EFFECTS OF THE PPAR-γ AGONIST ROSIGLITAZONE ON COGNITION IN TG2576 MICE

By

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Summary of Thesis:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder for which there is no cure. At the neuropathological level, AD is characterized by the presence of large numbers of amyloid-beta containing plaques (Aβ-plaques), and neurofibrillary tangles comprised mostly of hyperphosphorylated aggregated protein tau. Both types of deposit are associated with neuroinflammation, synaptic and neuronal cell loss. Accumulating evidence indicates a role for metabolic dysfunction in the pathogenesis of AD. Type 2 diabetes increases the risk of developing AD and several post-mortem analyses have reported evidence of insulin resistance in Alzheimer brain tissue. Insulin-based therapies have emerged as potential strategies to slow cognitive decline in AD, these include the use of insulin sensitizers, such as rosiglitazone, which mediates its effects on insulin sensitivity via the peroxisome proliferator-activated receptors-gamma (PPAR-γ) receptor. While the results of insulin sensitizers on cognition in animal models of AD have been largely positive, the impact of these compounds on cognitive decline in AD patients has been variable.

Animal experiments provide a unique opportunity to examine the specific conditions and mechanisms by which insulin sensitizer's impact on AD-related pathology. This thesis details experiments conducted in a popular Amyloid Precursor Protein overexpressing transgenic mouse model of amyloid pathology that over-produces human Aβ. The aim of these experiments was to determine if chronic dosing with rosiglitazone ameliorated phenotypic behavioural deficits in transgenic mice, and lowered specific biomarkers associated with Aβ over-production. The results indicate that rosiglitazone largely does not reverse phenotypic behavioural alterations in these mice, nor does it reduce total Aβ levels. From this preclinical data, it is concluded that rosiglitazone is likely not a suitable therapeutic treatment for use in human patients with AD.

"I've seen things you people wouldn't believe. Attack ships on fire off the shoulder of Orion. I've watched C-beams glitter in the dark near the Tannhauser Gate. All those moments, will be lost in time, like tears in rain. Time to die."

Roy (Rutger Hauer) in 'Bladerunner' (Ridley Scott)

CONTENTS

ABBREVIATIONS

~	Approximate
µl	Microliters
5HT	5-Hydroxytryptophan
ACH	Amyloid Cascade Hypothesis
AD	Alzheimer's disease
ADDLS	Aβ-derived diffusible ligands (Soluble Aβ Oligomers)
ADL	Activities of daily living
AG1024	Insulin receptor tyrosine kinase inhibitor
AICD	APP Intracellular cytoplasmic/C-terminal domain
AKT-1	RAC-alpha serine/threonine-protein kinase-1
aMCI	Amnesic MCI
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APOEε2	Apolipoprotein E Allele 2
APOEε3	Apolipoprotein E Allele 3
APOEε4	Apolipoprotein E Allele 4
APP	Amyloid Precursor Protein
APP_{SWE}	APP Swedish mutation
ARC	Activity-regulated cytoskeletal protein
ATL	Anterior temporal lobe
ATP	Adenosine Triphosphate
ATP	Adenosine triphosphate
Aβ	Amyloid Beta (Beta (β)-amyloid)
Aβ-Plaques	Amyloid Beta Plaques
BAD	Inhibitor of Bcl-2
BBB	Blood Brain Barrier
Beta-secretase	β-secretase (β-site APP cleaving enzyme 1)
BIN1	Bridging Integrator 1
BL	Bottom Left
BL6HJL	Black 6 Swiss James Webster
BP	Base pair
BPS	Behavioural and neuropsychiatric symptoms
BR	Bottom Right
C83	Membrane-bound C-terminal fragment
C99	C-terminal fragment of 99 amino acids in length
CA1	Cornu Ammonis-1 area of the hippocampus
CA3	Cornu Ammonis Area 3
CAA	Cerebral amyloid angiopathy
CaMKII	Calcium calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate response element binding protein

CDKN2A, p16INK4A	Cyclin-dependent kinase inhibitor 2A, (CDKN2A, p16Ink4A)
CK2	Casein kinase II
CLU	Clusterin gene
CNS	Central nervous system
COC	Cross of Changes
COX-2	Cyclooxygenase-2 enzyme
CR1	Complement receptor 1
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
Cu	Copper
Cu2+	Copper ions
CXCL1 and CXCL2	Neutrophil-recruiting chemokines
DAB	3,3'-Diaminobenzidine
DG	Dentate gyrus
DLBT	Dementia of the Lewy body type
DLS	Dorsolateral striatum
DMN	Default mode network
DMNS	Delayed non-matching to Sample
DMS	Delayed matching to Sample
DMS	Delayed matching to sample
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DR6	Death Receptor-6
DS	Down syndrome
DVD	Digital video disc
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENIS	Early-Intervention Strategy
EOAD	Early-Onset Alzheimer's disease
EOFAD	Early-Onset Familial AD (Autosomal dominant AD)
EPM	Elevated plus maze
ERK	Extracellular signal-related kinase
ERPs	event-related potentials
FAD	Familial Alzheimer's disease
FAD	Familial AD
FDG	Fluorodeoxyglucose (18F) or fludeoxyglucose (18F) (INN).
Fe	Iron
FGF2	Fibroblast growth factor 2
fMRI	Functional magnetic resonance imaging
FoxO	Forkhead Box O
FTD	Frontotemporal dementia
FTDP-17	frontotemporal dementia with parkinsonism (chromo. 17)
G6Pase/G6P	Glucose 6-phosphatase
GABA	Gamma-aminobutyric acid
GDS	Global Deterioration Scale
GFAP	Glial fibrillary acidic protein
GLUT-1 ;GLUT-2; GLUT-4	Glucose transporter-1, 2, and 4 respectively
GSK	GlaxoSmithKline
GSK3	Glycogen synthase kinase 3
GSKα	Glycogen synthase kinase α
GWAS	Genome-Wide Association Studies
hAPP	Human Amyloid precursor Protein
HKI	Hexokinase

HPA	Hypothalamic-Pituitary-Adrenal
HRP	Horseradish peroxidise
IDE	Insulin-degrading enzyme
IGF 1, IGF2	Insulin-like growth factors I, and II
IL (e.g. IL-1β)	Interleukin (e.g. Interleukin-1β)
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate protein 2
ISF	Interstitial fluid
K+	Potassium ion
K+	Potassium
kDa	Kilodaltons
Kg	Kilogram
LINS	Late-Intervention Strategy
LOAD	Late-onset Alzheimer's disease
LRP	Low-density lipoprotein receptor-related protein
LRP	Lipoprotein receptor-related protein
LTD	Long-term depression
LTM	Long term memory
LTP	Long-term potentiation
MANOVA	Multiple analysis of variance
MAP	Mitogen-activated protein
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MAPT-17	Microtubule-associated protein tau gene (chromosome 17)
MCI	Mild Cognitive Impairment
MCT4	Monocarboxylate transporter 4
MGS	Muscle glycogen synthase
ml	Milligram
MMP-1	Matrix metalloproteinase-1
MRH	Metabolic reserve hypothesis
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Matching to sample tasks
MTL	Medial Temporal Lobe
Na+	Sodium ion
N-APP	Amino-terminal fragment of APP
NEP	Neprilysin
NFT	Neurofibrillary tangles
NGS	Normal Goat Serum
NIA	U.S. National Institute on Aging
NIH	U.S. National Institutes of Health
NIRKO mice	Brain/neuron-specific insulin receptor knockout mice
nMCI	Non-amnestic MCI
NMDA	N-Methyl-D-aspartic acid
NMDR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2–	Superoxide
p38MAPK	Mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDAPP	TG mice overexpressing hAPP V717F
Pde6brd1 (RD)	Retinal Degeneration Mutation
PDGF	Platelet derived growth factor-β
PDK	Phosphoinositide-dependent kinase
PDPK1	phosphoinositide-dependent kinase 1
PET	Positron emission tomography
PFC	Prefrontal cortex
PI3K	phosphatidylinositol 3-kinase
PiB	11C-Pittsburgh Compound B
PICALM	Phosphatidylinositol binding clathrin assembly protein gene
PKB	Protein kinase-B
PPAR	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome proliferator-activated receptor-gamma
PPRE	Peroxisome proliferator response element
PR	Preference ratio
PSAPP mice	Cross hAPP mutant mice with those expressing mutant PSEN1 transgenes
PSEN1	Presenilin-1
PSEN2	Presenilin-2
RAGE	Advanced glycation endproducts
RCAN1	Regulator of calcineurin 1
ROS	Reactive oxygen species
SAD	Sporadic Alzheimer's disease
sAPPα	Soluble APP fragment
SEC	Socioeconomic Status
SEM	Standard error of the mean
SGZ	Sub-granular zone, dentate gyrus
SJL	Swiss James Webster
SNPs	Single Nucleotide Polymorphisms
SOR	Spontaneous Object Recognition
STM	Short term memory
STZ	Streptozotocin
SVZ	Sub-ventricular zone dentate gyrus
T1D	Type 1 diabetes Miletus
T2D	Type 2 diabetes Miletus
TG	Transgenic mouse
Tg2576	TG mice overexpressing APP695 (KM670/671NL)
TG-A	PPAR-delta agonist GW610742
TG-B	PPAR-γ agonist GW855266X
TG-C	Transgenic Non-Drug Treated
TGF-α	Transforming growth factor alpha
TG-R	Transgenic Rosiglitazone Treated
TL	Top Left
T-Maze FCA	T-maze forced choice alternation task
TNF-α	Tumor necrosis factor-α
TR	Top Right
Tyr	Tyrosine kinase activity receptor
TZD	Thiazolidinedione
UK	United Kingdome
VCR	Video Recorder
Wk	Weeks
WM	Working memory
Wnt	Lipid-modified signaling glycoproteins

WT	Wildtype mouse
WT-C	Wildtype Non-Drug Treated
WT-R	Wildtype Rosiglitazone Treated
Zn	Zink
α-secretase	Alpha-Secretase
βAPPs	soluble βAPP fragment
β-secretase	Beta-Secretase
γ	Gamma
VPC	Visual Paired Comparison Task
NLR	Novel Location Recognition Task

ACKNOWLEDGEMENTS

MY interest in dementia research and neuroscience in general, stems from my childhood experience of watching a relative suffer from Alzheimer's disease, a common cause of dementia that in most cases derives from a background of biological, environmental, and social risk factors. At the time I remember thinking that maybe if I knew enough about the brain, this devastating disease could be prevented. I do believe that it can be at least slowed down, although much of what we think we know about the disease is at worst wrong, and at best an oversimplification. Alzheimer's, like most forms of dementia, is a complex brain disorder and currently attempts at treating it are like trying to fix a car engine without knowing fully how the engine works to start with.

This thesis details the experimental studies I conducted as part of a "Collaborative Awards in Science and Engineering" (CASE) PhD studentship, which was co-funded by the Biotechnology and Biological Sciences Research Council (BBSRC), and GlaxoSmithKline Pharmaceuticals, Harlow, UK. All experimental studies were conducted during August 2006 to December 2009, at the School of Psychology, Cardiff University. Writing this thesis would not have been possible without the help, advice, and encouragement of a large group of people. I give thanks to My PhD supervisor, Professor Mark Good, Cardiff School of Psychology, and special thanks to Kate Dresser, for her kind advice, support and generosity. My thanks also go to Professor Rob Honey, Cardiff School of Psychology, and to my long suffering wife Rachel, for her love, support, and patience throughout this project (someone really ought to start a support group for partners/spouses of those writing a PhD). I am also grateful for the outstanding animal husbandry by staff at Cardiff University. Finally, I am mindful of the sacrifice made by the animal subjects in this study.

John Anderson, January 2013

1. GENERAL INTRODUCTION: AETIOLOGY OF AD

ALZHEIMERS DISEASE is considered as a complex, progressive and irreversible neurodegenerative disease of the brain, and represents the most common form of dementia in the elderly population. Emerging data demonstrate pivotal roles for brain insulin resistance and insulin deficiency as mediators of cognitive impairment and neurodegeneration, particularly AD. In this context AD is increasingly being referred to as type of brain diabetes in which endogenous brain-specific impairments in insulin and Insulin like growth factor signalling account for the majority of AD-associated abnormalities. However, AD emerges from a complex background of genetic, psychological and environmental factors, all of which can influence its expression and development.

This introductory chapter has two principal aims. The first is to set the general context by providing a review of the aetiology of AD, including its prevalence and incidence, genetics, clinical features, molecular pathology, diagnosis and treatment. When discussing the clinical manifestation of AD, special consideration will be given to examining the nature of memory loss in AD, as well as impairments related to behavioural disinhibition and anxiety as these form the context to specific experiments detailed in Chapter 3 of this thesis. Indeed, understanding the memory deficits experienced by patients is essential to designing and assessing the efficacy of novel pharmaceuticals for the treatment of such impairments [1], one of the principal concerns addressed by this thesis. Thus, the second aim is the take what may at first glance seem to be the jangling discords of AD aetiology, and relate them to the concept of AD as a metabolic disorder. In this context, the focus of discussion revolves around recent attempts to use rosiglitazone (an insulin sensitizer) as a means of ameliorating cognitive (learning and memory) deficits, and similar deficits in preclinical animal models of select pathological components of AD. Indeed, of particular concern throughout this chapter is how the concept of AD as a metabolic disorder relates to the overproduction and aggregation of the amyloid-beta protein in

the context of the amyloid cascade hypothesis (ACH). This is important because the ACH is still regarded as the dominant theory of AD causation, and as such, forms the basis for many preclinical treatments for AD, including the use of insulin sensitizers. In this way I hope to achieve a symphony of words that captures something of the immense and sprawling complexity that is AD. I believe this to be the correct context within which to fully appreciate the specific experiments detailed in Chapters 3 and 4 of this thesis. These experiments test the hypothesis that rosiglitazone ameliorates specific behavioural and neuropathological deficits associated with a popular preclinical animal model of mutated Amyloid Precursor Protein, which is linked to a rare familial (inherited) form of AD in humans. Discussion of preclinical animal models in AD and the specific phenotypic alterations associated with the model used in experimental studies in this thesis is discussed in Chapter 2. Finally, Chapter 5 provides a discussion of the main experimental findings from this thesis, and considers these in relation to the wider scientific literature.

1.1 PREVALENCE AND INCIDENCE

In 1906, the German physician Alois Alzheimer gave a seminal lecture to his peers in Tübingen, Germany on the first case of the neurodegenerative disease that Kraepelin some years later named Alzheimer's disease (AD) [2, 3]. In this single case, Alzheimer described the results of his clinical observations and post-mortem studies on a 56-year-old-female patient known as Auguste D, who at the age of 51 had been admitted to the state asylum in Frankfurt suffering from memory and language deficits, auditory hallucinations, delusions, paranoia, and aggressive behaviour [4]. When Auguste D died in April 1906, her brain was sent to Alzheimer for examination where he duly observed an atrophic brain with striking neurofibrillary tangle (NFT) pathology, as well as the presence of unusual deposits in the cerebral cortex that were refractory to staining; now known as amyloid-beta plaques (Aβ-plaques). In the following year, Alzheimer published his findings in a short paper that marked the start of our understanding of the disorder. However, being exclusively associated with dementia before the age of 65 years (early-onset), a diagnosis of AD remained a relative rarity for many years after Alzheimer. In the early 1970's the disorder was recognised as a major cause of dementia in those over the age of 60/65 years (late-onset), and eventually, a diagnosis of AD was formally adopted in medical nomenclature to describe individuals,

of all ages who displayed a characteristic symptomatic pattern based on cognitive deficits typified by memory and language impairment [5]. Today this unitary concept of AD has fragmented into an increasing number of genetic and clinical subtypes.

According to Alzheimer's Disease International, nearly 36.5 million people worldwide had a form of dementia in 2010, with the overall predicted increase in prevalence over the next 30 decades estimated to reach approximately 66 million by 2030, and then 115 million by 2050 [6]. Much of this increase is considered to reflect demographic ageing, a process that shows the successes of improved health care over the last century in many countries [7]. The fastest growth in the elderly population is taking place in low and middle income countries such as China, India, and their south Asian and western Pacific neighbours [7]. Because the prevalence of dementia doubles about every five years after age 65, and after age 85 the risk reaches nearly 50 %, it is estimated that there will be significant disparity in the regional prevalence of dementia by 2030, with developing regions showing a 40% increase, Europe, 63%, North America, between 77-146%, and in the different regions of Latin America, 107%, and Asia 111% increase [6]. Indeed, whilst approximately 58% of the world's dementia cases currently reside in these countries, by 2050, low and middle income countries are expected to account for 71% of the world's prevalent dementia cases [6]. Currently the number of new dementia cases (i.e. its incidence), is estimated to be around 7.7 million annually [6], with this figure predicted to increase further as populations age. About 3.6 million (46%) of these new cases are expected to impact in Asia, 2.3 million (31%) in Europe, 1.2 million (16%) in the Americas, and 0.5 million (7%) in Africa [8, 9].

Although there are many different subtypes of dementia, AD is considered the most prevalent, accounting for between 60-80% or more of the total, with a greater proportion in the higher age ranges [10-12]. However, the pathological hallmarks belonging to more than one subtype can also be co-present within many patients, particularly those with advanced late-onset dementia, making mixed dementia fairly common in this population [13]. Most cases of mixed dementia still reflect the pathological hallmarks of AD together with vascular dementia [14]. Presently, the number of persons believed to have AD worldwide is approximately 26.6 million, with the overall prevalence of the condition predicted to quadruple in line with dementia generally, to 106.2 million by 2050 [15]. Currently, about 48% of all AD cases reside in low and middle income countries, most of which are set to see the largest increases in AD over the next several decades [6, 15]. Although AD is more common in women

than men in late-life, this is usually attributed to the longer lifespan of women compared to men [16]. Over 90% of AD cases are late-onset (LOAD), where the condition affects people over the age of 60-65 years [13]. Age appears to be the most influential risk factor for AD (and other dementias). The prevalence of AD increases exponentially with age, rising from 3% among those 65–74 years to almost 50% among those 85 years or older [17, 18].

Less than 10% of cases AD are early-onset (EOAD), developing between the ages of 30-60, or 65 years [19]. Approximately 60% of EOAD cases are classed as familial (EOFAD) [20]. Of these early-onset familial cases, approximately 13% are inherited in an autosomal dominant manner with at least three generations affected [21, 22]. Autosomal dominant AD is mostly associated with a number of single gene disorders involving specific point mutations or single base substitutions all of which are extremely rare and account for less than 1% of AD cases overall [23] (see section 1.1.2). Although there is evidence for genetic factors affecting disease risk in many Alzheimer's cases [24], and first-degree relatives of patients with the late-onset disease have approximately twice the expected lifetime risk for developing AD, most cases are said to be of sporadic (SAD) origin because they are rarely consistent with a pattern of Mendelian transmission [25]. Rather, the risk of developing SAD appears to be modulated by an interaction between multiple risk factors, including age, susceptibility genes, and environmental factors [26, 27].

1.2 Genetic determinants and Risk Factors

Three causative genes have been associated with autosomal dominant familial AD; the gene on chromosome 21 for the Amyloid Precursor Protein (APP) [28 , 29], and the genes on chromosome 14 and 1 for the Presenilin 1 and Presenilin 2 genes (PSEN1, and PSEN2 respectively)[30-32]. Within the APP gene there is a specific region which encodes the Aβ peptide, with mutations in this gene shown to alter the subsequent processing of Aβ or its propensity to undergo fibrillogenesis, leading to early deposition of the peptide and EOFAD [29]. Mutations in the presenilin genes that cause EOFAD also lead to changes in Aβ processing and its deposition [29]. It has been hypothesised that presenilin protein comprises a key component of the catalytic site in γ-secretase [33], one of the protease enzymes involved in the production of Aβ, although this process is tightly gated by at least two other membrane proteins [34].

Taken together these observations strongly support the hypothesis that the generation of Aβ is central to AD pathogenesis. Indeed, whilst the identification of specific genes related to autosomal dominant AD has only had a minimal impact clinically, in terms of basic research aimed at understanding underlying disease mechanisms, the identification of these mutations has had greater impact and led to the creation of the Amyloid Cascade Hypothesis (see section 1.6.1). However, it should be said that kindreds with autosomal dominant AD have also been described with none of the point mutations associated with the above genes, indicating that other factors may be causally related to the disorder in some cases [35, 36]. There are also alternative theories of AD causation. Thus, research has shown that familial APP mutations may confer a gain or loss of function that in the presence of trophic factor withdrawal, resulting in the amino-terminal fragment of APP (N-APP) binding to the Death Receptor-6 (DR6), the induction of caspase-6-dependent neuronal axon pruning, Wallerian degeneration and thus, neuron death [37]. Identification of mutated genes isolated from FAD kindreds has also enabled the development of various transgenic animal models [38-41]. Although none of the models fully replicates the full human disease [42], each has contributed significant insights into the pathophysiology of Aβ in AD, as well as the testing of pre-clinical therapies.

1.2.1 Production and Clearance of Aβ

1.2.1.1 Production of Aβ

Aβ derives from the Amyloid Precursor Protein (APP), a large transmembrane protein expressed in many tissues and concentrated in the synapses of neurons [43]. APP is found in most cell types, including neuronal and glial cells, and contains a large extracellular region, a single transmembrane domain, and a small cytoplasmic tail [44]. Like other members of its protein family, APP can undergo regulated intra-membrane proteolysis by α, β and γ secretases to generate secreted and cytoplasmic fragments [43, 45]. However, only APP has a transmembrane Aβ forming region, allowing it to produce Aβ peptides [46]. APP is cleaved via two main pathways. Cleavage of APP via α-secretase represents a non-amyloidogenic pathway, and involves APP being cleaved in the middle of its Aβ region to produce a soluble APP fragment (sAPPα) which has neuroprotective properties [47]. This process leaves a membrane-bound C-terminal fragment (C83), that can be further cleaved by the intra-membrane γ-secretase complex (of which the presenilins form key components to yield a 3-kDa Aβ fragment

(P3) [48]. Under normal circumstances this non-amyloidogenic pathway accounts for about 95% of APP processing [49]. In the amyloidogenic pathway, β-secretase provides the initial (potentially neurotoxic) cleavage of APP in its extracellular domain (N-terminus), which generates a non-amyloidogenic fragment (sAPPβ), and a membrane bound C-terminal fragment of 99 amino acids in length (C99). C99 is then susceptible to a second intra-membrane cleavage by γ-secretase, which generates the 4-kDa Aβ peptide and an intracellular domain (AICD) product, both of which are released from the membrane into the cytosol [50, 51].

In non-neuronal cells, α-secretase cleavage predominates whereas in neurons the β-cleavage pathway predominates [52]. Depending on the point of cleavage by γ-secretase, three principal forms of Aβ are produced that have a chain of 38, 40, or 42 amino acid residues (Aβ38, Aβ40, and Aβ42, respectively). This along with research showing that the production and release of Aβ into the extracellular space positively correlates with the level of neuronal and synaptic activity [53-56], suggests that brain Aβ may be largely neuronal in origin. Furthermore, it has been suggested that synaptic vesicle recycling through coupled endoexocytosis may be the primary mechanism mediating the activity-dependent production and release of Aβ in the brain [54, 57]. The relative amount of Aβ42 formed is noteworthy because it is far more prone than the more abundantly produced Aβ40 to aggregate, and form the insoluble amyloid protofibrils and fibrils common to some forms of senile plaque [58-60]. Aβ peptides are capable of aggregation and self-assemble into dimer, trimer and higher-order soluble oligomers [46, 60, 61] (also called ADLLs or "Aβ-derived diffusible ligands" [62]). Soluble Aβ describes any form of Aβ that is soluble in aqueous buffer, and remains in solution following high speed centrifugation. Whilst the formation of Aβ oligomers has been extensively studied by using synthetic forms of Aβ, research relating to endogenous formation has been limited [60, 63-65]. Nevertheless, endogenous soluble Aβ is considered to have the same potential for forming different assembly species, which may be produced via different pathways [64, 66]. The mechanisms of formation may even differ between pathways [46]. If soluble aggregates are not degraded or cleared from the brain, they form the "seeds" from which Aβ-plaques develop [67, 68]. However, both APP and Aβ are normal constituents of many cells; they have been implicated in a number of important physiological roles in the periphery, and central nervous system (CNS).

Nominal Function of APP and Aβ

APP functions as a receptor since its homologues bind both extracellular ligands and intracellular adaptor proteins [69]. Consequently, APP has been implicated as a regulator of synapse formation [70], a regulator of neural plasticity [71], and as a bona fide iron oxidase [72]. As a natural product of cellular metabolism, Aβ is present in the interstitial and cerebrospinal fluid (CSF) of humans throughout life [60], where its normal physiological role is uncertain. However, several potential physiological activities have been suggested for Aβ, including the activation of kinase enzymes involved with phosphorylation processes [73, 74], (antioxidant) protection against oxidative stress [75, 76], regulation of cholesterol transport [77, 78], anti-microbial activity potentially associated with the peptides pro-inflammatory activity [79], and as a transcription factor for AD-associated genes [80], and apoptotic genes [81]. Although higher Aβ concentrations have been postulated to boost synaptic glutamate in the hippocampus [82, 83], it is not clear whether this effect can also occur at nominal physiological concentrations. However, research suggests that in low concentrations, Aβ does enhance neuronal activity via its ability to act as a regulator of release probability at hippocampal synapses [84]. Indeed, research conducted in animals suggests that low doses of Aβ enhance memory retention in behavioural tasks (T-maze foot-shock avoidance), as well as the in vivo enhancement of acetylecholine production in the hippocampus [85]. Furthermore, at sub-nanomolar doses, Aβ markedly enhances Long Term Potentiation (LTP) in hippocampal slices [85, 86], with LTP being a cellular process that many consider to underpin memory consolidation [87]. However, higher concentrations of Aβ have the opposite effect; depressing LTP and promoting LTD or 'Long Term Depression' (see review, [50]).

1.2.1.2 Clearance of Aβ

At any given time the amount of Aβ in the brain is thought to be determined by the steady-state balance between its in vitro production and release, and its degradation and clearance [88]. The canonical view is that any chronic imbalance between its production and clearance will result in the accumulation of Aβ in the interstitial fluid (ISF) [89], and eventually its deposition in the brain parenchyma as senile plaques [49, 88, 90, 91]. In terms of its in vitro degradation, several metallopeptidase protease enzymes have been implicated, the most important of which are probably neprilysin (NEP) [92], and insulin degrading enzyme (IDE) [92], both of which possess a zinc-

binding motif. NEP is the most dominant protease, and due to its presynaptic localization and extracellular position of its catalytic site [93, 94], may be the best candidate for regulating the amount of Aβ in the synaptic cleft. NEP is capable of degrading monomeric and oligomeric forms of Aβ [95], and examination of human post-mortem tissue suggests that the expression of NEP is inversely related to the extent of AD pathology [96-98].

IDE is the principle protease responsible for the clearance and inactivation of insulin, and as such is ubiquitously expressed in the body where it has a subcellular localization, being primarily located in the cytosol and peroxisomes [99-101]. The highest expression of IDE occurs in the liver, testes, muscle and brain [102], where in the latter it is found in multiple neurons, but particularly in neocortical pyramidal cells [103]. Structurally IDE is composed of four homologous domains which together resemble a clamshell-like arrangement enclosing a large central chamber for engulfing the substrate. Substrates must enter the chamber, and it is believed that a hinge-like conformational change allows substrate binding and product release [104]. IDE exists as an equilibrium mixture of monomers, dimers, and tetramers, with dimers thought to be the predominant and most active state [105, 106]. In addition to its central involvement in insulin metabolism, IDE degrades other small peptides, including glucagon [103], insulin-like growth factors I and II (IGF1 and 2 respectively) [107], transforming growth factor alpha (TGF-α) [105], and in a non-selective manner, extracellular forms of monomeric Aβ [108, 109]. Nevertheless, IDE does not apparently metabolise Aβ dimers and trimers [109], and incubation of these secreted oligomeric species with the enzyme does not rescue the inhibition of synaptic plasticity [110]. Furthermore, given its subcellular location, IDE would not appear to be appropriately placed to degrade monomeric Aβ. However, a small but significant proportion of IDE is secreted from cells through an unconventional protein secretion pathway [111], and it is this component of IDE that is functional in relation to Aβ degradation where it appears to be routed via detergent-resistant membrane complexes into exosomes for secretion along with Aβ [112]. IDE is also unusual among zinc metallopeptidases in that it exhibits allosteric kinetic behaviour in which small peptide substrates such as Aβ may increase the activity of the enzyme toward the same or other small peptides [106]. Animal research has indicated that IDE expression responds to Aβ accumulation in the brain [113], and mice with a homozygous deletion of IDE have elevated endogenous brain Aβ [114, 115]. Indeed, in post-mortem tissue obtained from AD

patients, levels of neuronal IDE are reduced in the hippocampus [116, 117]. However, whilst in the cortex IDE protein levels normally diminish with of age, for unknown reasons, cortical IDE expression in AD is elevated [118].

Aβ can be cleared from the brain parenchyma through the interstitial fluid and Virchow–Robin perivascular space, a space surrounding perforating arteries and veins that is considered to play the role of a lymphatic vessel [88] (see review [119]). Aβ also likely interacts with the low-density lipoprotein receptor-related protein (LRP) [119], or with P-glycoprotein [120] in order to cross the blood brain barrier (BBB). There is also an efflux of Aβ from the blood into the cerebral parenchyma mediated by the receptor for advanced glycation end products (RAGE) [121]. In addition to these processes, glial cells may also have a role in clearing Aβ deposits from the brain (see section 1.5.4).

1.2.2 APP Gene Mutations

Mutations within the APP gene on chromosome 21, account for 10-to-15% of EOFAD cases [35, 122], with several different missense mutations having been discovered in exons 16 and 17 [123]. All these mutations alter APP processing, Aβ production, or alter the propensity of Aβ to aggregate into β-sheet amyloid fibrils. Missense mutations are often referred to as 'point mutations' because they involve a single nucleotide being changed, resulting in a codon that then codes for a different amino acid. Examples of point mutations in the APP gene include the "Dutch" (E693Q) [124], London (V717I) [28], "Indiana" (V717F) [125], "Florida" (I716V) [126], "Iowa" (D694N) [127], "Arctic" (E693G) [128], and "Swedish" (K670N/M671L) mutations [129]. The Swedish mutation is located just outside the N-terminus of the Aβ domain of APP, it favours β-secretase cleavage in vitro [130], and is associated with an increased level and deposition of Aβ42 in AD brains [131]. The Dutch and Iowa mutations are both located in the Aβ forming domain of APP, and accelerate Aβ40 fibril formation in vitro [132, 133]. Both these mutations are also associated with "cerebral amyloid angiopathy" (CAA), a condition associated with the deposition of Aβ in cerebral blood vessels and to a lesser extent, veins [134]. The Dutch APP mutation, in particular, is known to result in cerebral haemorrhages and dementia in patients with AD [124], whereas the Iowa mutation is associated with severe CAA and widespread NFT formation as well as an unusually extensive distribution of Aβ40 in Aβ-plaques in the AD brain [127]. The Arctic mutation is also located inside the Aβ domain, but makes APP less available to α-secretase cleavage, and thus increases β-secretase processing of APP, favouring

intracellular Aβ production in vitro [135, 136]. Whilst this mutation is likewise associated with severe CAA, this is generally in the absence of cerebral haemorrhage, abundant parenchymal Aβ deposits, and NFTs [137]. The London mutation is located in the transmembrane domain of APP, altering γ-secretase cleavage to produce an increase in the level of Aβ42 and/or, the ratio of Aβ42/Aβ40 in vitro [138]. The London mutation is associated with extensive parenchymal Aβ deposition, moderate CAA, and abundant Aβ-plaques and NFTs in AD brain [139, 140]. Both the Indiana and Florida mutations are also located in the transmembrane domain of APP, with the former associated with mild CAA, and large numbers of NFT's and Aβ-plaques in the AD brain [141], and the latter affecting γ-secretase cleavage causing an increased Aβ42 concentration and Aβ42/Aβ40 ratio in vitro [126, 138]. In summary, mutations close to the β-secretase site enhance the production of both Aβ40 and Aβ42, whereas mutations close to the α-secretase site result in impaired α-secretase activity, an increase in γ-secretase activity, and thus, an increase in secreted Aβ [142]. Mutations next to the γ-secretase site lead to a selective increase in Aβ42 over the Aβ40.

Additional evidence implicating the APP gene in the parthenogenesis of AD comes from the fact that nearly all those with Down syndrome (DS) develop the neuropathological hallmarks of AD after the age of 40 years, with more than half showing clinical evidence of cognitive decline [143]. The development of DS is caused by the presence of an extra copy of human chromosome 21 [144, 145], with the presumed association between DS and AD being the lifelong overexpression of the APP gene and thus the resultant overproduction of Aβ [20]. However, recent research suggests that people with DS are also trisomic (having three copies) for a gene that produces a protein called "Regulator of Calcineurin 1" (RCAN1), leading to excess production of this protein. In DS this can initiate a chain reaction which results in the death of neurons in the hippocampus [146], leading to disrupted memory for events and autobiographical experiences, as well as impairments in spatial navigation [147-149]. In addition to the loss of hippocampal neurons, RCAN1 also results in the loss of cortical neurones [146]. Despite only having the usual two copies of the RCAN1 gene, some AD patients also have elevated levels of the RCAN1 protein, perhaps in response to stroke or hypertension [150]. Although the mechanisms by which RCAN1 mediates its deleterious effects remain unclear, research has suggested a possible role in mitochondrial degradation [151].

1.2.3 Presenilin Gene Mutations

Over 176 different missense mutations in the PSEN1 gene have been identified in approximately 390 families with a familial history of AD [152], and represent the most common cause of EOFAD accounting for between 18 and 50% of all autosomal dominant cases [153]. Defects in the PSEN1 gene are known to cause the most severe forms of AD, with most mutations displaying complete penetrance and an age of onset around 30 years, but can be greater than 58 years [25]. By contrast, only 12 missense mutations have presently been identified in the PSEN2 gene [154], with these mutations being a rare cause of EOFAD [25]. Missense mutations in the PSEN2 gene may be of lower penetrance than those relating to PSEN1, with the former possibly being subject to the modifying action of additional genes or environmental influences [26, 155]. The clinical features of PSEN2-affected families appear to differ from the clinical features of PSEN1 in that the age of onset in PSEN2 family members is generally older than some family members with PSEN1 mutations, ranging from 45-to-88 years [25]. Thus, a minority of late-onset cases appear to be associated with PSEN2 mutations [156], a fact further supported by recent research showing that some genetic polymorphisms of the PSEN2 gene may confer an enhanced risk of sporadic AD [157].

PSEN genes are expressed mostly in neurones and glia; their exact function and role in AD is uncertain [25]. However, because both PSEN1 and PSEN2 genes encode for transmembrane presenilin proteins that form the major components of the atypical aspartyl protease complexes responsible for the γ-secretase cleavage of notch proteins and APP [158, 159], PSEN mutations are normally assumed to confer pathogenesis in AD by affecting the production of Aβ. For example, a gain of-function phenotype has been suggested with some PSEN1 mutations due to the observed increase in Aβ42 production, accompanied or not, with reduced Aβ40 production, thereby leading to an increased Aβ42 vs. Aβ40 ratio in mice and humans [160, 161]. Concordant with this, the deposition of Aβ42 in Aβ-plaques has been suggested to be an early preclinical event that occurs in PSEN1 mutation carriers [161]. However, it also appears that some disease-causing PSEN1 mutations do not increase production of Aβ42 or affect the production ratio of Aβ42/Aβ40 [162], suggesting that different PSEN1 mutations mediate pathological effects via Aβ-independent means. Indeed, recent research using cell cultures has demonstrated that PSEN1 mutations associated with EOFAD can cause defective lysosomal proteolysis/autophagic function in fibroblasts providing one possible mechanism by which the abnormal accumulation and deposition of proteins

can occur in the AD brain [163]. Additionally, PSEN1 mutation may introduce aberrations in the intracellular Ca^{2+} mobilization in astrocytes [164], possibly contributing to aberrant neural-glial communication in AD. PSEN2 is expressed in a variety of tissues; in the brain, is expressed primarily in neurons [165]. The cause of pathogenesis in PSEN2 mutations is less clear, particularly since this presenilin protein is a less efficient producer of Aβ than PSEN1 [166]. However, although not well understood, PSEN2 mutations linked to EOFAD have been reported to modify the way in which γ-secretase processes APP, with some mutations having been linked to an increase in the rate of Aβ production [167]. Nevertheless, a truncated version of the PSEN2 protein derived from differentially spliced mRNA exons does not apparently affect the ratio of Aβ peptides produced [168].

1.3 Risk factors related to SAD

Although advancing age is the most significant risk-factor for SAD, the relationship between aging and disease risk remains unclear. Physiological function declines with aging, even among the most robust sectors of the older population, although the degree to which this decline is attributable to true biological factors or social or lifestyle factors that accompany older age is not entirely clear [169]. However, it is certainly true that there is substantial heterogeneity in patterns of aging [170]. That is, while many older people continue to show expected patterns of decline in health and functional ability with advancing age, others appear more resilient to various physiological (e.g., infection, neuropathology), emotional (e.g., bereavement), or environmental challenges [169]. Thus, in relation to dementia, it may not simply be the number of years lived that matters most, but rather a person's resiliency in the face of various challenges or perturbations that act to bring about the dementia state sooner rather than later. Indeed, the resilience argument seems to make sense of the perplexing observation that in aged individuals neuropathological examination has detected those in whom the microscopic features typically associated with AD are present, and yet a clinical history of dementia is absent. In 2010 the U.S. National Institutes of Health (NIH) published an independent state-of-the-science conference report [171], which was aimed at providing healthcare providers, patients, and the public with an assessment of currently available data on prevention of AD and cognitive decline with age. Amongst other things, this report included a systematic

review of the risk factors associated with cognitive decline and AD, with one of the main summary points being that that firm conclusions cannot be drawn about the association of any modifiable risk factor with cognitive decline or AD [171]. Current epidemiological views of AD regard age, family history of AD, and specific inheritable genetic factors as non-modifiable risk factors for dementia, whereas potentially modifiable factors include those related to cardiovascular disease (smoking, hypertension, diabetes, and obesity), lifestyle and psychosocial factors (depression, physical activity and alcohol consumption), and cognitive reserve (education and occupational attainment). It is clear that mild cognitive impairment (MCI) also represents a risk factor for dementia, although the degree to which this entity represents a non-modifiable/modifiable risk factor is uncertain.

1.3.1 Genes Associated with SAD

In comparison with EOFAD, the genetic basis of LOAD appears more complex; probably because the aetiology of these cases depends on genetic susceptibility at multiple gene levels, as well as the interaction between these and the various non-genetic risk factors already discussed (see reviews, [25, 26, 172]). Recently, genome-wide association studies (GWAS) have had considerable successes in identifying a number of gene variants with single nucleotide polymorphisms (SNPs) that may increase the risk of developing LOAD. These include specific gene loci relating to "Clusterin" (CLU), "Phosphatidylinositol-binding clathrin assembly protein" (PICALM), and "Complement receptor 1" (CR1) [173, 174], as well as "bridging integrator 1" (BIN1) [175]. All of these genes have been independently replicated by subsequent research confirming their status as risk modifier genes for LOAD [176-178]. However, all SNPs associated with these genes in the above studies have had small estimated effect sizes, with odds ratios reported in the range of 1.1 to 1.5 (with little overlap among studies). Nevertheless, such odds ratios are likely to be inaccurate, and the true effects may be much smaller [179]. How the contributions of CLU, CR1 and PICALM loci potentially add up to AD in late life remains to be clarified, although current research supports existing hypotheses about the roles of Aβ, lipid, chaperone and chronic inflammatory pathways in AD pathogenesis (see review, [180]). It is evident that scientists still know very little about the roles of many of these genes, including how they relate to AD. For example, whilst BIN1 and PICALM have been implicated in APP processing and Aβ toxicity [180, 181], other studies have suggested a link with

tauopathy [182], although a recent study of biomarkers related to cerebrospinal fluid (CSF) Aβ42 fragments, and phospho-tau (the pathological state of tau), has failed to find a correlation with these biomarkers and gene variants [183].

1.3.1.1 Apolipoprotein E Gene Polymorphisms

Despite the continued successes of GWAS, only one gene, "apolipoprotein E" (APOE) on chromosome 19, has the unequivocal status of a "susceptibility" gene for LOAD in multiple patient populations [184-186]. APOE exists in three different isoforms (epsilon; 2, 3 and 4), with the frequencies of these three alleles being highly variable in different populations [187-189]. Carrying the APOEε4 allele is a risk factor for AD [190], and in particular LOAD [191-194], whereas the APOEε2 allele is associated with decreased risk [195]. Indeed, even within in the GWAS studies reported above, the association between APOEε4 and AD dominates the results [26]. Furthermore, even SNPs near the APOE gene had larger effect sizes in these studies compared to CLU, PICALM and CR1. Nevertheless, APOEε4 has incomplete lifetime penetrance, even in the highest-risk APOE genotypes [196], and the fraction of genetic variance for risk in LOAD attributed to APOE is estimated to be only 10 to 20% [197, 198]. Given that oligogenic segregation analyses (i.e. the prioritizing for whole-exome sequencing studies to identify families more likely to harbour rare variants), support the presence of at least 4 to 6 additional major genes [199, 200], it is likely that additional risk loci remain to be discovered.

APOEε4 acts in a dose-dependent manner: carriers of two APOEε4 alleles have a higher risk and earlier onset of AD compared to heterozygous subjects [184, 186, 201, 202]. However, whilst these studies help to explain some of the variation in the age at which AD develops, how APOE ε4 confers this risk is not known. APOE is one member of a family of proteins that help carry cholesterol and other types of fat in the bloodstream. Prevailing evidence suggests that the differential effects of APOE isoforms on the brain in AD are multifactorial, containing Aβ dependent, and independent effects (see review, [203]). For example, it is evident that Aβ aggregation and clearance play the major role in AD pathogenesis. APOE colocalizes with parenchymal (brain) and vascular Aβ deposits, with numerous studies having demonstrated that it can physically interact with Aβ peptides in an isoform-specific manner, affecting the physical/conformational properties of Aβ and thus potentially enhancing plaque formation [204, 205]. Alternatively, the physical interaction

between APOE and Aβ likely affects the efficiency of Aβ clearance from the brain, either across the BBB [206], or by modulating cellular uptake through receptor-mediated endocytosis [207]. Indeed, in vivo studies using functional neuroimaging methods such positron emission tomography (PET) in conjunction with amyloid (Aβ) tracers such as 11C-Pittsburgh Compound B (PiB), have shown an APOEε4 dose-dependent increase in fibrillar Aβ burden in cognitively healthy individuals [208]. Subsequent research has also found that Aβ burden measured using PiB-PET correlates with a family history of LOAD, perhaps accounting for the known increased risk [209]. It is unclear whether increased Aβ deposition is an early initiating event in LOAD [210], although longitudinal studies of those with MCI suggest an increase fibrillar Aβ burden is predictive for future conversion to AD [211-213].

APOE likely modulates disease risk for AD through pathways not directly linked to Aβ. As the major apolipoprotein of the brain, APOE is important for cholesterol homeostasis by serving as a ligand in receptor-mediated endocytosis of cholesterol-containing lipoprotein particles (see review, [214]). Cholesterol is a major constituent of the neuronal membrane and the synapse, and impaired redistribution of lipids and cholesterol might affect neuronal plasticity [180]. There is increasing evidence that abnormal cholesterol metabolism may be a key component of a pathological cascade leading to AD [215]. Moreover, APP processing through γ-secretase takes place in the cholesterol-rich membrane, with high intracellular cholesterol favouring the amyloidogenic processing pathway of APP [180]; the degree of lipidation of APOE seems to affect the clearance rate of Aβ [206]. Taken together this suggests that APOEε4 likely contributes to AD pathogenesis by modulating the metabolism and aggregation of Aβ by directly regulating brain lipid metabolism and synaptic functions through APOE receptors (see, [216]). APOE is also associated with circulating levels of cholesterol as well as atherosclerosis, and might serve as a common convergence point with other risk factors such as diabetes through a vascular component [215]. However, since recent research in elderly individuals has reported high APOE concentrations in the blood irrespective of allele type indicate an increased risk of death from cardiovascular disease [217], it is difficult to see how APOEε4 may confer a differential impact on increased risk of AD in this singular regard.

APOE has also been implicated in neural injury and repair processes long before its association with AD [214]. In this regard, transgenic animal studies of native murine APOE knockout mice which express similar levels of human APOEε3 or APOEε4 in the

brain, has shown that expression of APOEε3, but not of APOEε4, was protective against age-related excitotoxic-induced neuronal damage [218] (but see, [219]). This may possibly explain the long-standing association between APOEε4 and poor outcome after brain to injury in humans [220]. Indeed, following brain injury, the expression of APOE is increased in both neurons and glial cells [219], and in clinical studies possession of an APOEε4 allele is associated with poorer outcome after head injury [221], as well as after acute stroke (see review, [222]). Interestingly, recent research has shown that even in healthy elderly adults, cortical thickness is associated with different APOE genotypes with significantly greater cortical thickness associated with APOEε2 carriers [223]. Indeed, one could speculate that part of the protective role of APOEε2 may be mediated by greater brain reserve. Cytokine and nitric oxide (NO) mediated neuroinflammation is also a significant pathological component of AD (1.7.4), and in cell culture and animal experiments APOE has been shown to be capable of suppressing pro inflammatory cytokine and NO production via a hithero unknown pathway [224]. This may represent another means by which different APOE alleles may modulate disease risk in Alzheimer's [203], and may be particularly relevant given that cardiovascular disease and diabetes are associated with chronic low-grade inflammation [225, 226]. Taken in aggregate, APOE clearly mediates its effects on AD risk via a range of mechanisms, none of which may be mutually exclusive.

1.3.1.2 Cardiovascular Risk Factors

Several reports in the literature suggest that cardiovascular risk factors predispose individuals to developing dementia, and more specifically AD, and vascular dementia (see review, [227]). Recent reviews of cohort studies suggest that smoking increases the risk for developing all forms of dementia, including AD [228-231]. However, recent reviews of the associations between dementia and hypertension [229, 232, 233], cholesterol [234], and obesity [229, 235, 236] reveal complex relationships, because although hypertension, increased cholesterol, and obesity in midlife all increase the risk for late-onset of dementia, blood pressure levels, cholesterol and body mass index decrease progressively before disease onset [237]. Thus, people with dementia often have lower blood pressure levels, cholesterol and body mass compared to non-demented persons at diagnosis. Although early primary prevention may be the most effective intervention with respect to these risk factors, it is clear from recent Cochrane reviews that neither statins [238] nor antihypertensive treatment [239] lower the

incidence of late-life dementia, although long-term trials from midlife onwards are lacking. Indeed, a recent Cochrane review has provoked controversy by concluding that there is insufficient evidence to recommend the widespread use of statins in the primary prevention of heart disease [240], although this is disputed by others [241].

1.3.1.3 Diabetes and Dementia

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [242]. The majority of diabetes cases fall into two broad categories. Type 1 diabetes (T1D) is the uncommon form, and results from autoimmune destruction of insulin-producing pancreatic islets [243]. T1D usually appears before the age of 40, and accounts for around 10% of all people with diabetes [244]. Type 2 Diabetes (T2D) is the most common form; it results from peripheral insulin resistance — a condition in which peripheral tissues such as muscle, liver, and the pancreas become resistant to the physiological actions of insulin [242]. Insulin resistance is central to metabolic syndrome, a cluster of high adiposity, abnormal glucose level, dyslipidaemia, hypertension, and high inflammation, which in the long-term is associated with poor health outcomes, including heart disease, stroke, and cancer [242, 245]. In most cases insulin resistance is linked to being overweight and morbidly obese (i.e. body mass index or increased abdominal obesity) [242, 243]. T2D usually appears in people over the age of 40, though South Asian people have a particular vulnerability in relation to developing insulin resistance where it often appears after the age of 25 [242, 244]. Both insulin resistance and diabetes result in primary hyperglycemia. Although insulin resistance in the absence of diabetes may not generate clinical symptoms, it is still often sufficient to cause pathologic and functional changes in various target tissues [242]. If increased insulin secretion (i.e. hyperinsulinemia), cannot overcome the insulin resistance, pre-diabetes or T2D usually ensues [242]. Acute life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the non-ketotic hyperosmolar syndrome [242]. Long-term complications include retinopathy, renal failure, peripheral neuropathy, sexual dysfunction, autonomic neuropathy and cardiovascular disease [242]. In the advanced stages of T2D there may also be damage to pancreatic islets leading to insulin deficiency and T1D. A number of prospective observational studies have reported an age-related association between T2D and dementia, showing that diabetes

increases the risk of late-onset dementia 1.3-to-3.4 fold (see, [229, 232, 246-248]). However, not all studies have confirmed the link between AD and diabetes [249].

1.3.2 Other Risk Factors Associated With SAD

1.3.2.1 Lifestyle and Psychosocial Factors

In Western countries lower socioeconomic status (SES) tends to correlate with lifestyle choices typically associated with the development of cardiovascular disease and metabolic impairment, including behaviours such as smoking, larger food portions, reduced physical activity, and increased alcohol consumption [250, 251]. However, in Asia, Latin America and Africa, chronic conditions such as cardiovascular disease, obesity and metabolic disorders such as T2D are all more likely to be associated with higher SES [252, 253]. For example, whilst in China medical services and economic prosperity have improved life expectancy, people are not necessarily healthier as improved SES in China is associated with a shift toward an unhealthy diet characterized by higher fat levels and greater amounts of added sugar as well as less exercise [254, 255]. In addition, some Asian and other ethnic populations have an increased susceptibility for developing metabolic disorders with age [256-259], although the basis for this remains unclear. Worldwide, the number of persons diagnosed with diabetes and in particular T2D, is on the increase, particularly in those low-to-middle income countries such as Asia, Latin America, and Africa which are also predicted to see the largest increases in the number of AD cases [6, 253]. Indeed, apart from demographic aging, the increase in diabetes may provide additional support for concerns regarding the projected rates of AD in these countries over the next several decades. There is also evidence to suggest that dietary intake of homocysteine-related vitamins (vitamin B12 and folic acid); antioxidants such as vitamin C and E; unsaturated fatty acids; and also moderate alcohol intake (especially wine) —could reduce the risk of AD [260, 261]. However, on the basis of the current data, it is not possible to make any general dietary recommendations [262]. Several cohort studies suggest that physical activity has a strong protective effect against dementia [229, 263, 264]. Making positive life-style changes (eating a healthy diet; engaging in regular exercise) may be the most effective way of protecting against age-related cognitive decline and dementia [27, 262, 265-267]. These likely reduce other risk factors associated with cardiovascular disease and stroke [268-270], as well as T2D [270-274].

1.3.2.2 The Metabolic Reserve Hypothesis

The metabolic reserve hypothesis (MRH), has been proposed in which a high metabolic brain reserve characterized by the presence of neuronal circuits which respond adaptively to perturbations in cellular and somatic energy metabolism, is posited to protect against declining cognition [275]. Lifestyle determinants of increased metabolic reserve are generally the same factors which protect against the development of metabolic disorders and cardiovascular disease (e.g. exercise, reduced caloric intake, and intake of specific dietary components that can promote neuroprotection). However, sedentary lifestyles and the effects of excessive caloric intake are well known in relation to the development of these disorders, and dementia [276] and decrease metabolic reserve in the brain [275]. The MRH postulates that the bidirectional relationship between metabolism and cognition is primarily mediated by alterations in central insulin and neurotropic factor signalling as well as central glucose metabolism, with downstream consequences for accumulation of neurohistologic lesions such as Aβ-plaques and NFTs [275]. MRH is supported by a range of epidemiological findings as well as the spectrum of individual cognitive trajectories during aging, with additional data from animal models identifying potential mechanisms for this relationship [275]. Identification of biomarkers for metabolic reserve may assist in generating a predictive model for the likelihood of cognitive decline with aging [275, 277]. Indeed, assessment of regional cerebral glucose metabolism via the radioactive labelled tracer (18F)Fluorodeoxyglucose (FDG) for use with PET (FDG-PET), has revealed that impaired cerebral glucose metabolism in temporoparietal association cortices is a reliable predictor of rapid progression to dementia in patients who show above expected levels of cognitive decline in aging (i.e. mild cognitive impairment), and could serve as a biomarker for the diagnosis of pre-symptomatic AD [278]. Frontal and temporoparietal metabolic impairment are also closely related to the progression of cognitive impairment in longitudinal studies; multi-centre studies suggest its use as an outcome parameter to increase the efficiency of therapeutic trials [275, 278].

1.4 Clinical Progression

Although the biological correlates of variability in relation to temporal progression have been investigated by many groups, including in relation to genetics [279-282], vascular factors [283, 284] and cerebrovascular disease [285-287], as well as MRI volumetric analysis of cortical atrophy [288-293], metabolic function [294, 295], and other factors [296, 297], the underlying reasons for such variations remain largely uncertain. This variability poses difficulties in relation to the treatment and assessment of patients over the duration of AD, although it can still be useful to consider its progression in terms of generalised stages. Currently the 7 stage Global Deterioration Scale (GDS) is the most popular means of charting the progressive decline of people with AD since it measures the cognitive, behavioural, and functional impairments of patients [298, 299]. GDS stages 1-2 do not deal with dementia, but deal with normal cognition, and normal aged forgetfulness respectively. Current diagnostic guidelines indicate that a portion of people at these stages will have prodromal dementia marked by the presence of abnormal biomarker patterns in the brain (e.g. those related to CSF levels of Aβ and phospo-tau), which may occur decades before more insidious symptoms appear [300]. Since there are no established diagnostic criteria currently in use for these biomarkers, these stages are not considered further here. GDS stage 3 represents the clinical construct known as Mild Cognitive Impairment (MCI) introduced by Flicker and colleagues [301] and the Mayo Clinic group [302-304], as a clinical adjunct for an intermediate stage of impairment between successful ageing and dementia [305]. MCI is briefly summarised here because many consider the particular animal model used in experiments in Chapters 3 and 4 of this thesis to most closely model the overproduction of Aβ in the very earliest stages of AD related, and thus, aspects of aMCI. Stages 4-7 then chart the general clinical progression of full blown AD. These are referred here as Mild (early), Moderate (mid), and Advanced (late) stage-AD, and are not considered in depth (see, [306]), although these stages are referred to when considering the nature of memory deficits and other affective and behavioural impairments in AD.

1.4.1 Mild Cognitive Impairment

According to popular guidelines by the Mayo Clinic, MCI can be divided into two broad subtypes: amnestic MCI (aMCI), and non-amnestic MCI (nMCI). As the most

common subtype, aMCI patients predominantly display impaired recall for recent events in relation to personal facts and autobiographical incidents in their spatio-temporal (i.e. episodic memory) [307]. The second (less common) subtype is non-amnestic MCI (nMCI), where instead of memory deficits the dominant impairment is in a non-memory domain such as attentional-executive function [302-304]. Both categories of MCI can be further subdivided into single and multi-domain. For example, patients could present with impairment in a single cognitive domain such as memory, but also have impairments in language and/or attentional-executive function. Whilst persons diagnosed with MCI have an increased risk of developing dementia compared to those without the condition [301, 304, 308], it is those with aMCI who are generally considered to be at highest risk of developing AD [308-310], whereas those diagnosed with nMCI are at risk of developing other forms of dementia [311]. Patients with objective memory loss (as verified via a psychological test), and/or impairments in multiple cognitive domains, have been shown to have less chance of improvement overall and thus a greater propensity to progress to AD within an assessment period [301, 302, 304, 308-310, 312, 313]. However, it remains unclear whether some nMCI cases may represent the early stages of atypical AD, where there is a slowly progressing focal syndrome characterised by impairment in one or more non-memory domains.

Consideration of MCI as a clinical adjunct for an intermediate dementia remains a controversial topic [314]. There are several definitions of MCI that are now used in the literature, many of which require rigorous field testing [303, 314]. Consequently, the frequency of MCI in the population has been found to vary considerably, both between and within definitions [315]. A more fundamental issue is the fact that numerous studies have now reported findings consistent with the view that most instances of MCI likely do not represent very early-stage dementia since most patients diagnosed with MCI either stay stable (i.e. non-progressers) or revert to baseline on follow-up assessment (e.g., [308, 312, 313, 316, 317]). However, in studies showing a return to baseline levels of cognition, the assessment of cognition has sometimes been limited to only a few domains. It could also be argued that the reason why so many MCI patients stay stable, is that some have mild disease and/or a higher cognitive/metabolic reserve, and thus, may take longer to transition from MCI to dementia [275, 318, 319]. It is also unclear in those MCI patients who do recover, whether or not the condition is intermittent, with essentially normal function interspersed with multiple relapses. Only longer assessment periods of two decades or more with incremental assessment

will likely resolve these issues. The pathologic substrate of MCI is not completely understood (see review, [311]). Nevertheless, it is generally accepted that many dementia patients do pass through a very early stage of the disorder marked by symptoms similar to those of MCI.

1.4.2 Clinical Syndrome of AD

1.4.2.1 Diagnosis and General Symptomology

More than 30 years have passed since the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's disease and Related Disorders Association (now called the Alzheimer's Association), established the most common criteria for AD [320]. Recently, expert international workgroups convened by the Alzheimer's Association and the National Institute on Aging (NIA), an agency of the U.S. National Institutes of Health (NIH), jointly issued four new criteria and guidelines for clinicians and researchers in relation to the diagnosis of AD [321]. These new criteria update, refine and broaden previous widely used guidelines so that they remain in line with research findings, particularly neuroimaging studies and studies relating to new biomarker patterns [300, 322-325]. Three of the new guidelines focus on the major stages of AD whilst the fourth updates the criteria for documenting and reporting Alzheimer's-related histopathological changes observed during an autopsy [326, 327]. Although these guidelines are expected to have a significant impact on AD research and clinical practice making a diagnosis, they have been criticised for being biased in favour of a specific theory of the pathophysiological origins of AD [328, 329]. The diagnosis of AD remains a difficult process that necessarily involves a number of different assessments, including a clinical review of the patient's medical history, physical examination, neuropsychological testing (i.e. via Mini-Mental State Exam), as well as nowadays, structural and functional neuroimaging [330]. Clinical assessment may take place in the home, in an outpatients department, in a day hospital over several weeks, or as a hospital inpatient.

In general people with AD experience two different categories of symptoms. The first are referred to as cognitive symptoms, and commonly disrupt memory, language and attentional-executive function [331]. In its common presentation, cognitive symptoms in AD relate to progressive amnesia followed by various combinations of focal cortical deficits such as such as aphasia, apraxia, agnosia, and impaired executive function [332]. The above pattern of neuropsychological changes largely reflects the

current view regarding the progression of NFT pathology in AD, which in most cases is symmetrical and initially thought to involve regions of the MTL, starting with the perirhinal cortex (also called the transentorhinal cortex), before spreading into the entorhinal cortex, hippocampus, amygdala, and thereafter the rest of the temporal lobes and basal forebrain structures (including all cortical association areas) [333-337]. The second are experienced by many but not all AD patients, and relate to "behavioural and neuropsychiatric symptoms" (BPS) [338]. Symptoms belonging to this latter category are diverse and variable between patients, but often involve apathy, agitation, anxiety, irritability, depression, and aberrant motor behaviour, disinhibition, delusions and hallucinations [339, 340]. The category of BPS is only considered in general terms here (see review, [341]), although reference is made to specific symptoms throughout this thesis. However, because behavioural disinhibition and non-conditioned anxiety are the most directly relevant to specific experiments detailed in this thesis, these symptoms are discussed further in section 1.6. Many people with AD and their families find BPS to be the most challenging and distressing effects of the disease. BPS is often a determining factor in a family's decision to place a loved one in residential care [338], and within long-term care facilities BPS continues to impact on the care and quality of life patients [338, 342].

Activities of Daily Living

Both cognitive symptoms and BPS impact on the activities of daily living (ADL) in dementia patients. Indeed, the impact on ADL is often used as a major criterion for the differentiation between MCI and early-stage dementia. There are two categories of ADL [343]. Basic ADL includes activities such as eating, bathing, dressing, toileting, volitional movement, and continence, and are usually intact in the early stages of AD. Complex (instrumental) ADL is dependent on intact memory, including attentional-executive functions (a major component of short-term working memory), and is associated with abilities that allow a person to live independently, including with respect to food preparation, housekeeping and laundry, managing financial matters, shopping, and using public transport. Instrumental ADL are typically impaired in the early stages of AD, with the impairment forming part of the very definition of the dementia syndrome [344, 345]. Whilst in general MCI is associated with intact ADL and thus, independent living [303, 304], recent research suggests that like early-stage AD, complex ADL is affected [346-349]. Furthermore, in MCI and AD there is also a

significant positive correlation between the presence and degree of BPS and ADL impairment [341, 350-355]. However, despite the disruption of instrumental ADL in MCI and early stage AD, most patients usually manage to live independently [356]. In the moderate dementia stage supervision is needed for most ADL when other cognitive domains are affected in a more obvious manner, and BPS symptoms where present, put increasing stress on care givers [306, 356]. Complete dependence of the patients, who by this time have also frequently developed neurological disturbances and incontinence, is typical of the late-stage of illness [356], with patients usually requiring a high level of care equivalent to that of a nursing home [306]. Overall, most AD patients are expected to survive for between 8 and 10 years with the condition after diagnosis, although the actual duration can range from 2 to 25 years [20]. However, the temporal progression of AD shows a pattern of high variability, with patients reported to transit the stages of the disease with time-courses ranging from months to decades [279, 357]. This has been supported by recent research demonstrating that the mean duration of the various stages of AD is comparable with their standard deviation (i.e. that that individual courses of progression may differ considerably between people, and from 'textbook' mean values [358]). The cause of death in AD is often the result of pneumonia or general inanition [20].

1.5 The Nature of Memory Loss in AD

Although once thought to be a simple concept, memory is now considered to be a collection of cognitive abilities, many of which are mediated by different systems and anatomical components within the brain. Memory research that began with neuropsychological studies of patients with focal brain lesions today also includes functional neuroimaging techniques such as PET, functional magnetic resonance imaging (fMRI), structural imaging methods such as MRI, as well as non-invasive electrophysiology techniques such as event-related potentials (ERPs) (see reviews, [359, 360]). In aggregate studies using these techniques have provided the rationale for the division memory phenomena into the specific categories outlined below [359]. In short these relate to Long-Term Memory (LTM), and Short-Term memory/Working Memory (STM, WM respectively). Since progressive impairment of LTM is often seen as the primary hallmark of AD, most of the focus of discussion in this section will be

on components of LTM. However, most types of memory are implicated in the primary amnesia common to AD, although not all forms are affected at the same time or to the same degree across the various stages of the disorder. Indeed, a large and growing body of research indicates that some components of WM are impaired in MCI as well as the early-stages of AD (see, [361, 362]), although the biological basis of these deficits remains poorly understood. Whilst the WM system is not covered in depth here (see, [363]), some components may be of relevance to the interpretation of specific behavioural experiments detailed in chapter 3 of this thesis. Thus, a short summary of WM in AD is provided following a discussion of LTM.

1.5.1 Long-Term Memory

LTM can be divided into two broad categories: Declarative memory for conscious (explicit) forms of recollection, and nondeclarative memory for unconscious (implicit) recall of information from previous experiences [364, 365]. Declarative memory can be further subdivided into episodic memory for autobiographical events in their spatio-temporal context (i.e. "What", "When" and "Where"), and semantic memory for factual knowledge devoid of its context of acquisition (i.e. landmark dates or personal semantics such as the date of ones birthday) [366, 367]. Both of these systems are compromised early on in AD. It widely accepted that declarative memory is critically dependent on the integrity of the MTL structures [368-371], although other cortical and subcortical structures are also important [372, 373]. Nonetheless, in humans bilateral damage limited to the hippocampus (dentate gyrus, CA fields and subiculum), and/or adjacent cortical regions is sufficient to produce severe anterograde amnesia, as well as temporally graded retrograde amnesia stretching back as much as 25 years [365, 374]. Two special types of declarative memory are also impaired in AD: spatial memory and recognition memory; both of these memory systems are discussed after episodic and semantic memory. The major subdivisions of LTM are summarised in Figure 1.1

1.5.1.1 Episodic Memory Impairments

The ability to encode and retrieve episodic memories, is supported by the circuitry of the MTL, including temporal-limbic structures such as the parahippocampal region (perirhinal cortex, and parahippocampal cortex or postrhinal cortex in rats, and entorhinal cortex), and hippocampus, the latter of which interacts extensively with a number of specific distributed cortical and subcortical structures [372]. Neocortical

structures forming part of this memory network include the prefrontal cortex and other areas that mediate working memory, effortful retrieval, source monitoring, and other processing functions essential to conscious recollection [375-378], as well as the parietal and temporal cortex —both of which are involved in complex perceptual processing essential to contents of recollection [379]. Other structures that appear to be involved in episodic memory include the fornix [380], mammillary bodies and mammillo-thalamic tract [381, 382], anterior dorsomedial nuclei of the thalamus [383], basal ganglia [384, 385], and retrosplenial cortex [386]. In both animals and humans, evidence from anatomical, neuropsychological, and physiological studies indicates that cortical components of this system have key functions in several aspects of perception and cognition, whereas the MTL structures have a central role in mediating the organization and persistence of the network of memories whose details are likely stored in those cortical areas [372]. However, accumulating evidence also implicates the hippocampus and perirhinal cortex in the process of perceptual binding as amnesic patients with damage to these structures have been shown to be impaired on perceptual tasks when discriminating between objects, faces and scenes with overlapping features [387, 388]. Nevertheless, the canonical view is that structures within the MTL, and in particular the hippocampus, have distinct functions in combining information from multiple cortical streams supporting the ability to encode and retrieve the details comprising episodic memories in humans [368], and similar episodic-like memories in other animal species [389, 390]. Conversely, selective damage in the hippocampus, MTL, and other structures of this large-scale memory system, or deterioration of these areas in AD compromises episodic memory [372].

Anterograde amnesia defined by the loss of recent episodic memory, is one of the earliest and most prominent symptoms experienced by most patients with AD [391]. Numerous studies have shown that patients with AD are impaired on episodic memory tests that use a variety of cognitive procedures (e.g., free recall, recognition, paired-associate learning) across virtually all sensory modalities (see review, [392]). However, the use of supra-span word lists (e.g. those which exceed working memory span) in multiple learning trials, and the delayed recall of these material have been shown to be particularly sensitive for detecting early changes in patients with respect to verbal episodic memory (see review, [393]). Evidence from many of these studies suggests that the episodic memory deficit of AD patients is due in large part, to ineffective consolidation or storage of new information [331, 393]. Episodic memory continues to

decline throughout the mid-stages of AD, and by the latter-stages of the disorder a profound retrograde amnesia for distant episodic memories is also evident [331]. In AD, the perirhinal cortex and entorhinal cortex are both bilateral anatomical sites associated with the most severe NFT deposition in the early-stages of the disorder [394-396]. Indeed, NFT accumulation has been shown to correlate with the degree of neuronal cell loss in AD [394, 397], as well as the loss of synapses [398], with the entorhinal cortex in particular considered to be most heavily damaged cortex in AD [336]. Since as part of the parahippocampal region, both the perirhinal and entorhinal cortex provide the main source of bidirectional connectivity between the neocortex and hippocampus [372, 399], the destruction of these areas likely plays a significant role in the memory deficits that often herald the onset of AD, and which likely characterize its continued deterioration throughout its clinical course [336, 400]. However, whether NFTs are causative in this respect remains uncertain, as the degree of neuronal apoptosis in AD has been shown to exceed NFT load suggesting the influence of additional factors [401]. Indeed, research from mice expressing wild-type human tau suggest that both neuronal and synaptic lesions can occur independently of fibular NFT formation, and may correlate best with the accumulation of pre-fibrillar oligomeric tau [402, 403]. However, in humans [404-406], and transgenic animal species (see reviews, [65, 407]), soluble Aβ species also correlate strongly with the extent of synaptic loss. Indeed, mounting evidence from human and animal studies suggests both the dysfunction and subsequent loss of synapses occur prior to frank neuronal degeneration [408], and that this is primarily mediated by diffusible soluble oligomers deriving from Aβ [60, 62-65, 408, 409] and phospo-tau [402, 403], making both types of aggregate attractive therapeutic targets in AD [410-412]. However, since the particular animal model used in the experiments detailed in Chapters 2 and 3 of this thesis expresses only the APP_{SWE} mutation, the primary focus in this thesis is on the role of Aβ isoforms in mediating the dysfunction and subsequent loss of synapses, and thus, the role of rosiglitazone in slowing down or reversing these changes and associated impairments in learning and memory.

Although fibrillar amyloid pathology presages the onset of symptoms in AD by years [413-419], it is not abundant in MTL in the early-stages of the disease, but is instead, more evident in the cortical regions comprising the "default mode network" [420, 421]. The default mode network (DMN) is comprised of a set of functionally interconnected cortical areas (posterior cingulate, inferior parietal lobule, lateral

temporal neocortex, ventromedial and dorsomedial prefrontal cortex) that project heavily to MTL structures [420], with abnormalities in this network associated with Aβ deposition [421, 422]. Ultimately the gradual degeneration and loss of synapses and neurons in AD results in the gross symmetrical atrophy of the cerebral cortex seen in end stage post-mortem brain tissue, something that can be detected and studied longitudinally by structural MRI [423, 424]. Additional mechanisms contributing to episodic memory impairment in AD stem from working memory and executive dysfunction deficits [425, 426], the latter of which may lead to increased sensitivity to interference due to decreased inhibitory processes, and thus, intrusion errors [427-429]. Since the frontal cortex plays an important role in the initial acquisition and encoding retrieval of episodic information in the absence of contextual cues, the recollection of source information (i.e. context), and assessment of the temporal sequence and recency of events [430-432], dysfunction of the frontal lobes can also contribute to episodic memory impairment [433, 434]. The recollection of source information will be explored further in Chapter 3 in relation to object place memory. In addition to these mechanisms, defective use of semantic information to bolster encoding may also be a factor [435-437].

1.5.1.2 Defective Semantic Memory

Although this thesis does not explicitly investigate semantic processing is a defining feature of human behaviour, central not only to language but also the capacity to access acquired knowledge in reasoning, planning, and problem solving tasks [438]. Semantic memory comprises a rich store of general knowledge about the world, including our understanding of words, pictures, objects, sounds, faces and events without connection to time and place [438]. Although semantic knowledge is acquired from specific experiences it is thought to be abstracted from the engram and generalised to a variety of different contexts. Cognitive neuroscientists have proposed many conceptual and mechanistic models for semantic memory storage and retrieval over the years, including those based on modality-specific components, attribute-specific components, and category-specific or meaning-specific components [439]. Most of these models have been informed by lesion/deficit observations and/or functional imaging studies, and less frequently by electrophysiology studies [440]. Each has focused on particular aspects of long-term semantic memory storage and retrieval; some detail multiple semantic memory subsystems (see, [441]).

Recent neuroimaging and neuropsychological studies are consistent with the view that modality-specific components of conceptual knowledge are likely involves a wide network of brain regions. For example, it seems that most of the actual content of semantic memory for objects is represented in neural systems that overlap with (or even correspond to), brain regions necessary for perceiving and using those objects [442-444]. Thus, current theoretical models of semantic memory tend to converge on the likelihood that it closely relates to perception and action, and that it is represented in the brain regions which overlap with (or possibly even correspond to), the regions responsible for perceiving and acting [444]. According to this view, the knowledge of what a cat sounds like would be stored in the auditory cortex, whilst an ability to recognize and imagine the visual features of a cat likely resides in the visual cortex. In support of a distributed network for semantic memory, a recent meta-analysis of functional neuroimaging studies by Binder et al., has suggested that long-term storage and recall of semantic information in the brain depends on the orchestration of a distributed but predominantly left-lateralized network of cortical areas, including posterior inferior parietal lobe, middle temporal gyrus, fusiform and parahippocampal gyri, dorsomedial prefrontal cortex, inferior frontal gyrus, ventromedial prefrontal cortex, and posterior cingulate gyrus, [445]. However, whilst specific features of conceptual knowledge are likely represented within a distributed network of brain regions, the ability to receive information in one modality and express it in another, to generalize across conceptually similar entities that differ in almost every specific modality, and to differentiate between entities that resemble each other in many modalities (all semantic abilities) — seem to depend on the anterior temporal lobe (ATL) [444]. Indeed, the ATL likely provides a major common convergence zone for many different types of semantic information [444]. However, recent research has also shown that patients with bilateral damage thought to be restricted primarily to the hippocampal region, acquired less factual knowledge than controls patients as well as exhibiting a temporally limited retrograde amnesia for factual information from the several years preceding the onset of memory impairment [446]. These results show that the hippocampus may also support semantic memory with its role in the acquisition and storage of semantic knowledge being time limited [446].

Impairments of semantic processing figure in a variety of brain disorders, including AD, semantic dementia, fluent aphasia, schizophrenia, and autism. In semantic dementia, clinicopathological studies consistently show that anterior and inferior

temporal regions bear the brunt of the damage across histopathological subtypes [447]. However, the neural correlates of semantic failure in other disorders are less clear. In AD, language impairment often occurs early in the course of the disorder, and deteriorates with progression of the illness to cause significant disability [331]. The impairment initially affects verbal fluency and naming (both of which require integrity of semantic concepts), prior to the breakdown in other facets of language [448]. Little is known about the underlying neuropathology of language impairments in AD. Semantic memory is progressively disrupted in AD, and is generally considered to reflect progression of neuropathology into the temporal, frontal, and parietal association cortices in which such semantic memories are thought to be diffusely stored. However, a recent review of functional neuroimaging studies by Verma and colleagues [448], has reported that semantic memory loss may occur in AD several years prior to diagnosis, and may reflect an altered state of cortical connectivity with respect to language networks possibly providing the structural basis for subsequent failure of semantic memory. Dysfunction of the right superior temporal cortex has also been implicated in AD, and may contribute to early semantic deficits [449]. Given that cognitively normal individuals with the APOEε2 allele have been reported to have specific thicker cortical volumes in the superior temporal cortex compared to those persons with the ε4 allele [223], one wonders perhaps, if those persons with the ε4 allele would show a dose dependent vulnerability to early decline of semantic memory due to reduced cortical thickness in this region.

Recent research has shown that patients with bilateral damage thought to be restricted primarily to the hippocampal region, acquired less factual knowledge than controls patients as well as exhibiting a temporally limited retrograde amnesia for factual information from the several years preceding the onset of memory impairment [446]. These results are interesting because they show that in addition to episodic memory, the hippocampus may also support semantic memory with its role in the acquisition and storage of semantic knowledge being time limited [446]. This probably explains why semantic memory deficits reportedly overlap with episodic memory impairments. Indeed, Leyhe et al. [307], have recently shown that in early-stage AD, both personal semantics (semantic information as it relates to autobiographical experiences [450]), and recall of autobiographical memory have a temporal gradient in patients with better preservation of childhood memories compared to early adulthood and recent life.

1.5.1.3 Spatial memory

How do we know where we are? Orientation in space and by implication, "spatial memory", is a key facet of our day-to-day existence as we follow familiar routes and navigate to a previous location, as well as explore and new environments [147]. The term "spatial" is somewhat ambiguous however, as it has different meanings and has been considered in numerous ways. For example, 'spatial competence' is associated with the processing of geometric (or metric) properties, including those related to distance and size, as well as dynamic properties such as velocity and strength. Clearly, the ability to navigate in the environment requires an understanding of all these properties, thus linking intuitive geometry with that of an intuitive physics [451]. In addition to navigation, spatial memory also forms a fundamental building block in relation to the psychological mechanisms supporting episodic memory and recognition memory processes [452]. As mobile organisms, humans must recognise objects despite updating their relative positions and orientations in the surrounding environment in order to efficiently interact with the world. Indeed, as the consequences of getting lost or poor recognition are potentially fatal, the ecological validity of spatial memory is highly conserved across species. Spatial memory impairment is particularly important in the context of this thesis because it forms one of the key phenotypic alterations associated with the particular animal model used as a vehicle in Experiments 1 and 4 of Chapter 3 in relation to the evaluation of the therapeutic impact of rosiglitazone in relation to learning and memory.

Spatial memory is supported by multiple parallel representations, including egocentric (body-centred) and allocentric (world-centred) representations, and those which update to accommodate self-motion [453]. Egocentric navigation fixates on the traversed direction with respect to own body (e.g. 'go straight ahead' or 'turn left'), and involves a self-to-object representational system. A fundamental aspect of egocentric navigation relates to "path integration", a form of "online" navigational strategy that involves an animal (including humans) starting at a fixed point, visiting several locations, and then returning directly to the original start point by processing self-movement information generated along the way [454]. However, as an individual moves through an environment, the positions of surrounding objects relative to the body constantly change. Thus, the concept of spatial updating is used to refer to the automatic cognitive process whereby the brain continuously computes the spatial

relationships between an individual and their surrounding environment as they move based on perceptual information about their own movements [455].

Spatial updating contributes to object and scene recognition by predicting the appearance of objects or scenes from novel vantage points, so that an individual can easily recognize them as they move [456]. However, observer movement does not necessarily automatically update representations of spatial layouts of scenes in small-scale (room-sized) environments [456, 457], raising questions about the effects of encoding points of view on the automatic spatial updating of representations of scenes. A form of viewpoint-independent spatial updating also occurs in humans (see, [458]), and may have a role in maintaining perceptual stability [459], as well encoding the spatial representations of object layouts [459, 460]. However, this may involve a fixed reference direction independent of the body [459], and may be an implicit learning process [461]. The updating of egocentric representations of location in order to accommodate self-motion (called path integration when the location in question is the start of the path), falls prey to cumulative error after relatively short paths, necessitating the incorporation of corrections from landmarks or celestial cues in long-distance orientation (e.g. [462, 463]). Allocentric navigation requires an object-to-object representational system that encodes information about the location of one object (or its parts) with respect to other objects in the environment [453]. This type of strategy is considered most useful in large and/or unfamiliar environments, and involves forming a mental representation of visible cues and landmarks when encoding the layout of a novel environment [464]. Current research indicates complementary roles for both egocentric and allocentric representations, with the former being most useful when allocentric representations are not of high fidelity [460]. In addition, both types of representations likely combine across learning and navigational situations, particularly since environments differ in respect of their familiarity, intrinsic structure, loco-motor demands, and the number and size of objects they contain [465].

Neural correlates of spatial and object-place memory

The hippocampus has long been implicated as a key neural correlate of spatial memory in both humans and animals [453], particularly since hippocampal lesions in many species have been shown to affect spatial recognition and memory for recently acquired allocentric representations [464, 466-469], as well as some allocentric

representations of places learned long ago (see review, [470]). At a cellular level, spatial recognition and spatial learning of position is supported by a subpopulation pyramidal cells within the hippocampus and entorhinal cortex, both of which contain a number of electrophysiological properties consistent with their role in forming allocentric representations of space, including head-direction, place and grid cells [466, 471]. In the 36 years since their discovery in rats [472], place cells have been documented in monkeys and humans [149, 473, 474]. In the hippocampus, place cells place cells fire when an animal is at specific locations in an environment (the cell's 'place field') [475, 476], and as the animal explores an open environment the ensemble of cells provides a stable representation of the animal's location that is independent of its orientation [477]. Thus, the pattern of place cell firing does not simply reflect direct sensory input because many of the cells continue to fire within their given place field irrespective of an animal changing the direction in which it is facing [478], or when the lights are tuned out [472]. Furthermore, the pattern of place cell firing has also been found to correlate with behavioural responses in some spatial memory tasks [466]. These and other studies show that place cells are encoding the more abstract concept of a place, in other words, a mental representation of where the rat "thinks" it is (see review,[147]). Although speculative, such representations are likely needed for mental imagery [147, 464]. Nevertheless, many studies have shown that place cells form part of a much broader circuit for dynamic representation of self-location in the brain (see review, [147, 466]). A key component of this network is the entorhinal grid cells, the activity of which is related to that of hippocampal place cells [479], and by virtue of their tessellating firing fields, may provide the neural elements of a path integration-based strategy [466]. However, the presence of grid (like) cells in a network of brain regions implicated in spatial cognition and episodic memory in both humans [480] and rats, [481], likely reflects a wider role for these cells in memory function [480]. Nevertheless, these two mesial temporal lobe regions have strong bidirectional interactions, probably explaining why they have a critical role in spatial and other forms of memory as well as their relation with the amygdala [482]. In summary, current research supports the role of the hippocampus in forming allocentric representations of space, whilst the entorhinal cortex likely mediates a path integration-based neural representation of the environment (see reviews, [464, 466]).

In addition to location representation at the single cell level, locations are also represented by distinct neural assemblies [373], with studies in both humans and rats

having identified patterns of neuronal firing within the hippocampus that represent specific goal locations in both real [483] and virtual spatial environments [484]. Consistent with the link between spatial and episodic memory, distinct patterns of hippocampal activation that are stable over time are have been suggested to represent specific episodic memories [485], although a thorough discussion of the results form experiments forming the basis for this are outside the bounds of this thesis. The presence of neural representations of the spatial environment is also not in itself, likely to be sufficient for successful place learning as rats, as rats with lesions of the intermediate hippocampus are unable to learn new spatial locations rapidly within a familiar environment despite the persistence of intact spatial representations and neuronal plasticity in the septal hippocampus [486]. This addresses an important distinction between forming accurate spatial representations and the subsequent translation of these into behavioural actions, such as navigation [373], the latter of which requires the intermediate hippocampus [486]. Indeed, whilst a thorough review of the lesion data supporting the conclusion that the hippocampus plays a pivotal role in aspects of recognition memory is outside the bounds of this thesis, the net conclusion from such studies is that the hippocampus is required whenever such memory involves remembering that a particular stimulus occurred in a particular place or when the memory contains a temporal or object recency component (e.g. [487, 488], but see, [489, 490]). Indeed, such processes are of relevance to the interpretation of Experiment 4 in Chapter 3 which deals with object-place memory.

Neuroimaging and other integrative methods in neuroscience now mean that it is possible to investigate how specific cells 'learn' about specific object–place or object–context associations [373]. Indeed, there is growing evidence from animal studies for the automatic encoding of object–context associations within the hippocampus from both lesion studies [491], and studies looking at the expression of immediate-early genes following task performance [492]. This suggests that learning about specific object–place or object–context associations may not always reflect a conscious declarative process. Although more speculative, two recent reviews of the neuropsychological literature have also suggested that the hippocampus may also mediate multiple cognitive processes through the process of relational binding and comparison in the presence or absence of conscious awareness (see reviews, [493, 494]). Of particular interest is the fact that associative representations in the hippocampus are thought to be built on previously formed representations of both the

space and context within which learning takes place [495], further bringing together models of hippocampal function that emphasise the structures role in either spatial [453] or relational memory processes [496].

Finally, many modern studies of spatial and recognition memory processes have shifted the focus away from the more traditional approach of studying the brain in terms of modular brain regions, to a more integrated approach whereby the emphasis is on the interactions between multiple cortical regions [373]. In this paradigm, functional disconnections of brain regions, using crossed unilateral lesions or temporary in-activations, have been particularly informative for addressing these issues. For example, using this approach it has been possible to determine that the perirhinal cortex interacts extensively with both the hippocampus [497], and medial prefrontal cortex to support learning about object–place associations [498]. Indeed, whilst the perirhinal cortex is known to play an important role in recognition memory generally [373, 499-501], it is worth pointing out that both the perirhinal and prefrontal cortex have been implicated in working memory processes [502-504], including executive functions in humans [505-507] and similar processes in non-human animals [508, 509]. Thus, it appears that the hippocampus, perirhinal cortex and prefrontal cortex form part of a neural system that supports memory for items and the contexts in which they occur [373]. These structures may perform this role irrespective of whether long-term or short-term memory processes are engaged [510].

Spatial memory deficits in AD

Visual impairment is a common symptom of AD and thus, considered to be one of the contributing factors to performance deficits in many visually mediated tasks (see, [511]). However, whilst, in general, research has shown that static visual acuity, stereo-acuity, dynamic visual acuity and motion direction discrimination are relatively intact, AD patients tend to perform significantly worse on tests of static spatial contrast sensitivity, visual attention, shape-from-motion, colour, visuospatial construction and visual memory [512]. Crucially, all these deficits are thought to be related to wider impairments in visuospatial memory in AD [513, 514], although recent neuroimaging research suggests that the loss of retinal ganglion cells, nerve fibre layer loss, and glaucoma may also play an important role mediating visual deficits in patients [511].

Individuals with AD exhibit deficits in a wide range of spatial, visuospatial, and visuoconstructual memory tasks, both in respect of immediate and long-term duration

(see reviews, [331, 361]). Visuospatial difficulties in AD are often characterized as showing more a rapid forgetting of information in serial position tasks, with the deficit in serial recall often attributed to more rapid decay and proactive interference between items [514]. Indeed, proactive interference is a particular issue in visuospatial working memory tasks even in those who are cognitively intact [515]. However, defective monitoring of STM and LTM, has also been shown to influence performance in short and long-term serial recall tasks [516], a function that has been associated with executive function [508, 517]. Nevertheless, consistent with the notion of largely intact implicit function, AD patients seem to do well on tasks involving priming (e.g. increased sensitivity to certain stimuli as a result of inconsequential prior exposure), although performance may depend on whether a given task requires perceptual or conceptual processing resources [518]. Interestingly, although semantic autobiographical memory is often preserved in AD until the moderate stages, semantic priming studies have yielded contradictory results [519]. Patients diagnosed with aMCI and early-stage AD exhibit a number of visuospatial difficulties in their day-to-day life, including topographical disorientation (e.g. getting lost whilst navigating in familiar or unfamiliar environments) [331, 520]. Recent research using a novel test of spatial memory (the four mountains test) in aMCI patients and those with early-stage AD, indicates that the core spatial memory deficit in both patient populations relates to an inability to represent topographical layout (even for very short durations) within the context of more general LTM impairments [521]. Indeed, performance on the four mountains test also differentiates AD from frontotemporal dementia (FTD) [521], a finding that has parallels with MCI research in that impairments in the allocentric component of spatial memory may be an accurate predictor of progression to AD [522, 523]. Taken together these findings suggest that topographical representation may be sensitive to the early pathological changes in AD and aMCI [514, 523]), and may be a consequence of MTL atrophy [521]. Together these findings suggest that evaluation of visuospatial processes may be a promising approach to finding predictive markers of AD in clinical populations.

As discussed already, object-place memory is also sensitive to the effects of hippocampal damage in humans and other mammalian species such as rodents [373, 524, 525], and in most cases this type of memory is also disrupted in early-stage AD with object-place deficits revealed via visuospatial tasks in which patients are asked to recall the spatial position of a target item [331]. This type of task is able to discriminate

between the cognitive deficits shown by patients with AD and those with depression [526], questionable dementia, and FTD [527]. Indeed, at least three MCI subgroups have been distinguished using visuospatial tasks of this nature [523]. Interestingly, the acquisition of these types of associations has been shown to require cholinergic modulation of perirhinal-prefrontal network interactions [528]. In addition, recent data with a transgenic mouse model exhibiting a selective elimination of the vesicular acetylcholine transporter in the hippocampus (which interferes with synaptic storage and release of acetylcholine), results in significant deficits in hippocampal LTP, as well as and selective deficits in a spatial memory task [529]. Indeed, since disruption of cholinergic signalling is known to be an early pathological change in AD due to the profound loss of the acetylcholine synthesis enzyme choline acetyltransferase and neurons in the forebrain's nucleus basalis of Meynert (the origin of major cortical cholinergic projections) [530], this potentially provides a basis for understanding the spatial memory deficits in the early-stages of AD.

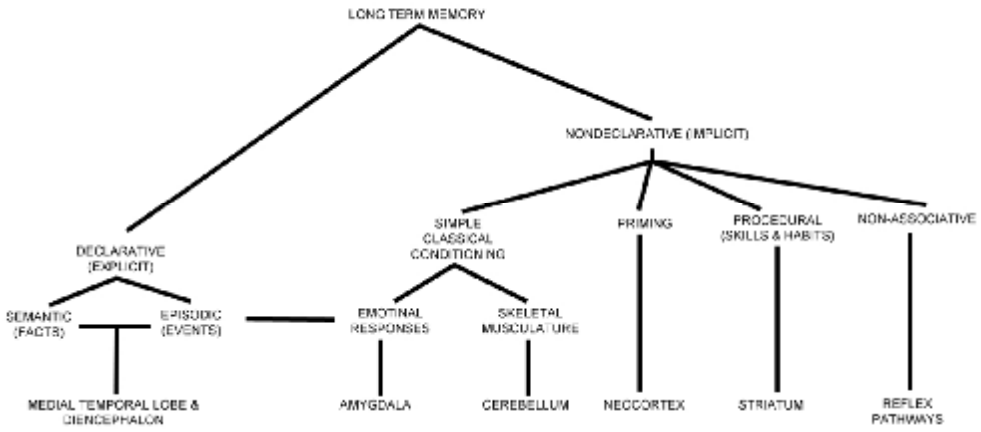

Figure 1.1: Major Divisions of Long Term Memory. Adapted from Squire, L.R. (2004). Memory systems of the brain: a brief history and current perspective. Neurobiology of Learning and Memory, 82(3), 171-7.

1.5.1.4 Recognition Memory

Recognition commonly refers to the cognitive process whereby something is identified as having been previously encountered (heard, seen, known, or felt) [531], but may also refer to a perception of truth, or the acknowledgment of something [532]. As such, recognition memory relates to the cognitive process of recognition, and is considered a subcategory of declarative memory (see review, [500]). Recognition memory can be subdivided into two component processes: delayed recollection (or retrieval) of the

details associated with the previously experienced/encountered event, object or person (episodic information), and an immediate familiarity for the feeling that a stimulus was previously experienced without explicit recollection [500]. These two processes are sometimes respectively referred to as "remembering" and "knowing".

Converging lines of evidence from research conducted with humans and animals suggests that recognition memory depends on the integrity of structures in the MTL [453, 464, 466, 477, 500, 501, 533-539], although there is uncertainty as to how these structures may differentially contribute to recollection memory processes [535, 540]. Currently, two different models (dual process verses single process models) potentially describe how recognition memory is organized across different senses. According to the dual process view, recollection and familiarity are supported by different anatomical substrates [500, 524, 541, 542]. In line with its role in episodic memory, recollection is thought to be hippocampal-dependent, and to support recognition of specific stimuli together with the contextual features of the learning event (i.e. where), whilst familiarity does not require the hippocampus and can support context-independent recognition of individual stimuli via the perirhinal cortex [500, 543-551]. However, the single process theory denies the independence of the two processes within recognition memory. In this theory, both familiarity and recollection are a continuous process, where the former is primarily thought of as a weak memory, and the latter is considered to be a type of strong memory [501]. The single process theory of recognition also has also accumulated experimental support in its favour, (see review, [501]), although it cannot fully explain some data obtained from lesion studies, particularly in rodents [546, 551-553].

Impairments in AD

There is considerable evidence that visual recognition memory is impaired in AD [331]. Aside from the potential impact of spatial and visuospatial memory deficits already discussed, these recognition deficits might concern defective strategies in relation to the forming, maintaining, and matching of the memory representation of the visual stimulus, especially when long inter-item lags occur [554]. Often this will involve a delayed matching to sample (DMS) recognition test. Basically, patients are first shown a study list of items to memorize (sample phase), then after a delay, the individuals are tested on their ability to discriminate familiar target items from those which are novel (test phase). Thus, the objective in DMS for the person to indicate via a simple yes/no,

whether or not the item was on the study list. Alternatives to DMS relate to delayed non-matching to sample (DNMS) paradigms where participants have to indicate which of the list of items are novel rather than familiar, and immediate matching to sample tasks in which sample and test phases are continuous with no delay.

In yes/no MS and DMS tasks, patient responses fall into four classes: hits, misses, false alarms, and correct rejections [555]. Based on counts of these responses, persons with AD and those with dementia of the Lewy body type (DLBT) generally show impaired memory performance for test items relative to controls, although DLBT patients exhibit a significant impairment relative to AD patients when DMS is used [556]. Indeed, further research using these and other cognitive tests in patient populations have shown that compared to AD, perception, attention, and working memory (WM: see section 1.5.3) are all disproportionately impaired in DLBT [557]. Recently, Viggiano, et al have used an elegant experimental paradigm to study the effects of repetition lag in AD [558]. In this paradigm, pictures were presented to mild- and moderate-AD patients and normal age matched controls at different levels of spatial filtering, allowing for the measurement of the amount of physical information required for identification of stimuli as a function of prior exposure and repetition lag. Viggiano, et al have shown that in the elderly, the magnitude of repetition priming did not differ as a function of inter-item lag, but instead, repetition-lag effects interacted with dementia severity, with the capacity for retaining memory traces over longer intervals worsening as the disease progresses [558]. Indeed, Viggiano, et al suggest that suggest that severe cortical degeneration may render AD patients unable to maintain their perceptual memories, and that dementia severity is a critical variable in the visual recognition memory assessment [558].

1.5.2 Nondeclarative Memory

Nondeclarative memory represents the second major division of LTM in mammals, and refers to the unconscious (implicit) recall of information from previous experiences. Nondeclarative memory phenomena mostly relate to forms of procedural memory for performing cognitive and sensorimotor skills (e.g. riding a bicycle), as well as habits, priming, simple associative learning (e.g. operant and classical conditioning), and non-associative learning (e.g. habituation and sensitization) [559]. Whilst early studies of memory-impaired patients with MTL damage led to the view that this brain region is critically involved in declarative memory [374], they also showed that

nondeclarative memory, immediate STM and WM processes, —were all relatively intact in these patients [359]. This fostered the prevailing view that these types of memory are largely independent of MTL function, depending instead upon on an array of distributed cortical and subcortical structures that includes regions of the pre-frontal cortex, cerebellum, and striatum [560, 561]. However, alternative formulations of memory systems exist which do not divide memory on the basis of consciousness (see, [562, 563]). Specific MTL structures such as the hippocampus may also mediate some forms of implicit learning [494], as well as under some circumstances STM [510, 564], particularly if the material to be learned exceeds the capacity of immediate memory, is difficult to rehearse, or if attention is diverted (see review, [510]).

Numerous neuropsychological studies have reported that many aspects of procedural memory are left intact in most AD patients [565], at least until the latter phases of the neurodegenerative process (see reviews, [518, 566]). For example, whilst explicit recognition of familiar or unfamiliar melodies is typically impaired in moderate-stage patients consistent with episodic memory impairment, implicit procedural memory for playing a musical instrument can be spared in musicians with AD [567]. Furthermore, unlike depressed patients, people with moderate AD can still develop a positive affective bias of judgment for previously heard melodies [568]. However, research has also revealed deficits in some domains, including amygdala-dependent fear conditioning and eye-blink conditioning that may be supported by inputs from the entorhinal cortex to the hippocampus [569, 570]. In addition as stated earlier, studies of priming have reported mixed findings in AD patients [518, 571-573] but see, [573]. Some variability across studies reflects differences in methodology, where some tests may involve a greater or lesser degree of competition between explicit and implicit memory systems, with the final effect of learning being better when all of the implicit memory capacity is engaged in the learning process [566]. AD patients also show impairments in conceptual implicit memory [574]. Since conceptual implicit memory tests do not require word production [574], deficits in these tasks may be related to MTL damage [575].

1.5.3 Working Memory Deficits

The basis for the current view regarding the anatomical subdivision of LT and ST memory originates from Milner (1966), who showed that whilst patients with MTL damage displayed declarative memory impairment, they also showed intact non-

declarative memory, and immediate STM, [374]. Indeed, this fostered the prevailing view that non-declarative and STM processes are largely independent of MTL function, depending instead on a wide array of cortical and subcortical structures which include amongst others, regions of the pre-frontal cortex, cortical association areas, cerebellum and striatum (see reviews, [359, 360]. However, it is important to point out that alternative taxonomies of these systems exist which do not divide LTM on the basis of consciousness (see reviews, [562, 563]).

STM is usually conceptualised in terms of WM, a model first proposed by Baddeley and Hitch (1974) as a response to the accumulation of experimental neuropsychological evidence that did not fit the previous Atkinson and Shiffrin model of STM [576]. Essentially WM is the system that actively holds multiple pieces of transitory information in the mind for a short period of time, where they can be manipulated in the course of completing various verbal and nonverbal tasks, and/or made available for further information-processing [577]. Thus, WM forms the basis for many day-to-day tasks, including retrieving from LTM, a familiar telephone number and then holding this information in mind whilst it is dialled (i.e. the initiation of a motor programme). Because WM requires the active manipulation of information or behaviours (i.e. "monitoring") as part of completing goal-directed actions, it is not exactly the same concept as STM. Nevertheless, correlational studies have not been able to consistently separate both constructs; there is evidence for a large or even complete overlap [578]. Thus, they probably refer to the system. In the original Baddeley and Hitch (1974) model [576], WM is comprised of three components: the central executive which functions as an attentional system that monitors on-going mental processes, and its two slave systems: the phonological loop and visuo-spatial sketchpad. These represent STM stores for phonological information and visuospatial information respectively [576]. More recently Baddeley has added a third slave system, the 'episodic buffer', which is considered to be a short-term multidimensional store for the temporary binding of episodic information; it forms an important interface between the subsystems of WM, LTM and the central executive [579].

1.5.3.1 Executive Dysfunction in AD

The central executive is probably the least understood but most complex component of WM and is unlikely to reflect a single unitary process [363]. Putative functions of the central executive include the control and monitoring of WM (e.g. [517]), cognitive

flexibility, multi-tasking and attentional control despite distractions and changing demands (e.g. [580, 581]), strategic planning, execution, and evaluation of a sequence of thoughts/actions to achieve a desired goal (e.g. [582, 583]); inhibition of inappropriate responses and capacity for self-regulation (e.g. [584, 585]); 'on-line' active construction of an theory of mind [586]; rule discovery such as sorting cards based on specific rules and fluid intelligence (e.g. [587, 588] and concept generation (e.g. [589, 590]). Perhaps, then, it is unsurprising that various neuropsychology and neuroimaging studies of executive processes have revealed links between many different brain areas, indicating a dynamic and flexible neural substrate for executive functions (see, [591]). However, strong consensus exists in the literature with respect to the importance of the frontal regions and more specifically, regions of the prefrontal cortex in a range of WM and executive functions [577, 592-596]. Several studies have examined the role of executive function in MCI and early-stage AD, and found a number of disruptions in executive processes [348, 426, 516, 581, 597-624], including in tasks related to response inhibition and task switching [625-627]. Indeed, multi-domain executive impairments may be predictive for conversion to dementia [628]. Defective self-monitoring [629], and control of attention [630] may well be predictive for which MCI patients will decline as opposed to stay stable across the assessment period. Furthermore, individual differences in executive function related to monitoring have been linked with memory accuracy in those over the age of 65 [517]. Deficits in WM [631] and in particular visuospatial executive function [632] may also distinguish those with MCI from persons exhibiting normal age related changes in cognition. Longitudinal studies investigating pre-diagnostic symptomatology and staging of AD also report that executive dysfunction is often present before diagnosis, with more rapid decline occurring 2-to-3 years before diagnosis [606, 612]. This evidence has led some to suggest that executive function may be the core underlying dysfunction associated with AD [606, 612]; others have proposed that there may be a subgroup of AD patients with a specific dysexecutive pattern of impairments (e.g. [599]).

Thus, it would appear that a significant proportion of people with AD and MCI have discernible executive deficits which cause significant impairment in many aspects of day-to-day living [614, 622]. Although overt atrophy of the frontal cortex is usually not an early-stage pathological change in AD, executive dysfunction in patients has often been ascribed to brain damage of vascular origin, including white matter changes [633,

634]. However, recent research suggests that hippocampal atrophy may be the root cause of impaired executive function in early-stage AD [635, 636].

1.5.3.2 Phonological Loop and Visuospatial Sketchpad in AD

A variety of data from psychological and neuroimaging studies have converged on the suggestion that phonological loop function is largely intact at the preclinical and early stages of AD, becoming more impaired as the disease progresses (see review, [361]). Since phonological loop function is not central to the behavioural experiments in mice detailed in Chapter 3 of this thesis, this component of WM is not considered further here. However, the visuospatial sketchpad may be important when considering the ST retention of visuospatial information in mice, particularly in Experiment 1 in relation to an assessment of rodent spatial WM. Indeed, in mild AD it is commonly accepted that memory impairment is often apparent in patients when visuospatial function is assessed. However, as visuospatial tasks also require processing by the central executive system [363], it is difficult to know the degree to which this slave system is compromised [361, 637]. Additionally, the damage to MTL structures involved with spatial and episodic memory in the early stages of AD may also partly explain some deficits in visuospatial WM in AD, particularly in circumstances where the material to be learned has exceeded WM capacity, if the material was difficult to rehearse, or if attention was diverted (i.e. [510]).

1.5.3.3 Episodic Buffer in AD

The episodic buffer is considered to be a multidimensional store that forms an interface between the subsystems of WM, LTM and the central executive [638]. As a multidimensional buffer, it allows a range of different subsystems to interact, despite their being based on different modes, with a major function of the buffer being to "bind together" different sources of visual, spatial, and verbal information with time sequencing information into integrated chunks or 'episodes' [639, 640] —a process once assumed to depend upon executive resources [638, 641]. The episodic buffer shares conceptual overlap with Tulving's original concept of episodic memory [366], although it differs from the latter in that it represents a temporary time-limited store for such information [640]. Here there is agreement with Cowan that the number of episodes or chunks that it can hold is somewhere in the region of four [642], with capacity differing from one individual to another as reflected by individual differences

in working memory span [363]. The episodic buffer has not been extensively studied in AD; suitable tasks need to be developed and validated (see review, [361]). However, experimental evidence indicates that both LT and ST memory binding deficits are evident in early-stage AD across a range of modalities [643-645]. Indeed, AD functional neuroimaging studies reveal large-scale functional abnormalities in brain networks which likely underpin deficits in relational binding [646].

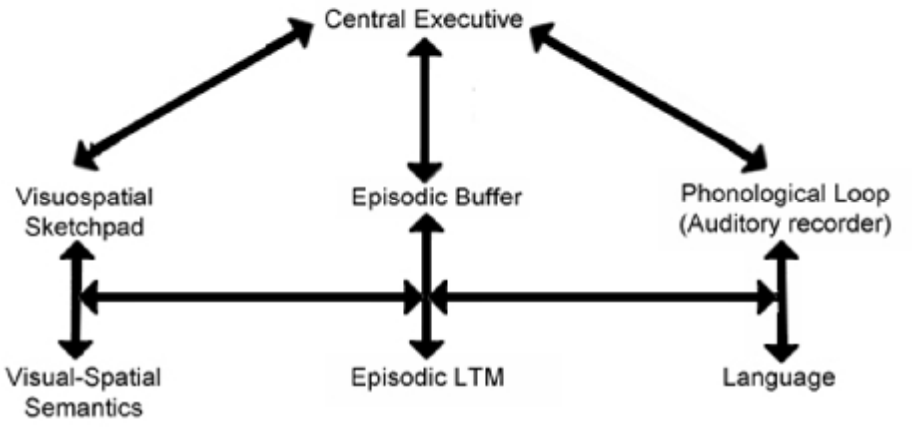

Figure 1.2: Major Divisions of Working Memory. Adapted from Baddeley, A. D., Eysenck, M., and Anderson, M. C. (2009). Memory. Hove: Psychology Press.

1.6 ANXIETY AND BEHAVIOURAL DISINHIBITION

Among the components of the limbic system, the amygdala is a fascinating structure that is involved in the processes of liking and disliking, and in the ways in which emotions drive actions (i.e. predicting which stimuli generate aversive events) and affect the salience of memories [647]. For example, the amygdala is central to the acquisition, storage, and expression of conditioned fear memory (i.e. Pavlovian fear conditioning) [648], a process that is widely accepted as dependent on LTP [649]. Indeed, when the normal function of the amygdala is disrupted it can often manifest as post-traumatic stress disorder, or as an anxiety disorder [650], the latter of which are amongst the most prevalent of all psychiatric disorders in that about 17 % of people will suffer from an anxiety disorder at some point in their lives [650, 651]. Besides AD, amygdala function is also adversely affected in several additional neurodegenerative disorders which compromise the structural integrity of the MTL, the most common of which is AD.

1.6.1 Circuitry and Function

The amygdalae are a group of nuclei (the amygdaloid complex) which are located deep within the rostro-medial part of each the temporal lobe in front of the HPC and underneath the uncus of the entorhinal cortex [652]. As such therefore, the amygdala has close interconnections with both cortical and subcortical areas [650], including the temporal cortex (perirhinal and entorhinal cortex), frontal lobe, insular, and cingulate association areas, with subcortical areas including the brainstem, hypothalamus, thalamus, hippocampus, and claustrum [653, 654]. The many components of the amygdaloid complex can be grouped into three principal divisions: the basolateral, cortical, and centro-medial nuclear groups. The largest part of the amygdala is the basolateral nuclear group and is reciprocally connected to many cortical areas in the per-frontal cortex, temporal, insular, and occipital regions [655]. Together with its close relations with the thalamus and projections to the striatum, the connectivity of the basolateral amygdalae likely belies its role in modulating memory, particularly for arousing or emotional events, during post-training periods of consolidation [656]. In particular, amygdala-pre-frontal cortex and amygdala-hippocampal circuitry has been broadly implicated in the processing of threatening-stimuli in humans [657], and conditioned fear responses in experimental mice (see review, [658]. Indeed, in primates the basolateral amygdalae nuclei are thought to be involved primarily in evaluating the emotional significance or context-dependent relevance of all stimuli, including social signals such as facial expressions [659]. Furthermore, recent evidence from electrophysiological recordings in rats has also suggested that the basolateral amygdalae is responsible for strengthening memories whose substrates are considered to be stored remotely in structures such as the hippocampus, striatum and cerebral cortex [652, 656]. Although there is insufficient space here to cover this topic in detail, recent research in rats has also supported the notion that the basolateral amygdalae likely plays a role in strengthening memories by increasing the number of neurons that come to best represent that event, and thus their cortical representations [660]. Furthermore, that traumatic, intrusive memories common to post traumatic stress disorder, might reflect abnormally extensive representational networks due to hyper-activity of the basolateral amygdalae consequent to the release of excessive amounts of stress hormones [660]. The cortical amygdala receives olfactory input directly from the olfactory bulb and indirectly from the olfactory cortex, with the cortical amygdala in turn projecting to the centro-medial amygdala and the hypothalamus [650]. This

amygdala -olfactory circuitry has been implicated in social buffering in male rats [661, 662], as well as odour recognition memory [663]. The centro-medial amygdala provides the main outputs for the basolateral complexes, and receives direct input from the cerebral cortex (largely restricted to fibers from the hippocampus, insula, and orbitofrontal cortex) [650]. However, because the amygdala is a bidirectional pathway that can relay information between association cortices and subcortical structures, it is in an optimal position to simultaneously influence the excitability of numerous brain regions at any given time [650]. Research has suggested that the centro-medial amygdala nuclei may be involved in allocating attention to stimuli of high significance as well as the initiation of situation-appropriate autonomic responses [659]. However, given the overlap in physiological and electrophysiological properties of the centro-medial amygdala and basolateral amygdalae neurons, it is more likely that processing is distributed across both nuclear groups with differences reflecting processing bias rather than a hallmark of mutually exclusive functions [659].

1.6.2 Behavioural Disinhibition and Anxiety in AD

Anxiety and behavioural disinhibition are both emotional components typically associated with BPS in AD [341, 664]. Behavioural disinhibition refers to the loss of control or restraint over social behaviours, aggression, hyperactivity, self-destructive behaviour and sexual disinhibition [665]. In addition, behavioural dysfunction in AD patients can also present as "cognitive disinhibition" on tasks of attention and memory [666, 667]. Anxiety is generally considered as a psychological and physiological state characterized by somatic, emotional, cognitive, and behavioural components linked with the unpleasant feeling of fear and concern [344]. Whilst anxiety is reported to be increased in up to 70% of AD patients [664], research into its time-course has revealed that patients generally display greater levels of anxiety at an early stage of the disease when cognitive function is more intact, followed by a progressive pattern of decreased anxiety in the presence of decreased cognitive function [668]. Anxiety in AD patients is usually diagnosed by assessing neuropsychiatric symptoms of excessive worry, restlessness, irritability, muscle tension, fear behaviour, and respiratory symptoms [669]. However, diagnosis of anxiety is often complicated by its comorbid presentation with depression and apathy [341, 670].

In mammals, anxiety and behavioural disinhibition are both components of emotion typically attributed to the neural circuitry supporting the amygdala and

prefrontal cortex (including sub-regions of the orbitofrontal cortex) (see reviews, [664, 671, 672]). In a recent study spanning several neurodegenerative diseases, behavioural disinhibition was most significantly associated with atrophy in the orbitofrontal cortex, anterior cingulate, and temporal lobes [673]. In addition, damage to the orbitofrontal cortex has also been linked to deficits in WM, and in particular executive function [506]. Amygdala atrophy is prominent feature in AD (see review, [664]), particularly in the early stages of the disorder where it relates to symptom severity [674]. Anxiety has also been linked to the differential effects of human APOE isoforms as well as and molecular changes within the amygdala nuclei [675, 676]. Furthermore, ApoE-/- mice expressing ApoE ε4 have been shown to demonstrate an age-dependent increase in anxiety on the elevated plus maze, and had significantly lower levels of microtubule-associated protein (MAP) 2-positive neuronal dendrites in the central nucleus of the amygdala [677]. AD pathology compromising the integrity of the amygdala and frontal regions has also been suggested as the root cause of patients inability to process fearful stimuli [678], as well as impairment in displaying enhanced emotional memory for negatively-valenced stimuli [569]. However, although severe amygdala dysfunction often manifests as components of Kluver-Bucy syndrome in humans and other primates [679] (including visual agnosia, hyperorality, hypermetamorphosis, blunting of fear or rage, and hypersexuality), pathological changes affecting the amygdala in AD rarely result in Kluver-Bucy syndrome [680]. The reasons for this are unclear although it is thought that the progressive functional disconnection hypothesised to occur between the MTL and other brain regions as AD [681, 682] may be sufficient to prohibit many of these abnormal behaviours from emerging in most AD patients [664]. It is interesting that recent findings support a theory of FTD as essentially a disorder of frontolimbic disconnection leading to unconstrained prefrontal connectivity [683], whereas AD is associated with frontal lobe hypometabolism [684], the latter of which has also been associated with impaired insight [684], and thus danger [685].

Although the role of the prefrontal cortex and amygdala in anxiety and behavioural disinhibition are widely acknowledged, it is less commonly understood that lesions of the ventral hippocampus can also produce behavioural disinhibition and, interestingly, reduced anxiety [686, 687]. Indeed, the role of the hippocampus in emotional responses is further supported by lesion studies in rodents, which show that lesions to the ventral-medial hippocampus result in behavioural disinhibition and anxiolytic behaviour on unconditioned tests of anxiety, including a version of the elevated plus

maze [686, 687, #9410, 688-690]. Thus, whilst the amygdala and prefrontal regions are increasingly implicated in a range of various antisocial, violent, and psychopathic behaviours [691-693], the hippocampus is also likely to be complicit in mediating many of these behaviours. Indeed, structural MRI has linked atypical anterior hippocampus asymmetries with those considered to be "unsuccessful psychopaths" (i.e. those who become institutionalized due to their poor behavioural control and aggressive tendencies) [694]. Hippocampal asymmetries in these individuals may reflect an underlying neurodevelopmental abnormality that disrupts hippocampal-prefrontal circuitry, resulting in affect dysregulation, poor contextual fear conditioning, and insensitivity to cues predicting capture by the 'long arm of the law'[694]. Taken together these findings implicate the neural circuitry supporting the amygdala, hippocampus and pre-frontal cortex in a range of maladaptive outcomes associated with anti-social and aggressive behaviours, as well as the unconditioned expression of anxiety and conditioned expression of fear. In this respect the pathological alterations observed in hippocampal-prefrontal circuitry in AD likely explain why affect behavioural dysregulation, poor contextual fear conditioning, and behavioural disinhibition, are all symptoms commonly experienced by AD patients.

1.7 MOLECULAR PATHOLOGY

At the microscopic level, AD is primarily defined by the presence of large numbers of diagnostic Aβ-Plaques and NFTs, both lesions of which are fairly robust and easy to detect [88]. Plaques and tangles are further associated with a diffuse loss of synapses and neurons (losses), as well as neuroinflammation and deficits in insulin signalling, the latter of which is considered separately (see section 1.9).

1.7.1 Amyloidosis and Aβ-Plaques

Amyloidosis refers to the physiological conditions wherein amyloid proteins and protein peptides aggregate to form plaque deposits in various organs and tissues. Many of these deposits contain amyloid fibrils formed from normally soluble proteins that have assembled into fibres dominated by a predominantly β-sheet conformation of their polypeptide backbone [695, 696]. Although amyloid is a naturally occurring protein, it is often associated with the pathological states common to 'amyloid diseases', including AD, T2D, and spongiform encephalopathies [697]. Amino acid

composition and sequence analysis of the proteins comprising a range of ex-vivo amyloid fibrils have revealed each amyloid disorder is associated with a particular protein or peptide [698]; AD is primarily associated with aggregation of the Aβ peptide. Like other amyloid-associated proteins Aβ has the ability to self-associate into varied assembly forms ranging from individual monomers to large insoluble amyloid fibrils found in some Aβ-plaques. Aβ can also form small intermediates known as soluble oligomers of varying molecular weights and sizes [46]. In addition, Aβ can also be deposited on the walls of cerebral blood vessels and veins during CAA [134]. CAA is found in approximately 25 to 90+ % of autopsied AD brains [699, 700], although its role in the progression of AD is not known. However, since CAA is associated with an increased risk of intracerebral haemorrhage, ischemic micro-infarcts, as well as non-haemorrhagic (white matter) lesions in the brain, it may induce a stepwise decline in cognition characteristic of vascular dementia [134].

1.7.1.1 Aβ-plaques and Their Topographical Distribution

In AD, the Aβ peptide is deposited as extracellular plaques although other proteins such as ubiquitin and metal ions such as Zink and iron have also been localised to these types of brain lesion [90]. The predilection site for the deposition of amyloid as Aβ-plaques is the cerebral cortex, in particular the isocortex which consists of the grey matter, or neuronal cell bodies and un-myelinated fibres [88]. Generally speaking, Aβ-plaques are not specific to AD, and are also found in other neurodegenerative disorders such as dementia with Lewy bodies, and dementia related to Down syndrome [49, 88, 90] as well as some persons with MCI, and aged persons evaluated as cognitively normal. Aβ-plaques are commonly, classified into diffuse and dense-cored plaques (neuritic plaques) based on their morphology and positive or negative staining with Thioflavin-S or Congo Red. Post-mortem identification and quantification of Aβ-plaques mostly relies on immunohistochemical staining methods utilising anti-Aβ antibody reagents [701].

Diffuse and Neuritic Plaques

Diffuse plaques are large 10-to-100μm areas of poorly defined Aβ deposition (principally shorter length Aβ isoforms such as those with 40 amino acids in length i.e. Aβ40) which tend to lack the fibrillar amyloid more commonly found in neuritic plaques [49, 90]. Due to defuse plaques being poorly immunoreactive, their number in

the brain depends on the quality of the immunohistochemistry, although in some brain regions they are the only type of Aβ deposit found [88]. Diffuse plaques do generally affect the structure of the surrounding neuropil tissue and consequently are not usually considered directly toxic [49]. This view is further supported by the fact that diffuse deposits can be found in large numbers in subjects whose intellectual status has been evaluated as normal [702, 703]. However, since diffuse plaques are often considered as pre-neuritic [704], the clinical status of these cases remains uncertain. Some consider persons with large numbers of diffuse plaques but whose intellectual status is normal —to be pre-symptomatic for full-blown AD [88, 327], although this view is not universally accepted [705]. Whilst it not known how long diffuse plaques may remain uncomplicated in the brain prior to conversion, some individuals are thought to have better cognitive reserve which may mask the subsequent signs of early dementia [88].

Neuritic plaques are larger (50–200μm) "focal" deposits of Aβ deposition that consist of a dense amyloid core with radiating fibrils that are often (although not exclusively), surrounded by a corona of dystrophic neuritic and glial cell processes together with reactive microglia and astrocytes [49]. Neuritic processes often contain paired helical filaments of hyperphosphorylated protein tau. The fibrillar amyloid contained in neuritic plaques is often identified via the Congo red or thioflavin S positive methods of detection [88]. In addition to the extracellular deposition of Aβ a growing body of evidence also suggests that Aβ-oligomers can accumulate intracellularly in AD, particularly in the neuronal cell body [88]. However, this "intracellular pool of Aβ" is controversial [88, 706], and it remains possible that it results from neurons internalizing extracellular Aβ deposits [707]. Nevertheless, the accumulation of intracellular Aβ via the uptake of extracellular peptides may provide a link between Aβ generation and synaptic dysfunction in AD [708]. Neuritic plaques are found mostly in layers II and III of the isocortex [709], the areal topography of which is non-random and dependent upon on the stage of the disease. Progression of amyloid pathology in the brain generally proceeds in a particular manner, which depending on the brain regions affected, can be classified into specific stages referred to as Braak stages (A-C) [710]. At Stage A, low densities of amyloid deposits are found in the isocortex, particularly in the basal portions of the frontal, temporal and occipital lobe. Very little amyloid found in the MTL and associated structures at this stage. Stage B shows an increase in amyloid deposits in almost all isocortical association areas with

only the primary sensory areas and primary motor field remaining almost devoid of deposits [88]. Here there is a mild involvement of the hippocampus as well as the entorhinal cortex. In stage C virtually all isocortical areas are affected, while deposits in the hippocampus generally show the same pattern as stage B although there is usually a sparing of the subcortical grey matter [88].

Until recently, cerebral Aβ burden could only be measured at autopsy and it is possible that in some of these clinicopathological studies there may have been a time lag between the last cognitive assessment and autopsy. Nowadays the build-up of amyloid in the brain in AD patients, those believed to be pre-symptomatic for AD, and in normal aged controls can be studied non-invasively by using tracers such as PiB-PET [711, 712]. However, whilst PiB- (negative amyloid scans) using this tracer indicate absence of AD with a high level of accuracy, positive amyloid scans in 10-to-30 % of healthy elderly volunteers (PiB+) make their predictive value less clear [713, 714]. Overall post-mortem studies have consistently shown that the distribution and density of Aβ-plaques (that is both diffuse and neuritic plaques), do not correlate well with the local extent of neuron death, synaptic loss, nor the degree of cognitive impairment in AD [715, 716], although as mentioned earlier, this is likely not the case for soluble oligomers of Aβ [60, 65]. Overall, the distribution of Aβ-plaques in AD does not correlate with that of neurofibrillary pathology [49], suggesting that these two types of protinaceous lesion may occur independently.

1.7.2 Neurofibrillary Tangles

Although this thesis does not involve research focusing on NFTs, they are an essential lesion in AD, and are therefore included here for completeness. In the CNS, tau proteins are best known for their ability to bind and help stabilize microtubules where they form vital components of the cell's cytoskeleton [717]. Axoplasmic transport (also called axonal transport) is a vital cellular process that involves the movement of mitochondria, lipids, synaptic vesicles, proteins, and other cell parts (i.e. organelles) to and from a neuron's cell body through the cytoplasm of its axon (i.e. axoplasm) [718]. In healthy neurons, microtubules guide the bi-directional transport tracts for nutrients and other molecules from the cell body down to the ends of the axon (anterograde transport), as well as from the terminals to the cell body (retrograde transport). Under normal circumstances tau stabilise microtubules. However, in AD individuals, tau molecules become hyperphosphorylated (phospo-tau) via a variety of intracellular

kinases, leading to their disassociation from microtubules [88]. Indeed, tangle-bearing neurons are said to be devoid of normal microtubules [88]. Due to malfunctions in axonal transport, this disrupts communication between neurons [561], and likely leads to the neuronal apoptosis and thus the development of dementia [41]. Like Aβ, dislocated phospo-tau is able to polymerize into a variety of assemblies, including small soluble tau oligomers [719, 720], which upon aggregation form paired bundles of helical filaments [717], the latter of which are the primary constituents of NFTs found inside the cell body (soma) of neurons, as well as in distal dendrites as neuropil threads [721]. However, other proteins such as actin and ubiquitin have also been localised to these lesions [88, 722]. A variety of silver impregnation staining techniques, have been typically employed to visualize NFTs, including the modified Bielschowski or Gallyas technique, or the fluorochrome dye thioflavin S technique. In addition to these, a number of immunohistochemical approaches have also been used to visualise NFTs, most of employing antibodies directed against fibular tau [88].

The topographical distribution of NFT pathology uses the Braak and Braak scheme of staging stages I to VI, with the spatial pattern being mostly predictable [334]. In the early stages of AD, NFT degeneration typically originates in the perirhinal cortex (particularly layer II neurons), and progresses through the hippocampus and amygdala. Eventually NFT pathology progresses to the association cortices, and finally, the deeper layers of the primary cortices [88]. However, variability in the staging of NFT pathology can be found in neuroimaging and neuropathological studies of AD patients [394, 723-726], and may reflect atypical presentations of the disorder [332, 727]. Although NFTs are usually conceptualised as intraneuronal lesions, when tangle-bearing neurons die the NFTs they contain become the extra-neuronal "ghost" tangles seen in the brains of individuals with advanced AD [728]. Unlike their intracellular counterparts, ghost tangles are glial fibrillary acidic protein (GFAP) and Aβ immunoreactive [729], and also contain significant amounts of blood proteins such as amyloid-P [730], and ubiquitin [722]. As discussed earlier, the staging pattern of tau pathology in most cases of AD likely helps to explain the correlation between the degree of amnesia and NFT burden early on in the disorder [394, 397].

1.7.3 Loss of Cells and Synapses

In the cortex the large and middle-sized pyramidal neurons are the most vulnerable in AD, whereas the very large Betz cells and the small granule cells are relatively spared

[88]. However, by using appropriately controlled methods with post-mortem tissue, it has been found that neuronal loss in AD is primarily "focal" with the global number of cortical neurons lost being generally insufficient to override the very high inter-individual variation [88, 731]. Focally, however, severe neuronal loss has been documented in a number of cortical regions, including layer II of the entorhinal cortex where the loss may reach 90 % of the normal neuronal population in the most advanced cases [732], in both CA1 and CA3 regions of the hippocampus [733, 734], in the superior temporal gyrus [401], and in the supra-marginal gyrus [735]. The loss of neurons is considered to be less extensive in the parietal and occipital lobes in AD, with cell loss predominantly found in cortical layers II and III [735]. There is also a substantial loss of neurons in the deep grey matter in AD, including the nucleus basalis of Meynert (innervating the isocortex in acetylcholine), substantia nigra in the midbrain (involved with dopamine production), locus coeruleus in the pons (main source of noradrenaline in the forebrain), and the raphe nuclei of the brainstem (innervating the isocortex in serotonin) [49, 88, 90]. It is no surprise then that the loss of neurones in AD is closely paralleled by widespread neurochemical changes, including disturbances in the cholinergic, glutamatergic, serotonergic and noradrenergic systems [530]. The reason for the focal loss of neurons in AD is still unclear [736], although as mentioned earlier it does correlate with the distribution of tangle-bearing cells [88, 332].

Dendritic spines are tiny protrusions along neuronal dendrites that constitute the major postsynaptic sites for excitatory synaptic transmission. These spines are highly motile and can undergo remodelling even in the adult nervous system [737]. Experiments conducted in animals suggest that the extent of spine remodelling is correlated with behavioural improvement after learning, implicating a crucial role of synaptic structural plasticity in memory formation [738-742]. Furthermore, recent research also suggests that a small fraction of new spines induced by novel experience together with most spines formed early during development and surviving experience-dependent elimination, are preserved in order to provide a structural basis for memory retention throughout the entire life of an animal [739, 741-743]. A loss or alteration of these structures has been described in patients with neurodegenerative disorders , including AD, and is considered to be a much better correlate of cognitive impairment in AD patient than Aβ-plaque burden (see reviews, [408, 744, 745]). As discussed earlier, mounting evidence from human and animal studies suggests both the

dysfunction and subsequent loss synapses is likely mediated by diffusible soluble oligomers deriving from Aβ [60, 62-65, 408, 409] and phospo-tau [402, 403], making both species of aggregate attractive therapeutic targets in AD [410-412].

1.7.4 Reactive Processes and Neuroinflammation

In the normal brain microglia and astrocytes have a highly characteristic morphology comprised of a small cell soma and thin, straight processes [746, 747]. Microglia are the main innate resident immunological surveillance and macrophagic cells of the CNS, and like peripheral macrophages comprise heterogeneous populations with distinct immunological and functional characteristics [748-750]. Under physiological conditions resting microglia poses a resting ramified morphology that does not occur in any other tissue related population of macrophages [747].

The inflammatory response is an early, non-specific immune reaction to tissue damage or pathogen invasion. Inflammation of the CNS is characterized by increased glial activation (both astrocytes and microglia), and concordant with pro-inflammatory cytokine concentration, blood-brain-barrier permeability (and thus, leukocyte invasion from the periphery), and recruitment of the complement system [751]. One key player that is believed to drive this neuroinflammatory process is interleukin-1-beta (ILβ), a pro-inflammatory cytokine that is known to be up-regulated in several neurodegenerative disorders, including Parkinson's disease, multiple sclerosis, and AD [752]. ILβ signals through the type I IL-1 receptor/IL-1 accessory protein complex, leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) dependent transcription of pro-inflammatory cytokines. This then leads to the activation of tumour necrosis factor-alpha (TNFα), IL-6, interferons, and the neutrophil-recruiting chemokines (CXCL1 and CXCL2) in glia [753]. Ultimately, ILβ signalling leads to the generation of reactive oxygen and nitrogen species (ROS), and thus, oxidative stress [754]. However, many of these processes are not inherently pathological since they are often form elements of the brains response to dealing with infection, damage, and thus recovery and repair. For example, some cytokines released form astrocytes have a role in restricting inflammation; protecting neurons and oligodendrocytes thereby helping to limit tissue degeneration and preserve function after CNS injury [755, 756]. Furthermore, the systemic inflammatory response represents a coordinated set of physiologic actions that serves to fight infection, heal wounds, and promote recovery from various external stressors by inducing sickness

behaviours (see review,[757]). Nevertheless, under certain conditions, such as in major trauma and neurodegenerative disease, an excessive pro-inflammatory response can auto-modulate glial phenotype with potentially disastrous consequences that can worsen prognosis and damage the surrounding neural tissue; including the loss of neurones and synapses (see review, [758]). Indeed, prolonged and widespread activation of microglia and astrocytes are apparent in AD brain, in which the severity of glial activation correlates with the extent of brain atrophy [759], and cognitive decline [760]. The majority of transgenic rodent models of AD also exhibit substantial reactive gliosis and accumulation of activated astrocytes in affected brain regions (see reviews, [761, 762]). These features are often observed before the appearance of plaques, and in those models which display NFTs and associated apoptosis of neurons, tangle pathology [763, 764], and neuronal death [764, 765].

1.7.5 Current Treatment

To date, only symptomatic treatments exist for AD, all of which attempt to counterbalance the neurotransmitter disturbance. Three cholinesterase inhibitors (CIs) are currently available and have been approved for the treatment of mild to moderate AD. A further therapeutic option available for moderate to severe AD is memantine, an N-methyl-D-aspartate receptor (NMDR) non-competitive antagonist [766]. Common treatments for BPS in AD include the use of many second-generation (atypical) antipsychotic drugs, and most at best only lead to a small-modest overall effect over placebo [341]. Indeed, many of the treatments for BPS in widespread use are associated with a number of adverse side-effects that present notable risks for people using these medications [767, 768]. The dementia antipsychotic withdrawal trial has also found no significant efficacy for drug versus placebo, thereby suggesting that people do not need to be maintained on antipsychotics [769]. Nevertheless, the usage of these medications is fairly widespread in many nursing homes, where the over-prescription of antipsychotic medications have raised concerns over their use as a 'chemical cosh' for placating difficult patients [768]. Treatments capable of stopping or at least effectively modifying the course of AD (referred to as 'disease-modifying' drugs), are still under extensive research or in phase I-III clinical trials. To block the progression of the disease these compounds have to interfere with the pathogenic steps responsible for the clinical symptoms, including most commonly, the deposition of extracellular Aβ-plaques and NFTs, inflammation, oxidative damage, iron

deregulation and cholesterol metabolism and metabolic dysfunction [412]. A comprehensive review of each of these lies outside the bounds of this thesis (see reviews, [412, 766, 770, 771]). However, a few salient points on anti-inflammatory therapies, anti-Aβ immunotherapies, and therapies related to insulin sensitizing drugs will be covered in the next section. Most of these therapies are heavily biased towards cases of 'pure' pathology, and instances of mixed dementia are likely to pose significant challenges to the treatment of dementia. Both plaque and tangle pathologies may themselves may represent a host response to a (as of yet) undetermined upstream pathophysiologic process in most cases of AD [772]. As such, the therapeutic targeting of these lesions, including their supposedly neurotoxic oligemeric intermediates may only succeed when the host response is directly deleterious [773].

1.8 PATHOGENESIS OF AD

During the past 20 years or so the Amyloid Cascade Hypothesis (ACH) has largely dominated academic and pharmaceutical led research, and today remains the lead hypothesis of AD causation. In short, the ACH which proposed that the aggregation of Aβ was the initial pathological event in AD that leads to the formation of Aβ-plaques and the subsequent induction of a deleterious biochemical cascade that initiates secondary pathologies such as inflammation, neurofibrillary pathology, and the death of synapses and neurons that ultimately results in dementia [774, 775]. This hypothesis is not reviewed in depth here (see, [776-782]). However, a few salient points are considered below.

1.8.1 The Amyloid Cascade Hypothesis

The identification of Aβ as the main constituent of diffuse and neuritic plaques [783], and genetic studies that identified mutations in the APP [28, 29], and presenilin genes [32, 155], leading to the accumulation of Aβ and early-onset FAD [28, 32, 784], were all critical findings that led to the articulation of the ACH. As discussed earlier, the mutations in these three genes all share a common effect of abnormally processing APP, with an average age of dementia onset at 50 years for APP mutations, 45 years for PS1 mutations and 52 years for PS2 mutations [785]. Although several hundred families with EOFAD carry at least one of the mutations mentioned above, it should be said that in the patient populations so far studied, no mutations have ever been found

in the "microtubule-associated protein tau" gene on chromosome 17 (MAPT-17), which encodes the tau protein [25]. For this reason the ACH considers the aggregation of tau and the formation of NFT pathology to be a downstream consequence of Aβ accumulation in the brain. Nevertheless, other neurodegenerative dementias do exhibit a pathological aggregation of tau (i.e. "tauopathies"), related to MAPT-17 mutation, most notably frontotemporal dementia with parkinsonism-17 (FTDP-17) [786]. Indeed, FTDP-17 has been associated with more than 40 mutations in the MAPT-17 gene [787]. Except for the age of onset and family history, no other pathological features appear to distinguish FAD from SAD [788, 789], and both are considered to share neuropathologic features that include Aβ-plaques and NFT pathology as well as the associated neuroinflammation [88, 790]. Although both types of AD may differ clinically (largely considered a result of variations in the staging of pathological lesions), the ACH tends lump both together as deriving from essentially the same pathogenic cascade. However, the steady state of monomeric Aβ in the brain is the result of a tightly controlled balance between production and removal, and SAD is considered to reflect defects in the clearance mechanisms for Aβ rather than in the enhanced synthesis which commonly occurs in EOFAD cases [782]. Indeed, it was recently demonstrated that the kinetics of Aβ production are similar between control and late-onset AD patients, the latter group of which were shown to have an impairment in the clearance of Aβ compared to controls, indicating that Aβ clearance mechanisms may be critically important in AD [791]. Among these mechanisms, interaction of Aβ with ApoE, decreased catabolism via reduced proteolysis, impaired transport across the BBB, and impaired CSF transport, all deserve special attention in the context of therapeutic intervention [779]. However, the ACH is not without its critics (see reviews, [780, 792-794]).

1.8.1.1 The Soluble Aβ Cascade Hypothesis

Over time, the ACH has undergone various alterations with respect to the description of the nature of Aβ as a means of initiating the deleterious events that underpin AD. The original idea that the plaques were pathogenic has currently fallen out of favour for several reasons. First, as indicated earlier, post-mortem studies have consistently shown that the distribution and density of Aβ-plaques does not correlate well with the local extent of neuron death, synaptic loss, nor the degree of cognitive impairment in AD [715, 716], and many AD patients with severely impaired memory show no plaques

at post mortem analysis. Second, many of the mouse models that contain mutated APP isolated from human FAD sufferers show memory deficits long before the plaques are observed in the brain [795]. Third, the distribution of Aβ-plaques in AD also does not appear to correlate closely with that of NFT pathology [88], and in most transgenic mouse models deriving form FAD kindreds, the overproduction/accumulation of Aβ does not lead to NFT formation or overt cell loss [42]. Forth, and perhaps most importantly, several therapeutics that were purported to reduce amyloid-β production or involve delivering antibodies targeting its aggregation into plaques, (i.e. plaque busting drugs), have failed in Phase III clinical testing, although many others are in various stages of development (see review, [778, 780]). In addition, recent advances in neuroimaging techniques in vivo (such as 11C-PiB retention) have shown the presence of robust plaques in otherwise cognitively normal people ([796, 797]. Although it is possible that some of these individuals may as stated be pre-symptomatic with better cognitive reserve, at a minimum these findings show that the presence of plaques does not necessarily equate with memory deficits. These observations have led to thinking that perhaps the insoluble plaques do not trigger the pathological events, and may be benign or even protective in nature [798]. However, since plaques affect the function of the DMN even in elderly persons who are healthy [799], and the failure to fully disengage this network during goal-orientated explicit memory tasks can impact on performance [800, 801], it remains possible that Aβ-plaques exert an effect on memory via this mechanism.

Thus, the ACH has been modified, and now suggests that synaptic toxicity and Aβ neurotoxicity and may be mediated by soluble species of Aβ that have self-assembled into dimer, trimer and higher-order oligomers [110, 802, 803] }, or the equivalent term of Aβ-derived diffusible ligands (ADDLs) [62] (see review, [407]. These oligomers are relatively long-lived, not converting into amyloid protein over periods of several days [64], and many groups have now identified a number of poorly characterized, but biochemically distinct forms of Aβ oligomers from brain tissue derived from transgenic mouse models related to FAD [804], and from human AD post-mortem brain tissue [805]. Studies have revealed high correlations between soluble Aβ levels and cognitive deficits in human AD patients [404, 405, 728, 806-809], as well as in transgenic mice containing one or more of the APP mutations related to FAD [810-813]. Indeed, species of Aβ oligomer have been found to impair memory in transgenic APP mice in the absence of plaques [804]. Furthermore, it has also been shown that these

transgenic mice have normal memory function only when behavioural testing co-insides with an episode of reduced Aβ oligomer formation [795]. The mechanism of the toxicity has been studied extensively from both the experimental and theoretical perspectives (see review, [65]), and include: the activation of inflammatory effects by interacting directly with the cell membrane [814]; induction of oxidative stress [815] through the formation of metal-Aβ complex [816, 817]; disruption of membrane receptors' function by intimate binding [818]; formation of membrane pore [819-822] and alteration of ionic homeostasis across the membrane [823, 824] and modification of the structure of deoxyribonucleic acid (DNA) by the process of attachment [825]. Aβ oligomers have been found to bind to synapses and exhibit significant toxicity *in vitro* and *in vivo* model systems (see reviews, [63-65]). More generalized neuronal impacts of Aβ oligomers include mitochondrial damage, proteasome inhibition, tau accumulation and hyperphosphorylation, impairment of fast axonal transport, endoplasmic reticulum stress, cell cycle re-entry and ultimately, cell death [65, 407]. In addition, the ability of synthetic Aβ oligomers to mimic the structure and activity of brain-derived species in many if not all of these components [58, 65, 185] validates their use for experimentation [60]. Some of these points are considered below in relation to impaired synaptic plasticity and synaptic loss.

Aβ oligomers and Impairments in functional synaptic plasticity

Once bound to synapses, Aβ oligomers have been found to instigate a variety of pathological processes, including by interfering with functional synaptic plasticity processes in the hippocampus [782]. At picomolar concentrations naturally secreted soluble Aβ oligomers can disrupt hippocampal LTP in acute brain slices and *in vivo* they can also impair the memory of a complex learned behaviour in rats [50]. Inhibition of LTP in acute hippocampal brain slices occurs within minutes of their application [62], a finding that has since been confirmed by other studies [826-828]. As a functional form of synaptic plasticity in the hippocampus, LTP is considered by many to underpin the process of memory consolidation [87]. Thus, the abolition of LTP has long been associated with memory impairment in various animal models [87, 829]. However, since astrocytes have been also been found to support hippocampal-dependent memory and LTP via interleukin-1 signalling [830], it is possible that reactive gliosis may also disrupt LTP. Disruption of functional plasticity by Aβ oligomers has been shown to include Long-term depression (LTD) of synapses, which

is promoted rather than inhibited [805, 831]. This is important because LTD is generally considered to reflect an activity-dependent reduction in the efficacy of neuronal synapses that can last hours or longer, following a long patterned stimulus. Thus, the overall impact of oligomers is depressed synaptic output.

At a morphological level, LTP is associated with dendritic spine growth, whereas LTD can induce the removal of postsynaptic 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptors and loss of spines (i.e. synapse pruning; see reviews, [832, 833]). Thus, under normal physiological conditions synaptic development, maintenance, and plasticity are frequently associated with changes in the morphology and number of dendritic spines (see reviews, [834, 835]). Indeed, dendritic spines are the major postsynaptic sites for excitatory synaptic transmission in the CNS, and spine re-modelling and the formation of new synapses are activity-dependent processes that provide a structural basis for memory formation [737, 836, 837]. Since a loss or alteration of these structures is well established in AD patients and forms a better indicator for cognitive impairment than Aβ-plaque burden [408, 744, 745], it was predicted that there should be a link between Aβ oligomers and synaptic loss in humans as well as in mouse models related to FAD kindreds [408, 831, 838]. This prediction has been confirmed [839], as have predictions that Aβ oligomers cause dysfunctional trafficking of ionotropic glutamate receptors and metabotropic glutamate receptors [839, 840]. The concentration of soluble Aβ oligomers has also been shown to predict synaptic change in AD [404], and oligomers accumulate with age in all the transgenic models examined so far, including mouse, rat, and *Caenorhabditis elegans* [841-844]. However, what promotes oligomer accumulation in SAD remains uncertain. In addition, since numerous transgenic mouse models overexpressing human APP FAD mutations (sometimes in combination with FAD-linked PSEN1 mutations), do not generally display NFT pathology or neuronal cell loss, the relevance of these *in vitro* experimental paradigms has been questioned [779]. Nevertheless, the Aβ oligomer hypothesis remains an attractive concept because it resolves the issues regarding the lack of correlation among deposited plaques, tau pathology and neuronal loss. However, many issues of which require clarification.

1.8.1.2 Aging, Senescence and Neuroinflammation

Aging is associated with a number of functional changes in the CNS, including "microglial priming", the process whereby microglial cells undergo a status change that

involves a dysregulated immune response to peripheral immune activation that results in an increased inflammatory profile of the CNS [845, 846]). In this context, microglial activation is often amplified and prolonged in the aged brain compared to adults, with the cause considered to reflect impairments in several key regulatory systems with age that make it more difficult to resolve microglial activation [846]. This impaired regulation and microglial hyper-activation following peripheral immune challenge has consequences, including exaggerated systemic neuroinflammation, sickness behaviour, depressive-like behaviour and cognitive deficits [846-849]. This probably explains why elderly patients exhibiting the signs of cognitive dysfunction are routinely screened for bladder infections, and it is also interesting that elderly patients suffering from pneumonia often present symptoms consistent with delirium. Work in rodents suggests that aged microglia are subject to replicative senescence (loss of mitotic ability after repeated rounds of replication), raising the possibility that old age, and perhaps other factors adversely affect viability and self-renewal capacity of microglia, resulting in the generation of senescent and/or dysfunctional cells (see review [850]. This may suggest that in some species at least, the brain's immune system may have a 'best before' date [850]. This priming may be particularly important in the context of Aβ accumulation in the brain since it can exacerbate the pathological mechanisms associated with AD (see reviews, [851, 852]. One possibility is that microglial senescence may drive an over-reaction to the presence of fibular Aβ in the cellular environment. Indeed, it may be the non-senescent microglia that exhibit the more neuroprotective side of these types of glial cell by releasing neurotropic factors and helping to metabolise Aβ and facilitate its removal from the brain [853, 854].

Aging is also associated with increased oxidative stress, something that can trigger a senescence program in astrocytes when faced with multiple types of stress. Astrocytic senescence is characterised in vivo by the expression of Cyclin-dependent kinase inhibitor 2A, (CDKN2A, p16INK4A), and matrix metalloproteinase-1 (MMP-1). In order to determine whether senescent astrocytes appear in vivo, a recent study by Bhat et al., used brain tissue from aged individuals, which was then examined for the presence of senescent astrocytes using INK4a and MMP-1 expression as markers [855]. Compared with foetal tissue samples, this study also reported a significant increase in p16(INK4a)-positive astrocytes in subjects aged between 35 to 90 years. Furthermore, the frontal cortex of AD patients harboured a significantly greater burden of p16(INK4a)-positive astrocytes compared with either non-AD adult control subjects of similar ages or foetal

controls, with the p16(INK4a)-positive astrocytes correlated with increased MMP-1 p16 [855]. However, perhaps the most exciting findings were that vitro Aβ42 triggered the senescence of astrocytes, driving the expression of p16(INK4a) and senescence-associated β-galactosidase [855]. Furthermore, that these senescent astrocytes produced a number of inflammatory cytokines, including interleukin-6 (IL-6), which seemed to be regulated by p38MAPK (a mitogen-activated protein kinase that is responsive to stress stimuli [855]. Although astrocytes have been previously shown to be key players in the inflammatory response in AD by releasing pro-inflammatory factors, Bhat et al., have been the first to link this to astrocyte senescence and have proposed that an accumulation of p16(INK4a)-positive senescent astrocytes may be the link between increased age and risk for sporadic AD [855]. However, for this to be more compelling it would have to be shown that astrocyte senescence *precedes* the accumulation of brain Aβ rather than just being a consequence of it. Furthermore, in aged persons who are cognitively normal but who have significant amyloid load in the brain it would be interesting to determine the degree of astrocytic senescence. Given that cellular senescence seems to be a more general facet of aging related to autonomous suppression of tumorigenesis as well as a means to mobilize the systemic and local tissue milieu for repair [856], it is also possible that the accumulation of Aβ in some elderly persons increases the chances of this becoming a runaway process that induces a neuroinflammatory cascade that ultimately results in neuronal cell death and dementia. Interestingly, although patients with comorbid cancer and dementia are more likely to die [857], limited research suggests that dementia is associated with a lower rate of cancer [858]. One could speculate that in AD this may be one sign of a possible senescence program in overdrive.

1.8.1.3 Oxidative Stress

Although reactive oxygen species (ROS) such as peroxides and free radicals are usually categorized as neurotoxic molecules, and typically exert their detrimental effects via the oxidation of essential macromolecules such as enzymes and cytoskeletal proteins, they are a natural by-product of cellular oxidative metabolism, and are generated in the mitochondria as molecules with unpaired electrons during the process of oxidative phosphorylation [772]. Since the brain is a metabolically active organ exhibiting high oxygen consumption, it has a robust production ROS such as superoxide (O_2^-) [859, 860]. Indeed, in normal physiological concentrations, ROS are known to be involved

in the functional changes necessary for synaptic plasticity and hence, for normal cognitive function [860]. Under normal physiological circumstances the amount of ROS produced are kept in check by an elaborate network of antioxidants [860]. However, these defensive mechanisms can fail, leading to neuronal oxidative stress. Thus, the detection of cellular damage to proteins, lipids, and DNA resulting from ROS is by definition, indicative of oxidative stress, with indirect evidence relating to the increased expression of (antioxidant) molecules involved in oxidant defence, including heme oxygenase, superoxide dismutases, glutathione transferases and catalase [859]. However, it is important to note that neurons displaying signs of oxidative stress may not necessarily be succumbing to oxidative stress, but rather, may be adapting by way of their oxidant defences [772]. Thus, in neurodegenerative disorders such as AD where oxidative stress is postulated to play a causal role, the associated mechanisms may be involved with maintaining a balance between oxidative stress and adaptation to this stress, ultimately reflecting the ability of living systems to dynamically regulate their defence mechanisms in response to oxidants [772].

Oxidative stress plays a major role in a number of pathological states, and has been clearly established as a contributor to disease and death in ischaemia-reperfusion injury, as well as several neurodegenerative diseases that include AD [859]. Since the aging process is also associated with an increase in the adventitious production of ROS, together with a concurrent decrease in the ability to defend against such ROS, it is not surprising that many studies have established oxidative stress and its associated cellular damage as significant contributors to neurohistologic lesions in AD, as well as mediators of early-neuronal vulnerabilities that place cells at risk of death [861]. However, in AD, there may be a number of contributory sources which further add to the background level of age-related ROS production and reduced defence. Most types of oxidative damage noted in AD (including glycation, protein oxidation, lipid peroxidation, and nucleic acid oxidation), result directly or indirectly from metal-catalysed hydroxyl radical formation. Therefore, it is not surprising that the loss of homeostasis of iron, copper and zinc in the brain, is accompanied by severe neurological consequences characterized with increased oxidative damage (see reviews, [862, 863]). Transition metals, such as iron and copper, can facilitate the generation of free radicals in vivo [864], as can manganese, zinc, and aluminium [865]. Indeed, the altered concentrations and distributions of these metals in the AD brain is likely suggestive of their importance in disease pathogenesis [866]. For example, Aβ

has an unusually high affinity for transition metal ions, which can accelerate Aβ40 and Aβ42 aggregation in vitro thereby contributing to their toxicity [867-869], although the oxidant effects of Aβ are now considered to be mediated via its interaction with redox-active metals such as iron and copper because chelation of Aβ significantly attenuates Aβ toxicity [870]. The relevance of this mechanism to disease pathogenesis is usually highlighted by the association of redox active metals with Aβ-plaques in AD, many of which contain relatively high amounts of Fe, Cu, and Zn [794, 863, 871], as well as iron-storage proteins such as ferritin [872]. In addition, soluble Aβ species have also been found to attach with specificity to particular synapses to cause oxidative stress, which occurs via a mechanism involving NMDAR activation and resultant calcium flux [873]. However, although Aβ may initiate oxidative stress at micromolar doses and reduce the antioxidant defence system, there is also evidence that oxidative stress increases the concentration of APP and thus, potentially the formation of Aβ peptides [874], although not all studies confirm this [875]. Indeed, it has been suggested that neurons respond to oxidative stress by increasing Aβ production [876], an increase that is associated with a consequent reduction in oxidative stress [877, 878]. In this context, Aβ has been shown to act as a potent antioxidant similar to superoxide dismutase [879, 880], effectively blunting oxidative stress in vivo [878, 881]. In addition, nanomolar concentrations of Aβ are known to block neuronal apoptosis following trophic factor withdrawal [75]. These and other studies (see review, [792]), have reported results consistent with the view that normal physiological concentrations of endogenous Aβ may have a neuroprotective role. Therefore, AD cases with APP mutations may lose effective antioxidant capacity due to mutation-driven protein dysfunction, while the Aβ deposits may reflect a host response to oxidative imbalance and an oxidative stress [772, 794, 882]. This is consistent with Aβ deposits beginning to appear around the age of 40 years in FAD [878].

Viewing endogenous Aβ as a protective response element provides a heuristically valid mechanism for why it is that the brains of elderly individuals when redox alterations are first manifest [878], can contain Aβ-deposits at loads comparable to most AD patients and yet remain cognitively intact [883]. Thus, while the production and deposition of Aβ may enable these individuals to starve off age-related redox imbalance, the presence of a profound and chronic redox imbalance in AD [859] may mean that even high levels Aβ are insufficient to negate the oxidative damage [793]. The cause of redox imbalance in AD remains unclear [862]. However, it is interesting

that studies of primary neuron cultures indicate a ferroxidase activity of APP, noting its up-regulation in response to increased iron stores [72]. Indeed, APP seems to facilitate the export of iron from cells likely in an attempt to minimize potential ROS generation by the presence of reactive iron [884]. APP has also been reported to prevent the release of iron (II) from heme further reducing the toxic accumulation of redox-active iron [885], and exogenous iron load reportedly promotes the α-secretase cleavage of APP, which generates a soluble, potentially neuroprotective Aβ peptide [862]. Thus, in addition to a less potent antioxidant effect of Aβ, it is possible that AD cases with APP mutations may also lose some functional ability with respect to the ability of APP to reduce the toxic accumulation of redox-active iron, leading to its accumulation in the brain and thus the generation of ROS. Also, it has not been determined whether in vivo Aβ species have differential effects on mitigating such oxidation (i.e. intracellular vs. extracellular), or if some FAD mutations may favour the production of species perhaps less adept at this antioxidant function.

Thus, from the studies summarised above, it is evident that whether or not Aβ levels become detrimental in the long-term, may well depend on multiple factors, including the production of ROS [853], and the degree of systemic neuroinflammation [851]. Indeed, glial cell senescence may be a key factor in this [850, 853, 855], as high concentrations of soluble Aβ have been shown to be sufficient to trigger a program of glial senescence in some individuals [855] — a process that is further associated with increased pro-inflammatory signalling [855], as well as further ROS production [886, 887]. Indeed, this could form a positive feedback loop as ROS are also known to trigger senescence in a wide range of cell types [888], including potentially astrocytes, as these cells are known to be highly sensitive to oxidative stress and develop senescence when faced with multiple types of stress [855]. However, it is also worth remembering that AD may develop even in those without significant Aβ deposition [705], suggesting the presence of additional Aβ–independent pathological roads to AD. Indeed, multiple "hits" from a variety of pathological mechanisms may be required before AD develops [889]. This also suggests that there may be more clinicopathological subtypes of AD that is currently recognised.

1.9 CONVERGENCE: AD AS A METABOLIC DISORDER

Accumulating evidence now suggests that may in many ways AD may represent a metabolic disorder, with pivotal roles for both brain insulin resistance and insulin deficiency, as mediators of cognitive impairment and neurodegeneration (see reviews, [890-893]). In this respect insulin resistance and insulin deficiency may both represent convergent age-related factors with other co-morbid health conditions such as metabolic syndrome and T2D, both of which are recognised as substantial risk factors of developing AD [890]. This final section briefly considers the roles of insulin resistance and insulin deficiency in the pathogenesis of AD.

1.9.1 Insulin Signalling in the Periphery and Brain

Insulin is nearly exclusively produced by the beta Islet cells (β-cells) of the pancreas, where it primarily acts as a metabolic regulatory hormone to stimulate the uptake of glucose by liver, muscle, and adipose tissue [242]. However, in order to achieve these effects, insulin and its trophic IGF, must firstly bind to their respective receptors, a process which results in phosphorylation and activation of intrinsic receptor tyrosine kinases, with the interactions between the phosphorylated receptors and insulin receptor substrate molecules then promoting the transmission of downstream signals which then affect physiological function[894]. Within most tissues glucose is stored as glycogen, although in adipocytes it is stored as triglycerides. The release of insulin from pancreatic β-cells occurs in response to peripheral blood glucose levels detected through the glucose transporter-2 (GLUT-2), although GLUT-2 is not itself stimulated or regulated by insulin [894]. From the close links that occur among insulin receptor sensitivity, pancreatic β-cell activity, blood glucose levels, and glucose use by insulin-sensitive tissues, under normal physiological conditions a classic negative feedback loop is formed [894]. In brief, insulin secreted by pancreatic β-cells induces a signalling cascade which results in the insulin-sensitive glucose transporter -4 (GLUT-4) being translocated from the intracellular vesicles inside muscle and fat cells, to the plasma membrane where they become available for absorbing glucose down its concentration gradient into muscle and fat cells fostering the lowering of blood glucose levels [895]. The subsequent detection of lower levels of glucose by the β-cells (via insulin-insensitive GLUT-2) then results in a decreased secretion of insulin [894]. The loss of pancreatic β-cells as in T1D or in animals treated with streptozotocin,

results in no or deficient insulin secretion and loss of blood glucose control (hypoinsulinemic, hyperglycemia) [894]. However, insulin levels are also indirectly affected when resistance to the normal physiological actions of insulin occurs as in metabolic syndrome, obesity and T2D [242]. In these scenarios, insulin is inefficient in activating its receptors controlling the uptake of glucose by muscle and other insulin sensitive tissues [894]. Although the molecular basis for this is still debated, research has implicated the down regulation of GLUT-4 in adipose tissue and skeletal tissue, a process known to cause impairments in glucose transport [894, 895]. Consequently therefore, blood glucose levels rise, causing the pancreatic β-cells to release more insulin until either glucose levels return to normal, or pancreatic insulin output reaches its maximum [894]. However, as mentioned earlier, if increased insulin secretion by the pancreas cannot overcome the insulin resistance, pre-diabetes or T2D usually ensues [242]. Furthermore, in chronic circumstances the near maximal output of insulin from pancreatic β-cells can lead to their loss, and thus, an absolute insulin deficiency that may require insulin replacement therapy [242].

Insulin and IGF enter the CNS by means of a saturable transport system at the BBB [896], where via the same phosphorylated-receptor cascades they subserve a variety of important functions, including feeding behaviour, inhibition of apoptosis, stimulation of neurogenesis and cell survival, metabolism, and regulation of the synaptic plasticity activities required for learning and memory (see reviews, [894, 897]). Insulin and IGF-1 receptors are widely expressed throughout the mammalian brain, and are expressed at particularly high levels in the hypothalamus, cerebellum, hippocampus, and cortex [898, 899], as well as in different cell types, including glial and neuronal cells [900]. Thus, signalling through insulin/IGF receptors is impaired, the structural and functional integrity of the CNS is compromised [894].

1.9.2 AD as a 'Type 3 Diabetes'

Insulin and IGF signalling pathways regulate glucose utilization, metabolism, and the adenosine triphosphate (ATP) synthesis needed for cellular homeostasis and dynamic modulation of a broad range of functions in the CNS [890]. Indeed, the first step to understanding AD as a metabolic disorder came with the realisation that the condition was associated with significant impairments in cerebral glucose hypometabolism [901-906], which worsens as the disorder progresses [907-910], and seem to represent a reliable marker of disease status [800, 903, 911-915]. These findings lend strong

credence to the view that impairments in insulin signalling likely have important roles in the pathogenesis of AD [916]. Indeed, a large body of evidence now indicates that insulin/IGF resistance [917-922], along with impaired brain energy utilization [923-926] are present in AD, and in particular AD brain tissue [916, 927, 928], and likely reflect fundamental components of AD since they can occur in early-stage AD patients even in the absence of T2D [897, 929]. Insulin and IGF polypeptides along with their associated receptor genes are all expressed in neurons [930-932], and glial cells [933-936], in brain regions considered to play critical roles in regulating and maintaining many aspects of cognitive function [894]. The highest levels of CNS expression of insulin and IGF polypeptides are found in the frontal cortex, hippocampus and hypothalamus —all structures known to be targeted by the pathological progression of AD [937, 938].

Insulin resistance and insulin deficiency in AD are also associated with significant disruption to signalling pathways regulating neuronal survival, energy production, and neuroplasticity [890], as well as abnormalities in the expression of genes and activation of kinases regulated by insulin IGF signalling [916, 924, 927, 939, 940]. For example, insulin and IGF regulated genes such as choline acetyltransferase, tau, and glyceraldehyde-3-phosphate dehydrogenase, which mediate cholinergic/cognitive, neuronal cytoskeletal, and metabolic functions, were all found to be suppressed in the AD brain in line with Braak Stage, whereas APP, GFAP, and microglial messenger ribonucleic acid (mRNA) transcripts all exhibited increasing expression with Braak Stage [940]. Insulin resistance mediated impairments in energy metabolism also lead to oxidative stress in AD, and subsequently the generation of ROS, DNA damage, and mitochondrial dysfunction —all of which drive pro-apoptosis, pro-inflammatory, and pro-APP and Aβ cascades [897]. The above effects are supported by research conducted with experimental animals in which brain insulin receptor expression and function were suppressed by intra-cerebroventricular injections of streptozotocin, leading to cognitive impairment and neurodegeneration with features that overlap with AD [941-947]. In essence, AD may be the manifestation of "Type 3 diabetes" [916, 940] with elements of insulin resistance and insulin deficiency [897]. In AD brains, deficits in insulin/IGF signalling are due to the combined effects of insulin/IGF resistance and deficiency [948]. The insulin/IGF resistance is manifested as by reduced levels of insulin/IGF receptor binding and decreased responsiveness to insulin/IGF stimulation, while the trophic factor deficiency is associated with reduced levels of

insulin polypeptide and gene expression in brain and cerebrospinal fluid [890, 916, 930, 939-941, 944]. This hypothesis is not covered in depth here as several critical reviews have recently been published on this topic and the reader is directed to these for detailed discussion [891, 948-950]. A few salient points are considered below.

1.9.3 Neurometabolic Coupling

Glucose metabolism fulfils important functions in adult brain related to neuroenergetics, neurotransmission, energy storage, biosynthesis and oxidative defence [894]. Indeed, compared to the rest of the body, the brain is the most energy demanding organ as it requires almost double the amount of energy in order to function correctly [894]. However, in contrast to the periphery, glucose transport into the CNS and the use of glucose by the majority of cells within the brain appears to be largely independent of insulin action in humans [894]. Insulin-insensitive GLUT-1 is the primary means by which glucose enters the brain across the BBB; it conveys about 50 times more glucose into the CNS than would otherwise enter [951]. However, glucose transport across the BBB is saturable but not active (i.e. is not energy dependant), rather, GLUT-1 is a facilitated diffusion system that bi-directionally transports glucose from the region of highest concentration to the region of lowest concentration [894]. Under normal physiological conditions glucose levels are roughly twice as high in blood as in brain interstitial and cerebrospinal fluids, thus driving glucose into the brain from the blood plasma [894]. In studies in which glucose levels are raised in the CNS (or isotopes of glucose are introduced into the CNS), the brain-to-blood efflux of glucose (which also occurs via GLUT1) can be demonstrated by the fact that any glucose not used by the CNS is returned to the blood [952]. For this reason glucose accumulation in the brain matches glucose use by the brain. Thus, the uptake, retention, and metabolism of glucose are nearly synonymous measures of CNS metabolic rate when glucose probes such as FDG-PET are taken up by the brain [894]. Glycogen synthase is the only enzyme able to synthesize glycogen (the main storage form of glucose in the body). Muscle glycogen synthase (MGS) is expressed in most tissues including brain tissue [953], and phosphorylation by several kinases, including glycogen synthase kinase 3 (GSK3) induces the inactivation of the enzyme [954]. Glucose 6-phosphatase (G6Pase) is an enzyme that hydrolyses glucose-6-phosphate resulting in the creation of a phosphate group and free glucose. In the periphery high levels of G6P allosterically activate MGS even when the enzyme is phosphorylated, this

being the primary mechanism by which insulin promotes glycogen accumulation in skeletal muscle [955]. However, due to the neuronal version of MGS being kept in an inactive state only astrocytes are able to store glucose in the form of glycogen in the healthy CNS [956]. This is important because only astrocytes can metabolise glycogen via aerobic glycolysis into lactate [956-958], the latter of which is believed to be the principal oxidative energy substrate used by cortical neurons to sustain their activity [959, 960], although this has been hotly debated [961, 962]. According to the Astrocyte-Neuron Lactate Shuttle hypothesis, glutamate overspill at tripartite synapses stimulates the process of aerobic glycolysis in astrocytes via the sodium-coupled reuptake of glutamate and ensuing activation of the Na+/K(+)-ATPase which triggers the uptake of glucose via GLUT1 [963], and the subsequent production and release of lactate (see reviews, [964, 965]). The main source of glucose for glycolysis is extracellular [959], although endogenous glycogen may serve as a transient source of glucose [965]. Lactate is exported to neurons where it is taken up by the proton-linked membrane carrier monocarboxylate transporter 4 (MCT4) [966], and subsequently used to generate the ATP necessary for synaptic activity [957, 967].

Measures of glucose levels in extracellular fluid in the rat hippocampus during memory tests reveal that glucose levels are dynamic, decreasing in response to memory tasks and loads; exogenous glucose blocks these decreases and enhances memory (as reviewed in [968]). Indeed, neural-glia metabolic coupling is known to undergo various plastic adaptations in parallel with the adaptive mechanisms that characterize synaptic plasticity. Spatial learning and working memory in rats increases the number of astrocytes [969, 970], and distinct sub-regions of the hippocampus are metabolically active at different time points during spatial learning tasks, suggesting that a type of metabolic plasticity (involving by definition, neuron-glia coupling), occurs during learning [971]. New learning initiates a cascade of events in the brain that can lead to short-term and long-term memories [972]. While short-term memories require post-translational modifications to existing proteins, the consolidation of long-term memories depends upon the activation of a gene cascade and downstream structural modifications in neurons that enable them to store the acquired information [973-975]. This includes the activation of cyclic adenosine monophosphate (cAMP) response element binding protein" (CREB) [972, 974, 976] as well as the translation at activated synapses of the immediate early gene activity-regulated cytoskeletal protein (ARC) [977-981]. ARC is believed to play a key role in actin cytoskeletal dynamics as well as

regulation of the membrane excitability via expression of AMPA receptors (see review, [982]). Furthermore, LTP and memory are also accompanied by synaptic structural changes associated with the phosphorylation of the p21-activated kinase-cofilin cascade involved in F-actin dynamics in dendritic spines [983-985], as well as the promotion of cytoskeleton assembly, and regulation of spine morphology [985-987]. Thus, LTM formation would appear to have a high metabolic demand within the underlying active neuronal network, much of which is critically dependent on the import of lactate into neurons [988]. Indeed, the organization of astrocytes in non-overlapping spatial domains indicates that they are uniquely positioned to shape the spatial distribution of the vascular responses that are evoked by neural activity (see review, [989]), and thus define the local availability of energy substrates by co-ordinating local blood flow with neuronal activity [990], a process that helps support neuronal functions [991]. However, this process also requires glucose and lactate to reach distal brain regions, a process accomplished via the astrocytic uptake of these energy substrates and their subsequent distribution and release through the astrocytic network via gap junctions (proteins CX30 and CX43) [992]. This process appears to be modulated by glutamatergic synaptic activity and AMPA receptors [992]. Indeed, the transfer of these energy substrates via this mechanism is able to sustain synaptic activity in the absence of extracellular glucose [992], suggesting an activity-dependent intracellular pathway for the delivery of energetic metabolites from blood vessels to distal neurons (see review, [968]). Cumulatively these data suggest that glial metabolic plasticity is likely concomitant with synaptic plasticity [964, 965], and that astrocytes play a central role in coupling synaptic activity to glucose utilization, a central physiological principle of brain function that provides a heuristically valid framework for experimental studies utilising FDG-PET [965, 993].

1.9.3.1 Metabolic Impairment in AD

AD is associated with cortical metabolic deficits and, in particular, significant impairments in cerebral glucose hypometabolism (as assessed via FDG-PET) [901-906]. Furthermore, the cortical hypometabolism becomes worse as the disorder progresses [907-910], and involves regions of the brain associated with the DMN, including the posterior cingulate, precuneus, parietal, temporal, and prefrontal brain regions [907, 994-999]. Regions comprising the hypothetical DMN are normally active during rest and tasks with low cognitive demand, but otherwise deactivated [1000]. Functional

changes in the DMN correlate with measures of cognitive performance [1001], and in AD, glucose hypometabolism in these regions represents a reliable marker of disease status [800, 903, 911-915]. Although one explanation for FDG hypometabolic patterns in AD patients reflects neuronal cell loss, the detection of these hypometabolic patterns in at-risk persons decades before clinical symptoms of dementia are evident [915, 1002, 1003] means that cell loss per se cannot be the root cause [1004]. Furthermore, in a MRI and FDG-PET study of asymptomatic individuals carrying mutations in the PSN1 gene for EOFAD, and normal matched controls, Mosconi et al [1005] reported extensive reductions in cortical glucose metabolism in the pre-symptomatic individuals in the absence of structural brain atrophy, again suggesting that cell loss is not a major contributor to low FDG-PET signals. Indeed, many studies have found that cortical glucose hypometabolism accurately predicts subsequent decline in MCI, and conversion to AD [915, 1006-1008]. For example, one recent study by Toussaint et al [1008], using a large cohort of normal elderly controls, aMCI patients, and patients with AD, reported that FDG-PET hypometabolic patterns in DMN regions strongly influenced the specificity of the discrimination between different stages of AD, progression to MCI, and conversion to AD. These findings are consistent with research showing that regional brain hypometabolism is likely a consequence of cortical disconnection, the early stages of which can occur in at-risk individual's decades before clinical symptoms of dementia are evident [681, 682, 1009]. In addition, although most studies have concluded that APOEε4 is not a major factor in the magnitude of cortical hypometabolism [1010-1012], a recent study has suggested that the E4 allele may be associated with a more global impairment glucose metabolism [1013].

1.9.3.2 Amyloid Deposition and Cortical Glucose Metabolism

The spatial relationship between amyloid deposition and impairments in cortical glucose metabolism are not completely understood. Several studies have shown an anatomical overlap between amyloid deposition and glucose metabolism in parieto-temporal, posterior cingulate and precuneus regions in AD, indicating and early metabolic vulnerability in amyloid plaque enriched brain regions [1014-1016]. By contrast, amyloid deposition in the frontal cortex, striatum and the thalamus has been reported to coincide with relatively spared metabolism [1015]. This suggests that metabolic dysfunction may not be driven by amyloid deposition in all brain regions

implicated in AD. Studies have also reported divergent findings with respect to associations between amyloid deposition and cortical metabolic dysfunction across clinical subtypes. For example, it has previously been shown that patients with EOAD show differently distributed glucose hypometabolism in the absence of a regionally specific pattern of amyloid deposition [1017]. Furthermore, a recent study by Lehmann et al [1018], of EOAD participants and those with other forms of atypical EOAD, has reported that hypometabolism patterns differed across AD variants (reflecting involvement of specific functional networks), whereas the amyloid patterns were generally diffuse and similar across variants. Furthermore, when compared with patients with typical AD, one study found that despite similar distributions of PiB retention in posterior cortical atrophy, these patients showed reduced FDG uptake in occipitotemporal regions suggesting that metabolic impairment rather than Aβ-plaque deposition drove the clinical presentation in these patients [1019]. However, a recent study by Ossenkoppele et al [1020], of 100 patients with AD and 20 healthy controls, reported an increase in amyloid burden via PET-PiB and modest metabolic dysfunction in the parietal cortex (FDG-PET) of younger patients with AD. Parietal amyloid load in this study was also found to be related to visuo-spatial functioning in younger patients, whilst metabolic impairment in the parietal cortex of patients was related not only to visuo-spatial skills, executive function, and attention [1020]. By contrast, in older patients, memory performance was associated with metabolic activity in the posterior cingulate, suggesting that clinical differences between younger and older patients with AD are related not only to topographical differentiation in downstream processes (i.e. metabolic dysfunction), but may also originate from distinctive distributions of Aβ accumulation [1020]. These findings are consistent with a previous study in non-demented elderly human subjects which also concluded that traceable Aβ deposition in the brain plays an important role in occurrence of neuronal dis-coordination in DMN and poor WM [1021]. However, this study found no correlation between DMN dis-coordination and glucose hypometabolism [1021].

Discrepancies between the above studies may reflect differences in cohort characterization, sample sizes, or the use of different methods for analysing PiB-PET data. However, the lack of correlation between amyloid deposition and cortical glucose metabolism in many studies suggests that fibrillar Aβ deposition may only account for a fraction of the clinico-anatomic heterogeneity in AD [1018]. This further suggests that processes other than fibrillar Aβ account for the region-specific

metabolic impairment in AD [1017]. Among the prime candidates in this regard are soluble Aβ oligomers, as cell culture studies have shown Aβ oligomers to impair energy metabolism in the brain by decreasing neuronal ATP levels and triggering hexokinase (HKI) detachment from mitochondria, thereby decreasing HKI activity in cortical neurons [1022]. Indeed, since HKI is a glycolytic enzyme that plays an important role in reducing mitochondrial ROS generation and apoptosis in neurons (and other cell types) [1023-1025]. Thus, Aβ could induce cortical metabolic function and cell death via increased oxidative stress [1022]. This could induce glial senescence, and more speculatively, perturb the normal function of astrocytes in meeting the energetic demands of neurons. Indeed, recent research suggests that Aβ does cause alterations of astrocytic metabolic phenotype, where they have been shown to impact on neuronal viability [1026]. However, observations from other neuroimaging studies show conversion to AD in individuals who do not have significant Aβ deposition, but who do have cortical glucose hypometabolism [705], suggesting other Aβ-independent mechanisms may be responsible.

1.9.3.3 Insulin Resistance and Metabolic Dysfunction in AD

CNS insulin receptors diverge from their peripheral counterparts both in structure and function [1027, 1028]. For example, neuronal insulin receptors are not considered to be involved with glucose metabolism [1029], although some research suggests that insulin stimulation may lead to a 15% increase in the uptake brain glucose in humans probably via an astrocyte-dependent mechanism [1030]. Indeed, using cell cultures containing Normal Human Astrocytes (basically human astrocytes which do not derive form pathological tissue), Heni et al., have recently shown that human astrocytes are insulin-responsive at the molecular level, expressing key proteins for insulin signalling, including the insulin receptor β-subunit, insulin receptor substrat-1, protein kinase B, and GSK- 3 [1031]. However, whilst Heni et al., reported gliogenesis and cell growth to be dose-dependent insulin phenomena in human astrocytes [1031] (a finding consistent with a previous study using cell cultures of rodent astrocytes [1032]), neither increased glucose uptake, nor lactate secretion appeared to depend on insulin stimulation [1031]. However, findings from rodent astrocyte-enriched cultures [1033, 1034], and some human clinical studies [1030] have produces inconsistent results with respect to the role of insulin stimulation in mediating glucose uptake and lactate secretion. Nevertheless, in line with previous findings [1034, 1035], Heni et al., did report that the

process of glycogenesis was insulin-dependent in human astrocytes in a manner similar to other glycogen-storing cell types [1031]. In aggregate, these results are consistent with the view that gliogenesis and glycogenesis are insulin dependent phenomena in human and rodent brain tissue, although there may be species differences with respect to the process of brain glucose uptake and lactate secretion. Together with the fact that fundamental differences between rodent and human astrocytes have been reported [1036], it is possible that these differences may explain some of the general variation between mice and men [1036]. Nevertheless, although speculative, insulin resistance or insulin deficiency may still impact upon the process of neuro-metabolic coupling in humans if astrocytic glycogenesis is impaired. Furthermore, the increased proliferation of astrocytes seen with higher insulin levels may suggest that those with hyperinsulinaemia could have greater numbers of astrocytes [1031], although this has not currently been verified. However, given the drop in responsiveness to normal physiological levels of insulin in other bodily tissues, it is perhaps more likely that insulin deficiency or insulin resistance results in fewer astrocytes (or greater numbers of senescent astrocytes). Although not directly relevant to this thesis, astrocyte senescence would be worth further investigation as differences in cerebral functions between lean and obese persons has been reported in the literature [1037], and could represent an additional cause of cortical hypometabolism in at-risk and early-stage AD patients.

1.9.4 Anti-apoptotic Mechanisms and Neurogenesis

Apoptotic mechanisms inhibited by insulin/IGF stimulation include the 'inhibitor of Bcl-2' (BAD), Forkhead Box O (FoxO), GSK-3β, and nuclear factor kappa B (NF-κB) [894]. Wnt proteins form a family of highly conserved secreted glycoproteins that act as regulatory signalling molecules for cell-to-cell interactions during developmental embryogenesis, as well as in the adult brain, experience-dependent synaptic plasticity [1038, 1039] via the regulation of acetylcholine receptor translocation to synapses [1039]. Wnt proteins bind to receptors of the 'Frizzled' and 'lipoprotein receptor-related protein' (LRP) families on the cell surface; via several cytoplasmic relay components these proteins activate beta-catenin (β-catenin), which then enters the cell nucleus and forms a complex with T-cell factor 1 to activate transcription of Wnt target genes [1040]. In the absence of Wnt proteins these target genes remain silent [1040]. GSK-3β is known to regulate Wnt signalling by phosphorylating β-catenin

thereby targeting it for ubiquitin and/or proteosome-mediated degradation [1040]. Both insulin and IGF-1 have equal potential to inhibit apoptosis in cells that are normally responsive to either IGF-I or insulin [1041]. Insulin/IGF resistance and/or deficiency of these, has been hypothesised to impact these functions in AD [897].

1.9.4.1 IGF-1 resistance and Neurogenesis

IGF-1 signalling is crucial for normal brain development, as well as the proliferation, survival, and differentiation of each of the major neural lineages, neurons, oligodendrocytes, and astrocytes, as well as possibly neural stem cells [1042]. Moreover, IGF promotes the function of fibroblast growth factor 2 (FGF2), which has an important role in maintaining neural stem cells in the undifferentiated state; it also plays a major role in activating the stem proliferation [1043-1047]. The sub-granular zone (SGZ) of the hippocampal DG, and sub-ventricular zone (SVZ) of the lateral ventricles, are two of the few brain regions that display regular neurogenesis throughout life [1048]. Whereas in the SGZ, stem cells differentiate into hippocampal DG granule cells, in the SVZ of the lateral ventricles the newly produced neurons migrate through the rostral migratory stream to the olfactory bulb where they become interneurons (see reviews, [1049, 1050]). Neuronal differentiation is initiated when the NeuroD1 gene is activated by paracrine Wnt3 produced by astrocytes [1051-1053]. NeuroD1 is a transcriptional factor that directly activates the insulin gene [1054-1056], and neuroD1-gene-deficient mice are known to lack the hippocampal DG region — a phenotype which causes a fatal functional impairment of the nervous system [1056, 1057]. Neonatal DT1 has been linked to homozygous mutations in the NeuroD1 gene as well as neurological abnormalities that included cerebellar hypoplasia, learning difficulties, sensory-neural deafness, and visual impairment [1058].

In aggregate, the above findings indicate a critical role of NeuroD1 in both the endocrine pancreas, and the CNS [894]. Although the initial claim in the 1960s that new neurons are critical for learning and memory [1059] is still controversial, subsequent analyses at the cellular, circuitry, system and behavioural levels over recent years has generated mounting evidence in favour of adult-born neurons providing a critical role in hippocampal and olfactory bulb functions (see reviews, [1060, 1061]). Indeed, from a functional point of view, hippocampal neurogenesis is considered to play an important role in structural plasticity and network maintenance with the

adult-born neurons likely delivering a unique processing capacity to pre-existing circuitry as opposed to merely replacing dying granule cells [1050].

1.9.4.2 Negative regulators of neurogenesis

As a biological process, neurogenesis is dynamically modulated by various physiological, pathological and pharmacological stimuli [1049, 1050]. Neurogenesis in the brain decreases with age (see review, [1062]), an effect that likely reflects an age-related decrease in Wnt3 protein and the number of Wnt3-secreting astrocytes [1053]. This suggests that the regulation of paracrine factors (or growth and differentiation factors), play a critical role in aging and neurogenesis [1053, 1062]. However, research in animals suggests that exercise may rescue impaired neurogenesis in the hippocampus of aged mice by increasing the de novo expression of Wnt3 [1053]. Other factors which serve as negative regulators of adult neurogenesis in the brain, and hippocampus in particular, include inflammation induced by injuries, neurodegenerative diseases and irradiation [1063], and insulin resistance [894]. Indeed, the hippocampus contains substantial amounts of immunoreactive insulin and insulin receptors [1064], while hippocampal neurons have been shown to release insulin under depolarizing conditions which then participates in memory processing through activation through its tyrosine kinase activity receptor (Tyr) kinase activity [899]. However, whilst insulin receptor-defective mice have been reported to develop cognitive impairments [1065], brain/neuron-specific insulin receptor knockout (NIRKO) mice have been reported to exhibit intact learning and memory despite a complete loss of insulin-mediated activation of phosphatidylinositol 3-kinase (PI3K), a loss of inhibition of neuronal apoptosis [1066], markedly severe neuronal insulin resistance, and reduced phosphorylation of Akt and GSK3β that leads to substantially increased phosphorylation of protein Tau [1066]. Nevertheless, whilst NIRKO mice develop an impaired sympathoadrenal response to hypoglycaemia [1067], these mice retain roughly 5% of their brain insulin receptor protein, with IGF-1 receptors unaffected [1066]. Furthermore, the insulin receptor and insulin-like IGF-1 receptor are structurally similar, with both found at the cell surface as heterotetramers that once activated, can phosphorylate and/or interact with the same intracellular protein substrates (including members of the insulin receptor substrate family) to activate many of the same downstream signalling molecules (including phosphatidylinositol 3-kinase PI3K) [1068, 1069]. Thus, it is possible that NIRKO mice are not IGF-1 resistant,

and that intact signalling through the IGF-1 receptor is able to functionally compensate for the loss of neuronal insulin receptors in this model.

Research in insulin-deficient rodents with streptozotocin (STZ) induced diabetes typically display learning and memory deficits [1070-1072], as well as impaired cell proliferation in the adult hippocampal DG [1073] due to a glucocorticoid-mediated mechanism [1074]. Indeed, glucocorticoids such as cortisol are increasingly recognized as having an important role in the pathophysiology of insulin resistance [1075-1079], and are associated with memory impairment in many AD patients [1080], as well as being predictive of hippocampal atrophy and memory deficits in AD patients [1081], and aged persons [1082]. Glucose tolerance also progressively declines with age in humans and is often accompanied by insulin resistance and a high prevalence of T2D [1083]. Indeed, glucose intolerance correlates with learning and memory deficits as well reduced vigilance and attention [1084], and numerous longitudinal studies have confirmed an association between T2D and cognitive impairment [1071, 1085], particularly in old age [1086, 1087] where it serves as a risk factor for AD [1088]. However, whilst T1D is also associated with cognitive impairments in humans, at least one longitudinal study of patients with T1D has failed to find substantial evidence of long-term declines in cognitive function despite relatively high rates of recurrent severe hypoglycaemia [1089]. However, given the fact that AD and T2D are also associated with insulin/IGF resistance [897], raised cortisol levels [897, 1090], and aberrant inflammation [752, 1091], it is perhaps not surprising that altered hippocampal DG neurogenesis has been reported in both conditions [897, 1092]). Indeed, dysfunctional neurogenesis resulting from subtle disease manifestations likely exacerbates early neuronal vulnerability to pathological processes common to AD [1092]. On the other hand, enhanced neurogenesis has been linked to environmental stimuli, physical activity, trophic factors and some cytokines — all of which may represent a compensatory response and thus, an endogenous brain repair mechanism [1092]. Whilst these factors can potentially enhance cell proliferation in the early stages of neurodegeneration, research conducted in animals has suggested that survival of newly generated neurons is progressively impaired in AD as pathological processes continue [1093].

1.9.5 Role of Insulin in Plasticity Processes

Insulin acts as a neuropeptide in the brain to regulate food intake, body weight, mood, cognitive function, memory, neuronal survival and synaptic plasticity [1094, 1095]. Indeed, the intranasal insulin administration has been associated with a number of positive effects on the brain, including improving learning and memory (particularly verbal memory), mood stabilizing effects in patients with AD and healthy volunteers [1096-1099]. The effect on memory in human AD patients [1100], and healthy males [1101] is also independent of changes in peripheral glucose levels. The positive effect of intranasal insulin administration on learning and memory has also been observed in diabetic and non-diabetic mice [1102, 1103]. Indeed, insulin signalling in the limbic system and hypothalamus has also been shown to be particularly important for spatial memory in rats [899, 1104, 1105].

The effects of insulin on cognition (particularly learning and memory) has also been supported by animal studies which show that insulin modulates the concentrations of neurotransmitters crucial for synaptic plasticity in the brain, promoting both excitatory glutamatergic and inhibitory transmissions mediated by gamma-aminobutyric acid (GABA)' [1106, 1107], as well as acetylcholine [1108]. In addition, cell culture research conducted in neurons and Xenopus oocytes expressing recombinant NMDARs, suggests that insulin potentiates NMDARs via delivery of new channels to the plasma membrane [1109]. Because insulin and insulin receptors are localized to glutamatergic synapses in the hippocampus, this suggests that insulin-regulated trafficking of NMDARs may play a role in synaptic transmission and plasticity, including LTP [1109]. Indeed, this is consistent with research showing that insulin enhances NMDAR-mediated synaptic transmission at hippocampal CA1 synapses [1110], as well as the regulation of AMPA receptor endocytosis to cause LTD of excitatory synaptic transmission in the hippocampus and cerebellum [1111-1113]. Recent research has also shown that neurons and synaptic plasticity in the hippocampus of mice lacking the insulin receptor substrate protein 2 (IRS-2) exhibit significant deficits in NMDA receptor-dependent synaptic plasticity [1114], and cumulatively these results suggest viable mechanisms for the underling memory and learning deficits associated with impairments in neuronal insulin signalling. Other research indicates that insulin may also play a regulatory role in the synthesis of the norepinephrine transporter, thereby modulating activity in CNS noradrenergic pathways [1115]. Clearly, in addition

to Aβ levels, insulin resistance and insulin deficiency have the potential to contribute to the further disrupt plasticity processes in the AD brain [891, 897].

1.9.6 Tau and Aβ Pathology

Tau is inappropriately hyperphosphorylated in AD by several proline-directed kinases, (including GSK-3β) [88], as well as increased activation of cyclin-dependent kinase 5 [1116] and c-Abl kinases [1117], leading to its misfolding and self-aggregation into the paired helical filaments found in NFTs, dystrophic neurites, and neuropil threads [1118]. Furthermore, besides fibrillar tau, pre-fibrillar tau (tau oligomers) can also aggregate [720], where in a similar manner to Aβ oligomers they contribute to neurodegeneration by causing synaptic and loss and neuronal death [719]. Combined with a dysfunctional ubiquitin-proteasome system [1119], the inflammation-driven process of ubiquitination of hyper-phosphorylated tau [1120] further causes its fibrillar accumulation, oxidative stress, and ROS generation —all of which promote neuronal apoptosis, mitochondrial dysfunction, and necrosis in AD [1121]. Since tau gene expression and phosphorylation are normally regulated by insulin and IGF stimulation in the brain [1122, 1123], impaired tau gene expression due to reduced insulin and IGF signalling [1124] and insulin deficiency (i.e. effective trophic factor withdrawal), has a number of pathological consequences in AD that include a failure to generate sufficient quantities of normal soluble tau protein (i.e. it accumulates as hyper-phosphorylated insoluble fibular tau instead), and attendant exacerbation of cytoskeletal collapse, neurite retraction, and synaptic disconnection [897].

Insulin resistance mediated impairments in energy metabolism have been linked to oxidative stress in AD and subsequently, the generation of ROS, DNA damage, and mitochondrial dysfunction, since all of these drive pro-apoptosis, pro-inflammatory, and pro-APP and Aβ cascades [897]. The concept that brain insulin resistance (with attendant oxidative stress and neuro-inflammation), promotes Aβ accumulation and toxicity is supported by experimental data [897]. For example, it is known that insulin stimulation normally accelerates the trafficking of Aβ from its point of origin at the trans-Golgi network to the plasma membrane, where insulin then stimulates its extracellular secretion [1125] and degradation by its IDE [1126, 1127]. Central insulin resistance/deficiency could in principle collapse this insulin-dependent means of degrading monomeric Aβ, allowing it to accumulate in the brain and oligomerise [1128]. Indeed, along with insulin/IGF and their concordant receptors [897], neuronal

IDE expression in post-mortem tissue obtained from AD are found to be reduced in the hippocampus [116, 117], and animal research conducted in transgenic mouse models containing APP mutations associated with FAD, has indicated reduced expression of IDE in the hippocampus due to raised glucocorticoid levels, and associated increase in Aβ levels [1129]. This is further supported by additional animal research which has also shown that IDE responds to Aβ accumulation in the brain [113], and mice with a homozygous deletion of IDE have elevated endogenous brain Aβ [114, 115]. However, whilst in the cortex IDE protein levels normally diminish with of age, paradoxically cortical IDE expression in AD brain tissue is found to be elevated [118], although longitudinal studies have not been conducted in this respect. These differential results for IDE in AD remain unexplained. However, whilst it is evident that insulin-independent factors are involved in the expression of IDE in the cortex, IDE expression itself may not be optimal in its capacity to degrade extracellular oligomeric Aβ [109], particularly if the oligomers form via intracellular pathways [64, 66]. More speculatively, it may also be the case that the increased IDE expression in the AD brain is a reflection of something akin to "IDE-resistance", where like T2D, higher IDE levels reflect a physiological rebalancing mechanism to bring escalating levels of Aβ back within an acceptable range. Indeed, rising Aβ levels may themselves reflect a similar phenomenon in some PiB+ persons, where the "Aβ-resistance" may reflect gene polymorphisms that result in reduced antioxidant capacity of the peptide in the face of early redox imbalance and oxidative stress. Furthermore, in some individuals who perhaps exhibit early glial senescence, the raised Aβ levels may be sufficient to cause central insulin resistance and thus the development of AD [897] (see next section). Conversely, it may also be the case that some PiB+ persons who do not develop AD are just simply 'resistant' to the detrimental effects of chronically high concentrations of Aβ [1130]. Again, although not directly relevant to the experiments detailed in chapters 3 and 4 of this thesis, it would be interesting to see if glial senescence is one of the factors which differ between PiB+ and PiB- phenotypes.

1.9.7 Causes of insulin Resistance/Deficiency in AD

Although the cause of cerebral insulin/IGF resistance in AD remains unclear, they likely reflect various mechanisms, none of which are likely to be mutually exclusive. Here I will mention two of the main factors which are considered to have a role in the induction of insulin resistance in AD, T2D and soluble Aβ oligomers.

Peripheral insulin/IGF resistance are defining features of T2D [242, 1131, 1132], as well as many cases of AD [890]. However, although these two diseases share some clinical and biochemical features suggestive common pathogenic mechanisms [1133, 1134], the correlation between T2D and AD [246, 1135] has yielded paradoxical results as not all studies have confirmed the association (e.g. [249]). Whilst this may possibly reflect co-morbid factors such as cerebrovascular complications and APOE genotypes [1136, 1137], findings in human post-mortem studies have also reported no significant increase in AD diagnosis among diabetics [1138], nor any significant differences in the densities of Aβ-plaques and NFT pathology in subjects with T2D compared with normal aged controls although peripheral insulin resistance is more common in AD than with normal aging [1139]. Thus, although diabetes is a risk factor for AD and other dementia T2D, these results indicate that in isolation T2D is unlikely to cause AD [897]. However, experimental mouse and rat models of chronic high fat diet feeding and diet induced obesity with associated T2D, do exhibit deficits in spatial learning and memory [1140, 1141]. However, whilst experimental obesity with T2D also causes mild brain atrophy with brain insulin resistance, neuro-inflammation, oxidative stress, and deficits in cholinergic function [1142, 1143], an important qualifier about these studies is that the associated brain abnormalities were typically modest in severity, and devoid of NFTs [897]. Therefore, observations both in humans and experimental models suggest that while obesity or T2D can be associated with cognitive impairment, mild brain atrophy, and a number of AD-type biochemical and molecular abnormalities in brain, (including insulin resistance and oxidative stress), they support the contention that in isolation they do not cause significant AD pathology [897]. Indeed, research conducted in transgenic mouse models of APP mice suggests that diet-induced insulin resistance likely promotes and aggravates a range of pre-existing pathologies linked to AD, including amyloidosis and neuroinflammation [1144-1148]. One mechanism by which brain insulin resistance, oxidative stress, neuro-inflammation, and cell death can be generated in AD is via disruption of the liver-brain axis, which in T2D can involve the transfer of toxic lipids (including ceramides) across the BBB [890, 948].

1.9.7.1 Aβ oligomers as a Cause of Insulin Resistance

Current evidence also suggests a role for the abnormal interplay between soluble Aβ oligomers and insulin signalling in the brain. Soluble Aβ oligomers inhibit insulin signalling [1149], and aside from their effect on plasticity and deterioration of synapse

function, composition, and structure leading to their loss [65]. A recent study by Zhao et al [1150] using mature cultures of hippocampal neurons has also reported that extracellular Aβ oligomers bound specifically to dendrites, leading to neuronal oxidative stress and a rapid but substantial loss of neuronal surface insulin receptors. Although the impact on IGF-1 receptors was not assessed in this study, removal of dendritic insulin receptors was associated with increased receptor immunoreactivity in the cell body, indicating the ectopic redistribution of receptors and subsequently a muted neuronal response to insulin (as measured by evoked insulin receptor tyrosine autophosphorylation) [1150].

A subsequent study by De Felice et al., [1151] using mature cultures of hippocampal neurons, has reported that soluble Aβ oligomers bind to synaptic sites on the dendrites of neurons, causing oxidative stress, and the removal of insulin receptors. Furthermore, that the down regulation of plasma membrane insulin receptors via a mechanism sensitive to calcium calmodulin-dependent kinase II (CaMKII) and casein kinase II (CK2) inhibition, occurred prior to the Aβ-mediated loss of dendritic spines [1151]. However, most importantly this study also showed that insulin prevented these effects; moreover, that rosiglitazone (an insulin-sensitizing drug used to ameliorate insulin resistance in T2D patients), enhanced the protective effects of insulin by preventing the loss of insulin receptors from dendritic plasma membranes, and thus the loss of synapses [1151]. Although the molecular identity of the synaptic binding sites for Aβ oligomers remain to be determined, they did not bind directly to insulin receptors in this study, nor did they compete with insulin for binding to insulin receptors [891, 1151]. Moreover, since insulin failed to block the binding of Aβ oligomers to dendrites in the presence of the insulin receptor tyrosine kinase inhibitor AG1024, it indicates an insulin signalling–dependent mechanism [1151]. Indeed, aside from its possible impact on causing the internalisation of insulin receptors (which within normal physiological limits may well be a natural regulatory mechanism), Aβ oligomers may also modulate other membrane proteins, including NMDAR by reducing their cell surface density [1152, 1153], and thus by implication their participation in nominal neuronal communication [891]. Therefore, it is plausible that Aβ oligomers may interact with specific (but so far undetermined) membrane-bound protein complexes that contain these receptors, which are then internalized via an unknown mechanism [891]. At the very least, the neuroprotective effect of rosiglitazone in the De Felice et al. study indicates that it requires specific membrane

protein components as well as insulin receptor-mediated intracellular signalling events [1151]. Recent research using cultured primary cortical rat neurons also suggests that rosiglitazone increases dendritic spine density and rescues spine loss caused by APOEε4 in primary cortical neurons [1154]. Although the above findings were reported after the experimental work for this thesis was conducted, they do support the rational for the use of insulin sensitizers in ameliorating components of AD pathology in preclinical mouse studies. This will be discussed further in section 1.9.8.

Two major conclusions can be drawn from the above studies. First, that the early neuronal damage in AD associated with Aβ oligomers may in part be triggered by the Aβ-related internalization of insulin receptors and a concomitant decrease in insulin signalling leading to insulin deficiency [891]. Second, that the effects of stimulating insulin signalling in neuronal cell cultures, supports the wider rational for a potential neuroprotective strategy involving insulin sensitizers an a therapy in AD (see next section). However, intracellular Aβ oligomers can also compromise insulin/IGF signalling in the brain by interfering with the association between phosphoinositide-dependent kinase 1 (PDPK1) and RAC-alpha serine/threonine-protein kinase (AKT1) to preclude AKT1 activation (key components of the insulin signalling cascade) [1155] (see review in, [891]). Indeed, Aβ peptides are generated intracellularly [1156], but can also be taken up into cells from an extracellular source [1157], and it is not clear which source of Aβ or Aβ-oligomers plays the pivotal role in mediating detrimental effects on neurones and insulin signalling, although it is possible that both scenarios are compatible with biological reality. For example, both targets (one being an extracellular Aβ-binding site on neuronal dendrites, the other an intracellular interaction between PDK and AKT), could be relevant to AD and Aβ, since Aβ oligomers could act via both pathways to impair insulin signalling [891]. Thus, it is uncertain which of these pathways is targeted by the therapeutic impact of improving insulin signalling. However, the fact that insulin treatment or activation of its downstream signalling pathway reduces intracellular Aβ production and increases its extracellular secretion by increasing competition between insulin and Aβ for IDE [1126], suggests that intracellular Aβ is more likely to be the key target of the insulin-mediated protective effect [891]. This is further supported by recent animal data showing that targeting intracellular Aβ by a specific γ-secretase inhibitor (or by immunotherapy) at an early stage of the disease can prevent synaptic dysfunction in

mouse models of AD [1158]. However, γ-secretase inhibitors and immunotherapy have failed to deliver quantifiable cognitive benefits clinically [412, 1159].

1.9.8 PPAR-γ Insulin Sensitizers: A Treatment for AD?

The findings discussed in this section provide a mechanistic explanation for why concentrations of insulin well as their receptor mediated signalling, are all markedly reduced in the AD brain [891, 897]. However, they also show that the vulnerability to Aβ-oligomers can be reduced or possibly even mitigated, by the use of intranasal delivery of insulin [894, 948, 1095-1097], as well as treatment with insulin sensitising compounds such as rosiglitazone. These are considered to mediate their beneficial effects in the periphery [1160] and CNS [1161], via the activation of the peroxisome proliferator-activated receptor-gamma (PPAR-γ).

1.9.8.1 PPAR-γ Receptors and Thiazoladinediones

PPARs represent a steroid hormone super family of ligand-inducible transcription factors that are expressed in fat cells, cells of the liver, muscle, heart, and inner wall (endothelium) and smooth muscle of blood vessels [1162]. As such, PPARs act as lipid sensors, enhancing insulin sensitivity by modulating whole body metabolism in response to dietary lipid intake, as well as the stimulation of mitochondrial function, and reduction of inflammatory responses [1163-1169]. There are three classes of PPARs, PPAR-α, PPAR-δ/β, and PPAR-γ, all of which are expressed in the adult brain, although PPARδ is most abundant, followed by PPAR-γ (see review, [1161]). PPAR-α is expressed in the hippocampal CA1 and dentate gyrus regions. The δ/β isoform is highly expressed in the dentate gyrus and CA1-3 regions of the hippocampus, and the γ isoform is expressed weakly in the brain but is present in the hippocampal dentate gyrus [1170].

In the periphery PPAR-γ is mainly expressed in adipocytes, where they regulate genes involved in adipocyte differentiation, fatty acid uptake and storage, and glucose uptake [1162]. PPAR-γ is bound and activated by several naturally occurring compounds, such as the eicosanoids 9- and 13-hydroxyoctadecadienoic acids [1171], and more recently nitroalkenes (i.e. nitrated lipids) [1172]. In addition, several high-affinity synthetic PPAR-γ agonists have been synthesized, including the thiazolidinedione (TZD) class of compounds [1173], or which Rosiglitazone and pioglitazone are the most popular in research settings, and have been developed for commercially (pioglitazone,

Actos; rosiglitazone, Avandia) for clinical use as insulin sensitizers in patients with T2D [1160]. However, TZDs have been shown to improve insulin sensitivity and glycemic control in diabetic individuals [1160], the mechanism of action is uncertain. Two popular mechanisms have been elucidated and have experimental support. Firstly, research has shown that at the insulin receptor level, rosiglitazone increases insulin sensitivity as the result of two convergent mechanisms: increased insulin receptor expression and insulin receptor activation [1174]. These effects were associated with a 40% increase in insulin-stimulated glucose uptake as a result of increased GLUT4 translocation to the plasma membrane in adipocytes, which happened without changes in the expression of GLUT4 at the mRNA or protein level [1174]. Additional evidence shows that rosiglitazone may improve insulin resistance in vivo by normalizing GLUT-4 protein content in adipose tissue, and increasing GLUT-1 in skeletal muscle and fat [1175]. Second, adipose tissue is also a source of free fatty acids and adipokines such as adiponectin and resistin [1176, 1177], and may have a role in preventing the development of insulin resistance [1178, 1179]. The production of adiponectin is decreased in obesity and subjects with T2D and coronary artery disease [1180], as well as women with polycystic ovaries [1181-1184]. Rosiglitazone stimulates adipocyte differentiation to adipocytes in which adiponectin is secreted leading to an increase in its blood plasma concentration [1185-1188]. Adiponectin improves glucose transport into cells and thus insulin sensitivity [1189, 1190]. Rosiglitazone has been shown to improve insulin sensitivity in patients with T2D, and impaired glucose tolerance via a mechanism that is probably related to changes in adiponectin production and adiponectin index [1191-1193]. Furthermore, adiponectin has also been shown to have anti-inflammatory properties [1194], and may be the mechanism by which TZDs are known to inhibit inflammatory cytokine production and thereby the development of atherosclerosis [1195]. However, the anti-inflammatory properties of rosiglitazone have also been linked to its direct activation of the PPAR-γ receptor and the down-regulation of early inflammatory response genes [1196].

TZDs have also been shown to promote mitochondrial function via PPAR-γ co-activator 1 family of proteins, and ATP production [1197, 1198], and inhibit pro-inflammatory gene expression via inactivation of NFkB-dependent promoters [1165, 1199]. Furthermore, this class of drugs has also been shown to reduce β-secretase activity [1197, 1200], as well as promote cortical spine density (via the PPAR-γ receptor) [1154]. Although little is known about the physiological role(s) of PPAR-δ in the brain

[1161], resent studies have also shown that agonists for this PPAR isoform can reduce amyloid toxicity in vitro [1201], reduce Aβ-plaque deposition and astrogliosis as well as increase the expression of neprilysin and IDE in APP mutant mice [1202]. Recent studies have also suggested PPAR-α may be an important regulator of cholesterol trafficking in macrophages [1203]. Thus, TZDs appear to mediate a diverse range of actions in the CNS, including those which may mitigate amyloid pathology, making them potentially useful as therapeutic agents for treating cognitive impairment in AD.

1.9.8.2 PPAR agonist treatments in AD

In addition to the cell culture research already mentioned, rosiglitazone has been shown to improve cognitive performance (i.e. learning and memory) and/or ameliorate pathological indices related to inflammation, insulin resistance, and phospho tau/Aβ, in range of experimental animal models [1164, 1167, 1204-1210], as well as different APP mutant models [1129, 1200, 1202, 1211-1217]. Indeed, similar effects have also been reported with pioglitazone [1218-1230]. However, all of these studies have reported beneficial results, with only one study [1231], reporting negative outcomes in animals. Thus, the majority of studies all support PPAR-γ as being a viable therapeutic target in AD [1166]. This rational is further supported by research demonstrating that many non-steroidal anti-inflammatory drugs (NSAIDs), which include ibuprofen, have a protective effect against developing LOAD [1232, 1233], and activate PPAR-γ [1234].

In terms of human studies with TZDs, the PPAR-γ agonist rosiglitazone (Avandia) has been most widely studied in human clinical trials, where in sharp contrast to animal studies, its effects in clinical trials undertaken with human AD patients have been mixed. A preliminary trial of rosiglitazone, 4 mg daily, vs placebo in 30 subjects with mild AD or aMCI showed better scores on tests of delayed memory and selective attention after 6 months of rosiglitazone treatment [1235]. A subsequent double-blind, randomized, placebo-controlled study of rosiglitazone in 511 patients over 24 weeks revealed no significant improvement in the treated group overall; however, improved cognition (Assessment Scale-Cognitive: ADAS-Cog) was observed in a subset of patients who did not possess an APOEε4 allele [1236]. More recently, a multi-centre randomized proof-of-concept clinical trial has been undertaken by GlaxoSmithKline (GSK) pharmaceuticals (the makers of Avandia), that applied FDG-PET for evaluation of metabolic therapy with an extended release version of rosiglitazone (rosiglitazone-XR) in 80 patients with mild-to-moderate AD [1237] over a 12 month period. Whilst

active treatment was associated with a sustained but not statistically significant trend from the first month with respect to an early increase in whole brain glucose metabolism, neuroimaging data indicated that active treatment did not lead to a significant decrease in the progressive decline in brain glucose metabolism [1237]. Furthermore, the rates of brain atrophy were similar between active and placebo groups, and measures of cognition (as assessed by the mini mental state Exam, ADAS-Cog, and the Clinicians' Interview-Based Impression of Change – Plus), also did not suggest clear group differences [1237]. Thus, whilst rosiglitazone was associated with an early increase in whole brain glucose metabolism, this was not associated with any biological or clinical evidence for slowing progression over a 1 year follow up in the symptomatic stages of AD [1237]. These results are consistent with the findings of two other phase 3 studies which evaluated the efficacy and safety of rosiglitazone in an extended release form (2 mg, or 8 mg) over a 48 week period as an adjunctive therapy to on-going acetylcholine esterase inhibitor treatment in 485 patients, and treatment in 496 patients adjunctive to donepezil [1238]. No statistically or clinically relevant differences between treatment groups were observed on the a priori primary endpoints in these studies, including the cognitive assessment via the 'Alzheimer's Disease Assessment Sub Scale', and 'Clinical Dementia Rating' scale [1238]. Also, consistent with the known profile of rosiglitazone treatment at 8 mg, both studies reported adverse events related to edema, although there was no evidence of an interaction between treatment and APOE status [1238].

One potential explanation of the limited efficacy of rosiglitazone is its poor bioavailability to the CNS. Although rosiglitazone passes the BBB, it undergoes active efflux through P-glycoprotein–dependent transport, limiting its concentration in the brain [1239], although pioglitazone has demonstrated CNS penetration and relevant biological activity in a transgenic mouse model of AD [1221]. However, this does not explain the apparent discord between the animal data evaluating the impact of these drugs on cognition (see next section and Chapter 2). Coupled with the disappointing results of treatment trials based on the amyloid hypothesis [1240] and yet the seemingly overwhelmingly positive results from animal studies of these treatments [412, 766], the discrepancy between human and animal studies demands urgent attention. Yet, it is of interest that a prospective randomized, open-controlled study of 32 patients with mild to moderate AD (26 patients) or MCI (6 patients), treated with pioglitazone (30 mg daily, n=15), or not (n=17, control group), recently reported that

pioglitazone resulted in cognitive and metabolic improvements in patients with AD and MCI with diabetes [1241]. Although these results require verification in a larger double-blind, randomized, placebo-controlled study, it does suggest that peripheral insulin resistance and or T2D may be a determining factor in the efficacy of TZDs in the treatment of AD. Furthermore, since none of the large phase 3 clinical trials so far reported with rosiglitazone were set up to track clinical changes according to whether individuals had concordant T2D or peripheral insulin resistance, and it is possible that a positive effect could have been masked in this patient population.

1.9.9 Summary of Strategy and Hypothesis

This thesis details experiments aimed at examining the impact of rosiglitazone on cognition and amyloid pathology in a popular transgenic mouse model. The mouse model used for these studies is generally known as Tg2576, and overexpress a genetic construct that contains a mutated form of the human APP (hAPP) gene associated with FAD kindreds [1242]. As a result, transgenic mice over-produce hAPP and consequently Aβ —a process that leads to the age-related development of amyloid pathology and cognitive deficits [1242]. Whilst not all clinical symptoms in AD patients find their parallel in such models, by using a variety of behavioural tasks it has been possible to observe some behavioural impairments in animals that exhibit similarities to those observed in AD patients [1243]. In addition, and importantly for this thesis, these mice have also been reported to develop spontaneous insulin resistance with age [1129, 1244] (see section 2.3.1.3).

The behavioural experiments detailed in this thesis (see Chapter 3) all use a dual treatment strategy. In the first set of experiments, a late intervention strategy was used to examine the impact of rosiglitazone on cognition and amyloid load in adult mice when drug administration was initiated at a point in adulthood when significant amyloid pathology, synaptic deficits, and behavioural impairment would already be expected in transgenic mice [1242, 1245, 1246]. Although Tg2576 mice are not considered to have AD, this strategy has some common ground with clinical studies in humans. For example, some studies have delivered rosiglitazone to patients with mild-to-moderate AD when pathological changes are established [1236, 1238, 1247]. However, whilst clinical studies evaluating the effectiveness of rosiglitazone in AD patients have reported variable results, pre-clinical studies conducted in Tg2576 have been more supportive and reported consistent findings suggesting that rosiglitazone

can ameliorate at least some cognitive and pathophysiological changes in hAPP mice when amyloid pathology is already established [1129, 1212, 1244]. However, it is important to point out, that the primary study upon which this thesis is based was conducted by Pedersen et al [1129]. In that study, rosiglitazone had only been administered to adult mice aged 8 months or older when amyloid pathology and synaptic deficits are already well established in Tg2576, and did not extend beyond a 6 month drug administration period. Critically, apart from the assessment of the impact of rosiglitazone on Aβ40 and Aβ42 levels, the behavioural assessment of these mice was limited to a de novo 8-arm radial maze task used to assess WM and reference memory errors. This is important because it is unclear whether rosiglitazone had a selective or wider range impact on cognition in the same animal model. Thus, in the first course of experiments, this thesis reports the outcome of late-intervention experiments following longitudinal administration of rosiglitazone from 8 months of age. Chapter 3 details the impact of late-intervention experiments on established age-related cognitive deficits across multiple domains (see Chapter 3). The behavioural battery used for this was comprised of tasks covering typical phenotypic changes in Tg2576 such as spatial and object recognition memory, as well as changes related to anxiety. Post sacrifice tissue analysis relating to late-intervention experiments can be found in Chapter 4, and relates specifically to the Elisa assessment of total amyloid load derived from single brain hemispheres, and Elisa assessment of plasma adiponectin protein levels (for drug penetrance in relation to an established physiological marker associated with PPARγ agonism). Based on the findings from Pedersen et al., [1129], the hypothesis was that longitudinal treatment with rosiglitazone would reverse an acknowledged spatial working memory deficit in Tg2576 mice, as well as reduce Aβ42 levels. None of the subsequent studies using rosiglitazone in Tg2576 by Denner et al., [1212], and Rodriguez-Rivera et al., [1215]) have reported outcomes for the impact of rosiglitazone on behavioural impairments across multiple cognitive domains with longitudinal (chronic) drug-administration.

At the time of its inception, the effects of chronic rosiglitazone on behavioural impairments in Tg2576 from an early age prior to the appearance of significant amyloid pathology were unknown. Indeed, apart from the current thesis, this is still the case as none of the subsequent studies in Tg2576 using rosiglitazone [1212, 1215] have addressed this issue, despite the fact that concerns have subsequently been raised by one pre-clinical study [1215], that rosiglitazone may have a limited therapeutic

window for ameliorating cognitive impairment in adult transgenic mice. Thus, in a second course of experiments, this thesis reports the outcome early-intervention experiments, all of which investigate the impact rosiglitazone on age-related cognitive deficits across multiple cognitive domains (using the same battery of tasks used in late-intervention experiments) following continuous dosing with rosiglitazone from 5 months of age (see Chapter 3). At this time amyloid and synaptic pathology would be minimal in these mice, and some measures of cognition would be comparable between transgenic and non-transgenic mice [1245, 1246]. The hypothesis in this course of experiments was that rosiglitazone should delay the onset of age-related cognitive deficits in transgenic drug-treated mice. Although it was not possible to conduct assessment of total amyloid levels for the early-intervention experiments, Chapter 4 details an experiment which assessed the impact of longitudinal rosiglitazone administration on the Aβ-related loss of dendritic spines in the hippocampal dentate gyrus. This has not been examined by any of the previous studies using PPAR treatments in preclinical mouse models of APP over-expression.

2. ANIMAL MODELS IN PRE-CLINICAL AD RESEARCH

2.1 INTRODUCTION

This thesis deals the preclinical assessment of rosiglitazone monotherapy, and details in Chapters 3 and 4 the various experimental manipulations undertaken with a popular transgenic (TG) mouse model of mutated human APP (hAPP) constructs associated with FAD kindreds. TG mice are widely used to model human diseases, and commonly involve the overexpression of a particular protein. Of these TG models, those that overexpress hAPP constructs are among the most frequently used and extensively characterised models in AD research. This chapter discusses the relevant necessary background information in relation to TG models. Since it is not possible here to provide an in-depth review of this rapidly expanding area of research, the reader is directed to several published reviews on the subject [42, 1248-1250]. This chapter will focus on hAPP mutants, and in particular, the particular model used in this thesis for experimental manipulations. Section 2.2 firstly summarises the major hAPP models used in AD-related pre-clinical research before in section 2.3 discussing the pathophysiological phenotypic changes associated with the Tg2576 model, which is the model of choice for the PPAR-γ intervention experiments detailed in Chapters 3 and 4. Section 2.4 then summarises the previously published research relating to the effects of rosiglitazone administration on cognition and other pathological indices related to Aβ in Tg2576.

The Tg2576 model was selected due to four main factors. *First,* Tg2576 expresses aspects of early-stage neuropathology and behavioural deficits which mimic some of those found in AD patients. As such, Tg2576 is widely considered to be a credible model of amyloid-induced pathology present in the very earliest stages of AD. *Second,* Tg2576 represents one of the most thoroughly characterised of all the hAPP mouse models, with fairly robust and in some cases age-related cognitive and behavioural

deficits [1245, 1246, 1251-1253] suitable for examining putatively therapeutic interventions. *Third,* some studies indicate that male TG mice may have a vulnerability for developing spontaneous peripheral insulin resistance with age [1129, 1244], such that these mice may provide us with a unique opportunity to examine more closely the specific conditions and the mechanisms by which insulin sensitizer's impact on measures of AD-related pathology at a very early stage of the process. Indeed, a number of studies using different hAPP models have reported that aspects of peripheral insulin resistance may drive or significantly complicate AD-related pathology in mice [1144, 1146, 1244, 1254, 1255], and that rosiglitazone ameliorates some of these factors and leads to cognitive improvement in Tg2576 [1129, 1215, 1244], and other hAPP mouse models [1213, 1214, 1216]. Thus, investigating factors which could impact on the development of pathological alterations early-on in the disorder is of considerable importance clinically, as well as in terms of understanding disease mechanisms. *Four,* that the onset and development of pathology and behavioural deficits in this model are compatible with aims of this thesis.

2.2 TG MODELS REPRODUCING AMYLOID DEPOSITION

TG modelling has been actively pursued on the basis of the ACH in AD research and has taken advantage of mutations in the APP, PS1 and PS2 genes that cause FAD [38-41]. Modelling is most often pursued in mice, for which the techniques of genetic modification have been particularly well developed. There are, however, a great many TG and knock out rodent models used in AD research, as well as viral vector driven models [1256], many of which express bi- and tri-genic constructs [40]. Together these models mimic a range of AD-related pathologies, although none fully replicates all the symptomatic and pathological changes associated with the human disease [42]. However, the failure of hAPP models to faithfully reproduce NFT pathology and neurodegeneration leave a question mark over the primacy of Aβ in both initiating and continuing to drive the pathological cascade in AD [705]. Thus, critics argue that most of these transgenic models are based on over-expression of exogenous APP, which does not apply to AD in humans [1257, 1258]. For instance, Robakis [1257] argues that since APP is metabolized to a large number of derivatives besides Aβ, some of which are reported to be neurotoxic (e.g., C-terminal fragments), disruptions of behaviour observed in these animals cannot unambiguously be explained by the soluble Aβ

oligomers. Nevertheless, despite these limitations the majority of these models have contributed significant insights into the pathophysiology of AD, and in particular, the effects of Aβ species in pathogenic cascades. Indeed, hAPP mouse models have been widely used in the preclinical testing of potential therapeutic modalities, and have played a pivotal role in the development of current Aβ-related immunotherapies for AD, some of which remain in clinical trials [42]. Thus, TG mouse modes will continue to play central roles in preclinical research related to AD.

2.2.1 Single, Double and Triple '3xTG' models

The first mouse models to develop Aβ-plaque pathology were generated by expressing human APP containing mutations associated with early-onset AD. Thus, in 1995 Games and colleagues published the first transgenic mouse (PDAPP) that over-expressed a minigene construct encoding human APP transgene associated mutation (V717F) [1259]. The platelet derived growth factor-β (PDGF) promoter was used in this model to drive the transgene containing V717F because, despite its name, it was known to be highly expressed in the central nervous system and to drive strong expression of exogenous transgenes in neurons [38]. PDAPP mice exhibited an age-dependent amyloid deposition in the brain along with thioflavin-S–positive plaques, including those resembling neuritic plaques with dense cores that were highly reminiscent of those seen in human AD. Dystrophic neurites, reactive astrocytes, and activated microglia were also found near these plaques, and the process was age-related in that plaque deposition was minimal at 6 months of age but readily apparent by 9 months, increasing dramatically by 12 to 15 months [1260]. Although PDAPP mice did not develop the NFTs or cell loss reminiscent of human AD, they were subsequently shown to develop age-related learning defects [1261] and synapse loss [1262]. Only a few years after the development of PDAPP, Ashe et al. [1263] taking a relatively similar approach published their Tg2576 (APPswe) model. The Tg2576 mouse line is now freely available, and is among the most studied TG models in AD research. Subsequently, many additional lines of mice expressing mutant human hAPP have been reported as developing similar amyloid pathology and cognitive deficits [1264-1266].

Double mutant mouse models have been developed which cross hAPP mutant mice with those expressing mutant PSEN1 transgenes (PSAPP mice). Such mice show accelerated amyloid deposition [1267-1269], largely due to the increase in Aβ42 production mediated by PSEN1 mutations [1270, 1271]. The pathology seen in hAPP and

PSAPP transgenic mice includes diffuse amyloid deposits as well as plaques containing fibrillar Aβ that resemble those found in human AD [1259, 1263, 1268, 1269]. Furthermore, the neuritic-type plaques in these transgenic mice are likewise surrounded by dystrophic neurites with associated reactive gliosis [1272-1274]. However, despite the robust amyloid deposition observed in APP and PSAPP transgenic mice, none of these models develops the widespread neuronal loss associated with human AD [1272, 1275], although astrocytic apoptosis has not been extensively studied in these models. Both neuronal cell loss and robust tau pathology (NFTs and neuropil threads) are also absent in mice expressing hAPP and PSAPP alone [38]. APP and PSAPP transgenic mice do develop vasocentric dense Aβ-plaques with associated structural microvascular damage and BBB abnormalities similar to those found in CAA [1276]. This type of pathology is largely absent in PDAPP mice [42]. In summary, the common pathological features of the PDAPP and Tg2576 mice (along with similar models), are the production of elevated Aβ levels, the formation of Aβ-plaques, dystrophic neurites, and gliosis. In addition, other neuropathological, electrophysiological, and neurochemical changes have also been found in these mice similar to those seen in AD patients [40].

When double mutant mouse models are combined with a phospho-MAPT tau mutation isolated from FTD kindreds, these so called triple 3xTG mutant mice display a much wider range of neuropathologies common to human AD (see review, [1250]), including dystrophic neurites surrounding neuritic plaques and more extensive NFT pathology and neuronal cell loss [1259, 1263, 1269]. For example, the 3xTG model harbouring the $PS1_{M164V}$, APPswe, and Tau_{P301L} mutations, typically displays an age-related progression of pathology that shows a regional distribution pattern of neurophysiological lesions similar to that seen in human AD patients, as well as Aβ deposits that precede tau pathology [1277]. In addition, behavioural deficits and synaptic dysfunction in this mouse model develops around 4 to 6 months, with NFT development (which occurs at a later age) associated with early intracellular Aβ deposition at 3 months [1277, 1278]. Nevertheless, none of the 3xTG models fully reproduce all of the pathological features of human AD [1279]. However, these x3TG models combine multiple genetic constructs from both FAD and FTD-17 in combinations that are not normally found in humans. Thus, the ultimate relevance of these models to human AD can be questioned [1280]. No-where is this more important than in the field of therapeutics in relation to immunomodulation targeting

the aggregating proteins Aβ and tau. This type of AD therapy is currently being assessed in many transgenic mouse models, and promising findings in animals have led to clinical trials in humans [770]. However, as mentioned in the introduction, there is a discrepancy between results obtained in TG murine models and those of on-going clinical trials [1281], highlighting the general limitations of these models. This is not helped by the lack of a commercially available animal model for SAD, although this is not one of the aims of the current thesis.

2.3 PHENOTYPICAL CHARACTERISTICS OF TG2576 MICE

Tg2576 mice overexpress hAPP harbouring the double hAPP mutation K670M/N671L related to FAD in Swedish kindreds (hAPPSwe), on a hybrid background strain of C57Bl/6J with Black 6 Swiss James Webster (B6SJL) [1263]. Tg2576 mice use a hamster prion protein promoter in order to drive strong expression of the hAPPSwe transgene. This model was created by microinjecting the human APP695 gene containing the double mutation K670N, M671L into B6SJLF2 zygotes using a hamster prion protein cosmid vector (basically, plasmid vectors that contain cos sites which circularizes the DNA in the host cytoplasm). The resultant mice from founder line 2576 were backcrossed to C57BL/6. In March 1999 Taconic Farms (Germantown, NY) received stock of Tg2576 from the Mayo Foundation [25], wherehence hemizygous males were backcrossed with C57BL/6NTac for derivation by embryo transfer. This colony is maintained by mating hemizygous male mice with B6SJLF1 female mice. Taconic received stock of Tg2576 from the Mayo Foundation in March 1999 [1282], wherehence hemizygous males were backcrossed with C57BL/6NTac for derivation by embryo transfer. The colony is maintained by mating hemizygous male mice with B6SJLF1 female mice. Since this time, the Tg2576 model has been made widely available to research institutions for non-profit research purposes enabling the formation of multiple Tg2576 mouse colonies, one of which is maintained by Professor M.A. Good, Department of Psychology, at Cardiff University.

Tg2576 mice express hAPP at levels more than 5-fold above the levels of the endogenous mouse APP [1263]. Because the APPswe mutation occurs close to the APP β-secretase processing site, it leads to increased β-secretase mediated cleavage and increased production of Aβ40 and Aβ42 with age [1263]. Soluble Aβ levels begin to increase from approximately 4 to 5 months of age in these mice; they become

significant by 6 to 7 months [1245, 1263, 1283-1285]. Between 6 to 10 months of age, insoluble forms of Aβ start to increase exponentially, a process that is associated with a slight reduction in soluble forms [1283]. Like PDAPP mice, Tg2576 mice show a robust age-related increase in Aβ in multiple brain regions, including the frontal and temporal cortices, and temporal-limbic regions such as the entorhinal cortex, hippocampus, presubiculum and subiculum, although the cerebellum is not significantly affected [1263, 1272, 1285, 1286]. By 9 to 10 months of age, there is significant deposition of Aβ, including as thioflavin-S–positive plaques similar to those found in AD [1263, 1287], gliosis, dystrophic neurites, abnormal tau-phosphorylation, and behavioural impairments [1283, 1288-1290]. However, whilst diffuse plaques are fairly abundant, the neuritic-like plaques in this model are not evident until around 12 to 23 months of age [1245, 1291]. Concordant with this, plaque associated microgliosis becomes evident from around 10 months of age, with reactive astrocytes present at 18 months [1245, 1272, 1273, 1292]. Accordingly, Tg2576 mice also show an inflammation response that includes the up-regulation of inflammatory markers such as IL-1β, TNF-α, Cyclooxygenase-2 (COX-2), complement protein C1q, and chemoattractant protein-1 [1293]. Neuroinflammation can be attenuated by the administration of non-steroidal anti-inflammatory drugs such as ibuprofen [1294, 1295], which is a PPAR-γ dependent effect [1295]. Significant structural but not physiological changes in cortical neurons have been reported in 12-month-old Tg2576 mice [1296], including increased dendritic lengths and volumes. However, extensive NFT pathology, neurodegeneration, and cell loss (including the loss of cholinergic neurons), is absent in this mouse model [1272, 1273, 1297, 1298], leading some to suggest that Tg2576 mice may model the pre-clinical aspect of AD [804, 1299]. Section 2.3.1 discusses the physiological phenotype of Tg2576. Since synaptic loss and metabolic impairments are the most directly relevant to this thesis, these are considered in most detail. Section 2.3.2 then briefly introduces the cognitive phenotype of Tg2576. However, in order to prevent unnecessary repetition of material, much of the detail relating to components of most relevance to the behavioural experiments used in this thesis, are reserved until Chapter 3.

2.3.1 Physiological Phenotype

2.3.1.1 Factors Related to Synaptic Loss

In Aβ-depositing mice it is known that "hyperactive" neurons in the hippocampus are found exclusively near Aβ-plaques, and appear to be due to a relative decrease in

synaptic inhibition as a result of soluble Aβ [82]. Although it is unclear how soluble Aβ may cause the heightened activity of these neurones, one theory is that it boosts synaptic glutamate in the hippocampus [82, 83]. Indeed, since plaque formation is itself associated with a transient drop in the concentration of soluble Aβ isoforms [1300], it is possible that this may allow any remaining soluble Aβ species to enhance neuronal activity via its ability to act as a regulator of release probability at hippocampal synapses [84]. However, since the majority of Aβ is considered to be neuronal in origin [53-56], this may then lead to even more soluble isoforms being produced, which subsequently cause the localised loss of synapses and depressed synaptic output typical of higher concentrations of the peptide Aβ. Thus, hippocampal neurons near plaques may "rev-up" before they eventually become hypo-active. There are however, likely to be multiple ways by which soluble Aβ species affect neuronal and synaptic activity, all of which have implications for network dysfunction [83].

Golgi-stained sections of human AD hippocampus and cortex reveal a significant decrease in dendritic spine density in many brain regions when compared to age matched controls [1301], and recent studies suggest that a failure in synaptic function underlies memory deficits in AD and ageing (see reviews, [1302, 1303]). Synapse dysfunction and age-related loss of dendritic spines also occur in mouse APP models (see reviews, [1304, 1305]), and correlate well with the earliest signs of cognitive impairment [1245, 1301, 1306, 1307]. Learning and memory impairments in Tg2576 mice in the absence of overt sensory or motor deficits also suggest that the hippocampus is functionally affected in APP overexpressing mice [1262, 1263], more of which will be discussed latter in relation to Experiments 1 in Chapter 3. Given the status of the hippocampus as a model system in studies of structural and functional neuro plasticity [833], most studies looking at the loss of dendritic spines in hAPP mice have focused on this brain region, and more specifically, the dentate gyrus and CA1 region. However, whilst most agree that spine density (number of spines per summed unit length of dendrite) is significantly decreased in the hippocampi of TG mice [1245, 1308-1312], the differences may be less obvious in other brain regions with age [838, 1303].

The Effects of Aging on Synaptic Loss

The loss of dendritic spines from the brain in Tg2576, particularly in the hippocampus, is a central component of the pathological phenotype of these mice. As such, this is critically important in Experiment 7 of Chapter 4, which assesses spine densitity in the

hippocampal dentate gyrus. The molecular layer of the dentate gyrus is one of the earliest regions to show age-related amyloid deposition and synapse loss in Tg2576 mice [1260, 1308, 1313]. Jacobsen et al. (2006) were amongst the first to report this [1245], where in 4 but not 2 month old TG mice, showed a significant decrease in spine density in the outer molecular layer of the hippocampal DG. This was also the time frame in which LTP deficits and contextual fear memory impairment emerged. Moreover, these deficits coincided with an increase in the ratio of pre-fibular Aβ42 and Aβ40, and remained stable between the ages of 12 to 18 months by which time, neuritic-like plaques and significant increases in reactive astrocytes and microglia were also observed [1245]. Using stereological methods at both the light and electron microscope level, Dong et al. [1308] reported similar findings in Tg2576 mice. Here, reduced synapse density (pre and post-synaptophysin-positive boutons) in the outer molecular layer of the DG was observed at both 6 to 9 months, and 15 to 18 months of age. A significant decrease in synapse density was also observed in layers II and III of the entorhinal cortex at 15–18 months of age [1308]. Moreover, decreases in synapse density were correlated with proximity to compact Aβ-plaques, providing evidence of a spatial relationship between synapse loss and neuritic-like plaques at the ultra-structural level in Tg2576 mice [1308]. However, this finding has not been confirmed by later studies [1303].

That soluble Aβ is likely the harbinger of synaptic loss in APP mutants is supported by several in vitro studies that have demonstrated oligomeric aggregates of Aβ reducing the number and/or length of dendritic spines in hippocampal neurons [1152, 1314, 1315]. Aβ oligomers generated in vivo reduce spine density in CA1 pyramidal neurons [1312], although the loss of spines and synapses is variable [1308, 1310-1312]. Using a Golgi-stain and light microscopy, Lanz et al. [1310] examined stretches of apical dendrites from five randomly selected completely filled CA1 pyramidal neurons in both PDAPP (between the ages of 2 and 11 months), and Tg2576 mice (between 2 and 20 months of age). This study reported that in the hippocampal CA1 region, both PDAPP mice and their wildtype (WT) littermates exhibited significant dendritic spine loss due to both age and hAPP overexpression, with both groups of mice exhibiting a significant loss of spines after the age of aged 2-months [1310]. Indeed, the genotype-dependent difference in spine density in the PDAPP mice was greatest at 2 months of age (17%), this was reduced to 8.8% by 5 months of age, and by 11 months no statistical difference in spine density was evident between the two genotypes, despite well-developed

plaque pathology [1310]. Like the PDAPP mice, Tg2576 mice also exhibited dendritic spine loss in CA1 hippocampal region due to both age and mutant hAPP overexpression, although a significant interaction between these variables was not detected [1310]. From 2 to 4.5 months of age, only the TG group showed a significant, albeit small reduction in spine density (6%), with the largest age-dependent loss occurring in both TG and WT groups between the ages of 4.5 and 11 months (18 to 19%) [1310]. No further reductions in CA1 spine density were observed between 11 and 20 month-old mice of either genotype, and overall from 2 to 20 months, aging produced a 23–26% loss of dendritic spines [1310]. Unlike PDAPP mice, the youngest age group of Tg2576 mice did not exhibit the largest decrease in spine density due to the APP transgene. Rather, by 4.5 months a 16% reduction in spine density was observed in TG mice compared to age-matched WT littermates, which increased to 14% by the age of 11 months although at 20 months both TG and WT mice had similar dendritic spine density in the CA1 region [1310]. This finding is consistent with the results of Dong et at al., (2007), who reported no significant genotypic differences with respect to CA1 dendritic spine density in 15 to 20 month old TG2576 and WT littermate mice, [1308]. These results are interesting for a variety of reasons. Firstly, they suggest that the synaptic loss in APP mutants develops in both the DG and CA1 long before overt Aβ-plaques are formed, confirming doubts with respect to a causal link between insoluble amyloid deposits, structural abnormalities of neurons and cognitive impairments [1310] (but see [1308].) In addition, the fact that genotypic differences in CA1 spine loss are nullified by increasing age [1310], suggests that some regions of the hippocampus may react differentially to the effects of Aβ as well as normal age-related processes.

The synaptic pathology in the hippocampus and in particular the DG of Tg2576 mice, probably underpins most of the spatial memory impairments observed in this mouse model, although this is speculative. As mentioned earlier, Jacobsen et al. [1245], has reported that Tg2576 mice exhibit an accelerated age-related decline in synaptic density in the DG region of the hippocampus, a process that was accompanied by a decline in basal AMPA receptor-mediated synaptic transmission, a deficit in the induction of LTP, and impairment in a contextual fear conditioning task prior to significant Aβ-plaque deposition. Indeed, the data from this study indicates that the perforant path input from the entorhinal cortex to the DG is compromised both structurally and functionally in Tg2576 mice, and this pathology manifests as memory defects before significant plaque deposition [1245]. These findings are further

supported by Chapman et al. [1316], who have previously shown that aged Tg2576 mice exhibit normal fast synaptic transmission and short term plasticity, but are severely impaired when it comes to in-vitro and in-vivo LTP in both the CA1 and DG regions of the hippocampus. Indeed, the disruption of LTP in this study also correlated with impaired performance in a spatial working memory task [1316]. As discussed previously, the primary pathogenic mechanism considered by most to be responsible for mediating these deleterious changes on hippocampal synapses and plasticity processes, are soluble Aβ oligomers. Indeed, one such species of this type Aβ*56, has been suggested as the primary oligomeric form that mediates synaptic changes and memory impairments in these mice [1317]. Evidence supporting this relates to a previous study by Lesne et al. [804], which reported that a transient reduction in Aβ*56 occurs in Tg2576 mice between 11.9 and 12.4 months of age when the rates of accumulation of insoluble, fibrillar Aβ are transiently elevated, but memory function (as assessed via a spatial memory task) is intact. Indeed, it was only when levels of Aβ*56 were not reduced, that memory function was impaired in Tg2576 mice [804]. Although longitudinal studies of Aβ*56 in Tg2576 mice are lacking, it is possible that other periods of low Aβ*56 occur with advanced age, which may help to explain discrepant findings in the literature with respect to reports of intact spatial memory in aged TG mice despite significant Aβ pathology [1285]. Tg2576 mice have also been shown to exhibit age related impairment in a variety of Pavlovian tasks, including contextual fear conditioning (conditioning to a particular context), and cued fear conditioning (conditioning to a cue such as a tone) [1245, 1318-1320]. Both the spatial memory and cued/contextual fear conditioning impairments are consistent with the hypothesis that hippocampal processing and its interaction with the amygdala, is aberrant in aged Tg2576 mice [1320], probably as a result of synaptic/plasticity changes [1245, 1321].

Microanatomy of Single Dendritic Spines in TG2576

Region-specific changes in the microanatomy of single dendritic spines over time have also been assessed in Tg2576 and other APP mutants [1311, 1312]. Middei et al., [1311] studied eight fully impregnated (randomly selected) hippocampal CA1 pyramidal neurons, or dorsolateral striatum (DLS) spiny neurons in Tg2576 and WT littermates aged between 3 and 15-months. This study reported that in each region, all mice showed a global reduction in the size of spines as a function of age. However, ageing

mutants exhibited smaller spines with shorter necks on CA1 pyramidal neurons, but larger spines with longer necks on DLS spiny neurons compared to their age-matched wild-type controls [1311]. Since robust synaptic strength is a feature of spines with large heads and long necks allowing accumulation of activated synaptic proteins [1322], the authors suggested that ageing-related alterations in size of DLS spines were likely a compensatory mechanism aimed at maintaining "procedural" abilities in these mice, at a time when cognitive abilities dependent on intact hippocampal function are compromised [1311]. On the other hand, the presence of spines with short necks and small head areas is a prominent feature that correlates with the hippocampal synaptic plasticity and/or basal synaptic transmission deficit in TG mice [1288, 1323], as well as episodic-like memory impairment [1324, 1325]. However, in contrast to other studies, age-related changes were exacerbated in TG mice relative to aged WT mice [1311].

2.3.1.2 Oxidative Stress and Metal Dyshomeostasis

Although not central to the research aims of this thesis, oxidative stress and metal dyshomeostasis are important pathophysiological components of the Tg2576 phenotype. Similar to AD patients, Tg2576 mice exhibit oxidative stress prior to significant Aβ deposition [1286, 1326, 1327], the latter of which also occurs with metal dyshomeostasis, since deposition of Cu, Zn and Fe metals are found in areas of amyloid plaque pathology [1328, 1329]. Furthermore, the degree of oxidative stress in Tg2576 mice has been shown to increase with age and correlate with the developmental pattern of β-secretase activity and Aβ-plaque formation [1327]. Interestingly, behavioural stress (via restraint stress) has also been shown to exacerbate both metabolic oxidative stress and other pathological hallmarks in these mice, which may result in an increase in corticosteroid levels [1330]. Although the increase in oxidative stress in these mice is usually attributed to redox interactions and increasing levels of soluble Aβ [873], studies assessing the temporal relationship between oxidative stress and soluble Aβ oligomers in Tg2576 mice are currently lacking. Presently therefore, the available evidence is consistent with the proposal that like human FAD sufferers, Tg2576 animals may overproduce a human form of Aβ that is less efficient at its antioxidant role [882], and a mutated form of hAPP that induces metal dyshomeostasis [862]. Although not directly related to the experiments detailed in this thesis, it would be interesting to see if murine levels of Aβ are up-regulated at a time of acute oxidative

stress as a possible compensatory response, or if these mice develop significant numbers of senescent glial cells with age as a result of oxidative stress.

Research suggests that therapeutic treatment of Tg2576 mice with antioxidants may mitigate oxidative stress and/or other pathological features associated with AD [1326, 1331, 1332]. However, studies of antioxidant treatments in AD patients have been rather disappointing [1333, 1334], placing the pathogenic role of oxidative stress in AD (at least as a treatment target in diagnosed patients) in doubt [1335]. Various transition metal chelators have also been trialed as possible treatments in Tg2576 mice and other APP mutants, and have frequently reported positive results [1336-1339]. To date several chelating agents have been investigated for their potential to treat neurodegeneration, and a series of 8-hydroxyquinoline analogues show the greatest potential for the treatment of these diseases in humans (see review, [1340]). However, developing chelators capable of crossing the BBB that are safe for humans poses significant challenges, although the approach is promising (see review, [1341]).

2.3.1.3 Spontaneous Insulin Resistance

Central to the justification for using rosiglitazone monotherapy in Chapter 3 of this thesis is research suggesting that Tg2576 mice spontaneously develop insulin resistance with age. Pedersen et al [1129, 1148, 1244] have probably provided the greatest single contribution to understanding the metabolic deficits in adult male Tg2576 mice in this context. In their initial study, this research group showed that TG mice exhibit increased sensitivity to physiological stressors, which was associated with aberrant hypothalamic-pituitary-adrenal (HPA) function, and regulation of blood glucose levels [1148]. Indeed, dysfunction of the stress responsive HPA axis is also evident in AD patients [1342]. More specifically, Tg2576 mice exhibited severe hypoglycaemia and death following food restriction, as well as sustained elevation of plasma glucocorticoid levels and hypoglycaemia following restraint stress [1148]. By 8 months of age male Tg2576 mice were reported to have lower basal serum insulin concentrations and exhibited a delayed insulin-induced reduction in blood glucose levels relative to WT counterparts, although basal levels of blood glucose and % glycosylated haemoglobin were both found to be similar between the two groups of mice [1244]. However, following an overnight fasting regime, TG mice were subsequently shown to have a greater rise in serum corticosterone levels as well as an excessive reduction in serum insulin concentrations [1244]. Furthermore, by 13 months

of age, Tg2576 mice became hyperinsulinemic suggestive of spontaneous peripheral insulin resistance, an effect that was prevented by the chronic oral administration of rosiglitazone starting at 9 months of age [1244]. Moreover, because insulin serum and glucocorticoid concentrations were maintained in transgenics at levels observed in WT mice following an overnight fast, these results provided evidence for a possible relationship between insulin resistance, impaired regulation of insulin and glucose levels, and aberrant stress responses in Tg2576 mice [1244]. As accumulation of soluble Aβ had been linked to memory deficits in Tg2576 mice, [841, 1284], Pedersen et al., suggested that insulin resistance in Tg2576 may contribute to cognitive dysfunction observed in these mice by increasing Aβ burden [1129]. In essence, the raised glucocorticoid levels in TG mice may have caused the deletion of insulin signalling components in the brain thereby initiating insulin resistance. Rational for this was based on experiments by Buren et al. [1343], who showed that in cultured adipocytes, raised glucocorticoid levels deleted components of the insulin signalling pathway leading to peripheral insulin resistance. Crucially, because IDE is up-regulated by insulin in neuronal cells [1344], Pedersen et al., hypothesised that central insulin resistance (involving insulin deficiency), would reduce IDE expression, and thus, cause a relative increase in Aβ levels that could lead to impaired learning and memory in TG mice [1129]. Indeed, because cortisol was previously reported to cause a reduction in the levels of IDE, and moreover, a specific increase in the levels of Aβ42 (but not Aβ40) in the brain [1345], a similar outcome was predicted in Tg2576 mice. Furthermore, by chronically administering rosiglitazone to TG mice, Pedersen et al., hypothesised that both central and peripheral insulin could be normalised along with IDE deletion, resulting in a reduction of Aβ42 and thus, improvement of learning and memory [1129]. Pedersen et al. tested their hypothesis in adult male Tg2576 mice and WT-littermates using a novel 8-arm radial maze task [1129], the results of which will be discussed in section 2.4.

Rodriguez-Rivera and colleagues [1215], have also reported age-related peripheral glucose-regulatory abnormalities and hyperinsulinemia in adult Tg2576 mice suggestive of peripheral insulin resistance. However, another group [1144], have found no convincing evidence for spontaneous peripheral insulin resistance in female Tg2576 mice. Whilst these discrepant results with female Tg2576 mice remain without resolution, studies in humans have shown that the sex steroid hormone estrogen may be protective against metabolic syndrome by contributing to the maintenance of

insulin sensitivity; estrogen deficiency can lead to development of T2D and insulin resistance in women [1346]. Taken in aggregate, current data suggest that peripheral glucose-regulatory abnormalities and hyperinsulinemia (both proxies for probable insulin resistance), may spontaneously develop in male Tg2576 mice [1129, 1148, 1215, 1244], but the spontaneous appearance of these physiological impairments in female TG mice is more uncertain [1144]. Further studies are required to robustly confirm the presence of an insulin resistant phenotype in male Tg2576 mice, and resolve discrepant data with respect to such impairment in female TG, although this is not addressed in the current thesis. In addition, it is interesting to note that, in contrast to the characteristic hypometabolism seen in AD patients, a recent study by Luo et al. [1347], using a combination of FDG-PET and functional fMRI in Tg2576 mice aged 7 and 19 months of age, has reported the presence of 'hypermetabolism' with respect to cortical glucose utilisation at the 7 month time point, which could not be accounted for by changes in vascular compliance. However, this hypermetabolic activity decreased with age such that, 19-month-old TG mice did not differ in terms of their cortical glucose utilisation from age matched WTs [1347]. These data are incompatible with central insulin resistance being present in TG mice, although this does not preclude the possibility that components of it may have been present in some brain regions (i.e. IDE hypo-function for example). However, the initial hypermotabolism revealed in this study may be an adaptive change to the overproduction of Aβ [1347], and could reflect aberrant behaviour in astrocytes, which in Tg2576 mice, have been shown to exhibit an age-related synchronous hyperactivity and long-range calcium waves [1348]. The extent to which these effects alter neural activity in rodents akin to the DMN is unknown; this is not addressed further in this thesis.

Diet Induced Insulin Resistance

Peripheral insulin resistance can be induced in rodents via dietary-induced obesity, a process which involves the chronic consumption of a high fat diet (see review, [1349]). This is much closer to the clinical situation as diet-induced obesity is a major pre-disposing risk factor for T2D in man. However, whilst dietary-induced obese animals exhibit moderate elevations in plasma insulin levels compared to control animals fed standard rodent chow, they do not typically develop diabetes (hyperglycaemia) [1349]. Both plasma glucose and insulin levels are increased following a glucose challenge i.e. the obesity is associated with impaired glucose tolerance and insulin resistance. Ho et

al. [1144], were amongst the first to investigate dietary-induced obesity in Tg2576. In 9 month old female TG mice, they discovered a high-fat diet led to increased levels of Aβ40 and Aβ42 peptide generation that corresponded with increased γ-secretase activities, decreased IDE activities (decreased IDE expression and activity), increased deposition of Aβ into plaques, and behavioural impairment in a spatial water-maze task relative to normal-glycemic TG mice. Furthermore, Ho et al., also observed a functional decrease in cortical insulin receptor-mediated signal transduction in the insulin-resistant TG mice, although in control studies no detectable alteration in total insulin receptor expression was found in the cerebral cortex of insulin-resistant Tg2576 mice relative to normal-glycemic TG mice [1144]. However, further exploration of the apparent inter-relationship of insulin resistance to brain amyloidosis revealed decreased insulin receptor autophosphorylation and reduced PI3K/pS473-AKT/Protein kinase-B (PKB) levels in the cortex. This was interesting for two reasons. First, AKT/PKB has been associated with an inhibitory role of on GSK-3α activity, the latter of which has been shown to promote Aβ peptide generation [1350]. Indeed, GSK-3α and pS9-GSK-3β phosphorylation (an index of GSK activation), positively correlated with the generation of brain C-terminal fragment of APP in the Ho et al., study [1144], which the authors noted provided a valuable index of γ-secretase activity in the brain tissue of insulin-resistant TG mice relative to normal-glycemic Tg2576 mice. Second, these results are also consistent with recent research suggesting that intracellular-Aβ oligomers in neurons can inhibit insulin receptor signalling in part by interfering with the association between phosphoinositide-dependent kinase (PDK), and AKT1, precluding AKT1 activation and eliminating the normally neuroprotective benefit of insulin [1155]. Indeed, given the aforementioned fact that total insulin receptor expression in the cerebral cortex was found in the Ho et al., study to be statistically comparable between insulin-resistant Tg2576 mice relative to normal-glycemic TG mice [1144], it suggests that this may have been the means by which functional insulin receptor deficits and thus, cognitive impairment was mediated in insulin-resistant Tg2576 mice. However, the authors could also not exclude the possibility that changes in body fat may have disregulated estrogen balance in these mice, possibly leading cognitive changes [1351], and insulin resistance [1346]. Another study conducted with female Tg2576 mice and WT-littermates fed a high fat diet [1146], has reported that whilst caloric intake of high-fat-diet-fed TG mice were similar to that of normal diet-fed TG or WT mice during 4 to 8 weeks (wk) of age, it increased sharply at 12 wk, and

went up further at 16 wk, which paralleled changes in the level of Aβ40 and Aβ42 in the brain of these mice and was associated with abnormal feeding behaviour with time. Indeed, the hypothalamus of high-fat-diet-fed TG mice had a significant decrease in the expression of the anorexigenic neuropeptide, brain-derived neurotrophic factor, at both the mRNA and protein levels [1146]. These findings suggest that the increased Aβ in the brain of high-fat-diet-fed Tg2576 mice is associated with reduced brain-derived neurotrophic factor expression, which led to abnormal feeding behaviour and increased food intake, resulting in obesity and insulin resistance in these animals[1146]. Moreover, limiting food intake in high-fat fed TG mice by pair-feeding a caloric intake identical with that of normal diet-fed mice, completely prevented the obesity and insulin intolerance of high fat-fed TG mice [1146], further supporting concerns on the potentially adverse long-term effects of allowing ad lib food access by default to most laboratory animals [1352].

Finally, chronic consumption of sugar in calorically sweetened beverages in humans is also a risk factor for obesity, as well as insulin resistance [1353], although much of the research in this aria has involved animal research conducted with hAPP mouse models other than Tg2576. For example, using the APPswe-PSEN1 model, Cao et al. [1254] have reported that a chronic 25-wk ad lib consumption of a 10% sucrose-sweetened water in TG (but not WT) mice form the age of 2 months, was sufficient in ~8 month old mice to induce increased body weight, glucose intolerance, hyperinsulinemia, and hypercholesterolemia, although frustratingly, this study did not report the sex of the mice used. Furthermore, these changes in TG mice were associated with the exacerbation of spatial memory impairment (as assessed via the Morris water maze test), and a 2 to 3-fold increase in insoluble Aβ-protein levels and deposition in the brain, although steady state levels of IDE did not significantly change in these mice, whereas there was a 2.5 fold increase in brain murine APOE levels [1254]. In aggregate, these results confirm two things. First, compared to WT littermates, APP mice have vulnerabilities for developing peripheral insulin resistance with age when the right environmental variables co-exist. Second, the development of peripheral insulin resistance in these mice can further exacerbate pre-existing amyloid pathologies, which may promote central insulin resistance. A similar exacerbation of amyloid pathology and decrease in central insulin signalling is also observed in TG mice which contain the double hAPP Swedish and London mutations ($APP_{SWE\text{-}LONDON}$), which also have a streptozotocin induced form of insulin-deficient diabetes [1145].

2.3.2 Behavioural phenotype of Tg2576

Any credible model of AD should ideally display not only key pathological characteristics of the disease, but also as many of the behavioural characteristics of the disease as possible, at least to the extent that this is realistic in a non-verbal animal. Whilst not all clinical symptoms in AD patients find their parallel in animal models, by using a variety of behavioural tasks it has been possible to observe some behavioural impairment's in animals that exhibit similarities to those observed in AD patients [1243]. As such, behavioural tasks are usually sub-divided into groups according to the type of memory process being tested. In general these fall into those assessing spatial memory, contextual memory (including episodic-like memory tasks), and those which examine working memory/novelty/loco-motor activity (see reviews, [1354, 1355]). The cognitive processes assessed by particular tasks within these categories can be differentially affected by the pathology in Tg2576 mice across the animal lifespan [1245, 1246, 1288, 1325, 1356]. Although the behavioural phenotype of Tg2576 mice also includes aspects of BPS [1250, 1252], most experimental studies fixate on cognitive impairments, and in particular, memory impairments. Spatial memory impairments and behavioural changes related to anxiety and object recognition are phenotypic features associated with Tg2576 mice, and are discussed in the introductions to individual Experiments in chapter 3.

2.4 PRE-CLINICAL STUDIES USING ROSIGLITAZONE

Several studies have now been published on the effects of rosiglitazone treatment in Tg2576 and reported beneficial effects on learning and memory as well as a reduction in pathophysiological components related to insulin resistance and $hAPP_{SWE}$ over-expression [1129, 1212, 1215, 1244]. This section briefly summarises these studies. However, particular emphasis is placed on the study by Pedersen et al. [1129], as this provides the primary impetus for the experimental work detailed in chapters 3 and 4.

2.4.1 Tg2576: Studies by Pedersen and Colleagues

However, these studies, only Pedersen et al. [1129], has reported the effects of rosiglitazone on cognition and Aβ-protein levels in adult Tg2576 mice following continuous administration of the drug over a period of several months. Having previously reported that dietary supplementation with rosiglitazone normalized the

insulin responsiveness of male Tg2576 mice [1244], this research group sought to test their hypothesis that increasing peripheral insulin sensitivity would improve the spatial learning and memory abilities of Tg2576 mice [1129]. Using a novel 8-arm radial maze task, Pedersen et al. determined the effects of rosiglitazone on the spatial learning and memory abilities of male Tg2576 mice using the following experimental design: Tg2576 mice subjected to a standard rodent diet supplemented with rosiglitazone (TG-R) at a concentration of 30 mg/kg, Tg2576 mice subjected to an un-supplemented (control) diet (TG-C), and wild-type mice subjected to an un-supplemented (control) diet (WT-C). The mice were placed on their respective diets beginning at 9 months of age, and by monitoring food intake and body weights over several weeks, they were able to determine that the TG-R mice were dosing themselves with rosiglitazone at ~4 mg/kg/day, a figure consistent with their previous findings [1244]. The initial behavioural testing was carried out when the mice were ~13 months of age, and had been subjected to their respective diets for ~15 weeks (roughly 3.4 months). Pedersen et al. reported that there was a significant attenuation of learning and memory deficits in the TG-R mice compared to TG-C counterparts, with TG-R mice exhibiting an improvement in spatial working and reference memory across test days similar to WT-C mice [1129]. In contrast, the TG-C mice made a consistent numbers of errors throughout the testing period, and did not exhibit improvement [1129]. These results suggested that rosiglitazone reversed the spatial learning and memory impairment Tg2576 mice.

In terms of the physiological measures, Pedersen et al. determined that rosiglitazone prevented abnormally high serum corticosterone levels from being reached in TG-R mice that had been fasted overnight [1129]. Because their behavioural paradigm involved repetitive overnight fasting that initiated abnormally high glucocorticoid levels in TG mice [1244], they hypothesized that abnormal regulation of glucocorticoid levels would contribute to the spatial learning and memory deficits of Tg2576 mice via a mechanism of lower IDE levels and concordant increase in Aβ-protein. The impaired performance of TG-C mice were found to correlate with higher levels of serum corticosterone relative to the levels found in TG-R and WT-C R mice [1129]. After ~19 weeks on rosiglitazone, Pedersen administered TG-R mice metyrapone (a drug that blocks glucocorticoid production). As a result, the beneficial effect of rosiglitazone on cognition was abolished [1129]. Since rosiglitazone was not considered to penetrate the BBB [1236, 1244], these data were interpreted by authors as evidence

for a significant role for peripheral insulin sensitivity in cognition [1129]. Finally, Pedersen et al. determined if rosiglitazone attenuated known glucocorticoid-induced changes in the brain. These studies were carried out on mice that had been sacrificed at ~16 months of age, following rosiglitazone administration for ~7 months. Pedersen et al. reported a statistically significant reduction in the IDE mRNA levels in the dentate gyrus, CA2/3, and CA1 regions of the hippocampus in TG-C versus WT or TG-R mice, although there were no reported differences in IDE mRNA levels among the groups in the frontal cortex [1129]. However, in TG-R subjects, rosiglitazone did not affect overall Aβ-plaque burden (thioflavin-S staining), nor did it significantly affect Aβ40 levels, although this study did report a significant reduction in the levels of Aβ42 in the frontal cortex of TG-R subjects versus TG-C mice [1129]. Pedersen et al. considered that due to the normalisation of glucocorticoid levels, rosiglitazone could have improved hippocampal IDE levels and ameliorated the frontal cortex IDE hypo-function in TG-R mice via increased insulin binding to its receptor and/or insulin receptor expression [1129]. However, the authors conceded that in TG-C mice, reduced uptake of insulin into the brain (itself a consequence of peripheral insulin resistance in humans [1357]), could have also contributed to the reduced levels of IDE observed in these studies [1129]. Thus, the beneficial effects of rosiglitazone on the TG learning and memory abilities (as well as the effects on IDE), could have been due to increased uptake of insulin into the brain independent of its effect on glucocorticoid levels [1129].

A striking outcome of the Pedersen studies is the discovery that fasting serum corticosterone levels in the Tg2576 mice were twofold higher than in control animals and furthermore, that this effect could be fully reversed by rosiglitazone treatment [1129, 1244]. This is of major significance given the well-documented detrimental effects of excess corticosteroids on cognition [1358], although glucocorticoids are necessary for normal learning and memory in human populations [1359]. However, since chronically raised glucocorticoid levels have been associated with antagonism of insulin actions and thus, over time the subsequent development of insulin resistance [1075] it provides a mechanistic (albeit provocative) explanation for the beneficial cognitive effects of rosiglitazone that does not depend on the drug gaining access to the brain. Nevertheless, several components of the work by Pedersen et al. [1129] demand critical appraisal, not least of which are concerns over the design and thus validity of the de novo behavioural task used in this study to assess spatial learning and memory (this is discussed in detail in section 3.3.6.1). There are also problems with the

conceptual framework suggested by Pedersen et al. Firstly, the authors correctly acknowledge that IDE indiscriminately degrades both monomeric Aβ42 and Aβ40 [1360], meaning that the selective reduction of Aβ42 in their study suggests a protease other than IDE was most likely responsible for this effect [1129]. Indeed, despite the recovery of IDE mRNA levels in the hippocampus of TG-R mice, this was not the location where a reduction in fibrilar Aβ42 was observed. A reduction of Aβ42 levels was only observed in the frontal cortex of TG-R mice, where IDE mRNA levels did not differ from other groups and the activity of IDE was reported to be less in TG-C mice [1129]. Whilst these data support earlier work showing that IDE hypo-function can contribute to the accumulation of fibrilar Aβ AD [1361, 1362], they fundamentally undermine the hypothesis that reduced IDE expression would in itself, lead to an increase in fibular Aβ, and thus memory impairment by this means. The authors also do not address why if raised glucocorticoids were responsible for the loss of IDE, activity of the enzyme was only significantly reduced in the frontal cortex [1129] despite the considerable expression of the glucocorticoid receptor in the hippocampus, and the established role of the latter in providing feedback to the HPA-axis [1363]. Thus, the exact relationship between the elevated glucocorticoid levels, IDE, and insulin resistance in the transgenic mice is unclear. Furthermore, whilst the pattern of IDE expression by Pedersen et al. [1129], is consistent with results obtained from human AD post-mortem tissue in that levels of neuronal IDE are reduced in the hippocampus [116, 117], they differ from the human studies in that IDE levels are typically elevated in cortical regions in AD patients [118]. Consistent with human clinical data, Leal et al. [114] reported a plaque-associated overexpression of IDE in the cerebral cortex of 16 month old Tg2576 mice with the peak of IDE expression in these mice coinciding with a sharp accumulation of soluble Aβ and massive Aβ deposition into plaques.

2.4.2 Tg2576: Studies by Other Research Groups

Two additional studies have reported effects on cognition (learning and memory) as well as on pathophysiological markers in Tg2576 [1212, 1215], but following a much shorter drug administration time. Using a contextual fear conditioning task, these studies determined the effects of rosiglitazone maleate (Avandia®, GlaxoSmithKline) pulverized into standard rodent diet at 30 mg/kg, on the learning and memory abilities of male Tg2576 mice as well as a group of WT littermates. Across both studies, mice in drug-treated groups received access to rosiglitazone for a 1 month period before

behavioural testing and subsequent sacrifice. In the first study, Rodriguez-Rivera et al. [1215], used the Tg2576 model to test the hypothesis that cognitive improvement in a contextual fear conditioning task would result following 1 month of PPARγ agonism with rosiglitazone, and would correlate with peripheral gluco-regulatory status. Rodriguez-Rivera et al. exposed Tg2576 mice and WT littermates to rosiglitazone initiated prior to, coincident with, or after the onset of peripheral gluco-regulatory abnormalities in TG mice aged 4, 8, and 12 months, respectively. Whereas in TG mice aged 5 months-old (peripheral gluco-regulatory abnormalities absent), and 13 months old (peripheral gluco-regulatory abnormalities present), rosiglitazone did not illicit improvement in a contextual fear conditioning paradigm after one-month treatment despite the drug reversing the peripheral gluco-regulatory abnormalities in the 13 month old mice, the drug did reverse the associative learning and memory deficits as well as peripheral gluco-regulatory abnormalities in 9 month old Tg2576 mice [1215]. These results suggested that the drug mediated cognitive improvement in this group did not correlate with peripheral gluco-regulatory abnormalities per se, but probably reflected age-dependent mechanistic differences underlying cognitive decline in this mouse model [1215]. Unfortunately, the impact of rosiglitazone treatment on total Aβ levels (which would have increased in the 13 month old mice) was not determined.

A subsequent study by Denner et al. [1212], administered rosiglitazone or control no-drug to 8 month old Tg2576 mice WT littermates (male and female). This study reported that regulation of hippocampal PPAR coincided with the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling following rosiglitazone mediated cognitive improvement in TG mice using a contextual fear conditioning task [1212]. Furthermore, in the hippocampal PPAR transcriptome of the Tg2576 mice, the authors found significant overlap between peroxisome proliferator response element (PPRE)-containing PPAR target genes and cAMP response element (CRE)-containing ERK/MAPK CREB target genes [1212]. Using quantitative mass spectrometry and bioinformatics on the hippocampal dentate gyrus, this study then identified many proteins related to synaptic plasticity and memory formation that were induced concomitant with rosiglitazone-mediated cognitive rescue and activation of PPAR and ERK2, and that these actions were reversed when hippocampal PPAR was pharmacologically antagonized, revealing a coordinate relationship between PPAR transcriptional competency and phosphorylated ERK that is reciprocally affected in response to chronic activation, compared with acute inhibition of PPAR [1212]. Thus,

the authors of this study concluded that the hippocampal transcriptome and proteome induced by cognitive enhancement with rosiglitazone harnesses a dysregulated ERK MAPK signal transduction pathway to overcome AD-like cognitive deficits in Tg2576 mice [1212]. This meant that whilst the PPAR signalling system may not be crucial for normal cognition, it can intercede to restore neural networks compromised by AD-like pathophysiological processes. However, these effects in Tg2576 mice have not yet been replicated, and the impact of treatment on IDE or total Aβ levels was again, not determined. In addition, the consumption of feed was not reported in the Denner et al. study, so it is not possible to say what daily dose of compound the mice were receiving. Nevertheless, the authors argued that these rosiglitazone-mediated effects are consistent with a growing body of research which suggests that rosiglitazone is able to cross the BBB in small quantities where it is able to activate CNS PPARγ [1165, 1239, 1364-1367], an effect which occurs in addition to its effects on glucocorticoid levels. Finally, both the Denner et al. [1212] study and that of Rodriguez-Rivera et al. [1215], included a WT control that was also administered rosiglitazone, and reported no adverse effects following 1 month of exposure. However, the effects of longer WT exposure to rosiglitazone have not been reported. This is of potential importance clinically, as not all AD patients have T2D, or indeed, peripheral glucose abnormalities. Finally, it remains an open question as to whether rosiglitazone has varied effects on performance in different cognitive domains in Tg2576 mice, and indeed other mutant APP mouse models. Currently, no studies have reported whether rosiglitazone has effects across multiple cognitive domains within the same study; most evaluating rosiglitazone have been 'one trick pony's' with respect to the assessment of memory. Thus, one of the aims of the current thesis is to assess rodent cognition in behavioural experiments (see Chapter 3) several cognitive domains, including spatial working memory, and anxiety.

Study	Age (months)/ Dose Rosiglitazone (RSG)/ Mode of Delivery	Sex (M/F)	Methods	Main Findings
Pedersen et al., (2004)	30 mg/kg in Diet. Dosing with RSG at 9 Months (MO), for 4 MO. Tested at 13 MO.	M	Serum corticosterone levels: enzyme immunoassay kit; Serum insulin levels: Rat Insulin ELISA kit.	At 8 MO, TG mice had lower basal serum insulin concentrations and delayed insulin-induced reduction in blood glucose levels relative to WT mice. Basal levels of serum corticosterone were similar between genotypes but overnight fasting caused a greater rise in serum corticosterone levels and an excessive reduction in serum insulin concentrations in TG mice. Deficits were absent in 13 MO RSG treated mice. Conclusion: Evidence for relationship between spontaneous insulin resistance, impaired insulin/glucose levels, and aberrant stress responses in TG mice.
Pedersen et al., (2006)	30 mg/kg in Diet. Testing at 13 MO after 15 weeks of RSG dosing, and then again at 14 MO (on diet for 19 weeks (wks). Final testing at 16 MO.	M	8-arm radial maze task. Enzyme immunoassay (corticosterone); Rat Insulin ELISA (insulin); Aβ ELISA (Aβ40 and Aβ42)	After 15 wks of RSG, recovery of spatial and working memory deficits was seen in 13 MO TG mice. However, no difference between groups when TG control mice were given short-term administration of the drug metyrapone at 14 MO. Evidence that increasing insulin sensitivity reduces Aβ load and improves the spatial learning and memory abilities of TG mice.
Rodriguez-Rivera et al., (2011)	30 mg/kg in Diet. TG mice assessed after 1 month treatment with RTZ initiated prior to, coincident with, or after, onset of peripheral gluco-regulatory abnormalities (4, 8, and 12 MO)	M and F	Fear conditioning, Glucose Tolerance Test, Fasted insulin measurement (endocrine multiplex assay)	Whereas 5- MO and 13 MO TG did not gain cognitive improvement after one-month treatment with RTZ, 9 MO TG mice exhibited reversal of associative learning and memory deficits. Peripheral gluco-regulatory abnormalities were improved in 9 and 13 MO TG with RTZ treatment; RTZ treatment had no effect on the normal glucose status of 5 MO TG mice. Conclusion: RTZ-mediated cognitive improvement does not correlate with peripheral gluco-regulatory abnormalities per se, but reflects the age-dependent mechanistic differences that underlie
Denner et al. (2012)	30 mg/kg in Diet. RSG Dosing started at 8 months, tested at 9 MO.	M and F	Fear conditioning	In the hippocampal PPARγ transcriptome, overlap between PPAR response element-containing target genes, and ERK-regulated cAMP response element-containing target genes. Within the TG dentate gyrus proteome, RSG induced proteins with structural, energy, biosynthesis and plasticity functions. RSG-mediated augmentation of PPARγ and ERK2 activity during TG cognitive enhancement reversed when hippocampal PPARγ was pharmacologically antagonized. Conclusion: hippocampal transcriptome and proteome induced by cognitive enhancement with RSG harnesses a dysregulated ERK MAPK signal transduction pathway to overcome AD-like cognitive deficits in TG mice.

Table 2.1: Major studies in Tg2576 using Rosiglitazone

2.4.3 PPAR-γ Studies Using Other hAPP Models

Several studies have investigated the use of rosiglitazone in APP mouse models other than Tg2576, which may shed light on the mechanisms underpinning CNS metabolic dysfunction, as well as the means by which rosiglitazone may have a beneficial effect on these pathologies. Escribano et al. [1214], have shown that $APP_{SWE-IND}$ mice also exhibit significant disruption to the HPA axis, whereby the hyper-secretion of glucocorticoids by 10 months of age results in a significant increase in their plasma corticosterone levels compared to WT littermates [1214]. Likewise, Escribano et al. have shown that rosiglitazone reversed an object recognition deficit in $APP_{SWE-IND}$ mice (which overexpress hAPP with the Swedish K670N/M671L and Indiana V717F familial AD mutations under control of the PDGF promoter) aged ~13 months, as well as reduced glucocorticoid levels (particularly corticosterone levels) [1214]. However, because rosiglitazone also reversed an object recognition deficit in young $APP_{SWE-IND}$ mice in this study when glucocorticoid levels were not increased, it suggests that the preventive effect of rosiglitazone on memory impairment cannot always be attributed to a plasma corticosterone lowering action [1214]. Indeed, PPAR-γ agonists are not reported to have analogous effects on glucocorticoids in humans [1368]. However, some studies have reported a possible relationship between the signalling associated with PPAR-γ activation and the glucocorticoid receptor [1369, 1370]. Furthermore, intra-hippocampal injection of the glucocorticoid receptor antagonist RU38486 in mice [1371], or in rats with glucocorticoid receptor gene deletion [1372], caused impairments in performance on avoidance and spatial memory tasks. Moreover, in $APP_{SWE-IND}$ mice it is apparent that the overexpression of APP and/or the presence of APP-derived fragments is sufficient to induce alterations in hippocampal glucocorticoid receptors independently of plasma corticosterone levels, a process which rosiglitazone reverses at different ages (again independently of glucocorticoid levels) [1214]. These results suggest that mutant hAPP mice manifest memory decline along with an early and lasting reduction of glucocorticoid receptors in the brain and hippocampus in particular - a process which rosiglitazone appears to reverse [152]. Indeed, in doing so, the drug may restore physiological control of the HPA axis to the benefit of learning and memory [1214]. However, this mechanism may require rosiglitazone to be freely available in the brain; since its bioavailability to the CNS is poor [1239], there may be insufficient drug in the brain to drive these glucocorticoid receptors effects.

Finally, it is interesting to note that whilst studies of rosiglitazone treatment in Tg2576 mice have not affected Aβ-plaque burden (by thioflavin-S staining), or Aβ40 levels [1129], another study by Yan et al. [1230] has reported that pioglitazone caused a statistically significant reduction in Aβ40 but not Aβ42 in the brains of Tg2576 mice. (again Aβ-plaque burden was not affected). However, in mice that express the double Swedish and Indiana hAPP mutations ($APP_{SWE-IND}$), rosiglitazone treatment has been shown to not only ameliorate a recognition and spatial memory impairment, but also reduce Aβ burden in the brain where it almost completely removes the abundant Aβ-plaques observed in the hippocampus and entorhinal cortex of 13-month-old transgenic mice, as well as reduce the number of neuropil threads containing phosphorylated tau [1213]. This shows that thiazolidinedione's such as rosiglitazone may have markedly differing effects on AD-like pathology in different APP mouse models. Recently, research has suggested that rosiglitazone, and lithium treatment may, in a TG mouse model that co-expresses APPswe and the exon 9 deletion of the PSEN1 gene ($APP_{SWE}PSN1$), mediate their beneficial effects on memory, Aβ-plaque burden and phospho-tau, by activating Wnt signalling in the brain [1216]. This mechanism possibly involves the increase in β-catenin, and inhibition of GSK-3β [1216]. Whether activation of this pathway occurs in Tg2576 mice is uncertain, as is the reason for why these drugs exert varying effects across different APP mouse models.

Study	Age/Dose/ Mode of Delivery	Sex (M/F)	Methods	Main Findings
Escribano et al., (2009)	5 mg/kg per day RSG administered orally to 1.5 MO APPswe-Ind mice for 10 weeks to prevent the cognitive impairment apparent in 4-MO Tg mice. In other studies, beginning at 9 months of age, hAPPswe-ind mice were daily treated for 4 weeks with rosiglitazone, at the same dose or with the vehicle. Age-matched non-transgenic also received the vehicle.	Unknown	Object recognition. Histological assessment of damage by staining with thionine (Nissl staining). Free floating tissue sections comprising the hippocampal formation were processed for immunohistochemistry (glucocorticoid receptor). Plasma corticosterone levels (enzyme immunoassay kit).	In mutant mice it was found that memory impairment in the object recognition test was prevented and reversed by chronic RSG treatment. An early down-regulation of glucocorticoid receptors GR occurred in the hippocampus that was not related to elevated plasma corticosterone levels, but was prevented by RSG. In parallel with behavioural studies, rosiglitazone also normalized GR levels in older animals. This effect may contribute to explain the attenuation of memory decline by PPARγ activation in an AD mouse model. No impairments were observed in the WT mice receiving RSG.
Escribano et al., (2010)	We treated 9-month-old transgenic mice by oral gavage with rosiglitazone maleate as a suspension in sterile water at a dose of 5 mg/kg/day or with vehicle for 4 weeks prior to testing (object recognition). Groups of animals underwent spatial reference learning in the Morris Water Maze test after 4 and 16 weeks of treatment.	M	Object recognition; Morris Water Maze test. Cortical Aβ42 and Aβ40 levels were measured by using a sensitive sandwich ELISA kit. For p-tau staining, slides were treated with methanol and H2O2 to inhibit endogenous peroxidase activity, followed by blocking with 3% milk in TBS.	Chronic treatment with RSG facilitated Aβ clearance. RSG reduced Aβ burden in the brain and almost completely removed the abundant Aβ-plaques observed in the hippocampus and entorhinal cortex of 13-month-old transgenic mice. In the hippocampus, neuropil threads containing phosphorylated tau were also decreased by the drug. RSG switched on the activated microglial phenotype, promoting its phagocytic ability, reducing the expression of prion inflammatory markers and inducing factors for alternative differentiation. The decreased amyloid pathology may account for the reduction of p-tau-containing neuropil threads, and for the rescue of impaired recognition and spatial memory in the transgenic mice.
Toledo and Inestrosa (2010)	9-month-old APPswe/PSEN1 were administered intraperitoneal (IP) injections of lithium chloride daily (3 mequiv. kg^{-1} IP, daily), dissolved in saline serum, or with rosiglitazone ($3mgkg^{-1}$) in sterile water by oral gavage. Control treatments included IP injections of saline solution or maleate in sterile water by gavage ($0.45mgml^{-1}$).	Unknown	Morris water maze Training was conducted up to 10 trials per day for a total of 4 days, until the criterion of 3 successive trials with an escape latency of < 20 s was met. Immunohistochemical procedures (Aβ-plaque quantification)/ Astroglial GFAP intensity. Immunoblotting (synaptic proteins).	Both RSG and Lithium Chloride significantly reduced (1) spatial memory impairment induced by amyloid burden; (2) Aβ aggregates and Aβ oligomers; and (3) astrocytic and microglia activation. They also prevented changes in presynaptic and postsynaptic marker proteins. Finally, both drugs activate Wnt signalling shown by the increase in b-catenin and by the inhibition of the GSK3. Conclusion: lithium and rosiglitazone, possibly by the activation of the Wnt signalling pathway, reduce various AD neuropathological markers.
O'Reilly and Lynch (2012)	6mg/kg/day in 50 ul administered in maple syrup. 7 MO APPswe/PSEN1 mice, on diet for 2 wks prior to testing and 2 wks during testing.	Unknown Reported	Morris Water Maze. Aβ ELISA (Aβ40 and Aβ42)	Accumulation of Aβ was accompanied by deficit in the reversal phase of learning in the Morris Water Maze, and that treatment with Rosi for a 4 week period, attenuated these changes.

Table 2.2: Major studies in other APP mouse models using Rosiglitazone

3. BEHAVIOURAL EXPERIMENTS

3.1 INTRODUCTION

THE AIM of the experiments in this chapter was to evaluate the changes in behaviour in adult male Tg2576 mice that might arise from continuous rosiglitazone treatment over a period of several months. Historically, the genesis of the programme of research detailed in this thesis originates from the need to further clarify the impact of rosiglitazone in adult male Tg2576 mice following the controversial 2006 publication of research by Pedersen et al. [1129]. It is important to clarify that, at that time no other study had yet been published on the impact of rosiglitazone in hAPP mutants, and only limited human clinical data was available — all of which was generally supportive of a beneficial effect of this drug in patients with early to moderate AD [1235, 1236]. As such, the overall lead hypothesis for this thesis was that rosiglitazone should ameliorate age-related learning and memory deficits as well as pathophysiological changes associated with the hAPP transgene in TG mice. However, subsequent to the 2006 publication or research by Pedersen et al., two other pre-clinical studies have been published which have also investigated the impact of rosiglitazone treatment on cognition and other pathophysiological measures related to hAPP over-expression in Tg2576 [1212, 1215]. As most of the experiments described in this thesis have investigated issues that have not been addressed by these recent studies, the results reported herein still make an original and timely contribution to our understanding of the impact of rosiglitazone on behaviour and amyloid load in adult male Tg2576 mice.

The experiments detailed in this thesis used animals that are subject to procedures classified as “Mild”. The relevant ethical approval had been obtained from the Local Ethical Review Committee (Cardiff University), and permission from the Genetic Modification Safety Committee and the Home Office. Since one of the primary

objectives of this study was to determine the impact of using PPAR-γ treatments to modify aspects of AD-related neuropathology and behavioural impairment, no other alternative to the use of TG mice was deemed viable. As stated earlier, the main disadvantage of the Tg2576 model is the lack of NFT development and neuron loss. However, this model does provide an excellent opportunity to assess the impact of therapeutic interventions on the pathology and behavioural changes associated with a theoretically early stage of AD pathogenesis and one of the leading putative cause(s) of AD: insulin resistance. Behavioural variability in female Tg2576 has been reported in several tasks [1356, 1373, 1374], likely occurring as a function of the estrous cycle [1351]. This is particularly true with respect to cognitive measures such as spatial memory in rodents [1351], as well as assessment of pathophysiological measures such as blood plasma insulin levels, and dendritic spine density in the DG of the hippocampus [1375, 1376], particularly in rodents [1377]) (see Chapter 4). Whilst the pharmacodynamics of many PPAR-γ compounds such as rosiglitazone are considered the same in clinical populations irrespective of sex [1378, 1379], variations in performance and physiology due to oestrogen could potentially confound the interpretation of any drug effects on behavioural performance or the physiological parameters being measured. For this reason, only male Tg2576 mice and WT littermates were used in the experimental studies detailed in this thesis.

3.2 Strategies and Hypothesis

None of the pre-clinical studies assessing the impact of rosiglitazone in Tg2576 mice have used a battery of cognitive and behavioural assessments covering multiple domains [1129, 1200, 1212, 1215, 1244]. Thus, it is unclear if rosiglitazone has a selective or wider range benefit on cognition in these mice. To address this issue, Experiments 1-4 in this chapter, comprise a battery of behavioural tasks that cover established phenotypic cognitive and other behavioural changes in adult Tg2576 mice. Experiment 1 involved the use of the T-maze forced choice alternation task (T-maze FCA task), a well-characterised and hippocampus-dependent test of rodent spatial working memory [1246, 1288, 1380]. Experiments 2 and 3 respectively then detail other behavioural phenotypes inTg2576 mice related to non-conditioned anxiety: the elevated plus maze (EPM) and marble burying task [1381, 1382]. Both of these tasks are popular methods of assessing anxiety-like behaviours in rodents [1383]. Experiment 4

then involved the assessment of behavioural changes related to object recognition. This involved the use of a *de novo* recognition task the design and rationale for which was based on the published research of Good and Hale [1384]. A detailed rationale and methodological procedures relating each of the above behavioural tasks can be found in the appropriate sections of individual experiments. Each of the above behavioural experiments is divided into two treatment strategies: a late intervention strategy and, an early-intervention strategy (see below). Finally, Experiments 5, 6 and 7 are detailed in Chapter 4, and report post sacrifice biochemical/physiological measures. Although Experiment 5 has data corresponding to the late and early-intervention conditions, due to time and financial constraints, it was not possible to do this for Experiments 6 and 7. As a result, these latter experiments relate to the late-intervention and early intervention strategies respectively.

3.2.1.1 Late-Intervention Strategy

Neuronal and behavioural deficits in Tg2576 mice appear to be established in a time-dependent manner, and can be temporally clustered into early deficits observed in 4- to 5-month-old animals, and late deficits that occur in animals after the age of 6-8 months [1245]. Early onset deficits in TG mice occur at ~4 months of age and include a decrease in hippocampal spine density in the DG, LTP, *in vivo* memory as measured by contextual fear conditioning task, and an increase in the ratio of Aβ42 compared to Aβ40 [1245]. Late deficits observed include spatial working memory impairment in tasks such as forced choice T-maze alternation [1288, 1385], which generally emerge between 6–8 months of age [1246, 1385]. At this time, insoluble forms of Aβ are increasing exponentially [1284, 1291], and hippocampal synaptic pathology is well developed [1245]. The initial studies in Tg2576 by Pedersen et al [1129, 1244], as well as most subsequent studies using this mouse model [1212] (but see [1215]), have administered rosiglitazone to adult mice aged 8 months or older when amyloid pathology and synaptic deficits are already well established. However, although all these have reported beneficial effects on learning and memory, the publication of research by Pedersen et al. [1129, 1244], has been controversial [1166]. Thus, further clarification of the impact of rosiglitazone in adult male Tg2576 mice was required. To achieve this, in the first set of experiments involve a late intervention strategy (LINS) in which chronic rosiglitazone administration took place from the age of ~9 months through to 18 months old (see figure 3.1). Following initial pre-drug conformation of

a cognitive phenotype at 8 months of age, it was predicted on the basis of findings from Pedersen et al. [1129, 1244], that rosiglitazone administration would then reverse this age-related cognitive and other behavioural deficits observed in male Tg2576 mice, as well as lower amyloid load.

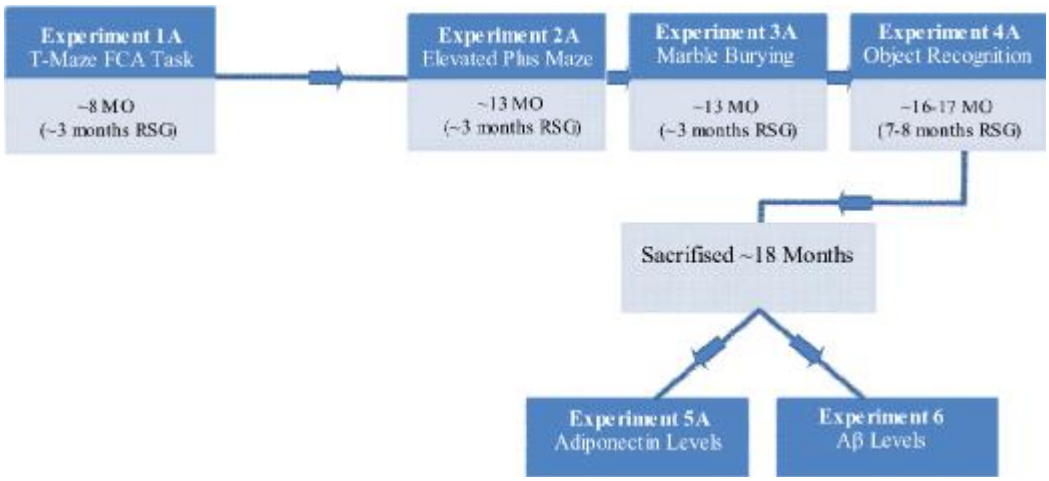

Figure 3.1: Summary of LINS Design. Key: MO= Month Old; RSG = Rosiglitazone. TG and WT mice were allocated to one of four experimental groups at ~9 MO [TG-non-drug (TG-N), TG-Rosiglitazone (TG-R), WT-non-Drug (WT-N) and WT-rosiglitazone (WT-R)] following completion of the pre-drug stage of Experiment 1 (this experiment was comprised of three stages see Figure 3.3), which assessed spatial working memory via the T-Maze FCA paradigm. Following the conclusion of Experiment 1 at 13 MO, the mice received Experiments 2 and 3 after a short break (Elevated Plus Maze and the Marble Burying task respectively), both of which assessed non-conditioned anxiety and behavioural disinhibition. The behavioural battery concluded with Experiment 4, which assessed memory for object recognition and object location. Following the sacrifice of the mice at the age of ~18 months, Experiments 5 and 6 were conducted. Experiment 5 assessed adiponectin protein levels from terminal blood plasma samples, whilst Experiment 6 assessed total Aβ levels (soluble + insoluble) extracted from homogenates of single brain hemispheres.

3.2.1.2 Early-Intervention Strategy

Some pre-clinical studies have raised concerns that rosiglitazone may have a limited therapeutic window for ameliorating cognitive impairments in adult hAPP mice [1215, 1386]. For example, as discussed in chapter 2, Rodriguez-Rivera et al. [1215], used the Tg2576 model to test the hypothesis that cognitive improvement in a contextual fear conditioning task would result following 1 month of PPARγ agonism with rosiglitazone and would be correlated with peripheral gluco-regulatory status. Rodriguez-Rivera et al. reported that whilst in TG mice aged 5 months-old and a separate cohort of TG mice aged 13 months, rosiglitazone did not elicit learning and memory improvement despite reversing peripheral gluco-regulatory abnormalities in the 13 months old mice. However, rosiglitazone treatment did reverse both peripheral gluco-regulatory abnormalities and associative learning and memory deficits (contextual fear conditioning) in 9 month old Tg2576 mice [1215]. These results are consistent with those of Nicolakakis et al. [1386], who using a different hAPP mouse model reported that in TG mice started on the PPARγ agonist pioglitazone at ~14 months for 6–8 weeks, physiological changes occurred (normalised glucose uptake in response to neuronal activity and attenuated astroglial activation), in the absence of recovery of a spatial memory deficit in these mice. Taken together these studies suggest that in order to be maximally effective, PPARγ agonists may have to be administered early, prior to the occurrence of significant amyloid pathology and synaptic deficits. However, to date none of the published studies conducted in Tg2576 mice have reported the impact of the drug on the appearance of late-stage age-related cognitive deficits following continuous administration of the drug from an early time point when amyloid and synaptic pathology in TG animals would be expected to be minimal [1242, 1245] and some measures of cognition would be comparable between transgenic and non-transgenic mice [1246]. Thus, in a second course of experiments, this thesis reports an early-intervention strategy (EINS) to investigate the impact of rosiglitazone administration on the established appearance of age-dependent cogitative deficits in 8 month old TG mice following continuous drug administration from the age of 5 months (see figure 3.2). It was predicted that rosiglitazone would delay the onset of age-related cognitive and behavioural deficits in adult TG mice. In each experiment, the result of experimental manipulations is presented chronologically: LINS data is presented first, followed by ENIS. To avoid repetition, discussion material related to each is reserved until after the presentation of both LINS and EINS results.

Figure 3.2: Summary of EINS Design. Key: MO= Month Old; RSG = Rosiglitazone. TG and WT mice were allocated to one of four experimental groups at 5 MO: TG-non-drug (TG-N), TG-Rosiglitazone (TG-R), WT-non-Drug (WT-N) and WT-rosiglitazone (WT-R), and run through the same battery of behavioural tasks used in the LINIS condition. However, in order to address potential concerns on T-Maze FAC performance due to the order in which this task and the anxiety tests were given to mice in the LINIS condition, the presentation of tasks was counterbalanced in ENIS such that anxiety related assessments were conducted first (Experiments 2B, 3B), followed after a short break by T-Maze testing (Experiment 1B) and then object recognition (Experiment 4B).

3.3 EXPERIMENT 1: SPATIAL WORKING MEMORY

3.3.1 Introduction

SPATIAL MEMORY is assessed in rodents by a variety of means, but most commonly via the T-maze, Y-maze, Morris water maze, radial arm maze, radial arm water maze, and Circular platform tasks such as the Barnes maze [1387]. All of these tasks measure a range different memory types in rodents, including 'reference' and 'working' memory. Reference memory can be thought of as retained information that is useful across all exposures to the task (i.e., on any day of testing). In the Morris water maze, for example, reference memory involves subjects learning the location of a fixed position platform or reward over the course of the test (e.g. a trial-independent representation), with successful completion of the task requiring amongst other things, the use of LTM in the process of navigating to the 'goal' location [1388]. In rodents, working memory is usually considered to relate to a representation of an object, stimulus, or spatial location that is typically used within a trial (such as T-maze forced-choice alternation), but not between sessions, to guide behaviour [1389].

3.3.1.1 Spatial Memory Impairment in Tg2576

Most studies have found that Tg2576 mice older than 14 months have significant memory impairment, although disagreement exists with respect to the exact time these deficits first become evident [1242, 1251, 1253, 1284, 1289, 1356, 1390]. Hsiao et al. [1242] reported that that in 9–10 month TG mice (sex not reported), spatial memory impairment was revealed via decreased alternation in a Y-maze paradigm, and impaired performance in a Morris water-maze task, whereas there was no deficit in these tasks at 2–6 months of age. This is broadly consistent with a study by Barnes et al. [1246], which reported that in male Tg2576 mice, deficits in a T-maze spatial navigation task generally emerges at approximately 6–8 months of age. Indeed, this study showed that whilst mutant male mice were able to process both intra-maze and extra-maze stimuli, they exhibited a deficit in forming an allocentric representation of their environment [1246]. It is of interest that during this time period, the production of insoluble and soluble forms of Aβ in cortical and hippocampal areas is increasing [1284, 1291]. However, using equal numbers of males and females, King and Arendash [1356] failed to find any overall TG deficit on Morris water-maze acquisition and retention from 3 months to 19 months. This finding was consistent with the outcome

of a previous study [1391], which reported that the combined data from equal numbers of aged males and females failed to deliver a significant TG deficit in either Y-maze alternation, water maze acquisition, passive avoidance, or active avoidance testing between the ages of 3 to 9 months old. However, results from other tasks in this study revealed substantive behavioural deficits in TG mice that were gender-dependent and sometimes progressive in nature. Thus, between 3 and 9 months of age, a progressive spatial impairment was observed in a circular platform task by TG males, as was a progressive deficit in visible platform testing for all TG animals [1391]. Other transgenic effects included both impaired water maze retention and circular platform performance in 3 month old TG females. This later effect was responsible for an overall (males + females) TG deficit in circular platform performance at 3 months of age [1391]. Further, sensorimotor testing revealed that TG males were more active in both open field and Y-maze at 3 months of age [1391]. Nevertheless, Westerman et al. [1284], reported that in Tg2576 mice aged between 4 and 25 months (sex not reported), a deficit in the Morris water-maze started to appear at around 6 months, whereas Ognibene et al. [1252] found that in TG mice (sex not reported) spatial memory impairment was shown in the Y-maze at 7–12 months. Spatial memory impairments have also been found in Tg2576 mice prior to 6 months when the brain is typically free of amyloid plaques and deposits [1356, 1391, 1392], providing one reason for seeking an early intervention strategy in Tg2576 mice. Thus, whilst not all studies have confirmed spatial memory defects in Tg2576 mice, those which have tend to report that they do not normally appear before 6 months but are usually present by 9 months.

Part of the variability in these studies is that the hAPP transgene may have interacted with sex differences in some spatial memory and other behavioural tasks. Additional factors which may have affected behavioural performance of mice in some tasks, including differences in the genetic background in Tg2576 and WT mice, and in particular the proportion of SJL background strain [1381]. Using in-bread strains also increases the risk of inadvertently "breeding in" unfavourable behavioural traits and/or physical defects which may affect behavioural performance. For example, the retinal degeneration Pde6brd1 (RD) mutation can be a major pitfall in behavioural studies using Tg2576 mice bred on a B6:SJL genetic background as this can potentially compromise tests of spatial memory and recognition memory processes [1393].

A previous study by Pedersen et al. in adult male Tg2576 has suggested that rosiglitazone is able to reverse the spatial memory deficit [1129], and is supported by

findings in other hAPP mouse models [1216, 1217]. In addition, rosiglitazone has also been shown to reverse a spatial memory deficit in aged rats fed a high fat diet [1211]. However, with the exception of Pedersen et al. [1129], all these studies have used the Morris water maze to assess spatial cognition. The Morris water maze is a classic task for testing spatial memory in rodents, and is acknowledged to be dependent on intact hippocampal function [1394-1396]. Indeed, the Morris water maze task was used in the original study characterizing the Tg2576 mouse [1242]. However, there are other popular ways of assessing spatial memory in rodents, and if rosiglitazone does reverse the spatial memory impairment in Tg2576 mice, it should do so whatever standard measure of rodent spatial memory is used.

3.3.1.2 T-Maze Forced Choice Alternation

T-Maze Forced choice alternation (FCA) and left-right discrimination tasks using the T-maze have been widely used to assess working memory [1389], and reference memory [1383, 1397, 1398], respectively in rodents. These tasks are based on the premise that animals have evolved an optimal strategy to explore their environment and obtain food with a minimum effort [1389, 1397]. Furthermore, in the absence of food-deprivation, a simple T maze can be used as described to assess spontaneous alternation, and is based on the willingness of rodents to explore a new environment [1397] (i.e. they prefer to visit a new arm of the maze rather than a familiar arm). All of these tasks are relatively easy to administer and depend on intact alternation behaviour [1398]. Damage to the hippocampus causes severe a spatial memory deficit [453], and the T-maze FCA version is widely considered to be dependent on intact hippocampal and prefrontal function in rodents [1380, 1396, 1399]. Indeed, the T-maze FCA task has been shown to be sensitive to the $hAPP_{SWE}$ mutation in an age-dependant manner [1246], probably as a function of its sensitivity to Aβ-related hippocampal dysfunction [1385]. A transgene-related impairment in the T-maze FCA task has been reported from the age of 8 months in Tg2576 mice [1246], as well as in TG mice aged 12 and 16 months [1246, 1251, 1288]. Furthermore, the likelihood of detecting significant impairment in Tg2576 mice relative to age-matched controls has been found to be highest with the use of T-maze FCA and the radial arm water maze tasks compared to other popular methods of assessing spatial memory in rodents [1400]. For all of these reasons the T-maze FCA task was considered a suitable means of assessing the effects of rosiglitazone treatment on spatial working memory in the present study.

3.3.1.3 Experimental Design

Experiment 1A (LINS) aimed to assess spatial working memory in adult male Tg2576 mice and WT littermates using the T-Maze FCA paradigm between the ages of 8 to 13 months. It was expected that Tg2576 mice would show a persistent deficit on this task throughout this time [1246, 1251, 1385]. This experiment consisted of three stages: a single pre-drug stage and two post-drug stages (see figure 3.3). All three stages involved the application of a separate T-maze FCA task. The pre-drug stage was conducted when mice were aged between 8 and 9 months. The objective here was to provide confirmation of phenotype on the T-maze FCA task. Following the completion of the pre-drug stage, groups of TG and WT mice were immediately allocated to drug-treated or non-drug treated conditions and started on their respective diets (see section 3.3.2.1). Post-drug 1 took place 10 days after the start of drug regimens, when the mice were aged between 10 and 11 months. The objective in this stage was to check for any early drug effects as this had not been determined in the original Pederson study [1129] upon which the current thesis is based. However, subsequent studies have reported effects on learning and memory in Tg2576 mice following 4 weeks of continued drug administration [1212, 1215]. Mice were then re-tested at post-drug 2 after ~3 months exposure to experimental diets. This stage took place when the mice were aged between 12 and 13 months (see figure 3.1). The aim of

Experiment 1B (EINS) was to assess if rosiglitazone exhibited a protective effect in relation to the age-related T-maze FCA impairment in 8 month old Tg2576 mice following continuous access to the drug from the age of 5 months. In both interventions, the same drug groups and controls were used, with the mice run in pseudorandom order across several staggered replications of no more than 18.

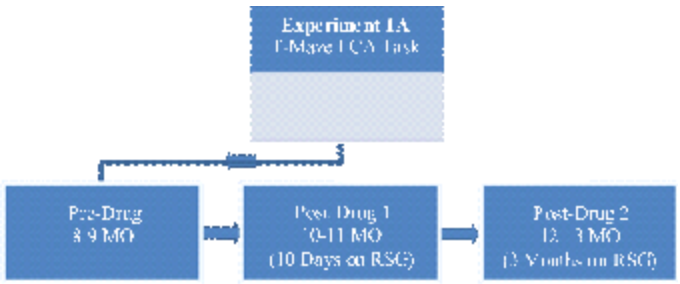

Figure 3.3 Experiment 1A. Starting with a pre-drug (no RSG) assessment at 8 MO, the mice were allocated to one of four experimental groups: TG-non-drug (TG-N), TG-RSG (TG-R), WT-non-Drug (WT-N) and WT-RSG (WT-R). Spatial working memory was then re-assessed at Post-drug 1after 10 days on RSG, and again at Post-drug 2 after ~3 months RSG administration.

3.3.2 Methods and Apparatus

3.3.2.1 Animals and Drug Administration

All mice were maintained on a 12-h light/dark schedule (lights off at 1500 hours), with ad libitum access to food comprised of standard pelleted rodent chow laboratory chow (RM1 Rodent, Expanded Pelleted, SDS, England), or the powdered equivalent (RM1 Rodent Expanded Ground, SDS, England). With the exception of Experiment 1, which required male mice to be water deprived for 22 hours prior to testing in order to maintain motivation to find a sucrose reward, all mice received ad libitum access to water. Where mice were water deprived they were maintained at 85% of free feeding weight, and weighed daily in order to ensure good general health. All behavioural experiments began at 09:00 each day during the light phase, and were completed in full compliance with the U.K. Animals (Scientific Procedures) Act 1986, and Home Office (United Kingdom) guidelines. The running order of mice during behavioural experimentation was counterbalanced for genotype and diet.

LINS Experiments

Animals used in LINS Experiments used 68 experimentally naive heterozygous male Tg2576 mice and 47 non-transgenic WT littermates (cohort 1), all of which were purchased from Taconic (Taconic Farms, Germantown, NY). Tg2576 mice contain a hamster prion protein promoter in their DNA insert in order to drive strong expression of the hAPPSwe transgene. This model was created by microinjecting the human APP695 gene containing the double mutation K670N, M671L into B6SJLF2 zygotes using a hamster prion protein cosmid vector. The resultant mice from founder line 2576 were backcrossed to C57BL/6. In March 1999 Taconic Farms (Germantown, NY) received stock of Tg2576 from the Mayo Foundation [25], wherehence hemizygous males were backcrossed with C57BL/6NTac for derivation by embryo transfer. This colony is maintained by mating hemizygous male mice with B6SJLF1 female mice. Taconic received stock of Tg2576 from the Mayo Foundation in March 1999 [1282], wherehence hemizygous males were backcrossed with C57BL/6NTac for derivation by embryo transfer. The colony is maintained by mating hemizygous male mice with B6SJL F1 female mice. Animals belonging to cohort 1 were received in the laboratory at the age of 2 months from Taconic Farms (USA), where they were initially housed in standard plastic mouse cages in same genotype groups of three or four. Each cage contained wood shaving bedding and cardboard tubes to provide some environmental

enrichment. The mice had been ear punched by Taconic Farms for identification prior to their arrival. At the age of 4 months all mice were singularly re-housed in standard mouse cages and their pelleted food exchanged for the powdered equivalent in preparation for experimental studies. Behavioural testing of mice from cohort 1 took place between the ages of 8 to 17 months. Cohort 1 mice were then immediately sacrificed at 18 months, and the necessary tissues harvested for biochemical analysis. The resulting tissue samples were labelled and stored at -20°C until further use. Independent genotyping of mice from cohort 1 were confirmed by GSK via a subsequent Aβ ELISA analysis following termination. The rationale for this was that any WT mice in the sample would produce a null result in the ELISA, as they contained no Aβ-plaques. Whilst this was an unusual means of confirming the genotype of these mice, it did establish that the mice had been assigned to the correct groups based on their genotype at receipt.

Drug Administration: Two previous studies have assessed the impact of rosiglitazone in WT mice [1212, 1215], and reported no significant impact of the drug on behavioural performance or peripheral gluco-regulatory mechanisms, although the impact of chronic exposure to the drug in WT littermates has not been determined. LINS Mice were allocated to drug-treated or non-drug treated groups and started on their respective diets between the ages of ~8-9 months after the completion of each pre-drug replication of Experiment 1A. Mice were allocated to experimental groups according to their mean pre-drug performance so that within genotype performance was matched across drug-treated and control groups (this would make post-drug performances easier to interpret). TG mice allocated to drug-treated conditions received powdered lab chow supplemented with research grade drug compound, either the partial PPAR-γ agonist GW855266X (TG-B, *n*=12) at 30mg/kg, PPAR-delta agonist GW610742 (TG-A, *n*=12) at 10mg/kg, or rosiglitazone at 10mg/kg (TG-R, n= 12). TG mice allocated to the control condition received un-supplemented powdered lab chow (TG-N, *n=12*). WT mice in the control condition also received un-supplemented powdered lab chow, whereas WT mice allocated to a drug-condition received powdered lab chow supplemented with rosiglitazone at a concentration of 10 mg/kg (WT-R, *n*=12). The decision to include a WR-R control was justified on the basis of pharmacokinetics and pharmacodynamics as both can differ among genetically engineered lines. Thus, the WT-R control provided a means by which target drug effects could be validated in relation to adiponectin levels, which should be similarly,

elevated across all PPAR treated groups (see chapter 4). TG-A and TG-B groups were run with the experimenter blind to the identity of the compound as part of an outsourced pilot study for GSK, the results of which were not intended to be disseminated as part of this thesis. Behavioural testing of TG-A and TG-B groups was abandoned midway through Experiment 1A due to concerns over the poor tolerability of these compounds in the mice. As a result, both these groups were dropped from the study and are not considered further here (these conditions are mentioned here merely for completeness). All mice were maintained on their respective treatment regimens until sacrifice. All PPAR compounds were supplied by GSK pharmaceuticals, Harlow UK.

EINS Experiments

The Tg2576 model has been made widely available to research institutions for non-profit research purposes, enabling the formation of multiple Tg2576 mouse colonies, one of which is maintained by Professor M.A. Good, Department of Psychology, at Cardiff University. The animals used in EINS Experiments were derived from this colony, and were comprised of 24 experimentally naive heterozygous male Tg2576 mice and 25 non-transgenic WT littermates (cohort 3), all of which were specifically bred from the colony maintained by Professor Mark Good at Cardiff University. The Tg2576 line was originally established by crossing a C57Bl/6j x SJL F3 founder twice into C57Bl/6j [1288]. Overexpression of the transgene was maintained in subsequent generations by crossing heterozygous Tg2576 to a C57Bl/6j x SJL F1 line, with each generation possessing differences in the relative %age of each of these background strains. It is estimated that the relative contributions are ~59% to 88% of C57Bl/6j; 12% to 41% of SJL [1288]. This cohort of mice was generated by pairing male heterozygous Tg2576 mice with female C57Bl/6j x SJL F1 mice, with the resulting offspring being either heterozygous Tg2576 mice possessing the mutation, or homozygous Tg2576 wild-type (WT) mice, at a ratio of roughly 50:50. Fifteen days after birth, the mice were ear-marked for identification (mouse ears are not normally thinned out sufficiently for ear punching before this time), and <0.05cm of distal the tail section was taken as a tail-biopsy for subsequent genotyping and PCR analysis (see chapter 4 for details) in order to determine genotypic status. Tail biopsies (<0.05cm) were removed using ethyl chloride local anaesthetic and a silver nitrate pen to seal the incision, with tissue samples stored at -20°C until further use. Following PCR

genotyping, at approximately 6 weeks of age, litters were weaned into standard plastic mouse cages of same-sex same-genotype littermate groups of 2 to 4 mice. At the age of 4 months all male mice were singularly re-housed in to standard plastic mouse cages and their pelleted food exchanged for the powdered equivalent. Female mice were maintained on the pelleted food as these were not used in primary studies.

Drug Administration: As stated previously, the TG and WT mice in the ENIS experiments were all allocated to experimental drug and non-drug groups at the age of ~5 months. The groups chosen were representative of those used in the LINIS condition except for the TG-A and TG-B groups. Thus, TG and WT mice allocated to the rosiglitazone condition received powdered lab chow supplemented with research grade rosiglitazone at a concentration of 10 mg/kg (TG-R, *n*=12; WT-R, *n*=13), whereas mice allocated to control groups again continued to receive un-supplemented powdered laboratory chow (TG-C, *n*=12; WT-C, *n*=12). As indicated in figure 3.1, mice in the EINS condition were maintained on their respective treatment regimens until sacrifice between the ages of 12 and 13 months. The necessary tissues were then harvested for biochemical analysis, with tissue samples again labelled and stored at -20°C until further use. Terminal tail biopsies (<0.05cm) were taken for independent genotyping (see chapter 4).

Preparation of Experimental Diets and Genotyping

Batches of lab chow dosed with PPARγ compound were prepared according to GSK standards. In brief, appropriate amounts of drug were weighed using an analytical weighing machine, and then added to a small amount of 1kg of powdered diet, and thoroughly mixed for 30 seconds using a standard coffee grinder. The contents from the grinder were then transferred to the remainder of the 1kg batch of feed, and mixed for a further 30 minutes in a standard food mixer. This ensured that the drug was distributed equally throughout the feed. Several batches of 1Kg drug supplemented diet were prepared in advance, and stored in a fridge at 2°C until required. All drug-supplemented diet was replaced in mouse food hoppers every 3 days to ensure no stale compound was consumed by the mice. For each mouse, the average daily food consumption was calculated over several weeks during periods of ad lib food and water access in order to determine the degree of consumption and thus, self-dosing in drug groups. Daily intakes, adjusted for body weight, were derived by dividing average intakes by the average body weight, and then multiplying the result by 30g (the

approximate weight of an adult mouse). Daily food consumption was approximately the same in each group across both interventions, ranging from ~4.5 to 6.3 ± 0.1 g/day/mouse. This meant that TG-R and WT-R mice were dosing themselves between approximately 4.5 mg/kg/day, to 6.3 mg/kg/day. The lower band of these figures was comparable with a previous report in Tg2576 using a similar delivery method [1129].

Genotyping Procedure

Tissue biopsies from mice were genotyped using the polymerase chain reaction (PCR) molecular biology technique in order to determine which mice harboured the human double APPswe mutation. PCR selectively amplifies specific deoxyribonucleic acid (DNA) regions exponentially for measurement using gel electrophoresis. In the in-house protocol used here, specific oligonucleotide primers were used to selectively amplify the nucleic acid sequence coding the 63 APPswe transgene. The selective DNA amplification in PCR occurs through enzymatic replication, and utilises the repeated thermal cycling of a DNA sample containing multiple reagents that include DNA polymerase, deoxynucleoside triphosphates (dNTPs) and oligonucleotide primers. Please see section 3.3.2.1 for details of mice used for genotyping procedures.

Methods: In preparation for the PCR DNA was first digested and extracted from the tissue samples. Defrosted tissue samples were digested by incubating in 600µl of TES cell lysis buffer (1M Tris HCl Buffer pH 8.0, 0.5M EDTA pH 8.0, 10% SDS, dH20) at 55°C for 20 minutes. Following this 1.5µl of 25mg/ml Proteinase K (Sigma, Cat. P2308) was applied to each sample, vortexed, and incubated overnight at 55°C. DNA was then extracted by adding 200µl of 5M ammonium acetate, which was vortexed and centrifuged for 10 minutes at 14,000 rpm to pellet the proteins. 650µl of supernatant was removed carefully so as not to dislodge the pellet, and transferred into 600µl of molecular biology grade isopropanol. This solution was mixed using inversion and centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded, and 150µl of ice cold 70% molecular biology grade ethanol was applied to the DNA-containing pellet, and then centrifuged for 2 minutes at 14,000 rpm. Following a repeat of the latter process, the supernatant was then removed and the samples dried at 37 °C until the ethanol was evaporated. DNA was re-suspended in 60µl of TE buffer (1M Tris HCl pH 8.0, 0.5M EDTA pH 8.0, dH20) and incubated overnight at 55°C. PCR was then carried out on the DNA samples within 24 hours or stored at-20°C until use.

PCR analysis: 1 µl of DNA sample was added to 24µl of master mix to make a 25µl solution. The samples and master mix were maintained on ice during this process. Master mix was prepared using 2.5µl 10x reaction buffer, 1.25µl 50mM $MgCl_2$, 1.25µl 10mM dNTPs, 0.25µl Primer 1501 (50 pmol), 0.25µl Primer 1502 (10 pmol), 0.25µl Primer 1503b (10 pmol), 1.5µl BIOTAQ Red DNA Polymerase (1µg/µl) and 16.75µl nuclease-free MilliQTM water (Millipore, USA). The DNA polymerase 10x reaction buffer and $MgCl_2$ were supplied in BIOTAQ Red DNA Polymerase kit (Bioline, Cat. BIO-21041). The oligonucleotide primers 1502 (5' 600bp) and 1503b (5' 450bp), were then used to amplify the APPswe transgene, while primer 1501 (5' 600bp) amplified the endogenous murine prion protein (PrP) with primer 1502. The 25µl solution for each sample was vortexed and centrifuged for 6 seconds at 4000rpm. Each sample was placed into the PCR cycle machine alongside one positive TG control (1µl of previously confirmed TG DNA, plus 24µl master mix), one negative WT control (also 1µl of previously confirmed WT DNA, plus 24µl master mix), and one blank control (comprised of 1µl nuclease-free water plus 24µl master mix). The blank control was a necessary means of detecting any potential contamination. The PRC cycler was programmed to carry out APPswe transgene amplification through initialisation at 72°C for 2 minutes followed by 36 cycles of: 1) denature at 94°C for 1 minute, 2) anneal and amplify at 62°C for 1 minute, and 3) incubate at 72°C for 2 minutes for elongation, with 2 seconds added to the elongation step per cycle. Samples were then maintained at 4°C in the PRC cycler until collected for electrophoresis.

Gel electrophoresis of DNA. Gel electrophoresis was used to separate out each of the DNA amplification products. A 17-tooth-combed 1.5% agarose gel was made using 3g NuSieve® molecular grade agarose (Fisher scientific, Cat. BMA50091) in 200ml of TAE buffer (40 mM Tris base, 1 mM EDTA pH 8.0, 0.01% v/v glacial acetic acid, dH_2O) with 20µl of SYBR® Safe DNA gel stain (Invitrogen, Cat. S33102). Once set the gel was placed into a RunOne system® gel tank with TAE buffer, then 10µl of DNA marker, 8µl of each sample and 8µl of each control was applied into separate tooth-comb slots. The DNA marker was made from the following: 10µl of 'DNA Marker' (Promega, Cat. G3161); 2µl "Orange G solution" (comprised of 0.25% Orange G, and 15% Ficoll in MilliQTM water). This was then vortexed thoroughly, and centrifuged for 10 seconds at 4000rpm. The gel tank was then run at 100V for approximately 20 minutes in order to drive the electrophoresis process. Results were obtained by observing and image of the

gel under UV light. A representative example of a gel electrophoresis can be seen in Appendix 2.

3.3.2.2 Apparatus

The T-Maze was constructed from three arms made of clear Perspex, each of which measured 9 cm wide and 13 cm high. The floor of the maze was made from brown melamine wood. The start arm was 52 cm long, with each of the goal arms measuring 26 cm long. The maze apparatus was housed in a quiet testing room on top of a green felt-covered table that was 92 cm high (see figure 3.4). The testing room was well illuminated and contained a variety of extra-maze cues (posters and laboratory benching). Guillotine doors were used to block the start and goal arms. Mice ran for a reward of 50 µl of 10% sucrose solution in tap water, which was placed in a food-well at the end of each goal arm. The arms of the maze were wiped down with a 1:20 dilution of Mr Muscle® glass cleaner with water, and dried with paper towelling, prior to placing each mouse in the apparatus. The experimenter wore surgical gloves throughout the experiment to reduce odour contamination as this could inadvertently provide a cue to animals during trials.

Figure 3.4: T-Maze apparatus insitu.

3.3.2.3 Behavioural Procedures

Mice received an initial habituation phase comprised of one 5 minute trial per day for 3 consecutive days, during which all arms of the maze were open and sucrose reward was placed only in the food wells. If the reward was not consumed within 5 minutes, the mice received a further 5 minutes in the T-maze. Mice were then tested over 8 consecutive days and received 6 pairs of runs per day, each of which was no more than a maximum of 10 minutes duration. On the first run of each trial (sample run), both goal arms were baited. However, a removable opaque Perspex door blocked access to one of the arms. The mouse was released from the start box and allowed to enter the

open goal arm and consume a sucrose reward (~50 µl 10% sucrose) for 10 seconds before being removed and replaced in the start box. A response was defined as an entry when the rear of the body (excluding the tail), passed the entrance to the arm. The maze (including both goal and non-goal arms) was then cleaned to obscure any potential intra-maze odour cues. On the second run of each trial (choice run), which followed within 5-10 sec of the first run, both goal arms were now open and the mouse was rewarded for correctly choosing the previously unvisited arm (now the only arm to contain a reward). If an incorrect choice was made, the entrance to the arm was blocked by a Perspex door and the mouse left in the non-baited arm for 15s before being returned to its home cage. The location of the sample arm (left or right) was varied pseudo randomly across the session such that mice received three left and three right presentations, with no more than two consecutive trials with the same sample location in each session. The order of left and right presentations was counterbalanced across days, and in the case of the LINS, across experimental stages, with mice run in squads of 8 in each replication so as to maintain an inter-trial interval (ITI) of approximately 21 min. In both the habituation and testing stages, mice were transported to the test room in individual home cages and acclimatised to the room for 10 minutes prior to each habituation and testing session taking place.

Scoring and Exclusion Criteria: During testing, T-maze performance was calculated as a %age of correct trials (successful alternations) of the six trials conducted per day. Mice were excluded from analysis due to morbidity/mortality, or if they spent more than three consecutive trials where they failed to move from the start position in the T-maze for the first 5 minutes (non-responsiveness). Based on this criterion, in Experiment 1A, 20 TG mice (TG-C, *n*=3; TG-R, *n*=3; TG-A, n=8; TG-B, n=6), and 5 WT mice (WT-C, *n*=2; WT-R, *n*=3) were excluded from cohort 1 due to mortality; 1 TG (TG-C), and 2 WT mice (WT-C, n=1; WT-R, n=1) were excluded for non-responsiveness. Although the mortality rate in Tg2576 is generally known to be greater over the first 12 month of life, the mortality rate in Experiment 1A seems particularly high, and is driven primarily by the high numbers of mice lost in the TG-A and TG-B conditions.

Thus, excluding TG-A and TG-B groups, the total numbers of mice in Experiment 1A were as follows: 17 TG mice (TG-C, n=8; TG-R, n=9), and 18 WT (WT-C, n=10; WT-R, n=8). In Experiment 1B, none of the mice were excluded due to mortality or non-responsiveness. Thus, the total numbers of mice in Experiment 1B were as follows: 24 TG mice (TG-C, n=12; TG-R, n=12); 25 WT mice (WT-C, n=12; WT-R, n=13). Although

one can see that strictly speaking, the rate of mortality is not comparable in the LINS and ENIS conditions, excluding the TG-A and TG-B groups in the LINIS condition, this could just have been chance as housing conditions and care remained the same across both cohorts of mice.

3.3.2.4 Statistical Analyses

All statistical analysis were carried out using an analysis of within-subjects variance (ANOVA) using the statistical package IBM SPSS Statistics v.21.00, with an alpha value (p) <0.05, taken as being statistically significant. Each of the three stages of Experiment 1A had previously been analysed as separate ANOVAs, each of which involved the 8 days of performance data being arranged in and analysed as, four test blocks, each of which contained the number of correct trials calculated across 2 days of testing. However, for the purposes of this study, Experiment 1A was analysed as a single three-way repeated measures ANOVA of the form: Genotype (TG or WT) and Treatment (drug-treated, or non-drug treated) as between-subject factors, and Stage (mean number correct choices calculated across the 8 days of testing) as the within-subject factor. The conclusions of this analysis were the same as that achieved via the separate blocked analysis of each stage, and in an overall ANOVA, there was no effect of block, and no interactions involving this factor (max F). However, since Experiment 1B was comprised of a single stage, the data was analysed as a three-way repeated measures ANOVA of the form: Genotype and Treatment as between-subject factors, and Block (4 test blocks, each containing the number of correct trials calculated across 2 days of testing), as the within-subject factor. Following significant interactions, tests of simple main effects were carried out using MANOVA syntax as described in Kinnear and Gray [1401] (p. 295, 350). Sidak correction was used for all multiple comparisons in order to guard against making Type 1 errors [1401]. In aggregate, these measures follow the conventional means for reporting statistical analysis in the sciences (p.373, [1402]).

ANOVA Assumptions: The assumptions of each 'within-subjects' ANOVA were also tested within the statistical package, and included tests for normality, homogeneity of variance, and sphericity. Normality was tested using the Shapiro-Wilk test; if this assumption was found to be violated ($p<0.05$), then the kurtosis value was checked. A perfect normal distribution will have a kurtosis value of '3.0', with skewness close to '0.' Kurtosis is positive if the tails are "heavier" than for a normal distribution (i.e. right 'positive' skewed distribution), and negative if the tails are "lighter" than for a normal

distribution (i.e. left 'negative' skewed distribution). If kurtosis is less than 3 it is platykurtic (flatter), and the lower it is, the flatter the distribution will be. If kurtosis is more than 3 it is classed as leptokurtoc (more peaked), with higher values representing a more 'pointed' distribution. Whilst ANOVA can tolerate data that is non-normal (skewed or kurtotic distributions) with only a small effect on the Type I error rate, platykurtosis can have a profound effect when group sizes are small [1401]. Thus, if platykurtosis was present, the ANOVA result was reported only after appropriate transformation of the data, or by using the less powerful non-parametric statistical tests (NPTESTS) which do not require the assumption of normality (see below). The assumption of homogeneity of variance was tested within SPSS by using Levene's test of equality of error variances. If this assumption was found to be violated ($p<0.05$), then non-parametric tests were used. However, if on these occasions the results of the non-parametric tests (see below) were of the same pattern of significance as reported by the parametric tests, the ANOVA result would be reported as ANOVA has been shown to be accurate despite violation in these circumstances [1401]. Finally, the assumption of sphericity was also tested in SPSS by using the Mauchly's test of sphericity. If this assumption is violated ($p<0.05$), then an epsilon correction is normally reported as the Greenhouse-Geisser result in the SPSS output table. However, if the Greenhouse-Geisser did not differ numerically from the value in 'sphericity assumed' then the latter was reported in order to prevent sections becoming un-wieldy due to stating partial degrees of freedom. If testing whether performance was above chance level in each group, one sample t-tests were used (appropriate for the number of conditions compared, the type of sample, and type of data).

Non-parametric tests: Since non-parametric tests are generally considered to be less powerful than parametric methods, these types of test were used when no parametric test was valid. The Mann-Whitney-U test was used in relation to a between-subjects design involving two independent groups, or the Kruskal-Wallis test (equivalent to the one-way ANOVA) for the comparison of data from more than two independent groups. However, since the Kruskal-Wallis test is an omnibus tests that cannot tell you which specific groups are significantly different from each other, determining which groups differed was accomplished by using an appropriate post-hoc test adjusted for multiple comparisons. Within IBM SPSS Statistics, the NPTESTS procedure offers the option of stepwise post hoc tests if the Kruskal-Wallis test is significant. The Wilcoxon signed-rank test was considered as appropriate test in

relation to a within-subjects design involving a one off comparison between two sets of scores from the same group (i.e. equivalent to the dependent t-test).

3.3.3 Results

3.3.3.1 Experiment 1A: LINS

Analysis Stages 1-3

Figure 3.5 shows the mean percentages of correct choices for mice in stages 1-3 of the T-Maze FCA task (the mean percentage of correct choices per test block in each stage can be found in Table 3.1). Inspection of this figure suggests that overall, Tg2576 were significantly impaired relative to WT controls in the FCA task in each stage, with no significant treatment effects. Thus, rosiglitazone agonism for approximately 3 months failed to reverse the spatial working memory deficit revealed by the T-Maze. Inspection of this figure also suggests that despite a reduction in the accuracy of WT drug-treated mice across post-drug stages, this was not significant. This description was confirmed by ANOVA of the form: Genotype and Treatment as between-subject factors, and Stage, as the within-subject factor (see section 3.3.2.4). This showed a main effect of Stage $F(2,60)=6.808$, $p=0.002$, a main effect of Transgene, $F(1,30)=28.701$, $p=<0.001$, a no significant effect of Treatment $F<1$, a significant two-way interaction of Stage by Genotype $F(1.30)=1.886$, $p=0.040$, no significant two-way Stage by Treatment interaction $F<1$, and no significant Stage by Genotype by Treatment interaction $F(2,60)=2.205$, $p=0.119$. Tests of between-subjects effects showed a non-significant Treatment by Transgene interaction $F(1,30)=1.211$, $p=0.280$. A follow-up test of simple main effects for the significant Stage by Genotype interaction revealed that TG mice were impaired relative to WT mice in each stage of the experiment: Pre-drug, $F(1,30)=7.381$, $p=0.011$; Post-Drug 1, $F(1,30)=24.125$, $p=<0.001$; Post-drug 2, $F(1,30)=31.561$, $p=<0.001$.

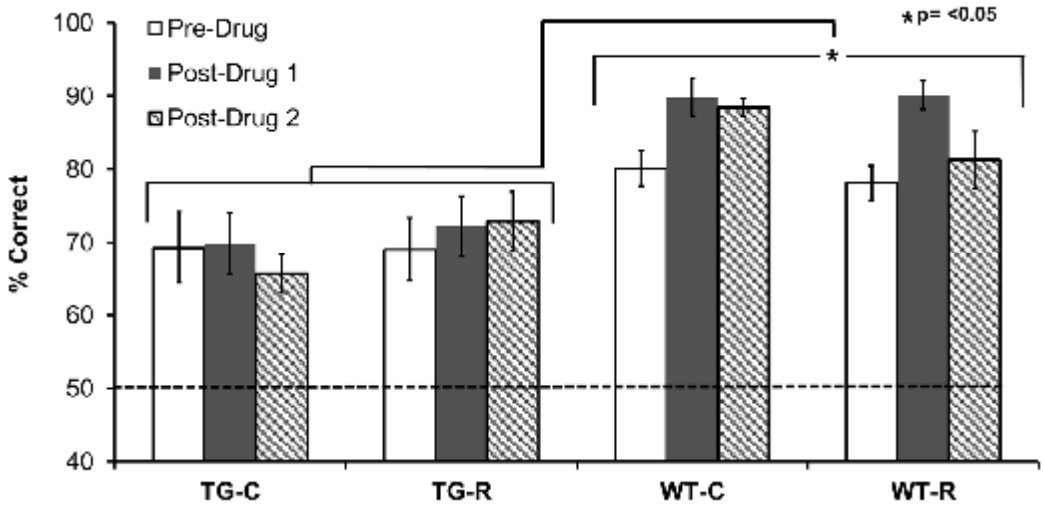

Figure 3.5: LINS T-Maze (Experiment 1A): Means (±S.E.M) with asterisk denoting significance at the 0.05 level. Dotted line represents chance levels of performance.

Stage/Group	Test Block 1 % Correct		Test Block 2 % Correct		Test Block 3 % Correct		Test Block 4 % Correct	
Pre-Drug	M	SEM	M	SEM	M	SEM	M	SEM
TG-C	60.42	6.63	68.75	5.84	75.00	6.10	72.92	7.17
TG-R	63.89	6.51	61.11	5.73	75.93	5.63	75.00	6.05
WT-C	65.74	5.63	76.85	4.77	86.11	4.81	91.67	3.67
WT-R	64.58	4.38	70.83	2.23	85.42	5.84	91.67	2.23
Post-Drug 1								
TG-C	59.38	5.98	66.67	4.72	77.08	6.25	76.04	6.38
TG-R	66.67	4.17	69.44	4.17	70.37	6.82	82.41	7.01
WT-C	85.19	4.56	86.11	4.39	94.44	2.78	93.52	1.85
WT-R	86.46	3.84	88.54	2.70	95.83	2.23	89.58	2.61
Post-Drug 2								
TG-C	60.42	3.43	71.88	4.71	63.54	5.21	66.67	3.52
TG-R	72.22	4.39	75.00	3.11	73.15	6.02	71.30	6.23
WT-C	83.33	3.93	81.48	1.85	94.44	1.96	94.44	2.41
WT-R	73.96	5.55	81.25	3.78	82.29	6.58	87.50	6.86

Table 3.1: LINS T-Maze (Experiment 1A): Mean percentage of correct choices per test block in each stage (each test block is the mean of 2 days data). As one can see from the data in this table, the means of both TG groups remain similar throughout all three stages of Experiment 1A indicating that there is likely to be no significant effect of rosiglitazone treatment on spatial working memory. Although this also remains the case for both WT groups, it is evident that there is more variability in the group means between drug-treated verses non-drug treated mice. Whilst this could indicate a mild but non-significant) negative drug effect, this could also just reflect natural variability in performance.

3.3.3.2 Experiment 1B: EINS

Figure 3.6 shows the mean performance of mice in the early-intervention group (see Table 3.2). These mice were given a single T-Maze FCA task (scores expressed as mean percentage of correct choices) following continuous drug administration from the age of 5 months. Inspection of this figure suggests that a significant overall transgene effect is once again evident indicating that continuous rosiglitazone administration from the age of 5 months did not delay the onset of the forced-choice alternation impairment. This description was confirmed by ANOVA with Genotype and Treatment as between-subject factors, and Block (4 test blocks, each containing the number of correct trials calculated across 2 days of testing), as the within-subject factor. This showed a main effect of Block $F(3,135)=10.726$, p=<0.001, a main effect of Transgene, $F(1,45)=6.504$, p=0.014, a main effect of Treatment $F(1,45)=6.504$, p=0.014, no significant two-way interaction of Block by Genotype $F(3,135)=2.611$, p=0.054, a significant two-way Block by Treatment interaction $F(3,135)=10.726$, p=0.001, and no significant Block by Genotype by Treatment interaction $F(3,135)=1.613$, p=0.189. There was no significant two-way Treatment by Transgene interaction $F<1$. A follow-up test of simple main effects for the significant Block by Treatment interaction revealed no significant differences between non-drug treated mice and drug-treated mice during any of the four test blocks: Test Block 1, $F<1$, p=0.846; Test Block 2, $F(1,30)=1.916$, p=0.176; Test Block 3, $F<1$; Test Block 4, $F<1$. The mean percentage of correct choices per test block can be found in Table 3.2. In aggregate, these results suggest that continuous dosing with rosiglitazone from 5 months of age was insufficient to prevent the onset of TG behavioural impairment on the forced choice alternation task.

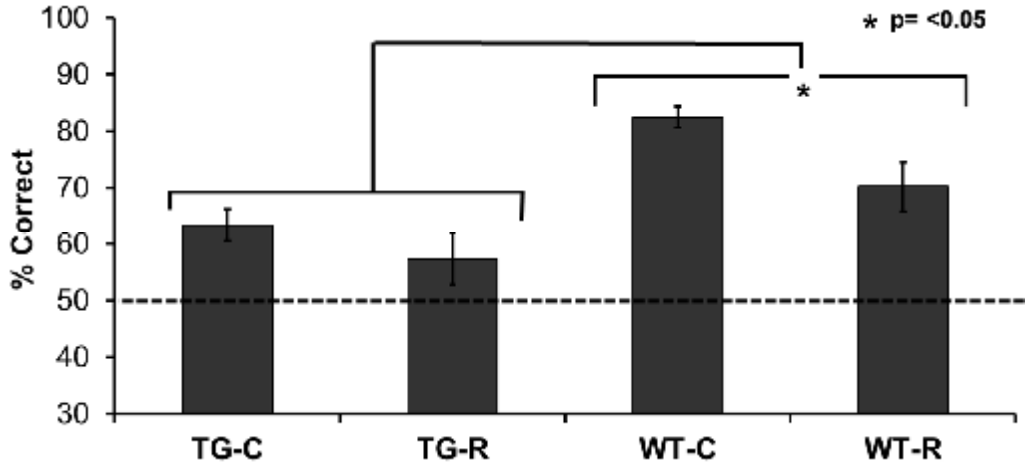

Figure 3.6: LINS T-Maze (Experiment 1B). Mean %age of correct choices. Overall TG mice are still significantly impaired to WT mice. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level. Dotted line represents chance levels of performance.

Group	Test Block 1 % Correct		Test Block 2 % Correct		Test Block 3 % Correct		Test Block 4 % Correct	
	M	SEM	M	SEM	M	SEM	M	SEM
TG-C	57.64	3.76	56.25	3.86	70.14	4.29	69.44	3.75
TG-R	55.56	4.02	54.86	7.21	61.11	6.10	57.64	6.52
WT-C	62.50	3.91	84.72	3.05	90.28	2.87	92.36	1.91
WT-R	67.31	5.12	69.23	3.70	69.87	6.23	74.36	6.22

Table 3.2: EINS T-Maze (Experiment 1B): Mean percentage of correct choices per test block (each test block is again the mean of 2 days data). As one can see from this table, the means of both TG groups again remain similar throughout all three stages of Experiment 1B indicating that there is no significant effect of rosiglitazone treatment on spatial working memory. Although this also remains the case for both WT groups, there is again evidence for variability in the WT-R group means relative to the WT-C mice. Again, this could indicate a mild (but non-significant) negative drug effect on spatial working memory.

3.3.4 Discussion

The results of Experiment 1A and 1B both indicate that the performance of adult Tg2576 mice remains significantly impaired relative to that of WT-C mice irrespective of when the treatment regimen is initiated. These findings lend further support to previous studies showing that adult Tg2576 mice are impaired in the T-maze FCA task [1246, 1251, 1288, 1385], although they also show that after ~3 months continuous rosiglitazone administration, the drug was unable to reverse or delay the age-related

spatial working memory deficit in TG mice revealed via the T-maze FCA task. These results contrast with several studies in the scientific literature, which have all reported that rosiglitazone can restore or enhance aspects of hippocampal function known to deteriorate in middle-aged rats [1403], aged rats [1207], models of T2D [1210], and perhaps most importantly, hAPP mouse models [1214, 1216, 1217]. For example, there are reports that rosiglitazone can enhance learning, hippocampal place cell activity, and synaptic plasticity in middle aged rats [1403], and in the $hAPP_{SWE-IND}$ model, reverse hippocampal glucocorticoid receptor down-regulation [1214], as well as removal of abundant amyloid plaques in adult TG mice [1213]. However, none of these dramatic effects on hippocampal function, have been observed in Tg2576, although studies have reported beneficial effects of rosiglitazone on ameliorating spatial memory deficits [1129], and learning and memory impairments associated with other behavioural tasks considered to involve recruitment of the hippocampus [1212, 1215]. Thus, although we have not studied hippocampal function on a neurophysiological level (i.e. synaptic plasticity and glucocorticoid receptor levels), comparison with the behavioural findings of some of the above studies are warranted, although this will largely be restricted to findings in Tg2576 relating to spatial memory, as these are the most relevant here. I should also be briefly comment on the observation that across both interventions, WT-R mice showed a persistent trend towards impaired performance relative to WT-C mice, although this was not significant within the 3 month administration period. This finding is consistent with those reported by other studies using much shorter drug administration times [1212, 1215]. However, it will be of interest to the reader to see how in the current study, this profile changes drastically in WT mice with continued administration of the drug over a longitudinal period.

3.3.4.1 Comparisons with Other Studies

Pedersen et al [1129] have studied the effects of continuous rosiglitazone administration in male Tg2576 mice. This study assessed spatial memory via a de novo 8-arm radial maze task [1129], and reported that the drug reversed a spatial learning and memory impairment in TG mice. Importantly, Pedersen et al. used a drug concentration of 10 mg/kg mixed with standard rodent laboratory chow, and delivered to animals in pelleted form for ~3.4 months. It is worth mentioning that this delivery method may have made it difficult to determine how much compound was actually reaching the mice (and indeed, how often it was replenished to avoid consumption of

stale compound), since it is possible that concentrations of the drug may have been unequal across food pellets. This is why in the current study we chose to use powdered diet, as this allowed us greater control over the delivery process (see protocol for experimental diets). In addition, unlike the current study, Pedersen et al. did not report any independent physiological parameters showing that the drug had achieved systemic circulation (i.e. adiponectin levels as in the current study). Nevertheless, it is noteworthy that the range of self-dosing reported in our study was not only comparable to that reported by Pedersen et al. [1129], but greater. Thus, although one possible reason why we did not observe a reversal of the spatial working memory deficit in the T-Maze is that the TG-R mice may have needed longer on the compound, as in both early and late-intervention experiments, mice had only received access to the compound for ~3 months before T-maze FCA testing was terminated. However, this explanation is unlikely for two reasons. First, the higher range of dosing in the current study would most likely have cancelled out the effects of a slightly shorter administration period. Second, one recent study has reported significant recovery of memory deficits in Tg2576 mice using a concentration of 10 mg/kg after only 1 month of continuous rosiglitazone administration [1215], a finding supported by other studies also using short duration administration periods of rosiglitazone in different hAPP mouse models [1216, 1217].

There are concerns over the experimental design of the de novo 8-arm radial maze task used to assess spatial learning and memory in the Pedersen et al. study [1129]. In short, the mice were initially placed in the central hub of the maze with all doors closed. All doors were then simultaneously opened when 4 arms were baited by an automatic pellet feeder. Four of the arms were consistently baited with 20 mg food pellets (1, 2, 4, and 7), whereas the other 4 arms were never baited. In addition to extra-maze cues (a door and computer), intra-maze cues included small pieces of white tape placed proximal to the floor of the baited arms. The mice were then allowed to continue exploring until all four baits in the food troughs had been consumed or until 5 min had elapsed. This procedure was repeated such that mice were subjected to 4 runs per day. During training (2 days) the mice were only allowed access to the baited arms, whereas during testing (3 days), the mice were allowed access to all 8 arms. The number of reference memory errors (entering an arm that was not baited), and working memory errors (entering an arm containing food that was previously entered), were recorded. Pedersen et al. observed that TG-R mice exhibited spatial

working and reference memory abilities similar to WT mice, with continued improvement across test days [1129]. By contrast, TG-C mice made consistent numbers of errors throughout the testing period, and did not exhibit improvement across these measures. The difficulty with this is that the nature of the cues controlling the performance of mice is uncertain meaning that it is impossible to determine what aspect of behaviour rosiglitazone modified the TG mice, or indeed, the nature of the neural systems engaged by the task. This leaves open the question of whether rosiglitazone was affecting spatial memory in this study or some other component of performance such as general arousal, motivation/impulsivity, or habituation processes. For example, during the test phase it is unlikely that mice entering un-bated arms provided a fair measure of 'reference memory'. Since these arms would most likely have appeared as novel spatial locations to the mice they would have encouraged further exploration. Thus, as no food was ever placed at these locations, one would predict that normal mice would habituate to them across the test days leading to progressively fewer entries. So, instead of measuring 'reference memory', entries into unabated arms may have measured short-term habituation to novel spatial locations. Although the FCA task also measures short-term habituation, unlike the Pedersen et al., study, the spatial location of the food varies systematically across trials between the left and right sides of the apparatus.

Two studies conducted with other hAPP models have also reported that rosiglitazone administration has a beneficial effect on spatial learning and memory [1216, 1217]. Toledo et al. [1216] reported that in 9 month old APP/PS1 mice given a daily gavage treatment of ground rosiglitazone maleate in a suspension in sterile water for 12 weeks (Avandia; concentration =3mgkg^{-1}), the drug reversed a spatial memory impairment in a modified Morris water-maze task, reduced Aβ aggregates and Aβ oligomers, as well as reduced astrocytic and microglia activation. In addition, Toledo et al. also showed that rosiglitazone prevented changes in presynaptic and postsynaptic marker proteins and activated a Wnt signalling pathway, the latter of which may have been responsible for mediating the positive effects in relation to reducing neuropathological markers associated with hAPP [1216]. However, it is of interest that the authors of this study do not report the numbers of male verses female mice used. This is of potential importance, because after 12 weeks of treatment, 'high variability' was observed between TG mice and WT-littermates on a standard Morris water maze task, with no clear difference emerging among any of the groups of animals [1216].

Thus, it is possible that this variability may in part have been due to changes in the oestrous cycle in female mice (if any were used), although incomplete reporting of experimental details makes this impossible to know for sure. In order to overcome the behavioural variability, Toledo et al. moved to a 'training-to-criterion' version of the Morris water maze[1216], which in in PDAPP mice had previously been shown to measure memory flexibility [1261], and exhibit greater sensitivity to hippocampal dysfunction [1261, 1404]. In brief, mice were trained to escape to a hidden platform inside a circular pool filled with lukewarm opaque water, and surrounded by salient distal objects mounted on walls of the test room. When a mouse reached a criterion of three consecutive trials with an escape latency of <20s, the platform was switched to a new location of 4 possible candidates, and the animal was retrained. Memory flexibility was measured by the number of trials needed to reach criterion at each location. Since mice were required to learn a series of successive spatial locations one at the time, it has been suggested that this task, may be an efficient means of reducing within-group variability —a major conundrum in behavioural neuroscience [1405]. Using this procedure, Toledo et al. [1216] found a significant difference between drug-treated verses non-drug treated TG mice, with the former showing a reversal of the deficit in the number of trials required to reach the escape criterion at each of the four platform locations. It is possible that in this instance, the lower drug concentration used by Toledo et al. would have produced a mild drug effect that only a more sensitive test of spatial memory was able to detect. However, the fact that a genotypic difference was not found in the standard Morris water maze task makes this uncertain. In principal this issue could have been tested in the current study by taking a second measure of spatial memory. This could have involved using the water maze task or an adapted version of a plus maze experiment in which mice are trained to find consecutive goal locations from multiple release points within the maze.

Finally, a recent study by O'Reilly and Lynch [1217] has also reported that in 7 month old female hAPP/PS1 mice and WT littermates, rosiglitazone maleate (Alpha Technologies, Ireland) at a concentration of 6mg/kg/day in 50µl of maple syrup for 2 weeks prior to behavioural testing, was sufficient to reverse the TG deficit in spatial memory. The study by O'Reilly and Lynch [1217] contains an obvious confound in that female mice were used to assess spatial memory and yet no attempt was made to check where the mice were in relation to their oestrous cycle at the time of testing. Nevertheless, the discrepancy between the findings from the current study, and those

from hAPP/PS1 mice, remain to be fully explained, although comparisons are complicated by the fact that the dynamics of hAPP pathology may be different in these models compared to Tg2576, and indeed, vary even with the same mouse model depending on the relative proportions of SJL F1 background [1374].

3.3.4.2 Factors Possibly Affecting the Outcome of Experimental Measures

Aside from the issues already discussed, two further other issues may potentially have contributed to the null findings in the present study. First, one could question the logic for supplying potentially diabetic TG mice with a sucrose reward during behavioural trials, particularly since in other hAPP mouse models the intake of 10% sucrose-sweetened water has been shown to induce insulin resistance and exacerbate memory deficits and amyloidosis [1254]. However, these effects are only observed with chronic ad libitum access [1254], which does not apply to the current experiment. Thus, consumption of the sucrose solution probably had a negligible effect on the overall performance of the mice. Second, it is possible that retinal degeneration due to the Pde6brd1 (RD) mutation may have affected T-maze performance in some mice [1393], but particularly the TG mice as Aβ pathology has also been linked to retinal degeneration in several hAPP mouse models [1406-1408]. However, it has been argued that Tg2576 mice show impaired spatial memory irrespective of retinal degeneration status [1406]. Whilst mice are able to use an allocentric representation to solve the T-Maze [1246], something that retinal degeneration could have potentially perturbed, mice and rats have been shown to solve the T-Maze via a number of different ways [1389, 1409, 1410], including with a sense of direction [1410]. Thus, to some extent, any visual problems could have been compensated for by the mice using non-visual types of information. The fact that despite any potential problems due to visual deficits, majority of WT mice (unlike most TG mice) were still able meet criterion across experimental stages, suggests that WT mice at least are able to use such information to acquire the task.

3.3.4.3 Late Intervention Rosiglitazone: Did we intervene too late?

In the remainder of this discussion, I want to briefly return to addressing some of the major findings of this experiment in relation to some theoretical concerns, and wider implications of using rosiglitazone to treat AD-like pathologies. Chief amongst these is the issue of whether or not administering rosiglitazone to adult mice (and indeed aged

humans) at a time when amyloid pathology is already well advanced in the brain is essentially, a late-intervention strategy that is 'too late'. In this regard, the findings from Experiment 1A are most consistent with those of Nicolakakis et al. [1386], who reported that in hAPP transgenic mice started on PPARγ agonist pioglitazone at ~14 months of age, physiological changes occurred (i.e. normalised glucose uptake in response to neuronal activity, and attenuated astroglial activation), in the absence of a recovery in the spatial memory deficit in these mice [1386]. Indeed, similar results have also been obtained with rosiglitazone in Tg2576 by Rodriguez-Rivera et al. [1215], who reported that rosiglitazone agonism for a 4 week period prior to behavioural testing led to the drug reversing peripheral gluco-regulatory abnormalities in 9 month old, and 13 month old Tg2576 mice, but only reversing learning and memory deficits in the 9 month old TG mice. It is interesting to note that Rodriguez-Rivera et al. used a contextual fear conditioning paradigm to assess memory in animals, and did not report the numbers of male and female mice used in their study. Although it is therefore possible that the failure of the drug to reverse learning and memory deficits in the 13 month old TG mice may have, as the authors themselves noted, reflected age-dependent mechanistic differences underlying cognitive decline in Tg2576 [1215], it is also possible that contextual fear learning may have been affected by differential activation of ventral hippocampal extracellular signal-regulated kinase between male and female mice [1411]. Both these studies show that it is possible to affect components of the pathological cascade common in AD without necessarily achieving cognitive improvement. Indeed, these findings mirror the inconsistency in the human clinical data as although some human clinical trials have reported rosiglitazone monotherapy to have beneficial effects on learning and memory in patients with mild-moderate AD has [1235, 1236], no significant effects of the drug have been reported in more recent phase 3 clinical data in patients with mild-to-moderate AD [1237, 1238]. This is despite the drug apparently eliciting an early increase in whole brain glucose metabolism [1237]. Indeed, across the human literature [412, 770, 771] and pre-clinical literature[1215, 1386], the conclusion seems to be that in order to be most effective PPARγ agonists may have to be targeted at the earliest preclinical stages of the disease. This is difficult to do in humans because it is currently not possible to reliably say which patient with aMCI will progress to AD from those who will not [311, 314, 1412].

Finally, it is worth mentioning that one of the possible reasons for why we report null findings with respect to spatial memory impairment compared to prior reported

studies in the scientific literature, is the differential activation of common neural structures. For example, a number of studies now suggest that brain regions such as the hippocampus can be differentially activated by behavioural tasks [510, 686], and that diverse behavioural effects may result when selective disruption occurs to particular hippocampal sub-regions. There is robust evidence for at least two distinct functional domains, although neuroanatomical, and neuropsychological studies suggest this may be an underestimate (see [686, 1413]). Selective lesion studies show that the hippocampus is functionally subdivided along the septo-temporal axis into dorsal and ventral regions, each associated with a distinct set of behaviours [686]. Dorsal hippocampus has a preferential role in certain forms of learning and memory, notably spatial learning, but ventral hippocampus may have a preferential role in brain processes associated with anxiety-related behaviours [686, 1413] (see introduction to Experiment 2). Thus, although speculative at this point, it is possible that depending on the fine distribution of PPARγ receptors in different brain regions, it may be the case that the weak CNS penetrance of rosiglitazone will only be sufficient to selectively affect certain functional domains within structures such as the hippocampus, whilst leaving others impaired. Whether or not a cognitive effect is seen will depend on how any given task functionally activates a particular brain region that may (or may not) have been affected on a physiological level by the drug in question. Indeed, this poses one possible reason for why it is that physiological changes may not necessarily be translated into cognitive improvement. Likewise, the same could apply to any possible neuroprotective functions of a drug when it is delivered early before significant amyloid pathology takes place in the brain.

3.4 Experiment 2: Non-conditioned Anxiety (EPM)

3.4.1 Introduction

3.4.1.1 The Elevated Plus Maze

A number of different tests are available for assessing anxiety in experimental animals, and rodents in particular. These tests are classically divided into conditioned and unconditioned tests. Unconditioned tests are based on the natural aversion of rodents to novel environments, which evoke a conflict between the animals exploratory and fear/defensive behaviours [1414]. Examples of popular non-conditioned tests of anxiety include the elevated plus maze (EPM), the marble burying task, and the forced swim test [1383]. Conditioned aversive tasks include the Pavlovian fear conditioning procedure, which assess learning and memory for associatively conditioned stimuli. Non-conditioned anxiety tests have certain advantages over conditioned tests, including being inexpensive and simple to apply because they require no previous training period, are based on the spontaneous behaviour of animals, and use a natural stimulus to induce anxiety states [1415].

The EPM is designed such that a cross-shaped platform is raised above floor level, with two arms constructed with high walls along the both sides (i.e. the 'closed' arms), whilst the remaining two arms have no enclosing along their sides (i.e. 'open' arms). As a result, this apparatus has physical properties such as height and lack of protection, that are believed to induce anxiety-like responses in rats [687, 1416], and mice [1383]. It is therefore considered an ethologically valid measure of anxiety in these animal species. Anxiety is reflected in the tendency for 'normal' mice to prefer spending more time exploring the 'safer' closed arms of the apparatus relative to the more risky 'open arms' [1417]. Thus, time spent in the open arms is a valid index of anxiety-like behaviour in rodents, including behavioural disinhibition [1418]. To put it another way, the EPM task is considered to have face value or 'construct validity' in terms of this and related behaviours (see below) being observable dependent variables in measuring an unobservable construct, such as anxiety and disinhibitory behaviour in rodents [1419]. This is further supported by the fact that anxiogenic drugs have been shown to reduce time spent on the open arms whereas anxiolytic drugs having the opposite effect (i.e. increasing the time spent on the open arms) [1420]. Related behavioural indices in this task also include the number of crossings between arms, or arm entries [1383]. These can also be used as ways of evaluating locomotor activity,

anxiety reactions and behavioural disinhibition [1383, 1421]. In addition, a number of ethological measures can also be used to assess anxiety reactions and behavioural disinhibition in the EPM, including head dipping (i.e. exploratory behaviour), stretch-attend postures (i.e. risk-assessing behaviour) and the degree of self-grooming (also a measure of behavioural disinhibition) [1419]. However, all of the behavioural measures mentioned above can also be affected (to a greater or lesser degree) by mouse strain differences, particularly with respect to locomotor activity [1422]. Nevertheless, anxiolytic drugs have been shown to alter patterns of exploratory activity in rodents by increasing the number of entries into (as well as the amount of time spent in) open arms of the EPM [1423-1425]. Thus, the EPM is a well characterised test for evaluating anxiety/disinhibitory behaviour in rodents [1383], and is used to assess these behaviours in the current study.

3.4.1.2 Anxiety and Behavioural Disinhibition in Tg2576

The neurological basis of emotional disturbances and BPS in AD is thought to reflect pathology in the MTL (particularly structures such as the amygdala and hippocampus), as well as frontal lobes [341, 1426]. Tg2576 mice similarly show pathology in these same brain regions [1242, 1291], suggesting that these mice likely exhibit a behavioural phenotype that includes emotional disturbances as well as some behavioural characteristics reminiscent of BPS in AD. However, these may not always correlate with amyloid deposition. For example, Jacobsen et al [1245] have detected an early decrease in spine density in the outer molecular layer of the hippocampal DG beginning as early as 4 months of age, which by 5 months coincided with a decline in LTP (following perforant path stimulation), and impairment in contextual fear conditioning task. At this time significant amyloid pathology is absent in TG mice [1245]. These results are in agreement with the findings from other studies [1427-1431], which have reported impaired fear conditioning to context and/or auditory cues in fear conditioning paradigms in Tg2576 mice. Several previous studies have also used the EPM and other tests to assess anxiety in Tg2576 mice [1252, 1381, 1382, 1390, 1391], and have revealed a phenotypic tendency for this model to display reduced anxiety/disinhibited behaviour. For example, although there are no published reports of behaviour in the EPM at an age earlier than 7 months, in 9 month old, and 17 month old Tg2576 mice and WT littermates, it has been reported that there is a greeter propensity for TG mice to spend increased time in the open arms as well as making

more open arm entries relative to WT mice [1382, 1390]. These results suggest that Tg2576 mice are less anxious/more disinhibited than WT mice. As such, these tendencies are referred to here as genotypic 'target' behaviours. In addition, using the EPM, 9 month old Tg2576 mice have also been shown to exhibit abnormal responses to unconditioned aversive or anxiogenic stimuli (e.g. by spending significantly more time visiting the open arms and making more entries into these open arms than controls [1382]). However, it is important to also mention the findings of Ognibene et al. [1252], who have reported that both these target behaviours in the EPM are non-significant trends in Tg2576 mice between the ages of 7 to 12 months. However, Ognibene et al., did report a number of other measures consistent with reduced anxiety in Tg2576 mice, including increased head dipping, less stretch-attend postures and increased self-grooming in the EPM, as well as preferential exploration of exposed regions of an open-field arena indicative of a disinhibited phenotype [1252]. Taken in aggregate, these studies are consistent with the view that TG2576 mice display behavioural responses associated with reduced anxiety and behavioural disinhibition, although it is evident that other factors can modify these behaviours, including most obviously background strain [1381].

Currently, no studies have reported outcomes for the assessment of rosiglitazone treated Tg2576 mice on the EPM task. However, rosiglitazone has been reported to reverse a contextual fear conditioning impairment in 9 month old Tg2576 mice, but not in mice aged 5, and 13 months of age (sex unreported) [1215]. Rosiglitazone has also been shown to reverse a contextual fear conditioning impairment in 20 month old male F344 rats [1207], although it had no effect hippocampal interleukin-1beta (IL1β) levels, markers of oxidative damage, or NMDA receptor expression. Thus, in order to assess the impact of rosiglitazone on non-conditioned anxiety as assessed by the EPM, in Experiment 2A (LINS), mice were given a single exposure to the EPM at ~13 months following a 2 day rest period after the conclusion of Experiment 1A. In Experiment 2B (EINS), mice received a single exposure EPM task at the age of ~8 months.

3.4.2 Methods and Apparatus

3.4.2.1 Animals and Drug Administration

Experiment 2A (LINS) used the mice from Cohort 1, which had previously undergone Experiment 1A. Experiment 2B (EINS) used the mice from Cohort 3 which were experimentally naïve (this because the order of spatial and anxiety experiments were

reversed in the ENIS condition). Please see section 3.3.2.1 for details of housing and drug administration.

3.4.2.2 Apparatus

The EPM was constructed from two open arms, each measuring 8cm wide x 50cm long, which ran from north to south, and two enclosed arms, measuring 8cm wide x 50cm long and 15cm high, running from west to east (see figure 3.7). The plus maze platform was elevated 90cm from the floor via a vertical black frame, and had a white laminate wood floor supported laterally by a black metal frame. The walls forming the enclosed arms were comprised of mat black coloured Perspex, with open arms containing an extremely short (1cm tall) clear Perspex walls to help prevent falls. The maze was placed inside a wooden arena (102cm2 and 43cm height) with a sawdust-covered floor to cushion the mouse in the event of a fall. The apparatus was located in quiet and brightly illuminated room comprised of multiple environmental cues, including shelves and a computer system. A camera was mounted on the ceiling and connected to a DVD player and monitor to ensure all sessions were recorded. A camera was attached to the ceiling directly above the maze, which was connected to a computer, a television monitor and video recorder (VCR). The camera input was used to visualise the maze activity on the television monitor, and each session was recorded using the VCR. Noldus® Ethovision tracking software on the computer was utilised to collect data using the camera input by manual scoring (see Scoring and Statistical Analysis).

Figure 3.7: EPM Apparatus. *Left*: The apparatus insitu. *Right*: A WT mouse exploring one of the open arms.

3.4.2.3 Behavioural Procedures

Testing in Experiment 2A and 2B took place over a single day, with mice transported to the test room in their individual home cages. Prior to the start of each trial, the EPM was cleaned thoroughly with a 1:20 dilution of Mr Muscle®, and the VHS video recording equipment was activated. The mouse was placed in the centre platform of the EPM facing a left-hand closed arm, after which the experimenter withdrew to the computer station at the edge of the room. The animal tracking software (Noldus© Ethovision) was then started, with the experimenter observing the behaviour of the mouse on the monitor, manually scoring as appropriate (see scoring Criteria). In the rare event that a mouse fell from the open arms, the animal was retrieved from inside the arena and placed back onto the centre platform of the EPM as previous. Each animal was given one session of five minutes (300 seconds) duration in the maze with free-access. When the session had expired, the animal was removed from the maze and placed back into its home cage prior to being returned to the holding room.

Scoring and Exclusion Criteria: Mouse behaviour within the EPM apparatus was scored manually using the Noldus© Ethovision software. Here, mice were scored for two measures: (i) the mean time spent in the open and the closed arms; (ii) the mean number of entries into the open and the closed arms. Each arm was assigned a specific letter on the computer keyboard, which was pressed upon entry into and exit from each arm. An arm entry was defined as when all four paws of the mouse passed the entrance of the arm, and an arm exit was defined as when all four paws crossed outside the arm. This criteria is comparable to that previously reported by Lalonde et al. [1390], and Ognibene et al. [1252]. Using this methodology, Noldus® Ethovision software automatically calculated the total duration spent in each arm and the total number of entries into each arm. The accuracy of this scoring method was checked for the first 3 mice tested per session. No further mice were excluded from the analysis due to mortality, although one WT mouse (from WT-C), was removed from the Experiment 2A due to a non-fatal injury which temporarily impeded its mobility. In addition, in Experiment 2B, 1 TG mouse (TG-C) met criterion for non-responsiveness (spending the whole 5 minutes at the start location). Thus, the total numbers of mice run in Experiment 2A were 18 TG mice (TG-C, n=9; TG-R, n=9), and 18 WT (WT-C, n=9; WT-R, n=9). In experiment 2B, the total numbers of mice run were as follows: 24 TG mice (TG-C, n=12; TG-R, n=12), and 25 WT mice (WT-C, n=12; WT-R, n=13).

3.4.2.4 Statistical Analysis

Raw data for each of the frequency and duration measures were analysed by 3-way ANOVA, which had Genotype and Treatment as the between-subject factors and Arm (open or closed) as the within-subject factor. Using IBM SPSS Statistics v.21.00 software with an alpha value (p) <0.05, taken as being statistically significant Any significant ANOVA interactions and non-parametric analysis were conducted as previously indicated in Experiment 1 (see section 3.3.2.4). As well as analysing the raw data, both the duration and frequency data in each experiment were converted into a preference ratio (PR). For the duration data the ratio was calculated for preference for spending time in open arms: = time spent in open arms/time spent in both open and closed arms. PRs above 0.5 indicate that the mouse was spending more time in the open arms than in closed arms. For the frequency data the preference ratio was calculated for making entries into open arms: = entries into open arms/Total number of entries). PRs above 0.5 indicate that the mouse was making more entries into open than closed arms. Analysis of PRs is less subject to the influence of individual variability in both duration data frequency of entries; it provides a measure of the extent of discrimination between the open and closed arms of the apparatus. All PRs were analysed by 1-way ANOVA, with PR: (Duration Open Arm, Frequency Open Arm Entries), as the dependent variable, Genotype and Treatment as factors.

3.4.3 Results

3.4.3.1 Experiment 2A: LINS

Mean Time Spent in Open and Closed Arms

Figure 3.8 shows the mean duration spent in the open and closed arms of the EPM. Inspection of this figure shows that there are no significant differences between any of the groups, although there is a tendency for WT mice spending more time in closed verses open arms of the apparatus (the TG mice slightly favour the open compared to closed arms). This description was confirmed by 3-way repeated measures ANOVA with **Arm** (duration spent in open and closed arms), Genotype, and Treatment as factors. This showed a non-significant effect of Arm $F(1,32)=0.933$, p=0.341, a non-significant effect of effect of Transgene, $F(1,32)=2.194$, p=0.148, a non-significant effect of Treatment $F(1,32)=1.614$, p=0.439, a non-significant two-way interaction of Arm by Genotype $F(1,32)=2.806$, p=0.104, a non-significant two-way Arm by Treatment

interaction $F(1,32)=0.398$, p=0.533, and a non-significant three-way interaction of Arm by Genotype by Treatment interaction $F(1,32)=0.049$, p=0.827. Tests of between-subjects effects showed that overall there was a non-significant two-way Treatment by Transgene interaction $F(1,32)=1.157$, p=0.290. In the absence of any significant interactions, no further statistical analysis was performed using the raw data. PRs were calculated for time spent in open arms (see Table 3.3). Despite this showing a trend towards TG mice spending more time in the open arms relative to WT mice, this was non-significant. A one-way univariate ANOVA confirmed this, with PRs (for time in open arms) as the dependent variable, and Genotype, and Treatment as factors. This showed a non-significant effect of Genotype $F(1,32)=2.554$, p=0.120, a non-significant effect of Treatment $F(1,32)=0.358$, p=0.554, and a non-significant Genotype by Treatment interaction $F(1,32)=0.017$, p=0.898.

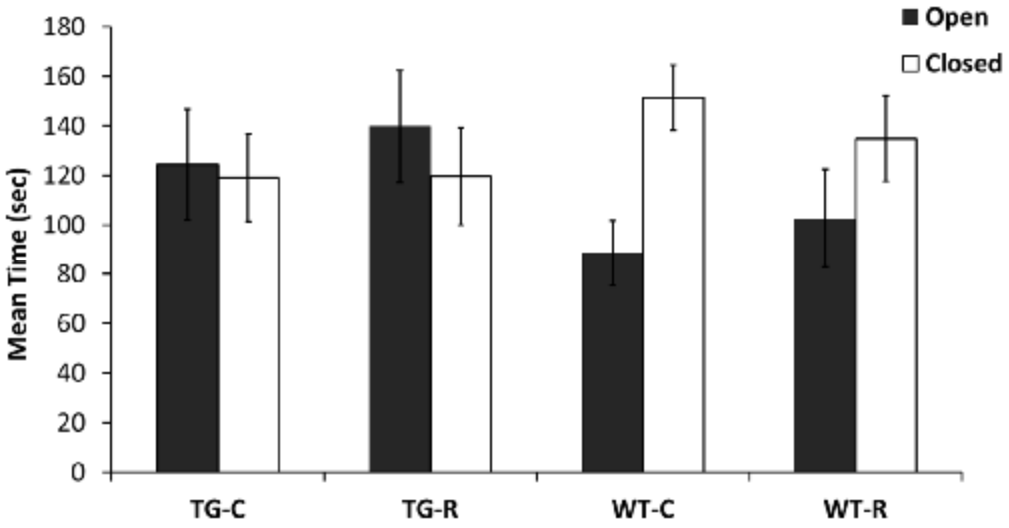

Figure 3.8: LINS EPM (Experiment 2A). Mean duration spent in open and closed arms. There are no significant transgene differences between groups with respect to the time spent in open verses closed arms of the EPM, and no significant effects of drug treatment. Values are means ±S.E.M.

	PR (Open Arms)	
LINS Group	M	**SEM**
TG-C	0.50	0.08
TG-R	0.53	0.08
WT-C	0.37	0.05
WT-R	0.42	0.07

Table 3.3: PR (duration) data for LINS EPM (Experiment 2A).

Mean Frequency of Entries into Open and Closed Arms

Figure 3.9 shows the mean frequency of entries into open and closed arms of the EPM. Inspection of this figure shows that there is no overall transgene effect with respect to the number of entries into open verses closed arms of the apparatus, or any significant differences due to rosiglitazone treatment. This description was confirmed by repeated measures ANOVA with Arm (frequency of open and closed arm entries), Genotype, and Treatment as factors. This showed a main effect of Arm $F(1,32)=14.518$, p=<0.001, a non-significant effect of effect of Transgene, $F(1,32)=0.007$, p=0.933, a non-significant effect of Treatment $F(1,32)=0.007$, p=0.933, a significant two-way interaction of Arm by Genotype $F(1,32)=4.422$, p=0.043, a non-significant two-way Arm by Treatment interaction $F(1,32)=0.380$, p=0.542, and a non-significant three-way interaction of Arm by Genotype by Treatment interaction $F(1,32)=1.332$, p=0.257. There was a no significant Treatment by Genotype interaction $F(1,32)=1.804$, p=0.189.

A follow-up test of simple main effects for the significant Arm by Genotype interaction revealed that TG mice made significantly more entries to open arms compared to closed arms $F(1,32)=17.482$, p=<0.001, whereas in WT mice the difference between the frequency of entries into open verses closed arms was non-significant $F(1,32)=1.458$, p=0.236. Thus, whilst this within genotype comparison confirmed the target behaviour in TG mice consistent their exhibiting a disinhibited phenotype, the seemingly poor discrimination in the WT mice between arms meant that the WT target behaviour of making more entries into closed arms verses open could not be confirmed. However, a between genotype comparison did show that WT mice made more entries into closed arms compared to TG mice $F(1,32)=4.793$, p=0.036, although no transgene effect was evident with respect to open arms $F(1,32)=1.068$, p=0.309. It is likely that larger mean for WT-R open entries likely contributed to the lack of a transgene effect with respect to open arm entries.

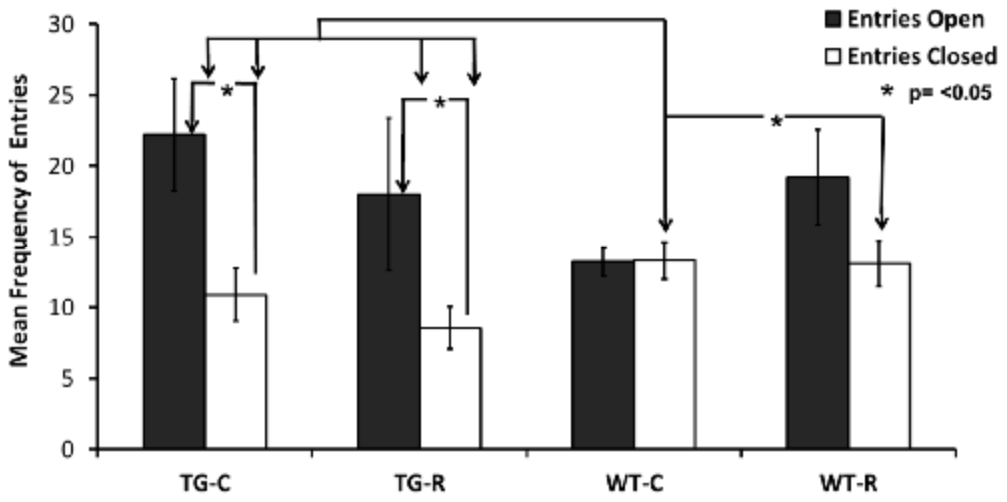

Figure 3.9: LINS EPM (Experiment 2A). Mean frequency of open and closed arm entries. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

PRs were calculated for the number of open arm entries (see Table 3.4). Inspection of this table suggests that there are no significant differences beyond an overall effect of genotype with TG mice making more arm entries than WT counterparts. A one-way ANOVA confirmed this interpretation, with PRs (total number of entries into open arms) as the dependent variable, and Genotype, and Treatment as factors. This showed a significant effect of Genotype $F(1,32)=5.417$, p=<0.026, a non-significant effect of Treatment $F=<1$, and a non-significant Genotype by Treatment interaction $F(1,32)=1.710$, p=0.200.

	PR (Open Arms)	
Group	M	SEM
TG-C	0.66	0.03
TG-R	0.61	0.06
WT-C	0.50	0.02
WT-R	0.56	0.05

Table 3.4: LINS EPM (Experiment 2A). Open Arm Entries PRs (frequency data).

3.4.3.2 Experiment 2B: EINS

Mean Duration Spent in Open and Closed Arms

Figure 3.10 shows the mean duration spent in the open and closed arms of the EPM for the early-intervention experiment. Inspection of this figure suggests that overall TG mice spent more time in open arms relative to WT mice, although this likely being driven by the large response in the TG-N group. This description was confirmed by 3-way repeated measures ANOVA with Arm (duration spent in open and closed arms), Genotype, and Treatment as factors. This showed a non-significant effect of Arm $F(1,44)=0.012$, $p=0.915$, no significant effect of effect of Transgene, $F(1,44)=0.069$, $p=0.794$, no significant effect of Treatment $F<1$, no significant interaction of Arm by Genotype $F<1$, no significant two-way Arm by Treatment interaction $F=<1$, and a significant three-way interaction of Arm by Genotype by Treatment interaction $F(1,44)=6.242$, $p=<0.005$. There was a non-significant two-way Treatment by Genotype interaction $F(1,44)=1.451$, $p=0.235$.

A follow-up test of simple main effects for the significant Arm by Genotype by Treatment interaction revealed that TG-R and WT-R mice did not differ with respect to the time spent in open arms $F(1,44)=1.964$, $p=0.168$, or closed arms $F(1,44)=0.596$, $p=0.444$. However, TG-C mice did spend significantly more time in open arms compared to and WT-C mice $F(1,44)=6.538$, $p=0.014$, whereas time spent in closed arms was not significant $F(1,44)=2571$, $p=0.116$. Thus, genotypic target behaviours were confirmed in TG-C mice when compared to WT controls. Indeed, within genotype comparisons showed that TG-C mice also spent significantly longer in the open arms compared to TG-R mice, $F(1,44)=5.704$, $p=0.021$, with the time spent in closed arms non-significant: $F(1,44)=2.136$, $p=0.151$. This suggests that rosiglitazone may have modified some component of anxiety/disinhibition in the TG-R mice. By contrast, WT-C mice did not differ significantly from WT-R in either the time spent in open arms $F(1,44)=2.488$, $p=0.122$, or closed arms $F<1$, suggesting that the drug did not significantly affect performance. Finally, within group comparisons showed that neither of the TG or WT groups showed a significant bias for time spent in open verses closed arms of the apparatus, although this was close with respect to the TG-C group: TG-R: $F(1,44)=1.150$, $p=0.289$, TG-C: $F(1,44)=3.717$, $p=0.060$; WT-R: $F<1$, WT-C: $F(1,44)=1748$, $p=0.193$. PRs were also calculated for time spent in open arms (see Table 3.5). Inspection of this table suggests that there is a tendency towards TG mice spending more time that WT mice in the open arms of the apparatus, although this is

not likely to be significant. A one-way univariate ANOVA confirmed this, with PRs as the dependent variable, and Genotype, and Treatment as factors. This showed a no significant effect of Genotype $F(1,47)=2.053$, p=0.159, no significant effect of Treatment $F<1$, but a significant Genotype by Treatment interaction $F(1,47)=6.146$, p=0.017. A follow-up test of simple main effects for the significant Genotype by Treatment interaction confirmed the findings in the raw analysis, revealing that compared to WT-C mice, the TG-C group spent more time in open arms of the apparatus, $F(1,44)=7.344$, p=<0.005, whereas neither of the drug treated groups differed significantly from each other on this measure, $F<1$. Within genotype comparisons then showed that TG-C mice spent significantly more time in open arms relative to TG-R mice $F(1,44)=4.537$, p=0.039, whereas the difference between both WT groups was again, non-significant $F(1,44)=1.851$, p=0.181. Therefore, TG-C mice are more disinhibited than WT-C mice, and rosiglitazone significantly reduces a component of the target behaviours in the TG-R mice.

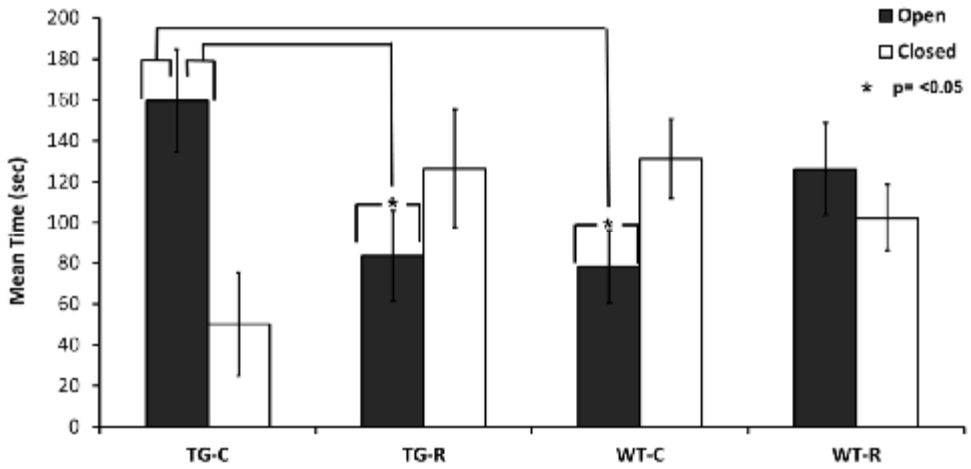

Figure 3.10: EINS EPM (Experiment 2B). Mean duration spent in open and closed arms. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

	PR (Open Arms)	
Group	M	SEM
TG-C	0.69	0.08
TG-R	0.44	0.10
WT-C	0.37	0.07
WT-R	0.53	0.08

Table 3.5: EINS EPM (Experiment 2B). Open Arms PR (duration data).

Mean Frequency of Entries into Open and Closed Arms

Figure 3.11 shows the mean frequency of entries into open and closed arms of the EPM. Inspection of this figure shows that there is an overall transgene effect, although the direction of this differs between genotypes. WT mice make significantly more entries into closed arms, whereas WT mice make significantly more entries into open arms. This is pattern is likely driven by the fact that unlike both drug-treated groups, the distribution of entries in non-drug-treated conditions shows a significant selection bias, with TG-C mice making more entries into open arms than closed, and WT-C mice making significantly more entries into closed arms than open. This interpretation was confirmed by an ANOVA with Arm, Genotype, and Treatment as factors. This showed revealed no significant effect of Arm $F<1$, no significant effect of effect of Genotype, $F<1$, no significant effect of Treatment $F(1,44)=2.792$, $p=0.102$, but a significant Arm by Genotype interaction $F(1,44)=11.536$, $p=<0.01$, no significant Arm by Treatment interaction $F<1$, but a significant Arm by Genotype by Treatment interaction $F(1,44)=6.657$, $p=<.05$. There was no significant Treatment by Genotype interaction $F<1$.

A follow-up test of simple main effects for the significant Arm by Genotype by Treatment interaction revealed that whilst the number of open arm entries was non-significant between non-drug treated groups, $F(1,44)=3.263$, $p=0.079$, WT-C mice did make significantly more entries into closed arms than TG-C mice, $F(1,44)=4.189$, $p=0.047$. Thus, although this analysis shows that the genotypic target behaviour was not confirmed in the TG-C mice (i.e. that they make more entries into open arms than WT mice), the behaviour of the WT mice was consistent with their exhibiting a more anxious/less disinhibited phenotype. TG-R and WT-R mice also did not differ with respect to the number of open arm entries $F<$, or closed arm entries $F<1$. Nevertheless, within genotype comparisons did show that TG-C mice made more entries into open arms than TG-R mice, $F(1,44)=4.330$, $p=0.043$, although the number of entries into closed arms was non-significant: $F<1$. By contrast, WT-C mice did not differ significantly from WT-R in either the number of entries into open arms $F<1$, or closed arms $F(1,44)=2.748$, $p=0.105$. Again, this suggests that rosiglitazone had modified some component of the disinhibited TG phenotype, but had a negligible effect on WT mice. Finally, whilst neither nether of the drug-treated groups showed a significant bias towards making more entries into one type of arm or another TG-R: $F<1$, WT-R: $F<1$, both non-drug treated groups did show a bias with TG-C mice making significantly more entries into open compared to closed arms, $F(1,44)=9.679$, $p=<.05$, and WT-C

mice making more entries into closed arms compared to open arms $F(1,44)=7.187$, p=0.010. Thus, within the non-drug control groups, significant arm biases were found in relation to the number of arm entries that were entirely consistent with genotypic target behaviours.

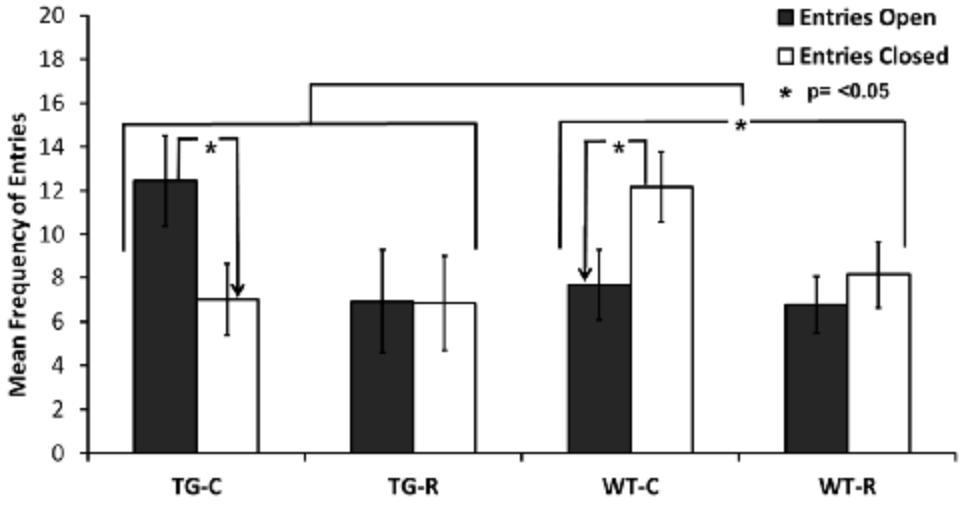

Figure 3.11: EINS EPM (Experiment 2B). Mean frequency of open and closed arm entries. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

PRs were also calculated for the number of open arm entries (see Table 3.6). In line with expected genotypic preferences, this did confirm that in the non-drug treated groups TG mice showed a significant preference for making more entries into open arms relative to closed than WT mice. This interpretation was confirmed by an ANOVA, with **PRs** as the dependent variable, and Genotype, and Treatment as factors. This showed a significant effect of Genotype $F(1,44)=4.704$, p=0.036, a non-significant effect of Treatment $F(1,44)=1.832$, p=0.183, and a significant Genotype by Treatment interaction $F(1,44)=8.180$, p=0.006. A follow-up test of simple main effects for the significant Genotype by Treatment interaction revealed that TG-C mice made significantly more entries into open arms compared to WT-C mice, $F(1,44)=12.138$, p==<.01, although the PRs for drug-treated groups did not differ statistically, $F<1$. Furthermore, and of considerable interest, a within genotype analysis showed that TG-R mice made significantly fewer entries into open arms compared to TG-C mice, $F(1,44)=8.521$, p=<.05, whereas the difference between both WT groups was not significant $F(1,44)=1.185$, p=0.282. This confirmed that rosiglitazone had reduced some component of anxiety/disinhibition in the transgenic mice.

	PR (Open Arms)	
Group	M	SEM
TG-C	0.66	0.04
TG-R	0.42	0.07
WT-C	0.38	0.04
WT-R	0.46	0.06

Table 3.6: EINS EPM (Experiment 2B). Open Arm Entries PRs (frequency data).

3.4.4 Discussion

During Experiment 2 mice were scored in the EPM according to two popular characteristics thought to correspond to anxiety and behavioural disinhibition in rodents: the total time spent in the open verses closed arms of the EPM apparatus, and the number of entries made into each of these arms. I will first discuss each of these measures in relation to the control (non-drug) treated mice before discussing in further detail the interesting findings in relation to rosiglitazone and its impact on both of these measures in the early-intervention group.

3.4.4.1 Evaluation of Experimental Controls

It is undoubtedly the case that across both late and early-intervention experiments, there is evidence to support the view that TG mice display disinhibited behaviours in the EPM, although this is most convincing for the early-intervention group. In Experiment 2A, no significant differences were found between with genotypes with respect to either the time spent in open and closed arms, or the number of entries into these arms (although weak trends in the direction of target behaviours are seen). Thus, in terms of target behaviour open arm entries, in the late intervention study TG mice are no more anxious or disinhibited than WT mice. However, the fact that compared to TG mice, the WT mice did make significantly more entries into closed arms, suggests that WT mice were the more anxious. Indeed, it is also apparent that unlike WT groups, both TG groups do show a significant bias in their arm selections, making more open arm entries than closed. This does suggest that some component of anxiety/behavioural disinhibition was present in TG mice that may have been absent in the WT mice (or at least, not present to the same degree). In Experiment 2B (early intervention) the situation is considerably different; both target behaviours were confirmed in non-drug treated controls. Thus, TG-C mice spent significantly more

time in open arms compared to WT-C mice, and made significantly more open arm entries than WT-C mice, although this latter finding was only evident when a preference ratio was calculated for the number of open arm entries divided by the total number of open and closed entries. Indeed, non-drug treated mice in both genotypes also displayed significant within group preferences, with TG-C mice making more entries into open verses closed arms, and WT-C mice showing the opposite preference. Both of these findings are consistent with previous studies using the EPM which show that TG mice display a disinhibited phenotype relative to WT mice (i.e. make more open arm entries and spend more time in the open arms [1381, 1382, 1390]). However, like the current study, not all studies have reported finding a significant genotypic difference with respect to these measures in the EPM [1252, 1381], although other measures have been reported which suggest that TG mice are comparatively disinhibited compared to WT controls [1252]. This suggests that a number of factors could affect the performance of mice in the EPM task. I will briefly summarise these.

Factors Possibly Affecting EPM Performance

With the exception of one study [1381], most studies have not reported the ratio of male to female mice used in experiments. The levels of oestrogen are known to influence anxiety related behaviours in humans [1351], as well as rodents (e.g. [1432, 1433]). Indeed, the numbers of female mice used is particularly important in the context of Tg2576 [1434] and other hAPP models [1435, 1436] where it has been reported that sex and/or endocrine factors (particularly those associated with reduced oestrogen signalling) strongly modulate cerebral β-amyloidogenesis in hAPP-transgenic mice leading to more extensive amyloid pathology than in age matched males. Thus, it is possible that some studies which have reported a significant genotypic difference with respect to the duration spent in open and closed arms, have used a higher proportion of female mice where oestrogen signalling happened to be transiently low. However, since this is not the case in the current study this cannot be considered a suitable explanation for why the findings in controls were more robust in the early-intervention data.

It is possible that variation in the genetic backgrounds of various TG2576 colonies used in studies may be responsible for some of the variance in reported outcomes relating to the EPM. For example, Lassalle et al. [1381], have investigated spatial memory and anxiety in Tg2576 mice relative to 3 genetic backgrounds of TG2576

differing by their genetic heterogeneity (homozygous verses heterozygous) and strain of origin (C57BL6, CBA, B6SJL F1) after only one generation backcrossing. Whilst Lassalle et al. report a significant transgene effect in terms of the time spent in the open-arms by TG mice relative to WT littermates, this difference is less pronounced in the B6SJL background in which WT mice also spend much time in the open-arms [1381] (note: the number of arm entries in this study was not assessed). That said, both the Tg2576 colony in Cardiff maintained by Professor Mark Good, and the mice purchased from Taconic [1282], originate from the same B6SJL background strain, although it is possible that small variations in the genetic makeup of these animals may have been responsible for the differential late, and early-intervention results. Indeed, it is possible that genetic differences in the late-intervention cohort could have reduced the sensitivity threshold of the EPM task, making it more susceptible to other factors. Finally, there are two methodological issues that could explain why the late-intervention data was not as robust as was the case in the early-intervention cohort. First, Experiment 2A (LINS) had fewer mice. Given that there were weak trends in the direction of target behaviours, fewer mice may have made the experiment more susceptible to individual variation responses that the PRs could not fully correct. Second, the mice in Experiment 2A were older (~13 months at test) than mice in Experiment 2B (~8 months at test). Whilst age may be one factor which could may changes in the behaviour of WT mice on a range of tasks [1437], limited evidence suggests that compared to mice with a predominant C57BL6 background, TG2576 and WT mice from a predominantly B6SJL F1 background may be less resilient to the effects of aging across some cognitive measures [1284, 1356]. However, this has not been investigated with respect to emotional behaviours in Tg2576 mice and WT littermates, particularly at the 13 month old time point. Indeed, whilst one study has reported a significant difference in 17 month old Tg2576 mice in some genetic backgrounds with respect to the total duration of time spent in open and closed arms [1381], the frequency of arm entries has not been assessed. Some research also suggests that anxiety measures in younger mice are less susceptible to pre-test conditions compared to the same variables in adults [1438]. Again, although this does not appear to have been systematically studied in Tg2576, since the mice in Experiment 2A had received prior exposure to searching for food in a similar apparatus (i.e. the T-maze), it is possible that that this may have reduced the sensitivity EPM to genotypic differences in the older mice. Indeed, the mice in Experiment 2B were not only younger adults, but

also experimentally naive. Nevertheless, the results of both interventions do support the view that TG mice exhibit less anxiety/greater behavioural disinhibition than WT both in young adult mice, and older mice.

3.4.4.2 Impact of Rosiglitazone on Anxiety and Disinhibition

Although the impact of rosiglitazone on anxiety and behavioural disinhibition is unknown, rosiglitazone has been shown to improve contextual fear conditioning in aged rats [1403], demonstrating that the drug may (in non-TG mice at least), affect some components of emotional memory in adult mice. However, it is evident from the results of the late-intervention experiment that there are no significant drug effects in any of the groups with respect to the primary methods of assessing anxiety and behavioural disinhibition in ~13 month old Tg2576 mice, in the EMP task despite being on the compound for between ~5-to-6 months. As such, the results of Experiment 1A are consistent with those of Nicolakakis et al. [1386], in that delivering PPARγ compounds to adult mice when amyloid pathology is well under way does not necessarily result in rescue the behavioural phenotype of hAPP mice. However, it is also apparent that in Experiment 2A the target behavioural effects in the EPM task were much weaker, and not conclusively incompletely demonstrated in controls. Thus, without confirming the behavioural phenotype in control mice, it is not possible to say anything further about what impact rosiglitazone might have had in ~13 month old mice. The results of Experiment 2B (EINS) confirm both sets of target behaviours in the TG and WT non-drug treated controls. Furthermore, unlike Experiment 2A, continuous rosiglitazone administration from the age of 5 months did significantly reduce the amount of time TG-R mice spent in open arms of the apparatus relative to same genotype control, as well as the number of entries into open arms of the apparatus. However, the drug did not seem to 'restore' the within group closed arm bias seen in the WT-C mice (although in WT-C mice this was only significant in relation to the number of entries into closed verses open arms). Thus, the current study is the first to report that when delivered early, rosiglitazone does affect the performance of TG mice in the EPM task. However, as to what component of the task the drug had affected, and what its mechanistic basis might be given the findings of experiment 1B, is more uncertain. In the final part of this discussion therefore, I will address these issues.

Does Rosiglitazone Make TG mice More Anxious?

Only two parameters were used in this study to detect differences in anxiety in the EPM. These parameters were the time spent in the open sections, and the number of open arm entries [1383]. Indeed, in this study, the number of entries into arms was probably the most sensitive of these parameters for assessing levels of anxiety and behavioural disinhibition across both cohorts of mice. This fits with several studies which have reported that Tg2576 mice have increased levels of locomotor activity in the EPM [1382] and other similar tasks [1439-1441]. Indeed, since at least some instances of clinical anxiety have been linked to hyperactivity of the septo-hippocampal system [1442]. For example, hyperactivity of this system has been linked to increased negative association of stimuli with a consequential increase in anxiety when the stimuli are subsequently presented [1442]. Thus, the hippocampal pathology observed in Tg2576 [1245, 1251, 1263, 1308, 1310] most likely explains the disinhibited phenotype of these mice. However, not all studies have found activity levels in Tg2576 to necessarily increase/decrease in the EPM in accordance with the traditional anxiety-like parameters such as the time spent in open and closed arms of apparatus [1441]. In this context, it is important to note that the number of entries in the EPM (and similar tasks), is a focus of considerable discussion, primarily because it is unclear as to whether this parameter is an 'activity index' (i.e. locomotor activity), or an 'anxiety index' [1441]. Indeed, studies have used increases or decreases in the frequency of entries parameter in the EPM and other similar tasks (i.e., elevated zero maze), as either anxiety or activity differences [1443-1446]. One possible reason for these contradictory interpretations is that entries parameter is only partially related to anxiety levels [1441]. For example, as a parameter of anxiety, the time spent in the open sections of the EPM can be affected by the number of entries, and is important to distinguish whether the differences observed in mice are therefore related to anxiety or activity levels [1441].

In order to address the above issues, Heredia et al. [1441], have recently proposed a new index of anxiety that they refer to as "Time by Entries" (TbE). This new parameter adjusts for the influence of activity on time in the open section of an elevated Zero-maze, although the principles can also be applied to the EPM. Heredia et al. used the following formula:

$$Tbe = \frac{\text{Time in Open Section}}{\sqrt{\text{Number of Open Arm Entries}}}$$

The square-root was used in order to maximize differences at low levels of activity, and minimize differences at high levels of activity or 'hyperactivity', and is based on the theoretical function of square-root as a half of parabola with a vertical directrix [1441]. If the entries increase (i.e. when an animal crosses the open section rapidly and continuously), this parameter minimizes the effect of the high levels of activity over time spent in the open section. Since time in the open section increases when there are high activity levels, Heredia et al. were able to re-evaluate the time spent in the open section of the maze by controlling for high activity levels with the TbE parameter. In addition, because TbE is a correction of time in an open area, it must be interpreted as the time spent in the open area [1441]. Thus, a high TbE rating is indicative of low levels of anxiety, while low TbE rating is indicative of high levels of anxiety [1441]. Using TbE in relation to the zero maze, Heredia et al. have assessed how individual housing, handling procedure and interaction between individual housing and handling procedure affect the baseline anxiety in Tg2576 mice and WT littermates [1441]. Tg2576 mice were randomly assigned to two experimental groups: 1) individually housed, unhandled, and 2) individually housed and handled. Heredia et al. reported that the handling procedure in wild type animals did not show any consistent effect on anxiety-like behaviour levels if the animals were not individually housed [1441]. With respect to Tg2576 animals, individually housed/handled Tg2576 mice were less anxious than the individually housed/unhandled animals, and Tg2576 handled animals showed a reduction in activity levels compared to the Tg2576 unhandled animals [1441]. Comparing Tg2576 mice with their WT controls, the results of Heredia et al. suggest that Tg2576 mice are less anxious than WT animals. *But*, after calculating TbE index, their results did not show significant differences for this parameter, and therefore, it was concluded that significant differences in time spent in the open section by Tg2576 mice are primarily mediated by the activity levels of this group [1441]. This means that differences in time spent in the open section between individually housed/unhandled Tg2576 mice and individually housed/unhandled wild type mice, were not due to differences in anxiety-like behaviour *per se*, but rather, reflected differences in activity levels [1441], probably as a consequence of the generally acknowledged hippocampal pathology in TG mice. One should say at this point, that in terms of the current study, whilst the mice from cohort 1 and 2 were all individually housed from a young age,

they were handled frequently by animal technicians (as part of husbandry and maintenance procedures), as well as by the researcher conducting the experiments, so effects should have been the same across both cohorts.

One has to wonder now, what the results would be if the TbE index is applied to the data in Experiments 2A and 2B. This data is summarised in Table 3.7. A subsequent comparison of the figures obtained for the TbE index in both the LINS and EINS EPM experiments were subjected to statistical analysis using a 1-way univariate ANOVA, with **TbE** as the dependent variable, and **Genotype**, and **Treatment** as factors.

	LINS TbE (Exp. 2A)		**EINS TbE (Exp.2B)**	
Group	M	SEM	M	SEM
TG-C	25.98	4.01	13.51	2.08
TG-R	36.45	4.72	7.64	1.50
WT-C	25.17	4.21	8.09	1.05
WT-R	24.53	4.00	10.66	1.01

Table 3.7: Group TbE Scores for EPM. Experiment 2A/B.

This showed that in both experiments there were no significant differences any of the groups. LINS: no significant effects of **Genotype** $F(1,32)=2.248$, $p=0.144$, of **Treatment** $F(1,32)=1.388$, $p=0.256$, or a **Genotype by Treatment** interaction $F(1,32)=1.714$, $p=0.200$. Similarly, EINS: no significant effect of **Genotype** $F(1,47)=0.23$, $p=0.727$, of **Treatment** $F(1,47)=0.27$, $p=0.870$, or a **Genotype by Treatment** interaction although this was close, $F(1,44)=3.349$, $p=0.074$. Both of these results are consistent with the findings of Heredia et al. [1441], and suggest that levels of anxiety were approximately similar across all groups in both experiments, with differences in time spent in the open section not reflecting differences in anxiety-like behaviour per se, but rather, differences in activity levels.

What else could explain the fact that rosiglitazone did, in the EINS data at least, significantly impact on performance in the EPM task? It is possible that the drug modified some motivational component of TG behaviour, leading to lower activity levels. If true, such a change has not been previously reported in the scientific literature, and does not in any case explain why a similar effect was absent in the late-intervention cohort. It is also possible that the drug may have in the EINS cohort,

prevented some component of hippocampal pathology in the TG mice such that this may have reduced the levels of hyperactivity. Indeed, the role of the ventral hippocampus in emotional processing is considered to be distinct from that of the amygdala (which is associated specifically with fear), as well as distinct from the role of dorsal hippocampal regions involved with spatial memory functions [80[686]. In this respect, Gray and McNaughton's theory of the septo-temporal axis [1447], can in principle, incorporate these apparently distinct hippocampal functions, and provide a plausible unitary account for the multiple facets of hippocampal function [686]. Thus, although speculative, it is at least plausible that rosiglitazone may have had an impact on some component of the ventral hippocampal function whilst leaving the dorsal regions either untouched, or if there were any physiological changes, maybe they were of insufficient magnitude to significantly impact upon spatial memory function as assessed via the T-Maze FCA task. Indeed, it is possible that amyloid pathology had progressed too far in the LINS cohort, for the drug to make a difference in either region, despite the fact that these 13 month old mice had received rosiglitazone agonism for ~5 months. Thus, in this case, it would appear that the late-intervention therapy really was 'too late' to make a significant difference. However, as stated earlier, it is also possible that variation between the cohorts in terms of the degree of SJL F1 background may also have had an impact on the pattern of results obtained.

3.5 Experiment 3: Marble Burying

3.5.1 Introduction

Marble burying behaviour has been a popular test of neophobia in rodents, where historically it has been used with mouse models of anxiety [1383, 1448]. This task is widely considered to tap into species-typical defensive reactions of rodents to spontaneously bury unfamiliar stimuli [1449]. Furthermore, marble burying is sensitive to the administration of anxiolytic and antipsychotic drugs, which are normally used to treat anxiety disorders or obsessive-compulsive disorder [1448, 1450]. Indeed, administration of such drugs in rodents has been shown to result in a reduction in burying behaviour in mice [1451-1453]. Currently, only one study has reported assessment of Tg2576 mice on the marble burying task. Using female Tg2576 mice and WT littermates aged 12.5, and a separate cohort of female mice aged 23 months, Deacon et al. [1374] reported no significant differences in marble burying between genotypes. The performance of male Tg2576 mice on this task has not been reported in the literature, and there are no reports investigating the effects of rosiglitazone on this measure of anxiety. It is worth just recapping that the anxiety tasks were counterbalanced across both LINIS and ENIS conditions, and were given to mice second in the LINIS condition, and first in the ENIS condition. Thus, in Experiment 3A (LINS), mice were given a single exposure to the EPM at ~13 months following a 2 day rest period after the conclusion of T-maze FCA testing (Experiment 1A). In Experiment 3B (EINS), the EPM task was delivered to 8 month old mice, exactly 2 days after the conclusion of the EPM testing (Experiment 2B).

3.5.2 Methods and Apparatus

3.5.2.1 Animals and Drug Administration

Experiment 3A (LINS) used mice the mice form cohort 1, which had previously undergone the T-maze and EPM task. Experiment 3B (EINS) used the mice from Cohort 3. These mice had received exposure only to the EPM. Please see section 3.3.2.1 for details of housing and drug administration.

3.5.2.2 Apparatus

Each marble burying arena was composed from a large white polycarbonate cage base 41cm long, 24.5cm wide and 12.5cm tall, filled with a level compact layer of sawdust

6.5cm deep (see Figure 3.12). Twenty mixed-colour glass marbles (1.5cm diameter) were placed on top of the sawdust layer equally distanced apart in a 4-column by 5-row arrangement. In comparison to a grouped-marble arrangement, this arrangement forced the mice to encounter the marbles more frequently, and prevented an alternative avoidance response rather than burying the aversive stimuli (see [1454]). A transparent Perspex lid 41cm long, 27cm wide and 1cm thick was placed securely on top of the cage base to prevent the mouse from escaping the arena. The lid was placed in a central position allowing for a 1.25cm gap either side for ventilation. Four marble burying arenas were placed on a table elevated 121cm from the floor, allowing 4 mice to be tested during one 30-minute session. A camera was attached to the ceiling directly above the arena to allow viewing and recording for documentation using a monitor and VCR. Each marble burying arena was photographed for documentation using a digital camera at the end of each session.

Figure 3.12: Marble burying apparatus. (*Left*) Initial setup of marbles. (*Right*): Typical WT result obtained after 30 minutes (non-buried marbles are displayed in transparent circles).

3.5.2.3 Behavioural Procedures

This test was given as a one day procedure. Each mouse received a single trial comprised of 30 min in a cage with a clear Perspex lid placed over the top to prevent escape (a small gap was present at the top and bottom of the lid to allow air circulation). When the session had expired, the animals were removed from the maze and placed back into their home cages prior to being returned to the holding room. The number of marbles buried was then recorded (see scoring method below), and the sawdust renewed for each session. Marbles were cleaned thoroughly with a 1:20 dilution of Mr Muscle® prior to being placed in each box. The experimenter wore latex surgical gloves at all times throughout the experiment, and all behavioural trials were recorded onto VHS video. Mice were run in the sequence as occurred in Experiment 1.

3.5.2.4 Scoring and Statistical Analysis

Using the criteria used in previous studies [1454], a buried marble was defined as a marble covered at least two thirds of their depth by sawdust. Thus, an unburied marble was over one third visible on the surface of the sawdust layer. No further mice were excluded from the analysis due to morbidity/mortality, and none of the mice met criterion for non-responsiveness (in this case, spending the whole 30 minutes at the start location). Thus, the total numbers of mice run in Experiment 3A were 18 TG mice (TG-C, n=9; TG-R, n=9), and 19 WT (WT-C, n=10; WT-R, n=9). In Experiment 3B, the total numbers of mice run were as follows: 24 TG mice (TG-C, n=12; TG-R, n=12); 25 WT mice (WT-C, n=12; WT-R, n=13). Since the dependent variable in this experiment had only one level, each Experiment was analysed by a single 2-way univariate ANOVA, which had **Marbles Buried** as the dependent variable, and **Genotype**; **Treatment** as factors. All statistical analysis were conducted using IBM SPSS Statistics v.21.00 software with an alpha value (P) <0.05, taken as being statistically significant. Significant ANOVA interactions and non-parametric analysis were conducted as previously indicated (see section 3.3.2.4).

3.5.3 Results

3.5.3.1 Experiment 3A (Late Intervention)

Figure 3.13 shows the mean number of marbles buried for each group in the marble burying task. Inspection of this figure shows that there is a significant transgene

effect, with WT mice burying more marbles than WT mice. However, there are no significant drug effects in either genotype. This description was confirmed by ANOVA, which had Marbles Buried as the dependent variable, and Genotype; Treatment as factors. This showed a significant effect of Genotype $F(2,33)=4.322$, p=<.05, no significant effect of Treatment $F<1$, and no Genotype by Treatment interaction $F<1$.

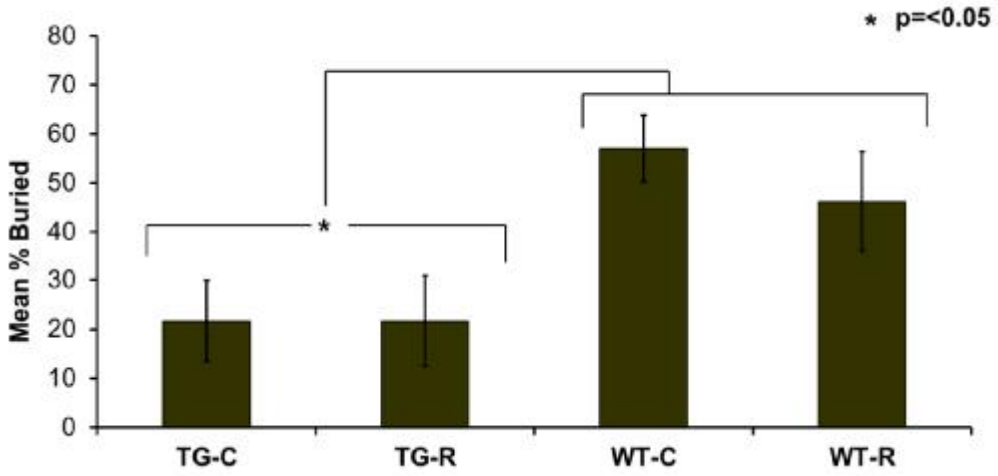

Figure 3.13: LINS Marble burying task (Experiment 3A). Mean %age of marbles buried for each group. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

3.5.3.2 Experiment 3B (Early Intervention)

Figure 3.14 shows the mean percentage of marbles buried for each group in the marble burying task. Inspection of this figure shows that there is a significant transgene effect, with WT mice burying more marbles than WT mice. However, there are no significant drug effects in either genotype. This description was confirmed by two-way univariate ANOVA, which had Marbles Buried as the dependent variable, and Genotype; Treatment as factors. This revealed a significant effect of Genotype $F(1,44)=14.328$, p=0.001, no significant effect of Treatment $F(1,45)=1.259$, p=0.268, and no significant Genotype by Treatment interaction $F<1$.

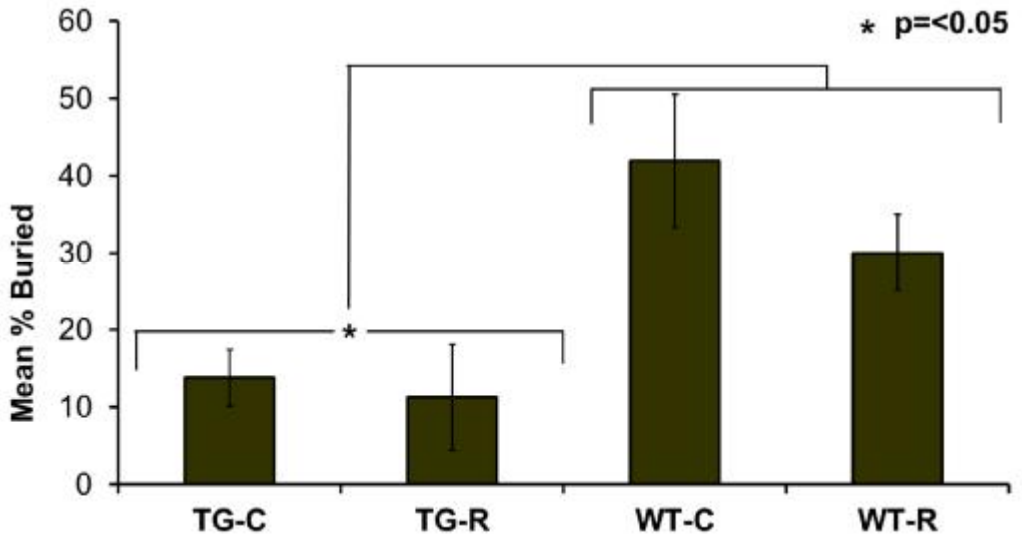

Figure 3.14: EINS Marble burying task (Experiment 3B). Mean percentage of marbles buried for each group. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

3.5.4 Discussion

The results of both Experiments 3A (mice aged 13 months) and 3B (mice aged ~ 8 months), show a significant transgene effect, with WT mice burying significantly more marbles than TG mice. This contrasts with the findings reported by Deacon et al. [1374], where no genotypic differences between either young (3 month old) or adult 12 month old) Tg2576 mice and WT littermates. There are three major factors which may help to may explain these discrepant findings. First, Deacon et al. assessed TG and WT mice at 3 months of age, and a separate cohort of mice aged 12 months of age. In both instances, female mice were used. This is potentially of significance because previous research has shown that progesterone and/or estrogen can influence impulsivity and/or fear in female rodents [1455-1457], and administration of estrogen and/or progesterone to ovariectomized rats decreases anxiety behaviours in the open field and EPM tasks [1456]. Consistent with this, research conducted in naturally cycling rats has shown when tested on a marble burying and conditioned fear tasks, rats in behavioural estrus showed less impulsive burying and freezing behaviour than diestrous rats [1458]. Indeed, when ovariectomized rats were administered progesterone, estrogen or vehicle, progesterone or both estrogen and progesterone, were found to decrease impulsive burying and freezing behaviour compared to vehicle [1458]. Thus, these results suggest that progesterone and/or estrogen may mediate

components of impulsive and/or avoidant behaviour in rodents, although these effects have not been verified in naturally cycling mice in the marble burying task. However, it seems reasonable to suggest that in the Deacon et al. study, behavioural estrus may have overlapped with the application of the marble burying task, such that the WT mice would have likely showed less impulsive burying behaviour than would have occurred in diestrous mice. Given that that in the current study, the results of Experiment 3 clearly show that TG mice bury less marbles anyway, behavioural estrus in some or all of the squads of mice tested in the Deacon et al. study could have accounted for the null finding. Second, methodological differences could also account for the discrepant findings. Deacon et al. used only 12 marbles, in contrast to the 20 marbles used in the methodology reported in the current experiment [1374]. This lower number of marbles may have further reduced their ability to detect genotypic changes in female Tg2576 mice. Third, strain differences were reported by Deacon et al. [1374], which may have also accounted for the differing results in the current study. More extensive research is required to characterise the performance of Tg2576 mice from different background strains before any firm conclusions can be drawn.

Most behavioural scientists would probably agree that marbles, by nature, are likely to be non-aversive to mice, something Njung'e and Handley [1454] confirmed in 1991 by showing that mice did not avoid the marble-containing side of a two compartment box. Thus, it is unlikely that either mice or rats are necessarily in a state of anxiety when faced with the sudden appearance of marbles in their environment. Indeed, whilst mouse marble burying has historically been used as a screening model for the detection of anxiolytics [1459], with benzodiazepine receptor agonists such as rapid-onset diazepam or chlordiazepoxide found to decrease the number of marbles buried [1460-1462], non-anxiolytic compounds such as classical antipsychotics, anxiolytics, psychostimulants, and certain classes of antidepressants (see review, [1449]) have also been found to modulate marble burying behaviour [1451, 1453, 1459, 1463]. This suggests that the predictive validity of this procedure for anxiety may be limited. Indeed, subsequent research has shown that mouse marble burying behaviour whilst genetically regulated, is not correlated with other anxiety-like traits in mice nor stimulated by novelty [1464]. Rather, it now appears to reflect a repetitive behaviour that persists/perseveres with little change across multiple exposures [1464]. In this way one can probably explain why considering the fact that TbE analysis of data in Experiment 2 showed that levels of anxiety were statistically comparable across groups,

marble burying behaviour nonetheless was found to differ significantly between genotypes in both the LINS and ENIS marble burying experiments. However, given the range of factors which can seemingly disrupt marble burying behaviour, one has to admit to the apparent uncertainty with respect to the likely neural mechanisms engaged by the marble burying task. Of key importance in this regard is whether the behaviour is a response of rodents to the presence of marbles [1449, 1465], or a non-intended consequence of digging behaviour [1462, 1466]. For example, marbles may appear buried due to mice engaging in digging behaviour in the general vicinity [1448]. Perhaps the most reasonable approach is to assume that marble burying simply measures species-typical digging behaviour [1448] that may in part be dependent on intact hippocampal function [1467]. Indeed, one interpretation of marble burying/digging is that it will likely be modified by any agent affecting hippocampal function, including the benzodiazepines and 5-HT active compounds [1447, 1468], although given that psychostimulants can also inhibit marble burying [1459, 1463], it is likely that brain regions other than the hippocampus are also involved in regulating this species-typical behaviour. Nevertheless, given the disruption to the hippocampal circuitry in Tg2576 mice, this may partly explain why the digging behaviour is disrupted in Tg2576. Furthermore, since burrowing/digging behaviour is not directly related to learning and memory, it cannot reliably predict changes in such aspects of cognition, although it may involve other aspects of cognition such as executive function or attention [1374].

Given these considerations, and the uncertainty as to whether or not rosiglitazone had modified some component of hippocampal function, it perhaps comes as no surprise that the drug did not modulate marble burying behaviour in either of Experiments 2A or 2B. However, having assessed mobility of mice in an empty arena prior to the marble burying task being run, could potentially have informed this issue. Nevertheless, the reasons for TG mice burying fewer marbles than WT is not likely to be a simple matter of the former being more active (i.e. they stop more infrequently than WT mice to dig). Furthermore, given the wide range of substances that can seemingly modulate marble burying behaviour, reducing locomotor activity (if indeed that is what rosiglitazone did in Experiment 2A), would be no guarantee that 'normal' marble burying behaviour would have been restored in the TG mice.

3.6 Experiment 4: Object Recognition Memory

3.6.1 Introduction

Object recognition memory is the ability of many animal species to discriminate the familiarity of previously encountered objects [1469]. As such, behavioural tasks such as delayed nonmatching-to-sample (DNMS) and spontaneous object recognition (SOR) for assessing object memory in non-human primates and rodents, have proved invaluable as animal models of specific aspects of human memory processes [1469]. Indeed, these tasks constitute the core means by which animal models can successfully access many key aspects of MTL amnesia first characterised in patient HM [525, 534]. Since it is impossible to do justice to the vast literature on this subject in the limited space available in this chapter, the reader is directed to the following reviews [500, 525, 1470]. This section briefly outlines the SOR task and some of its variants, as these are now common behavioural tests used to measure object recognition in animals, and rodents in particular. A *de novo* version of the SOR task is used to assess object recognition memory in the current study, specific details of which are summarised at the end of this section.

3.6.1.1 Spontaneous Object Recognition Tasks

The spontaneous object recognition task originates from the work of Ennaceur and Delacour [1471], and exploits the natural tendency of rodents to explore novel stimuli in preference to familiar stimuli. Typically, the SOR task is run in an open field arena, although recent efforts to address certain controversial aspects of the literature have prompted the introduction of a novel Y-shaped apparatus for testing SOR [1472] (these are not considered here). In the open field test, the spontaneous loco-motor or exploratory activity of mice is usually studied in circular or square arenas, which vary in size depending on the experiment concerned and whether the environment is divided into distinct quadrants or sections [1355]. The SOR paradigm is similar in principle to the DNMS task. A single SOR trial consists of sample and choice phases, separated by a variable retention delay [525]. In the sample phase, the rodent is introduced into the testing apparatus, which contains two identical junk objects (A1 and A2). The rodent is allowed to freely explore these objects for a limited amount of time before being removed from the apparatus. At the end of the retention delay, the animal is reintroduced to the apparatus, which now contains a triplicate copy of the

sample object (A3) and a novel object (B) to which the animal has had no prior exposure. Under normal circumstances, rodents will preferentially explore the novel object in this choice phase, and this behaviour is taken as the index of recognition of the familiar sample object [525]. For this reason, the SOR task is sometimes referred to as Novel Object Recognition task (NOR) [1473], as a means of differentiating it from other variants which assess spatial memory (see below). SOR is also known as the visual paired comparison (VPC) task in studies with humans and monkeys [1469]. Because SOR uses the natural exploratory behaviour of animals, unlike DNMS it does not require a pre-training phase (i.e. reward-based learning of the non-matching-to-sample rule). Aside from the advantage of allowing object recognition to be studied in a more natural manner without the potential complications of interpretation introduced by nonmatching-to-sample acquisition (or motivational considerations), the task is much quicker and simpler to run than DNMS [1473]. Furthermore, by varying the length of the interval between sample and test phases, SOR tasks can be used to assess either LT or WM processes [1474]. Indeed, the task is incredibly versatile in its simplicity. With slight modifications for example, the SOR can be used to assess object-in-place memory (see figure 3.15). In the spontaneous Novel Location Recognition task, the index of stimulus recognition is this time taken to be a measure of the greater time spent exploring a familiar object that has switched its spatial location in the choice phase [1474]. In this instance, the natural propensity of rodents is to spend more time exploring the familiar object that has been moved to a different location, versus the familiar object that remains in its previous location [1474]. For all of these treasons, SOR tasks have often become the test of choice for behavioural neuroscientists. As a result, SOR tasks have contributed greatly to our current understanding of the neurobiological basis of object recognition memory [525], as well as the interactions of multiple brain regions (perirhinal cortex, hippocampus and medial prefrontal cortex) mediating object-in-place memory [498, 1475].

3.6.1.2 Recognition Memory in Adult Tg2576 Mice

Probably the most systematic contribution to understanding object recognition memory in Tg2576 mice using SOR analogues has been undertaken by Good and colleagues. In their initial study with male and female mice, Hale and Good [1253], used a standard square shaped arena to assess object novelty, recency discrimination, and object-location memory in Tg2576. Using a standard two-item object array and

delays between the sample and test stages of up to 2 minutes, 30 minutes and 24hrs, this study found that 14-month old Tg2576 mice were able to detect novel objects as well as age-matched WT mice [1253]. In addition, TG mice also exhibited a normal recency effect by exploring less the object most recently encountered compared to an object encountered earlier in a trial [1253].

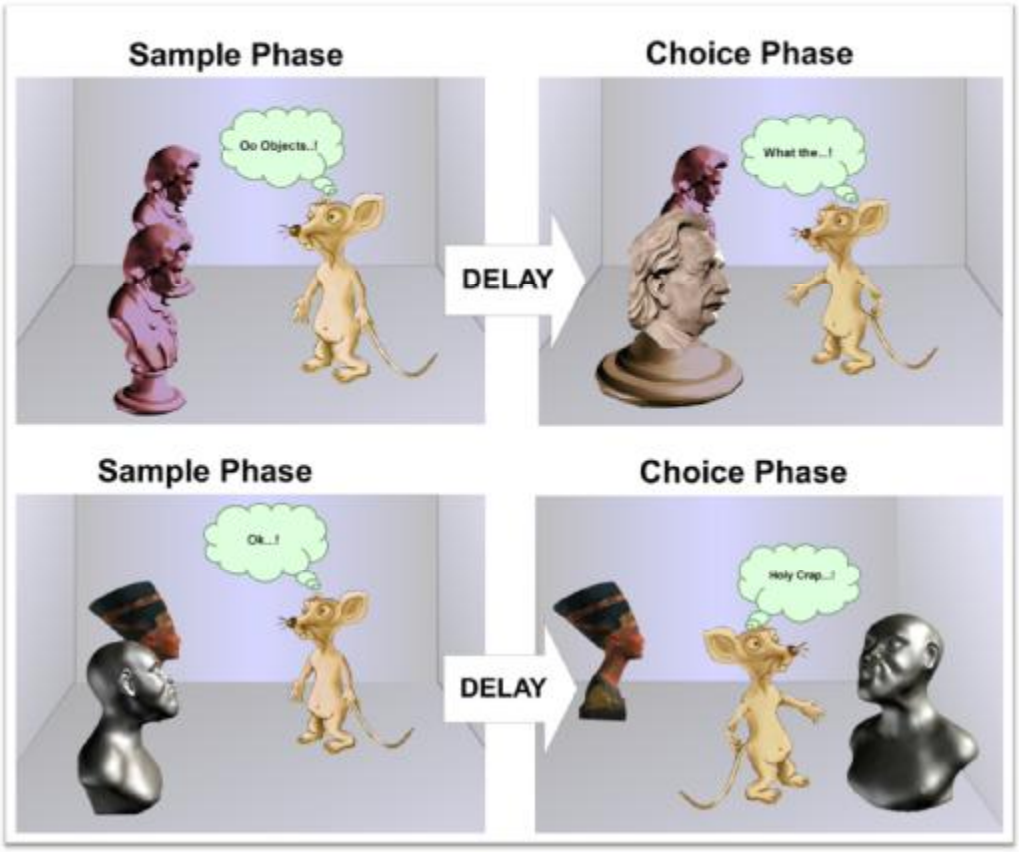

Figure 3.15: Two-trial SOR tasks**.** (*Top panel*) 'Novel object' Recognition. (*Bottom Panel*) Novel Location Recognition.

Consistent with studies of visuospatial recognition memory in AD patients Hale and Good showed that Tg2576 mice were impaired in detecting a change in the relative positions of an array of four familiar objects [1253]. In this test, object-location memory was assessed by using a procedure previously devised by Dix and Aggleton [1476] in which rats were firstly familiarised to a set of four objects (square configuration), in the centre of a square arena. Then following a retention delay, subjects were given a choice phase in which two identical copies of the sample objects had been exchanged across diagonal spatial locations (i.e. topological transformation),

while the remaining copies of sample objects stayed in the same locations as previous [1476]. Dix and Aggleton suggested that whilst standard novel location tasks involving the relocation of familiar objects to novel positions may reflect memory for the spatial organization of objects in the arena, only the modified object-location task described above would require memory for specific object-location associations. Thus, the results of the Hale and Good suggested that adult TG mice are impaired in forming a representation of the spatial organization of objects in an arena, but not impaired in discriminating familiar from novel items *per se* [1253].

In order to further investigate the extent to which the hAPPswe mutation selectively disrupts processes specific to memory for location (as opposed to object identity), Good and Hale [1384] undertook a further study of object recognition memory with a cohort of 16 month old male Tg2576 mice. In order to determine whether the size of the object array interacted with object recognition memory in Tg2576 mice (i.e. made object novelty detection harder), object novelty and object–location tasks both used a four-item object array. In line with their previous findings, Good and Hale showed that adult Tg2576 mice retained normal object novelty discrimination [1384], although in contrast to their previous paper this was only examined using a two minute retention interval (it remains possible that novelty discrimination may have been impaired with longer delays). Also consistent with their previous study, Good and Hale found that Tg2576 mice displayed impaired memory for the location of objects when 2 objects out of 4 underwent diagonal topological transformation [1384]. Good and Hale also tested spatial novelty in subjects by using a manipulation that involved moving two familiar objects to novel locations that had not been previously occupied by any of the objects. This manipulation involved changing the metric relationships between the objects and the arena walls for two displaced objects, whilst the remaining objects were positioned in the same locations as previous. Of importance here is the fact that subjects were counterbalanced across two different sample array configurations. If animals were allocated to an expanding array condition, the objects were initially presented in the centre of the arena and then, during the choice trial, two objects were moved to the corners of the arena. If on the other hand subjects were allocated to the contracting array condition, the objects were initially presented in the four corners of the arena and then during the choice trial, two objects were moved to the centre of the arena. Interestingly in this experiment both control and Tg2576 mice preferentially explored the familiar objects moved to

previously unoccupied (novel) locations [1384]. In order to test the generality of their findings, Good and Hale used a spatial novelty manipulation that kept the metric properties of the array (i.e. object–object distance and shape of the landmark array) consistent between the sample and test trial. Again, the results of this experiment showed that both control and Tg2576 mice preferentially chose to explore familiar objects when they were moved to previously unoccupied locations [1384].

Overall, these results indicate that aged Tg2576 mice are able to form representations of the identity of objects as well as exhibit memory for the spatial organization of objects in an arena. In contrast, conjunctive memory for specific object–location associations is severely impaired in aged TG mice [1384]. Such conjunctive processes are the very basis of episodic memory for "what," "where," and "when" in humans, as well as similar processes in other animal species [367]. Indeed, using similar methodology to their previous studies with female and male mice, Good et al. [1324] have shown that TG but not WR mice aged 10-12 months old are impaired when it comes to forming an integrated memory of the spatio-temporal context in which objects were presented. These results demonstrate that male and female wild-type, but not APP-mutant, mice are able to form an "episodic-like" memory of the spatio-temporal properties of objects supporting the hypothesis that aberrant APP processing contributes to impairments in event memory [1324]. In a recent review, Palmer and Good [1321], summarised converging evidence from animal and human studies which suggests that an early target of amyloid pathology is synaptic activity in the DG (dentate gyrus)/CA3 network which probably disrupts pattern separation processes required for the formation of episodic memory.

Supporting Studies of Recognition Memory in hAPP Mice

A study by Ognibene et al. [1252], with 7-12 month old Tg2576 mice (sex un-reported) has also reported findings consistent with those of Good and Hale [1253]. In this study the object recognition task was comprised of seven consecutive stages (each 5 minutes in duration), each of which was separated by a three minute retention interval. In the first stage subjects were habituated to an empty circular open field arena. In stages 2-4 subjects were then familiarised to five objects placed in the centre of the arena, such that four objects formed a square, with the fifth object at its centre. Throughout stages 2-7 the frequency and the time spent exploring objects were measured for each subject. During stages 5-6, the central object then exchanged places with one of the peripheral

ones, a topological manipulation similar to that used by Good and Hale [1253]. In stage 7 one of the five familiar objects was then substituted with a novel object. The results of Ognibene et al. showed that whilst object novelty detection was comparable across TG and WT groups, Tg2576 mice failed to discriminate displaced objects and were therefore impaired in forming a representation of the spatial organization of objects in the arena [1252]. These results corroborate the findings of Good and Hale [1253]. In a subsequent longitudinal study Middei et al. [1325], further assessed object novelty and object-location memory in adult and aged Tg2576 mice (sex not reported). Whilst this latter study also utilised the seven-stage object recognition task, the procedure was modified at stages 5-6 to examine object-place memory by moving two familiar objects to different spatial locations, a manipulation similar to the spatial novelty manipulation used by Good and Hale [1384]. However, it is important to note that whilst Good and Hale moved two familiar objects to two novel (previously unoccupied) locations in the choice trial, the study by Middei et al., only re-positioned one of the two spatially displaced objects to a novel location, as the second displaced item was moved to a position that had previously contained the first displaced object [1325]. Using this modified scheme Middei et al., tested mice first at 7 months and then again at 14 months of age. Middei et al. showed that seven month old Tg2576 mice failed to explore objects that had changed spatial location, although along with WT controls, TG mice showed a normal preference for object novelty [1325]. However, at 14 months of age, both controls and Tg2576 mice displayed behavioural abnormalities because both groups failed to react to objects that had been displaced, or object novelty [1325]. In considering the differences between this study and that of Good and Hale [1384], it is interesting to note that Good and Hale used a four-item array, whereas Middei et al. used a five-item array [1325], the latter of which may have placed extra cognitive demands on older subjects, perhaps accounting for why 14 month old transgenic and WT mice failed to discriminate spatially shifted objects, as well as novel from familiar objects. An additional factor may have been that some aged subjects may have developed subsequent visual impairments related to the effects of age on genetically pre-disposed retinal degeneration processes (see [1406]).

SOR paradigms have been used by several groups to test object novelty detection in hAPP mice including the Tg2576 model, and reported contrasting results with that of the studies discussed thus far. For example, Taglialatela et al. [1477], found that using a two object array, object novelty discrimination was intact in 5 month old Tg2576 mice

(males and females) when subjects were tested following a two-minute retention period, but was impaired when testing took place after 4 hours, and 24 hours. The authors of this study addressed the fact that their results contrast with the findings of Good and Hale [1253], and suggested that the discrepancy is possibly due to the latter having a higher proportion of the SJL background in there mice [1477]. This is plausible because differences in background strain are known to make a difference to the cognitive phenotype of Tg2576 mice [1381]. Studies using other mouse models of hAPP over expression have also revealed deficits in object novelty detection. For example, a recent study by Simón, et al. [1478] used a two-item object array in a box shaped arena to assess object novelty detection in 2, 4 and 8 month old $hAPP_{SWE-IND}$ mice, and reported that object novelty detection was intact in transgenic and littermate controls when subjects were aged 2 months, but impaired in $hAPP_{SWE-IND}$ mice at 4 and 8 months of age. Whilst this study also apparently used Tg2576 mice in behavioural studies, frustratingly the authors do not clearly indicate which behavioural tests had been undertaken with Tg2576 mice and when [1478].

Escribano, et al. [1214], have used a standard SOR task to evaluate object novelty in $hAPP_{SWE-IND}$ mice that received oral rosiglitazone at a dose of 5 mg/kg per day starting at 1.5-month-old mice for a total of 10 weeks. Rosiglitazone rescued object novelty detection in $hAPP_{SWE-IND}$ mice at 4 and 10 month of age (following 4 weeks of drug administration [1214]. Whilst it was unfortunate that this study did not examine whether rosiglitazone therapy modified Aβ levels, the authors did provide immunohistochemical evidence that linked the restorative effects of this drug to prevention of an early decrease in hippocampal glucocorticoid receptors. In untreated mice this decline coincided with the onset of behavioural deficits [1214]. In a subsequent study [1213], Escribano et al., confirmed the restorative effects of rosiglitazone on object-novelty detection, but also showed that the drug facilitated Aβ clearance by reducing plaque burden in the brain, as well as reducing the number of neuropil threads containing phosphorylated tau [1213]. Furthermore, rosiglitazone treatment led to activation of microglia, promoting phagocytic ability, and reducing the expression of pro-inflammatory markers [1213]. This study provides further insights into the mechanisms for the potentially beneficial effect of rosiglitazone on amyloid pathology and cognitive deficits. Importantly for this thesis, the effects of rosiglitazone on recognition memory in Tg2576 mice, has not been examined.

3.6.1.3 Experiment Rationale and Design

In experiment 4 object recognition memory was assessed in Tg2576 and WT littermates using a de novo SOR (called the 'Cross of Changes' or 'COC'). Two versions of this task are used in this thesis. First there is the Absolute Novelty version of the COC task, where in the choice trial mice have to discriminate two different novel objects within a segmented open field arena from two familiar objects appearing in the same spatial locations as per the sample trial (see Figure 3.16a). Since Tg2576 and WT mice have been shown to exhibit comparable preferences for object novelty in both 2 and 4 item arrays [1253, 1324, 1384], it was predicted that Tg2576 and WT mice should similarly exhibit a comparable preference for object novelty in the Absolute Novelty task. In the second version of the COC task, in the choice phase mice are required to discriminate between two different but familiar objects occurring in the same locations as per a previous, and the same novel object that appears in two familiar spatial locations. This generates a potential spatial mismatch during the test for the novel stimulus in a way that the absolute novelty task does not (see Figure 3.16b). Thus, the second version of the COC task is referred to as Spatial Mismatch task.

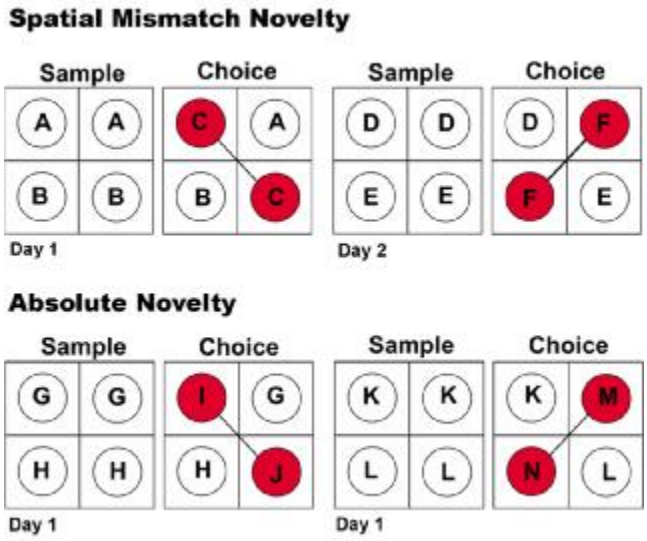

Figure 3.16: COC Task. (*a*): Spatial Mismatch version. This shows the typical setup, with letters different denoting the placement of different objects. Red circles represent the diagonal plane in containing the novel object. (*b*): Absolute Novelty version. Red circles again represent the diagonal plane in containing the different novel objects. As can be seen, the direction of the switch in both versions of the COC task are counterbalanced across test days, with different objects being used of separate days. Note: although we are primarily interested in object novelty in these tasks, the familiar objects involve regency effects.

Although none of the SOR paradigms currently reported in the literature have involved the placement of the same novel object in two different spatial locations simultaneously, it seems reasonable to assume that 'recognition' in the context of the Spatial Mismatch task, would likely require the hippocampus for encoding and short-term recall of specific object-location associations [487, 1475]. Indeed, research in rats has previously shown that individual hippocampal neurons develop responses to specific stimuli in the places where they have differential significance [495]. Thus, in the Spatial Mismatch task, the differential significance with respect to the novel object could be reflected in separate familiar/novel discriminations taking place in both the top and bottom horizontal axis of the apparatus generating a greater net amount of exploration time with respect to the novel object relative to both familiar objects. Given that an early target of amyloid pathology in Tg2576 is synaptic activity in the DG (dentate gyrus)/CA3 network (see review by Palmer and Good [1321]), and the fact that specific object-place associations are disrupted in Tg2576 [1384], one hypothesis would be that although TG and WT mice can detect object novelty, only the WT mice should be able to respond to the differential significance of the novel object given its mismatched spatial locations, and thus generate a greater net amount of exploration time relative to the summed response to familiar objects. Thus, Experiment 4A (LINS) involved mice being given the Spatial Mismatch Novelty COC task between the ages of 16-17 months. By this time the mice had been on compound for between ~8-to-9 months. It is important to say that an initial pilot experiment for the relative novelty task had been conducted with a separate cohort of ~12 months old non-drug treated Tg2576 mice and WT littermates prior to its use in Experiment 4A. However, there was insufficient time to run the Absolute Novelty task with the LINS cohort due to a prior agreement with GSK to conclude behavioural studies in order to honour the agreed time-table for completing the necessary biochemical analysis (much of which required pre-booking of equipment and staff resources at GSK, Harlow).

In Experiment 4B and 4C (EINS), mice were given both versions of the COC task between the ages of ~10 and 12 months of age. The Relative Novelty task (Experiment 4B) was given to mice first, followed two days later by the Absolute Novelty version (Experiment 4C). The two day gap between these tasks was used in order to ensure that animals remained sensitive to object novelty, as mean contact time with objects tends to decrease in TG and WT groups across test days.

3.6.2 Methods and Apparatus

3.6.2.1 Animals: and Drug Administration

Experiment 4A (LINS) used mice from cohort 1, which had previously undergone the T-maze and both anxiety related tasks. Experiment 4B and 4C (EINS) used the mice from Cohort 3. These mice had received exposure to both anxiety tests first, followed by the T-Maze. Object recognition assessment was the last behavioural experiment conducted with both cohorts. Please see section 3.3.2.1 for details of housing and drug administration.

3.6.2.2 Apparatus

Open Field: How location is defined within the context of this experiment was an important consideration. For example, most object-location SOR paradigms use an un-partitioned open field arena in which particular target locations are primarily usually intended by researchers to be distinguishable on the basis of intra-maze and/or extra-maze cue(s). Furthermore, because in general each target location within an un-partitioned arena is often visible to subjects from any other position in the apparatus, one may consider that the ability to resolve multiple locations may vary according to how salient the cues are from different positions and the degree to which cues themselves span multiple areas. In order maximise the ability of the mice to form sufficiently robust representations of locations within an apparatus, in Experiment 4 animals received exposure to objects in a novel open field rectangular arena (83 cm x 58 cm x 35 cm), where the internal space was partitioned into four separate compartments of approximately equal size by means of internal walls (each the same height as the perimeter wall) that formed a plus configuration. Distinct extra maze cues were distinguishable from each compartment, with transit between internal compartments accomplished via 2 internal doorways (each 15 cm high and 15 cm wide) located at the bottom of each internal partition where it joined the perimeter wall (see figure 3.19a). The outer walls, floor and internal partitions of the arena were all constructed from commercially available (5mm) white foam card (see Figure 3.17.a). The arena was located on top of a table 50 cm from the floor, in the centre of a (novel) quiet testing room that contained a variety of extra-maze cues, including posters on the walls, and a computer. Finally, the middle of each arena contained a circular object zone (see Figure 3.17.b), each of which measured 10cm in diameter. This was

marked out on the floor with black permanent marker, with the centre point of each zone clearly delineated by a small cross. Via a camera suspended from the ceiling 90 cm above the arena mid-point, each object zone was transposed onto to a computer monitor (RISC-PC), before the floor of the arena was covered with approximately 2 cm of aspen wood chip bedding. These transposed object zones would be used to score object-mouse interactions (see method).

Recording equipment and Objects: The camera was attached to a Panasonic video recorder (Secaucus, NJ, Model Number NV-MV20), the computer monitor, and PC (Acorn Computers, Cambridge, England). All behavioural sessions were recorded onto VHS video, and the movement of animals tracked via Ethovision software (Noldus, Wageningen, Holland). Objects were obtained from a variety of sources, and were constructed from materials that could not be easily gnawed by the mice, and were nonporous (e.g., toddler proof reinforced plastic). The shape and size of objects varied in order to make them as geometrically interesting and distinct as possible. All objects were free standing and mounted onto the blank side of marble effect dominoes (1cm thick) in order to make them hard to displace by the mice (see figure 3.17 c). None of the objects exceeded the boundary of an object zone, and none were taller than 20 cm. All objects were of a proportion that made it difficult for the animals to climb or rest on during the experiment, and each was represented in quadruplet.

Figure 3.17: Object Recognition Arena. (*a*): Left: The arena insitu. Top right: A TG mouse exploring one of the objects. Bottom Right: A view of one of the objects used in experiments from the perspective of a mouse. (b) Right: Objects insitu within the object zones. Left: A closer view of the one of the object zones showing the centre marking. (c) Some of the objects used within the apparatus for object recognition studies.

3.6.2.3 Behavioural Procedures

The Spatial Mismatch and Absolute Novelty tasks were both four day procedures. Each task involved two days of habituation (each day = 1 ten minute trial), immediately followed by two consecutive test days. Each test day involved a 10 minute sample trial, followed ~2 minutes later by a 10 minute choice trial. Both Tasks utilised the same habituation method. Mice were run in four staggered replications, with each containing no more than 12 mice. Each replication contained mice representative of all experimental conditions, and as always were run with the experimenter blind to genotype and condition. Within each replication, the mice were run in squads of 6. Mice were transported to the testing room 30 minutes before the start of testing in order to habituate. During this time the mice remained in their home cages in order to maintain familiarity with kin (mice were arranged in the holding room in the order in which they were run during experiments). At the start of each day of experimentation, the video recorder was started in the test room, and left to fun continuously. This prevented errors in relation to forgetting to initiate recording during individual trials. The recorder was checked periodically to ensure that it remained on, and tapes renewed as necessary to ensure complete coverage.

Habituation Days: On the first habituation day, mice were exposed to the arena in devoid of any objects. The mice were placed into the corner of a chamber facing the perimeter wall, and allowed to freely explore each of the chambers for 10 minutes. The start chamber chosen for each mouse was counterbalanced across groups and session. When the habituation session had expired, the mouse was removed from the apparatus and placed into its home cage before being transported back to the animal holding room. Before the next trial was initiated, any mouse droppings were removed from the arena, and the woodchip surfacing agitated in each compartment in order to disrupt any odour trails left by the previous occupant. On the second habituation day, the woodchip surfacing was brushed aside to reveal the circular object zones in each chamber. One set of identical objects were then placed directly over the centre point for the top-left (TL) and top-right (TR) zones (i.e. A, A^1), whilst a different set of identical objects were placed over the centre point for the bottom-left (BL) and bottom-right (BR) zones (i.e. B, B^1). All objects were placed facing outwards into the arena. The marble bases of each object were then re-covered with wood chip so that neither bases nor object zones were visible. If necessary the wood chip covering adjusted so that it was distributed approximately equally in each compartment.

During each habituation trial, a mouse was placed into the corner of the chamber diametrically opposite to one used on day-1 (again facing the perimeter wall), and allowed to explore for 10 minutes. With the experimenter secreted behind a canvas screen to prevent any visual disruption to subjects, the computer tracking software was started and used to record the movements of the animal. Exploratory behaviour manually scored by the experimenter (see scoring and statistical analysis). When the session had expired, mice were removed from the arena as previous. The objects were then removed from the arena and immersed in dilute Mr. Muscle cleaner (1:20 dilution in water), and dried with a clean cloth. Fresh (identical) copies objects were then replaced in each of the object zones as previous. Prior to the start of a trial the woodchip flooring was agitated in each compartment to disrupt odour trails. Stock objects were always kept out of the line of sight of the mice.

Sample and choice trials (Spatial Mismatch Novelty): For the sample and choice trials, the same basic procedure was used as in the habituation stages, except that now three object types were used (see Figure 3.16a). In the sample trial each mouse was presented with two copies of an object in the top and bottom zones as per the 2nd day of habituation. The subject was then placed into the corner of a chamber diametrically opposite to the one that was used on the second habituation day as previously described, and allowed to explore for 10 min. The experimenter then retreated behind a canvas screen prior to starting the tracking software and scoring the exploratory behaviour of the mouse recorded (see scoring and statistical analysis). When the trial had expired the subject was removed from the arena and placed back in its holding cage. During a subsequent 2 minute interval, all objects were removed from the arena and cleaned as previous. Fresh copies of the sample objects were replaced in the object zones as previous, except that now, one object in each set was replaced with a copy of the same novel object such that the change always took place across diametrically opposite locations i.e. TL: A, TR: C; BL: C, BR: B. The mouse was then placed back into the arena in the same position as used in the sample trial, and allowed to explore for a further 10 min. The animal's exploratory behaviour was again scored. When the choice trial was over, the mouse was placed back into its holding box and transported back into the animal holding room. All objects were then removed from the arena, washed as previous, and then fresh copies used to set up the arena for the next sample trial. Again, wood chip covering was agitated as previous, and adjusted so that it remained equally distributed in each compartment. All mice received 2 consecutive days of

testing on each trial type, with different sets of objects used on each day. The type of object used in the sample phase, the type used as a novel cues, and the left–right positioning of the novel cues, were each counterbalanced between groups and across days. Although on any one test day subjects were placed into the same corner on both the sample and test, the corner of entry was counterbalanced across days. Thus, across both days of testing each subject experienced placement in a chamber during a choice trial where the novel object had, and had not been placed. The experimenter wore latex surgical gloves at all times throughout the experiment to prevent odour contamination of the apparatus/objects.

Sample and choice trials (Absolute Novelty Task- Experiment 4C only): At the end of the relative novelty task, the animals received a 2-day break before commencing the absolute novelty task. The same basic methodology was used as previous, except that during choice phases, two different novel objects were placed in 2 different but familiar locations (see figure 3.16b). The type of objects used in the sample phase, the type used as a novel cues, and the left–right positioning of the novel objects, were each counterbalanced between groups and across days. All behavioural sessions were recorded onto VHS tape and scored as previous (see below).

3.6.2.4 Scoring and Statistical Analysis

Each object had been previously been assigned a specific scoring zone on the computer monitor and black and white observation monitor both of which matched the size and position of the corresponding object zones on the arena floor (as relayed by the camera suspended in the ceiling above the apparatus). A separate button a keyboard computer was used to identify each object zone on the monitor screen, and pressing the relevant key signified the beginning or end of investigative behaviour. Object exploration was therefore defined as the time an animal spent attending to objects within the zone boundaries [1471]. In addition, object exploration was also scored if an animal made head/whisker contact with an object in transit as this was taken to represent velocity based wiskering [1479]. Object exploration was not scored if the animal was in contact with but not facing the object or if it sat on the object, or used it as a prop to look around or above the object. EthoVision recorded the total exploration time for each target zone, and signified the end of the trial.

Mice were excluded from this experiment if they died prior to completing both sample and choice phases across the two test days, or if they had any mobility related

injuries or other health related issues which might otherwise affect their performance in this task. Mice were also excluded if they met criterion for non-responsiveness, which in this case, involved any animal, on either of the sample trials, failing to move from the start position for the entire duration of the trial, or failing to explore the two sets of sample novel objects for 5 seconds or less. This reduced the risk of any choice trial responses from being unfairly influenced by either low object contact times, or incomplete representation of both sets of sample objects. One additional criterion came into effect specifically for object recognition tests, and involved assessment of the repetitive spinning behaviour which can affect some mice (most commonly TG). On any given trial, any mice that exhibited a tendency to spin repetitively were excluded from behavioural trials if the spinning behaviour lasted for longer than 10 seconds on more than one occasion at the boundary of a scoring zone, as this would otherwise artificially inflate some object contact scores. Why some mice spin in this manner is not well understood, but it may be an unfortunate side-effect of continued inbreeding. Although spinning behaviour was observed in some mice in both experiments, it was not severe enough to meet criteria for exclusion and in most instances took place outside the vicinity of the scoring zone. No further mice were excluded from the analysis of Experiment 4A due to any of the above criteria. However, in order to maintain as robust a non-drug control as possible, two additional age-matched WT mice were added to the WT-C group (these mice belonged to a different cohort, and had received prior exposure to the T-Maze, and EPM) in order to maximise numbers. Thus, the total numbers of mice run in Experiment 4A were 18 TG mice (TG-C, n=9; TG-R, n=9), and 21 WT (WT-C, n=12; WT-R, n=9). No mice were excluded from the Experiment 4B and 4C due to mortality/morbidity, or repetitive circling behaviour. Two mice (1 TG-R, and 1 WT-C) were excluded for non-responsiveness. Thus, in Experiment 4B/4C, the total numbers of mice run were as follows: 23 TG mice (TG-C, n=12; TG-R, n=11); 24 WT mice (WT-C, n=11; WT-R, n=13).

Habituation data in each was only used as a dry run on scoring and for the purposes of assessing mice for early exclusion. Analysis of raw contact times for sample and choice data were analysed separately by 3-way repeated measures ANOVA. In order to verify that any differences in object contact times between groups was not due to any gross changes in locomotor activity per se, the data from across both sample stages was analysed by 3-way ANOVA with Day (Total object contact time Day1, and Day 2), Genotype, and Treatment as factors. Raw contact times from across both choice trials

were analysed by 3-way ANOVA, which had Genotype and Treatment as the between-subject factors, and Object (Familiar vs. Novel) as the within-subject factor. To ensure interrater reliability, an independent experimenter who was also blind to the animal assignments and object contingencies rescored 20% of all test phases from the original video footage. The rescored results significantly correlated with the original scores ($r=.82$, $p=0.01$) indicating robust interrater reliability. To ensure that we were sensitive to differences between groups, raw data from across choice trials were also converted into a preference ration ratio of the form: time spent exploring novel object(s)/time spent exploring all objects. PRs above 0.5 indicate that the mouse is exploring the novel object(s) more than the remaining familiar object(s), whereas PR scores below 0.5 indicate that mice are exploring familiar objects(s) more than the novel object(s). PR data was analysed by 1 way univariate ANOVA, with Genotype and Treatment as factors. Finally, independent sample t-tests were used when comparing the PRs of each group to chance. All analysis were conducted using IBM SPSS Statistics v.21.00 software as previous, with an alpha value (P) <0.05, taken as being statistically significant. Any significant ANOVA interactions and non-parametric analysis were conducted as previously indicated in Experiment 1 (see section 3.3.2.4).

3.6.3 Results

3.6.3.1 Experiment 4A (LINS): Spatial Mismatch Task

Sample Trial Data

Table 3.8a shows the mean contact times in seconds made by WT and Tg2576 mice with 2 pairs of novel objects in the sample stages of each test day, in the Spatial Mismatch Task. Inspection of these data indicates that overall, both Tg2576 and WT mice explored two sets of novel objects at approximately the same rate across sample stages, although all groups showed a decline in contact time with objects across the 2 sample trials. This description was confirmed by ANOVA with Day (Total object contact time Day1, and Day 2), Genotype, and Treatment as factors. This showed a main effect of Day $F(1,35)=42.799$, p=<0.001, non-significant effect of Genotype $F(1,35)=1.879$, p=0.179, no significant effect of Treatment $F(1,35)=0.217$, p=1.579, no significant Day by Genotype interaction $F(1,35)=0.341$, p=0.563, no Day by Treatment interaction $F(1,35)=0.073$, p=0.789, no Day by Treatment by Genotype interaction $F(1,35)=2.288$, p=0.139, or genotype by treatment interaction, $F(1,35)=1.240$, p=0.273. Thus, any differences in object contact times between groups in the subsequent choice stages were not likely to be caused by gross changes in object exploration *per se*.

(a)

	Sample (Day 1) Contact Times (Sec)		Sample (Day 2) Contact Times (Sec)	
Group	M	SEM	M	SEM
TG-C	39.7	4.49	22.9	4.49
TG-R	33.5	5.34	20.1	5.34
WT-C	38.4	4.02	26.0	4.02
WT-R	39.0	4.39	19.6	4.39

(b)

	PR. Novel Objects	
Group	M	SEM
TG-C	0.43	0.04
TG-R	0.62	0.04
WT-C	0.67	0.04
WT-R	0.55	0.04

Table 3.8: LINS Object Recognition (Experiment 4A). Spatial Mismatch Task (*a*) Raw object contact times for novel objects used in both sample stages. (*b*) Mean choice trial preference ratio for novel objects.

Choice Trial Data

Figure 3.18 shows the mean total object contact times in seconds made by WT and Tg2576 mice across choice trials in the Spatial Mismatch Task. Inspection of this figure suggests that a significant transgene effect exists between non-drug control groups, with WT-C mice spending more time exploring novel objects compared to TG-C mice. However, the total amount of time spent exploring familiar objects remains comparable between these groups. No transgene effect exists between drug-treated groups; each spends a similar amount of time exploring both classes of object. The relative time spent exploring either class of object does not differ between TG groups. The aforesaid also applies to the comparison of the relative time spent exploring novel, and familiar objects, in both WT groups. Nevertheless, the exploratory trends vary within groups. Whilst the TG-R group, like WT-C mice, appear to spend a greater amount of time exploring novel over familiar objects (i.e. they discriminate between novel and familiar objects), neither the TG-C nor WT-R groups appear to explore one type of object over another. However, both TG-C and WT-R groups do exhibit trends in their exploratory behaviour suggestive of some degree of discrimination taking place (PR comparisons will further inform the status of these trends). Thus, rosiglitazone primarily seems to have affected the exploratory *preferences* of drug-treated animals, rather than the total time spent exploring a particular class of object *per se*.

The above interpretation was confirmed by an ANOVA, with Object, Genotype, and Treatment as factors. This confirmed a significant effect of Object $F(1,35)=10.588$, $p=0.003$, no significant effect of effect of Genotype, $F(1,35)=0.878$, $p=0.355$, no significant effect of Treatment $F(1,35)=0.391$, $p=0.536$, no significant two-way interaction of Object by Genotype $F(1,35)=3.244$, $p=0.080$, no significant two-way Object by Treatment interaction $F(1,35)=0.469$, $p=0.498$, and a significant three-way interaction of Object by Genotype by Treatment interaction $F(1,35)=10.740$, $p=0.002$. The Treatment by Genotype interaction was not significant $F<1$. A follow-up ANOVA for the Object by Genotype by Treatment interaction revealed a significant transgene effect exists between non-drug control groups, with WT-C mice spending more time exploring novel objects compared to TG-C mice, $F(1,35)=5.981$, $p=0.020$, although the total amount of time spent exploring familiar objects remains comparable between these groups $F(1,35)=3.016$, $p=0.091$. When compared to WT-C mice, this demonstrates a relative impairment in the TG-C mice with respect to total exploration time of novel objects. Despite the trend in the TG-R group for spending more time exploring novel

objects compared to WT-R mice, any relative difference in total exploration time of either class of object is non-significant: Novel: $F(1,35)=0.008$, p=0.927; Familiar F(1,35)=1.471, p=0.233. Further comparisons show that the relative time spent exploring novel and familiar objects did not differ between TG groups: Familiar objects, $F(1,35)=2.859$, p=0.100; novel object, F(1,35)=1.645, p=0.208. Likewise the relative time spent exploring novel and familiar objects did not differ between WT groups: Familiar Objects, $F(1,35)=2.811$, p=0.103; Novel Objects, $F(1,35)=0.493$, p=0.487. In terms of within group exploratory preferences, the TG-R group, like WT-C mice, appear to spend a greater amount of time exploring novel over familiar objects (i.e. that discriminate between novel and familiar objects), TG-R, $F(1,35)=6.149$, p=0.018; WT-C, $F(1,35)=18.398$, p=<0.01, neither the TG-C or WT-R groups differ statistically in terms of their within group preference for exploring one type of object over another: TG-C, $F(1,35)=1.485$, p=0.231; WT-R, $F(1,35)=1.590$, p=0.216. However, both TG-C and WT-R groups do exhibit trends in their exploratory behaviour suggestive of some degree of discrimination taking place (see PRs analysis). Rosiglitazone primarily seems to have affected the exploratory *preferences* of drug-treated animals (i.e. the ratio of time each spends exploring one type of object over another), rather than the total time spent exploring a particular class of object *per se*.

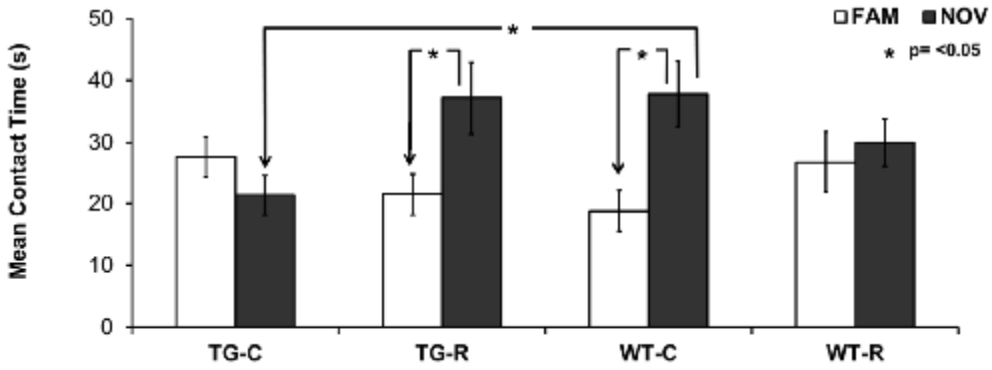

Figure 3.18: LINS Object Recognition (Experiment 4A). Mean contact times the novel object, and two familiar objects across both choice stages of the Spatial Mismatch Task. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

The raw contact time data were converted into a preference ratio as indicated in section 3.6.2.4 (see Table 3.8b). Inspection of this table suggests that TG-C mice are significantly impaired relative to WT-C and TG-R groups, and that the WT-R group is

impaired relative to the WT-C group. This description was confirmed by a one-way ANOVA with PR as the dependent variable, and Genotype, and Treatment as factors. This revealed a main effect Genotype, $F(1,35)=6.462$, p=0.016, no significant effect of Treatment, $F<1$, and a significant Genotype by Treatment interaction, $F(1,35)=13.154$, p=0.001. A follow-up test of simple main effects for the significant Genotype by Treatment interaction then revealed that the TG-C group was impaired relative to the WT-C group $F(1,35)=20.522$, p=<0.001, whereas neither drug-treated groups differed significantly, $F(1,35)=0.549$, p=0.464. Further comparisons then showed that compared to TG-C mice, the TG-R group spent significantly more time exploring novel over familiar objects, $F(1,35)=9.289$, p=0.004, as did WT-C mice in comparison to the WT-R group, $F(1,35)=4.109$, p=0.050. Finally, in order to test whether groups successfully showed above-chance performance on choice trials, the Mean PRs for each group were compared to chance levels (i.e. 0.50= no discrimination) using one sample t-tests. This statistical analysis revealed that the only the WT-R group failed to perform at above chance levels: TG-C, ($T(8)=-6.430$, p=<0.001; TG-R, ($T(8)=2.630$, p=0.030; WT-C, ($T(11)=4.468$, p=0.001; WT-R, ($T(8)=1.644$, p=0.555. These results are important because they indicate that the impairment in the TG-C mice is a relative one as these mice do discriminate in favour of exploring familiar objects over novel ones. By contrast, the impairment in the WT-R group appears to reflect a more general impairment in discrimination between objects as the PR score was not above chance levels (although it was close). In aggregate, these results indicate complex relationships within groups with respect to the exploration of objects in the Spatial Mismatch Task.

3.6.3.2 Experiment 4B (EINS): Spatial Mismatch Task

Sample Trial Data

Table 3.9a shows the mean contact times in seconds made by WT and Tg2576 mice with sample stage novel objects in the Spatial Mismatch task. Inspection of these data indicates that overall, both Tg2576 and WT mice explored two sets of novel objects at the same rate as control mice across both sample stages, although all groups showed a decline in contact time with objects across the 2 sample trials. This interpretation was confirmed by a 3 way repeated measures ANOVA with Day, Genotype, and Treatment as factors. This showed a main effect of Day, $F(1,43)=8.284$, p=0.006, no effect of Genotype $F<1$, no significant effect of Treatment $F<1$, a no Day by Genotype interaction $F<1$, no significant Day by Treatment interaction $F<1$, and no significant Day by Treatment by Genotype interaction $F(1,43)=0.103$, p=0.750. The genotype by treatment interaction was not significant, $F<1$. Thus, any differences in object contact times between groups in the subsequent choice stages were not likely to be caused by gross changes in object exploration *per se*.

(a)

	Sample (Day 1) Contact Times (Sec)		Sample (Day 2) Contact Times (Sec)	
Group	M	SEM	M	SEM
TG-C	38.4	4.94	29.4	5.08
TG-R	38.7	3.58	29.3	3.25
WT-C	35.5	3.65	28.7	3.83
WT-R	34.4	4.74	31.9	3.95

(b)

	PR. Novel Objects	
Group	M	SEM
TG-C	0.40	0.03
TG-R	0.69	0.01
WT-C	0.67	0.02
WT-R	0.48	0.03

Table 3.9: LINS Object Recognition (Experiment 4B). Spatial Mismatch Task. (*a*) Raw object contact times for novel objects used in both sample stages. (*b*) Mean choice trial preference ratio for novel objects.

Choice Trial Data

Figure 3.19 shows the mean total object contact times in seconds made by WT and Tg2576 mice across choice trials in the EINS Spatial Mismatch Task. Inspection of this figure suggests that a significant transgene effect exists between non-drug control groups, with WT-C mice spending more time exploring novel objects over familiar ones compared to TG-C mice, the latter of which seem to show the opposite discrimination. Discounting the likely significant discrimination in the TG-C mice for the familiar object, the pattern of responses across groups looks very similar to that indicated by Figure 3.18, with Rosiglitazone treatment recovering function in the TG-R mice, but having a detrimental effect on discrimination in the WR-R mice as this is the only group which does not appear to show a preference for either the novel or familiar object. The total amount of time spent exploring familiar objects again looks statistically comparable between groups. The above description was confirmed by a ANOVA, with Object, Genotype, and Treatment as factors. This showed a significant effect of Object $F(1,43)=17.957$, p=<0.001, a no significant effect of effect of Genotype, $F<1$, no significant effect of Treatment $F(1,43)=3.794$, p=0.058, no significant two-way Object by Genotype interaction, $F<1$, a significant two-way Object by Treatment interaction $F(1,43)=6.089$, p=<0.05, and a significant three-way Object by Genotype by Treatment interaction $F(1,43)=41.090$, p=<0.001. The Treatment by Genotype interaction was not significant $F(1,43)=1.433$, p=0.238.

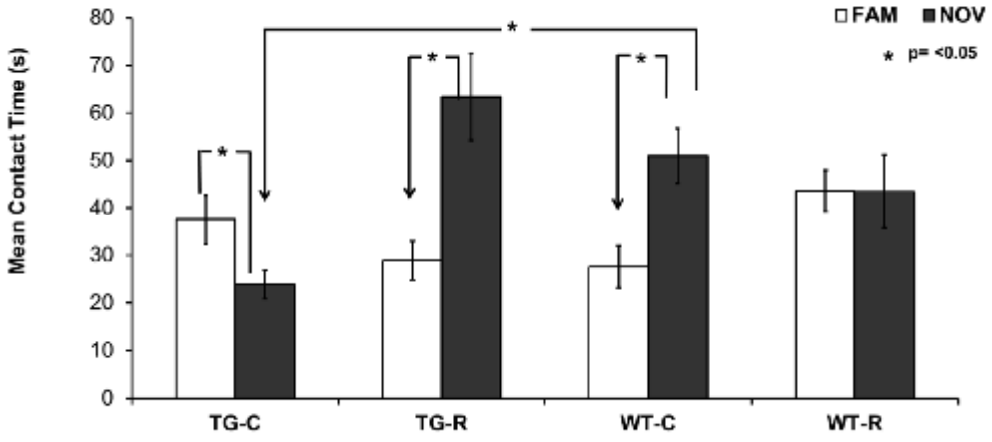

Figure 3.19: EINS Object Recognition (Experiment 4B). Mean contact times the novel object, and two familiar objects across both choice stages of the Relative Novelty Task. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

A follow-up test of simple main effects for the significant Object by Genotype by Treatment interaction revealed a significant transgene effect between non-drug control groups, with WT-C mice spending more time exploring novel objects compared to TG-C mice, $F(1,43)=7.815$, p=0.008, although the total amount of time spent exploring familiar objects remained comparable between these groups $F(1,43)=2.262$, p=0.140. Compared to WT-C mice, this demonstrates a relative impairment in the TG-C mice with respect to total exploration time of novel objects. No transgene effect exists between the TG-R and WT-R groups with respect to the total time spent exploring familiar objects although this was close, $F(1,43)=3.929$, p=0.054, whereas TG-R mice did spend significantly more time exploring novel objects relative to WT-R mice, $F(1,43)=4.555$, p=0.039. Further comparisons showed that TG-R mice spent longer exploring novel objects relative to TG-C mice $F(1,43)=16.704$, p=0.<001, although the relative time spent exploring familiar objects did not differ between these groups, $F(1,43)=2.811$, p=0.232. Neither WT group differed with respect to the time spent exploring novel objects, $F<1$, although the WT-R group did spend significantly more time exploring familiar objects relative to WT-C mice, $F(1,43)=5.321$, p=0.026. Both the TG-R and WT-C groups spent significantly more time exploring novel over familiar objects, TG-R, $F(1,43)=35.370$, p=<0.001; WT-C, $F(1,43)=17.171$, p=<0.01. Again, WT-R mice did not exhibit a significant bias for exploring novel over familiar objects, $F<1$. However, unlike Experiment 4A, the raw contact time data did establish that the TG-C group spent significantly more time exploring familiar over novel objects, TG-C, $F(1,43)=6.379$, p=0.015 (remember this was only confirmed in the PR analysis in Experiment 4A). These results again suggest that rosiglitazone affected the exploratory *preferences* of drug-treated animals.

The raw contact time data were converted into a preference ratio as indicated in section 3.6.2.4. This data is displayed in Table 3.9b. Inspection of this table suggests that TG-C mice are significantly impaired relative to WT-C and TG-R groups, and that the WT-R group is impaired relative to the WT-C group. This interpretation was confirmed by ANOVA, with Genotype, and Treatment as factors. This revealed no significant effect Genotype, $F(1,43)=1.804$, p=0.186, a significant effect of Treatment, $F(1,43)=5.108$, p=0.029, and a significant Genotype by Treatment interaction, $F(1,43)=16.287$, p=<0.001. A follow-up test of simple main effects for the significant Genotype by Treatment interaction then revealed that again the TG-C group was

impaired relative to the WT-C group $F(1,43)=41.719$, p=<0.001. However, in contrast to Experiment 4A, the WT-R group was significantly impaired relative to the TG-R group, $F(1,43)=21.204$, p=<0.001 (likely as a consequence of the heightened mean novelty response in the TG-R group). Further comparisons then showed that compared to TG-C mice, the TG-R group spent significantly more time exploring novel over familiar objects, $F(1,43)=47.977$, p=<0.001, as did WT-C mice in comparison to the WT-R group, $F(1,43)=16.505$, p=<0.001. Finally, in order to test whether groups successfully showed above-chance performance on choice trials, the PRs for each group were compared to chance levels (i.e. 0.50= no discrimination) using one sample t-tests. This analysis revealed that all groups except the WT-R group made a significant discrimination; TG-C, ($t(11)=-3.633$, p=0.004; TG-R, ($t(10)=10.000$, p=<0.001; WT-C, ($t(11)=10.652$, p=<0.001; WT-R, ($t(12)=-0.457$, p=0.656. These results affirm that TG-C mice discriminate in favour of exploring familiar objects over novel ones, whereas the WT-R group do not discriminate indicating a more systemic impairment in discrimination between objects. In aggregate, these results confirm that rosiglitazone significantly alters the preference of TG mice in favour of exploring novel over familiar objects, whilst it impairing recognition memory more generally in the WT-R mice.

3.6.3.3 Experiment 4C (EINS): Absolute Novelty Task

Sample Trial Data

Table 3.10 shows the mean contact times in seconds made by WT and Tg2576 mice with 2 pairs of novel objects in the sample stages of each test day, in the Absolute Novelty Task. Inspection of these data indicates that overall, both Tg2576 and WT mice explored two sets of novel objects at the same rate as control mice across both sample stages. However, whilst most groups showed a slight decline in contact time with objects across the 2 sample trials, the WT-C mice showed a slight increase. This interpretation was confirmed by ANOVA with Day, Genotype, and Treatment as factors. This showed a no significant effect of Day $F<1$, no significant effect of Genotype $F(1,43)=2.161$, $p=0.149$, no significant effect of Treatment $F<1$, no significant Day by Genotype interaction $F<1$, no significant Day by Treatment interaction $F<1$, and a no significant Day by Treatment by Genotype interaction $F(1,43)=2.694$, $p=0.108$. There was also no significant genotype by treatment interaction, $F<1$. Thus, any differences in object contact times between groups in the subsequent choice stages were not likely to be caused by gross changes in object exploration *per se*.

(a)

	Sample (Day 1) Contact Times (Sec)		Sample (Day 2) Contact Times (Sec)	
Group	M	SEM	M	SEM
TG-C	32.9	4.51	26.0	3.35
TG-R	31.4	3.32	28.8	4.33
WT-C	34.3	6.07	40.7	8.75
WT-R	41.7	8.79	33.8	2.69

(b)

	PR. Novel Objects	
Group	M	SEM
TG-C	0.58	0.04
TG-R	0.64	0.03
WT-C	0.69	0.03
WT-R	0.53	0.05

Table 3.10: EINS Object Recognition (Experiment 4C). Absolute Novelty Task (*a*) Raw object contact times for novel objects used in both sample stages. (*b*) Mean choice trial preference ratio for novel objects.

Choice Trial Data

Figure 3.20 shows the mean total object contact times in seconds made by WT and Tg2576 mice across choice trials in the Absolute Novelty Task. Inspection of this figure suggests a significant transgene effect between non-drug control groups, with WT-C mice spending more time exploring novel objects compared to TG-C mice. However, the total amount of time spent exploring familiar objects were statistically comparable between these groups. No transgene effect exists between drug-treated groups; each spends a similar amount of time exploring both class of object. Furthermore, only the WT-C group appears to spend significantly more time exploring novel objects compared to other groups. However, the exploratory trends do differ within these groups. The TG-R group, like WT-C mice, show a significant within group preference for exploring novel over familiar objects (i.e. they successfully discriminate between these classes of objects). By contrast, neither TG-C nor WT-R groups appear to exhibit a significant preference for exploring novel over familiar objects, although the TG-C mice are clearly trending in this direction (the PRs will further inform the status of this trend). Overall, these observations suggest that rosiglitazone may have had at most, a mild effect on the ability to detect absolute novelty in TG mice, whereas the drug has again clearly impaired the performance of WT-R mice.

The above description was confirmed by a 3 way repeated measures ANOVA, with Object, Genotype, and Treatment as factors. This showed a significant effect of Object, $F(1,43)=23.425$, p=<0.001, a significant effect of effect of Genotype, $F(1,43)=5.929$, p=0.019, a non-significant effect of Treatment $F(1,43)=0.60$, p=0.807, no significant Object by Genotype interaction, $F<1$, no significant Object by Treatment interaction $F(1,43)=2.333$, p=0.134, and a significant Object by Genotype by Treatment interaction $F(1,43)=6.351$, p=0.016. The Treatment by Genotype interaction was not significant, $F<1$. A follow-up test of simple main effects for the significant Object by Genotype by Treatment interaction revealed a significant transgene effect between non-drug treated mice, with the WT-C group spending more time exploring novel objects compared to TG-C mice, $F(1,43)=8.287$, p=0.006, although the total time spent exploring familiar objects remained comparable between these groups $F<1$. Compared to WT-C mice, this again demonstrates a relative impairment in the TG-C mice with respect to total exploration time of novel objects. There was no transgene effect between the TG-R and WT-R groups with respect to the time spent exploring novel objects, F<1, or familiar objects, F(1,43)=3.917, p=0.054. Likewise, neither TG group differed

significantly from each other in terms of the total time spent exploring novel objects, F<1, or familiar objects, F<1. However, the WT-C group did spend significantly more time exploring novel objects relative to the WT-R mice, F(1,43)=5.104, p=0.029, although neither WT group differed with respect to the time spent exploring familiar objects, F(1,43)=1.436, p=0.237. Within group comparisons showed that only the TG-R and WT-C groups spent significantly more time exploring novel over familiar objects, TG-R, *F*(1,43)=6.730, p=0.013; WT-C, *F*(1,43)=19.849, p=0.<001; WT-R, F<1, although the TG-C mice did show a similar but non-significant pattern of response in favour of the novel objects: TG-C, *F*(1,43)=3.353, p=0.074.

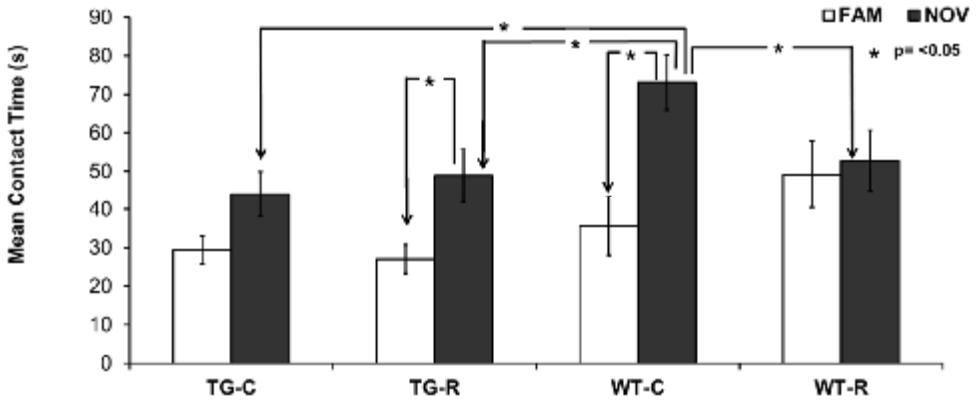

Figure 3.20: EINS Object Recognition (Experiment 4C). Mean contact times for the Absolute Novelty Task. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

Raw contact time data were converted into a preference ratio as indicated in section 3.6.2.4. This data is displayed in Table 3.10b. Inspection of this table again suggests that only the TG-R and WT-C groups exhibited a statistically significant preference for exploring novel over familiar objects. This description was confirmed by ANOVA with Genotype, and Treatment as factors. This revealed no significant effect Genotype, *F*<1, no significant effect of Treatment, *F*(1,43)=1.336, p=0.254, and a significant Genotype by Treatment interaction, *F*(1,43)=7.753, p=<0.01. A follow-up test of simple main effects for the significant Genotype by Treatment interaction then revealed that the PR score of both TG groups did not differ significantly, *F*(1,43)=1.250, p=0.270. The TG-R group also did not differ significantly from the WT-R group, although this was close, *F*(1,43)=3.429, p=0.071. However, WT-R mice were significantly impaired relative to

their WT-C counterparts $F(1,43)=8.265$, p=0.006, suggesting that rosiglitazone had detrimentally impacted on the ability of these WT mice to respond to novel objects in their environment. TG-C mice were significantly impaired relative to WT-C mice, $F(1,43)=4.348$, p=0.043. Finally, in order to test whether groups successfully showed above-chance performance on choice trials, the mean PRs for each group were compared to chance levels (i.e. 0.50= no discrimination) using one sample t-tests. This statistical analysis confirmed that only the TG-R and WT-C groups were responding at above chance levels in the absolute novelty task: TG-C, (t(11)=-1.773, p=0.104; TG-R, ($t(10)=4.183$, p=0.002; WT-C, ($t(10)=5.590$, p=<0.001; WT-R, ($t(12)=0.492$, p=0.632. In aggregate these results suggest that both TG-C and WT-R mice likely exhibit impairment in absolute novelty, although the PR analysis does suggest that unlike the WT-R mice, TG-C mice are discriminating to some degree in the expected direction.

3.6.4 Discussion

In this experiment object recognition memory was assessed in Tg2576 and WT littermates using a de novo COC task. Two versions of this task were used. In the Absolute Novelty version the choice trial required mice to discriminate two different novel objects from two different familiar objects within a segmented open field arena. Since Tg2576 and WT mice exhibit comparable preferences for object novelty in 2 and 4 item arrays [1253, 1324, 1384], it was predicted that TG and WT mice should exhibit a comparable preference for object novelty in this task. In the spatial Mismatch Task, the mice were required to discriminate between two different familiar objects, and the same novel object appearing in two different spatial locations. Thus, unlike the Absolute Novelty task this generates a potential spatial mismatch during the test for the novel stimulus leading to a disruption of specific familiar/novel discriminations in both the top and bottom horizontal axis of the apparatus. This should lead to a greater net amount of exploration time with respect to the novel object relative to familiar objects. However, whilst both TG and WT mice can detect object novelty, it was hypothesised that only the WT mice should be able to respond to the mismatched spatial locations, and thus make a significant bias for exploring novel objects over familiar ones.

It is evident from both Experiments 4A and 4B, that TG-C mice do exhibit impairment in the Spatial Mismatch task, although the nature of the impairment in is unclear because the mice do make a significant discrimination in favour of familiar

objects (this was only confirmed via PR scores in the LINS mice, and reflected in both PR scores and raw contact time data in the EINS experiment). The reason for this preference in TG mice is unclear. It could represent random noise. On the other hand, it could be a consequence of the experimental design. For example, TG mice evidently can tell that an object is novel (otherwise behaviour would be distributed randomly), but in the absence of a robust ability to form specific object place associations in a segmented arena, it is possible that they treat the two copies of the novel object as a singular entity. Thus, since both familiar objects are different, these may incur a greater overall contact time. It is of interest that whilst Tg2576 mice are able to form representations of the identity of objects (as well as exhibit memory for the spatial organization of objects in an arena), conjunctive memory for specific object–location associations is severely impaired in aged TG mice [1384]. However, these studies have been conducted in an open field non-segmented arena, and it is possible that in the Spatial Mismatch Task, specific object–location associations are more vulnerable to the APP_{SWE} mutation.

It is of interest that whatever underpins the TG impairment in the Spatial Mismatch task, this is reversed in the TG-R mice both in aged mice (LINS mice were aged 16-17 months at test), and young adult mice (ENIS mice aged ~10 and 12 months of age). Given that it is uncertain as to what the mechanism of 'impairment' is in non-drug treated TG mice the mechanism for this recovery is unclear in the TG-R mice, especially considering that Experiment 1 no improvement in spatial working memory was detected in either the LINS or EINS cohorts. However, in both cases these mice had been on compound for much longer when tested in the Spatial Mismatch task, so it is possible that a longer period of drug agonism (LINS:~8-to-9 months; EINS 7 months) may have allowed recovery of some component of hippocampal function. This remains untested in TG2576 mice, although in separate studies that I have not been able to include in this thesis, I have conducted both versions of the COC task with a cohort of 12 month old hippocampal lesion and sham BL6 mice. In that experiment I found that the Spatial Mismatch task was sensitive to hippocampal lesion, where the Absolute Novelty Task was not. These results are preliminary, and require replication. One should also say that although the effects of rosiglitazone have not been studied in Tg2576 mice prior to the current study, the drug has been found to significantly impact upon object recognition processes in other hAPP mouse models [1213, 1214], although these have used standard 2-item object arrays in a standard

rectangular (non-segmented) open field arena. However, whilst the impact of rosiglitazone was clearly unable to rescue the spatial working memory deficit in Experiment 1, it is possible that differential changes in this brain region led to an improvement of recognition memory. Likewise the changes induced by the drug in the WT-R mice leading to the impairment of recognition memory in both the Spatial Mismatch and Absolute Novelty tasks are also uncertain, although Experiment 7 of Chapter 4 will discuss this issue in more detail.

The finding in the current study that TG-C mice were impaired in the EINS Absolute Novelty Task was suppressing given that previous research has reported 'absolute' object novelty detection to be intact in Tg2576 mice [1252, 1253, 1324, 1325, 1384], although others have reported contradictory findings [1477]. Whilst differences between studies with may possibly involve differing proportions of the SJL background in there mice, it is also possible that the type of open field arena used as well as the number of objects used, is also important. For example, although previous research has shown that absolute object novelty is comparable in TG2576 mice and WT littermates with 2 and 4 object arrays [1253, 1384], this has not been assessed in an apparatus where by its design configurable cues would have been harder to derive for the whole object array (such as in the Absolute Novelty Task), and possibly the environment was more conductive to forming specific object place relationships (by nature of the fact that the arena was segmented into 4 zones, each of which were associated with distinct as well as overlapping extra-maze cues. The novelty discrimination may therefore have been harder in the Absolute Novelty task, possibly explaining why TG-C mice failed to make an above chance discrimination. However, whether rosiglitazone agonism was responsible for slightly adjusting the TG-R response enabling them to make an above chance discrimination is uncertain in this experiment remains uncertain. Thus, it remains possible that rosiglitazone had a marginal effect on absolute novelty detection in TG-R mice, or we may just have been unlucky with the responses in the TG-C group, such that these mice would most times show a significant novelty discrimination in this experiment (indeed, the trend is clearly there in the raw contact time data for this group). These findings require further investigation and robust verification. Nevertheless, it is evident that rosiglitazone is able to reverse components of object recognition deficit in Tg2576 mice although the nature and mechanism of reversal is unknown.

3.7 CHAPTER DISCUSSION

The overall lead hypothesis for this thesis was that rosiglitazone should ameliorate age-related cognitive deficits and pathophysiological changes associated with the hAPP transgene in TG mice. As discussed in the introduction to this chapter, this was primarily based on the controversial publication of research by Pedersen et al. [1129, 1244]. Here a dual treatment strategy was used to assess the study hypothesis. In the first course of behavioural experiments we sought to determine if chronic rosiglitazone agonism from the age of ~8-9 months would reverse specific behavioural deficits in TG mice related to spatial working memory (T-Maze FCA), anxiety (EPM and Marble burying task), and object recognition memory (Spatial Mismatch task). At this time, insoluble forms of Aβ are increasing exponentially [1284, 1291], and hippocampal synaptic pathology is well developed [1245]. The outcome of these studies suggest that whilst the drug was unable to reverse the spatial working memory deficit in TG mice, or moderate behavioural disinhibition in the EPM task, the drug did significantly alter the preference of aged TG mice for exploring two identical copies of a novel object placed in two different but familiar spatial locations. With the exception of the object recognition data, these result fit with other research suggesting that administration of PPARγ compounds largely do not reverse cognitive deficits in adult Tg2576 mice when amyloid pathology is already well developed even if they are able to moderate some aspects of the underlying physiological abnormalities in these mice [1215, 1386]. As such these findings fit with recent clinical studies in humans [1237, 1238, 1247, 1480]. However, in these human studies it is of interest that explicit object recognition skills were not assessed in patients receiving rosiglitazone or pioglitazone agonism. None of the clinical or pre-clinical research has also indicated serious adverse effects of rosiglitazone on cognition, although in preclinical studies none have assessed the long term impact rosiglitazone administration. However, adverse health effects of rosiglitazone administration have been reported in the human literature, although these are contested (see discussion in [1481]). On the basis of the results of the LINS behavioural experiments, the study hypothesis must be largely rejected, although the object recognition data is the one exception.

In a second course of experiments, this study used an early-intervention strategy (EINS) strategy to investigate the impact of rosiglitazone administration on the established appearance of age-dependent cogitative deficits in 8 month old TG mice

following continuous drug administration from the age of 5 months. At this time significant amyloid pathology and synaptic deficits were absent in TG mice, and it was predicted that rosiglitazone would delay the onset of age-related cognitive deficits in adult TG mice. Again we derive inconsistent results on this issue. Whilst the EPM and object recognition data (Spatial Mismatch Novelty in particular) suggest longitudinal rosiglitazone agonism from a young age may be sufficient to moderate some components of phenotypic changes in Tg2576 mice, it clearly is unable to rescue the primary working memory deficit of these mice, or impact upon marble burying behaviour, or absolute novelty detection (although the latter may have been mildly benefited). Given the object recognition data for the Spatial Mismatch task in the EINS cohort, it would have been of interest to have perhaps re-tested the EINS mice on the T-Maze FCA task. Thus, the hypothesis for the EINS cohort cannot be accepted in its entirety and the physiological basis for the beneficial effects of rosiglitazone on TG mice are uncertain. In this respect it will be of interest to evaluate this in the context of the final two experiments of this thesis (see Chapter 4).

4. PHYSIOLOGICAL MEASURERS

4.1 INTRODUCTION

THE AIM of the experiments in this chapter was to provide physiological evidence for the behavioural studies and thus, the study hypotheses detailed in Chapter 3. The two biochemical measures used in this study use tissue samples obtained at sacrifice. These relate to the assessment of adiponectin protein levels from terminal plasma samples (Experiment 5A and 5B), and assessment of total Aβ levels isolated from single brain hemispheres (Experiment 6) and the assessment of dendritic spine density in the dentate gyrus of the hippocampus form slices of lightly fixed brain tissue (Experiment 7). As the rationale for many of these measures has been outlined in previous chapters, I will provide a brief summary of the main points for each experiment.

Before discussing Experiments 5-to-7 in detail, I want to address from the outset the major limitation of this chapter. It will be evident from the above that there is an absence of important histological validation of the mice used in experimental studies, including whether their pathology had developed according to expected timescales. The absence of such descriptive information was unavoidable due to financial and motivational limitations imposed upon the Author by the commercial partner (GSK). However, whilst there is a clear need for these experiments to be conducted in the future, it is worthwhile to remember that the major deficit in spatial working memory was not recovered in rosiglitazone treated TG mice (see Experiment 1), and that the deficit overlapped with that in the TG control mice. Indeed, although this may not have been the case with Experiments 2 and 4, the results of Experiment 7 will further support the likely view that key pathological physiological changes related to the over-production of Aβ (loss of dendritic spines) were still very much in evidence in TG mice despite chronic exposure to rosiglitazone. In addition to histological validation, there

was also a disappointing lack of available funds from the commercial partner to support wider exploration of other relevant biochemical markers such as IDE, IGF-1, and GLUT-4. These could have widened the scope of possible changes elicited in the treatment groups. Although many of these were among the original aspirations of the study, I was unable to follow through with these for the reasons discussed.

4.2 Experiment 5: Plasma Adiponectin Levels

As mentioned previously, the genesis of the programme of research detailed in this thesis originates from the need to further clarify the impact of rosiglitazone in adult male Tg2576 mice following the controversial publication of research by Pedersen et al [1129]. It is notable that whilst the Pedersen et al and several other studies have reported beneficial effects of rosiglitazone on the cognitive and/or some pathological aspects of the Tg2576 mice [1129, 1212, 1215, 1244], none of these studies has attempted to verify the systemic penetration of the drug by looking for its impact on a predictable physiological marker other than peripheral glucose responses. Thus, in this thesis both intervention strategies verify the systemic penetration of rosiglitazone by validating against terminal plasma adiponectin levels [1208], which should be similarly, elevated across all PPAR treated groups. In addition, several studies have also reported that adiponectin protein as an anti-inflammatory effect [1194, 1482-1484], something which may also confer some measure of benefit to reducing the pro-inflammatory profile of Tg2576 mice (this has not been reported in the literature).

4.2.1 Methods and Apparatus

4.2.1.1 Animals: and Drug Administration

Experiment 5A (LINS) used mice the mice from cohort 1. To recap, cohort 1 mice had been sacrificed by cervical dislocation between the ages of 18 and 19 months, wherehence the necessary tissues harvested for biochemical analysis. The resulting tissue samples were then labelled and stored at -20°C until further use. Experiment 5B (EINS) used the mice from Cohort 3. To briefly recap, these mice were sacrificed between the ages of 12 and 13 months and the necessary tissues harvested for biochemical analysis in the same manner as above. In brief, terminal blood samples (200 µl) were rapidly obtained by cardiac puncture, and plasma was separated by centrifugation (120× g for 20 min) at room temperature and stored individually at

–20°C until assay. Please see section 3.3.2.1 for further details of housing and drug administration.

4.2.1.2 Analytical Procedures

Plasma adiponectin levels were measured using a mouse adiponectin immunoassay (RandD Systems, Quantikine Acrp30 kit). All samples, standards, and controls were assayed in duplicate as per the manufacturer's instructions. In short, all reagents and samples were brought to room temperature before use. All reagents, standard dilutions, and samples were prepared as directed in the product insert. Any excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desiccant pack. This was then resealed. 50 µL of Assay Diluent was added to each well, followed by 50 µL of Standard, Control, or sample. The plate was then sealed (with the attached a plate sealer), and incubated at room temperature for 3 hours. After this time had expired each well was aspirated and washed, repeating the process 4 times for a total of 5 washes. 100 µL of Conjugate was then added to each well. The plate was then covered with a fresh plate sealer, and incubated at room temperature for 1 hour after which the plate was aspirated and washed 5 times. 100 µL Substrate Solution was then added to each well, and incubated at room temperature for 30 minutes. During this stage the plate and contents were protected from light. 100 µL of Stop Solution was then added to each well. The plate was then read at 450 nm within 30 minutes, with the wavelength set for correction at 540 nm or 570 nm.

4.2.1.3 Statistical Analysis

Raw data for each of the levels of plasma adiponectin were analysed by 1 way ANOVA, which had [**Adiponectin**] (concentration adiponectin) as the dependent variable, and **Genotype** and **Treatment** as factors. All analysis were conducted with IBM SPSS Statistics v.21.00 software with an alpha value (P) <0.05, taken as being statistically significant Any significant ANOVA interactions and non-parametric analysis were conducted as previously indicated in Experiment 1 (see section 3.3.2.4). Experiment 5A was conducted with samples from 15 TG mice (TG-C, n=7; TG-R, n=8), and 16 WT (WT-C, n=9; WT-R, n=7). The differences in numbers of samples compared to the previous experiment reflect the inability to obtain sufficient blood samples for analysis from some mice. Experiment 5B the total numbers of mice run were as follows: 23 TG mice (TG-C, n=12; TG-R, *n*=12); 24 WT mice (WT-C, n=11; WT-R, n=13).

4.2.2 Results

4.2.2.1 Experiment 5A: LINS Cohort

Figure 4.1 shows the mean levels of adiponectin protein levels derived from terminal blood plasma levels for the LINS cohort. Inspection of this figure suggested that overall rosiglitazone treated mice displayed significantly higher adiponectin levels relative to non-drug treated mice. This interpretation was confirmed by ANOVA with Adiponectin concentration (ng/mg) as the dependent variable, and Genotype, and Treatment as factors. This revealed a no significant effect Genotype $F<1$, a significant effect of Treatment, $F(1,27)=45.166$, $p=<0.001$, and no significant Genotype by Treatment interaction, $F<1$. Thus, in the late stage cohort we conclude that the delivery method succeeded in the drug reaching systemic penetration.

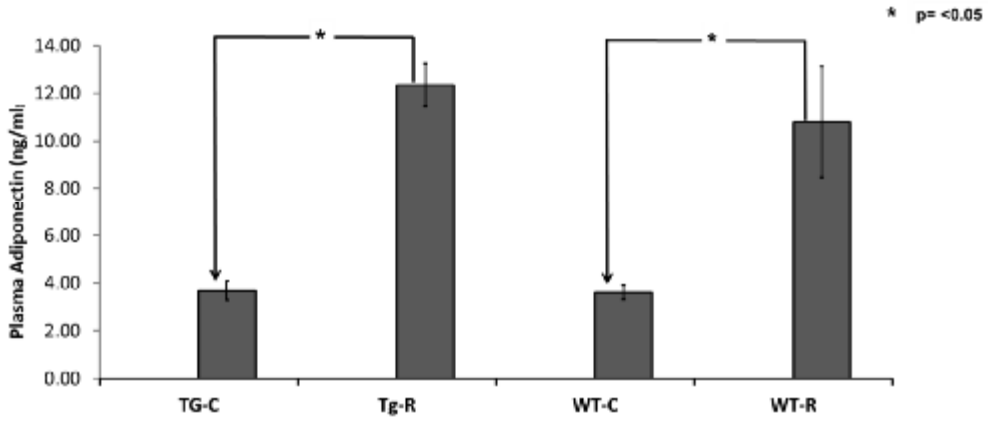

Figure 4.1: LINS Plasma Adiponectin Levels (Experiment 5A). Mean levels of plasma adiponectin isolated from terminal blood samples. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

4.2.2.2 Experiment 5B: EINS Cohort

Figure 4.2 shows the mean levels of adiponectin protein levels derived from terminal blood plasma levels for the EINS cohort. Inspection of this figure suggested that again, rosiglitazone treated mice displayed significantly higher adiponectin levels relative to non-drug treated mice overall. This interpretation was confirmed by ANOVA with Adiponectin concentration (ng/mg) as the dependent variable, and Genotype, and Treatment as factors. This revealed a non-significant effect Genotype $F(1,45)=1.243$, $p=0.271$, a significant effect of Treatment, $F(1,45)=70.848$, $p=<0.001$, and a non-significant Genotype by Treatment interaction, $F<1$. Thus, in the early-stage cohort we

again conclude that the delivery method succeeded in the drug reaching systemic penetration.

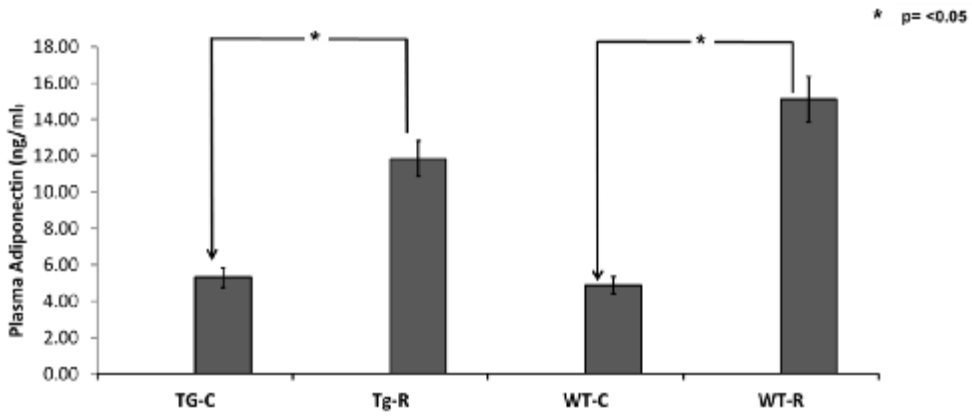

Figure 4.2: EINS Plasma Adiponectin Levels (Experiment 5B). Mean levels of plasma adiponectin isolated from terminal blood samples. Values are means ±S.E.M with asterisk denoting significance at the 0.05 level.

4.3 EXPERIMENT 6: TOTAL AB LEVELS

Several studies have reported that rosiglitazone significantly impacts upon Aβ42 levels and or amyloidosis in hAPP mouse models [1129, 1213, 1217]. Since Aβ represents the primary instrument of synaptic deficits thought to underlie the behavioural phenotype of Tg2576 mice [1245, 1309, 1312, 1321], this study assessed total Aβ levels extracted from single brain hemispheres. Thus, on the basis of Pedersen et al, it was predicted that rosiglitazone would lower amyloid levels in the drug-treated Tg2576 mice. However, whilst the assessment of adiponectin levels are applicable to both LINS and EINS cohorts, unfortunately time and cost issues limited the assessment of total Aβ levels to the LINS cohort only. In both cases, this assessment involved the use of Enzyme-linked immunosorbent assay (ELISA). Since both these measures reflect relatively small nature of the adiponectin and Aβ experiments, results relating to these are covered in the same discussion. Finally, this chapter also summarises the genotyping procedure used as the primary means of establishing the genetic identity of mice used in both LINIS and EINS studies.

4.3.1 Methods and Apparatus

4.3.1.1 Animals: and Drug Administration

Experiment 6 (LINS only) used mice the mice from cohort 1. To recap, these mice had been sacrificed by cervical dislocation between the ages of 18 and 19 months, wherehence the necessary tissues harvested for biochemical analysis. In brief, brain hemispheres were extracted fresh and divided along the central sagittal access with a scalpel such that the two brain hemispheres were separated. One hemisphere was chosen for the Aβ ELISA analysis. The remaining brain hemisphere was snap frozen in liquid nitrogen for autoradiography (unfortunately cancelled by GSK due to lock of funding). The hemispheres chosen for each condition were counterbalanced across mice from within each treatment group to avoid any hemispheric bias. All tissue was stored at -20°C until further use. Please see section 3.3.2.1 for further details of housing and drug administration.

4.3.1.2 Analytical Procedures

Total human Aβ levels (the soluble and insoluble Aβ protein extracts) expressed by the APPswe mutation in Tg2576 mice were quantitatively measured in brain samples by ELISA using commercial colorimetric immunoassay kits for human Aβ40 (Invitrogen, Cat.# KHB3482) and human Aβ42 (Invitrogen, Cat.# KHB3442). This sandwich ELISA uses microtiter strips with wells coated with a monoclonal antibody specific for the NH2-terminus of human Aβ. The protocol involved a series of diluted standards of known concentration being assayed alongside the experimental samples in order to create a standard curve to accurately measure sample concentrations of Aβ40 and Aβ42 peptides. The intensity of the coloured end reaction was then read by a plate-reader, with the light being directly proportional to the concentration of human Aβ present in the sample.

In preparation of the ELISA, the human Aβ40 and Aβ42 standards were reconstituted from powder with standard reconstitution buffer (55 mM sodium bicarbonate, pH 9.0), as indicated by the manufacturer, and diluted 1:20 in BSAT-DPBS (0.2 g/l KCl, 0.2g/l KH_2PO_4, 8.0 g/lNaCl, 1.150 g/l Na_2HPO_4, 5% BSA, 0.03% Tween-20 in MilliQTM water, pH 7.4). The tissue samples maintained on ice, were also diluted 1:20 in BSAT-DPBS containing 1:100 protease inhibitor cocktail (Calbiochem, Cat.# 539134). Serial dilutions of the standards in standard diluent buffer (provided in handy

kits) were then made for the standard curve: 500 to 7.81 pg/ml for the Aβ40 standard, and 1000 to 15.63 pg/ml for Aβ42 standard. Samples were then diluted further with standard dilution buffer in order to make two sample dilutions for the ELISA to in order to ensure detection within the standard curve range. The corresponding dilution required had been previously piloted because it varies with Aβ type (i.e. soluble and insoluble), isoform (i.e. Aβ40 and Aβ42), and with age (Aβ increases with age, and the mice used with in this asses were considered elderly). For example, a whole hippocampus from 12 month old TG2576 mice aged 12 months, would normally need to be diluted 1:400 and 1:800 in order for soluble Aβ to be detected within the standard curve range. Once prepared, 50µl of each diluted standard and sample were applied to the antibody-coated microtiter strip wells, in duplicate, with 50µl of rabbit anti-human Aβ40 or Aβ42 detection antibody. Following a 3 hour incubation period on a shaking plate at room temperature, liquid was then discarded from each well and washed four times with working wash buffer (25x concentrate wash buffer 1:24 in deionised water). 100µl of the secondary antibody (1:100 anti-rabbit Ig's-HRP 100x concentrate in HRP diluent) was then applied to each well using a multi-channel pipette and incubated for 30 minutes on a shaking plate at room temperature. Following washes (as described above), 100µl of stabilised chromogen was then applied to each well, and incubated for approximately 20 minutes at 25°C in the dark. This blue-coloured reaction was then stopped by applying 100µl of stop solution into each well and the absorbance was measured at 450 nm using a colorimeter plate reader (FLUOStar Optima, BMG Labtech). Optima 2.0 software directly calculated Aβ concentrations of each sample based on the absorbance values relative to the absorbance values of the standard curve (based on known concentrations of standard serial dilutions). Only values within the standard range were used. Aβ protein concentrations were calculated by taking the mean value of duplicates, and expressed as pg of Aβ per µg of protein (this required the use of protein concentrations obtained in the BCA assay).

4.3.1.3 Statistical Analysis

Raw data for each of the estimates of total Aβ levels were analysed by 2 way multivariate ANOVA, which had [Aβ isoform] (Aβ40/42), and Treatment as factors. All analysis was conducted with IBM SPSS Statistics v.21.00 software with an alpha value (P) <0.05, taken as being statistically significant Any significant ANOVA interactions and non-parametric analysis were conducted as previously indicated in

Experiment 1 (see section 3.3.2.4). Experiment 6 was conducted with samples from 18 TG mice (TG-C, n=9; TG-R, n=9), and 21 WT (WT-C, n=12; WT-R, n=9).) Note: The WT samples were only used to verify genotype (none of these showed any trace of human Aβ).

4.3.2 Results

4.3.2.1 Experiment 6: LINS Cohort

Figure 4.3 shows the mean levels of Aβ40 (Figure 4.3 A) and Aβ42 (Figure 4.3 B), both of which were derived from single brain hemispheres. Inspection of this figure suggests no significant differences between drug-treated and non-drug treated groups with respect to total levels of either Aβ40 or Aβ42. This interpretation was confirmed by ANOVA, which had [Aβ isoform] (Aβ40/42), and Treatment as factors. This gave a significant effect of Aβ isoform, $F(1,16)=8.742$, $p=<0.05$, a no significant effect of Treatment, $F<1$, and no significant Aβ isoform by Treatment interaction, $F<1$. Thus, I conclude that rosiglitazone had no impact upon either Aβ40 or Aβ42 levels.

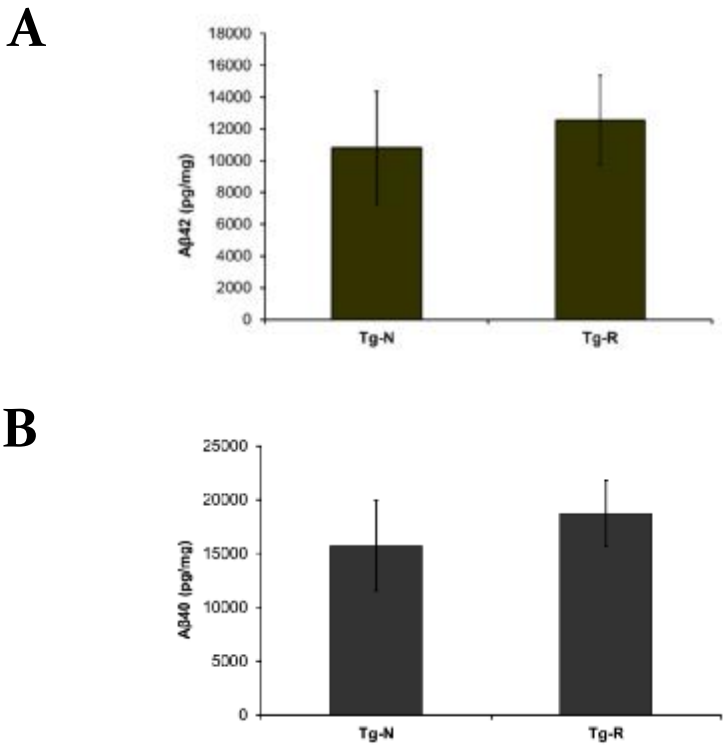

Figure 4.4: LINS Total Aβ Levels (Experiment 6). (A) Mean levels of total Aβ40 (B) Mean levels of total Aβ42 Both Aβ isoforms isolated from whole single brain hemispheres. Values are means ±S.E.M.

4.3.3 Discussion

4.3.3.1 Experiment 5: Adiponectin Levels

As a means of validating against the primary method of drug administration in this study, both Experiments 5A and 5B show that rosiglitazone had reached systemic penetration in both cohorts of mice. This is consistent with other studies which have found that rosiglitazone increases peripheral levels of this protein [1485-1487]. Adiponectin is an important adipocyte-derived hormone that regulates metabolism of lipids and glucose, and its receptors (AdipoR1, AdipoR2, T-cadherin) appear to exert actions in peripheral tissues by activating the AMP-activated protein kinase, p38-MAPK, PPAR alpha and NF-kappa B [1483]. Indeed, adiponectin has been shown to exert a wide range of biological functions that could elicit different effects, depending on the target organ and the biological milieu [1483, 1488]. This includes potent anti-inflammatory effects [1194, 1482]. There is also substantial evidence to suggest that adiponectin receptors are expressed widely in the brain and adiponectin levels are known to be expressed in regions of the mouse hypothalamus, brainstem, cortical neurons and endothelial cells, as well as in whole brain and pituitary extracts [1483]. However, it should be said that adiponectin signalling in the brain is still poorly understood, and the function of adiponectin receptors may well vary depending on their location in the brain [1489].

The impact of increasing adiponectin levels in hAPP mice has not been explored. This is of potential significance as anti-inflammatory treatments have been linked to beneficial effects in some AD patients [412, 766], and rosiglitazone may impart its anti-inflammatory effect via peripheral and central mechanisms that involve adiponectin signalling, [1164, 1490-1493], although neither of these is likely to be mutually exclusive. Anti-inflammatory effects associated with adiponectin have also been reported via a heme oxygenase-1-dependent pathway on stellate macrophages (Kupffer cells) [1194]. The peripheral mechanism at least, probably involves the PPAR-γ-mediated production of adiponectin protein (a hormone) in adipose tissue [1482], and it is conceivable that any centrally mediated anti-inflammatory role of this protein could be beneficial in AD. However, given the robust increase in adiponectin levels in drug treated mice in the present study, it is disappointing that any impact on cognition was extremely limited. Indeed, although adiponectin is considered to exert a potent anti-inflammatory effect, this view is being challenged in light of recent reports of elevated levels of this protein in various inflammatory disease states, including arthritis,

preeclampsia and end-stage renal diseases [1483]. Indeed, it has been shown that adiponectin induces production of the pro-inflammatory mediator IL-6 and activation of NF-κB in human synovial fibroblasts and adhesion molecule expression in endothelial cells [1484, 1494, 1495]. However, it is not uncommon for many proteins such as adiponectin to exert sometimes opposite effects in different tissues (i.e. something can be pro-inflammatory or not depending on the circumstances). Thus, raising adiponectin levels could have contradictory effects in AD, and indeed hAPP models, (as well as WT mice).

4.3.3.2 Experiment 6: Total Aβ Levels

Despite the fact that several studies have reported that rosiglitazone significantly impacts upon Aβ levels and or amyloidosis in hAPP mouse models [1129, 1213, 1217], the finding in the current study that there was no significant impact of the drug on either Aβ40 or Aβ42 levels is disappointing. Nevertheless, it remains possible that we may have reduced the soluble portion of Aβ40/42, although we did not explicitly test this. I have already discussed the inconsistencies with the findings of Pedersen et al [1129], in relation to a differential impact of rosiglitazone on Aβ42 but not Aβ40 levels. However, it is evident that other studies have also provided contrasting results to the current study in relation to the purported effects of the drug on ameliorating insoluble aggregates of Aβ [1213, 1217]. These discrepancies may partly reflect methodological differences between studies, as well as differences in the amyloid dynamics across hAPP models. However, further investigation is required to in order to clarify this issue. Nevertheless, I have confidence in the findings of the present study that rosiglitazone does not impact on Aβ levels in Tg2576 mice, although I cannot be certain that some regional differences in amyloid pathology may have existed as a result of rosiglitazone agonism (again the assessment method did not allow for regional analysis). If there were some regional differences, this in itself could potentially explain the limited impact of rosiglitazone in reversing the cognitive phenotype of the Tg2576 mice used in this study.

4.4 Experiment 7: Dendritic Spines Density

4.4.1 Introduction

Dendritic spines are tiny protrusions along neuronal dendrites that constitute the major postsynaptic sites for excitatory synaptic transmission in the CNS. These spines are highly motile and can undergo remodelling even in the adult nervous system [737]. Experiments conducted in animals suggest that the extent of spine remodelling is correlated with behavioural improvement after learning, implicating a crucial role of synaptic structural plasticity in memory formation [738-742]. Furthermore, recent research also suggests that a small fraction of new spines induced by novel experience together with most spines formed early during development and surviving experience-dependent elimination, are preserved in order to provide a structural basis for memory retention throughout the entire life of an animal [739, 741-743]. Thus, spine re-modelling and the formation of new synapses are activity-dependent processes which provide a structural basis for memory formation [737, 836, 837].

A loss or alteration of dendritic spines has been described in patients with neurodegenerative disorders, including AD [838], where in the latter they are considered to be a much better correlate of cognitive impairment in patients than Aβ-plaque burden (see, [408, 744, 745]). Mounting evidence from human and animal studies suggests both the dysfunction and subsequent loss synapses in AD is mediated by diffusible soluble Aβ oligomers [60, 62-65, 408, 409]. Once bound to synapses, Aβ oligomers have been found to instigate a variety of pathological processes, including by interfering with functional synaptic plasticity processes in the hippocampus [782]. For example, at picomolar concentrations naturally secreted soluble Aβ oligomers can disrupt hippocampal LTP in acute brain slices and *in vivo*, where in the latter they have been shown in rats to impair the memory for complex learned behaviour [50]. Disruption of functional plasticity by Aβ oligomers has been shown to include LTD, which is promoted rather than inhibited [805, 831]. Thus, in summary, the overall impact of oligomers appears to be depressed synaptic output. However, phospo-tau oligomers have also been linked to detrimental effects on neurones and synapses [402, 403], highlighting that both species of oligomer are attractive therapeutic targets in AD [410-412]. On the basis of the amyloid hypothesis, it was predicted that there should be a link between Aβ oligomers and synaptic loss in mouse models related to FAD kindreds [408, 831, 838]. This prediction has been confirmed [839], as have related

predictions that Aβ oligomers cause dysfunctional trafficking of ionotropic glutamate receptors and metabotropic glutamate receptors [839, 840]. The concentration of soluble Aβ oligomers has been shown to predict synaptic change in AD patients [404], and oligomers are known to accumulate with age in all the transgenic models examined so far, including mouse, rat, and *Caenorhabditis elegans* [841-844]. However, what promotes oligomer accumulation in SAD remains uncertain, and numerous transgenic mouse models overexpressing human APP FAD mutations (sometimes in combination with FAD-linked PSEN1 mutations), have shown that these models do not generally display NFT pathology or overt neuronal cell loss, thus questioning the relevance of these *in vitro* experimental paradigms [779].

Transgenic models have become crucial to understanding the role of Aβ in AD pathology, as well as for testing novel therapeutic strategies. However, in order to test the effects of candidate therapeutic treatments, it is necessary to recognize the type, extent, and onset of pathologies in each model. In the Tg2576 mouse model (originally termed APP 695SWE), amyloid plaques do not develop until 18 months [1242], although both LTP deficits in hippocampal CA1 and DG and spatial memory deficits in a modified water maze are evident before this, suggesting impaired synaptic plasticity. Jacobsen et al., were the first to characterise this across the lifespan of Tg2576 [1245], and show that decreased dendritic spine density, impaired LTP, and behavioural deficits occurred months before overt plaque deposition at 18 months of age. Indeed, a decrease in spine density in the outer molecular layer of the dentate gyrus (DG) was found as early as 4 months of age in these mice, and by 5 months there was a significant decline in LTP in the DG after perforant path stimulation and impairment in contextual fear conditioning [1245]. Moreover, an increase in the Aβ42/Aβ40 ratio was first observed at these early ages, although total amyloid levels did not significantly increase until ~18 months of age, at which time significant increases in reactive astrocytes and microglia were also observed [1245]. Importantly, Jacobsen et al., show that the perforant path input from the entorhinal cortex to the DG is compromised both structurally and functionally in Tg2576, and this pathology is manifested in memory defects long before significant plaque deposition. Indeed, the DG/CA3 network is considered to be central to the proper encoding of episodic memory [1496], and disruption of the CA3/DG pattern of activation in Tg2576 likely represents the primary pathophysiological basis for memory impairment in these mice [1321].

4.4.2 Methods and Apparatus

4.4.2.1 Animals and Drug Administration

Experiment 7 (EINS) used the mice from Cohort 3. To recap, these mice were sacrificed between the ages of 12 and 13 months, and the necessary tissues harvested for biochemical analysis. In brief, the mice were first culled via cervical dislocation and checked for dead prior to the heads being decapitated with a sharp scissors. The brains were then rapidly dissected and sectioned along the coronal axis on a vibratome into 250µm slices in 0.1m phosphate buffered saline (PBS) at room temperature[a]. Brain slices were collected into a segmented Petri dish containing PBS in its wells. Following collection, PBS solution was removed from each well and replaced with 1.5 % paraformaldehyde (PFA) for 10-15 minutes. Following pre-stain fixing, the PFA was removed and each slice washed three times with PBS in preparation for labelling with DiD stock solution containing 7.5 µl of DiD crystals dissolved in ethanol (V-22887, Molecular Probes, Eugene, OR.) per 1 ml of PBS. The stock solution was covered with silver foil (to prevent photo bleaching) and placed on a generic solution mixer until use. All solutions were at room temperature when used.

4.4.2.2 DiD labelling and mounting

PBS solution was removed from each well and replaced with 1ml of DiD stock solution. The Petri dish was then covered with laboratory cling film, tin foil, and then placed on a tissue rocker on low speed for 16-20 hours[b]. This method encourages DiD crystals to

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[a] Preparation of fresh slices in ice cold solutions causes many dendritic spines to disappear. Although spines re-form during a recovery period (typically 1hr whilst incubated in ringer solution), research has shown that the new expression of spines and spine proteins is abnormal (Kirov, Petrak, Fiala, & Harris 2004; Cheng, Huang, Lin, Chow, and Chang 2009). In addition, in transgenic mouse models such as Tg2576, there may also be potential recovery complications due to differences in brain plasticity mechanisms mediating spine recovery. However, because cutting brain slices in solutions at room temperature prevents cold induced spine loss (Bourne, Kirov, Sorra & Harris 2007), this potential problem can be avoided. Finally, pilot data has indicated that profusion fixed tissue does not differ quantitatively (i.e. number of cells and spines detected) from fresh tissue cut at room temperature (Anderson J, unpublished pilot data).

[b] Placing the Petri dish in a water bath at 37°C for 2-3 hours also works. In this case, heating speeds up dye diffusion and gives a slightly deeper penetration of the dye into the brain slice. However, this method also produces more crystal clumping and a higher occurrence of artefact staining, which collectively make confocal imaging problematic. Heating for longer than 2-3 hours was found to give too intense a stain making confocal imaging impossible. With a weaker DiD stock solution this method can be used to label cortical neurons, although there appears to be more staining artefacts generated with this method.

repeatedly wash across the surface of the tissue maximising the total number of collisions between different parts of the brain slice and the dye crystals. Although this method requires a longer time for dye diffusion along cell membranes, it produces a sparse labelling of granule cells whose apical dendrites intersect with or are very near the slice surface, with minimal artefacts and dye clumping (i.e. multiple crystals settling in fewer positions).

Following incubation with the DiD stock solution, the tissue was transferred to a low intensity lighting lab and washed 3 times in PBS before being post-fixed in 4% PFA for 10-15 minutes to preserve staining and improve tissue stability. During this time the tissue was kept covered with tin foil to prevent dye bleaching. The PFA was then removed and the tissue washed three times in PBS before being mounted onto glass microscope slides with the glycerol-based mounting medium Fluromount (containing DABCO as an anti-fade reagent; Sigma-Aldrich, USA). Slides were then cover slipped and sealed with clear nail varnish. All imaging took place within 2 days of cover slipping when background staining was minimal.

4.4.2.3 Laser confocal microscopic imaging of cells and image analysis

Hippocampal granule cells were imaged using Zeiss 510 Meta confocal laser scanning microscope (LSM 510 META). All images were taken using the Plan-C-APOCHROMAT 63 × water immersion lens (1.2 corrected). We used 2048 × 2048 pixels for frame size without zooming. A 633 nm Helium/Neon laser (at 5%) was used to visualize fluorescence emitted by DiD. The configuration parameters were as follows: (1) filters, channel 3 band pass 560–615 nm, (2) pinhole diameter, 108 µm, (3) beam splitters, MBS-HFT UV/488/543, DBS1 mirror, DBS2 NFT, DBS3-plate. Serial stack images of randomly stained dendrites were imaged from brain slices with a step size ranging from 0.2 to 0.5 µm. Stack images were imaged if the dendritic segment could be traced back to a cell body, with samples obtained from both apical and basal dendrites (this ensured we sampled cells from within the granule cell layer).

Following collection of confocal imaging, all z-stacks were de-convolved with the Autoquant X 2.1.1 software (Media Cybernetics Inc., USA) using the blind adaptive point spread function (PSF) algorithm to remove noise associated with spherical

aberration[c] (see, [1497]). All stacks were analysed in black and white. All de-convolved z-stacks were imported into Imaris x64 6.3.1 software (Bitplane AG, Switzerland), and the dendritic segments measured in 3D. Only samples measuring between 70 and 120 um from the proximal end of the dendrite were used in the analysis. Each image stack was then imported into NeuronStudio x64 0.9.86 software (freely available from Mount Sinai School of Medicine, New York, USA), and the dendrite then projected and traced in 3D so that the default spine detection and classification module of NeuronStudio could be used to count the number of dendritic spines along the length of dendrite (see Figure 4.5). In short this uses a Rayburst-based spine analysis routine, a processes which counts and classifies spines into either, Stubby, Thin, or Mushroom within concentric 50 µm circles at increasing distance from the soma (Sholl analysis). This classification scheme makes use of each spine's head to neck diameter ratio, length to head diameter ratio, and head diameter to determine its proper type. An operator then inspected each cell, and made minor corrections as needed using the NeuronStudio interface. In order to measure the spine density of the sample the total number of spines were then divided by the total length of the dendrite sample (micrometers). The NeuronStudio software is a robust programme for and the accurate counting of dendritic spines, and unlike other semi-automated methods, has been peer reviewed for robustness [1498, 1499].

4.4.2.4 Statistical analyses

Curvature spine ratios of dendritic segments in the Tg2576 vs Wt groups in the Sholl analysis data were compared by 1-way univariate ANOVA with **Spine Density** as the dependent variable, and **Genotype,** and **Treatment** as factors. Sidak tests were used in tests of simple main effects following significant interactions. The null hypothesis for each test was rejected at a threshold of 0.05. All data are presented as mean ± SEM.

[c] Although application of the blind algorithm on the complete z-stack produces good results, splitting the stack into smaller segments of interest and then applying the deconvolution algorithm works better and reduces the effects of overshadowing of more intense stained sections on less intensely stained structures (Anderson J, unpublished pilot data). This may also help ensure that the adaptive PSF is sensitive to changes in *z*-distance and ensure that spine density measurements are as accurate as possible, (e.g. by allowing more spines to be imaged in the z-axis than is usually possible).

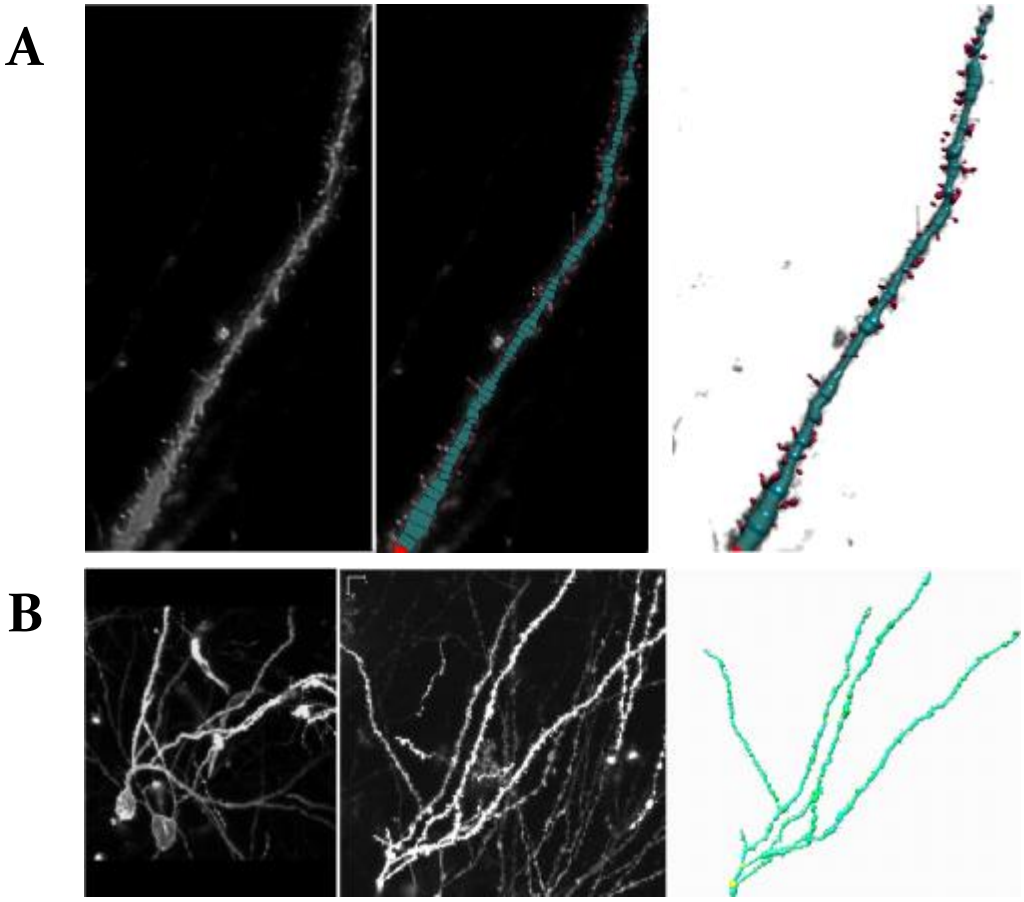


Figure 4.5: Experiment 7 (Confocal Imaging). (A) Reconstruction of dendritic spine samples in NeuronStudio. *Left*: a deconvolved stretch of dendrite. Middle: Automated Scholl identification of dendritic spines and model tracing of the stretch of dendrite. *Right*: The model tracing and spines without the background image. (B) Reconstruction of a second sample obtained from a WT mouse.

4.4.3 Results

Figure 4.6 shows the mean spine density obtained from confocal data stacks of segments of mouse apical and basal dendrites belonging to hippocampal granule cells across treatment conditions. Inspection of this figure indicates two important findings. First, that TG mice have significantly lower dendritic spine density in the dentate gyrus granule cell population relative to the WT-C group, and that rosiglitazone does not change this. Second, that the WT-R mice have significantly lower dendritic spine density in the dentate gyrus granule cell population relative to the WT-C group, reflecting an impairment that is similar to that observed in the TG population. The above interpretation was confirmed ANOVA, which revealed

significant effect of Genotype $F(1,130)= 38.034$, p=<0.001, a significant effect of Treatment $F(1,130)= 35.851$, p=<0.001, and a significant Genotype by Treatment interaction $F(1,130)= 45.665$, p=<0.001. A follow –up test of simple main effects for the significant Genotype by Treatment interaction revealed that neither of the TG groups differed from each other, $F<1$, with both TG groups exhibiting a significant loss of dendritic spines relative to WT-C mice, TG-C verses WT-C, $F(1,130)= 84.791$, p=<0.001; TG-R verses WT-C, $F(1,130)= 81.220$, p=<0.001. Further comparisons showed that the DG spine density in WR-R group was significantly lower than in the WT-C group, $F(1,130)= 81.220$, p=<0.001, but was not significant when compared to either of the TG groups, TG-R verses WT-R, $F<1$; TG-C verses WT-R, $F<1$. These results show that chronic rosiglitazone agonism for an approximate 12 month period failed to recover the loss of hippocampal DG dendritic spines in TG mice, but did cause a significant loss of spines in this brain region in WT-R mice in par with the loss seen in both TG groups.

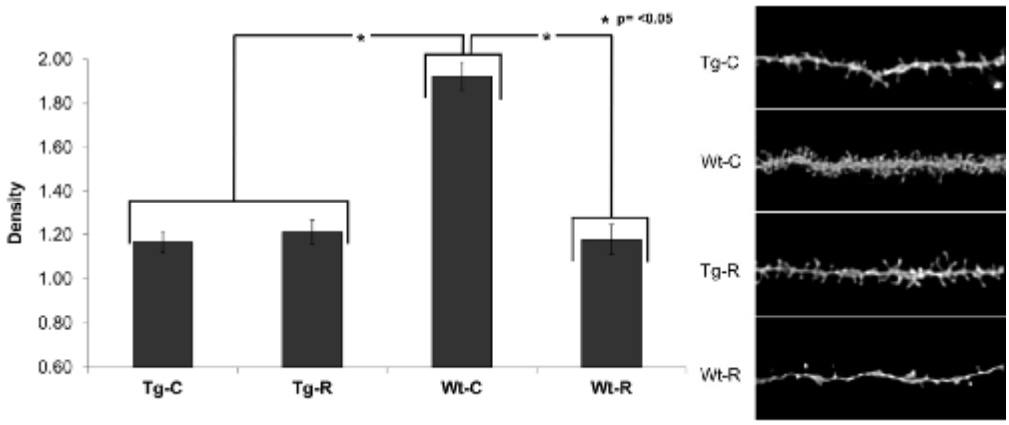


Figure 4.6: Experiment 7 (Spine Density Results). (*Left*) Graph showing mean dendritic spine density for each group. *(Right*) Examples of tissue samples from each group.

4.4.4 Discussion

Although the method of staining hippocampal slices in this experiment was novel, and lacking in more extensive validation, the results obtained with respect to the reported loss of dendritic spines in the dentate gyrus of Tg2576 mice are in line with those reported by previous studies using this mouse model [1245, 1308, 1309]. Despite encouraging research showing that rosiglitazone may have a protective role on cortical dendritic spines [1154], as well as a protective effect on spines by boosting insulin signalling [1151], I find that long term rosiglitazone agonism did not significantly

recover the Aβ related loss of dendritic spines in the hippocampal dentate gyrus. Whilst it is possible that changes in spine density did take place in other regions of the hippocampus, and indeed the cortex (these regions were not investigated), I am inclined to believe that if these effects exist in vitro they are probably of limited value as recovery of the behavioural phenotype in my experiments was limited. This at least is consistent with the larger double blind clinical studies in humans with mild to moderate AD which show that Rosiglitazone administration does not recover function across a range of cognitive domains [1238, 1247, 1480].

The surprise finding of significant loss of dendritic spines in the rosiglitazone treated WT mice probably goes some way to explain in mechanistic terms why this group exhibited impairment with chronic exposure. Nevertheless, the reasons for this are unclear. One possibility is that we induced a hyperisnsulinemic/Hypoglycemic state in these mice by providing them with access to diabetic medication when they essentially may have had normal blood glucose and insulin levels. In this context it is unfortunate that the planned measures of these biomarkers were cancelled due to financial restrictions imposed by the commercial partner. Another possibility is that we may have boosted IGF-1 levels in these mice. At least one previous study has shown that IGF-1 promotes Aβ production by a secretase-independent mechanism [1500]. If in this effect had occurred in the hippocampus of WT-R mice as a result of chronic rosiglitazone treatment, it could have resulted in an increase in murine Aβ levels that subsequently had an adverse impact on synaptic function in these mice. It is interesting that both TG rosiglitazone treated groups exhibited an elevation in total Aβ levels in Experiment 6, although this was not significant. The results of Experiment 7 at least suggest that measurement of murine Aβ may be important when rosiglitazone (and indeed other PPARg agonists) are used in preclinical studies using hAPP mutants.

5. SUMMARY

ANIMAL studies provide us with a unique opportunity to study the effects of therapeutic agents on limited disease mechanisms common to AD in the early stages. This thesis dealt with the preclinical assessment of chronic rosiglitazone administration in WT and TG2576 mice. Tg2576 mice contain the APPSWE transgene which results in the overproduction, accumulation and deposition of Aβ, neuroinflammation, the loss of dendritic spines in the hippocampal dentate gyrus, and learning and memory deficits. As such, the lead hypothesis for this thesis was that in the LINS condition, rosiglitazone should reverse the established age-related learning and memory deficits and pathophysiological changes (i.e. lower Aβ levels). In the EINS condition, rosiglitazone should prevent the onset of these changes when drug administration was initiated from 5 MO prior to significant Aβ pathology taking place, as well as prevent the loss if dendritic spines in the dentate gyrus.

Experiment 1 of this thesis shows that rosiglitazone failed to recover or prevent the onset of a robust spatial working memory deficit in TG mice, as revealed by the FCA T-maze task. However, in Experiment 2 (EPM), I did show that rosiglitazone was able to prevent at least some component of behavioural disinhibition in the TG mice, although it did not have the same effect on TG mice in the LINS condition. Likewise, in Experiment 3, the drug did not reverse or prevent the behavioural deficit as revealed by the Marble Burying task. Whilst both Experiments 2 and 3 were intended to assess anxiety and behavioural disinhibition, discussions of both experiments address concerns that these tasks may measure different things, which in the case of the Marble Burying task, still remain unclear. Finally, in Experiment 4 I also found that rosiglitazone did reverse a behavioural impairment related to the detection of a novel object that appears in two mismatched spatial locations along with two different familiar objects. It is not possible to say whether or not in the EINS condition the drug prevented the deficit because the nominal age at which the TG deficit occurs is

currently unknown. Nevertheless, the same result in both conditions indicates that rosiglitazone had a robust beneficial effect on object recognition memory in the TG mice. Thus, in summary, although rosiglitazone did not reverse of prevent the onset of the spatial memory impairment (the major deficit in these mice), the drug may have had a limited impact on reducing behavioural disinhibition and object recognition memory. The limited impact of rosiglitazone in rescuing the behavioural phenotype of the TG mice in this thesis supports the contention that in order to be most effective, PPAR agonists may have to be delivered to patients early, before significant Aβ pathology occurs. However, this would mean delivering the drug to at risk individual's (i.e. individuals with aMCI) decades before overt dementia symptoms appear. This is problematic for a number of reasons, not least of which because current bio-markers are not able to pinpoint with exact certainty those persons who are destined to develop dementia from those who will not. Also, the particular age used in the current thesis for early drug monotherapy, make a human comparison problematic, as it would likely require delivering the drug in childhood.

In Experiment 5, the significant elevation in murine adiponectin levels show that the delivery method for the drug provided an effective means by which rosiglitazone could reach systemic penetration. It is interesting to note that in T2D (which is associated with chronic low grade infection), adiponectin levels are typically decreased [1178, 1180], with higher levels being associated more with a reduced risk for T2D [1186]. The status of adiponectin levels in AD is largely unknown, although one research group has recently reported that in women, increased plasma adiponectin levels serve as an independent risk factor for the development of both all-cause dementia and AD [1501]. In this context, an elevation in adiponectin levels could be a sign of that pro-inflammatory processes have outstripped the natural capacity of the system to deal with the infection/damage (i.e. response is maximal, but insufficient to overcome the problem). In addition, some chronic inflammatory conditions may involve a loss of anti-inflammatory efficacy. Either way, the result may be that more anti-inflammatory proteins (including adiponectin) have to be produced in order for the system to try and keep neuroinflammation in check. This suggests that in some people at least, a form of 'adiponectin resistance' could develop. The results of Experiment 5 in this thesis are disappointing in that artificially raising adiponectin levels did not seem to translate into a robust rescue of the behavioural phenotype of TG mice. Nevertheless, on first glance, it is not unreasonable to think that the elevations in adiponectin with

chronic exposure to rosiglitazone may have been one of the factors responsible for the limited restoration of the cognitive phenotype, particularly if it dampened down some of the central inflammation processes. However, recent research has started to question whether or not endogenous adiponectin is able to influence CNS pathways, particularly since it may not pass the BBB [1489], so it is unclear whether raising peripheral levels of this protein would have an impact upon CNS function in Tg2576 or an adverse effect in WT mice. Nevertheless, CSF adiponectin has been detected after an i.v. injection of full-length adiponectin in C57Bl/6J mice, and that both systemic and i.c.v. administration of adiponectin reduced serum glucose and lipid levels and decreased body weight [1487]. Indeed, that study was instrumental for suggesting that adiponectin could be a centrally acting signalling molecule; a position further substantiated by subsequent research showing that peripherally administered adiponectin stimulated AMPK in the hypothalamus of mice, to increase food intake and decrease energy expenditure [1488]. However, whether endogenous adiponectin might have a similar effect in humans is not yet clear. In addition, the identification of adiponectin receptors on brain endothelial cells (and the finding of a modified secretion pattern of centrally active substances from BBB cells) does provide one mechanism by which adiponectin could conceivably evoke effects on energy metabolism [1489]. In summary, the effects of elevating adiponectin levels in hAPP models may have limited value, (beneficial in TG, and possibly detrimental in WT), require further investigation.

It is also clear that in this thesis, rosiglitazone not only failed to lower total Aβ levels (see Experiment 6), but also failed to prevent the Aβ-related loss of dendritic spines in the hippocampal dentate gyrus (see Experiment 7). This is in spite of rosiglitazone having been systemically present from the age of 5 MO. Although a number of concessions have been discussed in relation to these findings, they do perhaps provide a mechanistic reason for why more robust rescue of the behavioural phenotype was not observed. As such, all of the above findings fit with those reported by the most recent large phase 3 clinical trials [1237, 1238], indicating that the drug does not significantly impact on dementia symptoms (although object recognition has not been robustly assessed). Thus, as far as big Pharma is concerned, rosiglitazone has been 'marked for death', despite incidental reports of beneficial effects of the drug in pre-clinical studies using hAPP models.

It is also evident from the experimental studies reported in this thesis that administering a drug such as rosiglitazone may have unintended side-effects. Thus, it is clear from the results of the experiments detailed herein, that the chronic exposure to rosiglitazone produces a significant behavioural impairment in the WT-R mice. At a pathophysiological level this fact could be reflected in the significant and somewhat shocking loss of dendritic spines from the hippocampal dentate gyrus. If this effect occurred across multiple brain regions, it would perhaps explain why the effect on cognition seemed to be have present to varying degree across all the behavioural domains assessed. Indeed, it is a shame that the study of additional biomarkers were cancelled by the industrial partner, as this could have greatly extended the ability of this thesis to pin down what had gone wrong in these mice (i.e. was it due to an elevation in IGF-1. Indeed, it would also have been interesting to see if this raised levels of murine Aβ. The only other study to have investigated the pre-clinical impact of the drug in WT littermates to Tg2576 mice has reported no adverse side effects, although administration of the drug was stopped after 4 weeks [1129, 1244]. One wonders if there was unreported data which showed similar results to my own for longer administration periods. I do not know if these detrimental effects would transfer to humans (a possibility in some people with aMCI for example), but, if they did, it would likely make learning and memory worse in the long-term. However, no adverse effects of the drug have been reported in other non TG rodent models [1165, 1204-1207, 1209-1211, 1239, 1365-1367, 1403], although these have not included chronic exposure in WT mice. Nevertheless, the findings from this thesis are likely to further add to the currently negative profile of this and similar drugs [1481]. In aggregate, the results detailed in this thesis do not support the use of rosiglitazone as a viable candidate for human trials as full rescue of the Tg2576 phenotype was not achieved. Nevertheless, the drug may have limited value as a research tool in pre-clinical studies.

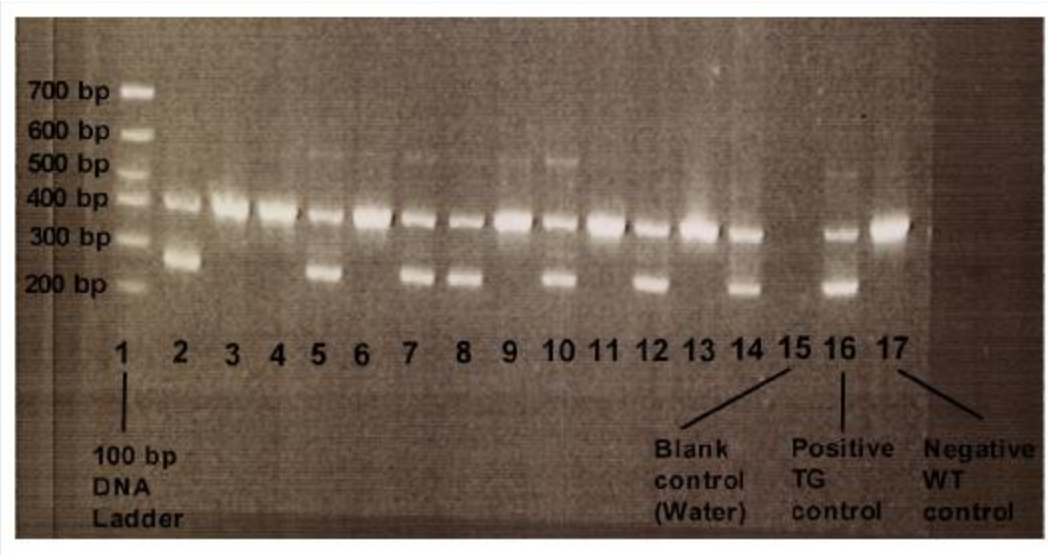
Final Thoughts

There are serious challenges ahead for neurodegeneration research and in particular, behavioural neuroscience in relation to animal models of specific pathological components common to AD. It is unfortunate that no commercially available model exists for SAD. This is important because within science, one is usually taught that arguing from the minority to the majority is bad practice. And yet, that is in a way, what we have to with hAPP models. Furthermore, whilst most (typical) AD involves

progressive amnesia followed by various combinations of focal cortical deficits (e.g. aphasia, apraxia, agnosia), and impaired executive function [72], it is of interest that the FAD mutations from which most animal models derive (including Tg2576) are almost exclusively associated with EOAD where atypical clinical phenotypes are much more common. Indeed, in atypical AD focal deficits other than memory impairment can dominate in the early-stages of the disorder, although some types are primarily amnesic in nature [72, 73]. In this respect, the clinical phenotype observed in AD patients seems to largely reflect the degree to which gross neuropathologies are either cortical predominant, or hippocampal predominant [72], although these distinctions are seldom considered in relation to TG models deriving from FAD kindreds. Incomplete reporting of methodological procedures with respect to the numbers of male vs. female mice used in experimental procedures is also a serious concern. This is bad practice and makes interpretation across studies difficult if not impossible in some instances. I also consider that similar stringent conditions should apply to the reporting of all pre-clinical studies conducted with hAPP mice, as is the case now with most human clinical data. This would help to reduce the bias towards just reporting positive results in the sciences, whilst 'bottom drawing' negative or null findings. Indeed, this is possibly one contributory reason for why big pharmaceutical led projects into neurodegeneration are on the decrease: it has become harder to push through to market marginally effective drugs.

Finally, it is evident that AD is an incredibly complex disorder, to treat. So far it appears resistant to the process of reductionist science. One feels that the current strategy of most pharmaceutical led endeavours to treat the disorder are possibly misguided in their over simplicity. The race to find the next 'block buster drug' may be an attractive financial incentive, but it can impede progress in targeting certain therapies to smaller groups of patients with particular genetic profiles. Indeed, whilst is likely that the treatment of AD will require poly pharmacy, given the high financial stakes involved when drugs fail clinical trials, this is not something that most commercial companies have the stomach for. There is also much about the brain we have yet to understand. Currently, trying to cure AD is somewhat like attempting to fix a car without knowing how much of the car works. Nevertheless, I have greatly enjoyed conducting the research associated with this thesis, and am hopeful that if we continue to integrate basic and medical research, real advances in the treatment of dementia will eventually be forthcoming.

APPENDIX 1



Representative photograph of gel electrophoresis showing PCR amplification of DNA from Tg2576 and WT mice. Lane 1 = 100bp DNA ladder; Lanes 2, 5, 7, 8, 10, 12 and 14 = double band corresponding to amplification of endogenous murine prion protein and APPswe transgene, therefore representing TG mice; Lanes 3, 4, 6, 9, 11 and 13 = single band corresponding to amplification of endogenous murine prion protein, therefore representing WT mice; Lane 15 = Blank (water) control, Lane 16 = Positive control (known TG sample), Lane 17 = Negative control (known WT sample).

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