evident in the WT controls, which has been previously reported in this AD model (Petrasek et al. 2018, Galeano et al. 2014). However, FTY720 was found to improve spatial memory in the MWM, with a significant increase in the amount of time spent in the platform-containing quadrant observed in the FTY720-treated APP transgenic group with respect to the vehicle-treated group of the same genotype. A trend toward increased time spent in the platform area was also observed in both WT and APP transgenic FTY720-treated animals with respect to their vehicle-treated counterparts. However, there did not appear to be a deficit in the latency of the APP McGill rats to reach the platform area/platform quadrant, which is contrary to other behavioural studies involving this model (Petrasek et al. 2018, Leon et al. 2010). Finally OF analysis was carried out to analyse locomotor activity in APP transgenic rats, which was not found to be significantly affected by either genotype or treatment, which is consistent with other behavioural studies involving this AD model (Galeano et al. 2014, Petrasek et al. 2018). This test was also conducted to assess anxiety-like behaviour, which is a feature of rodent AD models (Webster et al. 2014). No significant effect of either genotype or treatment was observed on time spent in the inner and outer zones, or the number of entries to these zones across all groups, but the literature on this is inconsistent (Petrasek et al. 2018, Galeano et al. 2014).

In terms of the biochemical analysis, we found that McGill APP transgenic rats express APP protein and the *app* gene in the hippocampus and cerebellum respectively, and that FTY720 attenuates this expression, suggesting that FTY720 is capable of reducing Aβ load, either directly or indirectly. We also found that neither genotype nor FTY720 treatment affects the expression of the *S1pr1* gene, indicating that the drug's effect on these receptors likely occurs at the level of the protein rather than the gene. In terms of markers of reactive astrocytosis, we found that neither GFAP nor Vimentin expression is affected by genotype or FTY720 treatment in the hippocampus, and a similar result was obtained from the RT-qPCR analysis on the genes of these proteins in the cerebellum. This is in contrast to another study which observed reactive gliosis in the hippocampus after 6 months in this model of AD (Hanzel et al. 2014). Next, we examined the levels of myelin proteins in the hippocampus of the transgenic rats, and we found no effect of genotype or treatment on the levels of MOG, but observed an increase in the expression of MBP in the WT FTY720-treated group, suggesting a myelin-stabilizing effect of FTY720. RT-qPCR analysis of *mog* and *mbp* both exhibited no effect of

genotype or treatment on their expression levels. Finally, we observed no significant difference in the expression of the Iba1 microglial marker in the hippocampus or the corresponding *aif-1* gene in the cerebellum, in terms of differing genotype or treatment, suggesting that microglia are not chronically activated in this model. This is again contrary to the literature, however McGill APP rats are 18 months of age at sacrifice in the study in question (Hanzel et al. 2014). Taken together, these results suggest that FTY720 can improve spatial memory, reduce the expression of APP in the APP McGill AD rat model and also stabilize myelin proteins in WT animals. However, FTY720 does not have any effect on inflammatory glial activation, or the expression of myelin proteins or genes in APP transgenics. Looking purely at the rat model of AD utilised in this study, it appears that at the time point selected for behavioural experiments and tissue harvesting (12 months), this model does not display a number of characteristic disease markers of AD animals, such as anxiety-like behaviour and reduced learning capacity in the MWM, as well as chronic astrocytic and microglial activation as a result of Aβ deposition and in conjunction with demyelination in the hippocampus. As has been suggested in other studies, this model may have greater use in studying early stages of AD (3-6 months) (Galeano et al. 2014).

2. Behavioural Testing in the McGill APP rat model of AD and the effect of FTY720.

The use of the OF, MWM and NOL behavioural tests is widely reported in assessing the behaviour of APP McGill rats of various ages (Leon et al. 2010, Galeano et al. 2014, Petrasek et al. 2018). These tests are primarily used for assessing cognitive domains such as reference memory, working memory, object recognition, locomotion and anxiety behaviours in rodent models of AD. Locomotion in OF studies involving this model have previously reported no effect of genotype on total distance travelled, which is consistent with our results, however total distance travelled has been shown to decrease with increased number of tests, so we were unable to observe this effect and the potential effect of FTY720 on this behaviour due to the fact that only one testing session was carried out (Petrasek et al. 2018). FTY720 has previously been shown to improve locomotion in MPTP-generated Parkinson's disease models, however, as AD is not predominantly a disorder of motor function, at least initially, we would not have expected to see an effect of FTY720 on this behaviour (Yao et al. 2019). Results published on anxiety behaviour in this AD model as examined by the OF test have proven to be inconsistent. A study involving the use of hemizygous McGill APP rats found that at 6 and 12 months, there was a significant reduction in the number of entries and the amount of time spent in the centre zone of the OF test in the APP transgenic rats, indicating an increase in anxiety-like behaviour in this group (Galeano et al. 2014). However, a similar study using the same model reported that both WT and McGill APP animals spent the majority of the OF test in the periphery and rarely entered the central zone of the maze, which was consistent with the results reported here, and indicates that anxiety behaviour is not a key characteristic of the spectrum of behaviours exhibited by this model (Petrasek et al. 2018).

In the MWM test, we observed an increase in the time spent in the platform-containing quadrant in FTY720-treated APP transgenics. The literature on MWM performance in this model is again, conflicting, with one study observing a reduction in the latency of both WT and

McGill groups to finding the platform with repeated testing but the McGill group performed significantly worse than WTs, while another study also observed a reduction in latency to the platform of both WT and McGill groups, with no difference in performance between the two groups (Petrasek et al. 2018, Galeano et al. 2014). We also observed no difference in the latency to the platform area in this study between WTs and transgenics. In a hippocampal Aβ-injection rat model of AD, it was found that the Aβ-injected animals exhibited a significant reduction in time spent in the target quadrant, which was rescued to control levels by FTY720. This suggests that the McGill model may not be the most optimal model for the study of the effect of FTY720 on spatial memory metrics, as other studies have shown that FTY720 can improve deficits in this behaviour that are induced by Aβ 1-42 intra-hippocampal injection (Asle-Rousta et al. 2013a).

In the NOL test, no significant difference in the propensity of the animals to spend more time exploring the displaced object over the stationary objects was observed either as a result of genotype or treatment, suggesting that reference memory is not impaired in this AD model. This result is consistent with another NOL test conducted on this model and similar Novel Object Recognition (NOR) tests in hemizygous McGill rats, but in total contrast to another study, which found profound impairment in recognition memory in both homo- and hemizygous McGill rats at 3 and 13 months of age (Petrasek et al. 2018, Galeano et al. 2014, Martino Adami et al. 2017, Iulita et al. 2014). From our results, it is evident that FTY720 was able to improve spatial memory in the MWM but not the NOL, suggesting that perhaps object recognition and spatial memory are encoded differently in the CNS i.e. are processed through differing circuits of neurons, and that perhaps the effect of FTY720 differs in these two behavioural tests due to differences in protein expression in the brain regions associated with these two behavioural metrics. It has been shown that the hippocampus, cortex and cerebellum are the three key brain regions recruited during the MWM task (Barnhart, Yang and Lein 2015). However, while both cortex and hippocampus have been identified as key processing regions for object recognition memory as well, it appears that the parahippocampal regions of the temporal lobe, including entorhinal, perirhinal and inferior temporal cortices are more closely linked to object memory (Hammond, Tull and Stackman 2004). Given that FTY720 acts through S1PRs, particularly S1PR1 and S1PR3/5, it is possible that the distribution/expression of S1PRs differs between the hippocampus and the entorhinal cortex and may account for the differences in behavioural outcome observed following FTY720 treatment in the MWM and NOL tasks.

A limitation of these behavioural studies may be that only animals of 12 months of age were utilised, and while a number of key AD pathology indicators are evident at this age in McGill rats, the full extent/spectrum of pathologies is not fully evident until 18 months of age, so treatment of rats with FTY720 at 12 months until 18 months may have yielded more significant data in terms of disease severity and the ability of FTY720 to ameliorate deficits in behavioural metrics. However, given the number of animals available to us, the use of animals of more than one age was not possible. All behavioural analysis taken together, there appears to be a significant discrepancy in the results being obtained from a number of different studies looking at this animal model, with little consensus. Caution must be taken when interpreting

the results of these behavioural studies as the reproducibility of behavioural components of this model appears to be variable.

3. FTY720 as a therapeutic for the reduction of Aβ.

Previous studies have indicated that the therapeutic benefit of FTY720 in relation to AD is specifically dependent on S1PR1 modulation and SphK activity in the CNS (Asle-Rousta et al. 2014). In the present study, we found that FTY720 can attenuate the expression of APP both at the protein and gene level, in the hippocampus and cerebellum respectively. It should be noted at this point that, ideally, hippocampal tissue would have been utilised for RT-qPCR and for possible immunohistochemical analyses, as this is one of the main neurodegeneration-relevant areas in AD. However, unfortunately the snap frozen rat brain hemispheres were likely not processed correctly for freezing, as initial cryostating of these frozen brains to generate hippocampal slices for immunohistochemical analysis revealed tissue damage. As a number of the hippocampi had already been sliced, it was decided that cerebellum would be used instead, as this is a region that has been associated with elevated Aβ oligomer deposition, particularly in the cerebellar cortex of AD patients (Larner 1997). While the effect of FTY720 on Aβ levels was not included in this study, we can extrapolate that if expression of its precursor is reduced, then the amount of precursor available for cleavage to Aβ42 is similarly reduced. A similar protective effect of FTY720 has previously been found in a number of studies. 3 month old 5xFAD mice treated for 2 months with 1mg/kg and 5mg/kg FTY720 showed reduced Aβ42 levels and therefore plaque burden (Aytan et al. 2016). Other studies have shown that FTY720 suppresses oligomeric Aβ-mediated cell death in mouse primary cortical neurons as well as decreasing Aβ production in a mouse neuroblastoma cell line (Doi et al. 2013, Takasugi et al. 2013). Interestingly, it has been shown that FTY720 does not directly bind to Aβ species (Joshi et al. 2017). A number of cellular and molecular targets have been implicated in the neuroprotective effects of FTY720. It has been suggested that SphK2 is required for FTY720's Aβ secretion-lowering effect, and that this effect occurs independently of FTY720's antagonistic effect on S1PR1s and subsequent intracellular GPCR-signalling cascades (Takasugi et al. 2013). Other studies have suggested that brain-derived neurotrophic factor (BDNF) is the key mediator promoting hippocampal neuroprotection from Aβ-mediated neuronal cell death and memory loss *in vivo* (Fukumoto et al. 2014, Doi et al. 2013). It has also been postulated that FTY720 may affect Aβ1-42 metabolism by altering microglial phagocytosis (Takasugi et al. 2013). Finally, another study has suggested that FTY720 produces an increase in GLUN2A-containing neuroprotective NMDARs, while concomitantly mobilizing extrasynaptic GLUN2B-containing –NMDARs to the synapse in cultured hippocampal neurons. Altered ratio of extrasynaptic:synaptic NMDARs decreases the calcium-responsiveness of these cultured neurons to soluble Aβ1-42, protecting them to detrimental alterations in calcium homeostasis associated with neurotoxic cell death (Joshi et al. 2017). Taken together, our findings suggest that FTY720 is capable of reducing amyloid-load in the McGill rat model of AD, but whether the mechanism is similar to other models of AD is yet to be elucidated.

4. Neuroinflammation in AD and the effect of FTY720.

In the present study, no significant effect of genotype was observed on the expression levels of the astrocyte and microglial markers GFAP, Vimentin and Iba1, which was an unexpected

finding, given that reactive astrocytosis has been reported in the McGill rat model at 6 months of age in the hippocampus, and microgliosis in the hippocampus at 13 months. It is possible that due to the sacrificing of the animals in this study at 12 months, this study missed out on seeing this effect, but there is evidence of increased microglial soma area and length, as well as microgliosis in the subiculum, at 6 months of age, so this is unlikely (Hanzel et al. 2014). Increased levels of the pro-inflammatory markers TNFα, IL-1β and COX-2 have also been observed, but were not examined in this study (Hanzel et al. 2014, Asle-Rousta et al. 2014). It is possible that changes in microglial and astrocytic morphology (activation state) and density may be hippocampal-region specific, and the homogenizing of the entire hippocampus for western blotting may cause this potential effect to be lost. Similarly no effect of genotype was observed on the expression of the genes for these proteins in the cerebellum, possibly due to significantly lower Aβ plaque burden in this area. FTY720 was not found to have any effect on the expression levels of these inflammatory markers either. Activation of primary mouse astrocytes by Aβ has been shown to be attenuated by FTY720 in vitro, and has also been shown to be correlated with increased phagocytosis of Aβ by GFAP$^+$ astrocytes (McManus et al. 2017). FTY720 has also been shown to reduce GFAP staining and numbers of activated (Iba1-positive) microglia in a 5xFAD mouse model of AD, however, this model has a much more severe AD phenotype than McGill rats owing to the presence of tau mutations (Aytan et al. 2016). Ideally, immunohistochemistry of for GFAP, Vimentin and Iba1 would be carried out, as this would allow visualization of increased number of astrocytes and microglia, as well as their activation states, as an indicator of whether Aβ deposition was causing the activation of these cells types, and western blotting may not have been the most optimal assay type to quantify this. Taken together, these results suggest that the McGill rat is not a suitable model for studying neuroinflammation in the context of astrocytic and microglial gliosis.

5. Myelination state and FTY720 in the APP McGill rat model of AD.

The breakdown of myelin and alterations in oligodendrocyte activity is understood to be one of the earliest potential signs of the onset of AD, preceding Aβ deposition and tau hyper-phosphorylation in a number of *in vivo* models of AD (Desai et al. 2009). It is thought the decline in the robustness of myelin with increased age leaves it vulnerable to stressors and pathologically aggregated protein species such as Aβ and NFTs causing cognitive decline (Bartzokis 2004). In the present study we did not observe any effect of genotype on the expression of either MOG or MBP in the hippocampus or *mog* and *mbp* in the cerebellum. No previous studies involving the McGill rat model of AD have been conducted in terms of examining myelination state and the potential for the inflammatory CNS environment produced in AD to negatively impact myelin, or myelinating oligodendrocytes. However, previous studies have implicated demyelination as a feature of Aβ-mediated neurodegeneration in other AD models (Mitew et al. 2010, Dong et al. 2018, Roher et al. 2002).

A number of mechanisms of Aβ-mediated alteration in oligodendrocyte and myelination profile have been identified, including through *PS1* mutation-mediated alterations in oligodendrocyte precursor cell (OPC) differentiation and MBP subcellular mislocation. Corpus callosum white matter damage and inflammatory demyelination as a result of direct Aβ deposition also contribute to an demyelinating AD phenotype as well as reduction in MBP, PLP

and cholesterol levels as a result of the presence of Aβ in CNS white matter, which negatively affects the synthesis and viability of myelin and axonal health (Desai et al. 2011, Jantaratnotai et al. 2003, Roher et al. 2002). Interestingly, we found that MBP expression is increased in WT animals treated with FTY720, which may be explained by the high lipophilicity of the drug. This feature of FTY720 may cause it to associate and accumulate around the fatty-lipid myelin sheath of myelinated axons, providing support to the myelin proteins contained within. 10nm FTY720 treatment for 72 hours has been shown to stimulate OPC differentiation and MBP expression in cultured rat OPCs, suggesting that FTY720 may have a stimulatory effect both on MBP expression and on maturation of OPCs to oligodendrocytes (Jung et al. 2007). However, no effect of FTY720 was observed on myelin protein expression in McGill rats. Taken together these results suggest that the McGill rat model of AD may not be a suitable model for studying hippocampal myelination in AD, but due to the lack of other studies using this model for this purpose, further study must be done in this area to corroborate these findings. It appears that FTY72O has a protective effect on baseline levels of myelination in WT animals but does not exert the same effect in the McGill model of AD.

6. Future Studies

A number of future studies could be carried out to corroborate and build on the work that has been done in this study, which includes but is not limited to:

- Given that other studies have identified BDNF as the neurotrophic factor likely mediating the neuroprotective effects of FTY720 against Aβ-mediated neurotoxicity, it may be worthwhile to carry out western blot and RT-qPCR experiments to determine whether FTY720 affects either protein or gene expression of BDNF in the McGill APP rat model.

- Immunohistochemistry would be a useful technique to employ in future studies with the McGill APP rat and FTY720 treatment, as this would enable the identification of any region-specific changes in GFAP and Iba1 expression levels in the hippocampus that cannot be detected by western blot, along with any morphological changes that may occur in conjunction with that. Staining for Aβ and being able to visualise whether there were any activated microglia surrounding Aβ plaques would also go a long way to identifying whether microglia were responding to Aβ-deposition in this model.

- Given that FTY720 was shown to reduce *app* gene expression in the cerebellum, future studies could assess any potential impact that early treatment of FTY720 may have on Notch signalling, which is crucial for endocrine development and is regulated by sphingosine 1- phosphate levels. A previous study has shown that FTY720 reduced Aβ1-42 plaque load without affecting Notch signalling, but expression of the Notch gene with FTY720 has not yet been studied (Takasugi et al. 2013).

- Future studies may be able to elucidate the role of peripheral macrophages in neuroinflammatory disease phenotype in the AD CNS, as it is thought that peripheral immune cells may enter the AD brain due to BBB leakiness. Western blot and

immunohistochemical analysis on infiltrating immune cells, for example, the CD3 T-cell marker, would be an additional study that could be performed.

7. Conclusion

Neuroinflammation has been described as a significant contributor to worsening disease severity in AD. It was initially hypothesised that FTY720 could ameliorate behavioural symptoms of AD in the McGill APP rat model of AD, namely spatial memory deficits in the MWM and NOL tasks, and locomotion and anxiety in the OF. It was also hypothesised that FTY720 treatment in this AD model could attenuate chronic activation of CNS-resident glial cells, astrocytes and microglia, in the hippocampus and cerebellum thus reducing the impact of a pro-inflammatory CNS microenvironment on Aβ-induced neurotoxicity. We also hypothesised that FTY720 treatment could attenuate the demyelination that has been shown to occur at very early stages of AD disease pathogenesis. Finally, we wanted to identify whether FTY720 treatment could directly affect APP expression in this model. Importantly, this study found that FTY720 reduces the expression of APP in the hippocampus, and the *app* gene in the cerebellum, thus highlighting FTY720 as a potential therapeutic for the amelioration of amyloid plaque burden in AD. Spatial memory was found to be improved in the McGill rat model in the MWM, but not NOL, indicating that FTY720 does have an effect on spatial memory in AD, but further studies are required to validate this finding. We conclude that neither locomotion nor anxiety were affected by genotype or FTY720 treatment, but findings on these behavioural metrics remain inconsistent in this AD model. We found no effect of genotype or treatment on astrocyte or microglial activation state, or myelination state in the McGill rat, however we found an increase in myelin protein expression in WTs with FTY720, suggesting that long-term FTY720 treatment in healthy animals may protect against age-related decline in myelin. In conclusion, we found FTY720 to be a useful therapeutic for the reduction of amyloid load and improvement in spatial memory, but not for the amelioration of reactive gliosis *in* vivo.

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