Characterising transgenic APP mutation mouse models of amyloid pathology for use in preclinical immunotherapy

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Contents

No disease-modifying compounds are available to halt disease progression in Alzheimer's disease (AD). Immunotherapy offers promising possibilities for the manipulation of Aβ levels which the amyloid cascade hypothesis proposes as the causative factor in AD. However, anti-Aβ antibodies have caused inflammation *in vivo.* An alternative antibody (2B3) which targets the β-secretase cleavage site of the amyloid precursor protein (APP) from which Aβ is cleaved has been shown to downregulate Aβ in human cell lines. The approach is thought unlikely to cause inflammation as the immune system is not relied upon for Aβ clearance. It was hypothesised that the administration of 2B3 to aged transgenic APP mutation mice would lower Aβ levels through the inhibition of Aβ production, with an associated lowering of cognitive deficits. Two murine APP mutation models [London APP(V717I) and Indiana PDAPP(V717F)] were characterised in order to identify cognitive deficits against which the ability of 2B3 to reduce deficits could be assessed. APP(V717I) mice were assessed in the marble burying task, the elevated plus maze and a foraging task assessing spatial working memory at 3, 6 and 19 months of age. The radial arm water maze was carried out at 10 months, before the T-maze non-matching to position task was administered at 11 months. Object recognition memory was assessed at 18 months. Similarly, PDAPP mice were assessed using the marble burying and elevated plus maze (9.5months), the T-maze (9-10 months), the foraging task (11 and 14 months) and the object recognition task (12 months). Whilst aged transgenic PDAPP mice displayed disrupted spatial working memory, no evidence of age-related cognitive decline was observed in APP(V717I) transgenic mice despite increases in Aβ pathology with age. 2B3 did not alter Aβ levels or spatial working memory in PDAPP(V717F) mice in a pilot study, whilst the *in vitro* downregulation of Aβ was successfully replicated in primary murine neurons. The findings indicate that transgenic models of neurodegenerative disease require thorough characterisation to optimise their use in pre-clinical research. Furthermore, the use of alternative immunotherapy in the treatment of AD remains a promising, but early stage avenue of study.

ADAM	a disintegrin and metalloprotease domain
AD	Alzheimer's disease
AICD	amyloid precursor protein intracellular domain
APP	amyloid precursor protein
Aβ	β-amyloid protein
BACE1	β-amyloid cleavage enzyme 1
BBB	blood brain barrier
BCA	bicinchoninic acid
CAA	cerebral Aβ angiopathy
CLU	clusterin
CNS	central nervous system
DAB	3,3'-Diaminobenzidine
dH_2O	distilled water
dNTPs	deoxynucleotide triphosphates
DMEM	Dulbeccos's modified eagle medium
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders edition four
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EPM	elevated plus maze
FA	formaldehyde
FAD	familial Alzheimer's disease
FCA	forced choice alternation
FGF	fibroblast growth factor
FTDP-17	familial frontotemporal dementia and Parkinsonism linked to chromosome 17
GFAP	glial fibrillary acidic protein
hAPP	human amyloid precursor protein
HBSS	Hank's buffered salt solution
HPC	hippocampus

HRP	horseradish peroxidase
IBO	ibotenic acid [(*S*)-2-amino-2-(3-hydroxyisoxazol-5-yl) acetic acid]
IDE	insulin degrading enzyme
IgG	immunoglobulin G
IN	intranasal
IP	intraperitoneal
LRP	low-density lipoprotein receptor-related protein
LTP	long term potentiation
Min	minute(s)
NEP	neprilysin
NFTs	neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders working group
NMDA	*N* -Methyl-D-aspartate
Nrg-1	neuregulin-1
NSE	neuronal specific enolase
OPD	o-phenylenediamine
PICALM	phosphatidylinositol binding clathrin assembly protein
PPF	paired-pulse facilitation
PSEN1	presenilin 1
PSEN2	presenilin 2
KO	knock-out
RAGE	receptor for advanced glycation end products
RAWM	radial arm water maze
SAD	sporadic Alzheimer's disease
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
TACE	enzyme which cleaves members of the TNF receptor family
TBS	tris buffered saline

Introduction

1.1 Overview of the general introduction

Currently available treatments for Alzheimer's disease (AD) are symptomatic only, and are not able to halt neurodegeneration or the decline in cognitive function over time. Treatments which target the driving factors in disease progression are greatly needed. Transgenic mouse models of AD-like pathology are valuable tools for investigating the potential disease-modifying characteristics of novel compounds. This introduction will summarise the neurobiological pathology and cognitive decline characteristic of AD, with a particular focus on Aβ, a 40-42 amino acid sized protein, and its role in the aetiology of AD. It is argued that Aβ is causal in AD progression, and is central in synaptic disruption and cognitive decline observed in patients and model systems. A range of other risk factors will be discussed, before presenting a short overview of transgenic mouse models of AD-like pathology. Following a presentation of the current treatments available for AD patients, a summary of the range of disease-modifying approaches currently under investigation is put forward. Whilst a range of steps in the Aβ cascade of pathology have been successfully targeted to lower Aβ levels in transgenic mouse models of AD, several of these approaches have not translated successfully to clinical trials or could have a number of side effects due to a lack of specificity in its targets. The concluding sections of the introduction will propose that an anti-APP β-secretase cleavage site antibody (2B3) which is able to reduce Aβ *in vitro* could provide a disease-modifying treatment with less of the side effects observed in pre-clinical studies and human trials. The introduction will hypothesise that the administration of this antibody will lower Aβ levels in transgenic APP mutation mouse models of AD-like pathology, and that such a reduction will reduce associated age-related cognitive deficits.

1.2 AD prevalence and cost

AD is a progressive neurodegenerative disease estimated to affect more than 493 000 people in the UK (Dementia UK report, 2010). It is the most common type of dementia, a class of disorders which reduce normal functioning of cognition and behaviour (Wimo & Prince, 2010). As a high number of patients are cared for by family members or friends in the community, the disease has far-reaching effects beyond that of the sufferers themselves. Carers report

high levels of strain, depression and distress (Marriot et al., 2000) and it is likely that the effect on carers of attending to an individual with AD in their home is highly underestimated (World Alzheimer Report, 2010). In addition to the disease being an emotional, cognitive and physical burden, it is estimated that the total annual cost of dementia in the UK is currently £23 billion (Dementia UK report, 2010). The problem is a growing one, as the number of people suffering from dementia is expected to rise by 154% over the next 45 years mirroring an expected increase in the proportion of aged members of the UK population (Dementia UK report, 2007). In line with this increase in patients, worldwide dementia costs are set to increase by 85% of current spending by 2030 (Wimo & Prince, 2010). With such a large scale health problem growing every year, there is a clear need for further understanding of the aetiology of the disease, the mechanisms of disease progression and potential treatments.

1.3 Cognitive and behavioural impairments

The earliest clinical manifestation of AD is subtle memory loss (Braak & Braak, 1995), affecting particularly semantic and episodic memory in 86-94% of patients (Dubois et al., 2007). Episodic memory impairment is also central feature of the core diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (American Psychiatric Association, DSM-IV-TR, 2000). This is followed by a progressive worsening of cognitive ability, which often targets specific functions such as language and perception (McKhann et al., 1984). Working memory and attentional control has been found to decline with disease progression (Becker, 1988; Baddley et al., 1991, Bellville, Chertkow & Gauthier, 2007), with short term memory deficits apparent with short and long delays (Grady et al., 2001). Impairments in recognition memory are apparent in a variety of domains including verbal, visual (facial, colours, patterns) and spatial stimuli (Moss, Albert, Butters & Payne, 1986). Non-cognitive changes are frequently observed in AD patients alongside cognitive decline. Firstly, behavioural changes including altered personality (Braak & Braak, 1995), outbursts of a verbal, emotional or physical nature and sexually inappropriate behaviour are common to varying degrees (McKhann et al., 1984). Secondly, neuropsychiatric symptoms and changes in emotional states are observed, such as depression, hallucinations and illusions (McKhann et al., 1984). Reports of 48% of AD patients suffering from increased anxiety, as well as frequent occurrences of emotional symptoms such as agitation, dysphoria and irritability highlight the wide range of behavioural and psychological problems in AD patients (Mega, Cummings, Fiorallo & Gornbein, 1996). Changes in sleep patterns are also frequent, with fragmented sleep during the night and increased tendency to sleep in daylight hours (Huitron-Resendiz et al.,

2002), with some patients suffering from insomnia (McKhann et al., 1984). These symptoms are accompanied by a reduced ability to carry out activities of daily living (McKhann et al., 1984). There have also been reports of increased seizures in AD patients (Amatinek et al., 2006).

1.4 Diagnosis of AD

Diagnosis of AD is based on the DSM-IV-TR and the National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders working group (NINCDS-ADRDA; McKhann et al., 1984; Dubois et al., 2007). Whilst confirmation of a diagnosis of AD can only be made following post-mortem histological identification of both Aβ plaques and NFTs, a range of methods are employed to diagnose patients with AD *in vivo* with up to 90% accuracy in specialised clinics (Brodaty et al., 2011). The diagnostic process relies on a number of tools, including neuropsychological tests such as the Cambridge Neuropsychological Test Automated Battery (CANTAB; Fray & Robbins, 1996) in combination with dementia assessments such as the Mini-Mental Test and the Blessed Dementia Scale (McKhann et al., 1984) to assess a range of cognitive functions. Central to this is the identification of an episodic memory impairment which has been present over the last 6 months, and which is of a progressive nature (American Psychiatric Association, DSM-IV-TR, 2000). This is normally demonstrated as a recall deficit following effective encoding which persists despite being cued (American Psychiatric Association, DSM-IV-TR, 2000). Other behavioural disturbances, such as depressive features, irritability, apathy and aggression can be measured using tools such as the Behavioural Rating Scale for Dementia, but these are not considered a part of the diagnostic framework and are not specific to Alzheimer's disease when compared to other dementias (Blazina et al., 1995). Medial temporal lobe atrophy and AD-specific patterns of activation are assessed using MRI and compared to controls. Finally, Aβ load measured using PET and alterations in CSF biomarkers, specifically low concentrations of Aβ42 with high phosphorylated or total tau, are used as AD-markers (Dubois et al., 2007; Brodaty et al., 2011). The emergence of biomarkers and use of imaging techniques in AD diagnosis have assisted an increase in diagnostic accuracy, which when using only the DSM-IV-TR and NINCDS-ADRDA guidelines to distinguish AD from other dementia cases was at an accuracy level of 23-88% (Dubois et al., 2007).

1.5 Pathology

AD results in widespread neuronal loss and subsequent reduction in size of the temporal and frontal lobes in particular (Mattson, 2004). This is linked to extensive cognitive decline in patients, often couples with changes in personality (Mattson, 2004). The core pathology of AD, as discussed below, is characterised at a cellular level by extracellular plaques and intracellular tangles, which are aggregates of the proteins Aβ and tau respectively (Hardy & Higgins, 1992).

1.5.1 Tau pathology

As the focus of the experimental work in this thesis is on altered APP processing and downstream changes in Aβ and cognition, an extensive overview of the tau pathology literature will not be presented. Nevertheless, a short overview of the contributions of tau pathology to AD is given.

The tau protein is a microtubule-associated molecule which is found in six different isoforms, ranging in size from 352 – 441 amino acids (Tolnay & Probst, 1999). The tau protein plays a central role in axonal transport through involvement in the assembly and stabilisation of microtubules (Tolnay & Probst, 1999). Once phosphorylated, tau is prevented from binding to microtubules, thus inhibiting microtubule functioning and normal axonal transport (Brion et al., 1991). As a pathological hallmark of Alzheimer's disease, intraneuronal neurofibrillary tangles (NFTs) composed of predominantly paired (but also straight) helical filaments of hyperphosphorylated and abnormally phosphorylated tau are numerous in AD brain tissue (Tolnay & Probst, 1999). Braak & Braak (1991) characterised the predictable way in which NFT formation spreads through brain areas, dividing the progression into six neuropathological stages. Pathology is initiated in the entorhinal cortex, progressing to the hippocampus with the emergence of mild cognitive impairment, and involves the neocortical association areas and severe dementia in the final stages (Braak & Braak, 1991).

The aggregation of phosphorylated tau into NFTs has been shown to be linked to cognitive decline and neurodegeneration independently of Aβ pathology. In tauopathy disorders such as familial frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), dementia is present in the absence of Aβ accumulation (Tolnay & Probst, 1999). This disorder is linked to mutations on the gene encoding tau, suggesting a central role for tau protein abnormalities in dementia (Tolnay & Probst, 1999). In support of this, the number of

NFTs in AD patients is correlated with cognitive impairment, as well as with neuronal loss (Tolnay & Probst, 1999; Gomez-Isla et al., 1997; Giannakopoulos et al., 2003).

1.5.2 Aβ pathology

Aβ is a 39-43 amino acid sized protein cleaved from amyloid precursor protein (APP), and is partly made up of the hydrophobic transmembrane domain of the APP COOH-terminal (Hardy & Higgins, 1992). Aβ protein monomers aggregate into oligomers, composing dimers, trimers and tetramers, before further aggregating into Aβ insoluble fibrils (Ward et al., 2000). These aggregates are evident in AD tissue as Aβ plaques, one of the classical pathological hallmarks which forms part of post-mortem diagnosis (McKhann et al., 1984). Aβ plaques are mainly made up of Aβ42, and are deposited in the extracellular space (Selkoe, 2001). Whilst the pattern by which Aβ pathology progresses through the brain is not as predictable as that of NFTs, there is nevertheless a general coherence in the development of pathology between patients. As characterised by Thal et al. (2002), Aβ pathology progresses through five stages, initiating in the neocortex. Phase 2 sees the CA1 area of the hippocampus, the entorhinal cortex, the amygdala and the occipital lobe develop Aβ plaques, progressing to subcortical regions such as the thalamus, striatum, hypothalamus, caudate nucleus and white matter by phase 3. Phase 4 and 5 are similar in that previously observed areas of pathology are more heavily affected, with the addition of regions such as substantia nigra and areas of the pons.

Whilst Aβ is a molecule which accumulates during normal aging, individuals with AD show elevated levels either due to overproduction, a failure of clearance, or a combination of the two (Klein, Krafft & Finch, 2001; Seubert et al., 1992; Shoji et al., 1992), with extensive data supporting the notion that Aβ plays a central role in the pathogenesis of AD (e.g. Selkoe, 2001; see Chapter 1, Section 1.5.2). The dominance of Aβ42 over Aβ40 in plaques and the observation that it aggregates more rapidly *in vitro* than Aβ40 (Jarrett & Lansbury, 1993) has led to the idea that Aβ42 may play the more central role in amyloid pathology, although both species have been shown to be neurotoxic *in vitro* (King et al., 2003; Mucke et al., 2000).

1.5.3 The interaction of tau and Aβ

In addition to the independent contributions of tau and Aβ aggregation to neurodegeneration, there is growing evidence of interactions between Aβ and NFT pathology. Whilst this interplay is not yet defined, NFT pathology in AD is increasingly seen as one of a number of cellular

responses to the increased levels of Aβ (Selkoe, 2001), an idea in line with the amyloid cascade hypothesis of AD (see Chapter 1, Section 1.5.2). In support of this, Aβ and tau colocalise in neurons with tangle pathology (Guo et al., 2006) and the exposure of hippocampal neurons to Aβ or fragments of Aβ increases phosphorylation of tau (Busciglio et al., 1995; Takashima et al., 1996). Indeed, a single intracerebroventricular infusion of an Aβ-clearing antibody has been found to clear both tau and Aβ pathology (Oddo et al., 2006), but this is specific to early stage, unphosphorylated tau (Oddo et al., 2004). The mechanism linking upstream Aβ to downstream tau in the cascade of pathological events could be tau-dependent disassembly of microtubule proteins initiated by soluble Aβ specifically, as such disassembly is not observed through exposure to insoluble Aβ or in tau-free model systems (King et al., 2006). Strong support for the importance of Aβ/tau interactions in the development of cognitive deficits comes from a study where endogenous tau knockout (KO) mice were crossed with human APP (hAPP) mutation mice. When tested in the Morris water maze at 4-7 months of age, hAPP mice with either one or two alleles of endogenous tau showed impaired acquisition of the task. In accordance with the hypothesis that Aβ/tau interaction is a central aspect of AD pathology, mice that carried either hAPP without endogenous tau, or the reverse, acquired the task successfully (Roberson et al., 2007). A recent study by Bush and colleagues (Lei et al., 2012) which demonstrated the emergence of behavioural deficits and Parkinsonism in tau-KO mice may offer indications of the mechanisms which link the hyperphosphorylation of tau and tangle formation to Aβ pathology. They propose that the main cargo of tau-dependent trafficking is APP, and that a reduction in the level of soluble tau results in lower levels of APP transported to the cell surface. As APP has been shown to play a role in iron export from the cell (Duce et al., 2010), an inhibition of this function could result in toxic intraneuronal iron accumulation.

1.5.4 Amyloid precursor protein (APP) processing

Aβ is cleaved from the single transmembrane polypeptide APP (Selkoe, 2001). There are 3 isoforms of 695, 751 and 770 amino acid residues, all of which are expressed throughout various cell types in the body (Selkoe, 2001). Neuronal cells express higher levels of the 695 isoform, however, and the levels of this form of APP are low in non-neuronal cells (Mattson, 2004). The N-terminal of the molecule protrudes into the extracellular space, whilst the 724-770 amino acids of the COOH-terminal of the protein are positioned intracellularly.

APP can be processed by two different pathways (see Fig. 1), with altered APP processing being central to AD (Mattson, 2004). The majority of APP is subject to proteolytic cleavage by α-secretase at residues 15 and 17 within the Aβ amino acid domain, producing the 83 amino acid sized C-terminal fragment of APP (C83) in addition to the N-terminal fragment sAPPα (Tanzi & Bertram, 2005). The enzymes responsible for α-secretase activity are likely to be the ADAM family of proteases, such as TACE, ADAM9 and ADAM10 (Buxbaum et al., 1998; Asai et al., 2003; O'Brien & Wong, 2011; Mattson, 2004). C83 can be further processed by γ-secretase to produce P3 and the APP intracellular domain (AICD; Cao & Sudhof, 2001 as cited in Tanzi & Bertram, 2005). This pattern of cleavage does not produce Aβ.

Alternatively, APP can be sequentially cleaved by the enzymes β- and γ-secretase, respectively. β-secretase has been identified as the transmembrane protein BACE1, whilst γ-secretase has been identified as a complex comprised minimally of the presenilins, γ-secretase, nicasterin, aph-1 and pen-2 (Edbauer et al., 2003; Francis et al., 2002). The sequential cleavage of APP results in the release of the N-terminal fragment sAPPβ and the C-terminal fragment C99. Importantly, cleavage of C99 by γ-secretase produces the 40-42 amino acid-sized Aβ protein, depending on the exact location of γ-secretase cleavage within the transmembrane domain, in addition to AICD.

The current model of APP processing proposes that APP can either be processed at the cell surface, or internalised through endocytosis and processed in endosomal compartments (O'Brien & Wong, 2011). This view of APP processing proposes that cell surface proteolysis involves α-secretase and γ-secretase, and does not result in Aβ production. In contrast, the processing of APP in endosomal compartments containing β-secretase and γ-secretase does lead to Aβ release. In support of this, inhibiting internalisation of APP lowers Aβ release by 80% (Koo & Squazzo, 1994). Furthermore, BACE1 is predominantly found in the Golgi apparatus, supporting the belief that β-secretase-cleavage of APP occurs following endocytosis (Luo & Yan, 2010). It is currently unclear what regulates whether APP is processed at the cell surface or following endocytosis, but a shift in this regulation could be instrumental in altering AD pathology.

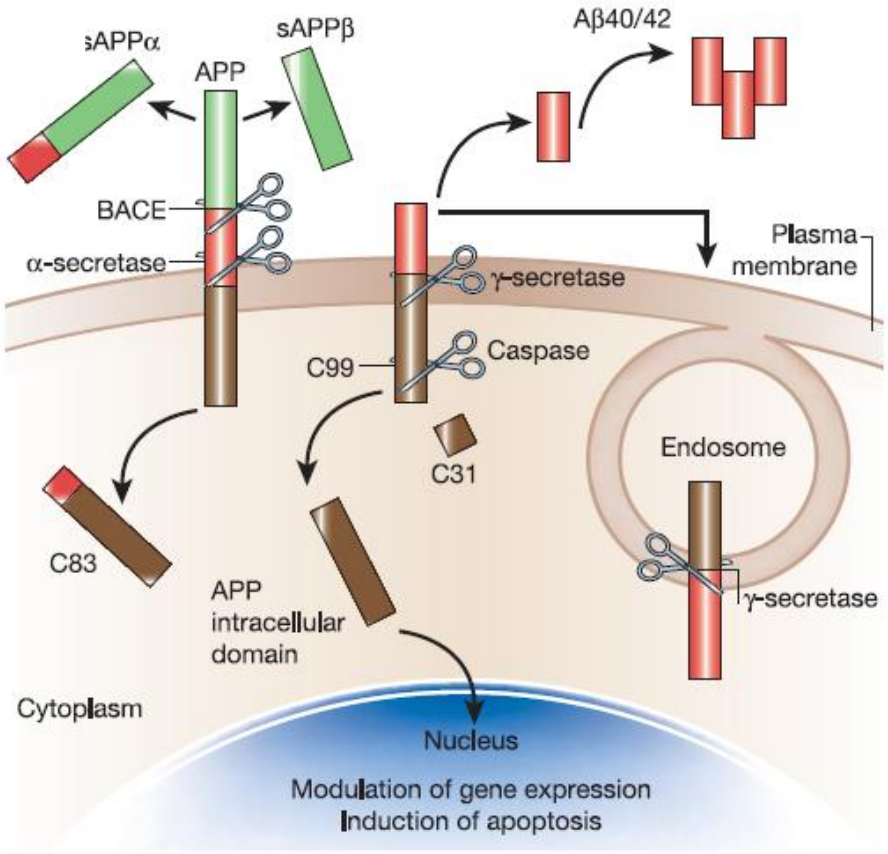

Fig. 1.1: A simplified schematic representation of APP processing by α-, β- and γ-secretase with the release of subsequent protein products as presented in Mattson (2004).

1.5.5 Neuronal loss and cerebral Aβ angiopathy (CAA)

The pathology of AD involves hallmarks beyond that of increased levels and deposition of Aβ and hyperphosphorylated tau, as neuronal loss and vascular damage caused by amyloid plaque formation are also common (Hardy & Higgins, 1992). Deposits of mainly Aβ40 are observed in small arterioles and capillaries in the cerebral cortex, but are rarely seen entering the white matter in subcortical layers (Selkoe, 2001). The contribution of CAA to cognitive decline or disease progression is unclear, as Aβ plaque load has not been found to correlate well with levels of angiopathy (Selkoe, 2001). In terms of neuronal loss, the hippocampi are heavily affected in AD patients (West, Coleman, Flood & Troncoso, 1994). Using imaging techniques, the hippocampal neural atrophy has been estimated to be 38% compared to age-matched healthy controls, with atrophy of the frontal lobes and the amygdalae at 15-16% of normal volume (Laakso et al., 1995).

1.6 Aetiology of AD

There are a number of hypotheses regarding the cause of AD. As the focus of the current work is on altering APP processing to downregulate Aβ production, the emphasis will be on the Aβ cascade hypothesis. Nevertheless, it is important to note that a variety of alternative hypotheses of AD aetiology have been put forward, highlighting the role of tau (Braak, Braak & Mandelkow, 1994; Rapoport, Dawson, Binder, Vitek & Ferreira, 2002), cholinergic dysfunction (Bartus, Dean, Beer & Lippa, 1982), oxidative stress induced DNA damage (Lu et al., 2004), mitochondrial dysfunction (Blass & Gibson, 1999; Castellani et al., 2002) and increased inflammation (McGeer & Rogers, 1992). The degree to which these are independent driving factors in aetiology, or act in combination with Aβ pathology (Mattson et al., 1992; Bush, 2003) is unclear.

1.6.1 AD risk

Age is a serious risk factor for AD, as patients are rarely diagnosed in the first 40 years of life with the exception of individuals with Down's syndrome. There are two subgroups of AD patients based on the age of onset of disease. The majority of AD patients suffer from the late onset form, defined as age of onset above 65 years. Only 1-5% of cases have an onset age below 65 years of age, referred to as the early onset form of AD (Tanzi & Bertram, 2005; McMurtray et al., 2006).

The risk of familial AD (FAD), which typically manifests itself as early onset AD, is dominated by genetic predisposition. A series of autosomal dominant mutations on several AD-linked genes inevitably lead to early onset AD in carriers. The first FAD *APP* mutation was identified by Goete et al. in 1991, but 31 other autosomal *APP* mutations have been identified since (O'Brien & Wong, 2011). In addition, 179 *presenilin1* (*PSEN1*) and 14 *presenilin2* (*PSEN2*) autosomal dominant mutations have been identified. Generally, these mutations have in common that they increase production of Aβ42 compared to Aβ40 (O'Brien & Wong, 2011).

By comparison, sporadic AD (SAD) is not linked to autosomal dominant mutations, but risk is nevertheless estimated to be 50-70% genetically determined (O'Brien & Wong, 2011). This is typically associated with late onset AD. The ε4 allele of the *APOE* gene has been strongly linked to AD, with a 3- to 15-fold risk increase with hetero- and homozygous carriers respectively (O'Brien & Wong, 2011; Harold et al., 2009). A group of smaller risk genes have been recently been identified using genome wide association studies (GWAS), which allow for

risk genes of smaller effect to be detected through the use of large sample sizes. These include variations on the *CLU* and *PICALM* genes (Harold et al., 2009), and underline the emerging complex genetic risk picture linked to SAD. GWAS studies with further increases in sample size are underway, opening up the possibility of further small risk genes to be identified. Mutations in these genes may not be sufficient to put an individual at risk when at low numbers, but may act together to increase the risk sufficiently to initiate AD pathology when in larger numbers. It is noteworthy that the pathology of SAD is very similar to that of FAD (O'Brien & Wong, 2011; Hernandez & Avila, 2008). This provides support to the idea that Aβ is central in the disease, despite potentially varying etiological triggers and genetic contributions.

A variety of lifestyle factors have also been identified to play a role in SAD risk, with medical conditions such as vascular disease, hypertension, obesity and stroke increasing AD risk (Breteler, 2000, Brodaty et al., 2011), as well as socioeconomic factors including low levels of education. Protective life style factors involve high levels of physical and mental exercise (Fratiglioni, Paillard-Borg & Winblad, 2004), with some indication that dietary elements such as wine consumption can reduce risk of AD by up to 50% (Lindsay et al., 2002). Not only do these studies provide a more balanced picture of the risk factors involved in the development of AD beyond genetic risk, but they indicate that there is potential for risk-reducing behaviour to influence AD prevalence.

1.6.2 The Aβ cascade hypothesis

The most influential theory of AD aetiology is the Aβ hypothesis (Hardy & Higgins, 1992). The hypothesis proposes that Aβ deposition is causative in the disease, and that other pathological characteristics associated with the disease, such as NFTs, neuronal loss, CAA and behavioural and cognitive deficits characteristic of dementia, are a result of this deposition.

The initial focus on Aβ as a central protein to disease progression was based on two sets of findings, beyond the identification of Aβ as the main component of plaques (Selkoe, 2001). Firstly, it was observed that individuals with Down's syndrome who live to 50 years of age have a higher incidence rate of AD than age-matched controls (Hardy & Higgins, 1992; Mann & Esiri, 1989). This combined with the discovery that the gene encoding APP is located on chromosome 21 of which individuals with Down's syndrome have an extra copy, indicated that Aβ may play a role in the initiation and/or progression of AD (Mann et al., 1989; Rumble et al., 1989; Hardy & Higgins, 1992).

The proposed mechanism by which Aβ induces tangle formation is through disruption of calcium homeostasis (Hardy & Higgins, 1992). Aβ is known to increase intracellular calcium levels, which plays a role in the phosphorylation of tau (Baudier & Cole, 1987). With abnormally high intracellular calcium levels driven by Aβ, abnormally high levels of phosphorylated tau protein may be produced, which may in turn aggregate into the paired helical filaments found in neurofibrillary tangles.

The amyloid cascade hypothesis has gained a substantial amount of support since being proposed in 1992, whilst remaining controversial (Tanzi, Moir & Wagner, 2004; Terry, 1996). The support for the central role of Aβ in AD comes from a variety of sources. Firstly, the identification of a number of autosomal dominant *APP* and the *presenilins* (*PSEN*) mutations in families with high numbers of AD sufferers implicated changes associated with APP and Aβ as critical elements in the development of AD (St George-Hyslop et al., 1987; Goete et al., 1991; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). Secondly, the generation of transgenic mouse models of AD-like pathology by the introduction of identified *APP* and *PSEN* mutations to the mouse genome demonstrated a causal link between alterations in APP processing and increased levels of Aβ (Games et al., 1995; Hsiao et al., 1996; Moechars et al., 1999b). Thirdly, the demonstration that immunisation with Aβ in transgenic mouse models which reproduce Aβ pathology similar to that seen in AD can not only lower Aβ levels and clear plaques, but improve cognition, suggests Aβ to be causally involved in the progression of dementia (Janus et al., 2000; Morgan et al., 2000; Wilcock et al., 2004; Rakover et al., 2007). Further evidence to suggest that Aβ pathology lies upstream of NFT pathology are the observations that the JNPL3 transgenic mouse line, which expresses a human tau mutation linked to frontotemporal dementia with parkinsonism-17 (FTDP-17), develops NFTs with age with no evidence of Aβ deposition (Lewis et al., 2000 as cited in Schwab et al., 2004). When crossing JNPL3 mice with Tg2576 mice, which develop Aβ plaques in the absence of NFTs based on the double Swedish *APP* mutations, the offspring develop comparable Aβ pathology to Tg2576 mice with exaggerated NFT pathology compared to the JNPL3 mice (Lewis et al., 2001). This indicates that NFT deposition is influenced by the presence of Aβ, whilst the NFT pathology does not influence Aβ plaque numbers significantly (Schwab et al., 2004).

As demonstrated by Pike et al. (1991), an early focus of the AD field was the neurotoxic properties of aggregated, fibrillar Aβ leading to neuronal death, with soluble Aβ considered less neurotoxic or in some instances, to have a trophic effect (Whitson et al., 1989; Whitson et al., 1990). Growing evidence has since demonstrated that the soluble, prefibrillar

forms of Aβ are more neurotoxic than insoluble, fibrillar Aβ (Klein, Krafft & Finch, 2001; Ashe, 2009; Teller et al., 1996). This modified version of the amyloid cascade hypothesis suggests a connection between non-fibrillar Aβ and neuronal apoptosis (Sponne et al., 2003). Aβ has been demonstrated to directly interfere with plasma membrane functioning, disrupting calcium homeostasis and causing neuronal death when in its pre-aggregated form (Pillot et al., 1999; Rhee et al., 1998; Lin et al., 2001). Non-fibrillar Aβ40 at low concentrations has also been shown to induce disruption of microtubule functioning through the generation of reactive oxygen species, leading to caspase activation and neuronal death (Sponne et al., 2003). Importantly, Walsh et al. (2002) identified Aβ oligomers as synaptotoxic *in vivo,* and found no such role for either Aβ monomers or fibrils. The observed disruption of long term potentiation (LTP), a proposed neuronal basis of learning (Bliss & Lømo, 1973), was found to be reversed if hippocampal slices were subjected to an anti-oligomeric Aβ antibody (Kayed et al., 2003). In 1996, Hsiao et al. showed that age-related deficits in spatial memory correlated with elevated levels of soluble Aβ40 and Aβ42 in transgenic mouse models of disease at 9-10 months of age. Similarly, a comparison between a series of APP overexpressing transgenic mouse lines found correlations between soluble Aβ42 levels and reduced numbers of presynaptic terminals, but no such correlation existed if soluble Aβ42 was substituted for either APP levels or plaque load in the analysis (Mucke et al., 2000). Oligomeric Aβ may also be driving memory deficits in AD patients (Klein, Krafft & Finch, 2001), and importantly, several groups have identified Aβ oligomers in tissue from AD patients (Roher et al., 1996; Enya et al., 1999; Pitschke et al., 1998).

The idea that soluble Aβ could be the predominantly neurotoxic form of the peptide fits with findings that point to strong correlations between soluble Aβ and cognitive decline (McLean et al., 1999; Oddo et al., 2006; Naslun et al., 2000) and synaptic loss (McLean et al., 1999; Lue et al., 1999). This addresses a controversial issue with the original amyloid cascade hypothesis (Klein, Krafft & Finch, 2001), namely the weak correlations observed between plaque load and cognitive deficits (Mucke et al., 2000; Cummings et al., 1996; Terry, 1996; Bartoo et al., 1997; Thal et al., 2002; Gomez-Isla et al., 1997; Lue et al., 1999; Holmes et al., 2008; McLean et al., 1999; Davis et al, 1999). Together, these studies have led to a refocusing of the Aβ cascade hypothesis in which soluble Aβ species are widely appreciated as disease-relevant, with fibrillar, insoluble Aβ formations potentially acting as a neutraliser of this toxicity as opposed to inducing neurotoxicity in themselves.

A second shift in the field to acknowledge is the growing awareness of intraneuronal Aβ as contributing to AD pathology in addition to extracellular Aβ, on which the focus has traditionally been. Oddo et al. (2006) found Aβ could be detected intracellularly prior to any extracellular presence, corroborating evidence from other groups observing intracellular accumulations of Aβ42 *in vitro* (Echeverria & Cuello, 2002; Tabira et al., 2002) and in transgenic mouse models of Aβ pathology (Wirths et al., 2001; Sheng et al., 2003). An interaction between the two pools of Aβ has also been reported, with extracellular Aβ42 leading to intracellular increases in Aβ42 (Glabe et al., 2001), as well as exchanges in the opposite direction (DeMattos et al., 2001; 2002b, Oddo et al., 2004; 2006). Aging transgenic Tg2576 mice which model Aβ pathology leads to intraneuronal accumulation of Aβ42 in synaptic compartments and neuronal processes (Takahashi et al., 2004), and intracellular Aβ has been linked to behavioural deficits in the APP/Tg mouse model of AD (Billings et al., 2005).

1.6.3 Synaptic disruption

Whilst the amyloid cascade hypothesis focused the attention of the field on Aβ being causative in disease progression (Hardy & Higgins, 1992), it was not in a position to provide detailed hypotheses regarding the mechanisms involved in the pathological cascade leading to neuronal death. There is increasing support for the idea that synaptic damage is both an early and important pathological event in AD patients (Mattson, 2004; Tanzi, 2005; Synder et al., 2005). This development is separate to the traditional "cholinergic hypothesis" which suggests that the impairment in cholinergic transmission following degeneration of the basal forebrain nuclei coupled with a reduction in the number of nicotinic receptors is an early and central part of AD pathology and disease progression (Scarpini, Scheltens & Feldman, 2003). There is controversy regarding whether this pathology is a driving force in the progression of the disease based around results suggesting that levels of acetylcholinesterase and choline acetyltransferase are stable during the early stages of AD (Scarpini, Scheltens & Feldman, 2003).

Aside from the role of acetylcholine in AD, extensive evidence supports the view that synaptic disruption is a central feature of AD. Brain regions which exhibit Aβ plaque pathology in patients frequently have a reduced number of synapses as well as display neuritic damage (Mattson, 2004; Dickson et al., 1995; Masliah et al., 1994), and synaptic loss correlates well with cognitive decline in patients (Terry et al., 1991; Sze et al., 1997; Lue et al., 1999; Tanzi, 2005). In PDAPP and Tg2576 transgenic mice, synaptic impairments are observed prior to

plaque deposition (Larson et al., 1999; Jacobsen et al., 2006) suggesting an early role for synaptic disruption. As APP is transported to synaptic terminals following endoplasmic reticulum and Golgi sorting (O'Brien & Wong, 2011; Koo et al., 1990), the synapse is a location with the potential for high levels of soluble Aβ (Gong et al., 2003; Buxbaum et al., 1998) and is thus at high risk for Aβ induced damage. In addition, synaptic activity has been found to regulate APP processing (Kamenetz et al., 2003), carrying with it the possibility that altered synaptic function could increase pathogenic processing of APP through an Aβ-mediated feedback loop.

Furthermore, there is evidence for the ability of Aβ oligomers to disrupt LTP in APP mutation mice *in vitro* (Lambert et al., 1998) and *in vivo* (Walsh et al., 2002), with the reversal of this effect observed in the presence of an anti-oligomeric Aβ antibody (Kayed et al., 2003). In addition, temporary disruption of a learned behaviour in Tg2576 transgenic mice using Aβ oligomers supports the idea that Aβ can disrupt synaptic functioning (Cleary et al., 2005). Snyder et al. (2005) found that application of Aβ42 to cultured neurons reduced the number of NMDA-receptors at the synapse, reducing sensitivity to glutamate. In support of this, a similar reduction in NDMA receptors can be seen in Tg2576 mice neurons (Snyder et al., 2005). AD patients have been found to have altered patterns of synaptic proteins, with changes in both the concentration, location and recycling of individual protein groups in regions vulnerable to AD pathology (Masliah et al., 2001).

There is evidence that the presence of Aβ at the synapse increases the vulnerability of the synapse to oxidative stress and excitotoxicity in addition to altering calcium homeostasis and energy metabolism (Mattson, 2004). Such changes are linked to activation of apoptotic signalling cascades, potentially offering pathways through which Aβ production can lead to neuronal death (Mattson, 2004). As neuronal apoptotic signalling pathways are predominantly initiated at the synapse, it emerges as a likely location for Aβ induced apoptosis (Mattson, 2004).

The growing evidence pointing to synaptic dysfunction and apoptosis induced at the synapse leads to the question of reversibility (Tanzi, 2005). There is need to investigate both whether cognitive function be restored in neurons which have been exposed to Aβ-induced impairments, and at what time point intervention is necessary to inhibit the initiation by Aβ of apoptotic signalling cascades (Tanzi, 2005).

1.7 Modelling AD: Transgenic mouse models of AD pathology and phenotype

An in-depth review of the two APP mutation mouse models utilised in this project will be described in their relevant experimental chapters. As an initial introduction, however, an overview of the role of transgenic mouse models in AD research will be presented. Whilst it is recognised that models based on mutations in the *presenilins* and *tau* genes are highly useful in a variety of contexts, the overview focuses on APP mutation models due to their central role in the current work. As the therapeutic approach to be assessed is based on altering APP processing to reduce Aβ production, models in which Aβ pathology is driven by APP mutations were a natural choice.

The aim of transgenic approaches to modelling Alzheimer's disease, or any other disease process, is to capture accurately a minimum of one feature of the disorder to allow for detailed investigation (Loring, 2000). Transgenic mouse models are created through the introduction of human genetic material into the mouse genome using site-directed mutagenesis of the gene of choice (Capecchi, 1989). Although this approach can be successful in a number of other species, the mouse is a commonly used species due to the large overlap between the human and mouse genomes (Mouse Genome Sequencing Consortium, 2002).

Whilst there are a number of transgenic mouse models of Alzheimer's pathology, no current model recapitulates all aspects of AD pathology as well as the typical cognitive deficits (Ashe & Zahs, 2010). Neuronal loss is a feature of AD which has been particularly challenging to recapitulate in model systems, whilst Aβ pathology in comparison is convincingly reproduced in a number of transgenic mouse models. Nevertheless, the introduction of transgenic mouse models of AD pathology to the field has been highly valuable. Whilst post-mortem examination of brain tissue from AD patients has tremendous value in classifying and characterising the pathological hallmarks of AD, transgenic mouse models of the disease pathology offers an insight into the temporal development of such hallmarks, as well as the association between different pathological key stages and cognitive deficits. Increased understanding of the temporal nature of pathological developments has helped dissociate Aβ plaque load and cognitive deficits, aiding the refocusing of the field's attention on soluble Aβ species. Nevertheless, it is important to critically evaluate the degree to which observations of transgenic mouse models of Aβ pathology are comparable to the human condition, given the differences in species as well as in the methods employed to assess cognitive function and behavioural change. This will be discussed in more detail in the relevant experimental chapters.

1.7.1 APP mutation mouse models

APP mutation mouse models of AD pathology are based on autosomal dominant mutations in *APP* isolated from FAD patients. Thirty-one mutations of *APP* have been identified (O'Brien & Wong, 2011), such as the Swedish double mutation around the β-secretase cleavage site (K670M/N671L, Mullan et al., 1992), the Arctic mutation (E693G, Kamino et al., 1992; Nilsbeth et al., 2001; Basum et al., 2008) in the middle of the Aβ fragment as well as the Indiana (V717F, Murrel et al., 1992) and London mutations (V717I, Goete et al., 1991), both around the γ-secretase cleavage site. These mutations have given rise to a range of APP mutation models based on different mutations using varying genetic background strains and genetic promoters, with the common outcome of elevation of Aβ (see Table 1).

Model	**Transgene mutation and promoter**	**Promoter**	**Aβ plaque pathology**	**Cognitive deficit**	**Reference**
Tg2576	APP Swedish (K670N/M671L)	Hamster *PrP*	Aβ plaques from 9-11 months, reduced synaptic density	Impaired spatial reference and working memory, object recognition memory (episodic like)	Hsiao et al. (1996)
PDAPP	APP Indiana (V717F)	*PDGFβ*	Aβ plaques from 6-8 months, loss of synaptic density, hippocampal atrophy	Impaired spatial reference and working memory, object recognition memory, cued fear conditioning	Games et al. (1995)
APP (V717I) or APP/Ld/2	APP London (V717I)	Murine *Thy1*	Aβ plaques from 10-12 months of age, altered synaptic functioning and c-fos expression	Impaired spatial reference memory and object recognition memory at 3-6 months, increased anxiety from 1 month	Moechars et al. (1999b)
TgAPP22	APP Swedish (K670N/M671L), London (V717I)	Murine *Thy1*	Aβ plaques from 18 months	n/a	Andra et al. (1996)
TgCRND8	APP Swedish (K670N/M671L), Indiana (V717F)	Syrian hamster *PrP*	Aβ plaques from 2 months	Impaired spatial reference and working memory	Chishti et al. (2001)
TgAPP23	APP Swedish (K670N/M671L)	Murine *Thy1*	Aβ plaques from 6	Impaired spatial reference memory,	Struchler-Pierrat et

			months, neuronal loss in CA1	seizures and stereotypic behaviour	al. (1997)
TgAPP/Sw /1 and 3	APP Swedish (K670N/M671L)	Murine *Thy1*	Aβ plaques from 18-25 months	Increased anxiety and impaired spatial reference memory	Moechars et al. (1999b)
TgAPP/ WT	No mutation, but WT APP over-expression	Murine *Thy1*	No Aβ plaques	Increased anxiety and spatial reference memory	Moechars et al. (1999b)
TgAPP/Fl TgAPP/Du	APP Flemish (A692G), Dutch (E693Q)	Murine *Thy1*	No Aβ plaques	Increased aggression, seizures	Kumar-Singh et al. (2000)
J20	APP Swedish (K670N/M671L), Indiana (V717F)	*PDGFβ*	Aβ plaques from 5-7 months of diffuse nature, neuritic plaques from 8-10 months	Impaired reference memory	Mucke et al. (2000)
TgAPP SweArc	APP Swedish (K670N/M671L), Indiana (V717F), Arctic (E693K (E22G)	*PDGFβ*	Aβ plaques from 2-3 months	n/a	Cheng et al. (2004)
TgAPP/ PS1	APP Swedish (K670N/M671L), PSEN1 (M146L)	*PDGFβ* and hamster *PrP*	Aβ plaques from 6 months	Impaired reference and working memory	Holcomb et al. (1998)
TgAPP/ PS1	APP Swedish (K670N/M671L), PSEN1 (A246E/TgPSEN1 5)	*PDGFβ* and hamster *PrP*	Aβ plaques from 9 months	Impaired reference and working memory, auditory trace fear conditioning	Borchelt et al. (1996)
3xTg-AD	APP Swedish (K670N/M671L), PS1 (M126V), Tau (P301L)	Murine *Thy-1*	Aβ plaques from 6 months, NFTs from 9 months	Impaired spatial reference memory	Oddo et al. (2003)
5xFAD	APP Swedish (K670N/M671L), London (V717I), Florida (I716V), PSEN1 (M146L/ L286V)	Murine *Thy-1*	Aβ plaques from 2 months, reduced synaptic density	Impaired spontaneous alteration	Oakley et al. (2006)

Table 1.1: Summary table of transgenic models of AD-like pathology adapted from Eriksen & Janus, 2007.

1.8 Currently available treatments

All available treatments for AD target the symptoms of the illness as opposed to the underlying causes, providing at best short-term improvements in cognition and behaviour (Schenk, 2002). Two types of pharmacological treatments are used in AD patients. Firstly, the acetylcholinesterase inhibitors donepezil hydrochloride (Aricept), rivastigmine (Exelon) and galantamine (Reminyl) enhance acetylcholine levels in an attempt to counteract the reduced levels of the neurotransmitter and its receptors (Scarpini, Scheltens & Feldman, 2003). Galantamine also increases cholinergic neurotransmission by increasing acetylcholine release and transmission pre- and post-synaptically respectively (Scarpini, Scheltens & Feldman, 2003).

These drugs are used in mild to moderate stage AD patients and provide similar benefits in terms of behavioural, functional and cognitive symptoms (Scarpini, Scheltens & Feldman, 2003). The response of individual patients to treatment is variable however, resulting in small average benefits across patients (Scarpini, Scheltens & Feldman, 2003). A newly available treatment for advanced AD, memantine (Ebixa), is a NMDA channel blocker designed to inhibit glutamate-driven disruption of calcium homeostasis and excitotoxicity (Scarpini, Scheltens & Feldman, 2003). Whilst these treatments can temporarily halt cognitive decline, they do not offer long term relief of symptoms and have a limited period of effectiveness within individual AD patients (Mayeux & Sano, 1999).

1.9 Treatment avenues under investigation

The wide range of data generated following the identification of mutations in *APP*, *PSEN1*, *PSEN2* and *APOE* collectively suggest that any disease-modifying treatment is likely to be more effective if levels of Aβ can be manipulated in some way (Tanzi & Bertram, 2005). This section will discuss the potential therapeutic targets in the Aβ cascade hypothesis which could be manipulated to alter the progression of AD beyond symptomatic treatment. Whilst no such treatment is currently available to AD patients, a wide range of possibilities are being explored in the scientific community, with several compounds proceeding to clinical trials.

Whilst treatments which targets causal factors involved in AD disease initiation and progression are greatly needed, improvements in treatment offers could be provided without fully halting or reversing the disease. As the majority of AD cases are SAD with an onset age above 65 years (McMurtray et al., 2006) involving years of neurodegeneration, a delay in onset or a slowing of disease progression could be sufficient to avoid late stage AD in a

number of patients. An analysis by Brookmeyer et al. (2007) predicted that a one year delay in onset and progression would result in a reduction in 9.2 million cases of AD by 2050.

Nevertheless, the growing evidence that AD-associated changes occur years prior to the emergence of clinical symptoms indicates that early intervention is likely to be most effective if a disease-modifying compound is to be used (Dubois et al., 2007). This is particularly relevant in the context of the amyloid cascade hypothesis, which would predict that an inhibition of Aβ levels and its associated toxicity will inhibit the development of downstream AD pathology. Based on this argument, several research groups have investigated forms of treatments designed to modify Aβ levels. These have included reducing Aβ production by selective increase or decrease in enzyme activity or enhancing Aβ clearance by a number of other strategies (Monsonego & Weiner, 2003). A short overview is provided of the research avenues investigated to date.

1.9.1 Secretase inhibition

The critical role of β- and γ-secretase in the production of Aβ makes these enzymes therapeutic candidates, in which reducing the activity of either secretase could prove beneficial in the aim of reducing Aβ levels.

APP is cleaved by β-secretase prior to γ-secretase cleavage, making it a suitable target to intervene in early Aβ production. In addition, β-secretase knockout (KO) mice produce virtually no Aβ whilst being generally healthy (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Scarpini, Scheltens & Feldman, 2003; Zouh et al, 2011). However, inhibition of β-secretase (BACE1) may result in the inhibition of similar enzymes such as BACE2 and cathepsin D, and later studies of BACE1 KO mice have suggested abnormalities such as shortened lifespan, hypomyelination and indications of schizophrenia-like behaviour (Dominguez et al., 2005; Hu et al., 2006; Willem et al., 2006; Savonenko et al., 2008). This is likely related to the fact that BACE1 has a number of other substrates in addition to APP, such as neuregulin-1 (Nrg-1), a neurotrophic factor which is essential in synaptic and neurotransmitter function, as well as neuronal migration and myelination (Luo & Yan, 2010). Thus, it is likely to be problematic to inhibit BACE1 as this inhibition will not be APP specific (Rakover et al., 2007; Van Dooren et al., 2005, De Strooper et al., 2010).

Despite β-secretase being a highly challenging target (De Strooper et al., 2010), at least 18 different β-secretase inhibitors have been developed (Luo & Yan, 2010). Several of

these have successfully lowered Aβ levels in transgenic mouse models of AD (Asai et al., 2006; Ghosh et al., 2007; Ghosh et al., 2008; Hussain et al., 2007; Iserloh et al., 2008), but none measured the effect of the inhibitor on behavioural or cognitive measures. One of these, CTS-21166 (CoMentis), has recently completed a phase I clinical trial demonstrating up to 80% reduction in plasma Aβ levels following intravenous administration (Luo & Yan, 2010). Similarly, May et al. (2011) reported significant reductions in plasma and CSF Aβ levels following administration of LY2911376, a non-peptidic orally available BACE1 inhibitor, to healthy volunteers. However, due to evidence from toxicology studies in rats showing an accumulation of unidentified biological material in the retinal epithelium and intracellularly within neurons and glia cells, the phase II clinical trial will not proceed with this compound. Thus, whilst BACE1 inhibition remains an interesting target for investigation, it is likely that the selectivity of the approach will need to be improved to ensure that side-effects are minimised.

Inhibition of γ-secretase is a therapeutic target due to its role in determining the length of the Aβ peptide (Scarpini, Scheltens & Feldman, 2003; De Strooper et al., 2010). As with strategies to target β-secretase, γ-secretase inhibitors have an innate challenge in that γ-secretase has a number of substrates besides APP, such as Notch (Scarpini, Scheltens & Feldman, 2003; Bermans & De Strooper, 2010; Tanzi & Bertram, 2005) and Nectin (Kim et al., 2002). Blocking Notch signalling is embryo-lethal, and a number of sources are indicating a role for γ-secretase in processing a number of transmembrane proteins (Kopan & Ilagan, 2004). Thus, successful inhibition of γ-secretase relies on it being APP-specific.

At least six γ-secretase inhibitors have been developed and are being tested in clinical trials or small scale human testing (Imbimbo, 2008). Semagacestat (LY-450139) progressed furthest of these (Bateman et al., 2009; Imbimbo, 2008, Siemers et al., 2007), but clinical trials and further development was halted in 2010 due to adverse effects in treated patients. These include accelerated cognitive decline and elevated levels of melanoma. A recent follow up of patients who were treated for 32 weeks prior to the disruption of the trial suggests irreversible acceleration of cognitive measures and a decreased ability to perform activities of daily living in treated patients compared to controls. The observation that Semagacestat negatively affects cognitive functioning in AD patients has received pre-clinical support from Bittner et al. (2009) who found the drug induced an irreversible loss of dendritic spines in wild type mice. These results suggest that blanket inhibition of APP secretases is likely to have unacceptable side effects, and that more specific approaches need to be considered. A subgroup of the currently available γ-secretase inhibitors appear not to affect Notch signalling (Petit et al.,

2003; Albright et al., 2008; Mayer et al., 2008), and thus could offer alternatives to Semagacestat with reduced side-effects.

1.9.2 Aggregation modulators

Aβ oligomers have been identified as synaptotoxic, whilst Aβ monomers show no such effect (Walsh et al., 2002). Therefore, inhibiting Aβ aggregation or β-sheet formation may provide sufficient neuroprotection to be used as a disease treatment (Tanzi & Bertram, 2005, Citron et al., 2004). Utilising aggregation-blocking compounds could attenuate Aβ toxicity and subsequently may inhibit neuroinflammation (Caughey & Lansbury, 2003). Efforts to limit neuroinflammation alone, such as through the use of non steroidal anti-inflammatory drugs, may also provide therapeutic benefits (Rogers & Lahiri, 2004). Aggregation of Aβ is dependent on interactions with heavy metals such as zinc and copper, and can be inhibited through administration of metal-complexing drugs such as clioquinol (Tanzi et al., 2004, Ritchie et al., 2003).

1.9.3 Increased clearance of Aβ

Apart from the use of antibody-mediated clearance, there are two pathways through which Aβ can be cleared from the brain, namely through proteolytic degradation or receptor-mediated transport to the periphery (Tanzi et al., 2004). The low-density lipoprotein receptor-related protein (LRP) has been found to be involved in transporting Aβ from the CNS to the plasma across the blood brain barrier (BBB), whilst the receptor for advanced glycation end products (RAGE) transports Aβ in the opposite direction. Modulation of their activity could aid clearance of Aβ from the CNS and potentially lead to degradation in sites such as the liver (Zlokovic, 2004). Indeed, LRP antagonists can lower efflux of Aβ from the CNS by 90% in mice models (Tanzi et al., 2004).

Alternatively, Aβ degradation can be increased within the CNS through enhancing the activity of zinc metalloendopeptidases such as insulin degrading enzyme (IDE) and neprilysin (NEP; Tanzi et al., 2004). These peptidases have a range of other substrates, however, including insulin, glucagon, β-endorphin, amylin, transforming growth factor, neuropeptide Y, enkephalin and substance P (Tanzi et al., 2004), indicating that upregulation of their activity is likely to produce noticeable side-effects. Despite these concerns, mouse models which either

overexpress IDE or are IDE-deficient provide *in vivo* support for an Aβ lowering or increase, respectively (Leissring et al., 2003; Farris et al., 2003), without reported side effects.

1.9.4 Immunotherapy

Immunotherapy involves the use of antibodies in the treatment of disease, and often relies on the activation of the immune system as an integral part of the treatment strategy. In the field of AD, immunotherapy has received much attention for its disease-modifying potential.

There are three main areas of anti-Aβ antibody-based immunotherapy (Schenk, 2002). Firstly, active immunisation with synthetic Aβ42 leads to the uptake of the peptide by antigen-presenting cells, followed by the activation of T-cells and B-cells, resulting in the production of anti-Aβ42 antibodies. These then activate the immune system in order to clear Aβ42 peptides. Secondly, active immunisation with a fragment of the Aβ42 peptide conjugated to a carrier protein leads to much the same sequence of events, with the exception of activation of T-cells by the carrier protein itself rather than the Aβ42 fragment. Thirdly, passive immunisation involves introduction of anti-Aβ42 antibodies, without the host being required to produce the antibodies itself.

The first demonstration of successful *in vivo* reduction in Aβ using immunotherapy was in 1999, when Schenk et al. passively immunised PDAPP transgenic mice with full length Aβ42. They treated mice either before AD pathology onset (6 weeks) or after (11 months) monthly for 11 months. Studying the effectiveness of disease-modifying treatment in aged mice is particularly clinically relevant, as evidence suggests that disease-related changes in the CNS are likely to be initiated years prior to clinical manifestation of symptoms (Dubois et al., 2007). Whilst aged mice showed reductions in Aβ pathology, a near total prevention of plaque deposition and associated gliosis was observed in young mice. This suggests that passive immunisation with Aβ42 has potential as both an early stage, preventative treatment as well as a late stage, disease-slowing alternative to acetylcholinesterase inhibitors. A drawback of the study is its lack of cognitive measures, thus making it impossible to infer whether a change in Aβ plaque load resulted in a corresponding change in cognitive function. Aβ lowering effects following active immunisation with Aβ42 have since been demonstrated by a number of other groups, as well as demonstrations that successful lowering of Aβ can be achieved using a non-toxic fragment of Aβ42 conjugated to polylysine in the Tg2576 model (Sigurdsson et al., 2001).

Importantly, there have also been reports of improved performance on spatial memory tests such as the Morris Water Maze following active immunisation with Aβ42 in TgCRND8 transgenic mice (Janus et al., 2000) and APP/PS1 mice (Morgan et al., 2000), as well as in Tg2576 mice on the radial arm water maze (Wilcock et al., 2004) and object recognition (Rakover et al., 2007). In the study conducted by Janus et al. (2000), a reduction in spatial reference memory deficits was observed in conjunction with a reduction in Aβ plaque load, but with no change in the overall Aβ levels, suggesting that functional improvements can be observed following small, potentially undetectable changes in overall Aβ levels. Such an interpretation would be in accordance with the synaptic deficit theory of AD, where synaptic dysfunction may be induced by high levels of Aβ at the synapse. Similarly, Morgan et al. (2000) saw inhibition of the development of a spatial reference memory deficit in the mice immunised over 8 months, whilst the reduction in Aβ plaque load was small. These data indicate that elevated Aβ levels and deposition can be tolerated to some extent, and leave cognitive function relatively unaffected. Similar observations have been seen in the human literature, where individuals who present with Aβ plaque pathology post-mortem were cognitively healthy individuals during life (Davis et al., 1999).

Passive immunisation studies with anti-Aβ antibodies have paralleled the success seen in active immunisation studies. Peripheral administration of anti-Aβ antibodies to PDAPP mice has also been shown to reduce Aβ plaque load by 81-93% depending on the antibody used (Bard et al., 2000) with only antibodies recognising the 1-16 amino acid sequence of Aβ being effective. Wilcock et al. (2004) treated Tg2576 mice weekly for either 3 or 5 months ending at the age of 28 and 22 months respectively. This led to a reversal of cognitive deficits as tested in the radial arm water maze. In contrast, 1 or 2 months of passive immunisation in 22 month old mice was not sufficient to alter spatial reference memory performance. Reductions in Aβ diffuse plaque load was observed after 5 months of treatment only, a pattern which was coupled with increased angiopathy and microhemorrhages. Reductions in cognitive deficits have also been observed despite a lack of Aβ change, with passive immunisation with m266, an antibody targeting Aβ13-28, in PDAPP mice improving performance on both the object recognition and holeboard learning and memory task (Dodart et al., 2002). Whilst passive immunisation in transgenic mice has not resulted in major side effects, there is evidence to suggest an increase in cerebral haemorrhages in APP23 mice (Pfeifer et al., 2002).

The majority of the immunisation studies conducted in transgenic mouse models of AD pathology use intraperitoneal injection as a means to deliver compounds to the CNS. The

following success in targeting both Aβ levels and improving cognition is surprising given the low permeability of antibodies to the BBB. Other studies have used passive immunisation through direct intraparenchymal infusion into the hippocampus and frontal cortex of Tg2576 mice (Wilcock et al., 2003). By using a direct delivery method where a controlled concentration of anti-Aβ is delivered, it is possible to investigate short term changes following administration. No changes were observed after 4 hours, but a reduction in diffuse Aβ emerged after 24 hours. Reductions in thioflavine-S-positive staining indicative of neuritic plaque load was not apparent until 3 days post-infusion, which coincided with staining for microglia activation. This suggests that anti-Aβ42 antibody-mediated clearance of Aβ consists of two phases focusing firstly on diffuse plaques, followed in the later stage by neuritic Aβ plaques clearance mediated by microglical activation.

The mechanisms by which immunisation with Aβ or anti-Aβ antibodies is effective are unclear. It has been suggested that reduced Aβ plaque burden is achieved through stimulation of microglial activity (Bard et al., 2000), the inhibition of Aβ aggregation (Solomon et al., 1996; Solomon et al., 1997; Frenkel, Katz & Solomon, 2000) or a combination of the two (Schenk, 2002). In support of microglial activation, it has been found that Aβ plaques are subjected to phagocytosis triggered by the Fc region on anti-Aβ antibodies bound to the plaques (Bard et al., 2000).

A third alternative for the mechanisms by which immunisation targets Aβ pathology is the “peripheral sink” hypothesis (Tanzi et al., 2004; DeMattos et al., 2004). This describes the idea that anti-Aβ antibodies bind and clear Aβ in the periphery rather than in the CNS, creating a concentration gradient across the BBB. The concentration of Aβ in the CNS is thereby reduced by passing from the CNS to the plasma. In support of this, DeMattos et al. (2001, 2002) demonstrated that PDAPP mice which were peripherally immunised with m266 showed reductions in plaque burden without any evidence of the antibody binding to plaques. This was paralleled by an increase in Aβ levels in the plasma.

Despite the promising results from immunisation studies in the animal literature, it has to date proved difficult to translate these findings to a clinical setting. Initially, a single dose phase I study actively immunising mild to moderate stage AD patients with AN1792, consisting of aggregated human Aβ42, reported good tolerability (Schenk, 2002). This was followed by a multiple dose study with similarly positive results and an antibody response in approximately half of the treated participants (Bayer et al., 2005). Whilst there were reports of two incidences of meningoencephalitis, these were either not related to the treatment or

occurred over 7 months following the last treatment. A second multiple dose study with over 70 treated patients was conducted successfully, before proceeding to a phase IIa clinical trial with 300 mild to moderate stage AD patients being treated with AN1792. After receiving between 1-3 doses over up to 3 months, dosing was terminated following reports of meningoencephalitis in 18 treated patients (6%), with no such incidences in the placebo group (Orgogozo et al., 2003). With the exception of 6 of these cases who developed permanent cognitive or neurological side effects, the majority of cases experienced no long term consequences. Despite good tolerability in the phase I trials, the side effects observed in the phase IIa trial show similarities to reports of cerebral haemorrhage and immune system activation in immunisation studies in mouse models (Pfifer et al., 2002). Follow up analysis of the interrupted trial revealed a developed antibody response in 19.7% of treated patients (Gilman et al., 2005). There was no evidence of a beneficial effect of antibody response on a battery of cognitive assessments with the exception of minor superior performance by responders on a neuropsychological test battery (Gilman et al., 200; Holmes et al., 2008).

A follow up of the 70 patients who took part in a phase I trial was concluded 6 years following treatment, of which 8 AD patients who received AN1792 underwent post-mortem histological analysis. The study showed that β-amyloid immunisation over time reduced the number of plaques in patients, but there was no evidence of an accompanying slowing of cognitive decline, as placebo and treatment groups had reached comparable levels of dementia at the terminal stages of life (Holmes et al., 2008). This result has been interpreted as a failing of preclinical data to translate into the clinical domain, as well as a failure of the Aβ cascade hypothesis, which would predict a reduction in Aβ plaque load to be beneficial in terms of rate of cognitive decline. Others have argued that the study provides further evidence that the destruction of Aβ plaques may not offer cognitive benefit if soluble, oligomeric Aβ is not also targeted, or may exacerbate conditions if it results in the release of pre-fibrillar Aβ from plaques to the extracellular space (Holmes et al., 2008).

Whilst active immunisation with AD patients has not been widely assessed, passive immunisation could be a viable alternative if adverse reactions such as meningoencephalitis persist. Passive immunisation may induce less side effects as the immune system is not called upon for antibody production, and provides a more reversible treatment should side effects emerge (Wilcock et al., 2004). Furthermore, passive immunisation sidesteps the issue of potential direct toxicity associated with particular residues of Aβ (Wisniewski & Koniezko, 2008). In addition, reports of variable antibody responses in the elderly population further

point to passive immunisation as potentially preferable as a treatment strategy in elderly AD patients (Wilcock et al., 2004). However, cerebral haemorrhage has also been observed in transgenic mice treated using passive immunisation (Pfeifer et al., 2002), suggesting that antibodies targeting Aβ through either passive or active immunotherapy could activate the immune system in a detrimental fashion. The unresolved issue of potentially increasing the level of toxic species of Aβ if using plaque-targeting antibodies, in association with the inflammatory issues observed when activating the immune system to clear Aβ, points to a need to develop alternative or more selective therapeutic strategies.

A potential reason for the lack of successful translation of disease-ameliorating strategies in mouse models of Alzheimer's disease pathology to human patients could be differences in the stage the pathology has progressed to at the time of compound administration. This was highlighted by Zahs & Ashe (2010), where the authors argue that the current mouse models of AD pathology model the early, asymptomatic stages of the disease specifically. Therefore, it could be more appropriate to classify preclinical treatment studies as preventative rather than initiated following the emergence of clinical symptoms, as would be more clinically relevant. In early stage drug trials, mild to moderate cognitive impairment patients AD patients are used, in which the disease process may have progressed beyond that modelled in transgenic mice. These treatments may prove more effective if tested longitudinally on pre-symptomatic individuals with a biomarker profile suggestive of early AD-associated changes. This highlights a need in the field for models which focus more specifically on the later stages of the disease, one that without doubt involves extensive cell loss, a feature which has not been successfully incorporated into current models.

1.9.5 Alternative immunotherapy to anti-Aβ antibodies

Whilst the focus of immunotherapy in AD research has been on anti-Aβ antibodies which bind various sites of Aβ42, there are groups which have utilised antibodies with alternative targets with the aim of lowering Aβ levels in the CNS. The benefit of such an approach is several-fold. Firstly, selecting alternative targets in the Aβ cascade results in valuable opportunities to test the Aβ cascade hypothesis. Secondly, using antibodies which do not bind Aβ may result in less activation of the immune system, as antibodies would not be bound to Aβ plaques. This could result in therapeutic strategies which are able to lower Aβ with limited medical side effects.

One alternative target in the Aβ cascade is BACE1. Monoclonal antibodies have been developed which bind to loops D and F, which are removed from the active site of the enzyme, but inhibits its ability to cleave APP (Zhou et al., 2011). This approach has been shown to reduce Aβ40 and Aβ42 production *in vitro* and *in vivo*, following stereotactic injection to the hippocampus and cortex of APPDutch mice at 3 months of age (Zhou et al., 2011). This approach has also been utilised by Atwal et al. (2011), who produced a monoclonal antibody with loop C, D and F of BACE1 as its substrates. In addition, a bispecific version of the antibody was created where half of the molecule was made up of the anti-BACE antibody and the other half a low affinity anti-transferrin receptor antibody. As transferrin receptors are present at the BBB, the merging of these two antibodies allows the molecule up to 10-fold higher penetration of the BBB as compared to the anti-BACE1 antibody in its original form (Yu et al., 2011). This type of antibody engineering may open possibilities for increased delivery of peripherally administered antibodies to human patients.

Focusing on altering APP processing, Thomas et al. (2006), Arbel et al. (2005) and Boddapati, Levites & Sierks (2011) developed antibodies which bind to the β-secretase cleavage site of APP rather than Aβ itself. The strategy is based on inhibiting BACE1 access to the cleavage site through steric hindrance, reducing the amount of APP processed by β-secretase cleavage, thereby lowering the production of Aβ (Thomas, Liddell & Kidd, 2011). APP can then undergo proteolytic cleavage by α-secretase as an alternative to amyloidogenic processing. This approach has several advantages. Firstly, it avoids issues relating to which form of Aβ to target in terms of neurotoxicity, as the production of the molecule itself is hindered. Secondly, given that the APP molecule is highly preserved through evolution, is present in most cells and tissues within humans and mice, and has a gene promoter region with characteristics of housekeeping genes (Salbaum et al., 1988), it is likely that the variety of molecules formed from processed APP serve important functions (Selkoe, 1991). It is therefore an advantage for a treatment strategy to minimise interference with the general processing of APP, avoiding only the production of Aβ40/42 specifically. Lastly, by avoiding enzyme inhibition, the approach will not interfere with ability of BACE1 to process other substrates aside from APP, an approach which has been problematic (Rakover et al., 2007; Van Dooren et al., 2005, Boddapati et al., 2011).

The potential of this approach for therapeutic treatment has been investigated by the two groups using both *in vivo* and *in vitro* assessments. Thomas et al. (2006) demonstrated that an antibody (2B12) against the β-secretase cleavage site reduced Aβ40 in a time- and

concentration dependent manner by up to 50% in human neuroblastoma (MOG-G-UVW) and astrocytoma (SH-SY5Y) cell lines expressing normal levels of APP, without binding Aβ itself. A similar antibody (2B3) with a higher specificity and affinity to the β-secretase cleavage site than 2B12 has demonstrated an ability to further reduce Aβ levels compared to 2B12 (Thomas et al., 2011). The characterisation of these antibodies has demonstrated that they remain bound to APP after 60 min incubation at pH 4.0 *in vitro*, suggesting that they will be unaffected by the similar pH level found in the endosomal/lysosomal system in which β-secretase cleavage of APP occurs (Thomas et al., 2011). Supporting the idea that the reduction in Aβ levels is based on inhibition of β-secretase cleavage is the finding that levels of the C-terminal fragment of APP (C99) produced following β-secretase cleavage are reduced if 2B3 is present (Thomas et al., 2011). Using hamster ovary cells overexpressing human APP, Arbel et al. (2005) showed that a similar antibody, BBS1, reduced secreted and intracellular levels of Aβ. Such demonstrations of effective downregulation of Aβ production *in vitro* are a first step in the evaluation of the antibody's therapeutic value. These results must be replicated *in vivo* using pre-clinical models of AD-like pathology in order to start building a convincing case for investing further research effort into anti-APP β-secretase cleavage site antibodies.

To date, Rakover et al. (2007) offers the only insight into the *in vivo* effect of anti-APP β-secretase cleavage site antibodies. Female transgenic Tg2576 mice received IP injections of BBS1 (or PBS) every two weeks from 6 to 12 months of age, at which point object recognition memory was assessed using the novel object recognition task. Whilst no differences in insoluble Aβ levels were observed as measured by both ELISA and immunohistochemical staining, BBS1 treated mice demonstrated a significantly higher discrimination ratio compared to PBS treated mice. Furthermore, the authors reported a reduction in microhemorrhages following BBS1 treatment. The effect of BBS1 on neuroinflammation was investigated using staining for F4/80 and GFAP, but BBS1 administration lowered F4/80 staining only.

Whilst these results are encouraging in the sense that an anti-APP β-secretase cleavage site antibody showed effects on biochemical and behavioural markers in a disease-limiting direction, there are a number of reasons why further research using this form of immunotherapy is desirable. Firstly, the lack of a reduction in any measure of Aβ pathology opens the possibility that the BBS1-linked reduction in microhemorrhages, neuroinflammation and object recognition memory could be related to a secondary effect of the antibody presence as opposed to the result of a significant downregulation of Aβ. Based on the Rakover et al. (2007) paper alone, the behavioural changes appear to have a stronger link with

alterations in vascular and inflammatory markers than with insoluble Aβ pathology. This link may be driven by changes in soluble Aβ, a measure thought to be more relevant in the context of cognitive deficits in transgenic APP mutation models. As soluble Aβ levels were not successfully measured in Rakover et al. (2007), further investigation of the role soluble Aβ species play in possible alterations in cognitive function would be beneficial.

Secondly, there is dispute in the literature regarding whether transgenic Tg2576 mice display deficits on the task used to assess the effect of BBS1 on cognitive function in Rakover et al. (2007). Whilst several groups have reported deficits using delay intervals between 5 min and 24 hours (Ribes et al., 2011; Yuede et al., 2009; Taglialetela et al., 2008; Bardgett et al., 2011), opposing patterns of results indicate that transgenic mice up to 16 months of age can discriminate between a novel and familiar object when there is no requirement to form a coherent representation of an object and its spatial location (Hale & Good, 2005; Good & Hale, 2007; Ognibene et al., 2005). A dispute regarding the presence of an object recognition deficit in Tg2576 mice used in Rakover et al. (2007) calls into question the utility of this task as a measure of changes in cognitive function following BBS1 treatment. Indeed, no wild type mice are included to indicate the baseline level of performance to be expected from healthy mice, making it difficult to determine the degree to which the BBS1 treatment reduced object recognition memory deficits. This is problematic due to the demonstration that PBS treated Tg2576 mice show a preference for the familiar over the novel object rather than equal exploration as expected if subjects have no memory of a previous encounter, indicating the interference of an additional factor such as anxiety or object bias on normal novelty seeking behaviour. Given that the increase in the discrimination ratio for mice treated with the high dose of BBS1 is significantly higher than the PBS treated mice, but not the mice treated with a low dose of BBS1 who are at chance level, it is likely that had PBS treated mice performed at chance level, no significant changes in cognitive function would have been observed.

Furthermore, the Rakover et al. (2007) paper provides no evidence that the BBS1 antibody penetrates the blood brain barrier after being administered in the periphery. It is therefore possible that the antibody did not arrive at its target, making it unable to lower neuronal Aβ levels. The changes in inflammatory and microhemorrhage markers may be driven by effects of the antibody in the periphery as opposed to interference with APP processing in the CNS.

Finally, it is worth noting that a single demonstration of *in vivo* effects of an anti-APP β-secretase cleavage site antibody will benefit from the parallel investigation of similar

processes. In addition, mice were treated from 6 months to 12 months of age in Rakover et al. (2007), demonstrating that early intervention using this approach can be beneficial, but providing little information of the effect of halting Aβ production once Aβ pathology is established. For the reasons listed, the current work set out to expand on the current knowledge of the effect of an anti-APP β-secretase cleavage site antibody as outlined further in the section below.

1.10 Thesis summary and aims

In this chapter, an overview of AD as a cognitive and biochemical disorder has been presented, as well as a discussion of immunotherapy as a treatment strategy in clinical AD research. The anti-β-secretase cleavage site antibody 2B3 and the use of transgenic mice to model AD have been introduced. Despite considerable focus of disease modifying treatments targeting the factors driving pathology in AD, limited success has translated to clinical trials. With AD growing in prevalence and only symptomatic treatments with limited effectiveness currently available, further efforts to identify effective compounds which translate successfully to a clinical setting are needed. There have been encouraging results of using anti-APP β-secretase site antibodies to reduce Aβ production *in vitro* (Thomas et al., 2006; 2011; Arbel et al., 2005; Rakover et al., 2007; Boddapati et al., 2011), but no such demonstration has been made *in vivo*. Importantly, a beneficial effect of antibody administration on cognitive functioning was reported (Rakover et al., 2007), although further exploration of the degree of cognitive improvement, biochemical change and antibody delivery to the CNS would be valuable. As such, the current work has several aims.

Firstly, the thesis aims to characterise two transgenic APP mutation mouse models of AD-pathology in terms of the behavioural and biochemical phenotype in order to establish baseline levels against which to assess whether the introduction of 2B3 to aged transgenic APP mice leads to a reduction in Aβ production and associated behavioural deficits. In addition, the thesis aims to replicate the *in vitro* reductions in Aβ levels following treatment with anti-APP β-secretase cleavage site antibodies (Thomas et al., 2006; Arbel et al., 2005; Boddapati et al., 2011) for the first time using a novel neuronal cell line. The work expands on Rakover et al. (2007) by treating mice at a clinically relevant late time point in terms of disease progression, as opposed to a preventative intervention starting at a young age. Furthermore, the use of a spatial working memory task for the evaluation of the cognitive benefit of 2B3 treatment expands assessments beyond object recognition memory allowing for an indirect evaluation of

the specificity of anti-APP β-secretase cleavage site antibodies *in vivo*. In addition, in order to understand the mechanism of action of a potential change in cognitive performance, it is desirable to obtain a measure of the effect of such a treatment on both soluble and insoluble Aβ levels.

Thus, Chapter 2 and 3 present behavioural characterisation work focused on the London APP(V717I) mutation model of AD, with parallel biochemical work presented in Chapter 4. In Chapter 5, behavioural characterisation of a second APP mutation model, the PDAPP(V717F) model is presented, followed by Chapter 6, where both *in vitro* and pilot *in vivo* attempts at reducing Aβ production and behavioural deficits using 2B3 are investigated.

Anxiety Phenotype of the London APP(V717I) Model

2.1 Introduction

This chapter investigates the onset and progression of anxiety-related behaviour in the London APP(V717I) mouse model of Aβ pathology. The main rationale for these studies was to establish whether transgene-related changes in anxiety could interact with assessment of learning and memory. Whilst information regarding the anxiety phenotype is available in the published literature, it was necessary to reassess the effect of the APP(V717I) mutation on anxiety behaviour because of the change in the genetic background strain. The first section of this chapter provides a brief outline of the pathology expressed by the APP(V717I) model. Experiments 1a and 1b therefore assessed the performance of (male and female) APP(V717I) mice at 3, 6 and 19 months on a marble burying task and the elevated plus maze (EPM) respectively.

2.1.2 The London mutation APP(V717I) model

In order to assess the ability of 2B3 to lower Aβ production *in vivo*, it was essential that a suitable model was selected. As described in Chapter 1, Section 1.9.5, the antibody treatment evaluated in this thesis was designed to lower Aβ production by blocking access to APP at the β-secretase cleavage site through steric hindrance. The Tg2576 mouse model of amyloid pathology (Hsiao et al., 1996) is one of the most widely used transgenic murine models in the field (Eriksen & Janus, 2007). It has been thoroughly characterised both in terms of biochemical pathology and learning and memory deficits (Hsiao et al., 1996; Morgan et al., 2000; Kawarabayashi et al., 2001; Irizarry et al., 1997a; Arendash et al., 2001; Kotilinek et al., 2002; Westerman et al., 2002: Zhuo et al., 2007; Barnes et al., 2004; Lesne et al., 2006; Chapman et al, 1999; Corcoran et al, 2002; Ognibene et al., 2005; Hale & Good, 2005; Good, Hale & Staal, 2007, Eriksen & Janus, 2007). Although this model is commercially available (and bred at Cardiff University), the two hAPP mutations in the Tg2576 model are located at the β-secretase cleavage site (K670M/N671L, Mullan et al., 1992), which results in enhanced cleavage of APP via this route. It is important to note that this is also the location to which 2B3 binds. As antibodies are highly specific in the recognition of their binding site, a change in amino acids within this sequence could greatly affect their ability to successfully bind to their

target molecule. In support of this, pilot Western blotting work suggested binding of 2B3 to APP from Tg2576 transgenic tissue was limited (Thomas & Kidd, unpublished data).

Given the potential limitation of using a mouse model with mutations at the β-secretase cleavage site, an alternative APP model was used. The selection of the London APP(V717I) mouse line was governed firstly by the fact that the APP(V717I) mutation is located at the γ-secretase cleavage site (and should therefore not interfere with the ability of the antibody to bind to the β-secretase cleavage site). Secondly, published work indicated that these mice display an early behavioural phenotype, in addition to a robust biochemical profile (Moechars et al., 1999b; Bronfman, Moechars & Van Leuven, 2000; Dewachter et al, 2000; Masliah et al., 2000; Dewachter et al., 2002; Van Dorpe et al., 2002; Postina et al., 2004; Van Dooren et al., 2005; Tanghe et al., 2010).

The heterogeneous APP(V717I) mouse model of Aβ pathology carries a single point mutation in *APP*, resulting in the substitution of valine for isoleucine at residue 717 (770 isoform), close to the γ-secretase cleavage site. Expression of this mutant allele is driven by the neuronal specific mouse *Thy1* promoter with expression levels of human APP 2 fold that of endogenous APP (Tanghe et al., 2010), with an increase in hAPP protein levels from 3 to 15 months of age (Dewachter et al., 2000). The mutation results in preferential cleavage of the APP molecule by γ-secretase closer to the C-terminal of the protein, thus increasing the levels of soluble Aβ42 compared to that of Aβ40 (Moechars et al., 1999b; Tanghe et al., 2010). An overview of Aβ pathology in the transgenic APP(V717I) mice obtained from published work is displayed in Table 2.1. As can be seen, there is considerable variation both with regards to the levels of Aβ reported at certain ages, as well as the relationship between levels of Aβ40 and Aβ42. Nevertheless, mice with the APP(V717I) mutation display an age-related increase in Aβ levels. As the cleavage of the APP protein is not thought to be dependent on age, it is believed that the accumulation of Aβ40-42 that accompanies aging in this model is due to the failure of clearance or degradation of the protein isoforms as opposed to increases in the overall levels produced (Dewachter et al., 2000, Tanghe et al., 2010).

Age (months)	Soluble Aβ40	Soluble Aβ42	Total SDS extracted (soluble) Aβ(40+42)	Insoluble Aβ40	Insoluble Aβ42	Total FA extracted (insoluble) Aβ(40+42)
3	2.5	0	0	0	0	0
4.5	1.7/5	1.3/0.6	n/a	n/a	n/a	n/a
5	7	n/a	n/a	n/a	n/a	n/a
6	2	5	n/a	n/a	n/a	n/a
6-9	2.5	0	5	0	~0	5
12	n/a	n/a	7000	n/a	n/a	125
15	15	10	21000	2000	750	310
16	25	12	n/a	4500	5000	n/a
16-18	4.6	0.5	n/a	n/a	n/a	n/a
22	150	100	n/a	16000	14000	n/a

Table 2.1: Summary table of reported approximated soluble and insoluble Aβ40 and Aβ42 (ng/g of tissue) values in transgenic APP(V717I) mice as measured using ELISA based on Dewachter et al. (2000), Dewachter et al. (2002), Etcheberrigaray et al. (2004), Postina et al. (2004), Willem et al. (2004), Van der Auwera et al. (2005) and Tanghe et al. (2010). N/A = not available. If two studies indicated different values at the same age, values were included side by side. SDS = sodium dodecyl sulfate, FA = formic acid.

In terms of the anatomical pattern of Aβ deposition, plaques develop first in the entorhinal cortex and subiculum before becoming evident in the frontal cortex (Tanghe et al., 2010). Aβ plaques appear from 10-12 months of age (Tanghe et al., 2010), although earlier reports from this group indicate a slightly later profile (12-15 months of age; Dewachter et al., 2000). Both diffuse and senile plaques are observed, which are surrounded by neuritic processes including hyperphosphorylated tau (Tanghe et al., 2010). In addition, neuroinflammation can be detected in association with plaques using immunostaining for activated astrocytes and microglia (Tanghe et al., 2010). In terms of vascular deposits, the Aβ deposits are similar in morphology, structure and localisation to that observed in human AD tissue (Van Dorpe et al, 2000; Tanghe et al., 2010).

The London V717I mutation was originally bred into the FVB/N background strain, and this strain has been used in the majority of behavioural work on this mutation (Moechars et al., 1999b; Dewachter et al., 2002; Van Dooren et al., 2005; Dewachter et al., 2009). These mice displayed a number of problematic physical and behavioural characteristics such as premature death, seizures and increased levels of anxiety (Moechars et al., 1999b; Ashe, 2009). These aspects of the phenotype clearly make the transgenic mice less suited for behavioural testing. Recently, the APP(V717I) mutation has been bred into the C57Bl/6 line, in which *APP* mutations do not result in the problems described above (Moechars et al., 1999b;

Ashe, 2009). Furthermore, C57Bl/6 mice are considered to be a good background strain for studies of learning and memory (Crawley et al., 1997).

In considering the published work on mice with the V717I mutation, much of the focus has been on characterisation of the biochemical changes, with little attention directed towards the assessment of the behavioural and cognitive phenotype. Indeed, the effect of the APP(V717I) mutation on changes in cognitive function with age is unknown. A summary of the behavioural profile of the APP(V717I) model is presented in Table 2.2, highlighting the changes in genetic background the V717I mutation was maintained on. Important to the current experiments are reports that transgenic APP(V717I) mice display an age-independent increase in anxiety compared to wild type mice from 4-8 weeks onwards when on the FVB/N background (Moechars et al., 1999b). Thus, it is necessary to assess whether a change in the genetic background influences the anxiety phenotype in these mice. It is also worth noting that whilst deficits in spatial memory have been reported at 3-6 months of age, the effect of the V717I mutation on cognitive functioning with age is unknown.

Type of assessment	Genetic back-ground	Construct assessed	Age	Deficit
Open Field	FVB/N	Anxiety	1-2, 3-4 and 5-12 months	Tg mice show increased anxiety compared to WT mice.
Morris water maze	F1 hybrid (C57Bl/6 & FVB/N)	Spatial reference memory	3-6 months	Tg mice show inferior performance compared to WT mice.
Novel object recognition	FVB/N	Object recognition memory	3-6 months	Tg inferior performance compared to WT after a 3 hour sample-test delay
Fear conditioning	n/a	c-fos expression following cued and contextual fear conditioning	n/a	Reduced increase in C-fos expression in the hippocampal CA1 and basolateral amygdalae of Tg mice compared to WT mice. Behavioural parameters not reported.
Electrophysiology (*in vitro*)	n/a	NMDA-dependent long term potentiation and NMDA receptor responses	n/a	Reduced NMDA receptor response in CA1 of Tg mice compared to WT mice. Impaired long term potentiation in CA1 of Tg mice compared to WT mice.

Table 2.2: Summary table of reported behavioural deficits as reported in Moechars et al. (1999b), Dewachter et al. (2002), Postina et al. (2004) and Dewachter et al. (2009). N/A = not available. Tg = transgenic, WT = wild type.

There is clearly a need to assess the behavioural profile of the APP(V717I) mice for several reasons. Firstly, the current behavioural analysis has been conducted within one research group only. In order to verify results, it would be beneficial to see the pattern of results replicated across laboratories. Secondly, due to the change in the genetic background that the mutation is maintained on from FVB/N to C57Bl/6, it is necessary to assess whether this change has altered the behavioural phenotype of the model.

The aim of the experiments presented in this chapter is to provide a developmental profile of potential changes in anxiety-related behaviour of London APP(V717I) mice. This was done to establish whether changes could interact with assessment of learning and memory, rather than as an attempt at establishing a behavioural phenotype against which to assess the effect of reducing Aβ through 2B3 administration. Anxiety in AD patients is measured using subjective report by the patient, or behavioural observation by carers or clinicians (Kogan,

Edelstein & McKee, 2000). This differs from the direct manipulation of rodent assessments of anxiety, in which animals are placed in an unfamiliar, exposed environment for behavioural observation. Furthermore, anxiety in AD patients is thought to be related to difficulties arising from cognitive deficits and confusion (Fisher & Noll, 1996 as cited in Kogan et al., 2000). It is therefore argued that the anxiety construct as measured in rodents holds limited translational value in terms of disease understanding, and therefore best serves as a feature of transgenic phenotyping, potentially independent of increases in Aβ levels with age. The experiments presented test the hypothesis that the *APP*(V717I) mutation would lead to age-independent disruption to anxiety-related behaviour. Experiments 1a and 1b assessed anxiety behaviour in wild type and transgenic APP(V717I) mice longitudinally at 3, 6 and 19 months in a marble burying task and the elevated plus maze (EPM) respectively.

The marble burying task is an unconditioned anxiety task based on the natural neophobic response mice exhibit when exposed to novel objects, and has been widely used to assess anxiety in mice (Thomas et al., 2009; Archer et al., 1987). When faced with negative stimuli such as food shock or noxious food, rodents will engage in burying behaviour referred to as "defensive burying" (Thomas et al., 2009). Whilst rodents also bury non-aversive objects such as marbles, it is traditionally thought that anxiogenic aspect of these objects is their novelty.

The EPM is an unconditioned anxiety task where an animal's unconditioned avoidance response to high, exposed locations is utilised to measure levels of anxiety (Pellow, Chopin, File & Briley, 1985; Walf & Frye, 2007). This anxiety-like response is expressed through a greater reduction in exploratory behaviour in exposed locations compared to non-exposed locations. Anxiety related to the exposed nature of the area is thought to contribute to anxiety-like behaviour rather than neophobia when faced with a novel space (Pellow et al., 1985). Support for the EPM being sensitive to behaviour related to anxiety comes from pharmacological manipulations showing sensitivity to anxiolytic and anxiogenic agents (Pellow et al., 1985, Belzung & Griebel, 2001). In addition, the task is considered an ethologically valid task due to the use of novel and exposed locations as anxiety inducing environmental elements which could be encountered in a natural setting (Walf & Frye, 2007, McHugh, Deacon, Rawlins & Bannerman, 2004). When wild type mice are exposed to the EPM, they are typically observed to spend approximately 25% of their time in the open, anxiogenic arms (Dawson & Tricklebank, 1995).

This study also considered whether gender influenced the emergence of a behavioural deficit or, indeed, the degree of disruption (c.f. McLean & Anderson, 2009; Johnston & File, 1991). As no gender information is available on this model, no predictions were made regarding the effect of gender on anxiety. However, transgenic mice were predicted to display higher levels of anxiety than wild type mice at all ages tested.

2.2 General method: Maintenance and breeding

All animals were health-checked weekly and maintained according to UK Home Office regulations and the Animal (Scientific Procedures) Act (1986). Standard cage material included a cardboard tube and a wooden gnawing block and approved nesting martial. Holding rooms were kept on a 12 hour light/dark cycle, with stable temperature and relative humidity levels at around 21^{o}C ± 2^{o}C and 60 ± 10% respectively. Mice were maintained on *ad libitum* access to food and water unless specified in the experimental protocol in the relevant chapters.

The APP(V717I) London mutation (Moechars et al., 1999b) is maintained on the inbred C57Bl/6 background. Wild type C57Bl/6 females (Cathays Park transgenic production unit, Cardiff University) were bred with heterozygous transgenic APP(V717I) males, a generous gift from Prof. Fred van Leuven, to produce an approximate 1:1 ratio of wild type and heterozygous transgenic offspring. Litter size is typically between 3-9 pups. Mice were weaned at 5 weeks and either group housed according to gender within a litter, or singly housed. In our hands, attrition rates were < 2%, and no observations of elevated aggression or general health issues were made. Average body weights did not differ between wild type and transgenic mice at any age. Locomotor activity was measured in an open arena at 3, 6 and 18 months, with no differences between transgenic and wild type mice in the percentage of time spent moving [3 months: (F (1, 36) = 2.28, p > .05); 6 months: (F (1, 36) = 2.80, p > .05); 18 months: (F (1, 36) = 3.34, p > .05)].

Genotyping of APP(V717I) mice

A tail or ear sample less than 2mm in size was obtained from mice at approximately 5 weeks of age or following culling and was stored at -20. DNA was digested (0.2% Proteinase K, 1M Tris HCl, 0.5M EDTA, 10% SDS) and extracted using 5M ammonium acetate followed by centrifugation at 14000rpm. Isopropanol was added to the supernatant followed by

centrifugation and two washes with 70% ethanol. DNA samples were incubated in 55°C TE buffer (1M Tris Chloride, 0.5M EDTA) and stored at -20°C until use.

Polymerase Chain Reaction (PCR) for APP(V717I)

The sense primer (Eurofins, MWG Operon, Ebersberg, Germany) NE1075 (5'-CCGATGGGTAGTGAAGCAATGGTT-3') and antisense primer NE1076 (5' – TGTGCCAGCCAACAGAGAAAAC-3') were utilised to target the *APP*(V717I) mutation. The master mix solution consisted of 1.25µl of 10x buffer (BIOTAQ Red DNA Polymerase, Bioline, London, UK), 0.625µl of 50x $MgCl_2$ (Invitrogen, Paisley, UK), 0.625µl of 10mM deoxynucleotide triphosphates (dNTPs; GE Healthcare, Little Chalfont, UK), 0.125µl of NE1075 and NE1076 at a concentration of 15pmol each, 0.75µl of Taq Red (Bioline), 8.5µl of DNAse- and RNAse-free H_20 was combined per sample, with 0.5µl of DNA. The thermocycling conditions were the following: 72°C for 2 minutes, 36 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, with 2 seconds added to the elongation step per cycle. Samples were stored at 4°C. The DNA product was separated on a 1.5% agarose gel electrophoresis using TAE buffer (40mM Tris-acetate, 1mM EDTA). Products were visualised using SYBR Safe (Invitrogen) and sized against a DNA ladder of product sizes 50, 150, 300, 500, 750 and 1000 base pairs (bp; (Promega, Southampton, UK). The *APP*(V717I) transgene DNA product is approximately 500bp, and appears in the transgenic samples only. This is depicted in Figure 2.1, visualised with a UV-transilluminator using Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, USA).

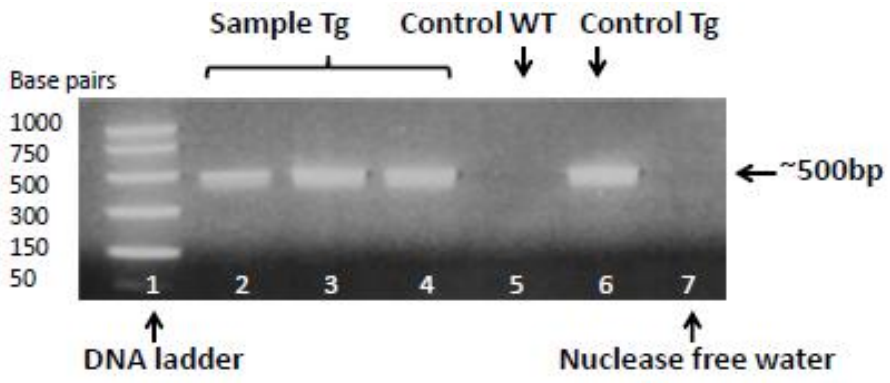

Fig. 2.1: Representative image displaying an electrophoresis gel of amplified genomic material from APP(V717I) transgenic and wild type mice. Lane 1 = DNA ladder displaying bp 1000 to 50. Lanes 2 – 4 = ~500bp fragment representing the *hAPP*V717I transgene in sample transgenic mice. Lane 5 = known wild type control sample. Lane 6 = known transgenic control sample. Lane 7 = nuclease free water control.

Samples which did not produce a DNA band for *APP*(V717I) were analysed for *beta-globin* to confirm DNA presence using the sense primer 1 (5'-CCAATCTGCTCACACAGGATAGAGAGGGCAGG-3') and the antisense primer 2 (5'–CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG-3') giving a 500bp fragment. The master mix solution consisted of 2.5µl of 10x PCR buffer (Invitrogen), 0.75µl of 50x $MgCl_2$ (Invitrogen), 4µl of 10mM dNTPs (GE Healthcare), 1.25µl of primer 1 and 2 (Eurofins MWG Operon) at a concentration of 10pmol each, 0.0625µl of Taq DNA Polymerase (Invitrogen), 14.6875µl of DNAse- and RNAse-free H_20 was combined per sample, with 0.5µl of DNA. The thermocycling conditions were as follows: 35 cycles of 94°C for 30 seconds, 60°C for 90 seconds, 72°C for 120 seconds, followed by 10 minutes at 72°C and storage at 4°C. The amplified DNA product was separated and visualised in an identical manner to that described above. The visualised DNA products can be seen in Fig. 2.2.

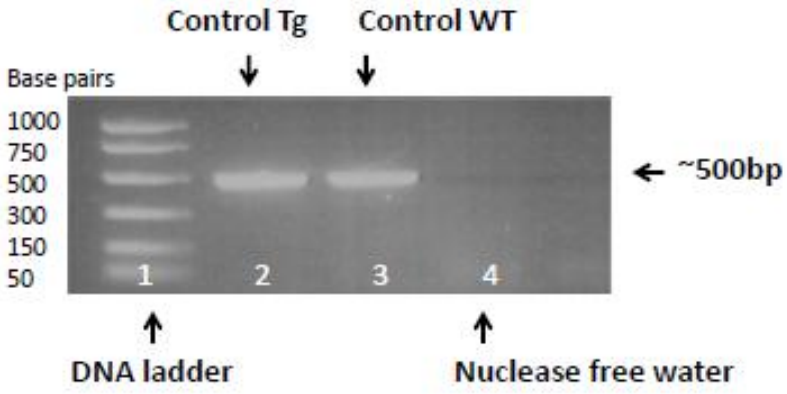

Fig. 2.2: Representative image displaying an electrophoresis gel of amplified genomic material from an APP(V717) transgenic and a wild type mouse. Lane 1 = DNA ladder displaying bp 1000 to 50. Lane 2 = ~500bp fragment representing the endogenous *beta-globin* gene in a known transgenic mouse sample. Lane 3 = known wild type control sample. Lane 4 = nuclease free water control.

Design

A longitudinal study of the level of anxiety-like behaviour exhibited by transgenic and wild type APP(V717I) mice at age points 3, 6 and 19 months was conducted. The assessment of anxiety using the elevated plus maze was conducted in parallel with measure of neophobia using the marble burying task. The order of administration of the EPM and the marble busying task was counterbalanced across subject groups. Both male and female mice were included at

all age points as a means of assessing whether the potential genotypic differences in marble burying performance were influenced by gender.

Subjects

A total of 21 heterozygous transgenic and 22 wild type London APP(V717I) mice on a C57Bl/6 inbred background strain were used (Moechars et al., 1999b), of which 19 were male (6 transgenic, 13 wild type) and 24 were female (15 transgenic, 9 wild type). All mice were tested at each time point, with the exception of 4 transgenic females which were tested at 3 and 6 months only. This was due to tissue collection and in one case, natural attrition of the colony. An experimental time line including sample size is presented in Figure 2.3.

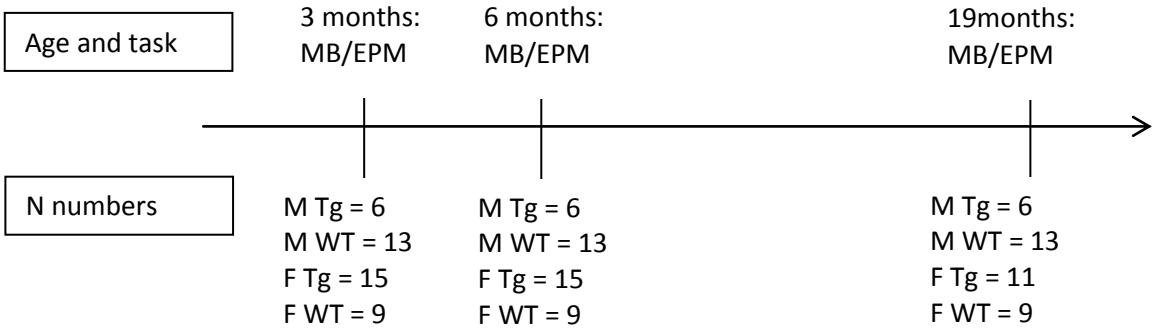

Fig. 2.3: Experimental time line indicating the age at which the marble burying (MB) and elevated plus maze (EPM) was carried out, as well as details of the sample sizes. The order of administration of the marble burying task and the elevated plus maze was counterbalanced at each age point. The overview is limited to the tasks discussed in the current chapter, and thus is not a full overview of the tasks administered to this cohort of mice.

General procedure of behavioural testing

All behavioural testing was carried out during the holding room light cycle. Mice were transported to test rooms in their home cages and allowed to acclimatise for at least 15 minutes prior to test initiation. Running order was counterbalanced for gender and genotype for all tasks. A camera was mounted in the ceiling of each test room and recorded all trials on a DVD recorder and monitor (VM-904K, Shiba Electrics Ltd, Hong Kong). The testing rooms contained a number of salient extramaze cues, and were lit from the ceiling. At every test point, the anxiety tests were performed in rooms novel to the animal. When tests made use of sawdust in the apparatus, this was not replaced between each mouse, but animal droppings

were removed and the sawdust raked by hand. In all other instances, the apparatus and test objects were wiped down with 70% ethanol wipes between trials.

2.3 Experiment 1a: Marble burying

2.3.1 Apparatus

In the marble burying task, mice were tested in an opaque box 13cm deep, 17.5cm long and 11cm wide. This was filled with 6cm of compacted sawdust on which 20 glass marbles (15mm diameter) were placed in a 4*5 pattern (see Figure 2.4). A transparent Plexiglas with ventilation gaps was placed over the box to avoid escape.

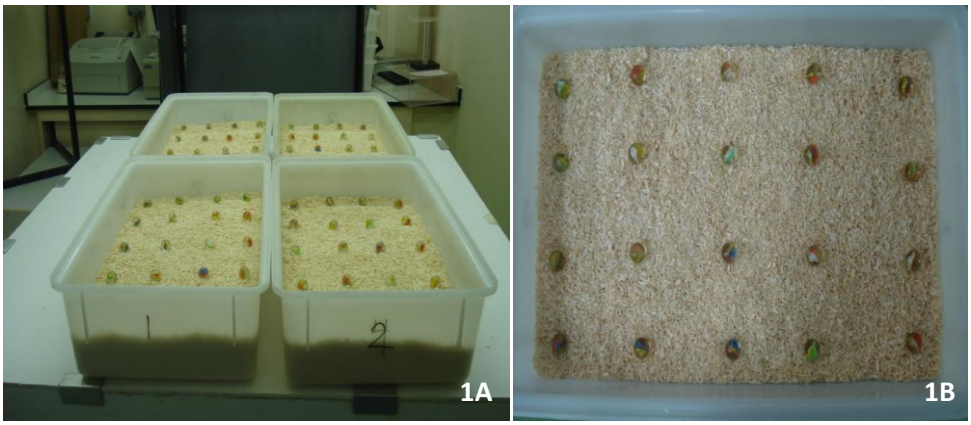

Fig. 2.4: 1A shows the marble burying set up within the test room. 1B shows the distribution of marbles within one test box.

Methods

Four mice were tested at a time in four boxes placed next to each other on a surface raised 1.1m above floor level for 30 minutes. The mice were placed in the middle of the box facing the eastern wall before the Plexiglas was fitted. None of the mice were able to see each other or the experimenter. Exposure times to the marbles were kept equal through initiating and completing trials in the same order. Marbles were not cleaned between trials unless obviously dirty.

Scoring and data analysis

Throughout the thesis, two-tailed significance testing was carried out with all data, with a significance value set at $p \leq 0.05$. Assumptions of Analysis of Variance (ANOVA) were tested and dealt with as detailed in the specific sections. For non-parametric analysis involving multiple independent variables, the alpha level was adjusted using the Bonferroni correction to avoid Type I errors.

In terms of Experiment 1a, the total number of buried and unburied marbles was recorded for each mouse upon their removal from the testing box. A buried marble was defined as at least two thirds of the marble covered with sawdust (Njung'e & Handley, 1991). The number of marbles buried was averaged across groups at each age point. The dependent variables at all ages were not normally distributed and violated the assumption of homogeneity of variance. As transformations did not aid in rectifying these violations, non-parametric Mann-Whitney U and Friedman's ANOVA analyses were used for data analysis.

2.3.2 Results

The average number of marbles buried by transgenic and wild type mice at the three different age points is presented in Fig. 2.5. Visual inspection suggests a lower number of buried marbles in the transgenic group compared to the wild type group. In order to test whether genotype affected the number of marbles buried at each age, a Mann-Whitney U test was carried out on the number of marbles at 3, 6 and 19 months with genotype as the independent variable. There was a significant effect of genotype at 3 months (U (21, 22) = 110.5, $p < .005$), 6 months (U (21, 22) = 88.5, $p < .0001$) and 19 months of age (U (17, 22) = 87.5, $p < .005$), with transgenic mice burying fewer marbles than wild type mice at all ages.

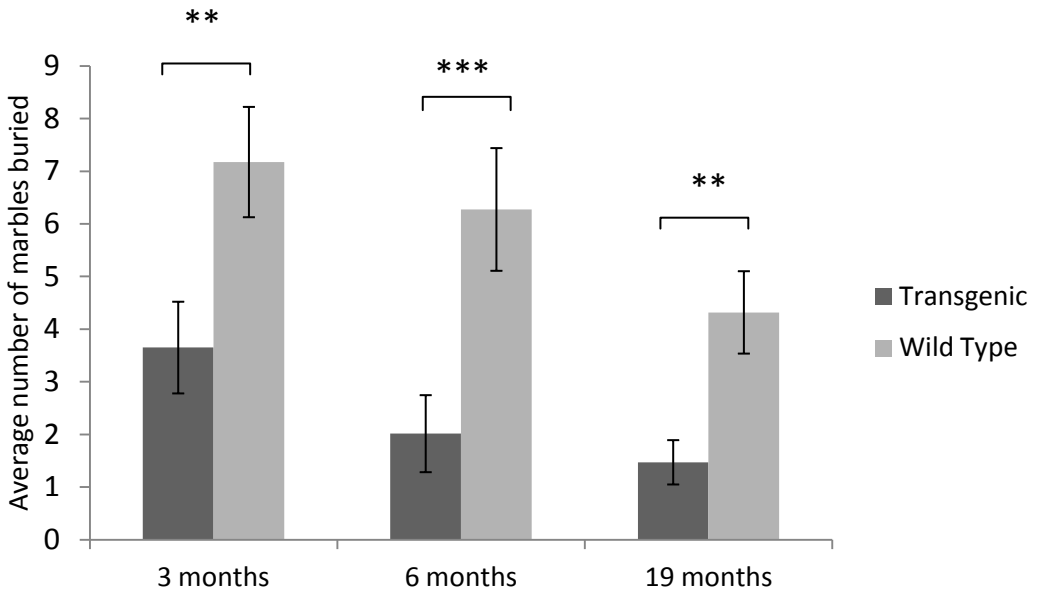

Fig. 2.5: The mean number of marbles buried for transgenic and wild type mice at ages 3, 6, and 19 months. ** = p<.005, *** = p<.001. Error bars ± 1 standard error of the mean (S.E.M.)

The average number of marbles buried by male and female mice of each genotype across the three age points is presented in Table 2.3. To test the effect of gender on the number of marbles buried, a Mann-Whitney U test with gender as the independent variable was performed. This revealed a significant effect of gender at 3 months (U (19, 24) = 76.5, p <.0001), 6 months (U (19, 24) = 141.5, p <.05) and 19 months of age (U (19, 20) = 99.5, p <.01), with males burying more marbles than females at all ages.

Age (months)	Gender	Wild Type		Transgenic	
		Mean	S.E.M.	Mean	S.E.M.
3	Male	**9.62**	1.25	**6.50**	1.80
3	Female	**4.00**	1.15	**2.43**	0.83
6	Male	**7.69**	1.74	**4.67**	2.06
6	Female	**4.43**	1.25	**0.88**	0.36
19	Male	**5.15**	0.68	**1.83**	0.83
19	Female	**3.11**	1.62	**1.27**	0.49
Mean	**Male**	**7.49**	0.79	**4.33**	1.01
	Female	**3.85**	0.76	**1.53**	0.36

Table 2.3: The mean and S.E.M. values for the number of buried marbles across gender and genotype at each age interval.

In order to assess the effect of genotype in the context of gender, the analysis was split by gender. In male mice, there was a significant effect of genotype at 19 months (U (6, 13) = 9.0,

p <.01), but not at 3 months (*U* (6, 13) = 21.5, *p* >.025) or 6 months of age (*U* (6, 13) = 32.0, *p* >.025), with transgenic males burying fewer marbles than wild type male at 19 months (see Fig. 2.6).

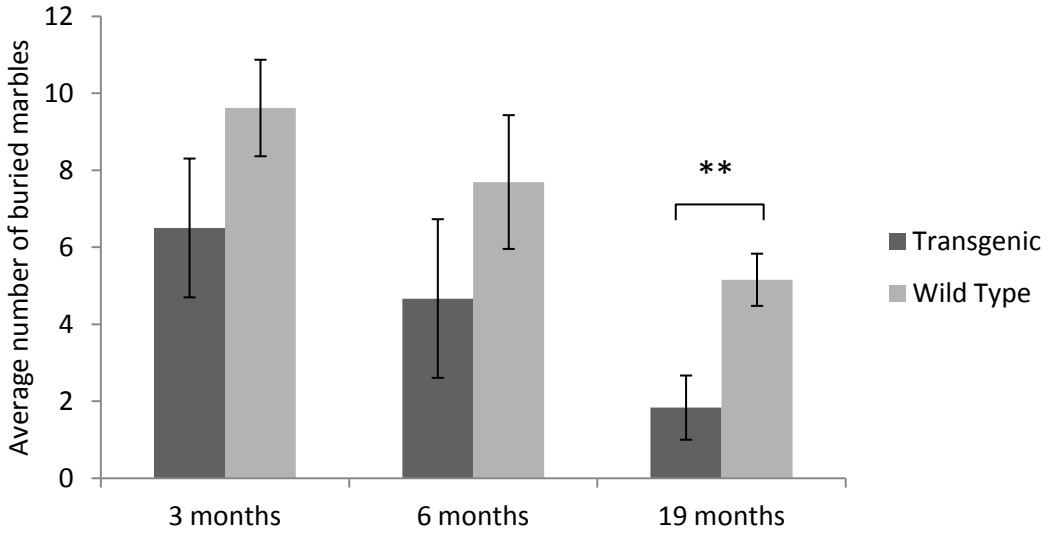

Fig. 2.6: The mean number of buried marbles for male transgenic and wild type mice at 3, 6, and 19 months of age. ** = *p* <.01. Error bars ± 1 S.E.M.

In terms of female mice (see Fig. 2.7), there was no significant effect of genotype on the average number of marbles buried at 3 months (*U* (9, 15) = 34.5, *p* >.025) or at 19 months of age (*U* (9, 11) = 40.0, *p* >.025), but at 6 months of age, female transgenic mice buried fewer marbles than wild type female mice (*U* (9, 15) = 9.0, *p* <.0001).

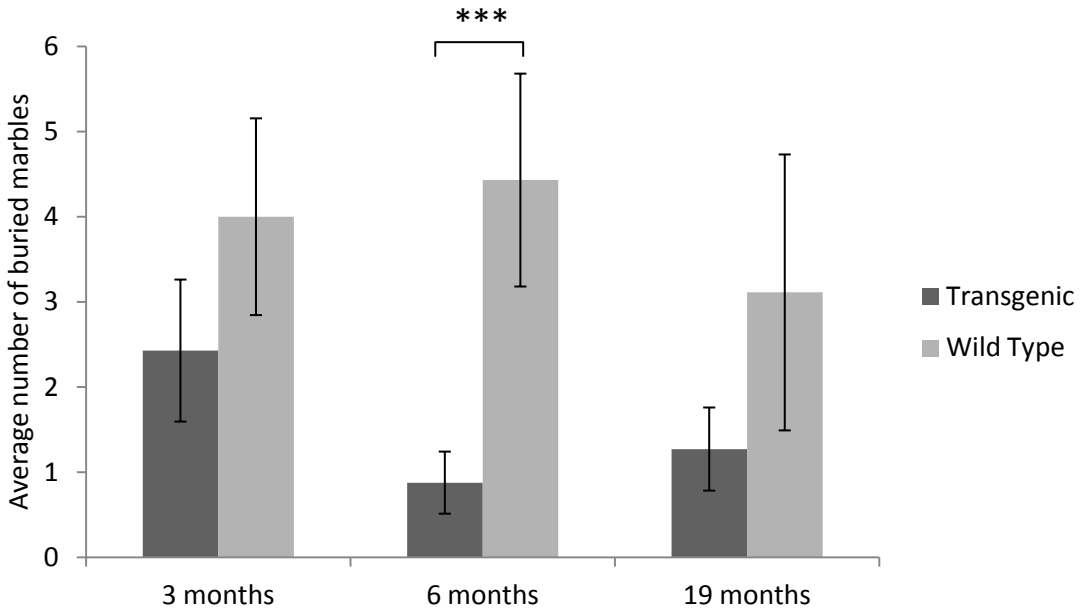

Fig. 2.7: The mean number of marbles buried for female transgenic and wild type mice at ages 3, 6, and 19 months. *** = $p < .001$ or more. Error bars ± 1 S.E.M.

In order to assess the effect of age on marble burying performance in the context of both gender and genotype, the data was split according to gender and genotype. The average number of marbles buried at each age was used as dependent variables in the non-parametric Friedman ANOVA test of repeated measures. As can be seen in Table 2.3, there was no significant difference between age points for any group [male wild type mice: ($\chi^2(2)=6.90$, $p > .0125$); male transgenic ($\chi^2(2)= 5.73$, $p > .0125$); female wild type ($\chi^2(2)= 2.53$, $p > .0125$); or female transgenic mice ($\chi^2(2)= 2.73$, $p > .0125$)].

2.4 Experiment 1b: EPM

2.4.1 Apparatus

The elevated plus maze consisted of four arms which are 70cm above floor level. The arms are 39.5cm long and 6.5cm wide, with a connecting area (6.5cm*6.5cm) in the meeting point between the arms (see Figure 2.8). Two of the arms (referred to as “closed arms”) are 16.6cm high and of dark material, whilst the other two arms (referred to as “open arms”) are 3cm high and of clear Plexiglas. The two open arms are positioned directly across from each other, as are the two closed arms.

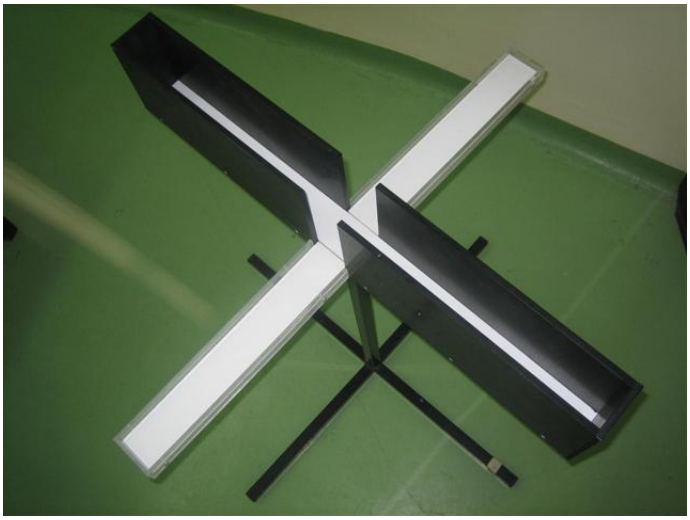

Fig. 2.8: The elevated plus maze free standing in testing room illustrating two open and two closed arms.

Methods

Mice were placed in the connecting area facing an open arm and left to explore the maze at their will for a period of 5 minutes based on literature suggesting this to be the optimum time period for detection of avoidance behaviours (Pellow et al., 1985). The experimenter was present in the room scoring the behaviour of the mice on a monitor facing away from the maze.

Scoring and data analysis

The level of anxiety each mouse exhibited was measured by the duration of time spent in the open and closed arms. This was manually scored whilst recording using EthoVision XT. An entry into an arm was defined as all four legs being positioned inside the arm. Similarly, exit out of an arm was defined as all four legs being positioned outside the arm. Information about the relationship between the amount of time spent in the open vs. closed arms of the elevated plus maze was captured by creating a composite variable expressing time spent in the open arms as a percentage of time spent in all arms using the formula [time in open arms/(time in open arms + time in closed arms)]*100. The data was analysed using repeated measures ANOVA.

2.4.2 Results

The percentage of time spent in the open arms as a percentage of time spent in any arm is presented in Fig. 2.9, and shows a preference for the closed arms in wild type mice which is not present in transgenic mice. This interpretation was confirmed by statistical analysis revealing a main effect of genotype (F (1, 35) = 9.9, $p < .005$), in which transgenic mice spent more time in the open arms (as a percentage of total time) than wild type mice. There was no significant between-subject effect of gender (F (1, 35) = 0.25, $p > .05$) or a gender*genotype interaction (F (1, 35) = 0.001, $p > .05$).

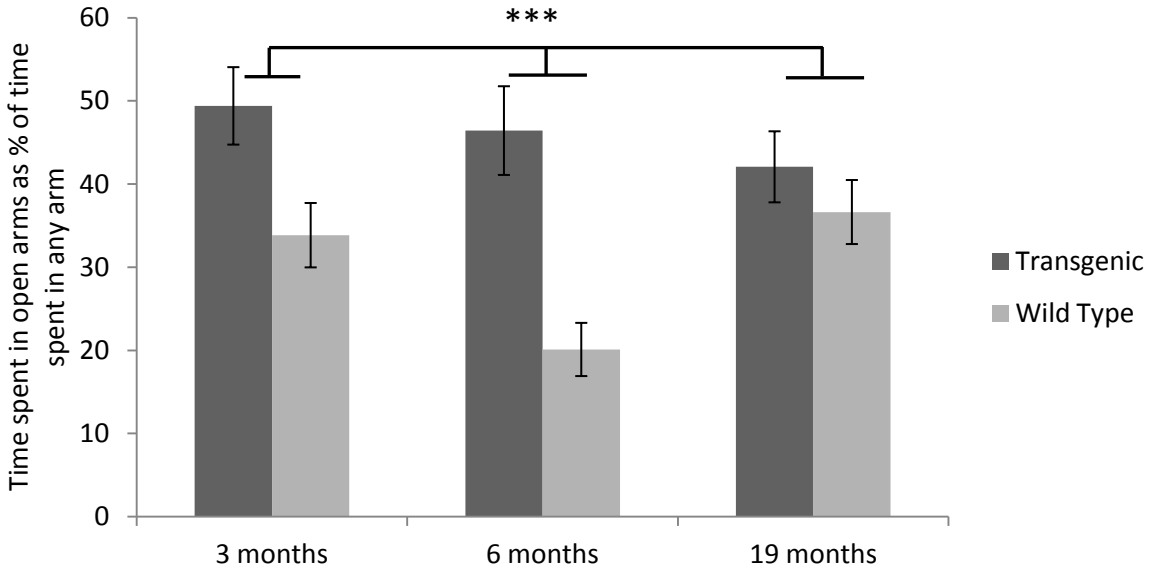

Fig. 2.9: The time spent in the open arms as a percentage of time spent in any arm for transgenic and wild type mice at ages 3, 6, and 19 months. Error bars ± 1 S.E.M. *** = $p < .005$.

In terms of the within-subject effects, there was an effect of age (F (2, 70) = 3.5, $p < .05$) on the time spent in the open arms as a percentage of time spent in any arm, driven by an increase in time spent in the open arms at 3 months compared to 6 months (F (1, 35) = 8.0, $p < .01$).

The means and S.E.M. values for the time spent in the open and closed arms are presented in Table 2.4, and mirror the pattern in the percentage data of a preference for the closed over the open arms in the wild type mice which was not present in the transgenic mice.

Age	Gender	Time in open arms (s)				Time in closed arms (s)			
		Wild Type		Transgenic		Wild Type		Transgenic	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
3	Male	**59.9**	8.4	**105.0**	19.6	**158.5**	9.9	**72.4**	22.7
3	Female	**92.9**	16.4	**102.8**	12.2	**139.9**	16.3	**114.6**	9.0
6	Male	**46.4**	6.1	**89.9**	29.6	**188.3**	11.1	**108.5**	26.9
6	Female	**42.9**	16.7	**104.0**	10.9	**183.5**	22.7	**112.8**	12.1
19	Male	**82.8**	10.9	**109.2**	28.2	**149.9**	10.9	**146.3**	30.2
19	Female	**93.6**	17.0	**104.5**	10.0	**151.4**	18.6	**148.3**	10.7
Mean	**Male**	**62.9**	5.5	**101.4**	14.3	**165.7**	6.6	**109.1**	16.3
	Female	**77.0**	10.4	**103.7**	6.6	**157.6**	11.3	**123.0**	6.8

Table 2.4: The mean and S.E.M. values for the time spent in the open and closed arms (s) across all gender and genotype groups at each age interval.

2.5 Chapter Discussion

The results from the longitudinal assessment of marble burying performance indicate a clear genotypic difference, with transgenic mice burying fewer marbles than wild type mice at 3, 6 and 19 months. In addition, a gender difference was identified, with male mice burying more marbles than female mice across genotype. Thus, transgenic mice are less anxious than wild type mice, with female mice showing the same tendency for reduced anxiety relative to male mice.

In terms of interactions between gender, genotype and age on marble burying behaviour, the general patterns of performance are stable across ages, despite only reaching statistically significant differences at certain age points. Female transgenic mice buried significantly fewer marbles than wild type mice at 6 months, but not 3 or 19 months of age. In contrast, male transgenic and wild type mice differed only at 19 months of age. The lack of significant genotypic differences within the genders at select age points may be influenced by a relatively high level of variance which appears to decrease with age. In addition, it is important to note the differences in sample size between gender/genotype groups, and the limitation this poses in terms of the conclusions one can draw regarding gender effects in the current study. Overall, transgenic mice display an anxiety-deficit compared to wild type mice of both genders, and this pattern of behaviour is present from 3 to 19 months of age with relatively little change (although see below for a discussion of the issue of repeated testing in anxiety assessments).

Although the marble burying task has been used as an assay of anxiety, recent evidence indicates that mice do not reduce their marble burying activity following habituation to the marbles in the home cage, following repeat testing or when given the opportunity to

avoid the marbles (Njung'e and Handley, 1991; Thomas et al., 2009). Indeed, mice buried a similar number of familiar and novel objects when comparing marbles to food pellets from the home cage. It has therefore been proposed that marble burying may be related to perseverative, obsessive-compulsive behaviour (Londei, Valentini & Leone, 1998; Albelda & Joel, 2011) or regulated by genetic background related to general digging behaviour (Thomas et al., 2009). It has been repeatedly established however that anxiolytic drugs such as diazepam reduce the number of marbles buried (Broekkamp et al., 1986; Njung'e and Handley, 1991; Ichimaru et al., 1995; Nicolas, Kolb & Prinssen, 2006). This suggests that neural pathways that are involved in anxiety-related behaviour are implicated in this task. It is therefore possible that marble burying is related to a complex set of behaviours, including anxiety-like behaviour, but in a way that is not driven by object novelty per se. To provide another index of anxiety APP(V717I) mice were also tested on the elevated plus maze.

The results from the EMP study show a clear genotypic difference with transgenic mice spending more time in the open arms than wild type mice. This indicates that transgenic APP(V717I) mice show reduced anxiety. This deficit was generally stable across age. There were indications of more variability in the wild type group, with an increase in anxiety from 3 to 6 months, and a numerical decrease in anxiety from 6 to 19 months. There were no contributions of gender to anxiety behaviour in the EPM. The results from the EPM experiment are clearly in accordance with those observed in the marble burying experiment. The stability of the reduction in anxiety-like behaviour in transgenic mice compared to wild type mice across two types of anxiety tests suggests that the tasks are assessing overlapping constructs.

The results of the behavioural characterisation of the APP(V717I) model contradict the published reports of increased anxiety APP(V717I) mice. Mutant mice showed a clear increase in anxiety behaviours across 1-12 months of age (Moechars et al., 1999b). The discrepancies between Moechars et al. (1999b) and the current experiments could be due to a number of factors. Elevated transgenic anxiety in Moechars et al. (1999b) was detected using an open field task to assess anxiety compared to the marble burying and EPM tasks used in the current study. Whilst the difference in tasks could account for the discrepancy, perhaps a more significant factor is the difference in the background strain (Moechars et al., 1999b; Crawley et al., 1997; Bucan & Abel, 2002; Wolfer & Lipp, 2000; Võikar et al., 2001). In Moechars et al. (1999b), the APP(V717I) model in maintained on an FVB/N background for the anxiety assessments, whilst a F1 hybrid generation of FVB/N and C57Bl/6 mice was used for the Morris

water maze task. When informally comparing the current cohort of APP(V717I) mice to Moechars et al. (1998; 1999b), it was notable that none of the reported aggression, seizures or premature death was observed in the current cohort of mice. It is therefore possible that the background change could have similarly affected the expression of anxiety phenotypes. Indeed, Võikar et al. (2001) found strain and gender differences on a battery of behavioural tests, including a stronger expression of anxiety in C57Bl/6 males than females when crossed into 129S2/SvHsd and tested in the elevated plus maze. Similarly, Holmes et al. (2003) report differential anxiety phenotype in serotonin transporter null mice when on different genetic backgrounds.

It is important to note that the effect of age on anxiety behaviour was confounded by repeated testing. As anxiety tasks, it is desirable for subjects to be unfamiliar with the task at the time of testing. Any effect of repeat exposure to the test was minimised through the use of novel test rooms at each test stage, as well as the delay of 3, then 13 months between testing points. It is however possible that the numerical reduction in genotypic difference at 19 months of age in both the marble burying and the EPM task is related to the higher level of handling and testing experience the cohort had undergone by this age in a series of other behavioural paradigms, in addition to potential habituation to the tasks themselves. However, there is a clear trend for the decrease in anxiety being stable both across age and tasks, suggesting that the transgenic anxiety deficit does not progress in parallel with Aβ pathology.

The assessments of anxiety in APP(V717I) mice was carried out with the aim of achieving a fuller phenotype of the model, as well as to investigate whether differences in anxiety exists between transgenic and wild type mice which may influence assessments of cognition. In terms of the translational value of behavioural assessments in mouse models of AD, it can be argued that measurements of cognitive performance are more relevant to the human condition than assessments of anxiety. Human patients are frequently found to suffer from elevated anxiety, and a high percentage of patients are on anxiolytic drugs like benzodiazepines (Blazer et al., 1991, Ownby & Carmin, 1998). The lack of consistency between the higher anxiety levels observed in patients and lowered anxiety levels as assessed in the marble burying task and EPM in the APP(V717I) model indicates that the tasks used measure different psychological constructs. Expressions of anxiety in patients could be influenced by an awareness of the progression of their disease and their own cognitive decline which is not possible to recapitulate in rodent models. Indeed, Harwood et al. (2002) identified a positive relation between the degree to which patients were aware of their cognitive deficits and

instances of depression and anxiety. Related to this, a high degree of disease awareness is related to elevated reports of hopelessness and feelings of life having reduced worth (Harwood & Sultzer, 2002). This highlights the need to view anxiety as assessed in mouse models of Aβ pathology as an important element of characterising the behavioural phenotype, with limited direct translational value in the context of immunotherapy.

When assessing transgene-related phenotypes in mouse models of AD-pathology, it is important to assess whether deficits are age-dependent or age–independent. As near zero levels of human Aβ can be detected at 3 months of age in this model (Dewachter et al., 2000), it is unlikely that the observed reduction in anxiety behaviour is related to an elevation in Aβ levels at this age. In addition, the anxiety deficit does not appear to exacerbate with age as Aβ levels increase. It remains possible therefore that the reduction in anxiety is related more to overexpression of hAPP than Aβ pathology. In this model, the *thy1* promoter drives stable hAPP expression from 2 weeks of age. Indeed, when comparing mice expressing non-mutant hAPP to both wild types and mice expressing the human APP(V7I7I) mutation, mice expressing any form of APP displayed increased anxiety and spatial learning deficits compared to non-transgenic mice (Moechars et al., 1999b). This underlines the ability of both non-mutant and mutant APP overexpression to affect anxiety-behaviours; although the mechanism(s) by which such overexpression of APP affects anxiety and cognition is unclear. Importantly, this phenotype is unlikely to be modified through a therapeutically induced change in Aβ levels, suggesting that anxiety-assessments may be unsuited to evaluate the effect 2B3 induced changes in behaviour mediated by reduced Aβ production. In order to identify an age-related cognitive phenotype in the APP(V717I) model which may be suited to assess the effect of 2B3 on cognition, the aim of the experiments reported in Chapter 3 were to characterise the onset and progression of cognitive changes in the APP(V717I) model.

Spatial and Object Recognition Memory in the London APP(V717I) Model

3.1 Introduction

The experiments reported in this chapter investigated the onset and progression of changes in spatial learning and memory the London APP(V717I) mouse model of Aβ pathology. The experiments aimed to establish the nature and developmental profile of spatial learning and object recognition memory.

As summarised in Table 2.2, Section 2.1.2, Chapter 2, APP(V717I) mice display a deficit in the Morris water maze at 3-6 months of age and a visual recognition deficit at the same age range (Dewachter et al., 2002). One interpretation of these results is that they may reflect structural or functional alterations in areas such as the hippocampus (spatial memory) and the perirhinal cortex (object recognition memory; Bannerman et al., 2003; 2004; Gray & McNaughton, 2000; Aggleton & Brown, 2006; Davis, 1998).

Given the results of the anxiety assessments, it is clear that in order to evaluate the effects of 2B3 on Aβ-related cognitive deficits it is first important to establish the cognitive profile of the V717I line. Earlier published reports of memory function in APP(V717I) mice have been conducted solely within the age range of 3 to 6 months. This relatively limited age range does not allow one to accurately pinpoint the age of onset and developmental profile of behavioural deficits. In addition, the discrepancies in anxiety-related behaviour between the published data and the findings reported in Chapter 2 raises the possibility that similar discrepancies could emerge in the cognitive phenotype. Similarly, the lack of information regarding the effect of gender on the APP(V717I) behavioural phenotype underlines the necessity of further characterisation of the model.

The aim of the experiments presented in this chapter was to provide a developmental profile of putative changes in cognitive function in London APP(V717I) mice. The experiments tested the hypotheses that the APP(V717I) mutation would lead to age-related deficits in spatial and object recognition memory. Both male and female mice were utilised to assess whether gender influenced the time of emergence of behavioural deficits or the degree of disruption to normal functioning. Moechars et al. (1999b) reported significant impairments in spatial learning in APP(V717I) transgenic mice using the Morris water maze at 3-6 months of age. In Experiment 3, spatial reference memory was examined using a radial arm water maze

task at 10 months of age, in which transgenic mice were hypothesised to display deficits in platform learning compared to wild type mice. The radial arm water maze was selected based on extensive evidence to implicate the hippocampus in successful performance in variations of spatial water maze tasks (Westerman et al., 2002; Hsiao et al., 1996; King et al., 1999). The hippocampus is throught to support episodic memory, an important cognitive process disrupted in AD patients (McKhann et al., 1984; Squire & Zola, 1998). Whilst the radial arm maze task does not directly assess episodic memory, the sensitivity of the task to hippocampal damage makes it relevant in the context of AD-related cognitive deficits. Furthermore, tasks sensitive to deficits in recognition of spatial locations in AD patients (Moss et al., 1986) have similiar task requirements to that of the radial arm maze task, although testing conditions vary significantly. In the context of preclinical work, the task has been used as a measure of cognitive dysfunction in APP mice (Gordon et al., 2001) that is sensitive to treatment intervention (Wilcock et al., 2004; 2006).

Experiment 4 and 5 assessed spatial working memory using a foraging task and the forced choice alternation non-matching to position T-maze task respectively. Performance on the non-matching to position paradigm tested in the T-maze is disrupted by hippocampal lesions (Deacon et al., 2002), and the task is sensitive to cognitive impairment in APP mutation mouse models (Chapman et al., 1999; Lalonde et al., 2002; Wriths et al., 2008; Corcoran et al., 2002). In terms of the relevance of spatial working memory paradigms to the cognitive profile of AD patients, it has been repeatedly demonstrated that short term or working memory impairments are widespread in individuals with AD (Baddely et al., 1991; Becker et al., 1988; Bellville et al., 2007; Grady et al., 2001; Moss et al., 1986). These impairments are often measured using delayed non-matching to sample paradigms which are similar to tasks like the T-maze in that sample information must be retained over a delay, prior to being utilised to identify the novel option (Grady et al., 2001). , The focus on visual stimuli in AD patient testing differs from rodent work in which spatial locations are to be recalled. Nevertheless, given the extensive medial temporal lobe, and in particular hippocampal, dysfunction in AD patients, the use of a delayed non-matching to position paradigm based on spatial information is highly relevant to AD (Squire & Zola, 1998).

In the foraging task (Pearce et al., 2005), mice were assessed longitudinally at 3, 6 and 19 months of age. The task is an adaptation of the radial arm maze which has been used extensively to assess spatial working and reference memory deficits in APP models (Dodart et al., 1999; Gordon et al., 2001). As such, it falls within the test battery relevant to working

memory deficits and hippocampal dysfunction in AD patients as discussed previously. Based on Moechars et al. (1999b), transgenic mice tested in the foraging task in Experiment 4 were predicted to perform at similar levels to wild type mice at 3 months of age, with a progressive decrease in spatial memory related performance compared to wild type mice evident at 6, and 19 months of age. In the T-maze at 11 months of age, transgenic mice were predicted to display a spatial working memory deficit. Object recognition memory at 18 months of age was assessed using novel object recognition in Experiment 6. Performance in the novel object recognition task relies on the perirhinal cortex (Aggleton & Brown, 1999), and offers an extension to the otherwise largely hippocampal-based task battery. Recognition memory deficits as observed in animal work has been suggested to be comparable to human semantic memory, defined as "knowledge for objects, concepts, faces and words" (Davies et al., 2004, p. 2441). AD patients have a well documented impairment in recognition memory, which applies to a variety of domains such as visual (including objects), spatial and verbal stimuli (Hof & Bouras, 1991; Moss et al., 1986), and perirhinal volume has been found to correlate positively with semantic performance (Davies et al., 2004). Experiment 6 tested the prediction that transgenic mice would show inferior discrimination of the novel and familiar objects after a 3 hour delay, but not after a 1 hour or 5 minute delay as demonstrated in Dewachter et al. (2002). An overview of the order of experiments and the sample size for APP(V717I) mice is presented in Fig. 3.1.

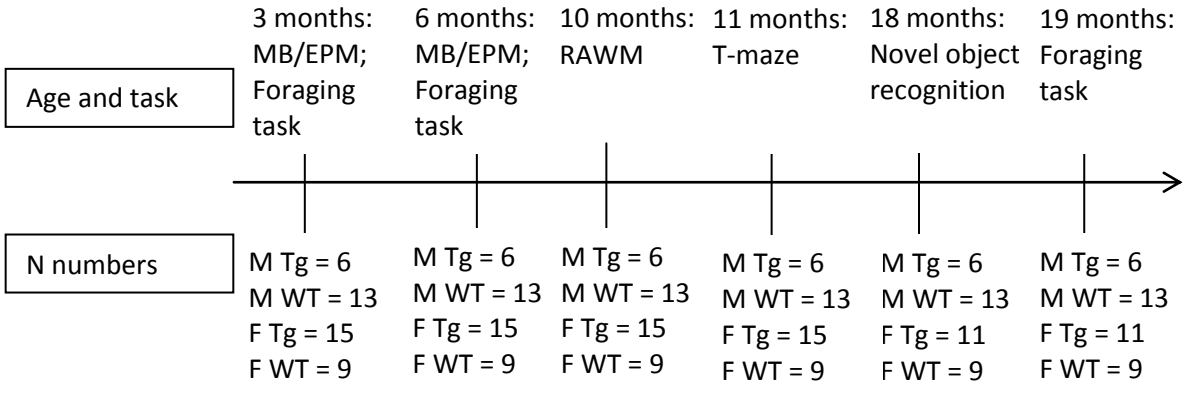

Fig. 3.1: Experimental time line indicating the age at which each test carried out in APP(V717I) mice, as well as details of the sample sizes. The overview includes the tasks described in Chapter 2, and thus provides a full overview of the tasks administered to this cohort of mice. At 3 and 6 months of age, the anxiety tasks were administered before the foraging task. The hippocampal lesioned mice are not included, but were assessed on the RAWM prior to the T-maze.

3.2 Experiments 3a and 3b: Hippocampal lesioned and APP(V717I) mice on the Radial Arm Water Maze

The radial arm water maze (RAWM) is an alternative version of the widely used Morris water maze. In a pool divided into 6 arms, mice are released from varying start arms and are required to learn the location of a stable escape platform in one goal arm. The task has been used to test spatial reference memory in several transgenic AD mouse models (Gordon et al., 2001; Wilcock et al., 2004; Wilcock et al., 2006) as well as in rats (Diamond, Park, Heman & Rose, 1999). As disruption to the hippocampus has been shown to disrupt spatial learning in C57Bl/6 mice in the Morris water maze and the radial arm maze (Aggleton & Brown, 1999; Logue, Paylor & Wehner, 1996; Cho, Friedman & Silva, 1998; Gerlai, 1998; Deacon, Bannerman, Kirby, Croucher & Rawlins, 2002), it is reasonable to predict that the RAWM is sensitive to hippocampal dysfunction. In order to confirm that the RAWM test procedure was sensitive to alterations in hippocampal function a preliminary study (Experiment 3a) was conducted with hippocampal lesioned mice prior to using the procedure on APP(V717I) mice (Experiment 3b). It was hypothesised that APP(V717I) mice would show impaired performance in the RAWM compared to littermate controls. In terms of gender, no direct information regarding male and female differences in APP(V717I) mice in spatial learning is available, but information from studies such as Clinton et al. (2007) suggest that female transgenic mice show poorer spatial learning in stressful conditions such as the Morris water maze compared to male 3xTg mice. It is therefore possible that APP(V717I) mice will show a similar impairment in spatial learning compared to males.

3.2.1 Design

Assessment of the effect of the APP(V717I) mutation on spatial reference memory was carried out at 10 months of age. Both genders were included in order to assess whether gender affects the expression of a transgenic phenotype in the RAWM. The effect of bilateral hippocampal lesions on spatial reference memory was established prior to evaluating APP(V717I) mouse performance.

Subjects

Experiment 3a: Fifteen male C57Bl/6 mice aged 5 months were tested in the RAWM, of which 7 mice had bilateral hippocampal excitotoxic lesions surgery 3 months prior to

behavioural assessment. Prior to administration of the RAWM task, mice had undergone extensive operant learning training to assess responding when cued ambiguously compared to unambiguously using Med Associates testing chambers. One mouse was excluded based on the histology indicating that the excitotoxic lesion to the hippocampus was restricted to unilateral damage. Thus, the behavioural data presented is based on 6 mice with bilateral hippocampal lesions and 8 non-surgical controls.

Experiment 3b: The subjects were those described in Chapter 2, Section 2.2, with 14 female transgenic APP(V717I) mice.

Apparatus

The RAWM consisted of 6 dark Perspex arms connected by a 37cm wide central area elevated 77cm above the floor (Figure 3.2). The maze was 28.3cm deep, with arms of 29cm length and 17.3cm width. A platform with a weighted dark plastic base (11.5cm*8.9cm) with a clear Perspex platform (17cm*8cm, 1cm thick) elevated to the total height of 19cm by a clear plastic rod was provided as a means of escaping the water for the mice. A visual cue consisted of a laminated white card (16.8cm*8.2cm) attached to the back wall of the goal arm 0.5 cm above the water line. The maze was filled 21cm high with 25-29^{o}C opaque water (~1% milk). The water was at approximately 1cm above the top surface of the platform to ensure that the platform was invisible to the mouse. Trials were video recorded.

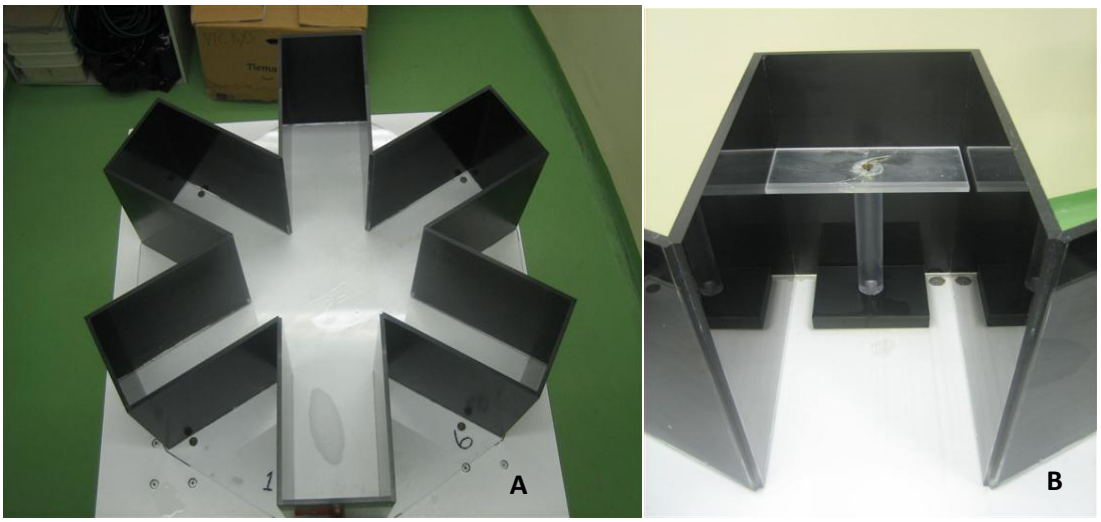

Fig. 3.2: Radial arms water maze apparatus without water and escape platform in A, with escape platform featured in B.

Method

Surgery

Mice were anaesthetised with Isoflurane [2-chloro-2-(difluromethoxy)-1, 1, 1-trifluoro-(ethane)] in O_2 during stereotaxic surgery. The skull was exposed by a scalp incision. Two holes were drilled on opposite sides of the midline at the appropriate coordinates (see Table 3.1). Three injections of 63mM ibotenic acid (IBO, Biosearch Technologies, USA) in PBS at a rate of 0.3µl per minute were made in each hemisphere using a 30G cannulae microinjection 5µl Hamilton #75 syringe (Hamilton Company, Reno, USA). Following each infusion, the needle was left in place for 1 minute before being retracted slowly. Upon completion, the wound was sutured and the animal was given a subcutaneous injection of glucosaline to aid rehydration. It was then placed in a 30^{o}C temperature controlled recovery chamber under monitoring until deemed alert and mobile. Once alert, the animal was place in the home cage with soaked diet and 500mg paracetamol added to the drinking water. Mice were perfused at 22 months.

Perfusion

Mice were given a 0.1ml intraperitoneal (IP) injection of 200mg/ml pentobarbital (Euthatal, Merial, Harlow, UK) to induce deep anaesthesia. Through the insertion of a cannula into the left cardiac ventricle, approximately 50ml of 0.1M PBS (pH 7.4) was pumped through the circulatory system. This was followed by approximately 100ml of 4% paraformaldehyde in 0.1M PBS (formaldehyde, FA). Following extraction of the brain, it was post fixed in 4% FA at 4^{o}C for 8 hours before being transferred to 30% reagent grade sucrose in dH_2O. The brain was left in sucrose until saturated indicated by it sinking (approximately 48 hours). Using a freezing microtome, 40µm coronal sections of brain tissue were cut and mounted on gelatinised slides in 0.1M PBS.

Four colleagues at Cardiff University kindly assisted in this work. The bilateral hippocampal lesions described were performed by Prof. Good. Perfusions of lesioned mice and controls were carried out by Dr. Reichelt, whilst the histological processing was performed by Mr. Anderson. In addition, Ms. Peatson assisted with the behavioural testing.

Site	Stereotaxic coordinates			
	Anterior-Posterior (-)	Lateral (±)	Ventral (-)	Volume (µl)
1	1.9	1.6	1.8	0.10
2	2.5	3.0	3.0	0.15
3	3.0	3.2	3.5	0.15

Table 3.1: The stereotaxic coordinates for bilateral hippocampal lesions outlined as mm from bregma (anterior posterior), from the midline (lateral) and from the skull surface (ventral).

Cresyl violet staining

Sections were dried at 20^{0}C for 24 hours before being stained. Staining involved immersing sections in xylene for 4 minutes before descending concentrations of ethanol was applied for 2 minutes each (100%, 90%, 70%) starting with 4 minutes in 100% alcohol. After 2 minutes in deionised water, 0.005% Cresyl violet was applied for 2 minutes, followed by 30 seconds in deionised water. An ascending series of alcohol was applied for 2 minutes each (70%, 90%, 100%), followed by an additional 2 minutes in 100% alcohol and two exposures to xylene, again of 2 minutes duration each. Slides were dehydrated in an ascending concentration of ethanol for 2 min each (50%, 70%, 90%, 100%, 100%) before being submerged in xylene for 4 minutes. Finally, slides were coverslipped with DPX Mounting media and air dried for 48 hours before sections were visualised using a Leica DMRB microscope. Images were captured using an Olympus DP70 camera and the programme analySIS-D.

Behavioural procedure

The procedure was adapted from Wilcock et al. (2004; 2006). The mice were given 4 days of testing, with 12 trials on each day and an inter-trial interval of 10 minutes. On the first day of testing, the platform was visually cued with white card on half of the trials. Each trial required the mouse to swim to the centre of the maze following release from a start arm, before making a choice to swim into any of the 6 arms. If the mouse made an incorrect choice (i.e. an arm where the platform was not located), the experimenter gently pulled the mouse back to the start arm by the tail and then released the mouse. A trial was either concluded when the mouse located the platform, or when 1 minute had passed without the platform having been located. In the latter case, the experimenter guided the mouse to the platform and allowed the mouse 30 seconds on the platform to enable the animal to encode the extra maze environment. The mouse spent the inter trial interval in a heated box set at 37^{o}C to avoid hypothermia. The location of the platform was kept constant for each mouse across all trials,

but was counterbalanced between two locations across groups. On each trial, the mice were released from a different start arm, all of which were counterbalanced across groups. On day 5, a probe trial was administered where the platform was removed, and the mouse was permitted to explore the maze freely for 1 minute.

Scoring and data analysis

An error was defined as the mouse entering an arm that did not contain the platform. Entry into an arm was defined as the point at which the back of the mouse (excluding the tail) crossed into the arm. Errors were counted manually by the experimenter, and noted down at the conclusion of each trial. An additional error was awarded if the mouse failed to locate the platform in the 1 minute available. During the probe trial, the amount of time spent in each arm as well as the number of entries into each arm was scored manually from video recordings. The error score obtained on each trial was averaged across each test day for each mouse, before the scores were averaged across test days to create a composite variable of total error average. For the probe trial, a score was calculated to represent the time the mouse spent in the goal arm as a percentage of time spent in any arm.

3.2.2 Results: Experiment 3a: Hippocampal lesioned mice

Histology

The maximum and minimum tissue damage obtained as a result of excitotoxic lesions is displayed in Fig. 3.3. In 4 lesioned animals, the entire hippocampus was removed. In 3 lesioned animals, the hippocampus was removed completely with the exception of the dorsal dentate gyrus bilaterally. There was also some (mostly unilateral) sparing of the ventral subiculum and CA1 in these 3 mice. Some cortical damage around the infusion site was evident in 5 out of 6 lesioned mice, predominately in the somatosensory and retrosplenial cortex, with some damage to the visual cortex. No hippocampal damage was present in any non-surgical control mice. Some cortical damage was evident, but as this was not focused around the craniotomy site, it is likely to be related to mechanical damage during tissue sectioning.

Maximum and minimum lesion size

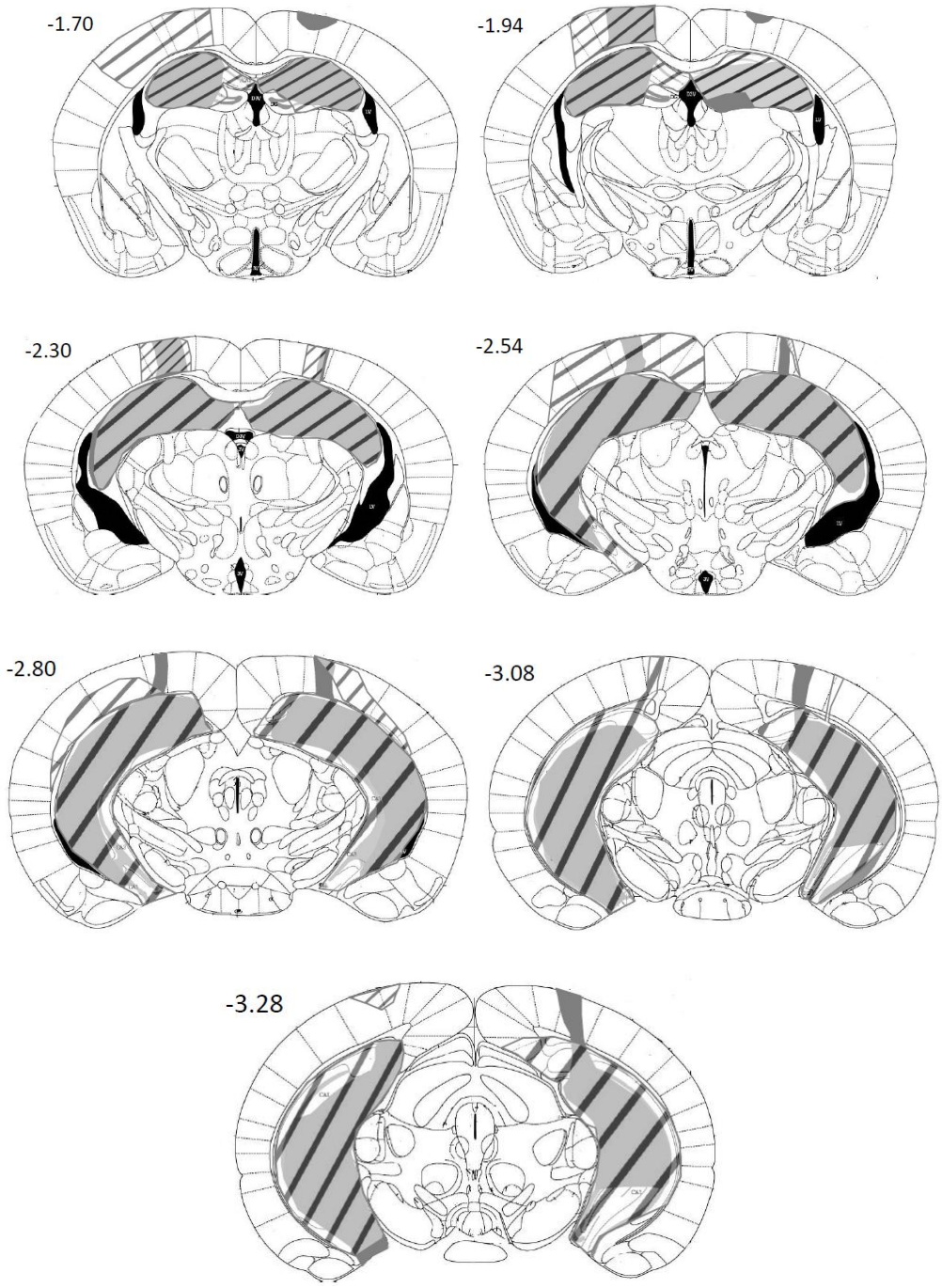

Fig. 3.3: The level of damage to the hippocampus and cortical areas around the infusion sites in hippocampal lesioned mice is summarised by the shading of the largest and smallest lesions in the cohort. Maximum sized lesion is indicated in striped grey, with the minimum sized lesion in dark grey. Each section is denoted as the distance from posterior from bregma (mm).

Behavioural results

Visually cued trials in hippocampal lesioned mice

There were no significant differences between lesioned and non-lesioned mice in the average errors on visually cued trials (F (1, 13) = 2.87, p > .05, see Fig. 3.4A) or in the average latency to complete visually cued trials (F (1, 13) = 2.50, p > .05; see Fig. 3.4B).

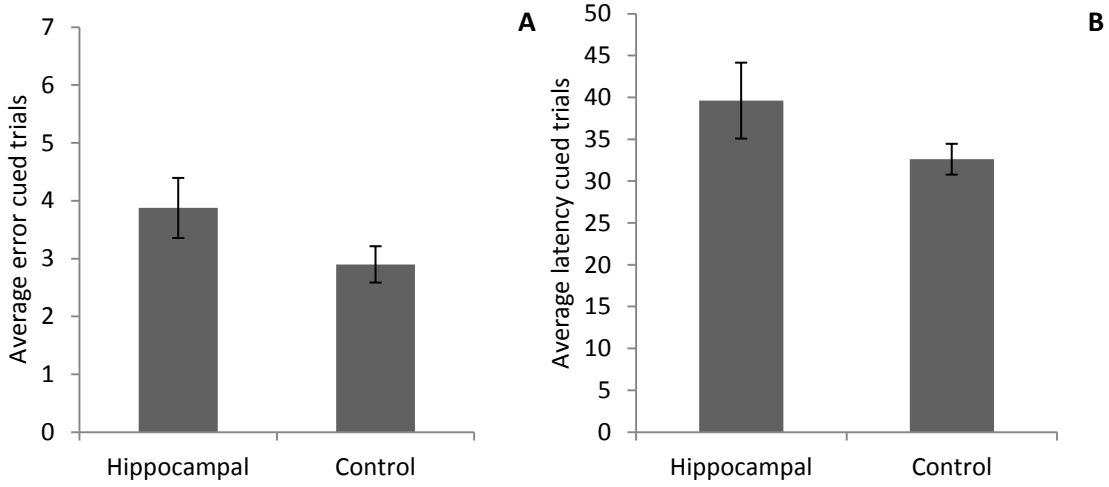

Fig. 3.4: Mean error (A) and latency (B) across visually cued trials on test days 1 – 4 for hippocampal lesioned and non-surgical control mice on the RAWM task. Error bars are ±1 S.E.M.

Error and latency in hippocampal lesioned mice

There was a significant main effect of test day (F (3, 39) = 9.51, p < .0001) in addition to hippocampal lesioned mice making significantly more errors across test days than non-lesioned mice (F (1, 13) = 27.23, p < .0001; see Fig. 3.5A). Hippocampal lesioned mice had higher latency scores than non-lesioned mice (F (1, 13) = 6.37, p < .05; see Fig. 3.5B).

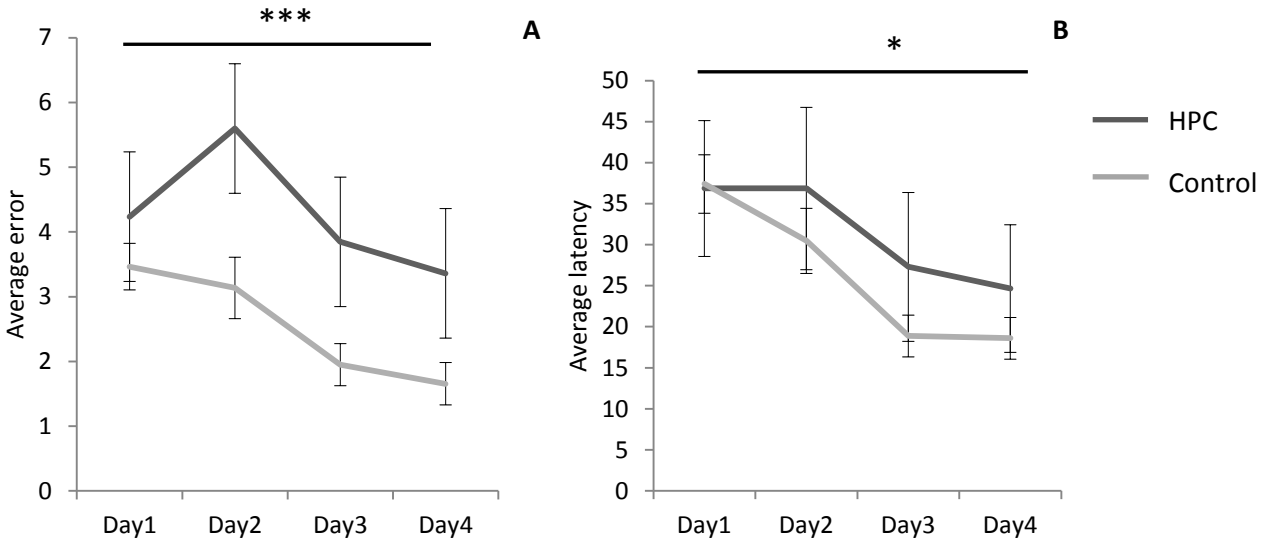

Fig. 3.5: Mean error (A) and latency in seconds (B) across trials on test days 1 – 4 for hippocampal (HPC) lesioned and non-surgical control mice on the RAWM task. The first test day does not include visually cued trials and is thus half the number of trials as the subsequent days. Error bars are ±1 S.E.M. * = $p < .05$, *** = $p < .0001$.

Results: Experiment 3b: APP(V717I) mice

Visually cued trials

On visually cued trials (see Fig. 3.6), there was no significant difference between transgenic and wild type mice on error scores (F (1, 38) = 0.02, $p > .50$), no effect of gender (F (1, 38) = 0.002, $p > .50$) and no gender*genotype interaction (F (1, 38) = 0.02, $p > .50$). This pattern was upheld when analysing latency scores on visually cued trials [genotype (F (1, 38) = 0.19, $p > .05$), gender (F (1. 38) = 1.49, $p > .05$), genotype*gender (F (1, 38) = 0.66, $p > .05$)].

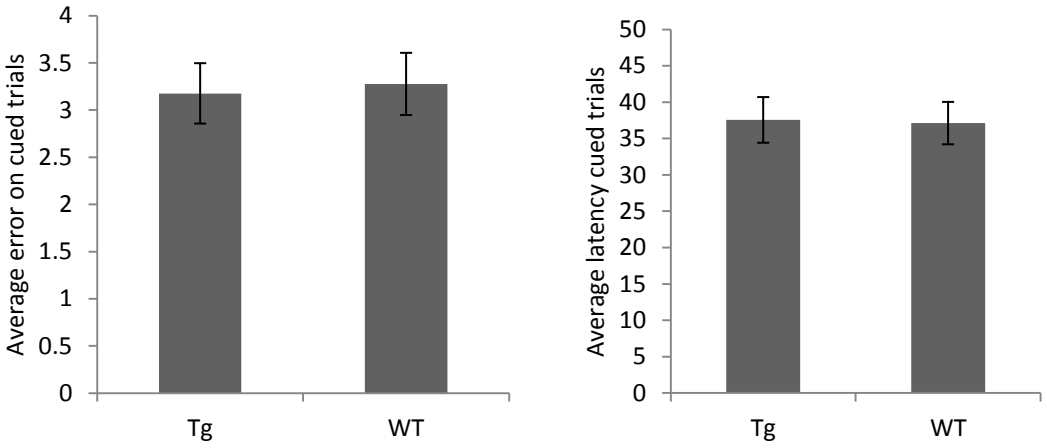

Fig. 3.6: Mean error and latency across cued trials for transgenic and wild type mice. Error bars ± 1 S.E.M.

Error and latency

Fig. 3.7A indicates that wild type and transgenic mice acquired the water maze task at the same rate. Indeed, ANOVA analysis confirmed that there was no significant main effects of genotype (F (1, 38) = 0.03, $p > .50$) or gender (F (1, 38) = 0.63, $p > .50$) on average latency across test daysAn effect of test day (F (3, 114) = 20.48, $p < .0001$) indicates learning across sessions. Additionally, there was no effect of genotype (F (1, 38) = 0.01, $p > .50$) or gender (F (1, 38) = 1.09, $p > .50$) on error scores across test days. Genotype and gender did not significant interact on either measure [latency (F (1, 38) = 2.04, $p > .50$), error (F (1, 38) = 2.04, $p > .50$)].

Probe trial

Fig. 3.7B shows the percentage time spent in the goal arm during the probe trial. This figure indicates that there was no difference between wild type and transgenic mice. This was confirmed by an ANOVA that revealed no significant main effects of genotype (F (1, 38) = 0.29, $p > .50$) or gender (F (1, 38) = 1.58, $p > .50$) and no significant interaction between these factors (F (1, 38) = 2.0, $p > .50$).

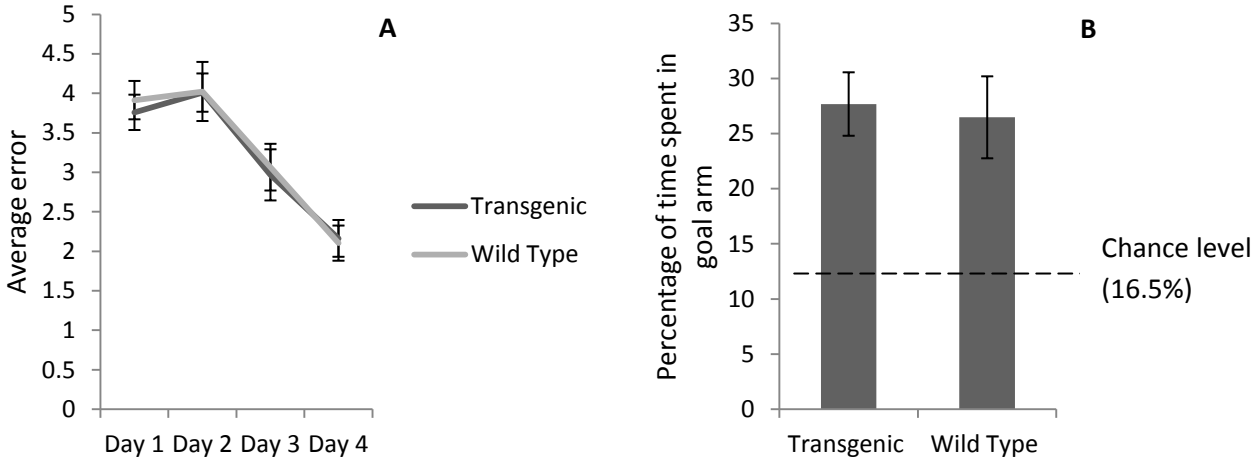

Fig. 3.7: A displays the mean errors made by transgenic and wild type within each test day. B displays the mean percentage of time spent in the goal arm during the probe trial. Error bars ± 1 S.E.M.

Due to the lack of effect of gender on these measures, the data presented in Fig. 3.7 have been collapsed across gender. For information however, average values split by gender and genotype is presented in Table 3.2

Genotype	Gender	Mean error		Mean latency		Probe trial (%)	
		Mean	S.E.M.	**Mean**	**S.E.M.**	**Mean**	S.E.M.
Transgenic	Male	**3.58**	0.38	**28.10**	1.39	**27.10**	3.89
Transgenic	Female	**3.07**	0.14	**24.77**	0.84	**27.92**	3.85
Wild Type	Male	**3.22**	0.25	**31.78**	1.47	**31.74**	5.17
Wild Type	Female	**3.35**	0.21	**28.17**	2.15	**19.24**	4.45
Mean	**Transgenic**	**3.38**	0.31	**26.84**	1.07	**25.73**	3.48
	Wild Type	**3.43**	0.28	**30.33**	1.30	**23.57**	3.68

Table 3.2: The mean and S.E.M. values for the mean latency and errors made across test days and the percentage of time spent in the goal arm during the probe trial presented split by gender and genotype.

3.2.3 Discussion

The performance of C57Bl/6 mice with bilateral hippocampal lesions compared to controls indicate that intact hippocampal functioning is necessary in order to acquire the platform location in the RAWM. Whilst the error scores of control mice decline with training, the error score of hippocampal lesioned mice is comparable on day 1 and day 4 of testing. The deficit in RAWM performance in hippocampal lesioned mice compared to controls is not present when the platform is cued, suggesting that the deficit is specific to the spatial learning requirements of the task. Therefore, the lack of deficit observed in APP(V717I) transgenic mice at 10 months compared to littermate controls indicates intact spatial reference memory and uncompromised hippocampal function.

This latter finding is in contrast to previous reports of a spatial reference memory deficit at 3-6 months of age (Moechars et al., 1999b; see Chapter 2, Section 2.5 for a discussion of the role genetic background may play). The lack of evidence of a spatial memory deficit in transgenic mice at 10 months of age suggests that this aspect of cognitive function is not affected by either APP over-expression or elevation of Aβ levels at this age. It is unclear what levels of soluble and insoluble Aβ are detectable in the central nervous system (CNS) of transgenic APP(V717I) mice by 10 months of age, but there is some evidence of soluble Aβ at younger ages indicating that at least trace levels are present (see Chapter 2, Section 2.1.2, Table 2.1). The absence of a behavioural deficit on this task may be due to insufficient Aβ pathology. Alternatively, it is equally possible that the task may be insensitive to subtle alterations in Aβ-induced synaptic deficits in APP(V717I) mice. The following set of

experiments was conducted to assess whether transgenic APP(V717I) mice display deficits on other spatial procedures, such as age-related deficits in spatial working memory using a foraging task (Experiment 4) and a forced choice non-matching to position T-maze task (Experiment 5).

3.3 Experiment 4: APP(V717I) mice assessed using the foraging task

3.3.1 Introduction

The foraging task is a spatial working memory task (Pearce et al., 2005), where a series of spatial locations are baited with rewards that can be retrieved in any order. Each location is always baited at the start of each trial. Efficient completion of the task requires the use of continuously updated information regarding which locations have been depleted of rewards at any given time during testing. The task is an adaptation of the 8-arm radial arm maze where animals are required to visit the baited half of the arms to obtain reward, but avoid the half of arms which are never baited (Olton & Samuelson, 1976; Olton & Papas, 1979; Floresco, Seamans & Phillips, 1997).

3.3.2 Method

Design

The longitudinal assessment of spatial working memory as measured in the foraging task was conducted in order to assess whether the presence of the APP(V717I) mutation led to higher error scores for transgenic mice than wild type mice, and whether such a deficit was age-dependent.

Subjects

The subjects were those described in Chapter 2, Section 2.2.

Apparatus

A 102cm*102cm sized arena with 45cm high walls painted a dark grey was filled with loose sawdust to a depth of approximately 2cm. The arena was placed on the floor, and contained

eight 3.5cm high circular pots with a diameter of 7.7cm placed in an hour-glass formation (Fig. 3.8). The pots were filled 3cm high with sawdust, and a coco pop reward [Kellogg's; rice, sugar and chocolate (4%)] placed in the centre of the pot, 1 cm from the bottom. Trials were DVD recorded and tracked using EthoVision XT (Noldus).

Fig.3.8: The foraging task arena with 8 baited pots arranged in the standard pattern.

Method

Pretraining and habituation

Testing was carried out in the same test room at each longitudinal testing point. Mice were food deprived to 90% of their body weight over approximately 2 weeks prior to testing in the arena. During this time, mice were individually trained to forage a baited pot in cages identical to their home cage. Each mouse was given three such attempts daily until proficient. The mice were habituated to the empty arena through 10 minutes of free exploration, followed by habituation involving exposure to the arena with two baited pots. Mice were habituated to the two pots until successfully foraging in both within 2 minutes. The number of habituation days required at 3 months of age was 4 days, whilst 2 days of habituation was sufficient at 6 and 19 months of age. On the first two days, the habituation duration was fixed at 10 minutes, regardless of when the pots were foraged. On any additional habituation days following that, habituation trials were terminated upon both pots being foraged.

Test phase

At test, the mice were placed in the middle of the arena facing north side of the maze. Mice were required to visit all pots and retrieve the reward in order for the task to be completed. If the task was not completed within 10 minutes, the experimenter ended the trial. Mice were tested over 4 consecutive days, with one trial on each day. The experimenter was present in the room scoring the performance on a monitor, facing away from the arena.

Scoring and data analysis

The dependent variable recorded in the test phase included the number of errors, return errors and consecutive errors. The detail regarding the coding of these errors is summarised in Table 3.3. Following testing, mice had individual data points excluded if during that trial, a pot was foraged in a way where the reward fell onto the arena surface.

Outcome measure	Definition	Example (error highlighted in bold)
Error	Jumping onto the edge of a pot from which the reward has previously been consumed and displacing sawdust through digging	Consume reward in pot 2, leave pot 2, **return to pot 2 and make an error**
Repeat error	Making an error as described above in a pot where (an) error(s) has/have previously been made	Consume reward in pot 2, leave pot 2, return to pot 2 and make an error, consume reward in pot1, **return to pot 2 and make an error**
Consecutive error	Making an error as described in "return error" in the same pot two or more times without making errors in other pots in the intervals between those errors	Consume reward in pot 2, leave pot 2, return to pot 2 and make an error, **return to pot 2 and make an error, return to pot 2 and make an error** (this would be counted as two consecutive errors)

Table 3.3: Overview of the types of errors recorded in the foraging task in terms of definitions and examples of implementation.

Perseveration, the repeated responding despite a lack of initial and/or further reinforcement, has been linked to hippocampal dysfunction (Dalland, 1976) and is commonly observed in Alzheimer's patients (Pekkala et al., 2008) as well as transgenic APP mutation mice models

(Kobayashi & Chen, 2005). Spatial perseveration was measured by identifying the subset of errors in which returns to an incorrect location were made (see Table 3.3). The number of each type of error made by individual mice was averaged across test days, before being averaged across gender/genotype groups at each age point. In addition to these "total" task error scores, it is possible to analyse the error scores made in the first half of the task as an indication of performance under simplified test conditions, in which task difficulty is lowered due to the high likelihood of encountering a baited pot by chance. The end of the first half of the task was defined as the point at which the 4th reward out of 8 was retrieved, at which point engaging with a baited pot was above 50% (the "first half of the trials"). In contrast, the more challenging phase of the trial was defined from the 4th reward consumption until task completion, where the probability of engaging with a baited pot was below 50% (the "second half of the task".

3.3.3 Results

A repeated measures ANOVA was used to analyse the number of errors as defined in Table 3.4. Repeat and consecutive error measures were analysed using Mann Whitney U and Wilcoxon Signed Ranks for between and within-subject analyses respectively, as these variables violated the assumptions of normality and heterogeneity of variance. These violations were not successfully rectified following transformation. For simplicity of presentation, only data for which significant group differences were observed are graphically presented. Mean and S.E.M. values for all conditions are presented in Tables 3.5-3.7.

Error

The overall number of errors averaged across test trials is shown in Fig. 3.9 (and Table 3.4). A repeated measures ANOVA conducted on the data from which these means were derived revealed a significant within-subject effect of age (F (2, 68) = 3.44, p < .05), reflecting an overall increase in error scores across groups from 3 to 19 months of age. There were no within-subject interactions between age, gender and genotype [Age*Gender; F (2, 68) = 1.07, p > .05; Age*Genotype; F (2, 68) = .56, p > .05; Age*Gender*Genotype; F (2, 68) = .24, p > .05)]. There was a significant between-subjects effect of gender (F (1, 34) = 6.75, p < .05) with females making more errors than males, but no main effect of genotype (F (1, 34) = 3.40, p > .05) or a gender*genotype interaction (F (1, 34) = 1.36, p > .05).

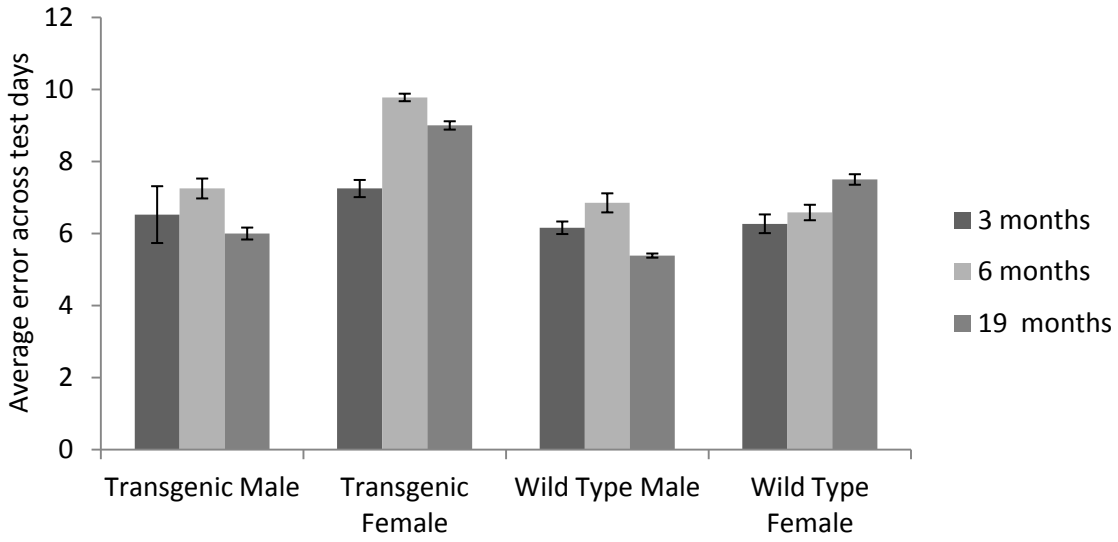

Fig. 3.9: Mean error across test days for transgenic and wild type mice of both genders at ages 3, 6 and 19 months. Error bars ±1 S.E.M.

		Error across trials				Error in the first half of the trials			
		Wild Type		Transgenic		Wild Type		Transgenic	
Age	Gender	**Mean**	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.
3	Male	**6.16**	0.17	**6.53**	0.79	**0.45**	0.02	**0.86**	0.02
3	Female	**6.27**	0.26	**7.25**	0.24	**0.67**	0.03	**0.56**	0.02
6	Male	**6.85**	0.55	**7.25**	0.28	**0.55**	0.02	**0.83**	0.05
6	Female	**6.58**	0.21	**9.78**	0.10	**0.69**	0.02	**0.76**	0.04
19	Male	**5.39**	0.06	**6.00**	0.17	**0.46**	0.03	**0.58**	0.02
19	Female	**7.50**	0.15	**9.00**	0.12	**0.49**	0.02	**0.84**	0.01
Mean	Male	**6.13**	0.24	**6.59**	0.21	**0.49**	0.02	**0.76**	0.05
	Female	**6.78**	0.21	**8.67**	0.43	**0.62**	0.04	**0.72**	0.05

Table 3.4: The mean error scores across trials and in the first half of the trials presented across age, gender and genotype with S.E.M. values.

In terms of errors made in the first half of the trials, Mann Whitney U comparisons between transgenic and wild type mice at 3, 6 and 19 months (summarised in Table 3.7) revealed a significant difference in error made at 19 months of age, with transgenic mice making significantly more errors in the first half of the trials than wild type mice (U (20, 22) = 119.0, p < .05, see Fig. 3.10 and Table 3.4). This genotypic difference was evident only when the data were collapsed across gender [Male: U (6, 13) = 33.0, p > .025; Female: U (9, 11) = 26.5, p > .025)].

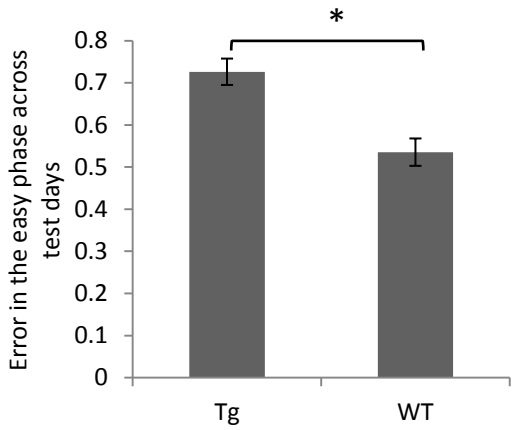

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Fig. 3.10: Mean error in the first half of the trials of transgenic and wild type collapsed across gender at 19 months of age. Error bars ±1 S.E.M. * = $p < .05$.

Repeat error

An analysis of the average repeat errors across whole trials revealed a genotypic difference at 6 months of age (U (20, 22) = 125.0, $p < .05$) with transgenic mice making more repeated errors than wild type mice (see Fig. 3.11A and Table 3.5). When analysing the effect separately for each gender, only transgenic female mice made significantly more repeat errors across trials than wild type female mice (U (9, 11) = 24.5, $p < .025$), with no differences between male mice of different genotypes (U (6, 13) = 34.0, $p > .025$).

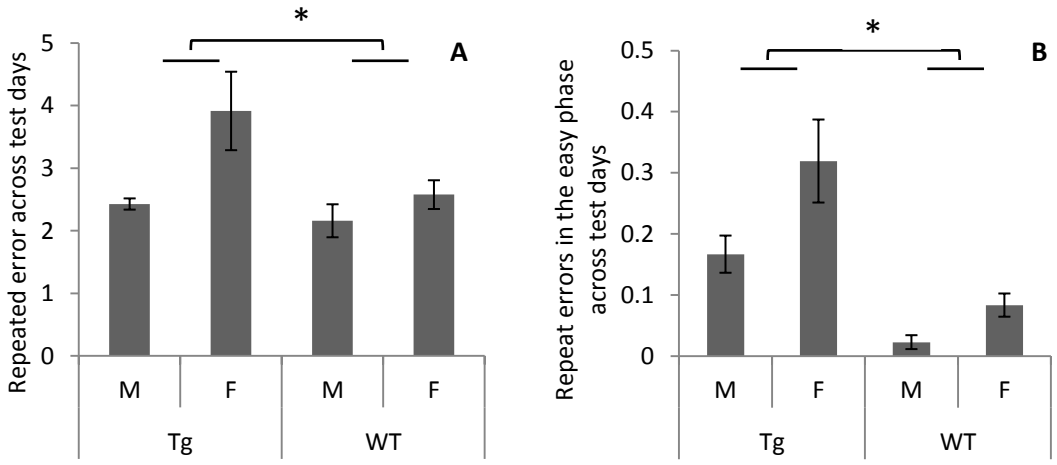


Fig. 3.11: Mean total repeat error (A) and repeat error in the first half of the trials (B) across test days for transgenic (Tg) and wild type (WT) mice of both genders (M = male, F = female) at ages 6 months of age only. Error bars ±1 S.E.M. * = $p < .05$.

Equally, in the first half of the trials, 6 month old transgenic mice made significantly more repeat errors (U (20, 22) = 139.5, $p < .05$; see Fig. 3.11B and Table 3.5). When analysed by gender, neither transgenic male nor transgenic female mice made more repeated errors in the first half of the trials than their wild type control group [male: (U (6, 13) = 22.0, $p > .025$); female: (U (6, 13) = 46.0, $p > .025$)].

		Repeat error across trials				Repeat error in the first half of the trials			
		Wild Type		Transgenic		Wild Type		Transgenic	
Age	Gender	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
3	Male	**2.39**	0.09	**2.53**	0.41	**0.02**	0.01	**0.09**	0.03
3	Female	**2.26**	0.11	**2.87**	0.11	**0.31**	0.10	**0.10**	0.01
6	Male	**2.46**	0.10	**2.50**	0.11	**0.02**	0.01	**0.17**	0.03
6	Female	**2.44**	0.10	**5.03**	0.04	**0.08**	0.02	**0.32**	0.07
19	Male	**1.63**	0.04	**2.25**	0.17	**0.04**	0.01	**0.00**	0.00
19	Female	**3.02**	0.14	**3.84**	0.13	**0.00**	0.00	**0.07**	0.01
Mean	Male	**2.16**	0.26	**2.43**	0.09	**0.03**	0.01	**0.09**	0.03
	Female	**2.58**	0.23	**3.91**	0.63	**0.13**	0.05	**0.16**	0.05

Table 3.5: The mean repeat error scores across trials and in the first half of the trials presented across age, gender and genotype with S.E.M. values.

Consecutive error

In the first half of the trials, 6 month old transgenic mice made significantly more consecutive errors (U (20, 22) = 155.0, $p < .05$; see Fig. 3.12B and Table 3.6) compared to wild type mice. When analysed by gender, no genotypic differences emerged within each gender [male: (U (6, 13) = 26.0, $p > .025$); female: (U (9, 11) = 49.0, $p > .025$); Table 3.7)].

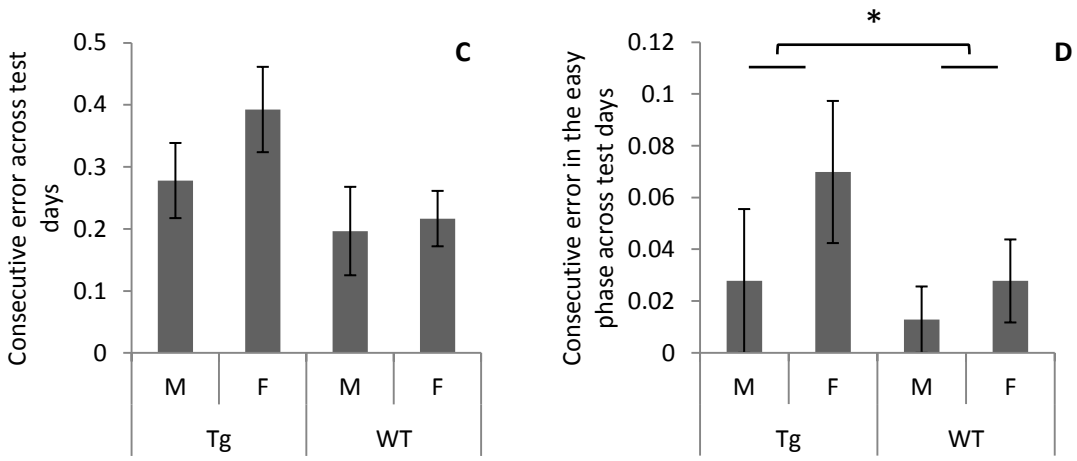


Fig. 3.12: Mean total consecutive error (C) and consecutive error in the first half of the trials (D) across test days for transgenic (Tg) and wild type (WT) mice of both genders (M = male, F = female) at ages 6 months of age only. Error bars ±1 S.E.M. * = $p < .05$.

Age	Gender	Consecutive error across trials				Consecutive error in the first half of the trials			
		Wild Type		Transgenic		Wild Type		Transgenic	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
3	Male	**0.06**	0.01	**0.17**	0.02	**0.00**	0.00	**0.00**	0.00
3	Female	**0.18**	0.02	**0.28**	0.01	**0.03**	0.01	**0.07**	0.001
6	Male	**0.30**	0.01	**0.29**	0.04	**0.00**	0.00	**0.08**	0.02
6	Female	**0.17**	0.01	**0.51**	0.03	**0.00**	0.00	**0.12**	0.03
19	Male	**0.23**	0.02	**0.38**	0.04	**0.04**	0.01	**0.00**	0.00
19	Female	**0.31**	0.03	**0.39**	0.03	**0.00**	0.00	**0.02**	0.01
Mean	Male	**0.20**	0.07	**0.28**	0.06	**0.01**	0.01	**0.03**	0.03
	Female	**0.22**	0.04	**0.39**	0.07	**0.03**	0.02	**0.07**	0.03

Table 3.6: The mean consecutive error scores across trials and in the first half of the trials presented across age, gender and genotype with S.E.M.

Variable (mean across test days)		Genotype difference				Genotype difference (collapsed across gender)	
		Male		Female			
		U	*p*	*U*	*p*	*U*	*P*
3 month	Error first half of the trials	16.5	0.045	66.5	0.952	182.5	0.233
	Repeat error	33.3	0.598	49.5	0.282	201.5	0.473
	Repeat error first half of the trials	29.0	0.166	64.5	0.823	191.0	0.188
	Consecutive error	33.5	0.531	51.0	0.286	163.0	0.063
	Consecutive error first half of the trials	39.0	1.000	57.0	0.374	197.5	0.143
6 month	Error first half of the trials	22.5	0.141	55.5	0.631	167.5	0.179
	Repeat error	34.0	0.660	**24.5**	**0.015**	**125.0**	**0.017**
	Repeat error first half of the trials	22.0	0.036	46.0	0.219	**139.5**	**0.013**
	Consecutive error	37.0	0.854	**27.5**	**0.021**	152.0	0.075
	Consecutive error first half of the trials	26.0	0.032	49.0	0.252	**155.5**	**0.017**
19 month	Error first half of the trials	33.0	0.581	26.5	0.074	**119.0**	**0.047**
	Repeat error	29.5	0.401	40.5	0.494	131.5	0.115
	Repeat error first half of the trials	33.0	0.323	36.0	0.098	171.0	0.664
	Consecutive error	32.0	0.516	48.0	0.906	165.5	0.523
	Consecutive error first half of the trials	33.0	0.323	45.0	0.366	171.0	0.434

Table 3.7: The test statistic and exact significance for the effect of genotype on variables from the foraging task is presented at each age time point both split by gender and collapsed across gender. Significant values are highlighted in bold (p = .05 for genotypic differences collapsed across gender, p = .025 for genotypic differences within each gender).

The effect of gender

To evaluate the effect of gender on measures of spatial working memory function in the foraging task, the data was collapsed across genotype. As can be observed from Table 3.8, the significant gender differences were limited to female mice having higher consecutive error

scores than males across the task at 3 months of age (*U* (19, 20) = 151.00, *p* < .05), as well as higher repeat error scores across the task at 6 (*U* (19, 20) = 137.00, *p* < .05) and 19 months of age (*U* (19, 20) = 115.00, *p* < .05). When analysed separately for each genotype, there were no significant gender differences.

Variable (mean across test days)		Gender difference				Gender difference (collapsed across genotype)	
		WT		Tg			
		U	*p*	*U*	*P*	*U*	*P*
3 month	Error first half of the trials	50.00	0.58	27.50	0.18	227.00	0.99
	Repeat error	47.50	0.48	31.00	0.29	206.50	0.61
	Repeat error first half of the trials	49.00	0.34	43.00	0.91	191.50	0.23
	Consecutive error	43.00	0.23	33.50	0.36	**151.00**	**0.04**
	Consecutive error first half of the trials	52.00	0.41	33.00	0.28	180.50	0.05
6 month	Error first half of the trials	53.00	0.74	39.50	0.86	197.00	0.59
	Repeat error	51.00	0.63	18.50	0.05	**137.00**	**0.04**
	Repeat error first half of the trials	49.50	0.44	41.50	1.00	176.50	0.21
	Consecutive error	43.00	0.29	26.50	0.22	195.00	0.55
	Consecutive error first half of the trials	52.00	0.41	40.00	0.89	182.50	0.19
19 month	Error first half of the trials	56.50	0.89	21.00	0.24	145.00	0.20
	Repeat error	38.00	0.18	21.50	0.27	**115.00**	**0.04**
	Repeat error first half of the trials	49.50	0.49	24.00	0.27	181.50	1.00
	Consecutive error	49.50	0.57	33.00	1.00	170.00	0.57
	Consecutive error first half of the trials	49.50	0.49	30.00	1.00	179.50	0.61

Table 3.8: The test statistic and exact significance for the effect of gender on variables from the foraging task is presented at each age time point both split by genotype and collapsed across genotype. Significant values are highlighted in bold (p < .05 for gender differences collapsed across genotype, p < .025 for gender differences within each genotype).

The effect of age

Within-subject analyses were carried out to assess the effect of age on spatial working memory function in transgenic and wild type mice separately. As seen in Table 3.9, there were no significant within-subject changes as a function of age on any measure of performance.

Variable (mean across test days)	Within-subject changes (across age points)			
	WT		Tg	
	X^2	*p*	X^2	*P*
Error first half of the trials	0.94	0.63	0.03	0.98
Repeat error	1.45	0.51	3.52	0.18
Repeat error first half of the trials	0.29	1.00	2.58	0.29
Consecutive error	3.65	0.17	2.98	0.23
Consecutive error first half of the trials	0.50	1.00	4.75	0.09

Table 3.9: The test statistic and exact significance for within-subject analysis of variables of transgenic and wild type performance from the foraging task is presented. WT= wild type, Tg = transgenic.

3.3.4 Discussion

The results from the longitudinal foraging task study indicate that there are few differences in spatial working memory performance between transgenic and wild type APP(V717I) mice at 3 months, with one of six measures of performance showing a significant transgenic deficit compared to wild type performance in male mice only.

At 6 months, more genotypic differences were evident, with transgenic mice making more repeat and consecutive errors than wild type mice, particularly in the first half of the trials. This finding suggests an effect of age on errors, with a general increase in errors between 3 and 6 months of age. However, when analysing within-subject changes in transgenic and wild type mice, no evidence of changes in performance as a function of age was obtained with the exception of an increase in overall error scores between 3 and 6 months. By 19 months of age, the majority of these genotype differences were no longer evident, with the exception of the emergence of transgenic mice making more errors than wild type mice in the first half of the trials when collapsing across gender.

Across age and genotype, female mice were observed to make significantly more errors than male mice, with a subset of performance measures indicating general differences between male and female mice at 3, 6 and 19 months of age. Thus, male mice appear to outperform female mice on a selection of variables in a manner that is not dependent on age or genotype. This trend does not uphold across the task measures however. More interestingly, genotypic differences in performance are influenced by gender, although the effect is limited to 2 measures of female performance at 6 months of age. This indicates that there are differences in the way males and females respond to the spatial working memory task, and raises the possibility that the phenotypic expression of the APP(V717I) transgene

may be different in male and female transgenic mice. Such an interpretation should be taken with caution however, as the effects of gender on spatial working memory in the foraging task are neither consistent across measures nor widespread in the data set. In addition, the unequal sample sizes between male and female APP(V717I) mice further encourages caution.

Overall, the APP(V717I) mutation leads to a subtle disruption of spatial working memory in transgenic mice. However, it is clear that these effects do not generalise across the variety of measures. Moreover, transgenic performance improved from 6 to 19 months of age in contrast to the expected pattern in a putative model of age-related Aβ-induced cognitive decline. This is despite 19 months being a relatively high age for transgenic mice, as well as an age where extensive Aβ plaque pathology has been reported (Dewachter et al., 2000). As spatial working memory has not been previously assessed in the APP(V717I) model, it is unclear how far these results generalise across laboratories and testing conditions.

Due to the subtle nature of the identified transgenic APP(V717I) deficit in spatial working memory, it is difficult to conclude that these transgenic mice show a robust memory deficit. In an effort to determine whether subtle changes in spatial working memory are manifested by this model in other tasks, Experiment 5 assessed memory function using a T-maze forced choice alternation paradigm. This task has well-replicated sensitivity to deficits in APP mutation models (Chapman et al., 1999; Lalonde et al., 2002; Wriths et al., 2008; Corcoran et al., 2002).

3.4 Experiment 5a and 5b: Hippocampal lesioned and APP(V717I) mice on the T-maze

3.4.1 Introduction

The T-maze forced choice alternation (FCA) task is a spatial working memory task where the mouse is placed in an elevated maze shaped as a T with a start arm and two goal arms. If given a choice between an already visited arm and a previously unexplored arm, a normal mouse tends to choose the unexplored arm (Deacon & Rawlins, 2006). This task is sensitive to hippocampal dysfunction (Deacon & Rawlings, 2006; Deacon et al., 2002), and APP mutations in mice (Chapman et al., 1999; Lalonde et al., 2002; Wriths et al., 2008; Corcoran et al., 2002). Experiment 5a investigated the effects of the APP mutation on the T-maze alternation task and Experiment 5b established that the procedure was sensitive to disruption of hippocampal function in mice. Transgenic APP mutation mice were hypothesised to show impaired delayed non-matching to position performance at 11 months of age.

3.4.2 Method

Design

APP(V717I) mice were tested at 11 months of age in order to assess whether the introduction of the APP(V717I) mutation is related to decreased performance in the T-maze FCA non-matching to position paradigm. Both genders were included in this study, as results from the foraging task (Experiment 4) indicate spatial information processing may be more sensitive to the APP mutation in female than male transgenic mice.

Subjects

Experiment 5a

The hippocampal lesioned and non-lesioned mice utilised were the same as those described in Chapter 3, Section 3.2.

Experiment 5b

The subjects were those described in Chapter 2, Section 2.2 assessed at 11 months of age.

Apparatus

The T-maze consists of one long start arm (52cm*8.5cm) and two shorter goal arms (26cm*8.5cm; see Fig. 3.13). The walls are made of clear Plexiglas (height 12.7cm). Two removable stop doors were used of either light or dark grey Plexiglas (13.5cm*8.6cm). At the end of each test arm was a well (8cm long, 2.3cm wide and 1.3cm high, with a groove of dimensions 7cm length, 1.5cm width and 1cm depth) of dark plastic material. These allowed for the use of a liquid reward of sweetened, condensed milk mixed 50:50 with distilled water without being visible from the choice point at the beginning of the goal arms. The T-maze was situated on a table (75cm*118cm) elevated 80cm above from the floor.

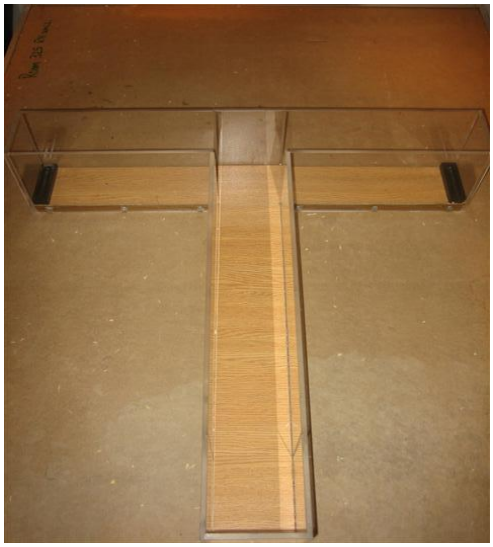


Fig. 3.13: The T-maze apparatus with two unbaited reward wells in place.

Method

Habituation

Mice were water deprived with daily access to water limited to 2 hours. The mice received 3 days of habituation. The mice were placed in the beginning of the start arm, facing into the maze. Both goal arms were baited with one drop of reward solution (approximately 30µl) in the wells. On day 1 and 2, the mice were given 10 minutes to explore the maze freely and consume the rewards. On day 3, the mice were given two habituation sessions of 5 minutes with baited wells in each session to ensure all mice were consistently consuming both rewards.

Test phase

The mice received 8 days of testing. On each day, the mice received a sample trial and a test trial. On the sample trial, both wells were baited, and a light grey door prohibited access to one of the goal arms. The mouse was placed at the beginning of the start arm, released, and was allowed to consume the reward in the only accessible goal arm. The mouse was then placed into the home cage whilst the maze and the visited well were cleaned. The light grey stop door was then removed, leaving both goal arms open. The mouse was placed in the start arm and was required to make a choice between the previously visited arm and the previously unvisited arm. The light grey door was then removed, leaving both goal arms open. The mouse

placed in the start arm and was required to make a choice between the previously visited arm and the previously unvisited arm. Mice were rewarded for visiting the unvisited goal arm. If the mouse entered the previously visited arm, a dark grey door was placed at the arm entry, confining the mouse to the goal arm for approximately 15 seconds before being returned to its home cage. If the mouse entered the previously unvisited arm, it was allowed to consume the reward before being returned to the home cage. Each mouse received 6 sets of trials per day with an inter-trial interval of approximately 20 minutes.

Scoring and data analysis

Entry into an arm was defined as the moment the back legs of the mouse crossed the entry line to the arm. Following each trial, the experimenter manually noted whether the mouse made a correct or incorrect choice. Mice were excluded from testing if they did not engage with the task, defined as requiring more than 5 minutes to move from the start arm on 8 or more trials. One female transgenic mouse was excluded during test day 2 on such grounds. For each test day, a percentage correct score across the six trials was calculated for each individual mouse. The percentage correct scores were then averaged across groups for each test day, before being averaged across test days.

3.4.4 Results

Experiment 5a: Hippocampal lesions

There was a significant differences between lesioned and non-lesioned mice in the average errors across test days (F (1. 13) = 39.00, $p < .0001$, see Fig. 3.14).

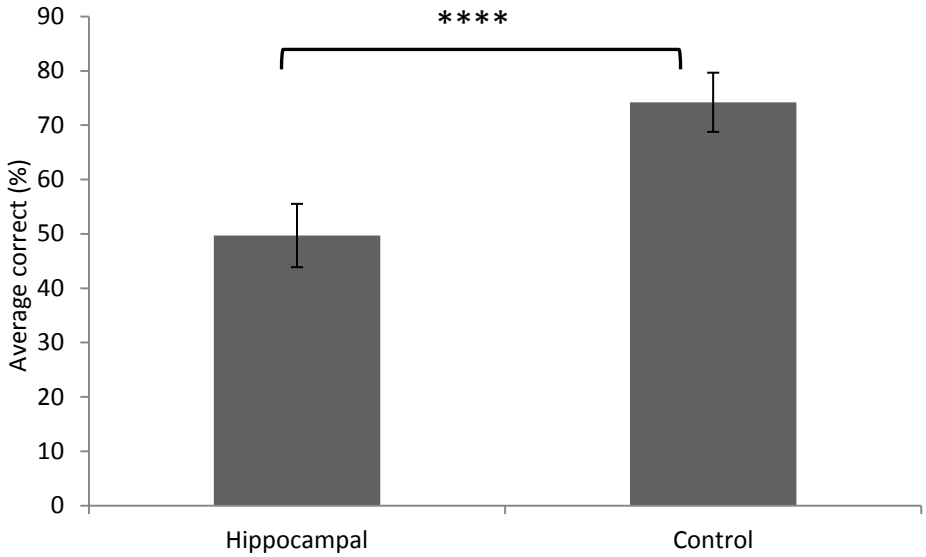


Fig. 3.14: Mean error across trials for hippocampal lesioned and non-surgical control mice on the T-maze. Error bars are ±1 S.E.M. ****= $p < .0001$.

Whilst there was no effect of test day on performance (F (7, 91) =1.37, $p > .05$), there was a significant interaction between test day and lesion status (F (7, 91) =4.14, $p < .001$; see Table 3.10). Simple effects analysis revealed that non-surgical control mice performed significantly better than hippocampal lesioned mice on the majority of test days [Day1: (F (1, 13) =36.83, $p < .0001$), Day2: (F (1, 13) =13.88, $p < .005$), Day3: (F (1, 13) =30.52, $p < .0001$), Day 5: (F (1, 13) =6.32, $p < .05$), Day 7: (F (1, 13) =13.17, $p < .005$)]. In contrast, control and lesioned mice did not differ on Day 4 (F (1, 13) =1.64, $p > .05$), Day 6 (F (1, 13) =4.46, $p > .05$) or Day 8 (F (1, 13) =4.18, $p > .05$).

Test day	Hippocampal		Control	
	Mean	S.E.M.	**Mean**	S.E.M.
1	**42.84**	5.36	**85.41**	4.91
2	**45.26**	10.91	**85.41**	4.91
3	**35.70**	4.69	**75.00**	5.45
4	**54.76**	5.14	**64.59**	5.84
5	**57.14**	5.35	**70.85**	2.72
6	**57.14**	5.35	**72.93**	5.39
7	**54.77**	3.33	**74.99**	4.45
8	**50.00**	3.94	**64.58**	5.83
Mean	**49.70**	6.29	**74.22**	5.44

Table 3.10: The mean percentage correct scores and S.E.M. values for hippocampal lesioned mice and non-surgical control mice presented by test day and averaged across test days.

Experiment 5b: APP(V717I) mice

An ANOVA assessing the effect of gender and genotype on the average percentage correct across trials revealed a significant main effect of genotype (see Fig. 3.15), with transgenic mice showing higher correct scores than wild type mice (F (1, 37) = 11.5, $p < .005$). A comparison of the average percentage correct score of wild type mice against chance using Mann Whitney U, due to violations of the assumptions of homogeneity of variance and normality, confirmed that wild type mice performed significantly above chance (U (22, 22) = 11.00, $p < .0001$). There was also a main effect of gender (F (1, 37) = 5.3, $p < .05$), with males showing a higher percentage correct score than females. However, there was no significant gender/genotype interaction (F (1, 37) = .33, $p > .50$).

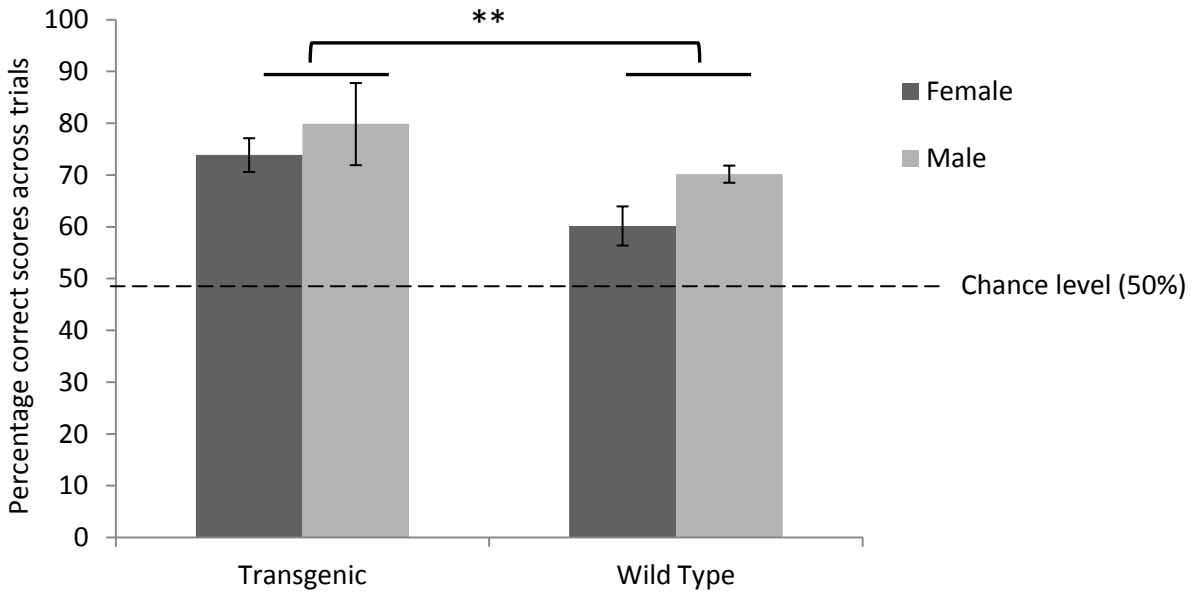


Fig. 3.15: Mean percentage correct across test days displayed for transgenic and wild type mice for both genders at 11 months of age. Error bars ±1 S.E.M. **= $p < .005$.

The pattern of performance in transgenic and wild type mice did not appear to be affected by test day (see Table 3.11). Using the Greenhouse-Geisser control for violations of the assumption of sphericity, there was no effect of test day on performance (F (7, 196.1) =1.96, $p > .05$), or any interaction between test day and genotype (F (7, 196.1) =1.03, $p > .05$), test day and gender (F (7, 196.1) =.57, $p > .05$) or test day, gender and genotype (F (7, 196.1) =.46, $p > .05$).

Test day	Wild Type				Transgenic			
	Female		Male		Female		Male	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
1	**57.42**	6.87	**69.23**	5.61	**64.10**	6.77	**72.23**	7.02
2	**57.40**	7.91	**74.35**	5.54	**73.08**	4.02	**75.02**	13.43
3	**57.41**	5.64	**67.95**	3.98	**82.05**	4.79	**86.10**	5.12
4	**66.67**	7.86	**73.06**	4.82	**73.08**	6.41	**88.88**	5.55
5	**68.56**	6.49	**73.08**	4.02	**83.32**	4.22	**80.55**	7.95
6	**61.12**	5.56	**69.23**	3.70	**74.35**	4.85	**75.00**	7.13
7	**55.56**	5.56	**64.10**	4.15	**74.35**	4.47	**80.55**	7.95
8	**57.41**	4.90	**70.52**	4.28	**66.67**	4.62	**80.57**	9.04
Mean	**60.19**	3.78	**70.19**	1.66	**73.87**	3.26	**79.86**	7.93

Table 3.11: The mean percentage correct scores and S.E.M. values for wild type and transgenic mice at 11 months of age presented by test day and averaged across test days.

3.4.4 Discussion

The results from the T-maze FCA non-matching to position paradigm show superior spatial working memory performance in 11 month old transgenic mice compared to wild type controls. Experiment 5b confirmed that this task was sensitive to extensive cell loss in the hippocampus of C57B/6 mice. The lack of disruption in APP mutation mice is surprising and indicates that the presence of the APP(V717I) mutation does not disrupt spatial working memory processes, despite such indications from the foraging task, but rather improved performance. It can be concluded that transgenic APP(V717I) acquire the spatial alternation task and in contrast to other APP mutation models (Chapman et al., 1999) appear to display improved performance.

In terms of gender, the T-maze data suggests that male mice regardless of genotype outperform female mice. This pattern is in line with the gender pattern from the foraging task, suggesting that female APP(V717I) show a spatial working memory deficit compared to males irrespective of the presence of the APP(V717I) mutation, as well as data from the 3xTg-AD model in which female mice are outperformed by males in the Morris water maze task at 12 months (Clinton et al., 2007). This has been shown to be due to female mice being more affected by stress than males, specifically at 12 months of age but neither at a younger nor older age (Clinton et al., 2007). As such, differences in stress-sensitivity could be involved in the gender differences detected in APP(V717I) mice the T-maze and the foraging tasks. It is worth noting however that the Morris water maze task is considered a more stressful paradigm due to the use of aversive rather than appetitive motivation. Furthermore, no evidence of a gender difference was found in the RAWM, indicating that the gender

differences in APP(V717I) mice are unlikely to be explained wholly by stress-sensitivity. Overall, the difficulty level of the tasks used needs to be appropriate for the control group used when assessing cognitive function. Thus, this gender information is important in guiding task selection and task difficulty manipulations, as the use of female APP(V717I) mice may require a lower task difficulty level than the use of males in order to be sensitive to genotypic differences. In addition, this pattern of cognitive differences may indicate that female transgenic APP(V717I) mice have more extensive Aβ pathology than males.

Overall, transgenic APP(V717I) mice do not show a strong, age-dependent decline in spatial working memory as assessed in the foraging task. With the T-maze results showing improved performance in transgenic mice compared to wild type mice, there is little evidence of a general spatial working memory deficit in this model. With evidence from two spatial working memory task and one watermaze navigation task suggesting a lack of clear spatial deficits in transgenic APP(V717I) mice at 3, 6, 10, 11 and 19 months of age, there appears to be little support for utilising these mice to assess the effects of 2B3 on spatial cognition.

Dewachter et al. (2002) provided evidence that APP(V717I) mice were impaired in a novel object recognition paradigm at 3-6 months of age. As there was a clear absence of a memory deficit associated with hippocampal pathology, the final experiment assessed non-spatial recognition memory in APP(V717I) mice, allowing for a wider assessment of cognitive function through the investigation of a perirhinal cortex-dependent task (Aggleton & Brown, 1999; Brown & Aggleton, 2001; Brown, Warburton & Aggleton, 2010).

3.5 Experiment 6: Assessment of novel object recognition in APP(V717I) mice

3.5.1 Introduction

Object recognition memory refers to the ability to recognise and respond to an object or situation in a way which indicates that it has been encountered previously, and has been found to be impaired in AD patients (Aggleton & Brown, 1999). Single novel object recognition when presented simultaneously with a familiar object has been shown to rely on the perirhinal cortex in monkeys (Meunier et al., 1993) and rats (Mumby & Pinel, 1994; Winters & Bussey, 2005), a structure which anatomically is located close to the hippocampus in the temporal lobe. Aggleton & Brown (2001) proposed a dissociation of hippocampal and perirhinal cortex functioning, with the perirhinal cortex supporting single object recognition, and the hippocampus processing more complex stimuli integrating features of objects, as well as the

temporal and spatial aspects of an encountered scene. This analysis raises the possibility that object recognition memory may be impaired in the absence of spatial memory deficits in the APP(V717I) model.

The novel object recognition task assesses object recognition memory function by allowing mice to explore a set of two identical objects. This is followed by a delay, after which the mice are presented with one copy of the previously encountered object and one novel object. Wild type mice preferentially explore a novel object over a familiar object unless the inter-trial interval is of a sufficient duration to disrupt recognition memory of the previously encountered object.

3.5.2 Method

Design

Assessment of the effect of the APP(V717I) mutation on object novelty detection as a characteristic of object recognition memory was carried out at 18 months of age. Both genders were included in order to assess whether gender affects the expression of a transgenic phenotype in the novel object recognition task. APP(V717I) transgenic mice are reported to display deficits in object recognition memory expressed as novelty preference after a 180 minute delay between sample and trial, but not after a 60 minute or 5 minute delay (Dewachter et al., 2002). Identical delay parameters were adopted in the current study in order to order to replicate the findings of Dewachter et al. (2002).

Subjects

The subjects were those described in Chapter 2, Section 2.2 assessed at 18 months of age.

Apparatus

Mice were tested in an 82cm*82cm arena with 40cm high walls of clear Perspex which were covered externally with white card. The laminate plywood base was painted white and divided into 16 equally sized 20.5cm*20.5cm squares, arranged in a 4*4 design (see Fig. 3.16). The arena was situated on a table (80cm*80cm) elevating it to 80cm above floor level.

The objects used for testing were a variety of household objects of materials such as glass and plastic. Examples include a tape measure, a funnel and a door stop and hair gel bottle. Objects were chosen based on pilot studies with C57Bl/6 mice and were explored equally. To minimise odour cues guiding the behaviour of the subjects, three copies of each object (two for the sample phase and one for the test phase) were used. Trials were recorded whilst the experimenter recorded the respective contact time of each object using an electronic dual timer.

Fig. 3.16: Object recognition arena with two identical objects in place in the sample phase.

Method

Habituation

The mice were habituated to the arena over three days. On days 1 and 2, the mice were permitted to explore the empty arena freely for 10 minutes. On habituation day 3, a single object that was not used in future testing was placed in the centre of the arena to assess whether mice had habituated to the novel environment sufficiently to explore an unknown object. The experimenter was visible throughout testing.

Sample and test phase

Counterbalancing was conducted for both spatial location of objects and the objects themselves, in addition to gender and genotype. In the sample phase, the mice were placed in the middle of the arena containing two identical objects and allowed to freely explore for 10 minutes. The objects were placed diagonally opposite each other in two of the middle 4 squares of the arena (see Fig. 3.16). After 10 minutes, the mice were returned to their home

cage for the duration of the delay (5 minutes, 60 minutes or 180 minutes). The objects and the arena were wiped with 70% ethanol wipes and the familiar object (identical to the two in the test phase) and the novel object (never before seen object) replaced the objects of the sample phase. The test phase was carried out in an identical fashion to the sample phase, the only difference being the presence of one familiar and one novel object. These objects are placed in the same spatial locations in both the sample and test phase. The type of object used in the sample phase and as a novel object, in addition to the spatial location of the novel object, were counterbalanced across groups. Hence, if a mouse encountered the novel object on the left in trial 1, it would encounter it on the right in trial 2. At each delay interval, two trials were run across two days, with each mouse tested at each delay interval (a total of 6 test days for each individual mouse). A two day break was given between each set of test days in an attempt to avoid habituation to novelty.

Scoring and data analysis

The amount of time spent exploring each object in the habituation, sample and test phase was recorded electronically by the experimenter. Exploration was defined as the mouse being within 2 cm of the object and interacting with it either by sniffing, biting, pushing or moving its nose in contact with the object. Exploration was not scored if the mouse was within 2 cm of the object, but did not face it, or if the object was used as a means to explore the extramaze environment. An electronic timer was used to manually record the duration of exploration.

For each delay interval, the time spent exploring the novel object on test day 1 and 2 was averaged. The same was done for the total time spent exploring any object on each test day. Using these average test day scores, a discrimination ratio was created by dividing the average novel score by the average total exploration score, and multiplying this by 100. A score of 50 would indicate an equal amount of time spent exploring the novel and familiar object across test days and the higher the score, the stronger the preference for the novel object.

3.5.3 Results

The contact times averaged across the two identical objects in the sample phase are presented in Table 3.12. In order to assess whether there were any sampling bias between groups, a repeated measures ANOVA with gender and genotype as factors was carried out.

There were no differences between transgenic and wild type mice on sampling time across delay conditions (F (1, 35) = 1.13, p > .05), but there was a main effect of gender (F (1, 35) = 11.31, p < .005) with female mice spending more time exploring the sample objects than male mice. Gender did not interact with genotype on this measure (F (1, 35) = 0.37, p > .05).

	Contact time (s) in sample phase							
	Wild Type				Transgenic			
	Male		Female		Male		Female	
Delay	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
5 min	**11.89**	1.11	**13.55**	2.08	**10.61**	2.79	**16.48**	1.78
60 min	**11.40**	1.44	**17.10**	2.33	**8.12**	1.08	**15.92**	2.17
180 min	**11.85**	1.25	**15.51**	1.55	**9.74**	1.38	**11.95**	1.20

Table 3.12: The mean contact time in seconds for transgenic and wild type mice of both genders when faced with a pair of novel objects over two habituation days (novel pairs presented on each day). Min = minutes.

The mean contact time for the novel and familiar object at each delay interval is presented in Table 3.13. There was a significant within-subject effect of delay (F (2, 70) = 9.97, p < .0001), in which the contact time at 5 minutes delay was significantly higher than at 60 minutes (p < .05) and 180 minutes (p < .0001). The effect of the difference in contact time at different test intervals is minimised however by the lack of interaction between delay and any other factor [delay*genotype: (F 2, 70) = 0.72, p > .05); delay*gender (F (2, 70) =0.62, p > .05); delay*genotype*gender (F (2, 70) = 0.76, p > .05)].

A significant main effect of object type revealed that novel objects were explored significantly more than familiar objects (F (1, 35) = 53.65, p < .0001). However, neither genotype, gender nor delay influenced this effect [object*genotype: (F (1, 35) = 0.69, p > .05); object*gender: (F (1, 35) = 1.04, p > .05); object*genotype*gender: (F (1, 35) = 0.16, p > .05); object*delay: (F (2, 70) = 1.37, p > .05); object*delay*genotype: (F (2, 70) = 0.62, p > .05); object*delay*gender: (F (2, 70) = 1.68, p > .05); object*delay*genotype*gender: (F (2, 70) = 0.95, p > .05)]. As in the sample phase, female mice were found to spend significantly more time exploring the objects in the test phase when collapsed across all other variables (F (1, 35) = 15.62, p < .0001).

Delay	Novelty status	Contact time (s) in test phase							
		Wild Type				Transgenic			
		Male		Female		Male		Female	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
5 min	Novel	**18.22**	0.94	**21.28**	1.67	**15.17**	1.10	**22.08**	1.50
	Familiar	**11.37**	0.80	**11.98**	0.99	**8.88**	0.33	**15.36**	1.14
60 min	Novel	**13.52**	1.12	**19.06**	2.08	**11.87**	1.79	**16.44**	1.44
	Familiar	**9.54**	1.16	**13.42**	1.90	**5.16**	0.52	**12.92**	1.04
180 min	Novel	**14.11**	0.99	**18.68**	1.32	**9.53**	1.79	**16.29**	2.24
	Familiar	**8.25**	0.59	**9.85**	0.82	**7.94**	0.87	**8.87**	0.62

Table 3.13: The mean contact time in seconds for transgenic and wild type mice of both genders when faced with a novel and a familiar object in the test phase (averaged across two trials). Min = minutes.

Fig. 3.17 shows the discrimination ratios of transgenic and wild type mice at each delay interval. For simplicity of presentation, data is collapsed across gender. An ANOVA assessing the effect of delay interval on the discrimination ratios in transgenic and wild type mice of both genders established no within-subject effect of delay (F (2, 70) = .19, p > .05) or any within-subject interactions with delay (delay*genotype: F (2, 70) = 1.67, p > .05; delay*gender: (F (2, 70) = 3.07, p > .05; delay*genotype*gender: F (2, 70) = 2.43, p > .05). There was no between-subjects effect of genotype (F (1, 35) = .54, p > .05), gender (F (1, 35) = .01 p > .05) or a gender*genotype interaction (F (1, 35) = .61, p > .05).

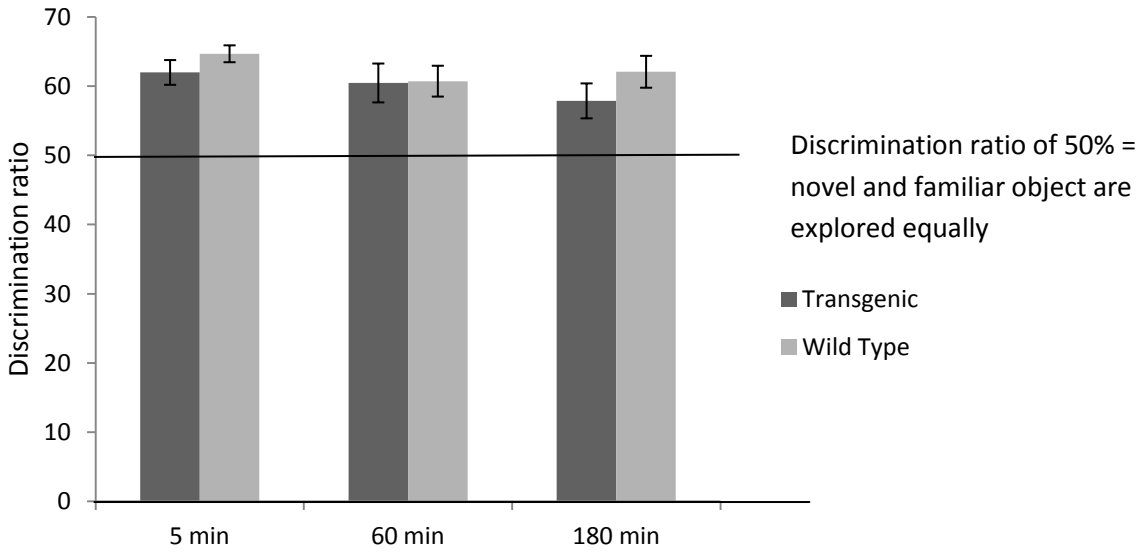


Fig. 3.17: Discrimination ratio (total time novel/total time any object*100) displayed for transgenic and wild type mice at delay intervals of 5 minutes, 60 minutes and 180 minutes. Error bars ±1 S.E.M.

3.5.4 Discussion

The results from the novel object recognition experiment indicate that there are no genotypic differences in object recognition memory between transgenic and wild type APP(V717I) mice of either gender at 18 months of age. Whilst male mice were observed to show lower contact times than female mice across the task, this difference did not interact with genotype. In addition, all groups showed a pattern of novelty preference, indicating that at both transgenic and wild type mice can discriminate between a novel object and one previously encountered after up to 3 hours delay. This indicates that the object recognition memory of transgenic APP(V717I) mice is not disrupted by Aβ pathology at 18 months of age, despite reports of extensive plaque pathology by this age (Dewachter et al., 2000; 2002).

It is worth noting that the object recognition task relies on novelty detection expressed as preferential exploration of a novel object over a familiar object. Thus, extensive exposure to novel objects may result in the cohort habituating to novelty, reducing the sensitivity of the paradigm to object recognition memory function. Whilst both transgenic and wild type mice in the current study demonstrated discrimination ratios above chance, it is possible that a cross-sectional design would enhance the sensitivity of the task in a manner which could preferentially benefit wild type performance. Furthermore, whilst pilot work was carried out to limit the potential variability introduced by objects of differential interest to subjects, a recent paper has highlighted the effect of the functional properties of objects (Heyser & Chemero, 2011). Specifically, objects that can be climbed were found to produce greater discrimination ratios in C57Bl/6 mice compared to those that can be touched only. As the current study utilised objects of both these categories, it is possible that this introduced further variability to the data. However, no systematic bias in wild type or transgenic performance would have resulted from the mixed use of objects due to careful counterbalancing of objects across genotype and gender groups, as well as delay conditions.

3.6 Chapter discussion

The aim of this chapter was to analyse the developmental profile of a range of cognitive processes in the APP(V717I) model in order to identify aspects of learning and memory in which transgenic mice show age-related deficits compared to littermate wild type controls. A secondary aim was the analysis of the effect of gender on the expression of the transgenic cognitive phenotype.

The results from a set of behavioural experiments assessing a range of cognitive constructs in APP(V717I) mice in this thesis largely point to a lack of a strong age-related behavioural phenotype in this model. This is in complete contrast with the published literature (Moechars et al., 1999b). In the RAWM test administered at 10 months (Experiment 3), no indications of a spatial navigation deficit observed. In terms of the longitudinal assessment of spatial working memory, the results from the foraging task do not indicate a transgenic spatial deficit at 3 or 19 months, although differences emerge on a subset of measures at 6 months of age (Experiment 4). These results are not corroborated by the T-maze experiment conducted at 11 and 14 months of age, where transgenic mice outperformed wild type mice in measures of spatial working memory (Experiment 5). Thus, the transgenic deficits observed in the foraging task can be considered at best transient and possibly a task-specific phenomenon. In terms of object recognition memory, transgenic and wild type mice display comparable performance in novelty detection in the novel object recognition task at 18 months of age (Experiment 6). Importantly, the lack of genotypic differences is consistently due to an ability of transgenic mice to successfully complete the tasks as opposed to poor performance of wild type mice.

In terms of gender, female mice made more spatial working memory errors than males regardless of genotype in both the T-maze and selected variables of the foraging task. This information is valuable when designing future experiments, as no previous information regarding gender is available in this model, and the task difficulty levels may need to be lowered for female mice compared to males. No such gender differences emerged from the RAWM or the object recognition task. Whilst it is not clear by which mechanisms gender influences performance, the emerging gender pattern raises the possibility that female transgenic mice display a more extensive Aβ pathology than transgenic males.

APP(V717I) transgenic mice were found to display lower levels of anxiety compared to wild type mice (Chapter 2). Therefore, the lack of a strong spatial memory deficit in transgenic mice could be due to an advantageously lowered anxiety enabling enhanced learning about the spatial information necessary to successfully master each task. Whilst this explanation could apply to the RAWM data, it is less likely to explain the general lack of deficit observed in the foraging task and the T-maze, as these tasks rely on different motivational manipulations. Nevertheless, the lack of a spatial memory deficit in transgenic APP(V17I) mice could be related to general alterations in motivation which enhance learning compared to wild type controls.

The differences in genetic background as discussed in Chapter 2, Section 2.5 is a potential factor in the discrepancy in results compared to Moechars et al. (1999b) and Dewachter et al. (2000). Importantly, small variations in genetic background can also affect spatial memory performance, as illustrated in Savonenko, Xu, Price, Borchelt & Markowska (2003). Despite the reduction of differences in genetic background through the breeding of FVB/N transgenic males with C57Bl/6 females, the noncongenic nature of the C57Bl/6 background has been shown to encourage the development of age-related spatial memory deficits in a manner not observed in congenic (more than 10 generations of sibling mating) strains of C57Bl/6 mice. Indeed, Savonenko et al. (2003) found that cognitive deficits evident in an APP_{swe} model on a noncongenic C57Bl/6 background were abolished when compared to either a congenic line of the same mutation or downstream congenic generations of the original noncongenic line. This highlights the importance of genetic background in behavioural assessments of cognitive deficits in AD-like mouse models, and could aid to explain the lack of a strong spatial memory phenotype in the current cohort of APP(V717I) mice.

There were a number of differences in the behavioural paradigms utilised between the current project and Moechars et al. (1999b) and Dewachter et al. (2002) that could contribute to differences in findings. These include both differences in the task themselves, as well as variations in testing protocol, such as under floor illumination in the object recognition task utilised only in Dewachter et al. (2002). The most important discrepancy in results between the groups in terms of age-related deficits is that of the water maze spatial navigation task. Here, two different versions of the water maze were used. Whilst Moechars et al. (1999b) utilise the standard water maze paradigm where a platform is located in a circular pool, the current study used a water maze with 6 arms which the animal has to discriminate between in order to locate the hidden platform. Thus, it could be argued that the RAWM has a higher difficulty level than the standard maze (Nilsson et al., 2004) as splitting the maze into 6 distinct zones inhibits the use of non-spatial strategies such as circling the edges of the pool. The RAWM requires the commitment of a choice whilst in the centre of the arena, thus reducing the likelihood of coming upon the platform by chance. If adopting this approach, there is the possibility that a lack of a spatial deficit in transgenic APP(V717I) mice is due a higher task difficulty level in the RAWM where wild type mice make sufficient errors to mask any transgenic-wild type differences as compared to the Morris water maze. This is unlikely however, due to a relatively low number of errors in both transgenic and wild type scores, in addition to data from the probe trial indicating successful learning in both groups. Therefore, the contrasting data between the two laboratory groups is unlikely to be due to differences in

task difficulty. Overall, differences in testing paradigms are not sufficient to explain the large discrepancies between the published literature and the current findings. The experiments outlined in this chapter cover a range of behavioural tasks across 3 – 19 months of age in both male and female mice without the detection of a strong age-related cognitive phenotype. In addition, the adopted tasks were verified through the use of C57Bl/6 mice with bilateral hippocampal excitotoxic lesions, demonstrating that the paradigms used were sensitive to hippocampal damage.

The lack of a recognition memory deficit in transgenic APP(V717I) mice may be due to insufficient delay between sample and test in the current work. The delay intervals were selected based on reports of an APP(V717I) transgenic deficit after a 3 hour delay, but not after 5 minute or 1 hour (Dewachter et al., 2002). An extension of the delay interval may have increased the task difficulty sufficiently for genotypic differences to emerge. It is worth noting however that whilst both transgenic and wild type performance on this task was above chance, mice did not show a strong novelty preference at the shortest delay of 5 minutes, suggesting that there is relatively little room for a decline in wild type performance as a function of increased task difficulty before they perform at chance level. The lack of a object recognition memory deficit is in line with data presented in Chapter 3 and 4 assessing both anxiety and spatial memory, demonstrating that the current cohort of APP(V717I) mice do not show a comparable phenotype to that reported in Dewachter et al. (2002) or Moechars et al. (1999b).

Whilst the longitudinal study of cognitive ability is powerful in that it allows detection of within-subject decline in performance with age, there may be potential beneficial effects of repeated testing on cognition which may mask cognitive decline. There are two variants of this argument. Firstly, a lack of deficit at the aged time point could be due to procedural learning from repeated testing. Secondly, repeated testing could have beneficial effects of cognition through acting as an environmental enrichment. Thus, lack of age-related aggravation in cognitive deficits in transgenic APP(V717I) mice from 6 to 19 months of age in the foraging task (Experiment 4) could reflect the effect of repeated testing rather than the absence of spatial memory decline. Environmental enrichment is widely recognised to be beneficial both at a behavioural and neural level in rodents, and is often assessed as the effect of enriched cage conditions on cognition (van Praag, Kempermann & Gage, 2000). In humans, cognitive exercise has been shown to be positively linked to performance on neuropsychological assessments in aged healthy individuals (Valenzuela & Sachdev, 2009). Thus, the argument

suggests that repeated testing in a number of tasks (including the foraging task) could offer sufficient cognitive exercise and environmental stimulation to protect transgenic mice from developing age-related cognitive deficits. The best way to control for this is through the use of parallel longitudinal and cross-sectional assessments of transgenic performance. In the absence of this control, it is desirable to minimise repeated testing to a limited number of test days. Between assessments in the foraging task at 6 and 19 months, the APP(V717I) cohort had undergone testing with the T-maze and RAWM assessment at 10-11 months of age. Including habituation days, the total number of days mice were exposed to testing rooms and equipment other than their home cage was 16, which is low considering the age span mice were tested at. In support of the argument that environmental enrichment, through repeated testing, could have attenuated potential genotypic differences at 19 months, Billings et al. (2005) have shown that learning slows the development of Aβ pathology if is occurs at a critical early age when assessing the 3xTg-AD model every 3 months from 2-18 months of age in the Morris water maze. The lack of behavioural effect is less relevant to the foraging task data due to the Billings et al. study using a reference memory design in which the location of the escape platform did not change between testing points compared to the foraging task as a working memory paradigm. Nevertheless, it is interesting to note that mere exposure to repeated testing can alter the development of pathology. In the current study, mice were not tested as repeatedly as in Billings et al. (2005). Furthermore, there have been reports of other cohorts of APP expressing transgenic lines which show a lack of cognitive profile at old age (24-26 months) following testing in an extensive battery of tests (Savonenk et al., 2003). Importantly, these mice showed a lack of cognitive deficit in parallel longitudinal and cross-sectional designs. Others have reported comparable spatial memory deficits in PDAPP transgenic mice tested extensively in a longitudinal as well as a cross-sectional design (Chen et al., 2000). These examples demonstrate that repeated testing need not attenuate age-related cognitive decline in APP mutation mouse models.

Whilst the relative similarity in performance between wild type and transgenic mice at 19 months could be due to procedural learning following repeated testing, this explanation is less likely due to the nature of the task. Firstly, each trial of the foraging task can be successfully solved in a number of ways. This means that the animal is not required to learn a set pattern of responding in order to obtain rewards. Indeed, the mice change their pattern of responding in each trial, such that the required behaviour to successfully complete the task changes in each trial based on the locations visited in the earlier part of that trial. Therefore, whilst opportunities to learn about the requirements of a successful trial are available, it is

unlikely that mice acquire a defined pattern of responding. Secondly, the foraging task involves only 4 test days at each age, with the 2nd and 3rd age points being separated in time by 13 months. With a stronger spatial working memory deficit indentified at 6 months compared to 19 months of age, it seems unlikely that transgenic mice benefitted more than wild type mice from repeated testing 13 months later.

The lack of a spatial working memory deficit in the T-maze is in contrast to the deficits identified in aspects of the foraging task, particularly at 6 months of age. It is therefore tempting to suggest that the two tasks either differ in the exact form of spatial working memory assessed, or in their sensitivity to spatial working memory deficits. Indeed, when viewing the total errors made across whole trials in the foraging task, arguably a more global measure of performance and potentially more comparable to the T-maze percentage correct score, there are no genotypic differences. Genotypic differences were restricted to subtypes of error and during parts of trials in the foraging task. Whilst this is not problematic in itself when put into the context of the whole task, it points to the versatility the foraging task offers in its measures of performance compared to the T-maze. In addition, the range of performance success as measured in the T-maze is naturally restricted, as chance performance allows a subject to score 50%. This leaves a relatively small window of opportunity for detection of genotypic differences.

The lack of age-related cognitive decline in transgenic APP(V717I) mice in both spatial and object recognition memory indicates that Aβ CNS levels were not sufficiently elevated in transgenic mice to impair cognition, potentially through an unknown protective factor of the C57Bl/6 background as compared to the FVB/N background. Overall, the experiments described in Chapter 3 demonstrate that APP(V717I) mutation model does not display detectable age-related deficits in learning and memory which could be utilised as a measure of the effect of Aβ-modifying treatment on cognition. However, in order to inform the behavioural data presented on the APP(V717I) model, the aim of the experiments reported in Chapter 4 was to provide a developmental profile of Aβ pathology in transgenic and wild type APP(V717I) mice.

Characterisation of Aβ Pathology in the London APP(V717I) Model

4.1 Introduction

This chapter will present the results of a developmental profile of Aβ pathology in the APP(V717I) model. The experiments aimed at identifying the age of onset and progression of Aβ pathology in the CNS of transgenic APP(V717I) mice.

There are a number of reasons why a biochemical characterisation of the APP(V717I) model was necessary. Firstly, in models of AD where potential behavioural deficits are thought to be driven by manipulations of gene expression and their biochemical effects, it is important to characterise the developmental profile of biochemical changes in parallel with the assessment of behavioural deficits. Knowledge of both aspects of disease progression within a model gives opportunities for increased understanding of how behavioural deficits and biochemical changes are linked, as well as increased sensitivity to the potential effects of a therapeutic manipulation at different levels of functioning of the CNS.

Secondly, there are no studies describing the biochemical effects of the introduction of the APP(V717I) mutation in C57Bl/6 mice, providing a direct comparison to the current cohort. Whilst a range of papers have reported measurements of Aβ in the APP(V717I) model (see Chapter 2, Section 2.1.2, Table 2.1 for an overview), all biochemical characterisation work has been carried out using the V717I mutation on the FVB/N background. The effect of this change is unknown, but studies such as Glazner et al. (2009) have reported differential development of Aβ pathology in the TgCRND8 when maintained on different genetic backgrounds (also see Savonenko et al., 2003). This further underlines the need to undertake a biochemical analysis to assess the compatibility of the data across background stains.

Thirdly, the importance of assessing the progressive elevation of Aβ was heightened in light of the lack of evidence for an age-related cognitive decline in the APP(V717I) model (Chapters 2 and 3). In addition, behavioural analysis of the model in Chapters 2 and 3 indicated a potential gender difference in expression of the transgenic phenotype, raising the possibility that Aβ pathology differs between males and females. Previous literature assessing the biochemical profile of the APP(V717I) mutation on the FVB/N background did not consider the potential influence of gender. Thus, this chapter presents work aimed to determine the levels of soluble and insoluble Aβ40 and Aβ42 as measured using ELISA, as well as the degree of Aβ

deposition in APP(V717I) transgenic mice of both genders compared to wild type mice at ages 3, 6, 9, 12 and 18 months. These ages were chosen in order to provide a direct parallel to the ages at which the behavioural work had been carried out.

On the FVB/N background, the transgenic mice develop elevated soluble Aβ40 and Aβ42 levels by 15 months of age, with higher levels of Aβ40 than Aβ42 (Dewachter et al., 2000; 2002). Some elevation has been reported as early as 3-6 months (Dewachter et al., 2000), but no plaque formation has been observed in this model until after 12 months (Dewachter et al., 2000). Thus, it was predicted that there will be a progressive elevation of soluble Aβ levels in transgenic mice compared to wild type mice at ages 3, 6 and 9 months, as well as significantly higher levels of soluble Aβ by 12 and 18 months of age. In terms of Aβ deposition, high levels of Aβ deposits were expected in transgenic brain tissue at 18 months, with a subset of transgenic mice potentially showing Aβ pathology at 12 months. No Aβ deposition was expected in wild type tissue at any age or in transgenic tissue at 3, 6, or 9 months of age. In the event that gender influences Aβ pathology in APP(V717I) mice on a C57Bl/6 background, female transgenic mice were predicted to display elevated Aβ pathology compared to male transgenic mice.

4.2 Experiment 8a: Aβ levels in brain homogenate of APP(V717I) mice

4.2.1 Introduction

The assessment of Aβ pathology using ELISA allows for the targeted measurement of human Aβ in brain homogenates, providing a quantifiable comparison between transgenic and wild type tissue at varying ages. Assessment focused on the left cortex as a representative sample for the rest of the brain as the APP(V717I) develop extensive pathology across the cortex (Dewachter et al., 2000).

4.2.2 Methods

Subjects

A total of 37 transgenic and 31 wild type APP(V717I) of both genders at varying age points were used, as outlined in Table 4.1. Group sample sizes ranged from 2-5 mice per group, and were identical across Aβ40 and Aβ42 analyses.

	3 months	6 months	9 months	12 months	18 months
Tg Male	3/4	3/3	3/3	5/4	4/4
Tg Female	3/3	3/3	4/4	4/4	4/4
WT Male	2/2	3/3	2/2	3/3	3/2
WT Female	2/3	3/3	3/3	4/4	5/5
Tg total	**6/7**	**6/6**	**7/7**	**9/8**	**8/8**
WT total	**4/5**	**6/6**	**5/5**	**7/7**	**8/7**

Table 4.1: Overview of the number of subjects included in the Aβ ELISA analysis split by gender and genotype at 3, 6, 9, 12 and 18 months of age. Soluble and insoluble Aβ sample sizes are denoted as soluble/insoluble respectively.

Enzyme-linked immunosorbent assays: General Sandwich ELISA

Brain dissection

Mice were culled by cervical dislocation, the brain removed immediately and the hippocampus and cortex were dissected bilaterally from the rest of the brain tissue before being snap frozen in liquid nitrogen. Samples were stored at -80^{o}C until further processing.

Protein Extraction

The left cortex sample of each mouse was homogenised in 2% sodium dodecyl sulphate (SDS) in dH_20 with 1% inhibitor cocktail (Millipore) at 1ml/75mg of wet tissue weight. The samples were homogenised using a Precellys 24-Dual (Bertin technologies, Montigny-le-Bretonneux, France) at 6000rpm for 2*30 seconds with a 30 second delay. The homogenate was rotated at 4^{o}C overnight, before being centrifuged at 100 000*g (28 300rpm) for 1 hour at 4^{o}C. The supernatant of soluble protein was diluted 1/5 in phosphate buffer (20mM Na_2HPO_4/NaH_2PO_4, 0.2mM EDTA, 0.4M NaCl, 0.2% bovine serum albumin (BSA), 0.05% CHAPS, 0.05% NaN_3 at pH 7) and stored at -20^{o}C. Following removal of the soluble fraction, an insoluble pellet remained to which 70% formic acid was added at 1ml/150mg of wet tissue weight. The insoluble pellet was centrifuged at 100 000*g (28 300rpm) for 1 hour at 4^{o}C. The supernatant of insoluble protein was diluted 1/20 in 1MTris Phosphate buffer (1M Tris, 0.5M Na_2HPO_4, pH 11) to neutralise the sample, and stored at -20^{o}C.

Bicinchoninic acid (BCA) protein assay

It is necessary to establish the amount of total protein present in each sample to standardise levels of individual proteins within a sample. The BCA Protein Assay Reagent bicinchoninic acid

kit (Pierce Thermo Scientific) was used to analyse the protein levels in the neat soluble fraction obtained in the protein extraction stage. Diluted bovine serum albumin standards (Thermo Scientific) were prepared ranging from 2 – 0.01mg/ml in a serial dilution. The standards, samples and negative controls were analysed in duplicate on a 96 well plate, with negative controls including dH_20 and 2% SDS. A volume of 200µl BCA Working Reagent consisting of 50 parts Reagent A (Pierce Thermo Scientific) and 1 part Reagent B was added, the plate was mixed thoroughly for 30 seconds and incubated at 37^{o}C for 30 minutes. The plate was read at absorbance 540nm using a spectrophotometer. Protein values in each sample were calculated using GraphPad Prism 4.0 from the standard curve.

Sandwich ELISA for soluble Aβ40

The anti-N-terminal human Aβ monoclonal antibody (MAb) 6E10 (Covance, Princeton, USA) was incubated on a 96 well microtitre plate (Greiner Bio-One, Frickenhausen, Germany) at a concentration of at a concentration of 0.167µg/ml diluted in carbonate/bicarbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) overnight at 4^{o}C. The plate was washed with PBST (137mM NaCl, 2.5mM KCL, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.05% Tween20) in between each stage, and all incubations were at room temperature (RT). Unbound sites were blocked with 1% non-fat milk powder in PBS for 30 minutes. Samples, standards and negative controls in PBST were incubated at 100µl per well in duplicate for 2 hours. Aβ40 standards (Invitrogen) ranged from 10-0.019ng/ml in doubling dilutions. Standards were made up in the identical background buffer to that of the samples, with a minimum dilution of 1/8 to that the background buffers did not inhibit the target signal. Negative controls included phosphate buffer and PBST. The detection antibody, BAM401AP (affinity purified, Autogen Bioclear, Calne, UK), an affinity-purified antibody specific to the C-terminal of the Aβ1-40 protein, was used as the detection antibody at a concentration of 0.45µg/ml in PBST for 1.5 hours. BAM401AP has been verified as specific through western blotting work within the laboratory group (Thomas, unpublished data). The secondary HRP-labelled anti-rabbit IgG (Pierce Thermo Scientific) was applied at 0.33µg/ml in PBST for 1 hour. The enzyme substrate, o-phenylenediamine (OPD), in 0.1M citrate-phosphate buffer (24mM citric acid, 51mM Na_2HPO_4, pH5) was applied and incubated for approximately 20 minutes in the dark. The reaction was stopped using 50µl 2.5M H_2SO_4 and read at 492nm using a spectrophotometer. The ELISA protocol provides a lower sensitivity limit of around 0.1ng/ml (Thomas et al., 2006).

Sandwich ELISA for soluble Aβ42

The measurement of soluble Aβ1-42 species from brain homogenate was carried out in an identical fashion to that described for the measurement of Aβ1-40 with the exception that the detection antibody was the affinity-purified anti-C-terminal human Aβ1-42 antibody (AB5739, Millipore, 0.5ug/ml in PBST). This antibody has been verified as specific through western blotting work within the laboratory group (Thomas, unpublished data). Aβ1-42 peptide standards (Invitrogen) at concentrations 10-0.019ng/ml in doubling dilutions provided a standard curve. All other conditions remain identical to that described in for the quantification of soluble Aβ40.

Sandwich ELISA for insoluble Aβ40/42

The measurement of insoluble Aβ1-40/42 species did not differ from the measurement of soluble Aβ1-40/42. However, due to the presence of formic acid in the samples, a 1/10 dilution of the sample was necessary to ensure that the concentration of formic acid did not inhibit the signal in the ELISA.

Data Analysis

Individual Aβ values were standardised to the total protein concentration of each sample, and all values are given as ng/mg of total protein. Aβ values were averaged across genotypes within each gender and age group. Due to low sample sizes in combination with a clear pattern of age-dependent increase in Aβ, which is in keeping with the hypothesised direction of effects, no statistical analysis of age was carried out without collapsing across gender.

4.2.3 Results

Average concentrations of soluble and insoluble Aβ40 and Aβ42 in transgenic samples are presented in Table 4.2. Wild type values were at near background levels at every age in both males and females. For simplicity, wild type values for each age/gender group are not presented, but the average values of each Aβ species for all WT mice are included.

Age	Gender	Soluble (ng/mg protein)				Insoluble(ng/mg protein)			
		Aβ40		Aβ42		Aβ40		Aβ42	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
3	Male	**0.00**	0.00	**0.10**	0.04	**0.08**	0.04	**0.01**	0.01
	Female	**0.01**	0.01	**0.02**	0.01	**0.04**	0.01	**0.05**	0.04
6	Male	**0.00**	0.00	**0.06**	0.01	**0.15**	0.09	**0.01**	0.01
	Female	**0.00**	0.00	**0.04**	0.02	**0.22**	0.03	**0.23**	0.01
9	Male	**0.00**	0.00	**0.03**	0.00	**0.07**	0.03	**0.01**	0.01
	Female	**0.02**	0.02	**0.03**	0.01	**0.21**	0.09	**0.04**	0.01
12	Male	**0.00**	0.00	**0.03**	0.01	**0.25**	0.05	**0.01**	0.01
	Female	**0.16**	0.05	**0.11**	0.06	**0.18**	0.08	**0.43**	0.25
18	Male	**4.78**	4.16	**2.34**	1.84	**0.23**	0.05	**0.97**	0.56
	Female	**23.95**	8.03	**16.69**	6.59	**3.65**	2.54	**9.79**	6.78
Mean	**WT**	**0.01**	0.01	**0.05**	0.01	**0.11**	0.02	**0.03**	0.12
	Tg	**3.13**	1.50	**2.10**	1.07	**0.54**	0.30	**1.22**	0.82

Table 4.2: Mean soluble and insoluble Aβ40 and Aβ42 concentrations for transgenic APP(V717I) mice of both genders at ages 3, 6, 9, 12 and 18 months. Mean WT levels across gender and age are provided for comparison.

Given the low levels of Aβ from 3 to 9 months of age, genotypic differences were statistically explored at 12 and 18 months only. Values of wild type and transgenic mice aged 18 months were collapsed across gender. Transgenic mice displayed significantly higher levels of insoluble Aβ40 (U (7, 8) = 7.00, $p < .02$) than wild type mice at 12 months of age, but no other genotypic differences were evident at this age [soluble Aβ40: (U (7, 8) = 17.50, $p > .05$); soluble Aβ42: (U (7, 8) = 25.00, $p > .05$); insoluble Aβ42: (U (7, 8) = 23.00, $p > .05$)]. By 18 months of age, transgenic mice were found to have higher levels of soluble Aβ40 (U (8, 8) = 4.50, $p < .001$) and Aβ42 (U (8, 8) = 7.00, $p < .01$), as well as insoluble Aβ42 levels (U (7, 8) = 6.00, $p < .01$) compared to wild type mice. No differences in insoluble Aβ40 levels was detected (U (7, 8) = 17.50, $p > .05$).

The effect of age on Aβ levels

In order to assess whether age influenced Aβ levels in transgenic mice, the data were collapsed across gender. Given that levels of Aβ rise in a non-linear fashion at 18 months of age, analyses were restricted to pairwise comparisons of the 18 month data with the earlier age points. The results are summarised in Table 4.3, and reveal significant differences in transgenic mice when comparing levels of insoluble Aβ40 at 3 and 18 months (U (6, 8) = 2.00, $p > .001$). Furthermore, transgenic mice differed significantly in measures of soluble Aβ when comparing tissue from 9 month and 18 month old mice [Aβ40: (U (7, 8) = 4.00, $p > .002$); Aβ42;

(U (7, 8) = 1.00, p > .001)], as well as when comparing 12 month and 18 month tissue [Aβ40: (U (8, 8) = 6.50, p > .002); Aβ42; (U (8, 8) = 5.00, p > .002)].

Pairwise comparison (age in months)	Soluble Aβ40		Soluble Aβ42		Insoluble Aβ40		Insoluble Aβ42	
	U	*P*	*U*	*p*	*U*	*P*	*U*	*P*
3 vs. 18	3.50	0.004	4.50	0.008	**2.00**	**0.001**	10.00	0.03
6 vs. 18	3.00	0.004	3.50	0.005	11.50	0.11	9.50	0.07
9 vs. 18	**4.00**	**0.002**	**1.00**	**0.001**	10.00	0.04	11.50	0.06
12 vs. 18	**6.50**	**0.002**	**5.00**	**0.002**	19.50	0.009	15.00	0.04

Table 4.3: The test statistic and exact significance for the effect of age in transgenic APP(V717I) mice on variables from ELISA analysis of Aβ pathology is presented collapsed across gender. Significant values are highlighted in bold.

4.2.4 Discussion

A biochemical analysis of soluble and insoluble human Aβ40 and Aβ42 in transgenic and wild type APP(V717I) tissue of both genotypes was carried out using ELISA. The numerical measurements of Aβ pathology show genotypic differences in Aβ pathology emerging from 12 months of age, indicating that Aβ pathology developed in an age-dependent manner in transgenic mice.

Statistical analysis revealed a general increase in transgenic soluble and insoluble Aβ40 levels compared to wild type mice. This effect was shown to be age-related, with no genotypic differences detectable at 3-9 months of age. By 12 months of age, transgenic mice show elevation of insoluble Aβ42 levels at 12 months of age. Further genotypic differences emerge in soluble Aβ40, soluble Aβ42 and insoluble Aβ40 emerging by 18 months of age. The effect to age on Aβ pathology was pronounced in transgenic tissue, with an absence of pathology in wild type tissue.

Comparing the current results to reported levels of Aβ in the APP(V717I) model is challenging due to the high variability and lack of gender specification in reported levels of soluble and insoluble Aβ40 and Aβ42 (see Chapter 2, Section 2.1.2, Table 2.1). Nevertheless, the soluble ELISA results reported in the current work overall appear higher than that of previous studies (Dewatcher et al., 2000; Willem et al., 2004; Etcheberrigary et al., 2004; Dewacther et al., 2002). This discrepancy emerges from 12 months of age, and is particularly evident in the levels of soluble Aβ in female transgenic mice. Only one study reports higher

soluble Aβ levels than the presented results (Tanghe et al., 2010). This is likely to be due to variations in the measurement protocols, as Tanghe et al. (2010) de-aggregated the Aβ samples prior utilising an ultrasensitive assay for increased sensitivity (Amorfix Aggregated Abeta Assay A4). In terms of the ELISA results for insoluble levels of Aβ, the levels presented in the current work are comparable to the literature (Tanghe et al., 2010; Willem et al., 2004).

Overall, the pattern of age-related increase in Aβ pathology in transgenic mice is in line with previous literature, where the elevation in Aβ levels has been found to be initiated around 12-16 months of age, with near background levels detected prior to that age (Dewachter et al., 2000; Willem et al., 2004; Tanghe et al., 2010). This underlines the observation that Aβ pathology does not develop until relatively late in this model compared to other APP mutation models such as the Tg2576 (Hsiao et al., 1996). This may be due to differences in the overexpression levels of APP, as transgenic Tg2576 mice displaying a 5-fold increase in hAPP compared to endogenous APP (Hsiao et al., 1996), whilst APP(V717I)transgenic mice produce 3-fold hAPP (Moechars et al., 1999b).

There is evidence of a gender effect in the development of Aβ pathology in APP(V717I) transgenic mice, with females displaying numerically higher levels of every species of Aβ measured compared to males at 18 months of age. The limited sample size means the value of statistical analysis is limited, indicating caution must be taken when interpreting the gender patterns observed. As no mention of gender has been made in the APP(V717I) literature, it is difficult to assess the way in which this pattern applies to previous work. A further discussion of the potential effect of gender on Aβ pathology is provided in the Chapter discussion.

4.3 Experiment 8b: Aβ deposition in the CNS of APP(V717I) mice

4.3.1 Introduction

Whilst analysis of brain homogenates using ELISA allowed for an easily quantifiable assessment of Aβ pathology across genotype, gender and age, it provided no information regarding the regional distribution of Aβ pathology. Therefore, immunohistochemical staining was utilised in parallel with ELISA techniques.

4.3.2 Methods

Subjects

Coronal brain sections from 6, 9, 12 and 18 month old transgenic and wild type APP(V717I) of both genders were used, with an sample size of 2-3 per genotype/gender/age group. Three month old mice were not included, as no Aβ deposition is expected at this young age, and ELISA results (4.2.3) suggested near background levels of Aβ in 3 month old mice.

Immunohistochemical staining for Aβ40/42

Mice were perfused as described in Section 3.2, Chapter 3, with the exception that sliced sections were stored in cryoprotectant (30% sucrose analytical grade, 30% Ethylene Glycol, 1% Polyvinylpyrrolidone in 0.1M PBS, ph 7.4) at -20^{o}C until use. Tissue sections from were rinsed three times in 0.1M Tris Buffered Saline (pH 7.4) before each treatment stage, except where otherwise specified, and all incubations were carried out at RT. As an antigen retrieval, sections were treated with 85% formic acid in dH_2O at 25^{o}C for 10 minutes, followed by Quench (10% Methanol, 10% H_2O_2, 40% dH_2O) for 5 minutes to block endogenous peroxidase. A 3% Normal Goat Serum (NGS) in TXTBS (0.1% Triton X-100 in TBS) block was applied for 1 hour. Without washing, this was followed by incubation with either the Aβ1-40 (AB5074P, Millipore) or the Aβ1-42 primary antibody (AB5078P, Millipore) at 1µg/ml with 1% NGS in TXTBS overnight on a stirrer. These have previously been shown to be specific to the stated Aβ species (Kamal et al., 2001). An anti-rabbit secondary antibody (Vector Laboratories Inc, Peterborough, UK) was applied at 7.5µg/ml with 1% NGS in TXTBS for 2 hours. The Dako Streptavidin ABC Complex (50% Solution A, 50% Solution B with 1% NGS in TXTBS) was applied for 2 hours, and two washes in 0.05M Tris buffer (pH 7.4) followed the standard TBS washes before sections were exposed to the peroxidase substrate 3,3'-diaminobenzidine (DAB) with nickel (Ni^{2+}) until a satisfactory degree of staining was obtained (DAB kit, Vector Laboratories). The reaction was stopped with 4^{o}C 0.1M PBS (pH 7.4) and sections were mounted on gelatinised slides before air-drying overnight. Slides were coverslipped as described in Section 3.2, Chapter 3.

4.3.3 Results

For ease of presentation, the full wild type staining profile is not presented due to the lack of Aβ40 or Aβ42 stained deposits in any sections at any age point. As illustrations of negative controls, 18 month old female wild type sections stained for Aβ40 and Aβ42, as well as an 18 month old female transgenic section not exposed to primary antibody are presented (Fig. 4.1). In addition, the immunohistochemical images from transgenic mice are presented at 12 and 18 months only, as no or fewer Aβ deposits were present in sections stained for either antibody at 6 and 9 months of age (data not shown).

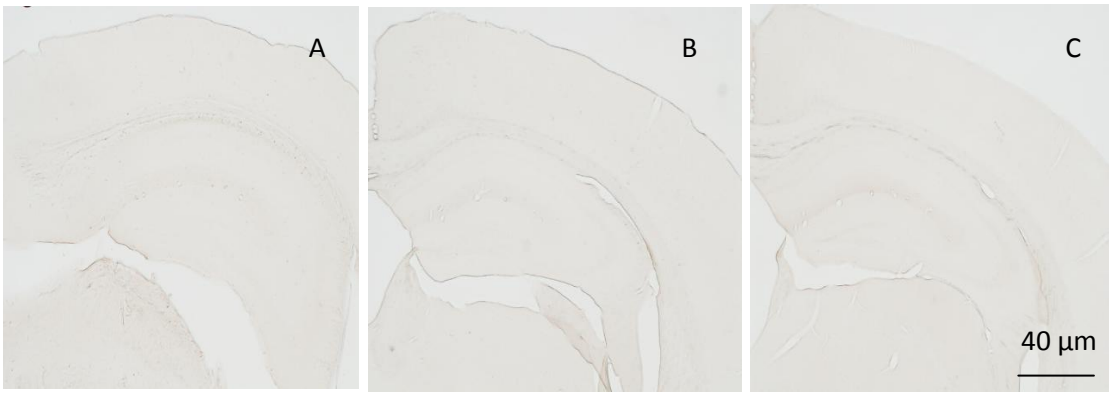


Fig. 4.1: Illustrative photomicrographs of coronal brain tissue sections from 18 month old wild type female mice stained for Aβ40 (A) and Aβ42 (B). 5.1C represents a tissue section from an 18 month old transgenic female mouse not exposed to an anti-Aβ antibody.

Photomicrographs of Aβ40- and Aβ42-stained deposits under high magnification are presented for illustration in Fig. 4.2. These are based on the hippocampus of 18 month old female transgenic APP(V717I) mice. Note the increased size of Aβ40 stained deposits compared to Aβ42, as well as the increased intensity of staining in Aβ40 sections compared to Aβ42.

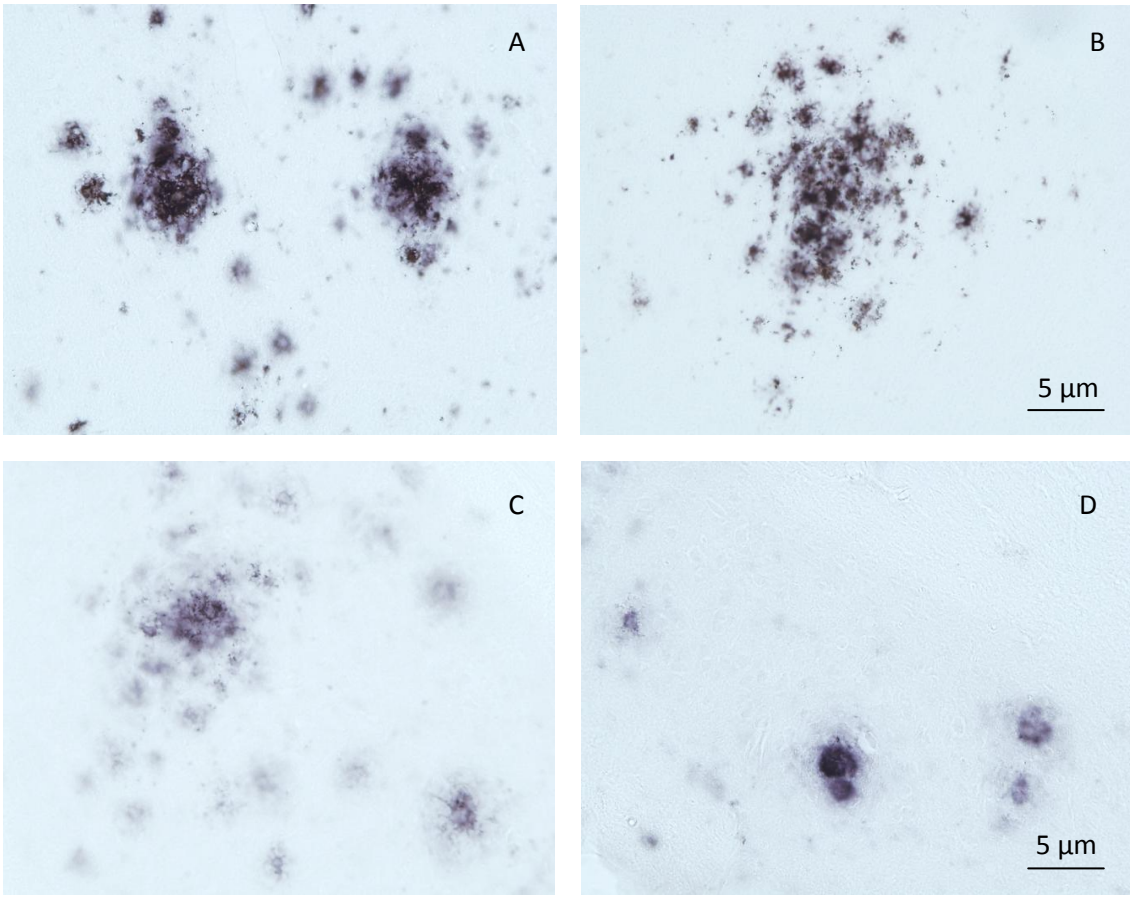


Fig. 4.2: Detailed photomicrographs of Aβ40 (A and B) and Aβ42 (C and D) stained sections displaying Aβ deposits in the hippocampus of an 18 month old transgenic female mouse.

Representative images of Aβ40 and Aβ42 stained coronal sections of transgenic APP(V717I) tissue from males and females at 12 and 18 months of age are presented in Fig. 4.3.

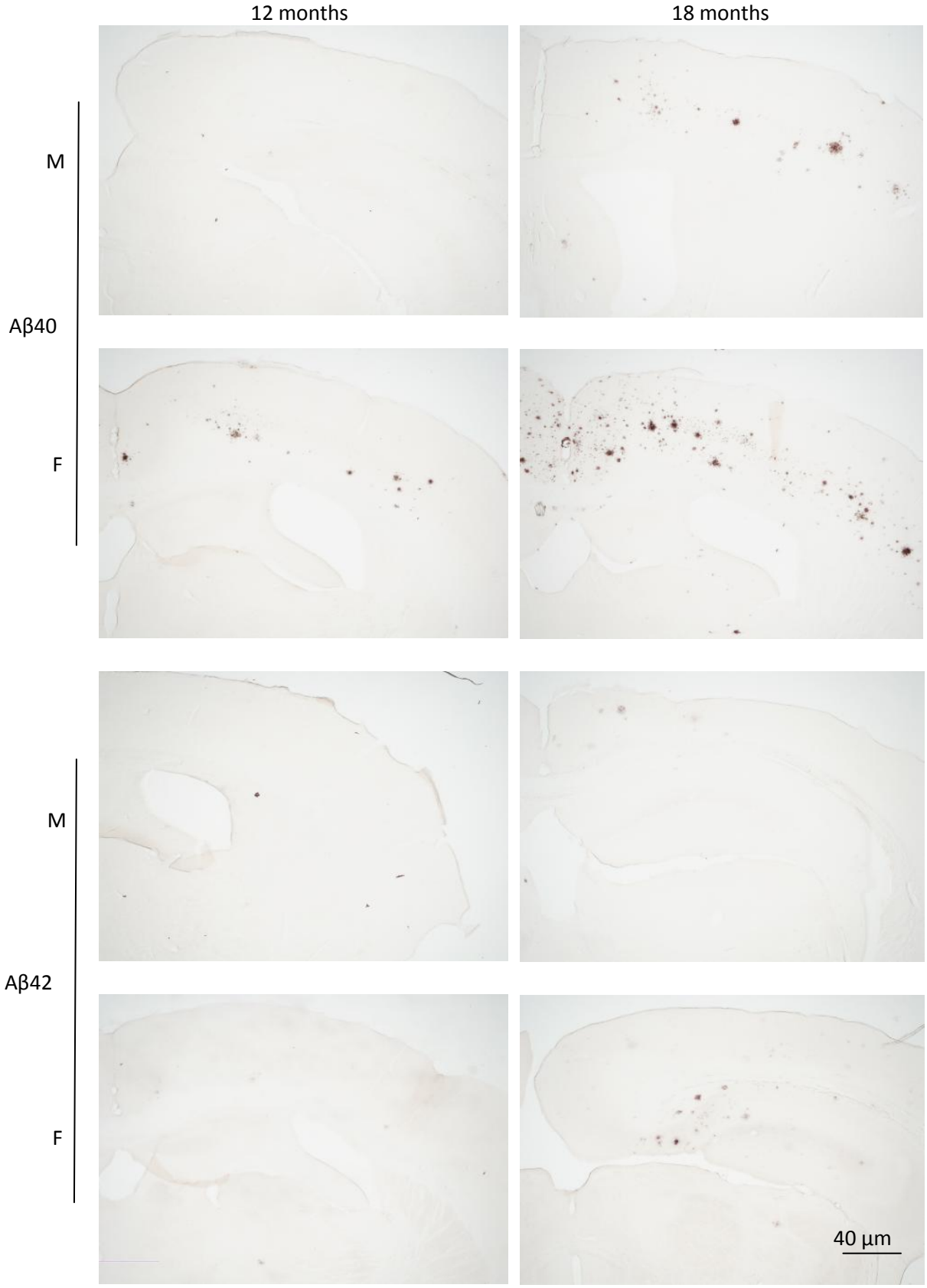


Fig. 4.3: Representative immunohistochemical photomicrographs of Aβ40 and Aβ42 stained coronal tissue sections of transgenic APP(V717I) from males and females at 12 and 18 months of age.

As can be seen from Fig. 4.3, Aβ40 stained deposits can be detected in APP(V717I) tissue at 12 and 18 months of age. Aβ42 stained deposits are less numerous, and are evident mostly at 18 months of age. Note the presence of Aβ42 staining in 18 month old female transgenic mice, but not male transgenic mice.

4.3.4 Discussion

From the immunohistochemical staining of APP(V717I) brain sections from transgenic and wild type mice of both genders at 6 – 18 months of age, it is clear that human Aβ staining is restricted to transgenic tissue. In addition, no sections without primary antibody displayed any level of staining, suggesting that the clustered dark staining observed in aged transgenic Aβ-stained sections was Aβ-specific.

Visual inspection of immunohistochemical sections suggests three conclusions. Firstly, Aβ40 deposition is heavier than Aβ42 disposition in transgenic APP(V717I) tissue, as previously shown in this model (Dewachter et al., 2000). Secondly, an age-related pattern of Aβ pathology is evident, with a higher number of Aβ deposits in tissue from aged mice. This pattern is influenced by gender, as female mice show pathology at 12 months of age with progressive decline by 18 months of age. In contrast, pathology in male transgenic tissue was limited to 18 months of age, indicating a delay in pathology onset compared to female mice.

4.4 Chapter discussion

This chapter investigated the developmental profile of Aβ pathology in the APP(V717I) model with the aim of identifying the age of onset and progression of Aβ pathology in brain tissue from transgenic APP(V717I) mice. Taken together, the results of ELISA and immunohistochemical analysis of Aβ pathology in the APP(V717I) model indicate that age-related elevation of Aβ is evident in transgenic mice both in terms of soluble and insoluble Aβ, and Aβ deposition, with higher levels of Aβ40 than Aβ42. This pathology is numerically more evident is female transgenic than male transgenic mice (although with a low sample size, this conclusion is not statistically assessed). This indicates that age is the key variable driving Aβ pathology in this APP mutation mouse model of AD-pathology, as would be expected in a model of neurodegeneration. Thus, these results are in accordance with the prediction that APP(V717I) transgenic mice would show an age-related elevation of Aβ (with higher levels of Aβ40 compared to Aβ42) emerging from 12 months of age. Importantly, these conclusions are

consistently supported by both ELISA and immunohistochemical analysis. This internal consistency in the evaluation of Aβ pathology increases confidence in the accuracy of the results.

In terms of gender differences, an interesting pattern emerges from the biochemical analysis of the APP(V717I) model where female transgenic mice display more extensive Aβ pathology than males when matched for age, indicting that female pathology develops earlier. Whilst statistical evidence for an effect of gender on the development of Aβ pathology is not available in the current study, the pattern has strong support in the literature. Female transgenic mice have frequently been reported to display both elevated Aβ pathology and impaired cognitive performance compared to transgenic male mice, with no such description of a gender difference in the reversed direction available (Sturchler-Pierrat and Staufenbiel, 2000; Bayer et al., 2003; Callahan et al., 2001; Lewis et al., 2001; Wang et al., 2003; Schuessel et al., 2004; Schafer et al., 2007). Remarkably, this effect is stable across APP mutation models despite differences in the mutation, promoter, genetic background and methodology used to assess cognitive and biochemical changes (Schuessel et al., 2004). This indicates that the gender differences observed across these studies is more likely to be linked to underlying, central aspects of the factors that differentiate the genders as opposed to artefacts related to the generation of one transgenic model (Schuessel et al., 2004). Moreover, these gender differences translate to the human clinical population, with females being at higher risk of developing AD than men (Fratiglioni et al., 1997; 200; Andersen et al., 1999; Jorm and Jolley, 1998). Female AD patients also show a different cortical distribution of Aβ plaques as well as increased overall levels (Kraszpulski et al., 2001). Whilst a lowering of estrogen in late life has been proposed as potentially modulating Aβ pathology in females, recent hormone replacement therapy has not supported this (Schafer et al., 2007). In addition, gender differences are observed in young female mice prior to a drop in estrogen levels (Schuessel et al., 2005; Schafer et al., 2007) suggesting that gender differences in Aβ pathology are unlikely to be explained by this factor alone. Recently, differences in male and female oxidative-stress response markers have been identified (Schuessel et al., 2004), with female AD patients having higher levels of upregulated oxidative stress enzymes than male patients. This could suggest that the level of oxidative stress in female AD patients is higher, or that female patients have increased sensitivity to oxidative stress resulting in a differential response. Whilst the mechanisms behind gender influences on Aβ pathology in AD patients and transgenic mouse models are unclear, it is emerging as a consistent factor across the field. Whilst little statistical evidence was obtained in the current study to support the interpretation of gender as an

influential factor in the development of Aβ pathology, it is worth noting that the numerical patterns in APP(V717I) female and male mice are in line with the gender literature in APP mutation models.

Importantly, Aβ pathology in transgenic APP(V717I) mice appears to develop at a relatively late stage, with little evidence of consistent Aβ pathology before 18 months of age. Other widely used transgenic APP mutation mouse models such as the Tg2576 model develop significant soluble Aβ levels by 6-7 months of age (Kawarabayashi et al., 2001; Westerman et al., 2002), with elevated insoluble Aβ levels at 6 and 10 months of age (Westerman et al., 2002). These differences could be related to a number of factors which are central to the development of transgenic models, such as the genetic mutation, promoter and level of overexpression of the transgene. Furthermore, differences in the genetic background mutations are maintained on have been found to influence Aβ pathology in APP mutation models (Galzner et al., 2009; Savonenko et al., 2003). The late development of Aβ pathology in the APP(V717I) restricts the use of the model for analysis of Aβ levels for 12-18 months, a substantially longer period of time than models such as the Tg2576 (Hsiao et al., 1996). The late Aβ phenotype of the APP(V717I) model is relevant in the context of behavioural deficits, as no age-related behavioural phenotype was detected between 3 and 19 months in the current work. As behavioural deficits are hypothesised to be driven by elevation of Aβ levels, it is reasonable to assume that a lack of high Aβ levels at 3 – 12 month is an important factor in the lack of a robust spatial memory deficit, or general cognitive phenotype, in this model. Furthermore, whilst there is an elevation of Aβ observed from 12 to 18 months in terms of both soluble and deposited Aβ, this is not sufficient to lead to robust disruption of cognitive processes.

Assessed across Chapters 2 – 4, the APP(V717I) model did not display consistent age-related cognitive decline, and the observed anxiety deficits were not found to develop with age. Furthermore, the presence of altered anxiety behaviour as early as 3 months of age strongly indicates that these changes are not related to Aβ toxicity, as levels of Aβ are near zero at this young age. This indicates that the changes in behaviour observed in anxiety assessments can be more readily attributed to stable factors which differentiate transgenic and wild type mice, such as APP overexpression, as opposed to Aβ pathology. In addition, the APP(V717I) model did not display elevation of Aβ levels until 12 – 18 months of age, a pattern consistently detected by both ELISA and immunohistochemistry. Furthermore, the Aβ pathology detected at this late age did not disrupt cognitive processes as assessed in Chapters

2 and 3, indicating that elevation of Aβ in itself is not sufficient to induce AD-like cognitive deficits in this model. Thus, the London APP(V717I) model does not appear to be a suitable model in which to assess the effects of 2B3 on both cognition and Aβ pathology. Chapter 5 is focused on the behavioural characterisation of an alternative APP mutation mouse model of AD-pathology with the aim of identifying deficits in cognition that may lend themselves to treatment manipulation.

Behavioural Characterisation of the PDAPP(V717F) Model

5.1 Introduction

This chapter investigates the nature of cognitive deficits in the PDAPP model of AD-pathology. The experiments presented aim to establish the types of learning and memory processes which may be affected by Aβ-pathology in the PDAPP model. This was necessary for three reasons. Firstly, in order to evaluate 2B3 as a potential Aβ modifying treatment of Aβ pathology. It is important that the effect of the antibody treatment is assessed on behavioural measures as well as on biochemical markers of Aβ pathology progression. Due to the lack of a strong behavioural phenotype in the APP(V717I) model, it was necessary to adopt an alternative APP mutation mouse model to achieve this. The PDAPP (V717F) model (Games et al., 1995) was chosen due to the location of the APP mutation it carries and its wide use in the AD pre-clinical field. Secondly, whilst the PDAPP model is one of the most widely used APP murine models in the field, the behavioural characterisation of the model has been limited to a few select tasks, with a lack of consistency across laboratory groups. Thirdly, changes in the genetic background strain of the V717F line raises issues of variability in the behavioural phenotype. Thus, the initial studies focused on characterisation of the behavioural profile of PDAPP mice. Experiments 9 and 10 assessed the anxiety behaviour in male PDAPP transgenic and wild type mice at 9.5 months of age. Experiment 11 examined spatial working memory at 9-10 months of age in the T-maze, and Experiment 12 used the foraging task to assess memory at 11 and 14 months of age. Finally, Experiment 13 assessed object recognition memory using the novel object recognition paradigm at 12 months of age.

5.1.1 PDAPP(V717F) mice

The transgenic PDAPP mouse model of Aβ pathology was developed by Games et al. (1995) and carries the familial autosomal dominant human Indiana APP mutation. This mutation manifests itself as a valine to phenylalanine substitution at residue 717 (based on the 770 isoform) of APP. In the London mutation model, valine is substituted with isoleucine at the same residue. The location of the mutation at the γ-secretase cleavage site rather than the β-secretase cleavage site is critical as it maintains a reduced likelihood of disrupting the antibody's binding capacity to the APP molecule. The transgene in the PDAPP model is driven

by the platelet-derived-β growth factor (PDGF-β) and contains a splicing cassette, resulting in expression of all three isoforms of hAPP (Games et al., 1995). APP expression levels have been indicated to be approximately 10-fold that of endogenous APP (Games et al., 1995). The PDGF-β promoter is said to target neuronal expression, although levels of both hAPP messenger RNA (Games et al., 1995) and hAPP protein levels have been observed in peripheral tissues such as the kidneys (Schenk et al., 1999).

The PDAPP model is frequently reported as being maintained on a triple background strain constituting the C57Bl/6, DBA/2 and Swiss Webster background strains (Janus & Eriksen, 2007), but it has recently been bred onto a pure C57Bl/6 background. Unlike the Tg2576 transgenic hAPP mouse model (Hsiao et al., 1996), the PDAPP mouse is viable when heterozygous and homozygous for the APP(V717F) mutation. The experiments reported in this thesis used heterozygous mice, thus the review of the pathological and behavioural profile of PDAPP mice will not include contributions from studies on homozygous PDAPP mice. It is worth noting, however, that gene dosage does play a role in both Aβ pathology and behavioural deficits. These are both more pronounced and present earlier in homozygous mice compared to heterozygous mice (Dodart et al., 1999; 2000).

In terms of pathological makers of disease, PDAPP mice reliably develop Aβ deposits with age (Games et al., 1995; Irizarry et al., 1997; Johnson-Wood et al., 1997; Masliah et al., 1996; Bard et al., 2000; Chen et al., 2000; Schenk et al., 1999). In heterozygous animals, no pathology is evident at 4-6 months, but deposits of human Aβ in the hippocampus, cerebral cortex and corpus callosum begin at 6-9 months of age (Games et al., 1995). Similarly, Dodart et al. (2000) reported numerous plaque deposits in transgenic mice in the hippocampus, the medial cingulated cortex and the corpus callosum at 6-7 months of age. From 4 to 8 months of age, hippocampal Aβ levels increase 17-fold, with a further 500-fold increase in transgenic PDAPP mice by 18 months of age (Johnson-Wood et al., 1997). By 12-15 months of age, considerable Aβ deposition is observed (Reilly et al., 2003). Whilst the majority of the dense and diffuse plaques are focused in the cerebral cortex and hippocampus, plaques have been reported in the frontal cortex and the cerebellum (Dodart et al., 2000). Soluble Aβ40 and Aβ42 levels rise from approximately 1600ng/g of cortex tissue at 12 months to 8700 and 22000ng/g by 15 and 18 months respectively (Schenk et al., 1999).

Transgenic PDAPP mice show alterations beyond Aβ pathology. Whilst no neuronal loss has been observed in PDAPP mice, the pathological profile does include synaptic loss, with a reduction in the density of synaptic and dendritic markers in the dentate gyrus (Games et al.,

1995; Masliah et al., 1996; Dodart et al., 2000). In addition, PDAPP mice display a reduction in the size of the hippocampus, the fornix and the corpus callosum (Dodart et al., 2000; Weiss et al., 2002; Redwine et al., 2003). Of these, a 25% reduction in the length of the corpus callosum appears to be stable across age and evident as early as 6 weeks of age (Redwine et al., 2003). The reduction in hippocampal volume is apparent prior to Aβ plaque deposition at 3-4 months of age, but not at 6 weeks of age (Dodart et al., 2000; Weiss et al., 2002; Redwine et al., 2003). This size reduction could be linked to overexpression of APP or gene dosage, as mice homozygous for the APP(V717F) mutation display a larger reduction in size than heterozygous mice (Dodart et al., 2000).

The PDAPP model is a widely used transgenic line that has undergone behavioural characterisation (Eriksen & Janus, 2007), and thus, arguably, could provide a more stable behavioural phenotype for drug manipulation. Several groups have investigated behavioural impairments in the PDAPP model, with varying results (summarised in Table 5.1). Generally, age-independent transgenic deficits in spatial reference memory are present from 3-4 months of age (Justice & Motter, 1997 as cited in Kobayashi & Chen, 2005; Brody & Holtzman, 2006; Dumas et al., 2008; Guilani, Vernay, Leuba & Schenk, 2009) with some evidence of performance decline with age (Huitron-Resendiz et al., 2002). Mirroring this are age-independent spatial working memory deficits detected 3, 6 and 9-10 months using the radial arm maze (Dodart, Meziane, Mathis, Bales, Paul & Ungerer, 1999; Morgan et al., 2000). Expanding on the spatial reference and working memory deficits of PDAPP mice, Chen et al. (2000) demonstrated that when mice are required to learn a series of spatial locations in the Morris water maze (training to criterion), transgenic mice show age-independent deficits in learning the first platform location compared to wild type mice. When learning the subsequent platform locations, young transgenic mice are not impaired, whilst older mice are, suggesting an increased sensitivity to interference or reduced ability to perform the “episodic-like component of the task” (Chen et al., 2000, p. 975). Thus, it appears transgenic PDAPP mice have age-independent deficits in spatial memory from a young age. More demanding spatial memory tasks may be sensitive to age-dependent changes in spatial information processing.

Object recognition memory has also been investigated in PDAPP mice. However, the literature is mixed. Some reports highlight an age-related transgenic deficits emerging at 9 months (Dodart et al. 1999). In contrast, reports demonstrate normal object recognition memory in transgenic mice with delays up to 4 hours between sample and test in mice aged 18-21 months of age (Chen et al., 2000). Transgenic PDAPP mice display deficits in emotional

learning at 11 months of age, where transgenic PDAPP mice show inferior cued fear conditioning to wild type controls (Gerlai et al., 2002). There are no published reports assessing anxiety behaviour in PDAPP mice.

Overall, one can conclude that PDAPP mice suffer dysfunction of multiple memory systems, including spatial memory and contextual memory, either through damage induced by elevated levels of Aβ or APP overexpression. As pointed out by Kobayashi & Chen (2005), the variability in the pattern of results in the literature could be related to the hybrid genetic background the mutation is maintained on. A differential contribution of each of the three genetic lines that makes up the genetic background could result in differences across laboratory groups (Dodart et al., 2002).

Assessment	Construct	Age	Deficit	Reference
Morris water maze	Spatial reference memory	3-4 months	Tg deficient compared to WT. Tg use spatial search strategies less than WT mice.	Justice & Motter (1997), Brody & Holtzman (2006)
Morris water maze	Spatial reference memory	3, 6 and 9-10 months	Tg deficient compared to WT.	Dodart et al. (2000) Justice & Motter (1997), Giuliani et al. (2009)
Morris water maze	Spatial reference memory	6 and 13-16 months	Tg impaired at learning a series of locations, but not one. At 13-16 months, Tg deficient at one location. Tg forget learnt locations faster than WT.	Dumas et al. (2008)
Morris water maze (multiple locations)	Spatial reference memory with an "episodic-like component"	6-9, 13-15 and 18-21 months	Tg deficient compared to WT at all ages. Increased sensitivity to interference from 13-15 months of age.	Chen et al. (2000)
Radial Arm Maze	Spatial working and reference memory	3, 6 and 9 months	Tg deficient compared to WT mice, but no motor activity differences.	Dodart et al. (1999)
Radial Arm Maze	Spatial working and reference memory	11.5 months	Tg deficient compared to WT.	Morgan et al. (2000)
Circular maze	Spatial reference memory	3-5 and 20-26 months	Tg mice show inferior use of spatial strategy at 3-5 months, and inferior use of all available strategies at 20-26 months.	Huitron-Resendiz et al. (2002).
Novel object recognition	Object recognition memory	3, 6 and 9-10 months	Tg deficient compared to WT at 9 months, but not at 3 or 6 months of age	Dodart et al. (1999)
Novel object recognition	Object recognition memory	6-9, 13-15 and 18-21 months	No Tg/WT differences at any delay interval.	Chen et al. (2000)
Fear conditioning	Context and cue conditioned fear	11 months	Tg show reduced cued, but not context, dependent conditioning.	Gerlai et al. (2002)
Electrophys. (*in vivo*)	CA1 potentiation/population spikes	3-4.5 and 24-27 months	Altered CA1 potentiation in young and aged transgenic mice compared to wild type mice.	Giacchino et al. (2000)
Electrophys. (*in vitro*)	Hippocampal LTP and PPF	4-5 and 27-29 months	Young Tg mice showed enhanced PPF and faster LTP decay. Old Tg mice showed reduced synaptic response, loss of field potentials, reduced PPF.	Larson et al. (1999)

Table 5.1: Summary table of reported behavioural analyses of heterozygous transgenic and wild type PDAPP mice at varying ages. Tg = transgenic, WT = wild type, Electrophys = electrophysiology, LPT = long term potentiation, PPF = paired-pulse facilitation. Justice & Motter, 1997 is referenced as cited in Kobayashi & Chen, 2005.

There is need for further characterisation of the PDAPP mutation model for several reasons. Firstly, the reported behavioural deficits are based on the mice maintained on the triple hybrid background strain of C57Bl/6, DBA/2 and Swiss Webster, whilst the cohort available for the current study was on a pure C57Bl/6 background. Given the strong influence background strain can have on behavioural phenotypic expression in transgenic models, it is important to establish whether the reported deficits remain once the mutation is moved to a single strain background (Savonenko et al, 2003; Crawley et al., 1997; Bucan & Abel, 2002; Wolfer & Lipp, 2000; Võikar et al., 2001). Secondly, the majority of the reported deficits in the PDAPP model appear to the age-independent, with some indication of a decline in performance with age. In order to assess the effect of 2B3 on cognition, it is essential that a cognitive deficit is selected that is likely to be driven by Aβ pathology rather than factors such as overexpression of APP.

Introduction Summary and Experimental Hypotheses

The aim of the experiments presented in this chapter is to provide a profile of changes in anxiety-related behaviour and cognitive functioning of PDAPP(V717F) transgenic mice at 9 – 14 months of age. No behavioural analysis was conducted at younger ages because younger mice were unavailable. The experiments presented test the hypotheses that the APP(V717F) mutation would lead to a reduction in anxiety-behaviours in transgenic mice compared to wild type controls when assessed in the marble burying task and EPM (Experiments 9 and 10 respectively), as well as inferior spatial working memory as assessed using the T-maze FCA (Experiment 11) and the foraging task (Experiment 12). In terms of object recognition memory, a mixed literature prevents a clear prediction, but given that the testing paradigm utilised in the current project resemble that of Chen et al. (2000) rather than Dodart et al. (1999), no object recognition memory deficit was predicted to be present in transgenic PDAPP mice at any delay interval in the novel object recognition task (Experiment 13).

5.1.2 Assessment of anxiety in PDAPP(V717F) model

Assessments of anxiety differences between transgenic and wild type mice in pre-clinical mouse models disorders are important due to the potential effect anxiety differences can play in learning and memory performance as assessed in other tasks. Galvan et al. (2008) reported transgenic PDAPP were more likely than wild type mice to show a lack of motivation to swim, a behaviour that could be linked to the water environment eliciting less anxiety in the transgenic

mice compared to the wild type mice. However, no information is available in the literature regarding direct measures of anxiety-like behaviours in the PDAPP model. Thus, in order to aid interpretation of behavioural results, the anxiety phenotype of transgenic PDAPP mice was compared to wild type controls.

5.2 General Method: Maintenance of the PDAPP(V717F) mouse model

The PDAPP(V717F) Indiana mutation is traditionally maintained on a triple mixed background compromising C57Bl/6, DBA and Swiss Webster (Eriksen &Janus, 2007). However, the colony used throughout this work was obtained directly from Eli Lilly (UK) and were maintained on a C57Bl/6 background. A colony of 18 heterozygous transgenic males and 22 wild type littermate male controls were a generous gift from Eli Lilly at the age of 9 months. Mice were housed as they were delivered, in a mix of group and single housing. A tail sample was collected at the end of the experimental procedures to verify genotype. In our hands, no observations of elevated aggression or general health issues were made. In terms of body weight, wild type mice were significantly heavier than transgenic mice from arrival at 9 months of age (t (38) = 2.74, $p < .05$). Attrition in wild type mice was 0% throughout, compared to an attrition rate of 10% in transgenic mice on *ad libitum* access to food and water. When food deprived to 90-95% of *ad libitum* body weight, the attrition rate of transgenic APP(V717F) mice increased to 16.7%. Locomotor activity was measured in an open arena at 9 months, with no differences between transgenic and wild type mice in the percentage of time spent moving (F (1, 35) = 2.29, $p > .05$), in the distance travelled (F (1, 35) = 3.23, $p > .05$) or in the average speed (F (1, 35) = 3.26, $p > .05$).

Polymerase Chain Reaction (PCR) for APP(V717F)

Two sets of primers were used in the APP(V717F) PCR master mix to target *APP* and *Actin*. This enabled the identification of carriers of the APP(V717F) mutation alongside identification of amplifiable genomic material in all samples. The primers 2010 (Eurofins MWG Operon; 5'-ATCTGGCCCTGGGGAAAAAAG-3') and 2011 (5' –GATGTCCTTCCTCCTCTGTTC-3') were utilised to target the *APP*(V717F) mutation, whilst the primers MusA-ActinF1 (5'-CACCACACCTTCTACAATGAGCTG-3') and MusA-ActinR1 (5'–TCATCAGGTAGTCAGTGAGGTCGC-3') targeted *Actin* (Eurofins MWG Operon). A master mix solution made up of 2.5µl of 10x buffer (BIOTAQ Red DNA Polymerase, Bioline), 0.5µl of 50x $MgCl_2$ (Invitrogen), 1.25µl of 10mM dNTPs (GE Healthcare), 0.05µl of each primer at a concentration of 100pmol each, 1.5µl of Taq

Red (Bioline), 14.05µl of DNAse- and RNAse-free H_20 was combined per sample, with 5µl of DNA. The thermocycling conditions and band visualisation was identical to that utilised for the APP(V717I) PCR (Section, 2.2, Chapter 2). The APP(V717F) transgene DNA product is approximately 900bp and appears in transgenic samples only, whilst the Actin DNA product appears as a band at approximately 500bp in every sample with genomic DNA (see Fig. 5.1).

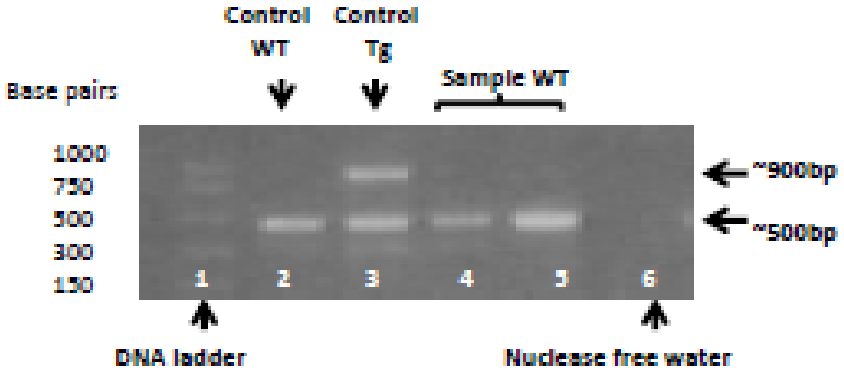


Fig. 5.1: Representative image displaying an electrophoresis gel of amplified genomic material from APP(V717F) transgenic and wild type mice. Lane 1 = DNA ladder displaying bp 1000 to 150. Lane 2 = ~500bp fragment representing the endogenous *actin* gene in a known wild type mouse sample. Lane 3 = ~500bp fragment representing the endogenous *actin* gene and ~900bp fragment representing the transgenic *APP(V717F)* mutation in a known transgenic control sample. Lane 4 and 5 = wild type samples. Lane 6 = nuclease free water control.

An overview of the order of experiments and the sample size for APP(V717F) mice is presented in Fig. 5.2.

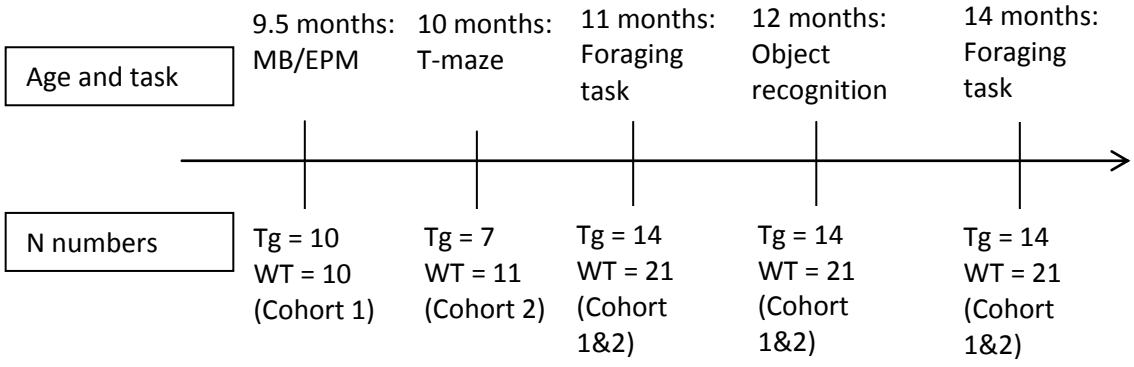


Fig. 5.2: Experimental time line indicating the age at which each test carried out in APP(V717F) mice, as well as details of the sample sizes. The cohort of mice used for marble burying and elevated plus maze testing differed from those tested on the T-maze. This is a full overview of the tasks administered to this cohort of mice up to 14 months of age.

5.3 Experiment 9: Marble Burying

The marble burying task is an anxiety task based on the natural neophobic response mice exhibit when exposed to novel objects, as described in Section 2.3, Chapter 2. Briefly, mice are exposed to 20 marbles, and their level of anxiety towards the marbles is measured as the number of marbles the mice bury during the 30 minute test time.

5.3.1 Method

Design

PDAPP model male mice were assessed at 9.5 months of age. For anxiety testing, delivery of test (marble burying and EPM) was counterbalanced across subject groups and conducted in novel testing rooms. The contribution of gender to the transgenic behavioural phenotype was not assessed, as female transgenic mice were not available for testing. For the same reason, the effect of age on anxiety behaviours in the model was not assessed.

Subjects

Ten transgenic male PDAPP mice aged 9 months with 10 littermate wild type controls were utilised.

Apparatus

The apparatus utilised was identical to that used for the APP(V717I) mice described in Section 2.3, Chapter 2.

Method

The testing procedure was identical to that used for the APP(V717I) mice described in Section 2.3, Chapter 2.

Scoring and data analysis

The approach taken to scoring and data analysis was identical to that used for the APP(V717I) mice described in Section 2.3, Chapter 2.

5.3.3 Results

The number of marbles buried by the mice was averaged within each genotype group (Fig. 5.2). Based on visual inspection, wild type mice appear to bury more marbles than transgenic mice. In order to test whether genotype affected the number of marbles buried, a Mann-Whitney U test was carried out. There was a significant effect of genotype (U (10, 10) = 22.0, $p < .05$), with wild type mice burying more marbles than transgenic mice.

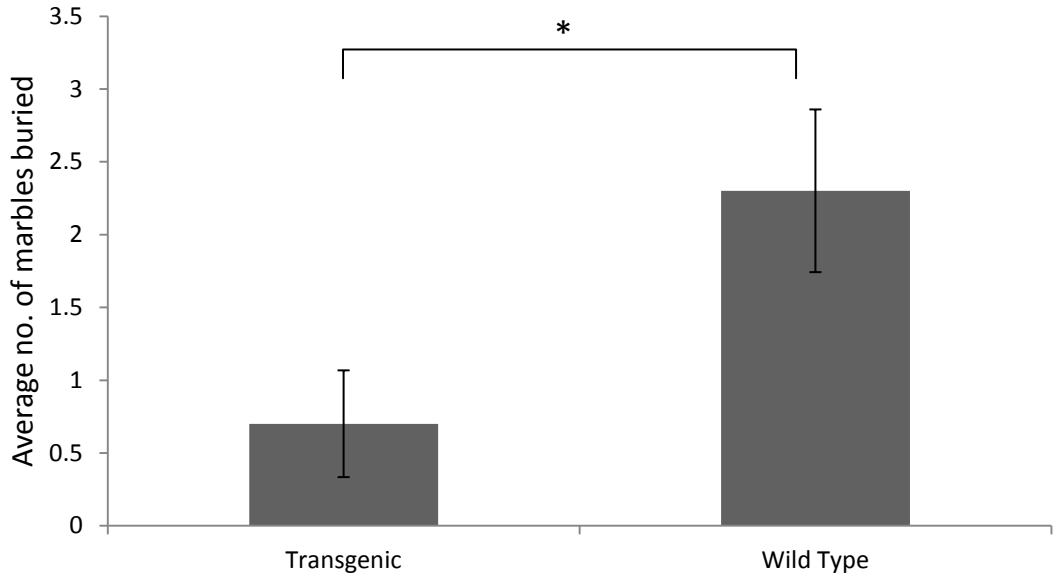


Fig. 5.2: The mean number of marbles buried for male PDAPP transgenic and wild type mice at 9.5 months of age. Error bars ±1 S.E.M. * = $p < .05$.

5.3.4 Discussion

It was predicted that wild type mice would bury more marbles than transgenic PDAPP mice at 9.5 months of age. The data supports this prediction, indicating that transgenic mice of this model display deficits in anxiety compared to wild type mice. Whilst no direct measure of anxiety behaviour in transgenic PDAPP mice is available in the literature, the present data may support the interpretation of the tendency of transgenic mice to show a lack of motivation to

swim in the Morris water maze (Galvan et al., 2008) as linked to the water environment eliciting less anxiety in the transgenic mice compared to the wild type mice.

As discussed in Section 2.3, Chapter 2, the marble burying task is controversial due to the undefined nature of the behaviour measured. Therefore, assessment of behaviour across two independent anxiety tasks was conducted in order to increase confidence in interpretation of the data presented in the marble burying task.

5.4 Experiment 10: EPM

5.4.1 Introduction

In order to validate the data obtained in the marble burying task, the EPM was administered in parallel. The validation of the EPM as a measure of anxiety-behaviour is presented in Chapter Section 2.4, Chapter 2.

5.4.2 Method

Design

The assessment of anxiety, as expressed as avoidance of novel, exposed spaces, was conducted in parallel with measures of neophobia using the marble burying task. Therefore, male mice were assessed at 9.5 months of age.

Subjects

Ten transgenic male PDAPP mice aged 9.5 months with 10 littermate wild type controls were utilised, as described in Section 5.3, Chapter 5.

Apparatus

The EPM apparatus used was identical to that described in Section 2.4, Chapter 2.

Method

The testing procedure adopted was identical to that described in Section 2.4, Chapter 2.

Scoring and data analysis

The approach taken to scoring and data analysis was identical to that described in Section 2.4, Chapter 2.

5.4.3 Results

As can be observed through inspection of Fig. 5.3, transgenic mice spent more time in the open arms as a percentage of time spent in any arm than WT mice (F (10, 10) = 18.94, p <.0001). The mean and S.E.M. values for the ratio variable are presented in Table 5.2.

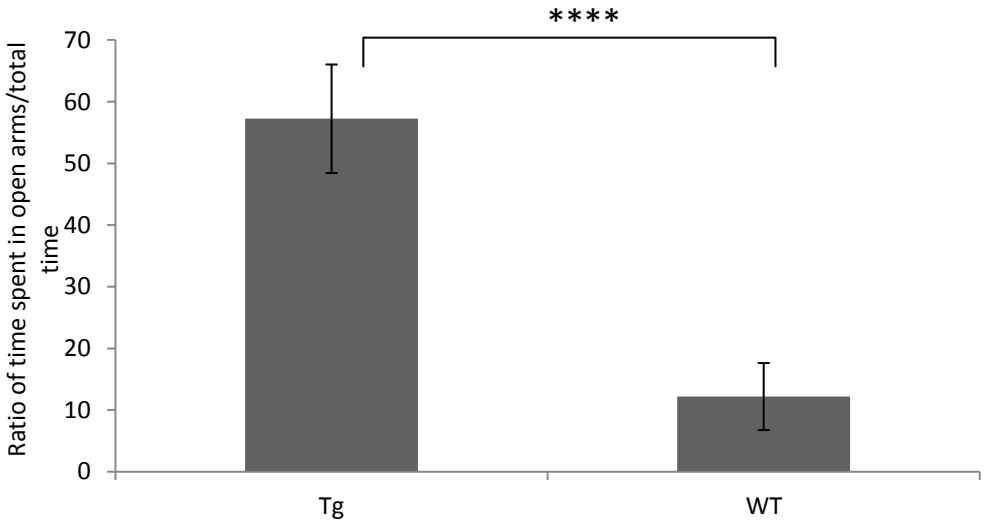


Fig. 5.3: The mean time spent in the open arms as a percentage of time spent in any arm for male PDAPP transgenic and wild type mice at 9.5 months of age. Error bars ±1 S.E.M. **** = p < .0001.

For simplicity of presentation, the time spent in the open and closed arms in seconds is not presented in graphs as the pattern of results mirrors that of the ratio variable. However, the means and S.E.M. values are presented for information in Table 5.2, and show a preference for the closed over the open arms in the wild type mice which is not present in the transgenic mice. Statistical analysis using ANOVA confirmed a significant main effect of arm type (F (1, 18) = 10.00, p < .005) in which more time was spent in the closed arm compared to the open arm. This measure interacted with genotype (F (1, 18) = 20.16, p < .0001), with wild type mice (but not transgenic mice, p > .05) spending significantly more time in the open compared to the closed arms (p < .0001).

Variable	Genotype	Mean	S.E.M.
Time in open arms	Tg	**133.77**	24.36
	WT	**29.50**	11.88
Time in closed arms	Tg	**97.92**	22.37
	WT	**236.24**	17.98
Ratio of time in open/closed arms	Tg	**57.24**	8.80
	WT	**12.21**	5.44

Table 5.2: The mean and S.E.M. values for the time spent in the open and closed arms (s), as well as the ratio of time spent in the open arms as a percentage of time spent in any arm.

5.4.4 Discussion

The results from the elevated plus maze test of anxiety at 9.5 months of age shows an anxiety phenotype in transgenic mice, with transgenic animals spending more time in the open, exposed arms than wild type mice. This result is both in line with the hypothesis and the data from the marble burying test, suggesting that reduced anxiety in PDAPP transgenic mice compared to littermate controls is a phenotype that can be observed across test paradigms with different response requirements.

5.5 Experiment 11: T-maze forced choice alternation non-matching to position task

5.5.1 Introduction

The T-maze task measuring spatial working memory has been described in Section 3.4, Chapter 3. It has been widely used to assess hippocampal function (Deacon & Rawlings, 2006), and results from Experiment 5b demonstrate disruption of wild type performance on the task following bilateral hippocampal lesions.

5.5.2 Method

Design

In order to assess the effect of the APP(V717F) mutation in PDAPP mice, transgenic and wild type mice were assessed on the standard T-maze forced choice alternation task using the non-matching to position paradigm. A set of trials in which a visual cue signalled reward was then administered. This was designed to assess whether a potential transgenic deficit performance could be ameliorated if rewards could be obtained without requiring the use of recent spatial

information to distinguish between previously visited and unvisited locations. Mice were assessed at 10 months of age.

Subjects

Eighteen male PDAPP mice were used, of which 7 were heterozygous to the APP(V717F) mutation and 11 were non-transgenic littermate controls.

Apparatus

The apparatus utilised in the standard trials of the T-maze FCA task was identical to that described in Section 3.4.3, Chapter 3. In the visually cued trials, a laminated, black and white vertically striped card of dimensions 12.7cm*8.5cm (see Fig. 5.4) was used. Each stripe was 0.95cm wide.

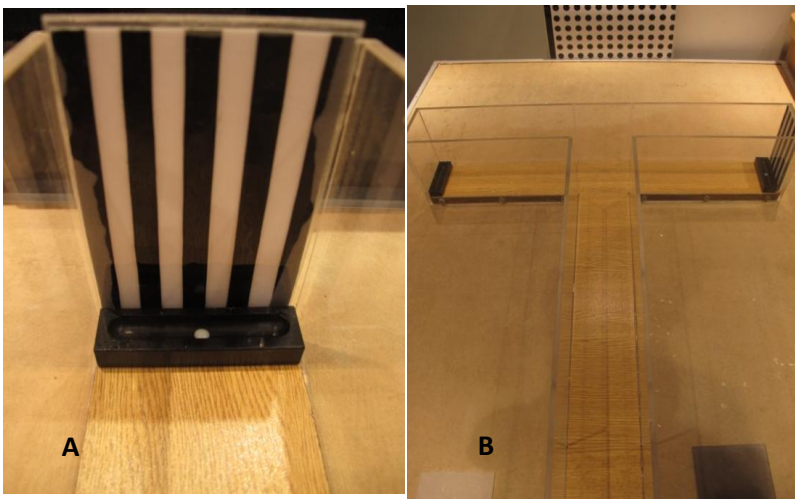


Fig. 5.4: A visually cued and baited reward well (A), with (B) demonstrating the T-maze apparatus with one baited and visually cued reward well in place.

Method

Mice received 8 test days of standard T-maze testing as described in Section 3.4.3, Chapter 3. Following a one day break from testing, 8 test days of visually cued trials were administered. The visually cued trials differed from standard trials only in the presence of a striped card inserted behind the well in the goal arm used on the sample trial. In the test trial, the visual cue was moved to the unvisited, baited goal arm. Thus, the mouse was not required to rely on

extramaze and/or intramaze cues or information regarding which arm was previously visited, as simply tracking the visual cue was consistently rewarded on each trial. This relies on the mouse forming an association between the reward and the visual cue.

Scoring and data analysis

Data was collected, scored and analysed as described in Chapter 4, Section 4.3.2.

5.5.3 Results

The average percentage correct responses (see Fig. 5.5) were analysed using a repeat measures ANOVA with test day and cue presence as within-subject factors, and genotype as a between-subjects factor. This revealed a significant difference in transgenic and wild type mice scores, with transgenic mice scoring lower than wild type mice in both visually cued and standard trials (F (1, 16) = 1115.541, $p < .0001$). There was a significant effect of cue presence in the direction of improved performance in visually cued trials compared to standard trials (F (1, 16) = 19.87, $p < .0001$), but transgenic and wild type mice did not differ in their response to the introduction of the visual cue [cue presence*genotype (F (1, 16) = 0.27, $p > .05$)].

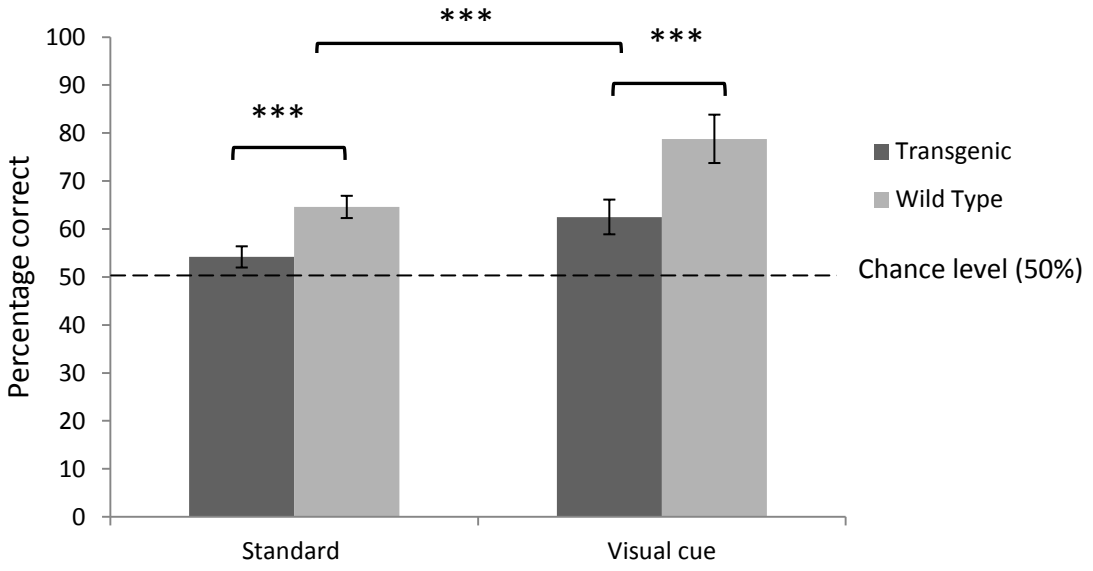


Fig. 5.5: Mean percentage correct across standard test days followed by visually cued test days for PDAPP transgenic and wild type mice at 10 months of age. Error bars ±1 S.E.M. *** = $p < .0001$.

There was a significant effect of test day mirroring an improvement in performance across test days (F (7, 112) = 2.54, p < .02; see Table 5.3), which was driven by significantly higher correct scores on test days 8 compared to test days 1 (p < .05). There were no interactions between test day and any other factor [genotype*test day: (F (7, 112) = 0.95, p > .05), test day*cue presence: (F (7, 112) = 0.98, p > .05), test day*cue presence*genotype: (F (7, 112) = 0.32, p > .05)].

Test day	Standard				Visual cue			
	WT		Tg		WT		Tg	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
1	**50.01**	5.40	**50.00**	5.03	**68.17**	4.74	**61.90**	5.99
2	**66.66**	6.48	**54.76**	5.50	**75.75**	5.67	**59.51**	6.14
3	**68.17**	7.05	**57.13**	6.13	**75.75**	5.67	**59.51**	10.83
4	**63.64**	4.81	**50.00**	4.39	**78.78**	5.54	**59.53**	4.95
5	**68.18**	3.92	**57.14**	4.17	**83.33**	5.02	**57.14**	8.02
6	**60.61**	6.07	**50.00**	5.16	**83.33**	5.02	**66.67**	8.13
7	**72.73**	7.41	**57.16**	6.46	**77.26**	6.06	**69.04**	7.65
8	**66.67**	7.05	**57.16**	5.95	**87.87**	3.95	**66.66**	6.29
Mean	**64.58**	2.21	**54.17**	2.32	**78.78**	3.62	**62.50**	5.04

Table 5.3: The mean percentage correct score (with S.E.M. values) for individual test days in transgenic and wild type PDAPP mice given 8 days of standard T-maze trials followed by 8 days of visually cued trials.

5.5.4 Discussion

The results from the T-maze study utilising standard trials followed by visually cued trials showed two general patterns. Firstly, wild type mice outperform transgenic mice regardless of the task difficulty level. Secondly, mice of both genotypes show improved performance in visually cued trials. This is in line with predictions that transgenic PDAPP mice would display impaired spatial working memory compared to littermate controls at 10 months of age, and that providing a visual cue to guide behaviour (thus removing the need to utilise recently acquired spatial information) would improve performance in both genotypes.

However, it is worth noting that in visually cued trials, wild type mice continued to outperform transgenic mice. Thus, transgenic mice appeared to benefit less from the introduction of the visual cue than wild type mice. This can be interpreted several ways. Firstly, in order for the visual cue to be efficient in lowering the task difficulty level, mice were required to form an association between the visual cue and the reward. Whilst this learning requirement is arguably less demanding than the use of recently acquired position information to locate rewards, it nevertheless requires learning. Thus, transgenic mice may be deficient in

learning simple associations compared to wild type mice. Alternatively, and perhaps more parsimoniously, transgenic mice may suffer from visual deficits that may limit their ability to encode the discriminative stimulus. As it is not possible to discount either of these explanations based on this data, it is necessary to treat transgenic PDAPP mouse performance deficits in behavioural tasks with caution until further information is available regarding the function of their visual system.

5.6 Experiment 12: Foraging task

5.6.1 Introduction

Based on results from the spatial working memory assessment of APP(V717I) mice presented in Section 3.3, Chapter 3, the foraging task appeared to be more sensitive to spatial working memory deficits compared to the T-maze task. Thus, PDAPP mice were also assessed on the foraging task to establish the generality of the behavioural phenotype across tasks.

5.6.2 Methods

Design

The initial stages of habituation were identical to that described in Section 3.3, Chapter 3, but an unforeseen adverse effect of food deprivation in this model restricted the further use of an identical methodology. Attrition in transgenic mice increased from 10% to 16.7% during food deprivation to 90-95% of *ad libitum* body weight, compared to 0% of wild type mice. The task was therefore altered from a food-reward to a liquid reward based task, as no such adverse effects of water-deprivation were observed when administering the T-maze task.

The liquid reward version of the foraging task was conducted in three phases. Phase A was identical in design to that described in Section 3.3, Chapter 3, with 8 liquid rewarded pots. This was carried out at 11 months of age. In phase B, the task difficulty level was lowered with a reduction in the number of pots from 8 to 6. This was carried out in an attempt to establish the level of task difficulty sensitive to a performance deficit in transgenic mice. A longitudinal assessment was conducted at 11 and 14 months of age in order to assess whether the presence of the APP(V717F) mutation led to higher error scores in the transgenic than wild type mice in an age-dependent manner. Phase C consisted of a control task in which only one pot was presented. The latter task served to assess whether transgenic and wild type mice

differed in their motivation or willingness to engage with the pots. This was carried out at 11 months of age.

Subjects

Thirty-five male PDAPP mice were used, of which 14 were heterozygous to the APP(V717F) mutation and 20 were non-transgenic littermate controls. One wild type mouse was excluded after 7 days of testing due to a lack of engagement with the task, and consistent inability to visit each pot in the arena. One transgenic mouse was excluded at the point of data analysis (see Section 5.6.4, Chapter 5). The data obtained from these mice were excluded from all analyses.

Method and Apparatus

Phase A

The colony was maintained on *ad libitum* access to food, but were water deprived to 90% of their body weight and given daily access to water for 2 hours. Eight pots which contained nothing except one drop (approximately 30µl) of sweetened, condensed milk (50:50 with dH_2O) were placed in the arena (Fig. 5.6) in an identical pattern to that utilised when testing the APP(V717I) mice (Section 3.3, Chapter 3). Using wooden cubes, pots were raised 8 cm above the level of the sawdust in order for the mice to be unable to visually locate the reward without climbing onto the edge of the pot, allowing the experimenter to score reward searching behaviour (Fig. 5.7). Mice were required to visit all pots and retrieve the rewards in order for the task to be completed. The mice were tested over 5 consecutive days, with one trial on each day.

Fig. 5.6: The 8-pot liquid version of the foraging task.

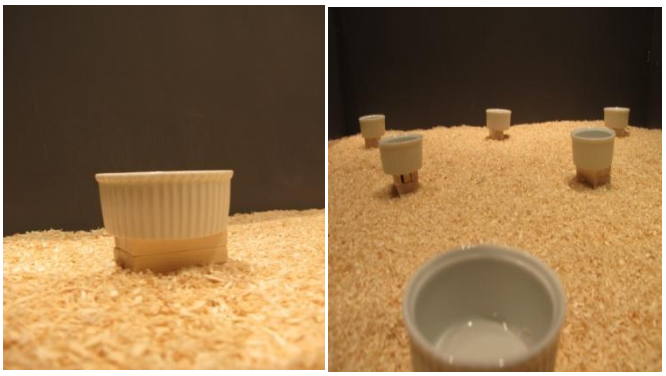


Fig. 5.7: The elevated reward pots in the liquid version of the foraging task.

Phase B

Immediately following 5 days of training in the 8 pot version of the liquid foraging task, the task difficulty level was reduced by decreasing the number of pots from 8 to 6. The pattern of the pots was altered to avoid locating pots in the centre of the arena in an attempt to minimise the effect variations in genotypic anxiety levels could play on task performance (see Fig. 5.8). Mice received 4 consecutive test days, with one trial on each day.

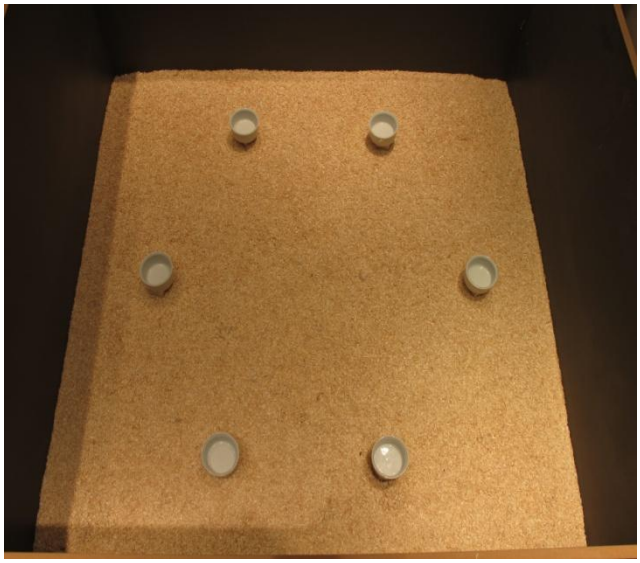


Fig. 5.8: The 6-pot liquid version of the foraging task.

Phase C

Immediately following testing in the 6-pot version of the foraging task, a control task was administered in which all but one rewarded pot was removed from the arena (see Fig. 5.9). Following consumption of the reward, subjects were left in the arena for 4 minutes. The number of errors made was recorded (returns to the single pot).

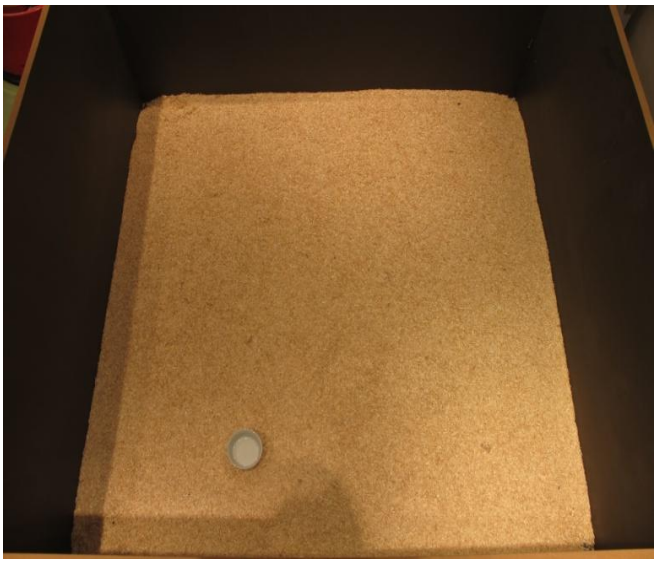


Fig. 5.9: The foraging control task.

Scoring and data analysis

The dependent variables of the task were scored and analysed as described in Section 3.4, Chapter 3 with the following changes. An error was defined as jumping onto the edge of a pot and lowering the head into the pot below the edge of the pot, as opposed to being defined by digging behaviour. For Phase C, only errors were analysed (as all errors were by definition also return and consecutive errors).

5.6.3 Results

One transgenic mouse was excluded from all analysis (Phase A, B and C) as an outlier due to performance scores being more than three standard deviations higher than the transgenic group mean. As all variables violated the assumptions of normality and homogeneity of variance which was not improved using transformations, they were analysed using Mann Whitney U and Wilcoxon Signed Ranks for between and within-subject analyses respectively.

Phase A: Error

(Returning to a previously visited pot)

Inspection of Fig. 5.10 suggests a slight increase in error scores across trials in PDAPP mice compared to the wild type group. However, statistical analysis revealed no significant differences between transgenic and wild type mice on error scores across trials (U (13, 21) = 107.50, $p > .05$) or during the first half of the trials when averaged across test days (U (13, 21) = 100.00, $p > .05$).

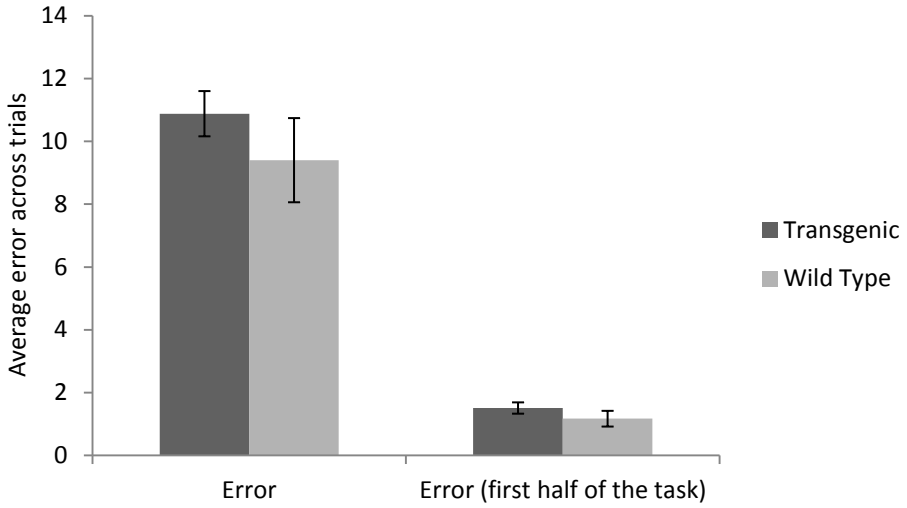


Fig 5.10: The mean error scores across trials and in the first half of the trials at 11 months of age for transgenic and wild type PDAPP mice. Error bars ±1 S.E.M.

Phase A: Repeat error

(Returning to a previously visited pot more than once)

As with the error scores, the repeat errors of transgenic PDAPP mice are numerically higher than wild type mice (see Fig. 5.11). However, there were no significant differences between transgenic and wild type mice on error scores across trials (U (13, 21) = 101.50, p > .05) or during the first half of the trials when averaged across test days (U (13, 21) = 83.50, p > .05).

Fig. 5.10: The mean repeat error scores across trials and in the first half of the trials at 11 months of age for transgenic and wild type PDAPP mice. Error bars ±1 S.E.M.

Phase A: Consecutive error

(returning to a previously visited pot twice or more without visiting any other pots in the interval between those errors)

The pattern of numerically superior wild type performance is also evident in the consecutive error scores (see Fig. 5.12). There was a non-significant trend towards wild type error scores being significantly lower than those of transgenic mice (U (13, 21) = 75.00, p = .055), and analysing consecutive error scores in the first half of the trials revealed no significant differences (U (13, 21) = 81.50, p > .05).

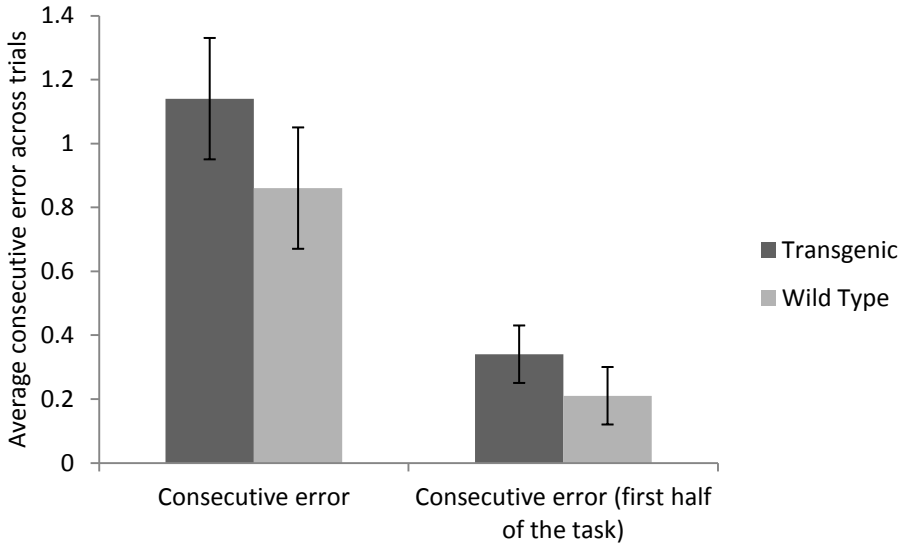


Fig. 5.12: The mean consecutive error scores across trials and in the first half of the trials at 11 months of age for transgenic and wild type PDAPP mice. Error bars ±1 S.E.M.

Phase B

For simplicity of presentation, an overview of the statistic and p value for all dependent variables is presented in Table 5.4 prior to a summary of analysis of each type of error measure.

Variable		Genotypic difference	
		U	*P*
11 months of age	Error	78.0	0.073
	Error first half of the trials	**68.0**	**0.025**
	Repeat error	84.5	0.118
	Repeat error first half of the trials	100.0	0.097
	Consecutive error	80.5	0.079
	Consecutive error first half of the trials	99.0	0.115
	Error in control task	90.0	0.178
14 months of age	Error	**53.5**	**0.005**
	Error first half of the trials	**35.5**	**0.001**
	Repeat error	**45.5**	**0.002**
	Repeat error first half of the trials	**32.0**	**0.001**
	Consecutive error	**49.5**	**0.003**
	Consecutive error first half of the trials	**33.0**	**0.001**

Table 5.4: The test statistic and exact significance for variables from the foraging task is presented at 11 and 14 months of age analysed for genotypic differences. Significant values are highlighted in bold.

Phase B: Error

From visual inspection of Fig. 5.13, transgenic mice appear to make more errors than wild type mice across the task and in the first half of the trials at both 11 and 14 months of age. Statistical analysis confirms that at 11 months, transgenic and wild type mice differed significantly in their error scores in the first half of the trials (U (12, 21) = 68.00, $p < .05$), but not in error scores across trials (U (12, 21) = 78.00, $p > .05$). At 14 months of age, transgenic mice made significantly more errors than wild type mice both across trials (U (12, 21) = 53.50, $p < .005$) and in the first half of the trials (U (12, 21) = 35.50, $p < .0001$).

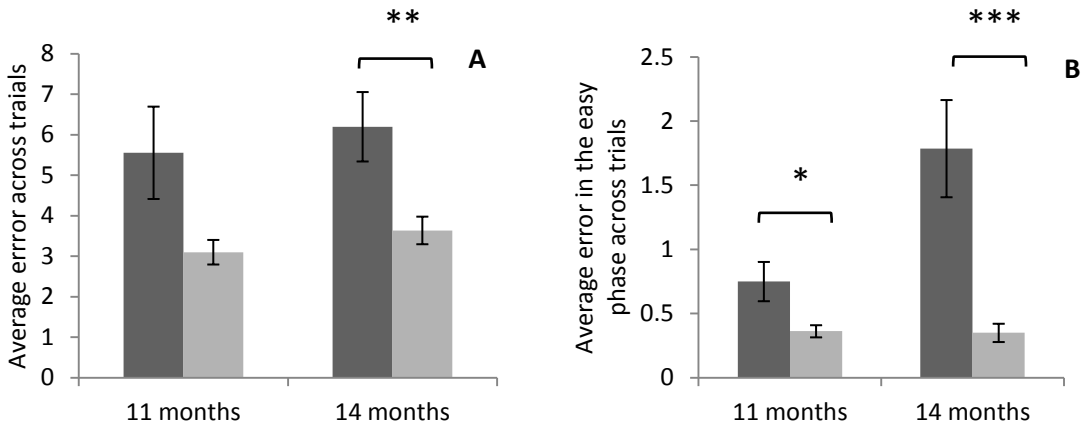


Fig. 5.13: Mean total error (A) and error in the first half of the trials (B) across test days for transgenic (dark grey bars) and wild type mice (light grey bars) at 11 and 14 months. Error bars ±1 S.E.M. * = $p < .05$, ** = $p < .005$, *** = $p < .0001$.

Within-subject comparisons of error scores at 11 and 14 months did not reveal significant increases in errors across trials (χ^2 (33) = -1.77, $p > .05$) or in the first half of the trials (χ^2 (33) = -1.51, $p > .05$) when collapsed across genotype. When analysing the within-subject changes in error scores from 11 to 14 months by genotype, no significant changes were observed in wild or transgenic mice across the task [wild type: (χ^2 (21) = -1.24, $p > .025$); transgenic: (χ^2 (12) = 1.22, $p > .025$)] or in the first half of the trials [wild type: (χ^2 (21) = -0.34, $p > .025$); transgenic: (χ^2 (12) = -1.83, $p > .025$)].

Phase B: Repeat error

From visual inspection of Fig. 5.14, transgenic mice appear to make more repeat errors than wild type mice across the task and in the first half of the trials at both 11 and 14 months of age. At 11 months, these differences are not significant across trials (U (12, 21) = 84.50, $p > .05$) or in the first half of the trials (U (12, 21) = 100.00, $p > .05$). At 14 months however, statistical analysis confirms that transgenic and wild type mice differed significantly in their repeat error scores across the task (U (12, 21) = 45.50, $p < .002$) and in the first half of the trials (U (12, 21) = 32.00, $p < .0001$).

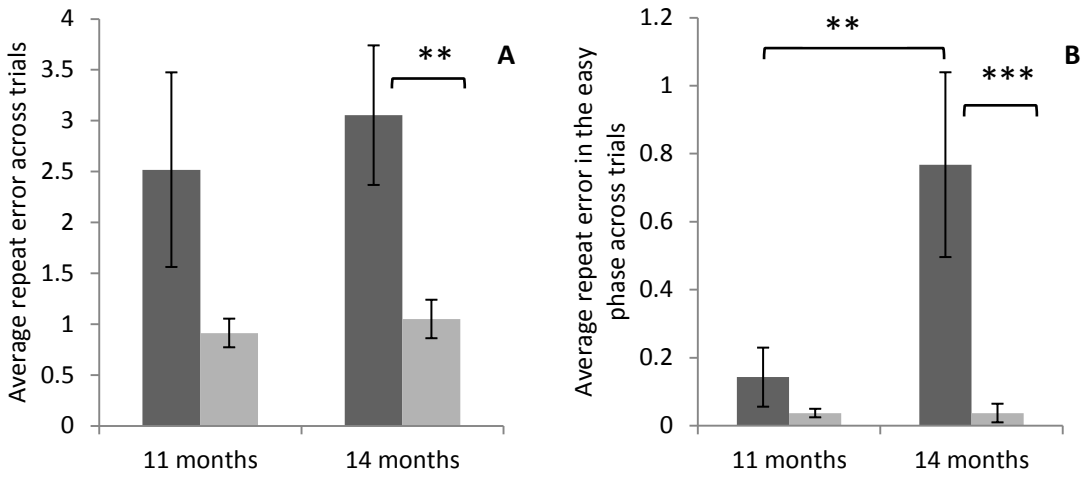


Fig. 5.14: Mean total repeat error (A) and repeat error in the first half of the trials (B) across test days for transgenic (dark grey bars) and wild type mice (light grey bars) at 11 and 14 months. Error bars ±1 S.E.M. ** = $p < .002$, *** = $p < .0001$.

Within-subject comparisons of repeat error scores at 11 and 14 months did not reveal significant increases in repeat errors across trials (χ^2 (33) = -1.03, $p > .05$), but there was a general increase in repeat error scores in the first half of the trials at 14 months of age (χ^2 (33) = -2.96, $p < .002$). When analysing the within-subject changes in repeat error scores from 11 to 14 months by genotype, no significant changes were observed in wild type mice across the task (χ^2 (21) = -0.22, $p > .025$) or in the first half of the trials (χ^2 (21) = -1.13, $p > .025$). Transgenic mice however displayed significantly higher levels of repeat errors in the first half of the trials at 14 months compared to 11 months of age (χ^2 (12) = -2.70, $p < .002$), but no significant difference in repeat errors across the task was observed (χ^2 (12) = -1.25, $p > .025$).

Phase B: Consecutive error

Fig. 5.15 illustrates the elevated levels of consecutive error scores in transgenic mice compared to wild type mice across measures. Statistical analysis reveals that genotypic differences are not significant across the task (U (12, 21) = 80.50, $p > .05$) or in the first half of the trials (U (12, 21) = 99.00, $p > .05$) at 11 months of age. Transgenic and wild type mice differ significantly in their consecutive error scores at 14 months of age, both across trials (U (12, 21) = 49.50, $p < .0001$) and in the first half of the trials (U (12, 21) = 33.00, $p < .0001$).

A

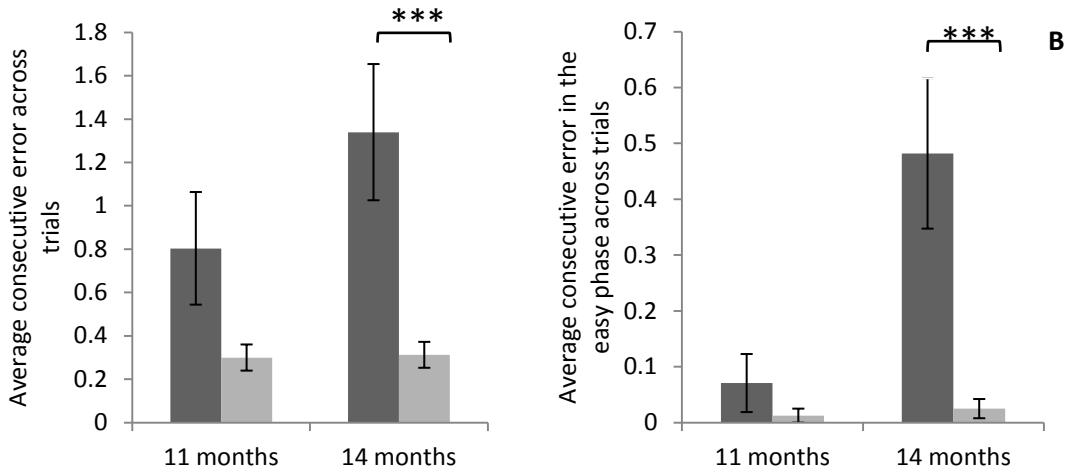


Fig. 5.15: Mean total consecutive error (A) and consecutive error in the first half of the trials (B) across test days for transgenic (dark grey bars) and wild type mice (light grey bars) at 11 and 14 months of age. Error bars ±1 S.E.M. *** = $p < .0001$.

Within-subject comparisons of consecutive error scores at 11 and 14 months did not reveal significant increases in consecutive errors across trials (χ^2 (33) = -0.87, $p > .05$) or in the first half of the trials (χ^2 (33) = -1.55, $p > .05$) when collapsed across genotype. When analysing the within-subject changes in consecutive error scores from 11 to 14 months by genotype, no significant changes were observed in wild type mice across the task (χ^2 (21) = -0.42, $p > .025$) or in the first half of the trials (χ^2 (21) = -0.001, $p > .025$), nor in transgenic mice across the task (χ^2 (13) = -1.79, $p > .025$) or in the first half of the trials (χ^2 (13) = -1.72, $p > .025$).

Phase C: Error

There were no significant differences between transgenic and wild type mice in the number of times they returned to the single, foraged pot during the 4 minute time interval (U (12, 21) = 90.00, $p > .05$; see Fig. 5.16).

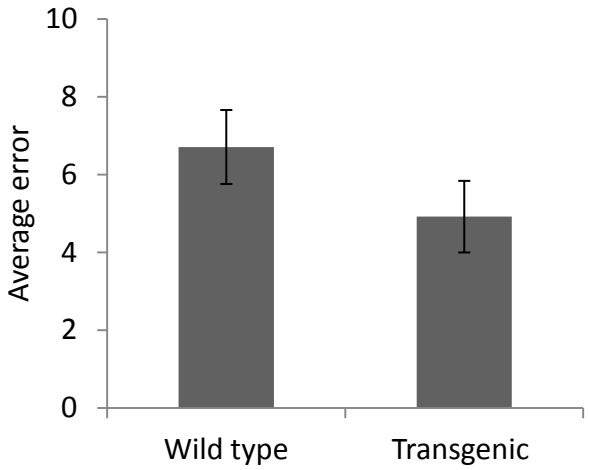


Fig. 5.16: Mean error for transgenic and wild type mice at 11 months of age in the one pot control task. Error bars ±1 S.E.M.

5.6.4 Discussion

It was hypothesised that the APP(V717F) mutation would result in lower spatial working memory performance compared to non-transgenic mice. Whilst limited evidence of genotypic differences in spatial working memory was evident in Phase A, support for the hypothesis was obtained in the critical stages of the experiment, namely Phase B, where the number of pots was reduced from 8 to 6. The lack of a difference in the number of errors made in the control task (Phase C) indicates that the performance differences observed between the transgenic and wild type group on the foraging task is not likely to be due to differences in hyperactivity, or differences in their general motivation to engage with the pots or consume the reward.

Using an appropriate level of task difficulty, the performance of transgenic mice decreased between 11 and 14 months, indicating that the task was sensitive to age-related deterioration of spatial memory. This cognitive decline was detected clearly in the first half of the trials, arguably the less demanding phase of the trials. Thus, wild type mice are able to perform the first half of the trials with limited errors at both 11 and 14 months. In contrast, the performance of transgenic mice deteriorated markedly in the first half of the trials by 14 months of age. Thus, the foraging task appears to be a suitable task for assessing the effect of 2B3 on cognitive performance in the PDAPP model, as a clear transgenic performance deficit can be measured which appears to be based on inferior cognitive processing in an age-dependent manner.

5.7 Experiment 13: Assessment of object recognition memory in PDAPP (V717F)

5.7.1 Introduction

Following evidence to suggest that transgenic PDAPP mice display impairments in spatial working associated with hippocampal function, the question of the degree to which deficits in learning and memory are restricted to this system becomes of interest. The literature on object recognition memory or visual novelty detection in the PDAPP model is varied, with reports of both age-dependent object recognition memory deficits (Dodart et al., 1999) and intact processing of visual novelty at 18-21 months of age (Chen et al., 2000). This discrepancy may be due to differences in the testing paradigm utilised by the two laboratory groups, with Dodart et al. (1999) introducing a spatial novelty aspect in the test phase by using a single item in the sample phase. As the testing procedure utilised in the current work aimed at isolating novel object recognition processes as opposed to a conjunction of novel object and novel place recognition, the procedure adopted was identical to that described in Section 3.5, Chapter 3 and similar to that of Chen et al. (2000).

5.7.2 Method

Design

PDAPP mice were assessed either with a 3 hour delay interval between the sample and the test phase, or with a 5 minute delay. The assessment of object recognition memory was carried out at 12 months of age, an age beyond that in which object recognition deficit can be detected as indicated by Dodart et al. (1999), and at which point Aβ plaque pathology is established (Reilly et al., 2003).

Subjects

In the 5 minute delay condition, 17 male PDAPP mice were used of which 7 were heterozygous APP(V717F) mice and 10 were non-transgenic littermate controls. In the 3 hour delay condition, 7 transgenic and 11 wild type littermate control mice were used.

Apparatus

The apparatus utilised in assessing object recognition memory in the PDAPP model was identical to that utilised when assessing the APP(V717I) model in Section 3.5.2, Chapter 3, with the exception of the objects used. As the mice were trained during the foraging task to jump onto objects to obtain a reward, the current experiment used taller objects (such as approximately 20cm tall bottles and ceramic figures) than those used with the APP(V717I) mice in an attempt to avoid mice climbing onto the objects.

Method

The testing procedure was identical to that described in Section 3.5.2, Chapter 3, with two exceptions. Firstly, only two delay intervals were used: 5 minute delay or 3 hours delay. Secondly, only one day of habituation to the arena with an object not used in future testing was employed, due to significant recent exposure to the test room and general arena testing through the foraging task.

Scoring and data analysis

The scoring and data analysis did not differ from that described in Section 3.5.2, Chapter 3.

5.7.3 Results

The contact times averaged across the two identical objects in the sample phase are presented in Table 5.5. In order to assess whether there was any sampling bias between groups, effect of genotype on contact time in the sample phase was analysed, with delay as a factor. Whilst there was no main effect of genotype (F (1, 29) = 1.04, $p > .05$), there was a main effect of sampling trials, in which contact time in sampling phase 1 was significantly higher than in sampling phase 2 (F (1, 29) = 10.98, $p < .005$). This effect appears to be driven by a trials*genotype interaction (F (1, 29) = 5.60, $p < .05$), in which transgenic mice explored the objects significantly more in sample phase 1 than in sample phase 2 (F (1, 31) = 13.707, $p < .001$). No further significant genotype*trials differences emerged [closest to significance: genotype difference in sampling phase 1 in the direction of increased sampling by wild type mice compared to transgenic littermates: (F (1, 31) = 3.90, $p > .05$)].

There were no differences in the sample contact times in the different delay groups (F (1, 29) = 0.67, p > .05), nor any interaction with delay and other factors [delay*genotype: (F (1, 29) = 0.70, p > .05), trials*delay: (F (1, 29) = 0.32, p > .05), trials*genotype*delay interaction: (F (1, 29) = 1.07, p > .05).

Trial	Delay	Contact time (s) in sample phase			
		Wild Type		Transgenic	
		Mean	S.E.M.	Mean	S.E.M.
1	5 minute delay	**22.10**	3.75	**27.46**	5.25
2		**16.06**	2.32	**13.50**	4.45
1	3 hours	**18.61**	2.20	**34.73**	10.25
2		**20.43**	2.33	**17.68**	3.78

Table 5.5: The mean contact time in seconds for transgenic and wild type mice when faced with a pair of novel objects over two habituation days (novel pairs presented on each day).

The mean contact time for the novel and familiar object at each delay interval during the test phase is presented in Table 5.6. A repeat measures ANOVA confirmed that transgenic and wild type mice did not significantly differ in the contact time at test (F (1, 31) = 0.10, p > .05), nor was there a genotype*delay interaction (F (1, 31) = 1.11, p > .05).

There was a main effect of object type, in which the novel object was explored significantly more than the familiar object (F (1, 31) = 5.86, p < .05), but genotype did not influence the amount of time spent exploring the novel and familiar objects [genotype*object: (F (1, 31) = 0.22, p > .05)]. Similarly, there were no interactions between delay, genotype and object type [object*delay: (F (1, 31) = 2.21, p > .05), object*delay*genotype: (F (1, 31) = 0.14, p > .05)].

Delay	Novelty status	Contact time (s) in test phase			
		Wild Type		Transgenic	
		Mean	S.E.M.	Mean	S.E.M.
5 minute delay	Novel	**14.90**	2.37	**15.80**	2.34
	Familiar	**12.46**	2.07	**15.66**	1.93
3 hours	Novel	**19.58**	3.05	**20.64**	4.33
	Familiar	**14.06**	1.59	**15.39**	5.03

Table 5.6: The mean contact time in seconds for transgenic and wild type mice when faced with a novel and a familiar object in the test phase (averaged across two trials).

Fig. 5.17 shows the mean discrimination ratio for transgenic and wild type mice. Inspection of this figure suggests there are no differences in performance between transgenic and wild type

mice in their ability to discriminate between the novel and the familiar object as measured in the discrimination ratio averaged across test days. ANOVA analysis confirmed that there was no main effect of genotype (F (1, 31) = 0.37, $p > .05$), delay (F (1, 31) = 1.66, $p > .05$), or a significant interaction between the two (F (1, 31) = 0.79, $p > .05$). When comparing discrimination ratios against chance, neither wild type nor transgenic mice differed significantly from 50% at the 5 minute delay (wild type: (F (1, 21) = 2.44, $p > .05$); transgenic: (F (1, 13) = 0.01, $p > .05$). When given a three hour delay, only wild type mice differed significantly from chance (wild type: (F (1, 21) = 4.65, $p < .05$); transgenic: (F (1, 13) = 2.62, $p > .05$).

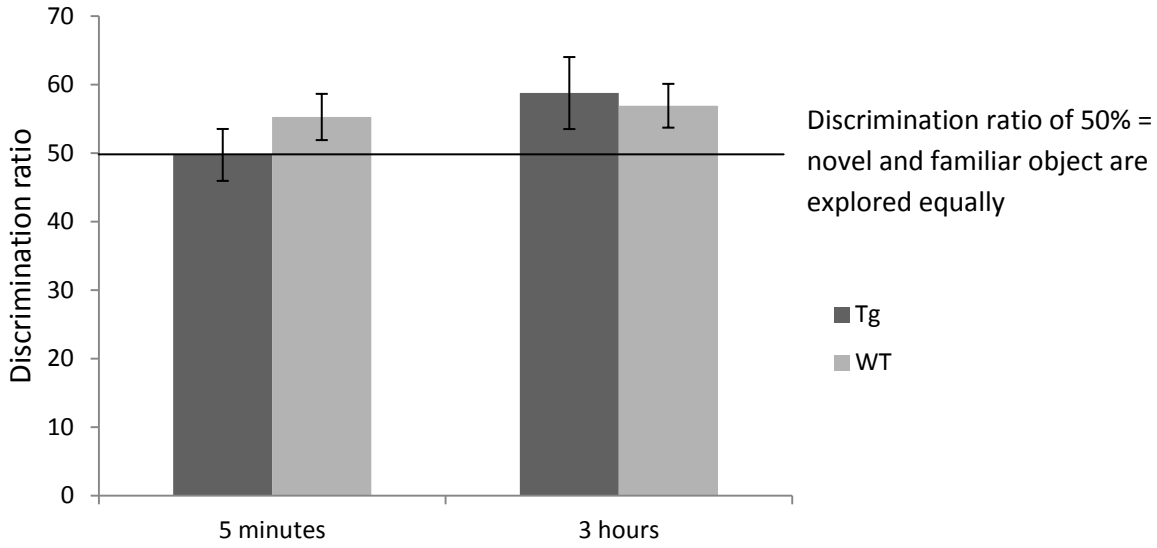


Fig. 5.17: Discrimination ratio (total time novel/total time any object*100) displayed for transgenic (Tg) and wild type (WT) mice at delay intervals 3 hours and 5 minute delay. Error bars ±1 S.E.M.

5.7.4 Discussion

The results obtained from the novel object recognition task suggests that harbouring the APP(V717F) mutation does not lead to differential performance at 12 months of age compared to wild type controls. The lack of a transgenic deficit compared to wild type mice is not a result of genotypic variations in the level of exploration of objects in the sample phase, but may be masked by a lack of discrimination between the novel and familiar object by wild type controls. This indicates that the object recognition memory of transgenic PDAPP mice is not disrupted by Aβ pathology at 12 months of age, despite reports of extensive plaque pathology

by this age (Dodart et al., 2000; Reilly et al., 2003). It is important to note however that the onset and degree of Aβ pathology may vary between colonies of PDAPP mice in different laboratories. In addition, it is possible that transgenic PDAPP mice would display a novel object recognition deficit compared to wild type controls in the event that wild type mice showed a stronger novelty preference.

This result is in line with those of Chen et al. (2000), who did not detect any differences between wild type and transgenic mice at any delay interval using a paradigm generally comparable to the current experiment. In contrast, Dodart et al. (1999) detected an age-related deficit in object recognition memory emerging at 9-10 months in transgenic PDAPP mice. However, Dodart et al. (1999) utilised a paradigm where one object is presented in the sample phase, followed by two objects in the test phase. In this scenario, spatial novelty is a factor in addition to object novelty. The novelty of the spatial location of an object has been shown to influence exploration in the Tg2576 model (Hale & Good, 2005; Good, Hale & Staal, 2007). Thus, Dodart et al. (1999) utilise a testing procedure with a lowered novelty detection difficulty level, which may disproportionately benefit wild type mice.

Whilst both Chen et al. (2000) and the current data point to a lack of genotypic differences in object novelty detection, there are differences between the data sets. Notably, the transgenic mice in the current study do not show a novelty preference in when there is a 5 minute delay, with transgenic mice spending a comparable amount of time exploring the novel and familiar object. No transgenic deficit is detected in this condition, arguably due to a relatively poor performance level of wild type mice. This is not observed in Chen et al. (2000), where both transgenic and wild type mice show a novelty preference in the no delay condition, which deteriorates at a comparable rate as the delay is increased. One explanation for this difference in general performance level could be variations in the sample phase. Whilst the current study allowed subjects 10 minutes to explore the objects in the sample phase, Chen et al. (2000) used a criterion of 20s of accumulated exploration time (maximum 20 minutes). However, this difference is unlikely to account for the relatively large differences in the results, as the average sample time for testing conditions listed in Table 5.13 reveal similar exploration levels to the 20s criterion of Chen et al. (2000). A second factor that differs between the current experiment and that of Chen et al. (2000) is the genetic background of the PDAPP mice. This factor is a continuing source of variation, and has been highlighted by Kobayashi & Chen (2005) as a potential explanation for differences between Chen et al. (2000) and Dodart et al. (1999) on the object recognition memory profile of the PDAPP model. The

change from a hybrid triple background to a pure C57Bl/6 background as in the current project offer increased potential for alterations to the behavioural phenotype of transgenic and wild type mice.

The performance of PDAPP and APP(V717I) mice described in Chapter 3 on the novel object recognition task differ in the degree of novelty preference, which was higher in APP(V717I) mice, despite the exploration times in the sample and test phase being higher for PDAPP mice. There are several factors which may influence the performance of wild type mice of both models in this task. As mentioned previously, the genetic background of both models is C57Bl/6. Nevertheless, neither model is likely to be congenic, and have arisen through a different mix of inbred strains. Furthermore, the objects used when testing the PDAPP model were not those utilised with the APP(V717I) mice. This was due to the novel object recognition task being administered after the liquid reward foraging task in PDAPP mice, resulting in PDAPP mice being trained to jump onto objects in an arena to obtain reward. It was therefore desirable to avoid objects which could be climbed, which was possible with the objects used to test APP(V717I) mice. This change in object characteristics could well have influenced performance in the task, as it has been shown that discrimination ratios are higher when mice can climb onto objects during exploration compared to objects which can only be touched (Heyser & Chemero, 2011).

5.8 Chapter discussion

The aim of this chapter was to provide a profile of potential changes in anxiety-related behaviour and memory of PDAPP(V717F) transgenic mice at 9 – 14 months of age. A predicted reduction in anxiety-behaviour was observed in 9.5 month old transgenic mice which was not evident in littermate controls (Experiment 9 and 10). In terms of spatial working memory, both the T-maze (Experiment 11) and the foraging task (Experiment 12) indicate transgenic deficits at 11 and 14 months respectively. In contrast, no genotypic differences in object recognition memory were observed, with the general performance level of novelty detection being relatively low across genotypes (Experiment 13).

In terms of anxiety behaviour, there are no direct data available in the literature on anxiety changes in transgenic PDAPP mice. Interestingly, the pattern of results observed is comparable to that observed in the APP(V717I) model (Chapter 2), as well as other models of Aβ pathology such as the Tg2576 line (Lalonde et al., 2003; Ognibene et al., 2005). This general trend towards lowered anxiety in transgenic APP mutation models suggests either that a range

of mutations in the APP protein exhibit a similar effect on anxiety behaviour through elevation of Aβ levels, or that overexpression of APP drives changes to anxiety behaviour without the involvement of Aβ pathology. A third alternative could involve a combination of both these factors. The observation that anxiety deficits in transgenic APP(V717I) mice are age-independent lend support to the notion that overexpression of APP may play a greater role than Aβ pathology. As no analysis of anxiety in the PDAPP model at a young age was possible, no conclusions can be drawn regarding the effect of age on changes in anxiety behaviour in this model based on the current data set. The novel object recognition task did not appear to be a sensitive task to assess object recognition memory in the PDAPP model, as limited novelty preference was evident in both transgenic and wild type mice.

The T-maze FCA and the foraging task revealed poorer performance in aged PDAPP mice. The detection of transgenic deficits across two separate tasks demonstrates that the behavioural deficit is not task-specific, whilst manipulations of the task difficulty level in the foraging task suggest that the lowered performance of transgenic PDAPP mice is related to deficits in spatial working memory as opposed to a general motivational or activity-related issue. It is important to note however that the continued deficit in transgenic mice following the introduction of the visual cue in the T-maze task could indicate a transgenic-specific issue with the visual system. Indeed, such issues have been reported in the Tg2576 model, and appear to be linked to a retinal degeneration gene found in 20% of inbred mouse strains (Garcia et al., 2003). When homozygous, the gene causes near blindness in carriers, confounding behavioural measures of cognitive performance (Garcia et al., 2003). Whilst this is an issue that one cannot dismiss based on the current data, the lack of a deficit in the recognition memory task would indicate that gross visual impairments are an unlikely explanation for spatial memory deficits in PDAPP mice. Furthermore, a number of previous studies of PDAPP mice in which visually cued platform locations have been utilised in the Morris water maze have found no genotypic differences in latency after 3 days of training, indicating intact sensorimotor function (Brody & Holtzman, 2006; Dumas et al., 2008). Interestingly, transgenic deficits in visually cued trials are evident on day 1 and 2, indicating that transgenic mice are impaired at learning the association between the cue and the platform, but the transient nature of this deficit does not implicate the visual system. In addition, whilst visually cued trials in the Morris water maze and the T-maze both rely on the ability of transgenic mice to form an association between the cue and the trial end point, the deficit in the appetitive task may reflect the nature of the prior training (alternation or position bias) or the nature of the reward.

When interpreting demonstrations of age-dependent spatial working memory deficits in transgenic APP mutation mouse models in the context of the amyloid cascade hypothesis, the prediction is that such deficits are related to elevations in Aβ levels in the CNS. Reports of pathological and biochemical analysis of PDAPP transgenic brain tissue confirms the elevation of soluble Aβ levels by 8 months of age (Johnson-Wood et al., 1997), with Aβ plaque deposition by 12-15 months of age (Reilly et al., 2003). It is therefore possible that the performance deficit detected in the foraging task in transgenic PDAPP mice at 14 months is related to Aβ pathology, although onset and degree of pathology in transgenic models may vary greatly between laboratories. Nevertheless, it is possible that the task could be utilised to assess the ability of 2B3 to reduce Aβ pathology and cognitive deficits *in vivo.* The aim of the experiments reported in Chapter 6 is to assess whether 2B3 can lower Aβ production and reduce spatial working memory deficits when administered to PDAPP transgenic mice.

The effects of 2B3 *in vitro* and *in vivo*

6.1 Experiment 14: The effect of 2B3 in primary neuronal cell cultures

6.1.1 Introduction

The aim of immunotherapy in AD research is to reduce the underlying pathology as well as the cognitive and behavioural symptoms of the disease using antibodies. Whilst several attempts at reducing Aβ pathology and cognitive deficits in transgenic mouse models have been successful using anti-Aβ antibodies (Schenk et al., 1999; Sigurdsson et al., 2001; Janus et al., 2000; Wilcock et al., 2004; Morgan et al., 2000), there has been limited success associated with translating the approach to human patients (Orgogozo et al., 2003; Gilman et al., 2005; Holmes et al., 2008). This is due both to a tendency for anti-Aβ antibodies to induce meningoencephalitis in patients, as well as to a lack of observed cognitive benefit associated with Aβ plaque clearance. As excess Aβ is deposited in insoluble plaques in the extracellular space, it is possible that antibodies which bind to Aβ excessively activate the immune system. An alternative approach is to use an antibody that binds to the β-secretase cleavage site of APP, a protein which can undergo proteolytic cleavage in a number of ways, to inhibit Aβ production through steric hindrance.

The use of anti-APP β-secretase cleavage site antibodies has been limited to date, but when assessed has proved successful both *in vitro* and *in vivo.* Rakover et al. (2007) demonstrated that administration of such an antibody to transgenic Tg2576 mice reduced deficits in the object recognition task, but without the hypothesised lowering of soluble or insoluble Aβ levels in the CNS. Thomas et al. showed that two different types of anti-APP β-secretase cleavage site antibodies, 2B12 (Thomas et al., 2006) and 2B3 (Thomas et al., 2011), could reduce Aβ40 and Aβ42 production in cell culture by 63% and 78% respectively. However, the effect was obtained using the human MOG-G-UVW and SH-SY5Y cell lines, which are not neuronal. It was therefore essential to determine whether the Aβ reducing effects of 2B3 could be replicated in primary neuronal cell cultures. By extending the types of cell systems the treatment has been tested in to one that more closely mimics neuronal cells, it is possible to assess whether the effects of 2B3 generalise across cell types. The main aim of this experiment was to use a primary neuronal murine culture to assess whether 2B3 reduced Aβ

production. Thus, primary neuronal cultures were developed using cortical tissue from foetal C57Bl/6 wild type mice.

6.1.2 Methods

The protocol for the generation, growth and differentiation of embryonic cells into neurons was based on methods generously provided by Dr. Kelly and Dr. Vinh, whilst the execution relied heavily on the expertise of Dr. Thomas, all at Cardiff University.

Generation and collection of foetal cortical tissue

Female C57Bl/6 mice were time-mated before being sacrificed at gestational day E16.5. Foetal cortical tissue was dissected from each hemisphere and the meninges were removed. Tissue was kept on ice in HBSS (Hank's Buffered Salt Solution) cell culture media.

Extraction, expansion and maintenance

Tissue samples were washed in media [DMEM (Dulbecco's Modified Eagle Medium), 1% Penicilin, 1% Streptomycin, 1% Fungizone] and centrifuged twice at 1000rpm for 3 minutes. After removal of excess media, 500µl of Trypsin (Worthington, Lakewood, USA, 10mg in HBSS) was added. The samples were incubated for 20 minutes at 37^{o}C, before 500µl of Trypsin inhibitor (Sigma-Aldrich, 20µg/ml in HBSS) and 500µl of DNAase (Worthington, 50µg/ml in HBSS) was added. Following 5 minutes incubation at 37^{o}C, 10ml of media was added before samples were centrifuged at 1000rpm for 3 minutes. Excess media was removed before 200µl expansion media [DMEM/F12 1:1, 2% B27, 20ng/ml Fibroblast Growth Factor (FGF), 20ng/ml Epidermal Growth Factor (EGF), 1% Penicillin, 1% Streptomycin, 1% Fungizone] was added. Cells were triturated lightly 3 times and counted, and were maintained at 37^{o}C in 24-well plates.

Cells were encouraged to proliferate whilst being regularly fed and passaged until approximately 25ml of cell-containing media was available. Cells were fed by replacing 40-50% of the media with fresh expansion media. The passaging procedure was identical to the extraction procedure from the first centrifugation onwards.

Neuronal differentiation

Once approximately 25ml of media with confluent cells was obtained, cells were differentiated. Glass coverslips were baked at 180^{o}C for 2 hours before one coverslip was added per well to a 24-well cell culture plate. Five hundred µl of 20µg/ml of poly-L-lysine made up in dH_2O was added to each well and incubated for 24 hours at 37^{o}C. Wells were washed 3 times in media (DMEM, 1% Penicillin, 1% Streptomycin, 1% Fungizone), excess media removed, then placed under UV light for 45 minutes. Following a 2 hour drying period, the cell passaging method was applied, using differentiation media (DMEM/F 12 1:1, 1% FBS, 2% B27, 1% Penicillin, 1% Streptomycin, 1% Fungizone) rather than expansion media. Approximately 75 000 cells in 40-50µl differentiation media were applied to each well and incubated for 4 hours at 37^{o}C before 500µl of differentiation media was added. Experimental manipulation of cells was carried out 24 hours after differentiation.

Neuronal detection using immunocytochemistry

In order to ensure differentiation of primary foetal cells into neurons, immunocytochemical staining using the neuronal marker Neuronal Specific Enolase (NSE) was carried out. In addition, a marker for glia cells (glial fibrillary acidic protein; GFAP) was used to assess the number of non-neuronal cells present in the culture. Immunocytochemical staining was carried out as described in Thomas et al. (2006).

Immunocytochemical staining using neuronal-specific markers confirmed the presence of multiple successfully differentiated neuronal cells. Whilst GFAP staining for glia cells showed labelling of some individual cells, the majority of the cell population was identified to be neuronal (data not shown).

Production of 2B3

2B3 is a monoclonal mouse IgG which binds to the β-secretase cleavage site of APP, the development of which has been described elsewhere (Thomas et al., 2011). The following sections will outline the methods adopted to produce 2B3 from 2B3-producing hybridoma cells stored in liquid nitrogen. This will include details of cell culture methods, concentration, purification, dialysis and quantification of IgG.

Cell culture

The 2B3-producing hybridoma cells were thawed in a 56^{o}C water bath before approximately 1.5ml of cell culture medium [RPMI1640 (Sigma-Aldrich, Dorset, UK), 2mM Glutamine, 10% Fetal Bovine Serum, 1% Penicillin, 1% Streptomycin] was added and resuspended prior to the addition to a further 30ml of media. The solution was centrifuged at 1000rpm for 5 minutes at room temperature (RT) and the supernatant subsequently removed. One ml of warm medium was added to the cell pellet and following resuspension, the cells were transferred to one well of a 24-well cell culture plate and grown at 37^{o}C in 5% CO_2. Cells were passaged when confluent by resuspension before the volume was divided between several wells with the addition of an appropriate volume of cell culture media depending on the size on the well or flask (Corning Incorporated, NY, USA) used for expansion.

When cells were grown to confluence, the media containing 2B3 was collected when approximately 50% of the cells were viable. The media was centrifuged at 1000rpm for 5 minutes at RT and the supernatant was collected and stored at -20^{o}C until use.

Concentration

Following filtering using 45µm Super Acrodisc 32 filters (Pall Life Sciences, Ann Arbor, USA), cell media of 2B3-producing hybridomas was concentrated using Amicon Ultra^{-15} centrifugal filter units (Millipore, Billerica, USA) with a 100 000 molecular weight cut off. Fifteen ml of media was centrifuged in a JS7.5 swing out rotor at 3000g at 4^{o}C. Following 1 hour of centrifugation, filtered media was discarded, and 15ml of fresh media was added to the top chamber to be centrifuged. This pattern continued until approximately 75ml had passed through one centrifugal filter unit. Several units were employed simultaneously, and the concentrated antibody was pooled after completion of centrifugation. Distributing media across several centrifugal units was carried out in an attempt to avoid high viscosity in the concentrated sample.

Purification

Concentrated media supernatant containing 2B3 was purified using an affinity chromatography MAb Trap^{TM} Kit (GE Healthcare) with the aim of both purifying and further concentrating the IgG solution. A sequence of buffers as well as the antibody sample was put

through a HiTrap Protein G column containing recombinant protein G which binds mouse IgG, but is genetically altered to lack the ability to bind albumin. Buffers were diluted 1:10 with sterilised dH_2O, excluding the neutralising buffer. The column was washed with sterilised dH_2O to clear ethanol residues following storage at approximately 1 drop per second, the constant rate throughout the procedure. The column was equilibrated with 3ml of binding buffer before the sample was applied. The supernatant from the column was collected in a numbered series of Eppendorf tubes containing 75µl neutralising buffer to preserve IgG activity. Ten ml of binding buffer was administered to remove molecules other than the antibody, before 5ml of elution buffer was passed through the column to release the antibody. A protein assay (see section 2.6.3) was carried out on the supernatant fractions in order to identify the fractions containing the antibody.

Dialysis

The antibody was dialysed in PBS (137mM NaCl, 2.5mM KCL, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, pH7.2) to ensure compatibility with the Tris and Glycine-based buffers of the MAb Trap^{TM} Kit. Slide-A-Lyzer Dialysis Cassettes (Pierce Thermo Scientific, Rockford, USA) of 0.5-3ml sample volume with a 10 000 molecular weight cut off were utilised. Cassettes were rotated in 2 litres of PBS at 4^{o}C overnight.

Quantification of 2B3 using ELISA

The methods utilised to measure 2B3 in cell culture media were based on the procedure used by Thomas et al. (2006, 2011) and was identical to that outlined in Section 4.2.2, Chapter 4 with the following exceptions. The 96-well plate was coated in an sheep anti-mouse IgG at a concentration of 1/4000 diluted in carbonate/bicarbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). Standards ranged from 210-0.10ng/ml in doubling dilutions. Doubling dilutions of the 2B3 sample were applied, from 1/1000 to 1/32,768,000. The detection antibody, HRP-labelled anti-mouse IgG (Pierce Thermo Scientific) was used at 1/6000 and incubated for 1 hour.

Administration of 2B3 to differentiated primary neurons and measurement of Aβ40 levels in media

Following differentiation, equal volumes of primary neurons from 4 independent embryos were passaged and placed into 3 conditions in a 24 well cluster plate and allowed to attach overnight. Cells were then incubated in differentiation media (DMEM/F 12 1:1, 1% FBS, 2% B27, 1% Penicillin, 1% Streptomycin, 1% Fungizone) only, or differentiation media containing 2B3 or an irrelevant mouse IgG (Sigma Thermo Scientific) at 10µg/ml at 37^{o}C for 48 hours at 37^{o}C. Media was collected and Aβ40 and Aβ42 levels were measured using ELISA methods as described in Thomas et al. (2006). Briefly, media was immunoprecipitated overnight at 4^{o}C using BAM401S (Alpha Diagnostics Int.) at 1:2000. This was followed by a 2 hour incubation with Protein A (Santa Cruz Biotechnology, Santa Cruz, USA) and 3 rounds of CHAPS buffer wash (150 mM NaCl, 50 mMTris, 1mM EDTA, 10mM CHAPS) and centrifugation at 3275g. Samples were washed in PBS and boiled for 5 minutes at 95^{o}C and centrifuged again at 3275g. The supernatant was used to measure Aβ40 using the same ELISA methodology as described in Section 4.2.2, Chapter 4.

Data analysis

The concentration of Aβ40 per sample was calculated in GraphPad Prism4.0 by the fitting of a non-linear regression curve to standard values and calculating unknown values from the curve. Individual Aβ values were standardised to the total protein concentration of each sample, and all values are given as ng/mg of total protein. ANOVA and post hoc analysis with Bonferroni correction was used to analyse the data, reflecting the independence of each primary cell preparation.

6.1.3 Results

Levels of Aβ40 following administration of either 2B3 or the negative controls are presented in Fig. 6.1. Inspection of this figure indicates a reduction in Aβ40 following 2B3 administration compared to controls. Statistical analysis confirmed a significant effect of treatment (F (2, 6) = 13.86, p < .005), with 2B3-treated levels significantly different from both the IgG (p <.05) and media control condition (p <.001). There was a 64.41% reduction in levels of Aβ40 in 2B3 cells compared to the cells treated with the irrelevant IgG, and a 73.96% reduction when comparing Aβ40 levels in 2B3-treated and media treated cells. There was no significant difference in Aβ40 levels between IgG and media treated cells (p >.05). Unfortunately, levels of Aβ42 were too low to be detected.

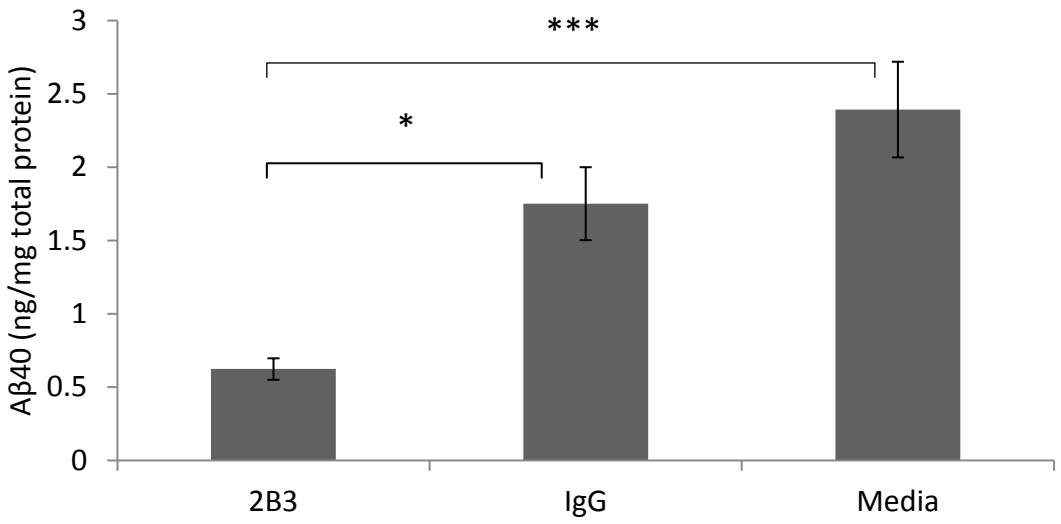


Fig 6.1: Levels of Aβ40 from primary murine cortical neurons after incubation with 2B3 or an irrelevant mouse IgG (10µg/µl) for 48 hours (ng/mg of total protein). *= $p < .05$, *** = $p < .001$. Error bars ±1 S.E.M.

6.1.4 Discussion

The results from the present experiment showed a significant reduction in Aβ40 levels following 2B3 administration compared to that either of an irrelevant IgG or a media control. This pattern of results is in line with predictions that neuronal cells will produce less Aβ if an anti-β-secretase APP antibody is present. Furthermore, the level of downregulation is highly comparable to that obtained using 2B3 and 2B12 in human astrocytoma and neuroblastoma cell lines (Thomas et al., 2006; 2011). In relation to the Aβ measurements obtained in aged APP(V717I) tissue in Chapter 4 (Section 4.2.3), the Aβ levels of the media-treated neurons are lower, as expected in wild type neurons not overexpressing APP. The lower Aβ levels in wild type APP(V717I) brain homogenate compared to that of primary neuronal cultures reflects a basic difference in tissue and cell culture preparations, as well as a difference in the concentration of the media obtained from primary neurons following immunoprecipitation. The experiment further demonstrated the need for an antibody that binds specifically at the β-secretase cleavage site of APP, as an irrelevant IgG does not produce a significant downregulation of Aβ40.

This is an important result as it is the first demonstration that the ability of 2B3 to reduce Aβ levels is transferrable from immortalised human cells to a primary mouse neuronal culture system. Thus, the antibody has been demonstrated to be effective in mouse neurons, suggesting that the application of the treatment to transgenic APP mutation models may be

equally successful in downregulating Aβ. Such a replication *in vivo* would open the possibility of assessing the effect of the antibody treatment on cognitive function, a central aspect of disease progression in humans. It is therefore an important measure against which to assess the potential utility of an anti-β-secretase cleavage site antibody to inhibit disease progression in transgenic APP mutation models.

6.2 Experiment 15: The effect of 2B3 on cognition and Aβ levels

6.2.1 Introduction

The hypothesis relating to *in vivo* 2B3 administration predicts a reduction in both Aβ levels and associated Aβ-related behavioural deficits following treatment. In order to test this hypothesis, PDAPP mice were administered 2B3 and tested on the foraging task described in Chapter 5.

Transgenic PDAPP mice aged 15 months of age were utilised in the current experiment for two interconnected reasons. Firstly, the treatment of AD at a time when the Aβ pathology is established represents more clinically relevant assessment than preventative treatment at a younger age, as AD diagnosis is not provided until the disease is established. Secondly, the use of aged transgenic mice offers model mice with AD-like symptoms in the form of a decline in cognitive function, allowing the assessment of potential changes on both biochemical and behavioural measures following 2B3 treatment.

The general aim of Experiment 15 was to evaluate whether 2B3 could inhibit Aβ production in transgenic PDAPP mice as detected by a lowering of Aβ levels in the CNS. Furthermore, the ability of 2B3 to reduce spatial working memory deficits of aged transgenic PDAPP mice in the foraging task was assessed. Eighteen 9 month old transgenic and 22 wild type littermate control PDAPP mice were generously provided by Eli Lilly to support this work. At the starting point of the current experiment, the colony had aged to 15 months. However, due to high attrition, the number of transgenic PDAPP mice available to partake in the experiment had been reduced to 12. With such a low sample size, the experiment should be considered a pilot study.

A central issue in immunotherapy which targets the CNS is access. The blood-brain barrier is a highly regulated membrane with tight junctions separating the central nervous system from the periphery. Whilst there are several transport routes across this barrier such as diffusion of lipid-soluble agents, receptor-mediated endocytosis and transport proteins

(Tam & Watts, 2010), the ability of IgG molecules to cross the membrane is a matter of debate. IgG levels in the CSF have been shown to be approximately 0.12% of that in serum (Felgenhauer, 1974). In order to assess the ability of 2B3 to inhibit β-amyloid production *in vivo*, it is essential that a method was adopted that ensured delivery of the antibody to its target, APP, within the CNS. Thus, a pilot experiment was carried out with the aim of assessing whether intracerebroventricular infusions using osmotic minipumps (Alzet, Cupertino, USA) could be used as a reliable method of antibody delivery to the brain.

6.2.2 Method

Design

A pilot study comparing the intensity and pattern of mouse IgG-related staining obtained through intracerebroventricular infusions using osmotic minipumps was conducted. One mouse received infusions of 2B3, whilst a control mouse received PBS infusions. In addition, the infusion cannula was placed into the left lateral ventricle only, leaving the right hemisphere as a within-subject control. An increase in non-specific mouse IgG related staining around the infusion site due to a potential inflammatory response would be detectable in tissue from both conditions, whilst 2B3-specific staining would be limited to the 2B3 condition.

In order to assess the effect of 2B3 on spatial memory in PDAPP mice, transgenic and wild type PDAPP mice were assessed in the 6-pot liquid version of the foraging task at 14 months of age prior to the surgical fitting of osmotic minipumps to allow for pre- and post-treatment performance measures. Transgenic mice were treated with 2B3 or a vehicle solution of differentiation media (DMEM/F 12 1:1, 1% FBS, 2% B27, 1% Penicillin, 1% Streptomycin, 1% Fungizone) concentrated and purified in an identical fashion to 2B3-containing differentiation media. Treatment was continuous for 14 days before Aβ levels were measured. In addition, four wild type mice were treated with vehicle to assess whether the presence of the osmotic minipump would alter their performance in the foraging task. On the 10^{th} to the 13^{th} day of treatment, spatial memory was assessed using the liquid reward version of the foraging task (see Chapter 5, Section 5.6.2). It was hypothesised that PDAPP transgenic mice treated with 2B3 would display lower Aβ levels than transgenic mice treated with vehicle and show an improvement in performance in the foraging task.

Subjects

Two C57Bl/6 male mice aged 6 months were utilised in the osmotic minipump pilot study, with one mouse in each treatment condition.

In the 2B3 treatment experiment, 19 wild type and 12 transgenic PDAPP (V717F) mice were assessed in the liquid version of the foraging task at 14 months of age (as reported in Chapter 5, Section 5.6.2). At 15 month of age, 12 male transgenic PDAPP mice underwent surgical fitting of osmotic minipumps. Half these mice were treated with 2B3 and half with vehicle. In addition, four wild type mice were treated with vehicle. Two of these wild type mice were utilised in biochemical analyses as negative controls.

Apparatus and method for the osmotic minipump pilot

2B3 was concentrated and purified to 1.35mg/ml, whilst PBS buffer served as the vehicle solution. Detailed methods relating to 2B3 production are outlined in Chapter 2, Section 2.3.

Treatments were administered using osmotic minipumps (Alzet, 1002) infusing 0.25µl of solution per hour continuously for 9 days. Pumps were filled and primed at 37^{o}C overnight. Mice were anaesthetised with isoflurane carried by O_2 during stereotaxic surgery. A 28G cannula from the brain infusion kit (Alzet, 0004760) was inserted into the left lateral ventricle at 3.4mm posterior, 10mm lateral and 3mm ventral from bregma. A 1.5cm long catheter linked the cannula to the pump positioned subcutaneously at the neck of the animal. Cyanoacrylate glue followed by dental cement secured the cannula to the skull before the wound was stitched. The mouse was given a subcutaneous injection of glucosaline to aid rehydration and was placed in a warm, secure environment to recover under monitoring. Mice were perfused on day 9 following surgery. 2B3 was detected in coronal sections by administering a 30 minute quench followed by a biotinylated anti-mouse IgG (Vector) at a concentration of 7.5µg/ml with 1% normal horse serum for 2 hours at RT. Incubation in the Dako Streptavidin ABC Complex and DAB staining was carried out as described in Section 4.3.2, Chapter 4.

Apparatus and method for the assessment of the effect of 2B3 in vivo

Assessment of spatial working memory as measured using the 6-pot liquid version of the foraging task was carried out described in Section 5.6.2, Chapter 5. Following 4 days of testing, mice were rested with *ad libitum* access to food and water for 3 days ensuring the return to *ad*

libitum weight prior to surgical fitting of osmotic minipumps. The minipumps were primed and implanted in an identical fashion to that described in the osmotic minipump pilot study 2B3 was concentrated to 1.98mg/ml, with the vehicle solution consisting of differentiation media (DMEM/F 12 1:1, 1% FBS, 2% B27, 1% Penicillin, 1% Streptomycin, 1% Fungizone) concentrated and purified alongside 2B3. After 7 days of recovery, mice were water deprived to 95% of their body weight. On day 9 following surgery, mice were given one habituation session of 10 minutes in the arena with two baited pots. This was followed by 4 test days in the foraging task on day 10 - 13 following surgery using the same methodology as that adopted in the test days prior to surgery. After completing all behavioural testing, mice were given *ad libitum* access to water and food and culled for tissue collection fourteen days following surgery.

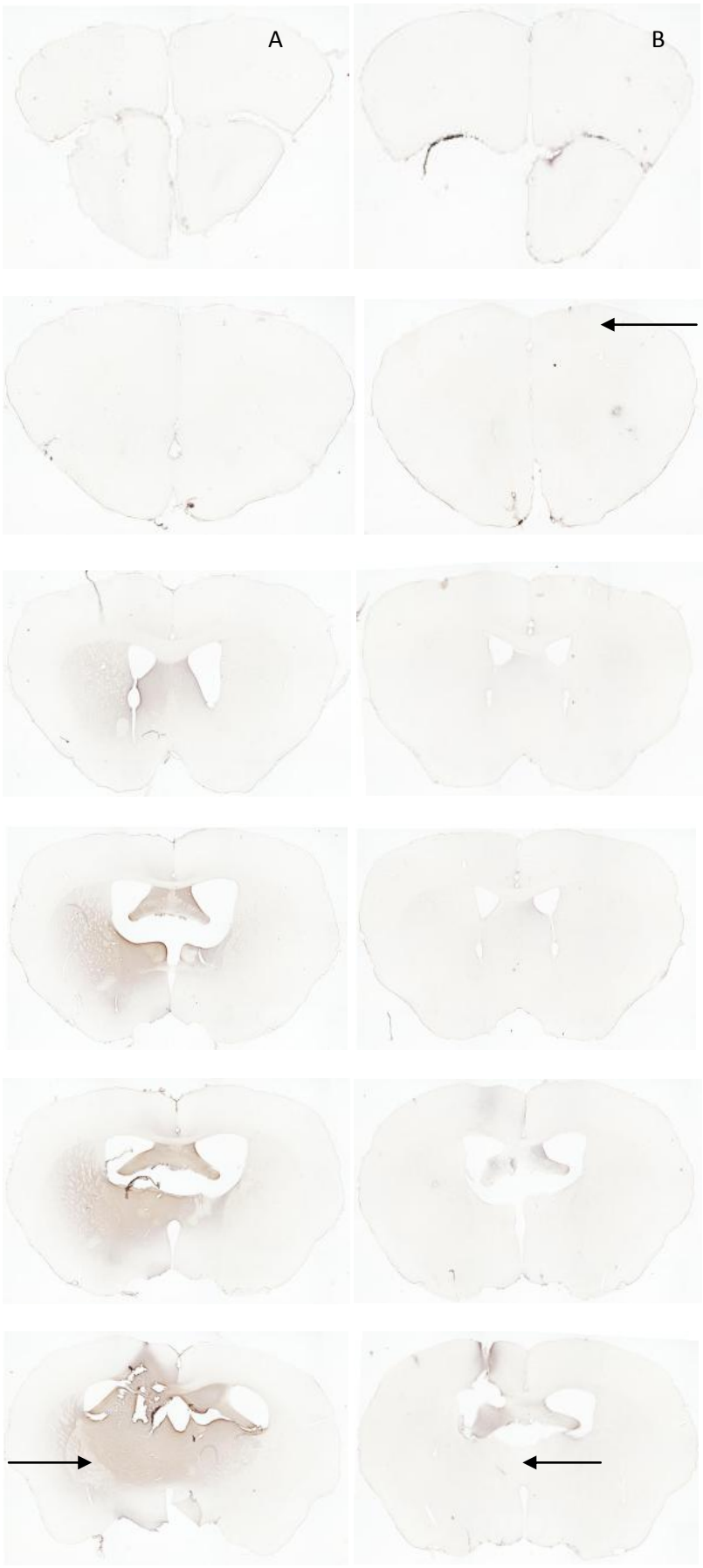
Data collection and analysis for the assessment of the effect of 2B3 in vivo

Performance in the foraging task was coded as described in Section 5.6.2, Chapter 5. Brain tissue was dissected to isolate the left cortex and hippocampus (as described in Section 4.2.2, Chapter 4), as these structures were located nearest the infusion site. Soluble and insoluble Aβ-40 and -42 were measured using ELISA (see Section 4.3.2, Chapter 4).

6.2.3 Results

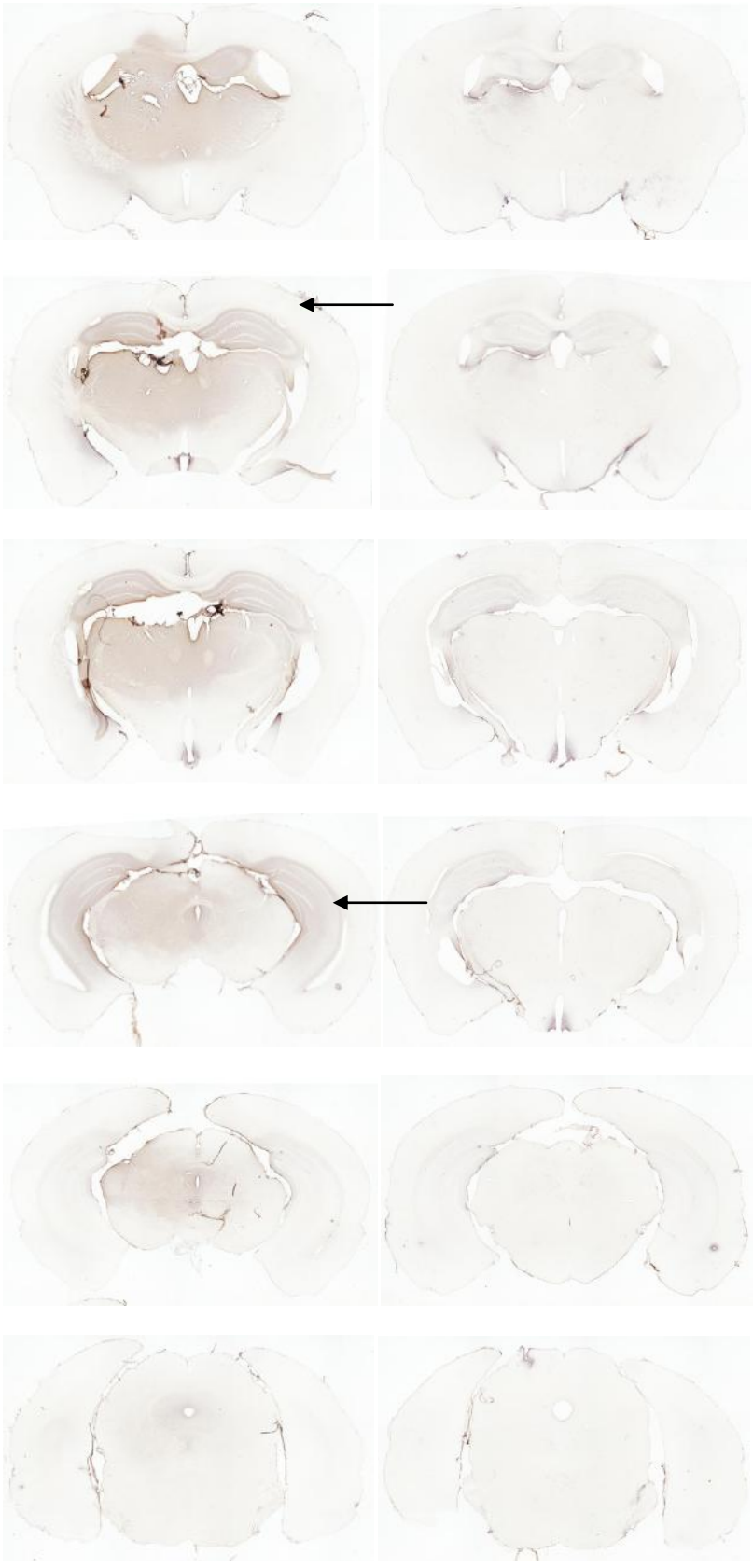
Osmotic minipump administration pilot

As can be observed from Fig. 6.2, infusion of 2B3 resulted in considerable intense staining around the infusion site than PBS infusions, in which only a limited increase in staining was detected. In addition, the mouse IgG staining in the 2B3 condition was detectable at large distances from the infusion site, with bilateral staining of subcortical structures including the hippocampus. No staining was evident throughout the cortex beyond the infusion site, including the frontal cortex. In contrast to the 2B3 condition, little staining was observed in the PBS infused tissue. At high magnification (x200), the staining in the 2B3 condition appears cellular, suggesting an internalisation of 2B3. This is not evident in the PBS condition, where the staining under high magnification is more general and appears to focus mainly on the extracellular matrix (Fig. 6.3).



No evidence of 2B3- or PBS-related staining in the frontal cortex

Stronger subcortical staining following 2B3 infusion compared to PBS infusion.



Staining in 2B3- and PBS-infused tissue is absent in the cortex, but is evident in the dorsal hippocampus and other subcortical structures in 2B3-infused tissue

Staining in 2B3-infused tissue evident in the ventral hippocampus

Fig. 6.2: Coronal sections of tissue infused with 2B3 (A) and PBS (B) via osmotic minipumps stained with an anti-mouse IgG secondary antibody.

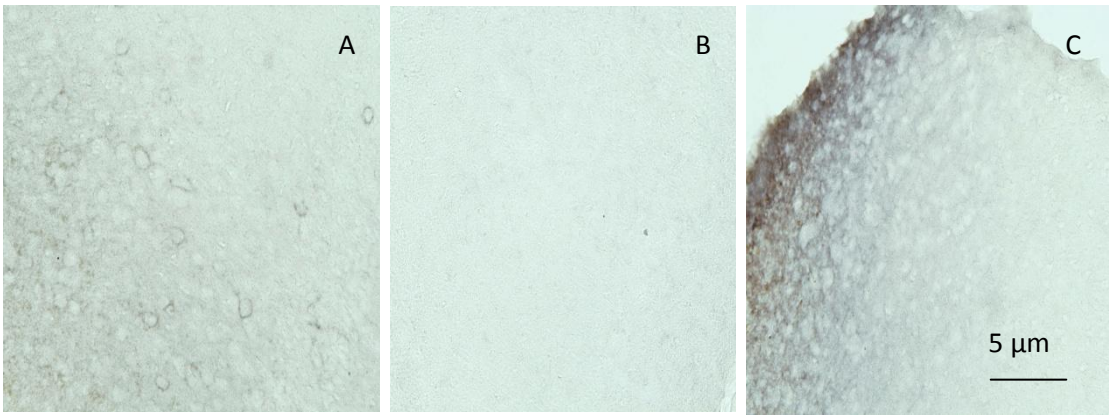


Fig. 6.3: Coronal section of cortical tissue infused with 2B3 (A) or PBS (B, C) using an osmotic minipump. A and B are comparable locations medial to the infusion site, whilst the PBS infusion site is displayed in C. All sections were stained with an anti-mouse IgG secondary antibody.

Behavioural results

One transgenic mouse was excluded from all analysis as an outlier due to performance scores being higher more than three times higher than the transgenic group mean. All variables were analysed using Mann Whitney U and Wilcoxon Signed Ranks for between and within-subject analyses respectively due to violations of the assumption of homogeneity of variance and normality which were not rectified by transformation. Mean and S.E.M. values for all conditions are presented in Tables 6.1-6.3.

Control groups

Firstly, the data were analysed to assess whether surgical implantation of osmotic minipumps altered wild type performance in the foraging task. Both between- and within-subject analysis of performance revealed no significant influence of osmotic minipumps infusing a vehicle on performance compared to pre-infusion performance or non-surgical wild type control mice (see Table 6.1). Thus, for comparison of transgenic and wild type performance, wild type mice were collapsed across control conditions.

Variable		Treatment difference			Within-subject changes			
					No pump WT		Vehicle WT	
		U	*p*		*U*	*P*	*U*	*P*
Before treatment	Error	22.50	0.718	Before vs. During	-1.01	0.331	-1.21	0.313
	Error first half of the trials	15.00	0.189		-0.80	0.425	-1.47	0.250
	Repeat error	21.00	0.600		-0.04	0.985	-1.21	0.313
	Repeat error first half of the trials	24.00	1.000		-0.01	1.000	-1.00	1.000
	Consecutive error	22.00	0.786		-0.30	0.801	-1.30	0.375
	Consecutive error first half of the trials	24.00	1.000		-0.45	1.000	-1.00	1.000
During treatment	Error	22.50	0.724					
	Error first half of the trials	25.50	1.000					
	Repeat error	24.50	0.885					
	Repeat error first half of the trials	24.00	1.000					
	Consecutive error	16.00	0.250					
	Consecutive error first half of the trials	24.00	1.000					

Table 6.1: The test statistic and exact significance for between- and within-subject analysis of variables of wild type performance from the foraging task is presented analysed for treatment differences. No pump WT = 15, vehicle WT =4.

Pre-treatment genotypic differences

Secondly, performance scores at 14 months (before treatment scores) as first presented in Section 5.6.3, Chapter 5, Phase B were analysed for genotypic differences of each transgenic treatment condition compared to wild type controls. This was carried out to ensure the continued significance of the transgenic impairment on the foraging task compared to wild type littermates following the subdivision of the transgenic mice. Between-subject analysis of genotypic differences was split by treatment, and revealed significant transgenic impairments on a subset of measures (see Table 6.2). The measures that remain sensitive to genotypic differences appear to be focused on the first half of the task, where the task difficulty is low compared to the second half.

Variable		Difference compared to WT controls			
		Tg 2B3		Tg Vehicle	
		U	*P*	*U*	*P*
Before treatment	Error	28.00	0.112	26.50	0.089
	Error first half of the trials	**19.00**	**0.018**	**7.50**	**0.001**
	Repeat error	28.50	0.119	22.50	0.045
	Repeat error first half of the trials	**19.00**	**0.003**	**4.00**	**0.001**
	Consecutive error	33.50	0.234	**10.00**	**0.002**
	Consecutive error first half of the trials	**19.00**	**0.005**	**2.00**	**0.001**

Table 6.2: The test statistic and exact significance for between- and within-subject analysis of variables of wild type performance from the foraging task is presented analysed for treatment differences. WT n=16, Tg Vehicle n= 6, Tg 2B3 n=6. Significant differences are highlighted in bold.

Analysis of treatment effects: Error

(returning to a previously visited pot)

Fig. 6.4A shows the average error scores of transgenic mice treated with 2B3 or vehicle prior to treatment, and following the surgical implantation of osmotic minipumps. Fig. 6.4B shows the errors during the first half of the trial. These suggest a decrease in error scores in transgenic mice treated with 2B3 that is not present in vehicle-treated mice.

In terms of pre-treatment values, no significant differences between the transgenic groups were present for total error scores (U (6, 6) = 17.00, $p > .05$) or error scores in the first half of the trial (U (6, 6) = 14.50, $p > .05$). In terms of measures taken during treatment, statistical analysis did not detect any significant differences between 2B3 and vehicle-treated mice on error scores across trials (U (6, 6) = 15.00, $p > .05$) or during the first half of the trials when averaged across test days (U (6, 6) = 15.00, $p > .05$). Average values across test days with S.E.M.s are presented in Table 6.3.

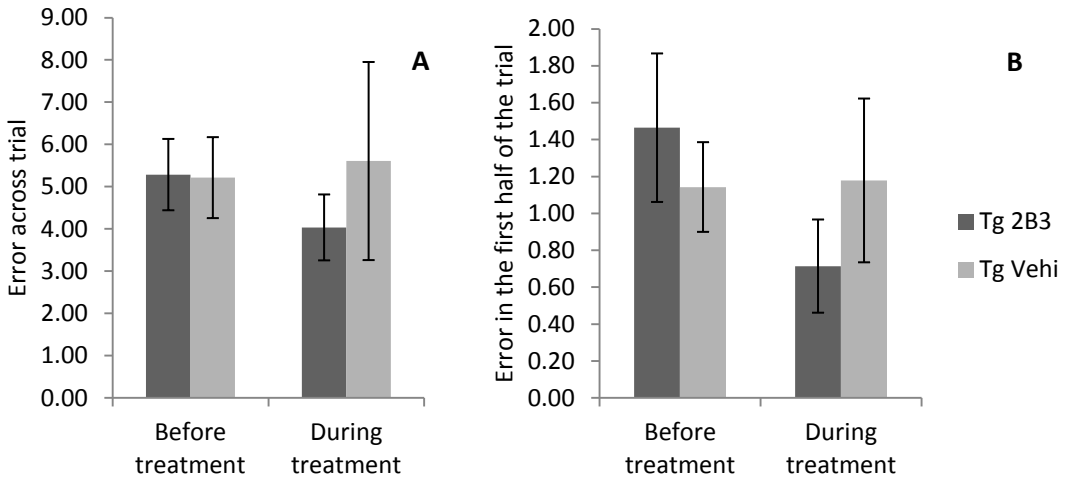


Fig 6.4: The mean error scores across trials (A) and in the first half of the trials (B) at 14-15 months of age for transgenic PDAPP mice prior to and during administration of 2B3 or vehicle. Error bars ±1 S.E.M.

In order to analyse the potential within-subject change in error scores before and during treatment, vehicle and 2B3-treated transgenic mice were analysed separately. This analysis did not reveal any significant difference in the before and during treatment scores of vehicle-treated transgenic mice across trials (χ^2 (6) = -0.31, p > .0125) or in the first half of the trials (χ^2 (6) = -0.11, p >.0125). Similarly, 2B3-treated animals did not differ significantly between before and during treatment performance on either the across trials (χ^2 (6) = -1.15, p >.0125) or first half measure (χ^2 (6) = -1.58, p >.0125). Analysis of the within-subject change in performance in wild type mice treated with vehicle revealed no significant changes (Across trials: χ^2 (4) = -0.73, p > .0125; in the first half of the trial: χ^2 (4) = -1.09, p > .0125). Similarly, wild type mice who did not undergo osmotic pump fitting did not significantly alter their performance across the two test trials (Across trials: χ^2 (14) = -0.60, p > .0125; in the first half of the trial: χ^2 (14) = -1.02, p > .0125).

Genotype	Treatment	Before treatment				During treatment			
		Error across trials		Error in the first half of the trials		Error across trials		Error in the first half of the trials	
		Mean	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.
Tg	2B3	**5.29**	0.84	**1.46**	0.40	**4.04**	0.78	**0.71**	0.25
Tg	Vehicle	**5.21**	0.96	**1.14**	0.24	**5.61**	2.34	**1.18**	0.44
WT	Vehicle	**3.75**	1.13	**0.44**	0.06	**3.19**	0.89	**0.31**	0.06
WT	No pump	**3.80**	0.39	**0.42**	0.16	**4.38**	0.71	**0.52**	0.16

Table 6.3: The mean error scores across trials and in the first half of the trials at 14-15 months of age for transgenic and wild type PDAPP mice prior to and during administration with 2B3 or vehicle.

Analysis of treatment effects: Repeat Error

(returning to a previously visited pot more than once)

In terms of pre-treatment values, no significant differences between the transgenic groups were present for total error scores (U (6, 6) = 16.50, $p > .05$) or error scores in the first half of the trial (U (6, 6) = 17.00, $p > .05$). As observed in the error scores, the trend for a reduction in repeat error scores during treatment with 2B3 (see Fig. 6.5) was not statistically significant compared to the performance of vehicle-treated mice across trials (U (6, 6) = 15.00, $p > .05$) or during the first half of the trials when averaged across test days (U (6, 6) = 16.00, $p > .05$). Average values with S.E.M. are presented in Table 6.4.

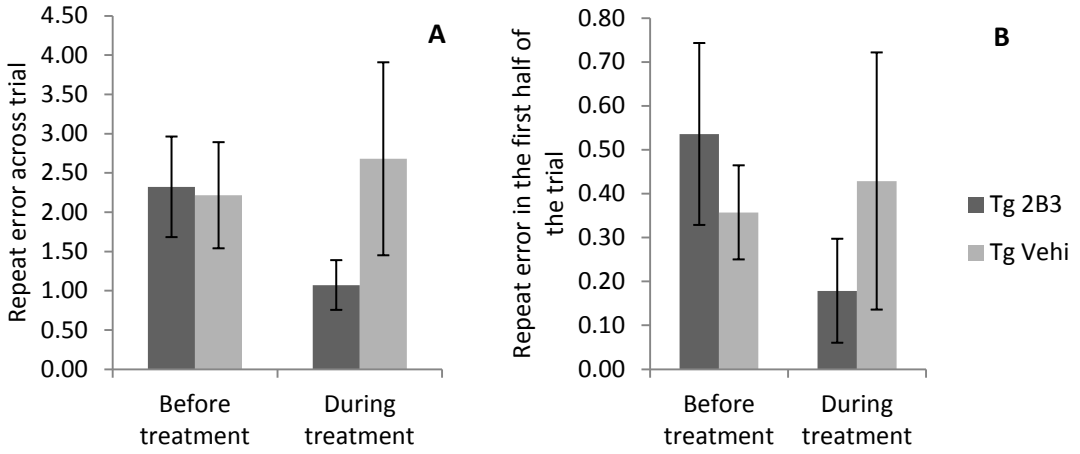


Fig 6.5: The mean repeat error scores across trials (A) and in the first half of the trials (B) at 14-15 months of age for transgenic PDAPP mice prior to and during administration of 2B3 or vehicle. Error bars ±1 S.E.M.

Within-subject analysis of changes in behaviour following administration of 2B3 or vehicle did not detect any significant differences between pre- and post-treatment in any condition. Specifically, vehicle-treated mice did not significantly alter their levels of total repeated error scores (χ^2 (6) = -0.11, $p > .05$) or repeated errors in the first half of the task (χ^2 (6) = -0.74, $p > .05$). Similarly, 2B3-treated mice showed no significant changes in total repeated error scores (χ^2 (6) = -1.58, $p > .05$) or repeated errors in the first half of the trials (χ^2 (6) = -1.84, $p > .05$).

Genotype	Treatment	Before treatment				During treatment			
		Repeat error across trials		Repeat error in the first half of the trials		Repeat error across trials		Repeat error in the first half of the trials	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Tg	2B3	**2.32**	0.64	**0.54**	0.21	**1.07**	0.32	**0.18**	0.12
Tg	Vehicle	**2.21**	0.68	**0.36**	0.11	**2.68**	1.23	**0.43**	0.29
WT	Vehicle	**1.38**	0.58	**0.00**	0.00	**0.88**	0.33	**0.00**	0.00
WT	No pump	**1.10**	0.25	**0.08**	0.06	**1.17**	0.34	**0.07**	0.05

Table 6.4: The mean repeat error scores across trials and in the first half of the trials at 14-15 months of age for transgenic and wild type PDAPP mice prior to and during administration with 2B3 or vehicle.

Analysis of treatment effects: Consecutive Error

(returning to a previously visited pot twice or more without visiting any other pots in the interval between those errors)

Prior to administration of 2B3 or vehicle, the two transgenic groups did not differ in total error scores (U (6, 6) = 13.50, $p > .05$) or error scores in the first half of the trial (U (6, 6) = 16.00, $p > .05$). During treatment, no significant reduction in consecutive error scores was observed in 2B3-treated compared to vehicle-treated mice in total consecutive error scores (U (6, 6) = 12.00, $p > .05$) or during the first half of the trials when averaged across test days (U (6, 6) = 15.00, $p > .05$; see Fig. 6.6). Average values with S.E.M.s are presented in Table 6.5.

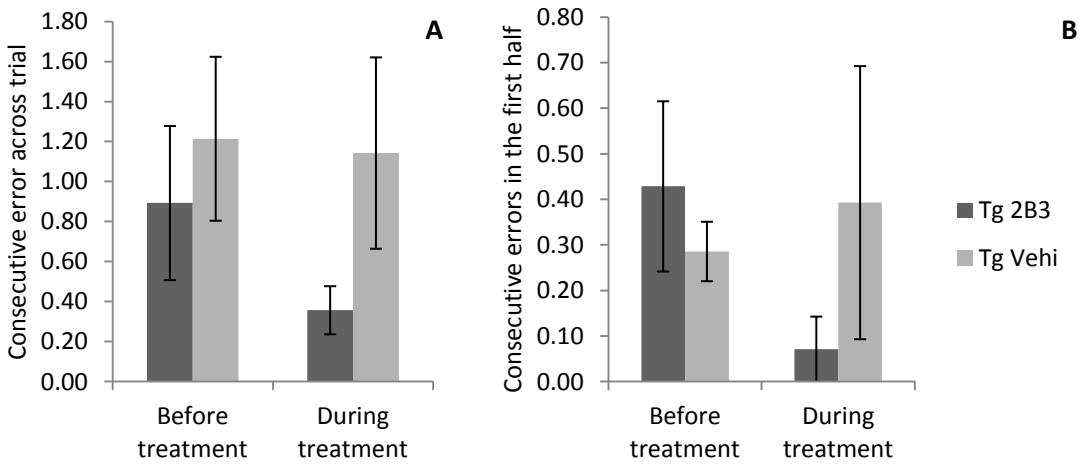


Fig. 7.6: The mean consecutive error scores across trials (A) and in the first half of the trials (B) at 14-15 months of age for transgenic PDAPP mice prior to and during administration of 2B3 or vehicle. Error bars ±1 S.E.M.

No significant within-subject changes were detected for vehicle or 2B3-treated transgenic mice in the total consecutive error scores [Vehicle: (χ^2 (6) = -0.68, p > .05) 2B3: (χ^2 (6) = -0.74, p > .05)] or the consecutive errors scores isolated from the first half of the task [Vehicle: (χ^2 (6) = -1.16, p > .05) 2B3: (χ^2 (6) = -1.84, p > .05)].

Genotype	Treatment	Before treatment				During treatment			
		Consecutive error across trials		Consecutive error in the first half of the trials		Consecutive error across trials		Consecutive error in the first half of the trials	
		Mean	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.	**Mean**	S.E.M.
Tg	2B3	**0.89**	0.39	**0.43**	0.19	**0.36**	0.12	**0.07**	0.07
Tg	Vehicle	**1.21**	0.41	**0.29**	0.07	**1.14**	0.48	**0.39**	0.30
WT	Vehicle	**0.25**	0.10	**0.00**	0.00	**0.13**	0.13	**0.00**	0.00
WT	No pump	**0.38**	0.11	**0.03**	0.02	**0.37**	0.11	**0.07**	0.05

Table 6.5: The mean consecutive error scores across trials and in the first half of the trials at 14-15 months of age for transgenic and wild type PDAPP mice prior to and during administration with 2B3 or vehicle.

Biochemical results

Analysis of treatment effects: Cortex

Measures of soluble Aβ40 and Aβ42 extracted from the left (treated) cortex are presented in Fig. 6.7, with insoluble values presented in Fig. 6.8. With a high level of individual variability in Aβ levels in transgenic mice regardless of treatment condition, there is no evidence of a systematic lowering of Aβ levels in 2B3-treated compared to vehicle-treated transgenic mice. Statistical analysis confirmed that neither soluble Aβ40 (U (6, 6) = 11.00, p > .05) nor Aβ42 (U (6, 6) = 11.00, p > .05), nor insoluble levels of cortical Aβ40 (U (6, 6) = 15.00, p > .05) or Aβ42 (U (6, 6) = 15.00, p > .05) levels differed significantly in transgenic mice following 2B3/vehicle administration.

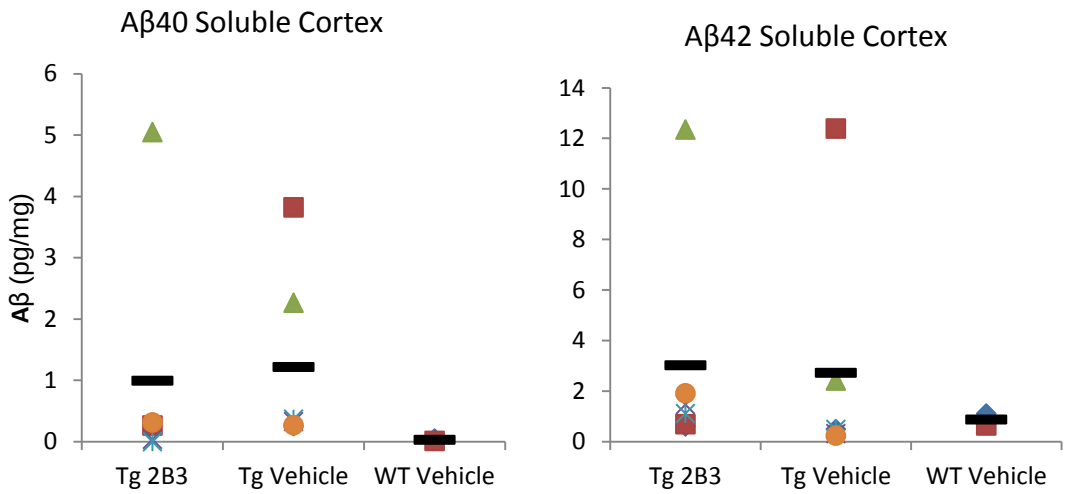


Fig. 6.7: Aβ40 and Aβ42 soluble values (pg/mg of total protein) from the left cortex of 2B3-and vehicle-treated PDAPP mice displayed as individual values with the mean denoted as a black line. Tg = transgenic, WT = wild type.

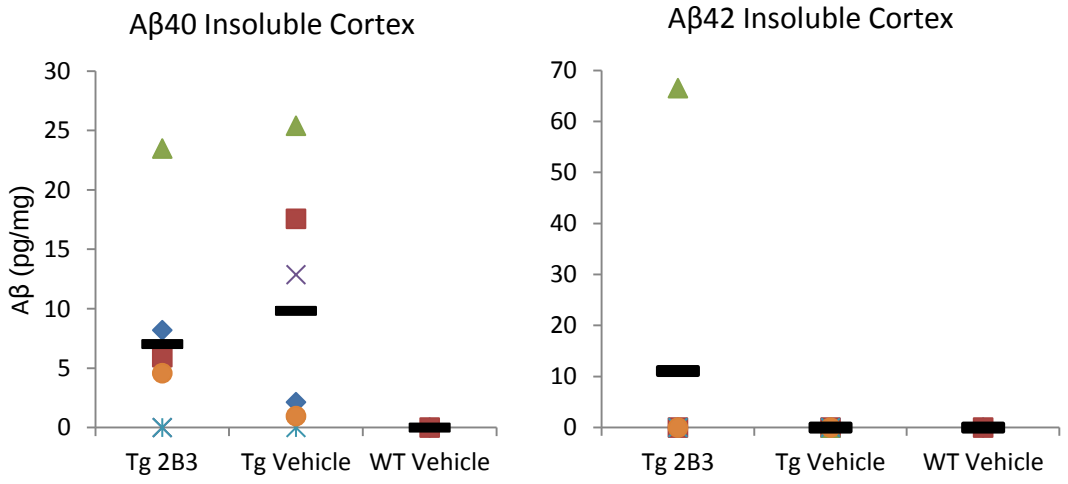


Fig. 6.8: Aβ40 and Aβ42 insoluble values (pg/mg of total protein) from the left cortex of 2B3 and vehicle-treated PDAPP mice displayed as individual values with the mean denoted as a black line. Tg = transgenic, WT = wild type.

Analysis of treatment effects: Hippocampus

Hippocampal measures of soluble Aβ40 and Aβ42 are presented in Fig. 6.9, with insoluble values presented in Fig. 6.10. As with cortical measures, statistical analysis provided no evidence for a lowering of Aβ levels in transgenic mice treated with 2B3 as compared to transgenic mice treated with vehicle [soluble Aβ40 (*U* (6, 6) = 11.00, *p* > .05; soluble Aβ42 (*U*

(6, 6) = 11.00, $p > .05$; insoluble Aβ40 (U (6, 6) = 15.00, $p > .05$; insoluble Aβ42 (U (6, 6) = 15.00, $p > .05$].

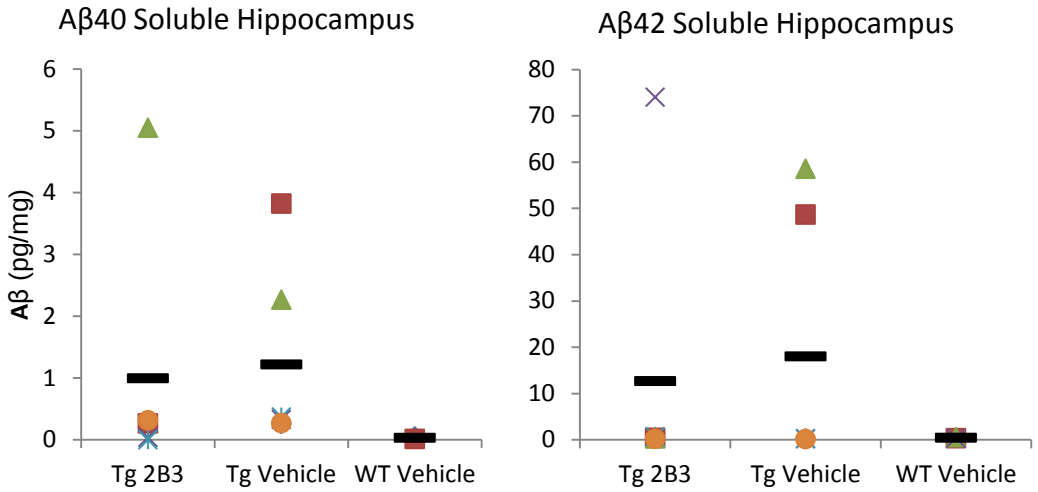


Fig. 6.9: Aβ40 and Aβ42 soluble values (pg/mg of total protein) from the left hippocampus of 2B3 and vehicle-treated PDAPP mice displayed as individual values with the mean denoted as a black line. Tg = transgenic, WT = wild type.

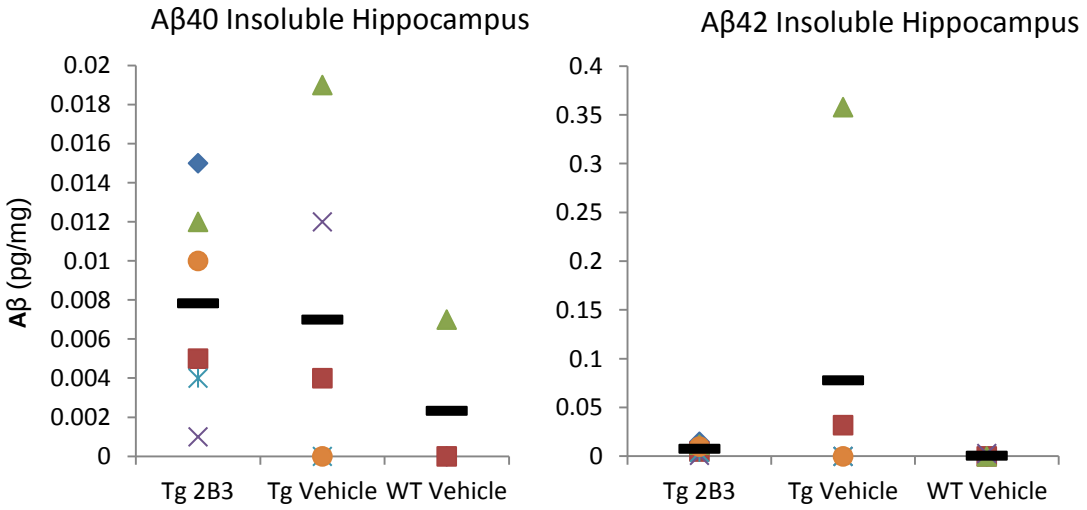


Fig. 6.10: Aβ40 and Aβ42 insoluble values (pg/mg of total protein) from the left hippocampus of 2B3 and vehicle-treated PDAPP mice displayed as individual values with the mean denoted as a black line. Tg = transgenic, WT = wild type.

The relationship between Aβ levels and behavioural measures

Non-parametric correlations were used to assess whether a relationship could be detected between measures of spatial working memory during treatment and Aβ pathology assessed by ELISA. As can be seen in Table 6.6, cortical measures of soluble Aβ40 were found to correlate positively with a subset of performance measures. In contrast, no significant relationship was observed between measures of spatial working memory in the foraging task and further measures of Aβ pathology.

Variable (during treatment)	Cortex Sol Aβ40	Cortex Sol Aβ42	Cortex Insol Aβ40	Cortex Insol Aβ42	HPC Sol Aβ40	HPC Sol Aβ42	HPC Insol Aβ40	HPC Insol Aβ42
Error	**0.443**	0.137	0.125	0.262	0.229	0.015	-0.132	0.023
Error first half of the trials	0.406	0.219	0.176	0.191	0.125	0.250	0.058	0.024
Repeat error	0.357	0.109	0.063	0.190	0.264	0.171	-0.229	-0.047
Repeat error first half of the trials	**0.520**	0.148	0.342	0.318	0.074	0.074	0.178	0.082
Consecutive error	**0.493**	0.001	0.202	0.282	0.230	-0.033	-0.184	-0.025
Consecutive error first half of the trials	0.270	0.045	0.368	-0.165	-0.180	0.090	0.240	-0.066

Table 6.6: Overview of the relationship between performance measures in the foraging task and ELISA measures of Aβ pathology as tested using Kendall's tau (τ). Significant correlations are highlighted in bold ($p < .05$). Sol = soluble, Insol = insoluble, HPC = hippocampus.

6.2.4 Discussion

The results from the administration pilot study indicate that intracerebroventricular infusion using osmotic minipumps offers a successful route of 2B3 administration. After 9 days of continuous infusion, 2B3 was detectable bilaterally across the subcortical structures, with no such staining evident in the PBS condition. Furthermore, whilst the administration method involved invasive surgery, animals tolerated the procedure exceptionally well and pilot work carried out at a different time point indicated no changes in behaviour or willingness to engage with behavioural tasks in animals with an osmotic minipump in place (data not shown).

The biochemical and behavioural results from Experiment 15 are in accordance with each other, with neither outcome measure identifying a significant effect of 2B3 administration. In terms of behavioural measures, no significant within-subject or between-subjects changes were observed, and measures of cortical and hippocampal Aβ levels did not reveal significant downregulation of Aβ in 2B3-treated mice compared to those receiving

vehicle. Aβ pathology appears to be positively related to a number of measures of spatial working memory performance, although this effect was restricted to cortical Aβ40. A larger sample size may have aided detection of connections between these measures.

There are a number of limitations with the present pilot study that may have influenced the pattern of results. Firstly, the sample size is a serious limitation, with low numbers in each treatment condition. However, it was considered a necessary and informative initial step in the evaluation of the antibody on a mouse line that displayed a robust behavioural phenotype. Nevertheless, it must be acknowledged that the low number of subjects limits the statistical power available to detect potential treatment effects. There are some interesting numerical trends in the behavioural data that warrant comment.

Consistently across the foraging task measures, and in particularly those from the first half of the test, the performance level of 2B3-treated transgenic mice is numerically very similar to wild type level. This was not the case for vehicle-treated transgenic mice, and 2B3-treated mice consistently made fewer errors than vehicle-treated mice. It is therefore possible that with an increased sample size, the behavioural pattern of improved performance in the foraging task in PDAPP mice treated with 2B3 would achieve reveal significant treatment effects. To investigate this, a post hoc power analysis carried out using StudySize2.0. Analysis indicated that on the basis of within-subject average error scores, the power of the present experiment was .31. Whilst this provides evidence that the current study is seriously underpowered, it is important to note that an increase in the sample size does not necessarily mean that the result would be significant.

A second factor that is worth considering is that of the age of the animals. The pre-treatment deficit in spatial working memory of transgenic mice may have been more pronounced had the cohort been older at testing. Whilst 14 month old wild type mice significantly outperformed transgenic mice in the foraging task, a larger numerical difference in performance scores would have offered a larger window of opportunity to detect significant treatment effects of varying magnitude. As a parallel to this point, Aβ pathology in 15 month old PDAPP transgenic mice was surprisingly low, with a high level of variability. This raises the possibility that a floor effect could mask treatment effects of 2B3. Verification of these results was sought both through re-genotyping and ELISA testing of samples at a range of concentrations, confirming accurate genotyping and assay sensitivity. As with the phenotypic profile of cognitive deficits, it is possible that further aging of the cohort would have resulted in higher levels of Aβ against which one could evaluate treatment effects.

In addition, questions have been raised regarding intracerebroventricular infusions and its status as a direct delivery method to the CNS. Pardridge (2005) points both to the slow diffusion of drugs to the brain parenchyma and the rapid absorption of substances in the CSF into the bloodstream to suggest that intracerebroventricular infusion should be classified as slow intravenous infusion as opposed to a direct delivery method to the CNS. Clearly, an antibody which does not reach its target cannot be expected to influence the processing of that target. However, pilot work showing a distribution of 2B3 across the infused hemisphere suggests success of delivery despite limitations of the speed of diffusion. Whilst immunohistochemical methods do not allow for assessment of the concentration of 2B3 in perfused brain tissue, the intensity of the staining indicates that a considerable volume of 2B3 was present in the hemisphere following 9 days of continuous infusion. It is therefore unlikely that delivery problems were an issue.

The duration of treatment in the current study was restricted as a result of the administration method. As such, the pilot study should be viewed as an acute treatment study. It is possible that significant treatment effects would have been detected had subjects received 2B3/vehicle administration for a longer period of time. Furthermore, it should be noted that wild type mice did not receive 2B3 infusion in the current study. As as pilot study, transgenic mice served as subjects in order to assess whether the administration of 2B3 would alter biochemical or behavioural measures in a system over-expressing APP and Aβ, as lowering Aβ levels in a healthy, balanced system such as wild type mice may be detrimental. In future work, the addition of 2B3-treated wild type controls would be desirable in order to assess whether any potential effect of 2B3 in transgenic mice is specific to elevation of Aβ levels as opposed to acting through a non-specific process comparable in wild type and transgenic mice.

6.3 Conclusion

This chapter investigated whether 2B3, an anti-APP β-secretase cleavage site antibody, could lower Aβ levels in primary murine neurons and whether such an effect could be replicated *in vivo*. Importantly, the effects of 2B3 on cognitive deficits in aged, transgenic PDAPP mice was also assessed, as potential AD therapeutics require alterations at the biochemical level to translate to cognitive and behavioural symptoms in order to be considered effective. Experiment 14 replicated the findings of Thomas et al. (2011) in demonstrating the ability of 2B3 to downregulate Aβ *in vitro,* and further established that such effects of 2B3 are not cell-

specific. Experiment 15 established the ability of osmotic minipumps to successfully deliver murine IgGs to the CNS, thereby bypassing potential restriction of access as determined by the BBB. In addition, the first attempt at assessing the effects of 2B3 *in vivo* was presented. Despite demonstrations of a downregulation of Aβ levels in primary murine neuronal culture, the lack of treatment effects of 2B3 *in vivo* does not support the hypothesis that an anti-APP β-secretase cleavage site antibody can lower Aβ pathology and associated cognitive deficits.

To date, no study has showed a lowering of both Aβ levels and cognitive deficits, with the only *in vivo* demonstration of the effect of an anti-APP β-secretase cleavage site antibody coming from Rakover et al. (2007). They succeeded in lowering impairments in novel object recognition following preventative, systemic administration of treatment with an accompanying reduction in inflammatory markers. Despite this, no Aβ-related alterations were observed. This indicates that alterations in Aβ pathology detectable using traditional ELISA and immunohistochemistry are not a necessary prerequisite to alterations in cognitive function. It follows therefore that anti-APP β-secretase cleavage site antibodies could provide benefits in terms of cognitive function following only small alterations in Aβ levels, as the synaptic deficit theory of AD would predict. This interpretation is in line with results from studies by Janus et al. (2000) and Morgan et al. (2000) who detected a reduction in spatial reference memory deficits in transgenic mice following active immunisation with Aβ42, but observed only limited alterations in Aβ levels. Similarly, passive immunisation studies using transgenic mouse models have encountered a parallel trend with a number of studies establishing ameliorating effects on cognitive deficits with smaller or non-detectable effects on Aβ pathology, particularly if the treatment duration was reduced to 1-2 months (Dodart et al., 2002; Wilcock et al., 2004).

There are a number of limitations of the *in vivo* experiment described in the current chapter. These include a limited sample size, a lack of range in terms of administration methods, duration of treatment, and the age of treated mice. In addition, alternative biochemical and behavioural outcome measures assessing the wider effects of 2B3 administration would be informative. Despite these limitations, a non-significant numerical trend was observed in the behavioural data with 2B3-treated mice showing indications of potential changes in spatial working memory following exposure to the antibody, despite the limited treatment period. Thus, the pilot study investigating the effects of 2B3 in transgenic PDAPP mice offers an interesting starting point for the further assessment of 2B3 *in vivo.* The potential future directions of this work will be discussed further in Chapter 7.

General Discussion

7.1 Overview

The main goal of this thesis was to provide a characterisation of two transgenic APP murine models of Aβ pathology with the aim of assessing the effect of 2B3, an anti-APP β-secretase cleavage site antibody, on Aβ pathology and associated cognitive deficits in these models. A brief overview of the results is provided followed by a more detailed description and discussion of the research findings.

7.2 Summary of findings

Currently available treatments for AD are symptomatic only, and do not succeed in altering disease progression in terms of age-related neurodegeneration or decline in cognitive function. Pre-clinical studies using transgenic mouse models of AD-like pathology have successfully lowered Aβ levels by targeting various steps in the Aβ cascade of pathology, but several of these approaches have either not translated successfully to clinical trials or could have a number of serious side effects due to a lack of specificity in the selected targets. Due to the large-scale health problem AD poses to society, there is great need for the development of novel disease-modifying treatments.

An anti-APP β-secretase cleavage site antibody (2B3) which is able to reduce Aβ levels *in vitro* could provide a disease-modifying treatment with fewer of the side effects observed in pre-clinical studies and human trials. Recent work has shown downregulation of Aβ levels in human non-neuronal cell lines following 2B3 administration (Thomas et al., 2011). It was therefore hypothesised that 2B3 administration *in vivo* would limit β-secretase processing of APP, lower Aβ levels in the CNS and reduce cognitive impairments. Prior to evaluating this hypothesis, it was necessary to identify and characterise a suitable APP transgenic mouse model. The London APP(V717I) and the PDAPP (V717F) models of AD-like pathology were characterised with the aim of identifying cognitive decline that could be related to escalating Aβ pathology with age. The work presented in this thesis is therefore based on two main hypotheses. Firstly, that aging of transgenic APP mutation mice would lead to impairments in measures of cognitive function associated with elevations of Aβ levels and secondly, that administration of 2B3 to aged transgenic mice would lower both Aβ levels and cognitive

impairments. The results of these investigations lend only partial support to the first of these predictions.

The analysis of anxiety-like behaviour in APP(V717I) mice as described in Chapter 2 aimed at establishing a developmental profile to assess whether potential genotypic differences were age-related. Due to the lack of available information regarding the influence of gender on phenotypic expression of genotypic differences, both male and female APP(V717I) mice were included throughout both behavioural and biochemical analyses. The results contradicted earlier reports of increased anxiety levels in transgenic mice, with wild type mice consistently displaying more anxiety-like behaviours than transgenic mice across age in two separate tasks. The contribution of gender was task-specific and largely independent of genotype, with female mice displaying lower anxiety levels than males in the marble burying task only. It was suggested that the differences in the pattern of results compared to available literature was most likely due to changes in the genetic background strain. Importantly, the emergence of anxiety deficits in transgenic mice at 3 months of age, prior to the development of Aβ pathology, suggested the influence of APP overexpression on marble burying and elevated plus maze performance. It was therefore necessary to widen the behavioural characterisation in order to identify an age-related cognitive phenotype in the APP(V717I) model against which 2B3 could be assessed.

Chapter 3 describes a set of experiments designed to detect potential transgenic deficits in spatial working memory, spatial references memory and object recognition memory. Moechars et al. (1999b) identified a spatial reference memory deficit at 3-6 months, but the developmental effects of the APP(V717I) mutation on spatial cognition were unknown. Across various age points and three tasks, there is little evidence to suggest that the APP(V717I) leads to impairments in spatial memory. On the T-maze, 11 month old transgenic mice outperformed wild type littermate controls, and inferior transgenic performance on selected variables in the foraging task at 6 months can be considered at best transient and task-specific. Furthermore, no genotypic differences in object recognition memory were detected. It was concluded that aging to 18 months of age did not result in impairments in spatial and object recognition memory in APP(V717I) transgenic mice on a C57Bl/6 background. This pattern of results may indicate that areas of the temporal and frontal lobe are not functionally impaired in this model. This interpretation is based on a combination of control experiments reported in Chapter 3, in which hippocampal lesioned mice were found to be impaired on the RAWM and the T-maze non-matching to position task, as well as

interpretation of published literature. The hippocampus is likely to be implicated in the foraging task given the spatial nature of the task in combination with evidence that performance is disrupted by lesions to the hippocampus in pigeons (Pearce et al., 2005). The T-maze non-matching to position paradigm has been shown to be sensitive to the prefrontal cortex in rats, indicating a similar sensitivity might be present in mice (Dias & Aggleton, 2008). In contrast, the contribution of the prefrontal cortex to performance on the foraging task has not been evaluated. A validation study of the foraging task using hippocampal and prefrontal manipulations would be desireable, and add strength to the conclusion that APP(V717I) mice do not display functional impairments in the hippocampus or prefrontal cortex. The lack of deficit in the object recognition task further underlines the intact nature of the medial temporal lobe in terms of the perirhinal cortex in this model. If one were to draw parallels to cognitive assessments of patients, the types of cognitive functions assessed across these tasks could be said to broadly relate to recognition and recall of objects and space, over long or short periods of time. It is possible that the differences in anxiety as reported in Chapter 2 could influence the performance in the tasks described. However, the use of both appetitive (T-maze, foraging task) and aversive (RAWM) task motivation should limit the degree to which such differences influence the overall performance profile. Furthermore, habituation for appetitive tasks was conducted until wild type mice, the more anxious of the genotypes, were reliably interacting with the testing apparatus without indication of fearfulness. Furthermore, it is unlikely that the longitudinal nature of testing in the foraging task would attenuate a cognitive deficit given that the task is one in which performance rather than acquisition is the outcome measure. Furthermore, the exact pattern of actions for successful performance is different on each trial, such that the effect of repeated testing is minimised. In light of these points, it is argued that the lack of cognitive deficits in APP(V717I) mice described in Chapter 3 are not artefacts of the choice of tests or the nature of testing.

The developmental profile of Aβ pathology in APP(V717I) was investigated in Chapter 4 in an attempt to inform the lack of age-related cognitive changes observed in the behavioural assays described in Chapter 2 and 3. Interestingly, there was evidence of elevated levels of Aβ in female transgenic mice compared to age-matched transgenic males. This is a common observation in the transgenic APP mutation model literature (Sturchler-Pierrat and Staufenbiel, 2000; Bayer et al., 2003; Callahan et al., 2001; Lewis et al., 2001; Wang et al., 2003; Schuessel et al., 2004; Schafer et al., 2007) which mirrors a trend in the current work towards female transgenic mice displaying a stronger behavioural phenotype than males. In terms of the developmental profile of Aβ pathology, the APP(V717I) model does not lead to

elevation of Aβ levels in transgenic males until 18 months, with females showing a comparable level at 12 months. This is relatively late compared to more established models such as the Tg2576 (Kawarabayashi et al., 2001; Westerman et al., 2002), and could explain the absence of detectable cognitive deficits prior to 12 months of age. However, several assessments of cognitive function were carried out between 14-19 months of age without transgenic deficits emerging. Overall, the results from Chapter 4 indicate that the level of Aβ pathology developed by 18-19 months of age in APP(V717I) transgenic mice on a C57Bl/6 background is not sufficiently extensive to disrupt spatial and/or object recognition memory. Interpreted in the context of the amyloid cascade hypothesis, the APP(V717I) model does not support the notion that elevation in Aβ levels is sufficient to initiate the pathological cascade resulting in cognitive decline when modelled in the mouse, possibly due to an interaction between protective features of the genetic background and the level of APP overexpression. The lack of an age-related behavioural phenotype in the APP(V717I) model highlighted the necessity for introducing an alternative APP transgenic model to the project.

Chapter 5 focused on the behavioural characterisation of the PDAPP APP(V717F) model of AD-like pathology. Whilst transgenic mice did not differ from wild type controls at 12 months of age on a measure of object recognition memory, genotypic differences were detected in both spatial tasks. A decrease in performance from 11 to 14 months of age indicated that the foraging task was sensitive to age-dependent deficits in cognitive function in transgenic PDAPP mice. Table 7.1 summarises the range of behavioural assessments carried out on both the APP(V717I) and the PDAPP model, and the presence or absence of transgenic deficits on each task.

Psychological/cognitive construct	Test	APP(V717I)		PDAPP(V717F)	
		Deficit	Age	Deficit	Age
Anxiety-like behaviour	EPM	√	3/6/19m	√	9.5m
	Marble burying	√	3/6/19m	√	9.5m
Spatial working memory	Foraging task	× /√/×	3/6/19m	√	11/14m
	T-maze FCA	×	11m	√	10m
Spatial reference memory	Radial Arm Water Maze	×	10m	n/a	n/a
Object recognition memory	Object recognition	×	18m	?	12m

Table 7.1: Summary of the impact of APP overexpression and/or Aβ pathology on cognition and behaviour in transgenic APP mutation mice as detected using a range of tasks in the current thesis. √ = pathology or APP overexpression causes deficit in the given task. × = no difference between transgenic and wild type mice on performance in the given task. ? = limited conclusions can be drawn due to poor wild type performance. n/a = not applicable as the task was not administered.

With a behavioural paradigm which is sensitive to PDAPP transgenic deficits, it was possible to assess the effect of 2B3 on cognitive decline and Aβ levels in aged transgenic mice, as described in Chapter 6. The ability of 2B3 to modulate Aβ pathology using murine neurons was established prior to intracerebroventricular infusions. Thus, proof of principle was obtained that 2B3 had the desired effect in cells comparable to those in the CNS of PDAPP mice. Despite this, acute treatment of 2B3 in aged transgenic mice at a point where cognitive decline was established did not result in significant changes in behaviour or Aβ levels. As this does not lend support to the prediction that 2B3 will alter Aβ pathology and associated cognitive deficits in APP mice, one is required to accept the null hypothesis that 2B3 was ineffective *in vivo.*

The lack of an effect of 2B3 *in vivo* could be based on experimental issues that resulted in sub-optimum conditions for the detection of significant effects. These include the concentration of 2B3, the duration of treatment, the administration method, the assay sensitivity to changes in Aβ concentration and the task sensitivity to changes in cognitive functioning. Perhaps most importantly, the limited sample size would have considerably reduced the statistical power. The second potential explanation for the lack of significant change following 2B3 treatment surrounds the efficacy of the antibody itself. The efficacy of 2B3 could be reduced if it was inhibited from binding APP at the cell surface, or was not internalised with APP to allow steric hindrance of β-secretase at the multiple intracellular sites, such as the endosomal/lysosomal system, where this cleavage is thought to occur (Thomas et al., 2006; Haas et al., 1992; Koo & Squazzo, 1992; Hartmann et al., 1997). However, the success reported by Rakover et al. (2007) in demonstrating biochemical and behavioural changes following administration of an anti-APP β-secretase antibody argues against such an interpretation.

It is important to highlight that the work with evaluating 2B3 *in vivo* is in its initial stages, and that the distance from pre-clinical studies to treatment of AD patients is clearly great. Beyond optimising conditions for the detection of significant effects of 2B3 *in vivo*, a number of challenges require attention. These include the degree of immune system activation and inflammation following 2B3 treatment and other potential side effects, the challenge of humanising the antibody, the administration method in human patients, the point in disease progression at which the treatment should be delivered to have optimum effect and the dosing regime in order to achieve safe efficacy. There are strong trends in the AD field that indicate that early intervention is considered key to clinical trial success, an approach which is supported by the continuing increase in focus on early biomarkers of AD to aid early diagnosis

or preventative treatment. The methods of administration in patients will clearly require another approach than surgical implantation as is possible in preclinical models. Whilst the degree to which antibodies can naturally pass the blood-brain-barrier is under discussion, the technical advances in bioengineering are demonstrating that bispecific antibodies (Atwal et al. (2011) and peptides capable of carrying large, hydrophobic cargo across the blood-brain-barrier (Skrlj et al., 2012). Such advances should be utilised by the AD research community to optimise peripheral administration of antibody therapies. Overall, there are substantial issues which will require attention should the antibody prove promising in pre-clinical work. Nevertheless, as a first step in a pre-clinical pilot, Experiment 15 offers some encouraging numerical trends.

7.3 Theoretical Implications

The utility of transgenic mutation models of AD-like pathology

The two APP mutation models analysed in this thesis are based on familial autosomal mutations in the same gene. Nevertheless, the profile of behavioural changes induced by the mutations is very different in the two lines. Whilst the PDAPP(V717F) model develops deficits in spatial working memory which were age-dependent, no such robust or age-related deficits were detected in the APP(V717I) model. The variety in the phenotypic expression of familial autosomal APP mutations in transgenic mouse models suggests that transgenic mouse models of AD-like pathology suffer from a level of variability that threatens their validity in the pre-clinical field. To support this argument, the genetic background strain on which the mutations are maintained have repeatedly been shown to strongly influence the phenotypic profile both in terms of behavioural deficits and pathological hallmarks of disease (Crawley et al., 1997; Võikar et al., 2001; Savonenko et al., 2003). Indeed, the numerous transgenic models vary in promoters, genetic background and the number and location of mutations. Nevertheless, they represent the most clinically relevant pre-clinical disease models available to the field and have been invaluable in examining AD-related disease progression, the temporal relationship between Aβ/tau aggregation and cognitive decline, the utility of intervention at varying stages of disease progression and the relationship between Aβ levels in the CNS and the plasma (Chapman et al., 1999; Oddo et al., 2003; Tanzi, 2005; Walsh et al., 2002; Mucke et al., 200; Klein, Krafft & Finch, 2000). In addition, the ability to isolate disease-related factors such as Aβ and tau pathology in separate models allows for the assessment of their individual contributions in the absence of any potential bilateral escalation. It has therefore been argued

that transgenic APP mutation mouse models of AD-like pathology are highly valuable for the pre-clinical study of disease mechanisms and progression, as well as the evaluation of novel therapeutic interventions (Wilcock et al., 2004; 2006; Schenk et al., 1999; Janus et al., 2000; Rakover et al., 2007; Eriksen & Janus, 2007). Essential to the successful use of transgenic models, however, is the careful analysis and selection of a model which best replicates the aspects of disease under consideration.

Whilst mouse models of AD-pathology have clear value, it is worth highlighting the issues in translating preclinical research to the patient population. Clearly, there are great species differences between mouse and man which place basic limitations on the degree to which preclinical conclusions can be applied to patients. Further discrepancies between transgenic mouse models of AD pathology and human patients emerge due to the way in which the transgenic mouse models are generated. Overexpression of APP is not a known feature of AD (with the exception of trisomy 21) and the subsequent Aβ pathology in mice could differ from human AD in important, aetiological ways. Indeed, overexpression of wild type APP has been shown to be sufficient to cause comparable cognitive deficits to those of mutant APP overexpression (Moechars et al., 1999b). This indicates that there are phenotypic elements to the transgenic mouse models which are a biproduct of model generation rather than a translationally-relevant disease feature. Furthermore, the lack of a mouse model which encompasses the full spectrum of AD pathology, including extensive neuronal loss with age, may be an important limitation if the interaction between separate pathological features is important for understanding drug effects in humans.

The nature of the behavioural outcome measures in preclinical work are also likely to differ from those used in clinical trials. The choice of task used to measure animal cognition in preclinical work are based on an attempt to measure dysfunction of specific area of the brain and/or a particular psychological construct which is relevant to the disease studied. However, these tasks have emerged from the animal literature in general rather than as specific preclinical tools in drug discovery and disease-related work, and as such do not necessarily have implicit translational value. Indeed, a number of the tools used as primary endpoints [e.g. Alzheimer's Disease Assessment Scale-cognition (Rosen, Mohs & Davis, 1984); Clinician Interview Based Impression of Severity-plus (Schneider et al., 1997); Blessed Dementia Scale (Erkinjuntti, Hokkanen, Sulkava & Palo, 1988) in clinical trials are heavily focused on the proficient use of language, with recall and recognition tasks often being word based or involving the naming of household objects. Clearly, the assessment of cognitive function using

language is not a translationally relevant concept. Other measures are based on the ability to function in daily living (such as turning on and off lights, self-feeding and maintaining hygiene standards). The measurement of deficits in spatial cognition is limited to the drawing of geometric shapes and limited questions regarding the ability of the patient to navigate familiar locations such as the neighbourhood and their homes. This spatial navigation can be more clearly linked to tasks such as water maze paradigms assessing spatial reference memory, but serve only a small part of patient assessment. Another aspect of patient testing which it may be possible to translate to preclinical work is the measurement of long term, autobiographical recall related to episodic memory (see Section 7.4 for a further discussion on the assessment of episodic memory in rodents). Overall, one could argue that the discrepancy between the results of clinical trials and preclinical data may be related to a discrepancy between the types of cognitive and behavioural testing carried out at the two stages of drug assessment.

Age-independent deficits in APP Models

As referred to in previous chapters, is important to establish the developmental profile of cognitive deficits in APP mutation mouse models of Aβ pathology prior to their use in pre-clinical studies. APP mutation models are based on the hypothesis that the overexpression of APP leads to elevated levels of Aβ with age, which is causative in the emergence of behavioural deficits. Due to the confounding variable of APP overexpression, it is problematic to link cognitive impairments with elevation of Aβ without analysing the temporal nature of the behavioural deficits. As observed in Chapters 2 and 3, an anxiety deficit in APP(V717I) mice was detected at 3 months, an age where ELISA and immunohistochemical methods do not detect Aβ pathology. The lack of escalation of that deficit with age, as also observed in the foraging task, makes a stronger case for the causative role of APP overexpression as opposed to increases in Aβ concentration. This highlights the need to assess the change in cognitive function with age in APP models. In addition, the use of control wild type mice overexpressing endogenous APP offers a method for dissociation of APP- and Aβ-driven behavioural changes. Indeed, Moechars et al. (1999b) demonstrated that both APP(V717I) transgenic and wild type APP overexpressing mice showed a similar profile of anxiety deficits and spatial reference memory impairments that was not present in non-overexpressing wild type mice. It is argued that a more widespread use of control mice overexpressing endogenous APP would offer an improvement of the current project, in addition to being beneficial to the field in general.

Immunotherapy as a treatment option for AD

If one accepts the proposal that a replication of Experiment 15, in which the conditions of 2B3 efficacy are optimised, would reveal improved memory in PDAPP mice, then along with the study conducted by Rakover et al (2007), this could be an important innovation in the field of immunotherapy for AD. The initial enthusiasm in the field regarding immunotherapy for AD was tempered by the failed clinical trials (Orgogozo et al., 2003; Nicholl et al., 2003; Pfeifer et al., 2002). The main issues were meningoencephalitis and a lack of cognitive benefit despite successful Aβ plaque clearance (Holmes et al., 2008). As 2B3 binds APP as opposed to Aβ, its substrate is arguably less permanent than deposited Aβ, as APP can undergo cleavage by α-secretase in the absence of β-secretase cleavage. The lack of binding to a substrate deposited in immunoreactive, extracellular plaques may hypothetically result in a reduced inflammatory response compared to traditional immunotherapy. The reduction in inflammatory markers observed by Rakover et al. (2007) following treatment of Tg2576 mice with an anti-APP β-secretase cleavage site antibody supports this argument.

The antibody approach used in this thesis offers an earlier intervention than other immunotherapy strategies through the inhibition of Aβ production, as opposed to post-production clearance of excess Aβ. 2B3 may therefore offer further cognitive protection through the inhibition of Aβ toxicity, which is thought to peak in the soluble Aβ form, prior to plaque deposition (Walsh et al., 2002; Mucke et al., 200; Klein, Krafft & Finch, 2000). It is possible that the issues observed in clinical trials using anti-Aβ antibodies will be reduced or absent using an anti-APP β-secretase antibody. Undoubtedly other issues will present themselves, such as the detection of AD at an early (presymptomatic) stage. Overall, immunotherapy as an approach for AD treatment remains in its initial stages, and requires extensive investment of research to further optimise efficacy and reduce side effects. As such, 2B3 and other anti-APP β-secretase antibodies remain potentially promising candidates.

7.4 Future directions

2B3 (or an alternative Aβ production downregulator) in vivo

The work carried out to test the central hypothesis of this thesis is in its infancy. There is clearly a need for optimisation and replication of Experiment 15 in order to assess whether the numerical trends observed in the behavioural measures can be replicated. To further this work, a range of treatment time points should be employed, in which preventative action in young transgenic mice is contrasted against the more clinically relevant late-stage treatment intervention. This relates to the issue of reversibility of Aβ-induced synaptic damage as

highlighted by Tanzi (2005). The question of whether cognitive function can be restored in neurons which have been exposed to Aβ-induced impairments, and at what time point intervention is necessary to inhibit the initiation by Aβ of apoptotic signalling cascades has not yet been answered.

The current work did not assess the effects of downregulating Aβ production on biochemical measures beyond Aβ levels. A range of biochemical markers of CNS functioning are available which could be utilised in future study. This may assess the effect of inhibition of Aβ production on inflammatory markers such as TNF-α, IL-1β, and S-100B (Craft, Watterson & van Eldik, 2005) as well as markers of synaptic density such as synaptophysin, synapsin, microtubule-associated protein-2 (Dawson et al., 1999), PSD-95 (El-Husseini et al., 2000) and neuronal calcium binding proteins such as calbindin D-28k and parvalbumin (Celio, 1990). This may provide a broader understanding of the direct effects of downregulation of Aβ production following varying durations of Aβ suppression. Furthermore, measures of products of proteolytic cleavage of APP by α-secretase (C83, sAPPα. P3, ICAD) in mice treated with 2B3 compared to controls would support the interpretation that a potential lowering of Aβ is the result of inhibition of BACE1 and subsequent enhancement of non-amyloidogenic cleavage.

As discussed in Chapter 1, Section 1.5.2, there is growing evidence to suggest that Aβ and NFT pathology are closely linked. King et al. (2006) suggests a mechanism by which an increase in soluble Aβ causes tau-dependent disassembly of microtubule proteins, placing tau downstream of Aβ in the disease cascade. Assuming the conditions under which 2B3 delivery could be optimised *in vivo* to replicate the knockdown of Aβ levels as observed in the primary neuronal cultures, it would be interesting to assess the effect of 2B3 in a model system which encompasses both Aβ and NFT pathology, such as the 3xTg model (Oddo et al., 2003). This would test whether modifications of Aβ levels could reduce both types of pathology despite only directly modifying APP processing. In addition, preventative action vs. treatment comparisons should be made evaluating the optimum intervention point for minimising or reversing the pathological interaction between Aβ and NFT.

Furthermore, it would be beneficial to expand the range of cognitive assessments of treated APP mutation transgenic mice and controls to assess whether a potential reduction in cognitive deficits can be generalised across various memory and learning paradigms. Alternatively, demonstrations of beneficial effects being restricted to select functions could indicate differences in the degree of reversibility of Aβ induced neuronal or synaptic damage

across structures and neuronal populations. The benefits of expanded cognitive assessment of transgenic mouse models are discussed further below.

Expanded cognitive assessment

The utility of transgenic APP mutation models could be increased through the expansion of the range of cognitive tests traditionally used and the increased use of translational cognitive tests that map more closely onto patient-based tests. In addition, the cognitive developmental profile should be established through both longitudinal and cross-sectional assessment. These points apply both to the field generally and the work presented in the current thesis.

The assessment of cognitive function in transgenic APP mutation models frequently utilises the Morris water maze, the radial arm maze and the object recognition task for the assessment of spatial reference-, spatial working- and object recognition memory respectively. Bussey et al. (2012) make a convincing case for the use of a cognitive battery of tasks assessing a variety of cognitive function, with a selection of tasks measuring similar processes. The administration of these tasks through the same touch-screen test chambers reduces the influence of the experimenter and apparatus variables on performance, and allows more direct comparison between tasks. When using this methodology, the order of administration of tasks must be carefully planned in order to avoid generalisation and transfer effects between tasks. A combination of longitudinal and cross-sectional design would be desirable.

In addition, attempts should be made to target aspects of cognitive decline that most accurately reflect the cognitive impairment observed in human AD patients. As an example, episodic memory deficits are an early diagnostic sign of AD in humans, and are assessed in tests such as CANTAB (Fray & Robbins, 1996). Episodic memory, the ability to recall an event in terms of its content and its temporal-spatial characteristics (Tulving, 1972), is thought to involve an element of conscious recall which calls into questions its application to animal cognition. Nevertheless, an analogue of episodic-like memory can be measured in rodents. These tasks require the recollection of “what” happened “where” and “when”, with the condition that all three aspects of an encountered event must be recalled (representing an integrated memory). Examples include an adaptation of the object recognition paradigm (Good, Hale & Staal, 2007) and a configural fear conditioning paradigm (Iordanova, Good & Honey, 2008) where temporal and spatial components are included. Thus, a greater focus on

the development of behavioural paradigms in mice with greater construct validity could offer a better platform on which to evaluate therapies.

7.5 Summary

Whilst work to develop disease-modifying treatments for AD has not been without obstacles, immunotherapy remains a promising possibility for the treatment of AD. The success and promise of immunotherapy up to this point is largely based on murine pre-clinical models, highlighting the essential role they have played in the field.

The work presented in Chapters 2 – 5 extends the current understanding of the behavioural phenotypes of the APP(V717I) and PDAPP(V717F) models, allowing a more comprehensive picture to emerge in terms of the developmental profile of cognitive and anxiety-like behavioural impairments. The evaluation of 2B3 as a treatment for Aβ-related pathology progressed with the novel demonstration of successful downregulation of Aβ production in primary murine neuronal cultures. The impact of 2B3 on the behavioural deficits in PDAPP mice revealed at best only numerical improvements. Clearly, a more extensive study is required to assess the impact of 2B3 on cognition in APP mice. Overall, however, the results are an important first step in the evaluation of novel immunotherapeutic approaches.

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