The Effect of Dietary Omega-3 Polyunsaturated Fatty Acids and Curcumin on Cognition and Pathology in a Mouse Model of Amyloid Pathology

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A thesis submitted for the degree of Doctor of Philosophy (PhD)

2011

Acknowledgments

It is my pleasure to take this opportunity to thank my colleagues, family and friends for their support over the last few years throughout my PhD. Firstly, my utmost appreciation goes to my primary supervisor Professor Mark Good for the years you have given to support, inspire and encourage me. Your enthusiastic approach and passion for research has taught me some valuable lessons for which I will use to help guide me for years to come. My sincere thanks also extend to my secondary supervisor Professor John Harwood and industrial supervisor Doctor Sue Plummer for your expertise and encouragement. It has been a pleasure working with all of you.

Thank you to the Biotechnology and Biological Sciences Research Council (BBSRC) and Cardiff University for my PhD sponsorship, and for the support provided by my industrial sponsors Cultech Ltd.

I would also like to thank numerous colleagues who have assisted me over the years. Thank you to the animal technicians – Clive, Pat and Kerry in particular, and the support given by the BNL team, particularly the man everybody knows and loves – Jeff Lewis. I really appreciate the time you always found to help me, and cater for my mouse-related needs (normally the feeding regime!) whenever I needed it. It was a joy to work with such lovely people in the dark days of the basement! Also thank you to the I.T. team and Dennis who would always get me out of a camera, laptop or maze-related pickle.

Thank you to Vicky Staal and Mariah Lelos for your early help in getting me started and teaching me the skills of the trade - behavioural tasks, genotyping and immunohistochemistry. I would also like to sincerely thank my good friend and colleague Cécile Bascoul-Colombo enormously for your support in teaching and assisting me with carrying out tissue collection, immunohistochemistry, ELISAs and lipid analysis. You were a truly valuable source of expertise throughout. I would also like to extend my thanks to 'Team Good' for our continual sharing of techniques and advice, particularly Amy Reichelt for your help with the Stroop task and Martha Hvoslef-Eide for all my unresolved work-related questions! It has been a joy to work with such great people.

With great gratitude, I would also like to thank all my friends for your immense support and patience, particularly while saying far too often 'Sorry, I'm hectically busy right now!' ...I can now return to the land of the living! In particular, I would like to thank my very good friends Emma Lydall, Joanne Morgan, Tracey Herlihey and Martha Hvoslef-Eide for being there on a daily basis if needed to keep me sane, with a plentiful supply of natter, tea and cake on those off-days which I would always welcome! My time in Cardiff would never have been the same without you all. My sincere thanks also go to Matt for your patience and support over the years in listening to my dramas or triumphs daily down the phone, and for the months of generally looking after me during my thesis-writing coma! Finally, thank you to my wonderful family Mum, Dad, Ad and Jen ...for your loving support and understanding. Especially thanks to Mum and Dad for allowing me to move back home when my funding ran out - although your kids get older, unfortunately we are never too old to move back in! My thesis is dedicated to you both.

Contents

Thesis summary

Previous studies have shown that dietary supplementation of curcumin or omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid can reduce behavioural deficits and β-amyloid (Aβ) pathology in several models of Alzheimer's disease (AD), including Tg2576 mice. However, no study to date had examined the effect of omega-3 PUFA and curcumin co-supplementation on behaviour or β-amyloid pathology in mouse models of AD. Further to this, no study to date had examined the effect of longitudinal omega-3 PUFA or curcumin supplementation provided from an early age before significant pathological development in the Tg2576 model. This was deemed important since human studies have suggested that lifelong dietary choices before disease development are an important factor in disease risk. Finally, although a plethora of studies has examined the effect of omega-3 PUFA supplementation, none has accurately examined its effect against an appropriate control diet. For example, some control diets contained differential levels of fatty acids such as excess total fat and omega-6 PUFAs. Such differences in the control diet may have contributed to the observed beneficial effects of dietary omega-3 PUFA supplementation, particularly since these dietary factors can exacerbate pathological processes related to AD.

Using this experimental design, chapter 3 found longitudinal dietary supplementation of omega-3 PUFA docosahexaenoic acid (DHA) from an early age in Tg2576 mice provided some protection from cognitive decline that was limited to later but not early stages of pathology. In contrast to previous reports however, Aβ pathology was unaltered by DHA supplementation. Providing DHA supplementation from an even earlier age, chapter 4 showed that DHA reduced behavioural deficits at an early age (prior to Aβ pathology), although interestingly, these effects were less robust at the later age. Chapter 5 examined longitudinal supplementation of curcumin, fish oil containing omega-3 PUFAs (DHA and eicosapentaenoic acid, EPA) and their co-supplementation from an early age in Tg2576 mice. The results consistently revealed no beneficial effects of dietary supplementation on behaviour or Aβ pathological measures. The findings from this thesis indicate that dietary supplementation of DHA relative to a suitable control diet can provide only limited protection against behavioural deficits in Tg2576 mice. In contrast, fish oil supplementation containing omega-3 PUFAs DHA and EPA provided no protection, indicating that DHA monotherapy may be a more advisable treatment. The null effects of curcumin supplementation at earlier stages relative to previous studies suggest that curcumin may only be effective during advanced stages of pathology, although further investigation is needed. Finally, the null effects of curcumin and fish oil/omega-3 PUFA co-supplementation suggest that this is not an optimal intervention strategy in reducing Aβ-induced changes.

Abbreviations

ABC	Avidin-biotinylated enzyme complex
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase domain-containing protein
AICD	APP Intracellular cytoplasmic/C-terminal domain
Akt	Protein Kinase B
ALA	Alpha (α)-linolenic acid
Alph1	Alphaprotein 1
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APPswe	APP Swedish mutation
ARA	Arachidonic acid
ARE	Antioxidant response element
ATPase	Enzymes that catalyze the decomposition of adenosine triphosphate
Aβ	Beta (β)-amyloid
BACE1	β-site APP cleaving enzyme 1 (β-secretase 1)
BAD	Bl-2-associated death promoter
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BIN1	Bridging integrator 1 gene
bp	Base pair
BSA	Bovine serum albumin
C83/α-CTF	α-carboxyl terminal fragment of APP
C99/β-CTF	β-carboxyl terminal fragment of APP
CA1	Cornu Ammonis-1 area of the hippocampus
CaMKII	Ca^{+2}/Calmodulin-dependant protein kinase
CDK	Cell-cycle dependant kinase
CIBIC	Clinical Interview Based Impression of Severity
CLU	Clusterin gene
COX	Cyclooxygenase
CR1	Complement receptor 1 gene
Cu+	Copper ions
DAB	3,3'-Diaminobenzidine
DG	Dentate gyrus
dH_2O	Distilled water
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DPA	Docosapentaenoic acid
DR	Discrimination ratio
DTA	Docosatetraenoic acid
EDTA	Ethylenediaminetetraacetic acid
EGb 761	Ginkgo biloba leaf extract
EGCG	Epigallocatechin gallate (green tea extract)
ELISA	Enzyme-linked immunosorbent assay
EOAD	Early-onset Alzheimer's disease

EPA	Eicosapentaenoic acid
EPM	Elevated plus maze
ERK	Extracellular signal-related kinase
ETA	Eicosatetraenoic acid
FAD	Familial Alzheimer's disease
FAME	Fatty acid methyl esters
FO	Fish oil diet
FO+C	Fish oil + curcumin diet
GFAP	Glial fibrillary acidic protein
GLC	Gas liquid chromatography
GSKα	Glycogen synthase kinase α
HIF-1	Hypoxia-inducible factor
HRP	Horseradish peroxidise
IDE	Insulin-degrading enzyme
IgG	Immunoglobulin G
IkBα	I-kappa-Bα (NfκB Inhibitor α)
IL (e.g. IL-1β)	Interleukin (e.g. Interleukin-1β)
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate 1
ITI	Inter-trial interval
K+	Potassium ion
KCl	Potassium Chloride
KH_2PO_4	Monopotassium phosphate
LA	Linoleic acid
LDL	Low-density lipoprotein
LOAD	Late-onset Alzheimer's disease
LOX	Lipoxygenase
LTD	Long-term depression
LTP	Long-term potentiation
LR11/SORLA1	Lipoprotein receptor 11
MANOVA	Multiple analysis of variance
MAP	Mitogen-activated protein
MCI	Mild cognitive impairment
$MgCl_2$	Magnesium chloride
MUFA	Monounsaturated fatty acids
Na+	Sodium ion
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NFTs	Neurofibrillary tangles
NfκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal Goat Serum
NMDA	*N*-Methyl-D-aspartic acid
NO	Nitric oxide
NPD1	Neuroprotectin D1
NSAIDs	Non-steroidal anti-inflammatory drugs
OB	Oil blend diet
OB+C	Oil blend + curcumin diet
P3	3 kDa fragment of APP
p38 MAP	p38 mitogen-activated protein kinase
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGF-β	Platelet-derived growth factor-β
Pen2	Presenilin enhancer 2
PFC	Prefrontal cortex
PHFs	Paired helical filaments
Phospho/p-JNK	Phosphorylated c-Jun N-terminal kinase
PI3-K	Phosphatidylinositol 3-kinase
PICALM	Phosphatidylinositol binding clathrin assembly protein gene
PPAR	Peroxisome proliferator-activated receptors
ppm	Parts-per-million
PrP	Prion Protein
PS1	Presenilin-1
PS2	Presenilin-2
PSD-95	Postsynaptic density protein 95
PUFA	Polyunsaturated fatty acids
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SAD	Sporadic Alzheimer's disease
SAT	Saturated fatty acids
S_D'	Stimulus presentation prime responses
SDS	Sodium dodecyl sulphate (protein extraction buffer)
SDS	Special Diet Services (diet manufacturer)
S.E.M.	Standard error of the mean
SJL	Swiss James Webster
SNAP-25	Synaptosomal-associated protein 25
TACE	TNF-alpha converting enzyme
Tg	Transgenic mouse
T-maze FCA	T-maze forced choice alternation task
TNF-α	Tumor necrosis factor-α
TTR	Transthyretin
w/v	weight/volume
WT	Wildtype mouse
α_1ACT	α-1 antichymotrypsin
βAPPs	soluble βAPP fragment
γ-CTF	Gamma (γ)-carboxyl terminal fragment of APP
ω-3	Omega-3
ω-6	Omega-6
ω-3/ω-6	Omega-3/omega-6 PUFA ratio

Chapter 1

General Introduction

1.0 Overview of the general introduction

The general introduction will first describe the characteristics of Alzheimer's disease (AD) and outline the rationale for studying the disease and developing therapies. The current literature surrounding its aetiology, pathology and potential therapeutic targets will then be described. An introduction into the importance of dietary factors in disease risk and progression will then be presented, with a particular focus on omega-3 polyunsaturated fatty acids (PUFAs) and curcumin compounds. The effect of these dietary compounds on AD pathology and related dysfunction is evaluated, in the context of *in vitro*, human and animal studies. The main aim of this thesis is to evaluate the effects of dietary intervention on brain pathology and cognition in a mouse model of amyloid pathology. The introduction therefore also includes an overview of animal models of AD and their relative strengths and weaknesses.

1.1 Alzheimer's disease

Alzheimer's disease is a neurodegenerative disorder, first described by Alois Alzheimer in 1906 (Berchtold & Cotman, 1998). This incurable and terminal disease is the most common form of dementia. Its prevalence in 2006 was estimated at 26.6 million people worldwide and is predicted to quadruple by 2050, affecting 1 in 85 people (Ferri et al., 2005; Brookmeyer et al., 2007). Alzheimer's disease has a huge socioeconomic impact, with 43% of cases estimated to need a high level of care which puts a great financial burden on society (Brookmeyer et al., 2007). As a result it is one of the most costly diseases in developed countries, estimated to cost $160 billion per year worldwide (Wimo, Jonsson & Winblad, 2006). Brookmeyer et al. (2007) estimated that if an intervention were to delay disease onset and progression by even one year, this would decrease the predicted 2050 prevalence by over 9 million, with the majority being high-level care cases.

Alzheimer's disease can be categorised in several ways, depending upon risk factors involved and the time of onset. These include familial and sporadic AD, or late-onset (senile) and early-onset (presenile) AD. Despite the different types of AD, pathology and clinical features appear the same (Selkoe, 2001). The stage at which pathology is present however is different, with pathology developing later and over a longer period in sporadic and late-onset AD compared with familial or early-onset AD (Anekonda & Reddy, 2005). As a result, early-onset AD (EOAD) is the term used for AD cases diagnosed before the age of 65 years, whereas late-onset AD (LOAD) is diagnosed after the age of 65 years (Brookmeyer, Gray & Kawas, 1998). The four main pathological traits of AD include: 1) the overproduction and accumulation of the beta-amyloid (Aβ) peptide which is neurotoxic and aggregates to form extracellular Aβ plaques, 2) the aggregation and hyperphosphorylation of tau proteins forming intracellular neurofibrillary tangles (NFTs), 3) neuronal cell loss, and 4) neuroinflammation and oxidative stress (Mattson, 2004).

Familial AD (FAD) is an autosomal dominant disease associated with genetic mutations of the amyloid precursor protein (APP), presenilin-1 (PS1) and presenilin-2 (PS2) genes (Mattson, 2004; Waring & Rosenberg, 2008). Numerous other genes are likely to be responsible but remain to be discovered (Jones, Harold & Williams, 2010). These APP and presenilin mutations alter APP processing to increase Aβ production (Scheuner et al., 1996; Citron et al., 1997; Wolfe et al., 1999). The APP gene has several mutations around the Aβ-dependant cleavage sites including APP K670N, M671L and V717I, which represent mutations at codons 670 and 671 in exon 16, and 717 in exon 17 of the APP gene (Mullan et al., 1992). FAD accounts for approximately 4 to 5% of all AD cases, and less than 10% of EOAD are due to autosomal dominant mutations (Waring & Rosenberg, 2008). Despite genetic mutations representing only 0.1% of all AD cases (Blennow, de Leon & Zetterberg, 2006), their role is central to FAD and has been key to furthering our understanding of the disease, particularly through the use of animal models.

Sporadic AD (SAD) accounts for the majority of cases and often develops in people after the age of 65 (therefore often referred to as LOAD), with increasing incidence in later years (Brookmeyer, Gray & Kawas, 1998). Although there is no known cause, there are several risk factors involved including a genetic component. It is well documented that the apolipoprotein E (ApoE) gene is implicated in SAD/LOAD. More specifically, the ApoE ε4 isoform accounts for up to 50% of late-onset cases (Saunders, 1993; Raber, Huang & Ashford, 2004).

Its precise role in pathogenesis remains inconclusive, although it is thought to alter APP metabolism or Aβ aggregation (Holtzman et al., 1999; Ma et al., 1999). It may also increase neuroinflammation and oxidative stress (e.g. Jofre-Monseny, Minihane & Rimbach, 2008). As almost half of LOAD sufferers do not carry the ApoE4 ε4 allele, other genetic or environmental factors must be involved. Indeed, research has claimed over 500 putative risk genes, with strong support for linkage on chromosomes 9, 10 and 12 (Pericak-Vance, 1997; Myers et al., 2000; Bertram, 2007). More recent genome-wide association studies have identified variants in four novel susceptibility genes (CLU, PICALM, CR1 and BIN1) providing compelling evidence for their association with AD risk (as reviewed in Hollingworth et al., 2011). Further support comes from their involvement in processes related to disease pathogenesis such as inflammation, amyloid clearance, lipid transport and metabolism, as well as endocytosis and intracellular trafficking (e.g. Jones, Harold & Williams, 2010; Hollingworth et al., 2011). In conclusion, genetic inheritance makes a significant contribution to the risk of AD, although it is clear that environmental factors must also may a substantial role in sporadic AD.

1.2 Clinical features of Alzheimer's disease

Alzheimer's disease is characterised by atrophy in multiple brain regions including the temporal lobe, parietal lobe, frontal cortex and cingulate gyrus (Wenk, 2003). The most commonly observed symptom of AD is cognitive dysfunction, including the inability to acquire new memories, a reduction in short-term and long-term memory, and loss of communicative abilities (Waldemar et al., 2007). Other behavioural symptoms such as confusion and emotional disturbances are observed, and these symptoms worsen in a time-dependant manner as the disease progresses. The precise behavioural phenotype of AD is detailed in conjunction with modelling the disease in animals, whereby similarities and dissimilarities are drawn (section 1.7).

The progression of AD is categorised into four stages; pre-dementia, early, moderate and advanced. Pre-dementia often goes undetected as symptoms are mistaken for normal age-related decline (Waldemar et al., 2007). Detailed diagnosis often reveals (amnesic) mild cognitive impairment (MCI), which frequently leads to the diagnosis of AD up to 8 years later (Arnáiz & Almkvist, 2003). A decline in short-term memory is the most common symptom of pre-dementia, in addition to subtle problems with executive functions (such as

attention, planning, and cognitive flexibility), semantic memory and sometimes apathy (Landes et al., 2001; Arnáiz & Almkvist, 2003; Bäckman et al., 2004). Early AD is diagnosed with increased learning and memory impairments, although individual cases do not follow the same progression of symptoms. For example, some sufferers exhibit agnosia, apraxia and communicative problems more than memory impairments (Förstl & Kurz, 1999). Short-term memory is most clearly affected at this stage, with loss of episodic, semantic and implicit memory to a lesser degree (Carlesimo & Oscar-Berman, 1992; Jelicic, Bonebakker & Bonke, 1995). At this early stage in the disease, AD sufferers can continue to perform tasks independently but may require assistance with difficult cognitive tasks (Förstl & Kurz, 1999).

Moderate AD is primarily associated with long-term memory impairment, such the inability to recognise close relatives (Förstl & Kurz, 1999). Loss of communication is also obvious, with patients showing difficulty with speech and fine motor skills such as writing (Frank, 1994; Taler & Phillips, 2008). Gross motor skills are also affected which commonly leads to falls. Other changes in behaviour are observed such as wandering, confusion, emotional problems (such as irritability, aggression, emotional lability, anxiety) and delusions (Waldermar et al., 2007). At this stage, sufferers find it difficult to maintain independence and often require caregivers to help perform daily activities (Förstl & Kurz, 1999). Advanced AD is the final terminal stage, whereby communication is severely affected and patients suffer from extreme apathy and fatigue, resulting in complete dependence upon caregivers (NIA, 2008). Eventually, bodily functions are lost and the disease results in death typically from secondary illness such as pneumonia (NIA, 2008).

Alzheimer's disease can often go undiagnosed for many years before symptoms develop enough to become apparent. This is likely to account for the limited effectiveness of treatments which begin after the patient becomes symptomatic. The need for earlier diagnosis and thus treatment is therefore imperative. The next section will overview the core features that characterise the pathogenesis of AD.

1.3 Alzheimer's disease: Pathogenesis

The theoretical characterisation of AD pathogenesis has evolved from early theories of cholinergic system disruption, to the now more generally accepted amyloid and tau hypotheses (Hardy & Allsop, 1991; Tiraboschi et al., 2004). The amyloid cascade hypothesis

proposes that the production and/or accumulation of β-amyloid is critical to the onset of changes that culminate in plaque formation, tau pathology and eventually, neuronal loss. Other pathological mechanisms include neuroinflammation and oxidative stress (Tuppo & Arias, 2005). Evidence suggests that these mechanisms are secondary to amyloid and tau pathology, but can further promote these pathological processes (Akiyama et al. 2000; Cai & Yan, 2007). The following sections will discuss how these factors lead to neurodegenerative loss and dysfunction in AD.

1.3.1 Amyloid and APP

The current literature has focused on the hypothesis that beta-amyloid (Aβ) is the major causative factor in AD pathogenesis - a view that has received support from genetic and histological evidence, along with animal studies (Hardy & Allsop, 1991). Masters et al. (1985) first recognised Aβ as the primary component of senile neuritic plaques, a major pathological hallmark of AD. Soon after, the first genetic mutations associated with AD were discovered on the APP gene localised on chromosome 21, which were found to promote the production of Aβ (Goate et al., 1991; Mullan et al., 1992). Further support for this proposition came from the observation that Down's syndrome 'Trisomy 21' sufferers, also associated with a genetic abnormality on chromosome 21, develop AD and Aβ plaques (Hardy & Allsop, 1991; Hardy & Selkoe, 2002).

Aβ is formed from proteolysis of the amyloid precursor protein (APP). APP is an integral transmembrane glycoprotein found in many tissues, particularly neuronal synapses. Its primary function is undetermined, although has been implicated in neural plasticity, synapse formation and repair (Turner et al., 2003; Priller et al., 2006). APP can be cleaved by three types of enzymes: α-, β- and γ-secretase. Importantly, only sequential cleavage by β- and γ-secretase produces Aβ in a process termed the "amyloidogenic pathway" (Boudrault et al., 2009). As illustrated in Figure 1.1, APP is firstly cleaved by β-secretase (e.g. BACE1) at its NH2-terminus, to form the N-terminus of the peptide and two cleavage fragments. These two fragments include soluble βAPP (βAPPs, the secreted ectodomain) and the β-carboxyl terminal fragment C99 (β-CTF, a membrane bound fragment). This cleavage by β-secretase is a prerequisite for Aβ formation. The C99 fragment is then further cleaved by γ-secretase (a complex of presenilin, nicastin, Aph1 and Pen2) to release AICD (γ-CTF) and the C-terminus

of Aβ from the membrane (Marlow et al., 2003). AICD interacts with transcription factors at the nucleus while Aβ is secreted into the extracellular space (Boudrault et al., 2009).

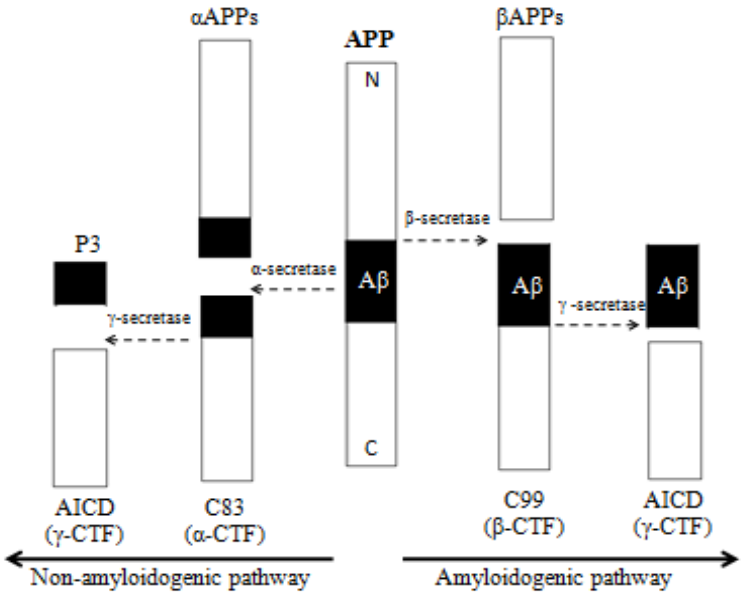

Figure 1.1 Amyloidogenic and non-amyloidogenic pathways of APP processing. The amyloidogenic pathway involves the sequential proteolysis of APP by β- and γ-secretase to produce the Aβ peptide. The non-amyloidogenic pathway involves APP cleavage by α- and γ-secretase which does not result in Aβ production.

Alternatively, APP can be first processed by α-secretase in the "non-amyloidogenic pathway" which prevents Aβ production (Figure 1.1). Here, α-secretase (e.g. TACE, ADAM9 and ADAM10 candidates) cleaves APP to form the αAPP soluble fragment (αAPPs, the secreted ectodomain) and the α-CTF, C83 (Lammich et al., 1999; Kojro & Fahrenholz, 2005). The C83 fragment can be further cleaved by γ-secretase to form P3 (a 3 kDa fragment) and AICD (Brunkan & Goate, 2005).

It is important to note that APP is metabolised in all cells and Aβ is produced at 'healthy' levels in non-demented individuals, although this can increase as a consequence of age likely due to an imbalance in oxidation status (Shoji et al., 1992; Meydani, 2001). However, Aβ production is significantly increased in patients with AD relative to aged individuals (Hardy & Allsop, 1991). One possible reason for this is that APP mutations in FAD generally cluster at or near β- and γ-secretase protease cleavage sites, thereby promoting Aβ production (Citron et al., 1992; Cai, Golde & Younkin, 1993). In sporadic AD, a combination of genetic

factors (e.g. ApoE ε4), environmental factors (e.g. diet) and comorbid medical conditions (e.g. type II diabetes) can cause accelerated formation of Aβ. Such examples are outlined in section 1.5. For example, hypercholesterolemia resulting from excess low-density lipoprotein (LDL) 'bad' cholesterol in the diet is a known risk factor that can increase amyloidosis (Refolo et al., 2000; Puglielli, Tanzi & Kovacs, 2003). Elevated cholesterol may accommodate Aβ production since cholesterol is an integral component of lipid rafts where amyloidogenic pathways of APP processing occur (Wahrle et al., 2002; Ehehalt et al., 2003; Vetrivel et al., 2004).

The overproduction of Aβ is central to the sequence of pathological events leading to AD development and progression (Näslund et al., 2000; Selkoe, 2000, 2001). Firstly, soluble monomeric Aβ aggregate to form oligomeric forms which are particularly neurotoxic and associated with cognitive decline in AD (Lue et al., 1999; Hardy & Selkoe, 2002). Secondly, Aβ folds incorrectly, accumulates and aggregates to form insoluble fibrils and deposits in the form of senile neuritic plaques which disrupt neuronal function (Hardy & Allsop, 1991). Finally, Aβ production can have downstream effects such as initiating the onset of tau pathology, neuroinflammation and oxidative stress, which together leads to further neuronal damage and Aβ production (Akiyama et al., 2000; Lewis et al., 2001; Hardy & Selkoe, 2002).

1.3.2 Tau and neurofibrillary tangles

Tau proteins are known as microtubule-associated proteins where their primary function is to regulate the assembly and stability of axonal microtubules in neurons by interacting with tubulin, the structural component of microtubules (Weingarten et al., 1975; Cleveland, Hwo & Kirschner, 1977). One of the characteristic hallmarks of AD is the abnormal aggregation of tau proteins leading to the formation of neurofibrillary tangles (NFTs) (Schneider & Mandelkow, 2008). Although the molecular mechanisms are not well understood, the tau hypothesis proposes that tau becomes saturated with phosphates in a process termed 'hyperphosphorylation', which prevents microtubule binding and results in its detachment from microtubules (Schneider & Mandelkow, 2008). This leads to microtubule instability and disintegration, and leads to disruption of axonal transport and synaptic degeneration (Mandelkow & Mandelkow, 1998; Hernández & Avila, 2007). Furthermore, the detached tau aggregate into self-assembled paired helical filaments (PHFs) which bundle together to form intracellular NFTs (see Figure 1.2; Hernández & Avila, 2007). Ultimately this obstructs

neuronal function via the inhibition of axonal traffic and disconnection of synapses, leading to further neurodegeneration (Mandelkow et al., 2003). Oxidative damage can also be induced which exacerbates neurodegeneration (Stamer et al., 2002).

The number of NFTs strongly correlates with the degree of cognitive decline and neurodegeneration in AD patients (Arriagada et al., 1992; Thal et al., 2000). The majority of NFTs are distributed in Aβ-affected brain regions, including the entorhinal region, hippocampus and frontal cortex (Arriagada et al., 1992). Although genetic tau mutations are not associated with AD, the identification of tau mutations in other neurodegenerative diseases, such as frontotemporal dementia, has highlighted its causative role in neurodegeneration (Poorkaj et al., 1998; Spillantini et al., 1998).

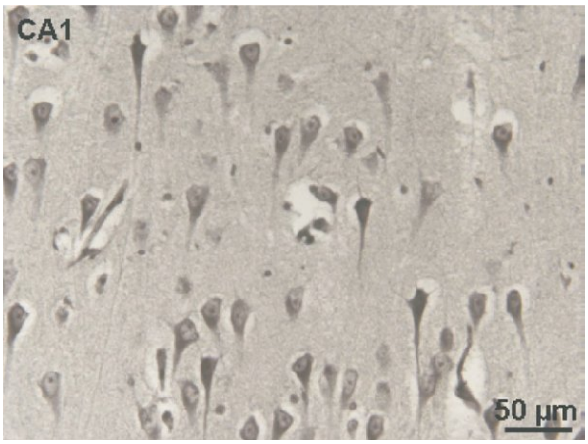

Figure 1.2 Section from an AD human brain showing pyramidal cells with tau neurofibrillary tangles within the hippocampal CA1 region (sourced from Schneider & Mandelkow, 2008).

1.3.3 Neuroinflammation

Neuroinflammation in the brain is a common feature of AD and is detected early before clinical onset (Engelhart et al., 2004). Most inflammatory pathways have been implicated in AD, as comprehensively reviewed in Akiyama et al. (2000) and Tuppo and Arias (2005). For example, upregulation of complement molecules, chemokines, cyclooxygenase, eicosanoids and cytokines such as IL-1β, IL-6 and TNF-α are detected in the AD brain (e.g. Kitamura et al., 1999; Xia & Hyman, 1999; Swardfager et al., 2010). Activation of inflammatory cells, microglia and astrocytes, are elevated and are often clustered at sites of Aβ deposition (e.g. Mrak, Sheng & Griffin, 1996; Ishizuka et al., 1997; Frautschy et al., 1998). Similarly, COX

expression is associated with NFT-affected sites (Oka & Takashima, 1997). This pattern of distribution suggests that these pathological hallmarks are involved in inflammatory development. Indeed, tau, Aβ and even APP have been shown to upregulate inflammation (e.g. Chong, 1997; Rogers et al., 1998; Suo et al., 1998). The development of these pathological hallmarks from early preclinical to advanced stages of AD therefore leads to a chronic state of neuroinflammation (Akiyama et al., 2000).

Chronic neuroinflammation has been shown to cause significant neuronal damage and behavioural dysfunction, highlighting its role in AD (e.g. Giulian et al., 1995; Heyser et al., 1997; Parachikova et al., 2007). Furthermore, inflammatory mediators can significantly increase pathological processes including amyloid pathology, tau phosphorylation, oxidative stress and can further perpetuate inflammation (e.g. Mackenzie, Hao & Muoz, 1995; McDonald et al., 1997, 1998; Griffin et al., 1998; Guo et al., 2002; Qin et al., 2003). This reciprocal relationship whereby inflammatory mechanisms both induce and are induced by AD pathology can therefore lead to a positive feedback cycle of greater neuroinflammation and amyloid/tau pathogenesis.

1.3.4 Oxidative stress

Oxidative stress is a disturbed redox state whereby antioxidant processes are unable to detoxify overproduced reactive oxygen species (ROS) such as free radicals and peroxides (Tuppo & Arias, 2005; Cai & Yan, 2007). These highly reactive molecules are free to interact with cells, driven by molecular instability, causing considerable damage to cells and results in a greater state of oxidative stress (Chauhan & Chauhan, 2006). Oxidative stress is a key feature of AD. Increased manifestations of oxidative stress are consistently shown in AD including lipid peroxidation, protein and DNA oxidation (Markesbery, 1997; Butterfield et al., 2001; Cai & Yan, 2007). These are detected from an early stage in AD and in multiple brain regions including the frontal, temporal, parietal and occipital cortices (Williams et al., 2006; Zafrilla et al., 2006). Manifestations are particularly marked in histopathologically-affected areas (Palmer & Burns, 1994).

It is generally accepted that oxidative stress significantly contributes to AD pathogenesis including the development of Aβ, NFTs and chronic neuroinflammation (e.g. Gamblin et al., 2000; Chauhan & Chauhan, 2006; Cai & Yan, 2007; Smith, Cappai & Barnham, 2007). For

example, it has been proposed that oxidative damage to DNA may result in altered protein synthesis, leading to abnormal tau and amyloid production in AD (Gabbita, Lovell & Markesbery, 1998; Markesbery & Lovell, 2006). The 'mitochondrial cascade hypothesis' also proposes that oxidative damage to mitochondria leads to amplification of ROS production which causes increased Aβ, tau pathology and apoptotic neuronal cell loss (Swerdlow & Khan, 2004; supported by Cardoso et al., 2004; Mancuso et al., 2007; Reddy, 2009). Conversely, Aβ, tau and neuroinflammation processes have also been shown to increase oxidative stress (e.g. Butterfield & Lauderback, 2002; Behl, 2005; Chauhan & Chauhan, 2006; Cai & Yan, 2007). Oxidative stress is therefore a potential target for AD therapeutic intervention. Current treatments under investigation include antioxidant compounds such as vitamin C, vitamin E and curcumin (Butterfield et al., 2002).

1.4 Therapeutic interventions for Alzheimer's disease

1.4.1 Aβ-based therapeutic strategies

A number of strategies are being investigated which target Aβ pathology. For example, active and passive anti-Aβ immunisation has shown some success in clearing Aβ and alleviating Aβ-induced cognitive deficits in mouse models of AD (e.g. Bard et al., 2000; DeMattos et al., 2001; Jensen et al., 2005), although clinical trials in AD patients have been halted due to complications (as reviewed in Hawkes & McLaurin, 2007 and Dodel et al., 2010). Other studies have reported inhibition of Aβ oligomerisation and aggregation by compounds such as apomorphine and metal chelators such as clioquinol (e.g. Lashuel et al., 2002; Walsh et al., 2005; Allana et al., 2009). Approaches that reduce Aβ production by modulating APP processing have been proposed, such as β- and γ-secretase inhibitors (Yin et al., 2007). For example, *Semagacestat* is a γ-secretase inhibitor and *Tarenflurbil* modulates γ-secretase to selectively lower Aβ42 for shorter less toxic Aβ peptides (Citron, 2004). Aβ production may also be reduced by altering dietary intake of cholesterol or saturated fatty acids. The latter are major components of lipid raft membranes where amyloidogenic APP processing occurs (Ehehalt et al., 2003). Further support for the role of lipid rafts in AD pathogenesis comes from Aβ-reducing effects of cholesterol-lowering drugs such as statins (Jick et al., 2000; Fassbender et al., 2001). Other strategies being investigated are anti-inflammatory and antioxidant compounds (Citron, 2004), which are examined in this thesis.

1.4.2 Tau-based therapeutic strategies

A number of approaches that could target tau pathology are being examined, such as the inhibition of kinases and phosphatases involved in tau phosphorylation (Kosik et al., 2002; Pei et al., 2003; Noble et al., 2005) and microtubule-stabilising agents to counteract the effects of hyperphosphorylated tau, such as *Taxol* (Parness & Horwitz, 1981; Boutté et al. 2005; Michealis et al., 2005). Tau aggregates could also be cleared using anti-tau immunotherapy (e.g. Asuni et al., 2007; Chai et al., 2011) or compounds which activate tau degradation pathways (e.g. Ravikumar, Duden & Rubinsztein, 2002). Tau aggregation could also be inhibited using anti-aggregation substances (e.g. Doody et al., 2008; Harrington et al., 2008; Bulic et al., 2010). Furthermore, due to the relationship between tau and Aβ development, tau has also been shown to be reduced by targeting Aβ production (e.g. Oddo et al., 2004). Although such therapies remain in their infancy of development, they are receiving greater attention and are on the verge of providing new AD treatment strategies within a few years (Medina, 2011).

1.4.3 Anti-inflammatory drug studies

Non-steroidal anti-inflammatory drugs (NSAIDs) such as *ibuprofen*, *aspirin* and *indomethacin* have been shown to reduce neuroinflammation, slow Aβ pathology and provide cognitive protection in AD models (e.g. Rogers et al., 1993; Lim et al., 2000; as reviewed in Cole & Frautschy, 2010). Epidemiological studies have also shown NSAIDs to lower the risk of AD and slow disease progression (in t' Veld et al., 2001; Zandi et al., 2002; McGeer & McGeer, 2007). Unfortunately however, these drugs have shown limited therapeutic effect in human clinical trials, perhaps due to the primary action of COX inhibition which is predominately expressed during preclinical not late stages of AD (McGeer & McGeer, 2007; Yermakova & O'Banion, 2001; Hoozemans & O'Banion, 2005). Furthermore, NSAID drugs that inhibit COX-1 activity are not suitable for chronic use due to side-effects such as gastrointestinal problems (Soll et al., 1991). Steroidal anti-inflammatory drugs, such as synthetic glucocorticoids, have had positive effects in reducing neuroinflammation in AD patients (Aisen et al., 1996); although this has not been supported in other clinical trials and epidemiological studies (e.g. Aisen et al., 2000; as reviewed in Akiyama et al., 2000). Their use is therefore not recommended, particularly since glucocorticoid administration has been shown to increase Aβ and tau pathology (Green et al., 2006; Wang et al., 2010). This

therefore warrants further investigation into other anti-inflammatory treatments. One such strategy is the dietary supplementation of natural compounds possessing anti-inflammatory properties. This is discussed further in section 1.6.

1.5 Alzheimer's disease risk factors

AD is a complex disorder with a number of risk factors, primarily including genetics (cf. section 1.1), age in LOAD (Hebert et al., 2003; Bermejo-Pareja et al., 2008) and health-related conditions such as cardiovascular disease (Stampfer, 2006; Rosendorff, Beeri & Silverman, 2007) and Type II diabetes or insulin malfunction (Farris et al., 2003; Luchsinger et al., 2004; Biessels et al., 2006). In addition to this, AD risk is influenced by environmental factors such as physical exercise (Larson et al., 2006; Scarmeas et al., 2009), mental stimulation and social engagement (Wilson et al., 2002; Bennett et al., 2006). Another important factor is diet, which will be considered in section 1.6.

1.6 Effect of diet in protecting against Alzheimer's disease

This section aims to overview the current literature surrounding the influence of diet in AD and its potential for therapeutic intervention. A general introduction to the forms of dietary intervention currently investigated will first be described, followed by a more detailed analysis of human and animal studies regarding the affect of omega-3 fatty acids and the polyphenolic compound curcumin on cognitive decline and AD pathology.

It is well documented that eating a healthy balanced diet can reduce the incidence of many chronic diseases, such as heart disease, and there is rising evidence that the same is applicable to AD (Scarmeas et al., 2009). Epidemiological evidence suggests that certain nutritional factors can increase or decrease the risk of AD, and clinical studies in patients and mouse models highlight the important contribution of diet to AD pathogenesis (Morris, 2004). For example, eating a Mediterranean diet is associated with reduced incidence of AD and dementia (Scarmeas et al., 2006, 2007, 2009). A number of dietary compounds have been found to have a beneficial effect in reducing the risk, symptoms and pathology of AD, including vitamin C and E, curcumin, garlic extract, Ginkgo biloba extract, green tea and red wine (Morris et al., 2004; Frank & Gupta, 2005). A common feature of these compounds is their antioxidant and anti-inflammatory actions (Akiyama et al., 2000). Moreover, these

compounds also have beneficial effects on Aβ-induced toxicity and restoring abnormal neurotransmitter release (e.g. Kim et al., 2004). These putatively therapeutic dietary supplements can be generally categorised into polyphenols, vitamins and fatty acids.

1.6.1 Polyphenols

Polyphenols are molecules containing multiple phenolic hydroxyl groups that are commonly sourced from fruit, plants and their extracts (Butterfield et al., 2002). They have potent antioxidant properties denoted by the number of hydroxyl groups on the aromatic phenyl rings (van Acker et al., 1996). The grape-derived polyphenol *resveratrol* and red wine have been shown to reduce oxidative stress, neuronal damage, AD risk and even Aβ and tau development (e.g. Orgogozo et al., 1997; Bastianetto, Zheng & Quirion, 2000; Wang et al., 2008, 2010). Similarly, green tea leaves and their extracts are highly abundant in polyphenols and have shown to be neuroprotective against Aβ toxicity, reduce oxidative stress and improve cognitive function (e.g. Serafini, Ghiselli & Fero-Luzzi Ghiselli, 1996; Choi et al., 2001; Kim et al., 2004; Weinreb et al., 2004). However, Foster et al. (1995) reported no significant benefits in AD patients. Polyphenolic Ginkgo biloba leaves and its EGb 761 extract are also neuroprotective against oxidative stress and Aβ toxicity, can improve cognitive function in AD patients and animal models, and increases non-amyloidogenic APP processing (e.g. Le Bars et al., 1997, 2002, 2003; Schindowski et al., 2001; Yao, Drieu & Papadopoulos, 2001; Stackman et al., 2003; Colciaghi et al., 2004). As a result, EGb 761 is currently approved for dementia treatment in Germany (Butterfield et al., 2002). Similar effects have been described for the polyphenolic compound curcumin, which is discussed in section 1.6.4.

1.6.2 Vitamins

Vitamin intake is thought to influence AD. Firstly, AD patients have low or deficient levels of vitamins B_{12}, C and E, which was correlated with cognitive decline (Levitt & Karlinsky, 1992; McCaddon & Kelly, 1994; Riviere et al., 1998; Perkins et al., 1999). The potent antioxidant vitamin E was found to alter processes involved in AD including reduced Aβ-induced oxidative stress, neuronal damage and behavioural deficits (Butterfield et al., 1999; Yamada et al., 1999; Yatin et al., 2000). Furthermore, vitamin E and high levels of combined vitamin C and E was related to reduced AD risk and incidence (Morris et al., 2002;

Engelhart, Geerling & Ruitenberg, 2002; Morris et al., 1998, 2005; Zandi et al., 2004). Sano et al. (1997) also reported positive effects of vitamin E supplementation in a clinical trial of AD patients. Similarly, supplementation of vitamin B_1 and B_{12} showed clinical improvements in AD and dementia patients (Meador et al., 1993; Mimori, Katsuoka & Nakamura, 1996; Nilsson et al., 2000). Although many studies have demonstrated potential use of these vitamins for AD treatment, clinical trials have produced mixed results. For example, vitamin B_1 treatment was shown to have no beneficial effect in AD (e.g. Nolan et al. 1991). Similar reports have been found with vitamin E (e.g. Petersen et al. 2005).

1.6.3 Omega-3 polyunsaturated fatty acids (PUFAs)

Omega-3 (ω-3) PUFAs are carbon chains with three or more double bonds, characterised by the first double bond in the n-3 position which is between the third and fourth carbons from the terminal methyl end (Boudrault et al., 2009). The three major omega-3 PUFAs are α-linolenic acid (ALA, 18:3ω-3), docosahexanoic acid (DHA, 22:6ω-3) and eicosapentaenoic acid (EPA, 20:5ω-3). The nomenclature of DHA for example, 22:6, indicates a 22-carbon chain with 6 double bonds. Omega-3 PUFAs are known as essential fatty acids as they are crucial for normal biological processes within the body, particularly brain health. Omega-3 PUFAs must be obtained through the diet as they cannot be synthesised *de novo* within the body; although long-chain omega-3 PUFA, such as DHA and EPA, can be formed from the short-chain ALA, and DHA can be derived from the shorter-chain EPA (DeMar et al., 2008; Gao et al., 2009). However it is important that high levels are consumed in the diet as conversion from ALA is inefficient (ranging from <0.2% to 8%; Gerster, 1998; Pawlosky et al., 2001; Brenna, 2002; Burdge & Caulder, 2005). Furthermore, brain uptake of preformed DHA is 7 times greater than DHA derived from ALA (Su et al., 1999).

DHA and EPA are predominantly sourced from fish oils and cold-water oily fish including salmon, mackerel and herring (Whelan & Rust, 2006). Fish do not synthesize omega-3 PUFA but obtain them from the algae or plankton in their diet (Falk-Petersen et al., 1998). Microalgae, such as *Crypthecodinium cohnii* and *Schizochytrium*, are therefore commonly grown to commercially produce DHA and EPA which are readily added to foodstuffs or capsuled for supplementation (e.g. Martek, 2007). Omega-3 PUFA can also be sourced at lower levels from vegetables, vegetable oils, fruits, grains and seeds such as flaxseeds (Whelan & Rust, 2006).

i) The importance of omega-3 PUFAs for brain health and cognitive status

Omega-3 PUFAs are crucial for normal brain health and cognitive status. DHA is the most abundant PUFA in the brain, where it is an integral component of neural membrane phospholipids and is particularly concentrated in synaptic membranes, myelin sheaths, synaptic vesicles and growth cones (Horrocks & Farooqui, 2004). It has great neuronal demand in the brain due to its high turnover in maintaining neural membranes (Farooqui & Horrocks, 2001). Specifically, DHA accounts for 10-20% of total brain fatty acid composition in humans and rodents (McNamara & Carlson, 2006), 8% of the brain dry weight (Muskiet et al., 2006) and 50% of the neuronal plasma membrane weight (Singh, 2005). In contrast, other omega-3 PUFAs including EPA and ALA comprise less than <1% of total brain fatty acids (McNamara & Carlson, 2006). Omega-3 PUFAs play a major role in neuronal growth and repair, neurotransmission, gene expression, membrane-bound enzyme and ion channel activities, intracellular and intercellular signalling, and synaptic plasticity (as reviewed in Horrocks & Farooqui, 2004; McNamara & Carlson, 2006).

Consistent with the importance of DHA in the brain structure and function, reduced brain DHA in healthy aged individuals has been associated with impaired neuronal and cognitive function (Horrocks & Yeo, 1999). Interestingly, animal studies have shown age-related dysfunction can be ameliorated with DHA supplementation (Favrelière et al., 2003). Epidemiological studies have also shown that consumption of omega-3 PUFAs and fish (containing omega-3 PUFA) is associated with a reduced risk of cognitive dysfunction in a middle-aged population (Kalmijn et al., 2004) and cognitive decline in an elderly population (van Gelder et al., 2007). Although inconsistent results have been reported (Kalmijn et al., 1997b; Morris et al., 2005), van Gelder et al. (2007) argued that this may be attributable to a shorter follow-up and less accurate information regarding omega-3 sources. Heude, Ducimetiere and Berr (2003) also reported a reduced risk of cognitive decline with higher plasma levels of omega-3 PUFAs. Similarly, reduced omega-3 PUFA intake or DHA deficiency in tissue sample measures was reported in neurological disorders including forms of depression, attention deficit hyperactivity disorders and schizophrenia (e.g. Mahadik et al., 1996; Burgess et al., 2000; Su et al., 2003). Interesting, omega-3 PUFA supplementation exerted clinical improvements in these disorders (e.g. Yoshida, Sato & Okuyama, 1998; Su et al., 2003; Richardson, 2006; Bélanger et al., 2009).

Similar to epidemiological studies, a randomized double-blind placebo-controlled multi-centre clinical trial reported DHA supplementation reduced age-related cognitive dysfunction in healthy aged individuals (Yurko-Mauro et al., 2009). However, this was not supported in similar clinical trials of DHA and omega-3 PUFA (EPA+DHA) supplementation using a number of doses matching or exceeding equivalent intake in epidemiological studies (Johnson et al., 2008; van de Rest et al., 2005, 2008; Dangour et al., 2010). However, it is important to note that clinical trials are much shorter than epidemiological studies, which may limit the detection of effects (Dangour et al., 2010; Quinn et al., 2010). For example, significant cognitive decline is not observed in healthy elderly individuals over short periods of time, and dietary interventions are relatively short in clinical trials compared to long-term dietary choices in epidemiological studies.

ii) The association between omega-3 PUFAs and Alzheimer's disease

There is strong evidence of a link between AD and omega-3 PUFA, particularly DHA. Firstly, DHA levels are markedly reduced in the brain (Söderberg et al., 1991; Prasad et al., 1998; Lukiw et al., 2005) and serum (Kyle et al., 1999; Conquer et al., 2000; Tully et al., 2003; Beydoun et al., 2007) of AD patients compared to age-matched controls. This is particularly the case in affected brain regions such as the frontal cortex and hippocampus (Söderberg et al., 1991). Although these reports are not always consistent (Skinner et al., 1993; Corrigan et al., 1998; Prasad et al., 1998), it is evident that DHA levels are altered in most cases in at least one area of the brain (Cunnane et al., 2007). Supporting this, Schaefer et al. (2006) reported high DHA plasma levels were associated with low AD risk. It is proposed that the DHA deficiency in AD may be attributable to low dietary intake, metabolic alterations or oxidative damage. For example, the stimulation of plasmalogen-selective phospholipase A^2 enzymes in AD may suggest increased release and metabolism of DHA, resulting in low levels (Farooqui, Rapoport & Horrocks, 1997; Farooqui, Ong & Horrocks, 2003). However, this has been considered unlikely as Lukiw et al. (2005) reported a reduction in DHA metabolites including neuroprotectin D1. In contrast, a reduction of brain DHA by oxidative processes has received strong support from evidence of increased lipid peroxidation products in AD, including DHA (Subbarao, Richardson & Ang, 1990; Praticò et al., 1998; Nourooz-Zadeh et al., 1999; Reich et al., 2001; Yao et al., 2003; Montine et al., 2004). Furthermore, these markers have been highly correlated with disease severity (Montine et al., 2002).

Epidemiological studies have shown high omega-3 PUFA or DHA intake to be associated with reduced incidence of AD (Friedland, 2003; Morris et al., 2003) and dementia (Barberger-Gateau et al., 2007). Further support for this has emerged from regular fish consumption, which is high in omega-3 PUFAs (Kalmijn et al., 1997a; Barberger-Gateau et al., 2002, 2007; Friedland, 2003; Morris et al., 2003; Albanese et al., 2009). Indeed, Huang et al. (2005) reported this association was applicable to fatty but not lean fish, which are particularly high in omega-3 PUFAs. However, it should be noted that these epidemiological findings on omega-3 PUFA and fish intake have not been consistently supported (Engelhart, Geerling & Ruitenberg, 2002; Devore et al., 2009). Studies in animal models of AD showing a therapeutic effect of omega-3 PUFA supplementation (reviewed in section 1.6.3 v) however provides support for the former results. Overall, these epidemiological results may explain why AD incidence is particularly high in Western societies, where the diet is omega-3 PUFA deficient. For example, the daily DHA intake in America is predicted to be ~80mg, which is less than half the intake reported in low-dementia groups (200mg/day) of epidemiological studies (Cole, Ma & Frautschy, 2009). Similarly, the high incidence of AD in Western societies may be related to the high intake of saturated fat, omega-6 PUFA and cholesterol, which appears to increase the risk of AD (Kalmijn et al., 1997a; Barberger-Gateau et al., 2007; Beydoun et al., 2007). Further supporting this, epidemiological studies show that a Mediterranean diet high in omega-3 PUFA is associated with lowered incidence of AD and MCI (Scarmeas et al., 2007, 2009).

iii) Potential mechanisms of omega-3 PUFAs in Alzheimer's disease

The following subsections will discuss the potential mechanisms by which omega-3 PUFAs could reduce the risk, incidence, pathology or symptoms of AD. This evidence has come from a mixture of *in vivo* and *in vitro* studies. Overall, this section demonstrates that omega-3 PUFAs have the potential to affect AD pathogenesis in a number of ways, including a reduction of disease processes and improvements in neuronal and memory function.

A. Affects on amyloid pathology

There is substantial empirical evidence that omega-3 PUFAs can reduce amyloid pathology both *in vivo* and *in vitro* (e.g. Lim et al., 2005; Lukiw et al., 2005; Oksman et al., 2006; Boudrault et al., 2009). Although *in vivo* studies cannot dismiss Aβ reduction through

indirect mechanisms, such as effects on neuroinflammation, several *in vitro* studies have shown omega-3 PUFA to reduce Aβ directly, as discussed below.

Sorting, transport and clearance of APP and Aβ

Omega-3 PUFAs increase levels of the Aβ/APP transport proteins transthyretin (TTR) and SORLA1 (LR11) both *in vivo* and *in vitro* (Puskás et al., 2003; Barceló-Coblijn et al., 2003; Ma et al., 2007). This may in turn reduce Aβ pathology as TTR can cleave Aβ, and inhibit Aβ aggregation and fibril formation through its sequestering and binding action (Stein & Johnson, 2002; Costa et al., 2008). In contrast, LR11 regulates intracellular transport of APP to reduce its processing into Aβ (Andersen et al., 2005; Cam & Bu, 2006; Spoelgen et al., 2006). Supporting this, LOAD sufferers have reduced LR11 which accelerate APP trafficking into endocytic Aβ-generating compartments thus increasing Aβ production (Dodson et al., 2006; Offe et al., 2006). Both genetic (inherited LR11 gene polymorphisms) and environmental factors (regulated by lipid intake) contribute to this reduction in LOAD (Ma et al., 2007; Rogaeva et al., 2007). Although a reduction in TTR by omega-3 PUFAs have not been consistently reported (Ma et al., 2007; Lim et al., 2005; Green et al., 2007), this highlights the potential involvement of other Aβ-reducing mechanisms.

Aβ degrading enzymes

Omega-3 PUFAs may reduce Aβ pathology by increasing the insulin-degrading enzyme IDE that degrades Aβ (Farris et al., 2003, 2004). Alterations to the IDE gene are associated with AD risk and IDE is reduced in patients with AD (Ertekin-Taner et al., 2004; Cook et al., 2003; Caccamo et al., 2005). Although no evidence has directly supported that increased dietary DHA can increase IDE (Green et al., 2007; Ma et al., 2007), Zhao et al. (2004) reported a low-DHA diet reduced IDE resulting in increased Aβ monomers. Under certain conditions, this suggests that a high DHA diet could increase IDE and therefore reduce Aβ. For example, IDE expression is regulated by PI3-K/Akt pathway, which is altered by DHA intake (Calon et al., 2004; Akbar et al., 2005).

APP processing and subsequent Aβ production

DHA supplementation reduced the γ-secretase PS1 component *in vivo* and *in vitro* and reduced Aβ levels (Green et al., 2007). Cole and Frautschy (2006) also found omega-3 PUFA limited γ-secretase activity by suppressing glycogen synthase kinase alpha (GSKα) via increasing PI3-K/Akt activity. DHA has also been shown to inhibit Aβ formation *in vitro* by inhibiting both α- and β-secretase activity (de Wilde et al., 2003). Supporting this, Lim et al. (2005) found a DHA-depleted diet to increase APP proteolysis by α- and β-secretase and increased Aβ levels *in vivo*. This was then reduced with DHA supplementation. In contrast however, α- and β-secretase has been reportedly unaffected by DHA supplementation *in vivo* (Green et al., 2007; Ma et al., 2007). DHA is also thought to reduce Aβ production by regulating the lipid microdomains where APP is processed (Lim et al., 2005; Boudrault et al., 2009). Non-amyloidogenic APP processing is believed to occur in caveolae, whereas amyloidogenic APP processing is believed to occur in lipid rafts (Ikezu et al., 1998; Ehehalt et al., 2003; Kawarabayashi et al., 2004). DHA is thought to encourage non-amyloidogenic pathways competitively over amyloidogenic pathways by affecting the relative proportion of these lipid microdomains, as well as their composition and functional properties (Shaikh et al., 2003; Ma et al., 2004; Hashimoto et al., 2005a; Eckert et al., 2011).

Aβ formation

Omega-3 PUFA can reduce the formation of oligomeric Aβ and subsequent mature fibrils *in vitro* (Hashimoto et al., 2009b). Although mechanisms remain elusive, DHA is thought to inhibit β-sheet transformation from α-helix monomers. Hashimoto et al. (2009b) reported DHA to prevent fibril formation by altering the morphology of Aβ fibres to highly unstructured and amorphous granular aggregates rather than normal ribbon-like structured forms. Similarly, Johansson et al. (2007) reported DHA to hinder Aβ fibrillisation by directly interacting with the Aβ peptide to stabilise oligomer species, thereby preventing Aβ-induced neurotoxicity.

B. Affects on tau pathology

DHA supplementation can reduce somatodendritic tau in an AD model expressing tau, APP and PS1 mutations (Green et al., 2007). The proposed mechanism was linked to a decrease in

Aβ levels (c.f., Oddo et al., 2004), and a reduction of tau phosphorylation as indicated by a reduction in early (but not late) phospho-tau epitopes and the tau kinase phospho-JNK (Green et al., 2007). Phospho-JNK, which is involved in tau phosphorylation, has also been reduced by DHA and EPA supplementation (Moon & Pestka, 2003; Xue et al., 2006). Since phospho-JNK is a member of the stress-activated MAP kinase family, its reduction may be related to a decline in inflammation by DHA (see Davis, 1999; Kyriakis & Avruch, 2001). Interestingly, diets low in omega-3 PUFA can increase tau pathology without affecting Aβ (Julien et al., 2010). In light of these studies, it is evident that tau pathology can therefore be modulated by omega-3 PUFA with mechanisms related to Aβ and neuroinflammation, and also independent of Aβ. Furthermore, omega-3 PUFAs may reduce tau pathology through its suppression of GSKα, which is involved in tau phosphorylation (Cole & Frautschy, 2006).

C. Modulation of inflammatory processes

Initial evidence that omega-3 PUFAs possess anti-inflammatory properties comes from clinical improvements in rheumatoid arthritis, psoriasis and ulcerated colitis following omega-3 PUFA treatment (as reviewed in Blok, Katan & van der Meer, 1996). The first mechanism whereby omega-3 PUFAs can reduce inflammation is by producing metabolites which exert potent anti-inflammatory effects, including resolvins, docosatrienes and protectins (Hong et al., 2003; Bazan, 2007; Dyall, 2010). For example, omega-3 PUFA metabolites can inhibit production and activity of cytokines, leukocytes, NfκB and COX (e.g. Marcheselli et al., 2003; Farooqui, Horrocks & Farooqui, 2007; Bazan, 2009). They can also agonise PPAR which is involved in the inhibition of inflammatory response genes (Chambrier et al., 2002; Yamamoto et al., 2005; Itoh et al., 2006). It is also important to note that these anti-inflammatory effects may reduce Aβ pathology as cytokines have been shown to increase β-secretase BACE1 expression, and PPARγ can increase Aβ uptake and clearance (Camacho et al., 2004; Sastre et al., 2003, 2006).

A second mechanism by which omega-3 PUFAs can reduce inflammation is by lowering the level of omega-6 (ω-6) PUFAs in the brain that can stimulate pro-inflammatory eicosanoid metabolites including prostaglandins, leukotrienes and thromboxanes (Wu & Meydani, 1998; Calder & Grimble, 2002; Horrocks & Farooqui, 2004; Farooqui, Horrocks & Farooqui, 2007). These fatty acids interact competitively for relative incorporation into brain phospholipids, availability for metabolism (release by phospholipases) and metabolism by

COX enzymes (Russo, 2009). The relative intake of these fatty acids therefore determines the relative production of anti-inflammatory omega-3 PUFA metabolites or pro-inflammatory omega-6 PUFA metabolites. High levels of omega-3 PUFA intake, specifically relative to omega-6 PUFA, would therefore have anti-inflammatory effects. The relative shift in the ω-6/ω-3 PUFA ratio by increasing omega-3 PUFA levels may explain the health benefits associated with high omega-3 PUFA intake and fish oils, which contain around seven times more omega-3 than omega-6 PUFA. A healthy ω-6/ω-3 ratio of 1:1 to 4:1 is recommended, although typical western diets currently provide skewed ratios of 10:1 to 30:1 (Simopoulos, 2002, 2003; Hibbeln et al., 2006) which may explain the increasing prevalence of AD in Western societies, since omega-6 PUFAs pro-inflammatory effects may acerbate AD pathology. This ratio should therefore be an important consideration during omega-3 PUFA intervention studies for AD.

D. Antioxidant effects

As outlined in section 1.3.4, oxidative stress is elevated in AD and promotes AD-related pathology and reduces brain DHA. Antioxidant protection may therefore restore brain DHA and reduce pathology. A number of studies have demonstrated the antioxidant properties of omega-3 PUFAs (as reviewed in Yavin, Brand & Green, 2002; Dyall, 2010). For example, omega-3 PUFA supplementation can reduce markers of oxidative stress such as protein carbonyls in humans (Mori et al., 2000) and animal models of AD with Aβ pathology (Hashimoto et al., 2002, 2005b; Calon et al., 2004). Furthermore, DHA supplementation increased antioxidant substances such as glutathione and antioxidant defence enzymes including catalase, glutathione peroxidise and glutathione reductase in hypercholesterolemic and Aβ-infused rats (e.g. Hossain et al., 1999; Hashimoto et al., 2002).

E. Affects on neural membrane structure and function

Synapses and dendritic spines are reduced in AD (Selkoe, 2002; Knobloch & Mansuy, 2008). This synaptic loss and resulting dysfunction is hypothesised to be the primary cause of AD cognitive changes, such as memory loss and is correlated better with synapse loss than Aβ plaques or NFTs (Montine et al., 2004). DHA plays a central role in neuronal structure and synaptogenesis, and its supplementation has been found to increase the number of synaptic proteins and dendritic spines (as reviewed in Wurtman et al., 2010). DHA supplementation

also reversed synaptic deficits following DHA depletion in an AD mouse model (Cole & Frautschy, 2006). Consistent with these beneficial effects on the brain, DHA supplementation can improve cognitive function in rodents and mild-to-moderate AD patients (Holguin et al., 2008a,b; Scheltens et al., 2008). Reduced brain DHA alters neural membrane activity by influencing neuronal conduction and transmission, neurotransmitters, enzymatic activities, receptors, ion channels and gene expression (as reviewed in Horrocks & Farooqui, 2004). Omega-3 supplementation however can correct these changes (as reviewed in Yehuda et al., 2002; Horrocks & Farooqui, 2004). For example, fish oil containing omega-3 PUFAs can normalise synaptosomal Na^+ and K^+-ATPase activity (e.g. Gerbi et al., 1994) and gene expression controlling signal transduction, ion channel formation, regulatory proteins and synaptic plasticity (e.g. Kitajka et al., 2002; Barcelo-Coblijn et al., 2003). Furthermore, DHA supplementation can improve neural membrane conduction and transmission by reducing the impact of excess omega-6 PUFAs and membrane-bound cholesterol, which can cause membrane rigidity (Yehuda et al., 2002).

F. Modulation of memory processes

Omega-3 PUFAs may directly influence cellular mechanisms of learning and memory processes by modulating putative synaptic mechanisms of memory, i.e., the induction of long-term potentiation (LTP) and long-term depression (LTD) (Chen & Tonegawa, 1997). These terms describe activity-dependent long-term increases (LTP) and decreases (LTD) in synaptic strength that may model synaptic processes mediating learning and memory (Bliss & Collingridge, 1993; Linden & Conner, 1995). Since memory is encoded by synaptic strength, LTP is considered the major cellular mechanism underling enhanced learning and memory (Bliss & Collingridge, 1993). Studies have found endogenously released DHA to block LTD and trigger or restore LTP in a concentration-dependant manner (e.g. Young et al., 1998; McGahon et al., 1999; Fujita et al., 2001). Evidence has shown DHA to influence LTP and LTD processes by directly altering the cellular mechanisms involved including potassium channels, the NMDA receptor and phospholipase A^2 (e.g. Nishikawa, Kimura & Akaik, 1994; Poling et al., 1995; Fujita et al., 2001; Calon et al., 2005). DHA may also influence synaptic plasticity by modulating the PI3-K pathway (e.g. Akbar et al., 2005), which is implicated in LTP induction (Sanna et al., 2002; Opazo et al., 2003). Since the level of endogenously released DHA is determined by phospholipid content and thereby dietary

intake, it is hypothesised that increasing dietary DHA could promote learning and memory processes.

G. Neuroprotection

The neuroprotective effect of DHA is well documented. Firstly, it can provide neuroprotection by reducing the toxic effects of Aβ, tau, inflammation and oxidative stress (as outlined previously; e.g. Wang, Chen & Su, 2010). Secondly, DHA has multiple neuroprotective effects by producing neuroprotectins such as NPD1, which is dramatically reduced in AD (Lukiw et al., 2005). NPD1 can downregulate pro-inflammatory genes, upregulate anti-apoptotic genes such as Bcl-2, Bcl-xl and Bfl-1, and protect against oxidative stress and Aβ-induced apoptosis (e.g. Lukiw et al., 2005; Bazan, 2005; Dyall et al., 2010). Consistent with this, DHA is reported to reduce markers of cell death, lower apoptosis and increase cell viability in neuronal and retinal cells (e.g. Kim et al., 2000; Rotstein et al., 2003; Florent et al., 2006). DHA can prevent neuronal apoptosis by increasing PI3-K/Akt signalling, inhibiting caspase-3 activity, reducing pro-apoptotic protein BAD and increasing phosphatides (e.g. Kim et al., 2000; Calon et al., 2004; Serhan et al., 2004; Akbar et al., 2005). Finally, DHA can have neuroprotective activity by potentiating activation of neurotrophic factors such as brain derived neurotrophic factor (Wu, Ying & Gómez-Pinilla, 2004).

iv) Omega-3 PUFA clinical trials in Alzheimer's disease and MCI patients

Although a number of epidemiological studies may suggest that omega-3 may provide some clinical benefit for AD and dementia, randomised double-blind placebo-controlled clinical trials were required to investigate these claims under more controlled conditions. Yehuda et al. (1996) first reported that a daily 'SR-3' supplement containing relatively high levels of omega-3 PUFA (ω-3/ω-6 ratio of 1:4) for 4 weeks relative to a typical western diet high in omega-6 PUFAs had multiple symptomatic improvements in AD patients including improved short-term memory, navigation, mood, cooperation, appetite and sleep. Otsuka (2000) then reported that a prescription of 900mg/day EPA for 3 to 6 months improved cognitive function in AD patients, but effects ceased after 6 months suggesting only short-term therapeutic efficiency. In another clinical trial, Chiu et al. (2008) found 1800mg/day DHA and EPA for 6 months to improve clinical condition (on a CIBIC-plus measure) in mild-to-moderate AD and

MCI patients compared to an olive oil placebo. The omega-3 PUFA supplement also improved cognition in MCI patients only, but no effects on cognitive decline could be attained as there was no cognitive change during the 6 month period.

In contrast to these promising findings, many clinical trials have failed to support beneficial effects of omega-3 in AD patients, although no adverse effects have been reported. Boston et al. (2004) reported no cognitive or clinical benefit from 500mg/day ethyl-EPA administration for 3 months in AD patients. Freund-Levi et al. (2006) found omega-3 PUFA supplementation (1700mg DHA and 600mg EPA) for 6 months did not delay cognitive decline in mild-to-moderate AD patients, although positive cognitive effects were observed in a subgroup of very mild AD patients. Similarly, Kotani et al. (2006) reported that 3 month supplementation of 240mg/day DHA (plus 240mg/day ARA) improved cognitive dysfunction in MCI and brain lesion patients, but not AD patients. In order to examine whether DHA treatment was only effective during early stages of pathological development in AD, Quinn et al. (2010) designed a study to include subjects with probable AD diagnosis. This large clinical trial found 200mg/day algal DHA supplementation for 18 months did not slow the rate of cognitive and functional decline in mild-to-moderate AD patients compared to soy or corn oil placebo.

Following this review of the current omega-3 PUFA literature, it is worth highlighting a number of common factors reported in these studies which may contribute to the observed effects. Firstly, higher doses of omega-3 PUFA supplementation were generally found to produce positive results in AD patients (Otsuka, 2000; Freund-Levi et al., 2006; Chiu et al., 2008), in contrast to relatively low doses showing no effect in AD patients (Kotani et al., 2006; Boston et al., 2004; Quinn et al., 2010). For example, the positive effect reported by Otsuka (2000) using 3-month EPA supplementation was almost double the EPA dose reported in the 3-month Boston et al. (2004) study. Interestingly, Freund-Levi et al. (2006) reported limited benefits in only very mild AD patients despite using the highest dose reported in clinical trials. This may suggest that extremely high doses may not be the optimal strategy. It is interesting that the relatively low dose supplemented in Quinn et al (2010) study was not beneficial, yet this was similar to levels reported as ‘protective’ in epidemiological studies and was higher than the average US dietary intake of omega-3 PUFA at 70mg/day. This therefore highlights that epidemiological findings are likely a result of an interaction of factors.

It remains unclear whether longitudinal supplementation is more effective than short-term, due to inconsistent results and a limited intervention period. The only study to report supplementation longer than 6 months was Quinn et al. (2010), reporting no benefits following 18-month treatment. However, the low dose and relatively short intervention period (compared to the period of high omega-3 PUFA intake in epidemiological studies) prevents the conclusion that longitudinal supplementation was ineffective. It is possible that positive effects may take longer to manifest or that omega-3 PUFAs may target AD at different stages of pathological development. In order to address this, longitudinal supplementation using a larger dose (perhaps similar to that used by Chiu et al., 2008) is recommended. Overall, EPA monotherapy is not recommended since the only study to report positive results found effects ceased over time. It is therefore possible that EPA and DHA co-supplementation is necessary for positive results, although this remains unclear due to inconsistent reports. Further examination of DHA monotherapy is therefore recommended (at larger doses than reported by Quinn et al., 2010) since DHA may be the most bioactive omega-3 PUFA, suggested by its importance in the brain and its positive effects in epidemiological studies. Furthermore, DHA appeared to account for the majority of positive effects in reducing pathological mechanisms of AD, as outlined in section 1.6.3 iii.

Although clinical trial results on omega-3 PUFA treatment were not overall convincing, it should be outlined that clinical trials have several limitations. For example, treatments are applied over only a short period of time which may limit the detection of later-stage or long-term effects. This may also prevent detection of effects as the level of clinical decline in which to observe effects is limited. Furthermore, treatments are applied once symptoms are present which does not assess the effect of early-stage treatment. Interestingly, some clinical trials reported beneficial effects of omega-3 PUFAs in very mild AD patients and MCI patients only (e.g. Freund-Levi et al., 2006; Kotani et al., 2006; Chui et al., 2008). This may suggest that omega-3 PUFAs are particularly effective when consumed during the very early stages of disease onset and may therefore influence early pathological processes. Clinical trials should aim to address these early stages (Cole & Frautschy, 2010), although the stage of diagnosis limits this. Studies using animal models of AD could therefore be used to shed some light on this.

v) Omega-3 PUFA studies in animal models of Alzheimer's disease

This section focuses on research carried out in rodents, although other studies have been carried out in higher species (e.g. Connor, Neuringer & Lin, 1990). This section provides an overview of studies investigating the effect of dietary omega-3 PUFAs in rodent models of AD. The main aim is to outline the experimental approach and results of these studies in order to examine the differences or similarities between their findings, and thus outline the theoretical justifications for the experimental designs used in this thesis. The following subsection will first outline the advantages of using animal studies to investigate dietary omega-3 PUFAs compared to human studies.

A. Human vs. animal studies

Animal studies offer a valuable opportunity to examine disease intervention in a tightly controlled manner, in contrast to human studies which have many confounding factors. For example, the use of prospective questionnaires for epidemiological results may be imprecise regarding omega-3 PUFA intake (Boudrault et al., 2009). Furthermore, the putative effect of omega-3 PUFA intake may result from simultaneous intake of other dietary components (Devore et al., 2009). For example, the reduced risk of AD with high fish consumption could be related to healthier lifestyle choices adopted by fish-eaters, not omega-3 PUFAs or fish intake *per se* (Arendash et al., 2007). Discrepancies in epidemiological results may also be related to food preparation, which is not taken into account. For example, fish may be beneficial but not if fried in saturated or transfats (Devore et al., 2009). Clinical trials are also problematic (despite increased control) as external factors such as diet cannot be controlled. Another limitation is the accuracy of measuring omega-3 PUFAs in serum as a marker of brain status or dietary intake, which has mixed views (Ma et al., 1995; Beydoun et al., 2007; Kuratko & Salem, 2009). In contrast, animal studies allow direct measure of omega-3 PUFAs in the brain, serum and even precise measures and control of dietary intake (including omega-3 PUFAs and complete nutritional content). Animal studies also allow longitudinal investigations which are impractical in human studies. This is particularly important when examining disease which develops over many years, since earlier exposures may fundamentally influence disease risk later in life (Launer, 2005). Animal studies have limitations however when applied to human disease due to their limited capacity to model disease states. Further details regarding animal models of AD are discussed in section 1.7.

B. Affect of omega-3 PUFA administration in rat and transgenic mouse models of Alzheimer's disease

A number of studies have examined the effect of omega-3 PUFA supplementation in rodent models of AD. Rat models are typically produced by the infusion of human Aβ40 into the cerebral ventricle, which causes accumulation of Aβ, oxidative stress, neuronal apoptosis, synaptic and cognitive deficits (Hashimoto et al., 2002, 2005a, 2005b, 2006). Interestingly, several studies have reported pre-administration of DHA or EPA before Aβ-infusion to protect against these Aβ-induced deficits (Hashimoto et al., 2002, 2006, 2009a). Hashimoto et al. (2005a,b) also reported amelioration of deficits with DHA administration following three generations of omega-3 PUFA deficiency. Overall these studies demonstrate the potential of EPA as well as DHA in reducing a number of Aβ40-induced deficits. Transgenic mice are also used to model aspects of pathology in AD by expressing genetic mutations that reproduce specific features of AD pathology such as increased Aβ and tau deposition (Hsiao et al., 1996; Oddo et al., 2003). An overview of these transgenic models is provided in section 1.7 and should be referred to for clarification of the models and their mutations referred to below. In particular, these genetically modified models are useful in examining the effect of intervention strategies on AD-like pathology.

Several studies have also examined omega-3 PUFA treatment in the triple transgenic mouse model, which possesses APPswe, presenilin-1 (PS1) and tau mutations resulting in accelerated Aβ and tau pathology (Table 1.1). All published studies have reported some positive effects including reductions in tau accumulation, tau phosphorylation, Aβ levels and JNK activation, as well as improved memory and neuronal function. Interestingly, a reduction in Aβ levels has not been consistently reported. Nevertheless, these studies have demonstrated the potential of omega-3 PUFA supplementation in ameliorating tau and Aβ, as well as their associated deficits. One of the major criticisms that can be levied against these studies is that the choice of experimental control limits the conclusion that omega-3 PUFA supplementation *per se* can result in beneficial effects. For example, Green et al. (2007) reported positive effects of DHA supplementation relative to a diet depleted in omega-3 PUFAs and containing almost double the amount of omega-6 PUFA. Similarly, Ma et al. (2009) reported positive effects of fish oil supplementation relative to a diet depleted in omega-3 PUFAs with high levels of saturated fat. Likewise, Arsenault et al. (2011) reported positive effects of fish oil supplementation relative to a diet high in omega-6 PUFA.

These differences in control diet may contribute to the apparent positive effects observed since diets with depleted omega-3 PUFA and high saturated, total or omega-6 fat can acerbate inflammation, oxidative stress, tau and Aβ pathology in transgenic models (e.g. Calon et al., 2004, 2005; Hooijmans et al., 2007; Ma et al., 2009; Julien et al., 2010). Furthermore, these factors can cause cognitive and neuronal dysfunction in healthy rodents (e.g. Gamoh et al., 1999; Greiner et al., 1999; Carrie et al., 2000; Moriguchi, Greiner & Salem, 2000; Ikemoto et al., 2001). To illustrate this point further, Green et al. (2007) reported the efficiency of DHA supplementation in reducing Aβ and tau pathology diminished after 6 months when co-supplemented with omega-6 PUFAs DPA or ARA, highlighting the detrimental effect of omega-6 PUFAs on pathology. In conclusion therefore, these studies show that omega-3 PUFA supplementation can be beneficial compared to conditions which may acerbate pathology or deficits. This may be particular relevant to AD patients in Western societies which have comparable diets. However, further examination into the effect of omega-3 PUFA supplementation compared to 'neutral' conditions is required to establish whether supplementation in a population consuming a healthy diet may ameliorate AD pathogenesis.

Several studies have also examined omega-3 PUFA treatment in double transgenic APPswe/PS1(dE9) mice expressing accelerated amyloid pathology (Table 1.2). All but one study demonstrated some positive effects including reductions in Aβ levels, deposits and plaque load, behavioural deficits, inflammatory markers and improved blood circulation. Although inconsistencies were found in amyloid load and the reduction of behavioural deficits, these studies demonstrate the potential of omega-3 PUFA supplementation ameliorating Aβ-induced deficits. Similar to studies in the triple transgenic model however, these effects may be attributable to differences in the control diet. For example, Oksman et al. (2006) found positive effects of fish oil relative to diets containing excess omega-6 PUFAs, and positive effects of omega-3 PUFAs relative to a diet containing excess omega-6 PUFAs and two diets almost depleted in omega-3 PUFAs. Similarly, Hooijmans et al. (2007) compared omega-3 PUFA supplementation to control diets containing higher levels of omega-6 PUFAs or excess saturated fat. Hooijmans et al. (2009) compared omega-3 PUFA supplementation to control diets containing relatively depleted omega-3 PUFAs and excess saturated fat. Consequently, it can be concluded that omega-3 PUFA supplementation can be beneficial compared to conditions which may acerbate pathology or deficits. Again, further examination is required under 'neutral' control conditions.

In contrast to these studies, Arendash et al. (2007) reported no positive effects of 13% omega-3 PUFA supplementation in APPswe/PS1(dE9) mice. It is argued however that this be related to the high levels of omega-6 and saturated fatty acids in the omega-3 PUFA diet relative to control (despite the high ω-3/ω-6 ratio), which may have masked the positive effects of omega-3 PUFA supplementation. Furthermore, the null effect may be related to the lack of omega-3 PUFA incorporation into the brain of transgenic mice, as indicated by unaltered levels omega-3 and omega-6 PUFAs in the cortex. This study cannot therefore rule out the possibility that beneficial effects may be observed when omega-3 PUFA penetrates the brain, as shown in other studies (e.g. Calon et al., 2004; Oksman et al., 2006; Arsenault et al., 2011).

A number of studies have also examined DHA supplementation in the Tg2576 mouse model, which expresses the APPswe mutation, amyloid pathology and Aβ-induced synaptic and behavioural deficits (Table 1.3). These studies are particularly relevant to this thesis as omega-3 PUFA supplementation was assessed in the Tg2576 model. These studies by Gregory Cole's research group demonstrated positive effects of DHA administration including reductions in Aβ pathology, synaptic deficits and oxidative stress, and improvements in processes underlying learning and memory including changes to CaMKII, drebrin and caspase activation. One study also reported reduced spatial memory deficits. The potential mechanisms for reduced Aβ were also reported including reduced APP processing and increased LR11. Importantly however, the majority of positive effects were relative to a DHA-depleted diet containing almost double the amount of omega-6 PUFA linoleic acid, and as argued above, this may have contributed to the apparent beneficial effects. In contrast, few effects were observed with DHA supplementation relative to the standard diet containing a similar level of omega-6 PUFA and low level of total omega-3 PUFA (~0.3%) and cholesterol. The only significant effect of DHA supplementation relative to the standard diet was a reduction in Aβ40. These results therefore suggest that DHA or omega-3 PUFA supplementation may not be beneficial in an AD model when relative to a normal baseline diet, and the most striking effects are observed against a poor-diet control group. In conclusion, further research is required to accurately examine whether omega-3 PUFA supplementation is beneficial relative to a baseline diet that controls for factors which may alter pathology and its associated deficits.

Table 1.1 Omega-3 PUFA intervention studies in triple transgenic (APPswe, tau + PS1) model mice

Study	Omega-3 PUFA diet	Control diet	Design	Reported effects
Green et al. (2007)	**DHA** ω-3/ω-6 ratio = 1:1 1.3% total ω-3 (DHA) 1.3% total ω-6 (ARA)	**TWD** ω-3/ω-6 ratio = 1:10 0.2% total ω-3 (ALA) 2.3% total ω-6 (ARA)	Age: From 3 months Length: 6 months	↓ Tau accumulation ↓ Aβ40 + Aβ42 (Soluble and intraneuronal) No effect on insoluble Aβ
Ma et al. (2009)	**HFBD + Fish oil (2.4%)** ~0.6% DHA 1.5% Cholesterol Safflower oil (high ω-6) No coconut oil = lower SAT	**HFBD** 0% total ω-3 1.5% Cholesterol Safflower oil (high ω-6) Coconut oil (high SAT)	Age: From 4 months Length: 5 months	Improved spatial memory in Y-maze ↓ JNK activation (inflammatory mediator) ↓ Tau phosphorylation (trend only) Improved insulin signalling (trend only)
Arsenault et al. (2011)	**Omega-3 PUFA fish oil** ω-3/ω-6 ratio = 1:2.8 9.5μM/g DHA ~2μM/g EPA, 0.5μm/g ALA 34μM/g ω-6 LA	**High omega-6 PUFA diet** ω-3/ω-6 ratio = 1:10.4 0μM/g DHA + EPA 7μM/g ALA 67μM/g ω-6 LA	Age: From 4 months Length: 8-10 months	↓ Phosphorylated tau (soluble, not insoluble) Improved N.O.R. memory in Tg + Non-Tg mice Improved entorhinal cortex neuronal function No effect on Aβ40/42 (soluble + insoluble) No effect on pre-synaptic marker synaptophysin No effect on post-synaptic marker PSD-95

HFBD = High fat bad diet, ω-3 = omega-3 PUFA, ω-6 = omega-6 PUFA, ↓ = Reduced, N.O.R. = Novel object recognition, μM = μmole, SAT = Saturated fat, TWD = Typical Western Diet, LA = linoleic acid (major omega-6 PUFA), ALA = α-linolenic acid (omega-3 PUFA).

Table 1.2 Omega-3 PUFA intervention studies in double transgenic APPswe/PS1(dE9) model mice.

Study	Omega-3 PUFA diet	Control diet	Design	Reported effects
Oksman et al. (2006)	**Fish oil** 2.2% fish oil supplement ω-3/ω-6 ratio = 1:1.4 22% ω-3, 30% ω-6	**Corn oil diet** ω-3/ω-6 ratio = 1:70 1% ω-3, 54% ω-6	Age: From 6 months Length: 3-4 months	↓ Aβ42 levels and microglial activation No effect on Aβ plaque load
		Soy oil diet ω-3/ω-6 ratio = 1:8 7% ω-3, 52% ω-6	Age: From 6 months Length: 3-4 months	↓ Aβ42 levels and microglial activation No effect on Aβ plaque load
Oksman et al. (2006)	**Omega-3 PUFA** 14% ω-3, 40% ω-6 0.5% DHA	**1) Soy oil diet** (as above) **2) Lipid neutral diet** 1% ω-3, 31% ω-6 **3) TWD** (1% cholesterol) 1% ω-3, 31% ω-6	Age: From 6 months Length: 3-4 months	↓ Aβ40 + Aβ42 (cf. diet 3 only) ↓ Exploratory deficits (cf. diets 1-3) No effect on spatial memory deficits in MWM No effect on Aβ plaque load
Hooijmans et al. (2007)	**Omega-3 PUFA** 14% ω-3, 40% ω-6 19% SAT	**1) Standard chow** 7% ω-3, 52% ω-6, 15% SAT **2) TWD** (1% cholesterol) 1% ω-3, 31% ω-6, 44% SAT	Age: From 6 months Length: 12 months	↓ Aβ deposits Improved blood circulation
Hooijmans et al. (2009)	**Omega-3 PUFA** 7% ω-3, 18% ω-6, 20% SAT (3% DHA, 0.8% EPA) ω-3/ω-6 ratio = 1:2.5	**1) Standard chow** 2.2% ALA, 16% ω-6, 38% S. ω-3/ω-6 ratio = 1:7.5, **2) TWD** (1% cholesterol) 0.5% ALA, 11% ω-6, 53% S. ω-3/ω-6 ratio = 1:22.5	Age: From 2 months Length: 6-13 months	↓ Aβ deposits (after 13 not 6 months treatment) ↓ MWM deficits (after 13 months cf. diet 2) Improved blood circulation (after 6m cf. diet 2) Improved blood circulation (after 13m cf. diet 2) No effect on open field, reverse MWM or circular hole board behaviour
Arendash et al. (2007)	**Omega-3 PUFA** 13% total ω-3 ω-3/ω-6 ratio = 1:3.8 20% SAT, 51% ω-6	**TWD** 3% total ω-3 ω-3/ω-6 ratio = 1:11.4 10% SAT, 31% ω-6	Age: From 2 months Length: 7 months	No effect on memory: MWM, RAWM, Y-maze, circular platform, platform recognition No effect in balance beam or string activity No effect on Aβ40/42 (soluble or insoluble)

TWD = Typical Western Diet, ω-3 = omega-3 PUFA, ω-6 = omega-6 PUFA, ↓ = Reduced, MWM = Morris water maze, RAWM = Radial arm water maze, cf. = compared to, SAT (or S.) = Saturated fat.

The Tg2576 studies outlined in Table 1.3 also provided evidence for the negative effects associated with diets depleted in omega-3 PUFA with high levels of omega-6 PUFA. The low-DHA diet relative to the standard diet resulted in spatial memory deficits, synaptic losses (PSD-95), and alterations to processes involved in learning and memory including reduced PI3-K signalling, increased caspase activation and reduced drebrin (Calon et al., 2004). Calon et al. (2005) similarly reported caspase activation, reduced NMDA receptor subunits, reduced CaMKII and increased pro-apoptotic BAD protein. Cole and Frautschy (2006) also reported increased protein carbonyls, and reduced drebrin, CaMKII and the PSD-95 synaptic marker. Furthermore, Lim et al. (2005) reported increased Aβ40 and APP proteolytic activity, and Ma et al (2007) reported reduced LR11. These results therefore support the potential contribution of depleted omega-3 and high omega-6 PUFA levels have to the effects observed when DHA supplementation is compared to such a diet.

Finally, another issue that requires further examination is the effect of omega-3 PUFA supplementation from an early age prior to pathological development. All transgenic studies outlined in this section provided treatment after pathology had developed, often during advanced stages (particularly in the Tg2576 model). The earlier the intervention is initiated then arguably the greater impact it may have on the initial and later stages of amyloid-induced pathology. This thesis therefore aims to examine the effect of omega-3 PUFA supplementation from an early age in Tg2576 mice, relative to a baseline control diet designed to control for other fatty acids which may have otherwise contributed to an apparent beneficial effect.

Table 1.3 Omega-3 PUFA intervention studies in APPswe Tg2576 model mice

Study	Omega-3 PUFA diet	Control diet	Design	Reported effects
Calon et al. (2004)	**High DHA (0.6%)** ω-3/ω-6 ratio 1:5 6.1% total fat 750ppm Chol. 2.5% ω-6 LA	**1) Low-DHA (<0.01%)** ω-3/ω-6 ratio 1:85, 4.9% LA 6.1% total fat, 750ppm Chol.	Age: From 17 months Length: 4-5 months	↓ MWM acquisition deficits, partially restored PI3-K pathway ↓ Post-synaptic marker drebrin and PSD-95 losses ↓ pro-apoptotic BAD activity, caspase activation, fractin/actin ratio No effect on SNAP-25, synaptophysin, synaptotagmin, + synaptobrevin
		2) Standard (0.09% DHA) ω-3/ω-6 ratio 1:7, 2.5% LA 11% total fat, 290ppm Chol.	Age: From 17 months Length: 4-5 months	No effects on any of above MWM not examined
Calon et al. (2005)	**High DHA (0.6%)** ω-3/ω-6 ratio 1:4 6.1% total fat 750ppm Chol. 2.8% ω-6 LA	**1) Low-DHA (<0.01%)** ω-3/ω-6 ratio 1:82, 4.9% LA 6.1% total fat, 750ppm Chol.	Age: From 17 months Length: 5.5 months	↓ NMDA receptor subunits (NR2A, NR2AB, NR1) losses ↓ Fodrin (caspase cleavage product) losses No effects on CaMKII, synaptophysin or SNAP-25 (in hipp.)
		2) Standard (0.09% DHA) ω-3/ω-6 ratio 1:9, 2.5% LA 11% total fat, 290ppm Chol.	Age: From 17 months Length: 5.5 months	No effect on any of above
Lim et al. (2005)	**High DHA (0.6%)** Same as Calon et al. (2004)	**1) Low-DHA (<0.01%)** Same as Calon et al. (2004)	Age: From 17-19months Length: 3-5 months	↓ total insoluble Aβ, Aβ42, Aβ40 + plaque burden ↓ C83 + C99 APP processing products No effect on BACE mRNA, ApoE, TTR expression, soluble Aβ
		2) Standard (0.09% DHA) Same as Calon et al. (2004)	Age: From 17-19months Length: 3-5 months	No effects on any of above except ↓ Aβ40 Plaque burden not examined
Cole & Frautschy (2006)	**High DHA (0.6%)** Same as Calon et al. (2004)	**1) Low-DHA (<0.01%)** Same as Calon et al. (2004)	Age: From 17 months Length: 5 months	↓ Aβ42 and Aβ40, carbonyls, fractin ↑ drebrin, PSD-95, CaMKII No effects on or GFAP or synaptophysin (although trend of ↑)
		2) Standard (0.09% DHA) Same as Calon et al. (2004)	Age: From 17 months Length: 5 months	↓ Aβ42 + PSD-95, CaMKII (statistical significance unknown) ↑ Aβ40 + carbonyls + fractin, synaptophysin (S.S.U.) No effect on drebrin or GFAP (S.S.U)
Ma et al. (2007)	**High DHA (0.6%)** Same as Calon et al. (2004)	**1) Low-DHA (<0.01%)** Same as Calon et al. (2004)	Age: From 17 months Length: 3.5 months	↑ LR11 losses
		2) Standard (0.09% DHA) Same as Calon et al. (2004)	Age: From 17 months Length: 3.5 months	No effect on LR11

ω-3 = omega-3 PUFA, ω-6 = omega-6 PUFA, ↓ = reduced, MWM = Morris water maze, Chol. = Cholesterol, S.S.U = Statistical significance unknown, LA = linoleic acid, hipp. = hippocampus

1.6.4 Curcumin

Curcumin (diferuloylmethane) [1,7-bis(4- hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a naturally-occurring polyphenolic compound extracted from turmeric, the powered rhizome of the plant *Curcuma longa*. Curcumin comprises 3 to 5% of turmeric and is its pharmacologically active component (Salvioli et al., 2007). It is a biphenolic compound composed of two aromatic rings (polyphenols) with hydroxyl groups which are connected by a β-diketone bridge (Figure 1.3). The β-diketone bridge features two α, β-unsaturated carbonyl groups (composed of double bonds or *dienone*) which can undergo Michael addition and is critical for some of the effects of curcumin (Weber et al., 2006). Curcumin is a spice commonly used as a yellow pigment for food colouring and a flavouring additive typically in Indian Cuisine. Curcumin has also been used for centuries in traditional Indian "Ayurvedic" medicine for its various indications (Chainani-Wu, 2003).

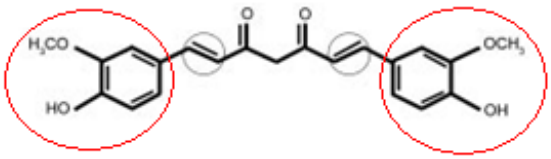

Figure 1.3 Structure of curcumin, highlighting the phenol groups (indicated by red circles) and the connecting β-diketone bridge (indicated by grey circles). Diagram adapted from Begum et al., 2008.

Several *in vitro*, animal and human studies have demonstrated potent antioxidant and anti-inflammatory properties of curcuminoids, as well as anti-viral, anti-fungal, anti-amyloid and anti-carcinogenic activities (e.g. Yang et al., 2005; as reviewed in Chainani-Wu, 2003). The ability for curcumin to reduce amyloid, inflammation and oxidative stress highlights it potential for AD treatment. Due to its extensive screening for cancer chemoprevention, curcumin has undergone thorough toxicological testing and preclinical investigation in several species including mice, rats, dogs, monkeys and humans, and is considered safe (Kelloff et al., 1996; Chainani-Wu, 2003; Cheng et al., 2001; Sharma et al., 2004; but see Lopez-Lazaro, 2008 for a cautionary use of curcumin). However, it has been suggested that very high doses of curcumin may lead to a switch from antioxidant to pro-oxidant effects which may have important therapeutic applications (Sandur et al., 2007a,b; Lopez-Lazaro, 2008). Despite this, a number of studies using mice and rats have shown no toxic effects of large doses around 5000ppm or 3500mg/kg body weight (Srimal & Dhawan, 1972; Kelloff et

al., 1996; Lim et al., 2001). This therefore shows potential for chronic use in AD, unlike NSAID intervention which is unsuitable long-term consumption due to safety issues (Rao & Knaus, 2008).

One potential issue with curcumin treatment however, is that it has poor bioavailability which may limit its pharmacological effect (Anand et al., 2007). This is attributed to poor absorption, rapid metabolism and rapid systematic elimination through the faeces (e.g. Pan, Huang & Lin, 1999; see Aggarwal & Harikumar, 2009). For example, curcumin is poorly absorbed into the bloodstream after oral consumption (Wahlstrom & Blennow, 1978; Kelloff et al., 1996; Cheng et al., 2001; Lao et al., 2006; Sharma et al., 2004). Even when administered via other administration routes such as i.p. injection, curcumin is rapidly metabolised and eliminated from the body (e.g. Pan, Huang & Lin, 1999), although tissue levels remain higher than oral dosing (e.g. Yang et al., 2005). Curcumin also has difficulty crossing the blood brain barrier (e.g. Pan, Huang & Lin, 1999). This could pose a potential problem for therapeutic use in AD where localisation to brain pathology is essential. Despite these issues, studies have proven curcumin to be biologically effective, particularly when provided in larger doses (Kelloff et al., 1996; Frautschy et al., 2001). Other attempts to increase bioavailability include provision of adjuvants such as piperine which block curcumin metabolism (Shoba et al., 1998) and special formulations such as lipid complexes or nanoparticles (Bisht et al., 2007; Maiti et al., 2007).

i) Potential mechanisms of curcumin in Alzheimer's disease

Although curcumin is not an essential nutrient required for general health, it has shown clinical benefit in health and disease states such as inflammatory conditions through its various biological activities (Sikora, Scapagnini & Barbagallo, 2010). With its favourable toxicity profile, curcumin is therefore being investigated as a treatment for various conditions, including AD. It has shown positive actions in reducing AD pathology, as detailed below.

A. Affects on amyloid pathology

A large number of studies have shown curcumin reduced amyloid accumulation both *in vivo* and *in vitro* (e.g. Frautschy et al., 2001; Lim et al., 2001; Ono et al., 2004; Yang et al., 2005;

Begum et al., 2008). *In vitro* studies have shown curcumin to inhibit Aβ40/42 fibril formation and aggregation, as well as inhibit Aβ extension and disaggregate pre-aggregated Aβ (Ono et al., 2004; Yang et al., 2005). Evidence suggests that curcumin may reduce Aβ indirectly through its antioxidant and anti-inflammatory properties (Begum et al., 2008), or by affecting ApoE, cholesterol and α_1ACT which regulate α- and β-secretases to be pro-amyloidogenic (as reviewed by Lim et al., 2001). Evidence has largely supported the role of curcumin in amyloid degradation and clearance, principally by activating resident brain macrophages such as microglia within and surrounding Aβ deposits (e.g. Frautschy et al., 2001; Lim et al., 2001) which are dysfunctional in AD (Fiala et al., 2007). This proposed amyloid-phagocytosing clearing effect was supported by Zhang et al. (2006) who found curcumin treatment to enhance Aβ uptake by macrophages of AD patients *in vitro*.

Evidence also suggests that curcumin can block Aβ aggregation through metal chelation (Baum & Ng, 2004; see Cherny et al., 2001, Ritchie et al., 2003 and Atwood et al., 2004 for effect of metals and chelators on Aβ). Curcumin may also reduce amyloid by its binding to aggregated Aβ, similarly to other Aβ-binding compounds such as Congo Red, Chrysamine G and RS-0406 (as reviewed in Yang et al., 2005; Garcia-Alloza et al., 2007). The binding of these compounds to Aβ prevents its polymerisation into oligomers and fibrils, thereby stabilising Aβ monomers and preventing Aβ aggregation (Podlisny et al., 1998; Nakagami et al., 2002). Since curcumin also labels Aβ and has similar structural properties to these compounds, it is thought that curcumin reduces Aβ pathology through the same processes. The curcumin metabolite tetrahydrocurcumin can also attenuate aggregated Aβ, although is less effective due to the loss of the dienone structure (Begum et al., 2008).

B. Affects on tau pathology

While curcumin can influence Aβ, the influence of this compound on tau pathology remains less clear. Garcia-Alloza et al. (2007) failed to report any effects of curcumin on tau pathology in a triple transgenic AD model. However, other evidence suggests potential for curcumin to alter tau pathology as curcumin altered tau-associated kinases such as CDK and ERK (as reviewed in Lin, 2004; Giri et al., 2004; Kim et al., 2008). Furthermore, the inhibition of Aβ fibril formation by several polyphenolic compounds was shown to also inhibit tau aggregation (Taniguchi et al., 2005). Although curcumin was not investigated in

this study, the latter shows that polyphenols have the potential for reducing tau pathology. Further investigation is therefore required.

C. Modulation of inflammatory processes

Curcumin is well known for its anti-inflammatory properties and may thus be effective in reducing the chronic state of neuroinflammation present in AD. Curcumin regulates several inflammatory mediators linked to AD including transcription factors (e.g. NFκB), cytokines (e.g. TNF-α, IL-1, IL-6 and IL-8), protein kinases (e.g. p38 MAP, IkBα, Akt), enzymes (e.g. LOX, COX, iNOS) and adhesion molecules (e.g. leukotrienes) both *in vivo* and *in vitro* (as reviewed in Chainani-Wu, 2003; Aggarwal & Harikumar, 2009). Furthermore, indices of inflammation such as GFAP, microglial activation and IL-1β are reduced by curcumin in animal models of AD (Frautschy et al., 2001; Lim et al., 2001; Begum et al., 2008) and other inflammatory disorders such as inflammatory bowel disease, arthritis and cardiovascular disease (Aggarwal & Harikumar, 2009). Interestingly, Yang et al. (2005) reported curcumin to be more effective at reducing amyloid pathology through its anti-inflammatory effects than high doses of NSAIDs.

Evidence suggests that the anti-inflammatory effects of curcumin can be concentration-dependant (e.g. Abe, Hashimoto & Horie, 1999; Grandjean-Laquerriere, et al. 2002; Chun et al., 2003) and some studies suggest its effectiveness is dependent upon large doses (e.g. Huang et al., 1991; Sharma et al., 2004). Furthermore, evidence suggests that anti-inflammatory effects are dependent upon curcumin supplementation prior to inflammatory development (e.g. Chun et al., 2003; Funk et al., 2006). This may suggest that in order for curcumin to reduce inflammation in AD, it may need to be taken in large doses prior to inflammatory changes. This was a major factor in considering the experimental design described in this thesis.

D. Antioxidant effects

Curcumin has potent antioxidant activities by targeting multiple sources of oxidative stress, even at small doses (Chan et al., 1998). Curcumin is a potent free radical scavenger of ROS including superoxide, peroxyl and hydroxyl radicals (Fujisawa et al., 2004). Curcumin also acts as a scavenger of NO-based free radicals such as nitric oxide and peroxynitrite, in

addition to iNOS (inducible nitric oxide synthase) which catalyses NO production (Sreejayan & Rao, 1997; Chan et al., 1998; as reviewed in Butterfield et al., 2002). Curcumin can also increase levels of the antioxidant glutathione (Jaruga et al., 1998; Piwocka et al., 2001) and activate the antioxidant response element (ARE) by affecting transcription factors such as Nrf2, HIF-1 and NFκB (Balogun et al., 2003; Calabrese et al., 2008). Furthermore, curcumin can inhibit enzymes involved in lipid oxidation including lipoxygenase and phospholipase D (Yamamoto et al., 1997; Began, Sudharshan & Rao, 1998; Skrzypczak-Jankun et al., 2000). As a result, curcumin can inhibit lipid peroxidation, protein oxidation and oxidative DNA damage (as reviewed in Butterfield et al., 2002). Such antioxidant effects have been demonstrated *in vivo* in animal models of AD (e.g. Frautschy et al., 2001; Lim et al., 2001; Begum et al., 2008). The reduction of lipid peroxidation in AD is particularly important as it otherwise exaggerates neurodegeneration by oxidising phospholipids (Asai, Nakagawa & Miyazawa, 1999; Butterfield & Lauderback, 2002).

Conversely, curcumin possesses pro-oxidant properties (Ahsan et al., 1999; Sandur et al., 2007a,b). This is well-documented in cancer studies whereby curcumin exerts pro-oxidant effects by generating ROS which arrests cancer cell proliferation and induces apoptosis (e.g. Javvadi et al., 2008; Kim & Lee, 2010; Lee et al., 2011). Although the mechanism mediating these opposing activities of curcumin is not fully understood, some evidence suggests that the switch between anti- and pro-oxidant activities may be concentration dependant (Aggarwal & Harikumar, 2009). However, other mechanisms must be involved as curcumin can induce opposing activity within the same dose study. Further research is therefore required to understand the oxidative properties of curcumin. This is particularly important if used to treat AD as pro-oxidant effects could exacerbate AD pathology.

E. Neurological effects and modulation of memory processes

There is accumulating evidence that curcumin is neurogenerative, neuroprotective and can influence memory processes. Firstly, curcumin administration can promote neurogenesis and development in the dentate gyrus of adult mice, as well as neurogenesis and synaptogenesis *in vitro* at low concentrations in neural progenitor and brain-derived adult neural stem cells (Kang, Cha & Jeon, 2006; Kim et al., 2008). This neurogenic effect may be particularly valuable in disorders such as AD whereby extensive neurodegeneration occurs. Interestingly however, Kim et al. (2008) found high concentrations of curcumin exerted cytotoxic effects

in vitro but not *in vivo*, highlighting the need for further investigation. Evidence supports a neuroprotective role of curcumin against age-related brain changes, traumatic injury and neurotoxic-induced effects, including administration of Aβ, kainic acid, lead and ethanol (Rajakrishnan et al., 1999; Kim, Park & Kim, 2001; Shukla et al., 2003; Yang et al., 2005; Bala, Tripathy & Sharma, 2006; Sumanont et al., 2006; Cole, Teter & Frautschy, 2007). These effects were closely linked with the antioxidant and anti-inflammatory properties of curcumin. The curcumin metabolite tetrahydrocurcumin was also neuroprotective against Aβ-induced toxicity (Begum et al., 2008; Mishra et al., 2011). Curcumin can also normalise memory and synaptic plasticity after injury (Wu, Ying & Gomez-Pinilla, 2006) and curcumin can inhibit JNK activation which may prevent LTP deficits (Chen & Tan, 1998; Begum et al., 2008).

ii) Curcumin studies in humans: epidemiological evidence

Epidemiological studies show urban and rural Indian populations have some of the lowest prevalence and incidence rates of AD and dementia worldwide (Chandra et al., 1998, 2001; Vas et al., 2001). Interestingly, Chandra et al. (2001) reported that a Northern Indian rural population in Ballabgarh had a 1 in 3.7 less risk of AD than an American population in the Monongahela Valley of Pennsylvania. Similarly, Ganguli et al. (2000) reported a 4.4-fold lower prevalence of AD in 70 to 79 year olds in Ballabgarh compared with the Monongahela valley population. They also calculated the probable frequency of AD in the Indian and American populations to be 0.7% and 3.1% in those aged 70 to 79 years, and 4.0% and 15.7% in those aged over 80 years, respectively. These studies therefore suggest that the American and Indian population had important differences in dementia risk factors. Consistent with this, Ganguli et al. (2000) reported a lower frequency of ApoE ε4 status in Ballabgarh (0.07) compared to the Monongahela Valley (0.11). This lower genetic risk (associated with AD) may be one factor that accounted for the reduced prevalence of AD in Indian populations.

However, another proposed factor is that of diet. Ng et al. (2006) reported that increased curry consumption was associated with better cognitive performance in a non-demented Asian population aged between 60 and 93 years (n=1010). Since the spice turmeric is used and consumed in large amounts in curries, this spice may be responsible for the observed effect. Although numerous alternative explanations could explain the results, these studies

have provided grounds for the initiation of controlled experimental studies into turmeric treatment for AD using its pharmacologically active substance, curcumin.

iii) Curcumin studies in humans: clinical trials

Two human clinical trials investigating curcumin supplementation in AD have been conducted. The first clinical trial was completed by the NIA in 2007 (ClinicalTrials.gov identifier: NCT00099710). This phase II, double-blind, placebo-controlled study used two doses of curcumin C3 complex® (2000 or 4000mg/day) in 33 mild-to-moderate AD patients over 24 weeks, followed by an extension study for another 24 weeks using one of the two curcumin doses (Ringman et al., 2005). Unfortunately however, no results have been published and the NIA assert that the study may be ongoing, extended or result submission has been delayed. No conclusions can therefore be obtained from this study.

In contrast, Baum et al. (2008) published results from a randomised, double-blind, placebo-controlled pilot clinical trial investigating 6-month curcumin supplementation (1g/day or 4g/day) in 27 AD patients. No cognitive protective effect was reported, likely due to the lack of cognitive decline detected during the short study period. However, Aβ40 serum levels were increased, perhaps suggesting that curcumin disaggregated Aβ deposits in the brain which were released into circulation. The interpretation of these results is limited and longer and larger trials are required to determine the efficiency of curcumin in AD. Another limitation of this study is that 120 mg/day standardized ginkgo leaf extract was supplemented in combination with curcumin and the placebo. Although the design has controlled for this in both the curcumin and placebo group, it is not possible to ascertain whether the outcome is related to curcumin alone or an interaction between the curcumin and ginkgo leaf extract. In conclusion, no human clinical trial provides strong evidence for a beneficial effect of curcumin on cognitive function or pathology in AD.

iv) Curcumin studies in rat and transgenic mouse models of Alzheimer's disease

Dietary curcumin treatment has been examined in two rat models of AD pathology. In Aβ-infused rats, Frautschy et al. (2001) found 1 month pre-treatment of 2000ppm curcumin reduced plaque pathology and protected against Aβ-induced lipid peroxidation, synaptophysin loss and microglia activation. Conversely, microglial labelling within and

around Aβ deposits was increased, suggesting phagocytic plaque clearance. They also reported that 2 month pre-treatment of 500ppm curcumin protected against Aβ-induced spatial memory deficits in the Morris Water Maze and PSD-95 loss. Interestingly, the 500ppm dose was found more effective at reducing plaque pathology than 2000ppm treatment. Ishrat et al. (2009) examined curcumin treatment in a sporadic AD rat model which expresses oxidative neuronal damage, neuronal dysfunction (such as cholinergic deficiency) and cognitive deficits caused by a cerebroventricular injection of streptozotocin (e.g. Prickaerts, Fahrig & Blokland, 1999; Sharma & Gupta, 2001). Ishrat et al. (2009) found 3 week oral post-treatment of curcumin (80mg/kg body weight) ameliorated cognitive deficits in the passive avoidance and water maze task, attenuated markers of oxidative stress, and increased glutathione levels.

Garcia-Alloza et al. (2007) examined curcumin treatment in 7.5 to 8.5 month old double transgenic APP_{swe}/PS1(dE9) mice, when amyloid pathology is well developed. Daily i.v. injection of curcumin (7.5mg/kg/day) for 7 days labelled senile plaques and cerebrovascular amyloid angiopathy, illustrating that curcumin can cross the blood-brain barrier and bind to amyloid deposits. Longitudinal *in vivo* imaging showed 7-day treatment reduced the size and number of plaques. In contrast, treatment failed to reduce soluble and insoluble Aβ40-42 and interestingly increased the soluble Aβ42:Aβ40 ratio. Although the latter is associated with neurotoxic effects (Walker et al., 2005), the authors suggest this may have simply been the immediate effects of curcumin. Curcumin treatment also reduced the size of dystrophic neurites immediately surrounding senile plaques and reversed dendritic morphological abnormalities (dendritic curvature) near and far from plaques, suggesting reduced toxicity caused by both aggregated and soluble Aβ. In summary, low dose short-term curcumin treatment reduces plaque pathology and the neurotoxic effects of Aβ on dendrites. Ma et al. (2009) investigated the effect of 4-month 500ppm dietary curcumin supplementation in 5-month old triple transgenic mice relative to a high-fat poor diet (high in saturated fat and omega-6 PUFA). Curcumin suppressed poor diet-induced tau phosphorylation, abnormal insulin signalling and pJNK (latter was non-significant). Furthermore, curcumin improved cognitive performance in the Y-maze following 2 months of dietary supplementation, but not after 1 month. No measures related to amyloid pathology (e.g. APP, PS1 or Aβ) were reported.

Lim et al. (2001) examined the effect of 6-month 160ppm low-dose and 5000ppm high-dose curcumin in Tg2576 mice from 10 months of age. Both doses significantly reduced inflammation (IL-1β) and oxidative stress (protein carbonyls), although low-dose curcumin also significantly reduced GFAP and microglia activation. Consistent with Frautschy et al. (2001) however, microgliosis was increased immediately outside and within plaques, suggesting amyloid phagocytosis. Indeed, low but not high-dose curcumin significantly reduced soluble and insoluble Aβ, and plaque burden. Consistent with unpublished findings by Chu, Lim and Cole (reported in Lim et al., 2001), high-dose curcumin suppressed amyloid clearance by glia in organotypic hippocampal slice cultures. This study reported no changes to APP production, suggesting that curcumin alters post-Aβ production mechanisms. In another study, Yang et al. (2005) examined the effects of a 5-month medium-dose (500ppm) curcumin supplementation in Tg2576 mice aged 17 months, relative to a control safflower oil-based diet. Curcumin supplementation significantly reduced insoluble Aβ by 85% and plaque burden by 33%. *In vitro* studies supported this, showing curcumin reduced amyloid aggregates and Aβ toxicity, even at micromolar concentrations. A 200µl i.v. injection of 50µM curcumin was also found to label plaques to a greater extent than dietary administration, suggesting curcumin can cross the blood-brain barrier but is less bioavailable following oral administration.

Begum et al. (2008) examined the effects of 4-month curcumin supplementation in 14.5 month old Tg2576 mice compared to control chow. Supplementation of tetrahydrocurcumin (TC), a curcumin metabolite, was also examined. Chronic feeding of 500ppm curcumin or TC resulted in a similar reduction of inflammatory markers IL-1β and GFAP. Curcumin was also found to reduce protein carbonyl levels, insoluble Aβ, soluble Aβ, plaque size and number. In contrast, TC failed to significantly reduce protein carbonyl levels, plaque pathology and insoluble Aβ, but was more effective at reducing soluble Aβ. This greater reduction by TC was correlated to a greater reduction in pJNK, a stress-activated kinase which is thought to influence Aβ (Borsello & Forloni, 2007). Curcumin also reduced pJNK, suggesting a potential mechanism for soluble Aβ reduction. Supporting the reduction of inflammatory markers by curcumin and TC, Begum et al. (2008) also reported that acute administration of these compounds by gavage, i.p. or i.m. reduced markers of inflammation and oxidative stress in lipopolysaccharide-injected C57BL6/J mice (a model of induced inflammation) compared to vehicle. These markers included IL-1β, iNOS, F2-isoprostanes and protein carbonyls. Curcumin was reported more effective than TC, and both studies

reported curcumin to reach the brain at higher levels. Since curcumin is naturally metabolised into TC, this highlights the use of curcumin for AD treatment.

In conclusion to these studies, it is evident that curcumin can effectively reduce multiple aspects of AD-like pathology. Although further investigation is required to examine the optimal treatment dose, it appears that 500ppm curcumin dosage was effective, as were lower 160ppm doses. The inefficiency of higher doses (5000ppm) to reduce amyloid pathology questions the mechanistic actions of curcumin and warrants further examination. Furthermore, all transgenic mouse studies have examined the effect of curcumin treatment during advanced stages of pathological development only, highlighting the need for examination at early stages which may unlock its full clinical potential in preventing or delaying the onset of pathology. Supporting this, several studies showed curcumin was more potent or only effective at reducing pathological measures such as inflammation when administered prior to its development (e.g. Chun et al., 2003; Funk et al., 2006). This could explain the limited results in clinical trials. This thesis therefore examined the effects of early administration of curcumin on Aβ pathology and cognitive deficits in Tg2576 mice.

1.7 Modelling Alzheimer's disease

The previous sections have outlined the rationale for exploring the putative beneficial effects of diet on AD pathogenesis. Clearly in evaluating these compounds on AD-like pathology a suitable animal model is required. This section will outline the use of animal models for Alzheimer's disease. Firstly, it will discuss the advantages and disadvantages of studying human disease in an animal model, followed by a summary of the different types of animal model used to study AD. Finally, a more detailed discussion of the Tg2576 model will be delivered in order to give an outline of the model utilised in the experimental chapters.

1.7.1 Why use animal models? The advantages and disadvantages

Studying human disease using animal models and human subjects both have their advantages and flaws. Neither alone can provide a comprehensive account, and so we depend on translational research which encompasses a bidirectional flow of information between animal and human research (Trojanowski et al., 2008). The study of human disease using human

subjects is ideal but limited due to the large number of extraneous factors such as environmental and genetic differences. Another constraint of human research is the limited time period which can practically be examined, which is a particular problem when investigating chronic diseases which develop over long periods of time. Another drawback is the detail or accuracy of data that can be obtained.

Animal models have therefore been developed which offer a valuable opportunity to investigate disease and intervention in a tightly controlled manner. For example, the development of transgenic models allows a large degree of genetic homogeneity in the sample population which may otherwise interfere with the research question. Environmental factors can also be significantly controlled and maintained for all subjects, such as dietary intake. Furthermore, the relatively short lifespan of animals allows rapid and comprehensive collection of longitudinal data, which is impractical in human studies. Moreover, animal studies allow more comprehensive collection of data, from biological samples to behavioural analysis, and increased control of when these data are collected. In contrast, the major disadvantage of using animal models to study human disease is their limited capacity to model the disease state and important physiological species-differences. Although models can encapsulate some of the main characteristics and symptomatic features of the disease, none can simulate the disease exactly in humans which limit the application of findings. Despite this, the ability to model specific aspects of AD pathology in animals is advantageous in allowing further understanding of pathological processes and potential intervention effects on specific pathologies.

1.7.2 Animal models of Alzheimer's disease

Animal models can be used to study AD by reproducing some behavioural and pathological changes observed in AD patients. A basic type of model uses non-transgenic rodents injected with pathological or pathological-inducing substances, such as Aβ, lipopolysaccharide or streptozotocin, into the cerebroventricular area of the brain. These substances induce features of AD such as amyloid pathology, neuroinflammation, oxidative damage, cognitive deficits and neuronal dysfunction (as outlined previously; reviewed in Yamada & Nabeshima, 2000). Tau pathology can also be induced by inhibiting phosphatase activity (e.g. by i.c.v infusion of okadaic acid) or through potentiating kinase activity in order to produce hyperphosphorylated tau and NFTs (Arendt et al., 1994, 1995). Although these non-transgenic models are useful

for investigating therapeutic effects on pathology, these models have limited face and construct validity.

The identification of genes associated with familial and sporadic forms of AD have made possible the development of transgenic mouse models, which arguably have greater face and construct validity. This approach inserts or deletes known human genetic mutations into the mouse genome. Deleted-gene 'knockout' models are used to explore gene function and role in AD pathogenesis (Spires & Hyman, 2005). More commonly, genes are inserted in order to induce pathological development and associated behavioural deficits. The three mutations commonly inserted include APP, tau and PS1 genes. Many types of AD model have been developed based on these mutations, including triple (possessing all three mutations), double and single transgenic mice. Importantly, these models develop pathology over time and so can be used to understand pathological processes, as well as examine potential therapeutic measures. A summary of the most common mouse models used to study AD are provided in Table 1.4 and will be described briefly in the following subsections. Other less commonly used models will also be discussed, although a more systematic review of AD models can be found in Spires and Hyman (2005).

i) Triple transgenic models

One of the most successful replications of AD neuropathy is the triple transgenic mouse model (3xTg-AD) which harbours $PS1_{M164V}$, APP_{swe} and Tau_{P301L} mutations (Oddo et al., 2003). It was the first model to develop both accelerated amyloid and tau pathology, allowing examination of their interaction which was particularly relevant to AD. This model displays an age-related progression of pathology which shows a regional distribution pattern similar to human AD brains, as well as Aβ deposits preceding tau pathology (Oddo et al., 2003). Likely to be initiated by intracellular Aβ deposition at 3 months, behavioural deficits and synaptic dysfunction develop around 4 to 6 months, with NFT development at a later age (Table 1.4; Oddo et al., 2003; Billings et al., 2005).

ii) Double transgenic models

A number of double transgenic mice carrying mutant PS1 and APP transgenes have been developed (Table 1.4). These mice display amyloid pathology and behavioural deficits, as

well as cerebral amyloid angiopathy, oxidative stress and dystrophic neurites (Holcomb et al., 1998, 1999; Arendash et al., 2001b; Garcia-Alloza et al., 2006a,b; Jankowsky et al., 2004a). Importantly, amyloid pathology and cognitive dysfunction develop at a greater rate and earlier age than single APP mutant mice, due to the PS1 mutation which alters APP processing to preferentially deposit high levels of Aβ42 (Borchelt et al., 1997; Holcomb et al., 1998; Westerman et al., 2002). Although this allows study of pathological development within a shorter time period, this may be problematic if examining behavioural changes in large cohorts or if aiming to target therapeutic intervention before pathological development. Double transgenic mice harbouring Tau and APP mutations are used model both tau and amyloid pathology, such as the JNPL3/Tg2576 cross (Lewis et al., 2001). Interestingly, amyloid pathology develops at the same age as Tg2576 mice yet tau pathology is enhanced compared to JNPL3 mice, thereby supporting literature suggesting that Aβ exacerbates tau pathology. Furthermore, models lacking amyloid pathology such as $PS1_{M146V}/Tau_{P301L}$ mice fail to produce LTP deficits despite expressing tau similarly to 3xTg-AD mice, which highlights the importance of APP mutations and Aβ pathology (Oddo et al., 2003).

Table 1.4 Summary of current mouse models used to study Alzheimer's disease. Table adapted and extended from Reddy & McWeeney 2006.

Mouse model	Pathological changes	Behavioural changes	References
APP mutations:			
Tg2576 ($APP_{swe/K670M/N671L}$)	Aβ deposits from 8–9 months	From 6 months	(Hsiao et al., 1996)
TgCRND8 $_{(APPswe+V717I)}$	Aβ deposits from 3 months, amyloid plaques from 5 months	From 3 months (e.g. MWM reference memory)	(Chishti et al., 2001)
$PDAPP_{(V717F)}$	Aβ deposits from 6-9 months	From 6-9 months	(Games et al., 1995; Chen et al., 2000)
APP23 (APP_{swe})	Aβ deposits from 3-6 months dependant on expression levels	From 3 months (e.g. MWM, hyperactivity, open-field, PA)	(Sturchler-Pierrat et al., 1997; Kelly et al., 2003; Van Dam et al., 2003)
C3-3 (APP_{swe})	Aβ deposits, ↑ Aβ40-42, plaques at 18 mon.	Not reported	(Borchelt et al., 1997)
PS1 mutations:			
PS1	↑ Aβ42	Not reported	(Duff et al., 1996)
$PS1_{P264L/P264L}$	↑ Aβ42	Not reported	(Flood et al., 2002)
Tau mutations:			
$JNPL3_{(P301L)}$	NFTs from 4.5-6.5 months	Motor and behavioural deficits from 4.5 months	(Lewis et al., 2000)
H Tau40	Axonal degeneration, no NFTs	Sensorimotor dysfunction	(Spittaels et al., 1999)
H Tau	↑ hyperphosphorylated tau at 6 months, tau redistribution (3 mon.), NFTs (9mon.)	Cognitive dysfunction from 12 months (e.g. Object recognition, MWM)	(Andorfer et al., 2003; Polydoro et al., 2009)
Double mutations:			
PS1(dE9) x Tg2576	Aβ deposits from 4-6 months, high Aβ42/Aβ40 ratio	Cognitive dysfunction (from 3m, before Aβ) (e.g. Y-maze, water maze, RAWM)	(Holcomb et al., 1998, 1999; Arendash et al. 2001b; Garcia-Alloza et al. 2006b)
PS1 x TgCRND8	Aβ deposits from 1 month	Not reported	(Chishti et al., 2001)
$PS1_{P264L/P264L}$ x Tg2576	Aβ deposits from 6 months, ↑ Aβ42	Not reported	(Flood et al., 2002)
$PS2_{N141I}$ x APP_{swe}	Aβ deposits from 5 months	From 8 months	(Richards et al., 2003)
JNPL3 x Tg2576	Aβ deposits from 8-9 months, accelerated tau pathology	Not reported	(Lewis et al., 2000)
Triple mutations:			
3xTg PS1/APP/Tau	Aβ deposits from 3-6 months, NFTs from 12 months	From 4 months (e.g. MWM, Contextual fear conditioning)	(Oddo et al., 2003; Billings et al. 2005)

RAWM = Radial arm water maze, MWM = Morris water maze, PA = passive avoidance, mon. = months.

iii) Single transgenic models: Tau, presenilin and ApoE mutations

Single transgenic models have been used to study pathological features of AD in isolation, which is advantageous in further understanding their role in AD. PS1 models are useful for examining the role of PS1 and the preferentially expressed Aβ42, although the lack of plaque development limits their application in modelling amyloid pathology (Flood et al., 2002). Models expressing human tau mutations have also been developed since APP models show limited tau hyperphosphorylation and fail to exhibit NFTs or significant neuronal loss (Takeuchi et al., 2000; Kurt et al., 2003). Although no tau mutations have been identified in AD patients, tau mutations associated with frontotemporal dementia were found to cause aggregation of hyperphosphorylated tau and NFT development similar to that observed in AD, and so were used to model tauopathy in AD (Götz, 2001; Andorfer et al., 2003; Lewis et al., 2000). Such tau-expressing models (Table 1.4) show age-dependant tau hyperphosphorylation, tau redistribution and accumulation, NFT formation and extensive atrophy (Andorfer et al., 2003). Tau models have also been developed using mutations of phosphatases and kinases involved in tau hyperphosphorylation (reviewed in Götz, 2001). ApoE models were developed to further understand the role of ApoE in AD since ApoE ε4 is a major genetic risk factor (Saunders, 1993; Strittmatter et al., 1993). Models include ApoE or ApoE allele overexpression or deficient (knock out) mice (reviewed in Spires & Hyman, 2005). Such models provide evidence for the importance of ApoE in neuronal and cognitive function, and Aβ development (Masliah et al., 1995, 1997; Tibolla et al., 2006).

iv) Single transgenic APP mutant models

The discovery of APP mutations on chromosome 21 in familial AD cases and its involvement in overproducing the principal pathological hallmark β-amyloid led to the development of the first transgenic AD models which reproduced aspects of amyloid pathology similar to that observed in AD (Chapman et al., 2001; Spires & Hyman, 2005). Table 1.4 presents a variety of single, double and triple transgenic models harbouring APP mutations. A review of each model is beyond the scope of this chapter, and so a more comprehensive review can be found in Reddy and McWeeney (2006) or Eriksen and Janus (2007). This section will briefly outline the development of APP models and their main characteristics. Due to its use in the experimental chapters, the Tg2576 mouse model will be detailed in the next subsection.

The pathological and behavioural phenotype of APP transgenic mice is dependent upon the APP missense mutation (Figure 1.4), background strain, APP primary structure (ranging from 563 to 770 amino acids in length) and the level of APP expression controlled under different gene promoters (Hsiao et al., 1996; Lamb et al., 1997). The most common APP mutations used to generate AD models include the APP_{V717} and the Swedish double APP ($APPswe_{K670M/N671L}$) mutations. Specifically, the human APP_{V717} gene mutation harbours the amino acids phenylalanine (F), glycine (G) or isoleucine (I) in substitution of valine (V) at codon 717 in exon 17 of the APP gene (Lamb et al., 1997; Figure 1.4). These mutations are referred to as the APP_{V717F}, APP_{V717G}, and APP_{V717I} mutation, respectively. The APPswe gene mutation is detailed in the Tg2576 model section (section 1.7.3). As illustrated in Figure 1.4, the majority of APP mutations occur close to or at the cleavage sites of APP, resulting in altered APP processing and Aβ production (Mullan et al., 1992). For example, mutations located around the β-secretase cleavage site are associated with a reduction in sAPPα products and increased Aβ (Lamb et al., 1997).

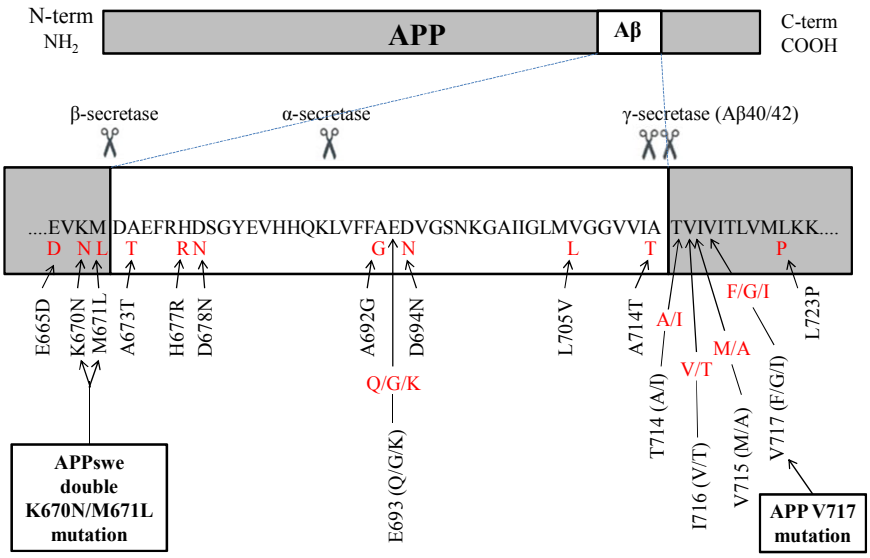

Figure 1.4 Amyloid precursor protein (APP) with the β-amyloid (Aβ) segment enlarged to show α-, β- and γ-secretase cleavage sites, the amino acid sequence (letters) and substitution sites (in red) of the APP gene associated with FAD mutations. The two most commonly used APP mutations, $APPswe_{K670M/N671L}$ and APP_{V717}, have been labelled. Information sourced from Van Dam & De Deyn (2006).

As shown in Table 1.4, the APP_{V717F} mutation was used to create the PDAPP model. This mutation is driven by the PDGF-β (platelet-derived growth factor-β) promoter in a Swiss Webster-B6D2F1 mouse strain (Games et al., 1995). APP is overexpressed and the mutation located around the transmembrane domain of APP (cleaved by γ-secretase) leads to a greater production of longer Aβ peptides terminating at amino acid 42(43), resulting in an elevated Aβ42:Aβ40 ratio (Suzuki et al., 1994; Lamb et al., 1997). This results in early and extensive AD-like neuropathy including plaque formation, synaptic density loss, phosphorylated tau, memory impairment, and plaque-associated neuroinflammation (Games et al., 1995; Chen et al., 2000; Lanz, Carter & Merchant, 2003). This model therefore shows amyloid pathology and neuronal dysfunction similar to AD (Masliah et al., 1996; Irizarry et al., 1997).

The Swedish double $APP_{K670M/N671L}$ mutation was used to create the Tg2576, APP23, C3-3 and TgCRND8 model (Table 1.4). APP23 mice express this mutation under the control of the murine Thy1.2 promoter on the C57BL6/DBA2 strain (Sturchler-Pierrat et al., 1997). The C3-3 model expresses this mutation on the C57BL6/C3H background strain, driven by the mouse prion promoter (Borchelt et al., 1997). This model develops plaques at a much later stage than the other models which have much earlier onset of amyloid pathology. The CRND8 model expresses both the APPswe and APP_{V717I} (London) mutation, driven by the Syrian hamster prion (PrP) promoter on the C57BL6/CH3 background strain (Chishti et al., 2001). This causes early amyloid development and cognitive impairment, but can cause premature death dependent upon background strain, thereby highlighting the importance of genetic background on the effects of APP overexpression.

These APP mutations therefore generate AD models that exhibit amyloid pathology and Aβ-associated neuropathy including senile plaques, synaptic changes, cholinergic degeneration, mitochrondrial abnormalities, neuroinflammation and hyperphosphorylated tau in an age- and distribution-dependant manner similar to that found in AD patients (Reddy & McWeeney, 2006). Plaque pathology is particularly characteristic of that observed in human patients, including fibrillar Aβ cores surrounded by dystrophic neurites, activated microglia and astrocytes (Sturchler-Pierrat, 1997; Calhoun et al., 1998). These APP mutations typically lead to cognitive dysfunction similarly observed in AD patients including memory deficits, executive dysfunction and behavioural changes to exploration and anxiety (e.g. Lalonde et al., 2002; Kelly et al., 2003; Van Dam et al., 2003). In contrast to AD patients however, these APP models fail to exhibit NFT development and neuronal loss (as reviewed in Reddy &

McWeeney, 2006). An exception to this is the APP23 model which shows some limited neuronal death in CA1 pyramidal neurons (Calhoun et al., 1998).

1.7.3 The Tg2576 mouse model

The Tg2576 mouse model is the focus of the animal models section as it is the AD model of choice used in the experimental chapters. The following subsections will detail the APP mutation, its resulting pathology and phenotypic characteristics, as well as justify the use of the model in the experimental chapters. Particular reference will be made to how accurately the Tg2576 model mimics AD in human patients.

i) The Swedish double APP mutation

The Tg2576 model expresses the human Swedish double $APP_{K670N/M671L}$ mutation which was discovered in a Swedish family with early-onset familial AD (Hsiao et al., 1996). As illustrated in Figure 1.4, this mutation harbours the amino acid asparagine (N) in substitution of lysine (K) at codon 670 (referred to as K670N), and leucine (L) in substitution of methionine (M) at codon 671 (referred to as M671L) in exon 16 of the APP gene (Mullan et al., 1992; Lamb et al., 1997). This 695-amino acid mutation in the Tg2576 model is controlled under the hamster prion protein promoter in a hybrid background strain of C57BL6/SJL (Hsiao et al., 1996). The resulting mutation leads to the overexpression of transgenic APP in the brain which is ~5.5 fold higher than endogenous mouse APP (Hsiao et al., 1996). The mutation occurs close to the β-secretase amyloidogenic processing site of APP, leading to increased cleavage by β-secretase and Aβ production (Figure 1.4; Mullan et al., 1992).

ii) Pathology

The Tg2576 model shows several features of amyloid neuropathy similar to AD patients. Firstly, they show an age-related increase in Aβ in multiple brain regions including the cortex (frontal, temporal and entorhinal cortex), hippocampus, presubiculum and subiculum, but limited in the cerebellum (Hsiao et al., 1996; Irizarry et al., 1997; Irizarry, Locascio & Hyman, 2001; Praticò et al., 2001; Bizon, Prescott & Nicolle, 2007). Soluble Aβ levels start to increase in the Tg2576 model from 4 to 5 months of age, which becomes significant by 6

to 7 months (Hsiao et al., 1996; Kawarabayashi et al., 2001; Westerman et al., 2002; Jacobsen et al., 2006; Bizon, Prescott & Nicolle, 2007). Between 6 to 10 months of age, insoluble forms of Aβ increase exponentially, mirrored by a slight reduction in soluble forms suggesting their conversion to insoluble forms (Kawarabayashi et al., 2001). Significant levels of insoluble Aβ are present in 70% of mice at 6 months and 100% at 10 months (Westerman et al., 2002). Accordingly, diffuse deposits and senile plaques start to develop between 9 to 12 months of age (Hsiao et al., 1996; Kawarabayashi et al., 2001; Bizon, Prescott & Nicolle, 2007). However, these insoluble forms of Aβ do not produce significant numbers of diffuse plaques until 12 months, and between 12 to 23 months these diffuse plaques increase and neuritic plaques with amyloid cores develop (Kawarabayashi et al., 2001). Consistently, Jacobsen et al. (2006) reported a detectable but modest scattered plaque load at 12 months which becomes significant by 18 months.

An amyloid burden similar to AD patients of 4 to 8% in affected regions was reported at 16 months by Irizarry, Locascio and Hyman (2001), although Kawarabayashi et al. (2001) reported comparable levels at 21 months. Between 2 and 8 months, Tg2576 mice have a 5-fold increase in Aβ40 and 14-fold increase in Aβ42 concentration (Scheuner et al., 1996). Consistently, Hsiao et al. (1996) reported that 11 to 13 month old mice had a greater Aβ40:Aβ42 ratio. This significant deposition of Aβ40 is similar to that found in ~33% of AD patients, although ~67% patients show very little Aβ40 deposition (Gravina et al., 1995). Also similar to ~33% of AD cases, Tg2576 mice show marked angiopathy (Gravina et al., 1995). Other parallels to the AD brain include Aβ deposits with fibres radiating from a central mass and some small deposits possessing an amyloid core with a 'Maltese cross' structural pattern (Hsiao et al., 1996). This model also shows multiple aspects of amyloid-associated pathology similar to aged AD brains including amyloid plaques surrounded by dystrophic neurites at 15 to 16 months, reactive astrocytes at 18 months and activated microglia from 10 months (Irizarry et al., 1997; Frautschy et al., 1998; Sasaki et al., 2002; Jacobsen et al., 2006). Importantly, this plaque-associated microgliosis is evidence of increased neuroinflammation, similar to AD patients. Consistent with this inflammatory response, other markers of inflammation have been reported in Tg2576 mice including IL-1β, TNF-α, COX-2, complement protein C1q and chemoattractant protein-1 (Lim et al., 2000, 2001; Sastre et al., 2006).

As well as neuroinflammation, the Tg2576 model shows a widespread pattern of oxidative stress similar but not identical to that observed in AD brains. Oxidative damage is one of the earliest events in the human AD brain (Nunomura et al., 2001). Mirroring this, lipid peroxidation was significantly elevated from 8 months of age in Tg2576 mice before plaque deposition (Praticò et al., 2001). Other oxidative markers increased globally in an age-dependant manner between 13 and 25 months (Pappolla et al., 1998; Smith et al., 1998; Lim et al., 2001). Some markers correlated with localisation or burden of plaque deposition, although others did not, suggesting some role of plaques in oxidative stress. The lack of oxidative markers at 4 months of age prior to the onset of Aβ development suggests the involvement of Aβ accumulation (Pappolla et al., 1998).

Tg2576 mice show an age-related disruption of neuronal processes emerging from an early age. Transgene-dependant reductions in CA1 and DG hippocampal dendritic spine density were observed from approximately 4 months of age in Tg2576 mice, with further reductions reported at 11-12 and 18-20 months (Figure 1.5; Lanz, Carter & Merchant, 2003; Jacobsen et al., 2006). Similar losses were observed in AD patients within this region (Scheff, Sparks & Price, 1996). These significant dendritic spine deficits were not observed at 2 months of age, suggesting a modulatory role of soluble Aβ (Lanz, Carter & Merchant, 2003; Jacobsen et al., 2006). Jacobsen et al. (2006) also reported impaired LTP and a basal synaptic transmission deficit at 4 to 5 months in the DG of hippocampal slices. Consistent with early changes, Klingner et al. (2003) reported cholinergic and noradrenaline neurotransmitter receptor alterations at 5 and 17 months of age. The early emergence of synaptic deficits in Tg2576 mice was central to the rationale for using this model in evaluating early dietary interventions presented in the experimental chapters.

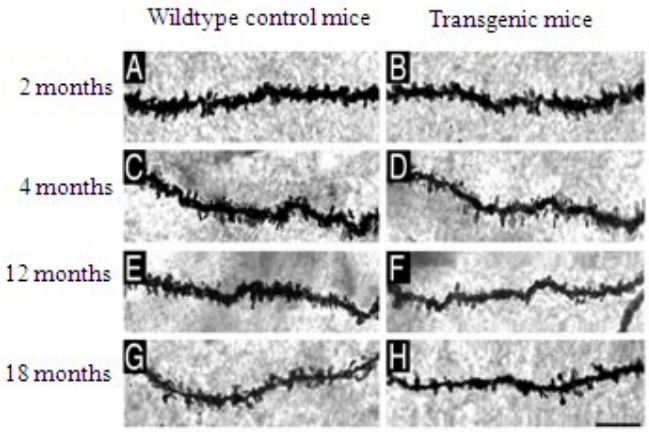

Figure 1.5 Loss of dendritic spine density in the DG region of the hippocampus in Tg2576 mice (B, D, F and H) relative to wildtype mice (A, C, E and G) at 2, 4, 12 and 18 months, respectively. Figure sourced from Jacobsen et al., 2006.

In contrast to reports of early synaptic deficits, Chapman et al. (1999) failed to find LTP impairments in Tg2576 mice aged 2 to 8 months, although confirmed CA1 and DG impairments at 15 to 17 months. Consistent with a later synaptic deficit, Stern et al. (2004) reported severe synaptic response impairment of cortical neurons at ≥14 months, which correlated with substantial plaque accumulation, but not at 8 to 9 months of age. Alpar et al. (2006) also reported reduced spine density in apical dendrites of pyramidal cells in the primary somatosensory cortex at 11 months. Furthermore, Spires et al. (2005) reported a ~50% spine density reduction, disrupted neurite trajectories and loss of presynaptic and postsynaptic markers (synaptophysin and PSD-95, respectively) in the vicinity of plaques in the cortex at 21 to 24 months. A 25% reduction of spine density was also observed not associated with plaques, suggesting widespread loss. Loss of presynaptic and postsynaptic structural elements was however not reported at the earlier age of 15 to 17 months in the hippocampus (Chapman et al., 1999). Pre- and post-synaptic alterations, such as reduced PDS-95 and GluR1, in Tg2576 neurons are supported *in vitro* (Almeida et al., 2005). The disrupted synaptic morphology and function reported in Tg2576 mice has been proposed as the main agent underlying at least some of the observed behavioural deficits outlined in section 6c iii (Ashe, 2001).

While outlining similarities between AD neuropathy in humans and the Tg2576 model, it is also important to highlight their dissimilarities. Unlike the human AD brain, Tg2576 mice do not show profound neuronal loss and tau pathology, such as extensive tau

hyperphosphorylation, PHF and NFT formation (Irizarry et al., 1997; Frautschy et al., 1998; Sasaki et al., 2002). Furthermore, Sasaki et al. (2002) reported that aged 18 to 29 month old Tg2575 mice had 10-fold more relatively large-size plaques than AD patients, and had giant plaques which are almost never observed in the human AD brain. Despite these differences, AD and potential therapeutic strategies as it shows multiple temporally-associated neuropathological changes (such as amyloid pathology, inflammation, oxidative stress, dystrophic neurites and synaptic dysfunction) and obvious behavioural deficits (reviewed in the next section) which are both progressive and characteristic of the disease. Moreover, Tg2576 mice show a limited extent of tau pathology by exhibiting tau hyperphosphorylation in neurites surrounding Aβ deposits (Puig et al., 2004). Furthermore, Tg2576 mice make for a suitable model to study pathological processes that characterise the early (or even pre-symptomatic; Ashe 2001) brain changes in AD and thus the potential benefit that may be gleaned from early intervention therapies.

iii) Behavioural phenotype

Human AD patients have impaired declarative memory (including semantic and episodic memory), recognition memory, visuospatial abilities and executive function, consistent with pathological disruptions to the medial temporal lobe, hippocampus, amygdala, parahippocampus (entorhinal and perirhinal cortices) and the cortex (as reviewed in Nestor, Scheltens & Hodges, 2004). Similarly, the Tg2576 model shows an age-related increase in Aβ-associated pathology in these brain regions which appear to correlate with a rather specific cognitive deficit controlled by these structures (Frautschy et al., 1998; Chapman et al., 1999; Arendash et al., 2001b; Lehman et al., 2003; Wilcock et al., 2004; Hale & Good, 2005). For example, the Tg2576 model is primarily characterised by impairments dependent upon hippocampal and medial temporal lobe function such as spatial, recognition and configural memory, as well as emotional disturbances (e.g. Hsiao et al., 1996; Corcoran et al., 2002; King & Arendash, 2002; Hale & Good, 2005; Lelos et al., 2011).

These deficits mimic symptoms observed in AD patients. For example, Tg2576 mice have impaired recognition memory concerning social information and object-location information (e.g. Hale & Good, 2005; Ognibene et al., 2005; Good & Hale, 2007; Good, Hale & Staal, 2007; Deacon et al., 2009). This is also reported in AD patients (Swainson et al., 2001; Willems, Adam & Van der Linden, 2002; Lee et al., 2003). Similarly, Tg2576 mice show

frontal lobe-dependant executive dysfunction in tasks, which is correlated to amyloid pathology in this region (e.g. Zhuo et al., 2007, 2008). Impaired executive function and attentional control has also been shown in AD patients (as reviewed in Collette, Van der Linden & Salmon, 1999; Foldi, Lobosco & Schaefer, 2002; Nagahama et al., 2003), which is related to pathology and significant neuronal loss in the frontal lobes (Arriagada et al., 1992; Salat, Kaye & Janowsky, 2001; Mintun et al., 2006). Despite the established pathology and cognitive deficits observed in AD patients and Tg2576 mice in the frontal lobe, this region has not been extensively studied in Tg2576 mice, unlike the medial temporal lobe and hippocampal memory domain. This is particularly interesting since frontal-lobe dysfunction has been reported before hippocampal-dependant deficits and pathology in Tg2576 mice (Zhuo et al., 2007). Further examination is therefore required.

Also similar to AD patients, Tg2576 mice show low susceptibility of certain structures, such as the striatum and cerebellum, to extensive Aβ pathology in the cortex and hippocampus (Middei et al., 2004; Zhuo et al., 2008). This results in a relative sparing of certain behaviours. For example, sensorimotor performance and certain forms of implicit memory, such as procedural memory/discrimination learning are unaffected, at least until late stages of pathology (e.g. Damasio et al., 1990; Hartman & Pirnot, 1995; Middei et al., 2004; Fleischman et al., 2005). Further detail of deficits in Tg2576 mice, particularly frontal and medial temporal lobe dysfunction, is detailed throughout chapters 3, 4 and 5.

The onset of behavioural and cognitive deficits in the Tg2576 model is variable. Generally however, behavioural deficits emerge from 6 to 9 months of age, coinciding with the appearance of amyloid pathology (e.g. Ognibene et al., 2005; Zhuo et al., 2008). Behavioural deficits in a number of cognitive domains are obvious from 9 months of age onwards when there is a marked increase in Aβ, particularly when Aβ is deposited to form plaques in the neocortex and hippocampus (Hsiao et al., 1996). Despite the majority of deficits occurring after 6 to 9 months of age, behavioural changes have been documented at an earlier age when the brain is free of amyloid plaques and deposits (King et al., 1999; Deacon et al., 2008). For example, studies have documented selective memory (passive avoidance, water maze and circular platform), sensorimotor (balance beam) and spontaneous behaviour impairments (hyperactivity, burrowing and nesting) at 3 months of age (King et al., 1999; King & Arendash, 2002; Deacon et al., 2008). Furthermore, a spatial memory deficit (Y-maze spontaneous alternation) was reported at 3 to 4 months (Hsiao et al., 1996; King et al., 1999),

and impaired contextual fear conditioning at 5 months (Dineley et al., 2002; Comery et al., 2005; Jacobsen et al., 2006). The appearance of such early deficits is supported by the presence of morphological and functional synaptic changes in the Tg2576 brain from 4 months of age, as detailed previously.

The discovery of behavioural and synaptic deficits at an early age is indicative of early-stage pathological events or mechanisms which precede substantial Aβ deposition, such as increased soluble Aβ or oxidative stress and inflammation. Indeed, Westerman et al. (2002) reported the presence of soluble Aβ from 4 to 5 months. An increase in soluble Aβ may therefore trigger early-onset morphological and functional synaptic deficits that lead to behavioural dysfunction (Jacobsen et al., 2006). The discovery of both behavioural and pathological changes during early stages of the disease provides justification for evaluating early intervention strategies using this model.

Although early changes are apparent which highlights the pathological role of soluble Aβ accumulation, it is also important to note that several measures of cognitive impairment are not demonstrated at an early age. For example, King et al. (1999) shows no impairment in Y-maze alternation, water maze acquisition, passive avoidance and active avoidance tests at 3 and 9 months. Similarly, Chapman et al. (1999) reported hippocampal LTP deficits at 17 to 19 months but not at 6 to 8 months, which correlated with impaired spatial working memory at the later age. This later onset of cognitive impairment has led to various conclusions regarding the form of Aβ that is responsible. For example, the onset of deficits from ~9 months implicates insoluble Aβ and amyloid plaques (e.g. Hsiao et al., 1996). In support of this, Westerman et al. (2002) reported that spatial memory deficits in the Morris water maze at 6 months correlated with an elevation of detergent insoluble Aβ aggregates. Furthermore, they reported that the presence of soluble Aβ in the absence of insoluble Aβ at 4 to 5 months was insufficient to cause spatial memory deficits before 6 months, therefore supporting the role of insoluble Aβ.

Interestingly, Westerman et al. (2002) also reported that the association of memory loss and insoluble Aβ levels was lost when correlated in older mice, suggesting that cognitive dysfunction in AD is related not necessarily to amyloid load but the conversion of soluble to insoluble Aβ. Similarly, Wang et al. (1999) reported the pathogenic importance of a progressive shift from soluble to insoluble pools of Aβ. Such theories are supported by the

neurotoxic properties of small intermediate Aβ assemblies such as oligomers and photofibrils (Lambert et al., 1998; Hartley, 1999). Similarly, Walsh et al. (2002) demonstrated the neurotoxic effect of Aβ oligomers in the absence of Aβ monomers and fibrils, which disrupted hippocampal LTP in rats *in vivo*. Furthermore, Lesné, Kotilinek and Ashe (2008) identified Tg2576 mice with plaques but markedly reduced levels of Aβ oligomers to have normal memory function. These studies suggest that preclinical and early clinical progression of AD is driven by accumulation of soluble Aβ and intermediate Aβ assemblies. Importantly, this may suggest that late-stage therapeutic intervention may not be the optimal strategy to ameliorate behavioural dysfunction. Overall, the precise roles of the diverse amyloid assembly states in AD-associated cognitive dysfunction remain unclear, with both amyloid plaques and soluble Aβ oligomers associated with impaired neuronal and cognitive function.

iv) Justification for use of the Tg2576 model

The Tg2576 mouse model was selected to investigate dietary intervention for AD in this thesis for various reasons including suitability and availability. Firstly, this model expresses multiple features of AD pathology and behavioural deficits which mimic that observed in AD. It is therefore thought to be a credible model of amyloid-induced pathology presented in AD. Secondly, the behavioural and pathological phenotype of this model has been extensively characterised which has uncovered robust behavioural deficits suitable for examining putatively therapeutic interventions. Thirdly, the onset and development of pathology and behavioural deficits in this model are compatible with multiple aims of this thesis, primarily including: 1) assessing both early (before pathological development) and longitudinal therapeutic intervention, and 2) further characterising the behavioural phenotype of the model, including the onset and variation of deficits historically associated with different neural structures. One potential disadvantage of the model is the lack of NFT development and neuron loss. However, this model provides an excellent opportunity to assess the impact of interventions on pathology and behavioural changes associated with a theoretically leading cause of AD pathogenesis.

1.8 Thesis aims

This introduction has highlighted a number of areas surrounding dietary supplementation and the Tg2576 model which require further research, and so the present study has multiple aims.

Despite considerable research into the effect of dietary omega-3 PUFAs on pathology and behaviour in animal models of AD, very few studies have attempted to examine the impact of this supplement in comparison to a suitable control diet which controls for the level of various other fatty acids, particularly the content of omega-6 PUFA. It is argued that such control is vital in order to understand the effect of omega-3 PUFAs, rather than its effect relative to a control diet with an unequal level of fatty acids or a high level of omega-6 PUFAs which may contribute to the observed effects. This would arguably provide a more valid assessment of its potential benefits in the context of a human population that maintains a relatively healthy diet. The first aim of the present study therefore was to investigate the impact of dietary omega-3 PUFAs and fish oil containing omega-3 PUFAs in comparison to a control diet which had an equal level of fatty acids and a 'neutral' blend of fatty acids in replacement of the additional omega-3 PUFAs/fish oil in the experimental diet. In particular, the level of omega-6 PUFA was controlled.

The second aim of the present study was to investigate the effect of dietary curcumin supplementation on behaviour and pathology in the Tg2576 mouse model. In particular, the present study examined the effect of curcumin when supplemented from an early age prior to the onset of pathology. This was particularly important as the current literature surrounding curcumin supplementation in AD models has examined intervention during late stages of pathology development only. Although these studies have shown positive effects, even earlier intervention was hypothesised to prove a more successful at reducing pathological changes that disrupt memory function. For similar reasons, supplementation of omega-3 PUFAs/fish oil was also examined from an early age. This was particularly important as no studies had examined early omega-3 PUFA treatment in the Tg2576 model, and no study had examined the effect of fish oil supplementation in the Tg2576 model at any time-point. Furthermore, the examination of behaviour was an important aspect of this research as only one study had reported the effects of dietary omega-3 PUFA supplementation on behaviour in the Tg2576 model.

A third aim of the present study was to investigate the effect of a combination of omega-3 PUFA (sourced from fish oil) and curcumin supplements. No published *in vivo* study to date has reported examination of these supplements in an AD mouse model. It was hypothesised that a combination of these supplements would have more potent effects than individual supplementation as previous research has outlined the potential synergistic effects of

combining treatments. More specifically, evidence shows that the efficiency of omega-3 PUFAs may be improved through its combination with curcumin as it provides antioxidant protection. Similarly, curcumin efficiency may be improved through its combination with omega-3 PUFAs as it may increase its bioavailability.

In particular, the present study aimed to investigate chronic treatment of these dietary supplements. Further investigation was required into the effects of longitudinal treatment as curcumin supplementation had been limited to 6 months in animal model studies. Although omega-3 PUFA supplementation has been more extensive in its time course, this has been limited to intervention at a later stage in transgenic models; highlighting the need for examination of chronic treatment from an early age. The potential importance of early intervention may be interpreted through human studies of dietary intervention, whereby short-term clinical trials of omega-3 PUFA have reported few positive effects in contrast to long-term epidemiological studies. The positive results from epidemiological studies may reflect long-term lifestyle choices of diet, perhaps suggesting that longitudinal dietary intervention may offer more positive effects. Furthermore, as AD is a long-term condition that develops over time, it is likely that longitudinal intervention would be required to provide therapeutic effects throughout the time course of the disease. Longitudinal treatment was therefore hypothesised to delay disease onset and reduce disease progression.

The overarching hypothesis under test in this thesis is that the pleiotropic actions of omega-3 PUFAs and curcumin on Aβ, inflammation and oxidative stress, will reduce behavioural and pathological indices of AD-like pathology in Tg2576 mice.

Chapter 2

Preparation of mouse colonies and dietary intervention

2.1 Introduction

Prior to presenting the results of the studies examining dietary supplementation in Tg2576 mice in chapters 3, 4 and 5, this chapter summarises the methods employed for generating and maintaining the mouse cohorts used, including genotypic analysis to identify transgenic mice harbouring the APPswe mutation. This chapter also outlines the experimental diets used in chapters 3, 4 and 5, including the methodology and results of the lipid analysis performed on the diets to ensure dietary content was accurate after production. Furthermore, this chapter outlines the statistical analysis design commonly employed throughout chapters 3, 4 and 5.

2.2 Preparation of mouse colony

2.2.1 Generation and maintenance of the Tg2576 colony

The Tg2576 model, possessing the human double APPswe mutation on a hybrid background strain of C57Bl/6 x SJL, was developed by Karen Hsiao and colleagues in 1996 (Hsiao et al., 1996). The transgene was generated by inserting human APP open reading frame into a hamster prion protein (PrP) cosmid vector and mutating the insert. The Tg2576 line was originally established by crossing a C57Bl/6j x SJL F3 founder twice into C57Bl/6j (Chapman et al., 1999). Overexpression of the transgene was maintained in subsequent generations by crossing heterozygous Tg2576 to a C57Bl/6j x SJL F1 line (Chapman et al., 1999). Each generation possessed differences in the relative percentage of each of these background strains, with an estimated 59% to 88% contribution of C57Bl/6j and 12% to 41% of SJL (Chapman et al., 1999). The separate mouse cohorts used in chapters 3, 4 and 5 were generated by pairing male heterozygous Tg2576 mice with female C57Bl/6j x SJL F1 mice. The resulting offspring were either heterozygous Tg2576 transgenic (Tg) mice, possessing the mutation, or homozygous Tg2576 wildtype (WT) mice, at a ratio of 50:50. The

possession of the transgene was detected through genotyping, as detailed in section 2.2.2. Genotype status (Tg or WT) was re-confirmed upon termination at the end of the study.

Following successful breeding, litters were weaned into same-sex mixed-genotype littermate groups consisting of 2 to 4 individuals at approximately 6 weeks of age. Only male mice were used in the experiments in order to reduce variability induced by gender differences, as reported in several tasks (e.g. King et al., 1999; Deacon et al., 2008, 2009). Mice were immediately ear marked for identification, and ear- or tail-biopsied for genotyping to determine genotype status. Tail biopsies (<0.05cm) were removed using ethyl chloride local anaesthetic and a silver nitrate pen to seal the incision. Tissue samples were stored at -20°C until further use. Mice were individually housed at 8 weeks of age following genotype results.

Mice were fed standard laboratory mouse chow (RM1 Rodent, SDS, England) until replaced with experimental diets (detailed in section 2.3) at either 2 months of age (in chapters 4 and 5) or 4 months of age (in chapter 3). Diets were allocated pseudo-randomly, controlling for genotype and littermate groups as far as possible. Mice were housed in a colony room maintained at a temperature of 21 ± 2°C and a humidity of 55% ± 10%. Colony rooms operated on a 12-hour light-dark cycle (lights on at 7:00 to 19:00) and behavioural experimentation took place during the light phase of the cycle as far as possible. The running order of mice during behavioural experimentation was counterbalanced for genotype and diet, and run blindly to the experimenter. All mice were experimented and maintained in full compliance with Home Office (United Kingdom) guidelines on the use of animals in scientific procedures.

2.2.2 Genotyping of the Tg2576 colony

2.2.2.1 Introduction

Tissue biopsies from mice were genotyped using the polymerase chain reaction (PCR) molecular biology technique to determine whether they harboured the human double APPswe mutation. PCR selectively amplifies specific deoxyribonucleic acid (DNA) regions exponentially for measurement using gel electrophoresis. In our protocol, specific oligonucleotide primers were used to selectively amplify the nucleic acid sequence coding the

APPswe transgene. Selective DNA amplification in PCR occurs through enzymatic replication by utilising repeated thermal cycling of a DNA sample containing multiple reagents including DNA polymerase, oligonucleotide primers and deoxynucleoside triphosphates (dNTPs).

2.2.2.2 Methods

Digestion and extraction of DNA

In preparation for the PCR, DNA was digested and extracted from the tissue sample. Defrosted tissue samples were digested by incubating in 600 µl of TES cell lysis buffer (1M Tris HCl Buffer pH 8.0, 0.5M EDTA pH 8.0, 10% SDS, dH_20) at 55°C for 20 minutes. Following this, 1.5µl of 25mg/ml Proteinase K (Sigma, Cat.# P2308) was applied to each sample, vortexed and incubated overnight at 55°C. Following digestion, DNA was extracted by adding 200µl of 5M ammonium acetate, vortexed and centrifuged for 10 minutes at 14,000 rpm to pellet the proteins. 650µl of supernatant was removed carefully not to dislodge the pellet, and transferred into 600µl of molecular biology grade isopropanol. This solution was mixed using inversion and centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded and 150µl of ice cold 70% molecular biology grade ethanol was applied to the DNA-containing pellet, and then centrifuged for 2 minutes at 14,000 rpm. Following a repeat of the latter process, the supernatant was removed and the samples were dried at 37 ºC until the ethanol was evaporated. DNA was resuspended in 60µl of TE buffer (1M Tris HCl pH 8.0, 0.5M EDTA pH 8.0, dH_20) and incubated overnight at 55°C. PCR was then carried out on the DNA samples within 24 hours or stored at-20°C until use.

PCR analysis and electrophoresis of DNA

1 µl of DNA sample was added to 24µl of master mix to make a 25µl solution. The samples and master mix were maintained on ice during this process. Master mix was prepared using 2.5µl 10x reaction buffer, 1.25µl 50mM $MgCl_2$, 1.25µl 10mM dNTPs, 0.25µl Primer 1501 (50 pmol), 0.25µl Primer 1502 (10 pmol), 0.25µl Primer 1503b (10 pmol), 1.5µl BIOTAQ Red DNA Polymerase (1µg/µl) and 16.75µl nuclease-free MilliQ™ water (Millipore, USA). The DNA polymerase, 10x reaction buffer and $MgCl_2$ were supplied in BIOTAQ Red DNA Polymerase kit (Bioline, Cat.# BIO-21041). The oligonucleotide primers 1502 (5'-

GTGGATAACCCCTCCCCCAGCCTAGACCA-3', 600bp) and 1503b (5'-CTGACCACTCGACCAGGTTCTGGGT-3', 450bp) were used to amplify the APPswe transgene, while primer 1501 (5'-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3', 600bp) amplified the endogenous murine prion protein (PrP) with primer 1502.

The 25µl solution for each sample was vortexed and centrifuged for 6 seconds at 4000rpm. Each sample was placed into the PCR cycle machine, alongside one positive Tg control (1µl of confirmed Tg DNA plus 24µl master mix), one negative WT control (1µl of confirmed WT DNA plus 24µl master mix), and one blank control (1µl nuclease-free water plus 24 µl master mix). The blank control was run as a means of detecting potential contamination. The PRC cycler was programmed to carry out APPswe transgene amplification through initialisation at 72°C for 2 minutes followed by 36 cycles of: 1) denature at 94ºC for 1 minute, 2) anneal and amplify at 62ºC for 1 minute, and 3) incubate at 72ºC for 2 minutes for elongation, with 2 seconds added to the elongation step per cycle. Samples were then maintained at 4ºC in the PRC cycler until collected for electrophoresis.

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

Figure 2.1 presents an example of a photographed gel under UV light that has undergone electrophoresis. The figure depicts the 100 bp DNA ladder (DNA marker) run alongside 13 samples, followed by the blank (nuclease-free water), positive (Tg) and negative (WT) controls. A single band at ~600 bp corresponds to amplification of the endogenous murine prion protein gene by oligonucleotide primers 1501 and 1502, which should be present in all tissue samples from Tg2576 transgenic and WT mice. The presence of the second band

corresponds to amplification of the APPswe transgene by oligonucleotide primers 1502 and 1503b, which confirms the tissue sample was collected from a Tg mouse. According to the bands in Figure 2.1, the samples corresponding to lanes 5, 6, 9, 10 and 13 were Tg mice and the samples corresponding to lanes 2, 3, 4, 7, 8, 11, 12 and 14 were WT mice.

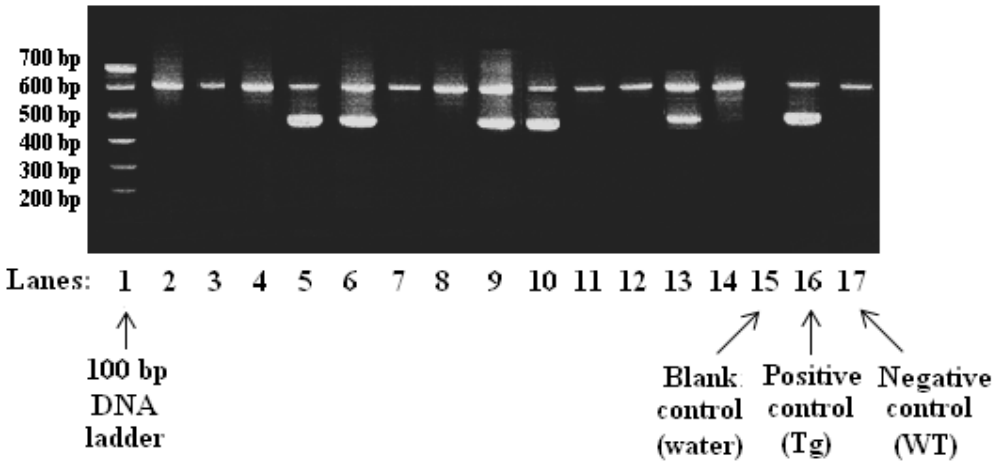

Figure 2.1 Representative photograph of gel electrophoresis showing PCR amplification of DNA from Tg2576 and WT mice. Lane 1 = 100bp DNA ladder; Lanes 2, 3, 4, 7, 8, 11, 12 and 14 = single band corresponding to amplification of endogenous murine prion protein, therefore representing WT mice; Lanes 5, 6, 9, 10 and 13 = double band corresponding to amplification of endogenous murine prion protein and APPswe transgene, therefore representing Tg mice; Lane 15 = Blank (water) control, Lane 16 = Positive control (known Tg sample), Lane 17 = Negative control (known WT sample).

2.3 The experimental and control diets

2.3.1 Introduction

Chapters 3 and 4 examined the effect of a diet supplemented with the omega-3 PUFA docosahexaenoic acid (DHA), whereas chapter 5 examined the effect of diets supplemented with fish oil (rich in omega-3 PUFAs DHA and EPA), curcumin or a combination of fish oil and curcumin.

In order to accurately examine the effect of dietary fish oil or DHA supplementation, the design of the experimental and control diets was an important consideration of the study. It is thought that the interpretation of omega-3 PUFA/fish oil/DHA supplementation findings have been confounded by the control diet in previous reports. For example, several studies in AD

mouse models have used a control diet which had an unequal level of total fatty acids relative to the experimental diet (e.g. Arendash et al., 2007; Green et al., 2007). The consequence of supplementing fish oil/DHA in the experimental diet without substituting the fatty acid content in the control diet prevents the conclusion that any effects are a result of fish oil/DHA specifically rather than merely increased quantities of fatty acids. In order to ascertain the effects of fish oil/DHA specifically, a suitable control diet should therefore contain an equal level of fatty acids as the experimental diet. This was controlled for in the fish oil/DHA dietary interventions assessed in the chapters 3, 4 and 5.

Several studies have however controlled for this matter, although omega-3 PUFA/fish oil/DHA supplementation has been primarily replaced by omega-6 PUFAs in the control diet, often resulting in a excessive ω-6/ω-3 ratio (e.g. Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006; Ma et al., 2007, 2009; Green et al., 2007; Arsenault et al., 2011). Since the metabolic effects of omega-6 PUFAs can be pro-inflammatory, the excess omega-6 PUFA content in the control diet may accelerate pathological development (as outlined in chapter 1). This approach is therefore comparing an omega-3 PUFA/fish oil/DHA diet with potential anti-inflammatory effects to a control diet with potential pro-inflammatory effects. Consequently, this design does not examine the effect of omega-3 PUFA/fish oil/DHA supplementation *per se*, but rather relative to a particularly poor diet which likely contribute to the beneficial effects. The supplementary fatty acids added to the control diet in chapters 3, 4 and 5 were therefore composed of an oil blend low in omega-6 PUFAs (and equal to that in the fish oil/DHA diet), in order to prevent an excessive ω-6/ω-3 ratio. The supplemented oil blend in the control diet was composed of fatty acids typically found in an average U.K. diet, primarily composed of 'neutral' saturated and monounsaturated fatty acids which were not expected to induce pro- or anti-inflammatory effects.

The majority of previous studies have shown ~0.6% DHA supplementation effective at reducing behavioural deficits and pathology in AD models (e.g. Calon et al., 2004, 2005, Lim et al., 2005; Hooijmans et al., 2007, 2009; Ma et al., 2009). Similarly, Green et al. (2007) found 1.3% DHA effective in a 3xTg model. In contrast, Arendash et al. (2007) reported 13% omega-3 PUFA supplementation (containing 5.71% DHA) to be ineffective in the APPswe/PS1 model, although this may have been attributed to a lack of incorporation into the brain or the high levels of fat. High levels of fat have been shown to increase pathology and so the 13% oil supplement may have been detrimental and overshadowed any positive

effects of DHA. Supporting this, Julien et al. (2010) reported that a 35% fat diet increased Aβ pathology in Tg2576 mice compared to a 5% fat diet. Based on these results, a high level of DHA or oil supplementation (e.g. 13%) was deemed inappropriate. Similarly, it was proposed that low levels of DHA supplementation (e.g. 0.06%) may not be suitable, as although it showed positive effects in previous studies, this was relative to a poor control diet with a high ω-6/ω-3 ratio. It could therefore be argued that higher levels of DHA supplementation may be required to be effective relative to a control diet with a relatively low ω-6/ω-3 ratio, as used in chapters 3, 4 and 5. It was therefore decided that the DHA supplemented diet used in chapters 3 and 4 would contain a 5% DHA-rich oil (containing 45% DHA, equating to 2.25% DHA of the diet dry weight), compared to a control diet containing a 5% 'neutral' oil blend. Results from Julien et al. (2010) lead us to conclude that this level of supplementation should not be detrimental. Furthermore, this level of DHA supplementation would provide levels compatible to human supplementation.

Previous studies examining fish oil supplementation in AD-model mice have reported positive effects of fish oil at a dose of 2.2% to 2.4% of total diet weight (Oksman et al., 2006; Ma et al., 2009). Ma et al. (2009) examined the effect of 2.4% fish oil in the 3xTg AD model relative to a particularly poor-diet high in saturated and omega-6 PUFA. The positive effects of fish oil reported in this study were therefore limited to fish oil supplementation relative to a poor-diet. Similarly, Oksman et al. (2006) examined the effect of a 2.2% fish oil supplemented diet (plus 2.4% soy oil) with an ω-6/ω-3 ratio of 1.4:1 compared to 5% soy-oil supplemented standard chow (ω-6/ω-3 ratio of 8:1) and 5% corn-oil supplemented standard chow (ω-6/ω-3 ratio of 70:1). Although the soy-oil diet allowed a more accurate examination of fish oil than the corn-oil diet, both control diets had excessive levels of omega-6 PUFAs which prevented the accurate examination of fish oil supplementation *per se*. Chapter 5 therefore aimed to examine the effect of a 2.5% fish oil supplemented diet relative to a 2.5% 'neutral' oil blend supplemented diet, containing similar levels of omega-6 PUFA.

In order to examine the effect of curcumin supplementation in chapter 5 it was important to select a dose that was previously reported as effective in reducing pathology and behavioural deficits. In addition to this, the dose must be safe and bioavailable *in vivo*. Frautschy et al. (2001) first showed positive effects of 500ppm and 2000ppm curcumin doses in the Aβ-infused AD rat model. Supporting this, studies in the 3xTg AD model (Ma et al. 2009) and Tg2576 model (Yang et al., 2005; Begum et al., 2008) showed a 500ppm curcumin dose to be

effective. Interestingly, Lim et al. (2001) reported that 160ppm curcumin was more effective than 5000ppm curcumin at reducing oxidative stress and inflammation in the Tg2576 model, and that only the 160ppm dose reduced amyloid pathology. Although further investigation is therefore required to discover the optimal treatment dose, it is advisable to provide dosages lower than 5000ppm and closer to 500ppm. Chapter 5 therefore examined a 500ppm curcumin dose since this dose appears effective at reducing pathological processes in a number of AD models including Tg2576 mice. Furthermore, 500ppm curcumin was the only reported dose to reduce spatial memory deficits (Frautschy et al., 2001). In order to accurately assess co-supplementation of curcumin (500ppm) and fish oil (2.5%) in chapter 5, a 2.5% neutral oil blend supplement was added to the curcumin diet to ensure that any observed differences in the combination diet was attributable to the fish oil and curcumin rather than curcumin with additional fatty acids. Although a curcumin alone supplemented diet would have been ideally been examined, the size of the mouse cohorts in chapter 5 limited the addition of this group. Also due to this issue, fish oil and curcumin co-supplementation was only examined in Tg2576 not WT mice as this condition was of theoretical interest. In all other conditions, both Tg2576 and WT control mice were used.

2.3.2 Overview of the experimental diets used in chapters 3 and 4

The experimental mouse diets used in chapters 3 and 4 were composed of a standard mouse chow (RM1 Rodent, Special Diet Services, SDS, Essex, UK) containing sufficient amounts of nutrients, including essential fatty acids. These diets were supplemented with either a 5% DHA-rich oil containing ~45% DHA (produced by DHASCO, Martek Biosciences Corporation, Columbia, MD, USA) or a 5% oil blend composed of lard, palm oil, olive oil and coconut oil (3:3:3:1 by weight, respectively). Diets were supplied in 5kg bags and stored in a refrigerated room at 4ºC or a -20ºC freezer throughout the course of the study, except when in use when one 5kg bag of each diet was stored in the mouse colony room in sealed containers at room temperature in an effort to limit lipid peroxidation. Limiting the oxidation of lipids, especially long-chain PUFAs such as DHA which are particularly vulnerable to oxidation, is essential when formulating or storing diets in order to maintain the level of fatty acids in the diet and prevent intake of oxidised fatty acids which may induce an oxidative state (e.g. Calon et al., 2004; Calon & Cole, 2007).

The composition of these food pellets were approximately equal with 12.8% crude protein, 7.2% crude oil, 3.9% crude fibre and 5.6% ash, with a calorific value of 3407-3409 kcal/kg, according to the manufacturer (SDS) specification. The fatty acid profile of each diet provided by SDS is presented in Table 2.1. The table shows that the DHA supplemented diet contained 2.25% DHA, with small levels of other omega-3 PUFAs. In contrast, the oil blend supplemented diet contained 0% DHA, with small levels of omega-3 PUFAs. The diets contained similar levels of omega-6 fatty acids. Importantly, SDS also reported that the diets contained no cholesterol. A lipid analysis was performed by a research associate (Dr. Cécile Bascoul-Colombo) on three diet samples taken from 3 diet bags to confirm the fatty acid profiles of these diets (excluding cholesterol levels). This was carried out 3 weeks after the date of arrival, whilst being stored in a refrigerated room at 4ºC. The methodology of this analysis is detailed in section 2.3.4. Results from the analysis of diet samples for chapter 3 are shown section 2.3.5, and results of diet samples for chapter 4 are shown in section 2.3.6.

Table 2.1 Fatty acid compositions of the DHA and oil blend diets, expressed as percentage of fresh diet weight. Information provided by the manufacturer (SDS, Essex, UK.)

Fatty acids	**Rodent chow + 5% DHA rich oil**	**Rodent chow + 5% oil blend**
Dodecanoic (Lauric) acid 12:0	0.10%	0.26%
Tetradecanoic (Myristic) acid 14:0	0.64%	0.24%
Myristoleic acid 14:1	0.01%	0.00%
Hexadecanoic (Palmitic) acid 16:0	0.87%	1.34%
Sapienic (Palmitoleic) acid 16:1ω-7	0.08%	0.08%
Octadecanoic acid (Stearic) 18:0	0.09%	0.31%
Oleic acid 18:1ω-9	2.04%	2.79%
Linoleic acid 18:2ω-6	0.95%	1.13%
α-Linolenic acid 18:3ω-3	0.08%	0.08%
Arachidonic acid 20:4ω-6	0.12%	0.11%
Docosapentaenoic acid 22:5ω-3	0.01%	0.00%
Docosahexaenoic acid 22:6ω-3	2.25%	0.00%
Total saturated fatty acids *	1.70%	2.15%
Total monounsaturated fatty acids *	2.13%	2.87%
Total polyunsaturated fatty acids *	3.41%	1.32%
Total omega-3 (ω-3) PUFA*	2.34%	0.08%
Total omega-6 (ω-6) PUFA*	1.07%	1.24%
Omega-3/omega-6 (ω-3/ω-6) ratio *	**2.19:1**	**0.06:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**0.46:1**	**15.5:1**

*Calculated from the manufacturer's specification.

According to manufacturer specifications, the DHA and oil blend diets contained equal levels of vitamins including 8150 iu/kg Vitamin A, 72 iu/kg Vitamin E, 7.66 mg/kg Vitamin B1 (Thiamine), 3.36mg/kg Vitamin B2 (Riboflavin), 4.3 mg/kg Vitamin B6 (Pyridoxine), 5.78

μg/kg Vitamin B12 (Cyanocobalamine), 3.47 mg/kg Vitamin C (Ascorbic acid), 9.71 mg/kg Vitamin K (Menadione), 0.57 mg/kg Folic acid (Vitamin B9), 54.75 mg/kg Nicotinic acid (Vitamin PP), 18.66 mg/kg Pantothenic acid (Vitamin B3/5), 1015.36 mg/kg Choline (Vitamin B4/7), 2115.75 mg/kg Inositol, 247.44 mg/kg Biotin (Vitamin H). The DHA and oil blend diets also contained equal levels of macrominerals (reported as a percentage of fresh diet) including 0.8% Calcium, 0.46% Phosphorus, 0.25% Phylate phosphorus, 0.22% sodium, 0.34% Chloride, 0.58% Potassium, 0.21% Magnesium; and equal levels of microminerals including 162.35 mg/kg Iron, 11.19 mg/kg Copper, 64.68 mg/kg Manganese, 14.57 mg/kg Zinc, 591.6 μg/kg Cobalt, 1067.36 μg/kg Iodine, 266.96 μg/kg Selenium and 9.36 mg/kg Fluorine. It is important to note that SDS reported that the diets contained no synthetic antioxidant such as butylated hydroxytoluene (BHT), but vitamins such as Vitamin E can provide antioxidant protection.

2.3.3 Overview of the experimental diets used in chapter 5

The experimental mouse diets used in chapter 5 were composed of a standard mouse chow (RM1 Rodent, Special Diet Services, SDS, Essex, UK) containing sufficient amounts of nutrients. According to the manufacturer (SDS) specification, the composition of the food pellets prior to supplementation were approximately 12.6% crude protein, 2.2% crude oil, 3.9% crude fibre, 5.6% ash and 0% cholesterol, with a calorific value of 2875 kcal/kg. In order to produce the experimental mouse diets, standard chow was supplemented with 7.5% of freeze-dried powder (produced by Cultech Ltd, Port Talbot, UK) containing the supplements. One third of the powder contained oil and so all experimental diets contained 2.5% oil. The control 'neutral' oil blend diet (OB) contained 2.5% of an oil blend composed of lard, palm oil, olive oil and coconut oil (3:3:3:1 by weight, respectively). The composition of this oil blend matched the control diet used in chapters 3 and 4. The fish oil diet (FO) contained 2.5% of fish oil comprised of 21% EPA and 15% DHA, equating to 0.53% EPA and 0.38% DHA in the fish oil diet. In contrast, the oil blend diet did not contain detectable levels of EPA or DHA. The freeze-dried powders produced for the curcumin diets contained 500ppm of curcumin with 2.5% oil blend (OB+C) or 2.5% fish oil (FO+C).

To ensure fatty acids in the diet were not affected by lipid peroxidation, additional efforts were taken to prevent oxidation. This included maintaining 2.5kg of diet in vacuum-packed

bags which were stored at 4ºC. Once a diet bag was opened for use, bags were sealed and maintained at 4ºC. In an extra effort to limit lipid peroxidation during supply, each mouse received fresh pellets every week in their homecage and pellets were examined approximately every 2 to 3 days to check for noticeable decomposition. Degraded pellets were replenished with fresh pellets. This method was in contrast to the approach taken in chapters 3 and 4 whereby mice received pellets in their food hopper which were topped up when food levels became low. Furthermore, the diets used in chapters 3 and 4 were stored in 5kg non vacuum-packed bags, maintained at 4ºC or frozen at -80°C until use when stored in the mouse colony room at room temperature in sealed containers. This was an important consideration of the study as it is well documented that long-chain PUFAs are particularly vulnerable to oxidation, which would reduce the amount of fatty acids in the diet and perhaps lead to an accumulation of oxidation products which could increase the state of oxidative stress *in vivo* (Calon et al., 2004; Calon & Cole, 2007).

The fatty acid profile for the diets provided by the manufacturer were presented as an average of all experimental diets and thereby lacked accuracy. Lipid analysis of diets (excluding cholesterol content) was therefore performed by a research associate (Dr. Cécile Bascoul-Colombo) on three samples taken from 3 bags of each diet, 3 weeks after arrival. The results are presented in section 2.3.7. Further to this, fatty acid composition of the diets was monitored over the course of the 12-month study to test for degradation of EPA and DHA. Samples were taken from each 2.5kg diet bag upon opening and finishing the bag, and stored immediately at -80ºC until analysis. Lipid analysis results revealed that over the course of 12 months, the reduction of EPA and DHA in the fish oil diets was below 5%.

According to manufacturer specifications, the diets contained equal levels of vitamins including 8142 iu/kg Vitamin A, 72 iu/kg Vitamin E, 7.51 mg/kg Vitamin B1 (Thiamine), 3.34mg/kg Vitamin B2 (Riboflavin), 4.2 mg/kg Vitamin B6 (Pyridoxine), 5.78 µg/kg Vitamin B12 (Cyanocobalamine), 3.47 mg/kg Vitamin C (Ascorbic acid), 9.7 mg/kg Vitamin K (Menadione), 0.55 mg/kg Folic acid (Vitamin B9), 53.5 mg/kg Nicotinic acid (Vitamin PP), 18.29 mg/kg Pantothenic acid (Vitamin B3/5), 1012.86 mg/kg Choline (Vitamin B4/7), 2040.75 mg/kg Inositol, 242.44 mg/kg Biotin (Vitamin H). The diets also contained equal levels of macrominerals (reported as a percentage of fresh diet) including 0.8% Calcium, 0.45% Phosphorus, 0.24% Phylate phosphorus, 0.22% sodium, 0.34% Chloride, 0.57% Potassium, 0.21% Magnesium; and equal level of microminerals including 160.96 mg/kg

Iron, 11.07 mg/kg Copper, 63.68 mg/kg Manganese, 14.2 mg/kg Zinc, 640.72 μg/kg Cobalt, 1066.36 μg/kg Iodine, 264.96 μg/kg Selenium and 9.24 mg/kg Fluorine. Therefore, the diets used in chapter 5 contained approximately the same levels of vitamins and minerals as the DHA and oil blend diets in chapters 3 and 4, with slightly higher levels of the micromineral Cobalt. It is also important to note that the diets contained no synthetic antioxidant such as butylated hydroxytoluene (BHT).

2.3.4 Lipid analysis of diets: Methods

2.3.4.1 Materials

Chemicals and solvents were purchased from Fisher Scientific (Loughborough, UK) and Sigma (Poole, UK). The internal standard fatty acid, pentadecanoic acid (15:0), was obtained from Nu-Check Prep Inc. (Elysian, MN, USA). Gas liquid chromatography was carried out on samples using a Clarus 500 gas chromatograph fitted with a Perkin Elmer 8500 flame isonisation detector and a 30m x 0.25mm internal diameter Elite 225 polar capillary column, all sourced from Perkin Elmer (Norwalk, Connecticut, USA).

2.3.4.2 Procedure

Lipid analysis to confirm the fatty acid profile of the diets was carried out on 3 samples of each diet, and the replicates were averaged for presentation. Lipids were extracted based on the protocol described by Garbus et al. (1963). Before lipid profiles could be analysed by gas liquid chromatography (GLC), lipid extracts had to be converted into fatty acid methyl esters (FAME) by acid-catalysed transmethylation. GLC analysis was then carried out on the samples. These steps are detailed below.

Lipid extraction

Diet pellets were crushed using a pestle and mortar, and 0.5g was added to 0.5ml MilliQ^{TM} water to achieve a 1g/ml aqueous phase. This was sonicated in 3.75ml chloroform-methanol (1:2, by volume) for 20 minutes at room temperature for homogenisation. 1.25ml of chloroform and 1.25ml of Garbus solution (2M potassium chloride in 0.5M potassium phosphate buffer, pH 7.4) was added, vortexed and centrifuged at 1500 rpm for 5 minutes.

The upper phase of the solution was discarded and the remaining lipid-containing lower phase was washed by applying 4.45ml of fresh upper phase solution (the upper phase of 'mixture solution': dH_2O-chloroform-methanol-Garbus, 4:10:10:5, by volume). Following vortex and centrifugation at 1500 rpm for 5 minutes, the upper phase was discarded and the lower phase transferred to a clean tube, comprising the first extract. The remaining lipids were extracted from the remaining sample by applying 3ml of mixture solution (mixed immediately before application). Following vortex and centrifugation at 1500 rpm for 5 minutes, the upper phase was again discarded and the lower phase (the second extract) was transferred to the first extract. This combined lower phase was then dried down under nitrogen at room temperature and resuspended in 250µl of chloroform-methanol, vortexed and transferred to a glass vial. This latter process was repeated and stored at -20°C until further use.

Methyl-esterification of lipids

Before fatty acids could be analysed by gas liquid chromatography (GLC), lipid extracts had to be converted into fatty acid methyl esters (FAME) by acid-catalysed transmethylation. Lipid extract samples were defrosted and vortexed, then dried down under nitrogen. To equalise volumes, 1ml of chloroform-methanol was added to dried samples and vortexed. 30µl was transferred to a tube containing 50µl of the internal standard fatty acid, pentadecanoic acid (C15:0) in toluene (1mg/1ml). The standard was used to calculate the amount of fatty acids in the samples during GLC. Following this, 3ml of methylation solution (2.5% [v/v] sulphuric acid in methanol-toluene, 2:1 by volume) was applied to each sample and incubated for 2 hours at 70°C. FAME were extracted by adding 2ml of 5% (w/v) sodium chloride in MilliQ^{TM} water and 3ml of HPLC grade hexane. After vortexing, the top layer containing FAME was transferred to a clean tube. This latter step was repeated for a second extraction. Following this, 3ml of 2% (w/v) potassium carbonate in MilliQ^{TM} water was applied to neutralise any remaining acidity and the top layer was transferred to a clean tube. Samples were then dried down under nitrogen and resuspended in 50µl of HPLC grade hexane, vortexed and transferred to GLC injection vials (Chromacol Ltd, UK). Samples were stored at -20°C until ready for GLC analysis.

Fatty acid analysis by gas liquid chromatography (GLC) and data analysis

GLC analysis was carried out by a research assistant (Dr. Irina Guschina). 10µl of FAME samples were analysed by GLC using a gas chromatograph, injected at a flow rate of 20ml/minute^{-1} with a split ratio of 20:1, using nitrogen as the carrier gas. The GLC followed a temperature programme whereby samples were initially heated to 170°C for 3 minutes, followed by heating to 220°C at a rate of 4°C per min^{-1} and maintained for 30 minutes. The chromatograms obtained showed peaks corresponding to different fatty acids which were identified by comparing retention times with those obtained from the standard fatty acids. Using the areas under the peaks, the percentage of each fatty acid was expressed as a percentage of total fatty acids. Using the data, the percentage of fatty acids as a percentage of fresh diet weight was also calculated using the milligrams of fatty acids per gram of diet (mg/g).

2.3.5 Results: Lipid analysis of diets used in chapter 3

Tables 2.2 and 2.3 shows the lipid analysis results of the diet samples used in chapter 3. The lipid analysis showed a similar pattern to the fatty acid composition provided by SDS. These data reveal that the main saturated fatty acids (SAT) in the diets were 16:0, followed by 14:0, with small amounts of 18:0, 12:0, 20:0, 22:0 and 24:0. The main monounsaturated fatty acids (MUFA) were 18:1ω-9, with small amounts of 16:1ω-7 and 20:1ω-9. Greater levels of polyunsaturated fatty acids (PUFA) was present than MUFA and SAT. The main omega-3 PUFAs of the DHA diet were of course 22:6ω-3 (DHA), representing 1.86% of fresh diet weight, followed by small amounts of 22:5ω-3 and 18:3ω-3. The only omega-3 PUFA in the oil blend diet was 18:3ω-3 (α-linolenic acid), which was also detected in the DHA diet at similar levels. Omega-3 PUFAs 22:6ω-3 and 22:5ω-3 were at undetectable levels in the oil blend diet. The main omega-6 PUFA was 18:2ω-6, representing 1.6% of the DHA diet and ~2.1% of the oil blend diet. As a result of the high level of DHA in the DHA diet and the similar levels of omega-6 in both diets, the DHA diet showed a greater omega-3/omega-6 ratio relative to the oil blend diet.

Table 2.2 Fatty acid compositions of the DHA and oil blend diets used in chapter 3, expressed as mean percentage of fresh diet weight ± S.E.M (Standard error of the mean). Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**Rodent chow + 5% DHA rich oil**	**Rodent chow + 5% oil blend**
Dodecanoic acid 12:0	0.04 ± 0.01%	0.05 ± 0.01%
Tetradecanoic acid 14:0	0.38 ± tr. %	0.08 ± tr. %
Hexadecanoic acid 16:0	0.86 ± tr. %	1.65 ± 0.07%
Hexadecenoic acid 16:1ω-7	0.08 ± tr. %	0.03 ± tr. %
Octadecanoic acid 18:0	0.07 ± tr. %	0.37 ± 0.01%
Oleic acid 18:1ω-9	1.66 ± 0.01%	2.87 ± 0.13%
Linoleic acid 18:2ω-6	1.60 ± 0.02%	2.06 ± 0.09%
α-Linolenic acid 18:3ω-3	0.17 ± 0.01%	0.20 ± 0.01%
Eicosanoic acid 20:0	0.01 ± tr. %	0.01 ± tr. %
Eicosenoic acid 20:1ω-9	0.01 ± tr. %	0.02 ± tr. %
Eicosadienoic acid 20:2ω-6	N.D.	0.01 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	N.D.
Arachidonic acid 20:4ω-6	N.D.	N.D.
Eicosapentaenoic acid 20:5ω-3	N.D.	N.D.
Docosanoic acid 22:0	0.01 ± tr. %	0.01 ± tr. %
Docosapentaenoic acid 22:5ω-3	0.01 ± tr. %	N.D.
Docosahexaenoic acid 22:6ω-3	1.86 ± 0.04%	N.D.
Tetracosanoic acid 24:0	N.D.	N.D.
Total saturated fatty acids	1.37 ± 0.01%	2.17 ± 0.09%
Total monounsaturated fatty acids	1.75 ± 0.01%	2.92 ± 0.13%
Total polyunsaturated fatty acids	3.64 ± 0.07%	2.27 ± 0.10%
Total omega-3 (ω-3) PUFA	2.04 ± 0.05%	0.20 ± 0.01%
Total omega-6 (ω-6) PUFA	1.60 ± 0.02%	2.07 ± 0.09%
Omega-3/omega-6 (ω-3/ω-6) ratio	**1.28:1**	**0.10:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**0.78:1**	**10.35:1**

N.D. = not detected, tr. = trace (less than 0.05%).

Table 2.3 Fatty acid compositions of the DHA and oil blend diets used in chapter 3, expressed as mean percentage of total fatty acid (by weight) ± S.E.M. Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**Rodent chow + 5% DHA rich oil**	**Rodent chow + 5% oil blend**
Dodecanoic acid 12:0	0.7 ± 0.1%	0.7 ± 0.1%
Tetradecanoic acid 14:0	5.6 ± 0.1 %	1.1 ± tr. %
Hexadecanoic acid 16:0	12.7 ± 0.1 %	22.4 ± tr. %
Hexadecenoic acid 16:1ω-7	1.1 ± tr. %	0.5 ± tr. %
Octadecanoic acid 18:0	1.0 ± tr. %	5.1 ± tr. %
Oleic acid 18:1ω-9	24.6 ± tr. %	39.0 ± 0.1 %
Linoleic acid 18:2ω-6	23.6 ± 0.1%	28.0 ± tr. %
α-Linolenic acid 18:3ω-3	2.5 ± 0.1%	2.7 ± tr. %
Eicosanoic acid 20:0	0.1 ± tr. %	0.2 ± tr. %
Eicosenoic acid 20:1ω-9	0.2 ± tr. %	0.3 ± tr. %
Eicosadienoic acid 20:2ω-6	N.D.	0.1 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	N.D.
Arachidonic acid 20:4ω-6	N.D.	N.D.
Eicosapentaenoic acid 20:5ω-3	N.D.	N.D.
Docosanoic acid 22:0	0.2 ± tr. %	0.01 ± tr. %
Docosapentaenoic acid 22:5ω-3	0.1 ± tr. %	N.D.
Docosahexaenoic acid 22:6ω-3	27.5 ± 0.3%	N.D.
Tetracosanoic acid 24:0	N.D.	N.D.
Total saturated fatty acids	20.3 ± 0.3%	29.4 ± 0.1%
Total monounsaturated fatty acids	25.9 ± 0.1%	39.7 ± 0.1%
Total polyunsaturated fatty acids	53.9 ± 0.3%	30.8 ± tr. %
Total omega-3 (ω-3) PUFA	30.1 ± 0.3%	2.7 ± tr. %
Total omega-6 (ω-6) PUFA	23.6 ± 0.1%	28.1 ± tr. %
Omega-3/omega-6 (ω-3/ω-6) ratio	**1.28:1**	**0.10:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**0.78:1**	**10.35:1**

N.D. = not detected, tr. = trace (less than 0.05%).

In summary therefore, the lipid analysis importantly confirmed the main difference between the two diets were the high level of omega-3 PUFA DHA in the DHA diet, and that both diets had similar levels of omega-6 PUFA. Although there was a slight difference in omega-6 PUFA levels between diets (0.47% diet weight), this was minimal compared with previous studies investigating omega-3 PUFA supplementation whereby the control diet contained high levels of omega-6 PUFA (e.g. Calon et al., 2004; Green et al., 2007). Furthermore, the analysis confirmed that the absence of DHA in the oil blend diet was compensated by "neutral" monounsaturated fatty acids and saturated fatty acids. Interestingly, the lipid analysis revealed that the difference between ω-3/ω-6 ratios of the two diets were not as wide as predicted by SDS, with the DHA diet containing a ratio of 1.28:1 (in contrast to their reported 2.19:1) and the oil blend diet a ratio of 0.10:1 (in contrast to their reported 0.06:1).

2.3.6 Results: Lipid analysis of diets used in chapter 4

Tables 2.4 and 2.5 shows the lipid analysis results of the diet samples used in chapter 4. This data reveals that the fatty acids in the diets were primarily composed from PUFAs, followed by MUFAs and SATs. The main SATs in the diets were 16:0, followed by 14:0, with small amounts of 18:0, 12:0, 20:0 and 22:0. The main MUFA was 18:1ω-9, with small amounts of 16:1ω-7, 20:1ω-9 (only shown in the lipid analysis) and 14:1 (only shown in SDS profile). According to our results, the main omega-3 PUFA of the DHA diet was of course 22:6ω-3 (DHA), representing 1.86% of fresh diet weight and 27.5% of total fatty acids, followed by 18:3ω-3 and small amounts of 22:5ω-3. The only omega-3 PUFA in the oil blend diet was 18:3ω-3 (α-linolenic acid), representing 0.20% of the fresh diet weight, which was importantly detected in the DHA diet at similar levels. Omega-3 PUFAs 22:6ω-3 and 22:5ω-3 were at undetectable levels in the oil blend diet. Similar to the DHA diet, 20:3ω-3 and 20:5ω-3 was also undetectable in the oil blend diet. The main omega-6 PUFA was 18:2ω-6, representing 1.6% of the DHA diet (by weight) and approximately 2.1% of the oil blend diet.

As a result of the high level of DHA in the DHA diet and the similar levels of omega-6 PUFA in both diets, the DHA diet showed a greater ω-3/ω-6 ratio relative to the oil blend diet. Importantly, this ratio difference was caused almost entirely by the DHA content. The difference between the ω-3/ω-6 ratios of the two diets were not as wide as predicted by SDS, with the DHA diet containing a ratio of 1.28:1 (in contrast to their reported 2.19:1) and the oil blend diet a ratio of 0.11:1 (in contrast to their reported 0.06:1). Although there was a slight difference in omega-6 PUFA levels between diets (0.47% diet weight), this was minimal compared with previous studies investigating omega-3 PUFA supplementation whereby the control diet contained excess levels of omega-6 PUFA (e.g. Calon et al., 2005; Green et al., 2007). In summary, the lipid analysis confirmed the main difference between the two diets was the high level of omega-3 PUFA DHA in the DHA diet, and the absence of DHA in the oil blend diet which was compensated by "neutral" monounsaturated fatty acids and saturated fatty acids, including 16:0, 18:0 and 18:1ω-9. Importantly, analysis also confirmed that both diets had similar levels of omega-6 PUFAs. Furthermore, the lipid analysis results of the DHA and oil blend diets were almost identical to the diets used in chapter 3, thereby allowing direct comparison of dietary design.

Table 2.4 Fatty acid compositions of the DHA and oil blend diets used in chapter 4, expressed as mean percentage of fresh diet weight ± S.E.M. Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**Rodent chow + 5% DHA rich oil**	**Rodent chow + 5% oil blend**
Dodecanoic acid 12:0	0.04 ± 0.01%	0.05 ± 0.01%
Tetradecanoic acid 14:0	0.38 ± tr. %	0.08 ± tr. %
Hexadecanoic acid 16:0	0.86 ± tr. %	1.65 ± 0.07%
Hexadecenoic acid 16:1ω-7	0.08 ± tr. %	0.03 ± tr. %
Octadecanoic acid 18:0	0.07 ± tr. %	0.37 ± 0.01%
Oleic acid 18:1ω-9	1.66 ± 0.01%	2.87 ± 0.13%
Linoleic acid 18:2ω-6	1.60 ± 0.02%	2.06 ± 0.09%
α-Linolenic acid 18:3ω-3	0.17 ± 0.01%	0.20 ± 0.01%
Eicosanoic acid 20:0	0.01 ± tr. %	0.01 ± tr. %
Eicosenoic acid 20:1ω-9	0.01 ± tr. %	0.02 ± tr. %
Eicosadienoic acid 20:2ω-6	N.D.	0.01 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	N.D.
Arachidonic acid 20:4ω-6	N.D.	N.D.
Eicosapentaenoic acid 20:5ω-3	N.D.	N.D.
Docosanoic acid 22:0	0.01 ± tr. %	0.01 ± tr. %
Docosapentaenoic acid 22:5ω-3	0.01 ± tr. %	N.D.
Docosahexaenoic acid 22:6ω-3	1.86 ± 0.04%	N.D.
Tetracosanoic acid 24:0	N.D.	N.D.
Total saturated fatty acids	1.37 ± 0.01%	2.17 ± 0.09%
Total monounsaturated fatty acids	1.75 ± 0.01%	2.92 ± 0.13%
Total polyunsaturated fatty acids	3.64 ± 0.07%	2.27 ± 0.10%
Total omega-3 (ω-3) PUFA	2.04 ± 0.05%	0.20 ± 0.01%
Total omega-6 (ω-6) PUFA	1.60 ± 0.02%	2.07 ± 0.09%
Omega-3/omega-6 (ω-3/ω-6) ratio	**1.28:1**	**0.10:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**0.78:1**	**10.35:1**

N.D. = not detected, tr. = trace (less than 0.05%).

Table 2.5 Fatty acid compositions of the DHA and oil blend diets used in chapter 4, expressed as mean percentage of total fatty acid (by weight) ± S.E.M. Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**Rodent chow + 5% DHA rich oil**	**Rodent chow + 5% oil blend**
Dodecanoic acid 12:0	0.6 ± 0.10%	0.7 ± 0.10%
Tetradecanoic acid 14:0	5.6 ± tr. %	1.1 ± tr. %
Hexadecanoic acid 16:0	12.7 ± tr. %	22.4 ± 0.07 %
Hexadecenoic acid 16:1ω-7	1.2 ± tr. %	0.4 ± tr. %
Octadecanoic acid 18:0	1.0 ± tr. %	5.0 ± 0.01 %
Oleic acid 18:1ω-9	24.6 ± 0.01 %	39.0 ± 0.13 %
Linoleic acid 18:2ω-6	23.7 ± 0.02%	28.0 ± 0.09 %
α-Linolenic acid 18:3ω-3	2.5 ± 0.1%	2.7 ± 0.01 %
Eicosanoic acid 20:0	0.1 ± tr. %	0.1 ± tr. %
Eicosenoic acid 20:1ω-9	0.1 ± tr. %	0.3 ± tr. %
Eicosadienoic acid 20:2ω-6	N.D.	0.1 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	N.D.
Arachidonic acid 20:4ω-6	N.D.	N.D.
Eicosapentaenoic acid 20:5ω-3	N.D.	N.D.
Docosanoic acid 22:0	0.1 ± tr. %	0.1 ± tr. %
Docosapentaenoic acid 22:5ω-3	0.1 ± tr. %	N.D.
Docosahexaenoic acid 22:6ω-3	27.5 ± 0.04%	N.D.
Tetracosanoic acid 24:0	N.D.	N.D.
Total saturated fatty acids	20.3 ± 0.01%	29.5 ± 0.09%
Total monounsaturated fatty acids	25.9 ± 0.01%	39.7 ± 0.13%
Total polyunsaturated fatty acids	53.8 ± 0.07%	30.8 ± 0.10%
Total omega-3 (ω-3) PUFA	30.2 ± 0.05%	2.7 ± 0.01%
Total omega-6 (ω-6) PUFA	23.7 ± 0.02%	28.1 ± 0.09%
Omega-3/omega-6 (ω-3/ω-6) ratio	**1.28:1**	**0.10:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**0.78:1**	**10.35:1**

N.D. = not detected, tr. = trace (less than 0.05%).

2.3.7 Results: Lipid analysis of diets used in chapter 5

Tables 2.6 and 2.7 present the results from the lipid analysis of diet samples from chapter 5. Abbreviations are used as follows: control oil blend diet (OB), fish oil diet (FO), curcumin diet (OB+C), and fish oil and curcumin diet (FO+C). This data reveals that the fatty acid composition of the diets was primarily composed from PUFAs, followed by MUFAs and SATs. The main SAT in the diets were 16:0, followed by 18:0, with small amounts of 12:0 (trace levels in FO and FO+C diets), 14:0, 20:0 and 22:0, and trace levels of 24:0. The main MUFA was 18:1ω-9, with small amounts of 16:1ω-7 and 20:1ω-9. The main omega-6 PUFA of all diets was 18:2ω-6 (linoleic acid), representing between 1.32% to 1.64% of fresh weight diet and 33.62% to 34.35% of total fatty acids, whereby the oil blend diets (OB and OB+C) contained the higher amounts relative to the fish oil diets (FO and FO+C). Relatively small

amounts of other omega-6 PUFAs (20:2ω-6 and 20:4ω-6) were contained in the diets at similar levels.

The main omega-3 PUFA of the fish oil diets (FO and FO+C) were 20:5ω-3 (EPA) and 22:6ω-3 (DHA), representing 0.53% and 0.34% of fresh diet weight respectively and ~13.6% and 8.62% of total fatty acids respectively. In contrast, the oil blend diets (OB and OB+C) contained EPA and DHA at trace or non-detectable levels. Relatively small amounts of 18:3ω-3 (ALA) was contained in the diets at similar levels, whereas 22:5ω-3 (DPA) was detected at small levels only in the fish oil diets. Importantly, fatty acid composition was almost identical between the oil blend containing diets, OB and OB+C, and almost identical between the fish oil containing diets, FO and FO+C.

As a result of similar levels of omega-6 PUFA in all diets and a high level of omega-3 PUFAs EPA and DHA in the fish oil diets (FO and FO+C), the fish oil diets showed a greater ω-3/ω-6 ratio relative to the oil blend diets (OB and OB+C). The ω-3/ω-6 ratio was 1.21:1 and 1.19:1 for the FO and FO+C diets, respectively, and 0.10:1 for both the OB and OB+C diets. Although there was a slight difference in total omega-6 PUFA levels between the oil blend and fish oil containing diets, this was minimal compared with previous studies investigating fish oil supplementation whereby the control diet contains high levels of omega-6 PUFA (e.g. Oksman et al., 2006; Ma et al., 2009). In summary, lipid analysis confirmed the main difference between the oil blend and fish oil containing diets was the high level of omega-3 PUFAs EPA and DHA in the fish oil diets, and their absence in the oil blend diets which was compensated by "neutral" fatty acids including predominately 18:1ω-9 MUFA, and smaller amounts of 12:0, 16:0 and 18:0 SAT, as well as 18:2ω-6 PUFA. Importantly, lipid analysis confirmed that both diets had similar levels of omega-6 PUFA, comprising ~34% of total fatty acids in all diets.

The ω-3/ω-6 ratio of the oil blend and fish oil diets were extremely similar to the ratios reported in chapters 3 and 4 of the oil blend and DHA supplemented diets. In contrast, they contained differential amounts of certain fatty acids such as EPA. Together, this may allow some comparison of results in the general discussion related to the effects of the ω-3/ω-6 ratio, the levels of supplementation and the presence of certain fatty acids in the diet.

Table 2.6 Fatty acid composition of the oil blend (OB), oil blend and curcumin (OB+C), fish oil (FO), and fish oil and curcumin (FO+C) diets used in chapter 5, expressed as mean percentage of fresh diet weight ± S.E.M. Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**OB**	**OB+C**	**FO**	**FO+C**
Dodecanoic acid 12:0	0.06 ± 0.01%	0.06 ± 0.01%	tr. ± tr. %	tr. ± tr. %
Tetradecanoic acid 14:0	0.05 ± tr. %	0.05 ± tr. %	0.10 ± tr. %	0.10 ± tr. %
Hexadecanoic acid 16:0	1.04 ± 0.03%	0.95 ± 0.02%	0.62 ± tr. %	0.61 ± 0.01%
Hexadecenoic acid 16:1ω-7	0.02 ± tr. %	0.02 ± tr. %	0.08 ± tr. %	0.08 ± tr. %
Octadecanoic acid 18:0	0.23 ± 0.01%	0.21 ± tr. %	0.10 ± tr. %	0.10 ± tr. %
Oleic acid 18:1ω-9	1.53 ± 0.04%	1.39 ± 0.03%	0.49 ± 0.01%	0.49 ± 0.01%
Linoleic acid 18:2ω-6	1.64 ± 0.06%	1.51 ± 0.01%	1.32 ± 0.01%	1.33 ± 0.02%
α-Linolenic acid 18:3ω-3	0.16 ± 0.01%	0.15 ± tr. %	0.16 ± 0.01%	0.15 ± tr. %
Eicosanoic acid 20:0	0.01 ± tr. %	0.01 ± tr. %	0.01 ± tr. %	0.01 ± tr. %
Eicosenoic acid 20:1ω-9	0.02 ± tr. %	0.02 ± tr. %	0.05 ± tr. %	0.05 ± tr. %
Eicosadienoic acid 20:2ω-6	0.01 ± tr. %	tr. ± tr. %	0.01 ± tr. %	0.01 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	tr. ± tr. %	tr. ± tr. %	tr. ± tr. %
Arachidonic acid 20:4ω-6	tr. ± tr. %	tr. ± tr. %	0.03 ± tr. %	0.03 ± tr. %
Eicosapentaenoic acid 20:5ω-3	tr.± tr. %	N.D.	0.53 ± 0.01%	0.53 ± 0.02%
Docosanoic acid 22:0	0.01 ± tr. %	0.01 ± tr. %	0.01 ± tr. %	0.01 ± tr. %
Docosapentaenoic acid 22:5ω-3	N.D.	N.D.	0.07 ± tr. %	0.07 ± tr. %
Docosahexaenoic acid 22:6ω-3	N.D.	N.D.	0.34 ± tr. %	0.34 ± 0.01%
Tetracosanoic acid 24:0	tr.± tr. %	tr.± tr. %	tr.± tr. %	tr.± tr. %
Total saturated fatty acids	1.41 ± 0.04%	1.29 ± 0.03%	0.84 ± 0.01%	0.84 ± 0.02%
Total monounsaturated fatty acids	1.58 ± 0.04%	1.43 ± 0.03%	0.62 ± 0.01%	0.62 ± 0.01%
Total polyunsaturated fatty acids	1.81 ± 0.06%	1.66 ± 0.02%	2.46 ± 0.03%	2.45 ± 0.06%
Total omega-3 (ω-3) PUFA	0.17 ± 0.01%	0.15 ± tr. %	1.64 ± 0.02%	1.62 ± 0.05%
Total omega-6 (ω-6) PUFA	1.65 ± 0.06%	1.51 ± 0.01%	1.36 ± 0.01%	1.36 ± 0.03%
Omega-3/omega-6 (ω-3/ω-6) ratio	**0.10:1**	**0.10:1**	**1.21:1**	**1.19:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**9.71:1**	**10.10:1**	**0.83:1**	**0.84:1**

N.D. = not detected, tr. = trace (less than 0.01%).

Table 2.7 Fatty acid composition of the oil blend (OB), oil blend and curcumin (OB+C), fish oil (FO), and fish oil and curcumin (FO+C) diets used in chapter 5, expressed as mean percentage of total fatty acid (by weight) ± S.E.M. Mean value of 3 replicates. Information sourced from the lipid analysis carried out at Cardiff University.

Fatty acids	**OB**	**OB+C**	**FO**	**FO+C**
Dodecanoic acid 12:0	1.32 ± 0.04%	1.37 ± 0.08%	0.03 ± tr. %	0.03 ± tr. %
Tetradecanoic acid 14:0	1.10 ± 0.01%	1.09 ± 0.01%	2.56 ± 0.01%	2.44 ± 0.03%
Hexadecanoic acid 16:0	21.63 ± 0.20%	21.60 ± 0.22%	15.71 ± 0.09%	15.69±0.12%
Hexadecenoic acid 16:1ω-7	0.49 ± tr. %	0.49 ± 0.01%	2.15 ± 0.01%	2.07 ± 0.01%
Octadecanoic acid 18:0	4.85 ± 0.04%	4.81 ± 0.06%	2.63 ± 0.02%	2.65 ± 0.02%
Oleic acid 18:1ω-9	31.87 ± 0.27%	31.68 ± 0.39%	12.42 ± 0.11%	12.55±0.11%
Linoleic acid 18:2ω-6	34.17 ± 0.39%	34.35 ± 0.19%	33.62 ± 0.32%	33.95±0.28%
α-Linolenic acid 18:3ω-3	3.34 ± 0.03%	3.35 ± 0.01%	4.02 ± 0.14%	3.84 ± 0.03%
Eicosanoic acid 20:0	0.29 ± tr. %	0.29 ± tr. %	0.26 ± tr. %	0.26 ± tr. %
Eicosenoic acid 20:1ω-9	0.51 ± tr. %	0.51 ± 0.01%	1.24 ± tr. %	1.23 ± 0.01%
Eicosadienoic acid 20:2ω-6	0.11 ± tr. %	0.11 ± tr. %	0.13 ± tr. %	0.14 ± tr. %
Eicosatrienoic acid 20:3ω-3	N.D.	0.03 ± 0.01%	0.04 ± 0.01%	0.04 ± tr. %
Arachidonic acid 20:4ω-6	0.03 ± tr. %	0.03 ± tr. %	0.81 ± 0.01%	0.80 ± 0.01%
Eicosapentaenoic acid 20:5ω-3	0.02 ± tr. %	N.D.	13.62 ± 0.15%	13.60±0.20%
Docosanoic acid 22:0	0.21 ± tr. %	0.21 ± tr. %	0.23 ± tr. %	0.24 ± tr. %
Docosapentaenoic acid 22:5ω-3	N.D.	N.D.	1.82 ± 0.01%	1.78 ± 0.03%
Docosahexaenoic acid 22:6ω-3	N.D.	N.D.	8.62 ± 0.10%	8.62 ± 1.13%
Tetracosanoic acid 24:0	0.07 ± tr. %	0.08 ± tr. %	0.09 ± 0.01%	0.09 ± tr. %
Total saturated fatty acids	29.47 ± 0.30%	29.44 ± 0.38%	21.51 ± 0.14%	21.40±0.18%
Total monounsaturated fatty acids	32.87 ± 0.28%	33.84 ± 0.41%	17.15 ± 0.13%	17.20±0.13%
Total polyunsaturated fatty acids	37.66 ± 0.42%	37.87± 0.21%	62.68 ± 0.73%	62.75±0.69%
Total omega-3 (ω-3) PUFA	3.48 ± 0.04%	4.54 ± 0.02%	41.08 ± 0.41%	42.81±0.40%
Total omega-6 (ω-6) PUFA	34.30 ± 0.39%	34.48 ± 0.19%	34.56 ± 0.33%	34.88±0.29%
Omega-3/omega-6 (ω-3/ω-6) ratio	**0.10:1**	**0.10:1**	**1.21:1**	**1.19:1**
Omega-6/omega-3 (ω-6/ω-3) ratio	**9.71:1**	**10.10:1**	**0.83:1**	**0.84:1**

N.D. = not detected, tr. = trace (less than 0.01%).

2.4 Statistical analysis

All statistical analysis reported throughout this thesis were carried out using SPSS 16 software, and results were considered significant when $p<0.05$. Parametric statistical analyses were carried out primarily using an analysis of variance (ANOVA) since differences between groups were examined under more than 2 conditions, which warranted the use of an ANOVA. Furthermore, parametric tests have greater statistical power than non-parametric tests. In order to use this statistical approach, the assumptions of a between-subjects ANOVA were met or tested, which included independence of observations, normal distribution (normality) and homogeneity of variance. The assumptions of a within-subjects ANOVA were also tested, which included normality, homogeneity of variance and sphericity.

Normality was tested in SPSS using the Shapiro-Wilk test of normality, and if this assumption was violated ($p<0.05$) then the kurtosis value was checked since an ANOVA is robust against violations of normality and can therefore be reported, except when platykurtosis is present (Kinnear & Gray, 2009). Since platykurtosis (a flat distribution; kurtosis value of 3) was not present under circumstances when the normality assumption was violated, the ANOVA result was reported without the need for transforming the data or using non-parametric statistical tests. The assumption of homogeneity of variance was tested in SPSS using the Levene's test of equality of error variances, and when the assumption was violated non-parametric tests were used (Mann Whitney-U test selected if a between-subjects design, or Wilcoxon signed-rank test or Kruskal Wallis test selected if a within-subjects design). Since the results of the non-parametric tests were the same pattern of significance as reported by the parametric tests on these occasions, the ANOVA result could be used since our confidence that the ANOVA result was accurate, despite violation, had been tested (Kinnear & Gray, 2009). Finally, the assumption of sphericity was tested in SPSS by using the Mauchly's test of sphericity, and if this assumption was violated ($p<0.05$) then an epsilon correction was made by reporting the Greenhouse-geisser result in the SPSS output table.

Statistical analyses were primarily carried out using an ANOVA with genotype (Tg, WT) and diet (DHA, OB; or OB, OB+C, FO, FO+C) as between-subject factors. Experiments requiring analysis of data with inclusion of within-subject factor(s) (e.g. day, block, Aβ isoform, response type) were carried out using a mixed factorial ANOVA design with between-subject factors of genotype (Tg, WT) and diet (DHA, OB; or OB, OB+C, FO, FO+C), and the within-subject factor(s). Significant between-subjects interactions were followed up with tests of simple main effects using MANOVA syntax, as described in Kinnear and Gray (2009), p.295. Significant within-subject interactions were followed up with tests of simple main effects and interactions using MANOVA syntax, as described in Kinnear and Gray (2009), p.350. Finally, significant interactions between mixed (between- and within-subject) factors were followed up with tests of simple main effects and interactions using MANOVA syntax, as described in Kinnear and Gray (2009), p.375. When testing whether performance was above chance level, an unpaired (independent) samples t-test was selected as this statistical choice was appropriate for the number of conditions compared, the type of sample and type of data.

2.5 Chapter Discussion

One aim of this chapter was to summarise the procedures used for breeding and identifying Tg2576 mice possessing the APPswe transgene, for use in experimental testing. A second aim of this chapter was to outline the experimental and control diet design employed in chapters 3, 4 and 5. Lipid analysis results confirmed that the diets contained very similar levels of fatty acids after production compared to the details provided by SDS. The DHA-rich diet and oil blend diets examined in chapters 3 and 4 contained 1.86% DHA and 0% DHA, respectively. The oil blend diet was indeed supplemented with a blend of 'neutral' oils including primarily MUFAs and some SAT. The levels of omega-6 PUFAs were very similar, as were levels of other fatty acids. This design therefore allowed more accurate examination of the effect of dietary DHA supplementation compared with previous studies (e.g. Calon et al., 2004; Green et al., 2007).

Similarly, the fish oil-containing diets and oil blend-containing diets examined in chapter 5 were primarily differentiated by the level of omega-3 PUFAs EPA and DHA. The fish oil diets contained 0.53% EPA and 0.34% DHA, which were absent from the oil blend diets. In compensation, the lipid analysis confirmed that the oil blend diets contained supplementary levels of MUFAs (primarily), with small levels of SAT and PUFA. Similar to the diets examined in chapters 3 and 4, the fish oil and oil blend diet contained very similar levels of omega-6 PUFAs. Importantly, combination of curcumin with the oil blend or fish oils had minimal effect on the fatty acid composition of the diets. Again, this design therefore allowed more accurate examination of the effect of dietary fish oil and curcumin supplementation compared to previous studies (e.g. Oksman et al., 2006; Ma et al., 2009). Importantly, SDS reported that the standard diet (RM1) before supplementation contained nil levels of cholesterol. The supplementary oil blend, DHA-rich oil and curcumin also contained no cholesterol, although the fish oil supplement was likely to contain small levels of cholesterol (although this was not measured). This difference in cholesterol levels should therefore be considered when interpreting the results in later chapters. The aim of the following chapters was to examine the effect of these diets on the behavioural phenotype and pathology of Tg2576 mice.

Chapter 3

The longitudinal effect of dietary omega-3 PUFA docosahexaenoic acid (DHA) supplementation on spatial learning and Aβ pathology in Tg2576 mice

3.1 Introduction

The aim of the experiments in this chapter was to test the hypothesis that longitudinal exposure to a diet supplemented with omega-3 PUFA DHA from an early age will protect Tg2576 mice from the development of cognitive abnormalities and associated Aβ pathology. Although it is well documented that DHA supplementation can reduce behavioural deficits and pathology in animal models of AD, these studies do not evaluate the effect of DHA supplementation *per se* due to the experimental design of the control diet. All studies investigating DHA supplementation in animal models of AD to date used a control condition with different levels of total fat, depleted levels of omega-3 PUFA or excess levels of omega-6 PUFA (resulting in a particularly high ω-6/ω-3 ratio) compared to the DHA diet.

For example, the five studies investigating DHA supplementation in Tg2576 mice carried out by Greg Cole's lab compared a DHA diet to a safflower oil-based diet depleted in DHA with a considerably high ω-6/ω-3 ratio (Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006; Ma et al., 2007). The 'high-DHA' diet contained 0.6% DHA, ~2.8% linoleic acid (18:2ω-6) and a low ω-6/ω-3 ratio of 4:1-5:1, whereas the 'low-DHA' diet contained 0% DHA, ~4.9% linoleic acid and a very high ω-6/ω-3 ratio of 82:1-85:1. Diets were matched in total fat (6.1%) and cholesterol (750ppm). The reported beneficial effects of DHA supplementation (e.g. improved cognition, increased post-synaptic markers, reduced Aβ pathology and increased LR11 levels; as outlined in section 1.6.3 v) were relative to the DHA-depleted diet, and so effects could therefore be attributable to the large difference in ω-3/ω-6 ratio or linoleic acid content, rather than DHA content.

These studies also compared the high-DHA and DHA-depleted diets to a standard control chow containing 0.09% DHA, ~2.8% linoleic acid and a low ω-6/ω-3 ratio of 7:1. The

similarities between the standard control and high-DHA diet would have made the standard diet a relatively suitable baseline diet to compare the effects of DHA supplementation. However, the control diet contained higher levels of total fat (11%) and lower cholesterol (290ppm) than both high-DHA and depleted-DHA diets, which may contribute to the reported effects. Interestingly, when compared to the control diet, beneficial effects of DHA supplementation on pathology and synaptic markers was limited to a reduction in total Aβ42 only, thereby suggesting that DHA supplementation, total fat and/or cholesterol had little effect in reducing pathology or deficits in Tg2576 mice. In contrast, major differences in pathology and synaptic markers were reported when comparing the control diet with the DHA-depleted diet, which would suggest that the differences observed were likely attributable to the high ω-6/ω-3 ratio or high levels of omega-6 PUFA linoleic acid in the DHA-depleted diet.

Similar issues were found in studies investigating the effect of DHA supplementation in other transgenic AD models. For example, Green et al. (2007) reported DHA supplementation to reduce Aβ and tau pathology in the 3xTg AD model by comparing a DHA diet containing 1.3% DHA (accounting for all omega-3 PUFA content), 1.3% total omega-6 PUFA and a ω-6/ω-3 ratio of 1:1, relative to a control chow depleted in DHA and omega-3 PUFAs (0% DHA, 0.2% total omega-3 PUFA), and containing higher levels of total omega-6 PUFA (2.3%) and ω-6/ω-3 ratio (10:1). Rather than a beneficial effect of DHA supplementation, the effects may have been attributable to the higher ω-6/ω-3 ratio in the control diet resulting from low levels of total omega-3 PUFAs and the high levels of total omega-6 PUFAs. In support of the potent effects of a high ω-6/ω-3 ratio, Oksman et al. (2006) reported that a diet deficient in DHA with a high ω-6/ω-3 ratio of 70:1 increased Aβ pathology in the APPswe/PS1 model, compared to mice fed a control diet also deficient in DHA but with increased levels of omega-3 PUFAs and a lower ω-6/ω-3 ratio of 8:1.

It is therefore clear that further research is required to accurately examine the effect of DHA supplementation relative to a suitable baseline diet. The following experiments therefore used a suitable baseline control diet whereby the primary difference between the DHA and control diet was the DHA content. Details surrounding the design of the experimental diets are described in chapter 2 and briefly outlined in section 3.2. Further investigation into the effect of DHA supplementation is also required as only one study has reported beneficial effects of DHA supplementation on reducing pathology (Lim et al., 2005) and behavioural deficits

(Calon et al., 2004) in the Tg2576 model, and this was relative to diets which do not accurately assess the effect of DHA supplementation.

Furthermore, this chapter aims to examine the effect of longitudinal DHA supplementation from an early age of 4 months in Tg2576 mice. This dietary intervention therefore aimed to target underlying pathology during its initial stages of development, as the presence of soluble Aβ levels have been reported from 4 to 5 months in the Tg2576 model (Westerman et al., 2002). In contrast, all studies investigating the effects of DHA supplementation in the Tg2576 model have been initiated during advanced stages of amyloid pathology from 17 months of age (Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006; Ma et al., 2007). Similarly, the investigation of omega-3 PUFA or DHA supplementation in double and triple transgenic mouse models of AD has primarily been limited to periods of established pathology (Oksman et al., 2006; Hooijmans et al., 2007, 2009; Ma et al., 2009). Although these late-stage interventions are particularly applicable to the potential treatment in AD patients whereby the disease is often not detected until more advanced stages, they do not examine the effectiveness of DHA as an early-stage preventative measure. Supporting this, Arendash et al. (2007) argued that because cognitive impairment is already well-established, the published studies failed to examine whether DHA is 'cognitively protective'.

To date, the potential effectiveness of early-stage intervention of DHA has been investigated by only three studies in transgenic models of AD. Green et al. (2007) and Arsenault et al. (2011) showed positive effects of DHA supplementation from 3 to 4 months of age on cognition, tau and Aβ measures in the triple transgenic mouse model, where pathology develops from 3 to 6 months (Oddo et al., 2003; Billings et al., 2005). Arendash et al. (2007) also examined the effect of omega-3 supplementation from 2 months of age in the double transgenic APP/PS1 model, where Aβ pathology is detected as early as 6-12 weeks (Holcomb et al., 1998). Although no positive effects of omega-3 PUFAs on behavioural deficits or Aβ pathology were reported in the latter study, this may be attributable to the lack of omega-3 PUFA incorporation into the brain of Tg mice. Overall, these early intervention studies show potential for slowing pathological progression and associated deficits. The potential effectiveness or necessity of early-stage intervention is further indicated by results from clinical trials which generally showed no beneficial effect of omega-3 PUFA treatment in patients with developed AD (Freund-Levi et al., 2006; Quinn et al., 2010). In contrast, the positive results from epidemiological studies suggest longitudinal dietary intake of omega-3

PUFA before the onset of disease may be necessary for effective results in AD and dementia (e.g. Kalmijn et al., 1997a; Morris et al., 2003; Huang et al., 2005; Barberger-Gateau et al., 2007).

Using a suitable baseline diet, it was hypothesised that longitudinal dietary DHA supplementation from an early age would slow the onset and reduce the progression of behavioural deficits and Aβ pathology in Tg2576 mice. In order to test this hypothesis, Experiment 1 assessed longitudinal spatial learning performance of non-transgenic (wildtype) and Tg2576 mice at 8, 12 and 16 months of age using a test sensitive to the APPswe mutation, the T-maze forced choice alternation task. Following this, Experiment 2 examined the effect of DHA on Aβ pathology in Tg2576 mice using an enzyme-linked immunosorbent assay (ELISA) detection method to measure levels of Aβ in the brain of mice sacrificed at 16 months of age. Finally, Experiment 3 examined the fatty acid profile of the mouse brain to confirm whether dietary DHA supplementation altered fatty acid levels, and importantly, to confirm whether dietary omega-3 PUFAs were incorporated into brain fractions.

3.2 Overview of the mouse cohort and dietary intervention design used

The experiments in this chapter used one cohort of male Tg2576 transgenic (Tg) and non-transgenic (wildtype, WT) mice. The cohort of Tg2576 and WT mice used in this chapter was generated and maintained as outlined in chapter 2, section 2.2. At 4 months of age, mice were taken off standard laboratory mouse chow and were fed experimental diets composed of standard mouse chow containing either a 5% DHA-rich oil (DHA; containing 1.86% DHA, 2.05% total omega-3 PUFA, 1.60% total omega-6 PUFA, and an ω-3/ω-6 ratio of 1.28:1) or the 5% oil blend enriched control diet (OB; containing 0% DHA, 0.22% total omega-3 PUFA, 2.07% total omega-6 PUFA, and an ω-3/ω-6 ratio of 0.10:1). Importantly, the difference in ω-3/ω-6 ratio was caused almost entirely by the DHA content, and the difference in omega-6 PUFA content between the two diets was minimal compared with previous studies investigating DHA supplementation (e.g. Calon et al., 2005; Green et al., 2007). Furthermore, the absence of DHA in the control oil blend diet was compensated by 'neutral' monounsaturated fatty acids and saturated fatty acids. Further details concerning these diets are outlined in chapter 2, section 2.3. It was thought that the different diets would not alter body weight in the mice, since this was not found between the fish oil diet

(containing DHA) and oil blend diet in chapter 5 (see chapter 5, section 5.2). The experimental schedule employed for chapter 3 is outlined in figure 3.1.

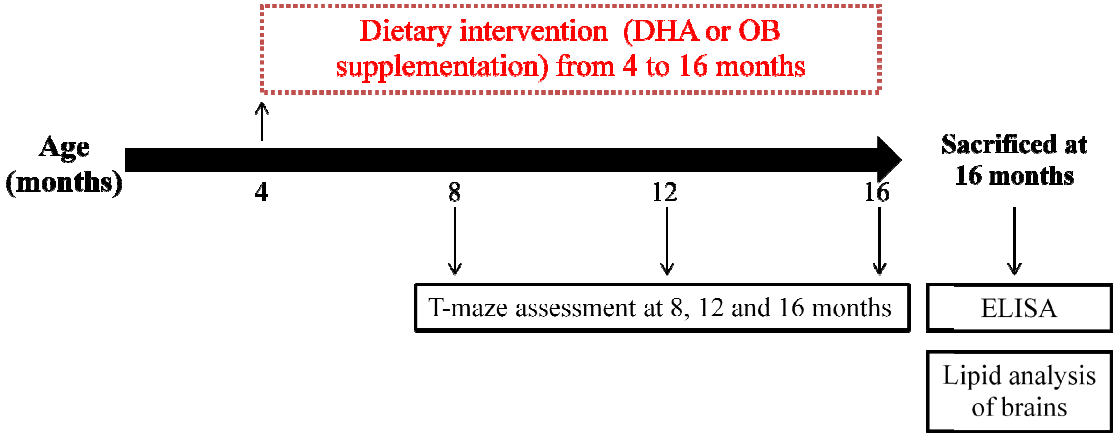

Figure 3.1 Experimental schedule employed in chapter 3.

3.3 Experiment 1: Longitudinal T-maze assessment

3.3.1 Introduction

This experiment aimed to test the hypothesis that longitudinal exposure to a diet supplemented with DHA from 4 months of age will improve memory function in Tg2576 and WT mice. These mice were assessed at 8, 12 and 16 months of age in order to examine whether early intervention delayed the onset or progression of memory impairment. These ages where selected as Aβ pathology is reportedly developed by 8 months of age, with diffuse deposits and plaques developing at 12 months of age, which are extensive by 16 months (e.g. Hsiao et al., 1996; Kawarabayashi et al., 2001; Westerman et al., 2002; Jacobsen et al., 2006; Bizon, Prescott & Nicolle, 2007). The alleviation of cognitive deficits at these ages may therefore indicate which stages of pathological development can be targeted by DHA supplementation. Previous studies have already demonstrated the potential for DHA supplementation to reduce cognitive deficits during late stages of pathological development in the Tg2576 model (Calon et al., 2004), the APPswe/PS1 model (Hooijmans et al., 2009) and the 3xTg model (Ma et al., 2009; Arsenault et al., 2011).

The assessment of spatial memory was selected because it is particularly affected in transgenic models of AD and is dependent on hippocampal function, which is particularly

compromised early in AD models and patients (see Ashe, 2001; Corcoran et al., 2002). Tg2576 mice show a rather specific learning and memory impairment in spatial memory tests, such as the T-maze, water maze, radial arm maze, radial arm water maze, Y-maze and circular platform, as well as versions of these tasks (Table 3.1). Importantly, these tasks measure different forms of spatial memory including 'reference' and 'working' memory, which can be differentially affected by pathology at different time points. Spatial 'reference' memory is where animals need to encode and recall a specific spatial location over the course of the test, which is trial-independent and probes long-term memory (Olton et al., 1979; Olton, 1986). Such tasks include the water maze or radial arm maze whereby an escape platform or reward can be found in a fixed location, and successful memory of this can be used to navigate to this 'goal' location to complete the task. Spatial 'working' memory is where animals use short-term memory of spatial information to solve the task, which is trial-dependent. Such tasks include the T-maze or Y-maze whereby a reward can be found in a previously unvisited location and so requires memory of the previously visited location. This is similarly required in tasks measuring species-typical behaviour such as spontaneous alternation, which measures the typical response for rodents to explore new environments and therefore switch between two maze arms on successive trials (Lalonde, 2002).

As shown in Table 3.1, these spatial memory deficits predominately emerge after 6 months of age in Tg2576 mice and are progressive, resulting in a relatively robust deficit at later stages. However, there is some evidence of early deficits at 3 months of age, and some later reports are inconsistent, thereby highlighting the need for further examination into the onset and progression of spatial memory deficits (which is further examined in chapter 4). For example, King and Arendash (2002) reported normal performance of transgenic mice in the circular platform and water maze at 3, 9, 14 and 19 months of age. In contrast, transgenic mice showed a stable deficit in the Morris water maze from 6 to 10 months of age onwards (Hsiao et al., 1996; Westerman et al., 2002; Adriani et al., 2006). Similarly, spontaneous alteration in the Y-maze and T-maze was reported normal at 3 to 4, 9, 12 and 14 months, whereas others report an impairment at 3, 7 to 12, 10, 12, 15, 17 and 19 months (Table 3.1). Such discrepancies could be due to methodological differences within tasks, differences in the sensitivity of various tests in detecting spatial memory deficits, or poor performance from controls.

The T-maze forced choice alternation (FCA) task was selected to assess the effect of DHA supplementation in this experiment as it is a robust test widely used to assay spatial working memory in rodents, and is sensitive to the APPswe mutation in an age-dependant manner and hippocampal dysfunction (e.g. Barnes, Hale & Good, 2004; Deacon & Rawlins, 2005). A transgene-related impairment in the T-maze had been reported from 8 months of age in Tg2576 mice, during the first point of assessment (Barnes, Hale & Good, 2004). A deficit has also been reported after 8 months of age, at 12 and 16 month assessment points (Chapman et al., 1999; Corcoran et al., 2002; Barnes, Hale & Good, 2004). It was therefore hypothesised that Tg2576 mice would show a deficit on this procedure at all ages. Based on previous evidence showing DHA supplementation to reduce cognitive deficits in rodent models of AD, it was hypothesised that DHA supplementation would reduce spatial memory deficits in Tg2576 mice. Furthermore, it was hypothesised that dietary DHA would improve T-maze performance in WT mice. Previous research has shown that omega-3 PUFA and DHA supplementation induced superior spatial learning and memory performance in healthy rodents (e.g. Jensen, Skarsfeldt & Høy, 1996; Suzuki et al., 1998; Lim & Suzuki, 2000a,b; Arsenault et al., 2011).

Table 3.1 Summary of hippocampal-dependant spatial memory measures in the Tg2576 mouse model.

Hippocampal-dependant spatial memory tasks																											
Behavioural task	**Age (months)**																										
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Morris water maze acquisition	N^{1}		N^{6}		Y^{1}			Y^{1}			$Y^{1,2}$			Y^{1}					Y^{6}								
					Y^{6}						Y^{6}																
Morris water maze retention (probe)	N^{1}				N^{1}			Y^{1}			Y^{1}			Y^{1}													
Water maze acquisition		N^{3}			N^{18}			N^{3}			Y^{2}		N^{16}					N^{16}									
		N^{16}						N^{16}					N^{18}														
Water maze retention (probe)		Y^{3}♀			N^{18}			N^{3}					N^{16}					N^{16}									
		N^{16}						N^{16}					N^{18}														
T-maze forced-choice rewarded alternation		N^{7}			N^{18}		Y^{12}	N^{4}	Y^{7}		N^{4}		Y^{10}	N^{15}	Y^{7}												
		N^{9}							Y^{12}		Y^{12}, N^{18}				Y^{9}												
T-maze spontaneous alternation											N^{4}					Y^{28}											
Y-maze spontaneous alternation		$Y^{1,3}$						$N^{3,16}$	Y^{1}		Y^{4}		N^{16}	Y^{19}				Y^{16}									
		N^{5}				Y^{14}																					
Appetitive Y-maze											Y^{4}			Y^{4}													
8-arm Radial arm maze																							Y^{17}				
6-arm Radial arm water maze																							Y^{11}		Y^{13}		Y^{13}
Paddling Y-maze		N^{4}							Y^{4}				Y^{4}								N^{4}						
Circular platform task		Y^{3}♀						N^{3}					N^{16}					N^{16}									
		N^{16}						N^{16}																			
Adapted circular platform acquisition						N^{8}																					
Redundancy cued water maze														N^{15}													
Closed field symmetrical maze																							Y^{17}				

Y= Transgene-dependant spatial memory deficit in Tg mice compared to age-matched WT controls, N = No transgene-dependant deficit, ♀ = gender-dependant deficit (females only). References ($Y/N^{\#}$) listed in Footnote 1.

Footnote 1. References (Y/N[#]) for Table 3.1: [1] Hsiao et al., 1996, [2] Adriani et al., 2006, [3] King et al., 1999, [4] Deacon et al., 2008, [5] Holcomb et al., 1998, [6] Westerman et al., 2002, [7] Chapman et al., 1999, [8] Pompl et al., 1999, [9] Corcoran et al., 2002, [10] Hale & Good, 2005, [11] Wilcock et al., 2006, [12] Barnes, Hale & Good, 2004, [13] Wilcock et al., 2004, [14] Ognibene et al., 2005, [15] Bizon, Prescott & Nicolle, 2007, [16] King & Arendash, 2002, [17] Asuni et al., 2006, [18] Zhuo et al., 2007, [19] Middei et al., 2004.

3.3.2 Methods

3.3.2.1 Design

One cohort of male Tg2576 transgenic (Tg) and non-transgenic (wildtype, WT) mice were evaluated in the T-maze forced-choice alteration (FCA) task at three ages. Experimentally naïve mice were first tested at 8 months of age, a period where behavioural abnormalities have been previously reported (e.g. Barnes, Hale & Good, 2004). This cohort was tested again at 12 months of age, when plaque development is typically at an early stage in this transgenic line, and once again at 16 months of age when plaque pathology is well established. This design therefore investigated transgene- and diet-dependant changes at these ages, in addition to whether these changes were age-dependant.

3.3.2.2 Subjects

The cohort of mice used in this experiment consisted of 25 Tg2576 (Tg) mice and 24 wildtype (WT) control mice. When tested at 8 months of age, 12 Tg mice received DHA diet (Tg DHA), 13 Tg mice received the oil blend diet (Tg OB), 13 WT mice received the DHA diet (WT DHA) and 11 WT mice received the oil blend diet (WT OB). By the second stage of experimentation at 12 months of age, the loss of 10 mice resulted in group numbers of 10 Tg DHA, 10 Tg OB, 10 WT DHA and 9 WT OB. By the third stage of experimentation at 16 months of age, the further attrition of 3 mice resulted in group numbers of 7 Tg DHA, 10 Tg OB, 10 WT DHA and 9 WT OB. The running order of mice was counterbalanced so that approximate equal numbers of each experimental group of mice were run in each batch (n=8), with several batches of mice being tested once per day over 14 consecutive days.

3.3.2.3 Apparatus

The T-maze was constructed from three arms each 9cm wide and 13cm tall in the formation of a 'T', and composed from clear Perspex (Figure 3.2). The long start arm was 52cm long and the two goal arms were each 26cm long, with the maze elevated 92cm from the floor. The maze was situated in a quiet, illuminated room surrounded by numerous distinct visual cues such as posters and shelving on the walls, benching and air conditioning ducts. Entrance to and exit from selected goal arms during the task were controlled using removable doors made from opaque Perspex. Approximately 50µl of a 10% sucrose solution in distilled water was used as a liquid reward during the task and placed in black-coloured recessed wells at the end of each goal arm.

Figure 3.2 T-maze apparatus, showing maze construction and recessed wells.

3.3.2.4 Procedure

Behavioural experiments were carried out by the author and a research assistant (Vicky Staal), with all data analysis carried out by the author. Mice were water deprived throughout this experiment in order to motivate them to consume the 10% sucrose reward. Water deprivation consisted of unrestricted access to water in their home cage for 2 hours each day, which was provided after completing the last training or test session of the day. Each mouse was checked daily for general health and weighed to ensure their body weight was maintained at over 85 to 90% of their *ad lib* weight. The experimenter remained in the room throughout all sessions and maintained a consistent position in the room throughout training.

The T-maze FCA experiment consisted of 4-day habituation to the testing room and apparatus, and a 10-day training (testing) period. Mice received one habituation session per day, which involved 5-minute free access within the T-maze in order to explore the maze. Each goal arm was baited with a 10% sucrose reward located in a recessed food well, and for the first 2 days of habituation, reward was also placed on the lip of the well. If the reward was not consumed within 5 minutes, the mice received a further 5 minutes in the T-maze. The maze was cleaned using a 1:20 dilution of Mr Muscle® glass cleaner with water, and dried with paper towelling, prior to placing each mouse in the apparatus. Mice were transported to the test room in individual home cages and acclimatised to the room for 10 minutes prior to each habituation and testing session.

Each mouse received a total of 10 days of training. After 5 consecutive days of training, the mice received a two-day break during which the mice received 48-hour *ad lib* water access. Each testing day was comprised of six trials whereby mice received a sample (forced-choice) trial and test (free-choice) trial, which were separated by a ~30 second interval. Each trial was separated by an inter-trial interval of approximately 10 minutes, during which the mouse was placed in their home cage. Before the start of each sample trial, the maze was cleaned and both goal arms were baited with ~50µl of 10% sucrose reward. Each mouse was placed at the end of the start arm and given access to one goal arm (left or right). The location of this 'sample arm' was selected pseudo-randomly by the experimenter, such that each mouse were exposed to three left and three right sample arms within each training session, with the restriction that each mouse received no more than two consecutive trials to the same sample location. Access to the remaining arm was blocked using an opaque Perspex door located at the start of the goal arm. Once the mouse had entered the sample arm, it was given 30 seconds to consume the reward and was then removed. The maze (including both goal and non-goal arms) was then cleaned to obscure any potential intra-maze odour cues.

Approximately 30 seconds after the sample trial, the mouse received the test trial whereby the Perspex door was removed providing access to both left and right goal arms, and the mouse was again placed at the base of the start arm. The mouse was presented with a choice of access to either the left or right arm, and once the choice had been made the mouse was confined to the arm using the Perspex door. Entry to the novel (non-sample) arm resulted in access to liquid reward and was recorded as a correct choice. An entry was defined as when the rear of the body, excluding the tail, passed the entrance to the arm. In contrast, entry to

the previously visited sample arm resulted in no reward and the mouse was restricted to this arm for 15 seconds. This was recorded as an incorrect choice. The mouse was then placed into their home cage until the next session.

3.3.2.5 Scoring and data analysis

T-maze performance was calculated as a percentage of correct trials (successful alternations) of the six trials conducted per day. Statistical analyses were carried out using an ANOVA with genotype (Tg or WT) and diet (DHA or oil blend) as between-subject factors, and day (1-10) as a within-subject factor. Following significant interactions, tests of simple main effects and simple interactions were carried out. A separate ANOVA analysed the data at 8, 12 and 16 months independently. In addition to this, a comparison of all three ages was carried out using a repeated-measures ANOVA. This analysis used the percentage of correct trials on the last day of acquisition (day 10) only to assess whether optimal learning performance varied with age in the Tg and WT groups across diet conditions. As attrition in the number of mice occurred over the period of testing, the latter analysis only included mice that were assessed at all three ages (Tg DHA n=7, Tg OB n=10, WT DHA n=10 and WT OB n=9).

3.3.3 Results: T-maze task

Figure 3.3 shows the percentage of correct trials for both Tg and WT mice fed the DHA or oil blend diet during acquisition of the T-maze FCA task at 8 months of age. An ANOVA with genotype, diet and day as factors revealed no significant effect of genotype ($F(1,45)=1.660$, $p>0.05$), diet ($F(1,45)=0.103$, $p>0.05$) or interaction between these factors ($F(1,45)=1.025$, $p>0.05$). As illustrated in Figure 3.3, these results indicate that Tg and WT mice performed equally well with no significant effect of 4-month dietary DHA supplementation. Statistical analysis also showed a significant effect of day ($F(9,405)=11.447$, $p<0.001$), a significant interaction of day by genotype ($F(9,405)=3.255$, $p<0.01$), no significant interaction of day by diet ($F(9,405)=1.823$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(9,405)=1.009$, $p>0.05$). Tests of simple main effects for the significant day by genotype interaction revealed that WT mice performed significantly better than Tg mice on day 9 ($F(1,47)=16.25$, $p<0.001$).

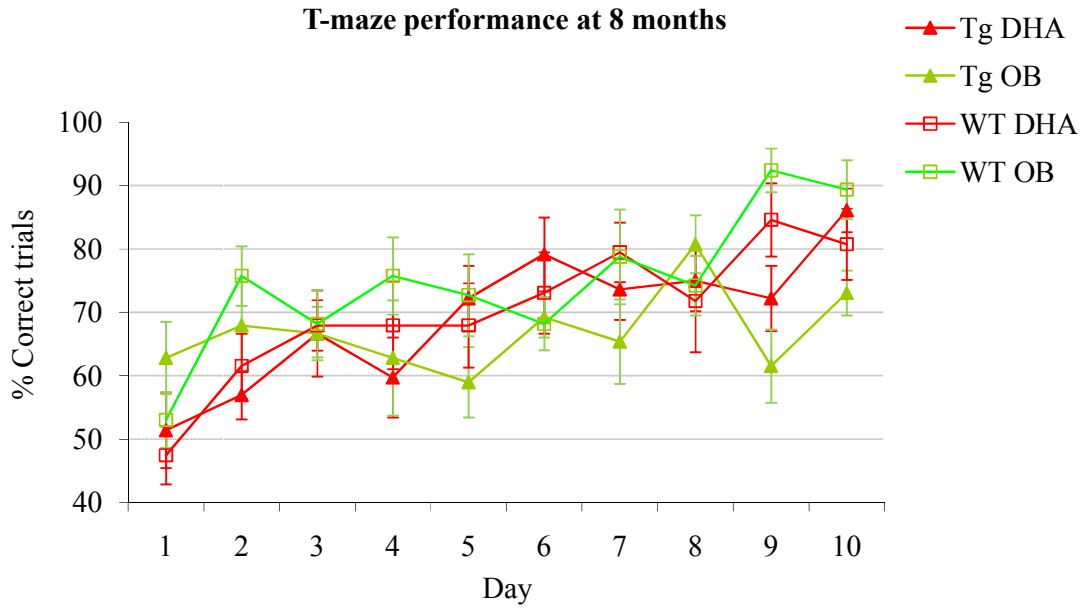

Figure 3.3 T-maze performance at 8 months of age. Mean percentage of correct trials over 10 days acquisition in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M (standard error of the mean).

Figure 3.4 shows the percentage of correct trials for both Tg and WT mice fed the DHA or oil blend diet during T-maze acquisition at 12 months of age. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,35)=16.918$, $p<0.001$), but no significant effect of diet ($F(1, 35)=2.806$, $p>0.05$) and no interaction of genotype by diet ($F(1,35)=0.556$, $p>0.05$). As illustrated in Figure 3.4, WT mice performed significantly better than Tg mice. However, there was no significant effect of 8-month dietary DHA supplementation on performance. Statistical analysis also showed a significant effect of day ($F(9,315)=10.950$, $p<0.001$), a significant interaction of day by genotype ($F(9,315)=1.920$, $p<0.05$), and no significant interaction of day by diet ($F(9,315)=0.707$, $p>0.05$) or day by genotype by diet ($F(9,315)=0.264$, $p>0.05$). A follow-up test of simple main effects for the significant day by genotype interaction revealed that WT mice performed significantly better than Tg mice on day 2 ($F(1,37)=5.12$, $p<0.05$), day 5 ($F(1,37)=5.79$, $p<0.05$), day 7 ($F(1,37)=21.57$, $p<0.001$), day 8 ($F(1,37)=11.19$, $p<0.01$), day 9 ($F(1,37)=30.80$, $p<0.001$) and day 10 ($F(1,37)=45.74$, $p<0.001$). This genotype effect was particularly evident on day 9 of training, which was likely due to the more rapid learning by WT mice on the last two

acquisition days compared with Tg mice which demonstrated slower acquisition and erratic performance (together with a better performance on day 10 than day 9).

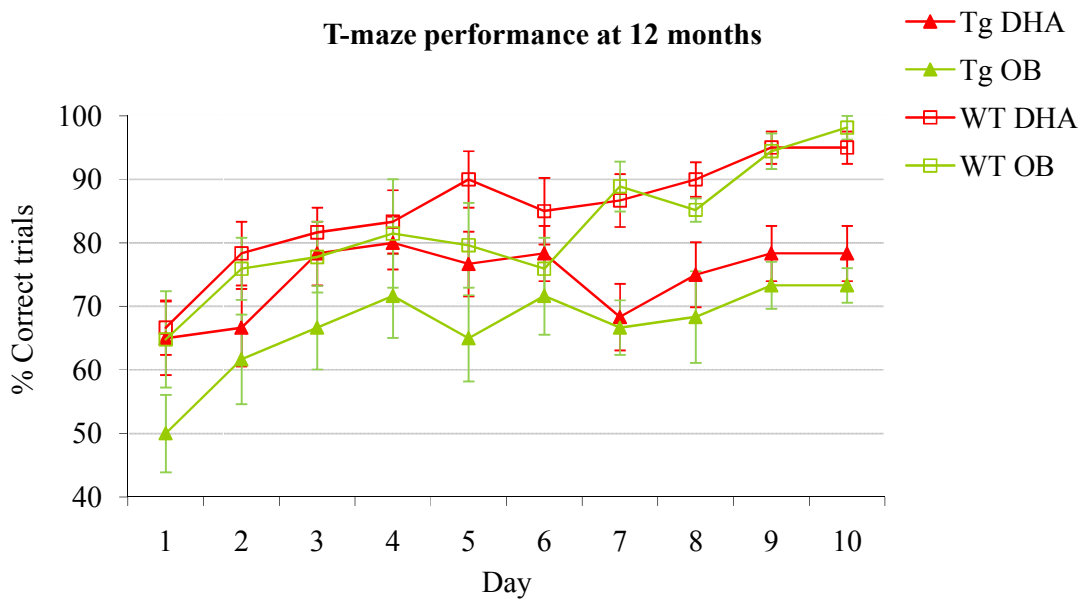

Figure 3.4 T-maze performance at 12 months of age. Mean percentage of correct trials over 10 days acquisition in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

Figure 3.5 shows the percentage of correct trials for both Tg and WT mice fed the DHA or oil blend diet during T-maze acquisition at 16 months of age. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,32)=21.409$, $p<0.001$), no significant effect of diet ($F(1, 32)=2.292$, $p>0.05$) and no interaction between these factors ($F(1,32)=1.363$, $p>0.05$). As illustrated in Figure 3.5, this analysis indicates that WT mice performed significantly better than Tg mice, and there was no significant effect of 12-month DHA supplementation on performance. Statistical analysis also showed a significant effect of day ($F(9,288)=6.106$, $p<0.001$) and significant interaction of day by genotype ($F(9,288)=3.349$, $p<0.001$), but no significant interaction of day by diet ($F(9,288)=0.924$, $p>0.05$). Follow-up tests of simple main effects for the significant day by genotype interaction revealed that WT mice performed significantly better than Tg mice on day 4 ($F(1,32)=22.22$, $p<0.001$), day 5 ($F(1,32)=8.94$, $p<0.01$), day 6 ($F(1,32)=7.17$, $p<0.05$), day 7 ($F(1,32)=21.63$, $p<0.001$), day 9 ($F(1,32)=13.06$, $p<0.001$) and day 10 ($F(1,32)=21.61$, $p<0.001$). Statistical analysis also revealed a significant three-way interaction of day by genotype by diet ($F(9,288)=2.541$, $p<0.01$). A follow-up test of simple interactions revealed a

significant genotype by diet interaction on day 3 ($F(3,32)=5.57$, $p<0.05$). Tests of simple main effects revealed a significant difference between Tg OB and WT OB mice ($F(1,32)=9.15$, $p<0.01$) and a significant difference between Tg DHA and Tg OB on day 3 ($F(1,32)=10.71$, $p<0.01$).

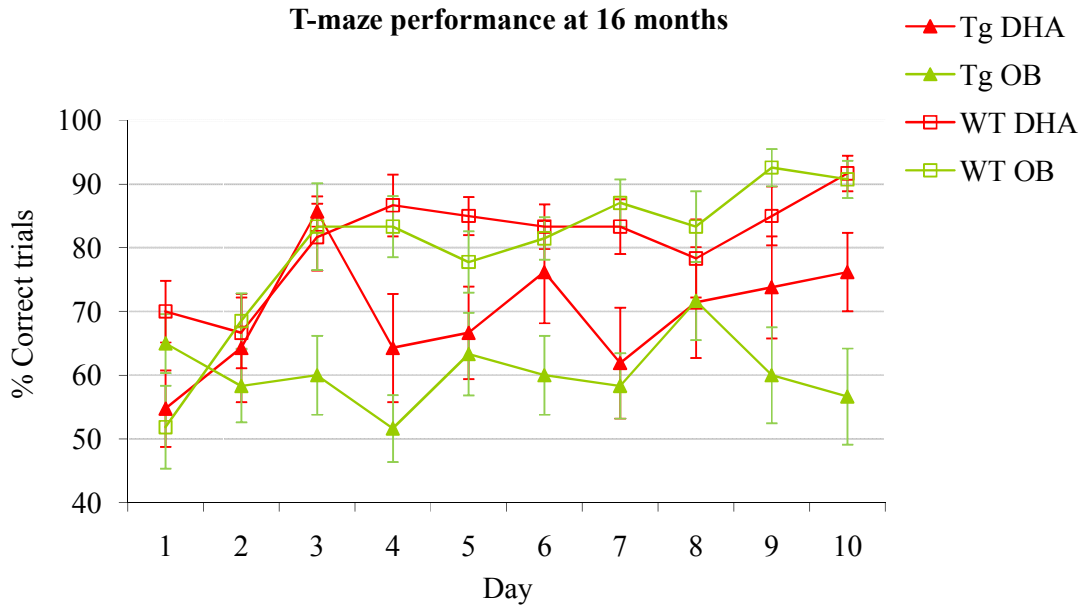

Figure 3.5 T-maze performance at 16 months of age. Mean percentage of correct trials over 10 days acquisition in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

Figure 3.6 shows the percentage of correct trials for both Tg and WT mice on DHA or oil blend diet on the last day of acquisition (day 10) of the T-maze task at 8, 12 and 16 months of age. This examined the effect of age, as well as genotype and diet, on learning. Due to mortalities throughout the course of experimentation several mice were excluded from this analysis including 3 Tg OB, 5 Tg DHA, 2 WT OB and 3 WT DHA. The last day of acquisition was used as it was thought to correspond to asymptotic performance, as shown in Figures 3.3 to 3.5. An ANOVA with age, genotype and diet as factors revealed a significant effect of genotype ($F(1,32)=36.920$, $p<0.001$), a significant effect of diet ($F(1,32)=4.478$, $p<0.05$) and a significant interaction of genotype by diet ($F(1,32)=4.926$, $p<0.05$). Following-up this interaction, tests of simple main effects revealed that Tg OB mice performed significantly worse than all groups including Tg DHA ($p<0.05$), WT DHA ($p<0.001$) and WT OB ($p<0.001$). There was no significant effect of age ($F(2,64)=2.905$, $p>0.05$), a significant interaction of age by genotype ($F(2,64)=3.469$, $p<0.05$), no significant interaction of age by

diet ($F(2,64)=1.164$, $p>0.05$) and no significant three-way interaction of age by genotype and diet ($F(2,64)=0.340$, $p>0.05$). Follow-up tests of simple main effects for the age by genotype interaction revealed Tg mice to perform significantly worse than WT mice at 12 ($F(1,34)=42.51$, $p<0.001$) and 16 months ($F(1,34)=22.39$, $p<0.001$), but not 8 months ($F(1,34)=3.92$, $p>0.05$), supporting the analysis at separate ages. These results suggest that T-maze performance was sensitive to age-related changes in Tg but not WT mice, suggesting that a decline in Tg performance was caused by Aβ-associated pathology. In contrast, the T-maze task was not sensitive to age-related cognitive decline in WT mice at these ages.

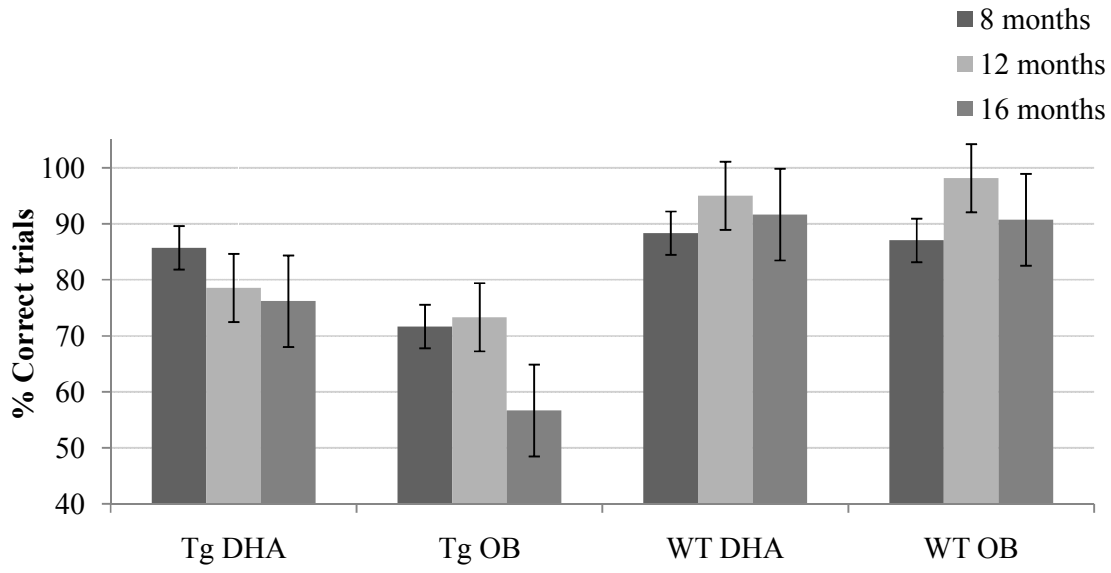

Figure 3.6 T-maze performance at 8, 12 and 16 months of age. Mean percentage of correct trials on the last day (day 10) of trial acquisition in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

In summary, the analysis of the T-maze FCA task showed that Tg2576 mice were impaired at 12 and 16 months, but not 8 months of age. Although DHA supplementation generally failed to enhance performance in Tg and WT mice, follow-up tests of a significant three-way interaction of day by genotype by diet at 16 months revealed Tg mice fed the oil blend diet to perform significantly worse than all groups on day 3. The transitory nature of these effects may suggest that it is attributable to sampling error or random fluctuations in performance. However, an analysis of the terminal levels of performance achieved by the end of training across all age groups in mice that completed the full set of tests also revealed a positive effect

of DHA diet on the performance of Tg mice. This was particularly illustrated in mice aged 16 months whereby Tg DHA mice achieved 76% correct compared to 57% correct reported in Tg OB mice. Tg OB performance was just above chance level (whereby a mouse can achieve 50% correct trials through chance alone), thereby suggesting limited learning or memory by Tg OB at 16 months, unlike the Tg DHA group. An unpaired samples t-test confirmed this by comparing the performance of Tg groups aged 16 months to chance levels (50%), revealing a non-significant difference for the Tg OB group ($t(18)=0.885$, $p>0.05$) but a significant difference for the Tg DHA group ($t(12)=4.260$, $p<0.001$).

3.3.4 Discussion

The T-maze FCA task revealed Tg2576 mice to be significantly impaired relative to WT control mice at 12 and 16 months of age, which supports previous findings of an age-dependant deficit (e.g. Chapman et al., 1999; Corcoran et al., 2002; Hale & Good, 2005; Barnes, Hale & Good, 2004; Zhuo et al., 2007). Although the lack of transgene-induced deficit at 8 months of age was in contrast to previous reports in the T-maze (Barnes, Hale & Good, 2004), our data revealed an impairment on day 9 of the task which perhaps suggested the onset of a modest deficit at this age.

Figure 3.6 showed Tg performance (particularly the Tg OB group) appeared significantly lower as a consequence of age. Using day 10 data it was calculated that Tg OB performance reduced by 15% from 8 to 16 months of age down to 56.6% correct trials, Tg DHA performance reduced by 9.5% down to 76.2%, WT OB performance increased by 3.7% up to 90.7%, and WT DHA performance increased by 3.3% up to 91.67%. These results therefore suggest that the APPswe mutation leads to an age-related decline in spatial learning likely resulting from increasing Aβ pathology with age. In contrast, the lack of mutation in WT mice and practice effects may have led to the increased performance observed. This general pattern suggests that DHA may provide subtle protection against age-related cognitive decline of spatial working memory in Tg2576 mice.

Supplementation of DHA provided limited protection against a decline in spatial working memory, albeit subtle, in Tg2576 mice at 16 months. This is supported by Calon et al. (2004) who reported that DHA supplementation reduced spatial learning deficits in 21-22 month old Tg2576 mice. In contrast, the present study found DHA supplementation did not benefit Tg

performance at 8 or 12 months of age. This may suggest that DHA may only be effective in the presence of advanced Aβ pathology. It is therefore possible that DHA targets plaque development, which is extensive at 16 months of age (Hsiao et al., 1996; Kawarabayashi et al., 2001; Bizon, Prescott & Nicolle, 2007). This may explain the lack of dietary effect at 12 months of age when plaque load has been reported scattered and modest (Jacobsen et al., 2006).

Although the results indicate modest positive effects of dietary supplementation during late but not early stages of pathology, it could be argued that the T-maze test was not sufficiently sensitive to detect more subtle dietary-induced behavioural changes at early stages. For example, despite the T-maze being a robust test widely used to assess spatial working memory, its 50% chance level results in a small window to detect subtle differences in learning and memory, and changes as a function of treatment. For example, transgene-dependant deficits in spatial memory are not detected in Tg2576 mice in the T-maze until 8 months, yet spatial deficits have been reported from 3 months in the Y-maze, circular platform and water maze task (King et al., 1999). It is therefore argued that performance should be further assessed using a wider range of cognitive tasks, particular more difficult tasks, in order to probe potential subtle effects. This was therefore examined in chapter 4.

This issue may also explain the lack of diet effect in WT mice in this study, which was unexpected as several studies have reported positive effects of omega-3 PUFA and DHA supplementation in normal healthy rodents. Supporting this argument, such studies have detected positive effects of dietary supplementation using more complex tasks including the Morris water maze and a multiple-alley maze (Jensen, Skarsfeldt & Høy, 1996; Suzuki et al., 1998; Lim & Suzuki, 2000a,b). In contrast to the latter tasks which provide multiple opportunities for error (e.g. several arm choices), the T-maze provides less opportunity for error with only 2 arm choices. Furthermore, it may be important to assess different forms of spatial memory, as highlighted in Gamoh et al. (1999) study which reported cognitive benefits of omega-3 PUFA supplementation in rodents which were limited to reference not working memory in the 8-arm radial maze. As the T-maze assesses working memory, it may therefore be important to assess dietary intervention using tests of spatial reference memory.

On another note, it is possible that performance in the T-maze or other spatial memory tasks could be confounded by visual ability as several AD mouse models harbouring the APPswe

mutation suffer from retinal degeneration related to Aβ pathology (see Garcia et al., 2004; Ning et al., 2008; Liu et al., 2009). Importantly however, Garcia et al. (2004) argue that APPswe mice show impaired spatial memory irrespective of retinal degeneration status. Moreover, intact novel item recognition in Tg2576 mice suggests at least some visual integrity (e.g. Good & Hale, 2007). Tests measuring visual ability have produced mixed results, with some deficits reported in a water maze task at 9, 14 and 19 months (King et al., 1999; King & Arendash, 2002), but not at 3, 6 and 14 months (King et al., 1999; King & Arendash, 2002; Zhuo et al., 2007). Moreover, no visual deficit was reported in the Morris water maze at 9-10 months (Hsiao et al., 1996) or radial arm water maze at 24 months (Wilcock et al., 2006). In order to rule out the possibility of retinal degeneration accounting for impaired performance otherwise attributed to memory dysfunction, future studies should assess the degree of visual integrity or screen for retinal degeneration gene status.

3.4 Experiment 2: Pathological assessment

3.4.1 Introduction

This experiment aimed to investigate the effect of longitudinal dietary DHA supplementation from an early age on later-stage Aβ pathology in Tg2576 mice. A vast body of literature has shown DHA to reduce Aβ pathology both *in vivo* and *in vitro* (e.g. Lim et al., 2005; Oksman et al., 2006; Green et al., 2007; Boudrault et al., 2009). As important as it was to assess the behavioural effects of DHA supplementation relative to a suitable baseline diet, it was equally important to assess the pathological effects to determine any changes to the underlying pathology of the disease. Although these measures normally correlate, one does not consistently predict the other, as demonstrated by Oksman et al. (2006) who reported DHA supplementation to reduce Aβ levels but did not improve spatial learning in the water maze. Conversely, Calon et al. (2004) reported the opposite. Based on previous literature and the longitudinal design of our dietary supplementation during early onset to later stages of pathology, it was hypothesised that DHA supplementation would reduce Aβ accumulation in Tg2576 mice. Furthermore, the T-maze results in experiment 1 may suggest that DHA could reduce plaque pathology to some extent, as suggested by some spatial learning improvements at 16 months of age.

In order to test this hypothesis, pathological analysis was carried out following behavioural testing in the T-maze task at 16 months of age when Aβ accumulation should be significant. Amyloid pathology can be assessed using immunohistochemistry and an enzyme-linked immunosorbent assay (ELISA) detection method. These techniques use specific human Aβ1-40 and Aβ1-42 detection antibodies to indicate the presence of these proteins in tissue samples. Immunohistochemistry is frequently used to assess the pattern of Aβ accumulation throughout the brain and can be used to quantify Aβ aggregates, although this is less precise than an ELISA (Kawarabayashi et al., 2001). In contrast, an ELISA is specifically used to quantify both soluble and insoluble Aβ levels in biological tissue samples, for example, from a whole brain or brain regions such as the cortex and hippocampus. Soluble Aβ exists in non-aggregated fashion in a non-fibrous form, whereas insoluble Aβ exists as diffuse and senile neuritic plaques of aggregated Aβ (Hardy & Allsop, 1991).

The ability to measure soluble forms of Aβ in addition to insoluble forms is particularly important as soluble, but not insoluble forms, are strongly correlated with markers of AD severity such as cognitive and synaptic impairment (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999; Näslund et al., 2000). Furthermore, post-mortem studies have revealed the presence of cortical Aβ deposits in humans without AD symptoms (Arrigada et al., 1992; Crystal et al., 1998), and Holmes et al. (2008) reported maintained AD progression when plaques were removed with Aβ immunisation. This therefore suggests a key role of soluble Aβ in AD pathogenesis.

For the purpose of this experiment, an ELISA detection method was therefore selected in order to measure both soluble and insoluble Aβ. Supporting this selection, Oksman et al. (2006) reported DHA to reduce total Aβ levels measured in an ELISA, but had no effect on plaque load measured by immunohistochemistry. Measuring both soluble and insoluble extracts may also shed light on the potential action of DHA. For example, selective reduction of insoluble but not soluble Aβ by DHA may suggest inhibition of Aβ aggregation. The specific measurement of Aβ40 and Aβ42 isoforms were also selected as these proteins are commonly assessed in Tg2576 intervention studies, and are the most common and pathogenic forms of Aβ in the Tg2576 model (Hsiao et al., 1996) and in AD patients (Kawarabayashi & Shoji, 2008). Using this highly sensitive ELISA measure, this experiment assessed Aβ accumulation in the cortex as this is one of the most pathologically affected brain regions (e.g. Hsiao et al., 1996). Although Aβ could have been measured in plasma samples, this may

not be representative of levels in the brain as Kawarabayashi et al. (2001) reported that the human APPswe transgene in Tg2576 mice is expressed at substantial levels in other organs and so plasma Aβ is likely derived from both the brain and peripheral organs.

3.4.2 Methods

3.4.2.1 Design and subjects

Soluble and insoluble human Aβ1-40 and Aβ1-42, expressed from the APPswe transgene, were quantified by ELISA in cortex samples of Tg2576 mice aged 16 months by a research associate (Dr. Cécile Bascoul-Colombo). This experiment analysed 3 Tg mice from each diet group (DHA and OB), following the T-maze FCA task at 16 months of age. WT mice were not systematically assessed as they do not express human Aβ. Analysis of one WT mouse from each diet group confirmed the absence of human Aβ.

3.4.2.2 Procedure

Tissue preparation

Mice were culled by cervical dislocation. The brain was immediately removed and maintained on ice while dissecting the cortex, hippocampus and cerebellum. The dissected tissue samples were immediately weighed and snap-frozen in liquid nitrogen. These samples were then stored at -80ºC until ready to be analysed.

Protein extraction

In order to extract soluble and insoluble proteins from the tissue, tissue samples were maintained on ice and homogenised using an autoclaved micropestle in freshly-made extraction buffer 1, added at a rate of 1ml per 150mg wet tissue weight. Extraction buffer 1 contained 2% (w/v) sodium dodecyl sulphate (SDS, Sigma) in MilliQ^{TM} water with 1% (v/v) protease inhibitor cocktail (Calbiochem, Cat.# 539134), pH 7.4. Samples were rotated on a rotating plate overnight at 4ºC (approximately 16 hours). Homogenates were then vortexed and centrifuged (Boeco U-32 R) at 21,000g RCF for 1 hour at 4ºC. Careful not to extract the protein pellet containing insoluble Aβ, the supernatant containing soluble Aβ protein (SDS

extracts) was removed and stored at -80ºC. In order to extract the insoluble Aβ, freshly-made ice-cold extraction buffer 2 was added to the protein pellet at the same rate as SDS, then homogenised and rotated overnight at 4ºC. Extraction buffer 2 contained 5M guanidine hydrochloride (Sigma, Cat.# 64505) in PBS containing 1% (v/v) protease inhibitor cocktail, pH 7.4. Homogenates were then vortexed and centrifuged at 16,000g RCF for 20 minutes at 4ºC. The supernatant containing the insoluble Aβ protein (guanidine extracts) was removed from under the lipid layer and stored at -80ºC.

Protein assay

A protein assay was carried out in order to measure the total volume of protein in each sample, which was necessary to express the amount of Aβ protein per total protein following ELISA measurements. Total protein concentration was measured using the bicinchoninic acid (BCA) method, with the Pierce® BCA Protein assay kit (Thermo Scientific, Cat.# 23227). This method combines the Biuret reaction (the reduction of Ca^{+2} and Cu^{+1} by proteins in an alkaline medium) with the colorimetric detection of Cu^{+} using a BCA-containing reagent. The resulting purple-coloured reaction is dependent on protein concentration, which exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a working range of 20 to 2,000 µg/ml. The absorbance readings of samples containing an unknown concentration of protein can be used to calculate quantitative measurements with reference to a standard curve based on known dilutions of a standard protein (bovine serum albumin, BSA) which is assayed alongside the samples (the soluble and insoluble Aβ protein extracts).

The assay was carried out as detailed in the kit instruction manual. In brief, protein samples maintained on ice were diluted with dH_20 to fall within the working range and to ensure no interference of substances including SDS and guanidine. Serial dilutions of the BSA protein with dH_20 were then prepared to create the standards (2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 µg/ml concentrations) for the standard curve. Using the microplate procedure, 25µl of each sample and standard were added to the 96-well Greiner 96 F-Bottom microplate, in duplicate. 200µl of BCA working reagent (50 parts BCA Reagent A with 1 part BCA Reagent B) were then applied to each sample and standard, and mixed thoroughly for 30 seconds. The plate was then covered and incubated for 30 minutes at 37ºC. Following a

cooling period at room temperature for 5 minutes, the absorbance was read at 570 nm using a microplate reader (FLUOStar Optima, BMG Labtech).

In order to calculate accurate protein concentrations of the samples, the duplicate absorbance values were averaged and the average blank replicate value was subtracted from all standards and samples. The standard curve was then obtained by plotting the standard absorbance readings versus its concentration in µg/ml. The protein concentration of each sample was then calculated using the program Microsoft Excel using the standard curve. Finally, the mg of total protein per mg of tissue was calculated by correcting for sample dilutions, converting µg/ml value to mg/ml, and using the tissue sample weight (mg). The total protein referred to the total soluble protein, since levels of total insoluble protein were negligible, as expected.

Enzyme-linked immunosorbent assay (ELISA)

Human Aβ levels, expressed by the APPswe mutation in Tg2576 mice, were quantitatively measured in samples (the soluble and insoluble Aβ protein extracts) by ELISA using commercial colorimetric immunoassay kits for human Aβ1-40 (Invitrogen, Cat.# KHB3482) and human Aβ1-42 (Invitrogen, Cat.# KHB3442). This sandwich ELISA uses microtiter strips with wells coated with monoclonal antibody specific for the NH2-terminus of human Aβ. Similar to the BCA assay, diluted standards of known concentration were assayed alongside the samples in order to create a standard curve to accurately measure sample concentrations of the desired proteins, Aβ1-40 and Aβ1-42. The intensity of the coloured end reaction is read by a plate-reader and is directly proportional to the concentration of human Aβ40 or Aβ42 present in the sample.

In preparation of the ELISA, the human Aβ40 and Aβ42 standards were reconstituted from powder with standard reconstitution buffer (55 mM sodium bicarbonate, pH 9.0), as indicated by the manufacturer, and diluted 1:20 in BSAT-DPBS (0.2 g/l KCl, 0.2g/l KH_2PO_4, 8.0 g/l NaCl, 1.150 g/l Na_2HPO_4, 5% BSA, 0.03% Tween-20 in MilliQ^{TM} water, pH 7.4). The samples, maintained on ice, were also diluted 1:20 in BSAT-DPBS containing 1:100 protease inhibitor cocktail (Calbiochem, Cat.# 539134). Serial dilutions of the standards in standard diluent buffer (provided in kits) were then made for the standard curve, from 500 to 7.81 pg/ml for Aβ40 standard and 1000 to 15.63 pg/ml for Aβ42 standard. Samples were then diluted further with standard dilution buffer to make two sample dilutions for the ELISA to

ensure detection within the standard curve range. The dilution required was previously piloted as it varies with Aβ type (soluble and insoluble), isoform (Aβ1-40 and Aβ1-42) and with age (as Aβ increases with age). For example, a whole hippocampus at 12 months of age for soluble Aβ needs to be diluted 1:400 and 1:800 to be detected within the standard curve range.

Once prepared, 50µl of each diluted standard and sample were applied to the antibody-coated microtiter strip wells, in duplicate, with 50µl of rabbit anti-human Aβ1-40 or Aβ1-42 detection antibody. Following a 3 hour incubation period on a shaking plate at room temperature, liquid was discarded from each well and washed four times with working wash buffer (25x concentrate wash buffer 1:24 in deionised water). 100µl of the secondary antibody (1:100 anti-rabbit Ig's-HRP 100x concentrate in HRP diluent) was applied to each well using a multi-channel pipette and incubated for 30 minutes on a shaking plate at room temperature. Following washes (as described), 100µl of stabilised chromogen was applied to each well and incubated for approximately 20 minutes at room temperature in the dark. This blue-coloured reaction was then stopped by applying 100µl of stop solution into each well and the absorbance was measured at 450 nm using a colorimeter plate reader (FLUOStar Optima, BMG Labtech). Optima 2.0 software directly calculated Aβ concentrations of each sample based on the absorbance values relative to the absorbance values of the standard curve (based on known concentrations of standard serial dilutions). Only values within the standard range were used. Aβ protein concentrations were calculated by taking the mean value of duplicates, and expressed as pg of Aβ per µg of protein, using the protein concentrations obtained in the BCA assay.

3.4.3 Results

Figure 3.7 presents the levels of soluble and insoluble Aβ1-40 and Aβ1-42 measured by ELISA in the cortex of Tg2576 mice fed the oil blend and DHA diet at 16 months of age. Individual values are shown in addition to the mean value (indicated by the black bar). The graphs show that the Tg OB and Tg DHA groups appear to have similar levels of soluble Aβ1-40 and soluble Aβ1-42, but levels of insoluble Aβ1-40 and Aβ1-40 were slightly lower in the Tg DHA group relative to Tg OB group. In order to test whether diet altered Aβ levels in the cortex of Tg2576 mice, an ANOVA with diet (OB, DHA) as a between-subject factor, and brain extract (soluble, insoluble) and Aβ isoform (Aβ1-40, Aβ1-42) as within-subject

factors was carried out. Statistical analysis revealed no significant effect of diet ($F(1,4)=0.301$, $p>0.05$), no significant interaction of isoform by diet ($F(1,4)=0.332$, $p>0.05$), no significant interaction of extract by diet ($F(1,4)=0.668$, $p>0.05$) and no three-way interaction of isoform by extract by diet ($F(1,4)=0.671$, $p>0.05$). These results therefore demonstrate that DHA supplementation failed to reduce Aβ pathology.

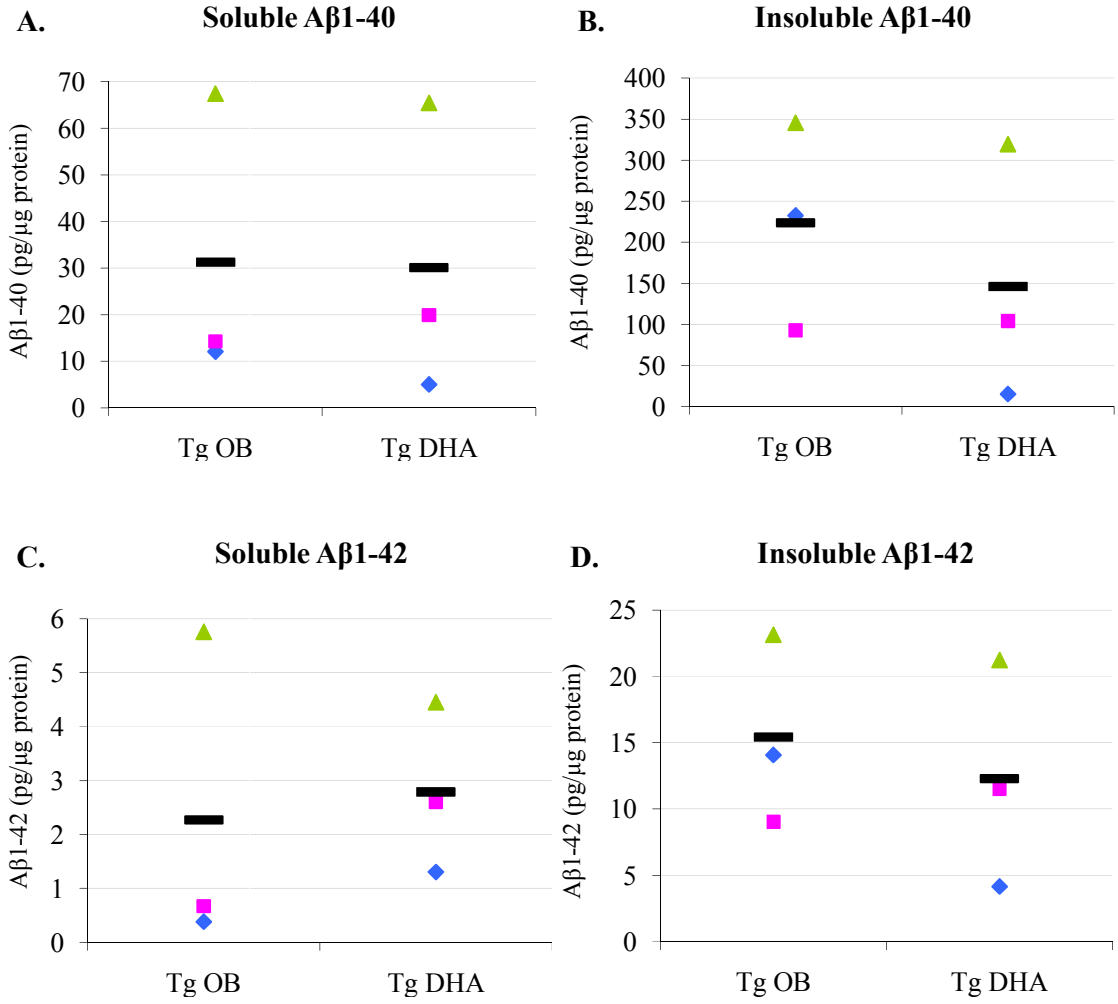

Figure 3.7 Levels of Aβ in the cortex of 16 month old male Tg2576 mice fed the oil blend (Tg OB) and DHA (Tg DHA) diet. Four species of Aβ were analysed by ELISA: A. Soluble Aβ1-40, B. Insoluble Aβ1-40, C. Soluble Aβ1-42, and D. Soluble Aβ1-42. Individual values are presented, in addition to the mean value (black bar).

Statistical analysis also revealed a significant effect of isoform ($F(1,4)=9.117$, $p<0.05$) caused by higher levels of Aβ1-40 than Aβ1-42, a significant effect of extract ($F(1,4)=11.386$,

$p<0.05$) caused by higher levels of insoluble Aβ than soluble Aβ, and a significant interaction of isoform by extract ($F(1,4)=10.386$, $p<0.05$). A test of simple main effects revealed this interaction to be caused by higher levels of insoluble than soluble Aβ for both the Aβ1-40 isoform ($F(1,5)=10.99$, $p<0.05$) and Aβ1-42 isoform ($F(1,5)=19.34$, $p<0.01$), and higher levels of Aβ1-40 than Aβ1-42 for both soluble extracts ($F(1,5)=7.27$, $p<0.05$) and insoluble extracts ($F(1,5)=11.88$, $p<0.05$).

3.4.4 Discussion

ELISA analysis of Aβ pathology confirmed the presence of soluble and insoluble Aβ1-40 and Aβ1-42 in brains of Tg2576 mice aged 16 months, consistent with previous reports (Hsiao et al., 1996; Kawarabayashi et al., 2001; Westerman et al., 2002). This analysis also corroborated evidence by Kawarabayashi et al. (2001) reporting higher levels of insoluble Aβ1-40 and Aβ1-42 relative to their soluble forms from 10 months of age. Greater levels of Aβ1-40 than Aβ1-42 levels were also found at 16 months of age, consistent with reports from Hsiao et al. (1996) in Tg2576 mice aged 11 to 13 months.

Inconsistent with the experimental hypothesis, the experiment revealed no significant effect of diet or significant interactions of isoform or extract with diet on Aβ levels. These results are in contrast to previous reports of beneficial effects of DHA supplementation effects on amyloid pathology. For example, DHA supplementation reduced total, soluble and insoluble Aβ1-40 and Aβ1-42, as well as Aβ deposition in the 3xTg (APP/PS1/Tau) model, the APPswe/PS1 model and the Tg2576 model (Lim et al., 2005; Oksman et al., 2006; Green et al., 2007; Hooijmans et al., 2007, 2009). It is important to note however that several factors may account for these discrepancies, such as differences in animal model and their underlying pathology, as well as the experimental design surrounding the diets and time of intervention. For instance, Lim et al. (2005) reported 0.6% DHA supplementation with an ω-6/ω-3 ratio of 4:1 reduced total (Aβ1-40 and Aβ1-42) insoluble Aβ levels in cortex samples of Tg2576 mice aged 22.5 months relative to a DHA-depleted diet with a ω-6/ω-3 ratio of 85:1, but not compared to a control chow containing 0.09% DHA with a ω-6/ω-3 ratio of 7:1. This may therefore suggest that a DHA-rich diet is only effective at reducing Aβ pathology when compared to a diet with excessive levels of omega-6 PUFA. Such issues will be further reviewed in the chapter discussion.

Although this experiment revealed no statistically significant effect on Aβ pathology as a function of diet, it is important to note that levels of insoluble Aβ1-40 and Aβ1-42 appeared reduced by DHA supplementation, suggesting that DHA may reduce insoluble Aβ accumulation when compared with a suitable baseline control diet. Before disregarding this non-significant effect, it is important to consider that the small number of mice examined in each group and the high variability of Aβ levels between individual mice may have limited the detection of a statistically significant effect of DHA supplementation. This is further supported by Chapman et al. (1999) who also reported that total levels of Aβ1-40 and Aβ1-42 was highly variable in the brain of six Tg2576 mice aged 17 to 19 months. This high variability in Aβ levels may therefore restrict the detection of subtle dietary effects, and therefore highlights the need for a larger number of mice per group when examining treatment effects on Aβ pathology. Further investigation into the effect of DHA on Aβ pathology in Tg2576 mice is thereby required, ideally using large cohorts.

3.5 Experiment 3: Lipid analysis of brains

3.5.1 Introduction

The aim of this experiment was to profile the fatty acid composition of the brain in 16-month old Tg2576 and WT mice in order to investigate the effect of the APPswe mutation and dietary DHA supplementation. Firstly, lipid analysis was important to discover whether composition of fatty acids in the brain was altered in Tg2576 mice relative to WT mice. Although it is well documented that AD patients have markedly reduced levels of DHA [22:6ω-3] in the brain relative to aged-matched non-demented controls (e.g. Söderberg et al., 1991), this was not reported in the frontal cortex of 20 to 22.5 month old Tg2576 mice fed a standard chow diet relative to WT mice (Calon et al., 2004, 2005). Similarly, levels of arachidonic acid (ARA [20:4ω-6]), linoleic acid (LA [18:2ω-6]), docosapentaenoic acid (DPA [22:5ω-6]), docosatetraenoic acid (DTA [22:4ω-6]), eicosapentaenoic acid (EPA [20:5ω-3]) and the ω-3/ω-6 ratio remain unaltered in the frontal cortex of aged Tg2576 mice (Calon et al., 2004, 2005; Lim et al., 2005). It was therefore hypothesised that Tg2576 mice would not have altered fatty acid composition in the brain relative to WT mice.

Determination of any transgene-deficits was particularly important for the interpretation of behavioural results in this chapter. For example, numerous studies have shown a loss of DHA

in the brain has been associated with cognitive dysfunction in AD (e.g. Pettigrew et al., 2001), in the ageing human population (e.g. Lauritzen et al., 2001) and in rodents (e.g. Gamoh et al., 1999; Greiner et al., 1999; Carrie et al., 2000; Moriguchi, Greiner & Salem, 2000; Ikemoto et al., 2001). As outlined in chapter 1, it is postulated that a loss of DHA has this effect because DHA plays an essential role in brain maintenance, structure and function. For example, DHA is the main PUFA comprising neuronal membrane phospholipids and is particularly concentrated in synaptic membranes, myelin sheaths, synaptic vesicles, astrocytes and growth cones (e.g. Horrocks & Farooqui, 2004). The brain is primarily composed of lipids, accounting for approximately 50 to 60% of the dry weight of the human brain, with DHA accounting for approximately 8% (Lauritzen et al., 2001; Muskiet et al., 2006). Furthermore, DHA accounts for 10 to 20% of total brain fatty acids in the adult human brain and over 17% in the rodent brain (Hamano et al., 1996; McNamara & Carlson, 2006). Alterations to DHA levels in the brain can therefore have a detrimental effect.

Secondly, lipid analysis of the brain was particularly important to ensure that dietary supplementation of DHA was incorporated into the brain fractions. Several studies have shown that DHA levels are increased in the frontal cortex of Tg2576 and WT mice as a result of 0.6% DHA supplementation (Calon et al., 2004, 2005; Lim et al., 2005). This pattern however has only been reported significant relative to DHA-deficient mice (induced by DHA-depleted diet), not relative to mice fed a standard diet. It would therefore be interesting to examine whether a diet supplemented with DHA would significantly increase DHA levels in the cortex of Tg2576 and WT mice relative to mice fed a suitable baseline control diet. Furthermore, it would be interesting to examine the effect of this diet on other fatty acids in the brain, as several studies have shown DHA deficiency and supplementation to alter levels of omega-6 PUFAs ARA, LA, DPA and DTA, as well as omega-3 PUFA EPA, in the brain of Tg2576 and WT mice (Calon et al., 2004, 2005; Lim et al., 2005). Based on previous evidence that brain levels of DHA are sensitive to dietary DHA intake, it is hypothesised that DHA supplementation would increase DHA levels in the brain of Tg and WT mice. Since omega-3 PUFA DHA competes with omega-6 PUFA for incorporation into the brain, it is also hypothesised that DHA supplementation would reduce omega-6 PUFA levels and the ω-6/ω-3 ratio.

Confirmation of dietary-induced alterations to fatty acid composition in the brain was particularly important for the interpretation of behavioural results. For example, Arendash et

al. (2007) argued that an omega-3 PUFA supplemented diet failed to enhance cognitive performance in APPswe/PS1 transgenic mice due to unaltered omega-3 and omega-6 PUFA levels in the brain relative to mice fed a standard diet, suggesting a lack of omega-3 PUFA incorporation into the brain. A lack of DHA incorporation into the brain may therefore explain the limited effects of DHA supplementation on behaviour and pathology in experiments 1 and 2.

3.5.2 Methods

Analysis of brain fatty acids were carried out on cortex samples from 16 month old Tg2576 and WT mice fed the DHA and oil blend diets from 4 months of age. The methodology used to analyse lipids in the brain was the same as detailed in the lipid analysis of diet samples featured in chapter 2, section 2.3. In order to collect brain samples, mice were culled by cervical dislocation. The brain was removed immediately and maintained on ice while dissecting the cortex, hippocampus and cerebellum. The dissected tissue samples were snap-frozen in liquid nitrogen and stored at -80ºC until ready to be analysed. Lipid analysis was carried out on 3 mice from each experimental group (Tg DHA n=3, Tg OB n=3, WT DHA n=3, WT OB n=3). Statistical analyses of fatty acid levels were carried out using an ANOVA with genotype (Tg, WT) and diet (OB, DHA) as between-subject factors. Tests of simple main effects followed-up significant interactions.

3.5.3 Results

Figure 3.8 presents the percentage of main fatty acid classes and the ω-3/ω-6 ratio of cortex samples analysed by lipid analysis from 16-month old Tg2576 and WT mice fed the DHA and oil blend diet. All groups revealed that saturated fatty acids composed the highest percentage of fatty acids in the cortex, followed closely by a relatively high level of polyunsaturated acids, and smaller levels of monounsaturated fatty acids. All groups also showed a higher percentage of total omega-3 PUFAs than total omega-6 PUFAs. Statistical analysis using an ANOVA with between-subject factors of genotype (Tg, WT) and diet (DHA, OB) revealed no main effect of genotype on any of these values (smallest, $F(1,8)=4.769$, $p>0.05$). Analysis also revealed that the total percentage of saturated fatty acids and polyunsaturated fatty acids were unaffected by diet (smallest, $F(1,8)=1.419$, $p>0.10$).

However, DHA supplementation significantly increased the total percentage of monounsaturated fatty acids relative to mice fed the oil blend diet ($F(1,8)=8.425$, $p<0.05$).

As expected, total omega-3 and omega-6 PUFAs were altered as a factor of diet. Results revealed mice fed the DHA diet had significantly higher levels of omega-3 PUFA ($F(1,8)=109.747$, $p<0.001$) and significantly lower levels of omega-6 PUFA ($F(1,8)=520.044$, $p<0.001$) relative to mice fed the oil blend diet. This significant effect of diet was also reflected in the ω-3/ω-6 ratio ($F(1,8)=383.839$, $p<0.001$), which ranged from 1.44:1 to 1.53:1 in mice fed the oil blend diet and 3.88:1 to 3.31:1 in mice fed the DHA diet. Analysis of these ratios revealed a significant genotype by diet interaction ($F(1,8)=9.691$, $p<0.05$). Following up this interaction, a test of simple main effects revealed WT DHA mice differed significantly from Tg DHA mice ($F(1,8)=14.03$, $p<0.01$) and WT OB mice ($F(1,8)=135.78$, $p<0.001$), and Tg DHA mice differed significantly from Tg OB mice ($F(1,8)=257.75$, $p<0.001$). This pattern of results is clearly displayed in Figure 3.8.

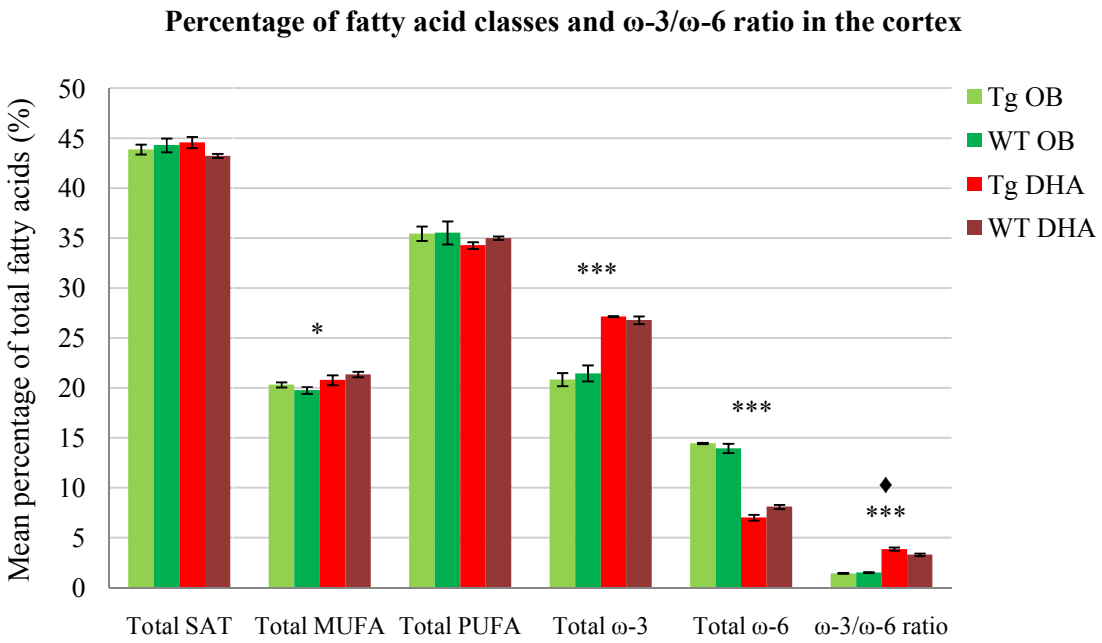

Figure 3.8 Main fatty acids classes and ω-3/ω-6 ratio in the cortex of Tg and WT mice at 16 months fed the DHA or oil blend diet. Results are presented as mean percentages of total fatty acids ± S.E.M. Significant effect of diet, * $p<0.05$, *** $p<0.001$; significant genotype by diet interaction ♦ $p<0.05$.

Table 3.2 presents the percentage of main fatty acids detected in cortex samples from 16-month old Tg2576 and WT mice fed the DHA and oil blend diet, indicating significant

effects of genotype and diet. No main effects of genotype were observed in fatty acid composition except for levels of 18:2ω-6 ($F(1,8)=26.672$, $p<0.001$), which was increased as a function of Tg status. Statistical analysis revealed significantly higher percentages of 20:3ω-3 ($F(1,8)=439.185$, $p<0.001$), 20:5ω-3 ($F(1,8)=816$, $p<0.001$), 22:5ω-3 ($F(1,8)=180.899$, $p<0.001$), 18:1ω-9 ($F(1,8)=26.969$, $p<0.001$) and 18:2ω-6 ($F(1,8)=10.714$, $p<0.05$) in mice fed the DHA diet than mice fed the oil blend diet. Of particular importance, DHA supplementation significantly increased the percentage of 22:6ω-3 DHA levels in the cortex ($F(1,8)=67.714$, $p<0.001$) by ~5% of total fatty acids. Analysis also revealed DHA supplementation significantly lowered percentages of 18:1ω-7 ($F(1,8)=12.566$, $p<0.01$), 23:0 ($F(1,8)=145.690$, $p<0.001$), 20:4ω-6 ($F(1,8)=358.385$, $p<0.001$) and 22:4ω-6 ($F(1,8)=3201.066$, $p<0.001$) in mice relative to the oil blend diet. Importantly, the observed reduction in arachidonic acid 20:4ω-6 (by 4.6-5.4%) and docosatetraenoic acid 22:4ω-6 (by 1.5-1.9%) was as expected.

Table 3.2 Fatty acid composition of cortex samples from 16 month-old Tg2576 and WT mice fed the DHA or OB diet. Presenting the main fatty acids (values >0.1%), represented as mean percentage of total fatty acids ± S.E.M.

Fatty acids	**Tg OB**	**WT OB**	**Tg DHA**	**WT DHA**
Hexadecanoic acid 16:0	19.2 ± 0.7	20.0 ± 1.0	20.4 ± 0.6	19.2 ± 0.1
Hexadecenoic acid 16:1ω-7	0.3 ± tr.	0.3 ± tr.	0.4 ± tr.	0.4 ± tr.
Octadecanoic acid 18:0	23.9 ± 0.2	23.7 ± 0.3	23.6 ± 0.1	23.5 ± 0.2
Oleic acid 18:1ω-9 ***	14.7 ± 0.2	14.4 ± 0.1	15.6 ± 0.3	16.0 ± 0.3
cis-vaccenic acid 18:1ω-7 **	3.4 ± tr.	3.3 ± 0.1	3.0 ± 0.1	3.1 ± 0.1
Linoleic acid 18:2ω-6 * †	0.6 ± tr.	0.4 ± tr.	0.6 ± tr.	0.5 ± tr.
Gamma-linolenic acid 18:3ω-6	N.D.	N.D.	N.D.	N.D.
α-Linolenic acid 18:3ω-3	N.D.	N.D.	N.D.	N.D.
Eicosanoic acid 20:0	0.2 ± tr.	0.1 ± tr.	0.2 ± tr.	0.2 ± tr.
Eicosenoic acid 20:1ω-9	0.9 ± tr.	0.8 ± 0.1	0.9 ± 0.1	0.9 ± tr.
Eicosadienoic acid 20:2ω-6	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
Eicosatrienoic acid 20:3ω-3 ***	0.3 ± tr.	0.3 ± tr.	0.9 ± tr.	0.8 ± tr.
Arachidonic acid 20:4ω-6 ***	11.2 ± 0.1	11.0 ± 0.4	5.6 ± 0.2	6.6 ± 0.2
Eicosapentaenoic acid 20:5ω-3 ***	N.D.	N.D.	0.4 ± tr.	0.3 ± tr.
Docosanoic acid 22:0	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.
Docosatetraenoic acid 22:4ω-6 ***	2.7 ± tr.	2.5 ± tr.	0.8 ± tr.	1.0 ± tr.
Docosapentaenoic acid 22:5ω-3 ***	0.1 ± tr.	0.1 ± tr.	0.4 ± tr.	0.3 ± tr.
Docosahexaenoic acid 22:6ω-3 ***	20.3 ± 0.7	21.0 ± 0.8	25.5 ± 0.1	25.3 ± 0.4
Tricosanoic acid 23:0 ***	0.2 ± tr.	0.2 ± tr.	N.D.	N.D.
Tetracosanoic acid 24:0	0.2 ± tr.	0.1 ± tr.	0.2 ± tr.	0.2 ± tr.
Nervonic acid 24:1	0.9 ± tr.	0.9 ± 0.1	0.9 ± 0.1	1.0 ± tr.

N.D. = not detected, tr. = trace (less than 0.1). Significant effect of diet, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significant effect of genotype, † $p<0.001$.

3.5.4 Discussion

Fatty acid composition in the cortex did not generally alter as a factor of genotype in Tg2576 mice aged 16 months, corroborating previous reports in the frontal cortex of Tg2576 mice aged 20 to 22.5 months fed a standard control diet or high-DHA diet (Calon et al., 2004, 2005; Lim et al., 2005). However, levels of omega-6 PUFA linoleic acid were significantly higher in Tg mice, although this did not alter the total level of omega-6 PUFA or ω-3/ω-6 ratio in the brain. Transgene effects on fatty acid composition however have been reported following intake of DHA-depleted diets, whereby Tg2576 mice were more susceptible to the effects of DHA deficiency than WT mice (Calon et al., 2004, 2005; Lim et al., 2005). These results suggest that this genotype effect is limited to DHA deficiency, as a genotype effect was not reported under normal control conditions or DHA supplementation. These results would therefore suggest that in the absence of DHA deficiency, Tg mice can incorporate dietary omega-3 PUFA into brain fractions as well as WT mice. This was supported in the current study, and in fact, this study interestingly revealed Tg DHA mice had a significantly higher ω-3/ω-6 ratio than WT DHA mice. This unexpected increase may be a result of a higher intake of DHA diet in Tg mice relative to WT mice. This explanation requires further investigation.

The fatty acid composition of the cortex in all mice was primarily composed of SAT, followed by PUFA and MUFA. Total SAT and PUFA were unaffected by dietary intake, although total MUFA was higher in DHA-fed mice relative to mice fed the oil blend diet (primarily driven by higher levels of omega-9 oleic acid). This was interesting as the oil blend diet contained higher levels of SAT and MUFA to compensate for the supplementary DHA omega-3 PUFA in the DHA diet. In contrast to the results, greater levels of PUFA and lower levels of SAT and MUFA was anticipated in the brains of DHA-fed mice. Also interestingly, the main PUFA in the cortex of all mice was DHA, despite the absence of DHA in the oil blend diet. This therefore suggests the conversion of omega-3 PUFA ALA (present in the oil blend diet) to DHA. Consistently, it has previously been reported that DHA levels in the brain can be maintained by the sole intake of dietary ALA, through conversion in the liver (Rapoport, Rao & Igarashi, 2007).

As predicted, dietary DHA supplementation led to a higher ω-3/ω-6 ratio in the cortex relative to mice fed the oil blend diet. Total levels of omega-3 PUFAs were also higher with

DHA supplementation, which was primarily contributed by elevations in DHA which increased by 5% of total fatty acids. Higher percentages of omega-3 PUFA eicosatrienoic acid (ETA), eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) were also observed with DHA supplementation. Interestingly the DHA diet also led to higher levels of omega-6 PUFA linoleic acid (LA) and omega-9 oleic acid. As predicted, the DHA diet induced lower levels of total omega-6 PUFAs, suggesting that DHA supplementation replaced omega-6 PUFA with omega-3 PUFA incorporation into brain phospholipids. This pattern of results is consistent with literature demonstrating that omega-3 and omega-6 PUFAs compete for metabolism and incorporation into brain phospholipids (Russo, 2009). The greatest reductions of omega-6 PUFAs were arachidonic acid (ARA) by 4.6-5.4% and docosatetraenoic acid (DTA) by 1.5-1.9% of total fatty acids. Lower levels of 18:1ω-7 and 23:0 were also detected with DHA supplementation. The reduction of ARA and DTA by DHA supplementation is consistent with reports which show the reverse effect whereby DHA is replaced by ARA and DTA in the brain of mice fed omega-3 PUFA depleted diets (Ikemoto et al., 2001; Greiner et al., 2003). Although the increase in DPA with DHA supplementation may appear contrary to reports which show an increase in DPA following omega-3 PUFA/DHA dietary depletion (Youyou et al., 1986; Salem et al., 2001; Greiner et al., 2003; Cole & Frautschy, 2006), these studies report omega-6 PUFA DPA rather than omega-3 PUFA DPA.

In summary, this is the first study in Tg2576 mice which has shown that DHA supplementation relative to a suitable baseline diet can increase total omega-3 PUFAs (particularly DHA) and reduce total omega-6 PUFAs (particularly ARA and DTA) levels in the cortex of Tg and WT brains. In contrast, Calon et al. (2004, 2005) and Lim et al. (2005) reported that DHA supplementation relative to a control diet did not alter DHA or omega-6 PUFAs ARA, DTA and DPA levels in the frontal cortex of Tg2576 or WT mice. Since their control diet contained a higher ω-3/ω-6 ratio (1:5) than the DHA diet (1:7), representing greater levels of total omega-3 PUFA or lower levels of total omega-6 PUFA, this may have prevented the detection of changes as a result of DHA supplementation since DHA can be converted from omega-3 PUFAs ALA or EPA. Studies in Tg2576 mice have however reported alterations of DHA, ARA, DTA and DPA levels with dietary DHA depletion, which was restored with DHA supplementation (Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006). The lack of transgene effect on fatty acid composition found in this study, paired with previous reports of oxidative stress in Tg2576 mice (Smith et al., 1998; Pappolla

et al., 1998; Praticò et al., 2001) together suggest that oxidative stress does not alter brain lipids in the Tg2576 model. Since DHA deficiency does alter brain lipids in Tg2576 mice (e.g. Calon et al., 2004), these results may suggest that dietary conditions contribute more to the loss of brain DHA in AD patients than oxidative conditions (contrary to Reich et al., 2001), further highlighting the importance of diet in AD. These results also question the potential role of Aβ pathology in altering brain lipids, contrary to previous reports (Hashimoto et al., 2002, 2005b, 2006).

3.6 Chapter Discussion

The aim of this chapter was to investigate the longitudinal effect of omega-3 PUFA DHA dietary supplementation from an early age on spatial memory and Aβ pathology in Tg2576 mice, relative to a suitable baseline control diet. Prior to the discussion of dietary effects, it was important to establish that Tg2576 mice showed a cognitive deficit and Aβ pathology. In agreement with the literature surrounding the Tg2576 model, results from experiment 1 revealed Tg mice to show an age-related decline in spatial memory between 12 and 16 months of age (Hsiao et al., 1996; Barnes, Hale & Good, 2004). This spatial memory deficit in the T-maze FCA task was not observed at 8 months of age, contrary to previous reports in this model (Chapman et al., 1999; Barnes, Hale & Good, 2004). Further to behavioural testing, brain analysis by ELISA detection confirmed the presence of soluble and insoluble forms of Aβ1-40 and Aβ1-42 proteins in the cortex of Tg mice aged 16 months of age. Consistent with previous reports, Aβ1-40 levels were higher than Aβ1-42 levels, and insoluble isoforms of Aβ were higher than soluble isoforms, which were expected at this age (Hsiao et al., 1996; Kawarabayashi et al., 2001).

Regarding dietary effects on spatial memory, experiment 1 revealed that DHA supplementation generally failed to enhance T-maze performance in Tg2576 or WT mice. Analysis of data at 16 months however provided some evidence of DHA ameliorating a transgene-related performance deficit in Tg mice. The results therefore indicate that DHA may provide subtle protection against cognitive decline caused by the APPswe mutation in aged Tg2576 mice at 16 months old. This is the first report providing evidence that DHA supplementation can reduce behavioural deficits in an AD model relative to a baseline control diet. This effect is somewhat supported by Calon et al. (2004) who reported DHA supplementation to reduce Morris water maze acquisition deficits in 21-22 month old Tg2576

mice, although this was relative to a DHA-depleted diet. Together with the data from experiment 1 showing no amelioration of spatial memory deficit at 8 or 12 months as a function of diet, this may suggest that DHA may only be effective during advanced stages of Aβ pathology. It could be argued that DHA targets plaque pathology which is extensive from 16 months of age, but scattered and modest at 12 months (e.g. Kawarabayashi et al., 2001; Jacobsen et al., 2006).

The results from experiment 2 revealed that dietary DHA failed to significantly alter levels of soluble or insoluble Aβ1-40 and Aβ1-42 detected by ELISA in cortex samples of Tg mice aged 16 months. In contrast, Lim et al. (2005) reported 3-5 month dietary DHA supplementation significantly reduced total insoluble Aβ by 77% and Aβ42 levels by ~49.1% measured by ELISA in Tg mice aged 22.5 months old compared to mice fed a DHA-depleted diet. Furthermore, they reported DHA significantly reduced plaque burden by 40.3%, as well as soluble Aβ by 38%, although this latter effect was not significant. It is important to consider here however that the lack of significant effects found in experiment 2 may be affected by the small number of mice analysed per group, and that future studies should aim to examine larger cohorts of mice.

Although the ELISA results in experiment 2 provide no support for previous findings, it is important to consider the potential effect of the experimental diets used. For instance, Lim et al. (2005) reported a reduction in Aβ pathology by DHA supplementation when compared to a diet deficient in DHA. In contrast however, when results were compared to a control diet containing 0.09% DHA with a low ω-6/ω-3 ratio of 7:1 (similar to their DHA diet at 5:1), DHA failed to reduce total soluble or insoluble Aβ levels, and total Aβ1-40 levels, although a significant reduction in total Aβ1-42 levels was observed. This therefore highlights that the apparent positive effects of DHA supplementation on Aβ pathology previously claimed by Lim et al. (2005) when compared to the DHA-deficient diet was likely driven by the high levels of omega-6 PUFA or the excessive ω-6/ω-3 ratio (85:1) rather than the addition of dietary DHA *per se*. In contrast, when compared to the control diet which more accurately assessed DHA supplementation, the results show limited evidence that DHA can reduce Aβ pathology measures, similar to the results of experiment 2.

However, it is also important to consider the effect of total fatty acid content in the diets when comparing the control and high-DHA diet in Lim et al. (2005) study. Here, their control

diet contained 11% total fat compared to 6.1% in the DHA diet. It is therefore possible that the apparent positive effects of DHA supplementation may be led by the reduction in total fatty acids. Some support for this comes from Julien et al. (2010) who reported that a high 35% fat diet increased Aβ pathology in Tg2576 mice compared to a 5% fat diet. In contrast to Lim et al. (2005) study, the DHA and control diets used in this chapter contained an equal level of fatty acids, thereby preventing a potential effect caused by differential levels of fatty acids in the diets. Under these conditions, DHA supplementation failed to alter Aβ pathology in Tg2576 mice.

Studies in other AD models have also shown DHA supplementation to reduce Aβ pathology, although it is argued here that the control diet is not suitably designed to assess the sole effect of DHA supplementation, but rather the effect of a diet with reduced omega-6 PUFA levels or ω-6/ω-3 ratio. For example, Oksman et al. (2006) reported that 3-4 month fish oil supplementation (0.4% DHA) reduced Aβ pathology in the APPswe/PS1 model at 10 months of age relative to a control diet (0% DHA). Although the study controlled for the total level of fatty acids in both diets, the different levels of omega-6 PUFAs (30% and 52%, respectively), omega-3 PUFAs (22% and 7%, respectively) and the ω-6/ω-3 ratio (1.41:1 and 8:1, respectively), prevented the inference that effects were a result of the omega-3 PUFA or DHA supplementation *per se* rather than the high omega-6 PUFA or ω-6/ω-3 ratio in the control diet.

Similarly, Green et al. (2007) reported that 3-, 6- and 9-month 1.28% DHA supplementation (ω-6/ω-3 ratio of 1:1) reduced soluble Aβ levels in the 3xTg mouse model compared to a control diet (0% DHA, ω-6/ω-3 ratio 10:1). Since the control diet contained almost double the amount of 18:2ω-6, it could be argued that the lower ω-6/ω-3 ratio of the DHA diet (driven by the reduced levels of 18:2ω-6) could have driven the Aβ-reducing effect, rather than the increased DHA content. Furthermore, this study found that replacement of approximately half the DHA content of the DHA diet with omega-6 PUFAs ARA or DPA (while maintaining the ω-6/ω-3 ratio at ~1:1) resulted in a loss of Aβ-reducing efficiency after 3 months in the DHA-ARA diet and after 6 months in the DHA-DPA diet. This highlights the potential impact of increased omega-6 PUFAs, rather than simply an increase in DHA levels. These studies therefore highlight how important the experimental design of the diets used in this chapter was in order to accurately assess the effect of DHA on pathology and behaviour. Our results therefore show that when a diet is supplemented with

DHA, relative to a suitable baseline diet with an equal level of total fatty acids and similar omega-6 PUFA levels, Aβ pathology is not affected and its affect on behaviour is somewhat limited.

The same argument relating to the important influence of experimental diet design can be applied to explain discrepancies between the amelioration of a spatial memory deficits by DHA supplementation reported in some studies (e.g. Calon et al., 2004) and the less convincing effect found in experiment 1. As Calon and colleagues used the same experimental diet design as Lim et al. (2005), it could be argued that the positive effect of DHA supplementation was driven by the increased levels of omega-6 PUFA and high ω-6/ω-3 ratio in the DHA deficient diet, rather than the addition of DHA in the DHA diet. Unfortunately, this study did not assess behaviour in Tg2576 mice fed the control diet containing a similar ω-6/ω-3 ratio, which prevented any potential comparisons to a diet which is argued to more accurately assess the effects of DHA supplementation. Similar arguments could be made in other AD mouse models investigating dietary DHA supplementation. For example, the improved cognition reported by Arsenault et al. (2011) in a 3xTg model by DHA supplementation relative to a DHA-depleted diet could again to attributable to the lower level of 18:2ω-6 rather than the addition of DHA *per se*.

The discrepancies between the results of previous studies investigating DHA supplementation and the results of experiment 1 and 2 of this chapter could also be caused by differences in age or experimental model. For example, this is the first study investigating effects of DHA supplementation from an early age during the initial stages of pathological development. Since previous studies have failed to examine dietary effects before 17 months of age and our study provides no evidence of positive effects at an early age (before 16 months), it is therefore possible that DHA is only effective at reducing behavioural deficits during advanced stages of pathology. However, since our T-maze experiment failed to detect a transgene deficit before 12 months in Tg2576 mice, this needs further investigation using more sensitive tests able to detect subtle differences in behaviour at an earlier age. This is important as behavioural deficits have been reported from as early as 3 months of age (e.g. Hsiao et al., 1996, King et al., 1999). Furthermore, it is also important to assess multiple forms of behaviour rather than simply memory as Oksman et al. (2006) reported DHA supplementation to reduce exploratory deficits in the APP/PS1 model, but failed to improve spatial learning the Morris water maze. Chapter 4 will address this issue, whereby Tg2576

mice are examined on a number of tasks from an early age to extend the behavioural profile of this model in order to 1) further establish the onset of behavioural deficits in Tg2576 mice, and 2) investigate the effect of DHA supplementation of the onset and progression of behavioural deficits detected.

The choice of experimental model may also have a large influence on the effect of dietary intervention. For example, positive effects of DHA supplementation have been shown in the APPswe/PS1 model on behaviour and pathology (e.g. Oksman et al., 2006; Hooijmans et al., 2007, 2009). Since this model shows an accelerated development of Aβ pathology and preferential deposition of Aβ1-42 (Borchelt et al., 1997; Holcomb et al., 1998), it may be possible that DHA is particularly effective against reducing Aβ1-42 isoforms or during periods of significant pathological development. This could be supported by our results which show DHA to be only effective at reducing a spatial memory deficit in aged Tg2576 mice, during advanced stages of Aβ aggregation and deposition at 16 months. Also supporting this theory, Lim et al. (2005) found DHA supplementation to reduce Aβ1-42 but not Aβ1-40 relative to both a control and DHA-depleted diet. Furthermore, the Aβ1-42 protein is more pathogenic or neurotoxic than Aβ1-40 (Wang et al., 1999; McGowan et al., 2005), which may explain why DHA supplementation appeared more effective in the APPswe/model than the Tg2576 model. Positive effects of DHA supplementation have also been reported in the 3xTg mouse model (e.g. Green et al., 2007; Ma et al., 2009; Arensault et al., 2011). Since Aβ and tau pathologies interact with each other to accelerate pathological development in AD, it is possible that part of the positive effect of DHA on reducing Aβ or cognitive deficits in this model is attributable to the effects on tau pathology reported in these studies.

Finally, the results of experiment 3 confirmed that brain levels of fatty acids were reflective of dietary intake in Tg2576 and WT mice at 16 months, whereby DHA supplementation increased DHA levels in the brain with compensatory reductions in omega-6 PUFAs and the ω-6/ω-3 ratio. Similar results have been reported following DHA supplementation, although these have been limited to a DHA diet relative to a DHA-depleted diet (e.g. Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006). Although experiment 3 confirmed incorporation of DHA in the brain at 16 months following 12-month DHA supplementation, it should be considered whether DHA was incorporated following 4- and 8-month dietary DHA supplementation during T-maze assessment at 8 and 12 months of age. It could be

argued that DHA was not incorporated into the brain fractions at 8 and 12 months, resulting in the lack of dietary effects observed, but incorporated at 16 months which was associated with cognitive improvement. However, this argument is unlikely as Lim et al. (2005) demonstrated incorporation of DHA into the Tg2576 brain following 3-5 months of 0.6% DHA supplementation. Furthermore, Ma et al. (2009) found fish oil supplementation (0.6% DHA) improved cognitive performance in the Y-maze after 2 and 3 months on the diet, but not after 1 month, suggesting incorporation of dietary fatty acids into the brain from early as 2 months supplementation.

Since DHA supplementation failed to significantly alter Aβ levels and yet some cognitive deficits were reduced, these results suggest that DHA may have improved cognitive or neuronal processes, or targeted pathological mechanisms such as neuroinflammation or oxidative stress. DHA supplementation may therefore have provided neuroprotection against Aβ-induced neurotoxicity, inflammation or oxidative damage. In support of this, *in vitro* studies have shown a neuroprotective effect of DHA in neuronal cells by reducing apoptosis and increasing cell viability (Akbar et al., 2005; Florent et al., 2006). In particular, this may be caused by the release of the DHA-metabolite neuroprotectin D1, which has been shown to protect against oxidative stress-induced apoptosis (Mukherjee et al., 2004). Furthermore, *in vivo* studies have shown DHA supplementation to improve synaptic membrane fluidity, reduce neuronal apoptotic products, lower lipid peroxide and ROS levels, and increase glutathione levels and glutathione reductase activity (Suzuki et al., 1998; Hashimoto et al., 2002, 2005b, 2006). Similar results have also been shown in the Tg2576 model including reduced postsynaptic marker loss, apoptosis, caspase activation and oxidative stress (Calon et al., 2004, 2005; Cole & Frautschy, 2006). However, it is important to note that these *in vivo* results were relative to DHA-depletion, so these effects may not be applicable to our study.

In conclusion, the results of this chapter suggest that dietary DHA supplementation had mild effects in reducing a spatial memory deficit in Tg2576 mice, which was relatively consistent with the lack of effect observed on Aβ pathology. The contrasting results of previous studies were likely attributable to their choice of control diet which was generally high in omega-6 PUFAs, depleted in omega-3 PUFAs, contained unequal levels of total fatty acids, and/or contained an excessive ω-6/ω-3 ratio. Since our study controlled for these factors, the results together suggest that DHA is only effective in robustly reducing transgene-related deficits and pathology when compared to a particularly poor diet. The observation of some positive

effects in reducing spatial memory deficits at 16 months may suggest that longitudinal DHA supplementation is particularly effective, or that DHA supplementation is effective during stages of advanced pathological development or during times of great cognitive dysfunction. However, it is argued that use of the T-maze FCA task, which had limited sensitivity in detecting transgene deficits at an early age, may have prevented the detection of more subtle dietary effects. It remains possible that a broader assessment of learning and memory may be more sensitive to dietary effects during stages of early pathological development. This was assessed in chapter 4. Since DHA supplementation failed to alter Aβ levels, it was hypothesised that the changes in behaviour caused by DHA supplementation were likely attributable to more general effects on cognitive or neuronal function, or a reduction in other pathological processes in the Tg2576 model such as oxidative stress or neuroinflammation. Further research is required to investigate this theory using a suitable baseline control diet.

Chapter 4

The effect of dietary omega-3 PUFA docosahexaenoic acid (DHA) supplementation on the behavioural phenotype of Tg2576 mice

4.1 Introduction

As a follow-up study to chapter 3, the main aim of the experiments reported in this chapter was to test the hypothesis that dietary DHA supplementation would improve the behavioural phenotype of Tg2576 mice when administered chronically from an early age. In addition, the selection of behavioural tests was expanded to include measures of spatial memory, anxiety and executive function which may be sensitive in detecting transgene-related deficits and diet effects.

Results from epidemiological studies highlight the importance of assessing intervention during early stages of disease development. For example, the results of the Rotterdam study reported an association between lifelong dietary omega-3 PUFA intake and reduced risk of early-stage (2 years after non-demented baseline) AD and dementia cases (Kalmijn et al., 1997a). In contrast, the results from the Rotterdam study at a later follow-up revealed no association in later-stage AD cases, 6 and 9.6 years after baseline (Engelhart et al., 2002; Devore et al., 2009). Devore et al. (2009) therefore suggested that omega-3 PUFAs may be protective against very early stages of dementia, potentially by targeting early pathological mechanisms or because pathology becomes too advanced in later stages to be substantially attenuated by omega-3 PUFAs. Supporting this, Devore et al. (2009) analysed the results from the Rotterdam study and found a modest but non-significant association (26% risk reduction) during short-term follow up (8 years after baseline), but no association (16% risk reduction) during long-term follow-up (9-14 years after baseline).

Westerman et al. (2002) reported the presence of soluble Aβ from 4 to 5 months of age in Tg2576 mice, which became significant at 6 to 7 months of age. Furthermore, studies have reported a reduction in dendritic spine density, impaired LTP and a deficit in basal synaptic transmission from 4 months of age in Tg2576 mice (Lanz, Carter & Merchant, 2003;

Jacobsen et al., 2006). The early manifestation of these transgene-related deficits highlights the need for earlier intervention. Little is understood about these early changes and their significance in the development of Aβ pathology and the onset of cognitive impairment in Tg2576 mice. Nevertheless, interventions that may interact with these early stages of neuronal pathology may have more profound effects on cognition. The experiments reported in this chapter therefore used a dietary supplementation procedure implemented from 2 months of age, when no behaviour or synaptic alterations have been observed in this model (e.g. Hsiao et al., 1996; Chapman et al., 1999; Lanz, Carter & Merchant, 2003; Jacobsen et al., 2006).

In order to accurately examine the effect of dietary omega-3 PUFA DHA supplementation, the experimental design of the DHA-rich and baseline control diet was an important consideration of the study. The same experimental diet design as chapter 3 was employed, whereby the baseline control diet was designed to have an equal level of total fat and total omega-6 PUFA content as the DHA-rich diet. Furthermore, the control diet was designed to be depleted in DHA but not omega-3 PUFAs, whereby levels of other omega-3 PUFAs were similar between diets. The resulting difference in the ω-6/ω-3 ratio was therefore attributable to the DHA content in the diets, and importantly, was not excessive. This design should therefore accurately examine the effect of DHA supplementation. In contrast, previous studies have provided evidence that DHA supplementation can ameliorate behavioural deficits, synaptic deficits and Aβ pathology relative to feeding a poor diet low in omega-3 PUFAs and high in omega-6 PUFAs, with an excessive ω-6/ω-3 ratio (e.g. Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006). This will be the first study to accurately examine the effects of DHA supplementation relative to a suitable baseline diet, and the first study to investigate early DHA intervention in Tg2576 mice.

In order to extend the behavioural profile of the Tg2576 model and assess the effect of dietary DHA supplementation, a battery of behavioural tasks were used to assess spatial memory, anxiety and executive function. Principally, spatial memory tasks were selected as this remains an important memory domain affected in mouse models of AD pathology. A novel 'foraging task' was developed to provide a more comprehensive analysis of spatial memory and performance. Further to this, anxiety was assessed using the elevated plus maze and marble burying task. These spatial memory and anxiety tests are believed to be amygdala- and hippocampal-dependant, and were therefore predicted to show deficits since

pathology is well established in these regions. Furthermore, a measure of prefrontal cortex (PFC) functioning was also assessed using a mouse-analogue of the biconditional discrimination task developed by Haddon & Killcross (2005). Importantly, this was one of the few experiments to directly measure PFC function in the Tg2576 model and was the first to assess the effect of DHA supplementation on PFC function. It was predicted that this task may detect a deficit since pathology is well established in the frontal cortex and PFC function is particularly affected in AD patients. Relative to a suitable baseline control diet, it was hypothesised that longitudinal dietary supplementation of DHA from an early age would slow the onset and reduce the progression of behavioural deficits in Tg2576 mice.

4.2 Overview of mouse cohort and dietary intervention design used

The experiments in this chapter used one cohort of male Tg2576 transgenic (Tg) and non-transgenic (wildtype, WT) mice. The cohort of Tg2576 and WT mice used in this chapter was generated and maintained as outlined in chapter 2, section 2.2. At 2 months of age, mice were taken off standard laboratory mouse chow and were fed experimental diets composed of standard mouse chow containing either a 5% DHA-rich oil (DHA; containing 1.86% DHA, 2.05% total omega-3 PUFA, 1.60% total omega-6 PUFA, and an ω-3/ω-6 ratio of 1.28:1) or a 5% oil blend (OB; containing 0% DHA, 0.22% total omega-3 PUFA, 2.07% total omega-6 PUFA, and an ω-3/ω-6 ratio of 0.10:1). Importantly, the difference in ω-3/ω-6 ratio was caused almost entirely by the DHA content, and the difference in omega-6 PUFA content was minimal compared with previous studies investigating DHA supplementation (e.g. Calon et al., 2005; Green et al., 2007). Furthermore, the absence of DHA in the control oil blend diet was compensated by "neutral" monounsaturated fatty acids and saturated fatty acids. Further details concerning these diets are outlined in chapter 2, section 2.3. The experimental and control diet design used in this chapter was the same as detailed in chapter 3. It was thought that the different diets would not alter body weight in the mice, since this was not found between the fish oil diet (containing DHA) and oil blend diet in chapter 5 (see chapter 5, section 5.2). The experimental schedule employed for chapter 4 is outlined in Figure 4.1.

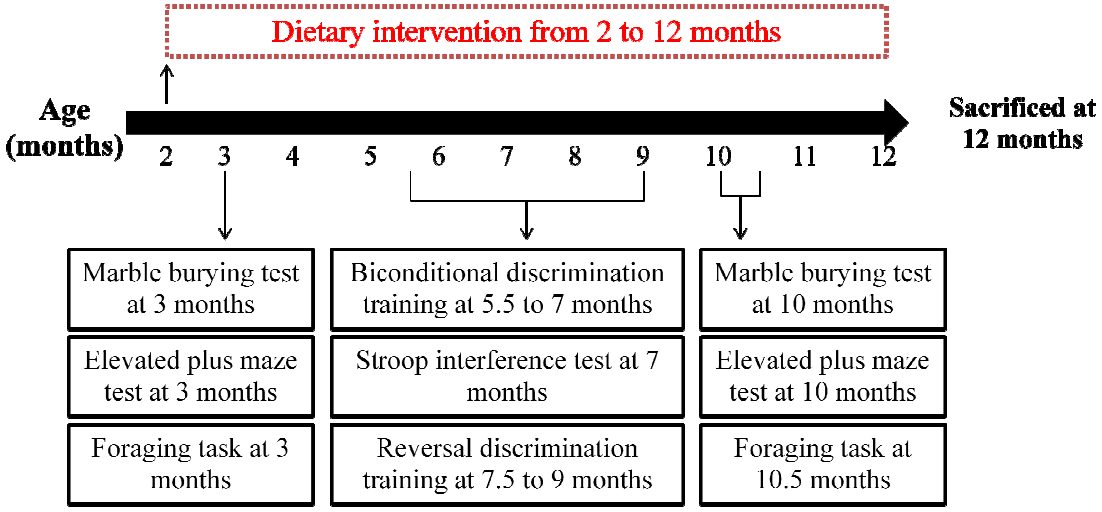

Figure 4.1 Experimental schedule employed in chapter 4.

4.3 Experiment 4: Anxiety measures

4.3.1 Introduction

This experiment aimed to evaluate anxiety in Tg2576 mice and examine the effect of DHA supplementation. This was deemed important as AD patients show emotional disturbances including fear-related memory disruption, apathy, depression, agitation, disinhibition and anxiety (Hamann, Monarch & Goldstein, 2000; Lesser & Hughes, 2006; Hollingworth et al., 2006; Spoletini et al., 2008). The neurological basis of emotional disturbances in AD is thought to reflect pathology in the medial temporal and frontal lobes, particularly in the amygdala and hippocampus (e.g. LeDoux, 2000; Kim & Jung, 2006; Sotres-Bayon, Cain & LeDoux, 2006; Maren, 2008; Truitt et al., 2009). Similarly, Tg2576 mice show pathology in these regions, which likely contribute to a range of emotional-related behavioural deficits observed in anxiety tests and fear conditioning experiments. For example, Tg2576 mice show passive avoidance deficits involving memory of footshocks at 3, 9 and 14 months, although not at the later age of 19 or 22 months (King et al., 1999; King & Arendash, 2002). Several studies have also shown impaired fear conditioning to context and/or auditory cues in contextual fear conditioning paradigms in Tg2576 mice from 5 months (Dineley et al., 2002; Barnes & Good, 2005; Comery et al., 2005; Dong et al., 2005; Jacobsen et al., 2006; Quinn et al., 2007). Although the latter findings were inconsistent across ages, the results suggest emotional dysfunction in Tg2576 mice similar to AD patients.

Tg2576 mice also show emotional-related behavioural deficits in anxiety tests such as the elevated plus maze which measures abnormal responses to unconditioned aversive or anxiogenic stimuli (e.g. Gil-Bea et al., 2007). In this test, the rodent is presented with an aversive or anxiogenic element within the elevated plus maze environment (two exposed open arms) which induces agoraphobic and inhibitory responses in normal rodents whereby a preference for the two 'safe' closed arms is observed (Pellow et al., 1985). Abnormal responses to the anxiogenic stimuli, such as increased exploratory activity in the open arms, correspond to reduced anxiety and behavioural disinhibition. The elevated plus maze is therefore a well documented test used to evaluate anxiety and disinhibitory behaviour in rodents (Pellow et al., 1985; Ognibene et al., 2005). Behavioural disinhibition is often associated with reduced anxiety and is defined as the inability to withhold inappropriate responding (Gil-Bea et al., 2007).

Previous studies have reported a deficit at 9 and 17 months of age in Tg2576 mice in the elevated plus maze, whereby Tg2576 mice spent increased time in the open arms and showed greater open arm entries relative to WT mice (Lalonde et al., 2003; Gil-Bea et al., 2007). These results suggest that Tg2576 mice are less anxious than WT mice. Furthermore, Ognibene et al. (2005) also reported a non-significant tendency for these deficits to emerge at 7 to 12 months of age. They also reported a number of ethological measures consistent with reduced anxiety, including increased head dipping (exploratory behaviour), less stretch-attend postures (risk-assessing behaviour) and increased self-grooming (behavioural disinhibition) in the elevated plus maze. However, there are no published reports of behaviour in the elevated plus maze at an age earlier than 7 months. This experiment therefore investigated the effects of the APPswe mutation on elevated plus maze behaviour at 3 and 10 months of age. Based on previous research it was hypothesised that Tg2576 mice would show a transgene-related deficit in the elevated plus maze.

Supporting these predictions, similar reports of reduced anxiety in the Tg2576 model has been shown in the open field test. The open field test is an anxiety test whereby large open spaces (an anxiogenic environment for rodents) normally induce agoraphobic and inhibitory responses such as reduced exploratory behavior consistent with increased anxiety (Pellow et al., 1985). It is well documented however that Tg2576 mice show increased activity in the open field test from 3 months, although some reports have been inconsistent (Chapman et al., 1999; King et al., 1999; King & Arendash, 2002; Lalonde et al., 2003; Deacon et al., 2008,

2009). Furthermore, Deacon et al. (2008) reported reduced rearing in the open field at 12 months, which was supported in the holeboard task. In contrast however, rearing behaviour in the open field was reported normal at 3, 8.5 and 21 months and increased at 22 to 23 months (Deacon et al., 2008, 2009). Despite these inconsistencies, other measures in the open field at the 22 and 23 months were consistent with reduced anxiety, including a higher latency to rear, the production of less faecal boli and failure to habituate (Deacon et al., 2009). In contrast, Deacon et al. (2008) reported normal defecation, latency to rear, latency to head dip and number of head dips in the holeboard test at 12 months. These inconsistencies highlight the need for further investigation into the onset and progression of anxiety-related behaviour.

The marble burying task is also frequently used to assess anxiety and screen anxiogenic drugs in rodents (Nicolas, Kolb & Prinssen, 2006; Crawley, 2008). In this test, rodents are presented with glass marbles in a digging medium in which they show spontaneous burying of these harmless novel objects (Poling et al., 1981; Archer et al., 1987). Although the normal response of burying the majority of marbles is often used as an index of anxiety, its validity remains questioned by some (as reviewed in Nicolas, Kolb & Prinssen, 2006). For example, some argue that marble burying behavior is dependent upon impulsive behavior or locomotory activity. Typically however, an abnormal response of leaving the marbles unburied corresponds to reduced anxiety. Since Tg2576 mice have been reported to have altered anxiety in tasks such as the elevated plus maze, open field and holeboard task (e.g. Lalonde et al., 2003; Deacon et al., 2008, 2009; Ognibene et al., 2005), we used the marble burying procedure in this experiment to extend the analysis of anxiety behaviour in Tg2576 mice.

To date, a review of the literature has presented only one study reporting marble burying assessment in Tg2576 mice at 3, 12 and 23 months of age. Here, Deacon et al. (2008) reported a lack of transgene-related deficit. However, the background strain of these mice differed from the cohort used in these experiments, whereby the Tg2576 cohort were the female offspring of Tg2576 hybrid BL6/SJL male mice crossed with F1 BL6/SJL females. In contrast, Tg2576 mice in our experiments were maintained by crossing heterozygous Tg2576 mice to C57BL/6j x SJL F1 mice. These background differences may therefore alter marble burying behaviour. Several studies have highlighted the effect of strain differences on behaviour, with particular reference to marble burying (Deacon et al., 2006; Thomas et al., 2009). Furthermore, Lasselle et al. (2008) outlined the important influence of genetic

background on the behavioural phenotype of the APPswe transgene in Tg2576 mice in particular. In light of the potential effects of background strain, together with evidence of anxiety-related deficits in Tg2576 mice in other tasks, gives cause to predict that Tg2576 mice would show marble burying deficits.

The second main aim of this experiment was to investigate whether early DHA dietary supplementation would modify the onset of anxiety deficits in Tg2576 mice. Although no studies have assessed DHA supplementation relative to a suitable baseline diet, several studies have shown that DHA improves cognitive function in hippocampal-dependant memory tasks including the Morris water maze, Y-maze and the novel object recognition task in AD mouse models (e.g. Calon et al., 2004; Hooijmans et al., 2009; Ma et al., 2009; Arsenault et al., 2011). In contrast, only one study has assessed DHA supplementation on a task related to anxiety. Here, Hooijmans et al. (2009) reported no effect of DHA on open field behavior at 8- and 15-month old APPswe/PS1 mice. Marble burying and elevated plus maze behaviour has been shown to be dependent on hippocampal function (Deacon & Rawlins, 2005; Barkus et al., 2009; Xiang et al., 2011), which has been reported dysfunctional in Tg2576 mice (e.g. Hsiao et al., 1996). Based on previous evidence showing DHA to reduce hippocampal-dependant memory deficits in AD models (Calon et al., 2004; Hooijmans et al., 2009; Ma et al., 2009; Arsenault et al., 2011), it was hypothesised that DHA supplementation may reduce a marble burying and elevated plus maze deficit relative to the oil blend diet.

4.3.2 Methods

4.3.2.1 Design and subjects

One cohort of male Tg2576 transgenic (Tg) and non-transgenic (wildtype, WT) mice were evaluated in the elevated plus maze and marble burying task at two ages. Experimentally naïve mice were first tested at approximately 3 months of age, before the onset of plaque pathology and major changes in synaptic morphology and function. This cohort was tested again at 10 months of age when plaque pathology was developing and where synaptic deficits should be clearly manifest. This design therefore investigated transgene- and diet-dependant changes at these ages, in addition to whether these changes were age-dependant. When tested in the elevated plus maze at 3 months of age, 14 Tg mice received DHA diet (Tg DHA), 14

Tg mice received the oil blend diet (Tg OB), 18 WT mice received the DHA diet (WT DHA) and 16 WT mice received the oil blend diet (WT OB). Group numbers were the same for the marble burying assessment at 3 months, except one Tg DHA mouse was excluded for circling behaviour. By the second stage of experimentation at 10 months of age in the elevated plus maze, the loss of 12 mice resulted in group numbers of 7 Tg DHA, 12 Tg OB, 15 WT DHA and 16 WT OB. These group numbers were the same for the marble burying assessment at 10 months. The running order of mice was counterbalanced so that one mouse from each experimental group was tested after another in the elevated plus maze as far as possible, blind to the experimenter. In the marble burying task, running order was counterbalanced so that approximately equal numbers of each experimental group was run in each batch (n=4). Mice were tested only once at each age.

4.3.2.2 Apparatus

Elevated plus maze

The elevated plus maze (EPM) was constructed from four arms each 40cm long and 6cm wide in a cross arrangement with a 6cm^2 central area, elevated 70cm above the floor. As illustrated in Figure 4.2, two arms provide a closed setting and two provide an open setting. The closed arms were surrounded by high-sided 15cm tall black Perspex walls, whereas the open arms had extremely short 1cm tall clear Perspex walls to prevent falls. The base of the maze arms were composed of white laminate wood which were supported by a black metal frame. The maze was placed inside a wooden arena (102cm^2 and 43cm height) with a sawdust-covered floor to cushion the mouse in the event of a fall. The apparatus was located in quiet and brightly illuminated room. A camera was attached to the ceiling directly above the maze, which was connected to a computer, a television monitor and video recorder (VCR). The camera input was used to visualise the maze activity on the television monitor and each session was recorded using the VCR. Noldus® Ethovision software on the computer was utilised to collect data using the camera input by 'manual scoring' (see procedure).

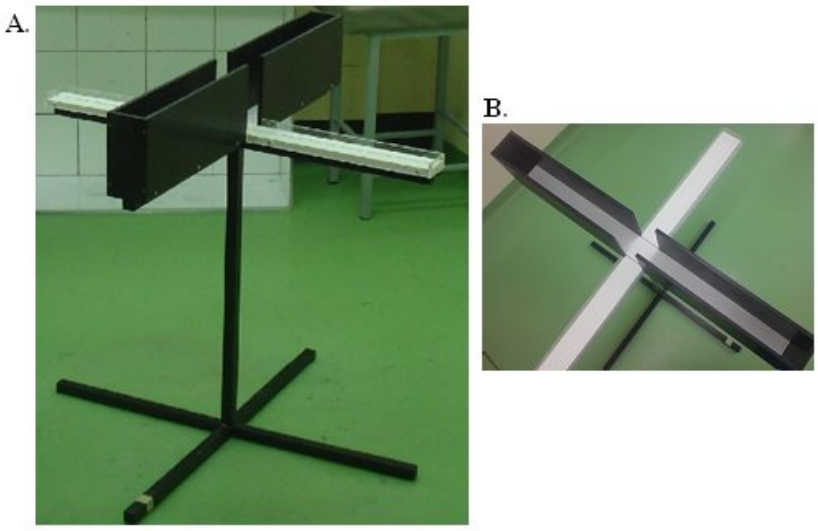

Figure 4.2 Elevated plus maze apparatus from two perspectives. A. Front-view, B. Ariel view.

Marble burying test

Each marble burying arena was composed from a large white polycarbonate cage base 41cm long, 24.5cm wide and 12.5cm tall, filled with a level compact layer of sawdust 6.5cm deep (Figure 4.3). Twenty mixed-colour glass marbles (1.5cm diameter) were placed on top of the sawdust layer equally distanced apart in a 4-column by 5-row arrangement. This arrangement, in comparison to a grouped-marble arrangement, forced the mice to encounter the marbles more frequently and prevented an alternative avoidance response rather than burying the aversive stimuli (see Njung'e & Handley, 1991). A transparent Perspex lid 41cm long, 27cm wide and 1cm thick was placed securely on top of the cage base to prevent the mouse from escaping the arena. The lid was placed in a central position allowing for a 1.25cm gap either side for ventilation. Four marble burying arenas were placed on a table elevated 121cm from the floor, allowing 4 mice to be tested during one 30-minute session. A camera was attached to the ceiling directly above the arena to allow viewing and recording for documentation using a monitor and VCR. Each marble burying arena was photographed for documentation using a digital camera at the end of each session.

Figure 4.3 Marble burying arena and marble arrangement

4.3.2.3 Procedure

Elevated plus maze

Mice were transported to the test room in individual home cages and the experimenter was present throughout all sessions in a maintained location. Each mouse received one 5-minute session in the maze with free-access, without habituation. The maze was cleaned with 1:20 dilution of Mr Muscle® Glass Cleaner between sessions to remove odours, urine and excrement. Each mouse was placed in the maze so that they faced the left-hand closed arm.

Noldus© Ethovision software on the computer was used to record mouse activity measures by the 'manual scoring' method. Here, each arm was assigned a specific keyboard key which was pressed upon entry into and exit from each arm. An arm entry was defined as when all four paws of the mouse passed the entrance of the arm, and an arm exit was defined as when all four paws crossed outside the arm. This criteria matched that used in Lalonde et al. (2003) and Ognibene et al. (2005) experiments. Using this methodology, Noldus® Ethovision software automatically calculated the total duration spent in each arm and the total number of entries into each arm. The accuracy of this manual scoring method was checked for the first 3 mice tested per session. The total frequency of entries and total duration spent in the open and closed arms were calculated. The latency to enter an open arm was also recorded by the experimenter on a notepad. Mice which did not enter the open arm at all were assigned a latency of 300 seconds (the total time spent in the maze).

Marble burying test

Mice were transported to the test room in individual home cages and the experimenter was present throughout all sessions out of sight. Each mouse received one 30-minute session in the marble burying arena with free-access, without habituation. Each mouse was placed in the middle of the arena at the start of the session, and carefully removed after 30 minutes using their home-cage cardboard tube to prevent any disturbance to the sawdust and marbles. The number of marbles buried/unburied was counted. Figure 4.4 shows two example photographs from the marble burying test. Using the criteria used in previous studies, a buried marble was defined as a marble covered at least two thirds by sawdust (e.g. Millan et al., 2002; Young et al., 2006). An unburied marble was therefore over one third visible on the surface of the sawdust layer. Between sessions, the sawdust in the arena was mixed to remove odours trails and the excrement removed. A handful of old sawdust was replaced with fresh after each session.

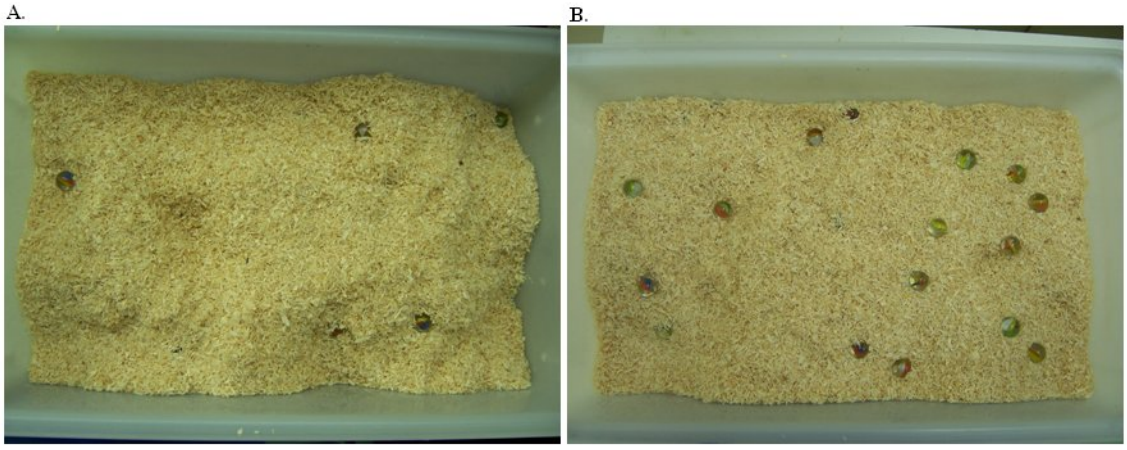

Figure 4.4 Example photographs from the marble burying test. A. Example from a WT mouse, showing 3 unburied marbles, 17 buried. B. Example from Tg mouse, showing 13 unburied marbles, 7 buried.

4.3.3 Elevated plus maze results

4.3.3.1 Elevated plus maze results at 3 months of age

Frequency of entries into the open and closed arms

Figure 4.5 shows the mean frequency of entries into the open and closed arms of the EPM for each experimental group at 3 months of age. An ANOVA with arm (open, closed), genotype

and diet as factors revealed a significant main effect of genotype ($F(1,58)=19.960$, $p<0.001$), no significant main effect of diet ($F(1,58)=0.878$, $p>0.05$) and a significant interaction of genotype by diet ($F(1,58)=11.547$, $p<0.001$). Statistical analysis also showed a significant effect of arm ($F(1,58)=58.91$, $p<0.05$), no significant interaction of arm by genotype ($F(1,58)=3.043$, $p>0.05$), a significant interaction of arm by diet ($F(1,58)=7.686$, $p<0.01$), and no significant interaction of arm by genotype by diet ($F(1,58)=1.750$, $p>0.05$). Follow-up tests of simple main effects for the significant arm by diet interaction revealed that frequency of entries into both open arms ($F(1,60)=5.72$, $p<0.05$) and closed arms ($F(1,60)=7.14$, $p<0.01$) altered as a consequence of diet. Overall these results are reflected in Figure 4.5, whereby all mice show a preference for closed over open arms and that this natural preference was exaggerated with DHA supplementation. However, as inspection of Figure 4.5 suggests, this appears to be predominately driven by the Tg OB group that showed little preference for the closed arms relative to open arms.

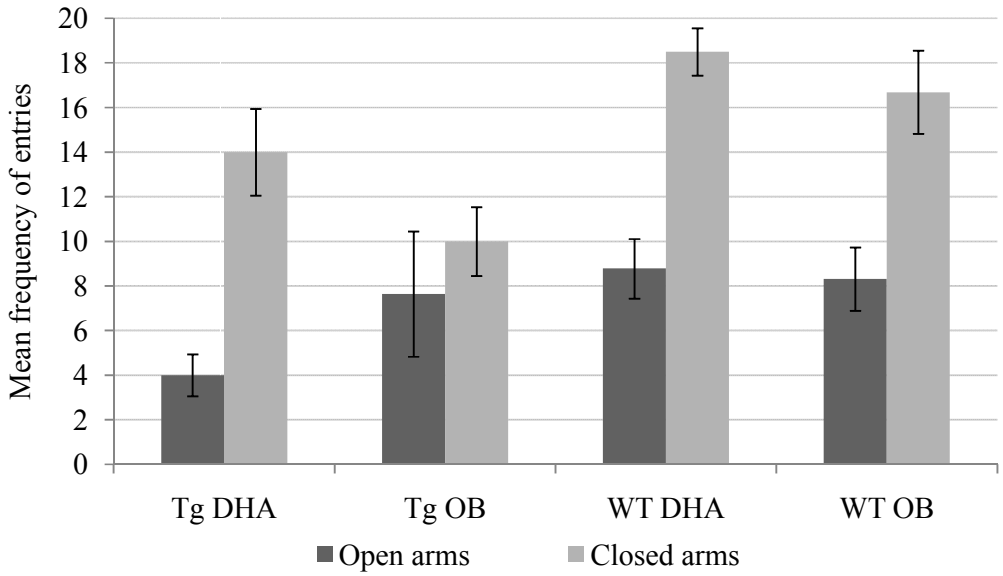

Figure 4.5 Mean frequency of entries into open and closed arms of the EPM at 3 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M (standard error of the mean).

Duration of time spent in open and closed arms

Figure 4.6 shows the duration of time spent in the open and closed arms of the EPM for each experimental group at 3 months of age. The figure shows that all mice had a preference for

closed over open arms. Interestingly, Tg mice appear to show a heightened normal response by spending more time in the closed arm. Furthermore, the time spent in the open arm appears reduced by DHA supplementation in Tg mice compared to all groups. An ANOVA with arm, genotype and diet as factors revealed a significant main effect of genotype ($F(1,58)=19.960$, $p<0.001$), no significant main effect of diet ($F(1,58)=0.878$, $p>0.05$) and a significant interaction of genotype by diet ($F(1,58)=11.547$, $p<0.001$).

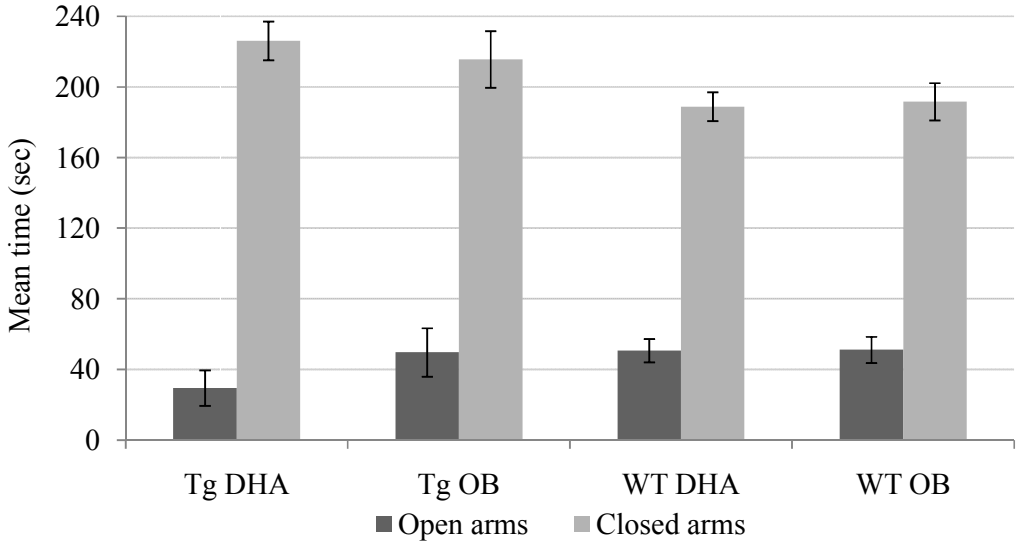

Figure 4.6 Mean duration spent in open and closed arms of the EPM at 3 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

Statistical analysis also showed a significant effect of arm ($F(1,58)=889.273$, $p<0.001$), a significant interaction of arm by genotype ($F(1,58)=20.536$, $p<0.001$), no significant interaction of arm by diet ($F(1,58)=3.417$, $p>0.05$), and a significant three-way interaction of arm by genotype by diet ($F(1,58)=6.332$, $p<0.05$). Follow-up tests of simple main effects and interactions revealed a significant genotype effect for both open and closed arm measures of duration ($F(1,58)=6.332$, $p<0.05$ and $F(1,58)=6.332$, $p<0.001$, respectively), a significant effect of diet for the open arm measure ($F(1,58)=5.15$, $p<0.05$), and a significant genotype by diet interaction for the closed arm measure ($F(1,58)=10.23$, $p<0.05$). Following up this interaction, tests of simple main effects revealed a significant difference between Tg OB and WT OB mice ($F(1,58)=35.78$, $p<0.001$) and a significant difference between Tg DHA and Tg OB ($F(1,58)=9.99$, $p<0.01$). Overall these results are reflected in Figure 4.6, whereby Tg groups appear to show a heightened normal response, supported by the main effect of

genotype in both open and closed arms. However, this appears to be predominately driven by the Tg DHA group, which is supported by the genotype by diet interaction. The results also reveal that the time spent in the open arm is lowered as a consequence of DHA, although this appears to be numerically driven by the Tg DHA group.

Latency to enter an open arm

Figure 4.7 shows the mean latency to enter an open arm of the EPM for each experimental group at 3 months of age. Transgenic mice, particularly the oil blend group, appear to show longer latencies to enter the open arms compared to WT mice. Statistical analysis using an ANOVA with genotype and diet as factors revealed a significant main effect of genotype ($F(1,58)=7.048$, $p<0.01$), no significant main effect of diet ($F(1,58)=1.220$, $p>0.05$) and no significant interaction of genotype by diet ($F(1,58)=0.320$, $p>0.05$).

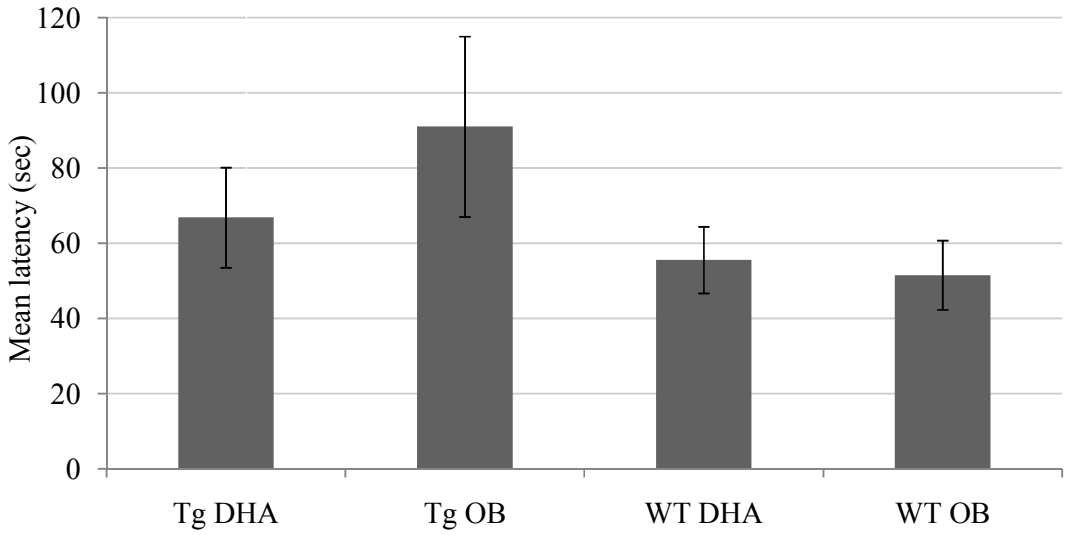

Figure 4.7 Mean latency to enter an open arm of the EPM at 3 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

In summary, Tg mice differ significantly from WT group at 3 months in the EPM. Although all mice showed a preference for the closed over open arm (both in duration spent and frequency of entries), as expected, some results indicate that DHA supplementation could exaggerate this preference.

4.3.3.2 Elevated plus maze results at 10 months of age

Frequency of entries into the open and closed arms

Figure 4.8 shows the mean frequency of entries into the open and closed arms of the EPM for each experimental group at 10 months of age. The graph shows that all mice show a preference for the closed over open arms, although this pattern is much weaker in the Tg OB group. An ANOVA with arm, genotype and diet as factors revealed no significant main effect of genotype ($F(1,46)=1.240$, $p>0.05$), a significant main effect of diet ($F(1,46)=4.787$, $p<0.05$) and no significant interaction of genotype by diet ($F(1,46)=3.010$, $p>0.05$). Statistical analysis also showed a significant effect of arm ($F(1,46)=67.379$, $p<0.001$), no significant interaction of arm by genotype ($F(1,46)=2.789$, $p>0.05$), no significant interaction of arm by diet ($F(1,46)=2.844$, $p>0.05$), and no three-way interaction of arm by genotype by diet ($F(1,46)=1.417$, $p>0.05$).

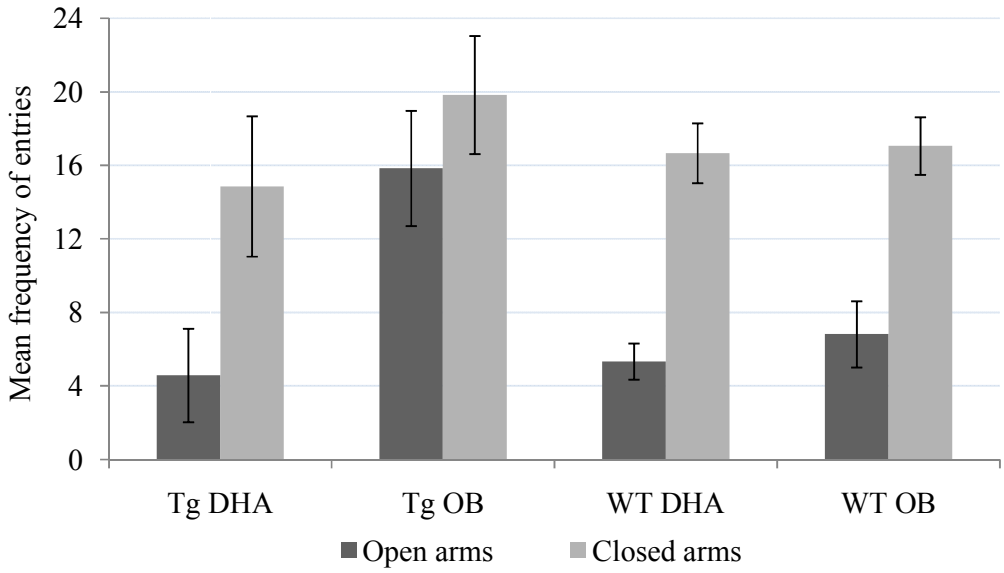

Figure 4.8 Mean frequency of entries into open and closed arms of the EPM at 10 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

Duration of time spent in open and closed arms

Figure 4.9 shows the duration of time spent in the open and closed arms of the EPM for each experimental group at 10 months of age. The figure shows that all mice preferred to spend

time in the closed arms, although Tg OB mice showed less preference, indicating that DHA ameliorated a deficit in Tg mice. An ANOVA with arm, genotype and diet as factors revealed a significant main effect of genotype ($F(1,46)=5.052$, $p<0.05$), no significant main effect of diet ($F(1,46)=0.240$, $p>0.05$) and no significant interaction of genotype by diet ($F(1,46)=2.996$, $p>0.05$). Statistical analysis also showed a significant effect of arm ($F(1,46)=98.296$, $p<0.001$), no significant interaction of arm by genotype ($F(1,46)=0.303$, $p>0.05$), a significant interaction of arm by diet ($F(1,46)=4.143$, $p<0.05$), and a significant three-way interaction of arm by genotype by diet ($F(1,46)=6.518$, $p<0.05$).

Follow-up tests of simple main effects and interactions revealed a significant diet effect for the open arm ($F(1,46)=4.18$, $p<0.05$) but a non-significant effect of diet for the closed arm ($F(1,46)=3.48$, $p>0.05$), and a significant genotype by diet interaction for both the open and closed arm measure ($F(1,46)=5.40$, $p<0.05$ and $F(1,46)=7.11$, $p<0.05$, respectively). Following up these interactions, a test of simple main effects revealed Tg OB mice spent significantly more time in the open arms than WT OB mice ($F(1,46)=8.90$, $p<0.01$) and Tg DHA mice ($F1,46)=9.18$, $p<0.01$), and Tg OB spent significantly less time in the closed arms than Tg DHA mice ($F(1,46)=8.90$, $p=0.005$). Figure 4.9 clearly depicts this difference between Tg groups and the overall preference for closed over open arms in all mice. The significant diet effect in the open arm appears to be driven by the Tg OB group, as is the main effect of genotype.

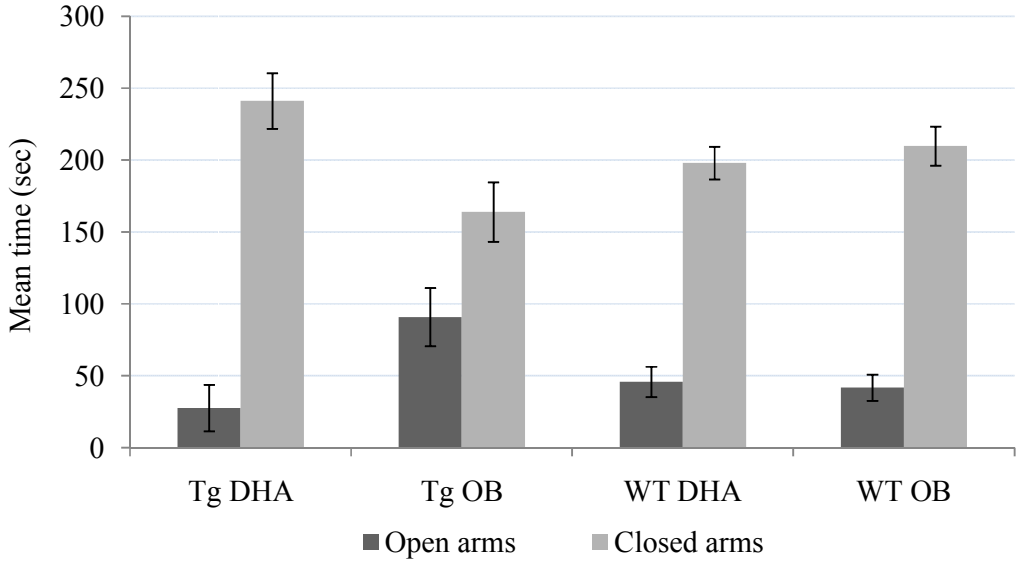

Figure 4.9 Mean duration spent in open and closed arms of the EPM at 10 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

Latency to enter an open arm

Figure 4.10 shows the mean latency to enter an open arm of the EPM for each experimental group at 10 months of age. This figure shows that mice had similar latencies, except WT mice fed the OB diet had longer latencies. Statistical analysis using an ANOVA with genotype and diet as factors revealed no significant main effect of genotype ($F(1,46)=0.154$, $p>0.05$), no significant main effect of diet ($F(1,46)=1.241$, $p>0.05$) and a significant interaction of genotype by diet ($F(1,46)=4.902$, $p<0.05$). Following-up this interaction, a test of simple main effects showed WT OB to have significantly longer latencies than WT DHA mice ($F(1,46)=4.20$, $p<0.05$).

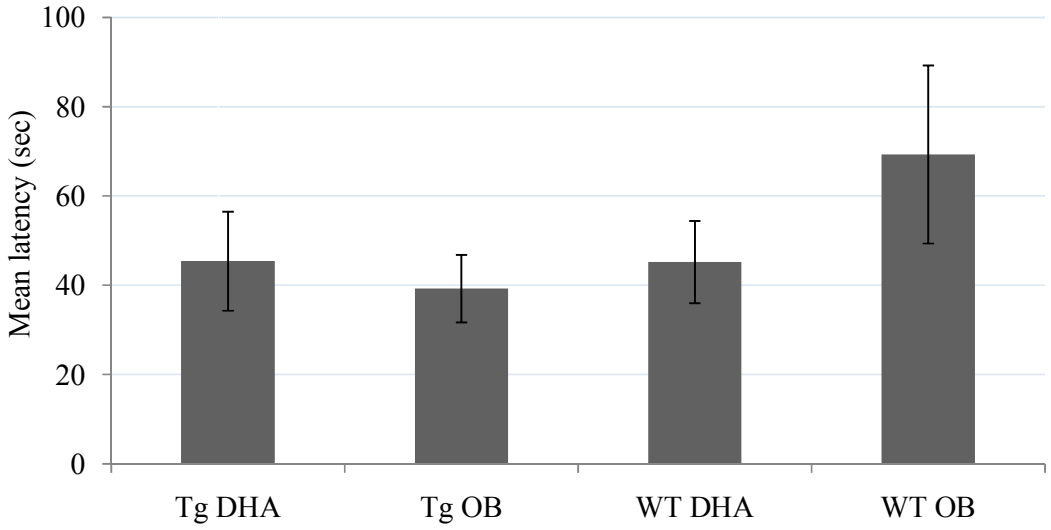

Figure 4.10 Mean latency to enter an open arm of the EPM at 10 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

In summary, at 10 months of age, both Tg and WT mice show a preference for the closed over open arms (both in duration spent and frequency of entries). However, this effect was considerably weaker in Tg2576 mice on the oil blend diet. DHA supplementation generally restored performance of Tg mice to that of control WT mice.

4.3.4 Marble burying test results

4.3.4.1 Marble burying results at 3 months of age

Figure 4.11 shows the mean number of unburied marbles in the marble burying test for each experimental group at 3 months of age. This figure shows that Tg OB mice showed abnormal marble burying compared to WT mice, and that DHA ameliorated this deficit. Statistical analysis using an ANOVA with genotype and diet as factors revealed a significant main effect of genotype ($F(1,57)=11.759$, $p<0.001$), no significant main effect of diet ($F(1,57)=3.361$, $p>0.05$) and a significant interaction of genotype by diet ($F(1,57)=5.940$, $p<0.05$). Following-up this interaction, a test of simple main effects revealed Tg OB left significantly more marbles unburied than WT mice ($F(1,57)=16.44$, $p<0.001$) and Tg DHA mice ($F(1,57)=8.75$, $p<0.01$). This effect is clearly illustrated in Figure 4.11, whereby Tg OB mice left an average of approximately 13 marbles unburied in contrast to ~7 marbles in the Tg DHA group which were comparable to WT mice (~6 and 5 unburied marbles). This effect was also demonstrated in ratio values (= [mean number of unburied marbles / total number of marbles available], where 0 = all marbles unburied, 0.5 = equal level of buried and unburied marbles, and 1 = all marbles buried). Here, Tg DHA = 0.35, Tg OB = 0.63, WT DHA = 0.28 and WT OB = 0.24, thereby showing that all groups except Tg OB have a higher buried to unburied marble ratio. These results indicate that the main effect of genotype was likely driven by the Tg OB group.

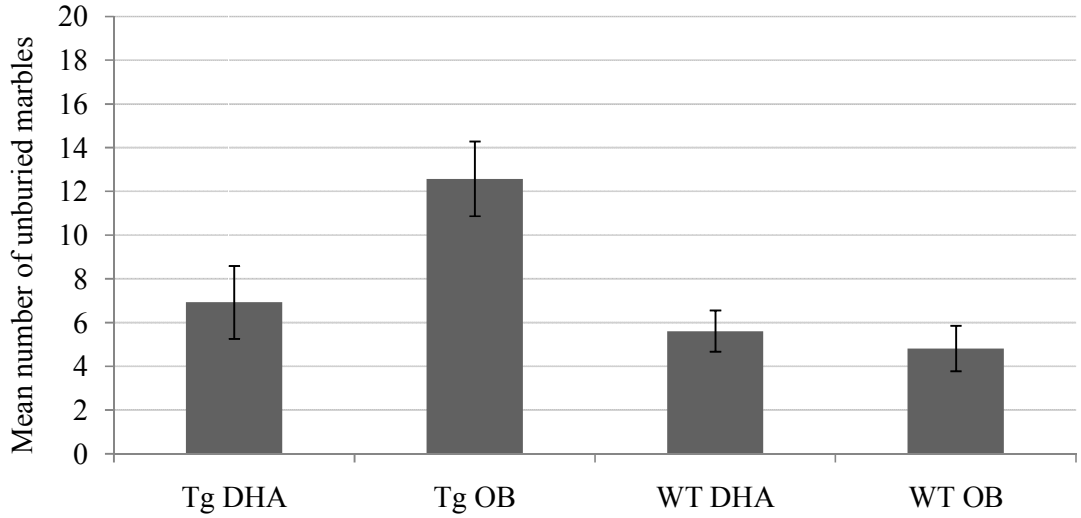

Figure 4.11 Mean number of unburied marbles (20 marbles in total) in the marble burying test at 3 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

4.3.4.2 Marble burying results at 10 months of age

Figure 4.12 shows the mean number of unburied marbles in the marble burying test for each experimental group at 10 months of age. Statistical analysis using an ANOVA with genotype and diet as factors revealed a significant main effect of genotype ($F(1,46)=5.507$, $p<0.05$), no significant main effect of diet ($F(1,46)=1.325$, $p>0.05$) and no significant interaction of genotype by diet ($F(1,46)=1.243$, $p>0.05$). These effects are clearly displayed in Figure 4.12, whereby Tg groups had similar results (~11 unburied marbles) and WT groups had relatively similar results (~5-7 unburied marbles). The diet appeared to have no effect in the Tg mice, although interestingly, DHA appeared to non-significantly alter WT performance. This effect is clearly illustrated in ratio values (number of unburied marbles/total marbles) whereby Tg DHA = 0.58, Tg OB = 0.55, WT DHA = 0.46 and WT OB = 0.27, thereby showing that Tg groups similarly had a higher buried to unburied marble ratio, and DHA appears to alter WT behaviour to deviate from the normal higher unburied to buried marble ratio.

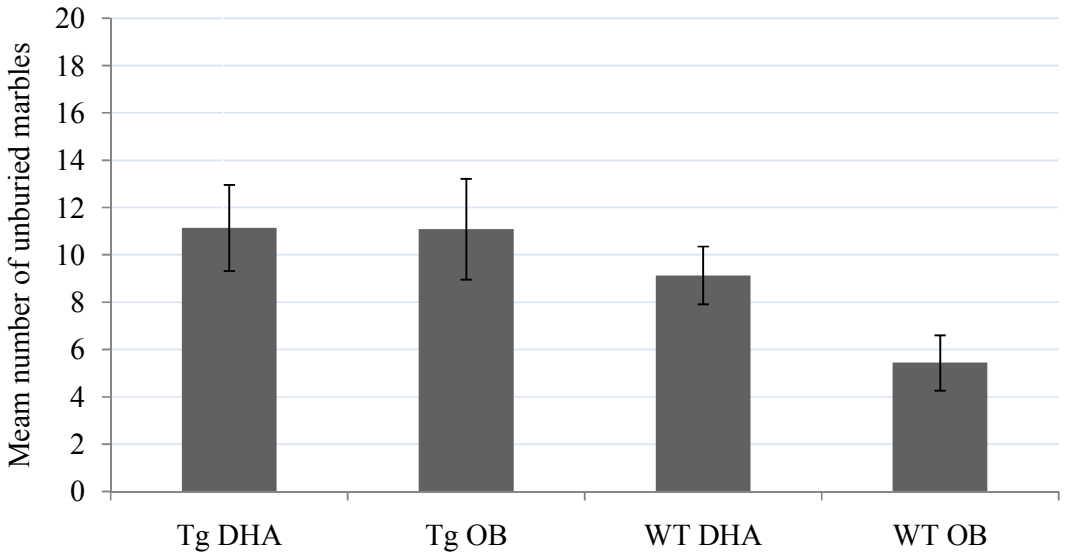

Figure 4.12 Mean number of unburied marbles (20 marbles in total) in the marble burying test at 10 months in Tg and WT mice fed the DHA or oil blend diet. Values are mean scores ± S.E.M.

4.3.5 Discussion

The results from the elevated plus maze study at 3 and 10 months of age showed that WT mice entered the closed arms more frequently than the open arms and spent more time in the closed arms during the 5-minute test (see also, Pellow et al., 1985; Lalonde et al., 2003).

Tg2576 mice however showed an abnormal pattern of EPM behaviour at both 3 months and 10 months of age, whereby preference for the closed arms was reduced compared to WT mice. This is consistent with other evidence for reduced anxiety and disinhibitory behaviour in Tg2576 mice (Lalonde et al., 2003; Ognibene et al., 2005).

Although Tg2576 mice showed behaviour associated with reduced anxiety, this was not consistently shown at 3 months. For example, young Tg mice entered the closed arms significantly less than WT mice, consistent with reduced anxiety, however, they spent significantly more time in the closed arms and less in the open arms. This latter result is consistent with a normal response of increased anxiety, which has been demonstrated in other AD models but not the Tg2576 model (e.g. España et al., 2010). Similarly, Tg mice had longer latencies to enter the open arm, consistent with a normal anxiety response.

At 10 months of age, Tg2576 mice showed a more consistent pattern of reduced anxiety across measures. For example, Tg mice fed the oil blend diet showed more entries into the open arms and fewer entries into the closed arms, in contrast to WT mice, which displayed a clear preference for the closed arms. Consistent with the arm entries measure, Tg OB mice spent more time in the open arms and a similar amount of time in the closed arms compared to WT groups. Tg mice also had shorter latencies to enter the open arm than WT mice fed the oil blend diet. Overall therefore, Tg mice showed altered anxiety at both 3- and 10-months compared to WT mice, and the pattern of altered anxiety is consistent with previous reports in the elevated plus maze in Tg2576 mice aged 10 months (Lalonde et al., 2003; Ognibene et al., 2005).

With regards to dietary effects, DHA supplementation appeared to enhance the natural preference for the closed arms at 3 months of age. More specifically, Tg mice fed the DHA diet showed reduced entries to the open arms and increased entries into the closed arms. A significant effect of diet was also found for the time spent in the open arm, which was reduced with DHA supplementation. This effect appeared to be particularly driven by the Tg DHA group, suggesting a beneficial effect of DHA supplementation. At 10 months of age, a main effect of diet was observed in the frequency of open and closed arm entries. Furthermore, Tg OB mice differed significantly from Tg DHA mice in the time spent in both the open and closed arms. Overall therefore, this study provides evidence that DHA supplementation can reduce anxiety-related deficits associated with the APPswe mutation in

Tg2576 mice. This effect was most apparent at 10 months of age when amyloid pathology is clearly evident in cortical and subcortical brain regions (see also Calon et al., 2004, 2005; Lim et al., 2005). Furthermore, this is the first experiment to provide evidence that DHA supplementation can ameliorate transgene-related anxiety deficits relative to a suitable baseline control diet. These findings compliment other published work showing positive effects of DHA supplementation on behaviour in AD mouse models (Calon et al., 2004; Hooijmans et al., 2009; Ma et al., 2009; Arsenault et al., 2011).

The results of the marble experiment showed a significant effect of genotype at both at 3 and 10 months of age, whereby Tg mice left significantly more marbles unburied than WT mice. This study therefore provides evidence that the APPswe mutation leads to behavioural deficits as early as 3 months of age. Importantly, WT mice on the control diet showed a higher ratio of buried to unburied marbles, which is consistent with normal marble burying behaviour in rodents (e.g. Deacon, 2006; Nicolas, Kolb & Prinssen, 2006). Furthermore, this behaviour is consistent across the ages whereby the ratio values (unburied/total marbles) for WT mice fed the control diet were 0.24 at 3 months of age and 0.27 at 10 months. Comparing the ratio values of Tg mice on the control oil blend diet also showed similar values at the two ages, with 0.63 at 3 months, and 0.55 at 10 months of age. This transgene-dependant deficit, consistent with a reduction in anxiety, was therefore non-progressive and age-independent.

This study is the first experiment to report a marble burying deficit in Tg2576 mice and stands in contrast to a previous report by Deacon et al. (2008) who tested similar mice at 3, 12 and 23 months of age. However, it is important to consider methodological differences that may explain the contrasting results. Firstly, mice at 3 and 12 months of age in Deacon and colleagues study were tested on a marble burying experiment using 12 marbles, in contrast to the 20 marbles used in our methodology. This lower number of marbles may have reduced their ability to detect genotypic changes. Secondly, and of particular importance, strain differences were reported by Deacon et al. (2008) which may have accounted for the differing results. The potential effect of background strain differences was demonstrated by directly comparing WT results in this experiment, where WT mice buried 15 marbles, to results from Deacon et al. (2008) at 23 months of age in the 20-marble task, where WT mice buried 12 marbles. The relatively low number of buried marbles reported by Deacon and colleagues may therefore account for the lack of genotype effect. Validation of a marble burying deficit in Tg2576 mice with a background strain identical to the cohort used in this

experiment therefore needs further investigation, and is assessed in chapter 5. It is important to note however, that the reduced anxiety-related deficit observed in this experiment is consistent to reports of reduced anxiety in Tg2576 mice in other tasks such as the elevated plus maze (e.g. Gil-Bea et al., 2007).

Although no significant main effect of diet was observed in the marble-burying experiment at both ages, a significant genotype by diet interaction was reported at 3 months of age. Tg OB mice differed from all groups and thus provided evidence that DHA supplementation reduced the marble burying deficit in young but not aged Tg2576 mice. In an attempt to explain this, it could be argued that DHA targets early but not late pathological changes. However, this is inconsistent with reports in the elevated plus maze in this chapter. In addition, Calon et al. (2004) reported that DHA supplementation ameliorated hippocampal-dependant deficits during advanced stages of pathology in APP mice. Comparisons to Calon et al. (2004) study however is somewhat limited by the differential use of task, time of intervention and use of control diet which was high in omega-6 PUFAs with an excessive ω-6/ω-3 ratio.

An important consideration of these marble burying results is interpretation of the reported deficits in Tg mice. As discussed in the introduction, alterations to marble burying behaviour is typically associated with anxiety-states (e.g. Crawley, 2008), although this interpretation remains controversial (Njung'e & Handley, 1991; Nicolas, Kolb & Prinssen, 2006). For example, locomotor activity may affect the degree of marble burying, and reports of hyperactivity in Tg2576 mice could therefore be directly related to the marble burying deficit. Evidence has both supported and refuted its involvement (as reviewed in Millan et al., 2002 and Nicolas, Kolb & Prinssen, 2006). Similar to locomotor activity, motor impairments related to a digging and a burrowing deficit reported by Deacon et al. (2006, 2009) in Tg2576 mice aged 22 to 23 months may contribute to marble burying deficits. Interestingly, similar to spatial memory deficits, digging deficits have also been associated with hippocampal function (Deacon, Croucher & Rawlins, 2002; Deacon & Rawlins, 2005). Alternatively, Millan et al. (2002) argued that marble burying behaviour reflects a form of impulsive behaviour. Although the biological basis of marble burying behaviour remains unclear, it is evident that Tg2576 show clear deficits that can be altered following DHA supplementation at an early age. The reduction of marble burying deficits by DHA may therefore reflect changes in anxiety, or behaviour such as impulsivity.

4.4 Experiment 5: Foraging task

4.4.1 Introduction

The foraging task was a novel cognitive test design based on the radial arm maze task. The radial arm maze measures spatial memory for previously visited locations within a maze composed of multiple arms radiating from a central platform (Olton, Collison & Werz, 1977). Rodents are motivated to perform this task by a food reward placed at the end of each arm (Olton & Samuelson, 1976; Olton, 1987), or selected arms depending on the version of task (as reviewed in Foreman & Ermakova, 1998). This task has been extensively used to assess spatial memory in animals and is particularly sensitive to hippocampal dysfunction (e.g. Olton & Paras, 1979; Ward, Stoelzel & Markus, 1999; He et al., 2002; Crusio & Schwegler, 2005). To date, only one study has reported radial arm maze deficits in Tg2576 mice at 24 months (Asuni et al., 2006). This is likely due to the slow acquisition displayed in mice on this task compared to other spatial memory tasks such as water maze paradigms (as reviewed in Foreman & Ermakova, 1998). As a result, the water maze and radial arm water maze tasks have been more extensively employed to assess transgene-related deficits in Tg2576 mice (e.g. Hsiao et al., 1996; Westerman et al., 2002; Adriani et al., 2006; Wilcock et al., 2004, 2006). Further to this, radial arm maze tasks results in longer testing periods due to the use of various delay-periods between choice trials, whereby animals are confined to the central platform using guillotine doors (see Foreman & Ermakova, 1998; Dudchenko, 2004).

Therefore a task based on the radial arm maze was designed which employed more rapid testing and potentially quicker acquisition in mice. Similar to the radial arm maze, the foraging task employed a food reward motivation as opposed to aversive escape motivation in the water maze which can induce stress and can affect performance (Harrison, Hosseini & McDonald, 2009). Unlike the radial arm maze however, the foraging task permitted free navigation as opposed to constrained search, similar to the water maze task. Such design features may probe different processes involved in spatial learning which are not tapped by the radial arm or water maze tasks individually (Hodges, 1996; Foreman & Ermakova, 1998). The foraging task also exploited the natural foraging tendencies of rodents. This may assess spatial memory more accurately than the radial arm maze as Spetch and Edwards (1986) reported that an open field version of the radial arm maze (particularly a version resembling more natural feeding behaviour) facilitated performance in pigeons compared to that of the

traditional radial arm apparatus. Unlike the radial arm maze, these environments allowed the pigeon to choose its own path between food sites. Therefore, this open-field foraging task version of the radial arm maze, that exploits natural foraging behaviour in mice, may probe spatial memory more accurately than the traditional radial arm maze. Consequently, this task may also be more sensitive in detecting treatment effects.

In brief, the foraging task presents rodents with 8 pots in different locations within an open field arena. Each pot is baited with food reward at the start of the session. Each mouse receives 10 minutes in which to retrieve the food rewards from the pots. Mice are trained to collect food rewards from pots in their home cage prior to testing. Efficient performance requires the mouse to recall the spatial locations of the pots within the maze that it has visited within each session in order to collect food rewards from unvisited pots. A number of measures are taken, such as the time taken to complete the task (collected all 8 rewards) and the number of return errors (a return is defined as when the mouse visits an already depleted pot). As this procedure provides an opportunity for the mouse to access multiple locations within the environment and thus increase the demand placed upon spatial memory it was anticipated that this procedure would be sensitive to the APPswe mutation and provide a means of further characterising the foraging strategies of mutant and WT control mice.

Mice were assessed in the foraging task at 3 and 10.5 months of age. Based on previous literature showing behavioural deficits from 3-4 months of age in the water maze probe trial, circular platform and Y-maze (e.g. Holcomb et al., 1998; King et al., 1999; King & Arendash, 2002), it was hypothesised that Tg2576 mice would show spatial memory impairments in the foraging task when assessed at both 3 and 10.5 months of age. Further supporting this, a rather robust spatial memory deficit has been reported from 6-9 months in the Morris water maze (Hsiao et al., 1996; Westerman et al., 2002) and from 8-10 months in the T-maze test (Chapman et al., 1999; Corcoran et al., 2002; Hale & Good, 2005; Barnes, Hale & Good, 2004).

Based on evidence from experiment 4, it was hypothesised that DHA supplementation would improve foraging task performance in Tg2576 mice at 3 and 10.5 months of age. This was further supported by Calon et al. (2004) which showed amelioration of Morris water maze acquisition (but not retention) deficits by DHA supplementation in Tg2576 mice aged 21 to 22 months. Similarly, Hooijmans et al. (2009) reported DHA supplementation to improve

spatial memory in the Morris water maze probe trial (but not during acquisition) in 15-month old APPswe/PS1 mice. Furthermore, Ma et al. (2009) reported a reduction of Y-maze deficits by DHA supplementation in a 3xTg model. In contrast however, Oksman et al. (2006) reported no effect of DHA supplementation on water maze performance in APPswe/PS1 mice. Supporting this, Arendash et al. (2007) reported omega-3 supplementation did not improve spatial memory performance in the Morris water maze, Y-maze, circular platform and radial arm water maze in the APPswe/PS1 model. Despite these inconsistencies in the APPswe/PS1 model, the prediction that DHA supplementation would reduce transgene-related deficits in Tg2576 mice was maintained based on previous results in Tg2576 mice by Calon et al. (2004) and the results of experiment 4.

4.4.2 Methods

4.4.2.1 Design and subjects

One cohort of male Tg2576 transgenic (Tg) and wildtype (WT) mice were evaluated in the foraging task at two ages following dietary supplementation of DHA or control oil blend. Experimentally naïve mice were first tested at 3 months of age, before the onset of plaque pathology. This cohort was tested again at 10.5 months of age when plaque pathology was developing. When tested at 3 months of age, 11 Tg mice received DHA diet (Tg DHA), 13 Tg mice received the oil blend diet (Tg OB), 18 WT mice received the DHA diet (WT DHA) and 16 WT mice received the oil blend diet (WT OB). By the second stage of experimentation at 10.5 months of age, the attrition of 8 mice resulted in group numbers of 7 Tg DHA, 10 Tg OB, 15 WT DHA and 16 WT OB. The running order of mice was counterbalanced so that approximate equal numbers of each experimental group of mice were run in each batch, with several batches of mice being tested once per day over 11 consecutive days.

4.4.2.2 Apparatus

The foraging task was carried out in an open field wooden arena ($102cm^2$ x 43cm tall), filled with a layer of sawdust 3cm deep and containing eight white ceramic pots in a 3 x 2 x 3 arrangement (Figure 4.13). Each pot was filled with sawdust and baited with a food reward hidden in the middle of the sawdust. Trial testing with another mouse cohort demonstrated

mice responded well to the food reward which was half a grain of Kellogg's® Cocopops. The arena was situated in a quiet, illuminated room surrounded by numerous distinct visual cues such as posters and shelving on the walls, benching and air conditioning ducts. A camera was attached to the ceiling directly above the arena to allow viewing and recording for documentation using a monitor and VCR. Experimental measures were recorded manually by the experimenter using a notepad.

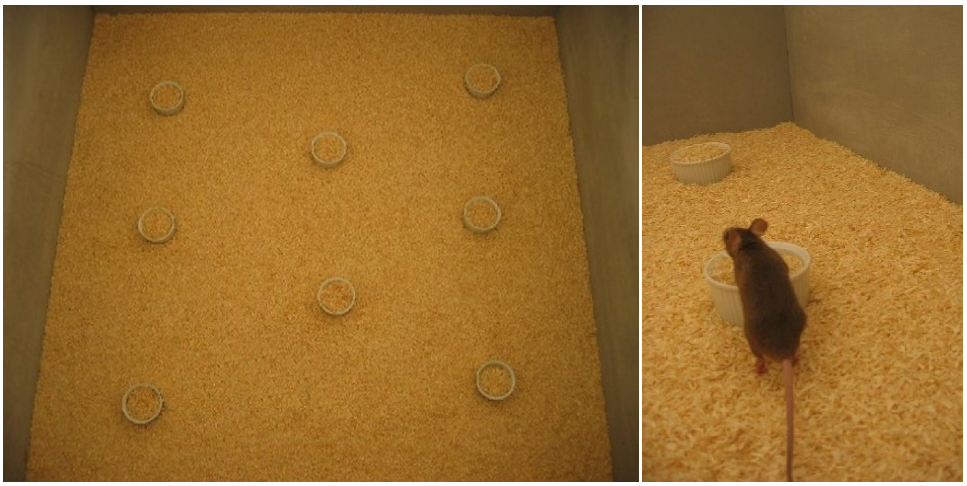

Figure 4.13 Foraging task apparatus showing pot arrangement within the open field arena, and a mouse attending a foraging pot during the task.

4.4.2.3 Procedure

Mice were placed on a food restriction regime throughout this experiment in order to motivate them to consume the food reward. Here, mice received a restricted amount of food per day varying between 2 to 5g of experimental mouse diet, provided approximately 2 hours after the experiment. The amount of food provided was individually calculated per mouse depending on their body weight profile. Each mouse was checked daily for general health and weighed to ensure their body weight was maintained above 85 to 90% of their *ad lib* weight. The experimenter remained in the room throughout all sessions and maintained their location out of sight. Mice were transported to the test room in individual home cages and habituated to the room for 10 minutes prior to each habituation and test session.

The foraging task consisted of an 8-day habituation and training period, followed by a 3-day testing period. The first 3 days of habituation and training involved 3 repeated exposures to a

baited pot in their home-cage to encourage foraging behaviour in the pot and the association of a food reward. Once mice successfully foraged within the pots, they received a 10-minute habituation session to the foraging arena without pots on habituation day 4. On habituation days 5 to 8, mice received a 10-minute habituation and training session in the arena containing 2 baited pots to encourage foraging in the pots. If the food reward was not consumed within 10 minutes on each day, mice received one extra session per day to ensure that all mice successfully foraged from both pots within 10 minutes. Between sessions, the sawdust layer in the arena was mixed and excrement removed to eliminate odour trails.

The 3-day test was conducted over consecutive days immediately following the habituation period. During each testing day, mice were given a maximum of 10 minutes to freely roam the arena containing 8 pots that were baited with half a grain of Kellogg's® Cocopops buried in the middle of the sawdust to 2/3^{rds} of the pot depth. Mice were removed from the arena after 10 minutes or once all 8 food rewards were successfully consumed. The experimenter recorded mouse activity in the arena including which pots in turn had foraging visits and when food rewards from each pot were consumed. A foraging visit was defined as when a mouse actively manoeuvred sawdust within the pot using its head or paws in order to search for food. The measures of behaviour included the total number of pots successfully foraged (i.e. the number of food rewards gained), the number of consecutive pots successfully foraged, the total number of return errors made (revisiting a pot after depleting the food reward), the number of consecutive return errors made, and the number of foraging errors made (visiting a baited pot without reward consumption). The time taken to complete the task was also recorded.

4.4.2.4 Data analysis

A separate ANOVA analysed the data at 3 and 10.5 months independently. Statistical analyses on the foraging task measures were carried out using an ANOVA with genotype (Tg, WT) and diet (DHA, OB) as between-subject factors, and day (1-3) as a within-subject factor. Tests of simple main effects were carried out following significant interactions.

4.4.3 Results

4.4.3.1 Foraging task: 3 months of age

Time taken to complete the foraging task

Figure 4.14 shows the mean time taken to complete the foraging task for both Tg and WT mice fed the DHA or oil blend diet at 3 months of age during days 1 to 3. The graph shows that Tg mice took more time to complete the task than WT mice, and the DHA diet generally failed to alter performance in both Tg and WT mice. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,54)=38.967$, $p<0.001$), no significant main effect of diet ($F(1,54)=2.358$, $p>0.05$) and no interaction between these factors ($F(1,54)=0.532$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,108)=12.137$, $p<0.001$), no significant interaction of day by genotype ($F(2,108)=0.236$, $p>0.05$), no significant interaction of day by diet ($F(2,108)=1.423$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=0.878$, $p>0.05$).

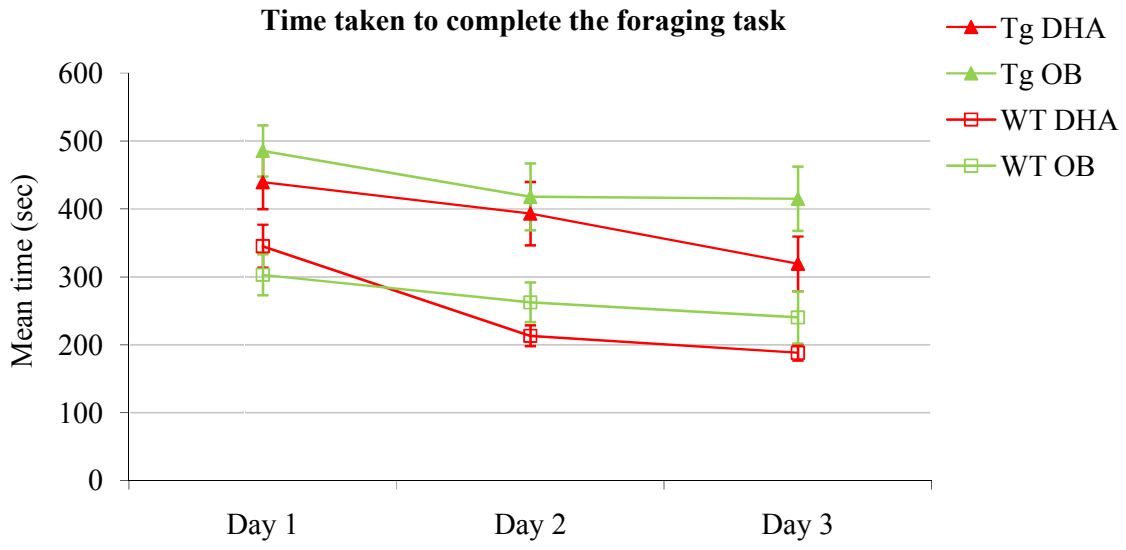

Figure 4.14 Mean time taken to complete the foraging task at 3 months of age (total time = 600 seconds). Values are mean scores ± S.E.M.

Number of pots successfully foraged (number of rewards gained)

The mean number of rewards gained in the foraging task across testing for both Tg and WT mice fed the DHA or oil blend diet at 3 months of age is shown in Figure 4.15. The graph

shows that Tg mice gained fewer rewards than WT mice, which successfully foraged from all pots to receive all 8 rewards. DHA supplementation appeared to improve Tg performance slightly, with no impact in WT mice. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,54)=15.406$, $p<0.001$), no significant main effect of diet ($F(1,54)=1.152$, $p>0.05$) and no interaction between these factors ($F(1,54)=0.1.152$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,108)=5.201$, $p<0.01$), a significant interaction of day by genotype ($F(2,108)=5.201$, $p<0.01$), no significant interaction of day by diet ($F(2,108)=2.101$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=2.101$, $p>0.05$). A follow-up test of simple main effects for the significant day by genotype interaction revealed Tg mice performed significantly worse than WT mice on days 1 and 2 (smallest, $F(1,56)=8.64$, $p<0.01$).

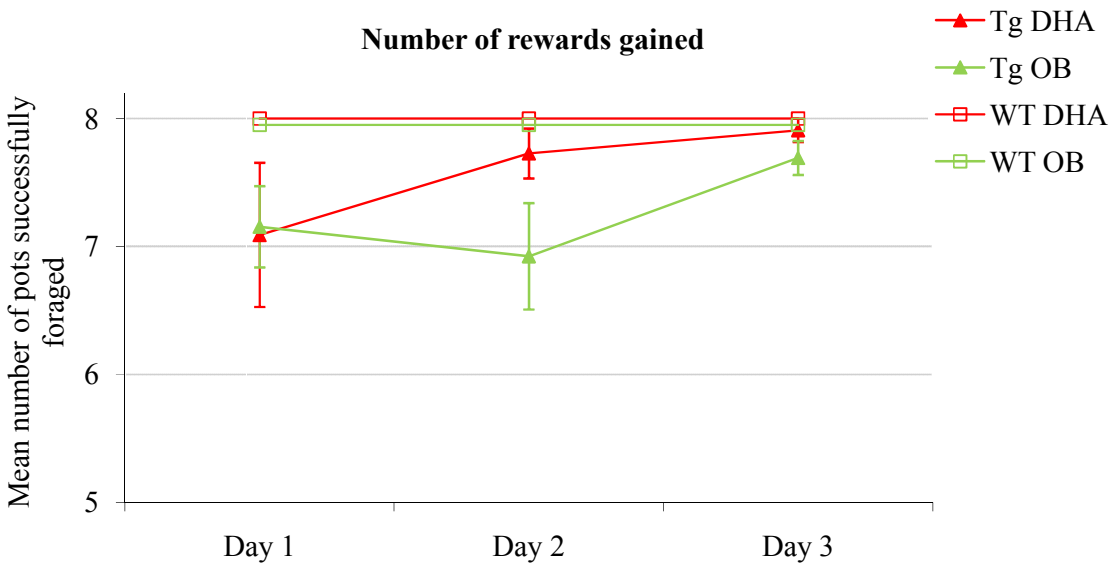

Figure 4.15 Mean number of rewards gained (number of pots successfully foraged) in the foraging task at 3 months of age (total time = 600 seconds). Values are mean scores ± S.E.M. S.E.M = 0 for WT groups.

Number of consecutive pots successfully foraged (number of consecutive rewards gained)

The mean number of consecutive pots successfully foraged by experimental groups during days 1 to 3 of the foraging task at 3 months of age is shown in Figure 4.16. The graph depicts that all mice made a similar number of errors across days, although 1-month DHA supplementation appeared to improve performance in Tg but not WT mice. An ANOVA with

day, genotype and diet as factors revealed no significant effect of genotype ($F(1,54)=0.837$, $p>0.05$), no significant effect of diet ($F(1,54)=2.188$, $p>0.05$), but a interaction between these factors ($F(1,54)=4.238$, $p<0.05$). Following-up this interaction, a test of simple main effects revealed Tg OB mice foraged from significantly less pots consecutively than WT OB mice ($F(1,54)=4.11$, $p<0.05$) and Tg DHA mice ($F(1,54)=5.63$, $p<0.05$). Statistical analysis also showed a significant effect of day ($F(2,108)=12.401$, $p<0.001$), no significant interaction of day by genotype ($F(2,108)=0.904$, $p>0.05$), no significant interaction of day by diet ($F(2,108)=1.925$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=0.595$, $p>0.05$).

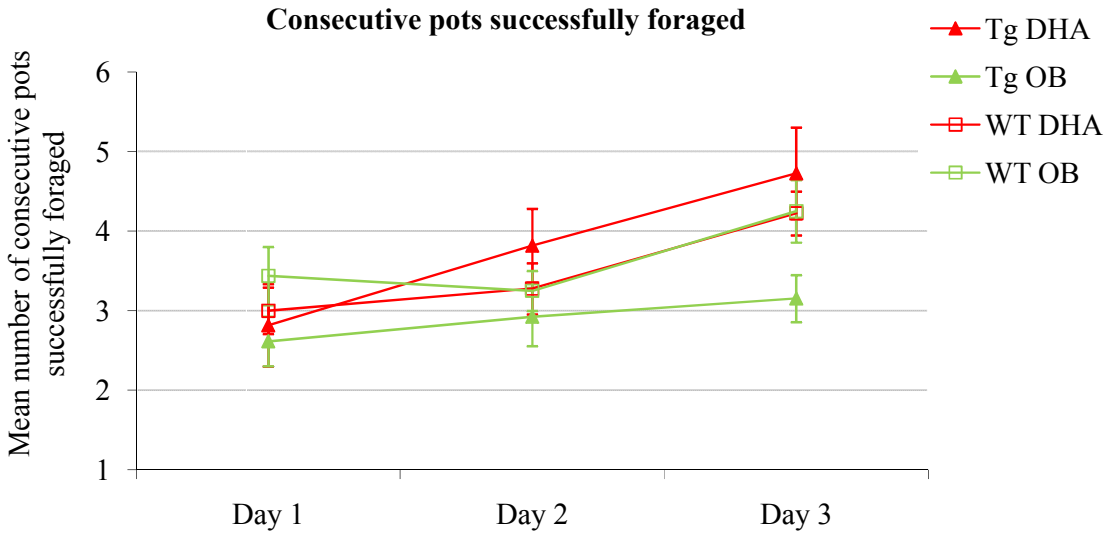

Figure 4.16 Mean number of consecutive pots successfully foraged in the foraging task at 3 months of age. Values are mean scores ± S.E.M.

Total number of foraging errors made

Figure 4.17 shows the mean number of total foraging errors made by experimental groups across training at 3 months of age. An ANOVA with day, genotype and diet as factors revealed no significant effect of genotype ($F(1,54)=1.199$, $p>0.05$), diet ($F(1,54)=0.115$, $p>0.05$) or interaction between these factors ($F(1,54)=2.714$, $p>0.05$). As illustrated in Figure 4.17, these results indicate that Tg and WT mice performed equally well with no significant beneficial effect of 1-month DHA supplementation compared to oil blend diet. Statistical analysis also showed no significant effect of day ($F(2,108)=0.406$, $p>0.05$), no significant interaction of day by genotype ($F(2,108)=0.011$, $p>0.05$), a significant interaction of day by

diet ($F(2,108)=3.482$, $p<0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=1.486$, $p>0.05$). A follow-up test of simple main effects for the significant day by diet interaction revealed no effect of diet on days 1, 2 or 3 (smallest, $F(1,56)=2.79$, $p>0.05$).

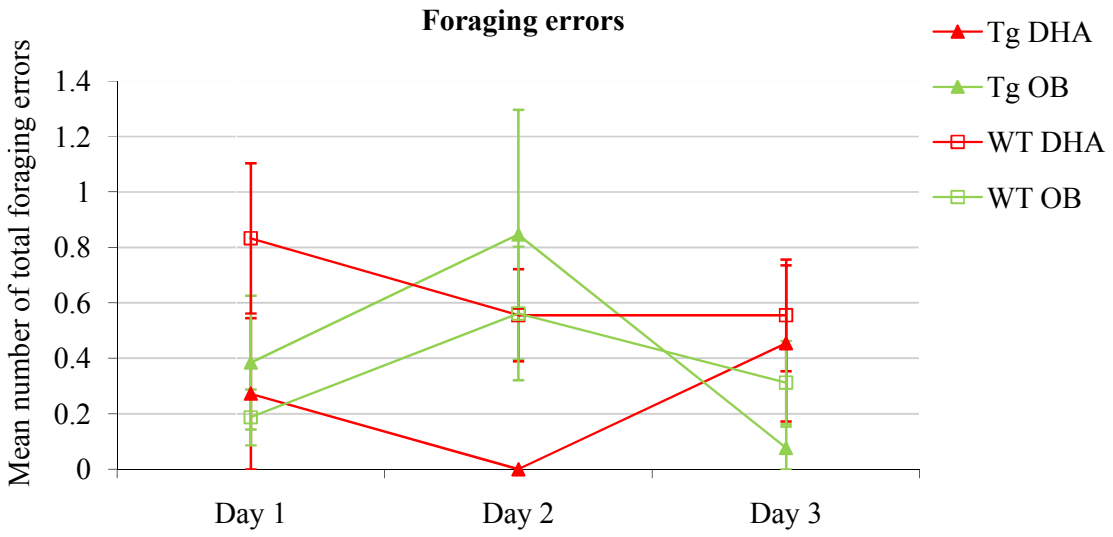

Figure 4.17 Mean number of total foraging errors made in the foraging task at 3 months of age. Values are mean scores ± S.E.M.

Total number of return errors made

Figure 4.18 shows the mean number of total return errors made by experimental during days 1 to 3 of the foraging task at 3 months of age. The graph shows that Tg and WT mice performed similarly, although Tg OB mice made more return errors than all groups. Furthermore, the results indicate that DHA supplementation appeared to benefit performance. An ANOVA with day, genotype and diet as factors revealed no significant effect of genotype ($F(1,54)=3.341$, $p>0.05$), a significant effect of diet ($F(1,54)=4.214$, $p<0.05$) and no significant interaction between these factors ($F(1,54)=0.620$, $p>0.05$). Statistical analysis also showed no significant effect of day ($F(2,108)=1.059$, $p>0.05$), no significant interaction of day by genotype ($F(2,108)=0.602$, $p>0.05$), no significant interaction of day by diet ($F(2,108)=0.180$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=0.744$, $p>0.05$).

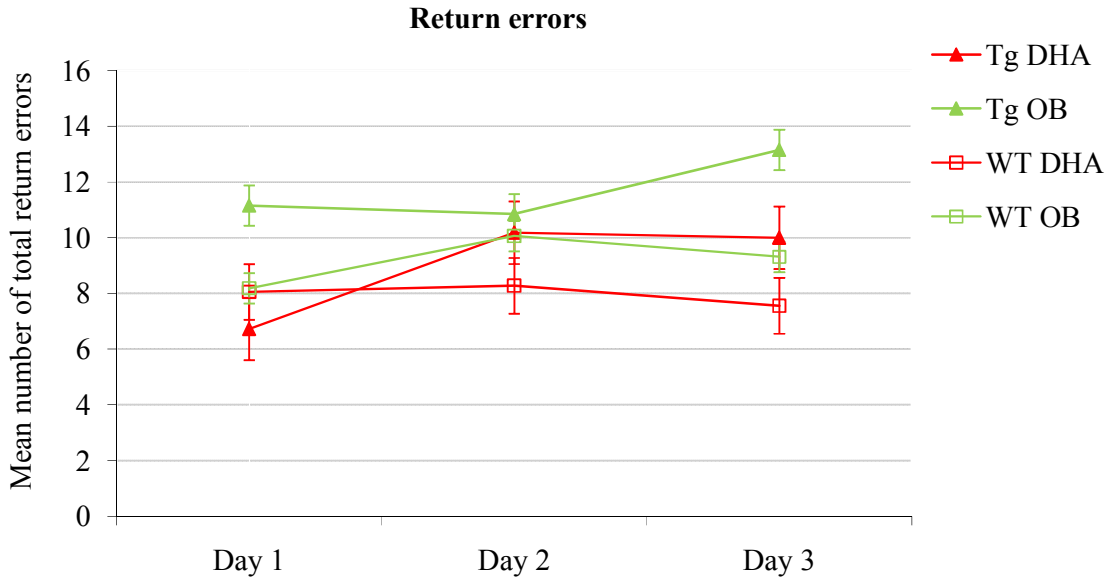

Figure 4.18 Mean number of total return errors made in the foraging task at 3 months of age. Values are mean scores ± S.E.M.

Number of consecutive return errors (2+) made

The mean number of consecutive return errors (2+) made by experimental groups during days 1 to 3 of the foraging task at 3 months of age is shown in Figure 4.19. The figure shows that Tg mice, particularly Tg OB, made more errors than WT mice. DHA supplementation also appeared to improve performance across days, particularly in Tg mice. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,54)=7.449$, $p<0.01$), a significant effect of diet ($F(1,54)=4.817$, $p<0.05$), but no significant interaction between these factors ($F(1,54)=2.414$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,108)=3.668$, $p<0.05$), no significant interaction of day by genotype ($F(2,108)=0.100$, $p>0.05$), no significant interaction of day by diet ($F(2,108)=0.410$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,108)=0.618$, $p>0.05$). The effect of day is clearly demonstrated in Figure 4.19, whereby errors decline over days.

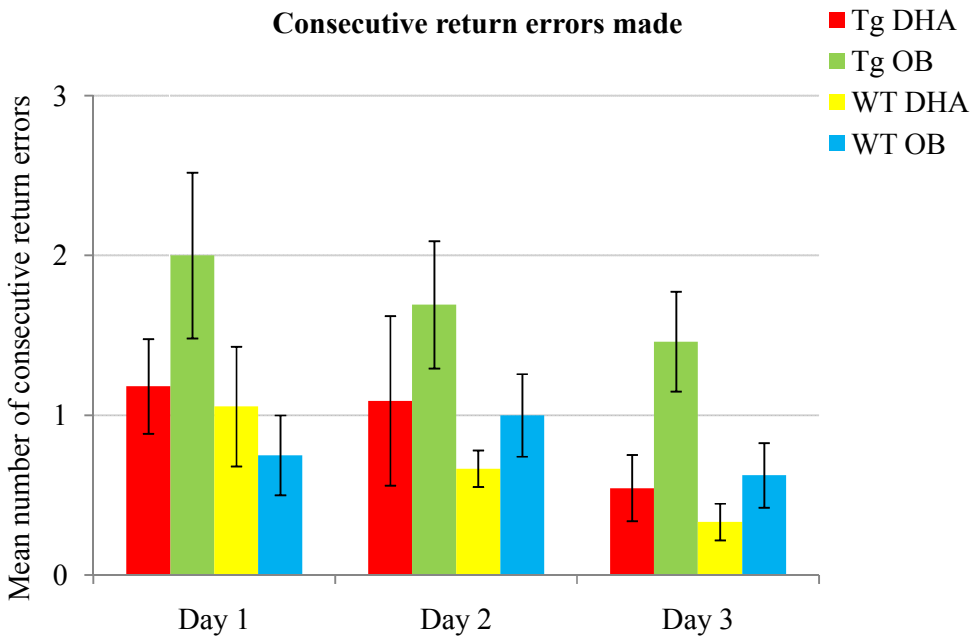

Figure 4.19 Mean number of consecutive return errors (2+) made in the foraging task at 3 months of age. Values are mean scores ± S.E.M.

4.4.3.2 Foraging task: 10.5 months of age

Time taken to complete the foraging task

Figure 4.20 shows the mean time taken to complete the foraging task for both Tg and WT mice fed the DHA or oil blend diet at 10.5 months of age during days 1 to 3. The graph illustrates that Tg mice took more time to complete the task than WT mice. Figure 4.20 also shows that DHA failed to alter performance in WT mice, but appeared to improve performance in Tg mice on days 2 and 3. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,44)=23.319$, $p<0.001$), no significant main effect of diet ($F(1,44)=1.105$, $p>0.05$) and no interaction between these factors ($F(1,44)=0.629$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,88)=42.888$, $p<0.001$), no significant interaction of day by genotype ($F(2,88)=1.780$, $p>0.05$), no significant interaction of day by diet ($F(2,88)=0.611$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,88)=1.833$, $p>0.05$). The difference between Tg groups is therefore not supported statistically.

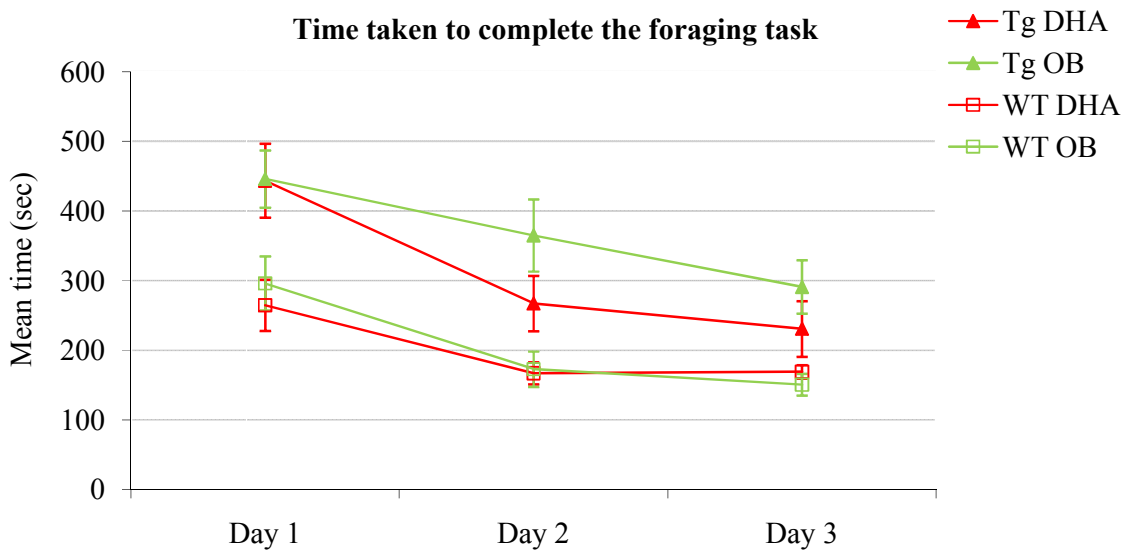

Figure 4.20 Mean time taken to complete the foraging task at 10.5 months of age (total time = 600 seconds). Values are mean scores ± S.E.M.

Number of pots successfully foraged (number of rewards gained)

Figure 4.21 shows the mean number of rewards gained in the foraging task for both Tg and WT mice fed the DHA or oil blend diet at 10.5 months of age. Overall, all mouse groups successfully foraged from the majority of pots to receive most rewards. An ANOVA with day, genotype and diet as factors revealed no significant effect of genotype ($F(1,44)=0.211$, $p>0.05$), no significant main effect of diet ($F(1,44)=1.042$, $p>0.05$) and no interaction between these factors ($F(1,44)=0.778$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,88)=4.833$, $p<0.01$), no significant interaction of day by genotype ($F(2,88)=2.286$, $p>0.05$), no significant interaction of day by diet ($F(2,88)=1.287$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,88)=1.515$, $p>0.05$).

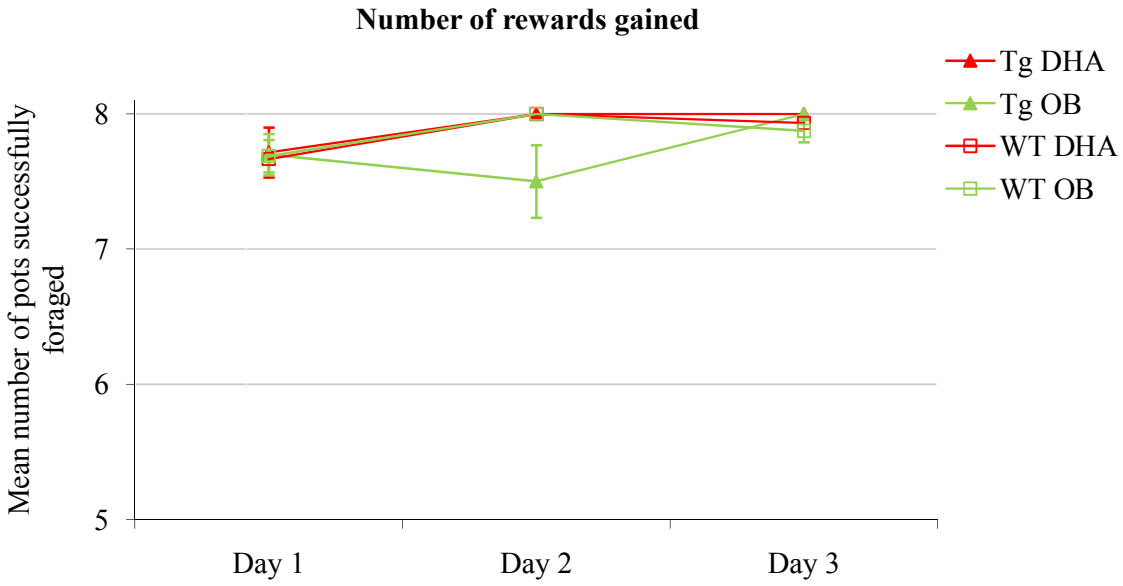

Figure 4.21 Mean number of rewards gained (number of pots successfully foraged) in the foraging task at 10.5 months of age (total time = 600 seconds). Values are mean scores ± S.E.M.

Number of consecutive pots successfully foraged (number of consecutive rewards gained)

Figure 4.22 depicts the mean number of consecutive pots successfully foraged by experimental groups across testing at 10.5 months of age. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,44)=4.372$, $p<0.05$), no significant effect of diet ($F(1,44)=1.158$, $p>0.05$) and no interaction between these factors ($F(1,44)=0.491$, $p>0.05$). As illustrated in Figure 4.22, these results show that Tg mice made less consecutive successful visits to pots than WT mice, and that diet failed to alter performance. Statistical analysis also showed a significant effect of day ($F(2,88)=7.991$, $p<0.001$), no significant interaction of day by genotype ($F(2,88)=0.265$, $p>0.05$), no significant interaction of day by diet ($F(2,88)=1.847$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,88)=2.366$, $p>0.05$). Unfortunately, the numerical difference between Tg groups on day 3, where DHA supplementation appears to improve Tg performance, is not supported by a significant interaction.

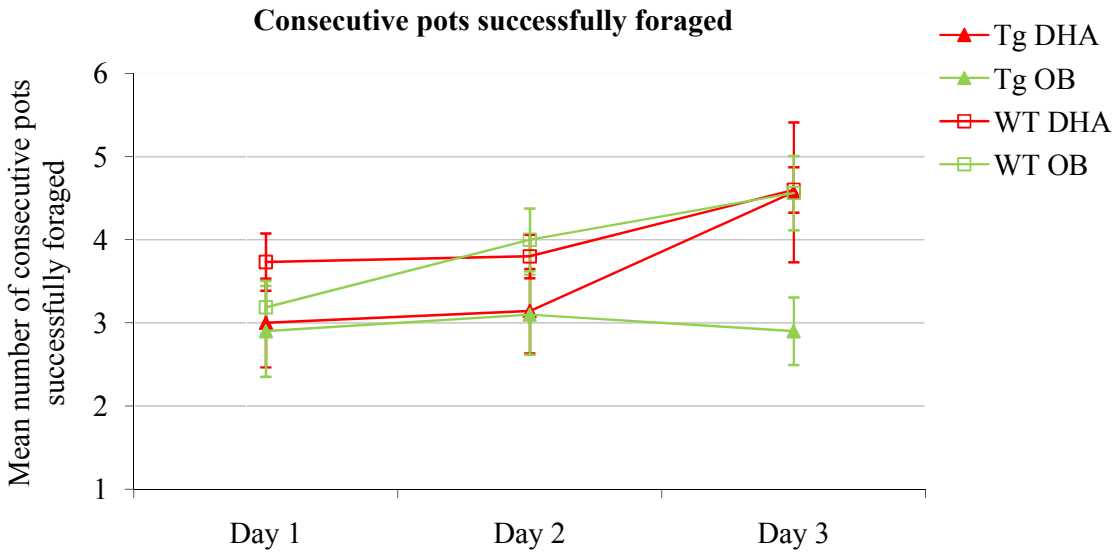

Figure 4.22 Mean number of consecutive pots successfully foraged in the foraging task at 10.5 months of age. Values are mean scores ± S.E.M.

Total number of foraging errors made

Figure 4.23 shows the mean number of total foraging errors made by experimental groups across days of the foraging task at 10.5 months of age. Tg and WT mice appear to perform equally well, with no effect of DHA supplementation compared to the oil blend diet. However, Tg mice fed the DHA diet appear to have made more errors than all other groups on day 1. An ANOVA with day, genotype and diet as factors revealed no significant effect of genotype ($F(1,44)=1.064$, $p>0.05$), diet ($F(1,44)=1.897$, $p>0.05$) or interaction between these factors ($F(1,44)=1.919$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,88)=8.054$, $p<0.001$), no significant interaction of day by genotype ($F(2,88)=1.149$, $p>0.05$), no significant interaction of day by diet ($F(2,88)=1.587$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,88)=2.672$, $p>0.05$).

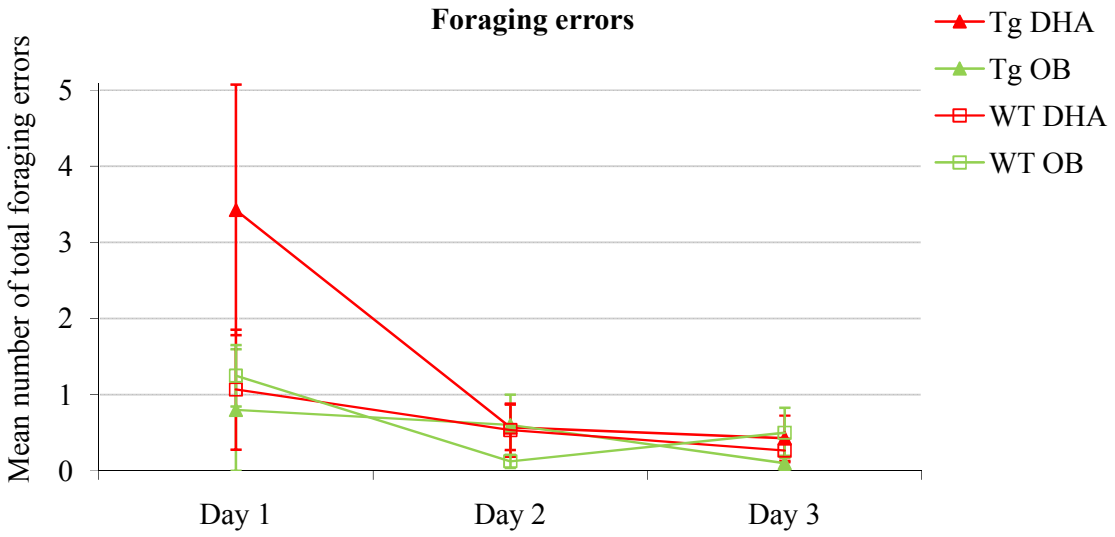

Figure 4.23 Mean number of total foraging errors made in the foraging task at 10.5 months of age. Values are mean scores ± S.E.M.

Total number of return errors made

Figure 4.24 illustrates the mean number of total return errors made for both Tg and WT mice fed the DHA or oil blend diet during days 1 to 3 of the foraging task at 10.5 months of age. The results demonstrate that Tg mice (particularly the Tg OB group) made more return errors than WT mice. The DHA diet failed to alter performance in WT mice, but appeared to improve performance across days in Tg mice. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,44)=13.817$, $p<0.001$), no significant main effect of diet ($F(1,44)=1.139$, $p>0.05$) and no significant interaction between these factors ($F(1,44)=0.604$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,88)=7.747$, $p<0.001$), no significant interaction of day by genotype ($F(2,88)=2.076$, $p>0.05$), and no significant interaction of day by diet ($F(2,88)=1.998$, $p>0.05$), but a significant three-way interaction of day by genotype by diet ($F(2,88)=5.097$, $p<0.01$). A follow-up test of simple interactions revealed a significant genotype by diet interaction on day 2 ($F(1,44)=5.39$, $p<0.05$). To follow up this interaction on day 2, a test of simple main effects revealed Tg OB mice made significantly more return errors than WT OB mice ($F(1,44)=24.91$, $p<0.001$), WT DHA mice ($F(1,44)=10.91$, $p<0.01$) and Tg DHA mice ($F(1,44)=7.94$, $p<0.01$). These results therefore show that DHA supplementation reduced the return errors deficit in Tg mice.

Figure 4.24 Mean number of total return errors made in the foraging task at 10.5 months of age. Values are mean scores ± S.E.M.

Number of consecutive return errors (2+) made

Figure 4.25 shows the mean number of consecutive return errors (2+) made by experimental groups during days 1 to 3 of the foraging task at 10.5 months of age. The figure shows that Tg mice, particularly Tg OB, made more errors than WT mice across days, and that DHA supplementation appeared to reduce the number of errors made, particularly in Tg mice. An ANOVA with day, genotype and diet as factors revealed a significant effect of genotype ($F(1,44)=15.995$, $p<0.001$), a significant main effect of diet ($F(1,44)=4.678$, $p<0.05$) and no significant interaction between these factors ($F(1,44)=1.931$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(2,88)=17.396$, $p<0.001$), a significant interaction of day by genotype ($F(2,88)=3.712$, $p<0.05$), no significant interaction of day by diet ($F(2,88)=1.667$, $p>0.05$) and no significant three-way interaction of day by genotype by diet ($F(2,88)=0.933$, $p>0.05$). A test of simple main effects revealed a significant effect of genotype on days 1, 2 and 3 (smallest, $F(1,46)=8.37$, $p<0.01$).

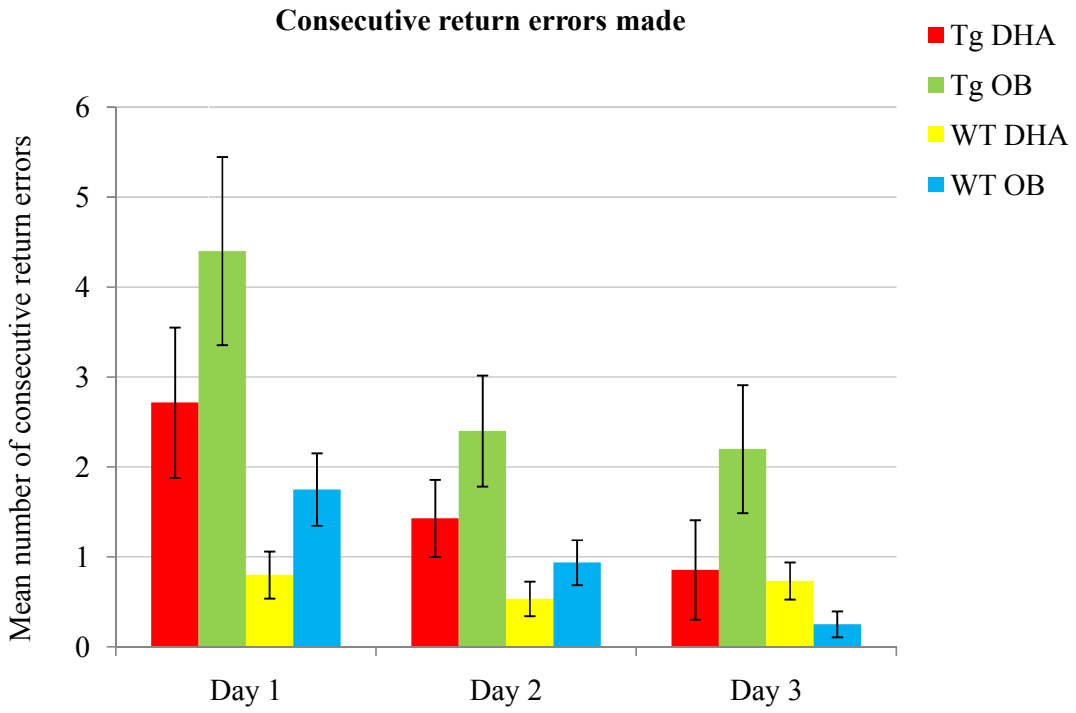

Figure 4.25 Mean number of consecutive return errors (2+) made in the foraging task at 10.5 months of age. Values are mean scores ± S.E.M.

4.4.4 Discussion

The results of the foraging task showed a significant effect of genotype at both at 3 and 10.5 months of age on a number of measures; whereby Tg mice took significantly longer to complete the task, made less consecutive successful visits to pots and made more consecutive return errors (2+). At 3 months of age, Tg mice also showed a transgene-related deficit by gaining less rewards than WT mice. In contrast, this was not shown at 10.5 months of age, although Tg mice made more return errors than WT mice at 10.5 months but not 3 months. Together, these measures suggest that Tg mice showed a spatial memory impairment, and the return error deficit may indicate perseverative or disinhibitory behaviour. In contrast to these transgene-related deficits, Tg mice made a similar number of foraging errors (whereby mice visited a pot without consuming the food reward) as WT mice at both 3 and 10.5 months of age. Importantly, this leads to the conclusion that Tg mice could forage as efficiently as WT

mice. This suggests that the observed deficits were not related to a general inability to forage successfully for food rewards.

This study therefore provides evidence that the APPswe mutation leads to a spatial memory deficit in Tg2576 mice as early as 3 months of age. This is consistent with previous reports of spatial memory deficits at 3 months of age in the Y-maze spontaneous alternation task and in a water maze task (Holcomb et al., 1998; King et al., 1999; King & Arendash, 2002). In contrast, other studies have not reported spatial memory and learning deficits around this age point in the Morris water maze and other water-maze paradigms (Hsiao et al., 1996; King et al., 1999; King & Arendash, 2002). It could be argued however that this lack of deficits may be specific to reference spatial memory, as opposed to the working spatial memory measured in this task. In contrast to this however, Tg2576 mice were not impaired at 3 months of age in tasks probing working spatial memory including the paddling Y-maze and Y-maze spontaneous alternation task (Hsiao et al., 1996; King et al., 1999; Deacon et al., 2008). However it could be argued that these Y-maze tasks, which have limited complexity by offering a choice of only two spatial locations (left and right arms), may not be sensitive enough to detect small but significant changes in spatial memory. Further investigation of working spatial memory using more putatively complex tasks is therefore required to understand the onset of transgene-related deficits in Tg2576 mice.

The spatial memory deficit reported in Tg mice at 10.5 months of age in the foraging task was consistent with a number of studies demonstrating spatial memory deficits from around 9 months of age in this model (e.g. Hsiao et al., 1996). For example, a reference spatial memory deficit in the Morris water maze and paddling Y-maze has been reported in Tg2576 mice from between 6 and 9 months of age (Hsiao et al., 1996; Westerman et al., 2002; Deacon et al., 2008). Similarly, progressive working spatial memory deficits have generally been reported in the T-maze FCA task from 8 months of age in Tg2576 mice (Chapman et al., 1999, Corcoran et al., 2002; Barnes, Hale & Good, 2004; Hale & Good, 2005). Together, the results of the foraging task at 3 and 10.5 months suggest that the spatial memory deficits in Tg mice is not necessarily associated with Aβ pathology as deficits were observed prior to Aβ pathological development. Instead, the observed deficit may be related to early synaptic deficits caused by the APPswe mutation (e.g. Jacobsen et al., 2006). The progressive worsening of the deficit by 10.5 months of age (as shown by a return errors deficit not observed at 3 months) may instead be associated with Aβ pathology.

Although the transgene deficits reported in this task suggest impaired spatial memory, it is important to consider alternative explanations for the deficits such as sensorimotor impairments including visual ability (as discussed in experiment 1), hyperactivity and digging deficits. Measures of hyperactivity in Tg2576 mice have been supported between 3 and 23 months in the open field task, although these results are inconsistent (Chapman et al., 1999; King et al., 1999; King & Arendash, 2002; Deacon et al., 2008, 2009; Lalonde et al., 2003). Hyperactivity could lead to an increase in pot visits, perhaps resulting in an increase in foraging or return errors. Conversely however, it could also be argued that hyperactivity in Tg mice could facilitate performance in the foraging task, such as completing the task in a quicker time. It is therefore thought that the deficits in the foraging task was unlikely a consequence of simple hyperactivity, but rather, it may exaggerate performance depending on spatial memory status. An additional factor that should also be considered is whether a simple digging impairment could account for foraging task deficits. Indeed, Deacon et al. (2006, 2009) reported a digging and burrowing impairment in Tg2576 mice aged 22 to 23 months assessed in several tasks. However, it was also reported that this impairment did not affect their ability to find and dig up hidden food pellets. Although these results cannot lead us to rule out the involvement of digging impairments, the fact that the deficit appeared to reflect a preseverative error strategy would suggest a more selective cognitive impairment.

Another important consideration is the potential use of olfactory cues by mice to solve the task. For example, mice could use olfactory trails such as urine to track its activity within the arena to guide responding to unvisited pots. Unfortunately this could not be controlled in this task, unlike tasks such as the T-maze. It is important to consider whether an olfactory impairment (rather than a spatial memory impairment) led to the observed transgene-deficit. In support of a spatial memory deficit, Deacon et al. (2009) ruled out a general olfactory impairment in Tg2576 mice aged 22 to 23 months, whereby Tg mice could find a hidden food pellet under woodchip bedding as easily as WT controls. In fact, they reported that Tg mice showed enhanced performance compared to controls, although this was not statistically significant. It was therefore concluded that an olfactory impairment was unlikely responsible for the foraging task deficit in Tg2576 mice. Another possibility is that Tg2576 mice failed to habituate to the arena as well as control mice. However, all mice reached the habituation criteria of successfully foraging within 2 pots in the foraging arena within a 5-minute period and thus this seems an unlikely factor underpinning the deficits in Tg2576 mice on this task.

The foraging task experiment showed several significant main effects of diet or genotype by diet interactions in mice aged 3 and 10.5 months age, suggesting that DHA supplementation ameliorated some performance deficits in Tg and WT mice. Improvements were restricted to the number of total and consecutive return errors made, and the number of consecutive rewards gained. At 3 months, significant diet effects were shown for the number of total and consecutive return errors made, with a selective improvement in Tg performance by DHA in the number of consecutive rewards gained. By 10 months, the results were less convincing, with positive effects of DHA shown only in the number of consecutive return errors measure, and positive effects of DHA selectively in Tg mice for the number of total return errors measure. A trend showed Tg DHA mice gained more consecutive rewards than Tg OB mice, although this was not supported statistically at 10.5 months. In contrast to these positive performance effects of DHA supplementation, no effects were found in several measures including the time taken to complete the task, the number of rewards gained, and the number of foraging errors. Overall however, these results provide some evidence that DHA improved spatial memory in Tg and WT mice, particularly at 3 months of age, with some selective improvements in Tg mice only. This would suggest that DHA can improve general cognitive function, as well as transgene-related deficits in Tg mice. These results are the first of its kind to provide evidence suggesting positive effects of DHA supplementation in reducing spatial memory deficits in Tg2576 mice at an early age relative to a suitable baseline diet.

These results are supported by the potent effects described by Calon et al. (2004) which reported DHA supplementation to reduce spatial memory and learning deficits in the Morris water maze in 21-22 month old Tg2576 mice, relative to a diet depleted in DHA with an excessive ω-6/ω-3 ratio. It is argued that the relative strength of the diet effects reported by Calon et al. (2004) were a likely consequence of the high levels of omega-6 PUFA and excessive ω-6/ω-3 ratio in their control diet, rather than the addition of DHA *per se*. Similar arguments could be made regarding positive effects of DHA supplementation in reports of other AD models (Hooijmans et al., 2009; Ma et al., 2009; Arsenault et al., 2011). For example, Hooijmans et al. (2009) failed to accurately examine DHA supplementation as the standard control diet was composed of almost double the amount of saturated fatty acids and the DHA diet contained supplementary levels of omega-3 PUFA which were not attributable to DHA. Similarly, the DHA diet in Arsenault et al. (2011) study contained supplementary EPA and half the amount of omega-6 PUFA relative to the control diet. Finally, Ma et al. (2009) examined the effect of fish oil supplementation containing DHA rather than the sole

effect of DHA, and was compared to a poor diet high in saturated fatty acids and omega-6 PUFAs.

4.5 Experiment 6: Biconditional discrimination training, Stroop interference test and reversal learning

4.5.1 Introduction

A plethora of evidence has shown the Tg2576 model to display a rather specific cognitive deficit in spatial, episodic and emotional memory primarily related to Aβ pathology in structures within the medial temporal lobe such as the hippocampus and amygdala (e.g. Hsiao et al., 1996; King & Arendash, 2002; Good, Hale & Staal, 2007). In contrast, cognitive function associated with the prefrontal cortex (PFC) is not well studied in the Tg2576 model. Interestingly, the results of the foraging task experiment revealed that Tg2576 mice displayed perseverative-like behaviour, which has been related to PFC damage (Barcelo et al., 1997; Munakata, Morton & Stedron, 2003; Carli et al., 2006). This therefore indicated that PFC-function may be disrupted in Tg2576 mice, and warranted further investigation, perhaps through the examination of executive function. Executive function is a common umbrella term used for a number of complex cognitive processes including planning, inhibition, cognitive flexibility, response-control and goal-directed behaviour (Binetti et al., 1996; Stauss & Knight, 2002; Swanberg et al., 2004; Schillerstrom, Horton & Royall, 2005). These skills underlie the ability to perform attentional, discrimination and reversal learning tasks. Such activities are dependent upon the integrity of the frontal cortex (as reviewed in Duke & Kasznick, 2000), although the more basic stimulus-response learning is also controlled by motor areas and the striatum (Spencer & Murphy, 2000; Partridge et al., 2002; Boettiger & D'Esposito, 2005).

It was also deemed important to assess executive function in Tg2576 mice and the effect of dietary intervention, since cognitive deficits related to the frontal lobe have been widely reported in human AD patients. Such deficits include executive dysfunction and attentional impairments (Collette, Van der Linden & Salmon, 1999; Perry & Hodges, 1999; Baddeley et al., 2001; Foldi, Lobosco & Schaefer, 2002; Swanberg et al., 2004; Baudic et al., 2006; Waltz et al., 2004). For example, Swanberg et al. (2004) reported that executive dysfunction was present in 64% of human AD patients. Furthermore, frontal lobe dysfunction has been

reported at early stages of disease development (e.g. Binetti et al., 1996; Sgaramella et al., 2001). Interestingly, cognitive function supported by cortical regions has not been exhaustively studied in the Tg2576 model, despite the age-related increase in Aβ pathology in the cortex. For example, Zhuo et al. (2008) reported that the prefrontal cortex (PFC) contained higher levels of Aβ42 compared to the hippocampus at both 3 and 6 months of age, indicating that this brain region may be highly vulnerable to the effects of amyloidosis and may suffer a greater functional impairment.

Consistently, some executive dysfunction has been reported in Tg2576 mice. Zhuo et al. (2007, 2008) reported reversal learning deficits in an attentional set-shifting test at 6 months of age, which corresponded to a three-fold increase in Aβ42 in the PFC compared with 3 months of age when no cognitive impairment was evident. In particular, Tg2576 mice were unable to successfully acquire a reversed stimulus-reward association which involved suppressing previously relevant rules and replacing them with new rules. Reversal learning is highly dependent upon PFC integrity, as demonstrated by lesion studies and frontal dementia patients (e.g. Duke & Kaszniak, 2000; Schoenbaum et al., 2002; McAlonan & Brown, 2003; Hornak et al., 2004). Reversal learning deficits in Tg2576 mice were also reported by Pompl et al. (1999) in the circular platform task at 7 months. Overall, this data suggests that employment of sparsely used PFC-dependant behavioural tasks could be an alternative tool for detecting early cognitive changes caused by Aβ-related pathology and probing the effectiveness of therapeutic strategies, such as dietary intervention. The first aim of this experiment was therefore to investigate PFC-dependant executive functioning in the Tg2576 mouse model from an early age at 5.5 to 9 months, when pathological changes have been reported in this region (Hsiao et al., 1996; Kawarabayashi et al., 2001; Zhuo et al., 2008). Secondly, this experiment aimed to investigate the effect of DHA supplementation on executive function in Tg and WT mice.

In order to assess executive function in Tg2576 mice, a mouse adaptation of the biconditional discrimination task and Stroop test in rats developed by Haddon and Killcross (2005) was used which was analogous to the human Stroop test. The human Stroop test (Stroop, 1935) is a common test used to assess executive functioning in AD patients (MacLeod, 1992). The test presents participants with a list of colour-related words (e.g. red, blue) which are coloured congruently (the red word is a red colour) or incongruently (the red word is a green colour), as shown in Table 4.1. Here, the test requires participants to follow one of two instructions:

1) to name the word presented, or 2) to name the colour of the word. Typically, participants take longer to respond to the incongruent presentations which induce different responses than the congruent presentations which induce the same response. This is caused by an 'interference effect' during incongruent presentations, whereby the over-learned response to read the word must be inhibited before the ink colour can be named (MacLeod, 1992).

Table 4.1 Presentations of congruent and incongruent word lists used in the Stroop test (Stroop, 1935). Congruent presentations invoke the same response regardless of instruction (read the word or state the colour of the word), whereas incongruent presentations invoke differential responses and the interference effect results in a reduced response time to state the correct answer for both instructions.

List 1 (Congruent)	List 2 (Incongruent)
Red	Red
Blue	Blue
Green	Green
Yellow	Yellow

Several studies assessing AD patients in the Stroop test have shown patients have difficulty in inhibiting these automatic competing responses during incongruent presentations, resulting in stronger Stroop interference effects than age-matched controls (Koss et al., 1984; Perry & Hodges, 1999; Amieva et al., 2002, 2004a,b). However, AD patients can display facilitated performance on congruent trials, possibly due to the lack of interference and lower level of executive functioning required (Spieler et al., 1996). Furthermore, AD patients show impairments in the reverse Stroop test, whereby patients show difficulty in shifting between task instructions (Perry & Hodges, 1999; Amieva et al., 2004b). It is thought that AD patients are impaired as they demonstrate difficulty in shifting attention to suppress previously learnt rules in order to learn new ones.

Haddon and Killcross (2005) developed a rodent specific PFC-dependant task that is analogous to the human Stroop test. In their study, rats were first subject to biconditional discrimination training whereby they received two auditory stimuli in one context (where each stimulus cued either a left or right lever-press response to gain reinforcement) and two visual stimuli in another context (where each stimulus cued either a left or right response to gain reinforcement). Following successful acquisition, rats were then subject to a Stroop

interference test which presented congruent and incongruent audiovisual compounds in each context, which cued either the same or different lever-press response learnt during training, respectively. To solve the response conflict during incongruent compound presentations, rats were required to use contextual cues to guide correct responding. Therefore, if tested in the context that was previously associated with auditory training, the rat should respond to the auditory stimulus in the compound rather than the visual stimulus. The test therefore requires inhibition of the previously learnt response. In relation to the human Stroop test, the audiovisual compounds are comparable to ink colour and words, and the contextual cues are comparable to the task instructions given (Haddon et al., 2008). Rats with medial PFC lesions were impaired in incongruent trials, but not in congruent trials or during biconditional discrimination training (Haddon & Killcross, 2005). Table 4.2 illustrates the basic experimental design used in this procedure.

Table 4.2 Basic experimental design of the Biconditional and Stroop interference test developed by Haddon & Killcross (2005).

Context	**Biconditional discrimination training**	**Stroop interference test**	
		Congruent compound	**Incongruent compound**
Context 1:	A1→R1, A2→R2	A1V1, A2V2→ A/V guide	A1V2, A2V1→ A guide
Context 2:	V1→R1, V2→R2	A1V1, A2V2→ A/V guide	A1V2, A2V1→ V guide

Key: A1 = Auditory stimulus 1; A2 = Auditory stimulus 2; V1 = Visual stimulus 1; V2 = Visual stimulus 2; R1 = Lever-press response 1 (Left); R2 = Lever-press response 2 (Right); A1V1, A2V2, A1V2, A2V1 = Audiovisual compound presentations; A/V guide = responses can be guided by either auditory or visual component of audiovisual compound; A guide = responses guided by auditory component of audiovisual compound; V guide = responses guided by visual component of audiovisual compound.

To investigate PFC-related executive function in the Tg2576 model, mice were tested in a modified version of biconditional discrimination training and the Stroop interference test outlined by Haddon and Killcross (2005). Tg2576 mice were also subject to reversal discrimination training, whereby the stimulus-response association learnt during the initial discrimination training was reversed. Based on previous evidence outlined in Tg2576 mice and AD patients (e.g. Zhuo et al., 2007, 2008; Perry & Hodges, 1999; Amieva et al., 2004b), it was hypothesised that Tg2576 mice would exhibit impaired performance during

incongruent trials in the Stroop test and reversal discrimination training, consistent with PFC dysfunction.

In contrast, it was hypothesised that Tg2576 mice would show intact performance during biconditional discrimination training and congruent test trials, as studies have shown intact or enhanced discrimination acquisition in similar tasks in AD patients (Spieler et al., 1996) and Tg2576 mice. For example, Zhuo et al. (2007) reported Tg2576 mice were able to learn the initial two-choice compound discriminations at 6 and 14 months. Similarly, Barnes, Hale and Good (2004) showed intact performance at 12 to 14 months in an intramaze brightness discrimination, a simple room discrimination, and a contextual biconditional left–right discrimination. These results suggest that acquisition of stimulus-reward associations may not be fully dependant on the PFC and instead dependent upon a region liberated from pathology (Zhuo et al., 2007). Indeed, the dorsomedial striatum has been shown to control executive function (Ragozzino, Jih & Tzavos, 2002; Ragozzino, 2003, 2007; Buckner, 2004; Palencia & Ragozzino, 2004), and the striatum is relatively spared from Aβ pathology in Tg2576 mice (as stated in Zhuo et al., 2008). Furthermore, compensatory morphological improvements in dorsolateral striatal spiny neurons have been reported (Middei et al., 2004). Together, this may explain how some executive function is intact in Tg2576 mice. Supporting this, striatal-dependant active avoidance performance and motor-response learning in the cross maze was intact or enhanced in Tg2576 mice aged 3 to 19 months (King et al., 1999; King & Arendash, 2002; Middei et al., 2004). Although one study has reported a deficit at 22 months in a cued T-maze discrimination task (Deacon et al., 2008), this may suggest that the striatum or its neural connections (such as the afferent corticostriatal connection) may be pathologically-affected during advanced stages.

Although no research has addressed the effect of DHA supplementation on PFC-dependant executive function in Tg2576 mice, it was predicted that DHA would reduce performance deficits based on results showing reduced cognitive deficits by DHA in Tg2576 mice in experiments 4 to 5 and previous research (e.g. Calon et al., 2004).

4.5.2 Methods

4.5.2.1 Design and subjects

One cohort of male Tg2576 transgenic (Tg) and wildtype (WT) mice were evaluated in this task from 5.5 to 9 months of age, during the development of Aβ pathology and prior to plaque onset. The task consisted of three sub-experiments including: Stage 1) biconditional discrimination task training at 5.5 to 7 months of age, Stage 2) the Stroop interference test at 7 months of age, and Stage 3) reversal discrimination training at 7.5 to 9 months of age. This design therefore investigated transgene- and diet-dependant changes in these PFC-dependant tasks.

The cohort of mice used in these experiments consisted of 8 Tg mice which received DHA diet (Tg DHA), 11 Tg mice which received the oil blend diet (Tg OB), 16 WT mice which received the DHA diet (WT DHA) and 15 WT mice which received the oil blend diet (WT OB). In addition to these reported mouse group numbers, 7 mice were excluded from the experiment; 5 died before completing the experiment (3 Tg DHA, 1 WT DHA and 1 WT OB) and 2 failed to respond during initial training (1 Tg OB and 1 WT OB). The running order of mice was counterbalanced so that approximate equal numbers of each experimental group of mice were run in each batch, which were tested twice per day during all experiments once in the morning and once in the afternoon. Mice were run blindly to the experimenter. Mice were maintained on water restriction throughout these experiments (as detailed in the procedure), and gradually reduced to 85 to 90% of their *ad lib* body weights over 7 days prior to the start of the experiment.

4.5.2.2 Apparatus

All three experiments were conducted within 16 identical Med Associates® (St. Albans, Vermont, USA) mouse operant chambers 12cm tall, 15cm wide and 14cm deep. Each chamber (Figure 4.26) was situated within a wooden sound- and light-attenuating box to prevent permittance of external stimuli into the chamber. Each chamber was composed of aluminium with two transparent polycarbonate walls and a ceiling. The front-facing polycarbonate wall was on hinges to function as a door. The floor consisted of 20 stainless steel bars 2.5mm in diameter and spaced 5mm apart, which were situated 4cm above a

removable aluminium box containing wood chippings designed to collect defecation. An additional 12.5cm^2 removable transparent Perspex sheet covered the bar flooring in half of the 16 boxes to create different contextual cues (bar floors = context 1; smooth floors = context 2). The apparatus was located in a quiet room.

Figure 4.26 Mouse operant chamber and set-up used for biconditional discrimination, Stroop task and reversal learning experiments. Photograph depicts Context 2 (smooth flooring) with transparent polycarbonate sheet used in 8 of the 16 chambers. Context 1 (bar flooring), used in the remaining 8 chambers, was identical but without the transparent polycarbonate sheet on the floor.

As shown in Figure 4.27, each chamber contained a magazine (2.5cm^2), two identical nose-poke manipulanda (10mm diameter) and two light-emitting stimulus lights (15cm height, above the nose-poke manipulanda) on one wall, and a 28V 100mA house light and sound-emitting speaker on the other wall. Nose-poke manipulanda were used as opposed to levers in the operant chamber as nose-poke responses are readily required by mice (Crawley, 2007). Each nose-poke manipulanda contained a yellow stimulus light and an infra-red photo beam to detect nose-poke entry. The recessed magazine also contained an infra-red photo beam which detected head entry responses and a yellow stimulus light which cued reinforcement delivery from a dipper dispenser attached to the magazine. The dipper delivered 0.04ml of one of two liquid reinforcements (dependant on the context), sourced from a container located immediately outside of the chamber, as shown in Figure 4.26. Liquid reinforcement was either a 20% sucrose solution flavoured with cherry Kool Aid (0.05% w/v) or a 10% maltodextrin solution flavoured with grape Kool Aid (0.05% w/v) (Cybercandy, London).

Pilot studies demonstrated that these liquid reinforcements could be easily discriminated and were well matched for motivational value under a water restriction regime.

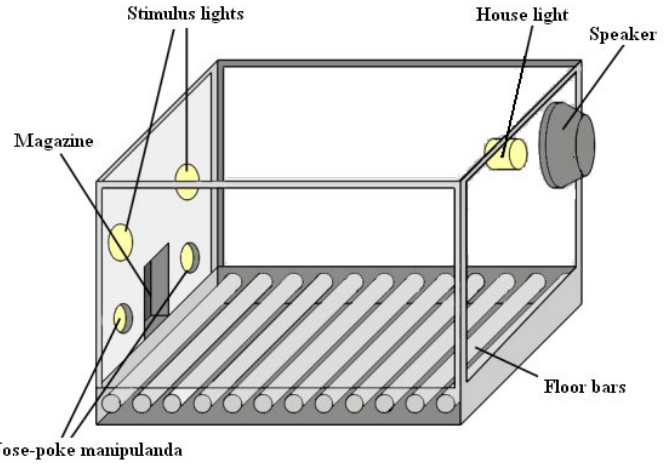

Figure 4.27 Layout of mouse operant chamber, showing location of magazine, nose-poke manipulanda, stimulus lights, house light and speaker.

The house and stimulus lights were used to deliver two types of visual stimuli to cue responding, including flashing illumination of the two stimulus lights (V1) or steady illumination of the house light (V2). Similarly, the speaker was used to deliver two types of auditory stimuli to cue responding, including a 2Hz tone (A1) or 2Hz buzz (A2). Each operant chamber was connected to a computer running MED-PC® software version IV (Med Associates Inc., Vermont, USA) which was programmed to control all stimulus presentations, and record nose-poke responses and magazine entries. The software automatically calculated the number of correct and incorrect responses.

4.5.2.3 Procedure

All mice received pretraining, biconditional discrimination training, the stroop interference test and reversal discrimination training. During all training and test experiments, mice received two sessions per day, once in the morning in one context and once in the afternoon in the alternative context. Sessions were separated by a 4-hour period in which mice were returned to their home cages. To strength the distinction of the contexts, reinforcement-context pairings were made whereby one liquid reinforcement (R1) was always provided in one context (C1), and the other liquid reinforcement (R2) was provided in the other context

(C2). Context order and reinforcement-context pairings were kept constant for each mouse during experiments. The combination of context, auditory and visual stimuli, nose-poke responses and reinforcement solutions were counterbalanced across mice and experimental groups as far as possible. Operant chambers were cleaned with 70% alcohol wipes after each session.

Pretraining

In order to form the association between the magazine and reinforcement delivery location, mice received two 20-minute sessions of magazine training for one day. During sessions the magazine light was illuminated to cue availability of liquid reinforcement which was delivered into the magazine on a random interval (RI) schedule of 30 seconds. Mice then received 7 consecutive days of two 36-minute reinforced nose-poke training sessions per day, once in the morning in one context and once in the afternoon in the alternative context. Here, mice were trained to nose-poke respond to the illumination of nose-poke manipulanda stimulus lights to gain reinforcement. During each session, mice received 12 randomized trials of 6 left and 6 right 120-second nose-poke manipulanda stimulus light illuminations. When presented with a left illumination, the correct response was to nose-poke the left manipulanda. When presented with a right illumination, the correct response was to nose-poke the right manipulanda. Following a correct response, reinforcement was immediately available and the magazine light was illuminated to cue the delivery. Each trial was separated by an average 50 second inter-trial interval (ITI), with a range of 40 to 60 seconds. The house light was not illuminated during training. Following successful nose-poke training, mice received nose-poke training on an RI-5, RI-10 and RI-15 schedule, each for one day, where reinforcement was available after an average of 5, 10 and 15 seconds respectively.

Stage 1: Biconditional discrimination training

Mice received 44 days of biconditional discrimination training, consisting of one 24-minute training session in the morning in one context (C1), and one session in the afternoon in the alternative context (C2). Each session contained 8 trials of 120-second discriminative stimulus presentations of auditory cues (A1 and A2) or visual cues (V1 and V2). Auditory and visual cues were only ever presented in separate contexts during training. Each trial was separated by a 50-second ITI, ranging from 40 to 60 seconds. Presentation of A1 in C1 or V1

in C2 would require either a left or right nose-poke response to receive reinforcement, and presentation of A2 in C1 or V2 in C2 would require the alternative nose-poke response. Both nose-poke manipulanda stimulus lights were illuminated for the duration of stimulus presentation. Reinforcement was available during the entire stimulus presentation and was delivered on a RI-15 schedule following a correct response. Correct responses were rewarded with 20% cherry-flavoured sucrose in one context and 10% grape-flavoured maltodextrin in the alternative context. The context, cue presentations and correct response-cue pairings were counterbalanced across mice and experimental groups.

An example of a typical biconditional discrimination training day for a mouse is shown in Table 4.3. This table shows that during morning trials in context 1 (bar floors [C1]), mice received random presentations of the auditory cues (buzz or tone [A1 or A2]) and were required to perform the correct nose-poke response (left or right [NP1 or NP2]) to receive reinforcement (e.g. cherry-flavoured sucrose [R1]). In this example, A1 cued an NP1 response and A2 cued an NP2 response. During afternoon trials in context 2 (smooth floors [C2]), mice received random presentations of the visual cues (stimulus lights or house lights [V1 or V2]) and were required to perform the correct nose-poke response (left or right [NP1 or NP2]) to receive reinforcement (e.g. grape maltodextrin [R2]). In this example, V1 cued an NP1 response and V2 cued an NP2 response.

Table 4.3 Experimental design for a biconditional discrimination training day. Pairings altered between mice subject to counterbalancing.

Session	**Context**	**Cue type**	**Correct response-cue pairings**
Morning	Bar floors (C1)	Auditory (A)	Buzz (A1) = Left nose-poke (NP1) Tone (A2) = Right nose-poke (NP2)
Afternoon	Smooth floors (C2)	Visual (V)	Stimulus light (V1) = Left nose-poke (NP1) House light (V2) = Right nose-poke (NP2)

Stage 2: Stroop interference test

Mice received 8 consecutive days of the Stroop interference test. Each day consisted of two 30-minute test sessions, one in each context. Each session contained 2 types of probe trial (congruent and incongruent) and 8 reinforced training trials, separated with an ITI of an average of 50s ranging from 40 to 60 seconds. The training trials were identical to the biconditional discrimination trials (Stage 1). Probe trials involved presentations of auditory

and visual training stimuli combined into audiovisual stimulus compounds. These included presentations of A1V1 (buzz and stimulus lights), A1V2 (buzz and house light), A2V1 (tone and stimulus lights) and A2V2 (tone and house light). Each compound was presented for 30 seconds and was presented in extinction whereby no reinforcement was available.

Presentation of these audiovisual compounds could be classified as either congruent or incongruent. Congruent trials contained compounds that require the same response, based on the previously learnt 'rules' of training in Stage 1. As shown in Table 4.4, presentation of an A1V1 compound would require a NP1 (left nose-poke) response, regardless of context, as both A1 and V1 cued NP1. Similarly, both stimulus elements in the A2V2 compounds would cue a NP2 (right nose-poke) response, regardless of context. Responses consistent with these previously learnt 'rules' were recorded as correct, whilst those that were inconsistent were recorded as incorrect.

Incongruent trials however contained audiovisual compounds that cued conflicting responses. For example, presentation of the A1V2 compound required both a NP1 response (cued by A1) and NP2 response (cued by V2). This response conflict was also presented with the A2V1 compound. During such trials, the mouse must resolve the response conflict by using the contextual information to guide correct responding. That is, the nose-poke response that was consistent with the current context. As shown in Table 4.4, presentation of the A1V2 compound in C1 (bar floors), where they had previously learnt the auditory discriminations A1 and A2, would require a response previously associated with the context – so NP1 response cued by the A1 element of the compound. However, presentation of the compound in C2 (smooth floors), where they had previously learnt the visual discriminations V1 and V2, would require a response previously associated with the context – so NP2 response cued by the V2 element of the compound. Responses inconsistent with the context, such as a response associated with the V2 element of A1V2 presentation in C1, was therefore recorded as incorrect.

Table 4.4 Experimental design for the Stroop interference test. Mice received one of each audiovisual compound in each context, which contained two congruent and two incongruent trials. Pairings altered between mice subject to counterbalancing.

Context + trial type	Audiovisual compound	Correct response
In C1 (Bar floors):		
Congruent	A1V1 (Buzz + Stimulus lights)	NP1 (Left)
Congruent	A2V2 (Tone + House light)	NP2 (Right)
Incongruent	A1V2 (Buzz + House light)	Guided by A1 = NP1 (Left)
Incongruent	A2V1 (Tone + Stimulus lights)	Guided by A2 = NP2 (Right)
In C2 (Smooth floors):		
Congruent	A1V1 (buzz + Stimulus lights)	NP1 (Left)
Congruent	A2V2 (Tone + House light)	NP2 (Right)
Incongruent	A1V2 (Buzz + House light)	Guided by V1 = NP1 (Left)
Incongruent	A2V1 (Tone + Stimulus lights)	Guided by V2 = NP2 (Right)

Stage 3: Reversal discrimination training

Mice received 56 days of reverse discrimination training, whereby the previously learnt stimulus-response discriminations in Stage 1 were reversed. This experiment was identical to the biconditional discrimination training, except mice received identical training sessions in the morning and afternoon in only one context and with only one discrimination (either auditory or visual stimulus) presentation. Allocation of the context (and therefore auditory or visual stimulus presentations) was counterbalanced so that approximately half of each experimental group received reversed auditory discriminations and half reversed visual discriminations. Table 4.5 shows the new rules of the reversal discriminations, whereby the previously learnt discrimination rules in Stage 1 were reversed. A response inconsistent with the new reversal rules were recorded as incorrect.

Table 4.5 The experimental design for the reverse discrimination test. Presenting the original discrimination rules previously learnt in Stage 1, which were reversed for reversal discrimination training in this experiment. Showing the correct responses required for each stimulus. Each mouse received either auditory or visual discrimination reversal. Pairings altered subject to counterbalancing.

Original discrimination rules	Reversed discrimination rules
A1 (Buzz) = NP1 (Left), A2 (Tone) = NP2 (Right)	A1 = NP2, A2 = NP1
V1 (Stimulus lights) = NP1 (Left), V2 (House light) = NP2 (Right)	V1 = NP2, V2 = NP1

4.5.2.4 Data analysis

Statistical analysis of data has been described throughout the results section, with further details in chapter 2, section 2.4. During the discrimination trainings (Stage 1 and 3), only the S_D' (stimulus presentation prime) responses were examined. The S_D' responses were the measure of performance in the variable time period prior to reinforcement on the RI-15 schedule during the 120-second stimulus presentation (S_D). Similar to Haddon and Killcross (2005, 2006), examination of these responses prior to reinforcement availability was a measure of discrimination performance uncontaminated by the presentation of reward.

4.5.3 Results

4.5.3.1 Pretraining results

In both contexts, all mice successfully learnt to collect liquid rewards from the magazine and produce nose-poke responses for reward.

4.5.3.2 Stage 1: Biconditional discrimination training results

Visual inspection of the mean number of correct and incorrect responses per minute for the visual and auditory discriminations revealed an obvious difference in response rates between groups. For ease of display, this has been shown in Figure 4.28 collapsing across stimulus types (auditory and visual) and day (1-44). Tg mice showed much higher response rates compared to WT mice, and mice fed the DHA diet also showed higher response rates, although both results appeared to be numerically driven by the Tg DHA group. Statistical analysis of this data using an ANOVA with between subjects factors of genotype (Tg, WT) and diet (DHA, OB) on the total number of responses per minute data revealed a significant effect of genotype ($F(1,46)=10.734$, $p<0.01$), a significant effect of diet ($F(1,46)=4.463$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=2.061$, $p>0.05$). Similarly, analysis of the total correct responses data revealed a significant effect of genotype ($F(1,46)=12.756$, $p<0.001$), a significant effect of diet ($F(1,46)=5.041$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=1.841$, $p>0.05$). Analysis of the total incorrect responses data revealed no significant effect of genotype ($F(1,46)=3.771$, $p>0.05$), a

significant effect of diet ($F(1,46)=1.909$, $p>0.05$) and no significant genotype by diet interaction ($F(1,46)=1.741$, $p>0.05$).

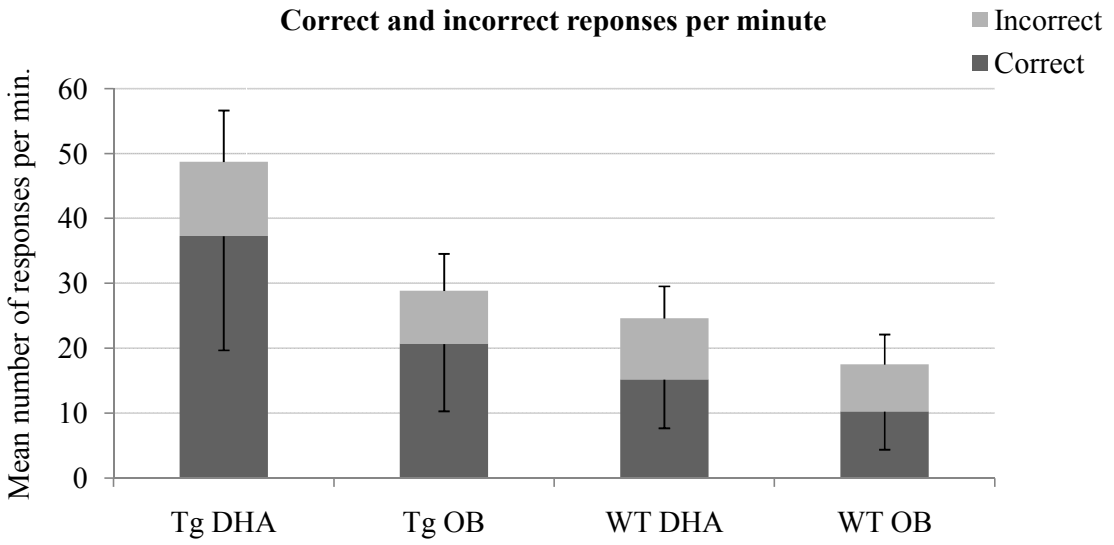

Figure 4.28 Mean number total S_D' responses per minute for each experimental group, displaying correct and incorrect response contributions, collapsed across stimulus types (auditory and visual) and days (1-44). S_D' responses = performance measure uncontaminated by reward delivery. Values are mean scores + S.E.M (correct responses) and - S.E.M (incorrect responses).

In order to account for this large difference in response rates between groups in evaluating acquisition of the discriminations, a discrimination ratio was calculated. Discrimination ratios (DR) were calculated using the following formula: [mean number of correct S_D' responses per minute / mean number of total S_D' responses per minute]. A DR of 0.5 was therefore equivalent to equal correct and incorrect responding, and was therefore not indicative of learning the auditory or visual stimulus discriminations. DRs were calculated for each trial and the data from the 44 acquisition days were averaged into eleven 4-day blocks for ease of presentation and analysis. Figure 4.29 shows the discrimination ratio data for each experimental mouse group over blocked days during acquisition of the biconditional discriminations. The data in this figure was collapsed across stimulus type (auditory and visual discriminations) to display overall group differences. Visual inspection of this figure shows that Tg mice, particularly those fed the DHA diet, successfully discriminated auditory and visual stimuli better than WT groups.

Statistical analysis of this data using an ANOVA with genotype (Tg, WT) and diet (DHA, OB) as between-subject factors, and blocked days (1-11) as a within-subject factor, revealed a significant main effect of genotype ($F(1,46)=22.781$, $p<0.001$), no significant main effect of diet ($F(1,46)=3.387$, $p>0.05$) and no significant interaction between these factors ($F(1,46)=3.174$, $p>0.05$). Analysis also revealed a significant effect of block ($F(10,460)=47.485$, $p<0.001$), a significant interaction of block by genotype ($F(10,460)=9.498$, $p<0.001$) and a significant interaction of block by diet ($F(10,460)=3.171$, $p<0.001$). A follow-up of simple main effects revealed a significant main effect of genotype at blocks 3 to 11 (smallest $F(1,46)=5.94$, $p<0.05$) and a main effect of diet at blocks 4, 7, 8 and 11 (smallest $F(1,46)=4.18$, $p<0.05$). Statistical analysis therefore confirmed that Tg mice showed superior discrimination acquisition relative to WT mice, and that DHA supplementation further enhanced performance.

Indeed, analysis also revealed a significant three-way interaction of block by genotype by diet ($F(10,460)=2.506$, $p<0.01$). Following up this interaction with a test of simple interactions, a near-significant interaction of genotype by diet was revealed at block 6 ($F(1,46)=4.01$, $p=0.051$), which was significant at block 11 ($F(1,46)=10.54$, $p<0.01$). Following-up this significant interaction at block 11, a test of simple main effects revealed Tg OB mice performed significantly better than WT OB mice ($F(1,46)=4.36$, $p<0.05$), and Tg DHA performed significantly better than WT DHA mice ($F(1,46)=29.24$, $p<0.001$) and Tg OB mice ($F(1,46)=11.27$, $p<0.01$). DHA supplementation therefore enhanced performance in Tg mice.

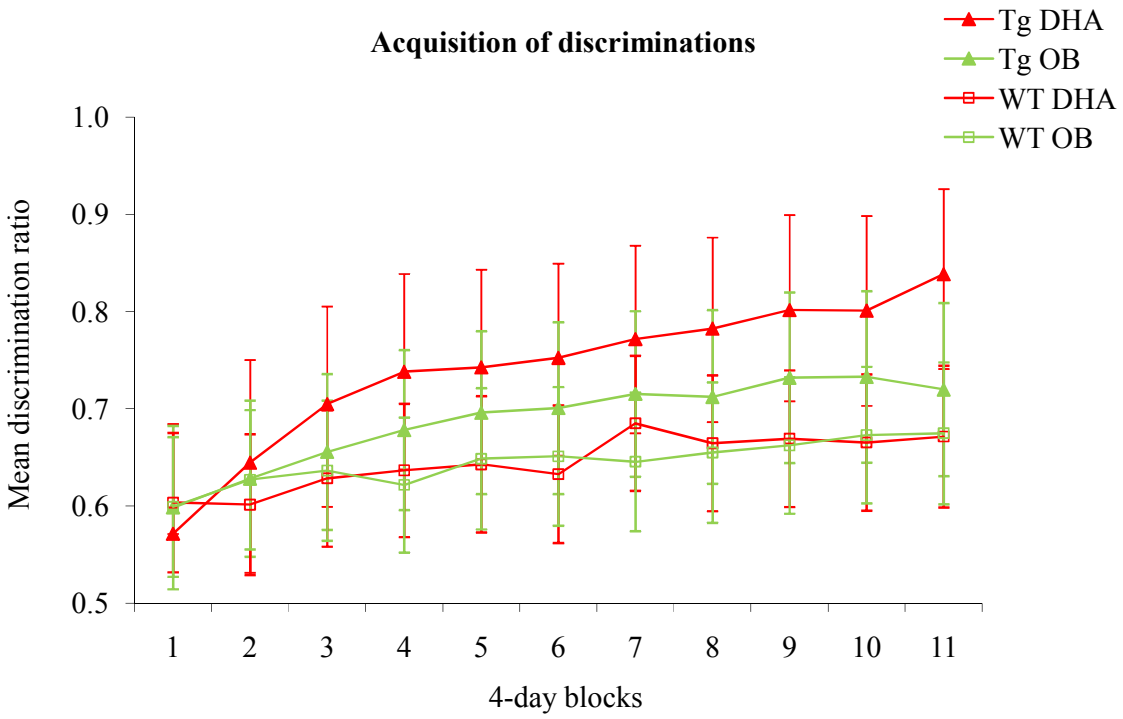

Figure 4.29 Acquisition of discriminations during the biconditional discrimination acquisition phase across 4-day blocks, collapsed across stimulus types. Graph showing the mean discrimination ratio (correct S_D' responses per minute/total S_D' responses per minute). S_D' responses = performance measure uncontaminated by reward delivery. DR of 0.5 = equal level of correct and incorrect responding. Error bars represent ± S.E.M.

Prior to proceeding to the Stroop interference test (Stage 2), it was important to ensure that all experimental mouse groups had acquired the biconditional discriminations. Visual inspection of Figures 4.30 and 4.31, showing the discrimination ratios for auditory and visual discriminations respectively for each group over blocked days, depicts that all groups performed above the 0.5 chance level. In order to test this, unpaired samples t-tests compared the auditory DR and visual DR to chance levels (DR 0.5) for each group separately using the 4-day blocks of data. Statistical analysis revealed all groups significantly discriminated the auditory stimulus presentations at blocks 1 to 11 (smallest $t(14)=18.219$, $p<0.01$), and the visual stimulus presentations at blocks 1 to 11 (smallest $t(14)=9.833$, $p<0.001$). This was particularly shown at the final block of training (block 11) for auditory discriminations (smallest $t(28)=15.039$, $p<0.001$) and visual discriminations (smallest $t(14)=27.346$, $p<0.001$). Thus, all mice produced significantly more correct than incorrect responses on both the auditory and visual discriminations during training, as shown in Figures 4.30 and 4.31.

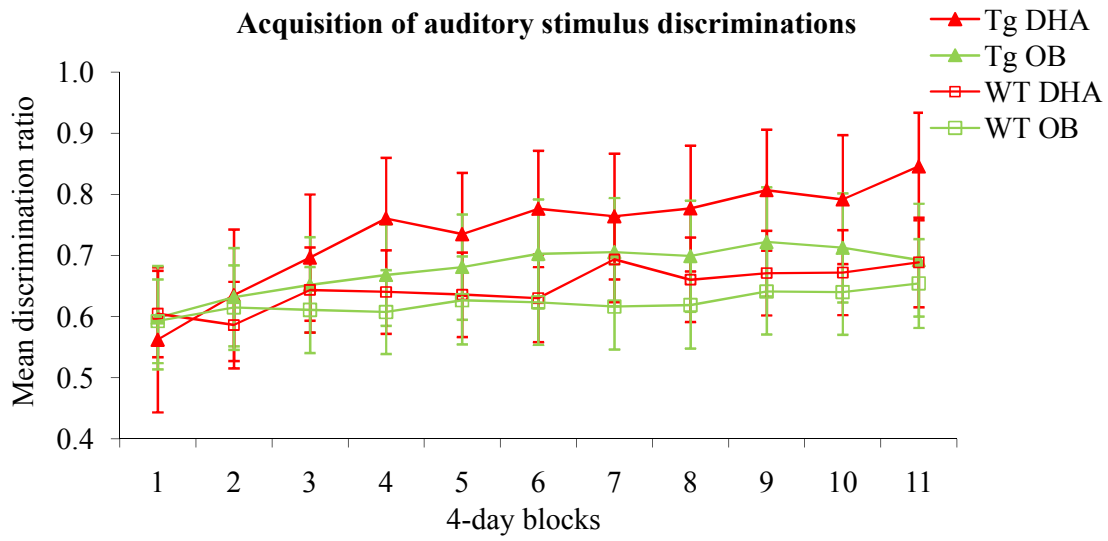

Figure 4.30 Acquisition of auditory stimulus discriminations during the biconditional discrimination acquisition phase across 4-day blocks. Graph showing the mean discrimination ratio (correct S_D' responses per minute/total S_D' responses per minute). S_D' responses = performance measure uncontaminated by reward delivery. DR of 0.5 = equal level of correct and incorrect responding. Error bars represent ± S.E.M.

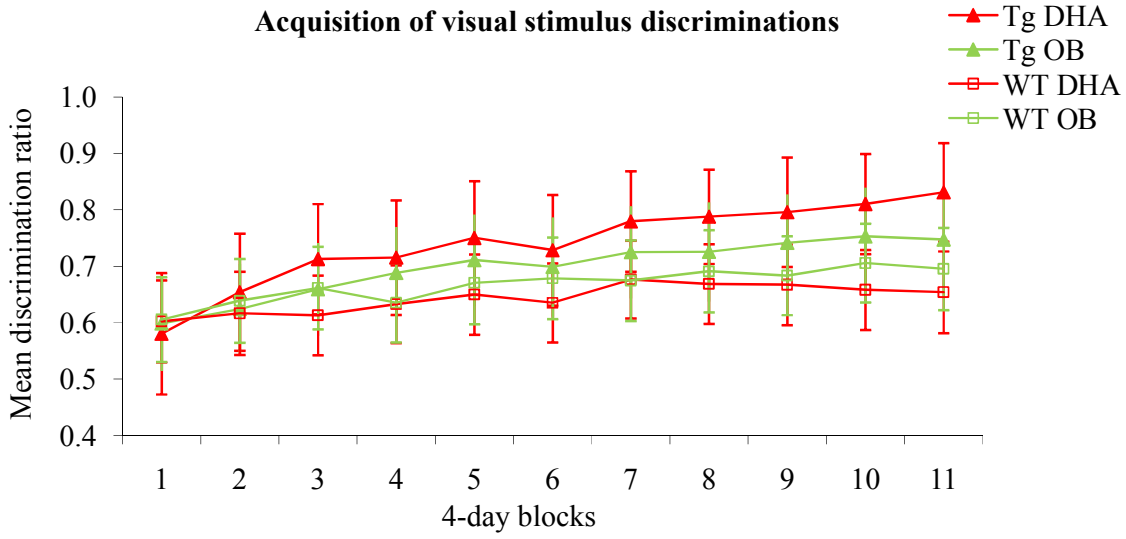

Figure 4.31 Acquisition of visual stimulus discriminations during the biconditional discrimination acquisition phase across 4-day blocks. Graph showing the mean discrimination ratio (correct S_D' responses per minute/total S_D' responses per minute). S_D' responses = performance measure uncontaminated by reward delivery. DR of 0.5 = equal level of correct and incorrect responding. Error bars represent ± S.E.M.

4.5.3.3 Stage 2: Stroop interference test results

Similar to the results of Stage 1, the mean number of correct and incorrect responses per minute for the training, congruent and incongruent trials of Stage 2 revealed a significant difference in response rates between groups. During training trials, Tg mice (particularly Tg DHA) showed much higher total response rates compared to WT mice, and Tg mice fed the DHA diet showed higher response rates than Tg OB mice (Figure 4.32). Statistical analysis using an ANOVA with between-subjects factors of genotype (Tg, WT) and diet (DHA, OB) on the total number of responses data revealed a significant effect of genotype ($F(1,46)=75.478$, $p<0.001$), no significant effect of diet ($F(1,46)=3.808$, $p>0.05$) and no significant genotype by diet interaction ($F(1,46)=1.122$, $p>0.05$). Similar analysis of the total correct responses data revealed a significant effect of genotype ($F(1,46)=12.048$, $p<0.001$), a significant effect of diet ($F(1,46)=4.396$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=2.630$, $p>0.05$). Analysis of the total incorrect responses data revealed no significant effect of genotype ($F(1,46)=0.595$, $p>0.05$), no significant effect of diet ($F(1,46)=1.690$, $p>0.05$) and no significant genotype by diet interaction ($F(1,46)=0.026$, $p>0.05$).

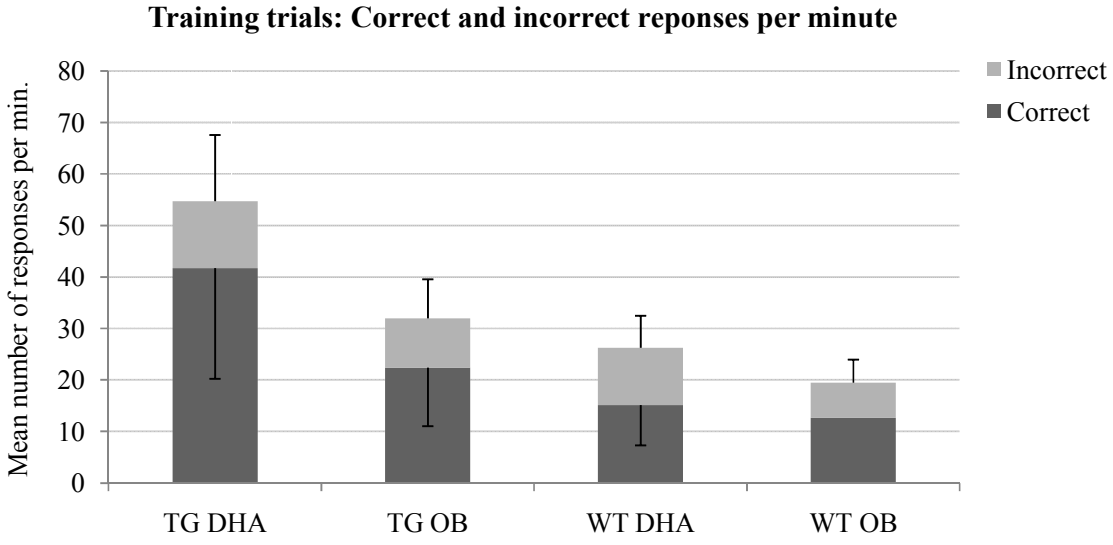

Figure 4.32 Mean number total S_D' responses per minute for each experimental group, displaying correct and incorrect response contributions during training trials, collapsed across days (1-8). S_D' responses = performance measure uncontaminated by reward delivery. Values are mean scores + S.E.M (correct responses) and - S.E.M (incorrect responses).

During congruent trials, Tg DHA mice appeared to show much higher total response rates compared to all groups, and WT OB mice showed lower responses than WT DHA mice (Figure 4.33). Statistical analysis using an ANOVA with between subjects factors of genotype and diet on the total number of responses data revealed a significant effect of genotype ($F(1,46)=5.842$, $p<0.05$), a significant effect of diet ($F(1,46)=4.053$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=0.731$, $p>0.05$). Similar analysis of the total correct responses data revealed a significant effect of genotype ($F(1,46)=8.990$, $p<0.01$), a significant effect of diet ($F(1,46)=4.052$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=1.045$, $p>0.05$). Analysis of the total incorrect responses data revealed a significant effect of genotype ($F(1,46)=4.951$, $p<0.05$), no significant effect of diet ($F(1,46)=1.358$, $p>0.05$) and no significant genotype by diet interaction ($F(1,46)=0.299$, $p>0.05$).

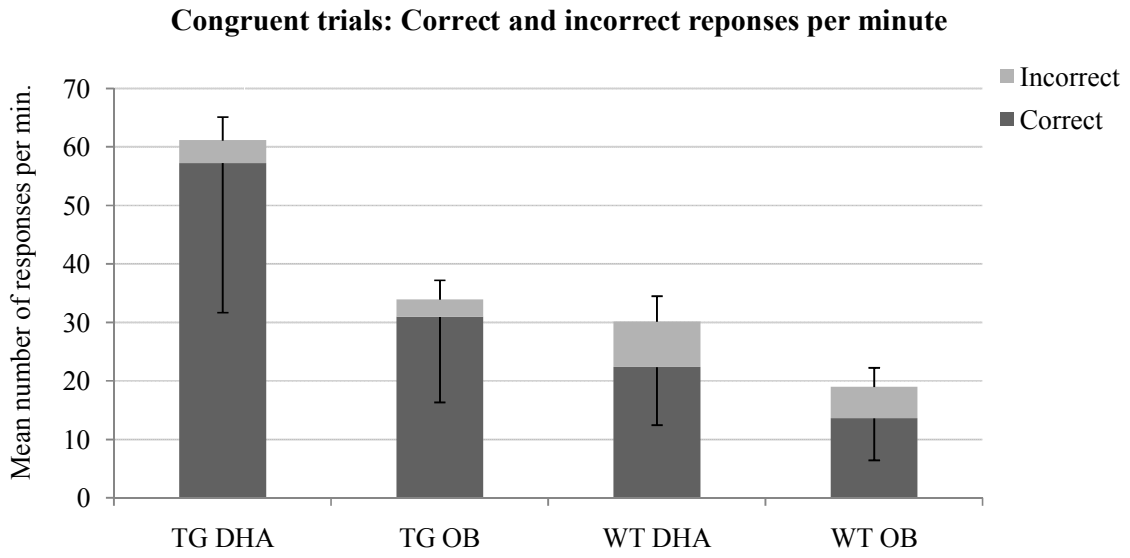

Figure 4.33 Mean number total S_D' responses per minute for each experimental group, displaying correct and incorrect response contributions during congruent trials, collapsed across days (1-8). S_D' responses = performance measure uncontaminated by reward delivery. Values are mean scores + S.E.M (correct responses) and - S.E.M (incorrect responses).

During incongruent trials, Tg mice (particularly Tg DHA) showed higher total response rates compared to WT mice, as shown in Figure 4.34. Furthermore, Tg DHA mice showed higher response rates than Tg OB mice, and WT DHA mice showed higher response rates than WT DHA mice. Statistical analysis using an ANOVA with between subjects factors of genotype and diet on the total number of responses data revealed a significant effect of genotype

(F(1,46)=4.232, p<0.05), no significant effect of diet (F(1,46)=2.206, p>0.05) and no significant genotype by diet interaction (F(1,46)=0.393, p>0.05). Similar analysis of the total correct responses data revealed a significant effect of genotype (F(1,46)=4.905, p<0.05), no significant effect of diet (F(1,46)=3.618, p>0.05) and no significant genotype by diet interaction (F(1,46)=0.790, p>0.05). Analysis of the total incorrect responses data revealed no significant effect of genotype (F(1,46)=2.291, p>0.05), no significant effect of diet (F(1,46)=0.225, p>0.05) and no significant genotype by diet interaction (F(1,46)=0.001, p>0.05).

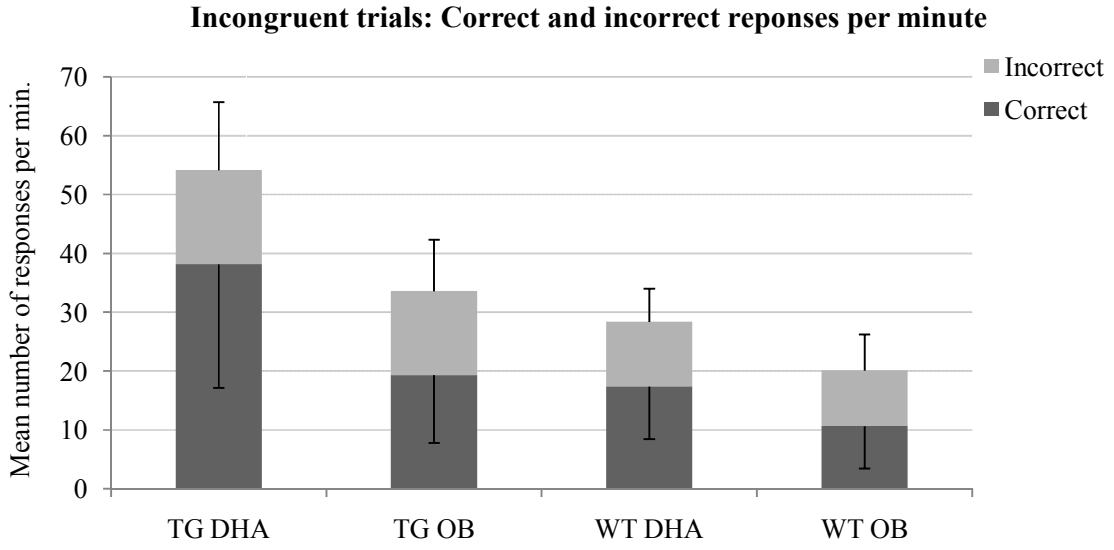

Figure 4.34 Mean number total S_D' responses per minute for each experimental group, displaying correct and incorrect response contributions during incongruent trials, collapsed across days (1-8). S_D' responses = performance measure uncontaminated by reward delivery. Values are mean scores + S.E.M (correct responses) and - S.E.M (incorrect responses).

Due to these differences in response rates, performance was analysed using discrimination ratios. Data was collapsed across testing days (1-8). Figure 4.35 illustrates that all mice showed higher discrimination ratios (therefore higher proportion of correct responses) for congruent compound trials relative to incongruent compound trials, which is consistent with Stroop interference literature. Interestingly, Tg mice appear to perform better than WT mice during congruent trials, although all groups appear to perform better than chance (DR 0.5), thereby demonstrating memory for the biconditional discriminations. Furthermore, Tg DHA mice appear to perform better than all groups during incongruent trials, and mice fed the DHA diet appear to be the only groups that performed better than chance, indicating memory

for the biconditional discriminations and its use to guide responding during presentation of incongruent compounds.

Statistical analysis using an ANOVA with genotype (Tg, WT) and diet (DHA, OB) as between-subject factors on congruent data revealed a significant main effect of genotype ($F(1,46)=19.929$, $p<0.001$) and diet ($F(1,46)=45.709$, $p<0.05$), but no significant interaction of genotype by diet ($F(1,46)=2.106$, $p>0.05$). This therefore confirms that Tg mice performed better than WT mice on congruent trials, and that DHA supplementation enhanced performance, although the latter appears to be predominately driven by Tg mice as WT groups show similar performance. Interestingly, the trend showing that Tg DHA was predominately driving the significant genotype effect was not statistically supported by a significant interaction. Similar statistical analysis on the incongruent data revealed a significant main effect of genotype ($F(1,46)=5.645$, $p<0.05$) and diet ($F(1,46)=16.433$, $p<0.001$), but no significant interaction of genotype by diet ($F(1,46)=1.771$, $p>0.05$). Similar to the congruent data, these results confirm that Tg mice performed better than WT mice on incongruent trials and that DHA supplementation generally enhanced performance across all groups. It is interesting to note that the effect of diet appeared to be predominately driven by the Tg DHA group, but this was not statistically supported by a significant interaction.

In order to test whether groups successfully showed above-chance performance on congruent trials, DR data from each group on the last day of training was compared to chance levels (DR 0.5) using an unpaired samples t-test. Statistical analysis revealed all groups differed significantly from chance; Tg DHA ($t(14)=21.723$, $p<0.001$), Tg OB ($t(18)=19.790$, $p<0.001$), WT DHA ($t(28)=26.614$, $p<0.001$) and WT OB ($t(30)=28.051$, $p<0.01$). This confirmed that all mice demonstrated memory for the biconditional discriminations. A similar analysis of the incongruent trials using an unpaired samples t-test showed all groups except WT OB mice differed significantly from chance; Tg DHA ($t(14)=15.537$, $p<0.01$), Tg OB ($t(18)=46.028$, $p<0.05$), WT DHA ($t(28)=27.399$, $p<0.05$) and WT OB ($t(30)=26.905$, $p>0.05$). This therefore shows that all mice except the WT OB group were able to successfully utilise the contextual information to guide responding during incongruent trials by the end of training. The statistical analysis reflects the pattern of results during incongruent trials shown in Figure 4.35, whereby Tg DHA mice performed particularly above chance levels, with Tg OB and WT DHA mice performing less well, and WT OB failing to perform significantly above chance.

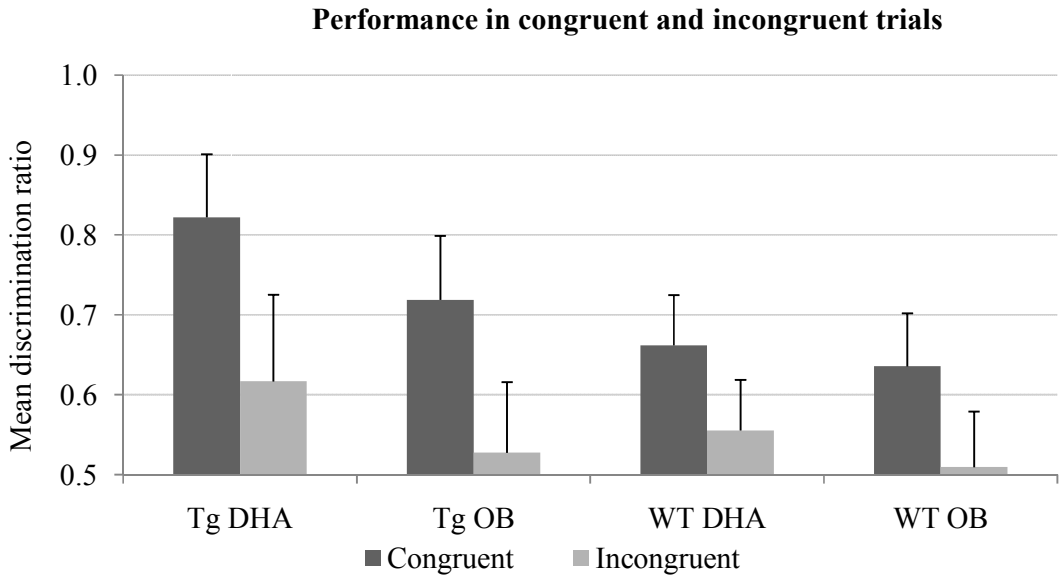

Figure 4.35 Mean discrimination ratio data for the congruent and incongruent compound probe trials, collapsed across testing days 1 to 8. Error bars represent + S.E.M.

Mice were given 8 reinforced training trials during the Stroop interference test to re-establish performance on the biconditional discriminations. Figure 4.36 depicts the average performance during training trials for each experimental group. Similar to Stage 1, the figure shows Tg mice performed better than WT mice, but with little effect of diet. Statistical analysis of this data using an ANOVA with between-subject factors of genotype and diet revealed a significant main effect of genotype ($F(1,46)=16.768$, $p<0.001$), no significant main effect of diet ($F(1,46)=01.147$, $p>0.05$) and a significant genotype by diet interaction ($F(1,46)=5.778$, $p<0.05$). To follow-up this interaction, a test of simple main effects revealed Tg DHA mice performed significantly better than WT DHA mice ($F(1,46)=18.28$, $p<0.001$). To examine whether experimental groups were correctly discriminating above chance levels, training trial DR data from each group on the last day of training was compared to chance levels (DR 0.5) using an unpaired samples t-test. Statistical analysis revealed all groups differed significantly from chance; Tg DHA ($t(14)=15.815$, $p<0.001$), Tg OB ($t(18)=12.049$, $p<0.001$), WT DHA ($t(28)=21.075$, $p<0.001$) and WT OB ($t(30)=11.740$, $p<0.001$). All groups therefore showed successful discrimination of auditory and visual stimulus presentations during testing.

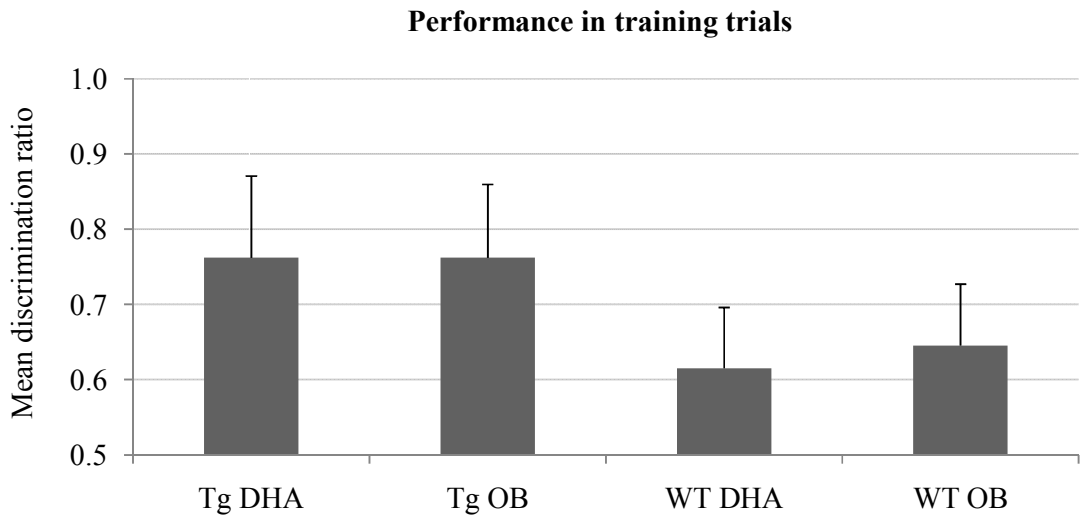

Figure 4.36 Mean discrimination ratio data (correct S_D' responses per minute/total S_D' responses per minute) for the training trials, collapsed across testing days. S_D' responses = performance measure uncontaminated by reward delivery. DR of 0.5 = equal level of correct and incorrect responding. Error bars represent + S.E.M.

4.5.3.4 Stage 3: Reversal discrimination training results

Similar to the results from Stages 1 and 2, the mean number of correct and incorrect responses per minute during the reversal training revealed an obvious difference in response rates between groups, as shown in Figure 4.37. For ease of display, the results have been collapsed across stimulus types (auditory and visual) and day (1-56). Tg DHA mice showed much higher response rates than all other groups. In particular, this was driven by higher correct response rates. All other groups showed relatively similar levels of correct responses (although WT OB showed fewer), and all groups showed a similar level of incorrect responding. Statistical analysis of this data using an ANOVA with between-subjects factors of genotype (Tg, WT) and diet (DHA, OB) using the total number of responses per minute data revealed no significant effect of genotype ($F(1,46)=2.313$, $p>0.05$), no significant effect of diet ($F(1,46)=2.474$, $p>0.05$) and no significant genotype by diet interaction ($F(1,46)=0.458$, $p>0.05$). Analysis of the total correct responses data revealed no significant effect of genotype ($F(1,46)=3.236$, $p>0.05$), a significant effect of diet ($F(1,46)=4.451$, $p<0.05$) and no significant genotype by diet interaction ($F(1,46)=0.966$, $p>0.05$). Analysis of the total incorrect responses data revealed no significant effect of genotype ($F(1,46)=0.584$,

p>0.05), no significant effect of diet (F(1,46)=0.129, p>0.05) and no significant genotype by diet interaction (F(1,46)=0.001, p>0.05).

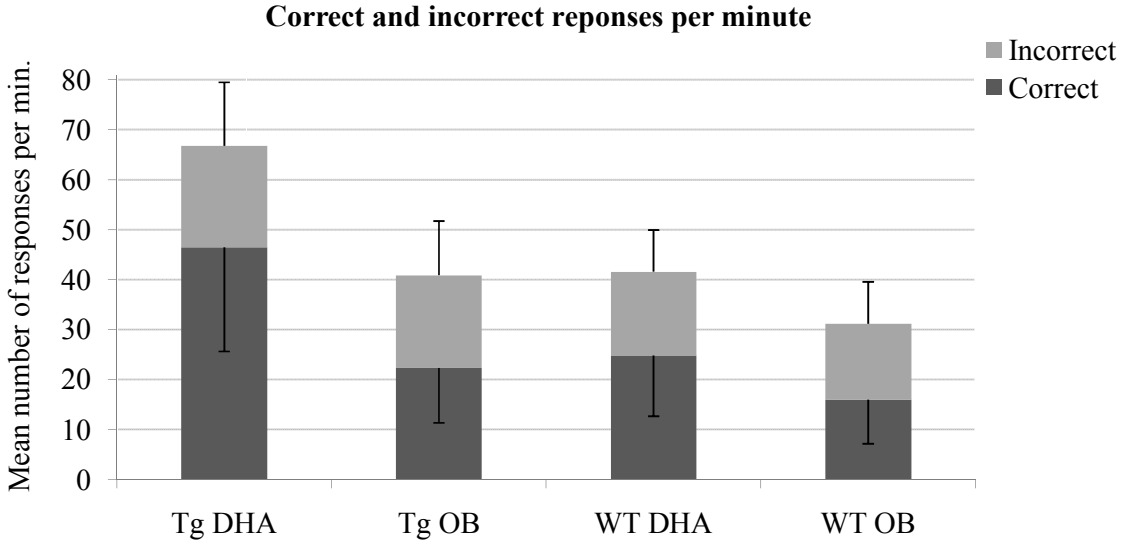

Figure 4.37 Mean number total S_D' responses per minute for each experimental group, displaying correct and incorrect response contributions, collapsed across stimulus types (auditory and visual) and days (1-56). S_D' responses = performance measure uncontaminated by reward delivery. Values are mean scores + S.E.M (correct responses) and - S.E.M (incorrect responses).

Due to these differences in response rates, performance was analysed using discrimination ratios. Data from the 56 acquisition days were averaged into fourteen 4-day blocks for ease of presentation and analysis. Figure 4.38 shows the discrimination ratio data for each experimental mouse group over blocked days during acquisition of reversed discriminations. The data in this figure was collapsed across stimulus type (auditory and visual discriminations) to show overall group differences. Visual inspection of this figure shows that Tg mice, particularly those fed the DHA diet, successfully acquired reversed discriminations better than WT groups, indicative of enhanced reversal learning.

Statistical analysis of this data using an ANOVA with genotype (Tg, WT) and diet (DHA, OB) as between-subject factors, and block (1-14) as a within-subject factor, revealed a significant main effect of genotype (F(1,46)=5.407, p<0.05), no significant main effect of diet (F(1,46)=1.946, p>0.05) and no significant interaction between these factors (F(1,46)=0.312, p>0.05). Analysis also revealed a significant effect of block (F(13,598)=42.615, p<0.001), a

significant interaction of block by genotype ($F(13,598)=4.147$, $p<0.001$), no significant interaction of block by diet ($F(13,598)=0.737$, $p>0.05$), and no significant three-way interaction of block by genotype by diet ($F(13,598)=0.540$, $p>0.05$). A follow-up of simple main effects revealed a significant effect of genotype at blocks 6 and 8 to 14 (smallest $F(1,46)=4.60$, $p<0.05$). Overall, statistical analysis confirmed that Tg mice were superior at acquiring reverse discriminations relative to WT mice, and that diet failed to alter performance. However, the overall effect of genotype appeared to be numerically driven by the Tg DHA group.

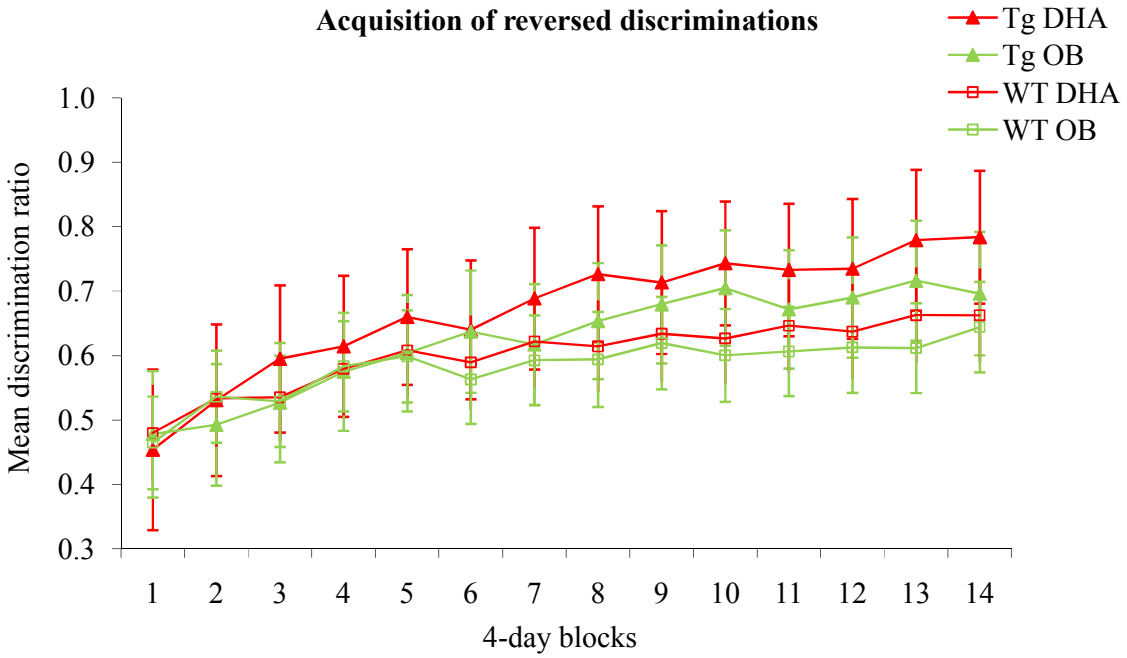

Figure 4.38 Acquisition of reversed discriminations during the reversal discrimination training across 4-day blocks, collapsing across stimulus types (auditory and visual). Graph showing the mean discrimination ratios (correct S_D' responses per minute/total S_D' responses per minute). S_D' responses = performance measure uncontaminated by reward delivery. DR of 0.5 = equal level of correct and incorrect responding. Error bars represent ± S.E.M.

4.5.4 Discussion

Stage 1: Biconditional discrimination training

Stage 1 of experimentation investigated the effect of transgene and DHA supplementation on performance in the biconditional discrimination task at 5.5 to 7 months of age. Interestingly,

inspection of correct and incorrect S_D' responses per minute revealed group differences whereby Tg mice showed significantly higher response rates than WT mice, and DHA supplementation also increased responses rates, although both results appeared to be predominately driven by the Tg DHA group. These results may be suggestive of hyperactivity in Tg2576 mice, which has been documented in tasks including the open field, Y-maze, automated activity boxes and the circular open field activity chamber (King et al., 1999; King & Arendash, 2002; Lalonde et al., 2003; Asuni et al., 2006; Deacon et al., 2008, 2009). Interestingly, DHA supplementation increased response rates in mice (particularly Tg mice), perhaps suggesting that DHA also increased hyperactivity. In order to compensate for these group differences which may otherwise bias interpretation of results, discrimination ratios (DR) were calculated and analysed for all experiments.

DR results from biconditional discrimination training showed that both Tg and WT mice fed the DHA and oil blend diet significantly acquired the auditory and visual discriminations from blocks 1 to 11. This lack of transgene deficit in Tg mice was as hypothesised, and was consistent with previous reports by Zhuo et al. (2007, 2008) who found 6- and 14-month old Tg2576 mice successfully acquired simple discriminations whereby 1 odour or digging medium was rewarded over the other odour or digging medium. This was also consistent with reports by Barnes, Hale and Good (2004) who found Tg2576 mice at 12 to 14 months to successfully acquire a contextual biconditional left-right discrimination in the T-maze, as well as an intramaze brightness discrimination and simple room discrimination.

When examining group differences during acquisition of the biconditional discriminations (collapsing across stimulus type), the results interestingly revealed Tg mice discriminated significantly better than WT mice. This effect was predominately driven by the Tg DHA group as Tg OB mice performed significantly worse than Tg DHA mice, although Tg OB also performed significantly better than WT. This therefore shows that Tg mice on the control oil blend diet were not impaired at acquiring the biconditional discriminations relative to WT mice, and DHA enhanced Tg performance. This lack of transgene deficit in Tg2576 mice was consistent with reports by Zhuo et al. (2007, 2008). This experiment presents the first evidence that DHA supplementation can enhance executive function in Tg2576 mice, which is somewhat supported by positive effects of DHA supplementation in other cognitive tasks (e.g. Calon et al., 2004).

Stage 2: Stroop interference test

Similar to Stage 1, the Stroop test results revealed a significant difference in response rates between groups. Tg mice had significantly higher responses during training, congruent and incongruent trials. DHA supplementation also resulted in significantly higher responses during training and congruent trials, with a non-significant trend in Tg mice during incongruent trials. In order to compensate for these group differences, discrimination ratios (DR) were calculated and analysed for all data.

Similar to the results found during biconditional discrimination training, all groups discriminated significantly above chance levels during the reinforced training trials in the Stroop interference test at 7 months. Similarly to Stage 1, Tg mice performed significantly better than WT mice and this effect appeared to be driven by Tg DHA mice. Indeed, Tg DHA (not Tg OB) mice performed better than WT groups. This therefore indicates that Tg mice on the control oil blend diet were not impaired at acquiring the biconditional discriminations relative to WT mice during the Stroop test, and DHA enhanced Tg performance to levels significantly better than WT groups. Diet failed to alter WT performance. This lack of transgene deficit was as predicted, and is consistent with previous reports by Zhuo et al. (2007, 2008) who found Tg2576 mice aged 6- and 14-months to successfully acquire compound discriminations whereby mice had to discriminate between simple odour stimuli in the presence of an irrelevant dimension (digging medium). This lack of transgene deficit was also corroborated by Barnes, Hale & Good (2004) who reported Tg2576 mice aged 12 to 14 months successfully acquired a contextual biconditional left-right discrimination in the T-maze, as well as an intramaze brightness discrimination and simple room discrimination.

Analysis of congruent trials during the Stroop interference test showed that all mouse groups could successfully discriminate above chance levels (equivalent to equal correct and incorrect responding) using the previously learnt stimulus-reward associations acquired in Stage 1. This lack of discrimination deficit in Tg2576 mice was consistent with reports by Zhuo et al. (2007, 2008) and Barnes, Hale and Good (2004). These results were also consistent with reports of AD patients in congruent trials of the human Stroop test (Koss et al., 1984; Perry & Hodges, 1999; Amieva et al., 2002, 2004a, 2004b).

Examination of congruent trial data also revealed Tg2576 mice performed significantly better than WT mice. This enhanced performance in congruent trials by Tg2576 mice is consistent with reports by Spieler et al. (1996) who found that AD patients displayed facilitated performance on congruent trials in the human Stroop test. One explanation for this facilitation in AD patients is that a deficit in attentional or inhibitory control of responses leads to immediate output of the dominant word pathway which is processed faster than colour processing. In contrast, age-matched controls have slower responses due to consideration of the instructions given during compound presentation, which is guided by inhibitory or attentional control. As a result, AD patients display lower response times which could be a result of disinhibitory behaviour rather than increased cognitive performance. The same argument could be applied to Tg2576 mice which show enhanced performance during congruent trials of the Stoop interference test. However, this genotype effect appeared to be driven by the Tg DHA group. Although this was not supported statistically, DHA supplementation was shown to significantly improve performance (which appeared to be numerically driven by the Tg DHA group). This therefore indicates that Tg mice fed the control oil blend diet were not impaired on the congruent trials, and similarly did not show enhanced performance unless fed the DHA diet. Based on the previous argument, it is unclear whether DHA supplementation increased cognitive performance or increased a tendency to show disinhibitory responses. The similar enhanced performance in biconditional discrimination training would support the argument for increased cognitive performance rather than the latter.

Examination of the incongruent trial data relative to congruent trial data revealed that all groups performed significantly better at congruent trials, consistent with Stroop test literature whereby the response-conflict caused by incongruent compounds leads to a reduction in correct responses (Stroop, 1935). This ‘interference effect’ was thereby present in all mice. Interestingly, analysis of incongruent trial data revealed that Tg2576 mice performed better than WT mice during incongruent trials, and WT OB mice failed to perform significantly above chance (in contrast to all other groups). Again, this result appeared to be driven by the Tg DHA group as Tg OB mice had similar responses to WT groups. Although no significant interaction was found to support this, DHA supplementation was shown to significantly improve performance. Overall therefore, Tg mice were not impaired in incongruent trials, and DHA supplementation enhanced executive function (particularly in Tg mice). These results suggest that Tg2576 mice had no difficulty in inhibiting automatic responses during

incongruent presentations and were able to successfully utilise the contextual information to guide responding during incongruent trials. This was contrary to predictions, since previous research has shown the PFC to be pathologically affected at 6 months, which was anticipated to affect executive function (Zhuo et al., 2008). The results were also inconsistent with human literature whereby AD patients show stronger interference effects than age-matched controls (Koss et al., 1984; Perry & Hodges, 1999; Amieva et al., 2002, 2004a, 2004b).

The result showing that all groups except the WT OB group could perform significantly above chance during incongruent trials is interesting and suggests that WT mice are unable to successfully utilise the contextual information to guide responding during incongruent trials. Similarly, Zhuo et al. (2008) reported poor performance in WT control mice during a reversal learning task at 14 months of age, but interestingly, not at 6 months of age. Importantly the results also suggest that DHA supplementation can improve WT performance and enable successful performance during incongruent trials. DHA supplementation therefore appears to enhance performance in both Tg and WT mice in this task.

Stage 3: Reversed discrimination training

Reversal training was carried out at 7.5 to 9 months of age. Similar to Stages 1 and 2, the reversal training results revealed a significant difference in response rates between groups. Statistical analysis revealed that DHA supplementation significantly produced higher correct responses. This appeared to be numerically driven by the Tg DHA group, similar to results in Stages 1 and 2, although this was not supported statistically. Unlike Stages 1 and 2, Tg mice did not show significantly higher response rates, although Tg DHA were numerically higher than all groups. In order to compensate for this difference, discrimination ratios (DR) were calculated and analysed for all data, consistent with previous data analysis.

DR results revealed that Tg mice acquired the reverse discriminations more effectively than WT mice. This enhanced learning was particularly shown on blocked days 6 and 8 to 14. Although no significant effect of diet or interaction was shown, the effect of genotype appeared to be predominately driven by Tg DHA mice, indicative of enhanced performance by DHA supplementation.

These results were inconsistent with previous reports of impaired reversal discrimination learning in Tg2576 mice at 6 months of age in versions of an attentional set-shifting test (see Zhuo et al., 2007, 2008). These results were also inconsistent with reports by Pompl et al. (1999) who found that Tg2576 mice aged 7 months showed a robust reversal learning impairment in an adapted circular platform task. Moreover, results were in contrast to reversal learning impairments reported in AD patients tested in the Stroop test (Perry & Hodges, 1999; Amieva et al., 2004b). In contrast to these studies however, the results were consistent with reports of intact reversal learning in Tg2576 mice at a later age. For example, Dong et al. (2005) reported intact reversal learning in a water-based T-maze task at 11 months. Similarly, Zhuo et al. (2007) reported no deficits at 14 months in reversed compound discriminations and during reversed intra-dimensional shifts (although it was argued that this was a result of poor WT performance). In an attempt to explain these differences, it could be argued that reversal learning deficits in Tg2576 mice may be associated with early stages of pathological development such as the increase in soluble Aβ, which is then lost during later stages when soluble Aβ is reduced and insoluble Aβ increases. Since soluble Aβ species are converted to insoluble forms during the time of experimentation at 7.5 to 9 months of age (Kawarabayashi et al., 2001), this may explain the transgene effect observed whereby Tg mice did acquire reverse discriminations. Further research is therefore required to examine the progression of PFC-related deficits and their underlying pathological cause.

Together the results from these experiments show that Tg2576 mice can successfully acquire biconditional discriminations and use information acquired during this initial training to guide responding in congruent trials of the Stroop test. Tg2576 mice could also successfully utilise the contextual information to guide responding during incongruent trials (in contrast to WT OB mice). Furthermore, Tg2576 mice showed intact acquisition of reversed discriminations at 7.5 to 9 months of age. Overall therefore, these results suggest that PFC-dependant executive function is generally intact. Further research to probe PFC-function longitudinally in Tg2576 mice at different ages is required to examine if and when deficits emerge. Further to this, these experiments also provide evidence that DHA supplementation can improve executive function, primarily in Tg2576 mice.

4.6 Chapter discussion

The aim of this chapter was to examine the behavioural phenotype of the Tg2576 model from an early age using a large battery of anxiety and cognitive tasks and to assess the effect of DHA supplementation on performance. Tg2576 and WT mice were examined in the elevated plus maze, marble burying task, foraging task, the biconditional discrimination task, the Stroop test and a reverse discrimination task. The tasks probed a wide-range of behaviours dependant on hippocampal, amygdala and prefrontal-cortex function.

Previous studies have reported early emerging deficits from 3 months of age in the Tg2576 model including impaired spontaneous alternation in the Y-maze, water maze retention in the water maze probe test, circular platform performance, memory retention of a footshock in the passive avoidance test, and hyperactivity in the open field and Y-maze (Hsiao et al., 1996; King et al., 1999). The absence of Aβ pathology reported before 4 to 5 months of age (Westerman et al., 2002) would suggest the involvement of other pathological processes. Supporting this, progressive synaptic deficits have been reported from 4 months of age, including a reduction in dendritic spine density, LTP impairment and basal synaptic transmission deficit in DG and CA1 hippocampal regions (Lanz, Carter & Merchant, 2003; Jacobsen et al., 2006). Moreover, a non-significant 8% reduction in dendritic spine density was also reported from 2 months of age (Jacobsen et al., 2006), with a significant 6% loss occurring between 2 and 4.5 months (Lanz, Carter & Merchant, 2003). The progressive worsening of such deficits until 18-20 months of age would suggest the involvement of Aβ-related pathology. Corroborating these early changes, Klingner et al. (2003) reported cholinergic and noradrenaline neurotransmitter receptor alterations at 5 months. Although the underlying mechanisms of these early changes remain unclear, it is evident that pathological processes begin from an early age which is related to early-emerging behavioural deficits.

Consistent with reports of early-emerging behavioural changes, experiment 4 revealed transgene-related deficits in the elevated plus maze and marble burying task at 3 months of age. Furthermore, experiment 5 revealed impaired performance in the foraging task at 3 months of age. These deficits were reported again between 10 and 10.5 months of age, suggesting a robust impairment in these tasks. Together these results suggest impaired hippocampal- and amygdala-function from an early age, before and during Aβ deposition. These changes therefore support the need for investigation of early-stage therapeutic

strategies. The transgene-deficit in the foraging task at 10.5 months was consistent with previous reports of spatial memory impairments around this age in several tasks such as the Morris water maze, T-maze, paddling Y-maze and appetitive Y-maze (Hsiao et al., 1996; Barnes, Hale & Good, 2004; Deacon et al., 2008; Adriani et al., 2006). The results of these experiments therefore highlight the potential use of 2 pre-existing tasks and 1 novel task in detecting transgene-related deficits from an early age in the Tg2576 model.

Experiment 6 was one of the few assessments of PFC-dependant function in Tg2576 mice, and used three tasks never before assessed in this model. Firstly, the results interestingly revealed increased response rates in Tg2576 mice, suggestive of hyperactivity which was consistent with previous reports in other tasks (e.g. King & Arendash, 2002). Analysis of discrimination ratio data from these experiments overall showed that PFC-dependant executive function is intact in Tg2576 mice at 5.5 to 9 months of age. The biconditional discrimination results were supported by previous studies, whereby Tg2576 mice showed intact acquisition of a simple discrimination, compound discrimination, contextual biconditional left-right discrimination, intramaze brightness discrimination and a simple room discrimination at 6 and 12-14 months (Zhuo et al., 2007, 2008; Barnes, Hale & Good, 2004). In contrast, intact Tg2576 performance during incongruent trials of the Stroop test was contrary to predictions. Intact reversal learning was also contrary to predictions and inconsistent with some literature of reversal learning impairments at 6 and 7 months of age (Zhuo et al., 2007, 2008; Pompl et al., 1999). In contrast however, some studies supported our results by showing no reversal learning deficits in other tasks at 11 and 14 months of age (Dong et al., 2005; Zhuo et al., 2007). Together, it was suggested that reversal learning deficits may be associated with early, not late, stages of pathology. This questions to what extent Aβ pathology affects executive function in Tg2576 mice. Overall, this highlights the need for further examination of PFC-function in Tg2576 mice.

With regards to DHA supplementation, further investigation was required in the Tg2576 model as previous studies failed to assess the effect of early-stage intervention or assess DHA supplementation *per se* relative to a suitable baseline control diet. The detection of synaptic changes and behavioural impairments from as early as 3 months of age in the Tg2576 model highlighted the need for early intervention strategies. This was supported by the experiments in this chapter. The second aim of this chapter was therefore to examine the effect of DHA supplementation by comparing it to a suitable baseline control diet which had very similar

levels of omega-6 PUFAs and total fatty acids, with compensatory increases in 'neutral' fatty acids to substitute the DHA content in the DHA diet. The primary difference between the diets was therefore the addition of DHA and an increase in the ω-3/ω-6 ratio caused by the DHA supplementation.

Experiments 4 and 5 revealed some evidence that DHA supplementation reduced transgene-related deficits in Tg2576 mice at 3 months of age in the elevated plus maze, marble burying task and the foraging task. These results therefore suggest that dietary DHA may target very early pathological processes emerging prior to Aβ development. Secondly, the results suggest that DHA may ameliorate dysfunction specific to hippocampal and amygdala regions, which are thought to control such behaviour (e.g. Hsiao et al., 1996; LeDoux, 2000; Deacon & Rawlins, 2005). Positive effects of DHA supplementation on PFC-dependant executive function were also found in mice aged 5.5 to 9 months in experiment 6. Although this effect was primarily generic to both Tg and WT mice, some results suggested that this was specific to Tg mice. In contrast to previous experiments, DHA supplementation interestingly enhanced Tg2576 performance when a transgene-deficit was not present. Moreover, DHA supplementation enhanced performance in Tg mice above WT levels. At the later age of 10 months, the positive effect of DHA supplementation in experiments 4 and 5 were less prominent. Although the elevated plus maze task provided strong evidence that DHA supplementation reduced deficits in Tg mice and enhanced WT performance, no diet effects were evident in the marble burying task. Some positive effects of DHA supplementation in reducing a transgene-deficit was also observed in the foraging task, although these were limited to a reduction in the number of total and consecutive return errors. Although no significant effects of DHA were found on the other measures, a non-significant reduction in Tg deficits was observed.

Together, these results suggest that DHA supplementation can provide some protection against transgene-induced deficits and improve normal cognitive performance at an early age in Tg2576 mice, but its efficiency reduced over time in 10-10.5 month old mice. Although these results may suggest that short-term dietary intervention is more effective than long-term intervention, the results from the T-maze experiment in chapter 3 conversely support the latter. Similarly, it could be argued that this chapter provides evidence that DHA targets early-stage pathological processes rather than later-stage pathology, although the results from experiment 1 again support the latter. Alternatively, the results from chapters 3 and 4 together

may suggest that intermediate stages of pathology (at around 10-12 months of age) are less altered by DHA supplementation. For example, DHA may effectively reduce soluble monomeric Aβ and amyloid plaques better than soluble oligomeric forms. However, DHA has been shown to effectively reduce oligomeric Aβ25-35 species *in vitro*, although oligomeric Aβ40 and Aβ42 have not been assessed (Hashimoto et al., 2009b). Further research into the effects of DHA on oligomeric Aβ40/Aβ42 would therefore be insightful.

In conclusion, the results from chapter 3 together with chapter 4 show some potential in using DHA supplementation as a therapeutic strategy for AD. However, these results have not been as robust as previous reports and it remains unresolved whether early-stage longitudinal intervention is an optimal strategy. Although a number of studies have provided strong evidence that DHA supplementation can ameliorate behavioural deficits and pathology in AD models, this has often been relative to a particularly poor control diet or a control diet which is not designed appropriately to assess the effect of DHA supplementation *per se*. Together, this may suggest that DHA supplementation may be particularly beneficial in replacement of a poor diet.

Since epidemiological evidence suggests that omega-3 PUFA or fish oil intake can reduce the risk of AD (e.g. Kalmijn et al., 1997a; Albanese et al., 2009), it is worth considering whether other aspects of these dietary sources are more important than DHA supplementation alone. For instance, fish oils contain a rich source of both omega-3 PUFAs EPA and DHA which may both be attributable to the positive effects observed in epidemiological studies. Furthermore, fish oil supplementation may be more important than algal sources of omega-3 PUFAs. Supporting this, several studies have reported positive effects of fish oil or EPA supplementation on behaviour and pathology in normal rodents or AD models (Suzuki et al., 1998; Song & Horrobin, 2004; Oksman et al., 2006; Hashimoto et al., 2009a; Ma et al., 2009; Arsenault et al., 2011). These aspects of omega-3 PUFA supplementation were therefore examined in chapter 5 to investigate whether this would be a more optimal strategy in targeting Aβ-induced behavioural deficits and pathology.

Chapter 5

The effect of fish oil and curcumin supplementation on the behaviour and pathology of Tg2576 mice

5.1 Introduction

The aim of the experiments reported in this chapter was to examine the effect of dietary supplementation with either fish oil (containing omega-3 PUFAs), curcumin, or their co-supplementation on the behavioural phenotype and pathology of Tg2576 mice. A number of epidemiological studies show dietary intake of fish to reduce the risk and incidence of AD (Kalmijn et al., 1997a; Barberger-Gateau et al., 2002, 2007; Morris et al., 2003; Larrieu et al., 2004; Huang et al., 2005; Schaefer et al., 2006; Albanese et al., 2009). Huang et al. (2005) provided evidence that oily but not lean fish were associated with risk, providing evidence that fish oils likely accounted for these positive effects. In support of this, several studies have shown fish oil to improve cognitive function in healthy rodents (Jensen, Skarsfeldt & Høy, 1996; Suzuki et al., 1998), reduce cognitive dysfunction and tau pathology in 3xTg AD models (Ma et al., 2009), and reduce Aβ pathology in the APPswe/PS1 model (Oksman et al., 2006). Fish oil supplementation can therefore target general cognitive processes and pathological mechanisms involved in AD.

A major nutritional feature of fish oils is the high levels of omega-3 PUFAs EPA and DHA. The findings in chapters 3 and 4 failed to provide compelling evidence of robust therapeutic benefit from DHA supplementation alone compared to an appropriate control condition. However, epidemiological findings implicate EPA content as important. Dietary supplementation of DHA and EPA was found to reduce synaptic deficits in an AD rat model (Kitajka et al., 2002) and reduce tau pathology, synaptic alterations and cognitive dysfunction in the 3xTg AD model (Arsenault et al., 2011). Moreover, Hashimoto et al. (2009a) demonstrated the importance of EPA alone in reducing Aβ-induced oxidative stress, cognitive dysfunction, synaptic alterations and neurotoxicity in an AD rat model. The EPA and DHA combination in fish oils therefore likely contribute to the positive effects observed in epidemiological and animal studies. Since fish oil supplementation has not been examined

in the Tg2576 mouse model, the experiments in this chapter investigated its effect on Aβ-pathology and its associated behavioural deficits in Tg2576 mice. Furthermore, since epidemiological studies are generally reflective of long-term intake, this chapter investigated longitudinal supplementation from an early age. In order to initiate treatment prior to pathological alternations, mice were given dietary supplementation from 2 months of age since no pathological, synaptic or behavioural deficits have been reported at that age in Tg2576 mice (Hsiao et al., 1996; Chapman et al., 1999; Lanz, Carter & Merchant, 2003; Jacobsen et al., 2006).

This chapter also aimed to investigate the effect of longitudinal curcumin supplementation from an early age in the Tg2576 model. Previous reports have shown 4 to 6 month dietary curcumin to reduce inflammation, oxidative stress, Aβ levels and plaque pathology in aged Tg2576 mice (Lim et al., 2001; Yang et al., 2005; Begum et al., 2008). In rat models of AD, 1-2 month dietary curcumin reduced plaque pathology, synaptic deficits, oxidative stress and cognitive dysfunction (Frautschy et al., 2001; Ishrat et al., 2009). Furthermore, Garcia-Alloza et al. (2007) reported 7-day i.v. injected curcumin was effective at reducing plaque pathology and dendritic abnormalities in aged APPswe/PS1 mice. Finally, Ma et al. (2009) found 4-month dietary curcumin reduced poor-diet induced (a high-fat diet rich in saturated fat and omega-6 PUFA) deficits including cognitive dysfunction, tau phosphorylation and abnormal insulin signalling in the 3xTg AD model. It is therefore evident that curcumin can target a number of pathological processes related to AD and their associated deficits. However, no study has examined the effect of curcumin supplementation prior to pathological development in transgenic models. For example, curcumin treatment was initiated in Tg2576 mice aged 10, 14.5 and 17 months when pathology is already manifest. The positive effects of curcumin raise the possibility that early intervention may prevent or delay the onset of cognitive and pathological changes induced by Aβ. This chapter will therefore present experimental evidence testing the hypothesis that early longitudinal treatment of curcumin in Tg2576 mice from 2 to 12 months of age will influence the cognitive and pathological phenotype in this APP model. This will be the first report of chronic curcumin treatment for greater than 6-months in any animal model of AD.

Since both fish oil and curcumin administration can reduce pathological processes of AD and associated deficits, it could be hypothesised that their co-supplementation may have synergistic beneficial effects. For example, these compounds may target differential

mechanisms which overall have more impact than individual supplementation. For instance, evidence showing curcumin to directly bind to aggregated Aβ may suggest that curcumin would be better at reducing plaque pathology than DHA (Yang et al., 2005; Garcia-Alloza et al., 2007). Alternatively these compounds may target similar mechanisms which may result in an exaggerated effect. Supporting this, synergistic effects in reducing AD-related pathology and deficits by combining supplements have been demonstrated with vitamin C and E combinations (Morris et al., 1998; Kontush et al., 2001; Zandi et al., 2004), herb combinations such as 'Zhokemei-to' (Williamson, 2001; Tohda et al., 2004), combined fish oil and the green tea extract EGCG (Giunta et al., 2010), and a DHA and catechin combination (Shirai & Suzuki, 2004).

Further to their potent pleiotropic effects individually, the author asserts that a combination of curcumin and fish oil may have synergistic effects by simply increasing their bioavailability. Humans and mouse studies have reported that lipid-curcumin formulations can provide higher plasma and brain levels of curcumin than unformulated curcumin, in addition to greater pharmacological effects in reducing oxidative stress and inflammation (Begum et al., 2008; Gota et al., 2010). Frautschy and Cole (2010) also developed a solid lipid-curcumin formulation which reportedly resolved bioavailability issues of curcumin (see Kelloff et al., 1996; Lao et al., 2006). The lipids present in the fish oil may therefore increase curcumin bioavailability and efficiency. Conversely, evidence also supports the idea that curcumin may increase bioavailability of omega-3 PUFA in the fish oils by providing antioxidant protection (Asai, Nakagawa & Miyazawa, 1999; Frautschy, Ma & Cole, 2009). Curcumin may not only maintain the level of omega-3 PUFA *in vivo* and prior to administration in the diets, but it may also limit the production of neurotoxic lipid oxidation products which can exacerbate pathology (Horrocks & Farooqui et al., 2004). Antioxidant protection may therefore be critical when administering lipids to pathological oxidative environments. Supporting this, Shinto et al. (2009) reported more effective stabilisation of cognitive decline in AD patients when fish oil supplementation was combined with the antioxidant alpha-lipoate in a pilot clinical trial. Curcumin and fish oil co-supplementation may therefore overcome factors that may otherwise limit their clinical efficiency.

This chapter therefore aimed to investigate the effect of a combination of dietary curcumin and fish oil on behaviour and pathology in the Tg2576 model. Supporting the potential advantages of this approach, Ma et al. (2009) reported this combination to have synergistic

effects in suppressing poor diet-induced activation of tau pathology, JNK, IRS-1 and cognitive deficits in 3xTg AD model mice. JNK and IRS-1 (insulin-resistant substrate 1) are involved in synaptic activity, insulin signalling, tau phosphorylation and Aβ-toxicity (as reviewed in Ma et al., 2009). Importantly, this effect was greater than reported in mice fed curcumin or fish oil alone, and the cognitive improvement in the Y-maze was reported after 1 month in contrast to 2 and 3 months in single compound treatments. Continuing their ongoing studies, Frautschy and Cole (2010) reported that this dietary combination can limit cognitive deficits and tau pathology in the Htau mouse model from 14 months of age when tau pathology and cognitive deficits are well developed (unpublished results). *In vitro* studies have also supported the synergistic effects of curcumin with omega-3 PUFAs DHA and EPA in reducing markers of inflammation and oxidative stress (Saw, Huang & Kong, 2010). This chapter provides the first report of curcumin and fish oil co-supplementation in the Tg2576 mouse model. Again, supplementation was initiated from an early age and was provided longitudinally throughout the course of pathological development until 12 months of age.

In order to investigate the effects of dietary supplementation from 2 months of age on behaviour and pathology in Tg2576 mice, behaviour was assessed using the marble burying and T-maze test. These measures were selected since chapters 3 and 4, as well as previous studies (e.g. Hsiao et al., 1996) have shown a rather selective hippocampal-related behavioural deficit in Tg2576 mice from an early age. Behaviour was first assessed from 8-9 months of age when Aβ pathology is developed and impairments are present in order to examine whether dietary treatment delayed the onset of deficits. Furthermore, both Tg2576 and WT mice were assessed to examine the potential treatment effects on general cognitive processes and pathological processes.

Ideally, more extensive behavioural testing would be carried out (similar to chapter 4) but the need for large cohorts of mice limited the number of behavioural tasks carried out. It was deemed particularly important to examine a large cohort (~20 mice per experimental group) since Tg2576 mice appear particularly susceptible to attrition and large numbers were required for multiple brain analysis (including immunohistochemistry, ELISA and lipid analysis). Moreover, it is argued that the small number of subjects in experimental groups used in previous studies reduced the validity of their findings (e.g. *n*=4-6 mice in Yang et al., 2005). This may have also contributed to the lack of DHA effect on Aβ measures in chapter 3 due to high variability; and so a large cohort of mice was examined in this chapter to prevent

such issues. Mice were culled at 12 months of age (following 10-month supplementation) for examination of Aβ pathology in the brain using both ELISA and immunohistochemical techniques. The 12-month time-point was selected in order to examine the effect on plaque-like 'amyloid body' pathology, since appreciable numbers of diffuse plaques were not shown histologically until 12 months of age (Kawarabayashi et al., 2001). Furthermore, this early period of amyloid deposition and beginning of plaque development between 6-12 months is thought to parallel early changes in the human AD brain (Funato et al., 1998).

It was hypothesised based on the evidence outlined above that supplementation of curcumin, fish oil and their combination would reduce behavioural deficits and Aβ pathology in Tg2576 mice. It was also predicted that co-supplementation of curcumin and fish oil would have a synergistic effect relative to their individual supplementation. Lipid analysis was carried out on brains at 12 months of age in order to examine dietary effects and ensure that omega-3 PUFAs were incorporated into brain fractions. This was important to determine as a lack of incorporation may explain a lack of diet effect on behavioural or pathology measures. Such an argument was suggested to explain the failure of omega-3 PUFA supplementation to reduce behaviour deficits in APPswe/PS1 mice (Arendash et al., 2007). Similar to chapter 3, it was hypothesised that fish oil supplementation would increase omega-3 PUFA levels in the brain.

5.2 Overview of the mouse cohort and the dietary intervention design used

The experiments in this chapter used one cohort of male Tg2576 transgenic (Tg) and non-transgenic (wildtype, WT) mice. The cohort of Tg2576 and WT mice used in this chapter were generated and maintained as outlined in chapter 2, section 2.2. At 2 months of age, mice were taken off standard laboratory mouse chow and were fed experimental diets containing either 2.5% oil blend (OB; containing 0% DHA and EPA, 0.19% total omega-3 PUFA, 1.65% total omega-6 PUFA, and an ω-3/ω-6 ratio of 0.10:1), 2.5% oil blend + 500ppm curcumin (OB+C; containing 0% DHA and EPA, 0.17% total omega-3 PUFA, 1.51% total omega-6 PUFA, and an ω-3/ω-6 ratio of 0.10:1), 2.5% fish oil (FO; containing 0.34% DHA, 0.53% EPA, 1.69% total omega-3 PUFA, 1.36% total omega-6 PUFA, and an ω-3/ω-6 ratio of 1.21:1), or 2.5% fish oil + 500ppm curcumin (FO+C; containing 0.34% DHA, 0.53% EPA, 1.67% total omega-3 PUFA, 1.36% total omega-6 PUFA, and an ω-3/ω-6 ratio of 1.19:1). Importantly, the diets containing fish oil supplementation (FO and FO+C) were similar in

lipid content, and the diets containing oil blend supplementation (OB and OB+C) were similar in lipid content. Furthermore, the difference in ω-3/ω-6 ratio between diets was caused almost entirely by the additional EPA and DHA content in the fish oil. Importantly, although there was a slight difference in total omega-6 PUFA levels between the oil blend and fish oil containing diets, this was minimal compared with previous studies investigating fish oil supplementation whereby the control diet contained high levels of omega-6 PUFAs (e.g. Oksman et al., 2006; Ma et al., 2009). Furthermore, the absence of DHA and EPA in the control oil blend diet was primarily compensated by "neutral" fatty acids including monounsaturated fatty acids and smaller amounts of saturated fatty acids. Further details concerning these diets are outlined in chapter 2, section 2.3. The experimental schedule employed for chapter 5 is outlined in Figure 5.1.

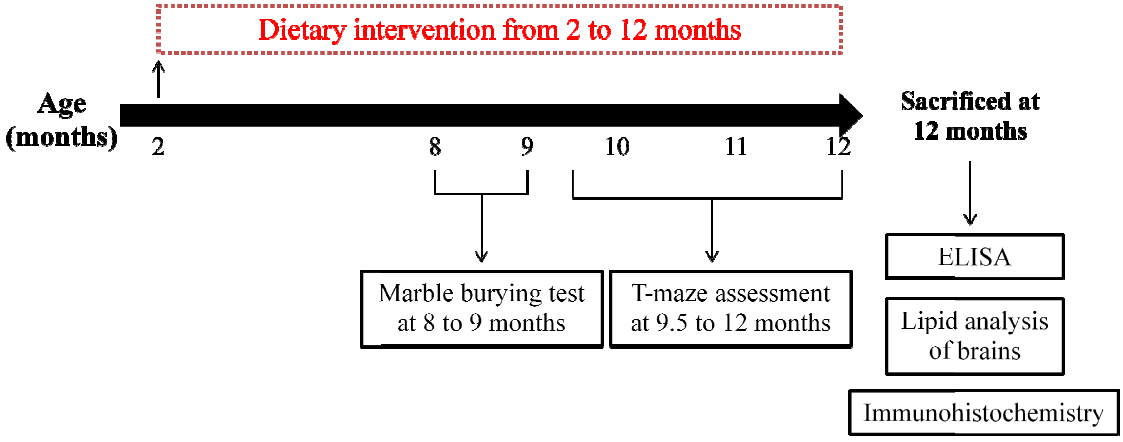

Figure 5.1 Experimental schedule employed in chapter 5.

Due to the large number of mice used in each experimental group and the practical issues of testing large cohorts, a control group of WT mice fed a FO+C diet was not included in the following experiments of this chapter. Importantly, although the Tg FO+C group could not be compared to a WT FO+C group, a comparison could be made to the Tg FO and Tg OB+C groups in order to ascertain any affects of fish oil and curcumin co-supplementation on Tg behaviour and pathology relative to a curcumin or fish oil diet alone. This comparison of Tg mice was particularly important, whereas a WT comparison was supplementary to the main experimental aims.

In order to detect whether genotype and diet altered markers of general health, mouse body weights were taken at least every month from 1 month of age and presented in Figure 5.2. As

can be observed, transgenic mice consistently had lower body weights compared to wildtype mice across their lifespan; a pattern that becomes more apparent with increasing age. Furthermore, dietary supplementation appears to not affect body weight. This apparent genotype effect is consistent with previous reports in the Tg2576 model (e.g. Toda et al., 2011) and in AD patients, which show weight loss compared to a non-demented population (Poehlman & Dvorak, 2000). This data suggests that pathological changes, particularly Aβ, lead to a weight loss phenotype. This may perhaps be caused by metabolic alterations or associated with low dietary intake. However, Toda et al. (2011) reported no differences in food intake or metabolic parameters (mean time course of oxygen consumption rates and respiratory quotients) in Tg2576 mice compared to controls at 6 to 7 months fed a standard diet. Further research into this phenotype is therefore required, and it is suggested that future studies examining dietary supplementation should monitor food consumption levels. A lack of obvious change detected in body weights between mice fed different experimental diets importantly suggested no difference in metabolic factors between diets and also suggest no particular aversion to specific diets.

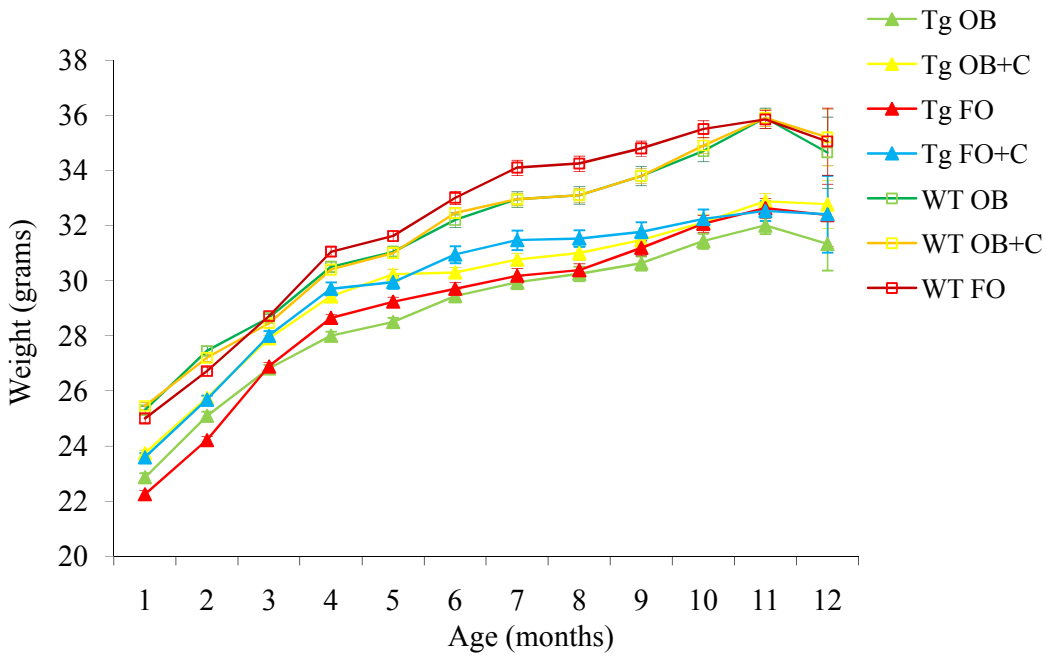

Figure 5.2 Body weight profile of Tg and WT mice from 1 month of age. Mice were fed standard chow from birth to 2 months of age, and then experimental diets from 2 months of age. Values are mean weight (grams) ± S.E.M.

Similar to the previous experimental chapters, a between-subjects ANOVA with factors of genotype (Tg, WT) and diet (OB, OB+C, FO, FO+C) was used to analyse the data. Further details and justification for this analysis is outlined in chapter 2, section 2.4. It is important to note however that the exclusion of the WT FO+C group in this analysis would likely alter the error term (the value of the F statistic; since this is fixed by the degrees of freedom) and therefore potentially result in a Type I or II statistical error (Kinnear & Gray, 2009). In order to validate the accuracy of the between-subjects ANOVA results, a one-way ANOVA with factor of group (Tg OB, Tg OB+C, Tg FO, Tg FO+C, WT OB, WT OB+C, WT FO) and post-hoc Tukey test was used to confirm the statistical results, since this statistical test would have an accurate error term. No discrepancies were found in the results of experiments 7 to 9 and so the between-subjects ANOVA results were reported. In contrast, the between-subjects ANOVA results for experiment 10 appeared inaccurate and differed from the one-way ANOVA analysis. The one-way ANOVA results were therefore reported in experiment 10 since these reflected the pattern of results more accurately and prevented error term problems.

5.3 Experiment 7: Marble burying assessment

5.3.1 Introduction

In order to assess the effects of early dietary intervention on the behavioural phenotype of Tg2576 mice, Tg and WT mice fed the experimental diets were assessed in the marble burying test at 8 to 9 months of age when pathology is present. Since experiment 4 (chapter 4) reported genotype and DHA-diet effects in this task, it was therefore argued that the marble burying test would be a suitable task to probe anxiety-related emotional deficits and should be sensitive to detect diet-induced changes. Based on the marble burying results in experiment 4 and reports of reduced anxiety in the elevated plus maze, open field and holeboard task (e.g. Lalonde et al., 2003; Deacon et al., 2008, 2009; Ognibene et al., 2005), it was hypothesized that Tg2576 mice would show deficits in the marble burying test at 8 to 9 months of age.

Although no study has examined the effect of fish oil supplementation in Tg2576 mice, Ma et al. (2009) reported fish oil to reduce a hippocampal-dependant spatial memory deficit in the Y-maze in the 3xTg AD model. Since the marble burying test is hippocampal-dependant, it was hypothesized that fish oil supplementation would reduce a marble burying deficit in

Tg2576 mice. Further support for this was provided by the positive effects of omega-3 PUFA DHA and EPA supplementation in reducing pathologically-induced behavioural deficits (e.g. Calon et al., 2004; Oksman et al., 2006; Arsensault et al., 2011). It was also hypothesized that curcumin supplementation would reduce marble burying deficits in Tg2576 mice since curcumin supplementation has been shown to reduce cognitive deficits in other animal models of AD (e.g. Frautschy et al., 2001; Ishrat et al., 2009; Ma et al., 2009). Similarly, it is hypothesized that fish oil and curcumin co-supplementation would reduce marble burying deficits in Tg2576 mice since studies have reported this combination to reduce cognitive deficits in the triple transgenic and Htau model of AD (Ma et al., 2009; Frautschy & Cole, 2010). Importantly, this is the first study to directly assess these supplements on behaviour (and specifically marble burying) in the Tg2576 model.

5.3.2 Methods

One cohort of male Tg2576 transgenic (Tg) and wildtype (WT) mice were evaluated in the marble burying task at 8 to 9 months, when plaque pathology was first developing. This design therefore investigated transgene- and diet-dependant changes. A minimum of 14 mice from each experimental group were tested (Tg OB n=14, Tg FO n=16, Tg OB+C n=14, Tg FO+C n=16, WT OB n=19, WT FO n=20, WT OB+C n=21). In addition to these numbers, 6 mice were excluded from the experiment due to circling behaviour which prevented reliable involvement in the task (WT OB n=1, Tg OB n=2, Tg OB+C n=1, Tg FO+C n=2). The running order of mice was counterbalanced so that one mouse from each experimental group of mice was tested after another as far as possible, which was run blindly to the experimenter. Mice were tested only once, with all testing occurring over 4 consecutive days. The apparatus and procedure used in this experiment was as described under the marble burying headings in chapter 4 sections 4.3.2.

5.3.3 Results

Figure 5.3 shows the mean number of unburied marbles in the marble burying test for each experimental group at 8 to 9 months of age. Statistical analysis using an ANOVA with genotype (Tg or WT) and diet (OB, OB+C, FO, FO+C) as factors revealed a significant main effect of genotype ($F(1,113)=41.162$, $p<0.001$), no significant main effect of diet ($F(3,113)=0.035$, $p>0.05$) and no significant interaction of genotype by diet ($F(2,113)=0.825$,

p>0.05). This genotype effect is clearly illustrated in Figure 5.3, whereby Tg groups left an average of approximately 14 marbles unburied in contrast to ~6 marbles in the WT groups. The experimental diets appeared to have no effect in Tg or WT mice.

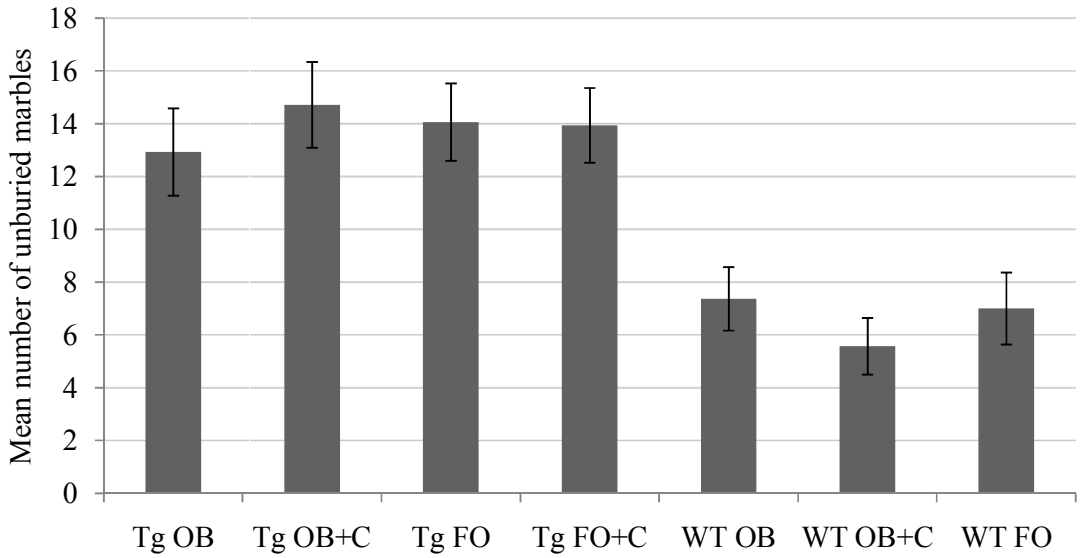

Figure 5.3 Mean number of unburied marbles (20 marbles in total) in the marble burying test at 8 to 9 months in Tg and WT mice fed the oil blend (OB), oil blend + curcumin (OB+C), fish oil (FO) and fish oil + curcumin (FO+C) diets. Values are mean scores ± S.E.M.

5.3.4 Discussion

The results of the marble burying experiment showed Tg2576 mice left significantly more marbles unburied than WT mice at 8 to 9 months of age. This study therefore corroborates previous results in experiment 4 providing evidence that the APPswe mutation leads to marble burying deficits in Tg2576 mice. This reduction in marble burying was also consistent with previous reports of reduced anxiety in Tg2576 mice in other anxiety-related tasks such as the elevated plus maze (e.g. Gil-Bea et al., 2007). Importantly, WT mice buried a higher number of marbles, which is consistent with normal marble burying behaviour in rodents (e.g. Deacon, 2006; Nicolas, Kolb & Prinssen, 2006).

Interestingly, this experiment provided no evidence that fish oil, curcumin or a combination of fish oil and curcumin affected marble burying behaviour in either Tg or WT mice. This

was in contrast to the prediction that that these supplements would reduce the marble burying deficit in Tg2576 mice. As outlined in the introduction of this experiment, multiple studies provide evidence that these supplements can reduce cognitive impairments in animal models of AD. However, these cognitive impairments were found in tasks assessing spatial memory, not anxiety-related behaviour (e.g. Frautschy et al., 2001; Ishrat et al., 2009; Hashimoto et al., 2009a; Ma et al., 2009; Frautschy & Cole, 2010).

It could also be argued that the lack of dietary effect in this experiment at 8 to 9 months is age-specific. For example, it is possible that the dietary supplements may have been more potent at earlier stages of pathological development. This argument may be supported for the fish oil supplement in particular as results from experiment 4 found that 1.86% DHA supplementation effectively reduced anxiety deficits at 3 months, with less convincing results at 10 months of age. It is possible that the reduced levels of omega-3 PUFA or DHA in the fish oil diet compared to the DHA diet in chapters 3 and 4 contributed to this lack of effect. Alternatively, it could be argued that the dietary supplements may be more effective at reducing behavioural deficits during advanced stages of pathology when there are greater levels of Aβ. Indeed, this could be supported by the majority of studies investigating omega-3 PUFA and curcumin supplementation in animal models of AD including Tg2576 mice, which have shown a robust reduction in Aβ pathology during advanced stages of disease (e.g. Frautschy et al., 2001; Lim et al., 2001; Calon et al., 2004; Lim et al., 2005; Yang et al., 2005; Cole & Frautschy, 2006; Begum et al., 2008; Garcia-Alloza et al., 2007). Unfortunately, the affect of age cannot be determined in this study as longitudinal behavioural assessment was not carried out. Longitudinal investigation of these supplements from early to advanced stages of disease in the Tg2576 model should therefore be carried out in future experiments.

5.4 Experiment 8: T-maze assessment

5.4.1 Introduction

This experiment aimed to assess the effect of supplementary fish oil, curcumin, and their combination from an early age of 2 months on spatial learning and memory in Tg2576 and WT mice aged 9.5 to 12 months. As detailed in experiment 1 (chapter 3), the assessment of spatial memory and learning was important as this is particularly affected in Tg2576 mice

and other models of AD. The T-maze forced choice alternation (FCA) task was selected as experiment 1 and previous studies have revealed Tg2576 mice to show deficits after 8 months of age, at 12 and 16 months (Chapman et al., 1999; Corcoran et al., 2002; Barnes, Hale & Good, 2004). Furthermore, experiment 1 found the T-maze task was sensitive in detecting dietary effects, albeit at 16 months. This robust task, sensitive to both the APPswe mutation and dietary effects, was therefore selected to examine the effect of the dietary supplements in Tg2576 and WT mice.

Firstly, it was hypothesised that Tg2576 mice would be impaired in the T-maze FCA task at 9.5 to 12 months of age based on results from experiment 1 and previous literature. Secondly, it was hypothesized that dietary supplementation of fish oil, curcumin, and a combination of fish oil and curcumin would reduce these spatial memory deficits in the T-maze. This hypothesis was based on evidence showing fish oil supplementation to ameliorate a spatial memory deficit in the Y-maze in the 3xTg AD model (Ma et al., 2009). This is further supported by studies of DHA and EPA supplementation showing reduced spatial memory deficits in rodent AD models (e.g. Calon et al., 2004; Hashimoto et al., 2009a; Hooijmans et al., 2009). The hypothesis was also based on studies reporting fish oil and curcumin co-supplementation reduced cognitive deficits in the triple transgenic and Htau model of AD (Ma et al., 2009; Frautschy & Cole, 2010). Furthermore, evidence has shown curcumin to reduce cognitive deficits in animal models of AD (e.g. Frautschy et al., 2001; Ishrat et al., 2009; Ma et al., 2009). This is the first experiment to assess these dietary supplements on spatial learning and memory in the Tg2576 mouse model.

5.4.2 Methods

One cohort of naïve male Tg2576 transgenic (Tg) and wildtype (WT) mice were evaluated in the T-maze forced-choice alteration (FCA) task at 9.5 to 12 months of age when plaque development is observed. Mice were placed on experimental diets from 2 months of age. This design therefore investigated transgene- and diet-dependant changes. The cohort of mice used in this experiment consisted a minimum of 16 mice per experimental mouse group (Tg OB n=16, Tg OB+C n=15, Tg FO n=16, Tg FO+C n=18, WT OB n=18, WT OB+C n=20, WT FO n=20). The running order of mice was counterbalanced so that approximate equal numbers of each experimental group of mice were run in each batch (n=8), run blind to the

experimenters, with several batches of mice being tested once per day over 8 consecutive days. Mice were maintained on water restriction throughout this experiment.

The apparatus and procedure was the same as described in chapter 3 section 3.3.2, except mice were tested for a period of 8 consecutive days rather than 10 days as performance stabilised by day 8. The experiment was carried out by the author with help from a research assistant (John Anderson), with all analysis carried out by the author. T-maze FCA performance was calculated as a percentage of correct trials (successful alternations) of the six sessions conducted per day.

5.4.3 Results

Figure 5.4 shows the percentage of correct trials for both Tg and WT mice fed the experimental diets during acquisition of the T-maze FCA task at 9.5 to 12 months of age. An ANOVA with genotype (Tg or WT) and diet (FO, OB, OB+C, FO+C) as between-subject factors and day (1-8) as a within-subject factor revealed a significant effect of genotype ($F(1,116)=68.273$, $p<0.001$), no significant effect of diet ($F(3,116)=0.567$, $p>0.05$) and no significant interaction between genotype and diet ($F(2,116)=0.165$, $p>0.05$). Statistical analysis also showed a significant effect of day ($F(7,812)=17.389$, $p<0.001$), a significant interaction of day by genotype ($F(7,812)=3.433$, $p<0.001$), no significant interaction of day by diet ($F(21,812)=0.917$, $p>0.05$) or day by genotype by diet ($F(14,812)=0.649$, $p>0.05$). A follow-up test of simple main effects for the significant day by genotype interaction revealed that WT mice performed significantly better than Tg mice on days 2 to 8 (smallest, $F(1,121)=17.98$, $p<0.001$). As illustrated in Figure 5.4, these results indicate that Tg mice perform significantly worse than WT mice, and that the dietary supplements failed to alter performance.

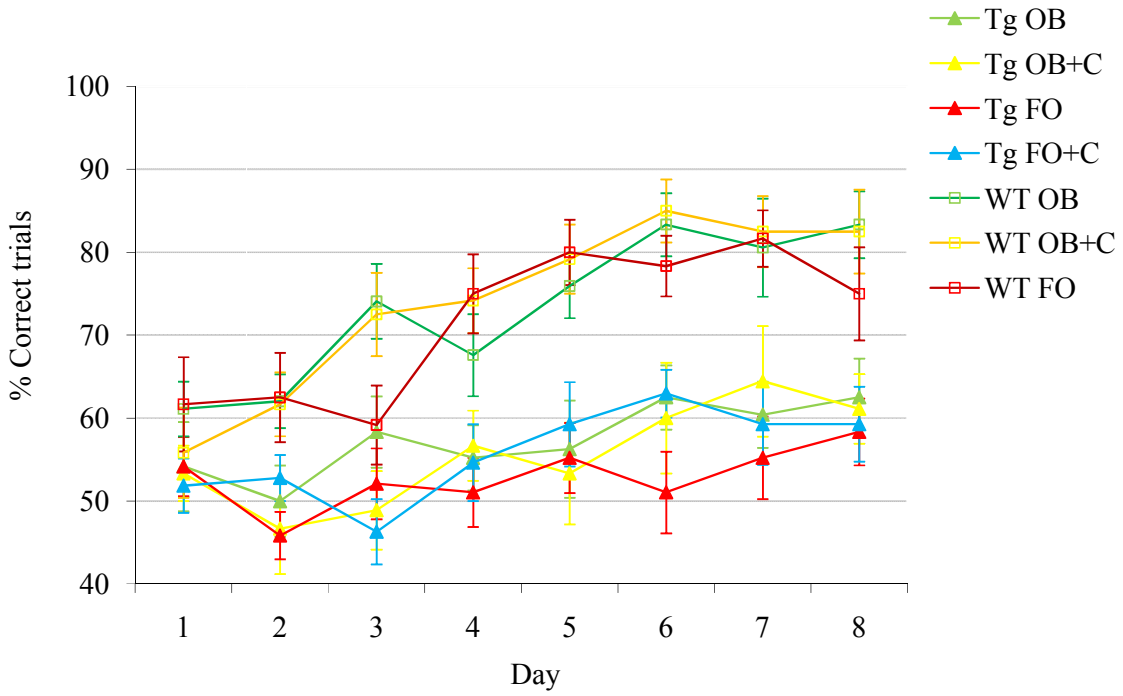

Figure 5.4 T-maze forced choice alternation task performance at 9.5 to 12 months of age. Mean percentage of correct trials over 8 days acquisition in Tg and WT mice fed the oil blend (OB), oil blend + curcumin (OB+C), fish oil (FO) and fish oil + curcumin (FO+C) diets. Values are mean scores ± S.E.M.

5.4.4 Discussion

The T-maze FCA task revealed Tg mice to be significantly impaired relative to WT control mice at 9.5 to 12 months of age, which supports previous findings (e.g. Chapman et al., 1999; Corcoran et al., 2002; Hale & Good, 2005; Barnes, Hale & Good, 2004; Zhuo et al., 2007). This experiment did not reveal any main effects of diet or interactions, thereby illustrating that the dietary supplementation of fish oil, curcumin or a combination of fish oil and curcumin failed to alter performance in Tg or WT mice. This experiment therefore did not provide any evidence that these supplements improved spatial working memory or learning. These results were not expected as previous studies have shown fish oil, curcumin and their combination to improve spatial memory in animal models of AD (e.g. Frautschy et al., 2001; Ishrat et al., 2009; Hashimoto et al., 2009a; Ma et al., 2009; Frautschy & Cole, 2010). A number of differences between these studies may have accounted for the discrepancies in results, such as the animal model and its underlying pathology, the experimental diets (e.g.

the amount of supplementation, the control diet) and the stage of experimentation. These factors will be considered in more detail in the chapter discussion.

Interestingly, this lack of diet effect was consistent with experiment 7 which showed that these dietary supplements failed to ameliorate marble burying deficits in Tg2576 mice at 8 to 9 months of age. Furthermore, the failure of the fish oil supplement in ameliorating the spatial memory deficit in Tg2576 mice was similar to the results in experiment 1 which showed that DHA supplementation failed to ameliorate T-maze deficits in Tg2576 mice aged 12 months (but showed some success at 16 months of age). It is therefore possible that these supplements may only be effective at reducing deficits during more advanced stages of pathology. This argument is supported by previous studies showing cognitive benefit of omega-3 PUFA/fish oil, curcumin, and its combination in animal models of AD during more advanced stages of pathology (e.g. Calon et al., 2004; Ma et al., 2009; Frautschy & Cole, 2010). Alternatively, it could be argued that the lack of diet effect in this experiment could be a result of the T-maze failing to sufficiently detect more subtle changes in spatial memory. Other spatial memory tasks, such as the radial arm maze which contains more arm choices, could therefore be employed in future research to probe perhaps more subtle effects caused by dietary supplementation.

5.5 Experiment 9: Pathological assessment

5.5.1 Introduction

This experiment aimed to investigate the effect of longitudinal dietary supplementation of curcumin, fish oil and their combination from an early age on Aβ pathology in Tg2576 mice aged 12 months. This study will provide the first evidence investigating the effect of early curcumin supplementation on Aβ pathology in Tg2576 mice. Previous studies have shown dietary curcumin (160ppm and 500ppm) to reduce amyloid plaque burden or deposits, as well as reduce soluble and/or insoluble Aβ levels in Tg2576 mice aged 16-22 months (Lim et al., 2001; Yang et al., 2005; Begum et al., 2008). Curcumin supplementation has also been shown to reduce amyloid plaque burden in the aged APPswe/PS1 mice and an Aβ-infused rat model (Frautschy et al., 2001; Garcia-Alloza et al., 2007). Interestingly however, Garcia-Alloza et al. (2007) found curcumin did not alter soluble and insoluble Aβ40 and Aβ42 levels in APPswe/PS1 model, but reduced plaque size and number. Supporting the Aβ-reducing

effect, *in vitro* studies have shown curcumin to inhibit Aβ40 and Aβ42 fibril formation and aggregation, Aβ extension and disaggregate pre-aggregated Aβ (Ono et al., 2004; Yang et al., 2005; Begum et al., 2008).

This study will provide the first evidence investigating the effect of fish oil supplementation, and fish oil plus curcumin co-supplementation on Aβ pathology in Tg2576 mice. Only two studies have examined the effect of fish oil in AD models, with Ma et al. (2009) failing to report effects of fish oil on Aβ pathology in the 3xTg model and Oksman et al. (2006) reported fish oil to reduce Aβ42 but not plaques in the APPswe/PS1 model relative to a poor diet. Similarly, only one study has reported the effects of fish oil and curcumin co-supplementation, but Ma et al. (2009) failed to report effects of fish oil on Aβ pathology in the 3xTg model. It is therefore evident that further investigation is required into the effects of these supplements on Aβ pathology.

In order to investigate whether supplementation of these diets reduced Aβ pathology in Tg2576 mice, brain samples of mice aged 12 months were analysed by immunohistochemistry and ELISA methods. In brief, immunohistochemistry is a biochemical method commonly used to visualise proteins in tissue sections. Indirect immunohistochemistry was used which process involves incubation of tissue sections in a primary rabbit antibody which binds to the target antigen (Aβ1-40 or Aβ1-42), which is followed by incubation with a biotinylated secondary anti-rabbit antibody which binds to the primary antibody. The secondary antibody is anti-rabbit so it is against the immunoglobulin (IgG) of the species in which the primary antibody was raised. Subsequent incubation in an avidin-biotinylated enzyme complex (ABC complex) and 3'3'-diaminobenzidine (DAB) is used to stain the secondary antibody, which therefore corresponds to staining of the antigen (Aβ1-40 or Aβ1-42) to allow visualisation in tissue sections.

ELISA analysis allows precise quantification of soluble and insoluble Aβ40 and Aβ42 levels in the hippocampus, whereas immunohistochemistry allows visualisation of Aβ40 and Aβ42 aggregate deposits throughout multiple brain regions. Both assessments would therefore ensure a comprehensive assessment of Aβ pathology. Furthermore, it was deemed important to assess plaque-like amyloid body deposits in addition to Aβ levels measured by ELISA as one biochemical method does not necessarily predict the results of another. For example, Garcia-Alloza et al. (2007) reported curcumin supplementation reduced plaque burden but

not soluble and insoluble Aβ40 and Aβ42 levels in the APPswe/PS1 model. Similarly, Oksman et al. (2006) reported fish oil supplementation reduced total Aβ42 levels, but had no effect on plaque load.

Levels of Aβ were measured in the hippocampus as this was one of the most pathologically affected brain regions in Tg2576 mice and correlates best with the behavioural assessments in this chapter which were hippocampal-dependant measures. It was deemed important to measure both Aβ40 and Aβ42 isoforms, as well as soluble and insoluble extracts as a reduction in one does not necessarily predict the other, and can shed light on the potential mechanisms of the intervention. For example, Lim et al. (2005) reported DHA to reduce total insoluble Aβ (both Aβ40 and Aβ42) and plaque burden, but not soluble Aβ, perhaps suggesting that DHA supplementation facilitates plaque clearance. Pathology was assessed in Tg2576 mice aged 12 months in order to assess Aβ pathology at a relatively early time point, but during a time when both soluble and insoluble species would be present, in addition to plaques. Although plaque pathology would not be substantial at 12 months, Kawarabayashi et al. (2001) reported that histological detection of diffuse plaques should be evident at this age, and so dietary effects may be observed. Furthermore, this period of early amyloid deposition and plaque development between 6 and 12 months was thought to parallel early changes in the human AD brain (Funato et al., 1998, as stated in Kawarabayashi et al., 2001). Important to the fish oil supplement in particular, this age is likely to mimic the time-point in epidemiological studies when positive effects of omega-3 supplementation has been reported in MCI and early AD patients.

Although this chapter has provided no evidence that the dietary supplements can ameliorate behavioural deficits in Tg2576 mice, it was important to evaluate the effect of the dietary supplements on underlying amyloid pathology as one does not consistently predict the other. For example, Oksman et al. (2006) reported DHA supplementation to reduce Aβ levels but not ameliorate spatial memory deficits. In contrast, Calon et al. (2004) and Arsenault et al. (2011) reported that DHA altered cognitive measures but not Aβ pathology. Although it may be hypothesised, based on the behavioural results, that the dietary supplements would fail to alter Aβ levels, it is possible that the dietary supplements may affect Aβ pathology without translation to the behavioural phenotype.

Based on the previous results surrounding curcumin supplementation on Aβ pathology in AD models, it was hypothesised that curcumin supplementation would reduce Aβ pathology in Tg2576 brains. Since only one study has reported fish oil supplementation to reduce Aβ42 but not plaque pathology in the APPswe/PS1 model (Oksman et al., 2006), it could be hypothesised that the same effect could be observed in Tg2576 mice. However, since fish oils contain omega-3 PUFAs, and omega-3 PUFA/DHA supplementation has been shown to reduce Aβ pathology in Tg2576 mice and other AD models (Lim et al., 2005; Oksman et al., 2006; Green et al., 2007; Hooijmans et al., 2007, 2009), it was hypothesised that fish oil supplementation would reduce Aβ pathology. Further supporting this, a reduction of Aβ40 and Aβ42 levels, as well as oligomeric species and mature fibrils by DHA has also been shown *in vitro* (Lukiw et al., 2005; Oksman et al., 2006; Hashimoto et al., 2009b). Finally, no study has directly assessed the effect of a combination of fish oil and curcumin on Aβ pathology in any AD model. Based on evidence from curcumin and fish oil/omega-3 PUFA/DHA supplementation studies individually, it was hypothesised that the combined supplement would reduce Aβ pathology in Tg2576 mice. Supporting this, Ma et al. (2009) provided evidence that fish oil and curcumin co-supplementation may target Aβ pathology by inhibition of JNK.

5.5.2 Methods

5.5.2.1 Design and subjects

Soluble and insoluble human Aβ1-40 and Aβ1-42, expressed from the APPswe transgene, were quantified by ELISA using left-hemisphere hippocampal samples of Tg2576 mice aged 12 months fed the oil blend, oil blend +curcumin, fish oil, and fish oil + curcumin diets. A minimum of 11 Tg mice were analysed from each diet group (Tg OB n=11, Tg FO n=12, Tg OB+C n=12, Tg FO+C n=14) following the T-maze FCA alternation task. The remaining mice from experimental groups were assigned for lipid analysis and immunohistochemistry analysis of brain samples. Immunohistochemistry was carried out on left-hemisphere brain samples from a minimum of 3 Tg mice and 1 WT mouse from each experimental group (Tg OB n=3, Tg FO n=4, Tg OB+C n=3, Tg FO+C n=4, WT OB n=1, WT FO n=1, WT OB+C n=1). Since WT mice do not possess the human APPswe transgene and therefore do not develop Aβ pathology, brain sections were used as negative controls for immunohistochemistry. WT mice were not systematically assessed using the ELISA protocol

due to their lack of pathology, although one WT mouse from each diet group was analysed to confirm this.

5.5.2.2 ELISA procedure

Mice were culled by cervical dislocation. The brain was immediately removed and maintained on ice while dissecting the cortex, hippocampus and cerebellum from the left and right hemispheres. The dissected tissue samples were immediately weighed and snap-frozen in liquid nitrogen. These samples were then stored at -80ºC until ready to be analysed. The hippocampus from the left hemisphere was analysed for Aβ pathology by ELISA, while the cortex from the left hemisphere was used for lipid analysis (experiment 10). The left-hemisphere sample was selected for ELISA analysis to match the left hemisphere selected for immunohistochemistry analysis. Samples from the right hemisphere were reserved for additional future research.

The samples were analysed as described in chapter 3 section 3.4.2, including protein extraction, protein assay and ELISA techniques. All aspects of data collection were carried out by the author with assistance from a research associate (Dr. Cécile Bascoul-Colombo).

5.5.2.3. Immunohistochemistry procedure

Tissue collection and preparation

In order to collect the brain samples for immunohistochemistry, mice received a lethal i.p. injection of 0.2ml sodium pentobarbitone (Euthatal®) and exsanguinated by intra-cardiac perfusion of 80ml 0.01M PBS pH7.4. Perfusion was carried out through cannula insertion into the left cardiac ventricle and incision to the right cardiac atrium, followed by perfusion with PBS using a perfusion pump for approximately 2 minutes until exsanguinated. The brain was immediately removed and maintained on ice to dissect the left and right hemispheres. The right hemisphere was dissected further to collect hippocampal, cortex and cerebellum samples for lipid analysis (experiment 10), then snap-frozen in liquid nitrogen and stored at -80ºC until use. The left hemisphere, used for immunohistochemical analysis, was fixed by transferring into cold 4% (w/v) paraformaldehyde in 0.01M PBS for 24 hours at 4ºC. Following this, the left hemisphere was transferred to 25% (w/v) reagent grade sucrose in

dH_2O for 48 hours at 4°C until the tissue sank. Tissue was then mounted on a freezing microtome at -25ºC and cut into 40µm coronal sections, which were stored in cryoprotectant (300g analytical reagent grade sucrose, 10g molecular biology grade polyvinylpyrrolidone and 300ml ethylene glycol in 500ml 1M PBS pH 7.4) at -20ºC until required for immunohistochemistry analysis.

Immunohistochemical staining and slide preparation

Immunohistochemical staining for Aβ1-40 and Aβ1-42 species was carried out identically using the procedure below but with different primary antibodies for Aβ1-40 and Aβ1-42 (as specified in the procedure). This process was carried out by the author with assistance from a research associate (Dr. Cécile Bascoul-Colombo).

Tissue sections were first washed 4 x 5 minutes to remove cryoprotectant using 0.1M TBS (1.2% [w/v] trizma base and 0.9% [w/v] sodium chloride in dH_2O, pH7.4). Antigen retrieval was then performed to reduce the effect of the PFA fixative by incubating the tissue in 85% (v/v) formic acid in dH_2O at 25ºC for 10 minutes. Following a wash in 0.1M TBS, tissue was incubated in quench (10% methanol and 10% hydrogen peroxide in dH_2O) for 5 minutes to reduce background staining caused by endogenous peroxidise activity. Following 3 x 10 minute washes in 0.1M TBS, non-specific binding of the primary antibody was blocked by incubating sections in 3% (v/v) normal goat serum (NGS) in TXTBS (0.1% Triton X-100 in 0.1M TBS, pH7.4) for 1 hour. This was then removed and sections were incubated in 0.1% (v/v) rabbit primary antibody with 1% (v/v) NGS in TXTBS overnight at room temperature on a stirrer. The primary antibody used to stain Aβ1-40 was rabbit anti β-amyloid 1-40 (Millipore, Cat.# AB5074P), and Aβ1-42 was stained using rabbit anti β-amyloid 1-42 (Millipore, Cat.# AB5078P).

Following this, sections were washed 3 x 10 minutes in 0.1M TBS, and incubated in 0.05% (v/v) goat anti-rabbit biotinylated IgG secondary antibody (provided in Vectastain® Elite ABC Kit) in TBS with 1% NGS for 2 hours at room temperature on a stirrer. After three 10 minute washes in 0.1M TBS, sections were incubated in an ABC complex (Vectastain® Elite ABC kit, Vector Laboratories, Cat.# PK-6101) for 2 hours at room temperature on a stirrer. This avidin-biotinylated enzyme complex (ABC) consisted of 0.25% (v/v) solution A (Avidin DH) and 0.25% (v/v) solution B (biotinylated peroxidise) into TBS with 1% (v/v) NGS.

Sections were washed 3 x 10 minutes in 0.1M TBS and further washed 2 x 5 minutes in 0.05M TNS (0.6% [w/v] trizma base in dH_2O, pH7.4) to remove free-floating biotin in the ABC complex and sodium chloride in the TBS which may interfere with 3,3'-diaminobenzidine (DAB) reactions. Sections were then incubated in fresh DAB solution (DAB Peroxidase Substrate Kit, Vector Laboratories, Cat.# SK-4100) for 1 to 3 minutes until stained a purple-brown colour. Importantly, DAB incubation was kept constant between samples for each Aβ-40 and Aβ-42 staining. The DAB solution consisted of 5ml dH_2O, 1 drop of buffer stock reagent, 2 drops of DAB stock reagent, 1 drop of hydrogen peroxide and 1 drop of nickel, added in respective order and mixed between reagents. The reaction was stopped by washing in cold PBS and stored in TBS at 4ºC overnight for mounting the next day.

Stained sections were then mounted on gelatine-coated slides, air-dried for 2 days and dehydrated in an ascending series of alcohol solutions and xylene (50% ethanol → 70% → 90% → 100% → 100% → xylene → xylene) each for 2 minutes. Sections were coverslipped using DPX (Di-n-butyl Phthalate in Xylene) mounting medium and air-dried in the dark ready for viewing under a light microscope.

Immunohistochemistry examination

Stained brain sections showing Aβ1-40 and Aβ1-42 deposition were observed using a Leica DMRB microscope and photographed using an Olympus DP70 camera. A lack of staining was confirmed in sections from WT samples, as expected. Representative sections of staining in each Tg experimental group were collated and shown in the results section.

5.5.3 ELISA Results

Figure 5.5 shows the levels of soluble and insoluble Aβ1-40 and Aβ1-42 measured by ELISA in the hippocampus of Tg mice fed the experimental diets at 12 months of age. Individual values are shown in addition to the mean value (indicated by the black bar). The figures show that all Tg groups appear to have similar levels of soluble Aβ1-40, insoluble Aβ1-40, soluble Aβ1-42 and insoluble Aβ1-42. In order to test whether diet altered Aβ levels in the hippocampus of Tg mice, an ANOVA with diet (OB, OB+C, FO, FO+C) as a between-subject factor, and brain extract (soluble, insoluble) and Aβ isoform (Aβ1-40, Aβ1-42) as

within-subject factors was carried out. Statistical analysis revealed no significant effect of diet ($F(3,45)=0.597$, $p>0.05$), no significant interaction of isoform by diet ($F(3,45)=1.026$, $p>0.05$), no significant interaction of extract by diet ($F(3,45)=111.884$, $p>0.05$) and no three-way interaction of isoform by extract by diet ($F(1,45)=1.791$, $p>0.05$). Diet therefore failed to alter Aβ levels, as illustrated in Figure 5.5.

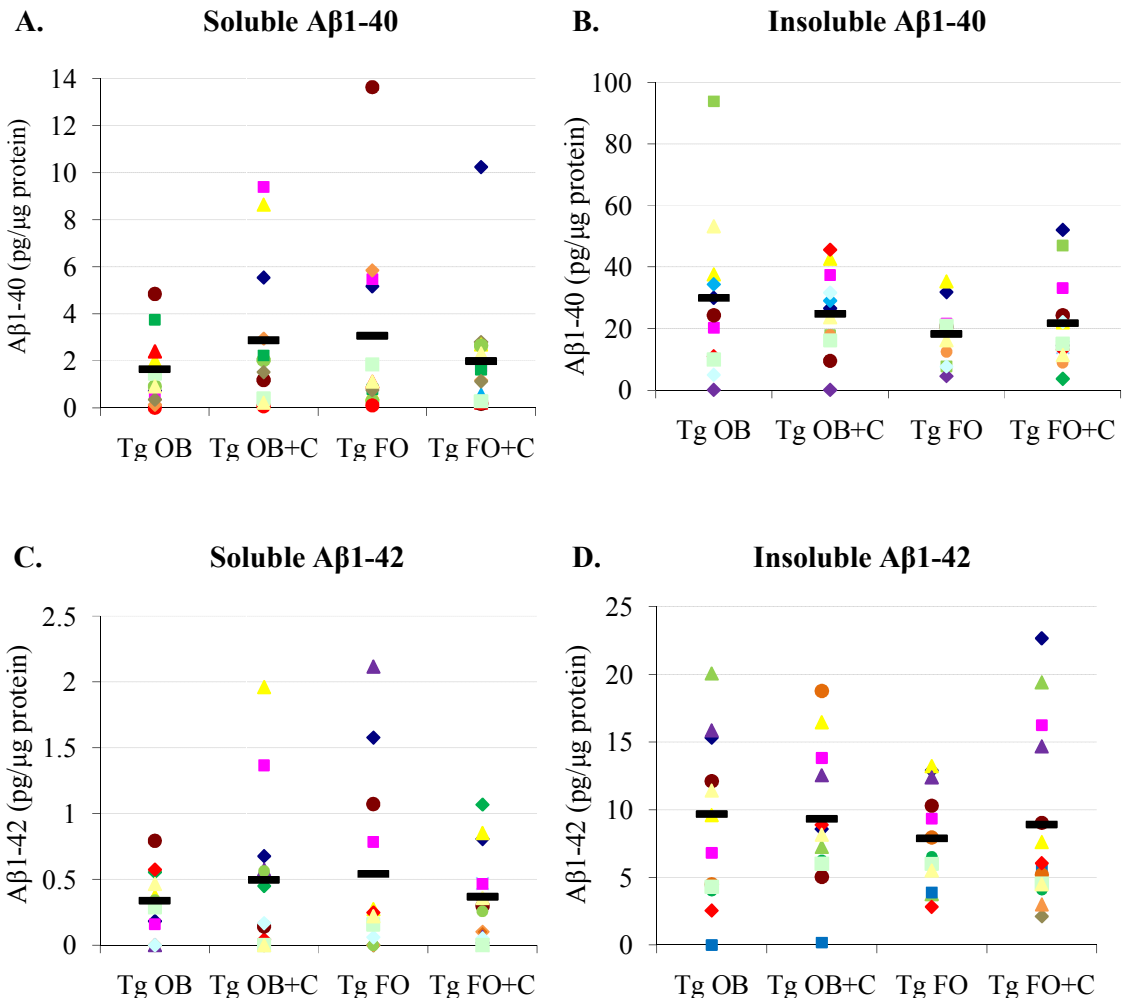

Figure 5.5 Levels of Aβ in the hippocampus (left-hemisphere) of 12 month old male Tg2576 mice fed the oil blend (Tg OB), oil blend + curcumin (Tg OB+C), fish oil (Tg FO), and fish oil + curcumin (Tg FO+C) diet. Four species of Aβ were analysed by ELISA: A. Soluble Aβ1-40, B. Insoluble Aβ1-40, C. Soluble Aβ1-42, and D. Soluble Aβ1-42. Individual values are represented, in addition to the mean value by a black bar.

Statistical analysis also revealed a significant effect of isoform ($F(1,45)=81.663$, $p<0.001$) caused by higher levels of Aβ1-40 than Aβ1-42, a significant effect of extract ($F(1,45)=105.784$, $p<0.001$) caused by higher levels of insoluble Aβ than soluble Aβ, and a significant interaction of isoform by extract ($F(1,45)=58.208$, $p<0.001$). A test of simple main effects revealed this interaction to be caused by higher levels of insoluble than soluble Aβ for both the Aβ1-40 isoform ($F(1,48)=107.34$, $p<0.001$) and Aβ1-42 isoform ($F(1,48)=137.37$, $p<0.001$), and higher levels of Aβ1-40 than Aβ1-42 for both soluble extracts ($F(1,48)=35.41$, $p<0.001$) and insoluble extracts ($F(1,48)=114.089$, $p<0.001$).

5.5.4 Immunohistochemistry results

In order to establish whether 10-month dietary supplementation of curcumin, fish oil or their combination affected plaque-like amyloid body deposition in Tg2576 mice, immunohistochemistry analysis for Aβ1-40 and Aβ1-42 was performed on 40µm brain sections from Tg mice aged 12 months, when amyloid plaques should be present. WT mice were also analysed to use as a negative control as these mice lack the human APPswe transgene and therefore Aβ pathology. Figure 5.6 presents representative examples of Aβ1-40 and Aβ1-42 staining of brain sections from WT mice. As expected, no amyloid deposits were observed throughout all brain sections including regions of the frontal cortex, cerebral cortex, hippocampus and the cerebellum (latter not shown).

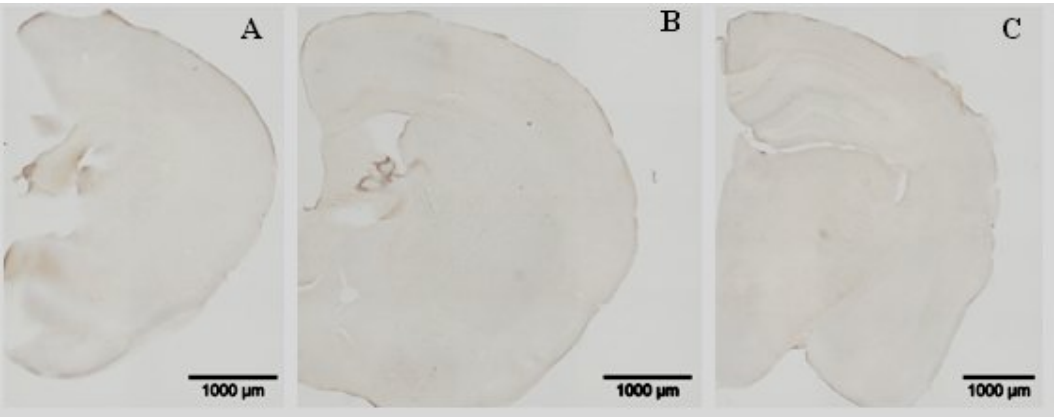

Figure 5.6 Representative example of 40µm brain sections from WT mice following staining for Aβ1-42 and Aβ1-40; A. Frontal cortex, B. Cerebral cortex, C. Cerebral cortex and dorsal hippocampus. No Aβ deposits were observed following Aβ1-40 or Aβ1-42 staining. Scale bar, 1000µm.

Figure 5.7 presents representative examples of Aβ1-40 immunohistochemical staining of brain sections from 12-month old Tg2576 mice fed the experimental diets. Overall Aβ1-40 load was highly variable between individual mice, with no apparent differences in the pattern of amyloid deposition or density as a result of dietary supplementation. Staining was observed in multiple brain regions including cortical and medial temporal lobe structures. In contrast, staining in the cerebellum was limited, with most brain sections devoid of staining (data not shown). Aβ1-40 deposition in the frontal lobe was particularly extensive relative to other brain regions, where staining revealed relatively large amyloid bodies scattered throughout the frontal cortex. Aβ1-40 was predominately deposited within the prelimbic, orbital and motor cortices, in addition to the piriform cortex in some cases. Scattered deposition was also present throughout the cerebral cortex and hippocampus, with some particularly large aggregates observed. Within the hippocampus, the majority of sections revealed a concentration of staining along the hippocampus fissure and the outer molecular layer of the dentate gyrus, extending to the CA1 and radiatum layer in some cases. Aβ1-40 deposition was also observed in the piriform, entorhinal, perirhinal, parietal and retrospenial dysgranular cortices, as well as the amygdala.

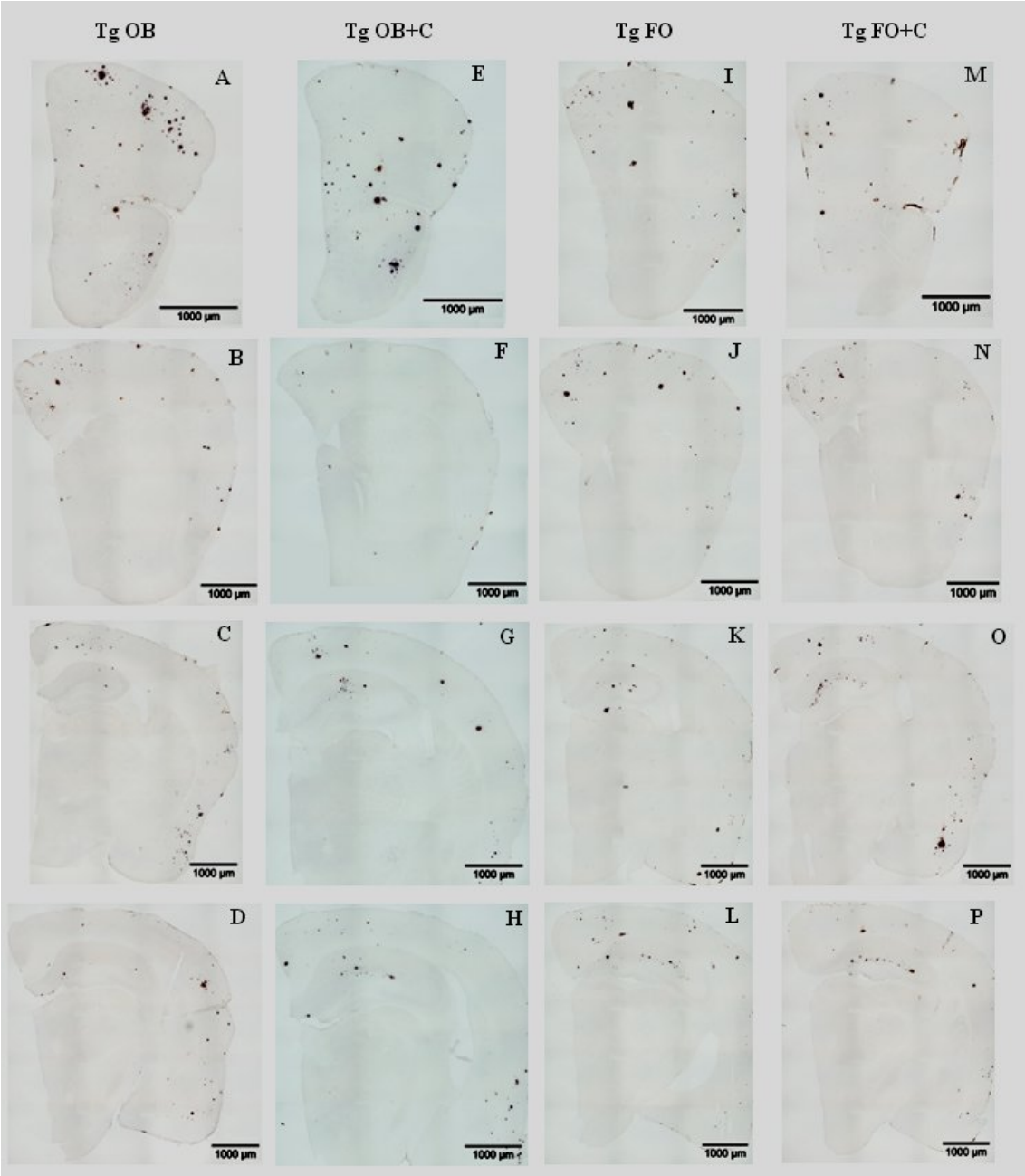

Figure 5.7 Immunohistochemical staining of Aβ1-40 in the frontal lobe, cerebral cortex and hippocampus of 12 month Tg2576 mice fed the oil blend (Tg OB; A,B,C,D), the oil blend + curcumin diet (Tg OB+C; E,F,G,H), the fish oil diet (Tg FO; I,J,K,L), and the fish oil + curcumin diet (Tg FO+C; M,N,O,P). Four representative sections shown for each Tg group. 40µm sections were immunostained for human Aβ1-40. Scale bar, 1000µm.

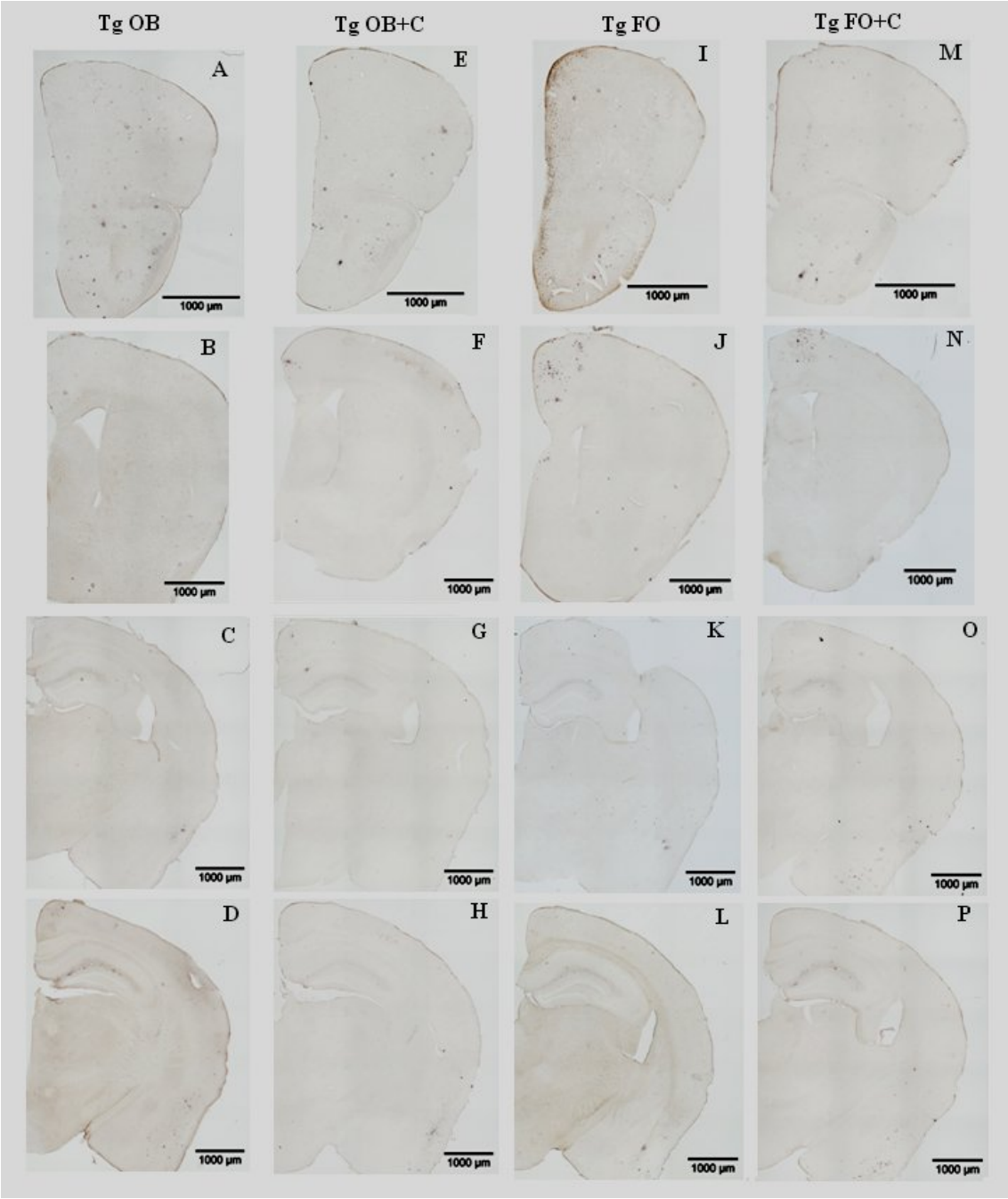

Figure 5.8 Immunohistochemical staining of Aβ1-42 in the frontal lobe, cerebral cortex and hippocampus of 12 month Tg2576 mice fed the oil blend (Tg OB; A,B,C,D), the oil blend + curcumin diet (Tg OB+C; E,F,G,H), the fish oil diet (Tg FO; I,J,K,L), and the fish oil + curcumin diet (Tg FO+C; M,N,O,P). Four representative sections shown for each Tg group. 40µm sections were immunostained for human Aβ1-42. Scale bar, 1000µm.

Figure 5.8 presents representative examples of Aβ1-42 immunohistochemical staining of brain sections from 12-month old Tg2576 mice fed the experimental diets. In contrast to

Aβ1-40 staining, Aβ1-42 load was relatively uniform between individual mice and deposition was not as extensive. Similarly to Aβ1-40 staining, analysis revealed no overt differences in Aβ1-42 deposition between Tg groups, indicating no effect of dietary supplementation. Aβ1-42 deposits were observed as small and diffuse amyloid bodies throughout the frontal, cortical and hippocampal regions. Staining was observed around the cingulate, motor, piriform, entorhinal and perirhinal cortices. Within the hippocampus, Aβ1-42 was concentrated along the hippocampal fissure. Little to no staining was observed in the cerebellum.

In summary, no systematic differences in amyloid staining was observed throughout brain regions in 12-month old Tg2576 mice fed the different experimental diets.

5.5.5 Discussion

Analysis of Aβ pathology by ELISA confirmed the presence of soluble and insoluble Aβ in brains of Tg2576 mice aged 12 months, consistent with previous reports (Hsiao et al., 1996; Kawarabayashi et al., 2001; Westerman et al., 2002). This experiment also corroborated evidence by Kawarabayashi et al. (2001) reporting higher levels of insoluble Aβ1-40 and Aβ1-42 relative to their soluble forms at this age. Greater levels of Aβ1-40 than Aβ1-42 levels were also found at 12 months of age, consistent with reports from Hsiao et al. (1996) in Tg2576 mice aged 11 to 13 months. Immunohistochemical analysis confirmed the absence of Aβ1-40 and Aβ1-42 deposits in WT mice and their presence in Tg2576 mice aged 12 months. Amyloid bodies were deposited throughout the frontal lobe, cortical and hippocampal regions for both isoform staining. Importantly, the observation of amyloid pathology within the hippocampus was consistent with reports of hippocampal-dependant behavioural deficits in Tg2576 mice in this chapter. Furthermore, Aβ1-40 staining was much more extensive than Aβ1-42 staining, which corroborated the ELISA results. Together, these results were consistent with previous immunohistochemical reports in the Tg2576 model (Hsiao et al., 1996; Frautschy et al., 1998; Kawarabayashi et al., 2001).

Interestingly, analysis of Aβ by ELISA revealed no significant effect of diet or significant interactions of isoform and/or extract with diet. Visual inspection of immunohistochemistry analysis confirmed this finding. These results consistently show that 10-month dietary supplementation of curcumin, fish oil, and a combination of fish oil and curcumin did not

reduce Aβ accumulation in the hippocampus of Tg2576 mice aged 12 months. Although these results did not support the hypotheses, they were consistent with results of experiments from this chapter which provided evidence that these dietary supplements failed to ameliorate behavioural deficits. Overall therefore, it could be concluded that relative to an oil blend diet, these supplements failed to alter the behavioural phenotype and Aβ levels measured by ELISA in Tg2576 mice.

This experiment provided the first evidence that fish oil, curcumin and a combination of fish oil and curcumin supplementation does not reduce Aβ pathology in Tg2576 mice. This was consistent with reports by Oksman et al. (2006) who reported fish oil supplementation failed to reduce plaque burden in APPswe/PS1 mice. The results of this experiment however were in contrast to numerous reports that supplementary diets containing omega-3 PUFA can reduce amyloid pathology in AD models (e.g. Lim et al., 2005; Hooijmans et al., 2007, 2009). Furthermore, the results of this experiment were also contradictory to previous reports that dietary supplementation of curcumin can reduce amyloid pathology in rodent models of AD including the Tg2576 model (e.g. Frautschy et al., 2001; Lim et al., 2001; Yang et al., 2005; Garcia-Alloza et al., 2007; Begum et al., 2008). It is important to note however that several factors may account for these discrepancies, such as the design of experimental diets, the time of intervention, and choice of AD model or age of subjects (corresponding to the stage of pathological development). For example, previous studies (e.g. Lim et al., 2001) of curcumin supplementation in the Tg2576 model was carried out after pathology had developed, at 10 months of age and over (in contrast to 2 months in our experiment), which could suggest that curcumin supplementation was only effective during advanced stages of disease when Aβ burden is particularly high. A more detailed outline of such discrepancies is reserved for the chapter discussion.

5.6 Experiment 10: Lipid analysis of brains

5.6.1 Introduction

The aim of this experiment was to profile the fatty acid composition in the brain of 12-month old Tg2576 and WT mice in order to investigate the effect of the APPswe mutation and dietary supplementation. Similar to chapter 3, it was important to examine whether composition of fatty acids in the brain was altered in Tg2576 mice relative to WT mice aged

12 months. No difference in several fatty acids has been reported in the frontal cortex of Tg2576 mice aged 20 to 22.5 months fed a standard chow, including omega-3 PUFAs DHA and EPA, omega-6 PUFAs ARA, DTA and LA, as well as the ω-3/ω-6 ratio (Calon et al., 2004, 2005; Lim et al., 2005). Similar findings were reported in chapter 3 in cortex samples of Tg2576 mice aged 16 months. It was therefore hypothesised that Tg2576 mice would not have altered fatty acid composition in the brain relative to WT mice. As outlined in chapter 3, determination of any transgene-deficits was particularly important for the interpretation of behavioural results as brain omega-3 and omega-6 PUFA levels are associated with cognitive function in humans and rodents, including AD patients and models (e.g. Gamoh et al., 1999; Greiner et al., 1999; Carrie et al., 2000; Ikemoto et al., 2001; Lauritzen et al., 2001; Moriguchi, Greiner & Salem, 2000; Pettegrew et al., 2001). An increase in brain omega-6 PUFA and reduction in omega-3 PUFA levels may therefore be related to impaired cognitive function.

Analysis of fatty acid composition in the brain was also important to ensure that dietary supplementation of fish oil altered levels of omega-3 and omega-6 PUFAs in the brain due to the high omega-3 PUFA content. Increased omega-3 PUFA content in the diet has been shown to be incorporated into brain fractions in healthy rodents and AD models (e.g. Jensen, Skarsfeldt & Høy, 1996; Calon et al., 2004, 2005; Lim et al., 2005; Oksman et al., 2006). chapter 3 also confirmed this with DHA supplementation relative to a suitable baseline control diet. Based on evidence that brain fatty acid levels are sensitive to dietary omega-3 PUFA intake (including fish oil sources; e.g. Jensen, Skarsfeldt & Høy, 1996; Suzuki et al., 1998), it is hypothesised that fish oil supplementation would increase omega-3 PUFA levels in the brain of Tg and WT mice. Since omega-3 PUFAs compete with omega-6 PUFAs for incorporation into the brain, it was therefore also hypothesised that fish oil supplementation would reduce omega-6 PUFA levels and the ω-6/ω-3 ratio. Confirmation of dietary-induced alterations to fatty acid composition in the brain was particularly important for the interpretation of behavioural results. For example, Arendash et al. (2007) argued that an omega-3 PUFA supplemented diet failed to enhance cognitive performance in APPswe/PS1 transgenic mice due to unaltered omega-3 and omega-6 PUFA levels in the brain relative to mice fed a standard diet, suggesting a lack of omega-3 PUFA incorporation into the brain. A lack of omega-3 PUFA incorporation into the brain may therefore explain the lack of fish oil effect observed in this chapter.

The analysis of brain fatty acids was also interesting in order to examine any effects of curcumin supplementation and co-supplementation with fish oil. Since curcumin has potent antioxidant properties, it could be hypothesised that curcumin may increase the levels of omega-3 PUFA in the brain by providing antioxidant protection against lipid peroxidation caused by oxidative stress in the Tg2576 model (Pappolla et al., 1998; Smith et al., 1998; Praticò et al., 2001; Frautschy, Ma & Cole, 2009). Although it may be argued that curcumin could similarly increase omega-6 PUFA levels, it is likely that curcumin would offer more protection to omega-3 PUFA levels since DHA is highly vulnerable to lipid peroxidation due to its six double bonds and high content in the brain (Yavin, Brand & Green, 2002; Montine et al., 2004; Frautschy, Ma & Cole, 2009). In contrast, omega-6 PUFA contain fewer double bonds and its peroxidisability is five times less than DHA, as mirrored in F2-isoprostane levels (Cosgrove, Church & Pryor, 1987; Nourooz-Zadeh et al., 1999; Reich et al., 2001). Furthermore, co-supplementation of curcumin with fish oil may also protect omega-3 PUFA content in the fish oil supplemented diet resulting in greater levels of omega-3 PUFAs in the brain.

5.6.2 Methods

Analysis of brain fatty acids were carried out on cortex samples from 12 month old Tg2576 and WT mice fed the experimental diets from 2 months of age. The materials and procedure used for lipid analysis of brain samples was the same as detailed in the lipid analysis of diet samples (see chapter 3, section 3.5.2). Lipid analysis was performed by a research associate (Dr. Cécile Bascoul-Colombo). Lipid analysis was carried out on cortex samples from the right-hemisphere from a minimum of 3 mice from each experimental group (Tg OB n=3, Tg FO n=4, Tg OB+C n=3, Tg FO+C n=4, WT OB n=4, WT FO n=4, WT OB+C n=4). Samples were collected differently from experiment 3 in chapter 3 as the tissue was being utilised for both lipid analysis and immunohistochemistry by dissecting the two hemispheres of the brain. In order to collect the tissue appropriately for both analyses, mice were injected with 0.2ml of sodium pentobarbitone (Euthatal®) and exsanguination was performed by perfusion with 0.1M PBS pH7.4, as detailed in experiment 9 (see section 5.5.2 for perfusion method). The brain was removed immediately and maintained on ice while dissecting the cortex, hippocampus and cerebellum from the right hemisphere. The dissected samples from the right-hemisphere were immediately snap-frozen in liquid nitrogen and stored at -80ºC until

ready to be used for lipid analysis. The left-hemisphere was prepared for immunohistological analysis (experiment 9).

5.6.3 Results

Figure 5.9 presents the percentage of main fatty acid classes and the ω-3/ω-6 ratio of cortex samples analysed by lipid analysis from 12-month old Tg2576 and WT mice fed the experimental diets. All groups revealed that saturated fatty acids composed the highest percentage of fatty acids in the cortex, followed closely by a relatively high level of polyunsaturated acids, and smaller levels of monounsaturated fatty acids. All groups also showed a higher percentage of total omega-3 PUFAs than total omega-6 PUFAs. Statistical analysis using one-way ANOVA with factor of group revealed no significant group differences in the percentage of total saturated fatty acids ($F(6,25)=1.667$, $p>0.05$), total polyunsaturated fatty acids ($F(6,25)=1.284$, $p>0.05$) and total monounsaturated fatty acids ($F(6,25)=1.362$, $p>0.05$).

As expected, significant group differences were found in the percentage of total omega-3 PUFAs ($F(6,25)=47.883$, $p<0.001$), total omega-6 PUFAs ($F(6,25)=87.566$, $p<0.001$), and the ω-3/ω-6 ratio ($F(6,25)=136.560$, $p<0.001$). A post-hoc Tukey test revealed that omega-3 PUFA, omega-6 PUFA and the and ω-3/ω-6 ratio altered as a factor of diet, whereby mice fed the oil blend and oil blend + curcumin diets differed significantly from mice fed the fish oil and fish oil + curcumin diets. Here, cortex samples contained higher levels of omega-3 PUFAs ($p<0.001$), lower levels of omega-6 PUFAs ($p<0.001$) and a higher ω-3/ω-6 ratio ($p<0.001$) in mice fed diets containing fish oil relative to the oil blend. The ω-3/ω-6 ratio ranged from 1.42 to 1.48 in the oil blend containing diets and 2.18 to 2.41 in the fish oil containing diets. Interestingly, Tg mice fed the fish oil containing diets had a higher ω-3/ω-6 ratio than WT mice fed the fish oil diet, although this did not reach significance. Therefore, these fatty acids did not alter as a factor of genotype or as a result of curcumin content.

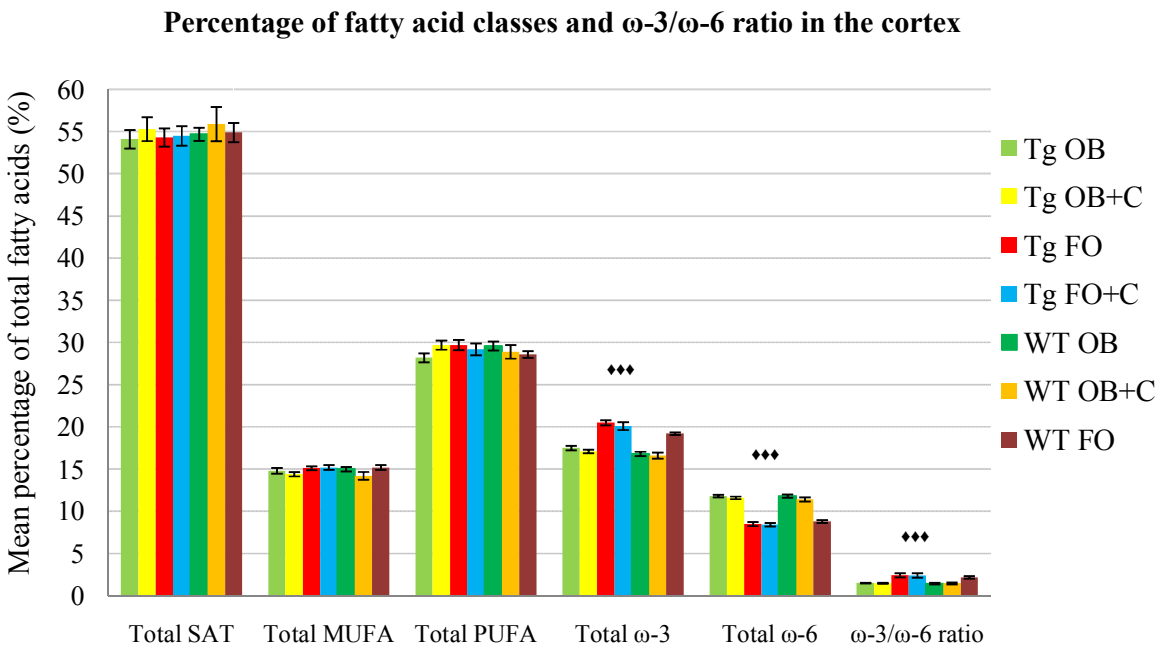

Figure 5.9 Main fatty acids classes and ω-3/ω-6 ratio in the cortex of Tg and WT mice at 12 months fed the oil blend, oil blend + curcumin, fish oil, or fish oil + curcumin diet. Results are presented as mean percentages of total fatty acids ± S.E.M. Significant effect of group, ♦♦♦ $p<0.001$.

Table 5.1 presents the percentage of main fatty acids detected in cortex samples from 12-month old Tg2576 and WT mice fed the oil blend, oil blend + curcumin, fish oil, and fish oil + curcumin diets, indicating significant effects of group. Statistical analysis revealed significant differences between groups for saturated fatty acids 18:0 ($F(6,25)=3.892$, $p<0.05$) and 23:0 ($F(6,25)=11.001$, $p<0.001$). Furthermore, polyunsaturated fatty acids LA 18:2ω-6 ($F(6,25)=4.097$, $p<0.05$), 20:3ω-3 ($F(6,25)=36.733$, $p<0.001$), ARA 20:4ω-6 ($F(6,25)=68.471$, $p<0.001$), EPA 20:5ω-3 ($F(6,25)=408.835$, $p<0.001$), 22:4ω-6 ($F(6,25)=82.636$, $p<0.001$), DPA 22:5ω-3 ($F(6,25)=728.377$, $p<0.001$) and DHA 22:6ω-3 ($F(6,25)=28.129$, $p<0.001$) altered as a factor of group.

A post-hoc Tukey test revealed mice fed oil blend-containing diets significantly differed to mice fed the fish oil-containing diets by having lower levels of 20:3ω-3 (smallest, $p<0.05$), 20:5ω-3 (smallest, $p<0.05$), 22:5ω-3 (smallest, $p<0.001$) and 22:6ω-3 (smallest, $p=0.006$), and higher levels 20:4ω-6 (smallest, $p<0.001$) and 22:4ω-6 (smallest, $p<0.001$). Interestingly, post-hoc analysis also revealed WT FO mice to have lower levels of 20:5ω-3 and 22:5ω-3 than Tg FO (smallest, $p<0.001$) and Tg FO+C mice (smallest, $p<0.001$), and lower levels of 20:3ω-3 than Tg FO mice ($p<0.05$). The results therefore suggest a genotype effect whereby

Tg mice incorporated these fatty acids at higher levels. Inspection of Table 5.1 suggests that mice fed curcumin-containing diets had lower levels of 23:0, which was supported statistically (smallest, $p<0.05$) with the exceptions of WT FO were not different to WT OB+C, WT OB were not different to WT FO+C, and Tg OB were not different to Tg FO+C. In addition, a number of other differences were found including higher levels of 18:0 in Tg OB relative to WT FO ($p<0.05$) and WT OB+C ($p<0.05$) mice, and lower levels of 18:2ω-6 in WT OB+C relative to Tg OB ($p<0.05$) and Tg FO+C ($p<0.05$) mice, and WT OB relative to Tg FO+C ($p<0.05$). Overall therefore, analysis confirmed that fish oil supplementation increased all omega-3 PUFAs except ALA and reduced omega-6 PUFAs ARA and DTA.

Table 5.1 Fatty acid composition of cortex samples from 12 month-old Tg and WT mice fed the oil blend, oil blend + curcumin, fish oil, or fish oil + curcumin diet. Presenting the main fatty acids (values >0.1), represented as mean percentage of total fatty acids ± S.E.M.

Fatty acids	Tg OB	Tg OB+C	Tg FO	Tg FO+C	WT OB	WT OB+C	WT FO
14:0	16.6 ± 0.6	18.2 ± 0.7	17.1 ± 0.6	17.2 ± 0.5	17.0 ± 0.5	18.9 ± 1.0	18.4 ± 0.6
16:0	17.5 ± 0.1	17.3 ± 0.3	17.6 ± 0.1	17.4 ± 0.2	17.7 ± tr.	17.0 ± 0.3	17.6 ± 0.2
16:1ω-7	0.40 ± tr.	0.50 ± tr.	0.50 ± tr.	0.50 ± tr.	0.50 ± tr.	0.50 ± tr.	0.50 ± tr.
18:0 ♦	18.5 ± 0.2	17.9 ± 0.2	18.2 ± 0.2	18.2 ± 0.2	18.1 ± 0.1	17.4 ± 0.3	17.4 ± 0.2
18:1ω-9	12.1 ± 0.2	11.7 ± 0.1	12.5 ± 0.1	12.6 ± 0.1	12.2 ± 0.2	11.7 ± 0.3	12.5 ± 0.2
18:1ω-7	3.0 ± 0.1	2.9 ± 0.1	2.8 ± tr.	2.8 ± 0.1	3.0 ± tr.	3.0 ± 0.1	2.8 ± 0.1
18:2ω-6 ♦♦	0.5 ± tr.	0.5 ± tr.	0.5 ± tr.	0.5 ± tr.	0.4 ± tr.	0.4 ± tr.	0.4 ± tr.
18:3ω-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:3ω-3	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.	N.D.	N.D.
20:0	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.
20:1ω-9	1.0 ± 0.1	1.0 ± tr.	0.9 ± tr.	0.9 ± 0.1	0.9 ± tr.	0.9 ± tr.	0.9 ± tr.
20:2ω-6	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.	N.D.	0.1 ± tr.	N.D.	N.D.
20:3ω-3 ♦♦♦	0.4 ± tr.	0.4 ± tr.	0.6 ± tr.	0.6 ± tr.	0.2 ± tr.	0.3 ± tr.	0.5 ± tr.
20:4ω-6 ♦♦♦	8.7 ± 0.1	8.5 ± tr.	6.7 ± 0.2	6.7 ± 0.1	8.9 ± 0.1	8.5 ± 0.2	6.9 ± 0.1
20:5ω-3 ♦♦♦	N.D.	N.D.	0.3 ± tr.	0.3 ± tr.	N.D.	N.D.	0.2 ± tr.
22:0	0.2 ± tr.	0.4 ± 0.2	0.2 ± tr.	0.2 ± tr.	0.3 ± tr.	0.4 ± 0.2	0.3 ± tr.
22:4ω-6 ♦♦♦	2.6 ± tr.	2.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	1.5 ± tr.
22:5ω-3 ♦♦♦	0.1 ± tr.	0.1 ± tr.	0.5 ± tr.	0.5 ± tr.	0.1 ± tr.	0.1 ± tr.	0.4 ± tr.
22:6ω-3 ♦♦♦	15.6 ± 0.2	15.2 ± 0.2	17.8 ± 0.2	17.5 ± 0.3	15.1 ± 0.2	15.1 ± 0.3	17.0 ± 0.1
23:0 ♦♦♦	0.5 ±0.1	0.4 ± tr.	0.3 ± tr.	0.3 ± tr.	0.6 ± 0.1	0.4 ± tr.	0.2 ± tr.
24:0	0.6 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.0 ± 0.3	0.8 ± 0.1
24:1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± tr.	1.0 ± 0.1	1.2 ± tr.

N.D. = not detected, tr. = trace (less than 0.1%). Significant effect of group, ♦ $p<0.05$, ♦♦ $p<0.01$, ♦♦♦ $p<0.001$.

5.6.4 Discussion

The results of this experiment revealed that fatty acid composition in the cortex did not generally alter as a factor of genotype in Tg2576 mice aged 12 months, corroborating evidence in chapter 3 in mice aged 16 months and previous reports (Calon et al., 2004, 2005;

Lim et al., 2005). However, results showed Tg mice fed the fish oil diets incorporated higher levels of 20:5ω-3, 22:5ω-3 and 20:3ω-3 (Tg FO only in latter) than WT FO mice, suggesting an effect of genotype. Despite this effect, total levels of omega-3 PUFAs were unaffected by genotype. Previous reports of a genotype effect whereby Tg2576 mice show reduced omega-3 PUFA incorporation following intake of DHA-depleted diets high in omega-6 PUFAs, therefore suggests that this transgene-effect is limited to an intake of a particularly poor diet high in omega-6 PUFAs (Calon et al., 2004, 2005; Lim et al., 2005).

The fatty acid composition of the cortex in all mice was primarily composed of SAT, followed by PUFA and MUFA, which were all unaffected by genotype and diet. Interestingly, the increased SAT and MUFA content in the oil blend-containing diets did not significantly alter brain levels of SAT and MUFA. Similarly to results in chapter 3, the main PUFA in the cortex of all mice was DHA, despite the absence of DHA in the oil blend diet. This therefore suggests the conversion of omega-3 PUFA ALA (present in the oil blend diet) to DHA *in vivo*. This is consistent with reports that DHA levels in the brain can be maintained by the sole intake of dietary ALA through its conversion in the liver (Rapoport, Rao & Igarashi, 2007).

As predicted, fish oil supplementation (FO and FO+C) led to significantly higher ω-3/ω-6 ratios in the cortex relative to mice fed the oil blend-containing (OB and OB+C) diets. This is consistent with literature demonstrating that omega-3 and omega-6 PUFAs compete for metabolism and incorporation into brain phospholipids (Russo, 2009) and so supplementation of diets containing omega-3 PUFA leads to greater incorporation of omega-3 PUFAs in replacement of omega-6 PUFAs. In accordance, total levels of omega-3 PUFA were significantly higher with fish oil supplementation, which was primarily contributed by significant elevations in DHA which increased by 2.1-2.7% of total fatty acids. Higher percentages of omega-3 PUFAs eicosatrienoic acid (ETA, 20:3ω-3), eicosapentaenoic acid (EPA, 20:5ω-3) and docosapentaenoic acid (DPA, 22:5ω-3) were also observed with fish oil supplementation. The higher level of DHA relative to EPA in the brain following fish oil supplementation (where EPA content was higher than DHA) would suggest the conversion of EPA to DHA *in vivo*. Also accordingly, fish oil supplementation lowered total levels of omega-6 PUFAs, caused by a reduction in arachidonic acid (ARA, 20:4ω-6) by 1.8-2.2% and docosatetraenoic acid (DTA, 22:4ω-6) by 1.2-1.3%. The reported effects on brain omega-3

and omega-6 PUFA content following fish oil supplementation was therefore consistent with previous reports (e.g. Jensen, Skarsfeldt & Høy, 1996; Suzuki et al., 1998).

With regards to curcumin supplementation and co-supplementation with fish oil, the results revealed no robust effects on fatty acid composition in Tg or WT mice. Some evidence suggested that curcumin altered levels of 23:0, although this was not fully supported. Aside from curcumin effects, a number of other differences were reported in levels of 18:0 and 18:2ω-6 which suggested interactions between genotype and dietary intake, although no consistent patterns would be ascertained. Overall therefore, fish oil supplementation but not curcumin supplementation appeared to alter fatty acid composition of brains. The lack of curcumin effect was contrary to predictions since curcumin was hypothesised to protect omega-3 PUFAs from peroxidation in the brain caused by increased oxidative stress in Tg2576 mice. However, the lack of effect could be explained by the fact that although oxidative stress is present in the Tg2576 model, this does not alter brain composition of fatty acids relative to WT mice when fed control diets (Calon et al., 2004, 2005; Lim et al., 2005; experiment 3).

In summary, this is the first study in Tg2576 mice which has shown that fish oil supplementation can alter fatty acid composition in the cortex of Tg and WT mice. The results also show that fish oil supplementation has a similar pattern of increasing total omega-3 PUFA (particularly DHA) and reducing total omega-6 PUFA (particularly ARA and DTA) as DHA supplementation, and can have this effect relative to a suitable baseline diet. In contrast, Calon et al. (2004, 2005) and Lim et al. (2005) reported that DHA supplementation relative to a control diet did not alter omega-3 and omega-6 PUFA levels in Tg2576 or WT mice, although the higher ω-3/ω-6 ratio in the control diet may have accounted for this effect. Overall, the altered brain lipid composition reported in this study would have anticipated complementary effects on behaviour (based on evidence outlined in the experiment introduction). This will be discussed in the chapter discussion.

5.7 Chapter Discussion

This chapter examined the effect of early-intervention longitudinal dietary supplementation of fish oil, curcumin and their combination on behavioural deficits and Aβ pathology in Tg2576 mice. Firstly, it is important to note that transgene-related deficits were observed in

Tg2576 mice in the marble burying test at 8 to 9 months of age and the T-maze FCA task at 9.5 to 12 months. These results therefore support studies showing impairments in Tg2576 mice related to tasks of hippocampal function and spatial memory (e.g. Hsiao et al., 1996; Westerman et al., 2002; Adriani et al., 2006). These results were also consistent with T-maze findings in chapter 3, and marble burying findings in chapter 4.

Despite predictions, results from these experiments consistently failed to show any effect of fish oil, curcumin or fish oil and curcumin supplementation on behaviour or pathology in Tg2576 mice. This was found in 2 behavioural measures sensitive to the APPswe mutation and 2 assessments of Aβ pathology including measures of Aβ40 and Aβ42 isoforms in soluble, insoluble and amyloid-body deposited forms. These consistent results therefore provide some evidence that these supplements are not suitable for treating Aβ-related pathological changes and its associated behavioural deficits, or for general cognitive improvement in healthy individuals. In contrast, studies have shown positive effects of all three supplements in animal models of AD. Firstly, Ma et al. (2009) reported positive effects of 2.4% dietary fish oil supplementation in the 3xTg AD model, which reduced cognitive deficits and tau pathology, as well as inhibited JNK activation and improved insulin signalling. A number of factors may explain this discrepancy, such as the differential use of control diets whereby fish oil reduced deficits induced by a poor diet high in omega-6 PUFAs and saturated fatty acids. This therefore does not provide evidence that fish oil *per se* can protect against deficits relative to a suitable baseline control diet, but may be instead be limited to conditions induced by a poor diet. In addition to this, the positive effects of fish oil reported by Ma et al. (2009) may be a result of targeting pathological processes which are absent in the Tg2576 model such as tau pathology.

In another study, Oksman et al. (2006) reported 2.2% fish oil reduced Aβ42 levels but not plaque pathology in the APPswe/PS1 model. Again, this discrepancy could be explained by the control diets which were high in omega-6 PUFAs, and so effects may be limited to a poor diet which can exacerbate pathology. The discrepancy could also be explained by the different animal model, as although both models express amyloid pathology, the APPswe/PS1 model shows an exaggerate state of pathology with greater levels of Aβ42. These results may therefore suggest that fish oil effects are limited to such conditions. It may therefore be likely that fish oil supplementation would reduce Aβ pathology and its associated deficits in advanced stages of pathological development in Tg2576 mice when

amyloid load is high. Indeed, such predictions are consistent with positive effects of omega-3 PUFA DHA supplementation in aged Tg2576 mice (e.g. Calon et al., 2004, 2005 Lim et al., 2005). If indeed fish oil targets large pathological loads, this may explain the lack of effectiveness of early-stage longitudinal intervention in this study. It is therefore evident that further investigation is required into fish oil supplementation, primarily concerned with its affects on late-stage pathology in Tg2576 mice. Since brain fatty acids were altered as a consequence of fish oil, this would suggest that sole increases in brain omega-3 PUFA and reductions in brain omega-6 PUFA were not effective in ameliorating deficits related to the APPswe mutation.

The results of this experiment were also inconsistent with a plethora of evidence showing positive effects of curcumin treatment in AD models, including Tg2576 mice. Discrepancies from reports in the APPswe/PS1 model (Garcia-Alloza et al., 2007) or the 3xTg AD model (Ma et al., 2009) may be attributable or limited to effects on different pathological processes such as tau pathology or accelerated amyloid deposition. Similar to the issues described above with fish oil supplementation, the effects of curcumin reported in Ma et al. (2009) was also relative to a poor diet and so may be limited to such conditions. Furthermore, curcumin effects reported in Garcia-Alloza et al. (2007) study may be attributable to differences in administration, whereby curcumin was injected rather than supplemented in the diet. For instance, injected curcumin has been shown to label Aβ plaques greater than dietary administration, suggesting greater bioavailability (Yang et al., 2005). Frautschy et al. (2001) also showed positive effects of dietary curcumin in an Aβ-infused rat model of AD, although the effects may be limited to injections of Aβ rather than natural forms and processes of Aβ accumulation in the brain.

Regarding curcumin supplementation specifically in the Tg2576 model, inconsistencies with Lim et al. (2001) reports may be attributable to dosage differences whereby 160ppm and 5000ppm curcumin was used. In contrast, Yang et al. (2005) and Begum et al. (2008) reported positive effects of curcumin while using the same 500ppm dose, although discrepancies may be due to the presence of an oil blend supplement in our experiment or the age of intervention. Since the oil blend contained neutral oils, it is thought unlikely that this would reduce the impact of curcumin. In fact, studies have shown increased bioavailability of curcumin in lipidated formulations, which may have been expected to increase potential effects of curcumin. It is therefore more likely that the different age of intervention may

account for the discrepancies, whereby late-stage intervention starting from 14.5 months (Begum et al., 2008) or 17 months (Yang et al., 2005) is more effective. Similar to fish oil supplementation, it may therefore be argued that curcumin supplementation primarily targets late-stage pathological processes. For example, curcumin may be most effective during periods of great amyloid burden, oxidative stress or inflammation; which would be consistent with its potent anti-plaque, antioxidant and anti-inflammatory properties. Overall, it is therefore evident that early-intervention longitudinal supplementation of curcumin is ineffective at reducing pathology and behavioural deficits in middle-age Tg2576 mice, while other evidence shows its effectiveness during advanced-stages. Further investigation into whether longitudinal early-stage intervention is more effective than late-intervention in reducing deficits during advanced stages of disease development is therefore required.

Alternatively to this hypothesis, it should be considered whether sufficient levels of curcumin were present in the brain of mice in this study following dietary intake in order to exert an effect. It is well documented that curcumin has bioavailability issues, particularly following oral dosing (e.g. Kelloff et al., 1996; Lao et al., 2006). This has been attributed to poor absorption into the bloodstream, difficulty crossing the blood-brain barrier, rapid metabolism and rapid systematic elimination through the faeces (Pan, Huang & Lin, 1999; Aggarwal & Harikumar, 2009). Despite this however, 500ppm dietary doses of curcumin have been reported effective in reducing deficits in animal models of AD, suggesting sufficient uptake in this study. Supporting this, Yang et al. (2008) reported 500ppm doses of curcumin to label plaques in the Tg2576 brain. Furthermore, metabolites of curcumin such as tetrahydrocurcumin have also been shown to have potent effects on pathology in Tg2576 mice (Begum et al., 2008). It was therefore assumed that curcumin bioavailability should not be an issue in this study explaining the lack of diet effect. Ideally it would have been advantageous to demonstrate this by measuring curcumin in the brain however tissue samples were unavailable for analysis.

Since curcumin bioavailability could be an issue following oral dosing, it could be argued that other administration methods may be more suitable for treatment. However, since curcumin is instable in solution which results in losses of 50% after 8 hours incubation in human blood (Wang et al., 1997), it is argued that *ad lib* dietary consumption is the best method to ensure maintained levels of curcumin longitudinally, which could not be practically achieved with injections. Supporting this need for frequent administration, Cheng

et al. (2001) reported serum concentration of curcumin peaks 1 to 2 hours after intake and declines gradually within 12 hours.

One of the most unexpected findings of this chapter was the results showing no beneficial effects of curcumin and fish oil co-supplementation. Although research into this supplement is in its infancy in AD models, positive findings were anticipated based on evidence that the supplements individually were effective in AD models and evidence indicated that their co-supplementation would likely increase their bioavailability. For example, Ma et al. (2009) reported that this combination supplement was more effective than fish oil or curcumin supplementation alone at reducing reported deficits in the 3xTg AD model. Together with Frautschy and Cole (2010) claiming that this combination supplement can reduce cognitive deficits and tau pathology in the Htau model (unpublished results), these are the only *in vivo* studies reporting effects of fish oil and curcumin co-supplementation in AD models. The experiments in this chapter are the first reports in the Tg2576 model and the first report on Aβ pathology *in vivo*. The discrepancies between the studies could therefore be explained by the animal model and underlying pathology, whereby the effectiveness of the supplement may be dependent upon targeting tau pathology. Alternatively, the differential results could be explained by the control diet used, whereby Ma et al. (2009) used a chow high in omega-6 PUFAs and saturated fat, perhaps indicating that the effectiveness of the combination diet is limited to such conditions.

Overall, it is therefore evident that early-stage longitudinal fish oil and curcumin co-supplementation does not reduce behavioural deficits or pathology in the Tg2576 model. Further investigation is required at more advanced stages of pathology, since conclusions from fish oil and curcumin individual supplementation suggests that these compounds may be more effective during periods of great pathological load. However, it is important to note that curcumin may not enhance the effectiveness of fish oil since lipid analysis revealed no alterations to brain fatty acids following co-supplementation relative to fish oil supplementation alone. The results of the chapter would also suggest that fish oil failed to increase the effectiveness of curcumin, although alterations to curcumin bioavailability by fish oil co-supplementation needs to be confirmed as this was not measured in this study. In conclusion, this chapter presents evidence that longitudinal early-stage supplementary fish oil, curcumin and their combination does not ameliorate behavioural deficits or pathology in the Tg2576 model. Further examination at more advanced stages of pathology is required

before we conclude that these supplements are not generally suitable for treating Aβ-related pathology and deficits. We can conclude however that treatment of these supplements from an early age does not slow the progression of Aβ pathology or behavioural deficits caused by expression of the APPswe mutation in middle-age Tg2576 mice.

Chapter 6

General discussion

Alzheimer's disease is the most common form of dementia with a growing incidence and a huge socioeconomic impact. Due to the complex aetiology of the disease there is no cure and current treatments have only limited symptomatic benefit. Investigations into new therapeutic strategies which target underlying pathological processes are therefore imperative. Furthermore, the discovery of treatments suitable for long-term use is also required in order to sustain its benefits throughout the aging process. Technological advances in diagnosis are leading to the possibility for earlier intervention, which may be a significant factor in delaying the progression and even preventing the onset of disease. Support for this comes from epidemiological studies which show long-term features such as genetic and environmental factors can reduce the risk and incidence of AD. In particular, there is growing support for the role of diet in modifying disease development and its potential for therapeutic use. There is a large body of evidence suggesting that fish oils and omega-3 PUFAs, such as DHA, can reduce the risk of AD, and *in vivo* and *in vitro* studies have demonstrated their capability to target pathological mechanisms. Similar evidence has been provided for the polyphenolic compound curcumin. The aim of this thesis therefore was to test the hypothesis that dietary DHA, fish oil and curcumin can reduce behavioural and pathological changes in a mouse model of AD-related amyloid pathology.

It has been argued in this thesis that although DHA supplementation has been examined in animal models of AD, none had accurately assessed the effect of DHA *per se* compared to a suitable baseline control diet - whereby the primary difference is DHA supplementation. The majority of previous studies used a control diet high in omega-6 PUFAs, saturated fatty acids and/or depleted in omega-3 PUFA. Therefore, the resulting conclusion that could be made from these studies was that DHA supplementation was beneficial relative to a poor diet that exacerbated pathological processes. A similar conclusion could be drawn from the two studies that have examined fish oil supplementation in transgenic AD models. These studies also used a poor diet high in omega-6 PUFAs and saturated fatty acids. Furthermore, only one of these studies reported effects on β-amyloid pathology and only one study assessed

effects on behaviour (Oksman et al., 2006; Ma et al., 2009). This highlights the need for further investigation of these supplements using a suitable baseline control diet that provide a more rigorous test of the hypothesis that these dietary supplements can ameliorate amyloid pathology and its impact on cognition. Moreover, these studies had only examined the effect of these supplements after pathology had developed, which may have limited the effectiveness of treatments and failed to examine whether these supplements would delay the onset of pathological development. The examination of early intervention would therefore be insightful. This issue was also a feature that needed addressing in curcumin supplementation studies, which showed positive effects in only aged AD model mice including the Tg2576 model. Finally, the co-supplementation of fish oil and curcumin may offer positive synergistic treatment effects, although its examination is in its infancy. Only one published study has reported positive effects, but this was relative to a poor control diet and failed to report effects of the diet on β-amyloid pathology.

A number of experiments were therefore carried out to test the putatively beneficial effects of dietary DHA, fish oil and curcumin supplementation, as well as fish oil and curcumin co-supplementation in the Tg2576 mouse model of AD. Particular emphasis was placed on using a suitable baseline control diet in order to accurately examine the effect of DHA and fish oils, in addition to examining longitudinal supplementation from an early age prior to pathological development. Importantly, both transgenic and wildtype mice were examined in order to determine transgene-dependant effects of diet.

Chapter 3 examined the effect of a DHA-rich diet containing 1.86% DHA supplemented from 4 months of age, when Aβ pathology is reported to first emerge. Results revealed spatial memory deficits in the T-maze at 12 and 16 months of age in transgenic mice, consistent with previous reports (e.g. Hsiao et al., 1996; Chapman et al., 1999; Barnes, Hale & Good, 2004). Results also revealed a mild amelioration of spatial memory deficits with DHA supplementation at 16 months of age, which was not found at 8 or 12 months. However, DHA failed to reduce Aβ accumulation in brains of Tg mice aged 16 months. The mild alleviation of spatial memory deficit by DHA was therefore likely related to a reduction of other pathological processes present in the Tg mouse related to Aβ pathology such as inflammation or oxidative stress. Since DHA supplementation altered levels of brain fatty acids at 16 months, whereby omega-3 PUFAs were increased and omega-6 PUFAs were reduced, this may have improved neuronal function in Tg mice, explaining the positive effect.

The presence of elevated DHA in the brain following DHA supplementation indicated that the lack of robust positive effect was not due to a lack of omega-3 PUFA incorporation, as shown with Arendash et al. (2007). However, it is unclear whether brain fatty acids were altered before 16 months, which may have explained the lack of dietary effect on behaviour at 8 and 12 months. Incorporation of DHA into brain fractions following 4-5 month supplementation in previous studies would suggest that this was not an issue (e.g. Calon et al., 2004).

In a second study, chapter 4 examined the effect of the same DHA-rich diet relative to the same baseline control diet, but was supplemented before the onset of any known pathological changes at 2 months of age. A large range of behavioural tests were used which aimed to be sensitive in potentially detecting smaller transgene and diet effects, which may have been an issue in the first study. Results revealed the presence of behavioural deficits from as early as 3 months of age, corroborating previous reports (e.g. King et al., 1999). Importantly, this suggested that deficits are present prior to pathological Aβ development; further highlighting the need for earlier intervention. The results also provided some evidence that DHA supplementation reduced a number of deficits including spatial memory and anxiety-related behaviour, as well as enhanced executive functioning; although similar to chapter 3, the reported effects were relatively mild. Interestingly, the effects were more robust at a younger age. Firstly, this demonstrated the importance of employing a battery of behavioural tasks when examining treatment effects since early-stage treatment effects were not reported in chapter 3 (likely due to the T-maze's lack of complexity and therefore sensitivity in detecting changes). Secondly, the results provide support that DHA does not simply target Aβ pathology, since deficits were reduced at 3 months before Aβ pathology emerged. In order to examine the effect of early DHA supplementation on Aβ pathology, future studies should aim to measure Aβ throughout the course of treatment. This would be difficult without large cohorts of mice, and so Aβ levels could perhaps be measured in the plasma. Although the pattern of plasma Aβ levels does not reflect brain Aβ levels, it is well documented that a decrease in plasma Aβ coincides with marked deposition in the brain (Kawarabayashi et al., 2001) and so an increase in plasma Aβ may suggest a reduction in the brain.

Chapter 4 results may suggest that DHA is more effective during early stages of pathology, which are similar to clinical trial results, showing omega-3 PUFA supplementation was only effective in people with very mild AD or MCI (e.g. Chiu et al., 2008; Freund-Levi et al.,

2006; Kotani et al., 2006). Alternatively however, since benefits were also observed during periods of significant Aβ load in Tg2576 mice, it is possible that DHA delayed the accumulation of Aβ during early stages, but lost efficiency over time. Similarly, Otsuka (2000) reported positive effects of EPA in a clinical trial of AD patients to cease after 6 months. If efficiency is lost over time, this would suggest that DHA treatment should be reserved for more advanced stages of pathology when more symptomatic benefit can be achieved. This requires further investigation. Overall therefore, the results of chapters 3 and 4 showed that DHA offers some protection from transgene-related deficits in the Tg2576 model relative to a baseline control diet, in contrast to its potent beneficial effects relative to a poor diet with omega-3 PUFA depletion, high omega-6 PUFA and/or an excessive ω-6/ω-3 ratio (e.g. Gamoh et al., 2001; Ikemoto et al., 2001; Calon et al., 2004, 2005; Lim et al. 2005; Hashimoto et al., 2005b). Although comparing DHA supplementation to DHA-depletion may reflect conditions in a number of AD cases which show depleted levels of DHA in the brain (e.g. Söderberg et al., 1991), these conditions are not reflective of all AD cases since not all show reduced DHA in the brain (Skinner et al., 1993). A reasonable conclusion from these studies therefore is that DHA deficiency and intake of high omega-6 PUFA should at least be avoided in AD, and that DHA may provide some protection under such conditions.

Chapter 5 examined the effect of 2.5% fish oil supplementation relative to a baseline control diet identical to the first two studies although it contained 2.5% rather than 5% oil blend. Diets were again provided before pathological changes at 2 months of age. Results revealed no beneficial effect of fish oil supplementation in two behavioural measures previously shown to be sensitive to the APPswe transgene and dietary DHA. Consistently, no effects were found on Aβ pathology at 12 months. The behavioural results were in contrast to Ma et al. (2009) in the 3xTg model, although this may suggest that fish oil targets pathological processes present in this model, such as tau (which was shown to be reduced with fish oil) rather than Aβ (which was not assessed by Ma et al., 2009). Furthermore, these results were in contrast to epidemiological studies showing fish intake to reduce the risk and incidence of AD. However, it is important to consider that epidemiological studies do not show a cause and effect relationship and a number of other extraneous factors may be involved. For example, this may suggest that other nutritional components of fish intake may be important or that healthier lifestyle choices are adopted by fish eaters that may account for the effect. These issues highlight the importance of highly controlled animal studies.

Since the same oil blend control diet was used in the DHA and fish oil experiments, it is possible to draw some conclusions between these studies. The results showed that the DHA-rich diet can provide some protective benefit whereas fish oil does not relative to the control diet. This may suggest that the higher levels of total omega-3 PUFAs or DHA in the DHA-rich diet were attributable for this effect, and that relatively lower levels of total omega-3 PUFAs or DHA in the fish oil diet had limited benefits. Although similar levels of omega-3 PUFAs or DHA in the fish oil diet have been reported beneficial in other studies, those results were in contrast to a poor diet (e.g. Calon et al., 2004). Furthermore, these results suggest that the amount of omega-3 PUFA is likely more important than the ω-3/ω-6 ratio alone since the DHA and fish oil diets had similar ratios. Alternatively, the comparison of DHA to fish oil results may suggest that a higher level of total fatty acids in the DHA-rich diet (5% oil compared to 2.5% oil in the fish oil study) provided more protection. However, this is unlikely since studies have shown that increased fat in the diet can exacerbate pathology in AD mouse models (e.g. Julien et al., 2010; Schroeder, Richardson & Virley, 2010), so it is more likely that the higher level of DHA is accountable.

With this in mind, it is interesting that the higher-fat DHA-rich diet had positive effects in contrast to the fish oil diet since it is well documented that the addition of dietary lipids, including omega-3 PUFA and DHA, can increase the production of oxidative products *in vivo* (Alexander-North et al., 1994; Allard et al., 1997; Kubo et al., 1997; Jenkinson et al., 1999; Song & Miyazawa, 2001; Grundt et al., 2003; Montine & Morrow, 2005; Corsinovi et al., 2011). It is possible that the composition of lipids in the DHA-rich diet (i.e. the high level of DHA) may therefore be accountable for the positive effects, despite its higher level of fat. For example, the fish oil diet was particularly rich in EPA (13.6% total fatty acids, 0.53% of diet weight) which was absent in the DHA-rich diet, and relatively low in DHA (8.62% total fatty acids, 0.34% of diet weight) compared to the DHA diet (27.5% total fatty acids, 1.86% of diet weight). Since Chen et al. (2009) revealed more extensive oxidation of EPA than DHA in mice, and DHA has been well documented as an antioxidant and can decrease lipid peroxidation (e.g. Hossain et al., 1998; Kubo et al., 1998; Maekawa, 1998; Green et al., 2001; Yavin, Brand & Green, 2002; Lim et al., 2005), the resulting production of lipid peroxides and state of oxidative stress may therefore be lower in the DHA diet relative to the fish oil diet. Therefore, despite the increased level of fatty acids in the DHA diet relative to fish oil diet, the potential increase in lipid-induced oxidative products may be lower than the fish oil diet and this production may also be outweighed by the antioxidant effects of DHA.

It would therefore be of interest to measure levels of various lipid peroxidation products in mice supplemented such diets in future studies. For example, an important measure of lipid peroxidation could include various isoprostanes, specifically F2-isoprostanes (an omega-6 PUFA ARA peroxidation product), F3-isoprostanes (an EPA peroxidation product) and F4-isoprostanes (a DHA peroxidation product). Another measure of lipid peroxidation could include the membrane lipid peroxidation product, 4-hydroxynonenal (HNE), which is increased in metabolic syndromes, cardiovascular and neurodegenerative diseases including AD (Mattson, 2009). Specifically, HNE can cause neuronal dysfunction by impairing membrane-associated glucose and glutamate transporter function and ATPases involved in cellular ion homeostatis, impairing ApoE antioxidant function, increasing inflammatory processes, altering cholinergic system function, and influencing β- and γ-secretase enzymes involved in Aβ production (as reviewed in Mattson, 2009). A profile of changes to these lipid peroxidation products may therefore shed light on the potential antioxidant roles of omega-3 PUFA and fish oil supplements in AD pathogenesis.

Providing further support for the argument that DHA may provide more antioxidant protection than fish oils, DHA depletion has been shown to increase oxidative stress (e.g. Lim et al., 2005) which highlights the important antioxidant role of DHA. Furthermore, fish oil intake has been shown to increase lipid peroxidation (e.g. Meydani, Natiello & Natiello, 1991). Although Higdon et al. (2000) reported fish oils rich in EPA not to increase lipid peroxidation, this measure was restricted to F2-isoprostanes not F3- or F4-isoprostanes. Future studies should therefore examine DHA and fish oil supplementation comparatively, with emphasis on measuring the level of various lipid oxidation products. These results question the potential role of EPA in AD treatment. Although beneficial effects of EPA has been reported in an Aβ-injected rat model (e.g. Hashimoto et al., 2009a), the majority of research into the beneficial effects of omega-3 PUFAs are primarily focused on DHA supplementation. It could be argued that the studies reporting some positive effects of EPA supplementation may be due to its conversion into DHA. Further examination of EPA relative to DHA supplementation is also therefore required since results from human studies have been inconsistent with regards to the beneficial effects of EPA monotherapy (e.g. Otsuka, 2000; Morris et al., 2003; Boston et al., 2004). The results of this thesis suggest that DHA monotherapy is more advisable than combined EPA and DHA therapy.

Chapter 5 examined the effect of curcumin and fish oil co-supplementation. Contrary to expectations, this supplement failed to ameliorate behavioural deficits or Aβ pathology in the Tg2576 model. This may suggest that fish oil supplementation increased the production of lipid peroxides which counteracted any beneficial effects of fish oil supplementation, and that curcumin failed to offer antioxidant protection. Similarly, the addition of the antioxidant vitamin E to omega-3 PUFA supplementation failed to reduce increased lipid peroxide products (e.g. Allard et al., 1997; Kubo et al., 1997). This is somewhat unlikely as curcumin is a potent antioxidant which has been shown to inhibit the oxidation of lipids (Asia, Nakagawa & Miyazawa, 1999; Ramírez-Tortosa et al., 1999). Instead, it could be argued that curcumin did provide antioxidant protection to the fish oil supplement, but this prevented the positive effects of lipid oxidation. For instance, oxidation of DHA is necessary for the generation of neuroprotective docosanoids such as neuroprotectin D1 (Bazan, 2005). Not only is NPD1 important for reducing oxidative stress, but it can also have an Aβ-lowering effect (Lukiw et al., 2005; Zhang & Bazan, 2010). Our efforts to limit lipid peroxidation with curcumin may therefore have limited the positive effects of one of the likely beneficial candidates in fish oil – the DHA. The measure of lipid peroxidation products (both neurotoxic and neuroprotective forms) following curcumin co-supplementation with fish oil, relative to fish oil alone, may therefore be informative in future studies.

This argument may also explain the lack of effect observed with curcumin supplementation in chapter 5. However, previous studies have documented positive effects of the same curcumin dose in aged Tg2576 mice (Yang et al., 2005; Begum et al., 2008). The lack of positive effect may therefore be related to the young age at which curcumin intervention was employed; i.e., curcumin may only be effective during more advanced stages of pathology. In support of this position, curcumin is well documented to target plaque pathology (e.g. Yang et al., 2005; Garcia-Alloza et al., 2007; Begum et al., 2008), which is most predominant during advanced stages. These results would therefore suggest that early intervention of curcumin does not alleviate behavioural deficits and Aβ pathology during mid-stage pathological development in the Tg2576 model, but together with previous reports, this would suggest that curcumin treatment is limited to late-stages. This however does not support the notion that early-stage intervention of curcumin is less effective than later-stage intervention at reducing pathology and deficits during advanced stages. Research into this would therefore be insightful. Another issue that may need further examination is whether the oil blend supplement added to the curcumin contributed to the lack of dietary effect observed.

This may be unlikely, since the control diet contained the same level of oil blend supplement. However, we cannot account for potential interactions between the oil blend and curcumin supplement, and therefore future examinations could compare this supplement to a curcumin alone treatment.

Another important issue that was not addressed in this study was the level of cholesterol in the diets. It has been well documented that cholesterol is associated with AD risk and can acerbate pathological processes (Notkola, 1998; Refolo, 2000; Morris et al., 2003; Puglielli, Tanzi & Kovacs, 2003). Since cholesterol was not measured in the diets, it cannot be ascertained whether differential levels of cholesterol in the diets may have contributed to the results. However, SDS reported that the supplementary oil blend, DHA-rich oil and curcumin formulation contained no cholesterol, although it was likely that the fish oil supplement contained small levels of cholesterol. It is therefore possible that the lack of positive effects of fish oil supplementation may have been caused by additional levels of cholesterol compared to the oil blend control diet. In contrast, positive effects of DHA supplementation (containing no cholesterol, according to SDS) were found when compared to the same oil blend control diet, suggesting that cholesterol in the fish oil diet could be a contributory factor. Future research examining the effect of such diets should therefore measure cholesterol, which is routinely done by some research groups (e.g. Calon et al., 2004, 2005; Lim et al., 2005; Cole & Frautschy, 2006).

Another question that may need further examination is whether the level of omega-6 PUFAs in the diets limited the efficiency of the DHA or fish oil supplementation, since Green et al. (2007) found high levels of omega-6 PUFAs DPA and ARA supplemented with DHA lost efficiency in reducing Aβ and tau pathology after 6 and 9 months, respectively. It may therefore be worth examining the effect of DHA or fish oil supplementation while containing the lowest level of omega-6 PUFA essentially required in contrast to the typical level of omega-6 PUFA intake. Another issue that needs addressing in future research is the limitations inflicted by a small sample size. Although this was addressed in chapter 5 with use of a large cohort, this was particularly a problem for the assessment of Aβ pathology in the DHA study in chapter 3. As a result, this may have prevented the detection of diet effects; which may have otherwise led to a considerably different conclusion regarding the potential mechanisms of DHA effect in Tg2576 mice. Future studies should therefore adopt a similar approach to sample size as used in chapter 4, which led to a reasonably confident conclusion.

Overall therefore, the results of this thesis suggest that DHA supplementation from an early age had some positive effects in reducing behavioural deficits in Tg2576 mice, particularly at a young age. Since DHA failed to alter Aβ pathology, this would suggest that DHA targeted other pathological processes such as inflammation or oxidative stress. Further research however is required to test such hypotheses, and to re-examine the effect on Aβ pathology using a large sample size. This thesis also presents robust evidence that fish oil, curcumin and their co-supplementation from an early age consistently failed to reduce behavioural deficits or Aβ pathology in Tg2576 mice. Future research should aim to examine whether other nutritional features of fish oil may be beneficial in AD models, and whether an omega-3 PUFAs DHA and EPA combination approach is as effective as DHA monotherapy. Further investigation is also required into whether co-supplementation of other antioxidants may enhance or limit the positive actions of fish oil or DHA, and perhaps whether low level doses of antioxidants with these oils may present an optimal approach. Furthermore, this study examined the effects of sardine fish oil and so further research could examine whether different types of fish oil (such as salmon, pollock and mackerel) have differential effects. The differential use of fish oil sources are rarely reported in published studies.

These results therefore provide some support for the positive effects of DHA in human studies of AD, but fail to corroborate the positive effects of fish oil reported in epidemiological studies. While comparing the results to human studies it is important to consider that the Tg2576 model is simply a model of amyloid pathology, which does not represent the complex aetiology and nature of disease pathogenesis present in AD cases. For example, the early emergence of cognitive deficits before Aβ aggregation in the Tg2576 model is not the case in AD patients where pathology often develops over many years prior to an identifiable impairment (Smith, 2002). Furthermore, Tg2576 mice do not express tau NFTs or significant neuronal loss. With this differing pathology, one should therefore not expect the same results with intervention strategies as achieved in an animal model. So although the results of this thesis suggest limited effectiveness of DHA, fish oil and curcumin dietary treatment on Aβ-related pathology, it does not shed light on whether such manipulations could be protective in models displaying a more comprehensive pattern of AD-like pathologies.

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