NEURAL NETWORKS IN A MOUSE MODEL OF AMYLOID PATHOLOGY

Alice Marie Palmer

Cardiff University

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Thesis Summary

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases. However, the aetiology of AD is still unclear and there is currently no cure. In order to develop new therapeutic strategies more basic research into the underlying mechanisms leading to cognitive impairment is needed. This thesis uses the Tg2576 mouse model to examine the effects of amyloid pathology on hippocampal network activity and memory, and to assess the therapeutic effects of exercise as a lifestyle intervention against amyloid-induced cognitive decline.

The hippocampal network is made up of different structural sub-regions, dentate gyrus (DG), CA1 and CA3, each of which plays a different role in memory encoding and retrieval. An analysis of c-fos expression was carried out followed by structural equation modelling to assess neural activity in each sub-region when Tg2576 and wild-type control mice explored a novel or familiar spatial environment. In young transgenic mice there was a reduction in DG engagement when encoding spatial novelty compared to controls. In aged mice deficits in DG engagement were seen in both genotypes. The influence of amyloid-induced hippocampal network alterations on cognition was assessed on a novel task designed to test configural integration of cues relating to episodic memory. An analysis of c-fos expression in normal mice revealed that this task was also DG dependent. Marked deficits in episodic-like memory were seen in older transgenic mice, however, in contrast to hippocampal activity, no deficits were reported in young transgenic mice compared to wild-types.

In the second section of this thesis the hypothesis that long-term voluntary exercise can reduce cognitive decline was tested. Exercise improved cognition in transgenic, but not wild-type mice, in tests of working and reference spatial memory. In addition, a deficit in configural memory for episodic information was reversed in transgenic mice by exercise. The effect of exercise on amyloid levels and DG neurogenesis was assessed, as these are putative molecular substrates which may be altered by exercise leading to improved cognition. However, exercise did not significantly influence either of these measures.

These data indicate sub-region specific changes in hippocampus network activity in the Tg2756 model of amyloid pathology, which may underlie deficits in spatial and episodic-like memory. Furthermore, prolonged exercise reduced cognitive decline in this model, and may be a useful therapeutic intervention to prevent or delay the onset of AD in at-risk patients. However, the precise molecular mechanisms by which exercise exerts its beneficial effect on memory remain to be established.

Abbreviations

Aβ: amyloid β

AD: Alzheimer's disease

ADAS: Alzheimer's disease assessment scale

ANOVA: analysis of variance

APOE: apolipoprotein E

APP: amyloid precursor protein

BASE1: β-secretase enzyme

BASE2: α-secretase enzyme

BCA: bicinchoninic acid assay

BDNF: brain derived neurotrophic factor

BIN1: bridging integrator 1

BrdU: bromodeoxyuridine

CA: cornu ammonis

CFI: comparative fit index

CLU: clusterin

COX-2: cyclooxygenase 2

CNS: central nervous system

CR1: complement component receptor 1

CSF: cerebro-spinal fluid

DAB: 3,3-diaminobenzidine

DG: dentate gyrus

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

DPX: di-n-butyl phthalate in xylene

EDTA: ethylene-diaminetetra-acetic acid

ELISA: enzyme-linked immunosorbent assay

Ent: entorhinal cortex

FAD: familial Alzheimer's disease

GABA: gamma aminobutyric acid

GFI: goodness of fit index

GluR1: metabotrophic glutamate receptor 1

GWAS: genome wide association study

IEG: immediate early gene

IGF-1: insulin dependent growth factor 1

IL-1β: interleukin 1β

IR: immunoreactive

Kb: kilobase

LTD: long term depression

LTP: long term potentiation

MCI: mild cognitive impairment

MLE: maximum likelihood estimation

MMSE: mini-mental state exam

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

MTL: medial temporal lobe

NFT: neurofibrillary tangle

NMDA: N-methyl-D-aspartate

NOR: novel object recognition

NR2A/B: NMDA receptor subunit 2 A/B

NSAID: non-steroidal anti-inflammatory drug

PBS: phosphate buffered saline

PBST: triton-X-100 PBS

PCR: polymerase chain reaction

PET: positron emission tomography

PFA: paraformaldehyde

pH: potential hydrogen

PiB: Pittsburgh compound B

PICALM: phosphotidylinositol-binding clathrin assembly protein

PPF: pulse paired facilitation

Prh: perirhinal cortex

PS1: presenillin 1

PS2: presenillin 2

PSD-95: post-synaptic density protein 95

RMSEA: root mean square error of approximation

RNA: ribonucleic acid

ROI: region of interest

SAD: sporadic Alzheimer's disease

SDS: sodium dodecyl sulphate

SEM: structural equation modelling

Sub: subiculum

TAE: tris acetate EDTA

TBS: tris base sodium chloride

TE: tris EDTA

TES: tris EDTA-SDS

Tg: transgenic

TNF-α: tumor necrosis factor-α

TNS: tris non-saline

TBST: triton-X-100 TBS

UV: ultraviolet

VEGF: vascular endothelial growth factor

Wt: wild-type

Chapter 1: General Introduction

1.1 Overview of Thesis

Alzheimer's disease (AD) is the most common form of dementia, affecting millions of people world-wide. With modern medicine increasing lifespan, AD is becoming a major medical, social and economic concern. After over 100 years of research much is now known about the pathology of AD, however the aetiology is still unclear and there is currently no cure for this neurodegenerative disease. In order to develop new therapeutic strategies more basic research into the underlying mechanisms leading to cognitive impairment is needed.

While a large body of work has examined the effects of amyloid pathology on synaptic function in mouse models, there has been very little work examining how this pathology influences network activity that underpins memory. The research described in this thesis uses a mouse model of excess amyloid production to assess the effects of this protein on hippocampal network activity (a major target of amyloid pathology in humans) and memory. The data revealed changes in dentate gyrus activity. Subsequent experiments examined the effects of physical exercise (a manipulation that influences dentate gyrus neurogenesis) on memory function in normal and transgenic mice.

Chapter 1 of this thesis reviews the literature covering background information pertinent to the experiments carried out in this thesis. Chapter 2 describes the production and maintenance of the Tg2576 transgenic mouse model used throughout these experiments. Chapters 3-5 present experimental data obtained using the Tg2576 model. More specifically, in chapter 3 analysis of immediate early gene (IEG) expression is used to assess the contribution of different hippocampal sub-regions to processing novel spatial information in the Tg2576 model. In chapter 4, memory for episodic information is examined in Tg2576 mice using a novel behavioural paradigm, and the relative involvement of the different hippocampal sub-regions in completing this task are assessed by analysing IEG expression in C57Bl6 mice. Chapter 5 presents data assessing the effects of long-term voluntary exercise on cognition in Tg2576 mice and the molecular processes which may be altered by exercise. The final chapter of this thesis discusses how the data presented here contributes to the current knowledge on hippocampal function and AD and how it may help to progress research in this field, with the ultimate aim of treating or preventing AD.

1.2 Alzheimer's disease

AD was first described over a century ago in 1906 by a German neurologist, the eponymous Dr. Alois Alzheimer (Alzheimer, 1907, Alzheimer et al., 1995). Alzheimer's observations were based upon one of his patients, Mrs Auguste Deter, a 51-year-old woman with abnormal behavioural symptoms including loss of memory, difficulties with comprehension, disorientation and hallucinations. Alzheimer conducted post-mortem histological analysis of the brain to identify the pathological hallmarks of the disease, brain atrophy, 'thick bundles of fibrils', and 'miliary foci', now known as neurofibrillary tangles (NFTs) and amyloid plaques.

A growing problem for society

AD affects approximately 820,000 people in the UK, with 163,000 new cases reported each year ('Dementia 2010' Alzheimer's Research Trust). The proportion of the population with dementia doubles with every 5 year age group and one third of people over 95 have dementia. As well as the huge personal cost of AD, this disease costs the UK economy £23 billion each year, a cost which is rising with the growing number of patients (Alzheimer's Society 2012). World-wide AD affects about 25 million people, with 4.6 million new cases each year (Wimo et al., 2007). Of these, approximately 60% of sufferers live in developing countries (Ferri et al., 2005). These figures are likely to double within a generation as modern medicine increases human life expectancy, making it even more crucial to find ways to tackle this disease.

Clinical symptoms

The most common clinical symptom of AD is cognitive impairment (Carlesimo and Oscar-Berman, 1992, Almkvist, 1996, Weiner et al., 2005, Waldemar et al., 2007). The earliest sign of the disease is minor forgetfulness which leads to a more pronounced deficit in short-term memory and in particular a deficit in episodic memory (memory for previous personal events) which often has a retrograde progression so that earlier childhood events are more easily remembered. Later on in disease progression patients also show a loss of verbal

memory, attention deficits, deficits in problem solving and skilled movement, confusion and disorientation. Eventually patients are no longer capable of carrying out even everyday tasks and become completely dependent. This deterioration is usually accompanied by emotional changes, with many patients becoming more aggressive, depressed or apathetic and some even experiencing paranoia and hallucinations (Burns et al., 1990, Perez-Madrinan et al., 2004, Apostolova et al., 2007). Many patients are initially diagnosed with mild-cognitive impairment (MCI), which is a less severe form of cognitive impairment than that seen in AD, but then frequently deteriorate to an AD diagnosis within 8 years (Arnaiz and Almkvist, 2003).

Types of AD: Familial and Sporadic

There are two main types of AD, early-onset or familial AD (FAD), which accounts for only 5-10% of AD cases, and late-onset or sporadic AD (SAD). Although the primary cause of these two diseases is different the similarity of pathology and cognitive symptoms identifies both of them as forms of AD.

FAD is an autosomal dominant form of the disease with onset before the age of 65. Mutations responsible for FAD have been identified in genes for the amyloid precursor protein (APP) and the presenilin genes (PS1, PS2) (Chartier-Harlin et al., 1991, Goate et al., 1991, Sherrington et al., 1995, Scheuner et al., 1996, Selkoe, 1997). APP is the protein which is cleaved to form the toxic amyloid β (Aβ) molecules found in senile plaques, and PS1/2 are subunits of the enzyme that cleaves APP to form the Aβ fragment (see section 1.3)

SAD onset is later in life, usually after 65, and is not inherited, although genetic risk factors have recently come to light. Of these, apolipoprotein E (APOE) has the highest linkage to AD, which is estimated to account for approximately 20% of the risk of SAD (Chapman et al., 2001a). There are three APOE isoforms, APOE-ε2, -ε3 and -ε4. The APOE-ε4 isoform leads to a higher incidence of SAD, whilst The APOE-ε2 isoform appears to be neuroprotective (Farrer et al., 1997, Corder et al., 1998). APOE proteins have been linked to amyloid pathology and are thought to be involved in clearance of Aβ from the brain (Kim et al., 2009). More recently, large genome-wide association studies (GWAS) have identified other genetic risk factors for AD including clusterin (CLU), complement component receptor 1 (CR1), phosphotidylinositol-binding clathrin assembly protein (PICALM) and bridging

integrator 1 (BIN1) (Harold et al., 2009, Lambert et al., 2009, Seshadri et al., 2010). The role of these genes in AD pathogenesis is still unclear, although there is evidence for a role of CLU and CR1 in amyloid clearance (Wyss-Coray et al., 2002, DeMattos et al., 2004).

Non-genetic risk factors may also influence the development of SAD in humans. These environmental risks include lifestyle factors such as diet, physical exercise and education (van Praag, 2009, Foster et al., 2011, Solfrizzi et al., 2011, Meng and D'Arcy, 2012), and also the presence of other health issues such as heart disease and diabetes (de Toledo Ferraz Alves et al., 2010, Holscher, 2011). However, the most influential risk factor is age; AD affects mainly individuals over 65 years of age, and the chance of developing AD doubles every 5 years after the age of 65, increasing to 50% by 85 years (Brookmeyer et al., 1998, Wimo et al., 2003).

Diagnosis

Accuracy of clinical diagnosis in AD is poor, particularly in the early stages (Sperling et al., 2011). The cognitive symptoms of AD show much overlap with other dementias, and it is only the post mortem histological findings of the pathological hallmarks of AD (amyloid plaques and NFTs) which can definitively diagnose AD at present (McKhann et al., 1984).

A report published in 1984 outlined criteria which are commonly used for clinical diagnosis of AD (McKhann et al., 1984). This report has since been updated to include recent advances in diagnostic criteria including the use of biomarkers for AD pathology (McKhann et al., 2011). Cognitive decline is assessed using a variety of neuropsychological tests, with the most commonly used being the Mini-Mental State Exam (MMSE: Folstein et al., 1975) and the Alzheimer's Disease Assessment Scale (ADAS: Rosen et al., 1984). These tests assess problem-solving abilities, attention, visuo-motor coordination and memory. To be diagnosed with AD patients must exhibit one or more cognitive deficits alongside memory impairments.

More recently, the development of clinical biomarkers has improved diagnosis of AD. These fall into two main categories, imaging biomarkers, using positron emission tomography (PET) scanning and magnetic resonance imaging (MRI), and cerebro-spinal fluid (CSF) biomarkers for changes in tau and Aβ levels (Hampel et al., 2008). PET scanning can be used to show the expression of the radiotracer Pittsburgh Compound-B (PIB) which has high affinity for Aβ aggregates and therefore can show amyloid expression in the brain (Klunk et

al., 2004, Jack et al., 2008). MRI can be used to detect brain atrophy as a measure of neurodegenerative pathology. In particular, atrophy of the hippocampus and surrounding medial temporal lobe (MTL) structures predicts cognitive decline and AD pathology (Gosche et al., 2002, Jack et al., 2002, Silbert et al., 2003, Whitwell et al., 2008). A further development in biomarkers is the detection of changes in CSF composition in patients with AD, which reflects biochemical changes in the brain (Fishman, 1992). AD patients show significantly decreased levels of $A\beta_{1-42}$ and increased levels of tau in their CSF compared to healthy controls (Sunderland et al., 2003). The use of these biomarkers alongside cognitive diagnostic tests has increased the accuracy of AD diagnosis, by improving differentiation between AD, normal aging, and other dementias, to greater than 85% (Andreasen et al., 2001, Blennow and Hampel, 2003).

Despite these advances, there is still a need for more accurate diagnosis of AD and development of biomarkers for earlier stages of disease pathology (especially pre-symptomatic markers), as many therapeutic strategies are aimed at preventing disease onset or slowing decline by acting during the preclinical phase.

1.3 Pathology of AD

There are three main pathological hallmarks of AD: neuronal loss, amyloid plaques and NFTs.

Neuronal loss

AD leads to neuronal damage and cell death, particularly targeting cholinergic neurons in the brain, which correlates with cognitive decline in AD (Davies and Maloney, 1976, Wilcock et al., 1982). Neuronal loss is the end-stage of the disease pathology, with several upstream events leading to this outcome including synaptic dysfunction, synaptic loss, oxidative stress and mitochondrial dysfunction occurring before cell death (Carter and Lippa, 2001, Marcello et al., 2008, Verri et al., 2012). The extent of neuronal loss can be clearly seen in AD patients using MRI to assess grey matter volume. Atrophy has been reported in MTL structures, and in particular in the hippocampus (Thompson et al., 2003, Whitwell et al.,

2007). However, atrophy appears once cognitive deficits are already apparent, suggesting that, whilst cell death may be the end-stage of pathology, it is not the main cause of cognitive deficits. The other two pathological hallmarks, amyloid plaques and NFTs, are thought to play a key role in causing cognitive decline in AD.

Amyloid pathology

The core component of senile plaques, as first described by Alzheimer in 1906, has since been identified as the Aβ peptide (Glenner and Wong, 1984, Masters et al., 1985). Aβ is a small protein (~4kDa) generated from proteolysis of the amyloid precursor protein (APP) (Glenner and Wong, 1984). The normal physiological function of Aβ is still unclear, although there is evidence that it may be involved in neuronal plasticity, synaptic function and repair (Turner et al., 2003, Priller et al., 2006). Several studies have shown that at high doses Aβ is a toxic molecule, leading to synaptic dysfunction in cultured neurons, and *in vivo* (Selkoe, 2008a). Aβ peptides aggregate in the brain to form neurotoxic oligomers and protofibrils leading eventually to formation of plaques The progression of amyloid pathology through the brain has been described in detail by Braak and Braak (1991). Amyloid pathology first affects neocortical areas, in particular the frontal, temporal and occipital lobes. Pathology then spreads to all cortical association areas and the hippocampus. The final stage of AD pathology includes amyloid plaques in primary cortical regions such as the primary motor and auditory cortices.

APP Processing

Aβ is generated from APP, a transmembrane protein which undergoes a series of enzymatic cleavage reactions leading to the production of different length protein fragments. APP cleavage can occur by two different pathways, the non-amyloidogenic pathway is involved in normal cell function, whereas the amyloidogenic pathway leads to the production of toxic Aβ molecules (for a review see Thinakaran and Koo, 2008). These two pathways are shown in **Figure 1.1**.

Aβ is produced by cleavage of APP by the β-secretase enzyme (BACE1) in the extracellular domain, and then subsequent cleavage of the inter-membrane region by γ-secretase. The γ-secretase enzyme is made up of different subunits, two of which are encoded by the PS1 and

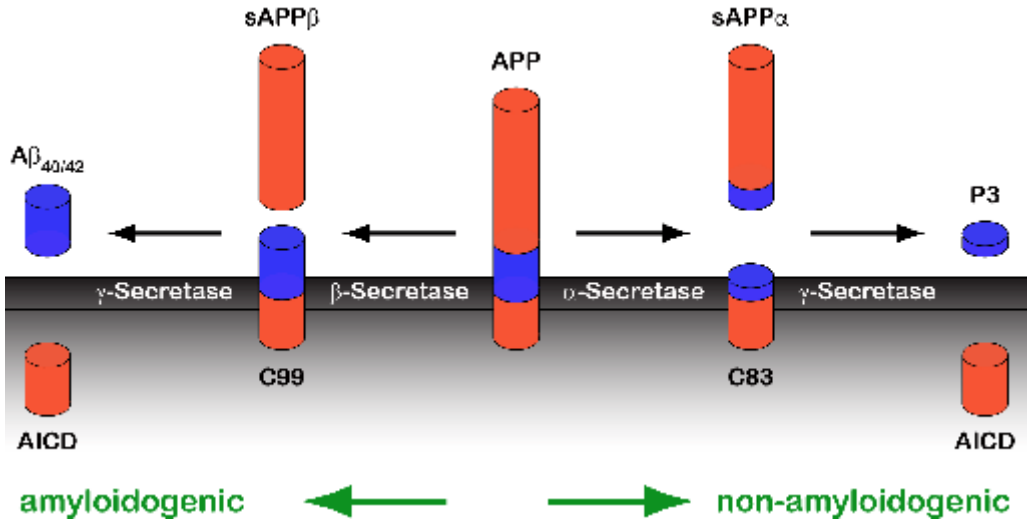

Figure 1.1: APP processing. APP can be processed via the amyloidogenic pathway through cleavage by β- and γ-secretase enzymes to produce toxic Aβ fragments. Alternatively APP can be processed via the non-amyloidogenic pathway through cleavage by α-secretase.

PS2 genes. γ-secretase produces Aβ fragments of 37-43 residues. In normal subjects the most common fragment is $A\beta_{40}$, however, in AD fragment lengths of the more toxic $A\beta_{42}$ are up-regulated (Wang et al., 1999, Naslund et al., 2000). Mutations linked to FAD increase the relative amount of $A\beta_{42}$ either by altering the β- or γ- cleavage sites on the APP gene, or by altering the PS1/2 subunits of the γ-secretase enzyme to increase $A\beta_{42}$ production (Chapman et al., 2001b).

An alternative route for APP cleavage is by the non-amyloidogenic pathway. The α-secretase enzyme (BACE2) cleaves APP in the middle of the Aβ fragment, precluding the production of toxic Aβ species. However, the relatively high levels of BACE1 in neuronal cells channels APP processing through the amyloidogenic pathway in the brain.

Tau pathology

Tau is a micro-tubule associated protein which is found widely within the brain and is involved in micro-tubule stability (Grundke-Iqbal et al., 1986, Iqbal et al., 1986). The phosphorylation state of tau protein regulates it's interaction with microtubules, and in a normal brain this state is in dynamic equilibrium. In AD the balance is shifted to produce hyperphosphorylated tau proteins which aggregate to form intraneuronal neurofibrillary tangles (NFTs). Aggregation into NFTs reduces binding to microtubules, leading to

cytoskeletal compromise and eventually cell death (Ballatore et al., 2007). Tau pathology begins in the trans-entorhinal cortical regions before progressing into the hippocampal formation, with the CA1 sub-region being affected the earliest. Eventually tau pathology spreads to the neocortex, beginning with association areas, but eventually also spreading to primary sensory and motor regions (Braak and Braak, 1991). Although mutations in tau have been linked to dementias including fronto-temporal dementia and Pick's disease (Poorkaj et al., 1998, Spillantini et al., 1998), none have been directly linked to AD. Despite this, the expression of NFTs is highly correlated with cognitive decline and neurodegeneration in AD (Arriagada et al., 1992, Thal et al., 2000).

Amyloid cascade hypothesis

The interplay between amyloid and tau pathology in AD, and how this ultimately leads to neuronal loss, is still not clearly understood. However, the most dominant theory of the past 20 years as to the cause and progression of AD is the amyloid cascade hypothesis.

Following the discovery of Aβ as the main component of senile plaques and the linkage of FAD to mutations in APP and PS1/2 genes, the amyloid cascade hypothesis was developed by Hardy and Higgins (1992). This hypothesis proposed that Aβ is the causative agent in AD pathology, which leads to the development of NFTs, cell loss, vascular damage and dementia as a direct result of Aβ deposition. This hypothesis is further supported by findings that Down syndrome patients, who carry a trisomy in chromosome 21, the site of the APP gene, develop amyloid plaques at an early-age (Masters et al., 1985, Robakis et al., 1987, Folin et al., 2003). Aβ is thought to lead to NFT accumulation, and not *vice versa*. Several lines of evidence support this conclusion; mutations in the tau gene leads to autosomal dominant fronto-temporal dementia without the appearance of amyloid plaques (Hutton et al., 1998); infusion of Aβ into tau mutant transgenic mice exacerbates NFT pathology in these mice (Lewis et al., 2000, Gotz et al., 2001); and in the 3xTg transgenic model harbouring mutations in both APP and tau genes amyloid pathology precedes that of NFTs (Oddo et al., 2003). Aβ may mediate tau toxicity in AD by activation of calpains which in turn activates kinases and caspases leading to hyperphosphorylation and cleavage of the tau protein into more toxic fragment (for a review see Wray and Noble, 2009).

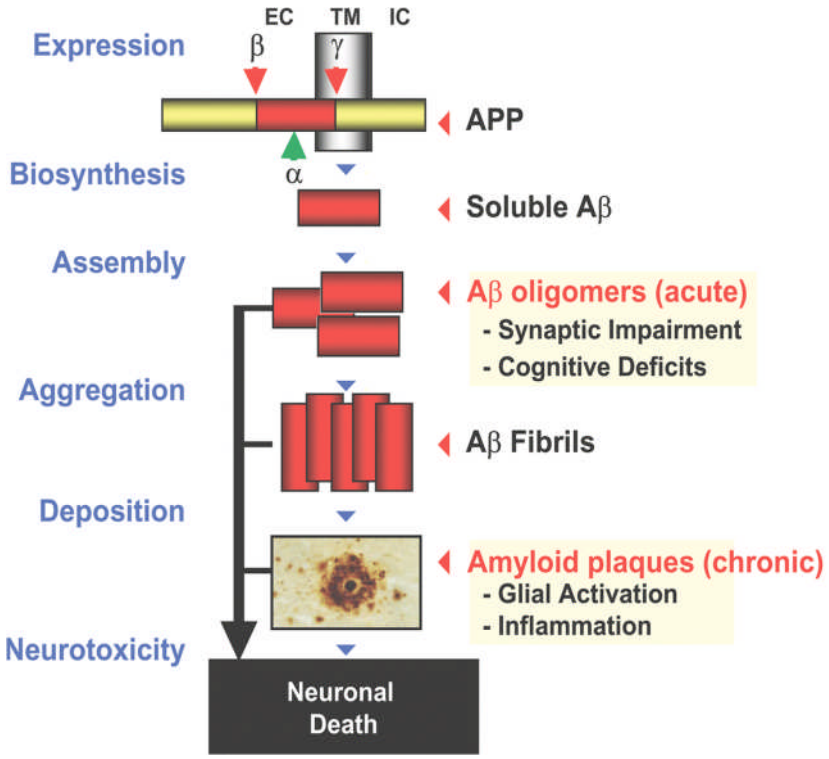

Figure 1.2: Amyloid cascade hypothesis. APP is cleaved to form Aβ molecules. These molecules aggregate to form soluble oligomers which can impair synaptic function and lead to early cognitive deficits in AD. Further aggregation of oligomers forms Aβ fibrils and eventually amyloid plaques. Plaques lead to glial activation and inflammatory responses in the brain. Together these processes lead to neurotoxicity and eventually cell death. *Figure reproduced from Biochemical Society Transactions.*

However, over the past two decades since the proposal of this hypothesis several experimental findings have caused controversy and led to revisions of the hypothesis. One of the key controversies is that amyloid plaque load shows a weak correlation with measures of cognitive dysfunction in AD patients (Terry et al., 1991). A better correlate with cognition, which also correlates poorly with amyloid load, is the loss of synaptic terminals (Terry et al., 1991). In a series of experiments, Selkoe and colleagues showed that soluble Aβ oligomers are themselves neurotoxic and cause synaptic dysfunction and loss of dendritic spines (the site of synaptic terminals) leading to cognitive decline (Walsh et al., 2002, Cleary et al., 2005, Shankar et al., 2007, Selkoe, 2008b). The level of soluble Aβ also shows a high correlation with synapse loss and cognitive symptoms in AD (Lue et al., 1999, McLean et al., 1999). These findings led to a new hypothesis, based on the original amyloid cascade, that it is not the amyloid plaques *per se* that cause the cognitive decline seen in AD, but rather the intermediate oligomers of Aβ that cause synaptic dysfunction leading to progressive

cognitive deterioration and eventually cell death (Klein et al., 2001, Selkoe, 2008a). An outline of this hypothesis is shown in **Figure 1.2**.

A recent review focusing on the newly identified risk factor genes for AD suggested a further amendment to the hypothesis to account for the difference in age of onset of FAD and SAD (Lambert and Amouyel, 2011). This review suggested that familial-linked mutations in APP and PS1/2 genes lead to increased production of Aβ, which occurs at an early stage in life. Risk factor genes for SAD such as APOE, CLU and CR1 lead to reduced clearance of Aβ and gradual accumulation of this protein in the brain, which does not reach toxic levels until a later time point. Both of these primary causes eventually lead to the pathological cascade highlighted by the more traditional amyloid hypothesis, with increased levels of Aβ in the brain leading to synaptic dysfunction, synaptic loss and eventually cell death.

Although there is still controversy as to whether all the assumptions made by this hypothesis are correct, given the wealth of evidence in favour of this theory the rest of this thesis focuses on amyloid pathology and Aβ accumulation as a primary step in AD pathogenesis.

1.4 Modelling AD in Transgenic Mice

Progress in the field of genetics over the past two decades has lead to the development of transgenic technology to produce mice with mutations: knock-in, or knock-out of specific genes of interest (Capecchi, 1989). These developments have opened up the field of AD research by providing animal models of familial forms of AD in which to investigate cognitive dysfunction and pathogenesis of the disease, and to test the efficacy of potential therapeutic agents.

As described in section 1.3, there are three core features of AD pathology; amyloid plaques, NFTs and cell loss. The amyloid cascade hypothesis suggests that the build up of amyloid in the brain is one of the primary stages in AD pathology, upstream of changes in tau proteins. Therefore the main focus here is on APP transgenic models, which model amyloid pathology, although a brief description of some models including both APP and tau mutations are included. Single tau mutant mice are used to model pathology in fronto-temporal dementia

(Ishihara et al., 1999, Lewis et al., 2000), but are a poor model for cognition as they often show severe motor impairment. In an effort to produce a model which successfully demonstrates all aspects of amyloid pathology, transgenic lines have been developed that express either single mutations in the APP gene associated with FAD, or multiple mutations including APP, PS1 and tau. A brief overview of the most commonly used models is described in **Table 1.1**, and a more detailed review of transgenic models of AD can be found in Spires and Hyman (2005).

Single mutations

The first transgenic mouse models for AD over-expressed the human APP (hAPP) gene in the CNS. This led to higher levels of APP in the brain, but did not cause plaque deposition. Following this a variety of transgenic lines were developed which combined hAPP over-expression with mutations identified in FAD patients. These models include the Swedish, APP_{695}K670N,M671L (Hsiao et al., 1996), Indiana, V717F (Games et al., 1995) and London, V642I (Moechars et al., 1999) mutations. These models show increased Aβ deposition, mimicking the plaques seen in human AD pathology. However, these models do not show NFTs or overt cell loss in the MTL. All of these models show memory impairment with onset of deficits occurring at different ages, that often precede plaque deposition. These findings added considerable support to the hypothesis that it is the accumulation of soluble Aβ in the brain that leads to synaptic dysfunction and early changes in memory function.

Multiple mutations

Mice expressing mutated hAPP combined with PS1 mutations demonstrate exacerbated amyloid pathology compared to single APP mutants and increased Aβ42/40 ratio, with earlier onset of both pathology and cognitive symptoms (Citron et al., 1997, Holcomb et al., 1998). These models allow assessment of molecular changes over a shorter time course, but have a limited window for assessing cognitive decline or the effects of preclinical treatment strategies. More recently a model has been developed that harbours three mutations (Oddo et al., 2003). The 3xTg model over-expresses mutant hAPP, PS1 and the Tau mutation P301L, and is currently the most successful model at mimicking the pathologies seen in human AD. These mice show early amyloid pathology followed later by the development of NFTs.

Table 1.1: Overview of commonly used transgenic mouse models of AD. References in bold are the original paper describing the model. 4m= 4 months of age.

Model	Pathology	Behavioural Impairments	Synaptic Changes	References
Tg2576 APP Swedish, 695.K670N-M671L, Hamster PrP promoter	4m: increased soluble Aβ 9-11m: Aβ plaques, gliosis, increased oxidative stress, dystrophic neuritis	4m: contextual fear conditioning 9-11m: object recognition, T-maze alternation, water maze	4m: reduction in spine density 5m: impaired basal transmission and LTP	**Hsiao et. al. (1996)**, Chapman et. al. (1999), Fitzjohn et. al. (2001), Kawarabayashi et.al. (2001), Westerman et. al. (2002), Hale and Good (2005), Jacobsen et. al. (2006)
PDAPP APP mini gene, Indiana V717F, PDGFβ promoter	6-8m: Aβ plaques, dystrophic neurites, gliosis, hippocampal atrophy	12m: object recognition, radial arm maze, water maze	5m: impaired basal transmission, LTP and PPF	**Games et. al. (1995)**, Hsiao et. al. (1999), Dodart et. al. (1999), Larson et. al. (1999), Chen et. al. (2000), Giacchino et. al. (2000)
APP23 APP Swedish, 695.K670N-M671L, Murine Thy-1 promoter	6m: Aβ plaques, neuronal loss in CA1, hyperphosphorylated Tau	3m: water maze 12m: Barnes maze 18m: passive avoidance	12m: impaired basal transmission	**Sturchler-Pierrat et. al. (1997)**, Lalonde et. al. (2002), Kelly et. al. (2003), Roder et. al. (2003), Prut et. al. (2007)
TgCRND8 APP Swedish, 695.K670N-M671L, and Indiana V717F, Syrian hamster PrP promoter	3m: Aβ plaques, increased Aβ42/40 ratio	2-4m: water maze, Barnes maze, object recognition	3m: increased LTP from	**Chishti et. al. (2001)**, Jolas et. al. (2002), Walker et. al. (2011), Francis et. al. (2012)
J20 APP Swedish, 770.K670N-M671L and Indiana V717F, PDGFβ promoter	5–7m: diffuse Aβ deposits 8-10m: Aβ plaques and gliosis	6m: water maze, object recognition	6m: reduction in hippocampal arc, calbindin and c-fos	**Mucke et. al. (2000)**, Palop et. al. (2003), Palop et. al. (2005), Escribano et. al (2009)
APP/PS1 APP Swedish, 695.K670N-M671L, PS1, M146L, PDGFβ, PrP promoter	3m: diffuse amyloid deposits 6-9m: abundant Aβ plaques	4-5m: water maze radial arm watermaze, Y-maze alternation	4m: impaired LTP 6m: impaired basal transmission 12m: decrease in dendritic spines	**Citron et. al. (1997)**, Holcomb et. al. (1998), Westerman et. al. (2002), Trinchese et. al. (2004), Knafo et. al. (2009)
3xTg APP Swedish, 695.K670N-M671L, PS1 M146V, Tau P301L, Thy1.2 promoter	3m: intraneuronal Aβ 6m: extracellular Aβ 12m: tau deposits	4m: water maze, contextual fear conditioning	6m: impaired basal transmission and LTP	**Oddo et. al. (2003)**, Billings et. al. (2005)
5xFAD APP Swedish 695.K670N-M671L, Florida I716V, London V717I, PS1 M146L-L286V, Thy-1 promoter	2m: Aβ accumulation and gliosis 9m: cell loss in cortex layer V and subiculum	4-5m: Y-maze alternation 6m: cross maze, reduced anxiety, water maze, contextual fear conditioning	4m: reduced synaptophysin 6m: impaired basal transmission and LTP 9m: reduced PSD-95 and syntaxin	**Oakley et. al. (2006)**, Ohno et. al. (2006), Kimura and Ohno (2009), Jawhar et. al. (2012)

A further model has been produced combining five different APP and PS1 mutations (Oakley et al., 2006). The 5xFAD model shows aggressive amyloid pathology and very early cognitive decline. The inclusion of multiple genetic mutations into one model allows the expression of pathology similar to that seen in human AD and also allows analysis of the interaction of amyloid and tau. However, critics suggest that these models do not reliably mimic human AD, as these combinations of mutations are not found in human patients.

Limitations of transgenic models of AD

The development of transgenic models has led to huge advances in AD research, particularly in establishing the molecular mechanisms underlying disease pathology. However, thus far, few treatment strategies which have been successful in reversing pathology or delaying disease onset in mouse models have made it into clinical use (see section 1.6). This lack of translation from basic science using animal models into human treatments highlights the inadequacy of current models of AD.

Although transgenic models do mimic several aspects of the pathology seen in human AD, so far no model has successfully demonstrated all three key features, amyloid plaques, NFTS and cell loss. Some models do show both amyloid pathology and changes in tau phosphorylation, but only the 3xTg model (which includes the P301L tau mutation) forms NFTs. Very few models show overt cell loss, with more cell loss reported in models combining several mutations (Oddo et al., 2003, Oakley et al., 2006). This may be due to difference between the susceptibility of mouse and human neurons to excitotoxic and oxidative damage. This means that whilst transgenic models do provide a useful tool for looking at the pathological changes that may be happening in AD, they are widely seen as only modelling preclinical or pre-symptomatic AD. It is thought that by the time AD is diagnosed in patients there is wide-spread cell loss throughout the brain. However, these models should not be disregarded as they provide a valid tool for probing the early molecular changes that lead to synaptic dysfunction and loss, which in turn underlie early cognitive changes preceding overt cell loss.

Comparison of findings between different transgenic mouse models developed in different laboratories is often difficult due to the confounding factor of background strain. This has limited the conclusions that can be drawn when comparing different mutations, as strain

differences, rather than mutation-derived differences, may be influencing the expressed phenotype (Linder, 2006). It is therefore important to analyse the effects of any given genetic mutation in multiple background strains to determine the precise influence of the transgene.

Another limitation of transgenic mouse models of AD is that the topography of amyloid pathology and type of plaque seen in these models does not match that of human AD patients. The progression of pathology seen in humans is from the neocortex, to the hippocampus, then to the basal ganglia, brainstem and cerebellum (Braak and Braak, 1991, Thal et al., 2002). In transgenic mice the localisation of Aβ deposits is mainly dependent upon the promoter being used, and is often highest in the hippocampus. This may lead to differences in the expression and progression of cognitive deficits between mouse models and humans as different brain areas are affected sequentially by AD pathology. Plaques found in APP transgenic mice are also qualitatively different to those seen in humans. In mice the Aβ peptide is SDS-soluble, whereas in humans very little amyloid is soluble in SDS (Kuo et al., 2001, Kalback et al., 2002). This is likely to be due to the vast number of post-translational modifications of the amyloid peptides seen in humans, which do not occur in mice (Kuo et al., 2001). Therefore treatments that successfully target plaque pathology in mouse models may not translate effectively to human patients.

Tg2576 model: the Swedish double APP mutation

One of the main aims of this thesis is to understand how amyloid pathology influences the activity and interaction of neural networks that contribute to memory processes. As noted above, subtle changes in memory function with age are often overlooked until they become serious enough to influence daily living, by which time overt cell loss is often detected. Although mouse models have their limitations they do provide an opportunity to examine neural network activity prior to extensive plaque formation and cell loss. The experiments reported in this thesis used the Tg2576 model to assess the effects of amyloid pathology on cognition and hippocampal activity. The following sections describe the main pathological and cognitive changes of Tg2576 mice.

The Tg2576 model was chosen as it is one of the most suitable models for the experiments carried out in this thesis. A single APP mutant model was chosen as the amyloid cascade hypothesis presents strong evidence in favour of Aβ being the causative agent in AD (see

section 1.3). Therefore, although this model does not develop NFTs or cell loss, it does show amyloid pathology which is arguably the most critical of the three pathologies in early AD. In addition, a single mutant model was chosen as these combinations of mutations, such as APP, PS1 and tau, have not been found in human AD patients, whereas the Swedish mutation used in the Tg2576 model is taken directly from a human FAD mutation (Mullan et al., 1992). Another attractive feature of the Tg2576 model is that it has been widely used in AD research and is one of the most well characterised APP mutant models, both in terms of the pathology and the development of cognitive deficits, described in more detail in the following sections of this review. The Tg2576 model is suited to the type of experiments described in this thesis is that it shows relatively late onset and slow progression of amyloid pathology (Kawarabayashi et al., 2001) compared to other models, allowing cognitive changes to be analysed over time and pre-plaque deficits to be assessed. This feature of the model is utilized in both chapters 3 and 4, for comparison of behaviour and hippocampal activity before and after the onset of amyloid pathology. In addition the relatively slow progression of pathology allows analysis of cognitive deficits over 10 months in the longitudinal exercise study presented in chapter 5.

The Tg2576 model was developed in 1996 by Hsiao and colleagues (Hsiao et al., 1996). This transgenic line over-expresses the human APP695 gene containing the double mutation K670N/M671L, first described in a Swedish family with FAD (Mullan et al., 1992). Transgene expression is driven by a hamster prion protein (PrP) gene promoter on a hybrid C57Bl6/SJL background (Hsiao et al., 1996). This mutation lies over the β-secretase cleavage site and leads to an increase of APP cleavage to produce toxic Aβ peptides (Mullan et al., 1992).

As described previously background strain can alter the phenotype expression in transgenic models. The Tg2576 model is maintained on an F1 hybrid background of C57Bl6 and SJL by repeated backcrossing into this strain (Hsiao et al., 1996). A study by Lassalle et. al. (2008) assessed the behavioural phenotype of mice harbouring the APP Swedish mutation on different background strains (C57Bl6, CBA and C57Bl6/SJL) and confirmed that the F1 hybrid strain gave the most consistent results.

Pathology expressed by Tg2576 mice

Tg2576 mice exhibit many features of amyloid-related neuropathology similar to human AD patients. Tg2576 mice have increased levels of soluble Aβ from 4 months of age and plaque deposition starting at 9 months, which become abundant by 12 months and progressively increase with age (Kawarabayashi et al., 2001). Between 2 and 8 months of age Tg2576 mice show a five-fold increase in $A\beta_{40}$ level and a fourteen-fold increase in $A\beta_{42}$ level, leading to an increased Aβ40:42 ratio (Hsiao et al., 1996). Plaques are seen first in the entorhinal cortex, spreading to the hippocampus, subiculum and then cortex (Hsaio 1996, McGowan 1999). Tg2576 mice also develop microgliosis from 10 months of age, astrogliosis from 18 months and dystrophic neurites from 15 months (Irizarry et al., 1997, Frautschy et al., 1998, Jacobsen et al., 2006). Plaque associated microgliosis provides evidence for inflammation as a result of increased Aβ, similar to that seen in human AD. Consistent with this, increases in inflammatory markers including TNF-α, COX-2 and IL-1β have been reported in Tg2576 mice (Lim et al., 2000, Sastre et al., 2006). Although this model does not develop overt tau pathology or NFTs, increased hyper-phosphorylation of tau has been reported in neurites surrounding amyloid plaques (Puig et al., 2004). The Tg2576 model does not exhibit any cell loss, the third key pathology of AD.

Cognitive deficits in Tg2576 mice

Tg2576 mice show a progressive decline in memory and other cognitive functions with age. Due to the nature of memory impairments in human AD and the nature of the pathology in both human and transgenic models, most behavioural characterisation has focussed on tasks that are sensitive to hippocampal cell loss in rodents – spatial memory and associative learning (see section 1.5).

Deficits on tasks designed to assess spatial memory have been reported from 6-10 months of age, coinciding with the build-up of soluble amyloid and the first appearance of amyloid plaques (Hsiao et al., 1996). Spatial working memory deficits have been reported from 9 months of age using forced choice T-maze alternation paradigms, with impairment increasing with further aging (Hsiao et al., 1996, Chapman et al., 1999, Corcoran et al., 2002, Barnes et al., 2004, Cacucci et al., 2008). Further investigation of the nature of these deficits revealed that, similar to human AD patients (Hort et al., 2007), they are due to reduced allocentric

representation of the environment, and not due to impaired processing of intra- or extra-maze cues (Barnes et al., 2004). Even earlier deficits in spatial working memory have been reported, before the onset of plaque pathology, at just 3-4 months of age using the Y-maze to assess spontaneous alternation (Holcomb et al., 1998, King and Arendash, 2002a).
Spatial reference memory impairments have also been reported in Tg2576 mice appearing between 6-10 months of age using hidden platform water maze paradigms (Hsiao et al., 1996, Westerman et al., 2002). Westerman et. al. (2002) reported a progressive decline in reference memory with age, demonstrating no deficit in learning at 4-5 months of age, slower acquisition by 10 months of age, and no learning at all by 24 months of age. Cognitive decline on water maze tasks is correlated with increased soluble Aβ protofibrils, but not plaque deposition (Hsiao et al., 1996, Westerman et al., 2002), supporting the hypothesis that soluble amyloid fragments cause early cognitive decline. However, a study by King & Arendash (2002a) presented conflicting findings to those reported above, showing no impairment in transgenic mice compared to controls on a hidden platform water maze task, even at 19 months of age. The different findings of these studies may be accounted for by differences in the criteria for inclusion of data into analyses, as King & Arendash used more stringent criteria to exclude mice they classed as 'non-performers'.

Tests of associative learning using fear-conditioning paradigms revealed interesting results in Tg2576 mice. Whilst Tg2576 mice show no deficit in cued fear-conditioning, which is not thought to depend upon the hippocampus, early deficits in hippocampus-dependent contextual fear-conditioning have been reported from 4 months of age, with a progressive decline with further aging (Dineley et al., 2002, Comery et al., 2005, Jacobsen et al., 2006). The finding of early behavioural impairments, which correlate with synaptic deficits (Jacobsen et al., 2006: see next section), support the hypothesis that early increases in soluble Aβ levels, which precede substantial plaque deposition, are a primary cause of cognitive decline in APP transgenic mice and AD patients (Selkoe, 2008a).

A series of papers by Good and Hale assessed aspects of both spatial and temporal memory in the Tg2576 model using object-in-place and object-recency paradigms. They demonstrated that at 14 months of age Tg2576 mice showed no deficits in detecting object novelty or the temporal order of object presentation, but were impaired in detecting changes in the relative spatial position of objects (Hale and Good, 2005). They went on to show that Tg2576 mice are impaired in an 'episodic-like' memory tasks in which normal animals remember both the location and temporal sequence of object presentation (Good et al., 2007b). A final study

further interrogated the nature of object-location processing deficits in Tg2576 mice, showing that whilst transgenic mice cannot form a memory for specific associations between which object is in which location within the arena, they can discriminate when an object is placed in an entirely new location (Good and Hale, 2007).

In addition to assessing hippocampus-dependent memory systems, many studies have also carried out tests of sensori-motor function to establish that deficits seen in memory tasks do actually reflect impaired memory, and not impairments in either sensory or motor performance as a result of either APP over-expression or Aβ accumulation (Hsiao et al., 1996, Holcomb et al., 1998, Chapman et al., 1999, Lalonde et al., 2002). It is widely reported that Tg2576 mice are hyperactive compared to controls from an early age, but generally no other deficits have been reported on tests of balance, reflexes, grip strength, agility and vision (Hsiao et al., 1996, Holcomb et al., 1998, Chapman et al., 1999, Lalonde et al., 2002). One study has reported conflicting findings of impaired sensorimotor performance in Tg2576 mice compared to wild-types, appearing at 14 months of age (King and Arendash, 2002a). However, as memory impairments are seen at the earlier time point of approximately 10 months of age, they cannot be attributed to sensori-motor impairment.

Overall these studies show, with only a few exceptions, the onset of deficits in Tg2576 mice in processing spatial and contextual cues from 3 months of age in certain tasks, as levels of soluble Aβ increase. Robust deficits in processing of spatial information are clearly evident by 9-10 months of age in Tg2576 mice, the point at which amyloid plaques are starting to develop. There is little evidence for deficits in processing of temporal information or for sensorimotor deficits in processing cue information.

Synaptic changes in Tg2576 mice

As previously described (section 1.3) synaptic changes are a prominent phenotype of AD in both humans and transgenic models, and correlate well with cognitive decline (Terry et al., 1991). Changes in synaptic morphology and plasticity have also been reported for the Tg2576 model.

Electrophysiological recordings have been made from the hippocampus of Tg2576 mice. Although every study assessing synaptic transmission and plasticity in the Tg2576 model have reported deficits compared to wild-type controls, the nature of the deficits vary widely

between studies. This may be dependent upon the protocol used for the slice or *in vivo* preparation and the type of stimulation used to induce long term potentiation (LTP) or long-term depression (LTD). LTP and LTD are the molecular mechanisms of synaptic plasticity thought to underlie learning and memory (Bliss and Lomo, 1973, Morris, 1989). These processes lead to long-term alterations in the strength of connections between neurons following repeated stimulation. LTP is dependent on activation of N-Methyl-D-aspartate (NMDA) glutamate receptors (Morris, 1989), which allow calcium into the neuron leading to downstream changes in the molecular composition of the synapse.

The earliest electrophysiology study in Tg2576 mice, by Chapman et. al. (1999), reported normal baseline synaptic transmission and pulse-paired facilitation (PPF: a measure of pre-synaptic function), but impaired LTP in both the CA1 and DG subfields of the hippocampus in old (15-17 months of age), but not young (2-8 months of age) Tg2576 mice. Furthermore, the synaptic deficits strongly correlated with performance on a forced-choice alternation T-maze task. In contrast to this, Fitzjohn et. al. (2001) reported impairments in baseline transmission in the CA1 region at 12 months of age in Tg2576 mice, but no changes in PPF or LTP up to 18 months of age in CA1. In addition, they did not find impairments on any measures in the DG up to 18 months. Another study by Jacobsen et. al. (2006) assessed synaptic transmission at an earlier time point, and demonstrated deficits in both basal transmission and LTP at only 4 months of age in Tg2576 mice, which coincided with a reduction in spine density in the DG and deficits in a contextual fear-conditioning paradigm. More recently, transmission in the mossy fibre pathway into the CA3 region has been assessed, and LTP deficits reported in 24 month old mice, but not in baseline transmission (Witton et al., 2010). Single cell recording studies have also shown degraded place cell firing in old (16 months old), but not young (3 months old) Tg2576 mice compared to controls, which correlated with performance on a T-maze alternation procedure (Cacucci et al., 2008).

In addition to electrophysiological data, alterations in synaptic protein expression have also been reported in Tg2576 mice, which may underlie the changes seen in transmission and plasticity. A reduction in the number and alteration in the morphology of both pre- and post-synaptic compartments in cultured neurons from Tg2576 mice was reported by Almeida et. al. (2005). In addition, levels of the post-synaptic markers PSD-95 and GluR1 were reduced in these neurons (Almeida et al., 2005). Assessment of the expression of the pre-synaptic marker synaptophysin has revealed mixed results, with one study reporting no change in expression (Jacobsen et al., 2006), another reporting increased synaptophysin expression

compared to wild-type controls at older ages (King and Arendash, 2002b), and a further study demonstrating reduced synaptophysin reactivity in neurons close to plaques in the hippocampus, but not in neurons located further away from plaque deposits (Dong et al., 2007).

Despite mixed results as to the precise nature of synaptic deficits in Tg2576 mice, it can be concluded from these studies that Tg2576 do show impairments in synaptic morphology, plasticity and transmission, which may underlie deficits in cognition.

1.5 Hippocampus and Memory

The hippocampus is a key target of pathology in both human AD patients and in transgenic models of the disease. The next section describes the anatomy and function of the hippocampus, and theories of how the different anatomical sub-regions contribute to memory. This review was undertaken in order to provide a conceptual background for the behavioural work undertaken in this thesis.

Hippocampus Anatomy

The hippocampus is part of the limbic system, and is found in the MTL. The hippocampus is highly connected with the parahippocampal cortical regions, which in turn receive input from the other neocortical regions (for reviews see Amaral and Witter, 1989, Witter et al., 2000, van Strien et al., 2009).

The hippocampus is made up of different sub-regions, of which the DG, CA1 and CA3 are the largest and also the best characterised. The hippocampus has a laminar structure with each sub-region made up of several layers. The deepest layer, made up of afferent and efferent fibres and inter-neurons is called the hilus within the DG, and the stratum oriens in the CA regions. Superficial to this is the principle cell layer, known as the granule layer in the DG and pyramidal layer in the CA regions and subiculum. The most superficial layer, containing apical dendrites, is the molecular layer in the DG and subiculum. In CA3 this layer is further divided into three, the stratum lucidum, stratum radiatum and stratum lacunosum-moleculare, and into two in CA1 which does not have the stratum lucidum. The

parahippocampal region lies next to the hippocampus and provides the main input and output pathways from this structure. This region is made up of the lateral and medial entorhinal cortex, perirhinal cortex and postrhinal cortex. In addition, the fornix is a major white matter tract which connects the hippocampus and parahippocampal structures to subcortical regions, such as hypothalamus and mammillary bodies, and to forebrain structures (Saunders and Aggleton, 2007).

There has been a wealth of studies describing hippocampal circuitry. A recent review by vanStrien et. al. (2009) described in detail all of the connections between hippocampal and parahippocampal regions in the rat. This system is well defined for the rat, and whilst much of the circuitry is the same for humans, it is likely that the system may be more complex again in humans. Hippocampal and parahippocampal circuitry is less well studied in mice, for example the postrhinal cortex has only recently been identified (Beaudin et al., 2012). However, for the purpose of this thesis only the simplified standard circuitry model is described, which has been verified in mice, rats and humans.

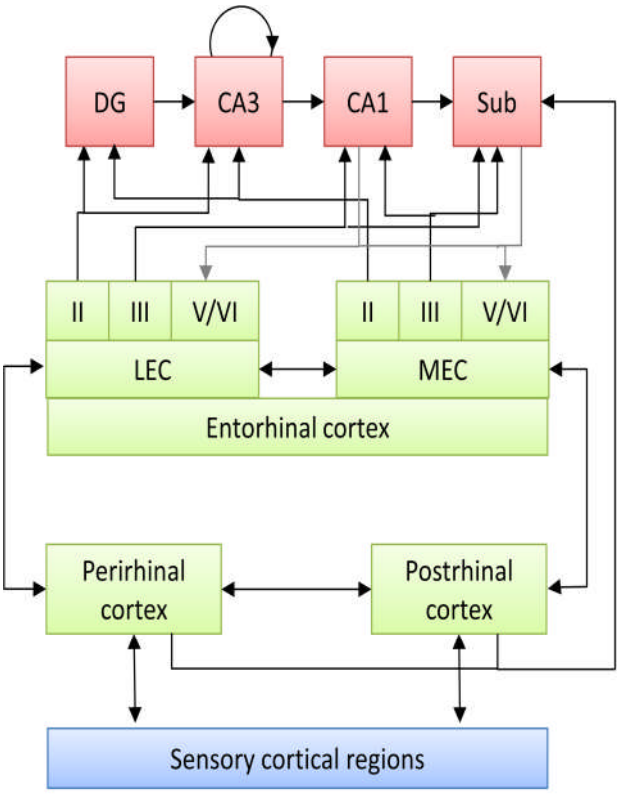

Figure 1.3: Hippocampus circuitry. Blue box indicates neocortical region, green boxes indicate parahippocampal regions and red boxes indicate regions in the hippocampus formation. DG: dentate gyrus, Sub: subiculum, MEC: medial entorhinal cortex, LEC: lateral entorhinal cortex. Adapted from van Strien et. al. (2009).

In the standard hippocampus model, shown in **Figure 1.3**, neocortical input arrives at the parahippocampal region in two parallel projection streams. Input via the perirhinal cortex to the lateral entorhinal cortex contains non-spatial information, whilst input via the postrhinal cortex to the medial entorhinal cortex contains spatial information (Hargreaves et al., 2005). Both of these connections are reciprocal. The entorhinal cortex projects to the hippocampus via the perforant pathway. This pathway projects to all sub-regions of the hippocampus, with projections from entorhinal cortex layer II projecting to the DG and CA3, and projections arising from layer III going to CA1 and the subiculum. A tri-synaptic pathway connects the hippocampal sub-regions in a unidirectional pathway, with mossy fibres originating in the DG projecting to CA3. From CA3 the Schaffer collaterals project onto CA1, and finally CA1 neurons project onto neurons in the subiculum. Output from the hippocampus is primarily from the CA1 and subiculum regions, which project back to layers IV/V of the entorhinal cortex. The other well characterised projections in the standard hippocampal circuitry are the recurrent collaterals in the CA3 sub-region, which project from CA3 pyramidal cells back onto the same parent cell.

Several neurotransmitter systems influence hippocampal information processing by regulating the level of activity within each sub-region. These include glutamate, GABA, acetylcholine, serotonin, dopamine and noradrenalin (Vizi and Kiss, 1998). Glutamate is the primary excitatory transmitter, and GABA is the primary inhibitory transmitter in the hippocampus, whilst cholinergic, dopominergic and serotinergic systems play a more regulatory role (Gulyas et al., 1999).

Theories of hippocampus function

It is now well accepted that the hippocampus plays a key role in learning and memory. Initial evidence for this came from patients with hippocampus damage, such as H.M. (Scoville and Milner, 1957). These patients show declarative memory impairments (semantic and episodic), but no deficits in implicit memory (e.g. procedural memory, priming effect). The precise way in which the hippocampus is involved in memory is still unclear, both in terms of the type of information which the hippocampus processes and the precise way in which it achieves this function. Several theories of hippocampus function have been proposed to account for experimental findings. The most well-known theories are described below, including cognitive map, relational and configural theory.

Cognitive map theory

One of the earliest theories which explicitly stated the function of the hippocampus is the cognitive map theory. This theory, proposed by O'Keefe and Nadel (1978), suggests that the key role of the hippocampus is to process spatial memory. More specifically, the fundamental role of the hippocampus is the construction and maintenance of spatial maps of the environment. One of the key pieces of evidence implicating the hippocampus in spatial memory was the discovery of 'place cells' in the CA3 and CA1 regions of the hippocampus (O'Keefe and Dostrovsky, 1971). These cells have firing patterns which are closely correlated with an animal's spatial location and only fire when an animal is in a specific part of an environment. More recently DG granule cells have also been shown to exhibit place specificity within a spatial environment, but, unlike CA3 and CA1 cells, they show multiple, small, irregularly spaced place fields (Jung and McNaughton, 1993). In addition, grid cells have been discovered in the entorhinal cortex, which demonstrate spatially selective firing in multiple locations within an environment in a regular 'grid' pattern (Hafting et al., 2005). This broad circuit of spatially selective cells strongly implicates the hippocampus in processing of spatial cues and location (Moser et al., 2008).

O'Keefe and Nadel (1978) proposed that the hippocampus stores representations of an environment, and can use this information to guide navigation and remember spatial contexts. Behavioural evidence supporting this argument is that specific lesions of the hippocampus in rodents severely disrupt performance on spatial navigation tasks. For example, on a radial arm maze hippocampal lesions increase the number of both working and reference memory errors (Jarrard, 1978, Olton et al., 1978), and in water maze tasks hippocampal lesions reduce task acquisition and lead to a reduction in the proportion of time spent in the target quadrant on a probe trial, when only distal cues can be used for navigation (Morris et al., 1982, Morris et al., 1990). In addition, MRI studies in humans have also implicated the hippocampus in spatial memory processing. In one study specific activation was seen in the right hippocampus when London taxi drivers were asked to recall complex routes through a familiar environment, which correlated with task performance (Maguire et al., 1997, Maguire et al., 1998). This pattern of hippocampus specific activation was not engaged by non-spatial tasks.

However, the cognitive mapping theory has come under some criticism since its proposal over 30 years ago, as it cannot account for all of the experimental data which been reported

since its proposal (for an examples see Cohen and Eichenbaum, 1991, Eichenbaum et al. 1999). For example, the discovery of non-spatial selective firing of hippocampal pyramidal cells suggests that the hippocampus must also play a role in processing non-spatial cues (e.g. Fried et al., 1997, Wood et al., 1999, Konkel et al., 2008). A more recent update of the cognitive map theory attempts to address some of these issues (O'Keefe, 1999). However, in this revision it is acknowledged that a purely spatial based cognitive map may be better suited to describe the function of the rodent hippocampus. In humans (and non-human primates) it seems more likely that whilst the right hippocampus is primarily involved in spatial memory processing, the left hippocampus may also process temporal and linguistic information.

Relational theory

The relational theory of hippocampus function was proposed by Eichenbaum and Cohen (1988, Eichenbaum et al., 1999) as an alternative to the cognitive map theory. This theory attempts to account for experimental evidence showing that the hippocampus also processes non-spatial information. This theory suggests that the hippocampus can process all manner of associations and sequences and links these into relational frameworks. Relationships involving spatial cues may play a large part of this, and account for the large number of spatially selective cells in the hippocampus, as many of the events in our daily lives involve some kind of spatial context. However, this is only one example of the type of relational processing which can be carried out in the hippocampus (Eichenbaum et al., 1999). An additional point which is central to relational theory is that the hippocampus enables the flexible representation of information stored there. This allows indirect associations and inferential judgements to be made about information gained across many episodes (Eichenbaum and Cohen, 1988, Eichenbaum et al., 1999).

A key piece of evidence in favour of relational theory, and opposing the cognitive map theory, was the discovery of non-spatial selective firing of hippocampal pyramidal cells (e.g. Fried et al., 1997, Wood et al., 1999, Konkel et al., 2008). Single cell recording studies reported cells which responded to specific task demands, and to the presentation of specific stimuli such as odours and objects. In addition, the relational theory can account for the behavioural evidence showing that lesions of the hippocampus impair sequence learning in rodents, and suggests how the hippocampus may bind temporal and spatial cues to form episodic memories (Eichenbaum et al., 1999). This is done using a 'memory space' system,

which contains a network of interconnections among representations of both common and rare events. There are both narrow codings, which represent specific events, and more broad codings, which may, for example, encode temporal sequences and therefore link events which occurred close in time. As these codings overlap specific detail about different events can be related as part of a whole episode.

Configural theory

A further theory of hippocampus function, which shares some features with the relational theory, is the configural memory theory proposed by Rudy and Sutherland (1989, 1995). This theory suggests that when inputs from different sensory modalities are presented together that are not linked directly, but are stored as individual inputs which are also linked to an independent shared, or configural, memory. Encoding and retrieval of configural memories is dependent upon the hippocampus. Configural theory proposes that episodic memory is a specific kind of configural memory, as it requires information from different modalities (spatial and temporal) to be stored and retrieved as a separate integrated memory from the individual inputs to define a specific prior event, and is therefore also hippocampus dependent (Rudy and Sutherland, 1989, Rudy and Sutherland, 1995, Aggleton and Brown, 1999, Ergorul and Eichenbaum, 2004, Good et al., 2007a, Iordanova et al., 2009). In contrast to this, elemental memory theory posits that sensory inputs are directly linked, rather than being linked to a separate independent memory, following the theory of association between simultaneous inputs (Hebb, 1949, Rescorla and Wagner, 1972, Rescorla et al., 1985). This type of processing is not disrupted by hippocampal lesions in rats (Iordanova et al., 2009), and is thought to be reliant on cortical structures (Iordanova et al., 2009, Rudy and Sutherland 1995).

This theory is particularly attractive as the predictions can be experimentally tested using a variety of behavioural paradigms, such as positive or negative patterning, transverse patterning and bi-conditional discriminations (Rudy and Sutherland, 1995). Each of these paradigms can only be solved by construction of a configural memory of the co-occurrence of stimuli, and not by simply forming representations of the constituent parts. However, a review of the literature assessing the impact of hippocampal lesions in rodents on each of these types of tasks has revealed mixed results, with some reports of configural impairments and other reports of no impairment. This has led to a further revision of the configural theory

which suggests that not all configural discriminations are hippocampus dependent, only those that have spatial or temporal learning requirements included as part of the discrimination; this type of memory was termed structural learning (Aggleton et al., 2007).

Another key theory related to configural information processing if the hierarchical relational binding theory (Bussey and Saksida, 2002, Bussey and Saksida, 2007, Cowell et al., 2010). This theory suggests that the hippocampus is not the only brain region which can process configural representations, and that instead there is a hierarchical increase in stimulus complexity throughout the MTL, and particularly across the ventral visual stream. The more caudal regions, such as the visual cortex, represent simple features of each stimuli, whilst more rostral regions, such as the perirhinal cortex, can process representations of more complex features by conjugating together different aspects of the stimuli. In this manner each region of the ventral visual stream can be seen as processing both elemental and configural representations, depending on whether it is compared to the more simple representation processed by more caudal regions of the more or the more complex conjugated representation of features processed by more rostral regions. Evidence in support of this theory is that configural tasks such as transverse patterning paradigms can be successfully learned by animals with hippocampal lesions, but not perirhinal cortex lesions (Saksida et al. 2007). However, a recent addition to this theory (Cowell et al., 2010), includes the hippocampus at the top of the ventral visual stream hierarchy, and suggests that the hippocampus is required for processing of the most complex conjugations of cues, such as those involving both spatial and temporal information (i.e. episodic information), which cannot be supported by structures lower down the hierarchy. In this manner the hierarchical relational binding theory comes to a similar conclusion as the structural learning theory, based on the currently available experimental evidence, that the hippocampus plays a key role specifically in configural binding of information with spatial and temporal contexts.

Role of the hippocampus in episodic memory

Both relational and configure theory suggest that the hippocampus can process episodic information, and there is now much experimental evidence implicating the hippocampus in episodic memory processing. This is of particular interest in the context of this theory as episodic memory impairments are also one of core cognitive deficits manifest in the early stages of AD (Almkvist, 1996, Bondi et al., 1999, Collie and Maruff, 2000). Episodic

memory is a type of declarative memory relating to the recollection of previous personal events. This form of memory was defined by Tulving (1972) as having three core components, memory for what happened, when and where, i.e. the spatio-temporal context of an event. This definition was later revised to include the requirement that it is only the subjective conscious recollection of these events that defines episodic memory (Tulving, 2002). In humans selective hippocampus damage leads to impaired declarative memory formation, with episodic memory more severely disrupted than semantic memory (Zola-Morgan et al., 1986, Rempel-Clower et al., 1996, Vargha-Khadem et al., 1997). Functional imaging studies have also shown hippocampus specific activation when subjects are presented with verbal or visual episodic memory tasks (Nyberg et al., 1996, Stern et al., 1996, Fernandez et al., 1998, Yancey and Phelps, 2001).

True episodic memory is difficult to model in animals due to the requirement of conscious recollection (Clayton et al., 2003, Suddendorf and Busby, 2003). However, leaving the conscious attributes aside it is possible to examine memory for the core components of episodic memory – that is, an integrated memory of when and where an event occurred. This type of memory, referred to in the literature as 'episodic-like', was first demonstrated in animals by Clayton and Dickenson (1998). They reported that scrub jays could not only remember where they had previously stored certain types of food, either perishable meal worms or non-perishable nuts, but also how long ago and retrieved the appropriate food which was still edible after different periods of time. The finding was translated into rodents by Babb and Crystal (2006) using a radial arm maze task. In this task rats were required to remember which arm different flavoured pellets were found in, and which were replenished after a certain time delay. Memory was assessed using taste-aversion methods, and showed successful retrieval of memories for which flavour food could be found in which arm after different time delays.

It is widely thought that the hippocampus is essential for episodic-like memory in animals, as demonstrated by lesion and inactivation studies (Aggleton and Brown, 1999, Day et al., 2003, Ergorul and Eichenbaum, 2004, Good et al., 2007a), and that its role in this type of memory in particular may reflect the need for storage and retrieval of memories formed by links between different sensory modalities, i.e. spatial and temporal modalities in the case of episodic memory. More recently, a novel test has been developed to test the mnemonic integration of cues containing episodic information using an operant box paradigm (Iordanova et al., 2008). In this task, described in more detail in chapter 4, rodents

spontaneously encode and recall a unique configuration of cues relating to a context and time of day that different auditory cues were presented. In the second stage of this procedure, one of these auditory cues is re-valued by pairing it with the delivery of a foot-shock. The memory for the unique configurations of cues is then tested by measuring conditioned freezing in each context/time of day configuration. This task has been shown to be sensitive to hippocampus lesions, inactivation and pharmacological blockade of hippocampal NMDA receptors (Iordanova et al., 2009, Iordanova et al., 2011).

Modelling Hippocampus Function

Several different models of how the hippocampus carries out its function to encode and retrieve memories have been proposed. These models are based on lesion studies, neurophysiological recordings, pharmacological and genetic manipulations.

One of the earliest computational models of hippocampus function was developed by Marr (1971). This model was based on mathematics using binary neurons and synapses to demonstrate how the hippocampus may store and retrieve patterns of information. Marr proposed that the CA3 region was ideally suited as the site of storage and retrieval of memories based on its recurrent connectivity. To allow the hippocampus to store the maximum number of memories a structure upstream of CA3 was required to de-correlate incoming information. Marr proposed that the DG region satisfied the requirements of this role. The CA1 region was suggested to play a role in recoding the memories retrieved from the CA3 region to allow reactivation of neocortical networks which were activated during the original episode.

Several more recent models have been based around Marr's theory, with the role of storage and pattern completion given to the CA3 region and the DG acting as a pattern separator (McNaughton and Morris, 1987, Eichenbaum et al., 1992, Squire, 1992, O'Reilly and McClelland, 1994, Shapiro and Olton, 1994, Treves and Rolls, 1994, McClelland et al., 1995, Rolls, 1996, Moscovitch et al., 2005, Rolls and Kesner, 2006). One of the most well established of these models is by Rolls and Treves. This model was first proposed in 1987, but has been updated numerous times as new empirical evidence has come to light (Treves and Rolls, 1994, Rolls, 1996, Rolls and Kesner, 2006). A recent review of this model, and experimental evidence to support it, can be found in Rolls and Kesner (2006). Within all of these models each hippocampal sub-region is thought to play a unique and specific role in

encoding and retrieval of information. Multi-modal information arrives at the parahippocampal regions from different sensory areas of the neocortex. The hippocampus is thought to play a key role in episodic memory by forming associations between these different inputs which can later be retrieved. A brief description of the roles of the DG, CA3 and CA1 sub-region is outlined below with empirical evidence to support these roles.

Role of the Dentate Gyrus

Computational and theoretical models propose that the major function of the DG is spatial pattern separation during encoding of new memories (McNaughton and Morris, 1987, O'Reilly and McClelland, 1994, McClellend, 1995). This process allows episodes containing similar or overlapping information to be distinguished as separate events and stored as separate representations in the CA3 network.

The anatomical connectivity of the DG makes this sub-region suitable as a pattern separator (Schmidt et al., 2012). During encoding of new memories information enters the hippocampus from the entorhinal cortex via the perforant path to the DG. There is a huge divergence of neuronal inputs from the ~112,000 pyramidal cells in layer II of the entorhinal cortex onto over 1,200,000 granule cells in the DG of the rat hippocampus. This leads to only sparse activation of cells being seen in the DG. Following this there is a convergence of DG inputs via mossy fibres onto ~250,000 CA3 pyramidal cells. Each CA3 cell receives input from ~72 DG granule cells, giving a low density of strong synapses onto these cells (West et al., 1991, Mulders et al., 1997). Therefore information from the neocortex via the entorhinal cortex is distributed among granule cells in the DG and can then form a new, more distinct, representation when it converges back onto the CA3 sub-region.

Since the proposal that the DG plays a key role in pattern separation (McNaughton and Morris, 1987, O'Reilly and McClelland, 1994, McClellend 1995), many studies have contributed experimental evidence in support of this theory. One of the strongest lines of evidence comes from DG specific lesions in rats (Gilbert et al., 2001, Rolls and Kesner, 2006, Goodrich-Hunsaker et al., 2008, Morris et al., 2012). Rats with DG lesions are impaired at discriminating between two objects with a small spatial separation on a delayed-match-to-sample test, but were unimpaired when the objects were separated by larger distances (Gilbert et al., 2001). In radial arm maze tasks rats with DG lesions were impaired in discriminations

between arms with a small separation on the maze, but could distinguish between those with a larger separation (Morris et al., 2012). In addition, transgenic mice lacking the NR1 subunit of the NMDA receptor specifically in the DG, therefore silencing LTP in this region, could not distinguish between two similar contexts, despite normal contextual fear-conditioning response (McHugh et al., 2007).

Electrophysiological data also support a role for the DG in pattern separation. DG cells fire in a spatially selective manner and have small and highly reliable place fields (Jung and McNaughton, 1993). Single-unit studies have also demonstrated sparse firing, with only 2-4% of cells being active in any given environment, and rate remapping in the DG which may facilitate pattern separation in this region (Jung and McNaughton, 1993, Leutgeb et al., 2007). Recording from DG granule cells is difficult, due to the sparse nature of their activity, therefore more recent studies have used immediate early genes (IEGs) to assess neuronal activity in the DG. These genes are expressed rapidly and transiently in response to neuronal activity (Dragunow and Robertson, 1987, Herrera and Robertson, 1996, Guzowski et al., 2001). Analysis of expression of the IEGs *Arc* or *zif268* when rats explored two different environments showed distinct ensembles of DG cells activated in each environment, in comparison, when rats explored the same environment twice the same ensemble of cells expressed *Arc* or *zif268* during both episodes (Chawla et al., 2005, Marrone et al., 2011).

Evidence from imaging studies also supports a role for the DG in pattern separation in humans. Recent developments in high resolution functional MRI now allow comparisons to be made between activity in the DG/CA3 region, CA1 and the entorhinal cortex. However, spatial resolution is still not high enough to distinguish between DG and CA3. Nevertheless, these studies have shown that the DG/CA3 region is selectively activated when participants are asked to distinguish between a sample stimulus and a highly similar stimulus, with many overlapping features, rather than a completely novel stimulus with few overlapping features (Bakker et al., 2008, Yassa et al., 2010b, Lacy et al., 2011).

Role of CA3

The CA3 region is thought to be critical for memory by acting as an auto-association network enabling episodic memories to be stored and retrieved. CA3 cells receive input from both the DG via mossy fibre afferents, and directly from the entorhinal cortex via the perforant path.

During encoding, CA3 cells are driven primarily by the sparse, but strong, input from mossy fibres. Synapse strength is modified via Hebbian learning mechanisms (LTP and LTD) so that information from different sensory modalities present in the same episode become associated. During retrieval CA3 cells are driven primarily by perforant path input. Evidence for a dissociation between the mossy fibre input for encoding and perforant path for retrieval comes from lesion studies using a navigation task in the Hebb-Williams maze. Disruption of DG-CA3 connections in rats impaired encoding, leading to increased number of navigational errors within each day, but spared retrieval as between day errors still decreased over test days (Lee and Kesner, 2004b, Jerman et al., 2006). In contrast, lesions of the perforant path input to CA3 caused the reverse with impairment in between-day retrieval, but not within-day learning (Lee and Kesner, 2004b).

Theoretical models propose that the CA3 region is important for encoding specific types of information linked to episodic memory (McNaughton and Morris, 1987, O'Reilly and McClelland, 1994, Treves and Rolls, 1994, McClelland et al., 1995, Rolls and Kesner, 2006). These include the rapid encoding of novel information and encoding associations between cues. Rapid encoding also enables the CA3 region to contribute to short-term and working memory by rapidly updating with new information. This may be done by maintaining the firing of neurons in the CA3 auto-associative attractor network using the excitatory recurrent collateral connections. An attractor is a network of, recurrently, connected neurons whose firing dynamics settle into a stable pattern. A stable attractor can maintain one memory in this way until a new input forces the attractor to represent a different memory (McNaughton and Morris, 1987, Treves and Rolls, 1994). Evidence for the role of CA3 in short-term memory comes from inactivation studies in which the NMDA receptor antagonist AP5 was injected specifically into the CA3 region. This impaired rats on a non-match to sample place task with a 10 second delay, but not when the delay was extended to 5 minutes or 24 hours (Lee and Kesner, 2003). Single unit recording data also support this idea; CA3 activity has been reported during the delay period in short-term memory tasks in rats (Hampson et al., 2000) and monkeys (Cahusac et al., 1989, Wirth et al., 2003). Further behavioural evidence for the role of CA3 in rapid encoding of novel information comes from sub-region specific lesions. CA3 lesions impaired performance on a one-trial contextual fear-conditioning task, suggesting that the rapid formation of memory for novel contextual cues was disrupted (Lee and Kesner, 2004a).

A further hypothesised role of the CA3 region is to encode associations between different inputs, i.e. paired associate learning. This function is not attributed to CA3 in all models, for example whilst Treves and Rolls (1994) propose CA3 is critical for encoding associations, O'Reilly and McClelland (1994) suggest paired associate learning occurs in the CA1 region via input from the Schaffer collaterals and perforant path. However, evidence from sub-region specific lesions showed that rats with CA3 lesions are impaired on object-place and odour-place associations, whereas rats with CA1 and DG lesions form these associations (Gilbert and Kesner, 2003). Similar impairments were seen in transgenic mice with CA3 specific knock-out of NMDA receptors in an odour-context task (Rajji et al., 2006). However, the CA3 region appears to uniquely contribute to associations involving spatial cues in rodents, and spatial and language cues in humans (Kesner et al., 2000), as CA3 lesions do not impair odour-object associations that contain no spatial cues (Gilbert and Kesner, 2003, Kesner et al., 2005). In contrast, rats with CA1 lesions do show impairment on this non-spatial association task (Kesner et al., 2005).

The CA3 region is also thought to be involved in cued recall (McNaughton and Morris, 1987, O'Reilly and McClelland, 1994, Treves and Rolls, 1994, McClelland et al., 1995, Rolls and Kesner, 2006). In particular, one of the fundamental properties of the CA3 recurrent collateral network is that during recall the whole memory can be retrieved from the presentation of a component of the memory. This process is known as pattern completion. Computational models predict that when input via the perforant path contains information about part of a previously experienced episode this activates CA3 cells previously driven by the same input. In turn these CA3 cells drive activity in the recurrent collateral network, activating other cells that stored information previously associated with the input or episode. CA3 specific lesions support a role for the CA3 region in pattern completion. Rats with lesions to the CA3 region are impaired on a location match-to-sample task when only a subset of the original cues provided during learning are present, but are not impaired when all cues are present (Gold and Kesner, 2005). In addition, transgenic mice with CA3 specific knock-out of NMDA receptors were impaired on a water maze task when only a sub-set of cues were present (Nakazawa et al., 2002). Electrophysiological recordings also support this hypothesis, showing that the CA3 population code shows only minor disruption after modest alteration of environmental cues, whereas the CA1 population code shows greater disruption (Lee et al., 2004a). This can be seen as pattern completion, in so far as the CA3 region

maintained a similar representation of the environment even when the relationship between cues was altered.

Role of CA1

From a computational point of view there is less agreement concerning the precise function of the CA1 region. Several theoretical models have proposed that CA1 plays a role in processing temporal information (O'Reilly and McClelland, 1994, Shapiro and Olton, 1994, Treves and Rolls, 1994, Rolls and Kesner, 2006), whilst other models attribute temporal processing to the CA3 region (Wallenstein et al., 1998, Eichenbaum et al., 1999, Lisman, 1999, Kesner et al., 2000). However, the majority of experimental evidence suggests that the CA1 region does play a role in temporal memory, in particular remembering order information and in temporal pattern separation. For example, rats with CA1 specific lesions are impaired on trace fear conditioning (Rogers et al., 2006), and an object-trace-odour task (Kesner et al., 2005). These data suggest that whilst the DG and CA3 regions may primarily process spatial associations, the CA1 can form associations between temporal contexts without a spatial component. In a sequence learning task on a radial arm maze both CA1 and CA3 lesions impaired performance (Gilbert et al., 2001, Gilbert and Kesner, 2006), suggesting that CA3 is also involved in temporal memory when a spatial context is involved, but only CA1 lesions impaired non-spatial temporal associations, such as odour-sequence tasks (Kesner et al., 2002). In addition, the CA1 region is critical in temporal pattern separation, as lesions of this region impaired performance on a radial arm maze task, in which normal rats remembered which arm was presented earliest in the sequence, only when the temporal separation between arms in the sequence was small (Gilbert et al., 2001).

It is now widely accepted that the CA3 region is critical for short-term memory, however a further role for CA1 in processing representations for intermediate-term memory (5 minutes-24 hours) has also been proposed (Squire, 1992, O'Reilly and McClelland, 1994, Treves and Rolls, 1994, Rolls and Kesner, 2006). Behavioural evidence for this comes from CA1 specific lesions that impaired performance in a delayed-non-match to place task on a radial arm maze when the delay was 5-minutes, but not when the delay was only 30 seconds, and retrieval of memories was impaired after a 24 hour delay (Lee and Kesner, 2003, Kesner et al., 2004, Lee and Kesner, 2004a). In addition, blockade of NMDA receptor signalling by

AP5 injection specifically into the CA1 region and disruption of perforant path input into CA1 also impaired performance on this task (Lee and Kesner, 2002, Vago and Kesner, 2008).

In conjunction with its role in intermediate term memory, the CA1 region is also thought to play a role in novelty detection over a longer time-scale than CA3. The anatomical connectivity of CA1 makes it suitable for this role; CA1 has two main input pathways, the Schaffer collaterals from CA3 and perforant path from the entorhinal cortex. Several computational models propose that CA1 can detect novelty using a 'match-mismatch' process by which new sensory information directly from the entorhinal cortex is compared to the stored representation from CA3 via the Schaffer collaterals (Lisman and Otmakhova, 2001, Hasselmo, 2005, Rolls and Kesner, 2006). Behavioural evidence in support of this proposal comes from disruption of the perforant path input from the entorhinal cortex into CA1, which causes deficits in spatial novelty detection using an object-location paradigm in rats (Vago and Kesner, 2008).

Another hypothesis as to the function of the CA1 region is that it may also be involved in recoding memories stored in CA3 by expanding these memories from the sparse encoding produced in the CA3 region to allow more accurate retrieval of the initial cortical inputs (Treves and Rolls, 1994, Lisman and Otmakhova, 2001, Rolls and Kesner, 2006). It is suggested that each CA1 cell receives information simultaneously from several CA3 cells. Therefore, each CA1 cell can represent larger episodes by combining the smaller parts stored by CA3 cells. According to several models (Squire, 1992, Treves and Rolls, 1994, McClelland et al., 1995, Rolls, 1996, Lisman, 1999), back-projections from the hippocampus, primarily CA1, to the entorhinal cortex and then to the neocortex are important for the long-term storage and retrieval of memories. Back-projections, as a result of previously learned pattern associations, bring back firing of cortical regions which were active during the original episode (Treves and Rolls, 1994, Rolls, 1996).

Whilst computational models have provided valuable insights into the putative mechanisms inherent in information processing carried out in the hippocampus, there has been little research examining how amyloid pathology impacts on these processes. This information is vital both in terms of understanding disease processes and understanding how therapeutic interventions may impact on disease mechanisms and neural systems. The next section

describes current therapeutic strategies for AD and focuses on a simple intervention that is considered to influence hippocampal function and cognition.

1.6 Therapeutic Strategies for AD

The final section of this review describes the current therapeutic strategies for AD, and in particular the effect of exercise as a putative therapeutic lifestyle strategy to improve cognition in AD.

Current therapeutic targets in AD

There is currently no cure for AD (Piau et al., 2011), however there are drugs available which may slow down and relieve symptoms of the disease. These drugs fall into two main categories, cholinesterase inhibitors and NMDA receptor antagonists (Lleo et al., 2006).

AD cell loss predominantly targets cholinergic neurons in the brain. Cholinergic cell loss correlates with cognitive decline in AD (Davies and Maloney, 1976, Wilcock et al., 1982). Cholinesterase inhibitors work by inhibiting the acetylcholine esterase enzyme which breaks down acetylcholine in the synaptic cleft. This leads to an increase in acetylcholine at synapses and increased cholinergic synaptic transmission (Wilkinson et al., 2004). However, cholinesterase inhibitors do not stop or reverse cell death and therefore are only beneficial during a small window when some cholinergic neurons are still present and functional in the brain. Commercially available cholinesterase inhibitors include donepezil hydrochloride (Aricept), rivastigmine (Exelon) and galantamine (Reminyl). Administration of cholinesterase inhibitors gives a small but significant improvement in cognition in most patients compared to placebos, measured on the MMSE or ADAS scale. These drugs can also reduce other psychiatric symptoms such as hallucinations, apathy and distractibility. Some reports suggest that using cholinesterase inhibitors reduced the level of care needed by patients and delays nursing home placement. However, beneficial effects usually only last for approximately four years before disease progression outweighs treatment effects (for a review of behavioural effects ofcholinesterase inhibitors see Lleo et al., 2006).

NMDA receptors are calcium channel glutamate receptors involved in synaptic transmission and plasticity and are involved in learning and memory processes (Morris, 1989). However, one of the causes of cell death in AD is excitotoxicity due to high levels of intracellular calcium brought about by over-activation of glutamate receptors (Michaelis, 1998). The only NMDA receptor antagonist currently commercially available is memantine (Ebixa). Memantine works by blocking glutamate transmission via NMDA receptors with non-competitive low affinity binding, therefore reducing excitotoxic cell damage in AD by reducing calcium influx when firing rates are high, but leaving the channel relatively open to allow synaptic transmission when stimulation is low (Lipton, 2004). Administration of memantine improves cognitive status and behaviour in patients with moderate-severe AD compared to placebos (Reisberg et al., 2003), however is has not yet been formally assessed whether it is equally beneficial in patients with mild AD. One study also reported that co-administration of memantine with donepezil gave greater cognitive improvement than either drug alone (Tariot et al., 2004).

Current research into new therapeutic targets has focused largely on reducing amyloid pathology, following the amyloid cascade hypothesis which proposes that Aβ accumulation as one of the primary events in AD pathogenesis (see section 1.3). Drugs have been developed which target the processing of APP to alter production of Aβ peptides. These targets include β- and γ-secretase inhibitors (Petit et al., 2001, Ghosh et al., 2008), and drugs that increase cleavage by α-secretase to promote non-amyloidogenic processing of APP (Bandyopadhyay et al., 2007). An alternative target is immunotherapy directly against Aβ peptides (Morgan, 2006). Studies in mouse models have shown positive effects of both active and passive immunisation with antibodies against Aβ in reducing amyloid load in the brain (Schenk et al., 1999, Bard et al., 2000). Drugs targeting Aβ pathways have reached clinical trials, but so far none have made it to commercial use (Alzheimer's Research Forum 2012, drugs in clinical trials).

Lifestyle interventions

Lifestyle factors including cognitive activity, physical activity and diet have been shown to reduce the risk of developing AD in later life, and can also slow the progression of cognitive decline in AD patients (Breuil et al., 1994, van Praag, 2009, Graff-Radford, 2011). There is a growing interest in these interventions as there is increasing evidence that by the time AD can

be clinically diagnosed it is too late for effective treatment of cognitive and neuronal pathology, at least using current treatments strategies. Therefore lifestyle interventions may provide a tool to reduce cognitive decline and improve the potential for therapeutic intervention at the stage of clinical diagnosis. At present the molecular mechanisms underlying the beneficial effects on cognition of these lifestyle interventions remain unclear, however, investigation of these mechanisms may provide novel drug targets for preventing AD in at risk individuals.

It is now widely reported that a lack of cognitive activity hastens cognitive decline, both in normal aging (Small, 2002) and in AD (Breuil et al., 1994). There is also evidence that cognitive activity in mid-life can reduce the risk of dementia in later-life (Fratiglioni et al., 2004, Wilson et al., 2007, Carlson et al., 2008), and that it can improve cognition in healthy older individuals (Gates and Valenzuela, 2010). It has been suggested that this effect is due to increases in cognitive reserve (Stern, 2002, 2006). Cognitive reserve is based on recruitment of alternative brain networks or more efficient use of brain networks as needed to compensate for pathology. More recently studies have shown the increasing cognitive activity in patients with mild to moderate AD, by engaging them in tasks requiring thinking, concentration and memory, can in fact slow down the rate of cognitive decline (Fratiglioni et al., 2004, Wang et al., 2012, Woods et al., 2012). Studies in rodent models using environmental enrichment to stimulate sensory and cognitive activity have shown similar results. Enrichment improves cognition in both normal animals, and in transgenic mouse models of AD (Arendash et al., 2004, Jankowsky et al., 2005, Lazarov et al., 2005). However, there are mixed results of the cellular effects of enrichment, with some studies showing no change in amyloid pathology (Arendash et al., 2004, Jankowsky et al., 2005), and others showing reduced amyloid pathology and up-regulation of gene transcripts for vasculogenesis and neurogenesis (Lazarov et al., 2005).

Recent evidence suggests that alterations in diet can benefit cognition, and in particular that caloric restriction can enhance learning and correlates with reduced risk of AD (Stranahan and Mattson, 2008, van Praag, 2009). Other dietary supplements have also been identified in epidemiological studies and controlled trials in humans and rodents which can reduce the risk of AD or slow down cognitive decline, including omega-3 fatty acid from fish oil, vitamins, flavanols from fruit and wine, curcumin and green tea (Kalmijn et al., 1997, Ringman et al., 2005, Gomez-Pinilla, 2008, Spencer, 2008). However, the study of dietary interventions is hampered by the fact that although natural products may be beneficial for cognition, the

specific active molecules are not always identified (van Praag, 2009). The mechanisms by which dietary supplements may improve cognition are still unclear, although there is evidence that they have anti-oxidant and anti-inflammatory properties, and may also have a mild influence on synaptic plasticity (van Praag, 2009).

Exercise and cognition

Recently the effect of physical activity on cognition in both normal individuals and AD patients has received much attention (van Praag, 2009, Graff-Radford, 2011). As this lifestyle intervention is a main focus of chapter 5 of this thesis, the literature related to cognitive and molecular changes with exercise is evaluated in greater detail below.

Exercise and human cognition

There is a wealth of literature which suggests that exercise may be particularly beneficial in reducing age-related cognitive decline (Kramer et al., 1999, Churchill et al., 2002, Heyn et al., 2004, Cotman et al., 2007, Angevaren et al., 2008, Hillman et al., 2008). Several epidemiological studies have assessed the influence of exercise on human cognition. Although some smaller studies have found no correlation between physical activity and improved cognition (Broe et al., 1998), larger studies generally support this conclusion. For example, a study in elderly women revealed that those in the highest quartile for physical activity had a reduced risk of cognitive decline over the 8 year follow-up period than those in lower quartiles (Yaffe et al., 2001). More specifically, people who were physically fit performed better on cognitive tasks designed to test working memory, reasoning, vocabulary and reaction time. One of the largest studies, carried out in Canada, assessed the influence of physical activity in over 9,000 elderly men and women (Laurin et al., 2001). This study found that not only did exercise reduce the risk of cognitive decline by approximately 40%, but that it also reduced the incidence of Alzheimer's disease by half. Another large scale study (~9,000 female participants) found that women with a history of physical fitness throughout life had a lower prevalence of cognitive impairment in later life than those that had been inactive (Middleton et al., 2010). Some studies have also used neurophysiological measures to assess the effects of exercise in humans. These studies showed positive outcomes, as measured by MRI and ERP (Colcombe et al., 2004, Hillman et al., 2008), and

also a reduction in age-related brain atrophy, particularly in the frontal, parietal and temporal brain regions (Colcombe et al., 2003).

Not only does physical exercise delay age-related cognitive decline, this intervention may also protect against brain damage caused by stroke, promote recovery after physical damage, act as an antidepressant and, more critically for this thesis, may be beneficial in delaying the onset and slowing progression of neurodegenerative disorders such as Parkinson's, Huntingdon's and Alzheimer's disease (van Praag, 2008).

Exercise and cognition in rodents

Studies have also been carried out in rodents to investigate the molecular changes underlying the beneficial effects of exercise on cognition. Similar to humans, exercise produces positive effects on cognition in rodents, with improved performance on tasks that rely upon an intact hippocampus, such as the Morris water maze, Y-maze, T-maze and radial arm maze (Fordyce and Farrar, 1991, van Praag et al., 1999a, Vaynman et al., 2004, van Praag et al., 2005, Schweitzer et al., 2006, Vaynman et al., 2006). Exercise also improves performance on tasks that do not in themselves require proficient physical activity, including contextual fear conditioning (Baruch et al., 2004), passive avoidance (Radak et al., 2001) and object recognition (O'Callaghan et al., 2007). Like humans, rodents show age-related cognitive decline that can be ameliorated with exercise (Samorajski et al., 1985, van Praag et al., 2005, Albeck et al., 2006). Interestingly, recent research has shown that exercise enhanced spatial pattern separation in young mice, but only had a mild effect on pattern separation in aged mice (Creer et al., 2010).

The advantage of rodent studies is that the experimenter has much greater control over specific factors, such as exercise regime, and this has therefore has been useful in assessing the type of exercise that is most beneficial to cognition. Although some studies have shown improvements after only one week of exercise (Vaynman et al., 2004, 2007), most have showed benefits only after a longer period of exercise (3-12 weeks) (van Praag et al., 2005, Schweitzer et al., 2006, O'Callaghan et al., 2007). There is also much debate as to the relative benefits of forced (treadmill) or voluntary (running wheel) exercise (Leasure and Jones, 2008). It has been argued that the stress induced by forced exercise may counteract any improvement in cognition created by the exercise itself (Narath et al., 2001, Yanagita et

al., 2007). Although some studies have shown beneficial effects of forced exercise on cognition (Ang et al., 2006, O'Callaghan et al., 2007), few studies have systematically compared forced and voluntary exercise. Those that have, have showed greater benefits to cognition with voluntary exercise compared to forced regimes (Yuede et al., 2009)

Exercise and Alzheimer's disease

There is mounting evidence that increased physical activity can delay the onset of AD and be beneficial even after clinical diagnosis of the disease (Churchill et al., 2002, Podewils et al., 2005, Rovio et al., 2005, Larson et al., 2006, Perez and Cancela Carral, 2008). A retrospective study assessing patients with AD, showed that patients that had been inactive (both physically and mentally) in mid-life compared to healthy controls, had an increased risk of developing AD, by 250% (Friedland et al., 2001). Several prospective studies also found similar results (Lytle et al., 2004, Podewils et al., 2005, Larson et al., 2006); one study showed that physical activity decreased the risk of developing AD by 60% (Laurin et al., 2001, Burns et al., 2008). Another study showed a correlation between physical activity and hippocampal volume in AD patients (Burns et al., 2008). Randomized control trials have been carried out in patients with AD or MCI and found that implementing an exercise program slowed or stopped cognitive decline compared to non-exercising control groups (Lautenschlager et al., 2008, Baker et al., 2010). However there are inconsistent findings in the literature, with some studies showing an inverse correlation between exercise and the risk of AD (Laurin et al., 2001, Rovio et al., 2005) and others finding no correlation (Broe et al., 1998, Wilson et al., 2002, Verghese et al., 2003). In addition, several studies have reported beneficial effects of exercise on improving mood and behaviour in AD patients, but no effect on cognitive decline (Heyn et al., 2004, Deslandes et al., 2009).

Further research has assessed the influence of exercise on people with a genetic disposition to develop AD: ApoE-ε4 carriers. Evidence showed that carriers benefit more from physical activity, in terms of cognition, than non-carriers (Schuit et al., 2001, Etnier et al., 2007), and that exercise correlated better with cognitive task performance in carriers than in non-carriers (Deeny et al., 2008). However, there is some discrepancy in the literature, as another study showed reduced effects of exercise in ApoE-ε4 carriers that already had symptoms of cognitive decline (Lautenschlager et al., 2008).

Exercise and transgenic models of AD

Although generally the literature from human studies suggests that exercise is beneficial to AD, there is still some debate and uncertainty. Therefore studies in transgenic mouse models of AD have been useful, both in establishing under what conditions exercise may be beneficial in improving cognition, and also in elucidating the putative mechanisms by which exercise improves cognition; whether exercise directly influences pathology or acts indirectly through other mechanisms to improve cognition.

Overall, a number of studies have reported beneficial effects of exercise on cognition in transgenic mouse models of AD. One of the first studies to assess the effects of exercise on AD transgenic mice used the TgCRND8 model, with exercise started 5 months before disease onset. Exercising transgenic mice resulted in improved rates of water maze learning compared to controls (although sedentary animals achieved equal performance from day 4 onwards), and reduced hippocampal and cortical amyloid load (Adlard et al., 2005). However, this study did not include any wild-type controls, so it is unclear whether cognitive deficits were completely restored to wild-type levels. Another study reported that short-term exercise (3 weeks) after disease onset improved reference memory in aged Tg2576 transgenic mice, but did not alter amyloid levels (Nichol et al., 2007, Parachikova et al., 2008). It was proposed that exercise was mediating beneficial effects on cognition by alterations in the immune and inflammatory profile of transgenic mice (Nichol et al., 2008, Parachikova et al., 2008). However, the effect of exercise on cognition varies between transgenic models and different regimes. For example, 9 months of exercise started before disease onset did not alleviate cognitive deficits in the APP23 model (Wolf et al., 2006), whereas improvements in cognition have been shown in the Tg2576 (Nichol et al., 2007, Yuede et al., 2009), APP/PS1 (Ke et al., 2011, Liu et al., 2011) and 3xTg model (Garcia-Mesa et al., 2011) with varying exercise regimes. Similarly whilst some studies suggest that exercise directly modifies amyloid pathology (Adlard et al., 2005, Yuede et al., 2009, Garcia-Mesa et al., 2011), others demonstrate no changes in amyloid levels (Wolf et al., 2006, Parachikova et al., 2008, Ke et al., 2011) and suggest alternative cellular mechanisms.

How does exercise improve cognition?

The general consensus in the literature is that exercise is beneficial in reducing cognitive symptoms of AD in both mice and humans. However, the molecular mechanisms underlying improvements in cognition with exercise are still unclear. The following section evaluates the putative mechanisms of action for the cognitive effects of exercise, derived from studies in rodents.

Synaptic Morphology and Plasticity

Similar to humans, aging rodents show reduced synaptic connectivity and plasticity in the hippocampus and cortex, which is correlated with cognitive decline (Barnes, 1994). Exercise has been shown to improve both long- and short-term synaptic plasticity specifically in the DG of mice and rats (van Praag et al., 1999a, Farmer et al., 2004, O'Callaghan et al., 2007). Exercise also increased levels of the pre-synaptic proteins synapsin and synaptophysin (Vaynman et al., 2006) and post-synaptic glutamate receptors NR2A, NR2B and GluR5 (Molteni et al., 2002, Farmer et al., 2004). Changes in molecular composition of synapses are accompanied by more gross changes in morphology. For example, exercise increases dendritic length, complexity and spine density, particularly in the DG, but also in CA1 and entorhinal cortex layer III (Eadie et al., 2005, Redila and Christie, 2006, Stranahan et al., 2007). Thus exercise can have clear beneficial effects of synaptic connectivity and function.

Neurogenesis

Neurogenesis is the process by which new neurons are generated from neural progenitor cells. This process occurs across the brain during pre-natal development, but is only found in the subventricular zone of the olfactory bulb and the subgranular zone of the DG in adults (Altman and Das, 1965). Adult neurogenesis in the DG has been linked to learning and memory, and theoretical models predict that adult neurogenesis increases the storage capacity of the hippocampus by enhancing pattern separation (for a review see Aimone et al., 2011). Exercise increases neurogenesis in the DG of both young and aged mice (van Praag et al., 1999a, van Praag et al., 1999b, van Praag et al., 2005, van Praag, 2008), which may underlie improvements in cognition (Leuner et al., 2006). More specifically increased neurogenesis

has been implicated in enhanced pattern separation (Clelland et al., 2009, Aimone et al., 2011), a hippocampal function which is also improved by exercise (Creer et al., 2010). Neurogenesis may facilitate increases in dentate LTP as a result of exercise as new neurons have an increased proportion of NR2B receptors, which have a lower induction threshold and are more highly excitable than mature DG neurons (Schmidt-Hieber et al., 2004, Ge et al., 2007).

Vascular effects

Exercise improves cerebro-vascular integrity by stimulating angiogenesis throughout the brain, but particularly in the cerebellum, striatum, cortex and hippocampus (Black et al., 1990, Swain et al., 2003, Ding et al., 2004, van Praag et al., 2005). More specifically, one study showed that exercise increased DG blood flow, and that this correlated with improvements in hippocampus-dependent learning (Pereira et al., 2007). Vascular effects of exercise may be brought about by increases in IGF-1 (insulin-dependent growth factor 1) and VEGF (vascular endothelial growth factors) (Cotman et al., 2007).

Growth factors

There is now increasing evidence that a dominant effect of exercise is an increase in the level of growth-factors, including VEGF, IGF and BDNF (brain-derived neurotrophic factor), which in turn promotes other downstream structural and functional changes in the brain described above (Cotman et al., 2007, Bekinschtein et al., 2011). BDNF up-regulation by exercise (Adlard and Cotman, 2004, Farmer et al., 2004, Vaynman et al., 2004) is of particular interest as this growth factor has been shown to play a role in synaptic plasticity, neurogenesis, neuronal growth and survival (Vaynman et al., 2006, Bekinschtein et al., 2011). Impaired BDNF release is associated with impaired memory function and abnormal hippocampal activity (Egan et al., 2003). In addition, BDNF polymorphisms have also been found to be a risk factor for AD (Ventriglia et al., 2002, Egan et al., 2003), and BDNF levels are 50-75% lower in AD patients than healthy controls (Murray et al., 1994, Peng et al., 2005).

Although it is still unclear exactly how exercise exerts its influence on cognition, it is likely that these putative mechanisms are linked and work together to improve neural system activity and thus cognitive function. However, research is still required to assess which of these mechanisms may be the most important, and which may act upstream of others as the primary change induced by exercise.

1.7 Aims of Thesis

This review has highlighted the current status of research into AD and hippocampus function. It is well documented that the hippocampus is a target of amyloid pathology, and that both humans and mouse models of AD show cognitive decline on hippocampal dependent tasks. However, at present there has been little research into precisely how hippocampus function is impaired in AD patients or transgenic mouse models, and how changes at the sub-region level may impact on specific cognitive behaviours. Relating hippocampal network changes at the sub-region level in AD to current theories of normal hippocampal processing of information may provide insight into more selective cognitive tasks for human patients which can aid earlier diagnosis of the disease. In addition, therapeutic strategies which target the most vulnerable hippocampal regions or specific cognitive domains related to the function of these regions may prove more effective in preventing or delaying the onset of cognitive decline.

With these overarching ideas in mind, this thesis presents three sets of experiments aimed at elucidating the effect of amyloid pathology on hippocampal processing and cognition, and whether these changes can be reversed by exercise. The aim of the first set of experiments is to assess neuronal activity in the Tg2576 model across the hippocampal network during behaviour, to establish how early amyloid pathology may alter functional interactions within this network. A contextual novelty paradigm was chosen as there is extensive literature implicating the hippocampus in processing this type of information. Given the early alterations in hippocampal plasticity and morphology in models of AD, particularly in the DG and CA1 regions, it is predicted that differences in hippocampal network interactions may be seen at an early age, possibly before the onset of overt amyloid pathology and behavioural deficits. The aim of the second set of experiments reported in this thesis is to establish the

effects of altered hippocampal activity in Tg2576 mice on a novel test of configural memory for episodic information, a cognitive function that is particularly dependent on the hippocampus and is affected early in AD. In addition, assessment of which hippocampal regions are involved in episodic-like memory processing on this task may provide an insight into which computational processes carried out by the hippocampus are impaired in Tg2576 mice. Finally, in the light of research reporting changes in hippocampal plasticity and neurogenesis with exercise, the aim of the final set of experiments presented in this thesis is to assess the influence of long-term exercise on cognition in the Tg2576 model, and to test putative molecular processes which may mediate exercise-induced improvements in cognition in this model. Behavioural paradigms were chosen to give a broad assessment of the influence of exercise on different aspects of memory and cognition, including anxiety, working- and reference-memory and episodic-like memory.

Chapter 2: Maintenance of the Tg2576 colony

2.1 Introduction

This chapter describes the methods employed for generating and maintaining the colony of Tg2576 mice which are used throughout this thesis. Tg2576 mice over-express the human APP_{695} gene containing the 'Swedish' mutation (Lys^{670}-Asn and Met^{671}-Leu) driven by a hamster prion protein (PrP) gene promoter on a hybrid C57B6/SJL background (Hsiao et al., 1996). The 'Swedish' mutation is heterozygous, therefore after generation of the colony polymerase chain reaction was used to identify the genotype of mice as either wild-type or transgenic; harbouring the APPswe mutation.

2.2 Breeding

Male Tg2576 mice were crossed with female C57Bl6 x SJL mice in order to maintain the hAPP genetic mutation on a stable background strain. At approximately 6 weeks of age, pups were weaned and the males were kept and individually housed. An ear-biopsy was then taken from each mouse for genotyping.

2.3 Housing Conditions

As all mice used in the experiments described in this thesis were male they were individually housed, as males from the Tg2576 strain tend to be aggressive and therefore cannot be group housed. All mice used in chapters 3 and 4 were housed in standard conditions in cages measuring L 48cm x W 15 cm x H 13cm with an opaque plastic base and a wire top. Mice were given *ad libitum* access to food and water, unless otherwise stated as part of a behavioural paradigm, and were kept on a 12hr light/dark cycle with all behavioural testing carried out during the light hours. Holding rooms were maintained at approximately 20^{o}C and 50% humidity. Mice used in chapter 5 were housed in similar conditions with the exception that they had larger cages, measuring L 42cm x W 27cm x H 13cm and had an exercise wheel in their cage (further details are provided in chapter 5).

2.4 Genotyping using polymerase chain reaction

Mice were genotyped for the APPswe mutation using polymerase chain reaction (PCR) to amplify the hAPP section of DNA. An ear biopsy sample was collected from each mouse at approximately 6 weeks of age and stored at -20^{o}C. To digest the tissue, samples were incubated at 55^{o}C in 600µl TES buffer (1M tris HCl, 0.5M EDTA, 10% SDS) for 20 minutes. 1.2µl of 25mM proteinase K was added to the solution and incubated overnight at 55^{o}C. DNA was extracted the following day by adding 200µl of 5M acetate to each sample and centrifuging at 14000rpm for 10 minutes to pellet the proteins. 650µl of the supernatant containing DNA was transferred to a fresh eppendorf containing 600µl of isopropanol than centrifuged at 14000rpm for 2 minutes to pellet the DNA. The supernatant was removed and the DNA was washed three times by adding 150µl of 70% ethanol and centrifuging for 2 minutes at 14000rpm then removing the supernatant. After washing, the DNA samples were placed in a drying room for 10-15 minutes to evaporate any remaining liquid. The DNA was then re-suspended by incubating with 60µl TE buffer (1M Tris HCl, 0.5M EDTA) at 55^{o}C overnight.

The following day, 1µl of each sample was mixed with 12µl of PCR master mix containing 1.25µl 10x buffer (BIOTAQ Red DNA Polymerase, Bioline), 0.625µl 50mM $MgCl_2$, 0.625µl 10mM dNTPs, 0.125µl each of primer 1501 (50pmol), primer 1502 (10pmol) and primer 1503b (10pmol), 0.75µl Red Taq polymerase (1µg/µl) and 8.375µl of RNAase free H_2O. This mixture was vortexed briefly and centrifuged at 4000rpm for 4 seconds then placed in the PCR cycler. Primer sequences for detecting the APPswe transgene were 1503b (5'-CTGACCACTCGACCAGGTTCTGGGT-3') and 1502 (5'-GTGGATAACCCCTCCCCC AGCCTAGACCA-3'). Control primer sequences for detecting the prion protein found in all animals were 1502 and 1501 (5'-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3'). DNA was amplified using 30 cycles as follows; DNA denaturing at 94^{o}C for 45 seconds, primer annealing at 60^{o}C for 30 seconds, strand elongation at 72^{o}C for 90 seconds. Samples were then incubated at 72^{o}C for a further 5 minutes to ensure all DNA strands were fully elongated then the reaction was terminated by incubation at 4^{o}C.

PCR products were separated by electrophoresis on a 1.5% agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.0), and visualized using sybr-safe (Invitrogen) staining. Samples were run alongside a 100bp DNA ladder, a negative control containing H_2O in place of the DNA sample, and a positive control for both transgenic and wild-type samples

containing DNA samples of known genotype. The APPswe transgene gives a fragment of ~300bp, seen only in transgenic animals. The prion protein gives a ~600bp fragment seen in both transgenic and wild-type animals indicating a successful PCR reaction. Examples of PCR bands used to identify wild-type and transgenic mice can be seen in **Figure 2.1**.

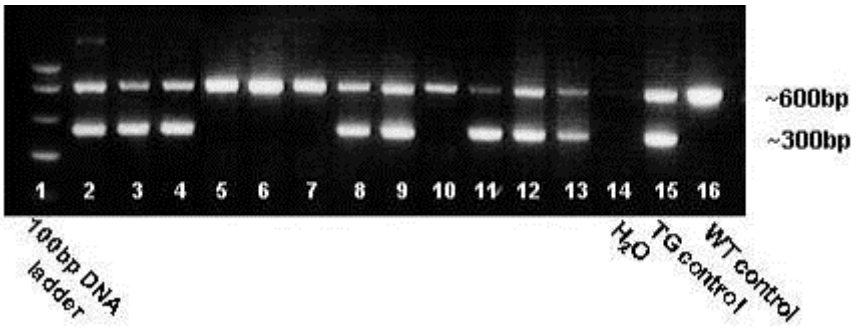

Figure 2.1: Example of bands produced by PCR to identify transgenic and wild-type mice. Transgenic mice produce two bands, of approximately 300 and 600bp, whilst wild-type mice only produce one band of approximately 600bp.

2.5 Experimental Cohorts

Three cohorts of Tg2576 mice were bred and used in the experiments described in this thesis. The first experimental cohort of mice were run on the configural learning task (data presented in section 4.3) at approximately 2.5 months of age and then sacrificed at 3 months of age for c-fos expression analysis (3.3). The second cohort of Tg2576 mice were tested on the configural and elemental learning tasks at approximately 11 months of age (4.4, 4.5) and the used for c-fos expression analysis at 12 months (3.4). The third experimental cohort was used in a longitudinal study of the effects of exercise on cognition, presented in chapter 5. Further details of the order of experiments which each group participated in can be found in **Table 2.1**. The exact number of mice used in each task can be found in the methods section of experimental chapters, as due to attrition rates these varied between experiments.

Table 2.1: Experimental Cohorts. Three cohorts of Tg2576 mice were bred for use in the experiments described in this thesis, with some cohorts used in more than one set of experiments. Details of which tests each cohort participated in are described here.

	Age (months)	Test	Mouse Numbers	Thesis Section
Cohort 1 56 mice	2.5m	Configural learning	24 mice – 12 wt, 12 tg	4.3
	3m	C-fos novelty task - tissue collection	38 mice – 20 wt, 18 tg (7 run on configural task)	3.3
Cohort 2 89 mice	11m	Configural and elemental learning tasks	109 mice -53 wt , 56 tg	4.4., 4.5
	12m	C-fos novelty task - tissue collection	48 mice, 22 wt, 22 tg (all run on configural task)	3.4
Cohort 3 93 mice	2m →	Exercise wheels provided	All 93 mice run on every task unless specified in methods of thesis section	5.2
	6m	Elevated plus maze		5.3
	7m	T-maze		5.4
	8m	Object-in-place		5.5
	10m	Foraging task		5.6
	11m	Configural learning task		5.7
		BrdU injection		5.9
	12m	Tissue collection		5.8, 5.9

2.6 Experimental Cohort Weights

The weights of each cohort of mice either at the age they were run on each task, or in the case of cohort 3 across the longitudinal study, are shown in **Figure 2.2**.

The data for cohort 1 can be seen in **Figure 2.2a**. There was little difference in weight between genotypes when mice were tested on either the configural learning task or when tissue was collected for c-fos expression analysis. This was confirmed by independent

samples t-test showing no significant difference in weight between genotypes for either task/age (configural: $t_{(22)}$=1.135, p=0.269, c-fos: $t_{(37)}$=1.903, p=0.065).

Figure 2.2b presents data showing the average weight of mice in cohort 2 when they were tested on the configural learning task and when tissue was collected for c-fos expression analysis. There was no significant difference in weights between genotypes when mice were tested on the configural learning, and elemental learning tasks at 11 months of age ($t_{(109)}$=0.824, p=0.412). However, by 12 months of age there was a significant effect of genotype on weight for the subset of mice in cohort 2 from which tissue was collected for the c-fos study, with wild-type mice being significantly heavier than transgenic mice ($t_{(46)}$=2.668, p=0.011). This difference in weight is unlikely to have impacted on the behaviour of the mice used in this study, as although the result was significant the actual difference in weight was only ~3g between genotypes.

The average weights of mice included in each group of the longitudinal exercise study are presented in **Figure 2.2c**. A three-way ANOVA was used to analyse differences in weight between genotypes and exercise groups in cohort 3 across the longitudinal exercise study (presented in chapter 5). This revealed a significant main effect of both month ($F_{(6,354)}$=290.727, p<0.001) and genotype ($F_{(1,59)}$=36.739, p<0.001), but not exercise group ($F_{(1,59)}$=2.388, p=0.128). There was a also a significant three-way interaction between these factors ($F_{(6,354)}$=3.025, p=0.007), and all two-way interactions were also significant (all p<0.01). Follow-up of the three-way interaction with tests of simple main effects revealed a significant increase in weight for all mice across months 3-9, but a plateau for transgenic mice from months 10-12 with no significant change in weight across these months (Wt: p<0.001 for all months, Tg: p<0.05 for months 3-9). There was no significant difference in weight between transgenic mice in either the exercise or sedentary group across the study (all p>0.05), whilst for wild-type mice the sedentary group were significantly heavier than the exercise group from month 4 onwards (month 3: p=0.821, month 4: p=0.025, month 5: p=0.13, months 6-12: p<0.01). There was also a significant difference between genotypes within each exercise group; within the sedentary group there was a significant difference in weights between genotypes at all months (all p<0.001), however, this genotypic difference was only seen in mice in the exercise group for months 3, 11 and 12 (month 3: p=0.03, month 4-10: p>0.05, month 11: p=0.016, month 12: p=0.004).

These data suggest that whilst there was little difference between weights of mice in the exercising group and transgenic sedentary mice up to month 10 of this study, sedentary wild-

type mice tended to be heavier than all other test groups. For month 11 and 12, wild-type mice in the exercise group also weighed significantly more than transgenic mice from either group, but still less than sedentary wild-type mice. However, despite carrying more weight, sedentary wild-type mice performed as well as their exercising counterparts on tasks involving mobility (see chapter 5), suggesting that this difference in weight did not influence task performance.

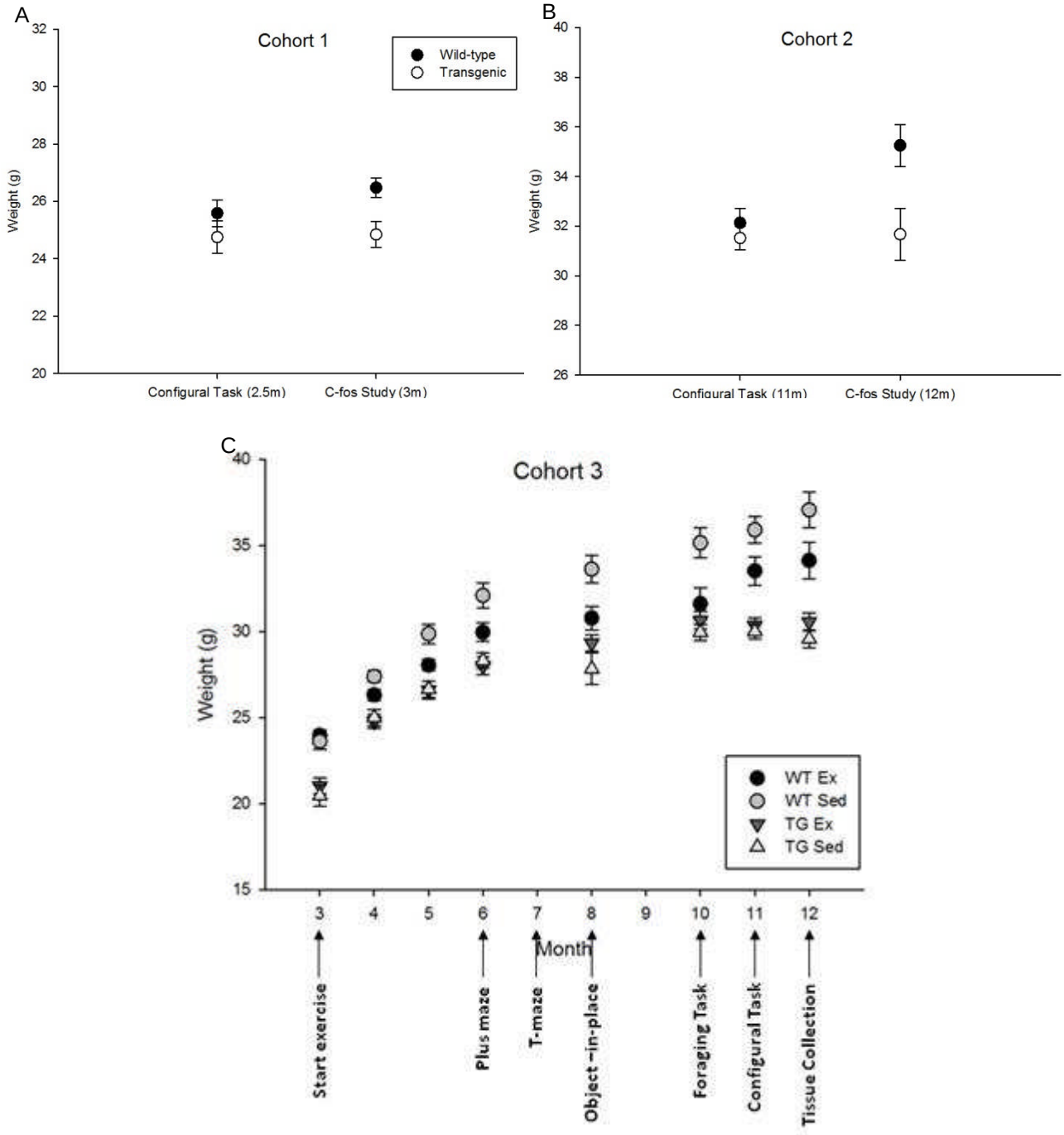

Figure 2.2: Experimental cohort weights. A: Cohort 1, tested at 2.5 and 3 months of age on the configural learning task and c-fos study. B: Cohort 2, tested at 11 and 12 months of age on the configural and elemental learning tasks and then used for the c-fos study. C: Cohort 3, used for a longitudinal study of the influence of exercise on cognition and pathology in tg2576 mice.

Chapter 3: Hippocampal activity in Tg2576 mice

3.1 Introduction

Whilst there is considerable evidence from transgenic models that excess amyloid production alters hippocampal morphology and synaptic plasticity (Chapman et al., 1999, Jacobsen et al., 2006), there is little evidence of how changes at the synaptic level may alter functional network activity, of the systems involved in learning and memory, to bring about the changes seen in behaviour (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002, Jacobsen et al., 2006). As reviewed in chapter 1, computational models of hippocampal function predict different roles for each sub-region in memory processing. However, the impact of amyloid pathology on the function of specific sub-regions has not previously been assessed. The main aim of this chapter is to address this question by analysing the expression of the immediate early gene (IEG) c-fos in the hippocampus of the Tg2576 model after exposure to a novel environment. It is expected that c-fos expression will be preferentially induced in hippocampal sub-regions involved in processing spatial novelty, and that transgenic mice will show a different pattern of activity to wild-type controls as a result of amyloid pathology.

Several studies using electrophysiological techniques have reported alterations in the DG and CA1 of APP transgenic models as a result of increased Aβ levels. Deficits in synaptic plasticity have been reported in the DG as early as 4 months of age and in CA1 at 12 months of age in the Tg2576 model (Chapman et al., 1999, Fitzjohn et al., 2001, Jacobsen et al., 2006). More recently, deficits in mossy fibre input to CA3 have also been reported in the Tg2576 model, although this was only assessed in 24 month old mice (Witton et al., 2010). Although electrophysiological measures provide clear synaptic physiology indices in hippocampal sub-regions, only a limited number of regions can be sampled at any one time. One of the key aims of the experiments described in this chapter is to compare activity between several regions across the brain at the same time point to see how they interact as a consequence of a learning experience. So far only one study has reported electrophysiological data from awake behaving APP transgenic mice, showing degraded spatial selectivity of place cells in the Tg2576 model (Cacucci et al., 2008), but the impact of

amyloid-pathology on simultaneous activity within the hippcampal network has not been extensively examined.

For the present set of experiments analysis of IEG expression was used to assess changes in neuronal activity, at single cell resolution, across multiple brain regions during a behavioural episode. IEGs are the first set of genes to be transcribed in response to neuronal activation (Tischmeyer and Grimm, 1999). There are two types of IEG; regulator IEGs which control the transcription of other genes, and effector IEGs which directly alter cellular and synaptic processes (Lanahan and Worley, 1998). IEGs which are of particular interest in the context of this thesis are the regulatory IEGs c-fos and zif268, and the effector IEG Arc. These IEgs have all been closely linked to learning and memory processes (Tischmeyer and Grimm, 1999, Kubik et al. 2007), and previous IEG studies linking IEG expression with behaviour have largely analysed either c-fos or zif268 (for reviews see Aggleton et al., 2012, Kubik et al. 2007). Although all three of these IEGS show similar expression patterns in response to behavioural activity there are subtle differences between these IEGs (Guzowski et al., 2001). C-fos expression, the most widely studied of these IEGS, is linked to neuronal activation during learning paradigms, whilst zif268 and Arc expression have been more closely associated with LTP processes (Abraham et al., 1992, Tischmeyer and Grimm, 1999, Kubik et al., 2007). Arc expression is found in the dendrites and the Arc protein is directly involved in regulating synaptic consolidation after behavioural experiences (Kubik et al,. 2007). Analysis of the expression of both c-fos, as a marker of overall neuronal activity, and either zif268 or Arc, as a marker of learning related plasticity, may provide a more detailed picture of neuronal network dysfunction in Tg2576 mice. However, for this thesis only c-fos expression was analysed, as pilot studies carried out using C57Bl6 mice indicated that the level of zif268 and Arc expression in the hippocampus were not sensitive to the behavioural paradigm used here. This may be due, in part, to the relatively high baseline level of zif268, and low level of Arc present in the hippocampus, making changes in expression of these IEGs less clear than changes in c-fos expression.

Studies of c-fos have shown that its expression within the brain is linked to neuronal activation (Dragunow and Robertson, 1987, Hunt et al., 1987, Morgan et al., 1987), and more specifically the activation of NMDA, AMPA and metabotrophic glutamate receptors (Vaccarino et al., 1992). Changes in c-fos expression have also been linked to learning and memory processes, with early studies reporting increases in c-fos expression after Y-maze (Tischmeyer et al., 1990) and avoidance learning (Nikolaev et al., 1992). In addition,

disruption of c-fos expression using either antisense oligonucleotides or a conditional CNS knock-out leads to disruption of spatial and associative learning, conditioned taste aversion and hippocampal LTP (Fleischmann et al., 2003, Yasoshima et al., 2006).

Few previous studies have assessed changes in IEG expression in transgenic APP models. Those which have largely reported reduced c-fos expression as a consequence of amyloid pathology. For example, decreased c-fos expression has been reported in all hippocampal sub-regions in transgenic mice expressing the APPswe/ind gene (Lee et al., 2004b), and more specifically reduced c-fos expression in the DG correlated with deficits in performance in the Morris water maze (Palop et al., 2003) and cross-maze learning (DeIpolyi et al., 2008). In the Tg2576 model Poirier et. al. (2011) assessed c-fos expression in the retrosplenial cortex, a structure in the cingulate cortex which is highly connected to the hippocampus. This study reported decreased levels of c-fos expression in the retrosplenial cortex of transgenic mice compared to wild-types from as early as 5 months of age, although c-fos expression increased above wild-type levels by 23 months of age. Although these studies generally show decreased IEG expression as a function of amyloid pathology, all of these studies have assessed baseline levels of IEG activity and not learning related induction of c-fos expression. This is of particular interest as the induction of IEGs during learning episodes is thought to be necessary for the production of new stable memories.

One study by Dewachter et. al. (2009) has assessed induction of c-fos expression after cued or contextual fear-conditioning in APPlon transgenic mice. This study reported impairments in c-fos induction in the basolateral amygdala and hippocampal CA1 region. However, the data did not distinguish between the cued or contextual conditioning paradigm, although historically contextual conditioning is thought to be hippocampus-dependent and cued conditioning is not (Kim and Fanselow, 1992, Phillips and LeDoux, 1992), and did not provide any behavioural measures to correlate with alterations in c-fos induction. A more recent study by Lelos & Good (2012), also assessed c-fos induction in the amygdala and hippocampus induced by cued fear conditioning. This study showed abnormal c-fos induction in the DG of 19 month old transgenic mice, and used structural equation modelling (SEM) to show functional disconnection between amygdala and hippocampal circuitry, despite no differences in behaviour between genotypes. However, although this study highlights changes in the circuitry underlying emotional processing and fear conditioning, it does not assess changes in c-fos expression in a task specifically designed to activate hippocampal networks. In this chapter, changes in hippocampal neural activity in the Tg2576

mice are assessed using a task designed to specifically induce hippocampal c-fos expression, as described below.

Several studies, mainly in rats, have assessed learning induced IEG expression in a variety of behavioural tasks. Here a summary of tasks that specifically activate c-fos expression in the hippocampus are described. Some of the earliest work assessing learning induced changes in IEG expression demonstrated dissociations between the hippocampus and perirhinal cortex. More specifically, increased expression of c-fos has been observed in the perirhinal cortex and area TE following exposure to novel stimuli, whereas changes in hippocampal c-fos expression are observed when novel spatial arrangements of familiar stimuli are presented (Zhu et al., 1995, Wan et al., 1999, Jenkins et al., 2004, Aggleton and Brown, 2005). Increased c-fos expression has also been seen in the hippocampus after re-arrangement of familiar room cues (Jenkins et al., 2004) and exposure to completely novel room cues (Vann et al., 2000, VanElzakker et al., 2008, Rinaldi et al., 2010). The study by Vann et. al. showed specific increases in dorsal hippocampus c-fos expression after exposure to a novel learning environment during a radial arm maze task. Another study reported increases in hippocampal c-fos mRNA expression after exploration of a novel environment (Hess et al., 1995), and more specifically increases in CA1 and DG c-fos expression with gross changes in environmental novelty, but only in CA1 after small modifications to a familiar environment (Sheth et al., 2008). Together these data suggest that spatial novelty can induce robust increases in c-fos induction in hippocampal sub-regions. These findings are in line with computational models which suggest that the hippocampus is involved in both spatial memory and detection of novel events (see section 1.5).

The studies described above analyse differences in activity in individual brain regions between test conditions by comparing mean c-fos cell count between different groups for each region, treating each region as an independent element. However, neural function is likely to rely heavily upon the interactions between different regions. To make full use of IEG mapping data structural equation modelling (SEM) can be used to account for the interactions between different neural elements of a network. Recently several studies have combined IEG expression data with SEM to suggest functional pathways and networks engaged during behavioural paradigms. Two of these studies are of particular interest as they assess hippocampal sub-region engagement during tasks related to novelty. Poirier et. al. (2008) used expression of the IEG zif268 to show differences in hippocampal recruitment during early- and late-training phases on a radial arm maze task. Although no overt

differences were seen in IEG expression between test groups, SEM revealed DG engagement via the tri-synaptic hippocampal pathway during early-training when the task is still novel, but not during late-training when the task becomes more familiar, favouring instead the direct perforant pathway from the entorhinal cortex to CA1. This difference in hippocampal network activation was further supported by evidence from Albasser et. al. (2010) who showed the same distinction between pathways, but this time with the tri-synaptic pathway recruited during exploration of novel objects, and the temporoammonic pathway recruited when exploring familiar objects. Both of these studies suggest that different networks of hippocampal sub-regions may be recruited when processing novel (trisynaptic pathway) or familiar (entorhinal-CA1) stimuli.

On the basis of previous studies, linking both spatial exploration and novelty to increased c-fos expression in the hippocampus, it was hypothesised that a task involving spatial novelty should induce robust increases in c-fos expression in the hippocampus. Therefore, the task chosen to assess hippocampus activity patterns in the Tg2576 model was exploration of an environment with either a familiar or novel extra-maze cues provided by two different test rooms. Spontaneous exploration of an arena in each room was used as this task does not have any specific learning requirements. Inclusion of a learning criterion, for example a radial arm maze task, would lead to behavioural deficits in transgenic mice compared to wild-types, and therefore differences in behaviour would confound any conclusions drawn from changes seen in c-fos expression. A study by Barnes et. al. (2004) demonstrated that Tg2576 mice can discriminate between rooms with different extra-maze cues using a food reward task.

The main aim of this experimental chapter is to assess spatial novelty induced changes in hippocampal c-fos expression in the Tg2576 model as a consequence of amyloid pathology. The experimental hypothesis was that Tg2576 mice will show altered hippocampal activity patterns when encoding of novel spatial stimuli as a result of amyloid-induced alterations in DG and CA1 synaptic plasticity and morphology.

3.2 Experiment 1: Activation of Hippocampal Neural Networks in C57Bl6 mice

Introduction

Previous research has shown that spatial novelty specifically increases hippocampal neuronal activity in rodents (Hess et al., 1995, Vann et al., 2000, Jenkins et al., 2004). These studies all used paradigms in which the rats had to learn about the spatial cues in order to solve a task, either a radial arm maze (Vann et al., 2000, Jenkins et al., 2004), or odour discrimination (Hess et al., 1995). The main aim of Experiment 1 was to assess whether spontaneous exploration of a novel spatial environment, in the form of a novel test room providing different extra-maze cues, would influence behaviour and c-fos expression in the hippocampus of C57Bl6 mice. It is hypothesised that mice exposed to spatial novelty will show greater c-fos expression in the hippocampus relative to those exposed to a familiar room.

C-fos expression was measured, using immunohistochemistry, in the hippocampal sub-regions, CA1, CA3 and DG, and also in the connecting parahippocampal regions. These regions were included as they provide the input and output pathways to and from the hippocampus and therefore may reveal upstream and downstream changes related to alterations in hippocampus activity. Counts of c-fos expressing cells in the hippocampus were divided into septal, intermediate and temporal regions as there is evidence that these regions of the hippocampus may have functionally distinct roles, with the septal and intermediate regions having a greater role in processing spatial information (Moser and Moser, 1998). C-fos expression in the auditory cortex was measured as a control. C-fos expression in this region should not be altered as a function of spatial novelty as background auditory stimuli were equal in both environments, therefore c-fos expression in this region should be equivalent between groups.

The second part of this analysis uses c-fos expression data to model hippocampal neural networks involved in processing novel and familiar spatial information through structural equation modelling (SEM). Structural equation models are multiple-equation regression models representing possible functional interactions between variables, allowing analysis of anatomically connected regions across whole networks. SEM was originally developed to analyse social and economic data, but its potential for neuroscience has been demonstrated as

a method of showing functional neural interactions using 2-deoxyglucose and fMRI data to measure neural activity (McIntosh and Gonzalez-Lima, 1991, Buchel and Friston, 1997). More recently, SEM has also been applied to c-fos expression data, using raw c-fos cell count data to compare theoretical models of neural activity within brain networks by assessing relationships between correlated (or uncorrelated) variables (Jenkins et al., 2004, Poirier et al., 2008, Albasser et al., 2010, Lelos and Good, 2012).

Methods

Subjects: 24 male C57Bl6 mice, aged 6-7 months were used. However, one mouse was found to have abnormal brain morphology and therefore this subject and its pair were removed from the analysis.

Apparatus: Two different test rooms, A and B shown in **Figure 3.1**, were used. Each room contained different equipment and visual cues along the walls, both were illuminated by overhead lighting. Identical square arenas were placed in the centre of each test room on a table 82cm above the ground. These were made of Perspex with a wooden floor painted grey, measuring 60 x 60cm, with 40cm walls. The arena was cleaned down with ethanol wipes between test sessions to remove odour cues. During all habituation and test sessions movement was tracked using Ethovision software connected to a video camera mounted above the arena.

Behavioural procedure: Mice were habituated to the testing routine and the arena in either room A or room B for three days. On each of these days mice were moved in their home cage to a quiet dark room for 30 minutes after which they were taken to test room A or B, placed into the centre of the arena and allowed to explore the arena for 15 minutes. After this they were returned to their home cages in the dark room for 90 minutes before being returned to the holding room. During habituation mice were yoked in pairs so that half of the mice were exposed to each room, either A or B.

On the fourth day the procedure was identical to the habituation days; subjects were moved into a dark room for 30 minutes, and then allowed to explore the arena for 15 minutes.

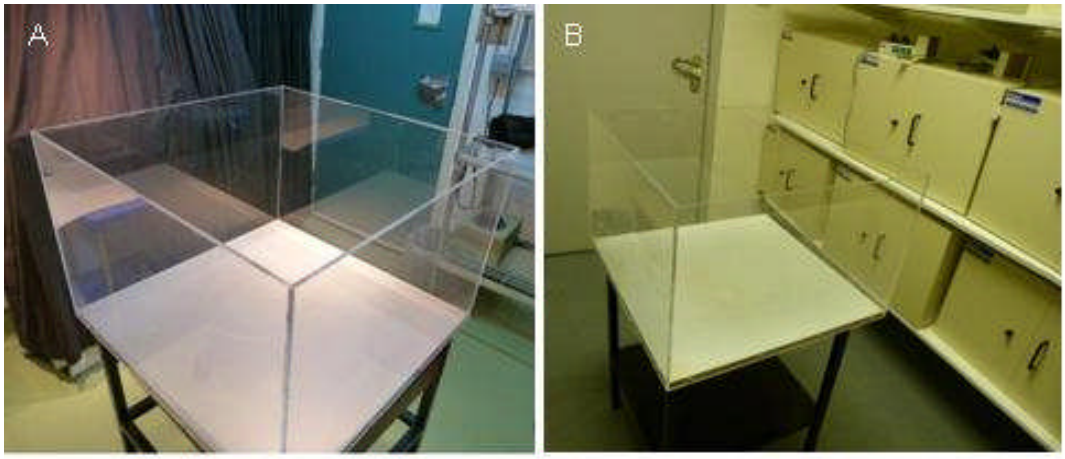

Figure 3.1: Test rooms A and B. Rooms were standard animal testing rooms of similar size containing different extra-maze cues around the room. Identical Perspex arenas were placed in the centre of each room.

However, during testing both mice within a yoked pair were exposed to the same room, therefore providing a novel room for half of the mice whilst presenting a familiar room to the other half. The room used on test day was counterbalanced across groups so each room, A or B, was used an equal number of times at test. After exposure to the arena mice were placed back in their home cage in a dark room for 90 minutes to reduce IEG activation by any other stimuli than those presented during the training procedure. After this period the mice were sacrificed and tissue was collected for histological analysis.

The movement of each mouse during testing was tracked using Ethovision software. Measurements of the distance travelled by each mouse and the amount of time spent moving during each session were recorded for each mouse, and then a mean for each group, novel or familiar, was calculated.

Histology: Mice were deeply anaesthetised by intra-peritoneal injection of 0.2ml of Euthatal (sodium pentobarbitone). Mice were then exsanguinated by insertion of a cannula into the left ventricle and perfusion with 50ml of 0.1M PBS (pH 7.4). This was followed by perfusion with 80ml of cold 4% paraformaldehyde in 0.1% PBS (PFA). The brain was then extracted and fixed for 3 hours in 4% PFA before being transferred to 30% sucrose solution overnight at room temperature. Brain tissue was then mounted onto a freezing microtome at -20^{o}C and cut into 40µm coronal sections. Tissue sections were stored at -20^{o}C in an ethylene-glycol based cryoprotectant until used for immunohistochemical analysis.

During processing the tissue from each yoked pair was stained in the same basket to reduce variation between the familiar and novel groups due to differences in staining intensity between each batch of tissue being processed. Tissue was transferred into baskets containing 0.1M PBS and washed 4 x 10 minutes with PBS to remove cryoprotectant before immunohistochemistry was carried out. Sections were blocked in 0.3% hydrogen peroxide in 0.1M PBST for 10 minutes on a stirrer at room temperature to inhibit endogenous peroxidise activity. After washing 4 x 10 minutes in PBST primary antibody for c-fos (c-fos (4) sc-52, 1:3000, Santa Cruz Biotechnology Inc.) was added and incubated for 48 hours at 4^{o}C. Sections were then washed 4 x 10 minutes in PBST, and incubated in goat-anti rabbit biotinylated IgG (1:200 in PBST, Vector Laboratories) with 1.5% normal goat serum for 2 hours at room temperature. Sections were washed 4 x 10 minutes in PBST then immersed in avidin-biotinylated enzyme complex (Vectastain Elite ABC, Vector Laboratories) made up of 1% reagent A and 1% reagent B in PBST for 1 hour at room temperature. Tissue was washed 4 x 10 minutes in PBST then 2 x 10 minutes in 0.05M Tris buffer and then stained with 3,3-diaminobenzidine (DAB) with nickel for 1-3 minutes (DAB substrate kit, Vector Laboratories). The reaction was stopped by addition of cold 0.1M PBS and sections were mounted onto gelatine-coated slides and allowed to air-dry overnight. Slides were dehydrated in an ascending series of alcohol concentrations and cleared in xylene before being cover-slipped using Di-n-butyl phthalate (DPX) mounting medium. An example of tissue stained for c-fos immunoreactivity using this protocol is shown in **Figure 3.2**.

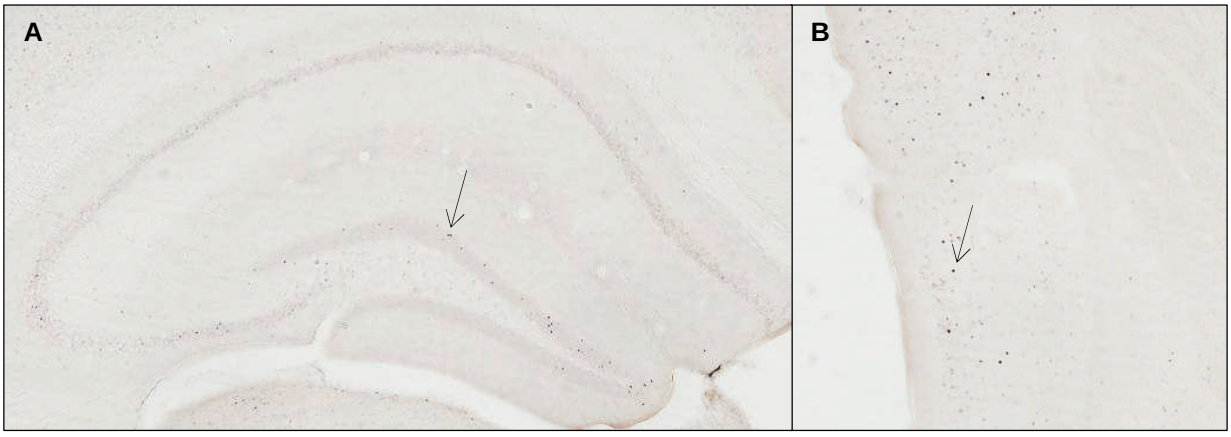

Figure 3.2: C-fos immunoreactivity in A: hippocampus and B: perirhinal cortex of a wild-type mouse. Arrows point to c-fos positive cells.

Cell Counting and Regions of Interest: Sections were photographed using a Leica DMRB microscope and Olympus DP70 camera with a 5x objective. The microscope was connected to a computer, and the images were captured and stored using AnalysisD software (Soft-Imaging Systems).

Cells were counted using ImageJ software. Cell counts were made for the fourteen regions of interest (ROIs) shown in **Figure 3.3** between the coordinates in **Table 3.2**. In some cases sections were excluded on the basis of poor staining or tissue tearing. For each section the regions of interest were defined by hand, and the number of immunoreactive cells within these regions was determined using a gray-scale intensity threshold. This threshold was defined for each yoked pair, using the first image from this pair to set the threshold at a suitable value for the level of staining and then maintaining the threshold for all subsequent images as described in Albasser et. al. (2010).

Cell counts were measured from 8 sections (four left, four right) for each ROI. For each subject a mean cell count was calculated for each ROI by averaging the 8 individual counts. For statistical analysis the individual ROIs were grouped into; septal hippocampus (DG, CA3, CA1), intermediate hippocampus (DG, CA3, CA1), temporal hippocampus (CA3, CA1), perirhinal cortex (rostral, caudal), entorhinal cortex (medial, lateral), subiculum (dorsal, ventral) and the auditory cortex.

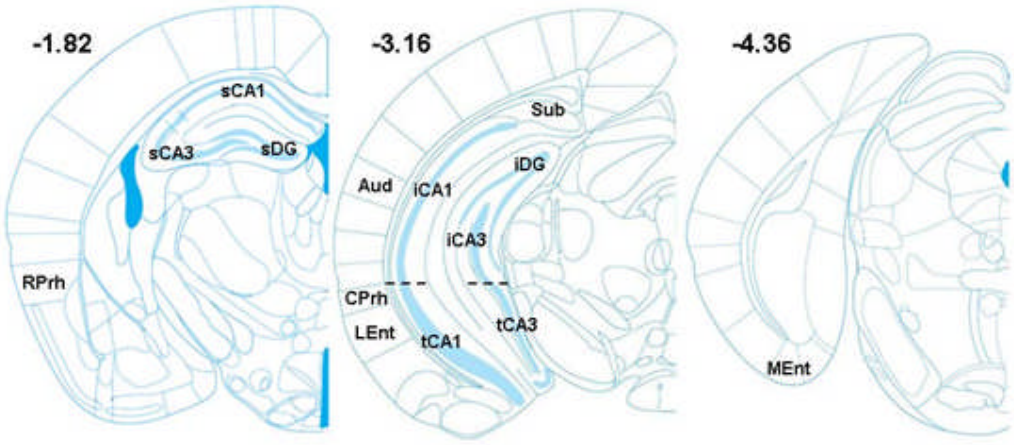

Figure 3.3: Regions of Interest. Location of each region of interest for which c-fos immunoreactive cells were counted (images taken from Franklin and Paxinos, 2007). sDG: septal dentate gyrus, sCA3: septal CA3, sCA1: septal CA1, iDG: intermediate dentate gyrus, iCA3: intermediate CA3, iCA1: intermediate CA1, tCA3: temporal CA3, tCA1: temporal CA1, RPrh: rostral perirhinal cortex, CPrh: caudal perirhinal cortex, Lent: lateral entorhinal cortex, MEnt: medial entorhinal cortex, Sub: dorsal subiculum, Aud: auditory cortex.

Table 3.2: Region of Interest Coordinates. Coordinates for the start and end section of each ROI with respect to Bregma. Each ROI was defined for tissue sections between these coordinates (taken from Franklin and Paxinos, 2007) and number of c-fos immunoreactive cells in this region was counted.

Region of Interest	Start Coordinate (from Bregma)	End Coordinate (from Bregma)
Septal DG	-1.46	-2.18
Septal CA3	-1.46	-2.18
Septal CA1	-1.46	-2.18
Intermediate DG	-2.46	-3.16
Intermediate CA3	-2.46	-3.16
Intermediate CA1	-2.46	-3.16
Temporal CA3	-2.92	-3.40
Temporal CA1	-2.92	-3.40
Rostral Perirhinal Cortex	-1.46	-2.18
Caudal Perirhinal Cortex	-2.92	-3.64
Lateral Entorhinal Cortex	-2.92	-3.64
Medial Entorhinal Cortex	-4.04	-4.48
Dorsal Subiculum	-2.80	-3.52
Ventral Subiculum	-3.28	-3.64
Auditory Cortex	-2.46	-3.16

Data analysis and statistics: Statistical analysis was carried out using SPSS software. Behavioural data were analysed using a repeated measures ANOVA, with test day as the within subjects factor and test group as the between subjects factor. An independent samples t-test was used to compare percentage change in exploratory activity. C-fos expression data, using raw c-fos immunoreactive (IR) cell counts, were also analysed using repeated measures ANOVA, with region as the within subjects factor and test group between subjects. In cases where the interaction was significant this was followed up with tests of simple main effects to look at the effect of one independent variable on the other within each group. To account for differences in the level of staining between yoked pairs a novelty ratio was calculated for each pair by dividing the cell count for the mouse in group novel by the total cell count from both mice in the pair. A value significantly different from 0.5 indicates a difference in c-fos induction between group novel and familiar. This was assessed using one-sample t-tests with Bonferroni correction for multiple comparisons.

Structural Equation Modelling

Structural equation models are multiple-equation regression models representing possible functional interactions between variables. SEM tests how well a pre-specified set of assumptions about the linear relationships between variables (i.e. the model) is able to reproduce the covariance matrix of the observed sample (i.e. is the model plausible given the data?). It is worth noting at this point that SEM can only be used to reject implausible models, and cannot prove that any particular model is correct.

The goal of SEM is to obtain a solution to a set of structural equations (derived from the model) which minimises the difference between the observed covariances from the data and those implied by the solution to the model. This process begins by giving starting values to the unknown parameters and then continuing with successive iterations until an optimal estimate is achieved. At each iteration the implied covariance matrix is compared to the observed matrix. The fit of each estimate can be assessed using a Chi^2 distribution comparing the observed and estimated covariance matrix.

There are two main considerations when constructing a model. The first is that the model must be based on anatomical connectivity. However, brain regions are highly interconnected and therefore a model must be reached which compromises between anatomical accuracy and the ability to interpret the model. The second is that if all connections in the model are reciprocal (as in many brain structures) there are too many unknown parameters and it is impossible to find a single numerical solution to the model. This type of model is known as being under-identified. Therefore when assessing models the most parsimonious model which is anatomical plausible and accounts for the data should be chosen. SEM also allows unmeasured variables to be incorporated into the model as residuals. These residuals, or error variances, can be thought of as accounting for the influence of brain regions not included in the model, and other sources of variation introduced by the experimental design.

Separate models were constructed to represent novel or familiar information processing. The aim was to assess whether there was any difference between groups for either a model exclusively representing processing of familiar information or a model exclusively representing processing of novel information.

Model Production: Path analysis of proposed neuronal network models was carried out using AMOS 6.0 software package. In SEM the number of regions in each model is limited by the number of subjects in each study, and in turn by the number of degrees of freedom in the model. In this study computational path analyses used maximum likelihood estimation (MLE: Kelloway, 1998) by an iterative method; the most consistent method of statistical estimation. MLE estimates all parameters in the model simultaneously.

An initial model was suggested for each analysis based upon anatomical connectivity and theory (Witter et al., 2000). After an initial estimation of fit, the model was adjusted to determine the best possible fit, while remaining anatomically viable. A best fit model was initially determined separately for mice in the familiar and novel conditions. Both data sets were then fitted individually to each model to determine if they gave a good fit. A comparison was then made between optimal models for each group using a multiple model comparison, tested by the χ^2 difference test. For multiple model comparison a null functional model in which all the path coefficients were set to be equal between groups was compared to an alternative functional model in which path coefficients were allowed to vary across groups If the χ^2 value was significantly different between models then the path coefficients which were allowed to vary between models were significantly different between groups. Comparisons of models in which path coefficients were either constrained or unconstrained across models serves as an omnibus test of changes in functional interactions. The significance of individual connections within each model was tested by constraining each path in turn and assessing the Chi^2 difference between models. A significant difference between the null model in which the path was constrained and the alternative model in which it was allowed to vary between conditions indicates a difference in functional interaction of this pathway between groups. However, if the omnibus test is not significant, caution must be used when interpreting differences in individual paths.

Model Fit: To determine how well each anatomically and theoretically driven model fits the data several indices of goodness-of-fit can be considered (Fan et al., 1997, Hu and Bentler, 1998). The χ^2 value assesses the difference between the matrix of covariances from the sample data compared to those produced by the model specifications. A non-significant χ^2 value suggests no difference between the two, and therefore the sample data fits the model. However, this method is limited by its reliance upon sample size (n). The power of the test

increases with sample size, therefore a study with a smaller n may fail to detect subtle differences between the covariance matrices. Therefore other fit indices can be used to assess the model, although it should be noted that path strength remains accurate even with smaller sample sizes (Bouchard et al., 2007).

In cases where n is small, as in this study, three additional suitable fit indices are the comparative-fit-index (CFI), the goodness-of-fit index (GFI), and the root mean square error of approximation (RMSEA) (Fan et al., 1997, Hu and Bentler, 1998). The CFI and GFI measures range from 0 to 1, where 1 indicates a perfect fit of the model with the data. The GFI assesses the proportion of variance accounted for by the estimated covariance matrix, and then compares this to the covariance matrix from the observed data. The CFI compares the sample covariance matrix to a null model, in which all variables are uncorrelated. Both GFI and CFI show a bias with smaller samples, making them more conservative fit indices. RMSEA determines how close a model is to the 'true' model by calculating the error of approximation between the model being tested and one with optimally chosen parameter estimates. It is based upon analysis of the residuals rather than path strength, with smaller values representing a better fit. To consider a model a good fit, and therefore to represent a plausible network activity pattern, χ^2 should be greater than 0.05, GFI and CFI greater than 0.9, and RMSEA less than 0.05 (Tabacknik and Fidell, 1996).

Limitations of SEM: Although SEM provides more detailed information regarding the functional interactions between brain regions, caution should be taken when interpreting results of this analysis. SEM can only be used to discount implausible models of functional connectivity, and cannot be used to prove that any particular model is correct. Another major caveat with SEM is that models only show correlations between brain regions and should not be interpreted as causality. Thus findings must be validated with other *in vivo* and *ex vivo* techniques to interpret causality. The direction of effect for pathways included in structural models is based upon the strongest anatomical connectivity between regions, although reciprocal connections have been described for most regions (van Strien, 2009). Including all reciprocal connections would make the models under-identified and therefore there would be more than one solution to each model. Another limitation of SEM is its reliance on sample size. Kline (2005) suggested that for SEM at least 10 samples were needed per free parameter. The experiments in this thesis do not have such large sample size and therefore

may be less statistically robust, however the use of fit indices including CFI, GFI and RMSEA make the conclusions more robust as these indices are more conservative for small sample sizes.

Results Experiment 1a: Behaviour

Exploratory activity during each test session was assessed using two measures of locomotor activity, the distance moved by each mouse during the test session and the time spent moving during each session. These data, summarised in **Figure 3.4 a,b**, show that exploratory activity in both groups habituated across days 1-3. On the final test day significant increases in activity were shown by group novel indicating that the mice were able to detect the novel environment. Repeated measures ANOVA of the distance moved during each test session showed a significant main effect of test day ($F_{(3,60)}$=8.277, p<0.001) but not group ($F_{(1,20)}$=0.002, p=0.968) and a significant interaction between these factors ($F_{(3,60)}$=5.807, p=0.001). Tests of simple main effects revealed a significant difference between mice in the novel or familiar group only on day 4 (day1-3: p>0.05, day 4: $F_{(1,20)}$=4.992, p=0.037).

The same pattern of exploratory behaviour was seen using measurements of the amount of time spent moving, with a significant main effect of day ($F_{(3,60)}$=6.767, p=0.001), but not group ($F_{(1,20)}$=0.005, p=0.945), and a significant interaction between these factors ($F_{(3,60)}$=4.019, p=0.011). Again tests of simple main effects showed a significant difference between groups only on test day 4 (day 1-3: p>0.05, day 4: $F_{(1,20)}$=4.114, p=0.046).

Exploratory behaviour was also expressed as a percentage change between test day 3 and 4 for each mouse, see **Figure 3.4c,d**. This reduces the variation due to individual differences in the overall level of activity between mice. There was an increase in exploratory behaviour between days 3 and 4 in group novel, which contrasts to the decrease seen in group familiar. Independent samples t-test revealed significant differences in percentage change for both the distance moved ($t_{(20)}$=5.038, p<0.001, **Figure 3.4c**) and the time spent moving ($t_{(20)}$=3.219, p=0.004, **Figure 3.4d**) between mice in the novel and familiar group.

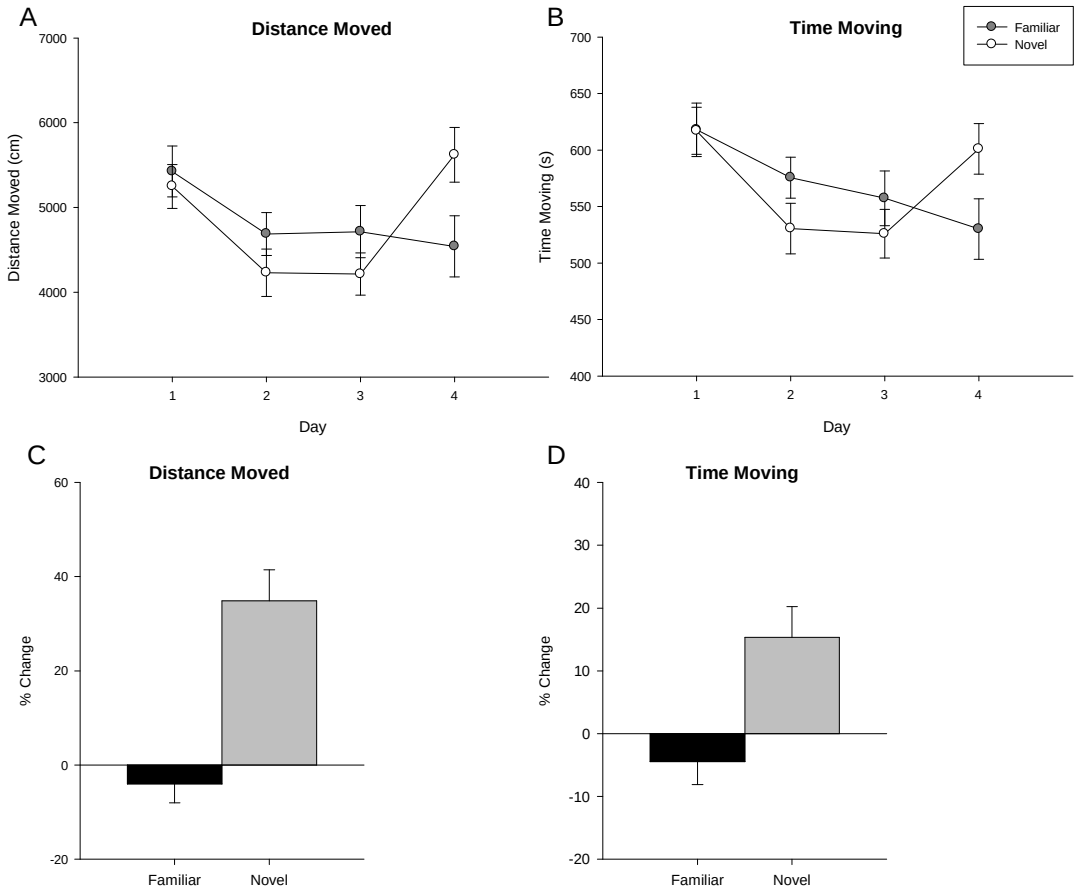

Figure 3.4: Locomotor activity in C57Bl6 mice. A**:** Distance moved during each test session. B: Time spent moving during each test session. Symbols represent the mean ± SEM (n=11). C: Percentage change in the distance moved on test day 4 compared to day 3. D: Percentage change in the time spent moving on day 4 compared to day 3. Bars represent the mean ± SEM.

Results Experiment 1b: C-fos Expression

Septal Hippocampus: C-fos expression data for sub-regions of the septal hippocampus are shown in **Figure 3.5a**. Numerically c-fos expression was increased in the DG and CA1 sub-regions in group novel compared to group familiar, but there was little difference between groups in the CA3 sub-region. The CA3 sub-region showed the highest levels of c-fos IR cells, whilst CA1 showed the lowest counts. Repeated measures ANOVA showed a significant effect of region ($F_{(2,40)}$=26.796, p<0.001) but not group ($F_{(1,20)}$=0.494, p=0.490) and no significant interaction between these factors ($F_{(2,40)}$=1.663, p=0.202).

Intermediate Hippocampus: **Figure 3.5b** shows c-fos expression data for the intermediate hippocampus. Inspection of this figure shows an increase in c-fos IR cell counts across all

three sub-regions in group novel compared to group familiar. The DG showed the lowest c-fos IR cell counts, whilst CA3 and CA1 were approximately equal. Repeated measures ANOVA revealed a significant main effect of region ($F_{(2,40)}$=10.978, p<0.001) and also group ($F_{(1,20)}$=7.410, p=0.013), but no significant interaction between these factors ($F_{(2,40)}$=1.294, p=0.285).

Temporal Hippocampus: C-fos expression data for the temporal section of the hippocampus are shown in **Figure 3.5c**. There was no significant difference in c-fos IR cell count in the novel group compared to familiar, although CA1 has higher cell counts than CA3. Repeated measures ANOVA revealed only a significant effect of region ($F_{(1,20)}$=9.804, p=0.005), but not test group ($F_{(1,20)}$=0.539, p=0.471) and no significant interaction between these factors ($F_{(1,20)}$=0.059, p=0.810).

Subiculum: C-fos expression data for the subiculum are shown in **Figure 3.5d**. Inspection of this figure shows little difference between group novel and familiar, but does show a higher IR cell count in the ventral subiculum compared to the dorsal. Repeated measures ANOVA showed a significant main effect of region ($F_{(1,20)}$=33.409, p<0.001) but not group ($F_{(1,20)}$-=0.921, p=0.349) and no significant interaction between these factors ($F_{(1,20)}$=0.035, p=0.853).

Perirhinal Cortex: **Figure 3.5e** shows c-fos expression data for the perirhinal cortex. There was no significant difference in c-fos IR cell count between group novel and familiar, or between regions. Repeated measures ANOVA revealed no significant effect of region ($F_{(1,20)}$=2.409, p=0.136) or group ($F_{(1,20)}$=0.003, p=0.956) and no significant interaction between these factors ($F_{(1,20)}$=1.492, p=0.236).

Entorhinal Cortex: C-fos expression data for the entorhinal cortex is shown in **Figure 3.5f**. There is little difference between groups novel and familiar, although there was a greater number of c-fos IR cells in the lateral compared to the medial entorhinal cortex sub-region. Repeated measures ANOVA showed a significant main effect of region ($F_{(1,20)}$=40.320, p<0.001) but not group ($F_{(1,20)}$=0.066, p=0.799) and no significant interaction between these factors ($F_{(1,20)}$=0.079, p=0.781).

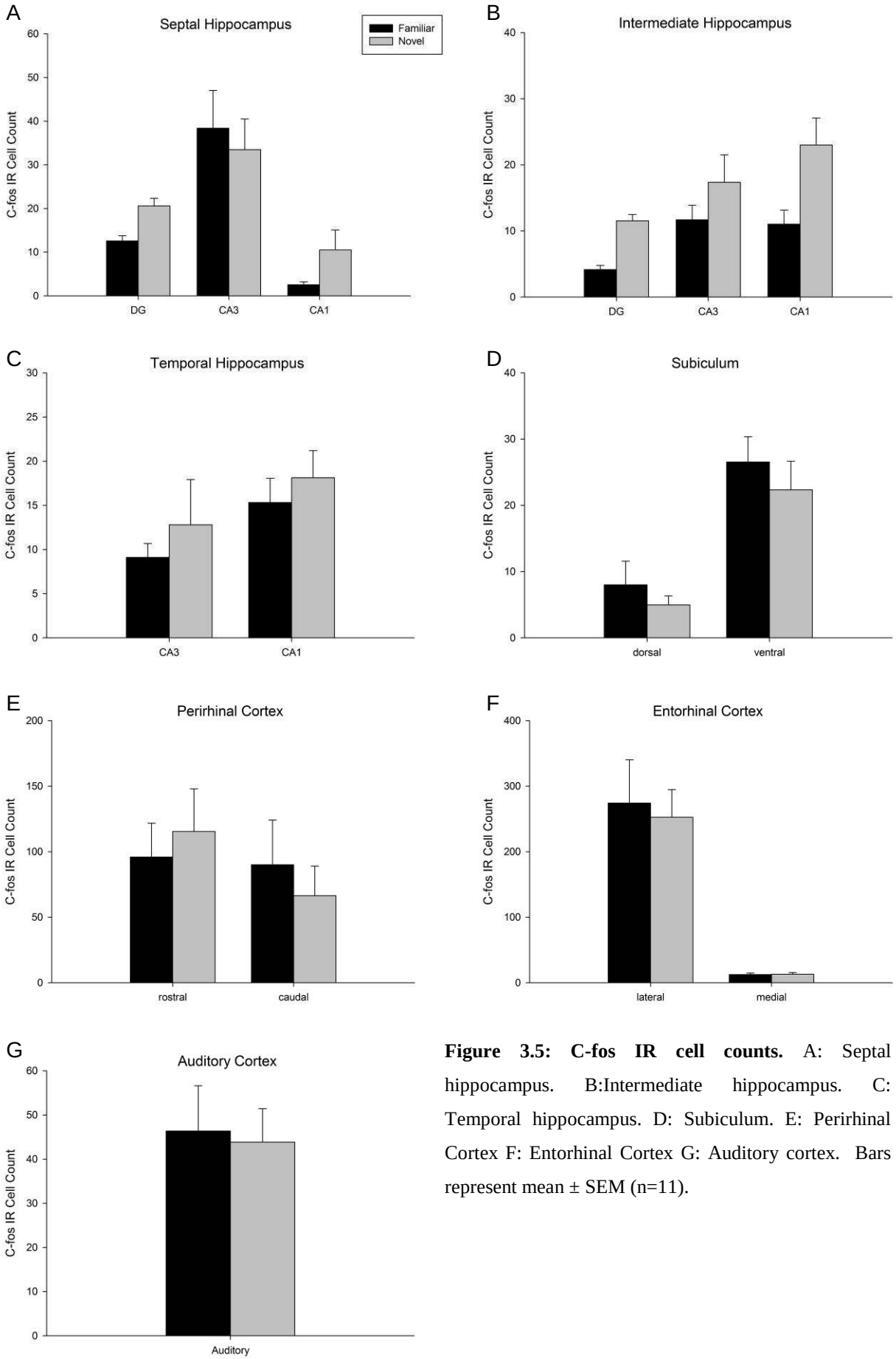

Figure 3.5: C-fos IR cell counts. A: Septal hippocampus. B:Intermediate hippocampus. C: Temporal hippocampus. D: Subiculum. E: Perirhinal Cortex F: Entorhinal Cortex G: Auditory cortex. Bars represent mean ± SEM (n=11).

Auditory Cortex: **Figure 3.5g** shows c-fos expression data for the primary auditory cortex. This region was used as control region as no changes in c-fos IR were expected in the auditory cortex between mice exposed to a novel or familiar spatial environment. Inspection of the figure shows this to be true, with no difference in c-fos IR cell count between group novel and familiar ($t_{(20)}$=0.197, p=0.846).

Novelty Ratio: A novelty ratio was calculated for each region to reduce the influence of differences in c-fos staining intensity between pairs, and any influence that testing on a different day may have had between pairs. This was calculated for each yoked pair based on the c-fos IR cell count for the mouse in group novel divided by the total IR cell count for the pair. Therefore values above 0.5 indicate increases in c-fos expression in response to novelty and values below 0.5 indicate decreases in c-fos expression in response to novelty. The mean ratio for each region is shown in **Table 3.2**. Inspection of this table reveals that novelty increased c-fos expression specifically in the DG and CA1 regions of the septal and intermediate hippocampus, but not any sub-region of the temporal hippocampus, subiculum or parahippocampal cortices.

Table 3.2: Novelty ratios for C57Bl6 mice. Ratios are based on the c-fos IR cell count for mice in group novel/total cell count across both groups. Numbers represent mean (SEM). Statistical comparison using one-sample t-test compared to 0.5, i.e. no difference in cell count between groups novel and familiar. Significant results are shown in bold, *p<0.05, **p<0.01, ***p<0.001. Increased c-fos expression was seen in the DG and CA1 regions of the septal and intermediate hippocampus.

	Septal Hippo			Intermediate Hippo			Temporal Hippo		Entorhinal		Perirhinal		Subiculum		Control
	DG	CA3	CA1	DG	CA3	CA1	CA3	CA1	lateral	medial	rostral	caudal	dorsal	ventral	auditory
Novelty Ratio	**.620*** (.020)**	.499 (.042)	**.738** (.059)**	**.732*** (.031)**	.579 (.045)	**.662** (.039)**	.511 (.073)	.547 (.062)	.545 (.063)	.505 (.041)	.545 (.040)	.491 (.082)	.442 (.037)	.446 (.047)	.485 (.041)

Results Experiment 1c: Structural Equation Modelling

Structural equation modeling was used to test models of functional hippocampal networks induced by exposure to either a familiar or novel spatial environment.

Correlations

Models of relationships between brain regions were derived for group novel and group familiar from the correlations across brain regions for the mean raw c-fos IR counts (**Table 3.3**). Inspection of this table shows more significant correlations in c-fos expression between brain regions in mice in group familiar than group novel, and a greater correlation between behavioural measures and c-fos expression in group familiar. Increased correlation gives increased power to test a model. Although very high inter-regional correlations suggest some degree of multi-collinearity between regions, this does not impact the predictive value of structural models.

Structural Models

To test differences between the two groups both data sets were first fitted to a baseline model which included all regions and all connections of the tri-synaptic pathway and perforant pathways (**Figure 3.6**). Initially pooled data from all the mice was fitted to this model. This model did not fit well ($\chi^2_{(8)}$=15.611, p=0.048, RMSEA=0.213, CFI=0.787, GFI=0.823), showing that although there may be anatomical connectivity, this does not necessarily mean a strong functional connectivity. Next each data set from group novel and group familiar were individually fitted to this model and then the model was adapted to give the best possible fit. Models were rejected if they did not have established anatomical connectivity between regions.

Table 3.3: Inter-region correlations of c-fos IR counts for C57Bl6 mice. Group familiar, standard test. Group novel, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, all significant correlations are shaded dark grey.

		dDG	dCA3	dCA1	cPrh	lEnt	dSub	distance day 4	time day 4	distance change	time change
dDG	r		0.365	0.445	0.631*	0.389	-0.326	-0.307	-0.406	0.061	-0.231
	p		0.270	0.170	0.037	0.237	0.327	0.359	0.215	0.859	0.494
dCA3	r	**0.594**		0.607*	0.799**	0.633*	-0.306	-0.512	-0.578	-0.243	-0.381
	p	**0.054**		0.048	0.003	0.037	0.359	0.108	0.062	0.472	0.248
dCA1	r	**0.541**	**0.959****		0.464	0.416	-0.283	-0.365	-0.389	0.304	0.131
	p	**0.085**	**0.000**		0.150	0.203	0.399	0.270	0.237	0.363	0.701
cPrh	r	**-0.287**	**0.109**	**0.101**		0.868**	-0.293	-0.676*	-0.765**	-0.463	-0.484
	p	**0.393**	**0.750**	**0.767**		0.001	0.383	0.022	0.006	0.151	0.131
lEnt	r	**-0.018**	**0.260**	**0.353**	**0.452**		-0.281	-0.648*	-0.737**	-0.494	-0.449
	p	**0.958**	**0.441**	**0.287**	**0.162**		0.402	0.031	0.010	0.123	0.166
dSub	r	**-0.271**	**-0.272**	**-0.151**	**0.038**	**0.439**		0.255	0.271	-0.050	0.152
	p	**0.420**	**0.418**	**0.658**	**0.913**	**0.177**		0.449	0.421	0.883	0.655
distance day 4	r	**0.074**	**-0.369**	**-0.270**	**-0.023**	**-0.092**	**0.452**		0.975**	0.560	0.353
	p	**0.829**	**0.264**	**0.423**	**0.946**	**0.788**	**0.163**		0.000	0.073	0.287
time day 4	r	**0.006**	**-0.464**	**-0.401**	**0.229**	**0.010**	**0.146**	**0.839****		0.622*	0.508
	p	**0.986**	**0.150**	**0.221**	**0.498**	**0.976**	**0.668**	**0.001**		0.041	0.111
distance change	r	**-0.341**	**-0.227**	**-0.109**	**0.223**	**0.448**	**0.773****	**0.498**	**0.359**		0.812**
	p	**0.305**	**0.503**	**0.750**	**0.510**	**0.167**	**0.005**	**0.119**	**0.278**		0.002
time change	r	**-0.329**	**-0.446**	**-0.335**	**0.137**	**0.439**	**0.580**	**0.395**	**0.489**	**0.820****	
	p	**0.323**	**0.169**	**0.314**	**0.688**	**0.177**	**0.061**	**0.230**	**0.127**	**0.002**	

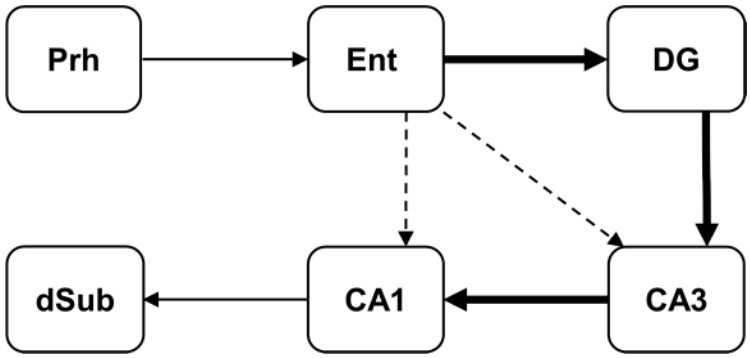

Figure 3.6: Baseline Model. This model was used as a starting point to derive best-fit models for each group. Prh: perirhinal cortex, Ent: entorhinal cortex, DG: dentate gyrus, dSub: dorsal subiculum. Bold arrows indicate the tri-synaptic pathway, dashed arrows indicate perforant path inputs directly to CA3 and CA1.

Group Familiar

The baseline model was a poor fit for data from group familiar and therefore was adapted to give a more optimal fit. The optimal model of neuronal activation in group familiar is shown in **Figure 3.7a**. This model proposes that when processing familiar information the most critical functional pathway is the perforant pathway directly to CA1 from the lateral entorhinal cortex. The DG and CA3 sub-regions do not play a key role in processing familiar information. The fit indices suggest that this is an excellent model for c-fos expression data from group familiar, as shown by the high CFI (1.000) and GFI (0.957) values, but low RMSEA (<0.001). However, the only significant path coefficient in this model is between the caudal perirhinal cortex and the lateral entorhinal cortex.

When data from group novel were fitted to this model, shown in **Figure 3.7b**, the fit indices revealed a considerably poorer fit, with CFI and GFI decreasing (CFI=0.461, GFI=0.860) and RMSEA increasing (RMSEA=0.182), although the chi-squared values remained non-significant. Therefore the optimal model for data from group familiar cannot explain the pattern of c-fos expression seen in group novel. For both data sets substituting the medial for the lateral entorhinal cortex, the ventral for the dorsal subiculum, or the rostral for the caudal perirhinal cortex yielded a poorer model fit.

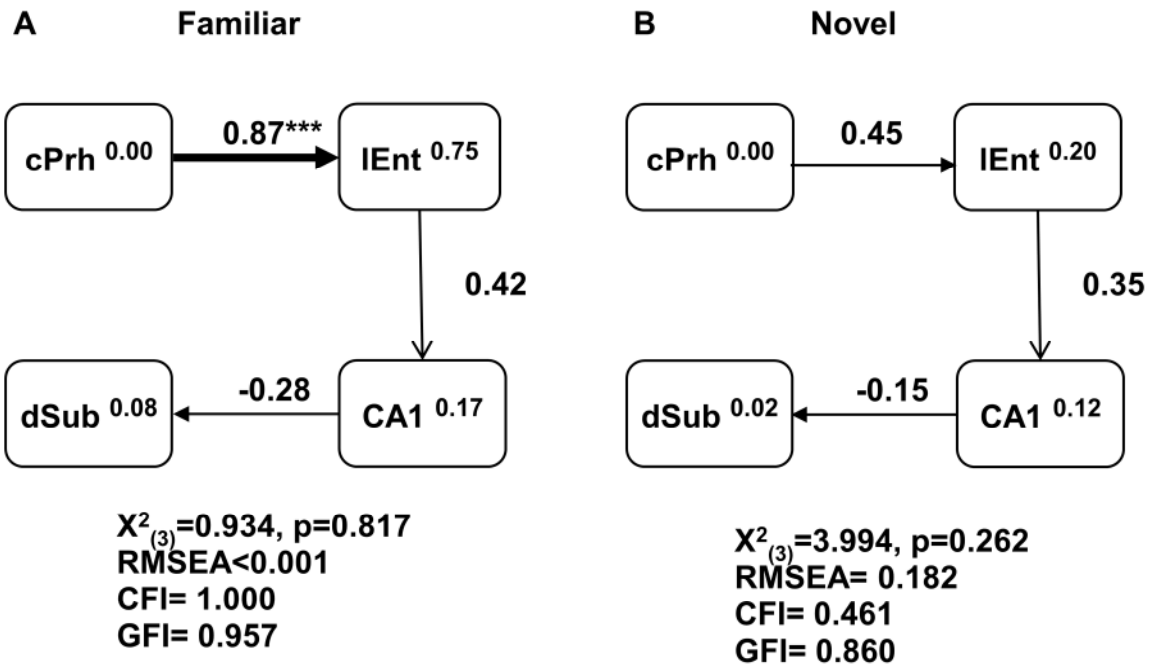

Figure 3.7: Optimal model for Group Familiar. This structural model is optimal for data from group familiar, but also shown is the fit of data from group novel. A: Fit of model to group familiar data. B: Fit of model to group novel data. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals (i.e. percentage of variance accounted for by each brain region). Bold arrows show significant pathways.

Group Novel

Fitting the baseline model to c-fos expression data from group novel revealed a high level of fit, therefore few modifications were required to find the optimal model. The optimal model for group novel, shown in **Figure 3.8a**, shows increased functional connectivity of all hippocampal sub-regions compared to group familiar, with both the tri-synaptic and the perforant pathways required in this model to optimally fit the data. This gives an excellent fit with high CFI and GFI (CFI=1.000, GFI=0.926) and low RMSEA (<0.001). Although only the pathway from CA3 to CA1 is statistically significant in this model the pathway from the DG to CA3 only marginally fails to reach conventional significance levels (r=0.594, p=0.054). Although tentative, this suggests that the tri-synaptic pathway may be the most important pathway when processing novel information, although both direct perforant pathways to CA3 and CA1 do contribute. Interestingly, unlike the model for group familiar, inclusion of an output pathway, to the dorsal subiculum, decreases the fit of this model.

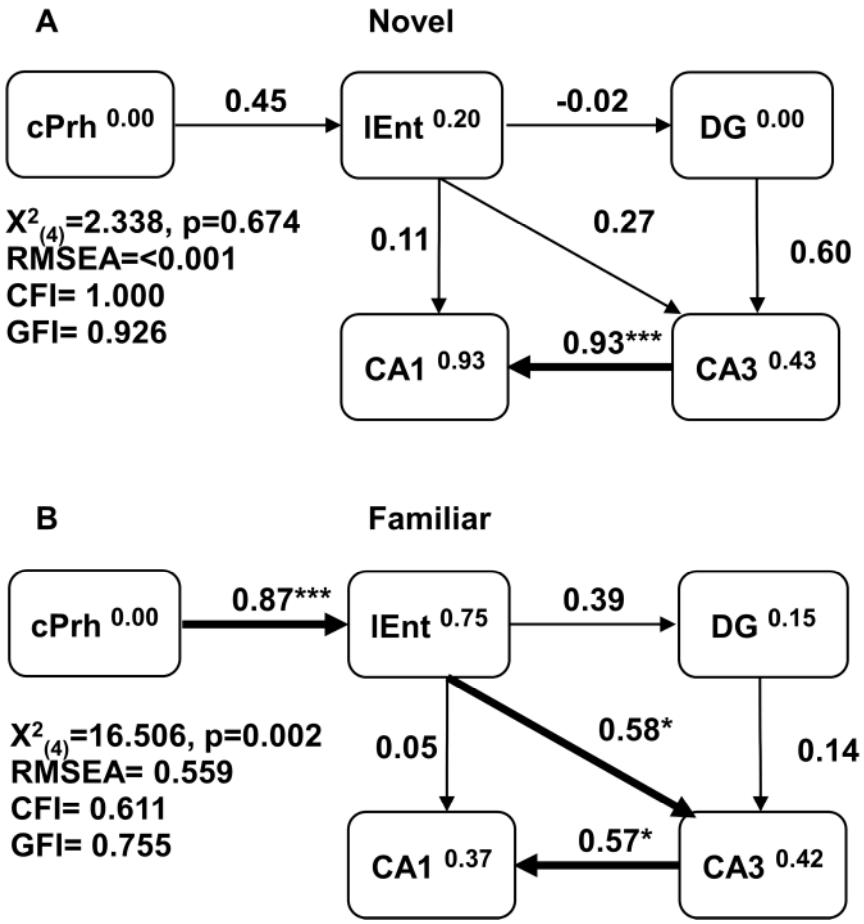

Figure 3.8: Optimal model for Group Novel. This structural model is optimal for data from group novel, but also shown is the fit of data from group familiar. A: Fit of model to group novel data. B: Fit of model to group familiar data. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

However, when the pathway from CA1 to the lateral entorhinal cortex is reversed the fit is only minimally reduced ($\chi^2_{(4)}$=3.265, p=0.514, CFI=1.000, GFI=0.898, RMSEA<0.001). This suggests that in the novel condition there may be functional connectivity in both directions though this pathway.

When data from group familiar was fitted to this model, shown in **Figure 3.8b,** the level of fit was considerably reduced, with a large decrease in CFI and GFI, increase in RMSEA and a significant chi-squared value ($\chi^2_{(4)}$=16.506, p=0.002, CFI=0.611, GFI=0.755, RMSEA=0.559). Interestingly, group familiar showed more significant pathways on this model than group novel despite the decreased overall fit.

Model Stacking

Comparison of path coefficients was made between mice in group novel and familiar using multiple group analysis with a Chi^2 difference test. Stacking data onto the familiar group model showed no significant difference in overall path strengths ($\chi^2_{(3)}$=4.268, p=0.234), however a significant difference was seen when data was stacked onto the optimal model for group novel which included additional pathways ($\chi^2_{(6)}$=28.175, p<0.001). Analysis of differences in individual path strength showed a significantly stronger interaction between CA3 and CA1 in group novel than group familiar ($\chi^2_{(1)}$=24.041, p<0.001), suggesting that this connection is more critical in the processing of novel compared to familiar information. Interestingly, despite the DG only being included in the optimal model for group novel and not group familiar, connections to this region did not differ in path strength between models.

Discussion

The data in experiment 1 have demonstrated increased exploratory activity in C57Bl6 mice in response to a novel environment, paralleled by specific increases in c-fos expression in the DG and CA1 hippocampal sub-regions of the septal and intermediate sections of the hippocampus. There was an overall increase in c-fos expression across the intermediate hippocampus in response to novelty; this overall effect of group was not seen for the septal hippocampus due to the lack of change in c-fos expression in the CA3 region. No difference was seen in c-fos expression between mice in group novel or familiar in the temporal section of the hippocampus or the parahippocampal cortex regions. Structural equation modelling based on c-fos expression data revealed different patterns of hippocampal network activation. Information about the familiar environment was processed primarily using a network involving the direct perforant path input into CA1 from the entorhinal cortex. In contrast to this, processing of novel spatial information also required the DG and CA3 sub-regions, activated via both the tri-synaptic pathway and the perforant path inputs directly into the CA3 and CA1 sub-regions.

The data presented here supports previous studies showing hippocampal engagement by spatial novelty. In particular, the specific increases in c-fos expression in the septal and intermediate hippocampus, but not the temporal section, supports the hypothesis of Moser & Moser (1998) that the hippocampus is made up of two anatomically and functionally distinct

regions. The dorsal region, including the septal and intermediate sections of the hippocampus, is thought to have a functional role in processing spatial information, whilst the ventral region, including the temporal section, processes emotional and temporal information. This same distinction has previously been reported in a c-fos expression study by Vann et. al. (2000) in which rats ran a radial arm maze task. Greater increases in c-fos expression were seen in the dorsal section of the hippocampus compared to the ventral section when rats encountered novel extra-maze cues.

Within the dorsal sections of the hippocampus sub-region specific increases in c-fos expression were seen. These data suggest that the DG and CA1 regions may be particularly critical for detecting spatial novelty. This idea supports several models of hippocampus function which suggest that the CA1 sub-region plays a specific role in detecting novelty by processing match-mismatch information to detect whether something has previously been experienced (Hasselmo and Schnell, 1994, McClelland et al., 1995, Lisman, 1999, Lisman and Otmakhova, 2001). From a conceptual point of view, the increased c-fos expression in the DG in the novel group compared to the familiar can be attributed to spatial pattern separation processes (Gilbert et al., 2001, Rolls and Kesner, 2006, Schmidt et al., 2012). This could be the case, as despite large alterations in the extra-maze cues, the arenas were identical, and therefore the test environments would share many common elements between the novel and familiar rooms. One must acknowledge that this is speculative in the absence of experimental evidence in which the common elements are systematically varied.

The lack of c-fos induction by novelty in CA3 in the present study is in contrast to other c-fos expression studies and may be due to the different tasks used in each study. Studies in which CA3 c-fos expression increased involved specific learning requirements, for example, remembering which arm to enter on a radial arm maze task (Vann et al., 2000, Jenkins et al., 2004). In contrast, studies more similar to the one used here, that allow exploration of a novel arena without specific task demands, do not show changes in CA3 c-fos expression (Sheth et al., 2008), and unreinforced viewing of novel configurations of stimuli does not significantly increase CA3 c-fos expression (Wan et al., 1999). According to computational models, the CA3 region is involved in retrieval of cues and short-term memory. For example, recall of previously visited arms or which arms are rewarded on a radial arm maze task would require activation of the CA3 recurrent collateral network and would therefore increase c-fos expression in this region (Rolls and Kesner, 2006). The task used here does not have any specific learning requirements and as such the degree of engagement of retrieval

processes supported by CA3 may be minimal. Additional experiments are required with mice to determine the conditions under which c-fos expression in the CA3 region is induced.

The optimal models of hippocampal network recruitment produced from the c-fos expression data are in broad agreement with those of other studies by Aggleton and colleagues (Poirier et al., 2008, Albasser et al., 2010). These latter two studies both assessed the relative contributions of hippocampal and parahippocampal regions to processing of novel or familiar information. The study by Poirier et. al. demonstrated different patterns of activation during the early- and late-phases of learning a radial-maze task. During the early phase many of the stimuli and task demands would still be novel to the rats, which then become familiar by the later phases of training. During early training the optimal model included all tri-synaptic pathway connections, whereas during the late-phase the entorhinal-CA1 perforant pathway became more critical. Interestingly, these two different models of hippocampal activation were produced despite no overall difference in c-fos expression between test groups. This finding has been paralleled in human MRI studies in which brain regions showed equivalent activation during different memory tasks, but were integrated into different functional networks (McIntosh, 1999, Maguire et al., 2000). The study by Albasser et. al. found the same dissociation between the tri-synaptic pathway for processing novelty and the perforant path directly to CA1 for familiarity on an object recognition task.

Overall the data presented here confirm that exposure to a novel spatial environment can selectively increase c-fos expression, and therefore neuronal activity, in the hippocampus. SEM analysis has also demonstrated qualitatively different patterns of hippocampal subfield engagement when mice process novel or familiar stimuli.

3.3 Experiment 2: Activation of Hippocampal Neural Networks in 3 month old Tg2576 Mice

Introduction

Experiment 1 validated the hypothesis that a novel spatial environment would induce changes in both behaviour and hippocampus c-fos expression in mice. Experiment 2 examines the effect of spatial novelty on behaviour and c-fos expression in 3 month old Tg2576 mice and their wild-type littermates. At 3 months of age there is little amyloid pathology present in Tg2576 mice. Increases in Aβ levels are detectable from 4-5 months of age (Kawarabayashi et al., 2001, Jacobsen et al., 2006), however, no behavioural memory deficits have been recorded at 3 months of age in spatial working or reference memory (Hsiao et al., 1996, Kawarabayashi et al., 2001, Westerman et al., 2002). More recently, a study by Jacobsen et. al. (2006) demonstrated deficits in contextual fear conditioning at 4, but not 2 months of age, suggesting that the first cognitive deficits may begin to appear between these two age points. This study also reported reductions in dendritic spine density and mild deficits in LTP in the DG, that coincided with the onset of cognitive deficits. Early reductions in dendritic spine density have also been reported in the CA1 region at approximately 4 months of age, which correlate with fear-conditioning deficits (Lanz et al., 2003, Perez-Cruz et al., 2011). These data suggest that at 4 months of age there are subtle alterations in synaptic morphology and plasticity despite no overt amyloid deposition. A recent study demonstrated that even at 2 months of age calcium regulation in the DG is impaired leading to alterations in mossy fibre transmission (Lee et al., 2012). This leads to the hypothesis that hippocampal neuronal activity, particularly in the DG and CA1 regions, may be altered at an early age before overt deficits in spatial memory and navigation are observed. The existing published data lead to the prediction that at 3 months of age Tg2576 mice will show a normal behavioural response to room novelty, although c-fos expression will reveal early alterations in neuronal activity in the DG and CA1 region.

Methods

Experiment 2 was carried out using an identical protocol to Experiment 1, unless otherwise specified.

Subjects: 18 transgenic Tg2576 mice and 20 wild-type littermates were used for this experiment.

Behavioural procedure: The behavioural procedure was identical to that described in Experiment 1, with the following exceptions. The test session was extended to 30 minutes, as pilot data suggested that a longer time period was necessary for Tg2576 mice to habituate to the test arena over the 3 day test period. Throughout testing mice were yoked into groups of four which were all tested on the same day. Each group contained two wild-type and two transgenic mice, and within each genotype one mouse was tested in the novel condition and the other in the familiar. Due to the unequal number of wild-type and transgenic mice there were two groups of only three mice, each with only one transgenic mouse.

Data analysis and statistics: Statistical testing was carried out using SPSS software. Behavioural data was analysed using a repeated measures ANOVA, with test day as the within subjects factor and test group and genotype as the between subjects factors. Two-way ANOVA was used to analyse percentage change in exploratory activity, with genotype and test group as between subjects factors. C-fos expression data, using raw c-fos immunoreactive cell counts, was also analysed using repeated measures ANOVA, with region as the within subjects factor both test group and genotype as between subjects. Significant Two-way or Three-way interactions were followed up with tests of simple main effects. To compare changes in activity patterns between genotypes as a function of test group a novelty ratio was calculated by taking the IR cell count in the each region for each mouse (wild-type and transgenic) in the novel group and dividing this by the total IR cell count for that mouse and the yoked mouse of the same genotype in group familiar. By calculating a ratio based upon yoked pairs any differences due to intensity of tissue staining between groups was removed, as well as any differences in overall cell count due to genotype. This leaves only those differences attributed to the test group, novel or familiar, and therefore should indicate difference between genotypes in the pattern of neuronal activity induced by novelty. To analyse these data repeated measures ANOVA was used, with region as the within subjects variable and genotype as the between subjects variable. Each novelty ratio was also compared to 0.5 to assess whether the ratio in this region had altered from chance as a result of exposure to a novel environment.

Structural Equation Modelling

Model Production: An optimal model was first produced for wild-type mice in either the novel or familiar test condition, and then data from transgenic mice in the same test condition was fitted to this model. If data from transgenic mice did not give a good fit to the wild-type model the model was adjusted to give an optimal fit for transgenic data. A comparison was then made between optimal models for each group using a multiple model comparison, tested by the χ^2 difference test.

Results Experiment 2a: Behaviour

Data showing exploratory behaviour in 3 month old Tg2576 mice are shown in **Figure 3.9**. Both wild-type and transgenic mice in the novel and familiar groups showed habituation over days 1-3, as demonstrated by a reduction in both the distance moved and the time spent moving during each test session. On test day 4, mice in the novel groups, irrespective of genotype, showed an increase in exploratory activity. There was also a trend for transgenic mice to show a higher level of exploratory activity than wild-type mice across all test days. Repeated measures ANOVA of the distance moved during each test session revealed a significant main effect of day ($F_{(3,102)}$=2.781, p=0.045), but not test group ($F_{(1,34)}$=0.001, p=0.970) and a main effect of genotype that failed to reach conventional levels of significance ($F_{(1,34)}$=3.764, p=0.061). There were no significant interactions between any of these factors (all p>0.05). Analysis of the amount of time spent moving during each test session also showed a significant main effect of day ($F_{(3,102)}$=25.640, p<0.001), but not genotype ($F_{(1,34)}$=1.293, p=0.263) or group ($F_{(1,34)}$=0.318, p=0.576). In contrast to the distance data, there was a significant interaction between day and test group ($F_{(3,102)}$=6.340, p=0.001), but all other interactions were not significant (all p>0.05). Tests of simple effects showed a significant difference between mice in the novel and familiar test groups only on day 4 (day 1-3: p>0.05, day 4: $F_{(1,34)}$=4.816, p=0.035).

Exploratory activity expressed as a percentage change between test days 3 and 4 can be seen in **Figure 3.9c,d**. Inspection of these figures shows a change in exploratory activity in opposite directions (numerically) for transgenic mice and wild-type mice in the familiar and novel test conditions. Two-way ANOVA for both the distance moved and the time spent moving showed a significant main effect of test group (distance: $F_{(1,34)}$=25.637, p<0.001,

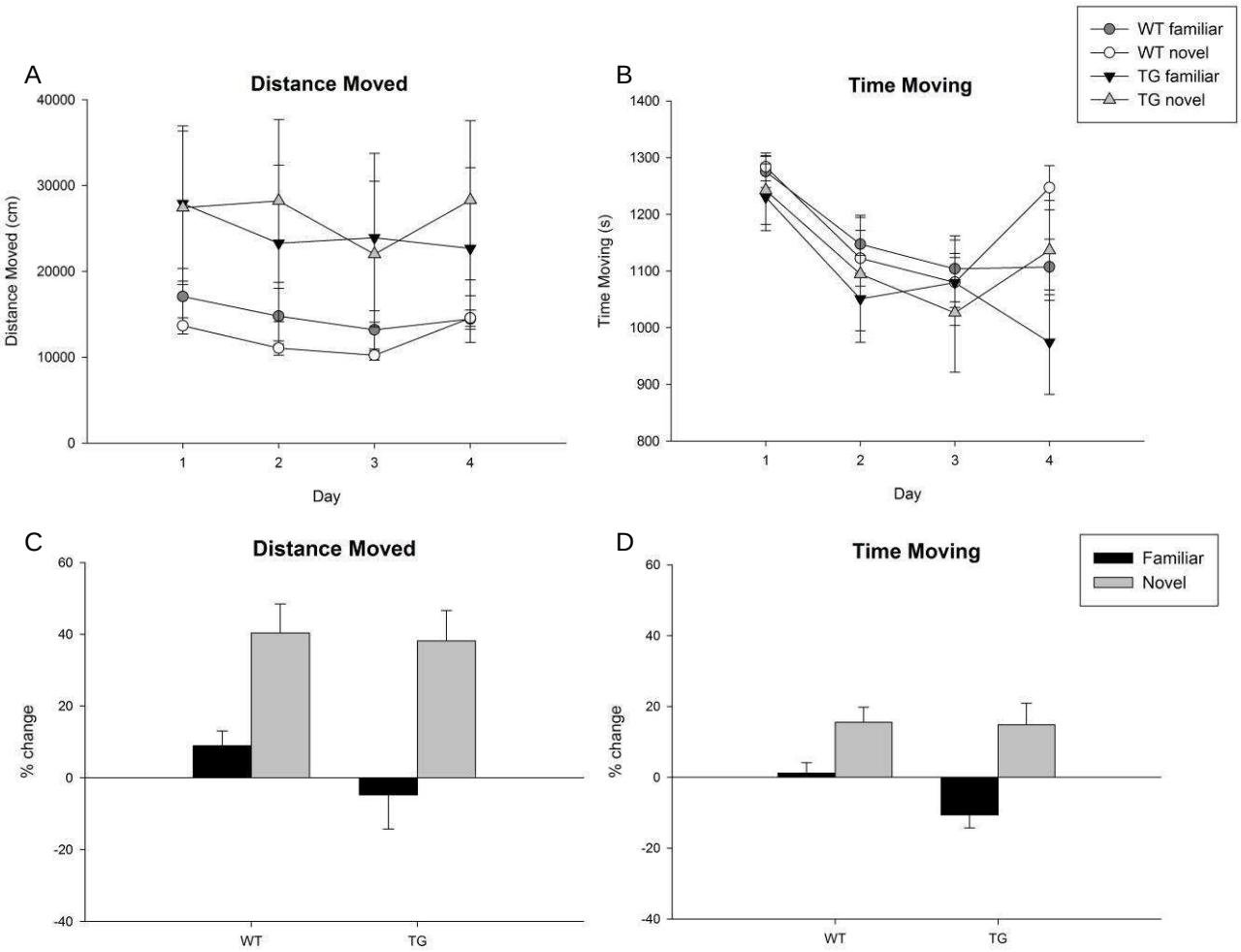

Figure 3.9: Locomotor activity in 3month old Tg2576 mice. A: Distance moved during each test session. B: Time spent moving during each test session. Symbols represent mean ± SEM (tg n=9, wt n=10 per group). C: Percentage change in the distance moved between test sessions 3 and 4. D: Percentage change in the time spent moving between test sessions 3 and 4. Bars represent mean ± SEM.

time: $F_{(1,34)}$=23.480, p<0.001), but not genotype (distance: $F_{(1,34)}$=1.544, p=0.223, time: $F_{(1,34)}$=2.517, p=0.122) and no significant interaction between these factors (distance: $F_{(1,34)}$=0.306, p=0.584, time: $F_{(1,34)}$=1.512, p=0.227). Together, these data indicate that at 3 months of age Tg2576 mice and their wild-type littermates both increased their exploratory behaviour in response to a novel spatial environment.

Results Experiment 2b: C-fos Expression

Septal Hippocampus: C-fos expression data of the raw c-fos IR cell counts for the septal section of the hippocampus can be seen in **Figure 3.10a**. In both wild-type and transgenic

mice there was a trend towards increased cell count between groups novel and familiar in all three sub-regions, although this increase was less marked in transgenic mice. However, repeated measures ANOVA revealed a significant main effect of region ($F_{(2,68)}$=27.939, p<0.001), but not genotype ($F_{(1,34)}$=1.922, p=0.175) and test group just failed to reach conventional levels of significance ($F_{(1,34)}$=4.096, p=0.051). All interactions between these factors were non-significant (all p>0.05).

Intermediate Hippocampus: C-fos expression data from the intermediate hippocampus is shown in **Figure 3.10b**. Wild-type mice show increased c-fos IR cells in all three sub-regions in response to novelty, whilst transgenic mice showed this effect to a lesser extent, notably in CA3 and CA1 regions. Transgenic mice had lower cell counts than wild-types in CA3 and even more prominently in CA1. Repeated measures ANOVA showed a significant main effect of region ($F_{(2,68)}$=9.250, p<0.001), but not genotype ($F_{(1,34)}$=1.835, p=0.184) and again test group just failed to reach significance ($F_{(1,34)}$=3.904, p=0.056). There was a significant interaction between region and genotype ($F_{(2,68)}$=6.083, p=0.004), but all other interactions were not significant (p>0.05). Follow up of this interaction showed a significant difference in IR cell count between wild-type and transgenic mice in the CA1 sub-region ($F_{(1,34)}$=4.881, p=0.034), whilst differences between genotype in the DG and CA3 were not significant (p>0.05).

Temporal Hippocampus: **Figure 3.10c** shows the c-fos expression data for the temporal hippocampus. There is little influence of either novelty or genotype on cell count in the CA3 sub-region. In comparison, in the CA1 region only wild-type mice showed increased IR cell count in response to novelty. Repeated measures ANOVA showed a significant main effect of region ($F_{(1,34)}$=70.463, p<0.001), but not genotype ($F_{(1,34)}$=1.858, p=0.182) or test group ($F_{(1,34)}$=2.902, p=0.098). There was also a significant interaction between region and test group ($F_{(1,34)}$=5.900, p=0.021), whilst all other interactions were not significant (all p>0.05). Tests of simple effects showed a significant difference between mice in group novel and familiar in the CA1 region ($F_{(1,34)}$=7.009, p=0.012), but not in CA3 (p>0.05).

Subiculum: C-fos expression data for the subiculum is shown in **Figure 3.10d**. There are few differences as a consequence of either genotype or test group, but there was a much greater number of IR cells in the ventral compared to the dorsal subiculum. Repeated measures ANOVA showed a significant main effect of region ($F_{(1,34)}$=140.649, p<0.001), but not

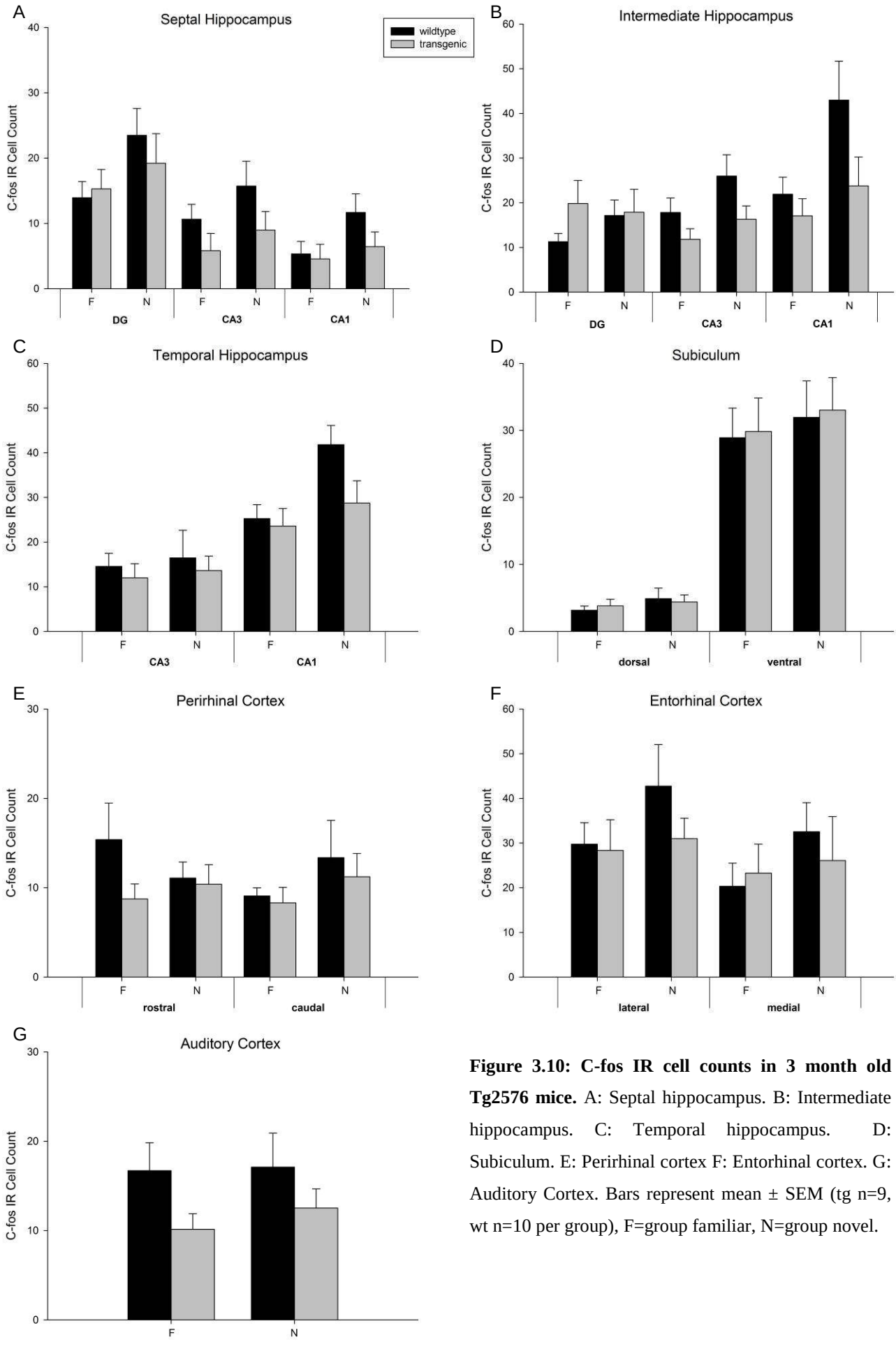

Figure 3.10: C-fos IR cell counts in 3 month old Tg2576 mice. A: Septal hippocampus. B: Intermediate hippocampus. C: Temporal hippocampus. D: Subiculum. E: Perirhinal cortex F: Entorhinal cortex. G: Auditory Cortex. Bars represent mean ± SEM (tg n=9, wt n=10 per group), F=group familiar, N=group novel.

genotype ($F_{(1,34)}$=0.038, p=0.846) or test group ($F_{(1,34)}$=0.586, p=0.449). There was no significant interaction between any of these factors (all p>0.05).

Perirhinal Cortex: **Figure 3.10e** shows c-fos expression data for the perirhinal cortex. There is little difference between genotypes or test group. Repeated measures ANOVA showed no significant main effect of region ($F_{(1,34)}$=0.405, p=0.529), genotype ($F_{(1,34)}$=1.241, p=0.273) or test group ($F_{(1,34)}$=0.247, p=0.622) and also no significant interactions between these factors (all p>0.05).

Entorhinal Cortex: C-fos expression data for the entorhinal cortex shows no significant difference between groups, **Figure 3.10f**. Repeated measures ANOVA revealed a significant main effect of region ($F_{(1,34)}$=6.914, p=0.013), but not genotype ($F_{(1,34)}$=0.444, p=0.510) or group ($F_{(1,34)}$=1.425, p=0.241). Again there are no significant interactions between these factors (all p>0.05).

Auditory Cortex: **Figure 3.10g** shows c-fos expression in the primary auditory cortex. There is no effect of novelty on cell count, and although there is a trend towards reduced cell counts in transgenic compared to wild-type mice, Two-way ANOVA showed no significant main effect of either genotype ($F_{(1,34)}$=3.658, p=0.064) or group ($F_{(1,34)}$=0.228, p=0.636), and no interaction between these factors ($F_{(1,34)}$=0.118, p=0.734).

Novelty Ratio: Novelty ratios were calculated as described in Experiment 1a, these are presented in **Table 3.4**. Analysis using paired t-tests (pairing within yoked groups) showed that there was no significant difference in novelty ratio between genotypes for any region (all p>0.05). Comparison of each region to 0.5 for transgenic mice showed no significant increase in c-fos expression in response to novelty for any region. In comparison wild-type mice showed increases in c-fos expression in the CA1 sub-region of the intermediate and temporal hippocampus and medial entorhinal cortex.

Table 3.4: Novelty ratios for 3 month old Tg2576 mice. Numbers represent mean (SEM). Statistical comparison using one-sample t-test compared to 0.5, i.e. no difference in cell count between groups novel and familiar. Significant results are shown in bold, *p<0.05, **p<0.01, ***p<0.001. In wild-type mice increases in c-fos expression were seen in the CA1 region of the intermediate and temporal hippocampus and the medial entorhinal cortex.

	Septal Hippo			Intermediate Hippo			Temporal Hippo		Entorhinal		Perirhinal		Subiculum		Control
	DG	CA3	CA1	DG	CA3	CA1	CA3	CA1	lateral	medial	rostral	caudal	dorsal	ventral	auditory
Tg	.524 (.083)	.590 (.089)	.540 (.096)	.438 (.067)	.575 (.061)	.535 (.065)	.526 (.048)	.555 (.038)	.537 (0.056)	.499 (.064)	.536 (.054)	.539 (.072)	.498 (.077)	.525 (.037)	.549 (.038)
Wt	.611 (.053)	.547 (.067)	.657 (.078)	.578 (.044)	.598 (.050)	**.640* (.044)**	.500 (.060)	**.623** (.025)**	.565 (.033)	**.629* (.054)**	.456 (.049)	.530 (.037)	.532 (.070)	.519 (.022)	.507 (.064)

Results Experiment 2c: Structural Equation Modelling

Group Familiar

Correlations

Table 3.5 shows the inter-region correlations for 3 month old Tg2576 mice in group familiar, and the correlation between c-fos expression in each region and behavioural measures. Transgenic mice show a greater number of significant correlations between regions, but no correlation with behaviour. In contrast, although wild-type mice show fewer inter-region correlations they do show a correlation between dorsal subiculum counts and the amount of time spent moving.

Structural Models

Fitting the baseline model (Experiment 1b, **Figure 3.6**) to pooled data including wild-type and transgenic familiar groups did not give a good model fit ($\chi^2_{(8)}$=11.647, p=0.168, GFI=0.841, CFI=0.945, RMSEA=0.159), so the model was adapted to fit each genotype. For wild-type mice the optimal model, shown in **Figure 3.11a**, matched that of C57Bl6 mice in group familiar, with the direct perforant pathway from the lateral entorhinal cortex directly to CA1 as the major hippocampal pathway in this model. The fit indices show this to be an excellent model of the data (GFI=0.915, CFI=1.000, RMSEA<0.001), although the only significant pathway is from the caudal perirhinal cortex to the lateral entorhinal cortex.

When data from transgenic mice in group familiar were fitted to this model the level of fit was poor ($\chi^2_{(3)}$=10.978, p=0.012, GFI=0.680, CFI=0.824, RMSEA=0.577), therefore the model was adapted to give a more optimal fit. The optimal model for transgenic mice in group familiar is shown in **Figure 3.11b**. This model largely matches that of wild-type mice, with the exception that in the transgenic model the caudal perirhinal cortex has been substituted for the rostral perirhinal cortex.

Table 3.5: Inter-region correlations of c-fos IR counts for 3 month old Tg2576 mice in group familiar. Wild-type, standard text. Transgenic, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, all significant correlations are shaded dark grey.

		dDG	dCA3	dCA1	rPrh	cPrh	lEnt	dSub	distance day 4	time day 4	distance change	time change
dDG	r		0.052	-0.105	0.643*	0.610	0.512	0.147	-0.462	0.029	-0.196	0.283
	p		0.887	0.773	0.045	0.061	0.130	0.686	0.179	0.937	0.588	0.428
dCA3	r	**-0.031**		0.921**	-0.018	-0.098	0.389	0.283	-0.178	0.256	0.379	0.300
	p	**0.937**		0.000	0.961	0.789	0.266	0.428	0.623	0.476	0.280	0.400
dCA1	r	**0.045**	**0.986****		0.068	-0.150	0.286	0.123	-0.009	0.326	0.218	0.027
	p	**0.909**	**0.000**		0.852	0.678	0.423	0.735	0.979	0.357	0.546	0.941
rPrh	r	**0.149**	**0.921****	**0.896****		0.545	0.629	0.025	-0.272	0.230	-0.409	-0.202
	p	**0.702**	**0.000**	**0.001**		0.103	0.051	0.946	0.447	0.522	0.241	0.575
cPrh	r	**0.746***	**0.126**	**0.214**	**0.137**		0.745*	0.699*	0.092	0.518	-0.023	0.200
	p	**0.021**	**0.746**	**0.581**	**0.725**		0.013	0.024	0.800	0.125	0.949	0.579
lEnt	r	**0.206**	**0.721***	**0.698***	**0.781***	**-0.011**		.717*	-0.196	0.448	0.158	0.250
	p	**0.595**	**0.028**	**0.036**	**0.013**	**0.978**		0.020	0.587	0.194	0.662	0.487
dSub	r	**0.371**	**0.892****	**0.921****	**0.868****	**0.464**	**0.775***		0.081	0.638*	0.344	0.360
	p	**0.325**	**0.001**	**0.000**	**0.002**	**0.208**	**0.014**		0.825	0.047	0.330	0.307
distance day 4	r	**0.131**	**-0.088**	**-0.145**	**0.160**	**-0.209**	**0.314**	**-0.049**		0.209	0.266	-0.042
	p	**0.736**	**0.822**	**0.710**	**0.681**	**0.590**	**0.410**	**0.901**		0.563	0.458	0.909
time day 4	r	**-0.013**	**0.109**	**0.103**	**0.357**	**-0.145**	**0.272**	**0.121**	**0.696***		-0.129	-0.187
	p	**0.974**	**0.780**	**0.792**	**0.346**	**0.710**	**0.480**	**0.757**	**0.037**		0.723	0.605
distance change	r	**0.023**	**-0.520**	**-0.469**	**-0.462**	**0.138**	**-0.408**	**-0.331**	**0.121**	**0.427**		0.834**
	p	**0.953**	**0.151**	**0.203**	**0.210**	**0.724**	**0.276**	**0.384**	**0.757**	**0.252**		0.003
time change	r	**-0.149**	**-0.285**	**-0.273**	**-0.127**	**-0.257**	**-0.233**	**-0.279**	**0.408**	**0.755***	**0.762***	
	p	**0.701**	**0.457**	**0.478**	**0.744**	**0.504**	**0.545**	**0.467**	**0.275**	**0.019**	**0.017**	

Table 3.6: Inter-region correlations of c-fos IR counts for 3 month old Tg2576 mice in group novel. Wild-type, standard text. Transgenic, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, all significant correlations are shaded dark grey.

		dDG	dCA3	dCA1	rPrh	cPrh	lEnt	dSub	distance day 4	time day 4	distance change	time change
dDG	r		0.640*	0.697*	0.197	0.027	0.173	0.118	-0.266	-0.369	-0.029	-0.177
	p		0.046	0.025	0.586	0.941	0.633	0.746	0.457	0.293	0.936	0.625
dCA3	r	**0.431**		0.906**	0.849**	0.674*	0.775**	0.781**	0.073	0.094	0.126	-0.051
	p	**0.247**		0.000	0.002	0.033	0.008	0.008	0.840	0.796	0.729	0.889
dCA1	r	**0.410**	**0.961****		0.640*	0.367	0.664*	0.588	0.093	0.050	0.085	0.073
	p	**0.273**	**0.000**		0.046	0.297	0.036	0.074	0.799	0.892	0.816	0.841
rPrh	r	**0.469**	**0.955****	**0.896****		0.883**	0.868**	0.943**	0.139	0.230	0.150	0.039
	p	**0.202**	**0.000**	**0.001**		0.001	0.001	0.000	0.701	0.522	0.678	0.916
cPrh	r	**0.418**	**-0.049**	**0.118**	**-0.038**		0.833**	0.921**	0.292	0.421	0.212	-0.157
	p	**0.263**	**0.901**	**0.762**	**0.923**		0.003	0.000	0.412	0.225	0.556	0.664
lEnt	r	**0.412**	**0.758***	**0.744***	**0.752***	**0.289**		0.899**	0.308	0.379	0.182	-0.084
	p	**0.270**	**0.018**	**0.021**	**0.019**	**0.451**		0.000	0.386	0.280	0.616	0.818
dSub	r	**0.292**	**0.428**	**0.634**	**0.366**	**0.719***	**0.471**		0.383	0.477	0.194	0.012
	p	**0.446**	**0.250**	**0.067**	**0.333**	**0.029**	**0.201**		0.275	0.164	0.591	0.973
distance day 4	r	**0.345**	**-0.167**	**-0.150**	**-0.109**	**0.158**	**0.284**	**-0.049**		0.964**	0.636*	0.366
	p	**0.364**	**0.667**	**0.701**	**0.780**	**0.684**	**0.459**	**0.900**		0.000	0.048	0.298
time day 4	r	**0.367**	**0.276**	**0.262**	**0.430**	**-0.082**	**0.420**	**0.083**	**0.710***		0.480	0.199
	p	**0.332**	**0.472**	**0.496**	**0.248**	**0.835**	**0.261**	**0.831**	**0.032**		0.161	0.582
distance change	r	**-0.277**	**0.353**	**0.493**	**0.404**	**0.274**	**0.486**	**0.631**	**-0.091**	**0.249**		0.684*
	p	**0.470**	**0.352**	**0.177**	**0.281**	**0.476**	**0.185**	**0.068**	**0.815**	**0.518**		0.029
time change	r	**-0.105**	**-0.209**	**-0.151**	**-0.224**	**0.407**	**-0.233**	**0.171**	**-0.568**	**-0.714***	**0.046**	
	p	**0.789**	**0.590**	**0.699**	**0.562**	**0.277**	**0.546**	**0.659**	**0.110**	**0.031**	**0.907**	

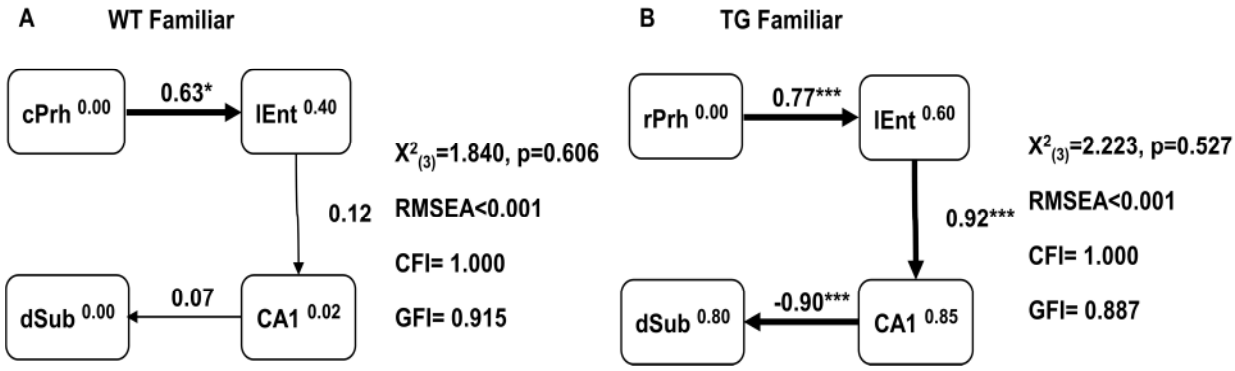

Figure 3.11: Optimal models for 3 month old Tg2576 mice in group familiar. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

Model Stacking

Direct comparison of data from wild-type and transgenic mice in group familiar by stacking onto the same model revealed a significant overall difference in path coefficients (wt model: $\chi^2_{(3)}$=9.653, p=0.022, tg model: $\chi^2_{(3)}$=14.242, p=0.003). This finding suggests that transgenic and wild-type mice use different functional networks when processing familiar information. Analysis of individual path strengths showed a significant difference between path strengths between the rostral perirhinal and entorhinal cortex ($\chi^2_{(1)}$=4.597, p=0.032) and between CA1 and the subiculum ($\chi^2_{(1)}$=6.870, p=0.009). Transgenic mice showed a much stronger interaction on both of these pathways compared to wild-type mice.

Group Novel

Correlations

Table 3.6 shows the inter-region correlations in c-fos expression and correlations with behavioural measures for mice in group novel. In contrast to group familiar, there are a greater number of significant correlations between regions in wild-type mice compared to transgenics. However, there are no significant correlations between c-fos expression in any brain region and behaviour for either wild-type or transgenic mice.

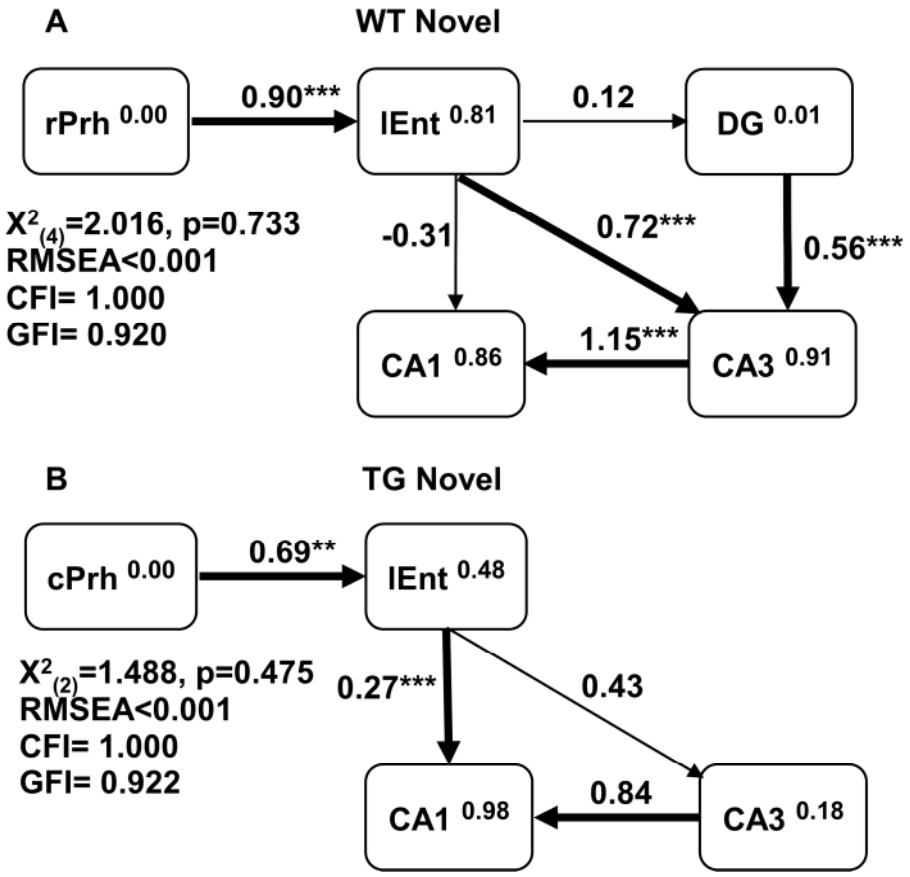

Figure 3.12: Optimal models for 3 month old Tg2576 mice in group novel. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

Structural Models

When the baseline model was fitted to pooled data from mice in group novel the fit was poor ($\chi^2_{(8)}$=27.954, p<0.001, GFI=0.754, CFI=0.825, RMSEA=0.372), therefore modification was needed to fit the model to the data set. The optimal model for wild-type mice in group novel is shown in **Figure 3.12a**. This model matches that of C57Bl6 mice, incorporating both the tri-synaptic and perforant paths, and has a high level of fit with the data as shown by high CFI and GFI (CFI=1.000, GFI=0.920) and low RMSEA indices (RMSEA<0.001). Again reversal of the pathway from the lateral entorhinal cortex to CA1 did not decrease the model fit ($\chi^2_{(4)}$=2.271, p=0.686, GFI=0.920, CFI=1.000, RMSEA<0.001) suggesting that there may be functional connectivity in both directions via this pathway, providing output from CA1 via the entorhinal cortex instead of the dorsal subiculum for novel information processing.

When data from transgenic mice in group novel were fitted to this model, the level of fit was less optimal ($\chi^2_{(4)}$=6.829, p=0.145, GFI=0.800, CFI=0.920, RMSEA=0.279). Adapting the model by substituting the rostral for the caudal perirhinal cortex improved the fit of the model to an excellent level ($\chi^2_{(4)}$=3.073, p=0.546, GFI=0.889, CFI=1.000, RMSEA<0.001). However, further adaptation of the model to remove pathways involving the DG gave the optimal model, shown in **Figure 3.12b** (CFI=1.000, GFI=0.922, RMSEA<0.001). The excellent fit of both of these models, however, suggests that the tri-synaptic pathway via the DG may still play a role in processing novel information in transgenic mice, but it is not relied upon as heavily as in wild-type mice, favouring instead the direct input into CA1 and CA3 from the entorhinal cortex.

Model Stacking

Data from wild-type and transgenic mice produced different optimal models of functional connectivity. Stacking data from transgenic and wild-type mice showed a significant difference in path coefficients (wt model: $\chi^2_{(6)}$=22.758, p<0.001, tg model: $\chi^2_{(4)}$=13.467, p=0.009). Interestingly, analysis of individual path strengths did not show a significant difference in connectivity with the DG, but did show a significantly stronger path strength between the rostral perirhinal and entorhinal cortex in wild-type mice ($\chi^2_{(1)}$=9.832, p=0.002), and between the entorhinal cortex and CA1 in transgenic mice ($\chi^2_{(1)}$=8.000, p=0.005). The difference in perirhinal to entorhinal cortex interaction matches that of mice in group familiar. The difference in entorhinal to CA1 path strength may reflect a shift in functional connectivity from processing by the DG to CA1 as the DG is not included in the optimal transgenic model. However, there is no significant reduction in path strength to the DG suggesting that this region is still engaged in a functional network in 3 month old transgenic mice.

Discussion

3 month old wild-type and transgenic Tg2576 mice showed increased exploratory activity in response to novelty, similarly to C57Bl6 mice. However, they did not show such robust changes in c-fos expression in the septal and intermediate hippocampus regions, with only a non-significant trend towards increased expression in response to novelty in these regions,

and only a significant difference seen in the intermediate CA1 region between genotypes. In addition, the temporal CA1 region also showed a significant increase in c-fos expression with novelty. Analysis of novelty ratios suggests that this increase in CA1 expression was driven primarily by increases in wild-type mice. Interestingly, despite differences in c-fos expression pattern between wild-type Tg2576 mice and C57Bl6 mice, the same SEM models fitted the data, with the tri-synaptic and perforant path inputs being critical when processing novel information, but only the direct entorhinal to CA1 inputs required for familiar information. Whilst transgenic mice engage similar networks as wild-types when processing familiar information, they show a different pattern of hippocampal network engagement when novel information is presented which does not require the DG sub-region.

In contrast to C57Bl6 mice there are few significant changes in c-fos expression for either wild-type or transgenic mice as function of novelty. The minimal differences seen between test groups, with only non-significant trends towards increased activity with novelty, may reflect the increased variation between subjects in the Tg2576 model compared to C57Bl6 mice due to a different background strain that in turn may alter attentional, motivational or sensorimotor processing (Lathe, 1996, Linder, 2006). Increased variation between subjects is also seen in behavioural measures, suggesting that c-fos expression may be reflected in behaviour. The increases in c-fos expression in the CA1 region match that seen in C57Bl6 mice and studies of novelty in rats (Wan et al., 1999, Vann et al., 2000, Albasser et al., 2010), again implicating this region in novelty detection, however, in this case increases were seen only in the intermediate and temporal region, and not the septal region. Increased c-fos expression in the temporal region of the hippocampus might suggest that mice with a C57Bl6/SJL background strain show a greater emotional response to novelty, likely to be a fear response to a novel environment, as this function has been attributed to the ventral section of the hippocampus (Moser and Moser, 1998).

It is interesting that although minimal changes in c-fos expression were recorded as a result of novelty in Tg2576 mice, SEM models were derived for wild-type mice which match those of C57Bl6 mice. This effect has been previously reported by Poirier et. al. (2008), who demonstrated different functional hippocampal engagement in two tasks despite equal overall IEG expression in each region. In 3 month transgenic mice, whilst the same pathways were used for processing familiar information, for processing novel information projections from the entorhinal cortex to the DG and then CA3 were less critical, suggesting some functional disconnection of the DG even at this early-time point. This is likely to be due to early

synaptic changes in the DG, such as those reported by Jacobsen et. al. (2006) as there is no amyloid deposition detected at this stage. However, the wild-type model for group novel, including the DG, did give a good, if not optimal, fit to the transgenic data suggesting that at 3-months of age the DG is not completely disengaged, with some synaptic function retained. This may explain why no significant differences in behaviour were reported between genotypes.

3.4 Experiment 3: Activation of Hippocampal Neural Networks in 12 month old Tg2576 Mice

Introduction

Having found minor differences in c-fos expression patterns between wild-type and transgenic Tg2576 mice at 3 months of age, before amyloid pathology, the next experiment, assesses whether excess amyloid production and pathology leads to differences in hippocampal neuronal activity. Therefore Experiment 3 tests Tg2576 mice at 12 months of age on the room novelty task. At 12 months of age Tg2576 mice have high levels of both soluble amyloid and plaque pathology (Hsiao et al., 1996, Kawarabayashi et al., 2001). In addition, robust behavioural deficits can be seen in tests of working and reference spatial memory (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002). At this age reductions in c-fos expression have been reported in the caudal retrosplenial cortex of transgenic mice compared to wild-types (Poirier et al., 2011). However, no studies have assessed c-fos expression in the hippocampus at this age. Synaptic alterations are also more widespread within the hippocampus at this age, with deficits in baseline synaptic transmission and LTP reported in the DG, and CA1 regions (Chapman et al., 1999, Fitzjohn et al., 2001). Reduction in synaptic proteins such as synaptophysin and PSD-95 have also been reported and dendritic spine density is further decreased (Lanz et al., 2003, Jacobsen et al., 2006). Therefore, the expectation is that there will be more widespread differences in c-fos expression between wild-type and transgenic mice, and that the pattern of hippocampal sub-region engagement may be further disrupted in transgenic mice and would include impaired activation of the DG and CA1 regions.

Methods

Experiment 3 was carried out using an identical protocol to Experiment 2.

Subjects: 22 Tg2576 mice and 22 wild-type littermates were used for this experiment.

Results Experiment 3a: Behaviour

The exploratory activity of wild-type and transgenic mice from each genotype, measured by the distance moved and the time spent moving, is shown in **Figure 3.13a,b**. Inspection of this figure shows that all tests groups habituated over days 1-3. However, there is only a trend towards an increase in exploratory behaviour in response to spatial novelty in wild-type mice, which is not seen at all in transgenic mice in group novel. Repeated measures ANOVA of the distance moved during each session showed a significant main effect of day ($F_{(3,96)}$=4.514, p=0.005), but not genotype ($F_{(1,32)}$=2.286, p=0.140) or group ($F_{(1,32)}$=0.807, p=0.376) and no significant interactions between any of these factors (all p>0.05). A similar result was seen for the amount of time spent moving during each session with a significant main effect of day ($F_{(3,96)}$=16.375, p<0.001), but not genotype ($F_{(1,32)}$=0.015, p=0.903) or group ($F_{(1,32)}$=0.376, p=0.544). Again there were no significant interactions between these factors (all p>0.05), although the interaction between test day and group narrowly failed to reach conventional levels of statistical significance ($F_{(3,96)}$=2.579, p=0.058).

Exploratory activity measured as a percentage change in the distance moved between test days 3 and 4 can be seen in **Figure 3.13c,d**. In wild-type mice there was an increase in exploratory activity in group novel, whereas in transgenic mice there was a decrease for both test conditions. Two-way ANOVA of the percentage change in distance moved revealed no significant main effects of either genotype ($F_{(1,32)}$=3.538, p=0.069) or group ($F_{(1,32)}$=1.346, p=0.254), and no significant interaction between these factors ($F_{(1,32)}$=2.014, p=0.165). Analysis of the percentage change in time spent moving showed a significant main effect of genotype ($F_{(1,32)}$=4.878, p=0.034), but not group ($F_{(1,32)}$=3.363, p=0.076), and no significant interaction between these variables ($F_{(1,32)}$=0.986, p=0.328).

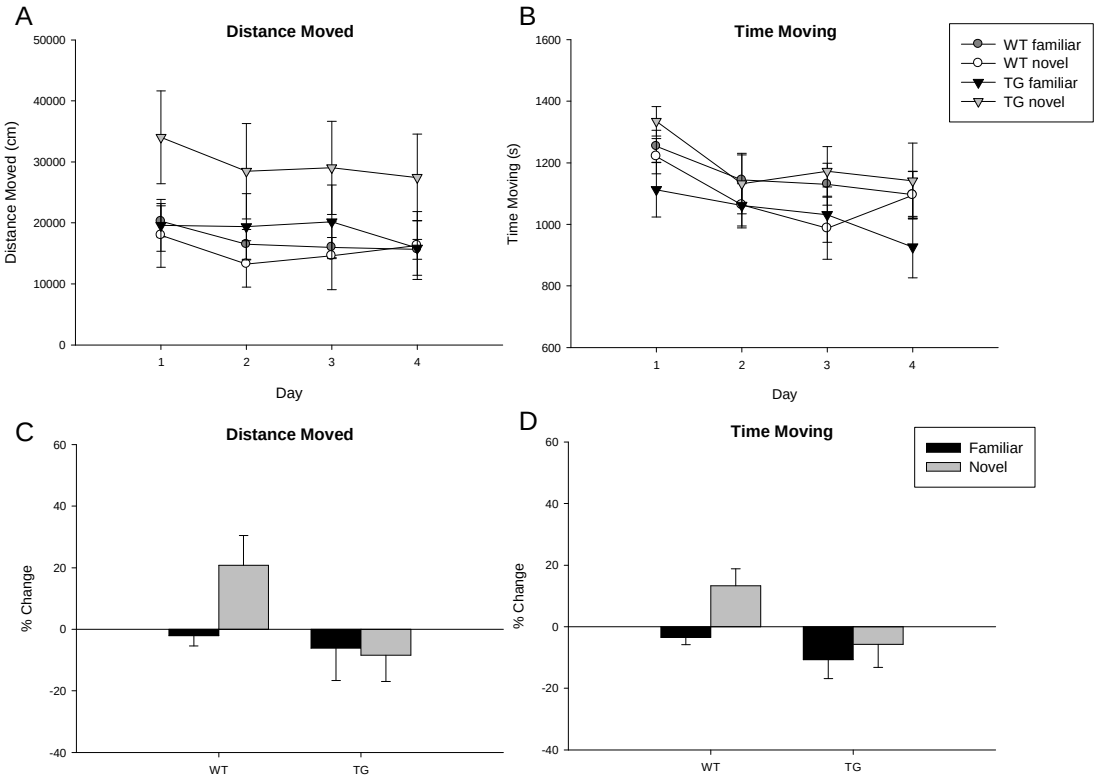

Figure 3.13: Locomotor activity in 12 month old Tg2576 mice. A: Distance moved during each test session. B: Time spent moving during each test session. Symbols represent mean ± SEM (tg n=11, wt n=11 per group). C: Percentage change in the distance moved between test session 3 and 4. D: Percentage change in the time spent moving between test session 3 and 4. Bars represent mean ± SEM.

Results Experiment 3b: C-fos expression

Septal Hippocampus: C-fos expression data for the septal hippocampus are shown in **Figure 3.14a**. Inspection of this figure shows a reduction in IR cell count in transgenic mice compared to wild-type across all regions and conditions, but no effect of novelty on either genotype. Repeated measures ANOVA showed a significant main effect of region ($F_{(2,80)}$-=18.539, $p<0.001$), and genotype ($F_{(1,40)}=4.620$, $p=0.038$), but not test group ($F_{(1,40)}=0.480$, $p=0.493$). All interactions between these factors were not significant ($p>0.05$).

Intermediate Hippocampus: C-fos expression data for the intermediate section of the hippocampus is shown in **Figure 3.14b**. There was little difference in cell count between mice in the novel and familiar conditions for either genotype, and only a non-significant trend towards a reduced cell count in transgenic mice compared to wild-types in the CA3 and CA1 sub-regions. Repeated measures ANOVA showed a significant main effect of region ($F_{(2,80)}$-

=5.327, p=0.007), but not test group ($F_{(1,40)}$=0.134, p=0.716) or genotype ($F_{(1,40)}$=2.186, p=0.147). There were no significant interactions between these variables (all p>0.05).

Temporal Hippocampus: **Figure 3.14c** shows c-fos expression data for the temporal section of the hippocampus. There was little difference in IR cell count between test conditions or genotype. Repeated measures ANOVA showed a significant main effect of region ($F_{(1,40)}$-=53.336, p<0.001), but not test group ($F_{(1,40)}$=0.103, p=0.750) or genotype ($F_{(1,40)}$=0.020, p=0.888), and no significant interactions between these factors (all p>0.05).

Subiculum: C-fos expression data for the subiculum is shown in **Figure 3.14d**. This figure shows no significant difference between test condition or genotype. Repeated measures ANOVA revealed a significant main effect of region ($F_{(1,40)}$=78.808, p<0.001), but not test group ($F_{(1,40)}$=0.142, p=0.709) or genotype ($F_{(1,40)}$=0.152, p=0.699), and no significant interactions between these factors (all p>0.05).

Perirhinal Cortex: C-fos expression data for the perirhinal cortex, shown in **Figure 3.14e**, shows little difference between c-fos IR cell count in group novel compared to group familiar for either genotype, and no difference between genotypes. Repeated measures ANOVA showed no significant main effects of either region ($F_{(1,40)}$=2.030, p=0.162), test group ($F_{(1,40)}$=0.252, p=0.619) or genotype ($F_{(1,40)}$=0.072, p=0.790). There were also no significant interactions between any of these factors (all p>0.05).

Entorhinal Cortex: **Figure 3.14f** shows c-fos expression data from the entorhinal cortex data. There is no significant difference between wild-type and transgenic mice in either test condition. Repeated measures ANOVA gave a significant main effect of region ($F_{(1,40)}$-=20.596, p<0.001), but not test group ($F_{(1,40)}$=0.484, p=0.491) or genotype ($F_{(1,40)}$=0.061, p=0.806), and no significant interactions between these factors (all p>0.05).

Auditory cortex: C-fos expression data for the auditory cortex, shown in **Figure 3.14g**, shows no difference between test groups or genotypes. Repeated measures ANOVA revealed no significant main effect of either genotype ($F_{(1,40)}$=0.548, p=0.464) or test group ($F_{(1,40)}$=0.783, p=0.381), and no interaction between these variables ($F_{(1,40)}$=0.531, p=0.470).

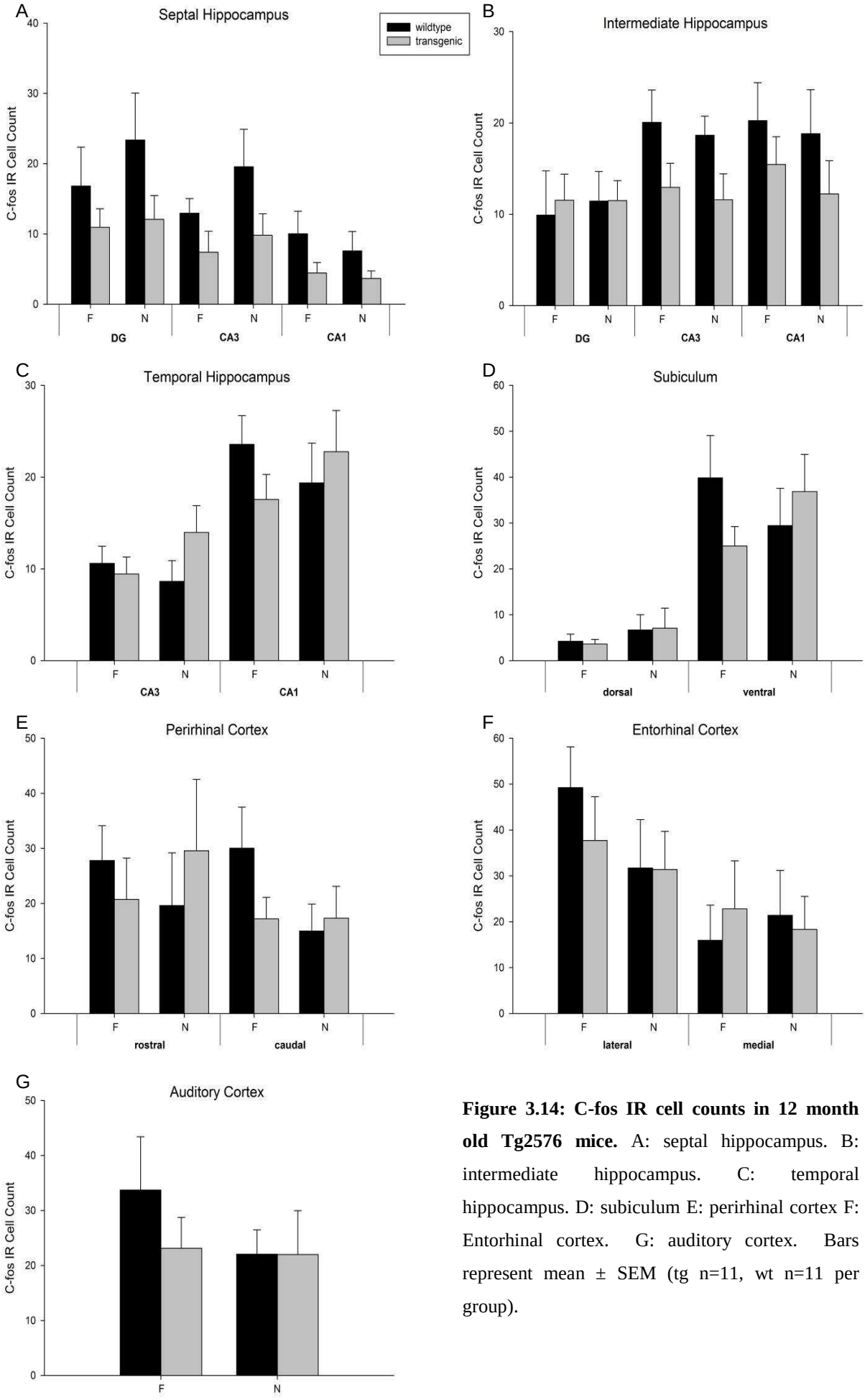

Figure 3.14: C-fos IR cell counts in 12 month old Tg2576 mice. A: septal hippocampus. B: intermediate hippocampus. C: temporal hippocampus. D: subiculum E: perirhinal cortex F: Entorhinal cortex. G: auditory cortex. Bars represent mean ± SEM (tg n=11, wt n=11 per group).

Table 3.7: Novelty ratios for 12 month old Tg2576 mice. Numbers represent mean (SEM). Statistical comparison using one-sample t-test compared to 0.5, i.e. no difference in cell count between groups novel and familiar. Significant results are shown in bold, *p<0.05, **p<0.01, ***p<0.001. No change in c-fos expression was seen in transgenic mice. In wild-type mice decreases in c-fos expression were seen in the lateral entorhinal, and perirhinal cortex and ventral subiculum.

	Septal Hippo			Intermediate Hippo			Temporal Hippo		Entorhinal		Perirhinal		Subiculum		Control
	DG	CA3	CA1	DG	CA3	CA1	CA3	CA1	lateral	medial	rostral	caudal	dorsal	ventral	auditory
Tg	.516 (.071)	.624 (.080)	.591 (.093)	.520 (.060)	.444 (.047)	.404 (.052)	.580 (.053)	.530 (.057)	.453 (.033)	.485 (.068)	.515 (.056)	.455 (.034)	.437 (.081)	.580 (.059)	.444 (.049)
Wt	.593 (.056)	.545 (.053)	.437 (.074)	.583 (.063)	.513 (.062)	.492 (.058)	.427 (.066)	.454 (.054)	**.368* (.051)**	.576 (.060)	**.285** (.067)**	**.322** (.044)**	.523 (.070)	**.406* (.041)**	.423 (.075)

Novelty Ratio: Novelty ratios, shown in **Table 3.7**, were calculated for each region as described in Experiment 1a. Comparison of ratio between genotypes using paired t-tests revealed a significant difference between genotypes only in the rostral perirhinal region ($t_{(11)}$=2.629, p=0.016), all other regions were not significant (all p>0.05), although the caudal perirhinal cortex and ventral subiculum only just failed to reach statistical significance. Comparison of the ratio in each region to 0.5 for each genotype revealed no significant changes in c-fos expression between group novel and familiar in transgenic mice. In contrast, wild-type mice showed significant decreases in c-fos expression in response to novelty in the lateral entorhinal cortex, rostral and caudal perirhinal cortex and the ventral subiculum.

Results Experiment 3c: Structural Equation Modelling

Group Familiar

Correlations

The correlations between c-fos expression in different brain regions and with behaviour for mice in group familiar are shown in **Table 3.8**. As with 3 month old Tg2576 mice there are a greater number of significant correlations between regions in transgenic mice compared to wild-type mice. There are no significant correlations between c-fos expression and behaviour in transgenic mice, but in wild-type mice DG c-fos expression correlates with both the time spent moving and the percentage change in time spent moving on day 4, and the c-fos expression in CA1 correlated with the change in the distance moved on day 4 of testing.

Structural Models

Fitting the baseline model to pooled data from mice in group familiar gave a poor model fit ($\chi^2_{(8)}$=19.636, p=0.012, GFI=0.802, CFI=0.827, RMSEA=0.263). Modification of this model gave the optimal model shown in **Figure 3.15a**. This model matches that of C57Bl6 mice and 3 month old wild-type mice in group familiar, with the direct pathway from the lateral entorhinal cortex to CA1 as the most critical hippocampal projection for this group. However, although this model is optimal, the fit indices indicate that the data from 12 month old mice does not fit this model as optimally as at 3 months of age, with reduced CFI and GFI

Table 3.8: Inter-region correlations of c-fos IR counts for 12 month old Tg2576 mice in group familiar. Wild-type, standard text. Transgenic, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, all significant correlations are shaded dark grey.

		dDG	dCA3	dCA1	rPrh	cPrh	lEnt	dSub	distance day 4	time day 4	distance change	time change
dDG	r		0.563	0.375	0.327	0.761**	0.411	0.736**	-0.249	-0.922**	-0.585	-0.960**
	p		0.072	0.256	0.327	0.007	0.209	0.010	0.552	0.001	0.127	0.000
dCA3	r	**0.728***		0.686*	0.543	0.246	0.736**	0.737**	-0.449	-0.604	-0.564	-0.494
	p	**0.011**		0.020	0.085	0.466	0.010	0.010	0.265	0.112	0.146	0.213
dCA1	r	**0.534**	**0.663***		0.258	-0.029	0.320	0.273	-0.698	-0.623	-0.865**	-0.657
	p	**0.091**	**0.026**		0.444	0.933	0.338	0.417	0.054	0.099	0.005	0.077
rPrh	r	**0.752****	**0.706***	**0.746****		0.399	0.620*	0.593	-0.573	-0.178	-0.494	-0.242
	p	**0.008**	**0.015**	**0.008**		0.224	0.042	0.055	0.138	0.674	0.214	0.563
cPrh	r	**0.477**	**0.469**	**0.752****	**0.656***		0.311	0.499	0.021	-0.697	-0.120	-0.688
	p	**0.138**	**0.146**	**0.008**	**0.028**		0.352	0.118	0.961	0.055	0.777	0.059
lEnt	r	**0.597**	**0.627***	**0.487**	**0.885****	**0.487**		0.756**	-0.369	-0.398	-0.373	-0.298
	p	**0.053**	**0.039**	**0.129**	**0.000**	**0.128**		0.007	0.368	0.329	0.362	0.473
dSub	r	**0.447**	**0.640***	**0.819****	**0.808****	**0.631***	**0.766****		-0.362	-0.611	-0.527	-0.594
	p	**0.168**	**0.034**	**0.002**	**0.003**	**0.037**	**0.006**		0.379	0.108	0.180	0.120
distance day 4	r	**0.312**	**-0.052**	**-0.169**	**-0.132**	**-0.124**	**-0.424**	**-0.539**		0.361	0.820*	0.352
	p	**0.350**	**0.880**	**0.620**	**0.698**	**0.716**	**0.193**	**0.087**		0.380	0.013	0.392
time day 4	r	**0.447**	**-0.170**	**-0.238**	**0.003**	**-0.094**	**0.035**	**-0.312**	**0.408**		0.593	0.933**
	p	**0.168**	**0.617**	**0.482**	**0.994**	**0.782**	**0.918**	**0.351**	**0.213**		0.121	0.001
distance change	r	**-0.455**	**-0.012**	**0.024**	**-0.206**	**-0.175**	**-0.366**	**-0.110**	**0.173**	**-0.738****		0.697
	p	**0.159**	**0.972**	**0.945**	**0.544**	**0.607**	**0.268**	**0.747**	**0.611**	**0.010**		0.055
time change	r	**0.265**	**0.214**	**0.114**	**0.332**	**0.058**	**0.346**	**0.110**	**0.162**	**0.326**	**0.036**	
	p	**0.430**	**0.527**	**0.738**	**0.319**	**0.864**	**0.297**	**0.748**	**0.635**	**0.328**	**0.917**	

Table 3.9: Inter-region correlations of c-fos IR counts for 12 month old Tg2576 mice in group novel. Wild-type, standard text. Transgenic, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, all significant correlations are shaded dark grey.

		dDG	dCA3	dCA1	rPrh	cPrh	lEnt	dSub	distance day 4	time day 4	distance change	time change
dDG	r		0.958**	0.992**	0.869**	0.929**	0.846**	0.826**	-0.207	0.525	-0.265	0.388
	p		0.000	0.000	0.001	0.000	0.001	0.002	0.656	0.226	0.566	0.390
dCA3	r	**0.668***		0.951**	0.826**	0.868**	0.780**	0.834**	-0.200	0.290	-0.326	0.176
	p	**0.025**		0.000	0.002	0.001	0.005	0.001	0.668	0.528	0.476	0.706
dCA1	r	**0.747****	**0.893****		0.877**	0.931**	0.854**	0.857**	-0.274	0.553	-0.196	0.409
	p	**0.008**	**0.000**		0.000	0.000	0.001	0.001	0.552	0.198	0.674	0.362
rPrh	r	**0.930****	**0.469**	**0.616***		0.910**	0.974**	0.935**	-0.295	0.693	-0.274	0.553
	p	**0.000**	**0.146**	**0.043**		0.000	0.000	0.000	0.521	0.084	0.552	0.198
cPrh	r	**0.776****	**0.380**	**0.470**	**0.703***		0.856**	0.792**	-0.179	0.847*	0.017	0.665
	p	**0.005**	**0.249**	**0.145**	**0.016**		0.001	0.004	0.702	0.016	0.970	0.103
lEnt	r	**0.614***	**0.614***	**0.567**	**0.425**	**0.690***		0.939**	-0.264	0.463	-0.207	0.332
	p	**0.044**	**0.045**	**0.069**	**0.192**	**0.019**		0.000	0.568	0.295	0.655	0.468
dSub	r	**0.882****	**0.617***	**0.720***	**0.913****	**0.671***	**0.581**		-0.233	0.319	-0.157	0.230
	p	**0.000**	**0.043**	**0.013**	**0.000**	**0.024**	**0.061**		0.615	0.486	0.737	0.620
distance day 4	r	**-0.513**	**-0.811****	**-0.686***	**-0.329**	**-0.001**	**-0.434**	**-0.368**		-0.274	0.789*	-0.426
	p	**0.158**	**0.008**	**0.042**	**0.388**	**0.998**	**0.243**	**0.330**		0.551	0.035	0.341
time day 4	r	**-0.137**	**-0.465**	**-0.360**	**0.078**	**0.047**	**-0.282**	**-0.031**	**0.456**		-0.166	0.947**
	p	**0.725**	**0.207**	**0.341**	**0.842**	**0.905**	**0.463**	**0.938**	**0.218**		0.722	0.001
distance change	r	**-0.368**	**-0.814****	**-0.521**	**-0.092**	**-0.122**	**-0.538**	**-0.244**	**0.729***	**0.701***		-0.380
	p	**0.329**	**0.008**	**0.151**	**0.814**	**0.754**	**0.135**	**0.527**	**0.026**	**0.035**		0.401
time change	r	**-0.186**	**-0.629**	**-0.363**	**0.069**	**-0.035**	**-0.434**	**-0.096**	**0.519**	**0.838****	**0.933****	
	p	**0.632**	**0.070**	**0.337**	**0.860**	**0.929**	**0.243**	**0.805**	**0.153**	**0.005**	**0.000**	

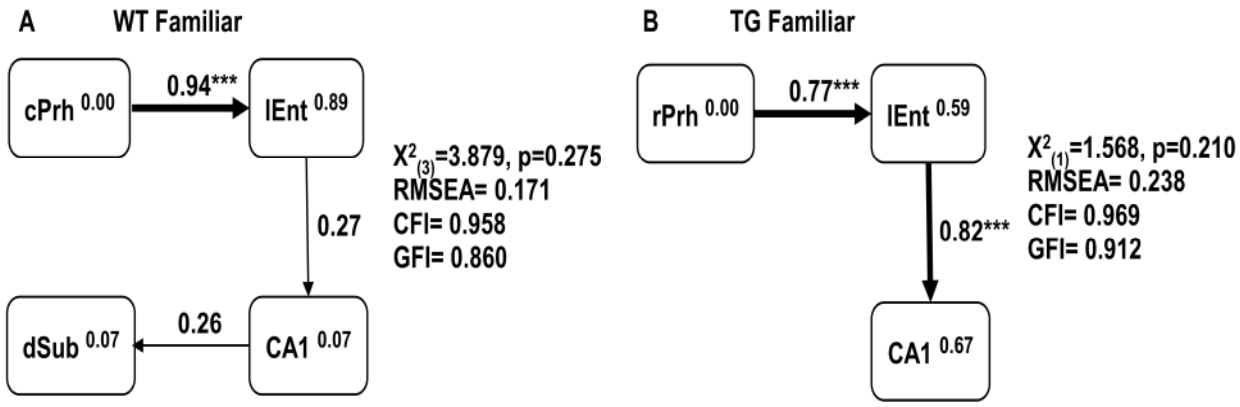

Figure 3.15: Optimal models for 12 month old Tg2576 mice in group familiar. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

and increased RMSEA compared to 3 months. This may be due to an increased variation in the data set due to the effects of aging. When data from 12 month old Tg2576 mice was fitted to this model the fit was poor, shown particularly by the significant chi-squared value ($\chi^2_{(3)}$=20.438, p<0.001, GFI=0.632, CFI=0.590, RMSEA=0.762). Modification of this model by changing the perirhinal cortex input from caudal to rostral, as in 3 month old mice, and removing the output via the dorsal subiculum gave a considerably better fit, see **Figure 3.15b**. However, with so few regions included in the model the degrees of freedom were reduced to 1, and therefore gave this model less predictive value, although all pathways are significant. When data from wild-type mice was then fitted to a model without projections from CA1 to the subiculum this also gave an excellent fit ($\chi^2_{(1)}$=0.057, p=0.812, GFI=0.996, CFI=1.000, RMSEA<0.001). These data suggest that the output from CA1 to the dorsal subiculum may be impaired by 12 months of age in normal mice, and that this impairment is increased in Tg2576 mice.

Model Stacking

Multiple group analysis revealed no significant difference in the overall structural weights between transgenic and wild-type mice in group familiar (wt model: $\chi^2_{(3)}$=4.931, p=0.117, tg model: $\chi^2_{(3)}$=0.118, p=0.943). In addition, analysis of individual pathways did not reveal significant differences in any pathway, although the connection from CA1 to the dorsal subiculum just failed to reach conventional significance levels ($\chi^2_{(1)}$=3.039, p=0.081). The lack of overall difference between structural weights suggests that both transgenic and wild-

type mice show similar functional interactions between these regions when processing familiar information at 12 months of age. However, the interaction between CA1 and the subiculum is less robust in transgenic mice.

Group Novel

Correlations

The correlations between c-fos expression in different brain regions and behaviour for mice in group novel are shown in **Table 3.9**. In wild-type mice there are significant correlations between all brain regions, and between c-fos expression in the the caudal perirhinal cortex and the time spent moving on day 4. In contrast, not all regions are significantly correlated in transgenic mice and only CA3 correlates with a behavioural measure, the change in distance moved. This pattern of greater correlation between brain regions in wild-type mice in group novel matches that of 3 month old Tg2576 mice.

Structural Models

Fitting the baseline model to pooled data from all mice in group novel gave a poor fit ($\chi^2_{(8)}$=51.873, p<0.001, GFI=0.714, CFI=0.752, RMSEA=0.511), therefore modification was required to fit the model to each data set. The optimal model for wild-type mice in group novel is shown in **Figure 3.16a**. Interestingly this model differs from that of C57Bl6 mice and 3 month old wild-type mice as the tri-synaptic pathway is not included in this model, with projections via the DG excluded from the model. This suggests that perhaps even in normal aging DG function is impaired and alternative pathways are predominantly used for processing novel information. This alternative pathway seems likely to involve the direct perforant pathway into the CA3 region and then the Schaffer collaterals to CA1, as these are the most significant pathways in this model. As with C57Bl6 mice and 3 month old wild-type mice, reversal of the lateral entorhinal to CA1 pathway only slightly reduced the model fit. The optimal wild-type model still does not give an excellent fit to the data. This may be due to increased variation between mice with aging, or may suggest alternative pathways outside the hippocampal projections modelled here, which could compensate for the functional disconnection of the DG. Fitting the wild-type model to data from 12 month old transgenic mice in group novel gave a good fit ($\chi^2_{(2)}$=1.879, p=0.391, GFI=0.921, CFI=1.000, RMSEA<0.001), which was only slightly improved by substituting the caudal for the rostral

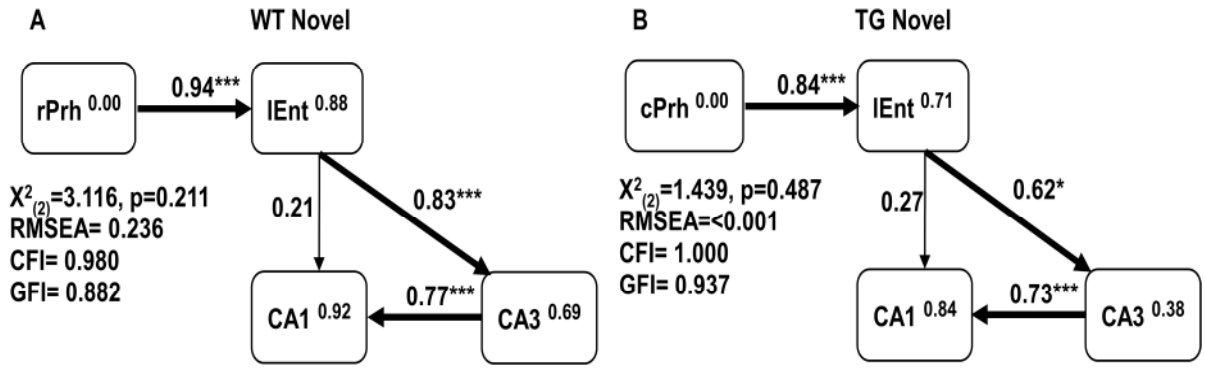

Figure 3.16: Optimal models for 12 month old Tg2576 mice in group novel. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

perirhinal cortex, **Figure 3.16b**. This model is an excellent fit and suggests that the early functional disconnection of the DG seen at 3 months of age continues at 12 months after the onset of amyloid pathology.

Model Stacking

Despite data from both wild-type and transgenic mice fitting the same optimal model in terms of hippocampal sub-region connectivity, stacking of the models revealed a significant difference in path coefficients (wt model: $\chi^2_{(4)}$=16.942, p=0.002, tg model: $\chi^2_{(4)}$=11.701, p=0.020). Comparison of individual path strengths showed that this was driven by a significant difference only in the rostral perirhinal to entorhinal cortex pathway ($\chi^2_{(1)}$=8.121, p=0.004), and not by a difference in functional interaction between hippocampal sub-regions. These data suggest that both wild-type and transgenic mice in group novel engage similar hippocampal networks, but that the input pathways from the perirhinal cortex may differ.

Discussion

In contrast to young Tg2576 mice, 12 month old mice did not show a significant increase in exploratory activity in response to novelty. There was a non-significant trend towards an increase in wild-type mice, whilst the behaviour of transgenic mice was not different between novel and familiar environments. This lack of behavioural change in response to novelty was paralleled by no differences in hippocampal c-fos expression for either genotype between

group novel and familiar, and differences in the parahippocampal cortices only for wild-type mice. There was, however, a reduction in c-fos expression in the septal hippocampus in transgenic mice compared to wild-types across test groups. Despite a different pattern of c-fos induction in wild-type mice SEM still revealed the same optimal model of network engagement for processing familiar information as in 3 month old mice, although this model did not give excellent fit indices. In contrast, the CA1 output via the subiculum was impaired in transgenic mice. When processing novel information there was no difference in hippocampal network engagement between genotypes at 12 months old, with neither genotype including the DG in the optimal model.

The reduction in c-fos expression across the septal hippocampus regions in transgenic mice compared to wild-types matches data from studies in other APP transgenic models which show reduction in baseline IEG expression (Palop et al., 2003, Lee et al., 2004b, DeIpolyi et al., 2008). This reflects a reduction in neuronal activity which is likely to be due to synaptic impairment (Chapman et al., 1999, Fitzjohn et al., 2001).

The lack of a behavioural change seen in this experiment was not expected as a previous study by Barnes et. al. (2004) demonstrated that at 12-14 months of age both wild-type and transgenic mice could discriminate between different test rooms. The main difference between these studies was that in the study by Barnes et. al. one of the rooms was associated with an appetitive reward. This leads to the suggestion that the deficit in response to novelty seen here may be due to impaired attentional processes with age; the mice do not attend to the different extra-maze surroundings when there are no learning requirements. There is evidence of some attentional deficits in the Tg2576 model, which are seen in both wild-type and transgenic mice (Middei et al., 2006, Zhuo et al., 2007). However, wild-type mice did show some, although non-significant, response to novelty, suggesting that amyloid pathology in transgenic mice may further reduce novelty detection.

The lack of a behavioural change makes the c-fos expression data difficult to interpret. Nevertheless, it seems likely that two processes are influencing c-fos expression; amyloid- and age-dependent changes. The different patterns of c-fos induction may account for these two processes independently. In wild-type mice there is no up-regulation of hippocampal c-fos expression, suggesting an age-dependent dysfunction in hippocampal activity. However, compensatory mechanisms seem to be at work in the parahippocampal cortices, where c-fos expression changes were observed. This may allow some detection of spatial novelty,

although not at the same level as in younger mice. In comparison, in transgenic mice there were no changes in c-fos induction in any region, suggesting that amyloid pathology caused more widespread deficits across the hippocampal and parahippocampal regions by 12 months of age.

Optimal SEM models were derived for each experimental group at 12 months of age that matched those of 3 month old Tg2576 and C57Bl6 mice to some extent. However, all of these models showed a reduced level of fit, making interpretation of the models less clear cut. The data from the 12 month models suggest that in both wild-type and transgenic mice connectivity of the DG is compromised. DG pathology has been widely reported in the Tg2576 model at this age (Chapman et al., 1999, Jacobsen et al., 2006), and, to a lesser extent, DG alterations with aging have been reported in non-transgenic mice (Kuhn et al., 1996, von Bohlen et al., 2006). However, the models derived in this experiment would not explain genotypic differences in behaviour reported in the Tg2576 strain (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002), as these models predict similar hippocampal engagement in both wild-type and transgenic mice. It seems likely therefore that compensatory mechanisms outside the hippocampus may be contributing to cognition in wild-type mice with aging. This idea is resonant of findings from MRI studies in humans, which show more wide-spread activity across different brain regions in aging humans (Cabeza et al., 1997, Cabeza et al., 2002, Maguire and Frith, 2003).

Overall, because the behavioural manifestation of novelty detection was relatively weak, few firm conclusions can be drawn from Experiment 3. Novelty detection is impaired by 12 months of age in both transgenic, and to a lesser extent, wild-type mice. Transgenic mice showed reduced c-fos expression in the septal region of the hippocampus as a consequence of amyloid pathology. Normal aging also altered c-fos induction in response to novelty in wild-type mice. However, the poor fit of SEM makes it difficult to identify with confidence the differences in hippocampal sub-region engagement as a consequence of amyloid pathology or aging.

3.5 Chapter Discussion

The experiments described in this chapter report changes in c-fos expression induced by exposure to a novel or familiar environment in C57Bl6 and Tg2576 mice at 3 and 12 months of age. The experiments have demonstrated changes in locomotor behaviour and hippocampal c-fos expression in response to spatial novelty in C57Bl6 mice, with differential hippocampal sub-region activity when mice process novel or familiar cues. A similar pattern of behaviour was seen in 3 month Tg2576 mice for both wild-type and transgenic mice, however less novelty induced c-fos expression was seen in hippocampal sub-regions. The same pattern of hippocampal sub-region engagement was seen in wild-type mice as in C57Bl6 mice. Interestingly, however, transgenic mice showed no evidence of engagement of the DG sub-region during the processing of room novelty information. In 12 month old Tg2576 mice there was little change in behaviour or hippocampus c-fos expression in response to novelty. The same pattern was evident in wild-type mice. SEM for 12 month old mice was less robust with a poorer level of fit of all models. Nevertheless, neither wild-type nor transgenic mice showed evidence for engagement of the DG in the novel condition.

The most interesting result from the present series of experiment, from a conceptual viewpoint, is the change in functional connectivity of the DG in transgenic mice as early as 3 months of age, despite normal reactivity to room novelty. This shows that the DG is compromised even before the onset of overt behavioural deficits. Alterations in the DG are likely to be due to early synaptic changes (Jacobsen et al., 2006), reflecting increased levels of soluble Aβ in the absence of plaque pathology at this age (Hsiao et al., 1996). These data lend support to the amyloid cascade hypothesis, more specifically the view that soluble oligomers contribute to the earliest cognitive deficits and disrupt normal neuronal activity (Selkoe, 2008a).

A previous study by Lelos & Good (2012) also used c-fos expression and SEM to assess functional connectivity in the Tg2576 strain. This study found specific alterations in the mossy fibre, DG to CA3, path strength between transgenic and wild-type mice in a cued fear-conditioning paradigm in which transgenic mice did not show a behavioural deficit. However, this study used 19 month old mice, so it is interesting that no age-related changes in this pathway were reported in wild-type mice, as seen in experiment 3. This may be because cued-fear conditioning is not a hippocampus-dependent task (Kim and Fanselow, 1992, Phillips and LeDoux, 1992), or may reflect the sensitivity of neural systems to aversive

stimuli, whereas the detection of spatial novelty is hippocampus dependent (Jenkins et al., 2004, Lee et al., 2005, Nyberg, 2005) and evident without the need for explicit reinforcement.

The DG appears to be more susceptible to amyloid pathology than other brain regions, as several studies have shown dentate specific changes in long-term potentiation (Chapman et al., 1999), neurogenesis (Dong et al., 2004) and spine density (Jacobsen et al., 2006) from an early age in the Tg2576 model. The data from the present series of experiments suggest that a similar DG dysfunction also occurs in normal aging. Several studies looking at the effect of normal aging in mice have shown deficits in dentate specific tasks and changes in the DG at the cellular level with aging, with alterations evident from 12 months of age (Kuhn et al., 1996, von Bohlen et al., 2006, Creer et al., 2010). A study by Froc et al. (2003) also showed reduced plasticity in the lateral perforant path input to the DG in C57Bl6 mice aged 12 months or more compared to younger mice.

Interestingly, although several studies have published findings of altered synaptic plasticity and reduction in dendritic spines at an early age in the CA1 region of Tg2576 mice (Lanz et al., 2003, Perez-Cruz et al., 2011), and we report a significant reduction in CA1 c-fos expression in transgenic mice compared to wild-types at 3 months of age, overall we did not find any difference in functional engagement within the network activated by novelty in young mice. However, there was a significant reduction in perforant path strength to the CA1 in group novel and a reduction in path strength to the subiculum in group familiar in young transgenic mice. In 12 month old Tg2576 mice, output from CA1 to the subiculum when processing familiar information did not fit the transgenic group data. These data suggest that although CA1 engagement when processing novel cues is not compromised as much as the DG, there are still subtle alterations in the activity of this hippocampal sub-region, coinciding with synaptic changes.

Data from C57Bl6 mice and 3 month old wild-type mice support theories based on computational models that propose a role for the CA1 region in novelty detection, and the DG in pattern separation (Treves and Rolls, 1994, Kesner et al., 2004, Lee et al., 2005, Rolls and Kesner, 2006). The CA1 region is anatomically suited to novelty detection via a match-mismatch process as it receives input directly from the entorhinal cortex, which putatively contains information about the current episode, and also receives information via the tri-synaptic loop from the CA3 region, which may contain information about previous episodes for comparison (Hasselmo and Schnell, 1994, McClelland et al., 1995, Lisman and

Otmakhova, 2001). Supporting this hypothesis and consistent with the data presented here, previous c-fos expression studies have found increases in c-fos in CA1 after presentations of novel configurations of familiar stimuli (Wan et al., 1999, Jenkins et al., 2004). However, data from an object recognition study in rats, in which different hippocampus sub-regions were lesioned, suggested that the CA3 region, with its recurrent collateral network, is critical to novelty detection (Lee et al., 2005). The authors suggest that when novelty detection occurs over a short time frame, i.e. a few minutes as in the object recognition study, the CA3 region is the most critical area for match-mismatch processing. However, when information is consolidated over a longer time period, as in the study presented here, the CA1 region is more critical to novelty detection. A study by Vago & Kesner (2008), in which the direct perforant path input into CA1 was disrupted, reported deficits in spatial novelty processing in lesioned rats. The authors conclude that this direct input pathway is critical only for match-mismatch processing of novel, and not familiar, information. However, the data we present here, supported by previous work from Aggleton and colleagues (Poirier et al., 2008, Albasser et al., 2010), argues against this conclusion, instead SEM suggests that the direct perforant pathway to CA1 is critical when processing both novel and familiar information, and that the additional engagement of the tri-synaptic pathway is critical for novel information.

Several experimental studies have demonstrated the role of the DG in spatial pattern separation (Gilbert et al., 1998, Schmidt et al., 2012) and this function is attributed to the DG in many models of hippocampal function (Treves and Rolls, 1994, McClelland et al., 1995, Wallenstein et al., 1998, Rolls and Kesner, 2006). The early changes in DG function in Tg2576 mice suggest that these mice should show early cognitive deficits in tasks with a greater overlap between stimuli, requiring greater spatial pattern separation. It has been suggested that contextual fear-conditioning relies heavily upon pattern separation processes in the DG to distinguish between two similar contexts (McHugh et al., 2007). Therefore, it is of note that the earliest cognitive deficits reported in Tg2576 are in contextual fear conditioning (Jacobsen et al., 2006, Perez-Cruz et al., 2011). The extent to which the current behavioural procedure engaged pattern separation is unclear. The rooms contained several features in common, such as the shape and material of the test apparatus, therefore, conceivably, pattern separation processes may have been important for novelty detection. Further experiments, particularly in young mice, in which the degree of common element is

systematically manipulated may help address this issue. Nevertheless, the data from the present experiments suggest that DG activity is engaged during context novelty detection.

In both 3 month old and 12 month old mice there is a distinction between the role of the rostral and caudal perirhinal regions in processing novel or familiar information. When wild-type mice process familiar information the optimal structural models included a path connecting the caudal perirhinal region to the entorhinal cortex, whilst in transgenic mice the optimal entorhinal input was from the rostral section of the perirhinal cortex. Interestingly when processing novel information this pattern of functional connectivity was reversed, with wild-type mice relying on rostral perirhinal to entorhinal connections and transgenic mice relying on connections from the caudal perirhinal cortex to the entorhinal cortex. In addition, one of the consistent differences between wild-type and transgenic mice across age groups is a difference in path strength between the rostral perirhinal cortex and the entorhinal cortex. The perirhinal cortex receives input from cortical, subcortical and parahippocampal regions, and also returns projections to many of these areas. However, the location of afferents and efferents to certain regions are not uniformly distributed across the perirhinal cortex and some are regionally selective to either the rostral or caudal section (Furtak 2007). For example, the rostral section receives significantly more input from the insular cortex, olfactory regions, amygdala and entorhinal cortex than the caudal region, and returns significantly more efferents to the insular, frontal and somatosensory cortices. In comparison the caudal region has relatively more afferents from the dorsal thalamus and postrhinal cortex than the rostral region and returns more efferents to temporal, piriform and postrhinal cortices. Selective differences in any of these connected regions may help to explain the differences in cell count and functional connectivity of the perirhinal sub-regions between genotypes, which may subsequently alter the relative strength of inputs into the entorhinal cortex. However, the aim of the experiments in this thesis was to assess differences in hippocampal networks, therefore c-fos expression was not analysed in all of the regions connected to the perirhinal cortex, so specific conclusions cannot be drawn about the exact change in interactions with the perirhinal cortex from distributed networks across the brain which may lead to altered input to the entorhinal cortex.

Overall the data presented in this chapter provide evidence for changes in hippocampal network engagement when processing novel information at an early-age in Tg2576 mice. The data are less clear in 12 month old mice, with both normal aging and amyloid pathology reducing the ability of these mice to detect room novelty.

Chapter 4: Episodic-like Memory in Tg2576 Mice

4.1 Introduction

In chapter 3, differences in hippocampal network activity patterns between transgenic and wild-type mice were reported in the context of a spatial novelty discrimination task. However, this task has very simple memory demands and therefore findings from this experiment may not be particularly relevant to deficits seen in more complex human memory processes that are disrupted in AD patients. The experiments reported in chapter 4 examine neuronal network changes induced by a behavioural analogue of episodic memory in mice and assess the effects of the Tg2576 mutation on memory for episodic and binary associative information.

As described in chapter 1 (section 1.5) one of the core cognitive deficits manifest in the early stages of AD is impaired episodic memory. Episodic memory relates to the conscious recollection of previous personal events and their spatial and temporal context (i.e. when and where an event happened: Tulving, 1972, 2002). Although episodic memory is difficult to model in animals (Clayton et al., 2003, Suddendorf and Busby, 2003), it is possible to examine memory for the core features of episodic memory – that is, an integrated memory of when and where an event occurred (see Crystal, 2010), often referred to as 'episodic-like' memory. Recently, it has been suggested that episodic memory may be a specific kind of configural memory (Iordanova et al., 2008, Iordanova et al., 2009), as it requires information from different modalities (spatial and temporal) to be stored and retrieved as a separate integrated memory from the individual inputs to define a specific prior event. The configural memory theory (Rudy and Sutherland, 1989, 1995) suggests that when different sensory inputs are presented together they are not linked directly, but are stored as individual inputs, which are all also linked to an independent shared or configural memory. Based on this theory, episodic memory can be viewed as a specific form of configural memory that relies upon hippocampal processing (Rudy and Sutherland, 1989, 1995, Aggleton and Brown, 1999, Ergorul and Eichenbaum, 2004, Good et al., 2007a, Iordanova et al., 2009). In contrast to this, elemental memory theory posits that sensory inputs are directly linked, rather than being linked to a separate independent memory, following the theory of association between simultaneous inputs (Hebb, 1949, Rescorla and Wagner, 1972, Rescorla et al., 1985). This type of processing is not disrupted by hippocampal lesions in rats (Iordanova et al., 2009).

While a decline in episodic memory is one of the earliest cognitive deficits in AD, the pathological events that cause this decline remain unclear. One candidate mechanism is the excess production or accumulation of Aβ. Very few studies have successfully tested episodic-like memory in mouse models of AD. Both of the studies which have explicitly assessed episodic-like memory in APP transgenic mice have used object recognition paradigms (Good et al. 2007b, Davis et al. 2012). However, these studies do not distinguish between representations based upon an integrated configural representation and those based on simple associations, which arguably may not be mediated by the hippocampus (Rudy and Sutherland, 1989, 1995, Iordanova et al., 2009, Cowell et al., 2010, Palmer and Good, 2011 for discussion). Thus far no studies have assessed whether configural processing of episodic information is impaired in transgenic models of AD. Indeed, most studies in APP transgenic models have favoured tasks which target either spatial or temporal memory and not the relationship between these two modalities (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002). Assessment of configural processing of episodic cues is of particular interest in the context of Alzheimer's disease and Aβ accumulation as this type of memory has been shown to rely upon the hippocampus, which shows early synaptic impairment in AD as results of amyloid pathology. In addition, this more complex representation episodic cues (as opposed to elemental binding of cues), provides a greater opportunity to pick up cognitive impairment which may be important for early detection in AD.

Recently, Iordanova et. al. (2008) developed a procedure that showed rats can form a configural memory of where and when auditory cues were presented. The same basic task can also be used to assess memory for simple associations between a cue and the location or the time of its presentation. In the Iordanova paradigm, rats were passively exposed to presentations of auditory cues (a tone or a clicker) in two different contexts (A and B). The context in which each auditory cue was presented was dependent upon the time of day. For example, the tone was presented in context A in the morning and context B in the afternoon and *vice versa* for the clicker. To establish whether the rats had formed a representation of this pattern, a conditioned (freezing) response was established to one of the auditory cues. During the test stage, in which no auditory cues were presented, rats showed more freezing in the context in which the conditioned cue had been presented at the specified time of day. An important feature of this procedure is that rats experienced the auditory cues equally often in context A and B and at each time point during exposure. This feature of the design helps to

rule out the possibility that simple binary associations were responsible for the observed pattern of freezing and, by default, indicated that rats had formed an integrated representation specifying the pattern of events. Consistent with configural theories, cell loss, reversible inactivation and disruption of NMDA-receptor dependent synaptic plasticity in the hippocampus disrupted the configural representation, but not binary associations involving the same episodic information (Iordanova et al., 2011).

This novel task is a particularly attractive behavioural paradigm for assessing episodic-like memory in the Tg2576 model for a number of reasons. Firstly, this task fulfils the key aim of this set of experiments in that it must be solved in a configural manner, and cannot be solved by simple binary associations of cues. In addition, this task can be readily adjusted to assess the separate components of episodic-like memory, temporal or spatial/contextual cues, to see whether there are underlying deficits in processing information from either one of these modalities or whether it is only the integration of these cues which is impaired in AD. Another attractive feature of this task is that memory formation relies upon the spontaneous integration of episodic information, as there is no reinforcement, either appetitive or aversive, during the encoding of these cues. In this way the task may closely reflect information processing engaged during human episodic memory formation, which occurs without a specific motivation to remember certain events. Another appealing feature of this task is that each of the components of this task, auditory stimuli, context and time of day, can be closely controlled. Although this feature of the task is not explored in this thesis, which instead follows the protocol outlined in the published literature (Iordanova et al., 2008, 2009), this allows manipulation of task difficulty, making this this task suitable for detecting a range of levels of cognitive impairment, and also for altering the pattern separation demands of this task which may be critical to episodic memory (Rolls, 2010, Holden and Gilbert, 2012, Schmidt et al., 2012). In terms of translation to human patients, this feature would make this task useful for making distinctions between patients with MCI, early or late stages of AD.

However, there are some caveats of this behavioural task in terms of the relationship between configural memory for episodic information tested here, and true episodic memory in humans, which may hinder translation of findings of this task to humans. The first, as outlined earlier, is that this task only assesses episodic-like memory, and does not test the conscious attribute of true episodic memory. The second is that there is some discrepancy in the literature as to the precise definition of the temporal aspect of episodic-like memory. Several studies of memory in rodents use recency or temporal order as measures of the

'when' aspect of memory (for example Babb and Crystal, 2006, Good et al. 2007b, Davis et al. 2012), whereas others use a time of day (i.e. morning and afternoon), in a similar way to this task (for example Amin et al., 2006, Zhou and Crystal 2011). It is unclear when using a time of day as the 'when' component of episodic memory whether the animals are using this component as a 'time-stamp', for example related to circadian cycles, or whether the animals are using other more contextual cues to distinguish the two sessions. However, it is argued here, that even if rodents are using contextual cues to establish a time of day this in many ways still relates to the way in which humans recall when previous events occurred. For example, when recalling an event from the previous week or month humans do not tend to use recency discrimination but instead often think about other cues which may have been present at that specific time. A final caveat of this task, which may preclude direct translation of findings to humans, is that it does not include single-trial learning. Single-trial or single-exposure learning is thought to be critical to episodic memory (McNaughton and Morris, 1987, Treves and Rolls, 1994, Lee and Kesner 2004a), as repeated experience of events is not necessary to stably encode memories in humans. However, pilot work revealed that rodents could not learn the complex configurations of cues used in this paradigm after a single exposure. Further work altering the salience of cues, as described above, may make this task easier, and therefore overcome this obstacle. Despite these shortcomings this task still represents an interesting and informative paradigm with which to assess memory in Tg2576 mice.

There is evidence that hippocampal synaptic plasticity contributes to configural memory, and excess Aβ production disrupts synaptic communication in the hippocampus in the Tg2576 model (Jacobsen et al., 2006). This leads to the prediction that Tg2576 mice will show a deficit in configural, but not elemental, memory for episodic information. The experiments in this chapter set out to test this hypothesis. Experiment 1 establishes that normal mice can form integrated configural memories using the paradigm designed by Iordanova et. al. Following this, Experiment 2 assesses configural memory for episodic information in 3 month old Tg2576 mice, revealing no deficit at this age. Experiments 3 and 4 assess memory for configural and elemental associations in 11 month old Tg2576 mice, respectively, demonstrating impairment in configural but not elemental processing of episodic cues in transgenic mice compared to controls. Finally, Experiment 5 uses c-fos expression analysis to investigate the neural systems involved in retrieval of configural and elemental

representations, in an attempt to establish which systems may be compromised in Tg2576 mice, contributing to the behavioural deficits reported on the configural memory task.

4.2 Experiment 1: Configural Learning in C57Bl6 mice

Introduction

The aim of Experiment 1 was to assess whether mice form spontaneous configural memories integrating episodic information of what happened when and where. The standard laboratory strain, C57Bl6, is used in this experiment, as this is the most well characterized strain of mice. The behavioural paradigm developed by Iordanova et. al. (2008) was used for this purpose. This paradigm has not been used with mice before and subtle changes were made to the apparatus to make the task more suitable for mice, including using mouse operant chambers and making the contexts more distinguishable from each other. Due to a high conservation in anatomy and memory processes between rats and mice, it is predicted that mice will be able to acquire configural representations of episodic information.

Methods

Subjects: 16 male 6 month old C57Bl6 mice were used in experiment 1. Animals were group housed in standard cages with free access to food and water and a 12hr light/dark cycle. Testing took place during the light-phase.

Apparatus: All testing was carried out in mouse operant chambers (L 15.9cm x W 14.0cm x H 12.7cm, Med Associates) connected to a standard PC running Med PC software (Med Associates Inc.).

Behavioural Procedure: The configural learning task was carried out as described in Iordanova et.al. (2008), and shown in **Figure 4.1**.

Stage 1, Exposure: Mice were passively exposed to a counterbalanced pattern of cue presentations for four days. Each mouse received two 5 minute sessions in the morning and afternoon periods; the two periods were separated by an interval of approximately 8 hours. In the first morning session, a mouse was placed in one of the contexts (e.g., striped context) and received 10 presentations of a 90dB auditory cue (e.g., tone), each for 10 seconds with an interval of 20 seconds between presentation. Each mouse was then returned to their home cage for a 5 minute period before being placed into the second context (e.g. blank context) where they received presentations of the remaining auditory cue (e.g. clicker). This procedure was repeated in the afternoon; however the auditory stimuli were reversed so the tone was presented in the blank context and the clicker in the striped context. Each context was cleaned thoroughly between trials, and the litter tray was emptied at the end of each morning and afternoon session.

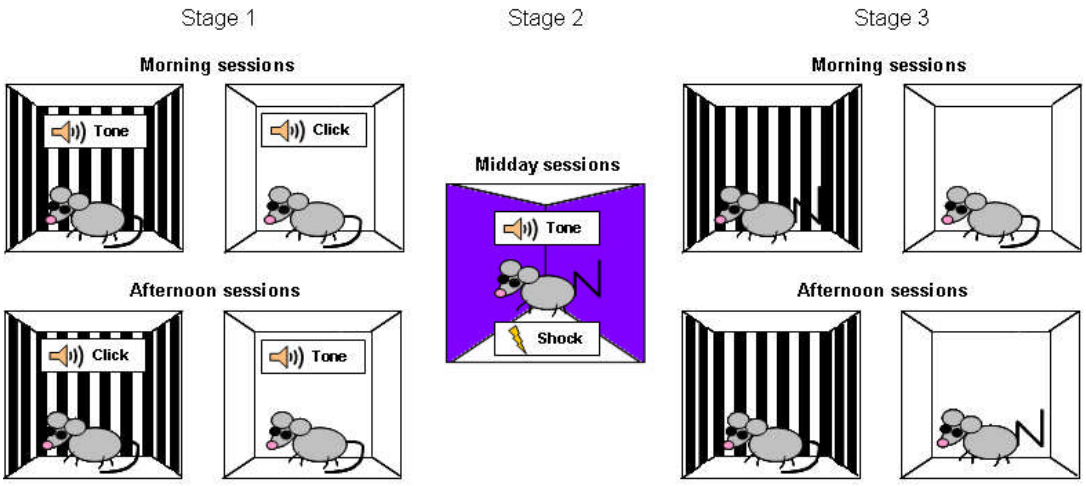

Figure 4.1: Task procedure. The task consists of 3 phases. Stage 1, Exposure: Mice are exposed to a pattern of cue presentations over four days containing information about what happened, where and when. Stage 2, Conditioning: To provide a measureable conditioned response (freezing), one of the auditory stimuli is re-valued by pairing with a foot-shock over two-days. Stage 3, Test: Mice were tested over the next two consecutive days in each context during morning and afternoon sessions and freezing behaviour was measured.

Stage 2, Conditioning: On days 5 and 6, the mice received conditioning trials during which one of the auditory cues was paired with foot-shock. Conditioning was carried out in a third context formed by a triangular-shaped insert in each operant box with purple wall-paper. Each mouse received two 90 second sessions per day carried out at midday with a 20 minute interval between sessions, which was spent in an adjacent holding room in a clean cage. In one session the mice received three 10 second presentations of one of the auditory stimuli (e.g. tone) that co-terminated with a 2 second foot-shock (0.4mV), with an interval of 20 seconds between presentations. In the second session, the mice received presentations of the remaining auditory cue (e.g. clicker), which was not followed by a foot-shock. The pairing of an auditory stimulus with foot-shock, and the order of presentation was fully counterbalanced across mice.

Stage 3, Test: Following conditioning, the mice were tested over the next two consecutive days in each context during morning and afternoon sessions. The test sessions lasted 3 minutes and no auditory cues were presented. The behaviour of the mice was recorded using a camcorder.

Conditioning test: On the final day the mice were tested for the level of freezing elicited by each auditory stimulus. This session was carried out in the same context and using the same parameters as the conditioning phase, with the exception that no foot-shocks were presented. Each trial was recorded using a camcorder.

Data analysis and statistics: Behaviour was scored using a 2-second time-lapse sampling procedure by an observer who was blind to genotype and test condition. Mice were scored as either moving or freezing (the lack of any visible movement except that of normal breathing) during each observation. The data were expressed as a percentage of observations with a freezing response and converted into a freezing ratio for each mouse for both the morning and afternoon sessions. The freezing ratio for the morning sessions was calculated as the percentage of time spent freezing in the correct context divided by the total amount of time spent freezing across both contexts. The ratio derived from the afternoon sessions was calculated in the same way, with the morning context again acting as the correct context. If an animal differentially expressed freezing according to the context and time of day, then the freezing ratio should be above 0.5 in the morning and below 0.5 in the afternoon.

Results

The results of this experiment are shown in **Figure 4.2**. C57Bl6 mice showed successful memory retrieval with a freezing ratio greater than 0.5 in the morning and less than 0.5 in the afternoon (H_0: $\mu=0.5$, morning: $t_{(16)}=2.811$, $p=0.009$, afternoon: $t_{(16)}=2.333$, $p=0.027$). Statistical analysis showed a significant difference between levels of freezing in the morning and afternoon (paired t-test, $t_{(7)}=3.134$, $p=0.0165$). This result indicates that mice can form configural memories of episodic information that enable them to distinguish between different patterns of context, time of day and auditory stimuli.

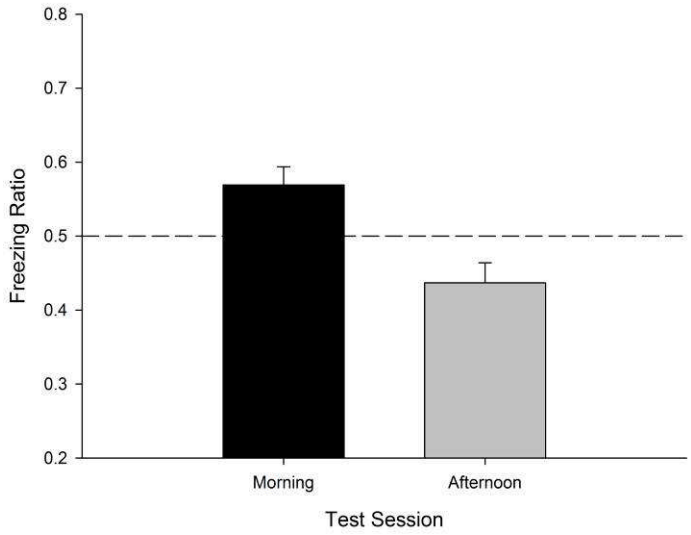

Figure 4.2: Configural learning in C57Bl6 mice. Freezing >0.5 in the morning and <0.5 in the afternoon indicates successful memory retrieval. Bars represent mean±SEM (n=16).

Discussion

The data presented in Experiment 1 showed that C57Bl6 mice can form configural representations involving spatial and temporal cues. Mice produced the expected pattern of behaviour, freezing more to the contexts which were previously associated with the conditioned auditory stimuli at the correct time of day. Interestingly, the behavioural procedure required few changes to the parameters to adapt its use for mice (Iordanova et al., 2008). The fact that a similar pattern of behaviour emerged in mice demonstrates the robustness of the task.

4.3 Experiment 2: Configural learning in 3 month old mice

Introduction

Having established a behavioural paradigm with which to assess whether mice can form a configural representation of episodic information, Experiment 2 examines the effect of the APPswe mutation on task performance in 3 month old Tg2576 mice to determine whether performance is influenced by pathology at this age. In the previous chapter it was reported that early alterations in hippocampal sub-region engagement are evident at this age, and previous studies have reported deficits in LTP and spine density in the hippocampus around this age point (Lanz et al., 2003, Jacobsen et al., 2006). However, these early alterations in hippocampus function did not have an impact on behaviour in the spatial novelty task presented in chapter 3, or in spatial navigation tasks used in previous studies to assess reference and working memory (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002). It was therefore of theoretical interest to determine whether early synaptic deficits reported in young transgenic mice were sufficient to disrupt configural memory for episodic information.

Methods

The testing procedure used in Experiment 2 was identical to that described in Experiment 1.

Subjects: Experiment 2 used male 2-3 month old Tg2576 mice. 24 mice were used in total, 12 transgenic mice and 12 wild-type littermates.

Results

The performance of young (2-3 month old) transgenic and wild-type mice on the configural (what-where-when) procedure is shown **in Figure 4.3a**. Inspection of this figure shows that both wild-type and transgenic mice performed similarly on this task. Both groups showed more freezing to the context in which the conditioned cue had previously been presented during the morning or afternoon session. A two-way ANOVA confirmed this interpretation

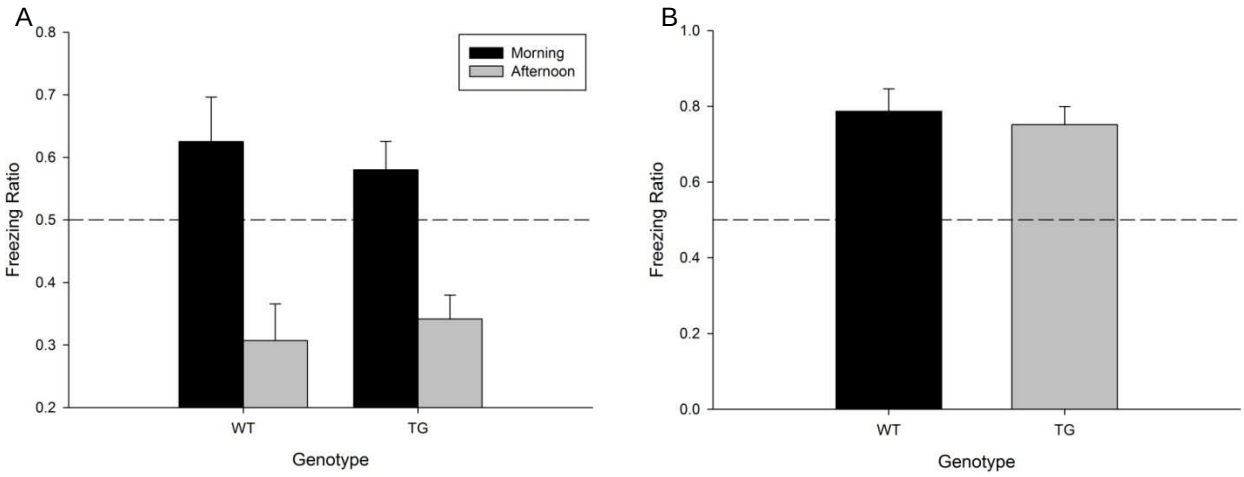

Figure 4.3: Configural learning in 3 month old Tg2576 mice. A: Configural learning in 3 month old wild-type and transgenic Tg2576 mice. Bars represent freezing ratio mean±SEM (wt n=12, tg n=12). Freezing ratio >0.5 in the morning and <0.5 in the afternoon indicates successful memory retrieval. B: Freezing ratio to the conditioned auditory stimuli compared to the unconditioned.

and revealed a significant main effect of time ($F_{(1,22)}$=40.108, p<0.001), but not genotype ($F_{(1,22)}$=0.007, p=0.935) and no interaction between the two factors ($F_{(1,22)}$=0.823, p=0.372). However, although the freezing ratio showed no difference in pattern of behaviour between wild-type and transgenic mice, transgenic mice showed an overall significantly greater level of freezing in both contexts compared to wild-type; wt: 3.54±1.06% of observations in conditioned context, 1.78±0.51% unconditioned; tg: 12.31±2.54% conditioned, 9.10±2.78% unconditioned (conditioned: $t_{(12,12)}$=3.186, p=0.004, unconditioned: $t_{(12,12)}$=2.588, p=0.017). As expected, at the final conditioning test both the wild-type and transgenic mice showed greater freezing to the auditory cue previously paired with foot-shock; **Figure 4.3b**, (H_0: μ=0.5, wt: $t_{(11)}$=4.846, p=0.001, tg: $t_{(11)}$=5.309, p<0.001).

Discussion

Experiment 2 revealed no deficit in the ability of young transgenic mice to form configural associations of episodic information. This finding suggests that episodic-like memory deficits are not seen before the onset of overt amyloid pathology, and survive alterations in hippocampus spine density (Lanz et al., 2003, Jacobsen et al., 2006), synaptic plasticity (Jacobsen et al., 2006, Perez-Cruz et al., 2011) and sub-region engagement at 3 months of

age (chapter 3). However, as a further reduction is seen in each of these measures with aging (Lanz et al., 2003, Jacobsen et al., 2006) it leaves open the possibility that deficits may be manifest at later ages. The lack of a behavioural deficit in 3 month old transgenic mice on this task matches findings on other tasks, such as the water maze and t-maze (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002). The only task for which deficits have been reported at an early age is contextual fear-conditioning (Jacobsen et al., 2006, Perez-Cruz et al., 2011). However, this finding is inconsistent with the data we report here, Tg2576 mice showed fear to both contextual and temporal elements of this task. It is likely that procedural differences can explain this discrepancy. In the task presented here the mice are pre-exposed to the different auditory cue-context-time combinations for four days prior to fear-conditioning, whereas in the classic contextual fear-conditioning paradigm, there is no extensive pre-exposure, and such exposure may protect against a deficit in processing contextual information.

Overall the data presented in Experiment 2 support the conclusion that at 3 months of age, before the onset of overt amyloid pathology, Tg2576 mice are able to form configural memories of episodic information.

4.4 Experiment 3: Configural learning in 11 month old Tg2576 mice

Introduction

In Experiment 3, 11 month old Tg2576 mice are tested on the configural learning task. At 11 months of age Tg2576 mice show robust amyloid pathology, with high levels of soluble amyloid and the appearance of plaques in the hippocampus and parahippocampal regions (Hsiao et al., 1996, Kawarabayashi et al., 2001). There are also marked deficits in synaptic plasticity in the DG and CA1 sub-regions and further reduction in dendritic spine density from that seen at 3 months of age (Chapman et al., 1999, Fitzjohn et al., 2001, Lanz et al., 2003, Jacobsen et al., 2006). In addition, by 11 months of age behavioural deficits are evident on several memory tasks including the water maze, t-maze, y-maze, and object recognition paradigms (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002, Good et al., 2007b, Yuede et al., 2009). Given the absence of a behavioural deficit on the

configural memory task in Experiment 2, the current study tested the prediction that a deficit would be evident in Tg2576 mice at 11 months of age.

Methods

Experiment 3 was carried out using an identical protocol to Experiment 1.

Subjects: 40 male 11 month old Tg2576 mice were used in total for Experiment 3, 20 wild-type and 20 transgenic mice.

Results

Figure 4.4a shows the results from 11 month old transgenic and wild-type mice. Inspection of this figure shows that unlike young transgenic mice, and age matched wild-type controls, 11 month old transgenic mice failed to discriminate between the contexts as a function of time-of-day. A two-way ANOVA revealed no significant main effect of either genotype ($F_{(1,35)}$=0.082, p=0.776) or time of day ($F_{(1,35)}$=2.004, p=0.166), but a significant interaction between these factors ($F_{(1,35)}$=8.908, p=0.005). Follow-up with tests of simple main effects

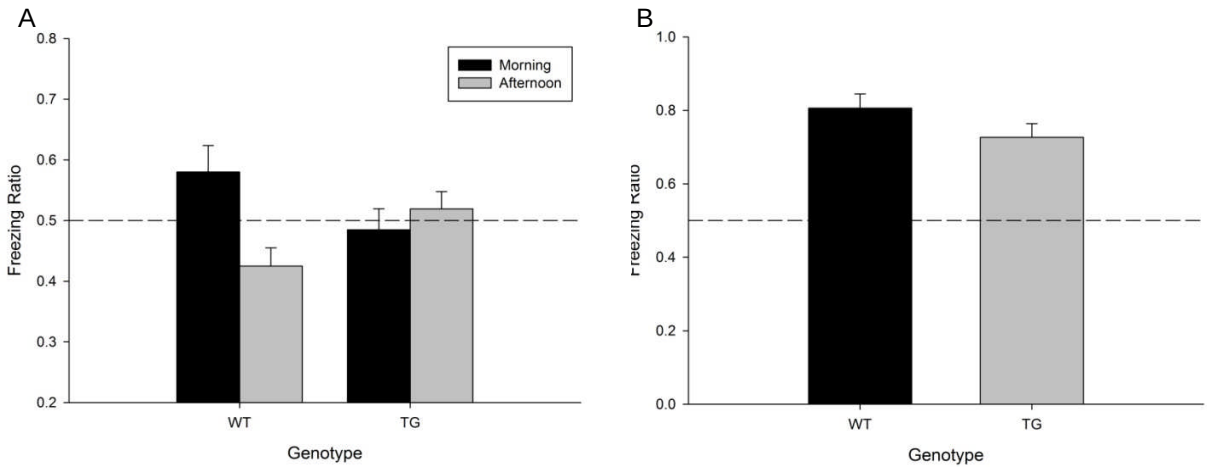

Figure 4.4: Configural learning in 11 month old Tg2576 mice. A: Configural learning in 11 month old wild-type and transgenic Tg2576 mice. Bars represent freezing ratio mean±SEM (wt n=19, tg n=18). Freezing ratio >0.5 in the morning and <0.5 in the afternoon indicates successful memory retrieval. B: Freezing ratio to the conditioned auditory stimuli compared to the unconditioned.

showed a significant effect of time-of-day for the wild-type mice ($F_{(1, 35)}$=9.682, p=0.004) but not for the transgenic mice ($F_{(1,35)}$=1.231, p=0.276). Importantly, the failure of older transgenic mice to discriminate between the contexts as a function of time-of-day was not caused by impaired discriminative conditioning of the auditory cues, see **Figure 4.4b** (H_0: μ=0.5, wt: t(18)=9.168, p < 0.001, tg: t(16)=7.737, p < 0.001). As with 3 month old mice, aged transgenic mice overall showed a significantly greater level of freezing than wild-type mice in both contexts; wt: 9.22±0.94% conditioned context, 7.01±0.84% unconditioned; tg: 17.90±3.44% conditioned, 18.19±3.16 unconditioned (conditioned: $t_{(20,20)}$=2.460, p=0.019, unconditioned: $t_{(20,20)}$=3.454, p=0.001).

Discussion

Experiment 3 reported that by 11 months of age transgenic Tg2576 mice were impaired compared to wild-type mice in forming configural memories of episodic information. This contrasts with the data presented in Experiment 2 for 3 month old transgenic mice, and indicates that the onset of amyloid pathology leads to a decline in configural memory for episodic information. When the performance of 3 and 11 month old wild-type mice is compared across experiments, 3 month old wild-types performed better, with a greater discrimination between contexts and time of day, suggesting some age-related decline in performance.

The data presented here supports data from other studies assessing spatial memory in showing a decline in cognition by 11 months of age (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002, Good et al., 2007b). In particular, our findings of deficits in episodic-like memory processing in aged Tg2576 mice replicates that report by Good et. al. (2007b), who showed similar deficits using an object recognition paradigm. Nevertheless, one would argue that the paradigm used here is a more accurate test of episodic-like memory than the object recognition paradigm, as it requires the use of an integrated configural memory. Configural association of cues is thought to be critical to stable episodic or episodic-like memory formation and, like episodic memory, is also reliant upon the hippocampus (Rudy and Sutherland, 1989, Alvarado and Rudy, 1995, Aggleton and Brown, 1999, Ergorul and Eichenbaum, 2004, Good et al., 2007a, Iordanova et al., 2009). In contrast, several studies have shown that elemental processing of binary associations is not

hippocampus-dependent (Rudy and Sutherland, 1995, Iordanova et al., 2009), and therefore can be separated anatomically from episodic memory.

4.5 Experiment 4: Elemental learning in 11 month old Tg2576 mice

Introduction

The data in experiment 3 revealed deficits in configural processing of episodic cues in 11 month old Tg2576 mice. Although evidence was presented that Tg2576 mice were able to process and form an association between an auditory cue and shock, it remains unclear to what extent the mice processed the contextual and temporal elements of this task. Experiment 4 examines whether the putatively configural deficit reflects a more rudimentary impairment in forming elemental associations between sensory events. Thus a second group of 11 month old mice were tested on the binary association versions of the task. Both spatial and temporal associations were tested using a what-when and a what-where paradigm. The formation of binary associations between cues is not thought to rely upon the hippocampus (Rudy and Sutherland, 1995, Iordanova et al., 2009), and therefore may be spared in aged Tg2576 mice. However, although the most prominent pathology is in the hippocampus of these mice, cortical pathology is also reported (Hsiao et al., 1996, Kawarabayashi et al., 2001) and thus prompted the following experiment.

Methods

Experiment 4 was carried out using an identical protocol to Experiment 1, unless otherwise specified.

Subjects: 68 male 11 month old Tg2576 mice were used in total for experiment 4, 36 wild-type and 36 transgenic mice. Of these 18 from each genotype were used for each behavioural condition; what-where or what-when.

Behavioural Procedure:

Experiment 4a: The procedure for the elemental associative tasks was identical to the configural task described previously with the following exceptions. For the what-when procedure the same auditory stimulus was presented in both contexts in the morning and afternoon (e.g., tone in both the blank and striped in the morning; clicker in both contexts in the afternoon). In the what-where procedure, one auditory stimulus was always presented in one context (e.g., tone in the blank context) in both the morning and afternoon trials. The remaining cue (e.g., clicker) was always presented in the alternative context (e.g., striped context) in both the morning and afternoon sessions. The cue-context or time-of-day assignments were counterbalanced across mice. The conditioning and test procedures were identical to those described for the configural task in Experiment 1.

Experiment 4b: To assess simple context discrimination, after a two week interval during which no testing was carried out, mice were tested for a further four days. The first two days of testing were carried out using a similar protocol to the conditioning phase of the configural task, described in Experiment 1. There were two main alterations to the procedure. First, one of the conditioning sessions was carried out in the striped context and the other in the blank context each day, and second, during each session mice received un-signalled foot-shocks in one but not the remaining context. Following this, the mice were tested over the next two days. Each day, around midday, mice were placed in each context (blank or striped) for 3 minutes, with a 3 minute interval between sessions, and freezing was scored in each context.

Results Experiment 4a: Elemental learning

There was no difference in performance between genotypes on the elemental learning tasks, shown in **Figure 4.5a**. A Two-way ANOVA revealed no significant difference between genotypes ($F_{(1,63)}$=0.189, p=0.666) or test ($F_{(1,63)}$=0.572, p=0.452) and no interaction between these factors ($F_{(1,63)}$=0.499, p=0.482). However, separate one-sample t-test analysis of what-when and what-where data comparing each freezing ratio to chance revealed that whilst wild-type mice were significantly above chance on both binary associations (when $t_{(14)}$=2.757, p=0.015; where $t_{(16)}$=2.354, p=0.032), transgenic mice were impaired on associations involving context but not time-of-day (when $t_{(17)}$=2.501, p=0.023; where, $t_{(16)}$=1.289, p=0.216).

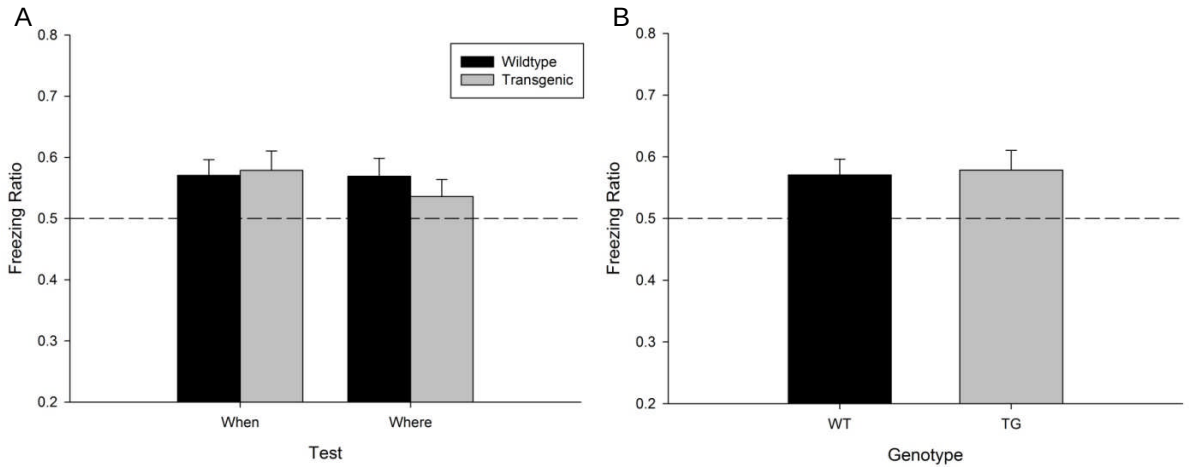

Figure 4.5: Elemental learning in 11 month old Tg2576 mice. A: Elemental learning; what-where, what-when. Bars represent freezing ratio mean±SEM (when, wt n=15, tg n=18, where wt n=17, tg n=17) Freezing ratio >0.5 shows successful memory retrieval. B: Context discrimination. Bars represent freezing ratio mean±SEM (wt n=12, tg n=12).

Results Experiment 4b: Context discrimination

The pattern of responding in the transgenic mice during the what-where test indicates a deficit in context processing. To confirm whether 11 month old Tg2576 mice were unable to discriminate between the contexts, all mice were given an additional period of training during which they received un-signalled foot-shocks in one but not the remaining context that were used during exposure, and freezing in each context was then recorded over the following two test days. Both wild-type and transgenic mice discriminated between the two contexts, and showed more freezing in the context paired with foot shock, **Figure 4.5b**. Both groups performed significantly above chance, (H_0: μ=0.5, wt: $t_{(15)}$=3.882, p=0.0015, tg: $t_{(15)}$=3.788, p=0.0018).

Discussion

Experiment 4 tested the performance of 11 month old Tg2576 mice on a task involving only elemental binary associations between an auditory stimulus and either a time-of-day or a context. The results suggest that elemental processing of temporal information, associating time of day with an auditory stimulus, is equivalent between wild-type and aged transgenic mice. However, the results of the contextual task were less clear. Although there was no significant difference in performance between wild-type and transgenic mice, only the wild-

type mice performed above chance level. These data suggest that Tg2576 mice may have a mild deficit in processing contextual cues, and linking them into an integrated memory. However, Experiment 4b demonstrated that aged Tg2576 mice were able to discriminate successfully between context cues and support the view that at the very least perceptual and associative mechanisms remain intact.

The latter finding draws into question any firm conclusion that the configural memory reflects a deficit in forming elemental associations. However, one must acknowledge that the two tasks are very different and that the mice had further opportunity to process contextual cues which may have masked a more subtle deficit in processing contextual cues on the simple discrimination task. Furthermore, the work by Good et. al. (2007b) came to the conclusion that spatial memory deficits may underlie episodic-like impairments. One should also not lose sight of the fact that the object recognition paradigm used by Good et. al. required the mice to remember the relative spatial location, whereas in the task presented here the mice are required to discriminate between two similar contexts – arguably these features may rely on slightly different memory systems. Indeed, there is some evidence to support this view. Context discrimination is supported by the perirhinal cortex (Corodimas and LeDoux, 1995, Bucci et al., 2000, Bucci et al., 2002), whereas novel object configurations are not (Wan et al., 1999, Aggleton and Brown, 2005). Furthermore, context-cue associations in the what-where task are disrupted by perirhinal cortex lesions in rats (Iordanova et al., 2009). The absence of an object recognition deficit in Tg2576 mice (Hale and Good, 2005) would suggest that perirhinal cortex activity is unlikely to be impaired in the Tg2576 model (Zhu et al., 1995, Wan et al., 1999, Aggleton and Brown, 2005, Winters et al., 2008), however, the level of disruption to this area in Tg2576 mice may be sufficient to disrupt context processing, at least under some conditions.

Behavioural studies have extensively reported deficits on spatial tasks in Tg2576 mice (Hsiao et al., 1996, Chapman et al., 1999, Westerman et al., 2002, Hale and Good, 2005), however, reports of temporal memory deficits are more varied with deficits in trace fear conditioning reported in 6 month mice (Ohno et al., 2006), but no deficit in recency judgement in an object recognition paradigm at 14 months (Hale and Good, 2005). In addition Good et. al. (2007b) reported deficits in spatial, but not temporal processing in 10-12 month Tg2576 mice in an object recognition based episodic-like memory task. The data presented here agree with that of Good et.al. showing that at 11 months of age temporal discriminations based upon the time of day are unimpaired in Tg2576 mice.

Overall is can be concluded that temporal associations are unimpaired and spatial aspects of elemental processing are only mildly impaired in the Tg2576 model. As such, the data suggest that gross deficits in processing either the temporal or contextual aspects of the configural memory task cannot account for the dramatic deficit seen Tg2576 mice at 11 months of age.

4.6 Experiment 5: C-fos expression during configural and elemental learning

Introduction

Experiments 3 and 4 demonstrated deficits in configural memory for episodic information in Tg2576 mice. The aim of Experiment 5 was to assess the contribution of hippocampal sub-regions to both configural and elemental learning using IEG analysis and SEM (as described in chapter 3). The aim of this analysis was to provide insight into how the tasks engaged activity in the hippocampus and thus provide a theoretical basis for understanding how the APPswe mutation may compromise this form of memory. Current computational models anticipate that the DG is important for pattern separation (Marr, 1971, Treves and Rolls, 1994, Gilbert et al., 2001, Rolls and Kesner, 2006, Schmidt et al., 2012), which may be engaged during the configural learning task to distinguish between the overlapping patterns of auditory, context and temporal cues. It is also predicted that the CA3 region will show altered c-fos expression in both the configural and elemental paradigms compared to controls. This region is hypothesised to play a role in pattern completion (Marr, 1971, Treves and Rolls, 1994, Gold and Kesner, 2005, Rolls and Kesner, 2006, Kesner, 2007), a function which would be necessary to recall which auditory stimulus was heard when only cues about the context and time of day are presented.

For this IEG analysis, comparisons were made between C57Bl6 mice in the configural and elemental, what-where, conditions. The what-when condition was not included as tissue can only be collected at one time-point, thus only giving an indication of neuronal activity during one specific behavioural episode, either the conditioned or unconditioned time-of-day. In contrast, when tissue was collected after a session in the configural and what-where

conditions each mouse had experienced one trial each in both the conditioned and unconditioned context, making the behaviour between groups comparable. To control for background c-fos expression levels a cage control group was included, and to control for c-fos expression as a result of handling, and not memory retrieval, a behavioural control group was also included.

Methods

Subjects: 47 male 6 month old C57Bl6 mice were used for this experiment in total, 16 in the configural condition, 16 in the elemental condition, 8 in the behavioural control condition and 7 in the cage control condition. Animals were individually housed in standard cages with free access to food and water on a 12hr light/dark cycle.

Apparatus: The apparatus used is described in Experiment 1.

Behavioural Procedure: The behavioural procedure was carried out for the configural and what-where elemental group as described in Experiments 1 and 4, with the exception that each mouse was placed into a quiet dark room 30 minutes prior to testing, during the interval between sessions and for 90 minutes after testing. On the final test days mice were sacrificed after this 90 minute period and tissue was collected for immunohistochemistry (see chapter 3 for details).

Mice were tested in groups of four and one mouse was sacrificed at the end of each test session, so that equal numbers of mice were sacrificed after the morning and afternoon session on both day one and day two of testing. Behavioural control mice were handled in a similar way to those in the test conditions. Mice were given one test session on each pre-conditioning day at midday during which they had two 5 minute trials with a 5 minute interval between. The same context was experienced during both trials and no auditory stimuli were presented. In this way the behavioural control mice had the same level of handling, but did not have to learn about context, cue or time-of-day. For the conditioning stage the behavioural control mice were exposed to the alternative context for two 90-second trials but no auditory stimuli or shock were administered. For the final test days, when tissue

was collected, behavioural control mice were exposed to two 3-minute trials with a 3-minute interval at midday with the same context for both trials. Behavioural control mice were sacrificed after this session, with half sacrificed on day 1, and half on day 2 of testing. In addition, as a control for baseline c-fos expression level, 7 mice were taken directly from their home cage and sacrificed.

Immunohistochemistry: Immunohistochemistry to detect c-fos protein was carried out as described in chapter 3, Experiment 1.

Cell Counts: Cell counts were made as described in chapter 3, Experiment 1. The same regions of interest were counted as detailed in chapter 3.

Data analysis and statistics: Freezing levels were scored as described in Experiment 1. Behavioural data were only analysed for the session after which each mouse was sacrificed. A comparison of the percentage of time spent freezing in the conditioned and unconditioned context is reported, with data pooled across mice sacrificed at each time point. A freezing ratio was calculated for each mouse by dividing freezing in the conditioned context by total freezing across both contexts. Data were analysed using Two-way ANOVA and one sample t-tests.

Raw c-fos expression data were analysed using Two-way ANOVA with region as a within-subject factor and test group as a between subject factor. Significant interactions were followed up with tests of simple main effects to assess differences between groups within each region.

Structural equation modelling: SEM was carried out as described in chapter 3, Experiment 1. An optimal model was first derived for either the configural or elemental test group and then c-fos expression data from all other groups was fitted to this model.

Results Experiment 5a: Behaviour

Due to technical problems behavioural data were scored from only a subset of mice; configural n=11, elemental n=14, behavioural control n=8. **Figure 4.6a** shows the percentage of time spent freezing in the conditioned and unconditioned context during the test session after which tissue was collected. Mice in the configural and elemental test groups showed greater freezing to the conditioned context, but mice in the behavioural control group showed equal freezing to both contexts. A Two-way ANOVA showed a significant effect of context ($F_{(1,30)}$=6.092, p=0.020), but not test group ($F_{(2,30)}$=1.251, p=0.301) and no interaction between these factors ($F_{(2,30)}$=1.290, p=0.290). This suggests that overall the mice spent a greater amount of time freezing to the conditioned context than the unconditioned context across all test groups. Inspection of the data suggests that this difference is driven by differences in the configural and elemental group, not the behavioural control group.

A freezing ratio was calculated for each group, shown in **Figure 4.6b**. Comparison of ratio between groups using a One-way ANOVA showed no significant difference between groups, ($F_{(2,30)}$=2.380, p=0.110), however when each group was compared to chance level, i.e. no difference in freezing between contexts, the configural group ratio was significantly above chance (H_0=0.5, $t_{(10)}$=2.487, p=0.032) , and the elemental group just missed conventional significance levels (H_0=0.5, $t_{(13)}$=2.077, p=0.058), whilst the behavioural control group was not different to chance level (H_0=0.5, $t_{(7)}$=0.573, p=0.585). These data show successful memory retrieval in both the configural and elemental test groups, and no difference in behaviour between contexts in the behavioural control group. There was no significant difference in overall level of freezing across both contexts between groups ($F_{(2,30)}$=1.251, p=0.535), with mean freezing percentage for the configural, elemental and behaviour group of 9.596±2.203, 14.72±2.641 and 10.63±2.617, respectively.

Results Experiment 5b: C-fos Expression

Septal hippocampus: C-fos expression data for the septal region of the hippocampus is shown in **Figure 4.7a**. There is a reduction in c-fos expression in the DG of mice in the configural group compared to other groups. In addition, in the CA3 region mice in the configural and elemental test groups had reduced c-fos expression relative to controls. Two-way ANOVA

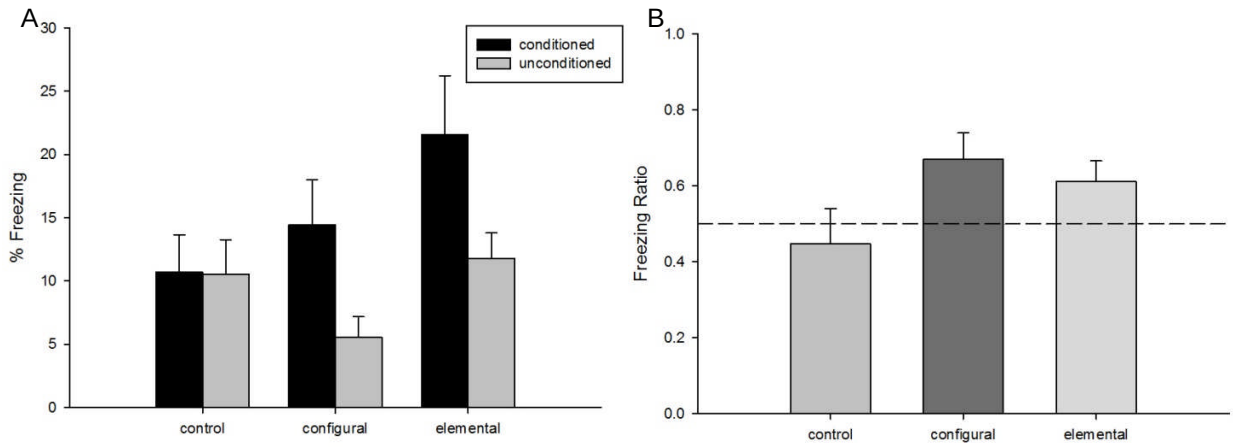

Figure 4.6: Freezing data from the final test session of each mouse in the IEG analysis. A: percentage freezing in the conditioned and unconditioned context at test. B: Freezing ratio of final test session. Bars represent mean±SEM (control=8, configural=11, where=14).

revealed a significant main effect of region ($F_{(2,86)}$=53.068, p<0.001), but not group ($F_{(3,43)}$=1.914, p=0.142), and also a significant interaction between these factors ($F_{(6,86)}$=2.790, p=0.016). Follow up of this interaction with test of simple main effects showed a significant difference in c-fos IR cell count in the DG between the configural and elemental group ($F_{(1,43)}$=5.051, p=0.030). In CA3 there was a significant difference between the behavioural control group and elemental group ($F_{(1,43)}$=4.237, p=0.046), and the cage control group and both the configural ($F_{(1,43)}$=4.845, p=0.033) and elemental group ($F_{(1,43)}$=5.721, p=0.021). In CA1 there was a significant difference in c-fos IR cell count between the cage control and elemental group ($F_{(1,43)}$=5.192, p=0.028). All other comparisons between groups within each region were not significant (all p>0.05).

Intermediate hippocampus: **Figure 4.7b** shows c-fos expression data for the intermediate hippocampus. The behavioural control group show greater c-fos expression in the CA3 and CA1 region than the configural or elemental test group. This was confirmed by Two-way ANOVA showing no significant main effect of either region ($F_{(2,86)}$=0.830, p=0.439) or group ($F_{(3,43)}$=2.827, p=0.050), but a significant interaction between these factors ($F_{(6,86)}$=3.277, p=0.006). Follow up of this interaction showed no significant differences between groups in the DG, although there was non-significant trend towards a difference between the configural and elemental group ($F_{(1,43)}$=3.732, p=0.060), but significant differences in CA3 between the behavioural control group and the cage control ($F_{(1,43)}$=10.048, p=0.003), configural group ($F_{(1,43)}$=8.392, p=0.006), and elemental group ($F_{(1,43)}$=7.367, p=0.009). In CA1 there were

also significant differences between the behavioural control group and the configural ($F_{(1,43)}$=4.658, p=0.036) and elemental group ($F_{(1,43)}$=6.835, p=0.012). All other comparisons between groups were not significant (all p>0.05).

Temporal Hippocampus: C-fos expression in the temporal hippocampus, **Figure 4.7c**, showed no difference between groups. Two-way ANOVA revealed a significant main effect of region ($F_{(1,40)}$=38.855, p<0.001), but not group ($F_{(3,44)}$=1.495, p=0.231), and no significant interaction between these factors ($F_{(3,40)}$=1.787, p=0.165).

Subiculum: C-fos expression data for the subiculum showed no differences between test groups, **Figure 4.7d**. Two-way ANOVA revealed a significant main effect of region ($F_{(1,40)}$=69.660, p<0.001), but not group ($F_{(3,44)}$=1.350, p=0.272), and no interaction between these factors ($F_{(3,40)}$=1.736, p=0.175).

Perirhinal cortex: **Figure 4.7e** shows c-fos expression data for the perirhinal cortex. Inspection of this figure shows a reduction in c-fos expression in the elemental test group compared to the behavioural control group in the caudal perirhinal cortex. Two-way ANOVA showed no significant main effect of either region ($F_{(1,40)}$<0.001, p=0.996) or group ($F_{(3,40)}$=0.996, p=0.405), but there was a significant interaction between these factors ($F_{(3,40)}$=3.798, p=0.017). Follow up of this interaction revealed a significant difference between the behavioural control group and elemental group in the caudal perirhinal cortex ($F_{(1,40)}$=4.764, p=0.035), and a trend towards a difference between the configural and elemental group, that failed to reach conventional significance levels ($F_{(1,40)}$=2.943, p=0.094).

Entorhinal cortex: C-fos expression data for the entorhinal cortex is shown in **Figure 4.7f**. This figure shows reduced c-fos expression in the cage control group in the medial entorhinal cortex compared to other groups. However, Two-way ANOVA showed a significant main effect of both region ($F_{(1,39)}$=9.021, p=0.005) and group ($F_{(3,39)}$=3.564, p=0.023), but no interaction between these variables ($F_{(3,39)}$=0.771, p=0.517). When the effect of group was followed up there was a significant difference between the behavioural control group and the cage control group ($F_{(1,39)}$=6.128, p=0.018), and elemental group ($F_{(1,39)}$=5.699, p=0.022), a difference between the cage control group and the configural group ($F_{(1,39)}$=4.927, p=0.032), and a significant difference between the configural group and elemental group ($F_{(1,39)}$=4.527, p=0.040). All other group differences were not significant.

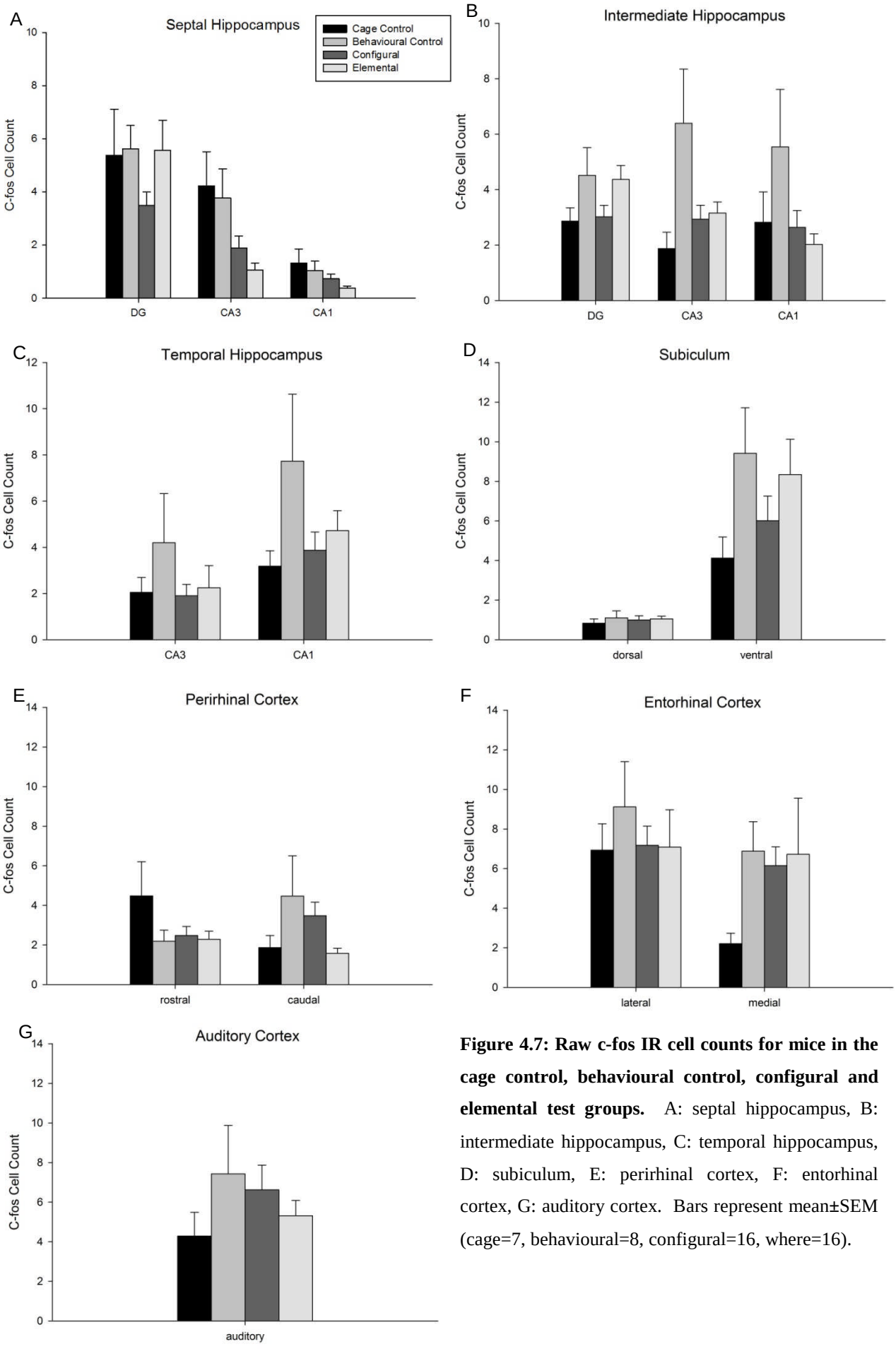

Figure 4.7: Raw c-fos IR cell counts for mice in the cage control, behavioural control, configural and elemental test groups. A: septal hippocampus, B: intermediate hippocampus, C: temporal hippocampus, D: subiculum, E: perirhinal cortex, F: entorhinal cortex, G: auditory cortex. Bars represent mean±SEM (cage=7, behavioural=8, configural=16, where=16).

Auditory Cortex: Finally c-fos expression in the auditory cortex did not differ between groups, **Figure 4.7g**. One-way ANOVA confirmed that there was no significant effect of group on c-fos IR cell count ($F_{(3,47)}$=0.793, p=0.505).

In summary, the most significant differences in c-fos expression between test groups were seen in the septal hippocampus. C-fos expression was significantly reduced in the DG of the configural group compared to the mice in the elemental group, and in the CA3 and CA1 region of both the configural and elemental groups compared to controls. A similar pattern was seen in CA3 and CA1 in the intermediate hippocampus, with reduced c-fos expression in the configural and elemental group compared to the behavioural control. The other significant difference was a reduction in c-fos expression in the caudal perirhinal cortex of mice in the elemental group compared to the behavioural control group.

Results Experiment 5c: Structural Equation Modelling

To assess the hippocampal pathways that may be involved in processing configural and elemental information SEM, based on raw c-fos IR cell counts, was used to produce models of hippocampal networks. The models focussed on connectivity within the septal region of the hippocampus, as this is both the region where the largest differences were seen in c-fos IR cell counts and was implicated in chapter 3 as being the region most affected by amyloid pathology.

Correlation Matrices

Models of relationships between brain regions were derived for mice in the configural and where group from the correlation matrices of each group, shown in **Table 4.1**. The behavioural measure shown here is the freezing ratio from the final test session before tissue was collected. Significant correlations were seen between CA3 and CA1 in both groups, whilst only the configural group showed correlations between cell counts in the DG and entorhinal cortex, and between CA1 and the subiculum. While the cell counts in any target region did not correlate with behaviour in the configural group, in the elemental group CA3 cell counts were significantly correlated with behaviour. After finding optimal models for both the configural and elemental group, the data from the two control conditions, behaviour

Table 4.1: Inter-region correlations of c-fos IR counts for mice in the configural and elemental group. Configural, standard text. Elemental, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, significant correlations are shaded dark grey.

		DG	CA3	CA1	Sub	mEnt	behaviour
DG	r		0.491	0.328	0.140	0.617*	-0.374
	p		0.054	0.215	0.618	0.019	0.257
CA3	r	**0.244**		0.796**	0.292	0.336	-0.357
	p	**0.362**		0.000	0.290	0.240	0.280
CA1	r	**0.370**	**0.681***		0.609*	0.418	-0.273
	p	**0.158**	**0.014**		0.016	0.136	0.417
Sub	r	**0.335**	**0.337**	**0.470**		0.466	0.287
	p	**0.222**	**0.219**	**0.077**		0.093	0.392
mEnt	r	**0.021**	**0.382**	**0.349**	**0.464**		0.013
	p	**0.944**	**0.178**	**0.221**	**0.095**		0.970
behaviour	r	**0.056**	**-0.646***	**-0.436**	**-0.140**	**-0.137**	
	p	**0.849**	**0.013**	**0.119**	**0.648**	**0.671**	

Table 4.2: Inter-region correlations of c-fos IR counts for mice in the control groups. Behavioural control, standard text. Cage control, bold text. Table shows Pearson's coefficient (r) and significance (p), where *=p<0.05, **=p<0.01 and ***=p<0.001, significant correlations are shaded dark grey.

		DG	CA3	CA1	Sub	mEnt	behaviour
DG	r		0.653	0.678	0.447	-0.405	-0.224
	p		0.079	0.065	0.267	0.32	0.594
CA3	r	**0.589**		0.853**	0.943**	-0.404	-0.058
	p	**0.164**		0.007	0	0.321	0.891
CA1	r	**0.482**	**0.955****		0.761*	-0.321	0.099
	p	**0.274**	**0.001**		0.028	0.439	0.815
Sub	r	**-0.673**	**-0.034**	**0.005**		-0.25	-0.147
	p	**0.097**	**0.943**	**0.991**		0.551	0.729
mEnt	r	**-0.382**	**0.438**	**0.439**	**0.575**		-0.126
	p	**0.398**	**0.325**	**0.324**	**0.177**		0.766

and cage controls, was fitted to the models. The correlation matrices for these groups are shown in **Table 4.2**. In the behavioural control group there were significant correlations in c-fos IR cell count between CA3, CA1 and the subiculum, but no correlations to behaviour. In contrast, in the cage control group the only significant correlation was between CA3 and CA1.

Configural Group Model

The optimal model derived for data from mice in the configural group is shown in **Figure 4.8a**. This model proposes that the data best fits a model in which the principal pathways involved are those in the tri-synaptic pathway from the medial entorhinal cortex to the DG, then to CA3 on to CA1. This model does not differ significantly from the data set ($\chi^2_{(3)}$=2.4, p=0.493) and gives an excellent fit according to the other indices (RMSEA<0.001, CFI=1.000, GFI=0.921). In contrast, when data from mice in the elemental condition were fitted to this model, although there was no significant difference between the covariance matrix predicted by the model and that produced by the data-set ($\chi^2_{(3)}$=3.7, p=0.297) the fit of the model was considerably reduced (RMSEA=0.131, CFI=0.828, GFI=0.888). Data from the cage control group also gave a poor fit to this model ($\chi^2_{(3)}$=11.8, p=0.008, RMSEA=0.698, CFI=0.633, GFI=0.688). Furthermore, data from the behavioural control group gave a similar excellent level of fit to the configural group data ($\chi^2_{(3)}$=1.1, p=0.782, RMSEA<0.001, CFI=1.000, GFI=0.927).

Elemental Group Model

The optimal model derived for the data from mice in the elemental test condition is shown in Figure 4.8b. This model, incorporating pathways from the entorhinal directly to CA3, and then on to CA1 and the subiculum, but without the DG, gave an excellent fit to the data from mice in the elemental group ($\chi^2_{(3)}$=2.2, p=0.528, RMSEA<0.001, CFI=1.000, GFI=0.929). In comparison the fit was poor for the configural group ($\chi^2_{(3)}$=5.1, p=0.162, RMSEA=0.234, CFI=0.893, GFI=0.833), the cage control group ($\chi^2_{(3)}$=3.5, p=0.323, RMSEA=0.164, CFI=0.964, GFI=0.815) and the behavioural control group ($\chi^2_{(3)}$=11.4, p=0.010, RMSEA=0.634, CFI=0.614, GFI=0.717). These data suggest that the DG connections, whilst being critical for modelling network activity in the configural test group are less important in

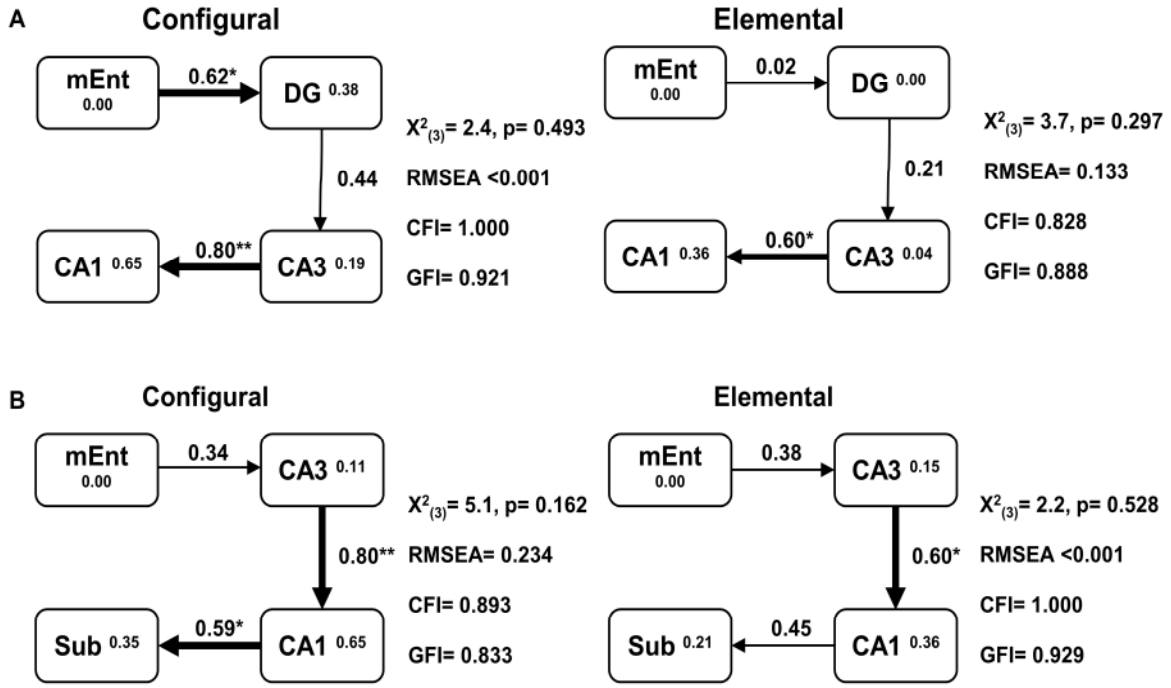

Figure 4.8: Optimal structural equation models of the configural and elemental groups. A: Optimal model for the configural group. B: Optimal model for the elemental group. Numbers over arrows show standardised path coefficients, numbers by regions are the residuals. Bold arrows show significant pathways.

the elemental group. This is supported by the significant difference seen in c-fos IR cell counts between these two groups in the DG.

Model Stacking

Multiple-group analysis was used to compare the strength of individual pathways between models. The data-sets were stacked onto the same model and each pathway constrained in turn to see which path strengths differed significantly.

Stacking data from the configural and elemental groups onto the optimal configural model (**Figure 4.8a**) revealed a trend towards an overall difference in structural weights, which just failed to reach conventional significance levels ($\chi^2_{(3)}$=7.433, p=0.059). Further analysis of individual pathways showed a significantly greater path strength between CA3 and CA1 in the configural group ($\chi^2_{(1)}$=5.378, p=0.020). When data from the behavioural control group were compared to the configural group on this model there was a non-significant trend towards a difference in structural weights ($\chi^2_{(3)}$=6.545, p=0.088). However, there was a significant reduction in path strength between the entorhinal cortex and DG ($\chi^2_{(1)}$=5.473,

p=0.019). Comparison of structural weights between the cage control and the configural group showed no significant overall difference in structural weights ($\chi^2_{(3)}$=2.670, p=0.445), and there were no significant differences in the weight of any individual pathway between these models.

Stacking data from the elemental and configural group onto the optimal model for the elemental group (**Figure 4.8b**) showed no significant overall difference in structural weights ($\chi^2_{(3)}$=5.459, p=0.141), but a significant difference in path strength between CA3 and CA1 ($\chi^2_{(1)}$=5.378, p=0.020). When data from the behavioural control group was compared to the elemental group on this model the difference in overall structural weights failed to reach conventional levels of significance ($\chi^2_{(3)}$=6.832, p=0.077), but there was a significant difference in the strength of the pathway from the entorhinal cortex to CA3 ($\chi^2_{(1)}$=3.881, p=0.049). Finally, stacking of data from the cage control and elemental group showed a significant difference in structural weights between groups ($\chi^2_{(3)}$=11.572, p=0.009). There was a significant difference between models in path strength connecting CA3 to CA1 ($\chi^2_{(1)}$=10.536, p=0.001).

Discussion

Experiment 5 revealed differences in hippocampal neuronal activity between configural and elemental learning paradigms using c-fos expression analysis. These data were then used to produce SEM models to suggest which hippocampal pathways are required for configural and elemental memory processing. Significant differences in c-fos IR cell counts were seen between mice in the configural and elemental condition in the DG of the septal hippocampus, the entorhinal cortex and a non-significant trend towards a difference in the caudal perirhinal cortex. Changes in hippocampal cell counts as a result of configural learning compared to other groups supports lesion and inactivation data showing the necessity of an intact hippocampus for this type of learning (Iordanova et al., 2009). Although the difference between the configural and elemental group in the perirhinal cortex is not quite significant this difference is still of interest as lesions to the perirhinal cortex disrupt performance on the elemental, what-where task (Iordanova et al., 2009).

The behavioural control group had the highest level of IR cells across almost every region. Mice in this group did not experience different combinations of cues during pre-conditioning,

and therefore may exhibit a higher level of c-fos activity as they are recalling less specific previous events. Alternatively, this difference may be due to a flaw in the experimental design, as although handling directly before sacrifice was identical, the behavioural control group had half the number of trials during pre-conditioning compared to the configural and elemental group. Therefore, although mice in the behavioural control had been extensively habituated to the handling procedure, the experience may still be a more novel to mice in this group than in the configural and elemental groups leading to higher c-fos activation. A final explanation may be that as the behavioural control group were not given any foot-shocks during training to condition fear, they would not have the same level of emotional response as mice in the configural or elemental condition. However, measures of overall freezing levels between groups revealed no difference between groups across sessions suggesting that this last explanation is unlikely, although in the configural and elemental group freezing was greater in the conditioned context compared to the unconditioned. As expected the cage control group generally showed the lowest level of c-fos IR cells across regions.

The data from the configural group highlighted a direct pathway from the entorhinal cortex to the DG and then to CA3. In contrast, the DG was not crucial to an optimal model for the elemental data. These results support the hypothesis that the DG is particularly important in configural learning. Arguably, a more complex pattern of stimuli is present in the configural than the elemental condition, and therefore pattern separation processes may be necessary to distinguish the unique combination of overlapping cues (Kesner et al., 2004, Rolls and Kesner, 2006, Schmidt et al., 2012). Although contextual fear-conditioning, which is similar to the what-where elemental task, has been suggested to require pattern separation (McHugh et al., 2007), the differences in pre-exposure to contextual cues may make this discrimination easier in our elemental task. Studies assessing pattern separation using spatial cues have manipulated the level of difficulty to demonstrate the increased or decreased requirement for pattern separation (Clelland et al., 2009, Creer et al., 2010). In a similar way, by increasing the number of cues to be associated, from a bi-conditional discrimination in the elemental task, to a configural association with three cues, the level of task difficulty was increased and may have made pattern separation more critical.

Evidence from lesion and inactivation studies show that the hippocampus is not required for elemental information processing (Iordanova et al., 2009, Iordanova et al., 2011). However, in these studies, hippocampal pathways are either no longer intact or not active and therefore alternative pathways must be used. The data presented here, showing excellent fit of a

hippocampal model to the elemental data set, suggests that when all brain regions are functional the hippocampus is engaged as well as other pathways supporting elemental processing. This may reflect that the hippocampus continues to process or monitor event information even though alternative pathways are ultimately involved in forming associative representations. The excellent fit of the behavioural control group data to the configural model suggest that incidental encoding of ongoing episodes may always engage the hippocampus when it is intact, leading to configural encoding of cues which are not necessarily task relevant.

The SEM analysis revealed that the CA3 sub-region of the hippocampus is implicated in both the configural and elemental group optimal models. This is likely due to the role of CA3 in pattern completion, which would, arguably, be necessary to recall both configural and elemental combinations of cues from presentation of only a subset of those cues (Kesner et al., 2004, Rolls and Kesner, 2006). The data presented here contrast to the findings of a recent paper, which used selective inactivation to show a specific role for the DG/CA3 in processing the spatial component of episodic-like memory, and CA1 in processing temporal information using an object recognition paradigm (Barbosa et al., 2012). The DG was not critical for processing the spatial component in the task used here. This difference is most likely due to the difference in task demand, object recognition vs. operant chamber cues. However, a more likely explanation is that in the paper by Barbosa et. al. no differentiation was made between DG and CA3 processing, as the inactivation encompassed both regions. Again this demonstrates one of the advantages of IEG analysis when assessing the role of specific sub-regions.

Overall, in Experiment 5 we reported differences in c-fos expression in hippocampus subregions implicating the DG more critically in configural than elemental memory processing.

4.7 Chapter Discussion

In this chapter a novel behavioural paradigm was used to test the effect of the APPswe mutation on episodic memory systems in the Tg2576 mouse model of amyloid pathology. In the task presented here the mice were required to create a separate configural memory of the unique combination of sound, context and time-of-day for each trial.

Experiment 1 used C57Bl6 mice to demonstrate that, like rats, mice can also form integrated episodic-like memories of which auditory stimulus, tone or click, is presented in which context, blank or striped, and at what time of day, morning or afternoon, as shown by a difference in freezing ratio between the morning and afternoon test sessions. Experiment 2 and 3 went on to show an age-dependent deficit in episodic-like memory processing in transgenic mice compared to wild-type controls. At 3 months of age both wild-type and transgenic mice were able to form an integrated configural memory for episodic information. In contrast, by 11 months of age, after the onset of amyloid pathology, whilst wild-type mice were able to form integrated configural memories, transgenic mice were impaired on this task. However, 11 month old wild-type mice did not perform as well as their 3 month old counterparts, suggesting that normal aging may have influenced performance on this behavioural paradigm. The effect of aging matches the data presented in chapter 3, which also showed age-dependent changes in wild-type mice. The findings of Experiment 4 are of particular interest, as they demonstrate that elemental processing of temporal cues is not impaired in Tg2576 mice at 11 months old, and spatial processing of cues is only mildly impaired. This suggests that the formation (or retrieval) of configural associations is highly sensitive to the APPswe mutation. Therefore, extrapolating to humans, the formation of configural memories for episodic information may be disrupted early in the course of AD, possibly reflecting impaired pattern separation processes supported by the DG, and lead ultimately to deficits in episodic memory.

The age-dependence of impairment in configural memory in Tg2576 mice supports the theory that Aβ production and accumulation is a key pathogenic process causing memory loss. This is supported by evidence of age-dependent memory impairments in the Tg2576 strain on other, more basic, memory tasks, such as contextual fear conditioning, t-maze and water-maze (Hsiao et al., 1996, Chapman et al., 1999, Jacobsen et al., 2006). However, accumulation of soluble β-amyloid into insoluble plaques does not occur until approximately 10 months of age (Hsiao et al., 1996). The data presented here show marked deficits in

episodic-like memory at just 11 month of age in Tg2576 mice, when only a few plaques are detectable. This finding suggests that it is unlikely that plaques are the primary cause of memory deficits and supports the recent prediction of the amyloid cascade hypothesis that it is not plaques, but the soluble oligomeric forms of amyloid that cause memory impairment (Hardy, 1992). This is verified by evidence that synaptic loss correlates more accurately with memory loss in AD than plaque load (DeKosky and Scheff, 1990, Terry et al., 1991), and that soluble, rather than fibrillar β-amyloid causes synaptic disruption (Selkoe, 2008a).

In Experiment 5 c-fos expression induced by configural and elemental learning was assessed, and these data were used to produce SEM models of hippocampal engagement. The major difference between the hippocampus sub-region engagement between configural and elemental tasks was the critical role the DG played in models of configural, but not elemental learning. It is tempting to relate the behavioural deficits reported in Tg2576 mice on this task to amyloid-induced alteration in DG activity, as functional connectivity of the DG is impaired in transgenic mice (see chapter 3). In the spatial elemental condition only a mild deficit in behaviour was detected, corresponding to the less critical role of the DG in the optimal model for this data set. Due to the complication of when to collect tissue for the temporal elemental condition it was beyond the scope of these experiments to assess c-fos expression in this condition. However, given the lack of behavioural deficit in this condition in Tg2576 mice it is predicted that the DG would not be involved in the optimal model of wild-type sub-region engagement in the temporal condition.

In conclusion, the data presented in chapter 4 have demonstrated that Tg2576 mice show an age-dependent decline in configural memory for episodic information. Furthermore, the analysis has revealed that the configural task relies upon the DG, a region that shows early alterations in APP models of amyloid pathology. These findings may have implications for assessing the efficacy of treatments in terms of how they influence neural network dynamics supporting memory and may facilitate their translation to clinical trials for AD.

Chapter 5: The effect of exercise on cognition in Tg2576 mice

5.1 Introduction

The data presented in chapters 3 and 4 revealed deficits in cognition and hippocampal network processing of information in Tg2576 mice. The experiments reported in chapter 5 address the issue of whether voluntary exercise can alleviate the cognitive deficit in Tg2576 mice.

There is much evidence to show that exercise can improve cognition in both rodents and humans (for reviews see Cotman et al., 2007, van Praag, 2009, Graff-Radford, 2011). Exercise is of specific theoretical interest as the results in chapters 3 and 4 indicate network changes in the DG in Tg2576 mice, and previous studies have shown that exercise can improve DG function (van Praag et al., 1999a, Colcombe et al., 2003, Eadie et al., 2005, Burns et al., 2008). There are several mechanisms by which exercise exerts these effects in the DG including; increasing neurogenesis (van Praag et al., 1999a, van Praag et al., 1999b), LTP (van Praag et al., 1999a, Farmer et al., 2004) and spine density (Eadie et al., 2005). Other more global changes that occur in the brain after exercise include improved vasculature and angiogenesis (Black et al., 1990, Swain et al., 2003, Ding et al., 2004, van Praag et al., 2005) and increased levels of growth factors including BDNF (Adlard and Cotman, 2004, Farmer et al., 2004, Cotman et al., 2007, Bekinschtein et al., 2011). Together these changes may contribute to alleviating the cognitive decline seen in AD. It has also been suggested that exercise may have more disease specific effects by altering amyloid levels and plaque load in the brain (Adlard et al., 2005, Nichol et al., 2008, Garcia-Mesa et al., 2011, Ke et al., 2011), although evidence for this is less conclusive (Wolf et al., 2006, Parachikova et al., 2008, Yuede et al., 2009).

Previous studies looking at the influence of exercise on cognition in APP transgenic models have largely shown positive results (Adlard et al., 2005, Nichol et al., 2007, Yuede et al., 2009). Several of these studies used short-term exercise to investigate the acute effects of exercise after disease onset. However, in the present study, the aim was to examine the effects of exercise from an early age, as network changes had been detected from a young age in mutant mice.

A study by Yuede et. al. (2009) assessed the effects of longer term (4 months) exercise on the Tg2576 model. However, this study did not include wild-type controls. Therefore, although they did show exercise-related improvements in cognition the authors could not conclude whether or not cognition was returned to wild-type level. Although most studies have shown improved cognition with exercise in AD models, there are mixed results on the effects of exercise on amyloid pathology (Adlard et al., 2005, Wolf et al., 2006, Nichol et al., 2008, Parachikova et al., 2008, Yuede et al., 2009, Garcia-Mesa et al., 2011, Ke et al., 2011). Therefore, in addition to examining the influence of exercise from an early age on cognition, the present study also assessed its effect on brain amyloid levels.

Another aim is to extend previous studies by assessing the influence of exercise on neurogenesis in Tg2576 mice. It has been proposed that one of the key ways in which exercise may help memory is through increasing neurogenesis (van Praag et al., 1999a, van Praag et al., 1999b), which may make memory capacity larger by increasing pattern separation (Clelland et al., 2009, Creer et al., 2010, Aimone et al., 2011, Sahay et al., 2011, Schmidt et al., 2012). Previous reports have shown that neurogenesis is decreased in Tg2576 mice (Dong et al., 2004), and other APP transgenic strains (Haughey et al., 2002). However, so far only one study has assessed the effects of exercise on altering neurogenesis in APP23 mice (Mirochnic et al., 2009), reporting that exercise did increase neurogenesis in old, but not young, mice.

In this chapter cognition is measured in Tg2576 mice after exercise using a selection of behavioural tasks. Cotman et. al. (2007) stressed the need for a more accurate analysis of the types of cognition improved by exercise, and in particular the need to use tasks which test aspects of memory that are more relevant to human memory processing. Therefore, the present series of experiment uses both standard spatial memory tasks, such as the t-maze and object-in-place paradigms, the elevated plus maze to test anxiety, and also a more novel foraging task. In addition, the present study also assesses the effect of exercise on configural memory for episodic information, using the task presented in chapter 4. Following behavioural testing, amyloid pathology and neurogenesis were evaluated in an effort to understand the putative mechanisms by which exercise influenced cognition.

5.2 Exercise performance

Introduction

A review of the literature suggested that the precise exercise regime is a critical factor influencing the outcome of the manipulation on cognition. Previous studies have shown varied results due to differences in type of exercise, intensity and duration of exercise and choice of controls (see for example van Praag et al., 2005, Vaynman et al., 2006, O'Callaghan et al., 2007, Leasure and Jones, 2008). The present study used voluntary exercise, using in-cage running wheels, as this is thought to be less stressful to the mice and has shown more beneficial effects on performance than forced treadmill exercise in several studies (Leasure and Jones, 2008, Yuede et al., 2009). As exercise is voluntary, the intensity of exercise cannot be controlled, but the level of exercise of each mouse can be recorded and correlated with measures of behaviour and pathology. In the present study mice were given access to wheels from 2-12 months of age. This time scale was chosen to assess the influence of an active-lifestyle on amyloid pathology and cognition, rather than the acute effects of exercise after disease onset. Finally, the control groups used in this study were provided with a static wheel, identical to those of the exercise group. This condition controls for environmental enrichment effects of the novel apparatus, which has also been shown to influence cognition (Lazarov et al., 2005, Costa et al., 2007, Gortz et al., 2008), and thus permitted a more direct assessment of the effects of exercise on performance.

Methods

Subjects: 90 male Tg2576 mice were used in this study. All animals were individually housed in large standard cages (L 42cm x W 27cm x H 13cm) containing an exercise wheel with free access to food and water and a 12 hour light/dark cycle. All mice were accustomed to regular handling before behavioural testing.

Running wheels: Running wheels (Wm Lillico & Son) with a 15cm diameter were provided in every cage from 2 months of age. 46 mice (23 wild-type, 23 transgenic) had freely moving

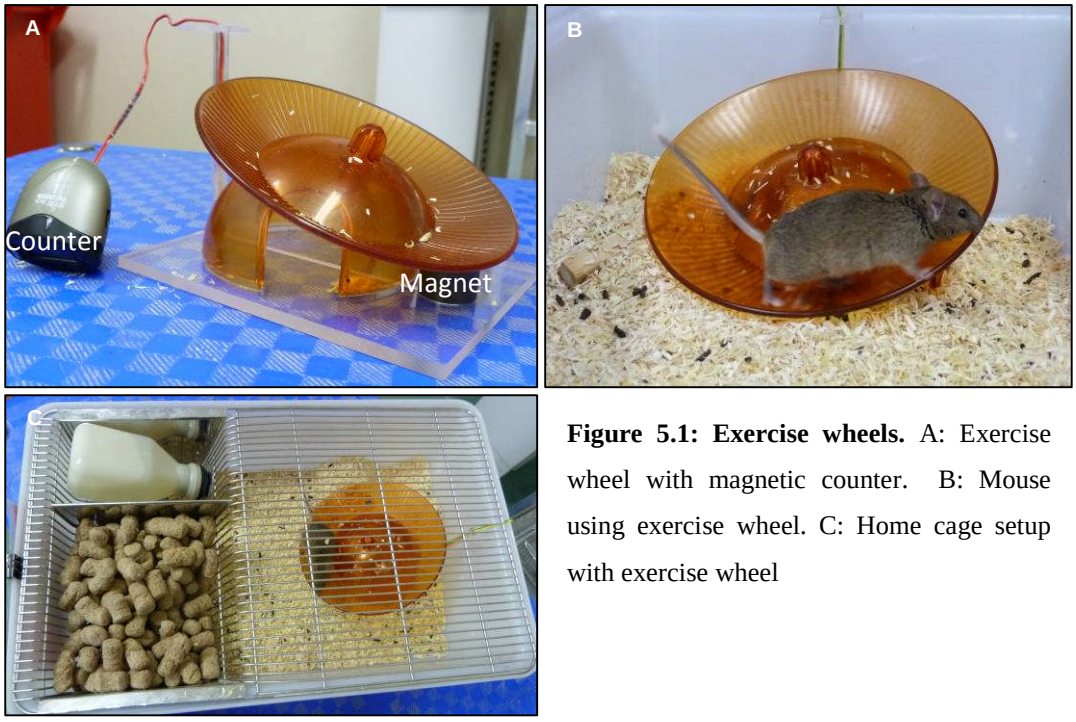

Figure 5.1: Exercise wheels. A: Exercise wheel with magnetic counter. B: Mouse using exercise wheel. C: Home cage setup with exercise wheel

wheels in their cages connected to magnetic counters (made in house) which recorded the number of revolutions. The other 44 mice (22 wild-type, 22 transgenic) had static wheels which were screwed to the base to stop rotation. Examples of these wheels are shown in **Figure 5.1**.

Results

To determine the level of exercise of each mouse in the running group the distance travelled was recorded daily for one week every month, and then the average distance run per day was calculated; these data are shown in **Figure 5.2**. During the first month of wheel activity wild-type and transgenic mice travelled, 10.57±1.24km/day (mean±SEM) and 8.31±1.15km/day, respectively. By 13 months of age the level of exercise had decreased for both genotypes to 2.91±0.68km/day for wild-type and 2.26±0.66km/day for transgenic mice. Analysis by Two-way ANOVA revealed a significant main effect of month ($F_{(10,440)}$=13.935, p<0.001), but not genotype ($F_{(1,44)}$=1.972, p=0.167) and no interaction between these factors ($F_{(10,440)}$=0.318, p=0.976). This shows that although running distance decreased over time as the mice aged, this decrease was equal for wild-type and transgenic mice as there was no difference between genotypes at any point.

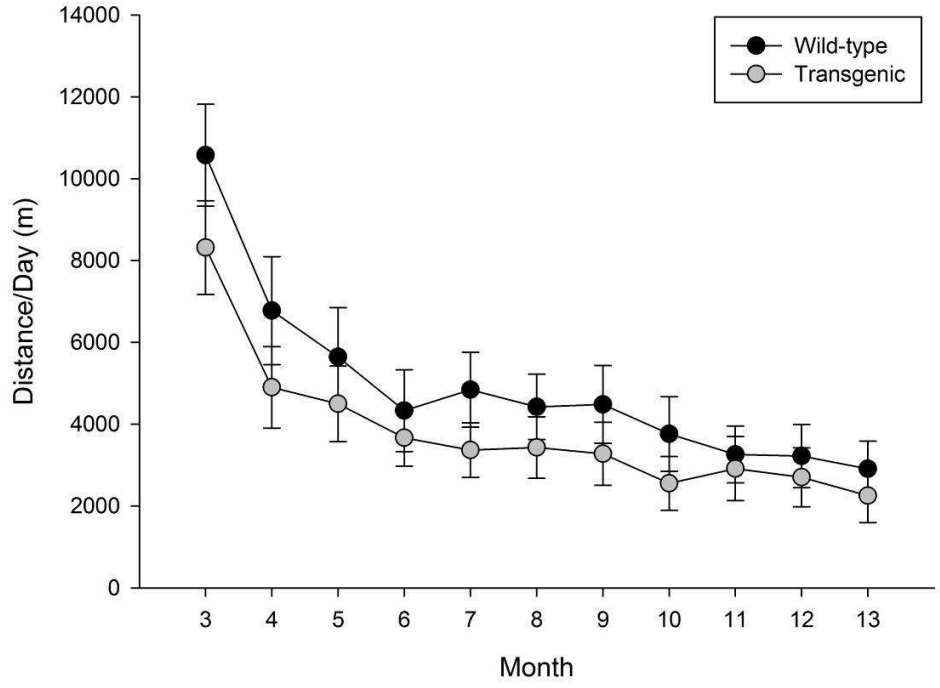

Figure 5.2: Exercise Measures. Distance run per day by wild-type and transgenic mice. Distance was measured for each mouse every day for one week per month. Points represent mean±SEM.

Discussion

Using the exercise paradigm outlined above there was no difference in overall exercise levels between wild-type and transgenic mice. During the first month of wheel access, when mice were 3 months of age, mice ran approximately 9km/day. Over time, as the mice aged and became habituated to the wheel, exercise level decreased to just over 2.5km/day by 13 months.

These data are comparable or even slightly higher than exercise levels seen in other studies, although not all studies report measures of voluntary exercise. In a forced (non-shock) exercise paradigm mice were exercised on a treadmill for approximately 650m/day for only 5 days/week (Yuede et al., 2009), which is much lower than the exercise levels seen here. Other studies in different APP transgenic mouse models have had varying exercise levels, with two studies on the APP/PS1 model using much lower levels of treadmill exercise, 600m/day and 330m/day (Ke et al., 2011, Liu et al., 2011), and a study in TgCRND8 mice showing comparable levels of voluntary exercise, 2-4km/night (Adlard et al., 2005).

In non-transgenic mice there have also been a mixture of results, with voluntary exercise in 3 month old C57Bl6 mice ranging from approximately 4.5km/day (van Praag et al., 1999a, van Praag et al., 1999b) to 23.5km/day (Creer et al., 2010). The distances run by mice in the present study are comparable to those in studies by van Praag, but much lower than reported by Creer, despite using similar wheels with a 'saucer' design.

Whilst there are a variety of exercise intensities across studies, the level of exercise in the present study falls in the middle of this range and is therefore comparable to other studies.

5.3 Experiment 1: The effect of exercise on anxiety in the elevated plus maze

Introduction

Experiment 1 used an elevated plus maze to assess the effects of exercise on anxiety and exploration in Tg2576 mice. The elevated plus maze was designed by Handley and Mithani (1984.) as a test of anxiety in rodents. It is currently the most popular test used to assess animal models of anxiety and the effects of pharmacological interventions. This test assesses anxiety based on spontaneous behaviour of rodents and the natural conflict between drive to explore a new environment, and the tendency to avoid unknown and potentially dangerous areas. A normal mouse will spend more time in the closed, safer, arms than in open and exposed arms (Lister, 1987). Previous work has shown that Tg2576 mice are less anxious than their wild-type littermates, showing disinhibition on this task and spending a greater proportion of time exploring the open arms (Lalonde et al., 2003). Although some studies have reported that exercise decreases anxiety in rodents (Binder et al., 2004, Salam et al., 2009), other evidence has shown that exercise enhances anxiety, via a mechanism putatively involving neurogenesis (Burghardt et al., 2004, Fuss et al., 2010, Onksen et al., 2012). This leads to the prediction that exercise may increase anxiety in transgenic mice, reducing the behavioural impairment compared to wild-type controls. This behavioural test was the first to be carried out, at 6 months of age, whilst animals were naïve, as prior handling and maze experience have been shown to alter basal anxiety levels on the plus-maze (Griebel et al., 1993).

Methods

Subjects: 89 male 6 month Tg2576 mice were run on the elevated plus maze. Of these 44 were wild-types, 23 in the exercise condition and 21 in the sedentary condition. 45 were transgenics, 23 in the exercise condition, 23 in the sedentary condition. Two sedentary transgenic mice were excluded from the analysis as they jumped off the maze during testing.

Apparatus: The elevated plus maze consists of four arms arranged in a cross constructed from plywood, and placed on a stand 75cm above the ground, **Figure 5.3**. Each arm measured 40cm x 7cm. Two opposite arms were open, with white floor surface and 1cm white walls. The other two opposite arms were closed, with a white floor and 15cm high black walls.

Behaviour: Mice were taken from their home cage and placed in the centre of the maze facing an open arm. They were allowed to explore the maze for five minutes, before being returned to their home cage. Each trial was recorded using an overhead camera connected to a DVD recorder and monitor. The maze was thoroughly cleaned with ethanol wipes between mice to remove odour cues.

Data analysis and statistics: The amount of time spent in the open and closed arms was scored manually by an observer blind to genotype and exercise condition. Entry into an arm was only counted when all four paws were in the arm. An exploration ratio was calculated for each mouse based on the time spent in the closed arms divided by the total amount of time

Figure 5.3: Elevated plus maze. The elevated plus maze consists of four arms, two open arms and two closed arms. Normal mice will spend a greater proportion of time in the 'safer' closed arms.

spent in both the closed and open arms (but not in the centre area). The number of entries into each arm was also scored. Two-way ANOVA, with exercise group and genotype as between subjects' factors was used to analyse exploration ratio and arm entry data. If the interaction was significant tests of simple main effects were carried out. Pearson's 'r' coefficient was used to assess the correlation between exercise and performance.

Results

Exploration ratio

Performance of exercised and sedentary Tg2576 mice on the elevated plus maze is shown in **Figure 5.4a**. Inspection of this figure shows a trend for reduced exploration ratio in transgenic mice compared to wild-types in both conditions, but a more marked reduction for those in the sedentary group. Two-way ANOVA revealed a significant main effect of genotype ($F_{(1,83)}$=8.298, p=0.005), but not of exercise ($F_{(1,83)}$=0.093, p=0.761) and no interaction between these factors ($F_{(1,83)}$=2.425, p=0.123).

Arm entries

Figure 5.4b shows the mean number of arm entries for each group of mice. Inspection of this figure shows little difference between genotypes or exercise condition. Two-way ANOVA showed no significant main effect of group ($F_{(1,84)}$=0.948, p=0.333) or genotype ($F_{(1,84)}$=0.407, p=0.525), and no interaction between these factors ($F_{(1,84)}$=0.023, p=0.881). This suggests equal exploratory behaviour between groups, which is not influenced by differences in locomotor activity. When arm entries were calculated as a ratio of entry into closed arms compared to total arm entries, as for the exploration ratio data, there was a significant main effect of genotype ($F_{(1,84)}$=7.003, p=0.010), but not exercise group ($F_{(1,84)}$=0.476, p=0.492) and no interaction ($F_{(1,84)}$=1.120, p=0.293). Means were; wild-type exercise 0.668±0.026 (mean ± SEM); wild-type sedentary 0.731±0.023; transgenic exercise 0.6113±0.047; and transgenic sedentary 0.598±0.039.

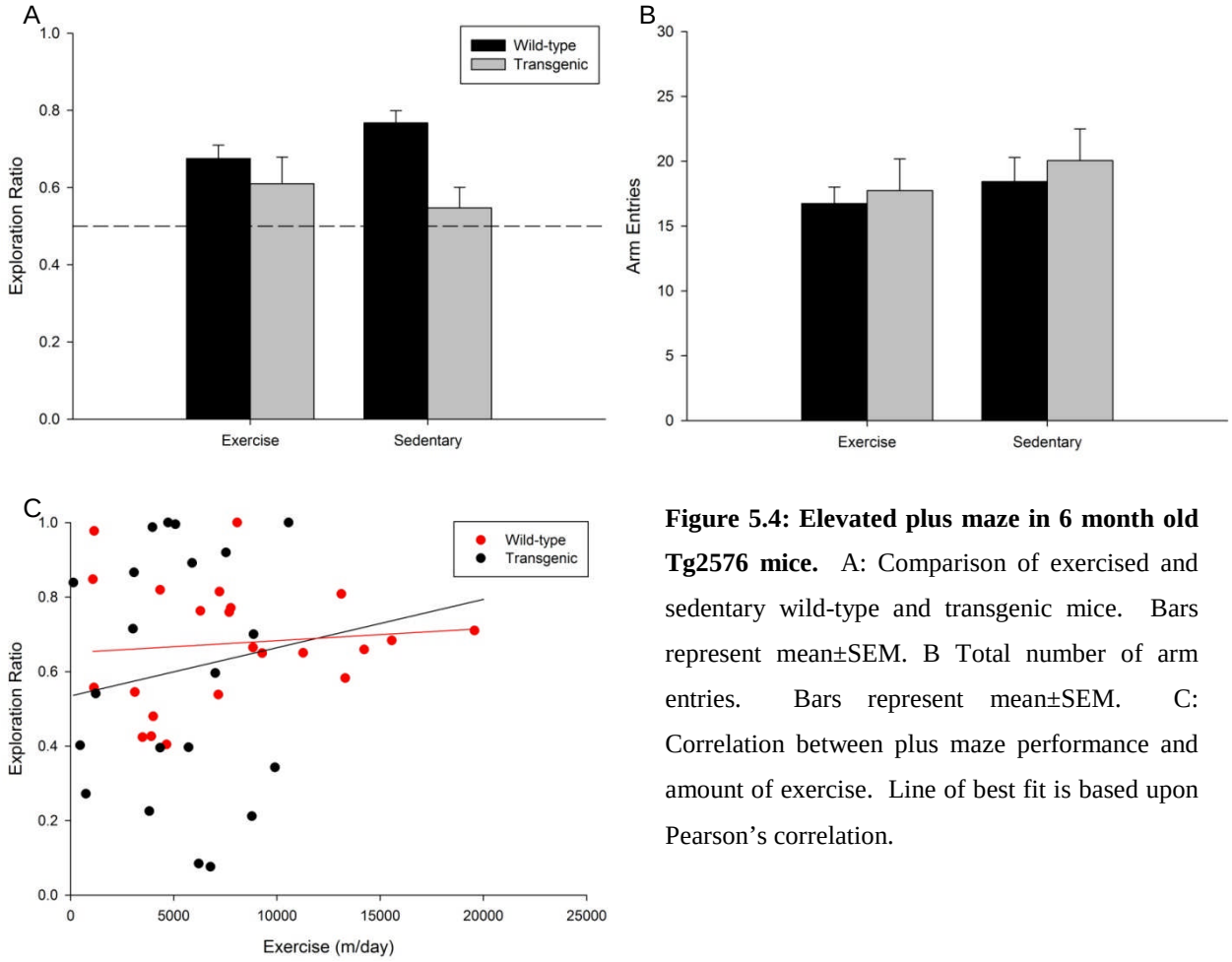

Figure 5.4: Elevated plus maze in 6 month old Tg2576 mice. A: Comparison of exercised and sedentary wild-type and transgenic mice. Bars represent mean±SEM. B Total number of arm entries. Bars represent mean±SEM. C: Correlation between plus maze performance and amount of exercise. Line of best fit is based upon Pearson's correlation.

Correlation with exercise level

To assess whether the level of exercise of individual mice affected their performance the correlation between these two factors was analysed using a Pearson's coefficient. The correlation can be seen in **Figure 5.4c**. There was no significant correlation between exercise and performance for either wild-type or transgenic mice (wt: r=0.095, p=0.668, tg: r=0.067, p=0.767).

Discussion

The data presented in Experiment 1 showed a significant difference in plus maze performance between wild-type and transgenic mice, with transgenic mice displaying a weaker preference for the closed arms overall. However, there was no significant effect of

exercise on performance for either wild-type or transgenic mice, and no correlation between exercise level and performance.

The difference seen between genotypes supports previous findings in the Tg2576 model, suggesting disinhibition and reduced anxiety in transgenic mice. Lalonde et. al. (2003) also reported a decrease in the ratio of closed compared to total arm entries and duration of time spent in the closed arm in transgenic mice compared to wild-types. However, the data in Experiment 1 extends these findings as this previous study tested Tg2576 mice at 17 months of age, when there would be severe amyloid pathology throughout the brain. Here it is shown that the same performance deficits can be seen as early as 6 months of age, when amyloid levels are elevated in the brain, but before plaque formation, supporting the hypothesis that soluble forms of Aβ lead to cognitive decline (Hardy, 1992, Selkoe, 2008b).

The lack of change in the total number of arm entries suggests that neither exercise nor genotype influence gross motor-activity, and that there is also no difference in the overall exploratory activity of these mice. This is supported by data from Yuede et. al. (2009), showing that exercise had no influence on general activity in Tg2576 mice.

Studies assessing the effects of exercise in other transgenic APP mutant models have shown positive effects of exercise, with exercise reducing anxiety in 3xTg and APP/PS1 mice, tested using open-field and dark-light box behaviour (Garcia-Mesa et al., 2011, Ke et al., 2011). However, no previous studies in APP transgenic models have tested anxiety using the elevated plus maze. Therefore, although the findings presented here of no change with exercise appear to contradict previous studies, the results are not directly comparable because of the difference in behavioural procedure. Studies assessing the effects of exercise on anxiety in C57BL6 mice have also had mixed results, one study reported increased anxiety with exercise (Onksen et al., 2012), whilst another showed reduced anxiety (Binder et al., 2004). The differences between these studies are likely to be due to differences in exercise regime and the behavioural paradigm chosen to assess anxiety. Although the literature assessing the influence of exercise on anxiety is inconclusive, the data presented here suggest that, at least in the Tg2576 model, exercise does not significantly alter anxiety in either transgenic or wild-type mice.

5.4 Experiment 2: The effect of exercise on working memory in the T-maze

Introduction

The aim of Experiment 2 was to assess the effects of exercise on spatial working memory in Tg2576 mice. Spatial working memory requires animals to use short-term memory of spatial information within each trial to complete the task. The forced-choice alternation T-maze paradigm was chosen as this task is specifically designed to test spatial working memory (Deacon and Rawlins, 2006). In this task, during the sample phase one arm of the maze is blocked off, forcing the animals to choose a particular arm, in which they receive a reward. In the test phase both arms are open and the animal must enter the previously unvisited arm to receive a further reward. In doing this they must remember which arm was previously visited. Each trial lasts no longer than 2 minutes, thereby interrogating only short-term memory. This task is dependent on hippocampal function, which is compromised in both AD patients and transgenic models (Ashe, 2001, Deacon and Rawlins, 2005). Previous studies of cognition in the Tg2576 model have shown trans-gene dependent impairments on this task from as early as 8 months of age (Barnes et al., 2004) and also at 10, 12 and 16 months (Chapman et al., 1999, Corcoran et al., 2002, Barnes et al., 2004). The deficits in Tg2576 mice are specific to the forced-choice alternation and do not reflect motor or visual deficits (Barnes et al., 2004). Previous studies have suggested that exercise in aged rats can reduce the number of spatial working memory errors in a radial maze procedure (Kim et al., 2010). Given the marked deficit that Tg2576 mice show with age on the T-maze alternation task, it is predicted that exercise will reduce the magnitude of this deficit.

Methods

Subjects: 89 male 7 month old Tg2576 mice were run on the T-maze. Of these 44 were wild-types, 23 in the exercise condition and 21 in the sedentary condition. 45 were transgenic, 23 in the exercise condition, 22 in the sedentary condition. Two days before testing, and throughout habituation and test days, all mice were water deprived to 85% of their original body weight, by allowing access to water for only 2 hours per day after testing.

Figure 5.5: T-maze. Mice are released in the start arm (nearest to camera), and must make a decision of which arm to enter to find a sucrose reward.

Apparatus: The t-maze was made of clear Perspex, with the start arm measuring 53cm, and each reward arm 26cm, all arms were 10cm wide and had 15cm high walls, **Figure 5.5**. Each of the shorter arms had a black plastic drinking trough placed at the end. The testing room had salient extra-maze cues on each wall made of coloured card and lanterns hung from the ceiling.

Behaviour: Throughout habituation and testing mice were given access to water for 2 hours a day, immediately after each test session. All mice were habituated to the maze for 3 days prior to testing. Habituation sessions lasted for 5 minutes per day during which each mouse was allowed to freely explore the maze. During these sessions both drinking troughs were baited with 15% sucrose solution. Once the animal had drunk the solution they were allowed to continue exploration of the maze for the remainder of the 5 minutes. If after 5 minutes the animal had not drunk the solution in both baited arms they remained in the maze for a further 3 minutes. If they still had not consumed the sucrose solution by the end of 8 minutes the mouse was habituated again later in the day.

At the start of each test trial the maze was cleaned thoroughly and both drinking troughs were baited with approximately 100µl of 15% sucrose solution. Each trial consisted of two phases, the sample phase and the test phase. During the sample phase one of the shorter arms was blocked off using an opaque Perspex door. Each mouse was taken from their home cage and placed at the end of the start arm. The mouse was allowed to explore the maze until they had drunk the sucrose solution in the unblocked arm. They were then removed from the maze

whilst it was cleaned to remove any odour cues and the second arm unblocked. The mouse was then returned to the end of the start arm for the test phase. During this phase the mouse was allowed to explore the maze until it entered one of the shorter reward arms, at which point the door was put in place behind the mouse to stop further exploration. Entry into the arm containing sucrose solution, i.e. the alternate arm to the sample phase, was counted as a correct trial, whereas entry into the un-baited arm, i.e. the same arm as the sample phase, was counted as an incorrect trial. Each mouse remained in the reward arm until they had drunk the sucrose solution or for the equivalent amount of time in the incorrect arm. After this the mouse was returned to their home cage. Testing lasted for 8 days with each mouse given six trials per day with approximately 10 minutes between each trial. The open arm during the sample phase was counterbalanced across trials.

Data analysis and statistics: Each trial was recorded as a correct or incorrect choice. The percentage of correct choices was then calculated for each mouse per day. Repeated measures ANOVA with day as the within subjects factor and genotype and exercise group as between subjects factors was used to analyse performance across days. Two-way ANOVA with genotype and exercise group as between subjects' factors was used to analyse data pooled across days. If any interactions were significant tests of simple main effects were carried out. Pearson's 'r' coefficient was used to assess correlation between exercise level and performance.

Results

Percentage of correct trials

Figure 5.6a shows performance of exercising and sedentary transgenic and wild-type mice across the 8 test days, and **Figure 5.6b** shows data combined across test days for each test group. Inspection of these figures shows task acquisition by wild-type mice, in both exercise and sedentary groups, across the first 5 days and performance reaching a plateau at approximately 75% correct choices. In contrast, transgenic mice, from either exercise or sedentary groups, showed little learning across days, with performance remaining only slightly above 50%. Three-way ANOVA showed a significant main effect of day ($F_{(7,595)}$=4.348, $p<0.001$) and genotype ($F_{(1,85)}$=40.277, $p<0.001$), but no significant main

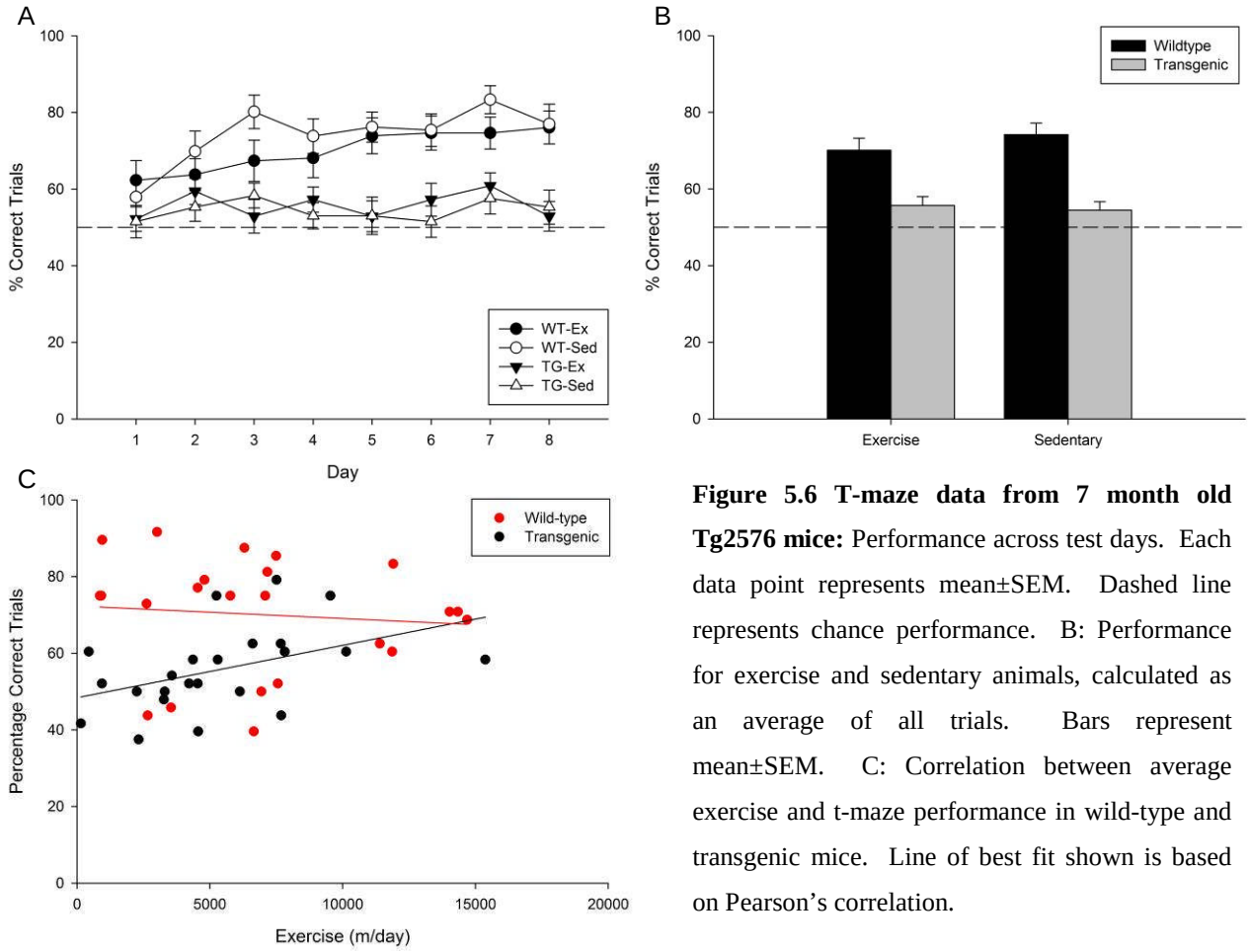

Figure 5.6 T-maze data from 7 month old Tg2576 mice: Performance across test days. Each data point represents mean±SEM. Dashed line represents chance performance. B: Performance for exercise and sedentary animals, calculated as an average of all trials. Bars represent mean±SEM. C: Correlation between average exercise and t-maze performance in wild-type and transgenic mice. Line of best fit shown is based on Pearson's correlation.

effect of exercise group ($F_{(1,85)}$=0.279, p=0.599). There was a significant interaction between day and genotype ($F_{(7,595)}$=2.391, p=0.020), but all other interactions were not significant (all p>0.05). Follow-up of this interaction with tests of simple main effects showed significant differences between wild-type and transgenic mice from test day 2 onwards, demonstrating acquisition of the task by wild-type, but not transgenic mice, regardless of exercise group (day 1: $F_{(1,85)}$=3.479, p=0.066, day 2: $F_{(1,85)}$=5.096, p=0.027, day 3-8: $F_{(1,85)}$>10, p<0.001).

Correlation with exercise

To assess whether the level of exercise of individual mice correlated with t-maze performance the Pearson's correlation coefficient was examined, **Figure 5.6c**. This showed a non-significant weak negative correlation in wild-type mice (r=-0.094, p=0.668), but a significant positive correlation in transgenic mice (r=0.440, p=0.036).

Discussion

The data reported in Experiment 2 revealed a deficit in spatial working memory at 7 months of age in transgenic mice compared to wild-type mice using a forced choice alternation T-maze paradigm. When data were analysed to compare differences between mice in the exercise or sedentary group there was no significant effect of exercise condition on any day, or when data were collapsed across days. Interestingly, however, when the performance of each individual mouse was compared to their exercise intensity there was a significant positive correlation between exercise level and performance in transgenic, but not wild-type mice. This correlation suggests that high intensity exercise may improve T-maze performance in transgenic mice, but that the average exercise intensity across the whole group was not high enough to significantly improve group performance.

These data extend previous findings assessing spatial working memory using the forced choice alternation T-maze in the Tg2576 model. Previous studies have only shown impairments on this task from 8-months of age (Barnes et al., 2004), but no deficit at 6 months (Zhuo et al., 2007). Here we have demonstrated robust deficits at just 7 months. This suggests that the increase in levels of soluble Aβ between 6 and 7 months is enough to cause cognitive impairment in Tg2576 mice when assessed using the T-maze.

None of the previous studies on the effect of exercise on spatial working memory in the Tg2576 model have used the T-maze to test this type of memory. Instead, the radial arm water maze has been used to demonstrate improved working memory after 3 weeks of exercise in 17 month mice (Nichol et al., 2007, Parachikova et al., 2008). Although in these studies the level of exercise was slightly less than in the experiment presented here, the difference in task may make changes in performance easier to detect even with low intensity exercise. It has been argued that the T-maze is not sufficiently sensitive to detect more subtle influences of therapeutics at this early age as the 50% chance level, and 80% wild-type performance level only gives a small window to detect changes. This is supported by data showing that although no cognitive deficits have been detected at 6 months of age in the Tg2576 model using the T-maze (Zhuo et al., 2007), cognitive deficits have been seen as early as 3 months using other spatial paradigms such as the water-maze (King et al., 1999). It is also possible that exercise did not influence cognition at this early-stage in the disease, as neither wild-type nor transgenic mice showed performance improvement with exercise. A study in APP/PS1 mice found improved spatial memory with exercise using a water-maze

task only in aged (24 months old), but not adult (7-8 months old) mice (Ke et al., 2011). Therefore it is necessary to probe spatial working memory at a later time point, and using a different task that may be more sensitive to small changes in cognitive function, this is carried out in Experiment 4.

Another study which has assessed alternation behaviour in the Tg2576 model found no effect of exercise on spontaneous Y-maze alternation at 9 months of age (Yuede et al., 2009). Spontaneous alternation is used to assess the natural preference of a mouse to explore a less recently visited environment, and therefore to alternate between arms on successive trials (Lalonde, 2002). As with the forced choice task, this requires mice to form a short-term memory of the arm most recently visited. Although the study by Yuede et. al. did not find any effect of exercise on alternation this finding is consistent with the results presented here, as the exercise intensity in the study by Yuede was lower than that used in this study, and the correlation presented here suggests that only high intensity exercise is enough to show improvements in spatial working memory.

Overall we have shown a significant correlation between exercise and performance in transgenic mice, however the average intensity of exercise was not enough to provide a group effect of exercise on spatial working memory using the T-maze alternation paradigm.

5.5 Experiment 3: The effect of exercise on spatial memory in an object-in-place paradigm

Introduction

Experiment 3 uses an object-in-place task to assess the influence of exercise on object-location memory in Tg2576 mice. Object recognition tasks are designed to test an animal's memory of previous events by measuring their spontaneous preference to explore novel items (or familiar items placed in novel locations) over more familiar ones (Ennaceur and Delacour, 1988). In the object-in-place version of this task, four objects are presented during the sample phase during which the mouse is allowed to freely explore the objects. At test the same four objects are presented, but with the location of two of the objects swapped. If a

mouse has correctly remembered the original spatial arrangement of the objects they will explore the displaced objects more than those in the same location, as the displaced objects provide the more novel stimulus. Lesion and IEG studies have shown that this object-in-place task is hippocampus dependent, unlike simple novel object recognition, which relies primarily on the perirhinal cortex (Aggleton and Brown, 2005, Barker and Warburton, 2011). Furthermore, previous research has shown that Tg2576 mice are unimpaired on a simple discrimination between a familiar, and novel object (Hale and Good, 2005), however, they cannot discriminate spatial novelty when an object is found in a different location (Hale and Good, 2005, Good et al., 2007b).

Methods

Subjects: 88 male 8-month Tg2576 mice were run on the object-location task. Of these 44 were wild-types, 23 in the exercise condition and 21 in the sedentary condition. 44 were transgenic, 23 in the exercise condition, 21 in the sedentary condition. One wild-type mouse and 2 transgenic mice, all in the sedentary group, were excluded from analysis as they did not interact with the objects during the sample phase.

Apparatus: A grey plywood arena, measuring 100cm x 100cm with 45cm walls, was placed on the test room floor with salient extra-maze cues around the walls of the test room. Four sets of four objects were used in this task. All objects were everyday items, shown in **Figure 5.7**. The objects and arena were cleaned with ethanol wipes after each phase of testing and between mice. The objects were placed equidistant from each other in the centre of the arena in a square formation, approximately 30cm from each other and the walls of the arena.

Behaviour: Each mouse was habituated to the arena during a 10 minute free exploration period on two consecutive days. On each habituation day a different set of four objects was placed in the arena to allow the mice to become familiar with object exploration.

Mice were tested over the next two consecutive days in groups of four including one transgenic and one wild-type mouse from each of the exercise and sedentary groups. Groups

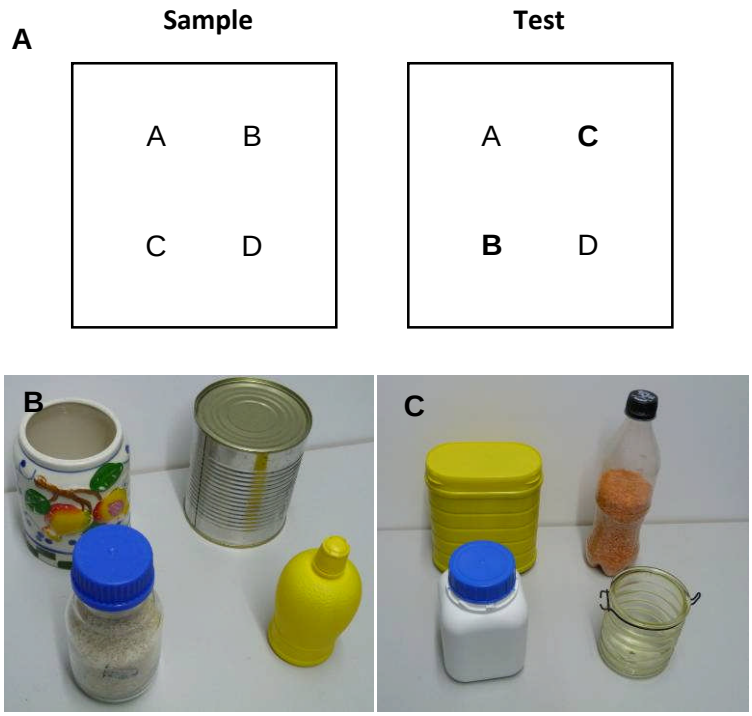

Figure 5.7: Object-in-place. A: Task design. During the sample phase mice are exposed to four objects in a specific configuration. After a 2 minute delay the mice are returned to the arena for the test phase, during which the configuration of the objects is changed, with objects B and C switching location. Wild-type mice show a preference to explore these objects in new locations over those in familiar locations. B and C: Object sets used during the sample and test phase across two days.

were determined by the level of exercise of each mouse, so that the highest exercising wild-type mouse was grouped with the highest exercising transgenic mouse and sedentary littermates. Each test day consisted of a sample phase followed by a 10 minute test phase with a 2 minute delay between sessions. During the sample phase mice were exposed to a new set of four objects in the centre of the arena which they were allowed to freely explore. Transgenic animals were given 10 minutes to explore the arena and objects. The amount of time spent actively exploring the objects was recorded during this session. The average object exploration time between the two transgenic mice in each group was used to yoke the behaviour of the wild-type mice, so that wild-type mice were removed from the arena during the sample phase after they had explored the objects for the same amount of time as transgenic mice. This yoking method was employed as it had previously been shown that transgenic mice show less object contact than wild-type mice in a given time period (Good and Hale, 2007).

After the sample phase mice, were returned to their home cage for a 2 minute interval while the objects and arena were cleaned with ethanol wipes, and the location of two of the objects diagonal to each other was switched, **Figure 5.7**. After this the mice were returned to the arena for the test phase, which lasted 10 minutes for all mice. All sessions were recorded using a camera attached to a DVD recorder and monitor. The same procedure was used for the second test day, but using a different set of four objects. The choice of object set and the location of objects in both the sample and test phase were fully counterbalanced between each group.

Data analysis and statistics: The amount of time spent exploring the objects in novel or familiar locations during each test session was scored manually by an observer blind to genotype. Object exploration was defined as the animal being within 2cm of the object and actively sniffing the object, not just using it as a prop to stand or lean on whilst looking around the arena. A discrimination ratio was calculated for each mouse as the amount of time spent exploring the objects in novel locations divided by the amount of time spent exploring all four objects during the test phase. This ratio was then averaged across the two test days. As a measure of the overall exploration level of each mouse the amount of time spent exploring all of the objects during each habituation session was scored, and averaged across the two days.

Exploration time and discrimination ratio were analysed using Two-way ANOVA, with genotype and exercise group as between subject factors. If the interaction was significant tests of simple main effects were carried out. The correlation between exercise level and performance was assessed using Pearson's 'r' coefficient.

Results

Exploratory activity

Overall object exploration during the two habituation sessions was scored and compared between groups. These data, shown in **Figure 5.8a**, reveal no effect of exercise on exploratory activity for either genotype, but a much lower level of object exploration in transgenic mice compared to wild-types. Analysis with Two-way ANOVA revealed a

significant main effect of genotype on object exploration ($F_{(1,84)}$=47.755, p<0.001), but no significant effect of exercise group ($F_{(1,84)}$=0.015, p=0.902) and no interaction between these variables ($F_{(1,84)}$=1.498, p=0.224). This finding validates our use of yoking exploration time between wild-type and transgenic mice during the sample-phase of the task so that they receive equal exploration of the objects and therefore the initial locations are all equally familiar.

Object-in-place task

The results of the object-in-place test are shown in **Figure 5.8b**. Inspection of this figure shows a discrimination ratio above chance for all wild-type mice and exercised transgenic mice, but not sedentary transgenic mice. Therefore all mice, except for the sedentary transgenic group, spent more time exploring the displaced objects in the novel location. Two-way ANOVA showed a significant main effect of genotype on performance

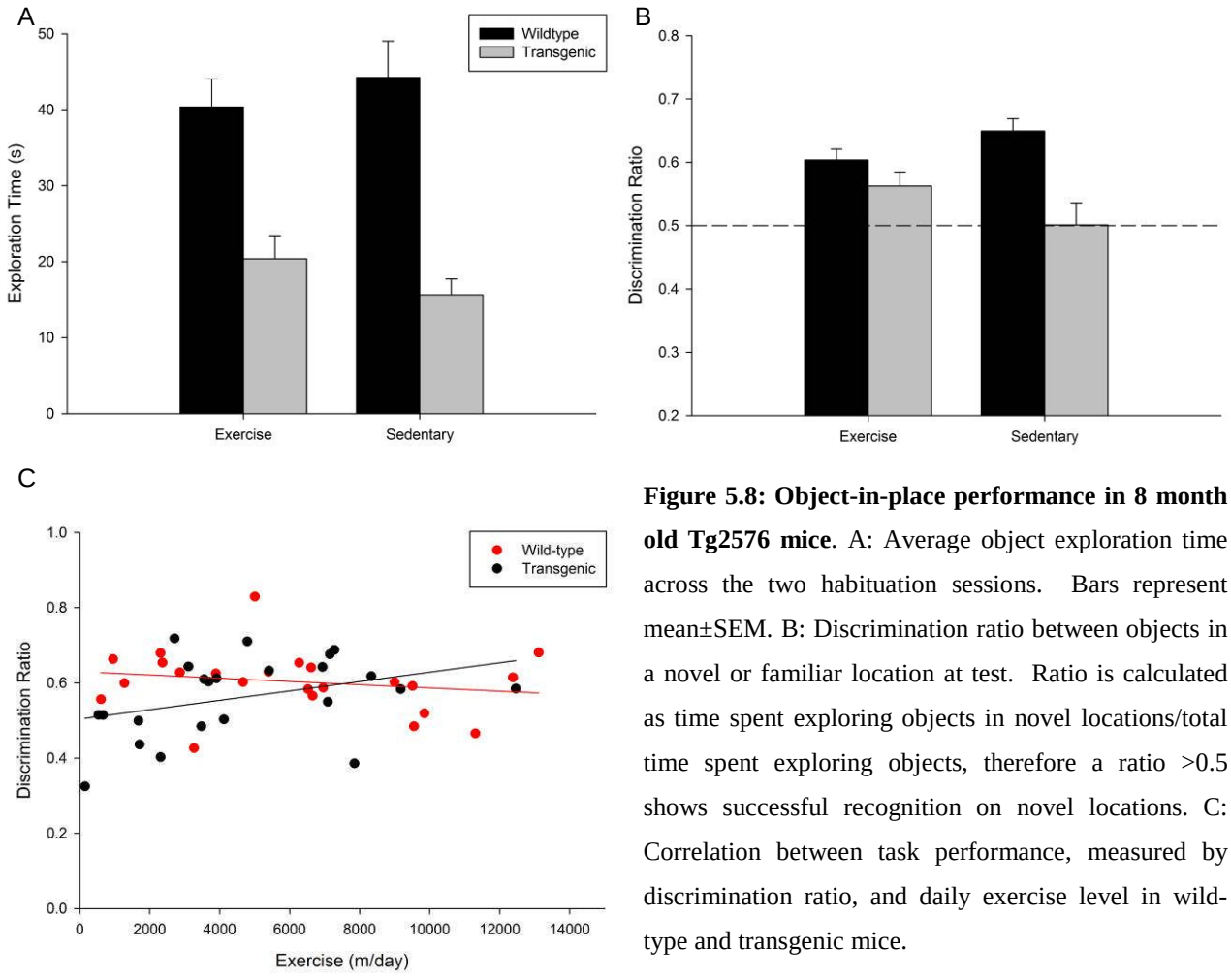

Figure 5.8: Object-in-place performance in 8 month old Tg2576 mice. A: Average object exploration time across the two habituation sessions. Bars represent mean±SEM. B: Discrimination ratio between objects in a novel or familiar location at test. Ratio is calculated as time spent exploring objects in novel locations/total time spent exploring objects, therefore a ratio >0.5 shows successful recognition on novel locations. C: Correlation between task performance, measured by discrimination ratio, and daily exercise level in wild-type and transgenic mice.

($F_{(1,81)}$=16.011, p<0.001), but no effect of exercise group ($F_{(1,81)}$=0.108, p=0.743). There was also a significant interaction between these two factors ($F_{(1,81)}$=5.126, p=0.026). Tests of simple main effects revealed a significant difference between genotype in the sedentary group ($F_{(1,81)}$=18.130, p<0.001), but not in the exercise group ($F_{(1,81)}$=1.645, p=0.203), but no significant effect of exercise within either genotype (both p>0.05) These data demonstrate that exercise improves recognition of novel spatial locations of objects in mice carrying the Tg2576 APPswe mutation.

Correlation with exercise levels

Figure 5.8c shows the correlation between the level of exercise and object discrimination for individual mice of both genotypes. Analysis using Pearson's coefficient showed a non-significant moderate positive correlation between exercise and performance on the object-in-place task in transgenic mice (r=0.367, p=0.085), and a non-significant weak negative correlation between exercise and performance in wild-type mice (r=-0.192, p=0.380). Therefore whilst exercise does enhance memory for object locations, the level of exercise is not linked with performance on this task.

Discussion

The data presented in experiment 3 revealed that exercise improved spatial memory in transgenic Tg2576 mice using an object-in-place paradigm, although there is no relationship between exercise intensity and task performance. However, exercise had no effect on overall exploration level, with transgenic mice in both exercise and sedentary conditions exploring objects significantly less than wild-type mice.

Very few other studies in APP transgenic mice have used object-recognition paradigms to assess the influence of exercise on spatial memory. This behavioural task was selected as it has advantages over other procedures that are more commonly used to assess spatial memory, such as the water-maze (van Praag et al., 1999a, Adlard et al., 2005, Nichol et al., 2007). Unlike the water maze, and other navigation based maze tasks, object recognition paradigms do not rely on the mobility of the mice, and therefore any influence exercise may have on fitness and mobility would not account for differences seen on this task. Object recognition

tasks also do not cause as much stress to the animals as there is no aversive or motivational stimulus required such as water in the water-maze and food-restriction in other land-based tasks. As stress has been shown to impact on normal cognition (Kim et al., 2006), has been identified as a risk factor for AD in humans (Black et al., 1991, Wilson et al., 2005), and also accelerates plaque deposition and cognitive decline in the Tg2576 model (Dong et al., 2004), it was important to keep this factor to a minimum during testing. Although no other studies have used object-in-place tasks to test the effects of exercise on spatial memory in Tg2576, a study by Yuede et. al. (2009) used a simple object recognition paradigm to test discrimination between novel and familiar objects after a delay. Their study found that voluntary, but not forced, exercise improved discrimination between objects in transgenic mice. It is interesting to note that although the study by Yuede showed no discrimination between the novel and familiar object after a 50 minute delay in sedentary transgenic mice at 9 months of age, it has previously been reported that even at 14 months of age transgenic mice can make this discrimination after a 24 hour delay (Hale and Good, 2005). It could also be argued that the object-in-place version of this task, rather than object-novelty, is more relevant to human Alzheimer's patients, who show deficits in processing spatial information (Alescio-Lautier et al., 2007, Hort et al., 2007, Iachini et al., 2009).

The improvement of performance in transgenic mice with exercise on this task suggests that exercise may be influencing hippocampal function. Object-in-place memory has been shown to rely specifically on the hippocampus (Aggleton and Brown, 2005, Barker and Warburton, 2011), therefore any improvement is likely to act via this structure. The mechanisms by which exercise might be altering hippocampal function are explored in Experiments 6 and 7, and discussed more fully in the chapter discussion.

5.6 Experiment 4: The effect of exercise on working memory using a foraging paradigm

Introduction

The foraging task is based upon the radial arm maze paradigm that measures spatial memory for previously visited locations, and is motivated by a food-reward placed in different locations (Olton et al., 1977, Olton and Papas, 1979). The standard radial arm maze has been extensively used to assess spatial memory in rats, and successful task performance has been

shown to be hippocampus-dependent (Olton and Papas, 1979, Ward et al., 1999, He et al., 2002). However, so far only one study has assessed spatial memory in the Tg2576 model using the radial arm maze, which showed deficits in spatial working memory at 24 months (Asuni et al., 2006). The standard radial arm maze is not favoured for assessing memory in mice as they show a very slow acquisition rate and the task requires long testing periods (Foreman and Ermakova, 1998). As a result, the water maze and radial arm water maze have been used more extensively to interrogate spatial memory deficits in Tg2576 and other APP mutant mice (Hsiao et al., 1996, Westerman et al., 2002, Adriani et al., 2006, Nichol et al., 2007, Parachikova et al., 2008).

The foraging task was designed to encourage faster task acquisition in mice and a more rapid test protocol. Similar to the standard radial-arm maze, the foraging task uses appetitive-rewards for motivation instead of aversive escape motivation, which can be stressful to the animal (Harrison et al., 2009). However, unlike the radial arm maze, the foraging task uses an open field arena allowing free exploration and navigation to find liquid-based rewards that are hidden in pots located around the arena. During each session mice are allowed to freely explore the arena to find rewards in each pot. Each pot is baited at the start of the session, but is not replenished during the test session. Therefore, the most efficient behaviour to obtain all of the rewards is to visit each pot only once, this requires the mice to form a memory of which pot locations have been previously visited and foraged. Performance deficits on this task have previously been demonstrated in 3 month and 10 month old Tg2576 mice (Hall and Good, unpublished data).

The foraging task provides measures of several different aspects of cognition. Measures of the amount of time taken to complete the task can show improved performance, but are susceptible to changes in motor function and also to anxiety. The total number of errors and the number of repeat errors provide an indication of spatial working memory. The numbers of return and consecutive errors provide an indication of perseverative and impulsive behaviour in the mice. Previous studies have demonstrated beneficial effects of exercise on spatial memory in APP transgenic models using a variety of tasks (van Praag et al., 1999a, Nichol et al., 2007, Yuede et al., 2009, Kim et al., 2010), leading to the prediction that improved performance should be seen on measures of the total number of errors and the number of repeat errors in the foraging task. In addition, reduced impulsivity with exercise has also been reported (Binder et al., 2004). Therefore, it is predicted that improved performance will also be seen on measures of the number of return and consecutive errors.

Methods

Subjects: 87 male 10 month old Tg2576 mice were run on the foraging task. Of these 44 were wild-types, 23 in the exercise condition and 21 in the sedentary condition. 43 were transgenic, 22 in the exercise condition, 21 in the sedentary condition. Two days before testing, and throughout habituation and test days, all mice were water deprived to 85% of their original body weight, by allowing access to water for only 2 hours per day after testing.

Apparatus: A square plywood arena measuring 100cm x 100cm, with 45cm high walls was used for this task. This was painted grey and the base was covered in a 1cm layer of sawdust. Foraging pots, consisting of a circular white ceramic pot 6.5cm diameter and 3.5cm deep, were mounted on wooden cube bases measuring 3x3x3cm, which were secured to the arena floor. The pots were arranged in the arena approximately 25cm apart in the configuration shown in **Figure 5.9**.

Behaviour: Throughout the training and test phase mice were water-deprived to approximately 85% of their pre-training weight, water was given for 2 hours immediately after testing each day. The first three days were reward training, during which the mice had to learn to forage the ceramic pots to gain a liquid reward of 15% sucrose solution. On these test days the mice were removed from their home cage and placed into a smaller empty cage. A ceramic pot with one drop of sucrose reward was placed in the cage and left there until the

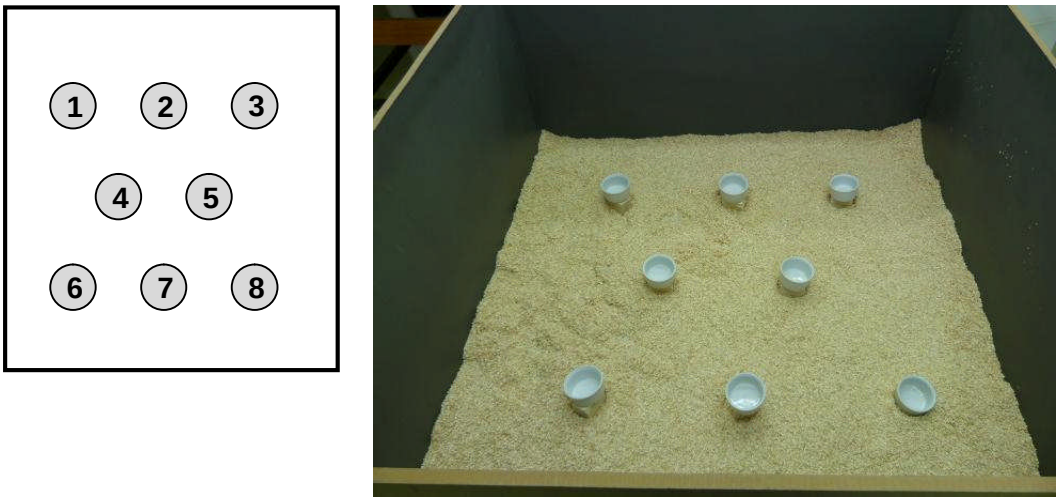

Figure 5.9: Foraging Task. 8 ceramic pots containing a 15% sucrose solution reward were arranged in an arena as shown. Mice were given 10 minutes to forage pots and consume rewards.

reward had been consumed and the mouse had left the pot. The pot was then removed, the sucrose replenished and replaced in the cage. This process was repeated until each mouse had consumed 3 rewards. The mouse was then returned to their home cage.

Mice were habituated to the test arena for the next two consecutive days, with one session per day, to ensure they foraged pots within the arena. During these sessions the arena was setup with two pots in the centre two positions (4 and 5) with a drop of sucrose reward in each. The mouse was placed into the centre of the arena and allowed to explore until they had consumed both rewards or 10 minutes has elapsed. After this the mouse was returned to their home-cage. This process was repeated on day two of habituation.

Mice were tested over the next five consecutive days with one session per day. During these sessions the arena was set up with eight pots each containing a drop of sucrose solution as shown in **Figure 5.9**. Each mouse in turn was taken from their home cage and placed in the centre of the arena always facing away from the experimenter. The mouse was allowed to explore the arena and forage pots until they had consumed the reward in all eight pots or until 10 minutes had elapsed before being returned to their home cage. The pots were then wiped down and the sucrose replenished before the next mouse. All test sessions were recorded using an overhead camera attached to a DVD recorder.

Data analysis and statistics: Each trial was scored for the order of pots foraged and whether a reward was found and consumed. A number of different measures were taken from this task. The time taken was calculated as the amount of the time from when the mouse consumed the first reward to the last reward. An error was scored if a mouse visited a pot after the reward was consumed: these were simply counted to give the total number of errors. Return errors occurred when a mouse immediately revisited a pot in which they have just consumed the reward. Consecutive errors occurred when a mouse immediately revisited a pot that they have just visited and found no reward. Repeat errors occurred when a mouse made more than one error in the same pot. This was calculated for each pot individually and then summed across pots.

Screening of data from the foraging task showed that all variables except for the time taken to complete the task were non-normal distributions. There were a large number of zero's in the samples, due to excellent performance of wild-type mice who completed the task with no

errors, therefore the data could not be transformed to fit the normal distribution. Therefore data were first analysed using a Two-way ANOVA, as ANOVA has been proven to be robust to violations of normality and there is no non-parametric equivalent of this statistical test. The results of this were then verified by assessing parametric (t-test) and non-parametric (Mann-Whitney test) comparisons between groups chosen *a priori*, with Bonferroni correction, to assess whether the violation of normality may have altered the interpretation of parametric analysis as the more conservative nature of non-parametric tests should account for this (Tabacknik and Fidell, 1996). Comparisons were made between wild-type and transgenic mice in either the exercise or the sedentary condition. Correlations between exercise and performance were assessed using Pearson's 'r' coefficient.

Results

Foraging task performance

Time Taken: The average amount of time taken each day to complete the task is shown in **Figure 5.10a**. Sedentary transgenic mice took longer than wild-type mice to complete the task, but there was little difference between exercising transgenic mice and wild-types. Two-way ANOVA revealed a significant main effect of both genotype ($F_{(1,83)}$=4.520, p=0.036) and exercise ($F_{(1,83)}$=4.182, p=0.044) on the time taken to complete the task, but no interaction between these factors ($F_{(1,83)}$=0.793, p=0.376).

Total number of errors: The average total number of errors for each group is shown in **Figure 5.10b**. Inspection of this figure shows a greater number of errors were made by transgenic than wild-type mice in both exercise conditions, but fewer errors were made by mice in the exercising transgenic group than the transgenic sedentary controls. Two-way ANOVA showed a significant main effect of both genotype ($F_{(1,83)}$=22.609, p<0.001) and exercise ($F_{(1,83)}$=4.356, p=0.040), but no interaction between these factors ($F_{(1,83)}$=2.635, p=0.108). To assess the influence of the violation of normality on the conclusions drawn from statistical analyses the results of t-tests and Mann-Whitney tests comparing wild-type and transgenic mice from either the exercise or sedentary group were compared. For the total number of errors both parametric and non-parametric analysis just failed to show a significant difference between genotypes for the exercising mice ($t_{(43)}$=2.328, p=0.050, $U_{(23,22)}$=156, p=0.054), but

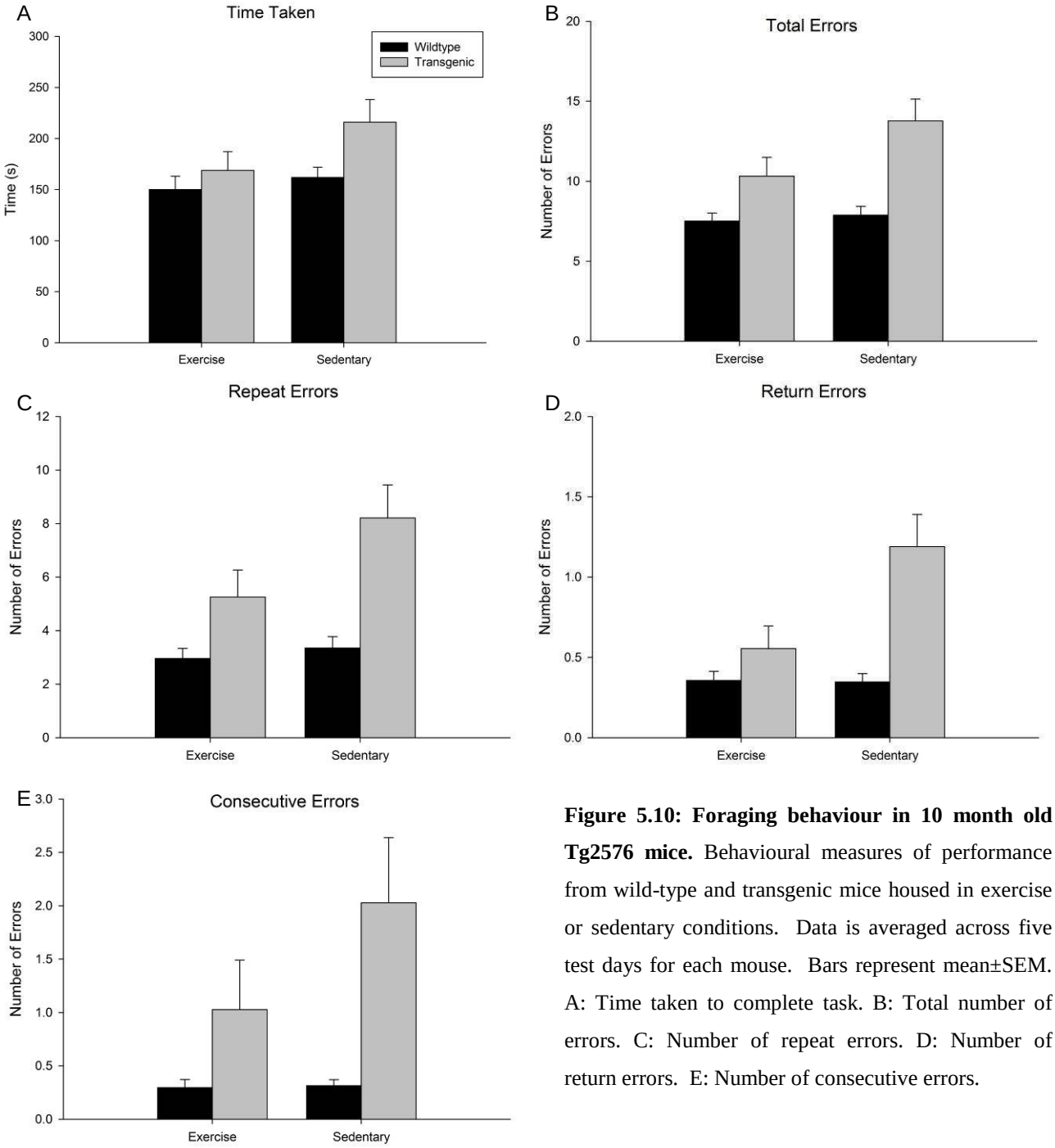

Figure 5.10: Foraging behaviour in 10 month old Tg2576 mice. Behavioural measures of performance from wild-type and transgenic mice housed in exercise or sedentary conditions. Data is averaged across five test days for each mouse. Bars represent mean±SEM. A: Time taken to complete task. B: Total number of errors. C: Number of repeat errors. D: Number of return errors. E: Number of consecutive errors.

did show a significant effect of genotype in the sedentary condition ($t_{(40)}$=4.292, p<0.001, $U_{(21,21)}$=69.5, p<0.001).

Repeat errors: **Figure 5.10c** shows the number of repeat errors made during the task. The pattern of results was similar to that of total errors, with exercising transgenic mice performing better than sedentary transgenic mice, but neither matching wild-type performance. The number of repeat errors showed a significant main effect of both genotype ($F_{(1,83)}$=20.727, p<0.001) and exercise ($F_{(1,83)}$=4.488, p=0.037), but no interaction between

these factors ($F_{(1,83)}$=2.519, p=0.116). Assessment of the effect of exercise using parametric and non-parametric tests revealed no significant difference between exercising wild-type and transgenic mice with parametric tests ($t_{(43)}$=2.255, p=0.058), but a significant difference using non-parametric tests ($U_{(23,22)}$=148, p=0.034). However, as non-parametric tests are the more conservative statistical test, and both genotype and exercise were significant using ANOVA it suggests that there is a difference between wild-type and transgenic performance in exercising mice, despite the t-test results. For sedentary mice there was no difference in outcome between the tests, with both showing significantly worse performance in transgenic mice ($t_{(40)}$=4.048, p<0.001, $U_{(21,21)}$=68.5, p<0.001).

Return errors: Inspection of **Figure 5.10d**, showing the number of return errors, again demonstrates improved performance in transgenic mice with exercise, almost to wild-type levels of performance. Two-way ANOVA showed a significant main effect of both genotype ($F_{(1,83)}$=16.981, p<0.001) and exercise ($F_{(1,83)}$=5.564, p=0.021), and also a significant interaction between these factors ($F_{(1,83)}$=6.067, p=0.016). Follow up of this interaction with tests of simple main effects revealed a significant effect of exercise between transgenic ($F_{(1,83)}$=11.503, p=0.001), but not wild-type mice ($F_{(1,83)}$=0.005, p=0.941), and also a significant difference between wild-type and transgenic mice in the sedentary condition ($F_{(1,83)}$=20.957, p<0.001), but not the exercise condition ($F_{(1,83)}$=1.423, p=0.236). Comparison of the effect of genotype within each exercise group using parametric and non-parametric analysis showed no significant effect of genotype in exercising mice ($t_{(43)}$=1.341, p=0.187, $U_{(23,22)}$=228.5, p=0.569), but a significant effect in sedentary mice ($t_{(40)}$=4.137, p<0.001, $U_{(21,21)}$=91, p=0.002).

Consecutive errors: The average number of consecutive errors made by each group is shown in **Figure 5.10e**. Again there is a trend towards improved performance with exercise in transgenic mice, although neither group improved to wild-type level. There was a significant main effect of genotype ($F_{(1,83)}$=10.757, p=0.002), but not exercise ($F_{(1,83)}$=1.446, p=0.233), and no interaction between these factors ($F_{(1,83)}$=1.303, p=0.257). Comparison of parametric and non-parametric analyses revealed no significant effect of genotype in exercising mice ($t_{(43)}$=1.595, p=0.236, $U_{(23,22)}$=175.5, p=0.142), but a significant difference in sedentary mice ($t_{(40)}$=2.966, p=0.010, $U_{(21,21)}$=73, p<0.001).

Correlation with exercise level

Figure 5.11 shows the correlation between exercise and performance on the foraging task.

Time Taken: Wild-type mice showed no correlation between the intensity of exercise and time taken to complete the task (r=0.055, p=0.804). In contrast, transgenic mice showed a moderate significant negative correlation between level of exercise and time taken (r=-0.434, p=0.043).

Total number of errors: Wild-type mice showed a moderate significant negative correlation between the level of exercise and the total number of errors (r=-0.422, p=0.045). For transgenic mice there was also a negative correlation between exercise and the total number of errors, but this result failed to reach significance (r=-0.392, p=0.071)

Repeat errors: Both wild-type and transgenic mice showed a non-significant negative correlation between exercise and the number of repeat errors (wild-type: r=-0.395, p=0.062, transgenic: r=-0.422, p=0.051), which just failed to reach conventional significance levels.

Return errors: Wild-type mice showed a non-significant negative correlation between exercise and the number of return errors (return: r=-0.234, p=0.283). In contrast, transgenic mice showed a robust negative correlation between exercise and return errors (return: r=-0.467, p=0.028).

Consecutive errors: Wild-type mice showed a non-significant negative correlation between exercise and the number of consecutive errors (r=-0.211, p=0.333), whereas this correlation was significant in transgenic mice (r=-0.445, p=0.038).

Discussion

Experiment 4 showed that exercise improved some aspects of performance on a spatial working memory task in Tg2576 mice. Exercise had little effect on wild-type mice, however, transgenic mice in the exercise group showed improvements compared to sedentary mice on measures of the time taken to complete the task, the total number of errors and the number of repeat, return and consecutive errors. Although exercising transgenic mice did not improve to match the performance of wild-type mice on all measures, there was a significant improvement to wild-type level on the number of repeat errors. The performance of each group on the total number of errors and the number of repeat errors suggests that exercise

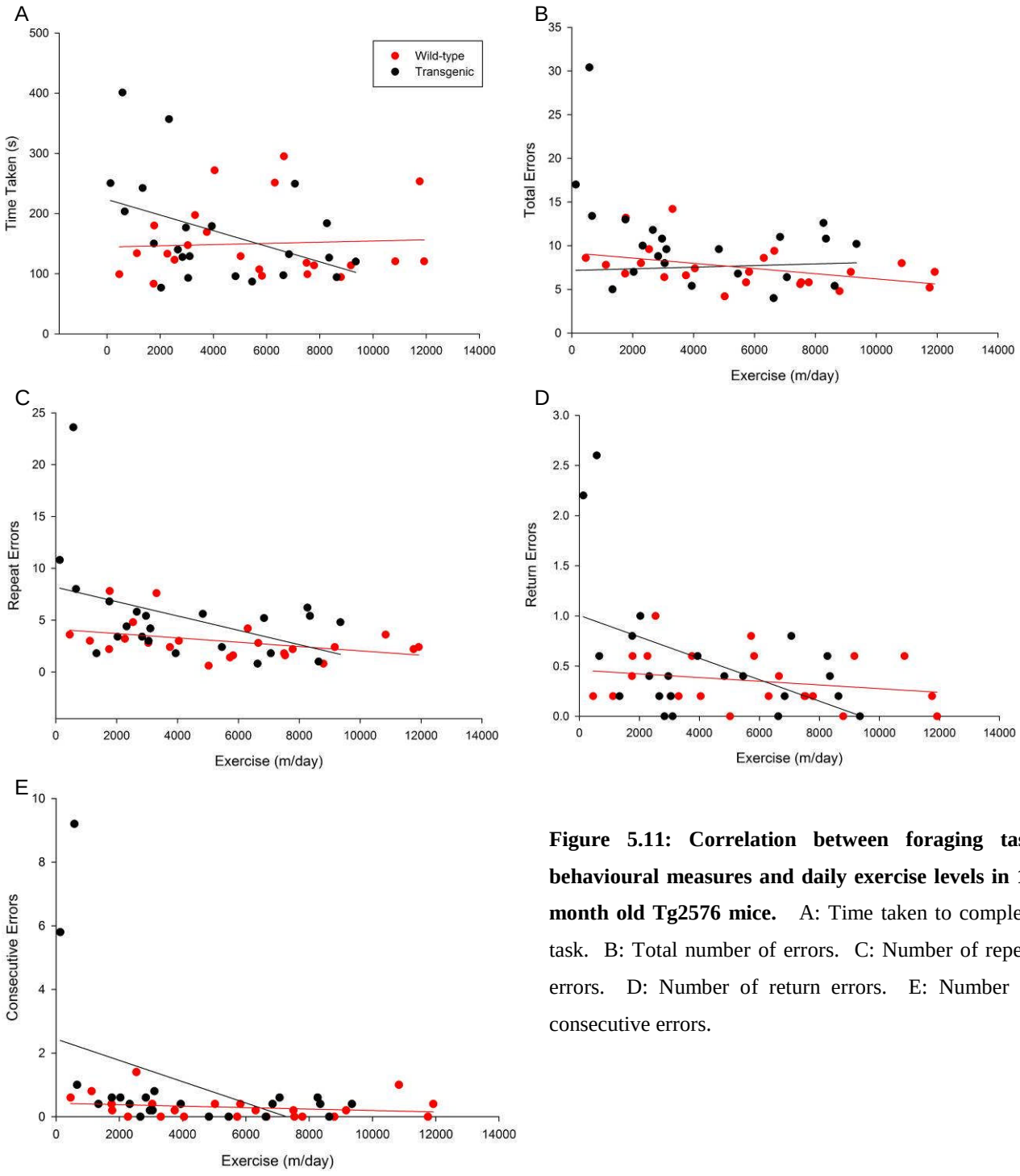

Figure 5.11: Correlation between foraging task behavioural measures and daily exercise levels in 10 month old Tg2576 mice. A: Time taken to complete task. B: Total number of errors. C: Number of repeat errors. D: Number of return errors. E: Number of consecutive errors.

improves spatial working memory in the Tg2576 model, whilst the improvement in measures of return and consecutive errors suggests that exercise also reduces perseverative and impulsive behaviour in these mice. There were also significant correlations between the individual level of exercise and performance for each mouse, although analysis of the scatter-plots indicates that this may be largely driven by the particularly poor performance of only a

few transgenic mice. Correlations were significant for transgenic mice on measures of the amount of time taken to complete the task and the number of return and consecutive errors. Interestingly, although there was no overall effect of exercise on performance between wild-type mice in the exercise or sedentary groups, there were significant correlations between exercise intensity and performance in wild-type mice for the total number of errors. The lack of a group difference, and any correlation in other measures, may be due to the fact that wild-type mice are performing near to ceiling level on this task. However, increasing task difficultly, by increasing the number of pots, may make the task too difficult for transgenic mice and produce a floor effect.

The beneficial effect of exercise on working memory shown here matches that of previous studies into the effects of exercise on memory in APP transgenic models. Other studies in the Tg2576 model have used the radial arm water maze and showed a beneficial effect of exercise on working memory deficits in 17 month old mice after 3 weeks of exercise (Nichol et al., 2007, Parachikova et al., 2008). These studies have shown improvement in escape latency across trials, with exercising transgenic mice performing as well as wild-type mice, whilst sedentary transgenic mice showed a much longer latency. A study using the 3xTg model of AD also showed similar positive results of exercise using a water maze task (Garcia-Mesa et al., 2011). Several other studies have used the water maze to assess the effect of exercise on long-term reference memory, but have not reported measures of short-term working memory on this task (Adlard et al., 2005, Wolf et al., 2006, Ke et al., 2011).

In Experiment 2 no overall effect of exercise on working memory in 7 month old Tg2576 mice was seen using the t-maze paradigm, although there was a correlation between exercise intensity and performance. Although this result seems to be contradictory to the improvement in measures of working memory presented here, this may be due to the limitations of the t-maze task. The foraging task arguably provides a greater number of measures of cognition, and also has a wider window for detecting deficits in the Tg2576 model, and thus greater opportunity to detect improvements. Whilst in the T-maze performance can only range form 50-100%, in the foraging task there is no limit to the number of errors a mouse can make in the 10 minute period. There is also an increased amount of handling required for the t-maze, therefore increasing the level of stress of the animals, which again may reduce performance on that task. The positive correlation seen in the t-maze between exercise and performance at 7 months of age suggests that it is likely to

be the choice of task, and not an effect of age which prevented any demonstration of improved working memory at this earlier age.

5.7 Experiment 5: The effect of exercise on configural memory for episodic information

Introduction

Chapter 4 described specific deficits in transgenic mice in the configural, but not elemental, integration of episodic information using a novel behavioural paradigm (Iordanova et al., 2008). This deficit suggests a specific impairment in associating the spatial and temporal context of an event, the key components of episodic-like memory. This deficit was age-dependent, with impairments seen in mice tested at 11 months, but not 3 months of age. Here we tested the effect of exercise on the performance of 11 month old Tg2576 mice on configural memory. Further details of this episodic-like memory task can be found in chapter 4. Although episodic memory deficits are one of the key cognitive impairments seen in AD patients, previous studies that have assessed the effect of exercise on cognition have not looked at an analogue of episodic memory in transgenic mice.

Methods

Subjects: 84 male 11 month old Tg2576 mice were run on the foraging task. Of these, 44 were wild-types, 23 in the exercise condition and 21 in the sedentary condition. 40 were transgenic, 20 in the exercise condition, 20 in the sedentary condition. One wild-type and one transgenic mice from the exercise group, and 3 transgenic mice from the sedentary group were excluded from the analysis as review of freezing data from day 9 showed that they did not learn the association between the correct auditory stimulus and foot-shock.

Apparatus and Behaviour: The apparatus used and behavioural training was identical to that in chapter 4, Experiment 1.

Data analysis and statistics: Freezing data were scored and analysed as described in chapter 4, Experiment 1. A repeated measures ANOVA was used to analyse freezing ratio data from test days 7 and 8, with session as the within subjects factor and group (wild-type exercise, wt ex; wild-type sedentary, wt sed; transgenic exercise, tg ex; transgenic sedentary, tg sed) as the between subjects factor. Tests of simple main effects were carried out if the interaction was significant. Freezing levels were analysed using Two-way ANOVA with genotype and exercise group as between subject factors. The correlation between exercise level and performance was assessed using Pearson's 'r' coefficient.

Results

Episodic-like memory

The effect of exercise on configural learning of episodic information in Tg2576 mice is shown in **Figure 5.12a**. Inspection of this figure shows that exercise improved memory for episodic information in transgenic mice. There is no difference in performance between exercising transgenic mice and wild-type mice in either condition, with all three groups showing a difference in freezing ratio between morning and afternoon session. In contrast, transgenic mice in the sedentary condition did not show a difference in freezing ratio between sessions. Two-way ANOVA confirmed this interpretation and showed a significant main effect of session ($F_{(1,75)}$=72.409, p<0.001), but not group ($F_{(3,75)}$=1.900, p=0.137), and a significant interaction between these two factors ($F_{(3,75)}$=3.894, p=0.012). Tests of simple main effects showed a significant difference between the morning and afternoon session freezing ratios in exercising and sedentary wild-type mice (ex: $F_{(1,75)}$=35.794, p<0.001, sed: $F_{(1,75)}$=39.793, p<0.001) and in exercising transgenic mice ($F_{(1,75)}$=12.341, p=0.001), but not in sedentary transgenic mice ($F_{(1,75)}$=2.589, p=0.112). These data show that transgenic mice in the sedentary condition performed significantly worse overall than exercising transgenic mice or wild-type mice in either condition.

Analysis of the percentage of time spent freezing showed that wild-type mice in the exercise and sedentary groups were freezing for 2.89±0.425% and 2.01±0.214% (mean±SEM) of observations respectively, whereas transgenic mice in the exercise and sedentary groups were freezing for 4.76±0.815% and 4.29±0.661% of observations respectively. There was a

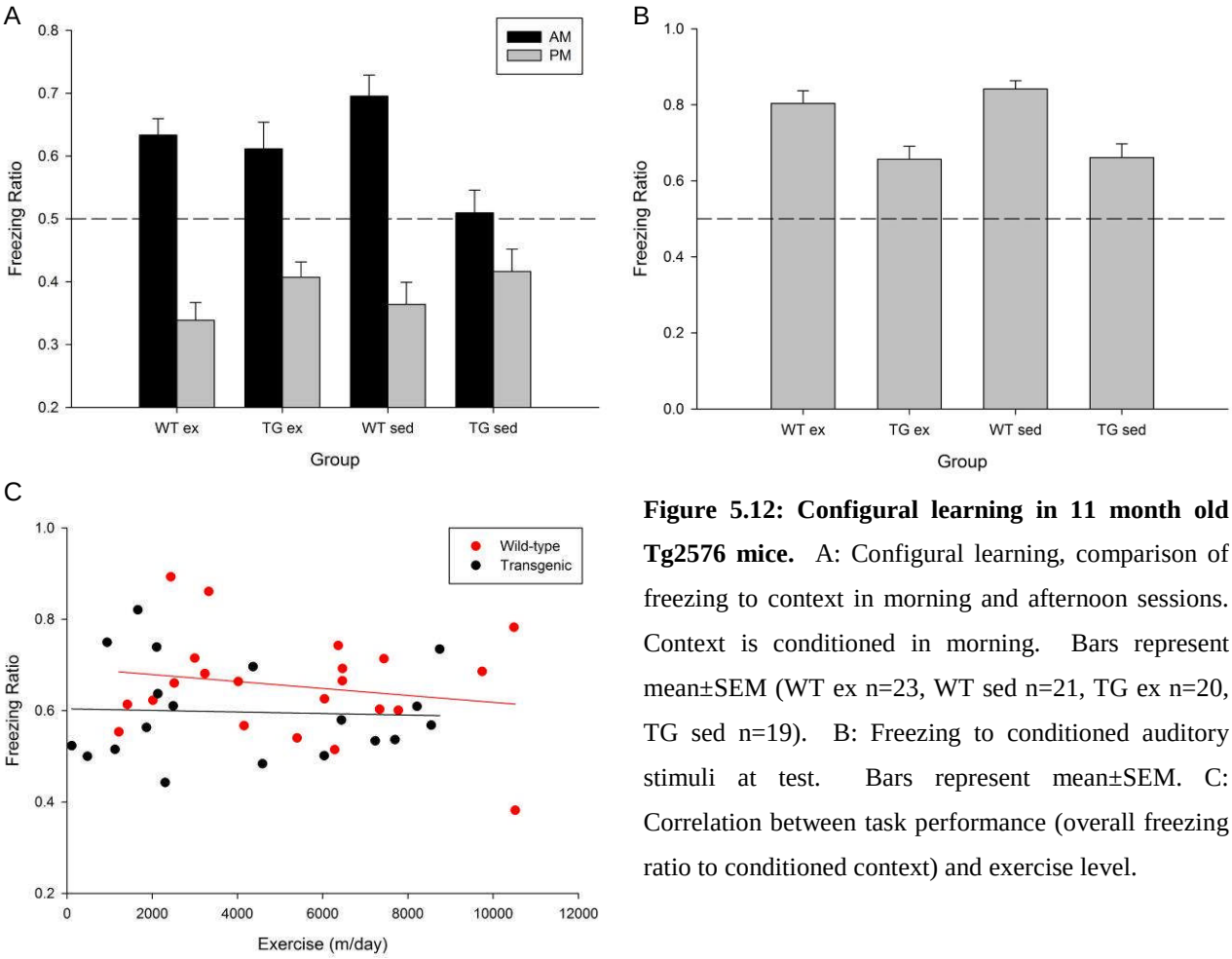

Figure 5.12: Configural learning in 11 month old Tg2576 mice. A: Configural learning, comparison of freezing to context in morning and afternoon sessions. Context is conditioned in morning. Bars represent mean±SEM (WT ex n=23, WT sed n=21, TG ex n=20, TG sed n=19). B: Freezing to conditioned auditory stimuli at test. Bars represent mean±SEM. C: Correlation between task performance (overall freezing ratio to conditioned context) and exercise level.

significant main effect of genotype ($F_{(1,75)}$=11.410, p=0.001), but not group ($F_{(1,75)}$=1.220, p=0.273) and no interaction between these factors ($F_{(1,75)}$=0.110, p=0.741). Therefore transgenic mice showed overall a greater level of freezing in all contexts compared to wild-type mice regardless of exercise condition.

Freezing Ratio

To assess whether the mice had associated the correct auditory stimulus with foot-shock during conditioning the level of freezing to the conditioned and unconditioned auditory stimuli was tested on day 9. Inspection of **Figure 5.12b** shows that, whilst all groups did make the correct association between auditory stimulus and foot-shock, this association was not as strong in transgenic mice. Two-way ANOVA showed a significant main effect of genotype ($F_{(1,75)}$=25.772, p<0.001) but not group ($F_{(1,75)}$=0.392, p=0.533), and no interaction between these factors ($F_{(1,75)}$=0.006, p=0.938).

Correlation with exercise level

The correlation between individual test performance and exercise level was calculated for mice in the exercise group using the combined freezing ratio from the morning and afternoon session to the conditioned stimuli as the measure of performance, **Figure 5.12c**. There was no significant correlation for either wild-type or transgenic mice, with wild-type mice showing a weak negative relationship between these factors (r=-0.204, p=0.361) and transgenic mice showing no relationship (r=-0.060, p=0.807) between performance and exercise level.

Discussion

Experiment 5 demonstrated that voluntary exercise improved configural memory for episodic information in 11 month old Tg2576 mice. Wild-type mice in both the sedentary and exercise conditions discriminated between the time-of-day and context in which an auditory cue was presented, although there was no effect of exercise on performance in wild-type mice. In contrast, transgenic mice in the sedentary condition were impaired and showed no discrimination between the two contexts and time of day at test. This deficit was rescued in transgenic mice in the exercise condition. However, there was no correlation between exercise level and performance for individual mice.

Interestingly, when the data presented in the current study are compared to that from 11 month old Tg2576 mice presented in chapter 4, it appears that all mice performed better in Experiment 5 than in the experiment from chapter 4. There was a greater difference in discrimination ratio between morning and afternoon session for wild-type mice, and also some learning in sedentary transgenic mice although this does not reach significance. For example, in chapter 4 the morning discrimination ratio for wild-type mice was 0.580±0.043 (mean±SEM) and in the afternoon it was 0.425±0.030. In comparison, here in Experiment 5 the mean morning and afternoon ratios, pooled across all wild-type mice, were 0.665±0.022 and 0.346±0.022 respectively. This improvement in learning in Experiment 5 could be explained in two ways. Firstly, the mice used in this exercise study had been exposed to a far greater amount of handling during previous tasks, Experiments 1-4, than those used in the initial episodic-like memory study in chapter 4. Therefore, these mice would be more accustomed to handling and would find this less stressful. As stress has a negative impact on

learning and memory (Dong et al., 2004, Kim et al., 2006) this may account for improved performance in the current study. The second explanation is that there may have been an environmental enrichment effect of having an exercise wheel in the cage, even when it is static. Several studies assessing the relative influence of exercise and enrichment in APP transgenic mice have shown that a locked wheel, or enrichment without exercise can improve cognition (Wolf et al., 2006, Nichol et al., 2007).

5.8 Experiment 6: The effect of exercise on amyloid levels in the brain

Introduction

Having demonstrated improvements in cognition in Tg2576 mice as a result of prolonged voluntary exercise, Experiment 6 examines whether exercise influenced amyloid pathology. The amyloid cascade hypothesis suggests that the primary cause of cognitive deficits in AD is the build up of toxic fragments of Aβ in the brain (Hardy, 1992, Selkoe, 2008a). Therefore it is possible that exercise may benefit cognition by directly altering Aβ levels in the brain. In Experiment 6 Aβ was measured in 12 month old mice, at which age there are high levels of soluble Aβ, and plaques of insoluble amyloid fibrils are beginning to form (Hsiao et al., 1996, Kawarabayashi et al., 2001). It is necessary to quantify the level of both soluble and insoluble amyloid species as a recent addition to the amyloid cascade hypothesis proposes that soluble Aβ, not plaques, is the cause of early cognitive deficits (Selkoe, 2008a). This is possible using enzyme-linked immunosorbent assays (ELISA). Here the level of $A\beta_{1-40}$ and $A\beta_{1-42}$ are measured in hippocampus and cortex samples from Tg2576 mice. Two species of Aβ are measured as although $A\beta_{1-42}$ is the most toxic fragment length in AD, $A\beta_{1-40}$ is much more prevalent in the Tg2576 model (Hsiao et al., 1996).

Methods

Subjects: No amyloid was expected to be present in wild-type tissue, therefore only a subset of wild-type mice were evaluated. Amyloid levels were measured in the hippocampus and

cortex for 10 wild-type mice, 5 from each of the exercise and sedentary groups. For transgenic mice in the exercising group, 19 cortex and 18 hippocampus samples were measured, and in the sedentary group 20 cortex and 19 hippocampus samples were measured. Data were excluded from 4 samples from transgenic mice in the exercising condition (1 soluble $A\beta_{1-42}$ hippocampus, 1 insoluble $A\beta_{1-40}$ hippocampus, 2 insoluble $A\beta_{1-40}$ cortex) as the amyloid level was too high to measure at the dilutions used for all other samples.

Tissue collection: Mice were deeply anaesthetised by intra-peritoneal injection of 0.2ml of Euthatal (sodium pentobarbitone) and then exsanguinated by insertion of a cannula into the left ventricle and perfusion with 50ml of 0.1M PBS (pH 7.4). The brains were then hemi-dissected and one hemisphere of each brain was maintained on ice while the hippocampus, cortex and cerebellum were dissected out. For each region the weight of the wet tissue was recorded before being snap frozen in liquid nitrogen and stored at -80^{o}C.

Protein Extraction: During protein extraction dissected tissue samples were removed from the freezer and maintained on ice. Extraction buffer (2% SDS in dH_2O with 1% Inhibitor Cocktail (Calbiochem #539134)) was added to each sample at a volume of 1ml/75mg wet tissue. Tissue was homogenised using a Precellys (Stretton Scientific) and rotated overnight at 4^{o}C. The next day the samples were centrifuged at 28300g for 1 hour at 4^{o}C. The supernatant containing the soluble protein fraction was removed and stored neat at -80^{o}C. 70% formic acid was added to the remaining tissue residue at a volume of 1ml/150mg of starting tissue weight. The samples were vortexed to break up the pellet and then centrifuged again at 28300g for 1 hour at 4^{o}C. Supernatant containing the insoluble protein fraction was removed, taking care to avoid the lipid layer, and stored neat at -80^{o}C.

Protein Quantification using Bicinchoninic Acid Assay: To quantify the concentration of total protein in each soluble protein sample a bicinchoninic acid assay method was used (Pierce BCA Protein Assay Kit, #23227, Thermo Scientific). This compares the amount of protein in each sample to known standards using a colour-change assay. Protein standards ranged from 2mg/ml of bovine serum albumin (BSA) to 0.01mg/ml. 10µl of each standard and 1µl of each sample were added to a 96 well plate in duplicate before 200µl of working

reagent (50:1 dilution of BCA reagent A:B) was added to each well. The plate was mixed for 30 seconds and then incubated at 37^{o}C for 30 minutes. The plate was allowed to cool for 5 minutes before the absorbance of each well was read at 540nm. Readings from the known standards were used to produce a standard curve from which the protein concentration of unknown samples was calculated in mg/ml.

Enzyme-linked Immunosorbent Assay: To assess the level of Aβ protein in the hippocampus and cortex of Tg2576 mice ELISA's were used to quantify soluble and insoluble β-amyloid levels. ELISA's were carried out using ready prepared kits designed to bind $A\beta_{1-40}$ and $A\beta_{1-42}$. (Human $A\beta_{1-40}$ ELISA kit, KHB3481, and Human $A\beta_{1-42}$ ELISA kit, KHB3441, both from Invitrogen). Each ELISA was carried out according to the kit instructions, described briefly here. The kit supplies plates pre-coated with monoclonal antibody specific to the NH_2 terminus of the human Aβ peptide. For the first stage 50µl of standards of a known Aβ content, controls and unknown samples from each mouse were pipetted into wells on the plate in duplicate. 50µl of a rabbit detection antibody specific to the COOH terminus of the Aβ peptide was added to each well and the plate was incubated for 3 hours at room temperature with agitation. The concentration of Aβ in each standard ranged from 500pg/ml to 7.81pg/ml with a 1:2 serial dilution in matched buffer to the unknown samples (i.e. dilutent buffer with 2% SDS for soluble protein samples and dilutent buffer with 70% formic acid for insoluble samples, matching the dilution factors of the samples - see below), giving seven standards in total. Control wells contained only matched dilutent buffer. After 3 hours the liquid in each well was decanted and the wells were washed 4 times before 100µl of secondary anti-rabbit antibody labelled with horseradish peroxidase was added and incubated for 30 minutes. After this second incubation liquid in the wells was decanted and they were washed 4 times. 100µl of chromogenic solution was then added to each well, which reacts with the horseradish peroxidise complex to produce a colour-change from clear to blue. The intensity of this change is directly proportional to the concentration of Aβ in the original sample. The colour change was stopped by adding 100µl stop solution to each well with the chromogen, and the absorbance of each well was measured at 450nm using a microplate reader.

A different dilution factor was used for soluble and insoluble proteins and for $A\beta_{1-40}$ compared to $A\beta_{1-42}$ as there are different levels of each of these peptides in each brain region.

The sample applied to each microplate needed to have a concentration that fell within the known standard concentrations. As there is a lot of variation in amyloid protein expression within the Tg2576 not all mice could be measured using one dilution factor; all samples were tested at one initial dilution factor and then any that fell outside the standard range were rerun at a higher dilution factor. Therefore the following dilution factors were used:

Soluble $A\beta_{1-40}$ cortex: 1:2000 (samples outside this range rerun at 1:8000)

Soluble $A\beta_{1-40}$ hippocampus: 1:400 (1:2000)

Soluble $A\beta_{1-42}$ cortex: 1:400 (1:1000)

Soluble $A\beta_{1-42}$ hippocampus: 1:400 (1:1000)

Insoluble $A\beta_{1-40}$ cortex: 1:1500 (1:5000)

Insoluble $A\beta_{1-40}$ hippocampus: 1:1200 (1:5000)

Insoluble $A\beta_{1-42}$ cortex: 1:800

Insoluble $A\beta_{1-42}$ hippocampus: 1:800

Data analysis and statistics: A curve was produced from the absorbance of the known standards plotted against the protein concentration using Graphpad Prism v.5 software package. The concentration of amyloid in each sample was calculated by reading it off the curve derived from the standards, and then multiplying this value by the dilution factor to give the concentration of the original sample. The amount of Aβ in each sample is expressed as nanograms of amyloid protein per milligram of total protein (ng/mg) as calculated from the BCA assay.

Two-way ANOVA with genotype and exercise group as between subject factors was used to analyse data from each brain region and amyloid species. Significant interactions were followed-up using tests of simple main effects. Correlations between amyloid level, exercise and behaviour were assessed using Pearson's 'r' coefficient.

Results

Effect of exercise on soluble $A\beta_{1-40}$

Within both the hippocampus and cortex, **Figure 5.13a**, there was no soluble $A\beta_{1-40}$ detected in wild-type mice, and no effect of exercise on amyloid level in transgenic mice. Two-way ANOVA showed a significant effect of genotype (hippocampus: $F_{(1,43)}=14.519$, $p<0.001$, cortex: $F_{(1,45)}=6.751$, $p=0.013$), but not exercise (hippocampus: $F_{(1,43)}=0.023$, $p=0.881$, cortex: $F_{(1,45)}=0.008$, $p=0.931$), and no interaction between these factors (hippocampus: $F_{(1,43)}=0.014$, $p=0.908$, cortex: $F_{(1,45)}=0.009$, $p=0.927$).

Effect of exercise on insoluble $A\beta_{1-40}$

The same results were found for the insoluble form of $A\beta_{1-40}$, **Figure 5.13b**; there was no amyloid in wild-type mice and no effect of exercise on amyloid levels in transgenic mice. Two-way ANOVA again showed a significant main effect of genotype (hippocampus: $F_{(1,42)}=6.594$, $p=0.014$, cortex: $F_{(1,43)}=10.244$, $p=0.003$), but not exercise (hippocampus: $F_{(1,42)}=0.108$, $p=0.744$, $F_{(1,43)}=0.062$, $p=0.805$) and no interaction (hippocampus: $F_{(1,42)}=0.158$, p0.693, $F_{(1,43)}=0.065$, $p=0.800$).

Effect of exercise on soluble $A\beta_{1-42}$

Assessment of levels of soluble $A\beta_{1-42}$, see **Figure 5.13c**, showed no effect of exercise on amyloid levels, and amyloid was only detected in transgenic mice. Two-way ANOVA of data from both the hippocampus and cortex again showed a significant effect of genotype (hippocampus: $F_{(1,43)}=8.288$, $p=0.006$, cortex: $F_{(1,45)}=10.501$, $p=0.002$), but not exercise (hippocampus: $F_{(1,43)}=0.414$, $p=0.523$, cortex: $F_{(1,45)}=0.001$, $p=0.979$) and no interaction between these factors (hippocampus: $F_{(1,43)}=0.371$, $p=0.546$, cortex: $F_{(1,45)}<0.001$, $p=0.988$).

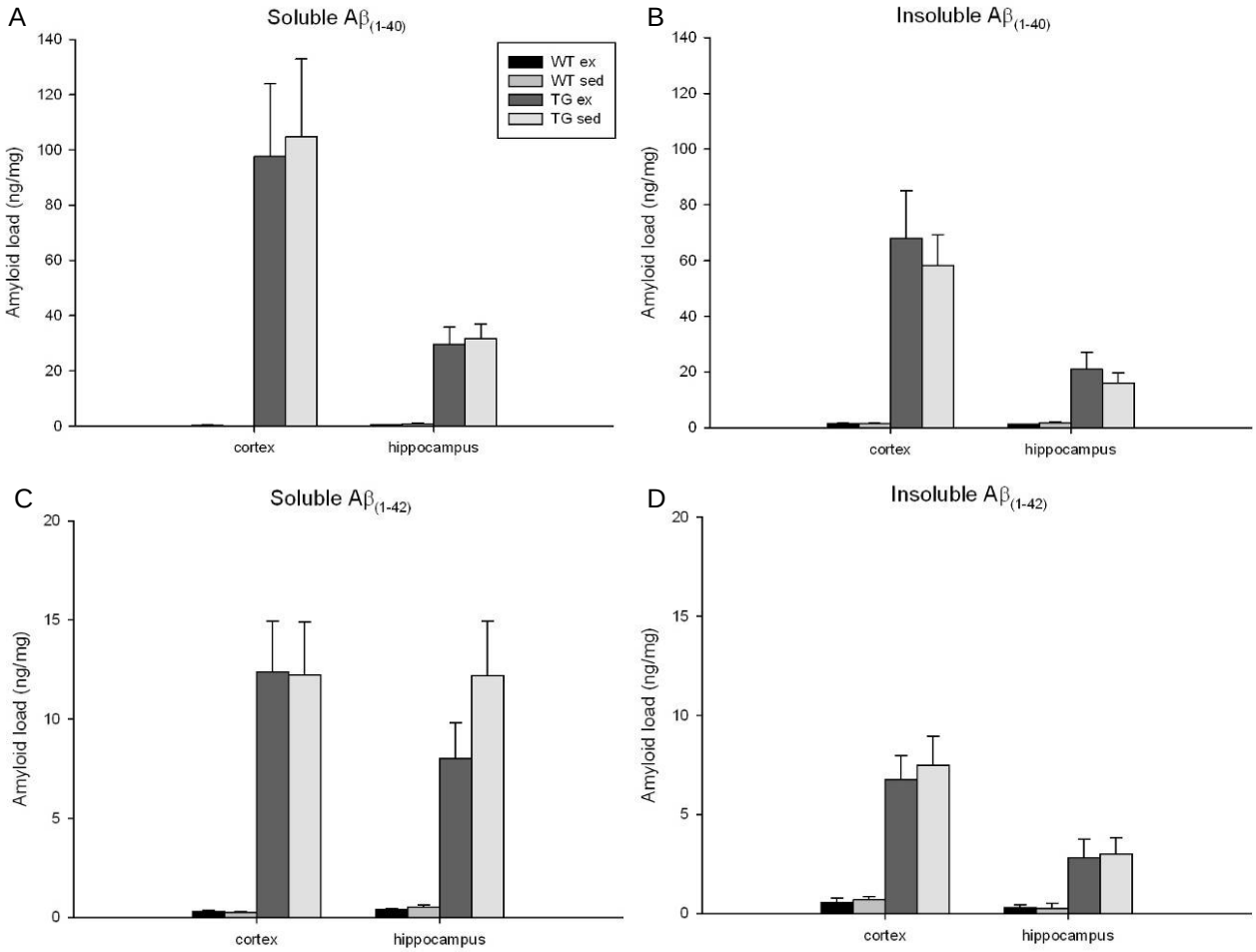

Figure 5.13: Amyloid load in the hippocampus and cortex of 12-13 month old Tg2576 mice. A: Soluble $A\beta_{1\text{-}40}$, B: Insoluble $A\beta_{1\text{-}40}$ C: Soluble $A\beta_{1\text{-}42}$, D: Insoluble $A\beta_{1\text{-}42}$. Bars represent mean ± SEM (WT ex: n=5, WT sed: n=5, TG ex: n=19, TG sed: n=20)

Effect of exercise on insoluble $A\beta_{1\text{-}42}$

Finally, the same pattern of results was seen for insoluble $A\beta_{1\text{-}42}$, see **Figure 5.13d**, with no effect of exercise on amyloid levels, and no amyloid detected in wild-type mice. Two-way ANOVA showed a significant effect of genotype (hippocampus: $F_{(1,45)}=4.287$, $p=0.044$, cortex: $F_{(1,45)}=11.441$, $p=0.001$), but not exercise (hippocampus: $F_{(1,45)}=0.003$, $p=0.957$, cortex: $F_{(1,45)}=0.048$, $p=0.828$) and no interaction (hippocampus: $F_{(1,45)}=0.008$, $p=0.930$, cortex: $F_{(1,45)}=0.020$, $p=0.888$).

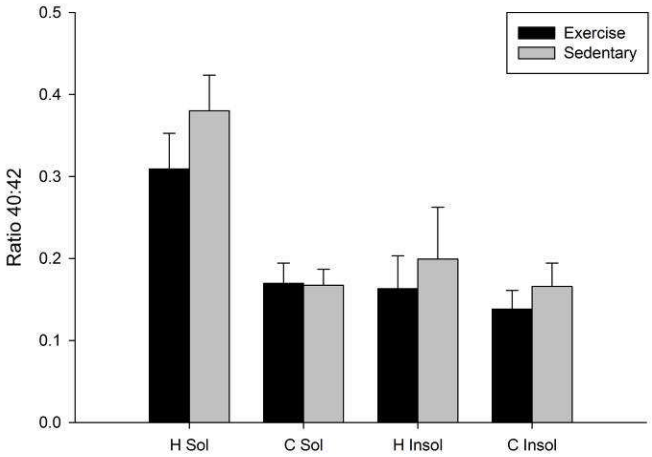

Figure 5.14: Ratio of $A\beta_{1-40}$:$A\beta_{1-42}$ in transgenic mice. H Sol: hippocampus soluble amyloid load, C Sol: cortex soluble amyloid load, H Insol: hippocampus insoluble amyloid load, C Insol: cortex insoluble amyloid load. Bars represent mean ± SEM.

Effect of exercise on the ratio of Aβ 1-40:1-42

The ratio of Aβ 1-40:1-42 was calculated for transgenic mice in the exercise or sedentary condition. These data, shown in **Figure 5.14**, revealed no change in ratio as a result of exercise, although it does show a higher ratio of soluble protein in the hippocampus than the cortex. Independent samples t-tests to compare mice from the exercise or sedentary conditions revealed no significant effect of exercise on the ratio of $A\beta_{1-40}$:$A\beta_{1-42}$ in either the hippocampus or cortex for soluble (hippocampus: $t_{(34)}$=1.425, p=0.163, cortex: $t_{(37)}$=0.076, p=0.939) or insoluble proteins (hippocampus: $t_{(34)}$=0.467, p=0.643, cortex: $t_{(35)}$=1.099, p=0.279).

Correlation with exercise level and task performance

Pearson's coefficients were used to investigate correlations between amyloid load, exercise level and task performance in exercising and sedentary transgenic mice. The results of this analysis are shown in **Table 5.1**. For both exercising and sedentary mice, there were significant correlations between amyloid load of the different length Aβ fragments and the soluble and insoluble proteins. There were no significant correlations between any form of amyloid and exercise level for mice in the exercise condition. For mice in the sedentary condition amyloid load did not significantly correlate with performance on any behavioural paradigm. However, for mice in the exercise condition amyloid load, particularly in the

Table 5.1: Pearson's Correlation Coefficients for amyloid level, exercise and cognition in transgenic mice. Data for exercising mice shown in standard text, data for sedentary mice shown in bold text. Dark grey shaded cells show significant correlations between variables, p<0.05=*, p<0.01=**, p<0.001=***.

		H Sol Aβ1-40	C Sol Aβ1-40	H Insol Aβ1-40	C Insol Aβ1-40	H Sol Aβ1-42	C Sol Aβ1-42	H Insol Aβ1-42	C Insol Aβ1-42	Plus maze	T-maze	Object-in-place	Foraging	Episodic memory
Exercise	r	-0.176	-0.087	-0.301	-0.364	-0.167	0.025	-0.099	-0.070	-0.099	0.639**	0.208	-0.344	-0.037
	p	0.485	0.724	0.241	0.151	0.521	0.918	0.686	0.776	0.696	0.003	0.394	0.162	0.886
hippocampus soluble Aβ1-40	r		0.745**	0.277	0.617*	0.522*	0.514*	0.612**	0.636**	-0.213	0.112	0.362	-0.082	0.431
	p		0.000	0.282	0.011	0.038	0.029	0.007	0.005	0.413	0.657	0.140	0.754	0.096
cortex soluble Aβ1-40	r	**0.348**		0.309	0.549*	0.012	0.795**	0.595**	0.473*	-0.506*	0.152	0.221	-0.238	0.622**
	p	**0.145**		0.228	0.022	0.965	0.000	0.007	0.041	0.032	0.535	0.362	0.342	0.008
hippocampus insoluble Aβ1-40	r	**0.486***	**-0.012**		0.090	-0.015	0.287	0.512*	-0.080	0.036	0.016	-0.169	0.177	0.106
	p	**0.035**	**0.961**		0.741	0.958	0.265	0.036	0.761	0.895	0.950	0.517	0.512	0.708
cortex insoluble Aβ1-40	r	**0.757****	**0.679****	**0.268**		0.013	0.627**	-0.001	0.735**	-0.341	-0.215	0.188	-0.198	0.548*
	p	**0.000**	**0.001**	**0.268**		0.964	0.007	0.998	0.001	0.196	0.407	0.469	0.462	0.034
hippocampus soluble Aβ1-42	r	**0.758****	**0.436**	**0.386**	**0.646****		-0.127	0.168	-0.053	0.325	-0.017	-0.061	0.126	0.076
	p	**0.000**	**0.055**	**0.102**	**0.002**		0.628	0.518	0.839	0.220	0.947	0.817	0.643	0.788
cortex soluble Aβ1-42	r	**0.503***	**0.654****	**0.108**	**0.566****	**0.876****		0.220	0.264	-0.373	0.177	0.229	-0.141	0.617**
	p	**0.028**	**0.002**	**0.661**	**0.009**	**0.000**		0.364	0.275	0.127	0.467	0.345	0.577	0.008
hippocampus insoluble Aβ1-42	r	**0.396**	**0.178**	**0.544***	**0.055**	**0.211**	**0.085**		0.143	-0.166	0.314	0.175	-0.047	0.248
	p	**0.093**	**0.454**	**0.016**	**0.818**	**0.372**	**0.723**		0.559	0.510	0.191	0.472	0.853	0.337
Cortex insoluble Aβ1-42	r	**0.217**	**0.333**	**0.214**	**0.308**	**0.655****	**0.739****	**0.081**		-0.351	0.102	0.354	-0.224	0.262
	p	**0.371**	**0.151**	**0.380**	**0.186**	**0.002**	**0.000**	**0.736**		0.153	0.677	0.137	0.372	0.311
Elevated plus maze	r	**0.180**	**0.196**	**-0.013**	**0.366**	**-0.050**	**0.015**	**0.059**	**-0.233**		-0.318	-0.069	0.105	-0.577*
	p	**0.489**	**0.435**	**0.961**	**0.135**	**0.844**	**0.953**	**0.817**	**0.352**		0.198	0.787	0.689	0.019
T-maze	r	**-0.013**	**-0.126**	**-0.114**	**0.118**	**-0.028**	**-0.062**	**-0.351**	**0.022**	**0.177**		0.481*	-0.210	0.229
	p	**0.959**	**0.597**	**0.643**	**0.621**	**0.906**	**0.794**	**0.129**	**0.925**	**0.481**		0.037	0.404	0.376
Object-in-place	r	**0.126**	**-0.113**	**0.040**	**-0.011**	**0.205**	**0.083**	**0.100**	**-0.128**	**0.137**	**-0.049**		-0.400	0.283
	p	**0.642**	**0.665**	**0.884**	**0.965**	**0.430**	**0.751**	**0.704**	**0.625**	**0.626**	**0.853**		0.100	0.271
Foraging	r	**0.074**	**-0.179**	**0.362**	**-0.093**	**-0.139**	**-0.257**	**0.228**	**-0.032**	**-0.471***	**-0.148**	**-0.346**		-0.253
	p	**0.764**	**0.450**	**0.128**	**0.695**	**0.560**	**0.274**	**0.333**	**0.894**	**0.048**	**0.534**	**0.173**		0.344
Episodic memory	r	**-0.216**	**0.104**	**-0.211**	**0.043**	**-0.178**	**-0.023**	**-0.331**	**-0.093**	**0.157**	**0.249**	**0.006**	**0.043**	
	p	**0.405**	**0.690**	**0.417**	**0.869**	**0.494**	**0.929**	**0.194**	**0.722**	**0.576**	**0.335**	**0.983**	**0.871**	

cortex, significantly correlated with performance on the configural memory task. Interestingly, these correlations were positive, suggesting that a higher amyloid load improved performance on this task. The level of soluble $A\beta_{1-40}$ in the cortex also correlated significantly with elevated plus maze behaviour in exercising mice, although this was a negative correlation, so that mice with lower amyloid levels showed better task performance.

Discussion

The data presented in Experiment 6 show no change in the overall levels of β-amyloid in the hippocampus or cortex of Tg2576 mice, and no change in the ratio of $A\beta_{1-40}$:$A\beta_{1-42}$ in either region as a function of exercise. There was also no correlation between amyloid level and exercise intensity for each mouse, suggesting that exercise intensity did not directly influence amyloid production or clearance. In addition, amyloid levels only correlated with behaviour on the configural learning task and elevated plus maze in exercising mice. The lack of any correlation between amyloid load and behaviour in sedentary mice suggests that amyloid is not a good predictor of the level of cognitive change in these mice. Several studies have suggested that although amyloid may be the primary cause of AD, a better predictor of cognitive decline in human patients is the loss of synaptic proteins (DeKosky and Scheff, 1990, Terry et al., 1991).

Previous studies assessing the influence of exercise on amyloid pathology have had mixed results. In Tg2576 mice few changes have been seen in amyloid levels as a result of exercise. One study showed that three weeks of wheel running in aged (17 month old) mice resulted in no change in the levels of insoluble $A\beta_{1-40}$ or $A\beta_{1-42}$ using ELISA, and no change in plaque load (Parachikova et al., 2008). Whilst another, using the same exercise regime and age of mice, showed no change in levels of $A\beta_{1-42}$ or plaque load, but a reduction in the level of soluble $A\beta_{1-40}$ using dot-blot assays (Nichol et al., 2008). A third study in Tg2576 mice used a longer exercise program, from 5-9 months of age, but still showed no change in levels of $A\beta_{1-40}$ or $A\beta_{1-42}$ using ELISA's as a result of exercise (Yuede et al., 2009). These data largely agree with the results presented here, exercise did not improve cognition by directly altering amyloid pathology in Tg2576 mice. However, the study by Nichol et. al. (2008) suggests that the choice of a more sensitive assay for amyloid detection may show some minor reductions in certain species of Aβ protein.

In contrast to findings in the Tg2576 model, studies in other APP transgenic models suggest that exercise can directly influence amyloid load. Two studies, both using 5-6 month exercise programs found reduced levels of Aβ in the TgCRND8 (Adlard et al., 2005) and 3xTg model (Garcia-Mesa et al., 2011). The differences in the mechanisms of action of exercise in different transgenic models are highlighted further by reports of no change in plaque load in APP23 mice after 9 months of exercise (Wolf et al., 2006), and decreases in insoluble Aβ measured by ELISA, but no change in plaque load, after 6 weeks of exercise in APP/PS1 mice (Ke et al., 2011). These varied findings suggest that whilst in some models exercise does directly influence amyloid pathology, there must also be non-amyloid dependent mechanisms by which exercise improves cognition in APP transgenic mice.

The significant correlation between performance on the configural memory task and amyloid load is of interest, as these correlations suggest that higher amyloid levels predict better task performance. This finding contradicts almost all other studies which have shown that increased amyloid correlates with reduced cognition in the Tg2576 model (Westerman et al., 2002). Again this may reflect the fact that amyloid load is a poor predictor of cognitive performance and that correlation is in fact due to chance. However, it is interesting that it is cortical, and not hippocampal amyloid load that correlated with performance. Configural memory for episodic information is hippocampus-dependent, whilst elemental information can be processed by extra-hippocampal cortical regions (Iordanova et al., 2009). It is possible that the high level of amyloid in the cortex disrupted cortical processing of information, which may disrupt processing of simple associative relationships and thereby promote configural information processing. Thereby reducing the conflict between configural and elemental processing which are likely to be recruited in parallel in the intact brain during this task.

Overall, no change in amyloid levels as a consequence of exercise in Tg2576 mice was seen, therefore other molecular mechanism must underlie the exercise-induced improvements in cognition. These mechanisms are further explored in Experiment 7, and discussed in section 5.10.

5.9 Experiment 7: The effect of exercise on dentate gyrus neurogenesis

Introduction

Experiment 6 showed that amyloid load was not altered by exercise in Tg2576 mice and cannot explain changes in cognition brought about by exercise. Therefore, Experiment 7 assesses whether neurogenesis may be a key factor linking exercise to changes in cognition. Previous evidence suggests that exercise increases neurogenesis in the DG of C57Bl6 mice, and that this correlates with improvements in cognition (van Praag et al., 1999a, van Praag et al., 2005). There is also evidence that neurogenesis is decreased in both AD patients (Lazarov and Marr, 2010, Winner et al., 2011) and in Tg2576 mice (Dong et al., 2004). Only one study so far has assessed the effect of exercise on neurogenesis in a transgenic APP model, the APP23 model, (Mirochnic et al., 2009), finding that exercise increased the number of new neurons in wild-type, but not transgenic mice, at 6 months of age, whilst at 18 months of age exercise mildly increased neurogenesis in both wild-type and transgenic mice compared to control housed mice. However, no deficit in neurogenesis was seen in the APP23 model between control housed wild-type and transgenic mice at any age. Therefore this experiment examines whether exercise increases neurogenesis in the Tg2576 model, which does show an age-related deficit in neurogenesis, and whether this correlates with the improvements in cognition reported in this chapter. Computational models, and experiments designed to test these theories, suggest that neurogenesis may improve memory specifically on tasks that require a high level of pattern separation (Clelland et al., 2009, Aimone et al., 2010, Creer et al., 2010, Aimone et al., 2011).

To assess the level of neurogenesis in Tg2576 mice the cell proliferation marker bromodeoxyuridine (BrdU) was used. This marker is a thymidine analogue which can be injected into rodents and is then incorporated into newly synthesised DNA during the S-phase of the cell cycle (Struikmans et al., 1997). BrdU can subsequently be detected using immunohistochemistry. As BrdU is not an endogenous protein it can only be used to label proliferating cells at a specific time-point, and the expression of this molecule can then be measured after a delay. BrdU was injected five weeks before tissue-collection. This time-point was chosen in order to assess the number of newly generated cells that survive to become incorporated into the functional DG network. The process of cell differentiation and maturation into functional neurons takes approximately 4 weeks (van Praag et al., 2002).

Cells that had differentiated into neurons, and not other cells such as glia, were distinguished by double-labelling with the neuronal marker NeuN.

Methods

Subjects and BrdU dosage: Neurogenesis was measured in the DG of 37 wild-type (18 exercise, 19 sedentary) and 38 transgenic mice (18 exercise, 20 sedentary) at 12 months of age. 5 weeks before tissue was collected mice were given intra-peritoneal injections of 50mg/kg of BrdU dissolved in saline (10mg/ml) for 5 consecutive days.

Tissue Collection: Mice were deeply anaesthetised by intra-peritoneal injection of 0.2ml of Euthatal (sodium pentobarbitone) and then exsanguinated by insertion of a cannula into the left ventricle and perfusion with 100ml of 0.1M PBS (pH 7.4). The brain was then extracted and hemi-dissected. One half was then fixed for 4 hours in 4% PFA at room temperature before being transferred to 30% sucrose solution overnight at room temperature. Brain tissue was then mounted onto a freezing microtome at -20^{o}C and cut into 40µm coronal sections. Tissue sections were stored at -20^{o}C in an ethylene-glycol based cryoprotectant until used for immunohistochemical analysis. Tissue was washed 4 x 10 minutes with 0.1M PBS (DCX) or 0.1M TBS (BrdU) to remove cryoprotectant before immunohistochemistry was carried out. For each mouse a sixth section was taken throughout the length of the DG.

Immunofluorescent labelling for BrdU and NeuN: DNA was denatured by incubating the sections for 30 minutes at 37^{o}C in 2M HCL. Tissue was rinsed 3 x 5 minutes in TBS (0.1M, pH 7.5) then quenched for 30 minutes in 3% hydrogen peroxide, 10% methanol in distilled water at room temperature. After rinsing in TBS, 3 x 5minutes, non-specific binding sites were blocked by 1 hour incubation in 0.3% TBS.T with 10% normal goat serum at room temperature. Sections were then incubated overnight in primary antibody (Rat anti-BrdU, 1:400, Abcam Ab6326, Rabbit anti-NeuN, 1:1500, Millipore ABN78) in 0.3% TBS.T with 10% normal goat serum at 4^{o}C. The next day, after washing 3 x 5 minutes in 0.3% TBS.T with 10% normal goat serum, secondary antibody (Alexa-488 goat anti-rat, 1:500 and Alexa-594 goat anti-rabbit, 1:500, both Molecular Probes A11006, A11012) was added with 10%

normal goat serum in 0.3% TBS.T and incubated for 2 hours at room temperature on a stirrer. Sections were washed 3 x 5 minutes in TBS then mounted onto gelatine-coated slides and allowed to air-dry. Slides were mounted by 5 minute immersion in xylene before being cover-slipped using Di-n-butyl phthalate in xylene (DPX) mounting medium.

Cell Counts: Sections were photographed using a Leica DMRB microscope with a 20x objective and Olympus DP70 camera. The microscope was connected to a computer, and the images were captured and stored using Leica Application Suite, Advanced Fluorescence software (v.2.6.0). For each mouse every sixth section from the brain was photographed and cell counts taken of the whole DG region on each section for BrdU+ cells. For double-labelled BrdU and NeuN sections images were overlaid and the number of BrdU+/NeuN+ cells was also counted. The total number of BrdU+, BrdU/NeuN+ cells was calculated by multiplying the number of cells counted for each mouse by six. An example of fluorescent staining for BrdU and NeuN is shown in **Figure 5.15**.

Data analysis and statistics: For double labelled BrdU and NeuN sections the percentage of new cells (BrdU labelled) that had differentiated into neurons (NeuN labelled) was calculated. For analyses of cell counts a Two-way ANOVA with group and genotype as between subjects factors was used. Tests of simple main effects were used to assess any significant interactions.

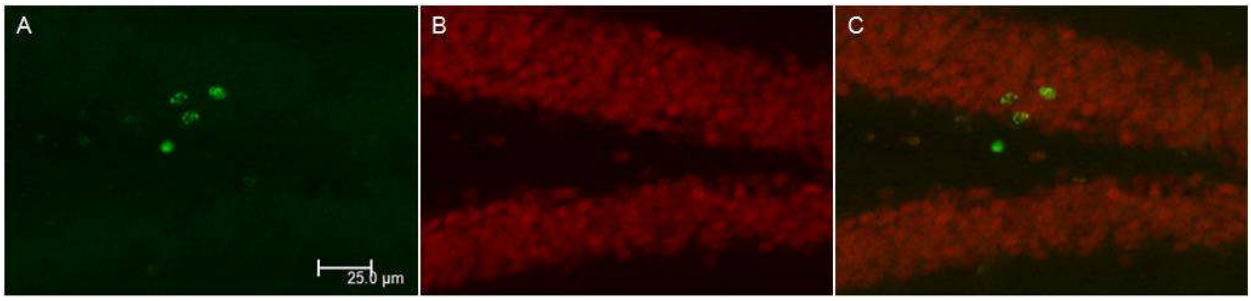

Figure 5.15: Expression of BrdU and NeuN in mouse dentate gyrus. A: BrdU expression (green). B: NeuN expression (red). C: Overlaid images showing double-labelled and single-labelled cells. All images taken using a 20x objective.

Results

BrdU expression

Example images of DG sections labelled for BrdU and NeuN expression are shown in **Figure 5.16**. The number of cells expressing the proliferation marker, BrdU, in the DG was counted and are shown in **Figure 5.17a**. Inspection of this figure shows a greater number of $BrdU^+$ cells in the DG of wild-type mice than transgenic mice, but very little effect of exercise on expression. This was confirmed by Two-way ANOVA which showed a significant difference between genotypes ($F_{(1,74)}$=8.269, p=0.005), but no effect of exercise ($F_{(1,74)}$=2.575, p=0.113) and no significant interaction between these factors ($F_{(1,74)}$=0.0231, p=0.880).

Figure 5.17b shows the number of double-labelled cells expressing both BrdU and the neuronal marker NeuN. These cells are newly proliferated cells that have differentiated into neurons, rather than glial cells. This measure gives a more accurate way of determining the level of neurogenesis than simply counting total number of $BrdU^+$ cells. The figure shows a much greater level of neurogenesis in wild-type compared to transgenic mice, and there is also a trend towards greater neurogenesis in exercising mice compared to sedentary controls. Two-way ANOVA showed a significant main effect of genotype ($F_{(1,74)}$=7.711, p=0.007), but the effect of exercise just failed to reach conventional significance levels ($F_{(1,74)}$=3.479, p=0.066) and there was no interaction between these factors ($F_{(1,74)}$=0.249, p=0.619).

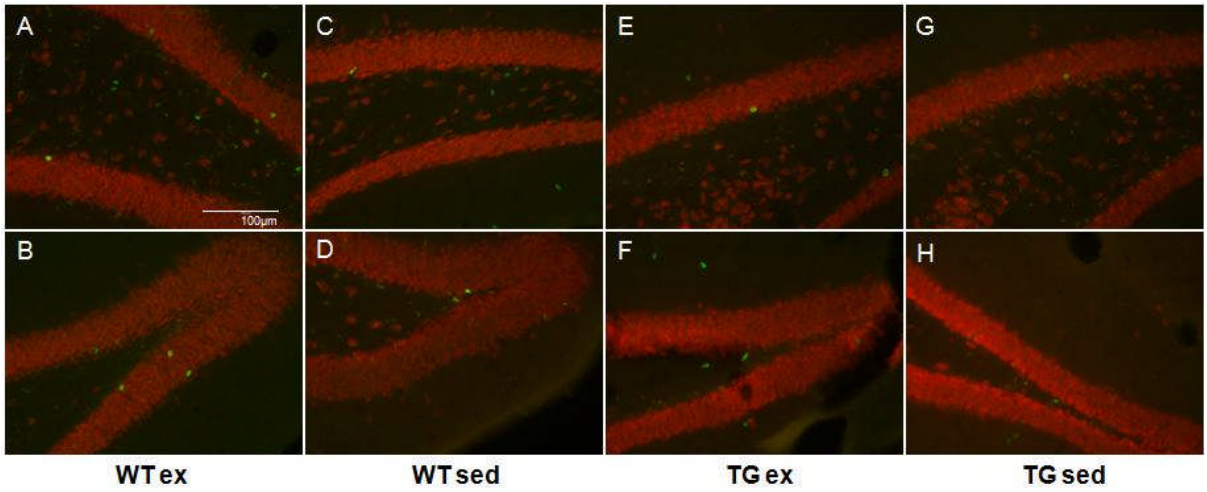

Figure 5.16: Example images of BrdU and NeuN expression in each experimental group. BrdU expressing cells are green, NeuN expressing cells are red. A/B: Wild-type exercise. C/D: Wild-type sedentary. E/F: Transgenic exercise. G/H: Transgenic sedentary.

To assess whether exercise or genotype influenced the fate of newly proliferated cells, i.e. whether they differentiate into neurons or glial cells, the percentage of $BrdU^+$ cells that also expressed NeuN was calculated. These data, shown in **Figure 5.17c**, reveal little difference between groups for this measure. Two-way ANOVA showed no significant main effect of either genotype ($F_{(1,74)}$=2.401, p=0.126) or exercise group ($F_{(1,74)}$=3.636, p=0.061), although exercise only just failed to reach conventional significance levels, and no interaction between these factors ($F_{(1,74)}$=0.125, p=0.724).

Overall these data show a reduction in overall cell proliferation, and more specifically in neurogenesis, in the DG of Tg2576 mice compared to wild-type controls, and also a non-significant trend towards exercise increasing neurogenesis.

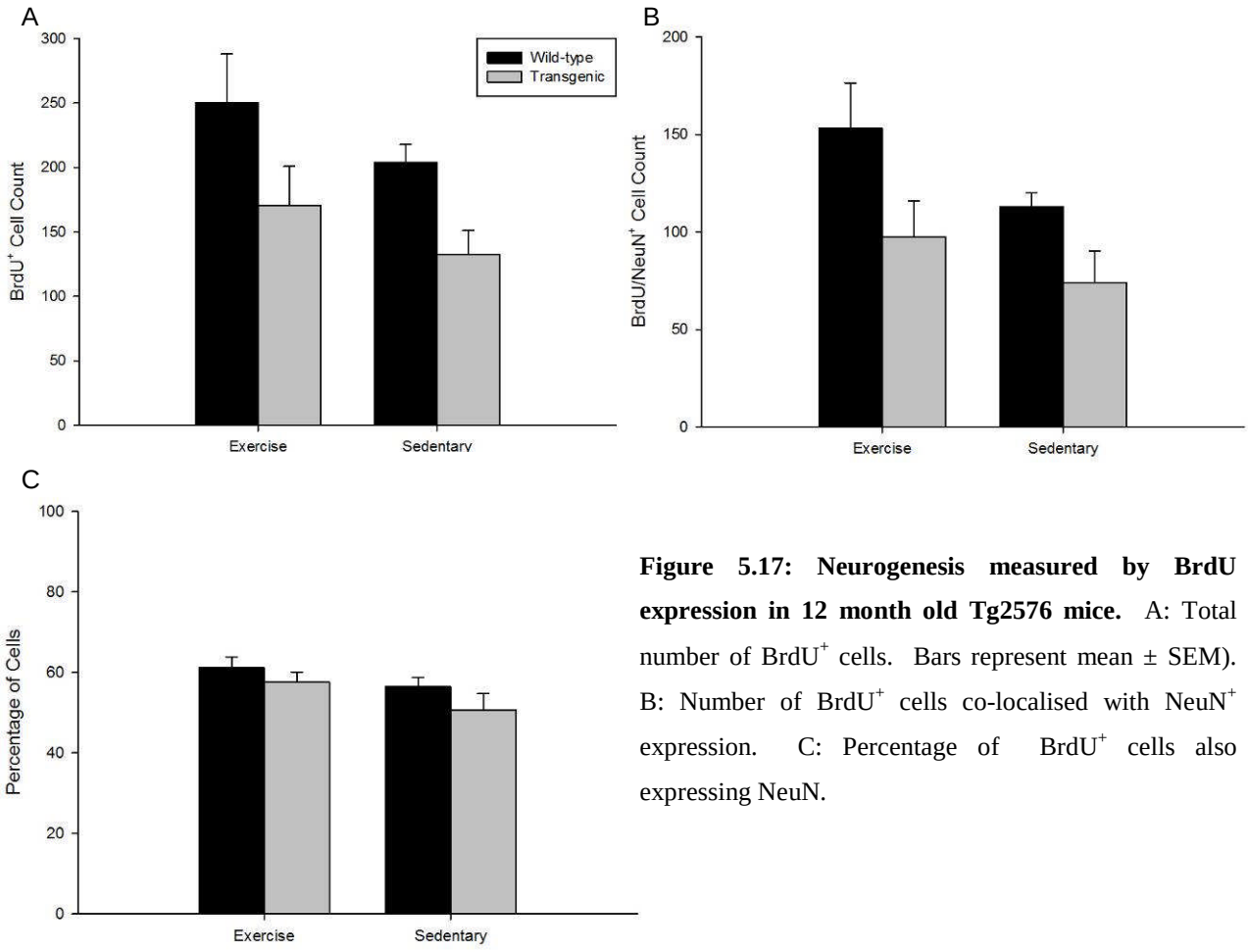

Figure 5.17: Neurogenesis measured by BrdU expression in 12 month old Tg2576 mice. A: Total number of $BrdU^+$ cells. Bars represent mean ± SEM). B: Number of $BrdU^+$ cells co-localised with $NeuN^+$ expression. C: Percentage of $BrdU^+$ cells also expressing NeuN.

Table 5.3: Correlation between neurogenesis, exercise and behavioural measures for 12 month old Tg2576 mice. Correlations are calculated using Pearson's coefficient for each group of mice; wild-type exercise, wild-type sedentary, transgenic exercise, transgenic sedentary.

Total BrdU$^+$ cells

		Exercise	Plus maze	T-maze	Object-place	Foraging	Episodic
Wild-type	r	0.058	-0.097	0.024	0.206	-0.030	-0.366
Exercise	p	0.819	0.703	0.924	0.412	0.907	0.135
Wild-type	r		-0.073	-0.063	0.004	0.179	-0.062
Sedentary	p		0.767	0.798	0.986	0.463	0.801
Transgenic	r	-0.235	0.055	0.069	0.173	-0.192	0.228
Exercise	p	0.347	0.833	0.787	0.493	0.461	0.396
Transgenic	r		0.188	0.318	-0.232	-0.119	0.302
Sedentary	p		0.455	0.171	0.370	0.617	0.239

BrdU/NeuN$^+$ cells

		Exercise	Plus maze	T-maze	Object-place	Foraging	Episodic
Wild-type	r	0.111	-0.202	-0.057	0.207	0.101	-0.312
Exercise	p	0.661	0.421	0.824	0.411	0.690	0.207
Wild-type	r		0.138	-0.101	-0.007	0.143	0.218
Sedentary	p		0.574	0.680	0.979	0.559	0.370
Transgenic	r	-0.302	0.030	0.050	0.202	-0.190	0.288
Exercise	p	0.223	0.910	0.844	0.421	0.464	0.279
Transgenic	r		0.172	0.234	-0.235	-0.154	0.131
Sedentary	p		0.495	0.321	0.363	0.517	0.616

% BrdU/NeuN$^+$ cells

		Exercise	Plus maze	T-maze	Object-place	Foraging	Episodic
Wild-type	r	0.010	-0.294	-0.247	0.056	0.452	0.250
Exercise	p	0.967	0.237	0.322	0.824	0.060	0.317
Wild-type	r		0.295	-0.033	0.063	-0.082	0.416
Sedentary	p		0.220	0.893	0.803	0.738	0.077
Transgenic	r	-0.348	-0.095	-0.037	0.171	0.014	0.219
Exercise	p	0.158	0.716	0.883	0.497	0.959	0.415
Transgenic	r		0.112	0.014	-0.236	-0.124	-0.208
Sedentary	p		0.659	0.952	0.363	0.602	0.424

Correlation between exercise and behaviour

Pearson's correlation coefficients for the relationship between measures of cell proliferation, exercise and behavioural performance are shown in **Table 5.3**. There are no significant correlations between any of these measures.

Discussion

Experiment 7 reports reduced neurogenesis in 12 month old Tg2576 transgenic mice compared to wild-type controls. In addition, there was a non-significant trend for increased neurogenesis with exercise in both wild-type and transgenic mice.

The genotypic difference seen here for both measures of total cell proliferation and neuron proliferation matches that shown by Dong et. al. (2004) in the Tg2576 model. Their study showed reductions in neurogenesis from as early as 3 months of age in transgenic mice compared to wild-type mice, and also a progressive decline in neurogenesis in both genotypes with age up to 9 months. Here we have shown that by 12 months neurogenesis is further reduced in both genotypes. A second paper assessing neurogenesis in this model showed that by 18 months of age no markers of neurogenesis are expressed in the DG of Tg2576 mice (Ihunwo and Schliebs, 2010). Together these data show a robust decline in neurogenesis with aging for both wild-type and transgenic mice, and a deficit in neurogenesis throughout life in transgenic mice compared to wild-types.

The trend for exercise increasing neurogenesis is mirrored by a trend for exercise to increase the percentage of newly generated cells adopting a neuronal cell type. The finding that exercise increased the percentage of cells adopting a neuronal cell type has previously been reported (van Praag et al., 1999b, Kobilo et al., 2011), although both studies showed a much higher percentage (~80%) of BrdU labelled cells adopting a neuronal phenotype. However, it is not clear from the data presented here whether exercise is increasing neurogenesis overall, increasing the survival of newly proliferated cells, or acting in both of these ways to increase the number of BrdU labelled cells. It would be possible to establish which of these mechanisms is occurring by either having a second cohort of mice from which tissue was collected 1 day after BrdU injection, or by also staining for doublecortin (DCX) expression. DCX is an endogenously expressed protein which can be used as a marker of immature neurons (Brown et al., 2003, Kronenberg et al., 2003). It is possible to compare the number

of new neurons expressing DCX, with those surviving for five weeks labelled with BrdU to assess what proportion of newly generated cells are surviving to maturity. This type of analysis has been carried out in previous studies, which have suggested that exercise increases both the level of proliferation and the survival of newly generated neurons (van Praag et al., 1999b).

There are several theories and experimental studies supporting the hypothesis that increased neurogenesis in the DG improves learning and memory (van Praag et al., 1999a, van Praag et al., 2005, Aimone et al., 2010). Newly generated cells are hyper-excitable compared to mature neurons (Schmidt-Hieber et al., 2004, Esposito et al., 2005, Ge et al., 2007) and are therefore preferentially activated in hippocampal networks (Ramirez-Amaya et al., 2006, Kee et al., 2007) and help to increase synaptic plasticity in the DG by lowering the threshold for LTP. The distinct properties of these cells have lead to the theory that neurogenesis is particularly important for supporting the pattern separation process function of the DG (Clelland et al., 2009, Aimone et al., 2011, Schmidt et al., 2012). These theories suggest that the reduction in neurogenesis seen in transgenic mice, compared to wild-types, may underlie some of the deficits seen in these mice on behavioural tasks. However, we found little correlation between neurogenesis and task performance for either wild-type or transgenic mice, and whilst memory performance was significantly improved by exercise, neurogenesis was not significantly altered (although one must acknowledge that there were trends in the appropriate direction). This suggests that in the Tg2576 model deficits in neurogenesis are not the primary cause of cognitive deficits, and that exercise acts by other molecular mechanisms to improve memory in transgenic mice. Other factors which may be influenced by exercise are discussed further in the next section.

5.10 Chapter Discussion

The experiments reported in this chapter tested the hypothesis that physical exercise would delay the onset, and/or reduce the magnitude of behavioural deficits in Tg2576 mice. Tg2576 mice and their wild-type litter mates were exposed to voluntary wheel-running for 11 months, from 2-12 months of age, alongside sedentary controls that were given access to a fixed wheel.

Although exercise did not significantly alter performance on any behavioural measure in wild-type mice, there were improvements in spatial memory, working memory and episodic-like memory in exercising transgenic mice compared to controls. It is interesting that although exercise did have beneficial effects on cognition in transgenic mice, there was no change in wild-type performance. Unlike the data presented here, several previous studies in rats and C57Bl6 mice have shown exercise-induced improvements in cognition (Rosenzweig and Bennett, 1996, van Praag et al., 1999a, van Praag et al., 2005). However, this may be due to differences in the background strain, as strain differences can lead to differences in learning and memory performance and alterations in cellular processes, such as LTP induction (Gerlai, 1996, Lathe, 1996, Crawley et al., 1997). This finding that exercise did not improve cognition in wild-type mice also parallels the findings of another study in 17 month old Tg2576 mice, that showed improved cognition in transgenic, but not wild-type, mice with short-term exercise (Parachikova et al., 2008). Neither of the other two exercise studies using the Tg2576 model incorporated a wild-type control condition for comparison (Nichol et al., 2007, Yuede et al., 2009). In terms of other APP models, many studies do not use wild-type control mice, and those that do show conflicting results with regards to cognition for wild-type mice. For example, in APP/PS1 Ke et. al. (2011) showed no difference on water maze, passive avoidance and exploratory activity tests, whilst Liu et.al. (2011) showed improved water-maze performance with exercise in both wild-type and transgenic mice.

Although the results from the present study demonstrated improvements in working memory with exercise at 10 months of age in Tg2576 mice in the foraging task, there was no improvement in spatial working memory at 7 months of age in the T-maze paradigm, and also no change in levels of anxiety as a result of exercise at 6 months of age. These data show some discrepancy with the current literature, which suggests that exercise does improve performance on both working memory and anxiety tasks in APP transgenic mice (Nichol et al., 2007, Garcia-Mesa et al., 2011, Ke et al., 2011). However, the difference in task choice and age at testing may explain the difference in results, and one study in APP/PS1 mice suggested that exercise may have a greater influence on cognition in older mice (Ke et al., 2011).

Having found improvements in cognition as a result of long-term voluntary exercise, brain tissue was collected from the mice to determine what molecular changes are influenced by exercise. No difference was found between exercising and sedentary mice in terms of amyloid levels, in line with several other studies (Wolf et al., 2006, Parachikova et al., 2008,

Yuede et al., 2009, Ke et al., 2011). This suggested that exercise may not be directly altering disease pathologenesis in Tg2576 mice. The second factor that was examined was the influence of exercise on neurogenesis. Although Tg2576 mice displayed reduced neurogenesis compared to wild-type controls, this deficit was not altered significantly by exercise despite improvements in cognition with exercise. This is an interesting finding as neurogenesis has repeatedly been shown to correlate with memory performance on tasks designed to test pattern separation (Clelland et al., 2009, Creer et al., 2010, Bekinschtein et al., 2011). There was also no correlation between learning and neurogenesis in either transgenic or wild-type mice. It could be suggested that the behavioural paradigms chosen for this study were not designed specifically to test pattern separation. However, it is argued that the configural memory task relies heavily on pattern separation processes to distinguish between similar patterns of the same set of context, temporal and auditory cues, and this task depends critically on the DG (section 4.6).

The experiments conducted as part of this chapter have not provided a plausible molecular mechanism to explain the changes seen in cognition in Tg2576 mice. There are, however, several other putative mechanisms that have not been examined in this thesis. Other studies have shown changes in spine density and synaptic proteins as a result of exercise (Molteni et al., 2002, Farmer et al., 2004, Eadie et al., 2005, Vaynman et al., 2006, Stranahan et al., 2007), paralleled by changes in LTP (van Praag et al., 1999a, Farmer et al., 2004). As Tg2576 mice have a reduction in spine density (Jacobsen et al., 2006), LTP (Chapman et al., 1999) and abnormal levels of synaptic proteins compared to wild-type controls (Almeida et al., 2005) the influence of exercise on any of these factors may help explain the improvement seen in cognition.

Tg2576 mice also show amyloid pathology surrounding brain vasculature (Kumar-Singh et al., 2005), which leads to perturbed vascular transport in the brain. Exercise improves blood flow and oxygen capacity, and also induces angiogenesis (Swain et al., 2003, Ding et al., 2004, van Praag et al., 2005, Cotman et al., 2007, Pereira et al., 2007). It is possible that the improved blood supply to the brain, and in particular the hippocampus, may help to alleviate cognitive deficits in the Tg2576 model.

A recent theory of molecular changes underlying exercise induced cognitive benefits is that physical exercise increases the level of tropic factors in the brain and in particular of brain-derived neurotrophic factor (BDNF), which may be the key factor leading to all of the

molecular changes listed above (Cotman et al., 2007, Bekinschtein et al., 2011). A recent paper by Bekinschtein et. al. discussed the possible link between BDNF and memory, and also how neurogenesis may be a mediating factor. However, the data presented here, showing improved memory without changes in neurogenesis suggests that whilst BDNF may alter both memory and neurogenesis, neurogenesis is not the mediating factor between increases in BDNF expression and increases in learning and memory.

In conclusion, the data reported in this chapter have demonstrated that physical activity is an effective lifestyle strategy to delay the onset and progression of cognitive decline brought about by excess amyloid production. However, how exercise is exerting its effect on cognition is still undetermined. Therefore further work is needed to target and identify the molecular changes in the brain in response to exercise to provide potential new drug targets for AD. Nevertheless, for those people with genetic risk factors for AD, promoting an active lifestyle may help delay or prevent disease-onset.

Chapter 6: General Discussion

Despite over 100 years of research into AD there is currently no cure, only symptomatic treatments. Although much is now known about the pathology of AD, the precise mechanisms leading to cognitive decline are still unclear. A further set-back in the development of successful therapeutics is the poor translation of effective treatments in animal models of the disease into human patients. This may be due to the inadequate choice of behavioural paradigms used to test the effects of treatments on cognition in mice, which do not relate well to human memory systems. Another growing concern is that, with current diagnostic criteria treatment may already be too late once the disease has been diagnosed, as inevitably this is already accompanied by marked neurodegeneration. The main aim of this thesis was to characterise the early stages of neuronal network dysfunction in transgenic mice that express a human early-onset AD mutation.

The data in chapter 3 of this thesis demonstrated differences in hippocampal sub-region engagement when Tg2576 mice explored an environment surrounded by either novel or familiar extra-maze cues. The examination of c-fos expression data in C57Bl6 mice showed engagement of the DG, CA3 and CA1 sub-regions via the tri-synaptic pathway when mice explored a novel room, compared to only CA1 engagement directly via the perforant path from the entorhinal cortex when exposed to a familiar environment. These data are consistent with data from other studies (Poirier et al., 2008, Albasser et al., 2010) and with computational models of hippocampal function (Marr, 1971, Treves and Rolls, 1994, McClelland et al., 1995, Lisman and Otmakhova, 2001, Rolls and Kesner, 2006). In 3 month old wild-type mice identical models of hippocampal engagement were observed. In transgenic mice at this age, the same entorhinal-CA1 model as wild-type mice was evident for mice exploring a familiar environment. In contrast, when mice explored a novel environment, unlike wild-type mice, the DG region was not engaged in Tg2576 mice, despite no overt difference in behaviour between genotypes. When 12 month old mice were run on the same task the results were unfortunately less clear. There was no significant change in behaviour in response to novelty and a poor fit to models of hippocampal engagement. However, it was tentatively concluded that by 12 months of age the DG was not engaged when encoding a novel spatial environment in either transgenic or wild-type mice, and that

the CA1-subiculum pathway was also impaired in transgenic mice when exploring a familiar environment. These data suggest both age-dependent and amyloid-dependent disruption of DG function. The main difference being the age of onset of these deficits is seen at a much earlier time-point in transgenic mice.

Our finding of early changes in DG function may be useful in the context of clinical diagnosis of AD, as we would predict, from models of hippocampus function, that one of the earliest cognitive deficits in AD may be a reduction in pattern separation (Marr, 1971, McNaughton et al., 1991, Treves and Rolls, 1994, McClelland et al., 1995, Rolls and Kesner, 2006). Developments in the spatial resolution of fMRI now allow analysis of brain activity localised to specific hippocampal sub-regions, which could help to distinguish people with altered DG activity that may be in the earliest stages of AD. Although normal aging also alters DG function and pattern separation (Holden and Gilbert, 2012) it would only be evident at a later age in cognitively normal individuals.

Chapter 4 of this thesis assessed configural memory for episodic information in Tg2576 mice using a novel behavioural paradigm exploiting memory for what, where and when a cue was presented. Episodic memory deficits are well documented in AD patients, and are one of the earliest signs of cognitive impairment, often seen before clinical diagnosis (Bondi et al., 1999). Episodic memory is also impaired in patients at risk for AD, and those with MCI (Saunders et al., 1993, Hodges et al., 2006). In this thesis age-dependent deficits in episodic-like memory are reported in Tg2576 mice. 3 month old transgenic mice performed as well as wild-types, whereas 11 month old transgenic mice, in which amyloid pathology is present, show a deficit in associating cues related to episodic-like memory, which may be due to deficits in associating contextual cues with cues from other modalities. The task presented in this thesis arguably assesses some aspects of human episodic memory, in terms of the spontaneous encoding of configural memory for an event (Rudy and Sutherland, 1995, Aggleton and Brown, 1999). Therefore this task may provide an effective tool to test therapeutic interventions for AD. However, further validation of this task is still required, for example assessing the efficacy of current AD drugs, such as memantine and cholinesterase inhibitors, in improving episodic-like memory using this paradigm.

At a conceptual level, episodic memory is thought to rely on pattern separation processes to separate the distinct elements of each episode and remove interference between episodes due

to overlapping contextual or temporal cues (Gilbert et al., 2001, Rolls, 2010, Holden and Gilbert, 2012, Schmidt et al., 2012). The findings of the c-fos expression experiment in chapter 4 support this conclusion, demonstrating that the DG region of the hippocampus is critical in the configural association of episodic information. As DG dysfunction occurs early in the pathogenesis of AD, this finding may explain why episodic memory is one of the earliest cognitive deficits reported in AD. It has also been proposed that if, as suggested here, episodic memory relies heavily on pattern separation, then behavioural interventions which structure daily living to reduce overlap and interference between episodic cues could potentially improve episodic and spatial memory in patients with early symptoms of AD or MCI (Holden and Gilbert, 2012).

A recent review by Gallagher & Koh (2011) examined the literature supporting the conclusions drawn here, that dysfunction in the neural circuits essential for episodic memory can be detected early on in the disease progression and may act as a new biomarker for AD. Evidence described in this paper suggests that one of the key differences between normal aging and AD is a loss of neurons in the entorhinal cortex, particularly layer II which projects to the DG and CA3 regions of the hippocampus. This loss is detectable even in the earliest stages of clinical AD using structural MRI (Gomez-Isla et al., 1996). Diffusion tensor imaging (DTI) has also shown degradation of the perforant path input from the entorhinal cortex into the hippocampus in AD patients, and to a lesser extent in normal aging (Yassa et al., 2010a). Degradation of the perforant path input to the DG has also been seen in APP transgenic models of AD, including the Tg2576 model (Reilly et al., 2003, Dong et al., 2007). The important role of entorhinal cortex pathology in AD was highlighted in a study by Harris et. al. (Harris et al., 2010), which used a transgenic mouse model which only expressed mutated APP in the entorhinal cortex. This localised expression led to downstream changes in plasticity in the DG, and spread of Aβ throughout the hippocampus.

The input from layer II neurons into the DG via the perforant path is critical for pattern separation. Studies of pattern separation in human subjects using fMRI have consistently implicated the hippocampus, and more particularly the DG/CA3 region, in this type of information processing (Kirwan and Stark 2007, Bakker et al. 2008). Furthermore, several studies have shown decreased pattern separation with aging in both rodents and humans (Toner et al., 2009, Creer et al., 2010), which was linked to altered DG/CA3 activity shown

by fMRI (Yassa et al., 2011). From a computational perspective, the reduction in DG activity driving the CA3 network leads to greater activity of the CA3 recurrent collaterals, shifting the function from pattern separation to pattern completion. This may explain the increase in BOLD signal detected in the DG/CA3 region. This effect is even more prominent in MCI patients compared to age matched controls (Yassa et al., 2010b). These data demonstrate that fMRI can complement structural MRI and DTI in linking structural changes to functional deficits in DG/CA3 information processing.

Although the experiments presented in this thesis did not show altered activity in the entorhinal cortex in Tg2576 mice, they did show altered functional connectivity of the DG, and impaired episodic memory on a task which requires DG engagement. Tg2576 mice do not show cell loss (Hsiao et al., 1996), therefore, unlike humans, the entorhinal cortex does not show atrophy. However, there is a reduction in the synaptic density in the entorhinal cortex layers II and II by 15 months of age (Dong et al., 2007). Despite no change in entorhinal cortex cell number, the perforant path connection to the hippocampus is degraded, as shown by decreased synaptic transmission and plasticity in this pathway, and a reduction in synaptic density by 6-9 months of age (Chapman et al., 1999, Jacobsen et al., 2006, Dong et al., 2007). This may lead to the reduction in DG engagement that is described in this thesis. From the data outlined above, it was also predicted that increased activity would be seen in the CA3 region as a result of reduced mossy fibre input from the DG, as seen in human fMRI studies. However, this was not detected in Tg2576 mice using conventional IEG analsyis.

These findings predict that DG alterations occur before the onset of measurable cognitive deficits, and even before a diagnosis of MCI. A further study of interest would be to assess DG activity and pattern separation in people at risk of AD, such as ApoE-ε4 carriers, or to carry out a prospective study in which a random sample of the population are tested and then the development of AD at a later age is assessed. Recent studies in ApoE- ε4 carriers have demonstrated deficits in episodic memory compared to non-carriers and volume loss in the DG/CA3 region (Nilsson et al., 2006, Mueller et al., 2008). However, more specific tests for deficits in pattern separation and functional activity have not been carried out. It is possible that cognitive tests of pattern separation and functional imaging, specifically of the DG, during these tasks may aid earlier diagnosis of AD, and therefore increase the window of opportunity for therapies targeted at pre-clinical stages of the disease.

Chapter 5 of this thesis reported experiments that examined the effects of long-term voluntary exercise on reducing cognitive decline and pathology in the Tg2576 model. Various measures of cognition were improved in exercising transgenic mice; both spatial working and reference memory, and, more importantly, episodic-like memory. These findings suggest that exercise can slow the progression of cognitive decline. We also assessed the effects of exercise on amyloid pathology and DG neurogenesis, but did not find any significant changes. Therefore, it is not possible to make any firm conclusions as to how exercise is mediating its effects on behaviour at the molecular level. The beneficial effects of long-term voluntary exercise are of particular interest as this is a life-style intervention can be carried out before the onset of clinical symptoms. Therefore, unlike drug-based therapies, a clinical diagnosis is not required, and this may be of particular use to those people at risk of AD, as a simple preventative strategy from an early age.

Although we have not been able to demonstrate a molecular mechanism underlying the effects of exercise, previous work suggests that exercise has both global effects in the brain on growth factors and angiogenesis (Cotman et al., 2007), and also specific effects on DG physiology and function (Eadie et al., 2005, Redila and Christie, 2006). In particular, exercise improved pattern separation in mice (Creer et al., 2010), which may explain the improved performance we have seen in configural memory. Establishing the precise mechanisms by which exercise exerts its influence on DG function may provide novel drug targets for AD, which could be used for older individuals for whom physical activity is more difficult. However, these targets are likely to still need earlier diagnosis of the disease, i.e. before the onset of cell loss.

Overall the work presented in this thesis may contribute to earlier clinical diagnosis of AD based on more specific tests of pattern separation related to episodic memory and assessment of DG function. It has also contributed to the growing literature showing that exercise is one of the most beneficial lifestyle strategies for reducing cognitive decline in AD, which may in future provide new drug targets for the disease. Finally, the configural memory paradigm presented in this thesis may help to provide a robust behavioural paradigm with which to further interrogate the nature of cognitive deficits in mouse models of AD, and assess the efficacy of putative therapeutics.

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