NSAIDS, Fatty Acids and Cholesterol as Modifiers of Pathology in Rodent Models of Alzheimer's Disease

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SUMMARY

Alzheimer's disease, the most common type of senile dementia, is characterised by β-amyloid plaques, neurofibrillary tangles and neuronal loss. Amyloid plaques are associated with the increased inflammation and oxidative stress.

Rodent models overexpressing APP are a useful tool to investigate both the processes and the consequences of amyloid generation, and are crucial for developing and testing hypotheses about treatments and risk factors. Tg2576 transgenic mice overexpress human "Swedish" mutated APP and show memory impairment followed by amyloid plaques pathology. GP56 rats also overexpress human "Swedish" mutated APP, but show no signs of AD-like pathology. In this thesis, I explored the influence of dietary and pharmacological modifications on pathogenesis of AD as tested in rodent models.

As epidemiological data implicated the role of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing the risk of AD, I investigated the role of ibuprofen as either prevention or treatment in transgenic mice. The results indicated that ibuprofen delayed the onset of behavioural impairment but only when it was administered early during the evolution of their pathology. Levels of Aβ and plaque pathology were not affected. Also consumption of n-3 fatty acids seemed to reduce the risk of AD, possibly by acting as an anti-oxidant. Thus I investigated the effect of DHA on learning ability and pathology in AD mice. Administration of DHA to young mice reduced the impairment in learning but did not alter Aβ levels or plaque pathology. Finally the role of high cholesterol diet was investigated in GP56 rats. Feeding rats for 8 weeks did not induce any amyloid-connected pathology.

The results indicate that modulation of inflammatory processes, or lipids can have modest ameliorating effects on behavioural impairments in rodent AD models, provided intervention is undertaken early, but suggest these may not be the most efficacious targets for therapeutic intervention.

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ABBREVIATIONS

AA	Arachidonic acid
Aβ	β amyloid
Aβ40	β amyloid 1-40
Aβ42	β amyloid 1-42
ACAT	acetyl-CoA: cholesterol-O-acyltransferase
AD	Alzheimer's Disease
ADDL	Aβ–derived diffusible ligands
ALA	α-linolenic acid
ApoE	Apolipoprotein E
APP	Amyloid Precursor Potein
BBB	Blood-brain barrier
BSA	Bovine serum albumin
C	Celsius
cm	Centimetre
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebral spinal fluid
C-terminal	Carboxy terminal
DAB	3,3-diaminobenzidine
DGLA	dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid

DNA	Deoxyribonucleic acid
DSM-IV	The Diagnostic and Statistical Manual of Mental Disorders – 4th Edition
E	East
ELISA	Enzyme Linked Immunosorbent Assay
EMEA	European medicine evaluation agency
EPA	Eicosapentaenoic acid
FA	Formic acid
FAD	Familial Alzheimer's disease
FCA	Forced choice alternation
FDA	Food and Drug Administration
fEPSP	filed excitatory postsynaptic potential
fMRI	Functional magnetic resonance imaging
g	Force
GFAP	Glial fibrillary acidic protein
GLA	γ-linolenic acid
GM1	Ganglioside 1
h	Hour
H_2O_2	Water
HDL	High density lipoprotein
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl Coenzyme A Reductase
ICD-10	The International Statistical Classification of Diseases and Related Health Problems, 10th revision
Il-1	Interleukin-1
Il-1α	Interleukin-1α

Il-1β	Interleukin-1β
Il-6	Interleukin-6
I/O	Input/Output
i.p.	Intraperitioneal
kDa	kiloDaltons
kg	Kilogram
L	Litre
LA	Linoleic acid
LDL	Low density lipoprotein
LPS	Lipopolisaccharide
LTP	Long term potentiation
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
M	Molar
mV	Millivolt
N	North
NE	North-East
NFTs	Neurofibrillary tangles
nm	nanometre

NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NW	North-West
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
PC	Personal computer
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
pg	Pictograms
PG	Prostaglandin
PPARγ	Peroxisome Proliferator-Activated receptor γ
PPF	Paired pulse facilitation
PrP	Prion protein
PS	Population Spike
PS1	Presenilin 1
PS2	Presenilin 2
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen species
Sec	Second
S	South
SAD	Sporadic Alzheimer's disease
SDS	Sodium dodecyl sulphate

SE	South-East
SPSS	Statistical Package for Social Sciences
SW	South-West
TNF-α	Tumour necrosis factor-α
μA	Microampere
μg	Microgram
μl	Microlitre
μm	Micrometer
V	Volt
W	West

Chapter 1

General Introduction

1.1. Introduction to the thesis

Alzheimer's disease (AD) is a fatal neurodegenerative disorder associated with the elderly. It is characterised by deterioration of cognitive functions accompanied by gradual loss of all memories, leaving suffering individuals to live in their own solitary world. The disease is prolonged, often lasting 8 to 12 years following diagnosis, which severely affects not only the afflicted people, but also their families and friends. As the percentage and the age of the elderly population in the world is constantly increasing, age-related disorders represent a progressively greater burden on western civilisations at the economic, social and medical levels. Current treatments are limited, and at best give only temporary alleviation of symptoms, with eventual incapacitation and death being inevitable. These considerations make the research for the cause of AD and the development of effective treatment of great importance.

AD, being a progressive neurodegenerative disorder, is characterised by large numbers of Aβ (β-amyloid) deposits, neurofibrillary tangles, neuronal cell loss, which is accompanied by glial cell proliferation, inflammation and oxidative stress. The presence of inflammation and oxidative stress is considered to be either a cause of AD pathology or an effect of Aβ deposition. Many therapeutic strategies emerged from the idea that inhibiting either neuroinflammation or oxidative stress will be beneficial.

There are two major forms of AD: early-onset familial and late-onset sporadic. Familial AD, which generally occurs in persons <60 years of age, is caused by mutation in three known genes; presenilins 1 and 2 (PS1 and PS2) and amyloid precursor protein (APP). Slower, but promising, progress has been made in studying the complex picture of late-onset AD, which is probably associated with susceptibility factors increasing risk but not guaranteeing onset of AD. There is currently only one fully established genetic factor – polymorphism in Apolipoprotein E (ApoE) gene. Research in this field suggests a crucial role of polymorphisms in many other genes (e.g. Interleukin-1 (IL-1) or Urokinase-plasminogen activator) as well as numerous environmental factors including diet (cholesterol or polyunsaturated fatty acids).

The aim of this thesis is to use transgenic models to evaluate possible treatment strategies (Non-Steroidal Anti-Inflammatory Drugs) and to assess the importance of susceptibility risk factors (high cholesterol diet or polyunsaturated fatty acids intake).

To study the correlation between AD and inflammation in more detail, I assessed the effects of chronic, oral administration of the widely used anti-inflammatory drug Ibuprofen, given to transgenic mice over-expressing APP and showing AD-like pathology.

I also explored the role of lipid metabolism and oxidative stress in AD pathology in more details in the same transgenic mice by administering chronic, oral supplementation of the major brain n-3 fatty acid – docosahexaenoic acid (DHA), which has anti-oxidant properties.

Considering the pivotal role of environmental factors for developing late-onset sporadic AD, I also wanted to determine whether chronic, oral administration of a recognised risk factor would exacerbate the phenotype of rats over-expressing mutated amyloid precursor protein but showing limited amount of AD-like pathology. To this end, I fed transgenic rats on a high-cholesterol diet and assessed behavioural, physiological and biochemical consequences.

1.2. Introduction to Alzheimer's disease

1.2.1. Epidemiology

Alzheimer's disease (AD) is one of the fastest growing neurological conditions in developed countries and is the leading cause of dementia. AD alone accounts for 65-70% of diagnosed dementia cases (Brookmeyer et al., 1998) and in 2000, there were 4.5 million persons with AD in the US population. By 2050, this number is predicted to increase by almost 3-fold, to 13.2 million (Hebert et al., 2003). Although estimates are subject to significant variation, the numbers are alarming and the importance of considering a wide range of therapeutic interventions aimed at either finding a cure or preventing disease progression is manifestly evident.

1.2.2. Diagnostic Criteria

To date, the diagnosis of probable or possible AD is based on neuropsychological examination using criteria such as insidious onset and progressive impairment of memory as well as loss of other cognitive functions (McKhann et al., 1984). Standardised sets of criteria for dementia (ICD-10 and DSM-IV) help to distinguish AD from other types of dementia or delirium, depression and normal aging.

As a support, a number of non-invasive imaging methods have been used to study patients with AD. These methods can measure brain functional activity (e.g., positron emission tomography [PET], single photon emission tomography, and functional magnetic resonance imaging [fMRI]), anatomy (e.g., computer-assisted tomography, and MRI), or chemical composition (magnetic resonance spectroscopy [MRS]). When combined with clinical, cognitive, and genetic evaluation, they can help to distinguish AD from other causes of dementia and to make a positive diagnosis (reviewed in Norfray & Provenzale, 2004).

Other ways of supporting the diagnosis come from biochemical analysis. The cerebrospinal fluid of AD subjects shows significant elevation of tau and reduction in the Aβ42, which are characterised in more detail in section 1.3 (Sunderland et al., 2003).

Definite diagnosis of AD can only be made after post-mortem examination of brain tissue for the presence of plaques and tangles. However, it has to be stated that the mere presence of plaques and tangles in the brain is not sufficient evidence for a conclusive diagnosis of AD as these have also been reported in cognitive healthy elderly (Davis et al., 1999). A confirmed diagnosis at early stages is crucial with the advent of new drugs to treat AD, as treatment may be most effective if initiated early in the disease course.

1.2.3. Clinical Symptoms

Dr Alois Alzheimer described for the first time symptoms of AD in 1907. He reported the case of a middle-aged woman who developed memory deficits and progressive loss of cognitive abilities accompanied by morbid jealousy. The post mortem analysis of her brain revealed amyloid plaques and neurofibrillary tangles (Yankner et al., 1996; Price et al., 1998).

The typical early symptoms of AD are mild memory loss (like episodic forgetfulness of day-to-day events) and subtle behavioural changes. As disease develops, people suffering from AD also lose other types of memory, e.g. memory for verbal and visual meaning, spatial and delayed memory, and finally short term and working memory is also affected. Most suffering people, even at early stages, show abnormalities in problem solving, communication skills, language, calculation and visual and spatial perception (reviewed in Forstl & Kurz, 1999).

Sufferers may also display a wide range of behavioural disturbances, which are often more distressing for carers than the cognitive problems. These include verbal and physical aggression, paranoid delusions, hallucinations, sleep disturbances, depression, apathy, social withdrawal, wandering and pacing, repetitive actions, inappropriate social behaviour (reviewed in Forstl& Kurz, 1999). In late stages of the disease, patients are often mute, incontinent and bedridden. They usually die of other medical conditions (e.g. pneumonia) 5-10 years after clinical onset (Price et al., 1998).

1.2.4. The role of genetic risk factors

There are two groups of AD patients that differ in age of onset, but have indistinguishable clinical and pathological features. The majority of AD cases are sporadic type of AD

(SAD), which is characterised by onset after 65 years of age (Katzman et al., 1998). Early onset autosomal dominant form of AD (familial AD) is connected to certain gene mutations and represents approximately 1% of diagnosed cases (Campion et al., 1999).

Studies of the familial AD (FAD) revealed mutations in genes encoding either APP, located on chromosome 21, or PS1 and PS2 located on chromosome 1 and 14, respectively (Goate et al., 1991; Mullan et al., 1992; Citron et al., 1992; Campion et al., 1999; Sherrington et al., 1996; Citron et al., 1997).

Significantly, the majority of the mutations in APP that are linked to FAD have been reported to alter the generation of Aβ (for relationship between APP and Aβ see section 1.3). For example, the "Swedish" APP mutation results in enhanced production of Aβ40 and Aβ42 (Citron et al., 1997). Furthermore, plasma Aβ42 levels from FAD patients with PS1 gene mutations are elevated, suggesting increased generation of what is thought to be the more amyloidogenic form of the Aβ peptide (Borchelt et al., 1996).

Most cases of AD are sporadic and they cannot be explained by identified genetic mutations. The ApoE ε4 allele has been genetically linked to SAD and has a gene dose effect on the risk and age of onset of the disease (Corder et al., 1993; Saunders et al., 1993). Individuals with two copies of the ε4 allele have a 50-90% chance of developing AD by the age of 85 years, and those with one copy have an around 45% chance (Corder et al., 1993). The mechanisms by which the ε4 allele conveys the development of AD are largely unclear but many studies have revealed isoform specific effects on neurodegeneration, Aβ deposition, fibrillisation, and neuritic plaque formation (reviewed in Bales et al., 2002).

1.2.5. Environmental risk factors

Most cases of AD are sporadic late-onset, and although there are no known definite causes of late-onset SAD, there are several known, non- genetic risk factors.

Risk factors of a medical nature include traumatic head/brain injury (Rasmusson et al., 1995), low cerebral perfusion (de la Torre et al., 1999), cerebral infarcts (Heyman et al., 1998), strokes and cardiovascular disease (Brayne et al., 1998), hypertension (Kokmen et

al., 1991), thyroid disease (Genovesi et al., 1996), viral infections (Grant et al., 2002) and depression (Palsson et al., 1999).

Dietary risk factors include a high cholesterol diet and high dietary fat intake (Luchsinger et al., 2002; Grant et al., 2002), low intake of n-3 fatty acids (Morris et al., 2003) and aluminium in drinking water (Flaten et al., 2001).

1.3. Neuropathological Hallmarks of AD

There are four major anatomical changes that occur in the AD diseased brain: cortical atrophy, degeneration of neurons, accumulation of extracellular amyloid plaques and accumulation of intracellular neurofibrillary tangles. The latter two, plaques and tangles, are the hallmarks of AD and their main histological components are two proteins, amyloid β-peptide (Aβ) and tau, respectively (reviewed in Selkoe, 2001).

1.3.1. Senile Amyloid Plaques

There are two major types of amyloid plaques in AD brain, neuritic plaques and diffuse plaques. Neuritic plaques contain dense bundles of amyloid fibrils and are surrounded by dystrophic neurites, astrocytes, and microglia. Diffuse plaques contain non-structural amyloid and are not surrounded by dystrophic neurites. Neuritic plaques may develop from diffuse plaques (reviewed in Selkoe, 2001).

The number of neuritic plaques has not shown a clear correlation with the severity of dementia in AD (Terry et al., 1991). Some cognitively preserved aged individuals have such a high density of neuritic plaques in brain that the diagnosis of AD would be made if the individual exhibited any clinical signs of dementia (Katzman et al., 1988; Price et al., 1999). However, these cases may represent preclinical AD.

1.3.2. β-amyloid peptides

β-amyloid peptides are the main constituent of senile plaques. They are formed by the proteolytical processing of amyloid precursor protein (APP). In healthy brain, the major cleavage product is 40 amino acids long β-amyloid (Aβ40), but some longer β-amyloid 42 is also produced (Selkoe, 2001). β-Amyloid 42 (Aβ42) seems to be the important

isoform in plaque formation, since it is less soluble due to its additional N-terminal β-pleated sheet structure, which increases its tendency for fibril formation and aggregation (Selkoe, 2001). The initial extracellular Aβ deposits are composed mainly of Aβ42, although the senile plaques contain both aggregated Aβ40 and Aβ42 (Iwatsubo et al., 1994). Many studies indicate that the aggregation of Aβ42 may be the critical event in the pathogenesis of AD (Younkin, 1995), particularly in the formation of plaques, but possibly also in cell death after intracellular Aβ accumulation.

Studies of Aβ neurotoxicity have traditionally focused on the effects of insoluble amyloid fibrils (reviewed in Selkoe, 2001), but recent work suggests that soluble, pre-fibrillar aggregates of Aβ may also confer toxicity to cultured neurons (Lambert et al., 1998; Klein et al., 2001; Lashuel et al., 2002; Walsh et al., 1997, 2002). Behavioural and morphological alterations found in several strains of APP transgenic mice prior to the apparently fibrillar amyloid plaques suggest that soluble monomeric or oligomeric Aβ species can mediate neuronal injury in these animals (Mucke et al., 2000). Moreover, microinjected intracerebroventricularly soluble oligomers were found to inhibit the late phase of long term potentiation (LTP) in the rat hippocampus *in vivo* (Walsh et al., 2002).

The research interest was not only shifted from insoluble Aβ to more soluble forms, but also from extracellular to intraneuronal. Increasing evidence indicates the role of Aβ accumulation inside the cells, in particular, neurons. Intraneuronal Aβ may trigger intracellular and/or extracellular Aβ fibrillogenesis (La Ferla et al., 1995; Walsh et al., 2000) and intraneuronal toxicity (Kienlen-Campard et al., 2002). Neurons in AD-vulnerable brain regions specifically accumulate amyloidogenic Aβ42 and this intraneuronal Aβ42 immunoreactivity appears to precede both neurofibrillary tangles and Aβ plaque depositions (Gouras et al., 2000). Aβ42 aggregates into oligomers within endosomal vesicles and along microtubules of neuronal processes. It occurs both in neurons in culture from APP transgenic mice (Tg2576, for details see section 1.7.2) and in Tg2576 and human AD brain (Takahashi et al., 2004), which is associated with pathological alterations within processes and synaptic compartments in Tg2576 mouse and human AD brains

Therefore, prevention or disruption of intracellular Aβ deposition could be one of the important therapeutic goals for AD. The discovery that the APP transgenic mice (Tg2576,

for details see section 1.7.2) demonstrate an initial intraneuronal Aβ deposition in the somata of the hippocampal CA1/subiculum neurons as early as 4 months of age, prior to the first presence of extracellular Aβ plaques at 9 months of age is an important finding (Shie et al., 2003). This presence of Aβ in the neuronal bodies may be one of the initial steps in a cascade of events leading to neuronal death and extracellular amyloid deposition or be responsible for dementia. Also the intraneuronal hippocampal Aβ might have disturbed the integrity of the hippocampal circuit, explaining the behavioural changes observed in young Tg2576 mice prior to any formation of amyloid plaques.

1.3.3. Amyloid Precursor Protein metabolism

Amyloid Precursor Protein (APP) is a type-I transmembrane protein (Selkoe, 2001). The major isoforms consisting of 695, 751, and 770 amino acid residues are expressed widely, with brain neurons expressing primarily the 695 isoform (Selkoe, 2001). Despite years of intense investigation, the normal functions of APP and its catabolites remain unclear. The APP holoprotein may be involved in cell–cell interaction, cell adhesion, protease inhibition (for the longer APP isoforms), and neurite outgrowth (King, 2004) and the secreted fragment APPs may have neuroprotective and neurotrophic properties (King & Scott, 2004).

The proteases involved in the proteolytical processing of APP into Aβ, are the α-, β- and γ-secretases (reviewed in Selkoe, 2001, see Figure 1.1). Studies shown that α-secretase is a zinc metalloproteinase and several candidates fulfil the criteria required of α-secretase (reviewed in Allinson et al., 2003).

The formation of β-amyloid peptides involves the cleavage of APP by β- and γ-secretases (Haass et al., 1992, Shoji at al, 1992). β-secretase cleaves APP just before the β-amyloid region, which releases a large amino-terminal part of APP and creates a 12 kDa carboxy terminal fragment (Citron et al., 2002). β-secretase was identified by five groups as a novel membrane-bound aspartyl protease and is also called as the β-site-APP-cleaving-enzyme 1 (BACE1) or membrane aspartyl protease (Memapsin2) (Lin et al., 2000; Vassar et al., 1999; Sinha et al., 1999; Yan et al., 1999; Hussain et al., 1999).

Processing of Amyloid Precursor Protein

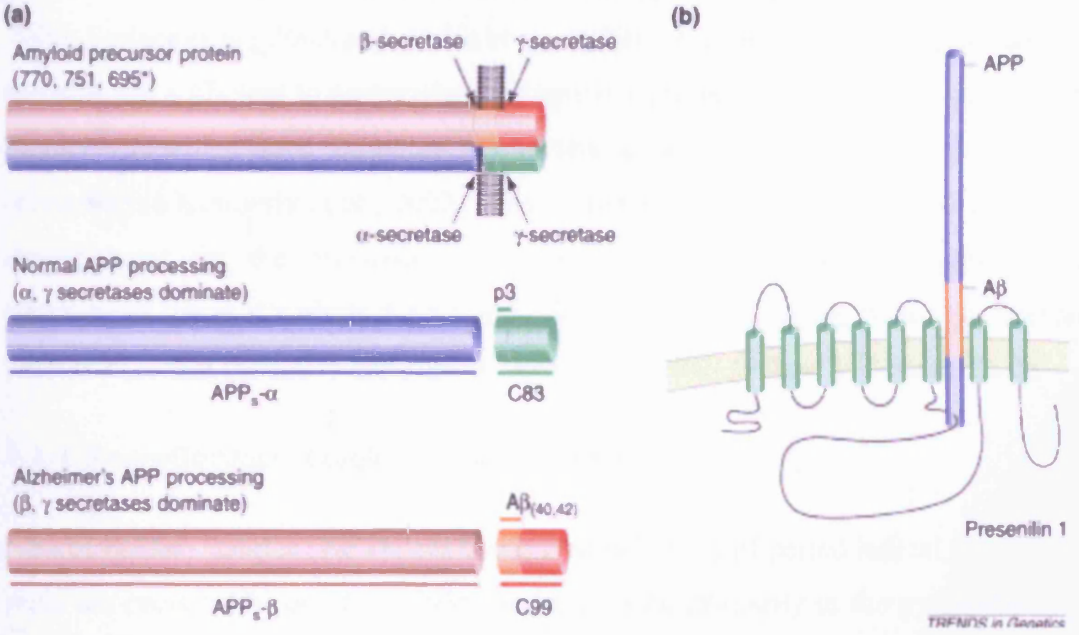


Figure 1.1. Processing of amyloid precursor protein.

(a) Normal processing of APP involves cleavage predominantly by the α-secretase, which cuts APP in the middle of the β-amyloid sequence, thus precluding the formation of Aβ. In the brains of FAD patients, processing by the β-and γ-secretases dominates, producing an excess of β-amyloid. Mutations to the APP gene cluster around the β- and γ-secretase sites, suggesting one mechanism by which APP processing can be misdirected towards producing the more toxic Aβ peptide.

(b) Presenilin has multiple membrane-spanning domains (green), two of which contain conserved aspartate residues that suggest aspartyl protease activity. *In vitro* and *in vivo* evidence that presenilins bind APP suggest a model like that shown in (b), in which presenilin cleaves APP into Aβ within the membrane. Mutations directed towards and around the putative aspartyl protease site lead specifically to overproduction of the highly amyloidogenic Aβ42 (adapted from Chapman et al., 2001).

The cleavage of the 12 kDa fragment by the γ-secretase generates the 4 kDa β-amyloid peptide. The γ-secretase can generate β-amyloid peptides ending at different residues, valine-40 or alanine-42 (Aβ40 and Aβ42) (Selkoe, 2001). γ-secretase catalyses intramembrane proteolysis of various type I membrane proteins, including the APP and the Notch receptor. Four membrane proteins are now known to be members of the protease complex: presenilin-1 or -2–nicastrin–aph1–pen2 (Esler et al., 2002; Li et al., 2000; Steiner et al., 2002 and Wolfe et al., 1999). Recent findings suggest that these four proteins are sufficient to reconstitute the active γ-secretase complex and that together they mediate the cell surface signalling of a variety of receptors via intramembrane proteolysis (reviewed in Kimberly et al., 2003). The β- and γ -secretases are obvious targets for drug development for the prevention and treatment of AD, but these proteases have a widespread tissue distribution and multiple substrates including Notch for presenilin γ–secretase, which creates difficulties (Citron et al., 2002; Selkoe & Kopan, 2003).

1.3.4. Neurofibrillary tangles and tau protein

Neurofibrillary tangles (NFTs) are comprised primarily of paired helical filaments formed from tau protein (Wood et al., 1986) and are found primarily in the pyramidal regions of the amygdala, hippocampus and neocortex. In non-AD patients, tau protein provides support to the cell's framework of microtubules. In AD patients, tau protein is hyper-phosphorylated. The extra phosphate is thought to cause microfilaments to become abnormally paired into helical filaments, which in turn form tangles. The cell then collapses and dies. A variety of kinases are capable of phosphorylating tau *in vitro* and these may represent a target for AD pharmacotherapy (reviewed in Hamdane et al., 2003)

Tangles predominantly affect those areas of hippocampus that are involved in processing learning and memory, which correlates with clinical deficits observed at early stage AD (Guillozet et al., 2003). It has been suggested that NFTs are related to the progression of AD (Braak et al., 1995; Guillozet et al., 2003), and a correlation between the number of NFTs and cognitive decline in AD has also been reported (Samuel et al., 1994; Nagy et al., 1995; Guillozet et al., 2003).

Unlike APP and the presenilins, no genetic links have been found between tau and AD (Buee et al., 2000). More than ten mutations have been described in the tau gene among

families with cases diagnosed as frontotemporal dementia with parkinsonism linked to chromosome 17 (Buee et al., 2000). Neuropathologically, these patients exhibit frontal and temporal lobe atrophy, filamentous tau pathology in neurons, gliosis, and neuronal loss (Buee et al., 2000).

1.3.5. Neuronal and synaptic loss

AD is characterised by degenerative changes in selected brain regions, including the temporal and parietal lobes and restricted regions within the frontal cortex and cingulate gyrus (reviewed in Wenk, 2003). The earliest neuronal cell loss is detected in the entorhinal cortex (Gomez-Isla et al., 1996), hippocampus (West et al., 1994) and in the basal nucleus of Meynert (Vogels et al., 1990). The exact mechanism of cell death in AD is unknown, but it may be linked to the neurotoxicity of Aβ, the development of NFTs, synaptic degeneration or apoptosis as evidenced by the expression of proteins related apoptotic pathways (Anderson et al., 1996; Su et al., 1997).

Along with (and possibly because of) the neuronal loss, AD is associated with degenerative changes in a variety of neurotransmitter systems. These include alterations in the function of the cortical cholinergic, serotoninergic, adrenergic and glutamergic systems in the hippocampal perforant pathway (reviewed in Wenk, 2003).

The degeneration of these systems may underlie specific aspects of the dementia associated with Alzheimer's disease.

1.4 Current theories underlying pathology of AD

Current theoretical characterisation of the pathological mechanisms underlying AD can be grouped into four main categories.

1.4.1. Amyloid Cascade Hypothesis

The amyloid hypothesis is the most powerful of the four hypotheses. It proposes that deposition of Aβ, the main component of senile plaques, is the causative agent of amyloid pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as direct results of this deposition (Hardy & Higgins, 1992). This amyloid cascade

is schematically illustrated in Figure 1.2. Key observations supporting β-amyloid as the initiating factor in AD include:

- Mutations causing AD increase the production of Aβ, or disturb the normal proteolytical processing of APP (reviewed in Selkoe, 1997). APP mutations at codons 716 and 717 lead to increased production of Aβ42 (Suzuki et al., 1994, Eckman et al., 1997), mutations at codons 670/671 (Swedish mutation) increase Aβ42 and Aβ40 production (Citron et al., 1992; Scheuner et al., 1996), and PS 1 and 2 mutations increase the production of Aβ42 (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996).
- ApoE4, a major risk factor for SAD, increases Aβ deposition (Holtzman et al., 2000).
- In Down syndrome (trisomy 21), the overexpression of chromosome 21-linked genes, including the APP gene, is related to the development of the neuropathological changes similar to AD around the age of 40 years (Iwatsubo et al., 1995).
- Transgenic mice exclusively overexpressing a FAD–linked mutant APPs develop first diffuse and then fibrillar Aβ plaques, associated with dystrophic neurites, astrocytes, and microglia. These plaques reproduce the major features of AD pathology, and cause in mice impairments in cognitive tasks. (Games et al., 1995; Masliah et al., 1996; Hsiao et al., 1996).
- Aβ can self-aggregate *in vitro*, and these aggregates can directly and indirectly mediate neurotoxicity (reviewed in Yanker, 1996).

Initially, investigators proposed that Aβ accumulation triggered a pathological cascade that ultimately produced the complete pathological and clinical symptoms of AD (Hardy & Higgins, 1992). Today, this hypothesis remains valid, except that it is less clear whether Aβ deposited as amyloid, or some other less well-characterized Aβ aggregate, initiates the cascade leading to neuronal death and dysfunction.

Small Aβ oligomers (Lambert et al., 1998), also referred to as Aβ-derived diffusible ligands (ADDLs), and protofibrils (Walsh et al., 1997, Lashuel et al., 2002) have emerged as alternative aggregated forms of Aβ that may mediate toxicity. Moreover, there is some evidence that intracellular accumulations of Aβ may also be neurotoxic (LaFerla et al., 1995, Gouras et al., 2000). Taken together these data support the hypothesis that AD results from the overproduction of the Aβ peptide(s).

Pathological Hypotheses of AD

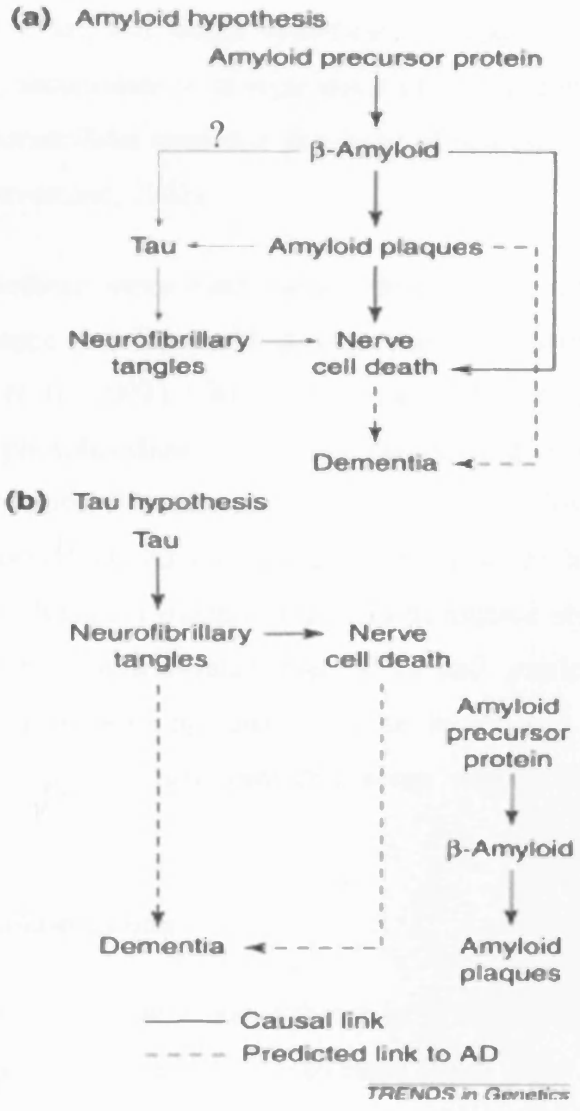


Figure 1.2. Amyloid-based versus tau-based hypothesis for AD pathology. According to the amyloid hypothesis, pathological processing of APP leads to increased brain concentration of Aβ, which in turn leads to amyloid plaque deposition. The plaques and/or more soluble Aβ can lead to neuronal death and tau pathology. Cognitive loss, culminating in dementia, can occur as a result of amyloid plaque formation, neuronal loss, direct effects of non-deposited Aβ, or some combination of these.

The tau hypothesis emphasises the role of tau and the consequent neurofibrillary tangles in producing cell loss and dementia. While amyloid pathway is more accepted, tau pathology is better correlated with dementia in humans (adapted from Chapman, 2001).

1.4.2. The Tau Hypothesis

The tau hypothesis argues that in AD, the normal role of tau in stabilising microtubules is impaired leading to tangle formation and neurodegeneration (reviewed in Mudher & Lovestone, 2002). The tau and tangle hypothesis is supported by the fact that tangles occur in the neuron, accumulate to occupy much of the neuron and apparently result in neuronal death, as extracellular tangles in the shape of neurons are abundant in late stages of AD (Mudher & Lovestone, 2002).

Support for this hypothesis arose from studies showing that tangles develop in neurons and that their abundance correlated with dementia severity (Samuel et al., 1994; Nagy et al., 1995; Guillozet et al., 2003). One of the assumptions of this hypothesis is that tau mutations alter tau phosphorylation, reducing the binding of tau to microtubules and leading to tangle formation (Mudher & Lovestone, 2002). However, tau mutations that lead to hyperphosphorylation and aggregation of tau do exist, but they do not cause AD (Hutton et al., 1998). Recent evidence from two transgenic studies (Gotz et al., 2001; Lewis et al, 2001) has demonstrated that NFTs and senile plaques formation are pathogenically related, illustrating that in some incidences amyloid influences the formation of NFTs. This in turn, provided some support for the amyloid cascade hypothesis.

1.4.3. The role of inflammation

The concept of inflammation as a major factor in AD has been based on post-mortem findings of immunological changes associated with the plaques, coupled with epidemiological evidence of a protective effect of anti-inflammatory agents (reviewed in McGeer & McGeer, 1995; Akiyama et al., 2000; Fassbender, 2000).

Microglial cells and astrocytes orchestrate the immunological reactions in AD. Highly activated microglial cells (the brain-resident cells of the mononuclear phagocyte system) cluster around plaques, express complement receptors and produce pro-inflammatory proteins (Akiyama et al., 2000). Microglia are normally the first cells in the brain to react to injury or infection and they recruit astrocytes by secreting proteins of the complement system, and through the release of cytokines, such as Interleukin-1β (IL-1β) and tumour

necrosis factor-α (TNF-α) (Eikelenboom et al., 1998). Astrocytes respond by releasing other complement proteins and acute-phase proteins, such as α1-anti-chymotrypsin and α2-macroglobulin, as well as neuronal growth factors and various cytokines – such as Interleukin-6 (IL-6) (Eddleston et al., 1993). Murine models of AD that overexpress APP and develop Aβ plaques also exhibit pro-inflammatory activation of microglia and astrocytes (Frautschy et al., 1998; Benzing et al., 1999; Stalder et al., 1999; Mehlhorn et al., 2000; Bornemann et al., 2001). Also IL-6 mRNA levels in the hippocampus and cortex are shown to be increased in both young and old APP transgenic mice as compared to non-transgenic mice (Tehranian et al., 2001). This increase in IL-6 mRNA levels is an early event in an Aβ-induced immune response cascade occurring before amyloid plaques can be detected and thus can represent a potential marker.

Microglia have been suggested to be responsible for either Aβ deposition (Frackowiak et al., 1992) or Aβ clearance (Bacskai et al., 2000; Chung et al., 1999; Jantzen et al., 2001). Microglia's role in neurodegeneration is not clear, although it has generally been assumed to be more negative. Studies of transgenic mice that overexpress mutant human APP and develop plaques have shown that crossing such mice with mice overexpressing a natural inhibitor of complement C3 results in a worsening of Aβ plaques load and more neuronal loss (Wyss-Coray et al., 2002). This result suggests that the inflammatory changes found in AD and mouse models, including activation of the classical complement cascade, may represent a beneficial response, at least in part.

There is evidence that the risk of AD is substantially influenced by polymorphisms in the inflammatory agents - IL-1α, IL-1β, IL-6, TNFα, α-(2) macroglobulin and antichymotrypsin (McGeer, 2001). It appears that AD is associated with alleles that promote increased transcription (McGeer & McGeer, 2001). This risk is increased when individuals carry AD-associated polymorphisms in two or more genes, as single nucleotide polymorphisms in the IL-1α and the IL-1β genes can give an almost four-fold elevated risk of contracting AD (Hedley et al., 2002).

Aβ peptide itself can induce a local inflammatory response, as fibrillar Aβ was shown to bind the complement and activate the classical complement pathway (Rogers et al., 1992). The activated complement products are suggested to play a key role in the recruitment and activation of microglia at the sites of fibrillar Aβ deposits (Eikelenboom et al., 1996).

In turn, these activated microglia produce multiple pro-inflammatory cytokines, which can start in turn a positive feedback loop that may trigger further dysregulation of the APP metabolism and local production of complement proteins and acute-phase proteins (Eikelenboom et al., 1996).

In summary, the idea that inflammatory processes are involved in AD pathogenesis is supported by genetic, pathological and epidemiological findings. But the problem is still to determine whether neuroinflammation is a cause or a consequence of the disorder.

1.4.4. The role of oxidative stress

The hypothesis that oxidative stress might be involved in AD pathogenesis was originally based on the free radical hypothesis of aging, which stated that age-related accumulation of reactive oxygen species (ROS) results in damage to major components of cells. The fact that age is a key risk factor in AD provides support for the free radical hypothesis, because the effects of the attacks by free radicals can accumulate over the years.

Oxidative stress is caused by an imbalance between the increased production of free radicals and the anti-oxidative defences (Halliwell, 1992). Free radicals are powerful harmful agents causing cell death or other forms of irreversible damage to major components of cells: nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins (Christen, 2000). Neurons appear to be particularly vulnerable to attack by free radicals, because their content of glutathione, an important natural antioxidant, is low, their membranes contain a high proportion of polyunsaturated fatty acids and brain metabolism requires substantial quantities of oxygen (Christen, 2000).

Oxidative stress can occur for a variety of reasons, some as common as the action of toxins from smoking or pollution (reviewed in Halliwell, 1997). This has been associated with numerous diseases including cancer, atherosclerosis, diabetes, and neurodegenerative disorders such as brain ischemia, Parkinson's disease, familial amyotrophic lateral sclerosis and AD (Halliwell, 1997)

Multiple lines of evidence have implicated oxidative stress and free radical damage to the pathogenesis and possible aetiology of AD (reviewed in Markesbery, 1997; Christen, 2000; Smith et al., 2000; Veurink et al., 2003).

The oxidation of mitochondrial DNA and, to a lesser extent, of nuclear DNA has been observed in the parietal cortex of AD patients (Mecocci et al., 1994). Protein oxidation has been observed in elderly individuals with and without AD, but appears to be more marked in AD patients in the regions presenting the most severe histopathological alterations (Hensley et al., 1995). Many studies have shown increased lipid peroxidation in the brains of AD patients, particularly in the temporal lobe, where histopathological alterations are very noticeable (Palmer et al., 1994; Lovell et al., 1995; Marcus et al., 1998). Montine (1998) and co-workers found that cerebrospinal fluid F_2-isoprostane concentrations are elevated in AD patients. These compounds are produced by free radical–catalysed peroxidation of arachidonic acid, independent of the cyclooxygenase enzyme. This discovery is important because it confirms that lipid peroxidation is elevated in AD, but also because it suggests the possible use of the quantification of cerebrospinal fluid F_2-isoprostane concentrations as a biomarker of this disease.

There is a dual relation between Aβ and the production of free radicals. Not only can the oxidative processes transform non-aggregated Aβ into aggregated Aβ *in vitro* (Dyrks et al., 1992), but also β-amyloid itself is a source of free radicals (reviewed in Veurink et al., 2003). It has been demonstrated that Aβ causes ROS accumulation in cultured hippocampal neurons (Mattson et al., 1995). It also induces lipid peroxidation (Butterfield et al., 1994). Aβ can cause excess production of superoxide radicals by interaction with vascular endothelial cells (Thomas et al., 1996). More recently, it has been shown that Aβ can elevate levels of hydrogen peroxide via its superoxide dismutase-like activity (Chan et al., 1999).

Collectively, this evidence suggests that assessing and reducing oxidative damage may be a beneficial clinical strategy to help prevent or delay the development and progression of AD. If, as appears more likely, oxidative damage is a downstream consequence of cell death caused by other specific mechanisms (hyperphosphorylation of tau), it is likely to be a key component in the cascade of pathology and pathogenesis and as such a potential target for neuroprotective therapy.

1.5. Treatment strategies

There is no cure for Alzheimer's disease. However, there are several drug treatments that may provide some symptomatic relief. Clinical treatments aim to provide support for surviving neurons and also seek to compensate for the partial loss of specific populations of cells, e.g. the cholinergic neurones by enhancing the function of remaining ones. This is the action of cholinesterase inhibitors, which can only produce a mild delay in the development of symptoms. Recent therapeutic approaches are directed to decreasing Aβ production or aggregation, or increasing its removal. Furthermore, more distal pathways are also targeted. Researchers are looking into modulating downstream events due to the presence of Aβ oligomers, such as free radical toxicity, inflammation, cell membrane damage, calcium dishomeostasis and excitotoxicity, or blocking the cellular response to injury through inhibition of neuronal apoptosis.

1.5.1. Cholinesterase inhibitors

Cholinesterase inhibitors enhance neuronal transmission by increasing the availability of acetylcholine at the receptors. They have been approved by the US Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) as treatment for AD for a long time, although they only provide symptomatic relief, not a recovery. Four inhibitors are currently available: tacrine, donepezil, rivastigmine, and galantamine (Cummings et al., 2002). These agents have been shown to produce improvements in global function and cognition. As such, they provide "cognitive enhancer' function and improve the individuals capacity to compensate for disability, rather than alleviating a specific component of the disease process *per se*. Secondary benefits include reduction in behavioural disturbances, temporary stabilisation of activities of daily living, delay of nursing home placement, and reduced demands on caregiver time (reviewed in Wynn & Cummings, 2004).

1.5.2. Vaccination

Despite very positive findings from APP mice models that immunization with synthetic Aβ or passive transfer of Aβ antibodies can markedly decrease the number and density of Aβ deposits in the brain (Schenk et al., 1999; Bard et al., 2000) and reverse impaired learning and memory (Dodart et al., 2002), the clinical trials in which Aβ42 synthetic

peptide was administered parenterally to individuals with mild to moderate AD had to be discontinued (Weiner et al., 2002; Hock et al., 2003). A phase II trial was discontinued when about 5% of the treated participants developed inflammatory reaction in the brain, although the preliminary evidence indicated an improvement in cognition scores over controls, including immunised patients that didn't produce any antibody (Weiner et al., 2002; Hock et al., 2003). Although initial clinical trials met problems, multiple studies are still investigating this treatment strategy further.

1.5.3. Memantine

Memantine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, was shown to have clinical benefit and good tolerability in AD. Due to its mechanism of action, memantine is considered a neuroprotective drug, whose value has been demonstrated in preclinical studies (Miguel-Hidalgo et al., 2002). Memantine exhibited efficacy and safety in trials in patients with moderate to severe AD (Reisberg et al., 2003). In October 2003, the FDA approved memantine for the treatment of moderate to severe AD.

1.5.4. Non- Steroidal Anti-Inflammatory Drugs (NSAIDs)

The pharmacological activity of NSAIDs is generally attributed to the inhibition of cyclooxygenases (COXs), enzymes necessary for the production of prostaglandins and leukotrienes from arachidonic acid. There are two kinds of COXs - COX-1 and COX-2, the constitutive and inducible forms of COX, respectively. They are known to be involved in inflammatory responses and normal neuronal functions.

The first indication that anti-inflammatory drugs might lessen the risk of Alzheimer's disease came from an observation that people with rheumatoid arthritis had an unexpectedly low prevalence of dementia (McGeer et al., 1990). It was an inventive idea, and more data have now accumulated, but the matter still remains unsettled. Up to now, there is compelling epidemiological evidence that long-term NSAID therapy has a dramatic effect on the risk of developing AD (Breitner et al., 1995; McGeer et al., 1996; Stewart et al., 1997; in t' Veld et al., 2001). Moreover, NSAID treatment delays disease onset, acts to ameliorate symptomatic severity, and slows disease progression (Rogers et al., 1993; Breitner et al., 1995, Rich et al., 1995, Stewart et al., 1997). Recently published

systematic review corroborated that NSAIDs do offer some protection against AD, particularly when taken long term (Etminan et al., 2003). Additionally, in humans, NSAIDs therapy results in a substantial reduction in the number of microglia associated with senile plaques, supporting the view that these cells are the targets of drug action (Mackenzie et al., 1998).

Although the NSAIDs data are encouraging, recommendations for chronic NSAID therapy in healthy elderly individuals are tempered by the documented risk of gastrointestinal bleeding and ulceration (James et al., 1999). To reduce the risk of gastrointestinal injury, new classes of NSAIDs have been developed, including selective inhibitors of cyclooxygenase-2 (COX2) and nitric oxide (NO)-donating NSAIDs.

These epidemiological findings have stimulated substantial interest in clinical trials of various anti-inflammatory agents to improve symptoms or delay progression in patients diagnosed with AD. The first study used indomethacin treatment for 6 months and provided slight but statistically significant improvement on a battery of cognitive tests, whereas placebo-treated patients declined (Rogers et al., 1993). However, this finding could not be reproduced in other studies using diclofenac/misoprostol (Scharf et al., 1999) prednisone (Aisen et al., 2000) or hydroxychloroquine (van Gool et al., 2001).

Although a lot of interest was placed in the newest group of NSAIDs - selective COX-2 inhibitors, studies have failed to support the hypothesis that they can slow the rate of decline in AD (Sainati et al., 2000; Aisen et al., 2003; Reines et al., 2004). The failure of selective COX-2 inhibition to slow the progression of AD may indicate either that the disease process is too advanced to modify in patients with established dementia or that COX-2 does not play a significant role in the pathogenesis of the disorder.

Epidemiological findings have stimulated substantial interest in the biological basis of the effect of this class of drugs, but the way that NSAIDs act to reduce the risk of AD is presently unclear. There are a variety of hypotheses, some of them conflicting with each other.

- The well-established function of NSAIDs is inhibiting COXs, both 1 and 2, and therefore, interleukin and prostaglandin formation. The prostaglandin (PG) H2 was shown to markedly accelerate the formation of dimers and higher oligomers of amyloid Aβ42

(Boutaud et al., 2002). Moreover, in studies *in vitro*, neurones treated with NSAIDs were protected against the toxic effects of Aβ42 and COX-1 selective inhibitors provided greater protection than did COX-2 selective inhibitors, suggesting that activation of COX-1 is required for Aβ4 -induced neurotoxicity (Bate et al., 2003). Another study suggested that expression of COXs may influence Aβ peptide generation through mechanisms that involve PG-E2-mediated potentiation of γ-secretase activity, further supporting a role for COX-2 and COX-1 in Alzheimer's disease neuropathology (Qin et al., 2003)

- Another target of NSAIDs action is the nuclear receptor and transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) (Lehmann et al., 1997; Landreth et al., 2001). The binding of NSAIDs to PPARγ results in the inhibition of proinflammatory gene expression (Delerive et al., 2001). PPARγ activation leads to inhibition of microglial-mediated neurotoxicity and cytokine expression elicited by Aβ fibrils both *in* vitro (Combs et al., 2000) and *in vivo* (Heneka et al., 2000).
- The potential neuroprotective properties of the NSAIDs were investigated by examining whether ibuprofen could reduce lipid peroxidation and superoxide radical generation. Lipid peroxidation and superoxide anion formation were induced in rat brain homogenate and ibuprofen significantly reduced this induction, suggesting possible neuroprotective features of this agent (Lambat et al., 2000).
- NSAIDs show direct nitric oxide radical scavenging activities in neuronal cells. Aspirin, mefenamic acid, indomethacin and ketoprofen directly scavenge generated nitric oxide radicals, while ibuprofen, naproxen or steroidal drugs have less marked or no scavenging effects. These results suggest that the protective effects of the former four NSAIDs against apoptosis might be mainly due to their direct nitric oxide radical scavenging activities in neuronal cells indicating neuroprotective activities against neurodegeneration (Asunama et al., 2001).
- NSAIDs protect neurons against glutamate toxicity. Ibuprofen significantly attenuated the decrease in dopamine uptake caused by glutamate, indicating preservation of neuronal integrity (Casper et al., 2000). Glutamate excitotoxicity has been implicated in Alzheimer's disease thus NSAIDs deserve further consideration as neuroprotective agents.
- NSAIDs have been shown to target directly γ-secretase. Eriksen (2003) and colleagues studied a whole range of NSAIDs in a human cell line and in APP transgenic mice. They showed that NSAIDs lower Aβ42 *in vitro* and *in vivo* and there is a significant

correlation between Aβ42 lowering and levels of ibuprofen. Moreover, flurbiprofen and its enantiomers selectively lower Aβ42 levels in broken cell γ-secretase assays, indicating that these compounds directly target the γ-secretase complex that generates Aβ from APP. Weggen (2001) and colleagues reported that a subset of NSAIDs, including ibuprofen, selectively suppressed the production of the amyloidogenic Aβ42 species both *in vitro* and in an acute treatment paradigm *in vivo*. *In vitro*, the decrease in Aβ42 secretion was accompanied by an increase in the Aβ (1-38) isoform, indicating that NSAIDs subtly alter γ-secretase activity. This group suggested that NSAIDs directly affect amyloid pathology in the brain by reducing Aβ42 peptide levels independently of COX activity.

- NSAIDs can lower amyloidogenic Aβ42 by inhibiting Rho, a small GTP-binding protein (G protein) that is involved in numerous cellular processes, including cell migration and survival, transcriptional regulation and vesicle transport (Zhou et al., 2003). They showed that increasing the activity of small G proteins in cultured cells selectively increased the concentration of Aβ42 while inhibition of the Rho effector Rho-associated kinase (Rock) reduced Aβ42 levels. Furthermore, treating a transgenic mouse model of Alzheimer's disease with Rock inhibitor reduced brain levels of Aβ42. Only the NSAIDs that were effective as Rho inhibitors lowered as well Aβ42 (Zhou et al., 2003).
- Gasparini (2004) and co-workers investigated the effects of compounds from every chemical class of NSAIDs on Aβ40 and Aβ42 secretion using *in vitro* studies and found that flurbiprofen and sulindac sulfide reduced the secretion not only of Aβ42 but also of Aβ40. Surprisingly, both COX-2 and COX-1 selective compounds significantly increased Aβ42 secretion. The levels of βAPP C-terminal fragments and Notch cleavage were not altered by any of the NSAIDs, indicating that γ-secretase activity was not overall changed by these drugs (Gasparini et al., 2004)
- Finally, NSAIDs have poorly defined effects on intracellular signalling pathways (Tegeder et al., 2001), including those used by cytokines (Baek et al., 2002).

The mechanisms through which NSAIDs act are likely to be complex and diverse. Although they have been examined extensively, no consistent conclusions can be drawn and further examination is clearly required.

1.5.5. Vitamin E and C

Antioxidants may protect the aging brain against oxidative damage associated with pathological changes of AD. As shown in recent cross-sectional and prospective study of dementia, the use of vitamin E and C supplements in combination is associated with reduced prevalence and incidence of AD (Zandi et al., 2004)

1.5.6. Oestrogen

Oestrogen replacement therapy for menopausal women has been shown to have potential both as a preventive and as a treatment for AD (reviewed in Cutter et al., 2003). Evidence from basic science demonstrates that oestrogen has multiple protective effects on neurons and neurotransmitter systems. However, the evidence for oestrogen's clinical role in the treatment and prevention of neuropsychiatric disorders is not well established (Cutter et al., 2003). Furthermore Henderson (2000) and colleagues failed to show that oestrogen given in standard doses can improve cognition in postmenopausal women with AD.

1.5.7. Statins

Statins are used to treat hypercholesterolemia and reduce *de novo* cholesterol synthesis by inhibition of the HMG-CoA reductase. Recently, two retrospective epidemiological studies indicated that there is up to a 70% lower prevalence and incidence of AD in subjects taking statins (Jick et al., 2000; Wolozin et al., 2000). The role of cholesterol in AD pathology is reviewed in section 1.6.3 and chapter 5.

1.5.8. γ-secretase inhibitors

Inhibition of production of amyloid peptides by inhibitors of γ-secretases has been suggested as the rational and most specific therapeutic approach. Unfortunately, γ-secretase complex is responsible for cleavage of various proteins, including Notch receptor. Recently, a γ-secretase inhibitor was developed that is able to reduce Aβ production without affecting Notch signalling, which raises the possibility that targeted γ-secretase inhibitor therapy might enter into clinical trials (Petit et al., 2001) Moreover, γ-secretase inhibitors were shown to reduce amyloid burden in animal models (Dovey et al., 2001).

1.5.9. Reversal or Prevention

When formed, amyloid plaques cause serious destruction to the surrounding brain tissue. This leaves the production of Aβ as the major target for consideration as therapeutic intervention. Usually the symptoms of AD do not appear until Aβ deposition in brain is extensive. Currently no pre-mortem diagnostic tool exists and substantial quantities of Aβ have already formed by the time AD is suspected or a diagnosis is made. By this time, the amount of Aβ already deposited may be sufficient to lead to an inevitable decline in neuronal viability and ultimate demise. In such situations the reduction, or even complete elimination, of Aβ production would not yield a 'cure', or possibly even much therapeutic benefit.

The scenario for the prevention, or early intervention, of Aβ deposition in persons who are pre-disposed to AD or suspected of having early signs of the disease is quite different from that presented above for reversal or cure. If the concept of giving any drugs as preventative proves to be a viable strategy, then questions arise regarding the appropriate time that administration of such drugs should be initiated. For example, should all persons over some pre-specified age be given the prophylaxis? Or should they be withheld until some suspicion of AD arises? Further what about members of families with the 'familial' form of the disease and the persons with Down syndrome?

Before these particular issues can be considered, we need to develop a better understanding of the pathology of AD.

1.6. Lipid metabolism in Alzheimer's disease

1.6.1. Overview

Lipids are major components of the mammalian diet. They consist of three main groups – neutral glycerides, phosphoglycerides and steroids. Neutral glycerides are primarily used to supply energy for cellular activities and are made up of a glycerol molecule esterified with one to three fatty acids. Triacylyglycerol is the major neutral glyceride. Fatty acids usually contain an even number of carbon atoms (16-20 are most common) bonded in a chain with a carboxyl group (COOH) at the end. If all the carbon atoms are joined by single carbon-carbon bonds, these fatty acids are called *saturated.* If they have one or

more double bonds between carbon atoms, they are called *monounsaturated* or *polyunsaturated* fatty acids respectively. The double bonds are counted from the methyl group determining the metabolic family, noted by n-x (n being the total number of carbon, x the position of the last double bond). Thus, for example, linoleic acid is named in the shorthand nomenclature 18:2, n-6. This compound has 18 carbon atoms, 2 double bonds and 6 carbon atoms from the last double bond to the terminal methyl group. A phosphoglyceride molecule consists of a glycerol bound to two fatty acids with a phosphate group at the third carbon atom. Phosphoglycerides are structural components of all cell membranes and differ from each other in the nature of the group attached to the phosphate. Steroids are lipids that include in their structure four rings of interconnected carbon atoms. The main steroid in mammals, cholesterol, is a major structural component of cell membranes and is used to synthesize other steroids, like the sex hormones (Lehninger, 2000).

1.6.2. Fatty Acids

Synthesis and structure

Although diet is a major source of fatty acids, most tissues synthesise fatty acids *de novo*. In addition, organs like the liver can also convert fatty acids from one form to another. However, humans cannot synthesise certain fatty acids *de novo*, such as linoleic acid (LA; 18:2, n-6), α-linolenic acid (ALA; 18:3, n-3) and their metabolites: γ-linolenic acid (GLA; 18:3, n-6), dihomo-GLA (DGLA; 20:3, n-6), arachidonic acid (AA; 20:4, n-6), eicosapentaenoic acid (EPA; 20:5, n-3), and docosahexaenoic acid (DHA, 22:6, n-3) (Yehuda et al., 2002). Because both n-3 and n-6 polyunsaturated fatty acids are required for good health, they are called Essential Fatty Acids and must be obtained from diet. LA is found in vegetable and nut oils such as sunflower, safflower, corn, soy and peanut oils. ALA is present in seed oils, such as linseed oil, rapeseed oil, soy oil and some nuts, particularly walnuts (Das et al., 2003).

LA is a precursor of the n-6 series of fatty acids, which include GLA and AA; ALA is a precursor of n-3 series of fatty acids - EPA and DHA. The main active components in both series are the longer chain polyunsaturated fatty acids (PUFA) – AA, DHA and EPA (Hansen et al., 1994). They are produced by desaturation and elongation using

microsomal enzymes from LA and ALA, respectively, or obtained directly from diet (AA - meats, eggs and prawns; EPA and DHA - deep-sea fish). The metabolic pathway for the synthesis of AA and DHA is shown on Figure 1.3 (Haag et al., 2003). During synthesis the n-3 and n-6 acids compete for the same enzyme systems (Sprecher et al., 1995).

Fatty acids in human brain

Lipids make up 50 to 60 percent of the dry weight of the brain, which represents the highest lipid content in human tissues after adipose tissue. Most of lipids are in the form of myelin surrounding neuronal axons. 35% of the total fatty acids of the brain are long chain PUFA, particularly DHA and AA (Sastry et al., 1985; Yehuda et al., 1999). Arachidonic acid is distributed in membrane phosphoglycerides throughout the body and is critically involved in second messenger pathways, cell signalling and eicosanoid pathways, whereas DHA is selectively enriched in synaptic plasma and retinal membranes (Sastry et al., 1985). The normal ageing process involves decreases in PUFA levels and changes in metabolism caused by decreased delta-6-desaturase activity (Horrobin et al., 1981), increased inflammatory responses (McGeer et al., 1995) and oxidative damage to the cell membrane (Markesby et al., 1997).

PUFA in human diet

A typical adult in Western society ingests 1-2g/day of ALA and the n-6/n-3 ratio of the diet is typically in the area of 8-16:1 (Lauritzen et al., 2001). The balance between the intakes of n-6 and n-3 fatty acids has been recognised by dieticians to be at least as important as the intake levels of individual fatty acids. This is because the metabolism of LA and ALA requires the same enzymes (they compete for them) and therefore if more n-6 is consumed, less long chain n-3 PUFA will be produced (Horrocks et al., 1999). Various health organisations now recommend a ratio of between 3:1 and 4:1 for dietary n-6/n-3 fatty acids (reviewed by Horrocks et al., 1999), though this does depend on the nature of the n-3 and n-6 fatty acids in the diet (Cunnane et al., 2003).

A typical daily supply of total very long chain n-3 PUFA in Western diets is estimated to be around 600 mg/day, with half of this amount coming from the diet (mainly fish) and the other half arising from endogenous production from ALA (Lauritzen et al., 2001).

PUFA synthesis from Essential Fatty Acids

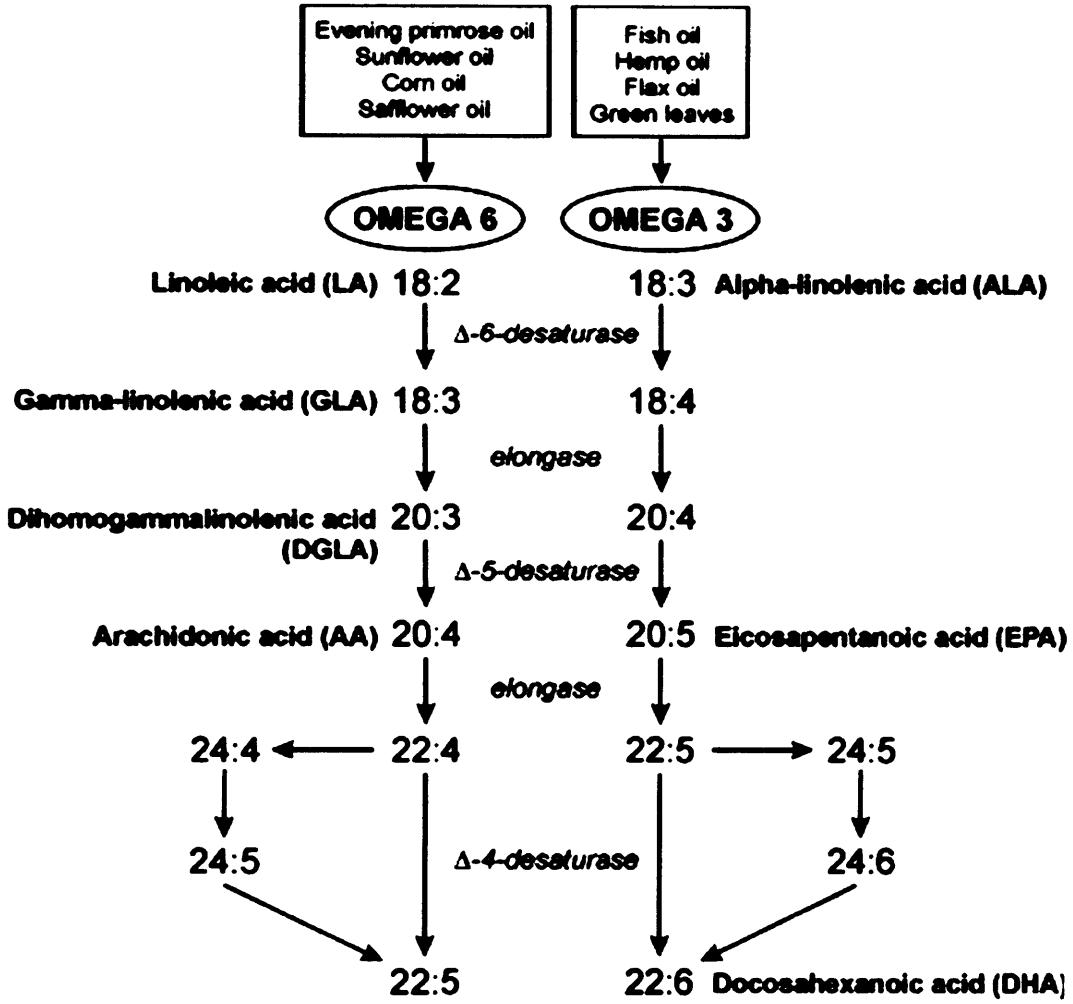


Figure 1.3. PUFA synthesis from parent Essential Fatty Acids.LA and ALA are desaturated and lengthened progressively by microsomal enzyme systems, to form important , very long chain PUFA – AA and DHA. Members of these two groups are inconvertible and they compete for the same enzyme systems in the cells (adapted from Haag et al., 2003).

Biological functions of PUFA in the brain

To date, a multitude of functions for DHA and other PUFA have been explored and there is a considerable evidence for their many roles in the brain. These include both physiological and patho-physiological functions:

- AA, DHLA and EPA are precursors of eicosanoids (e.g. prostaglandins, thromboxanes and leukotriens), which are local hormones that participate in a number of physiological effects (e.g. parturition initiation, platelet aggregation, renal electrolyte regulation, blastocyte implantation and activation of immune cells) as well as patho-physiological conditions (inflammation). Eicosanoids derived from n-6 PUFA generally have pro-inflammatory properties and activate immunological responses, while the ones derived from n-3 (mainly EPA) usually have anti-inflammatory features (Haag et al., 2003). Thus, high intake of n-6 PUFA can lead to aggravated inflammatory conditions. The n-3 PUFAs may also have anti-inflammatory effects by suppressing synthesis of inflammatory cytokines such as IL-1β, IL-1α and TNF in humans (Endres et al., 1989).
- Modification of the PUFA content in a membrane may be associated with changes in its physical properties such as fluidity, flexibility and permeability, which in turn influence the activities of membrane-associated molecules (Suzuki et al., 1998). PUFAs sustain cell membrane fluidity, i.e., the degree of freedom for molecules to move in the membrane. The ageing process is associated with a decrease in membrane fluidity and a decrease in the level of PUFA, particularly in hippocampus and cortex (Yehuda et al., 2002). Changes in membrane fluidity have been shown to affect various membrane functions, including neurotransmitter release (e.g. glutamate). McGahon et al. (1999), in three published studies, postulated that age-related impairment in LTP is linked closely to a decrease in membrane fluidity affecting glutamate release. Eight week dietary supplementation of DHA, ALA, LA or AA reversed the age-related decrease in glutamate release and the impairment in LTP *in vitro* in the rat dentate gyrus. By increasing the fluidity of membranes, PUFAs also affect the activity of many major membrane-bound enzymes, like the Na/K-ATPase, which functions in maintaining neuronal membrane potential. The activity of this enzyme in rat brain nerve endings was depressed by 40% in n-3 deficient rats, compared with the n-3 adequate group (Bourre et al., 1989).

- The n-3 PUFAs are essential for regulating neuronal excitability. Vreugdenhil et al. (1996) reported that extracellular application of either DHA or EPA caused a shift of the inactivation state towards a more hyperpolarising direction for both sodium and calcium currents in freshly-isolated rat hippocampal neurons from CA1 region of the hippocampus. Furthermore Xiao et al. (1999) reported that both DHA and EPA applied extracellulary to mouse brain slices reduced the frequency of electrically evoked action potentials in hippocampus regions and also hyperpolarised the resting membrane and raised the stimulatory threshold of action potentials. This stabilising membrane excitability function for n-3 PUFAs could be useful in the treatment of epileptic seizures.
- The PUFAs can influence various neurotransmitters levels in the brain. Delion and colleagues (1996, 1997) reported lower dopamine levels in the cerebral cortex in the n-3 deprived rats, coupled with increased serotonin levels, accompanied by a low ratio of n-3 to n-6 PUFA in the membrane. Furthermore, when Chalon (1998) and co-workers increased the ratio of n-3 to n-6 PUFA in rat diet, this resulted in increased dopamine levels in the cerebral cortex.
- DHA appears to be crucial in brain development. Martin (1998) and co-workers showed selective accumulation of DHA in synaptic growth cones during neuronal development. The outgrowth of neurites induced by nerve growth factor was promoted by DHA and suppressed by AA (Ikemoto et al., 1997) and dietary n-3 PUFA deficiency decreased nerve growth factor content in rat hippocampus (Ikemoto et al., 2000). DHA deficiency caused a reduction in the size of neurons in the CA1 region of the hippocampus, hypothalamus and cortex, which could be linked to a loss in the optimal function of neurons (Ahmad et al., 2002).
- The presence of PUFAs in the brain seems to be beneficial for preventing neuronal death. Lauritzen et al. (2000) showed that LA prevented neuronal death in animal models of global ischemia, even when administered acutely after the insult. Okada et al. (1996) showed that chronic administration of DHA to rats protected hippocampal neurons from damage caused by either hypoxia or ischemia and significantly improved the spatial learning deficit following hypoxic conditions. DHA also protected neural cells from induced apoptotic death (Akbar et al., 2002).
- PUFAs increase the number of receptors expressed on the cell membrane, including the insulin receptor, possibly by increasing membrane fluidity. Insulin receptors are on neurons and are believed to have a role in cognitive function, including learning

and memory (Zhao et al., 1999). These insulin receptors are affected by deficiency of n-3 fatty acids (reviewed by Das, 2002). Also, AA has been shown to stimulate glucose uptake when applied directly to cultured cerebral cortical astrocytes (Yu et al., 1993).

PUFA in cognitive function and AD

Adequate supplies of both AA and DHA are critical for optimal growth and functional development of the brain in infants (reviewed by Horrocks et al., 1999; Haag et al., 2003). First the placenta and then breast-feeding are the main sources of long chain PUFA for growing children, but these fatty acids are absent in most commercial infant formulas (Jensen et al., 2000). A meta-analysis of 20 published studies confirmed the association of breast-feeding with significantly higher scores for cognitive development compared with the infants who received formula feeding (Anderson et al., 1999).

In recent years, numerous studies have examined the influence of nutrition on ageing processes, particularly cognitive decline. For example, a study performed among aged men has shown that a high n-3 PUFA intake (via fish consumption) was inversely associated with cognitive impairment (Kalmijn et al., 1997).

The role of DHA and other PUFAs has also been widely investigated in AD patients. Concentrations of essential fatty acids were found to be abnormal in plasma and red blood cell phospholipids from AD patients compared to age-matched controls (Corrigan et al., 1991). A particularly marked decrease in DHA content of phosphatidylethanolamines (a major class of brain phosphoglycerides) has been observed in frontal grey matter and hippocampus of AD patients (Soderberg et al., 1991). Yehuda et al. (1996) reported that an essential fatty acid mixture SR-3 (ALA and LA, in a ratio of 1:4) improved the quality of life (mood, appetite, sleep) of AD patients. Further clinical studies suggested that low serum DHA might be a significant risk factor in the development of AD in elderly patients (Kyle et al., 1999). Plasma levels of DHA, total n-3 fatty acids, and the ratio n-3/n-6 were lower in AD patients, patients suffering from other types of dementia and people with cognitive impairment when compared to normal patients. This study implied that a decreased level of plasma DHA was common in all types of cognitive impairment and not unique to AD (Conquer et al., 2000). Finally, two major epidemiological studies performed by Morris (2003) and co-workers revealed that intake of saturated or trans-

unsaturated (hydrogenated) fats may increase the risk of developing AD and that consumption of fish or DHA reduces the risk of incidence of AD to 60% compared to a people on low n-3 diets.

Although the numerous studies reviewed above show that levels of DHA and other PUFA in the central nervous system during development and in adulthood could alter cognitive functions in humans, the precise mechanism of action of these fatty acids is still largely unknown.

1.6.3. Cholesterol

Cholesterol is the most prevalent steroid in humans and is an essential structural component of cell membranes (Lehninger, 2000). Elevated levels of cholesterol are known to increase the risk of cardiovascular diseases such as heart attack and stroke, but accumulating evidence suggests that cholesterol may contribute to Alzheimer's disease as well.

Cholesterol in the brain

The brain accounts for only 2% of the whole body mass but it contains almost a quarter of the total cholesterol. Relatively little is known about cholesterol homeostasis in the brain, as most of the studies on cholesterol metabolism have been performed in peripheral cells (reviewed in Dietschy & Turley, 2001, see Figure 1.4.). Lipoproteins circulating in plasma are the main source of cholesterol for peripheral cells, but they cannot cross the blood-brain–barrier (BBB) (Dietschy & Turley, 2001). Although some researchers suggest that a small fraction of lipoproteins could cross the BBB (Koudinov et al., 1996), it still means that the brain is almost entirely dependent on cholesterol synthesized *de novo* (reviewed in Dietschy & Turley, 2001). In fact, it has been established that cholesterol used by oligodendrocytes to produce myelin is made locally in the brain and is not transported from the periphery (Jurevics et al., 1995). It is also known that neurons form functioning synapses only in the presence of astrocyte derived cholesterol (Mauch et al., 2001).

Cholesterol metabolism in the brain

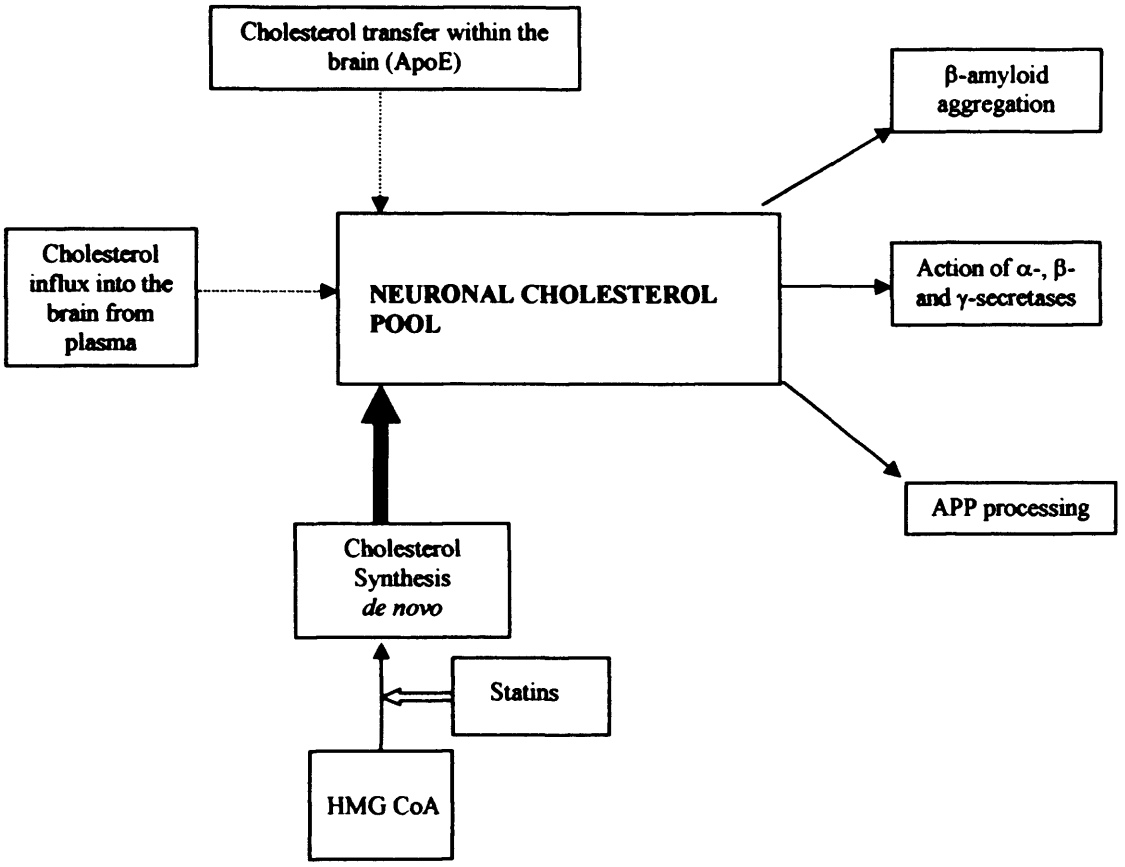


Figure 1.4. Schematic view of cholesterol homeostasis in neurons. Neurons maintain cholesterol homeostasis mainly through *de novo* biosynthesis (indicated by a thick arrow) but also to a lesser extent through internalisation of lipoproteins, obtained from either plasma or other parts of the brain (indicated by dotted arrows). Statins reduce *de novo* synthesis of cholesterol by inhibition of HMG-CoA reductase. The intracellular pool of cholesterol affects APP processing, Aβ aggregation and the action of all secretases cleaving APP.

Cholesterol is the product of a multienzyme pathway that begins with the action of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) synthase and reductase (Lehninger, 2000). The latter enzyme is the target of statins, which are widely used to treat hypercholesterolemia. Cholesterol homeostasis is maintained by the interplay between synthesis, uptake and catabolism. Excess cholesterol is eliminated by acetylation or oxidation. In the brain, cholesterol when converted to 24-hydroxycholesterol can cross the BBB (Bjorkhem et al., 1998), and this is a putative way for it to be removed from the brain.

Brain cholesterol turnover is much lower than that of the periphery (Andersson et al., 1990). As it is generally assumed that lipoproteins cannot cross the BBB, Edmond et al. (1991) has suggested that an increase in cholesterol in liver and plasma does not change the concentration of cholesterol in brain. However, animal studies brought contradictory results (reviewed in chapter 5). To support animal results, some studies show that a fraction of lipoproteins could cross the BBB (Koudinov et al., 1996),

Cholesterol in the periphery is transported by lipoproteins. The brain contains its own fraction of lipoproteins in cerebrospinal fluid, which could be responsible for local re-distribution of cholesterol (Dietschy & Turley, 2001). Apolipoprotein E (ApoE) is the main transport protein in the brain for cholesterol. Intracellular cholesterol is absorbed onto ApoE leading to the formation of ApoE lipid complexes, which transport this sterol from regions high in cholesterol to regions low in cholesterol (Zhang et al., 1996; Mahley et al., 1988).

As an integral component of all membranes, cholesterol affects markedly their ordering, rigidity and fluidity. In general, higher cholesterol levels enhance the rigidity of the membrane and make it less permeable to small water-soluble molecules (Yeagle et al., 1991).

The cell membrane is not considered a homogenous mixture of lipids and proteins any more, but also contains particular areas, called lipid domains (reviewed by Simons & Ehehalt, 2002; Lai, 2003). Lipid domains contain particularly high concentrations of sphingolipids and cholesterol. Lipid rafts (which are presumed to be too small and transient to be observed *in situ*) and caveolae are the main types of lipid domains. The

latter are morphologically identifiable and have been recognised as stable flask-like invaginations of the plasma membrane (Lai, 2003). Lipid domains play a central role in many cellular processes, including membrane sorting and trafficking, cell polarisation and signal transduction (Simons & Ikonen, 1997). Lipid domains have been identified in most mammalian cells, including neurons and glia and have been implicated in pathogenesis of many diseases such as viral infections, AD and prion diseases (Simons & Ehehalt, 2002). Cholesterol plays a crucial role in composition, function and generation of lipid domains and, when present in either markedly low or high amounts, changes functioning of these structures (reviewed Simons & Ehehalt, 2002; Lai, 2003).

Cholesterol and AD Epidemiology

There have been a number of epidemiological studies that have pointed to a relationship between cholesterol and the development of AD. Sparks (1990) and co-workers were one of the first groups to suggest a possible link between cholesterol and AD. They found that patients with critical coronary artery disease also showed deposition of Aβ similar to that seen in AD patients. The link between cholesterol and AD became stronger when a genetic linkage study by Strittmatter et al. (1993) identified a variant of the gene that encodes the cholesterol-carrying ApoE as a late-onset AD risk factor. Approximately 40% of AD patients have at least one ApoE ε4 allele, and being homozygous for ApoE ε4 allele increases the risk of AD 4-fold. The leading hypothesis for the mechanism by which ApoE ε4 increases the risk of AD is that ApoE binds Aβ. This in turn, will either promote the aggregation of Aβ or reduce clearance of aggregated Aβ (reviewed Wolozin, 2004).

Further, cross-sectional analysis has described an association of atherosclerosis (for which hypercholesterolemia is a recognised risk factor) with AD in the Rotterdam Study (Hofman et al., 1997). Many other longitudinal studies in various populations have supported this discovery, suggesting inter-relationships between elevated cholesterol levels, ApoE genotype and late-life cognitive impairment or AD risk (Jarvik et al., 1995; Notkola et al., 1998; Evans et al., 2000; Kivipelto et al., 2001; Launer et al., 2001).

Recently, two retrospective epidemiological studies indicated that there is up to a 70% lower prevalence and incidence of AD in subjects taking statins (Jick et al., 2000;

Wolozin et al., 2000). Statins are used to treat hypercholesterolemia and reduce *de novo* cholesterol synthesis by inhibition of the HMG-CoA reductase. Whether statins reduce cerebral Aβ by lowering cholesterol in the brain or by another mechanism remains an open question.

The role of serum cholesterol in cognitive performance in an aging population has also been studied. Teunissen et al. (2003) reported that relatively high ratios of the cholesterol precursors - lanosterol and lathosterol, indicative of a high rate of endogenous cholesterol synthesis, are associated with relatively low memory performance. No research has been done on the role of these precursors in the neurodegenerative processes.

Although these are interesting results, epidemiological studies cannot reveal the cause of disease. They can only suggest that cholesterol and AD pathogenesis are in some way linked.

Cholesterol and the processing of APP

There is growing biochemical evidence from studies in vitro that excess cholesterol may promote amyloid plaque formation. Although many studies have been performed, the results indicate various mechanisms and no unifying conclusion can be drawn. Here is a short overview of interactions between cholesterol, APP and β -amyloid.

- APP and Aβ are both associated with lipid domains (Bouillot et al., 1996; Lee et al., 1998; Refolo et al., 1991). Lipid domains have been proposed to regulate APP processing by favouring the clustering of APP and both γ- and β-secretases (Ehehalt et al., 2003; Ikezu et al., 1998; Lee et al., 1998). Also the physical association of Aβ to ganglioside (GM1), another component of lipid domains, facilitates the aggregation of soluble Aβ in the form of amyloid fibrils (Yanagisawa et al., 1995). Another study suggested that Aβ associated with lipid domains adopts a different conformation, acting as a "seed" for amyloid plaque formation (Mizuno et al., 1999). Various studies confirmed that caveolae-like lipid domains contain several proteins important in the pathophysiology of AD including APP, Aβ, ApoE, PS1 and possibly both α- and β-secretases (Anderson et al., 1998; Cole et al., 1999; Ikezu et al., 1998; Lee et al., 1998; Nishiyama et al., 1999;

Sambamurti et al., 1999). This clustering may help in promoting the specific interactions between all of them.

- The cell cholesterol levels can actively regulate APP processing and Aβ generation. An increase in cell cholesterol content up-regulates, whereas a decrease down-regulates, Aβ generation (Fassbender et al., 2001; Frears et al., 1999; Simons et al., 1998). Cholesterol depletion in rat hippocampal neurons leads to a reduction of the C-terminal fragment, suggesting that β-secretase cleavage could depend on cholesterol (Simons et al., 1998). Furthermore, secretion of the APP ectodomain generated by α-secretase pathway was shown to increase in response to cholesterol depletion (Bodovitz et al., 1996; Kojro et al., 2001). Thus, the presence of cholesterol seems to promote the amyloidogenic (β- and γ-secretase) pathway while inhibiting the non-amyloidogenic (α-secretase) pathway of APP processing (Bodovitz et al., 1996; Kojro et al., 2001; Wahre et al., 2002).
- Intracellular cholesterol distribution regulates APP processing and Aβ generation (Puglieli et al., 2001). This group suggested that the dynamic equilibrium between free cholesterol bound to the membrane and cholesteryl-esters regulates the generation of Aβ. A selective increase in cholesteryl-esters is sufficient to up-regulate the generation of Aβ. This group also established that blockade of the enzyme acyl-coenzyme A cholesterol acyltransferase (ACAT), which catalyses the formation of cholesteryl-esters from cholesterol, reduced both cholesteryl-esters and Aβ synthesis in a dose-dependent manner (reviewed in Puglieli et al., 2003).
- Cholesterol affects membrane physical properties such as ordering, rigidity, and fluidity (Yeagle et al., 1991). APP is a transmembrane glycoprotein and the secretase cleavage sites lies within, or near, its intramembranous domain. Thus, it is possible that a change in membrane cholesterol levels would affect the way APP resides or function in the membrane.
- Cholesterol has indirect effects via the ApoE ε4 allele, a susceptibility gene of SAD (Strittmatter et al., 1993). ApoE ε4 allele, compared with ApoE ε2 allele and ApoE ε3 allele is less effective in neuronal repair, increases Aβ fibrillogenesis and decreases Aβ clearance (reviewed by Weisgraber & Mahley, 1996). The ApoE ε4 allele is associated with higher cholesterol levels (Sing et al., 1985) and a recent study has shown that ApoE ε4 promotes the efflux of cholesterol from neurons less efficiently than ApoE ε2 and

ApoE ε3 (Michikawa et al., 2000). However, it is also possible that ApoE contributes to the pathology of AD by effects on general lipid metabolism (Roher et al., 1999; Wood et al., 1999).

Taken together these results suggest that cellular cholesterol levels, as well as the distribution of cholesterol throughout the cell, could mediate APP-Aβ processing. These interactions suggest also that there must be a link between cholesterol and AD.

Cholesterol in AD patients

Although there is a confirmed association between cholesterol and AD, the role of this steroid in the pathogenesis of this disease is not clearly understood. Individuals with elevated levels of plasma cholesterol have an increased susceptibility to AD, apparently influenced by the ApoE ε4 genotype (Jarvik et al., 1995, Kuo et al., 1998). Moreover, AD patients have increased levels of total serum and low-density lipoprotein (LDL) cholesterol (Jarvik et al., 1995, Kuo et al., 1998) along with a reduced level of high-density lipoprotein (HDL) in their plasma (Kuo et al., 1998, Fernandes et al., 1999), as compared to age-matched controls. This metabolic profile (high plasma cholesterol with high LDL, and low HDL) is commonly found in patients with atherosclerosis. Atherosclerosis has also been shown to correlate with an increased risk of AD, with higher risk being associated with more advanced arteriosclerosis (Hofman et al., 1997).

AD patients in early stages of dementia have increased levels of 24S-hydroxycholesterol in the cerebro-spinal fluid (Papassotiropoulos et al., 2002) and in plasma (Papassotiropoulos et al., 2000). 24S-hydroxycholesterol can cross the BBB into the circulation and reflects cholesterol homeostasis in the brain (Papassotiropoulos et al., 2000). As 24S-hydroxycholesterol is elevated in AD and vascular dementia patients, it could suggest that brain cholesterol turnover is increased during neurodegeneration.

Although higher amounts of cholesterol accumulate in the dense cores of amyloid plaques in the brain of AD patients (Mori et al., 2001), data on the cholesterol content of the brain tissue of Alzheimer's patients have been ambivalent. Mason et al. (1992) found that the cholesterol content was lower in the temporal gyrus of autopsied brains of AD patients in contrast to control subjects. However, Sparks et al. (1997) showed a small but significant

increase in frontal cortex grey matter of AD patients with the ApoE ε4 genotype compared with ApoE ε4 control subjects. Technical differences (e.g. different tissue preparation and different brain region analysed) in the studies could account for the lack of consistent results. Additionally, mRNA levels of HMG-CoA reductase in brain were found to be indistinguishable between AD samples and control samples (Yasojima et al., 2001).

Clearly an understanding of cholesterol biosynthesis in the brain and its role in AD requires further investigation. It is possible that cholesterol on its own could trigger AD pathology but also that cholesterol homeostasis failure may be a result of developing AD pathology.

1.7. Animal Models of Alzheimer's disease

Historically, models of AD were focused on drug or CNS lesion-induced behavioural deficits based on specific neuroanatomical lesion or neurotransmitter deficit associated with the disease. The beginning of transgenic technology and identification of AD-associated genes has revolutionised animal models of AD allowing a better understanding of pathology and treatment of this disease. Gene-targeted and transgenic mice have proven to be invaluable for studying the pathogenesis of AD. Transgenic animals display neuropathological and behavioural features of AD including amyloid Aβ and amyloid deposits, neuritic plaques, gliosis, synaptic alterations and signs of neurodegeneration as well as memory impairment.

1.7.1. Rational for animal studies

The key purpose for attempting to replicate AD neuropathology in animals is to gain:

- A better understanding of the impact of certain types of AD pathology on specific brain systems (e.g. temporal lobe versus frontal lobe systems);
- A better understanding of the physiological processes which are responsible for the formation of AD neuropathologies;
- An increased understanding of how specific pathologies, or the development of pathologies, affect physiological functions and cognition;

- An opportunity to evaluate potential therapies on specific aspects/features of AD and cognition.

1.7.2. Mouse Models

Transgenic mice show some of the characteristic AD pathology, such as an age-dependent formation of Aβ plaques. However, they usually (until recent multiple transgenic development, see below) lack both the tau pathology (neurofibrillary tangles) and the neurodegeneration. Importantly, many of these transgenic lines develop age-dependent deficits in some relevant behavioural tests and thus provide an animal model not only for amyloidosis but also for the cognitive deficits of AD patients. The best-characterised AD mouse transgenic models are:

PDAPP mouse

Games (1995) and colleagues created the first successful transgenic mouse model of AD using a transgene construct of the human APP gene containing the V717F mutation under a PDGF-β promoter. It developed many of the pathological hallmarks of AD, including neuritic plaques surrounded by dystrophic neurites, astro- and microgliosis, and synapse loss, but no neurofibrillary tangles. Behavioural studies with the PDAPP mouse have been reported independently by two groups (Dodart et al., 1999; Chen et al., 2000) and describe similar early cognitive deficits at a time point before amyloid plaques could reliably be detected. Redwine et al. (2003) recently described a magnetic resonance imaging (MRI) and stereological analysis of PDAPP mice. Hippocampal volume (mainly dentate gyrus) was reduced in PDAPP mice before Aβ plaques could be histologically identified.

APP Tg2576 mice

First reported by Hsiao and colleagues (Hsiao et al., 1995), to date this has probably been the most widely reported AD transgenic mouse model. This mouse over-expresses a human APP with the K670N/M671L ("Swedish mutation" - a lysine at residue 670 is exchanged for asparagine and methionine at residue 671 is exchanged for leucine) mutation (APP695) under control of the prion protein promoter to ensure high CNS expression. Initially this transgene was engineered into FVB/N mice, resulting in an early

lethality before any amyloid pathology was manifested (Hsiao et al., 1995). Creating a new line in a different genetic background (C57BL/6J×SJL cross) resulted in better survival and the emergence of thioflavin-S-positive amyloid plaques with age (onset approximately 9–12 months) (Hsiao et al., 1996). Extensive neuropathological studies provided evidence of an age-related increase in amyloid deposits, neuritic dystrophy, astrogliosis, reactive microglia and abnormal tau phosphorylation (Hsiao et al., 1996, Irizarry et al., 1997, Frautschy et al., 1998, Kawarabayashi et al., 2001). Since genetic background is known to influence susceptibility to excitotoxic cell death, attempts to backcross these mice onto a 'pure' C57BL/6J line were undertaken but resulted in early lethality (Carlson et al., 1997).

Several laboratories have reported deficits in learning and memory in aged, but not young Tg2576 mice, including spatial reference memory deficits in the water maze (Hsiao et al., 1996; King et al., 1999; Westerman et al., 2002) and spatial working memory deficits in the radial-arm water maze (Morgan et al., 2000) and T-maze (Chapman et al., 1999, King et al., 1999). Westerman (2002) and colleagues reported that cognitive changes in Tg2576 mice might be more related to soluble forms of Aβ rather than plaque. By examining mice at several ages, they reported an inverse correlation between overall water-maze performance (mean probe score) and insoluble Aβ.

In electrophysiological studies of the hippocampus, these mice showed normal synaptic transmission but exhibited reduced hippocampal LTP in CA1 and the dentate gyrus (Chapman et al., 1999). It was proposed that these alterations in synaptic plasticity underlie some of the cognitive deficits in AD. In similar studies Fitzjohn (2001) and co-workers found impaired synaptic transmission but normal levels of LTP. This data favours excitotoxicity rather than a direct deficit in the LTP process as the major correlate of the cognitive dysfunction in this mouse strain relevant to AD. A variety of inflammatory and oxidative stress markers have also been studied in the Tg2576 mice to examine the involvement of these processes in AD-like pathology. Tg2576 mice show the same type of oxidative damage that is found in AD, and this damage directly correlates with the presence of Aβ deposits (Smith et al., 1998) and increased lipid peroxidation precedes Aβ plaque formation (Pratico et al., 2001). IL-1β and TNF-α immunopositive microglia and IL-6 immunoreactive astrocytes surround fibrillar Aβ deposits (Benzing et

al., 1999) and a number of cytokines is induced in a surrounding of plaques (Mehlhorn et al., 2000).

APP23 mouse

Developed at Novartis and first reported by Sturchler-Pierrat et al. (1997), the APP23 mouse uses a human APP751 construct containing the K670N/M671L mutation, under the control of a Thy1 promoter for high neuronal expression. These mice have been characterized extensively in terms of pathology, and recapitulate many of the features reported in the PDAPP and Tg2576 mice, namely neuritic plaques accompanied by local astro- and microgliosis, with a regional expression largely confined to neocortical and hippocampal areas (Sturchler-Pierrat et al., 1997). Interestingly, neurodegeneration has been reported in the hippocampal CA1 region of 14–18-month-old mice with an apparent correlation with plaque load (Calhoun et al., 1998). The APP23 mouse is also a good model of cerebral amyloid angiopathy (Winkler et al., 2001). Behavioural characterization studies showed similarities to other APP transgenic lines (PDAPP, Tg2576 lines) with age-dependent and amyloid-related cognitive changes (Lalonde et al., 2002; Kelly et al., 2003; Van Dam et al., 2003).

PSAPP mouse

Shortly after the identification of mutations to the PS1 gene as a genetic cause of some cases of familial AD (1995), Duff and colleagues reported on a mouse line overexpressing the PS1 gene containing the M146L mutation (Duff et al., 1996). Although these mice do not appear to develop plaques, they show an increase in extracellular Aβ42 levels. Subsequently, the crossing of the Tg2576 with the PS1 transgenic mice resulted in a novel mouse line (PSAPP) that showed greatly accelerated plaque pathology and associated gliosis (Holcomb et al., 1998). In contrast to the 9–12 month onset of plaque deposition seen in the Tg2576 line, by 3 months of age thioflavin-S-positive plaques can be detected in the cingulate cortex of PSAPP mice and by 6–8 months of age, amyloid deposition and plaque size increased and extended to most cortical regions (McGowan et al., 1999). Despite the extensive amyloid pathology by 12 months of age, no clear evidence for neurodegeneration or neurofibrillary tangles was evident. The clearest age-related impairment in spatial learning and radial arm maze

working memory (both tests performed in a water maze) emerge at 15–17 months of age, a time point where there is intense amyloid deposition and signs of local oxidative stress and neuroinflammation (Arendash et al., 2001). This age of onset seems later than that described for the PDAPP and Tg2576 lines, which display lesser amyloid pathology. Initially, Holcomb et al. (1998) described reduced Y-maze alternation in 12–14 week PSAPP and Tg2576 mice compared to wild-type controls, a finding that clearly suggests no relationship to Aβ pathology, but these results could not be replicated (Arendash et al., 2001).

CRND8 mouse

The CRND8 mouse encodes a double-mutated form of hAPP695 under the control of the prion protein promoter. The combination of V717F and K670N/M671L APP mutations results in extremely high levels of Aβ42, such that thioflavin-S-positive amyloid deposits became evident in all mice by 3 months of age (Chishti et al., 2001). These plaques are associated with dystrophic neurites and gliosis, although it is unknown at the present time whether neurodegeneration occurs in this mouse model. In terms of cognitive status, at 11 weeks of age, the CRND8 mice show impaired acquisition of spatial learning in a Morris water maze, although cued learning and swim speeds were similar between groups (Chishti et al., 2001). CRND8 mouse seems like a particularly valuable model, but the early lethality is a problem in this line.

JNPL3 (tau) mouse

Although tau mutations do not cause AD, an attempt to create a tau-overexpressing mouse has been made (Lewis et al., 2000). The transgene contains the most common tau mutation, associated with frontotemporal dementia and Parkinsonism (FTDP-17 mutation P301L) under the control of the mouse prion promoter. Hemizygous JNPL3 mice express the transgenic tau at equivalent levels to endogenous tau, while homozygous mice express double this amount. From 6–7 months, a neurological phenotype becomes manifest in hemizygous JNPL3 mice, consisting of delayed righting reflex, hypolocomotion and muscular weakness (Lewis et al., 2000). Within 4 weeks of symptom onset, the mice become moribund. In terms of pathology, in contrast to the APP transgenic mice, the JNPL3 mice develop neurofibrillary tangle (NFT) pathology, confirmed by tau

immunostaining, silver staining and electron microscopy. No amyloid plaques have been identified.

An attempt to cross the JNPL3 mouse with the Tg2576 mouse was described by Lewis et al. (2001). The resulting double transgenic mouse (tau/APP) developed Aβ deposits at the age similar to Tg2576; however, relative to JNPL3 mice, the double mutants exhibited neurofibrillary tangle pathology that was substantially enhanced in the limbic system and olfactory cortex. These results indicate that APP/Aβ influences the formation of neurofibrillary tangles and this mouse is the first mouse model to exhibit these two cardinal signs of AD pathology.

3xTg-AD

In a search for manifestation of all of the hallmarks of AD in murine models, a triple transgenic model of AD was generated (Oddo et al., 2003). The triple transgenic mice (3xTg-AD) harbour three mutant transgenes: PS1M146V, APPSwe, and tauP301L. The 3xTg-AD mice develop an age-dependent and progressive neuropathology that includes plaque and tangle pathology. Further analysis of these mice has shown that, despite equivalent overexpression of human APP and tau, Aβ pathology precedes conformational or hyperphosphorylation changes in the tau protein (Oddo et al., 2003). These results support the amyloid cascade hypothesis, which predicts that Aβ deposition is the earliest pathological trigger of AD. However, pathology in these transgenic mice is due to three independent transgenes, and it cannot be used directly to infer what might be the primary causation in human AD in the absence of mutations.

1.7.3 Rat Models

All known rodent models are generated in mice strains, as the transgenic techniques for creating mice are far more advanced than for rats. An advantage of using a rat strain to investigate APP overexpression is that this species has been well characterised in terms of basic learning and memory processes and a rat APP overexpressor may, therefore, offer a broader practical base to evaluate the effects of the APP mutation on different cognitive systems.

GP56 Rats

A transgenic rat (GP56 line) was created by Vanessa Marshall in Paul Chapman's lab as a part of her thesis (Marshall, 2002). Transgenic rats were created in Sprague-Dawley background and overexpressed a human APP with the "Swedish mutation" (APP695, the same as Tg2576 mice) under control of the prion protein promoter to ensure high CNS expression. They had an estimated 12 copies of the transgene (Tg2576 have 90 copies) and APP was expressed at levels approximately 55-61% of that displayed by the Tg2576 mouse. As measured by ELISA the amount of SDS-soluble Aβ40 and Aβ42 was higher than in controls, and it did not appear to increase as a function of age. There was very little evidence that deposited Aβ was present in the GP56 rat brains at any age tested.

These levels of soluble amyloid were insufficient to induce performance differences between transgenic and control rats on a range of behavioural tasks sensitive to the same mutation in mice, from 8 to 24 months of age. Hemizygous GP56 rats were tested on a battery of tasks, including a forced-choice alternation task in the T-maze, spatial matching to position task in the water-maze and finally at the age of 24 months, on a standard reference memory water-maze task.

All studies were performed in hemizygous rats. As the pathology was limited, crossing two transgenic hemizygous rats generated homozygous transgenic rats. Homozygous rats were not characterised behaviourally but there was some indication that their Aβ levels are higher then in hemizygous rats and might increase with age.

1.7.4. Electrophysiological studies of hippocampus

Memory loss is a major symptom of AD. Long Term Potentiation (LTP) of synaptic transmission in the hippocampus is the primary experimental model for investigating the synaptic basis of learning and memory (Kandel & Abel, 1995). It was first reported by Bliss and Lomo (1973) and it is a prolonged increase in the efficiency of synaptic transmission following brief, high frequency stimulation, i.e. a stimulus evoked at a constant amplitude and low frequency results in a larger synaptic response after a short period of high frequency stimulation. This effect occurs within milliseconds and can last for hours in vitro and days in the awake animals (Bliss & Collingridge, 1993). LTP is

mainly induced by the activation of the *N*-methyl-d-aspartate (NMDA) receptor complex. It is a subtype of glutamate receptor that allows electrical events at the postsynaptic membrane to be transduced into chemical signals, which are thought to activate both pre- and postsynaptic mechanisms to generate a persistent increase in synaptic strength (Bliss & Collingridge, 1993). There is strong evidence suggesting that NMDA receptors are involved in spatial learning in the hippocampus (Collingridge & Singer, 1990). Chronic intraventricular infusion of an NMDA receptor antagonist dose dependently caused a parallel impairment of spatial learning in water maze and in perforant path LTP *in vivo* (Davis et al, 1992). Furthermore, studies on Tg2576 mouse have demonstrated a correlation between behavioural deficits and impairment of both CA1 and dentate gyrus LTP (Chapman et al., 1999). Thus for my PhD I decided to try to show correlation of behaviour and its impairment with electrophysiology LTP measurements *in vivo.*

1.8. Overview of structure of the PhD

Well-characterised transgenic animal models of AD were used to evaluate potential treatment strategies using NSAIDs and to determine the importance of susceptibility risk factors (high cholesterol or polyunsaturated fatty acids intake) on behavioural, electrophysiological and biochemical measurements.

Each chapter raises a different question about AD-like pathology assessed in animal models, while reporting on the use of similar techniques. NSAID treatment questioned whether it is possible to prevent/treat cognitive deficit and synaptic physiology in transgenic mice. High cholesterol diet was tested for the potential of inducing amyloid pathology in the transgenic rat model. DHA supplementations tested the potential of n-3 fatty acids to prevent development of AD-like pathology in transgenic mice models.

The methods I concentrated on were: 1) behavioural assessment of cognitive function by using tasks like forced choice alternation task in the T-maze and standard reference Morris water maze; 2) electrophysiological analysis of hippocampal plasticity *in vivo* and 3) biochemical and histological assessment which included ELISA measurements and immunostaining.

Two animal models were used for experiments. APP Tg2576 mice demonstrate well-established and well-documented deficits in cognitive tests, and GP56 rats which fail to

show any impairment in a battery of tests (reviewed in chapter 1.7). Therefore, for testing agents that were supposed to prevent/treat AD-like impairment in rodent models, Tg2576 mice were used. Whereas for testing agents that were supposed to induce AD-like pathology, GP56 transgenic rats were chosen. For the studies with cholesterol, homozygous rats were chosen. Although not well characterised, they had a greater potential for developing pathology (higher and increasing with age Aβ levels).

Behavioural tests were chosen to be the most descriptive for the respective transgenic models e.g. for Tg2576 mice Forced choice alternation task in the T-maze was chosen as it was validated by Paul Chapman's laboratory (Chapman et al., 1999) and it shows constant significant difference between transgenic and non-transgenic littermates. For GP56 rats, the standard reference memory version of the Morris water maze was chosen. Marshall (2002) had previously demonstrated that Sprague-Dawley rats failed to move in the Forced Choice Alternation task in the T-maze and that spatial matching to position task in the water-maze was too difficult even for non-transgenic rats.

1.9. Aims of this PhD

This thesis reports a comprehensive study of the role of an APP transgenic mouse and an APP transgenic rat model of AD as tools for testing new treatment strategies (NSAIDs) and validating susceptibility factors (cholesterol and DHA).

To summarise, the aims of this thesis are:

1. To determine the effect of Ibuprofen treatment on behaviour, synaptic plasticity and basic neuropathology of Tg2576 mice in two paradigms – prevention versus treatment.
2. To inspect the role of high-cholesterol in behaviour and synaptic plasticity impairment and basic amyloid neuropathology in GP56 rats.
3. To study effects of early DHA supplementation on behaviour and basic neuropathology of Tg2576 mice (prevention study).
4. To rationalise the importance of a multidisciplinary approach to investigating neurodegeneration.

Chapter 2

General methods

2.1. Behavioural testing

Measuring learning and memory in behavioural tasks in rodent models of Alzheimer disease can provide insight into both the mechanisms of the disease and the basic processes of learning and memory. It also allows testing effects of various treatments and susceptibility risk factors.

2.1.1. Forced Choice Alternation Task in the T-maze

Theoretical background

The Forced-Choice Alternation (FCA) task performed in the T-maze is a well-established behavioural test that measures a spatial working memory, sometimes referred to as an "episodic-like memory". Most of the earlier work, which established both the memory requirements for FCA and the relevance of the hippocampus and the prefrontal cortex, was conducted in rats (Rawlins et al., 1982; Aggleton et al., 1990). As Tg2576 transgenic mice show pathological changes both in the prefrontal cortex and the hippocampus, the FCA task was successfully adapted to be used in mice and detect memory deficits (Chapman et al., 1999).

Subjects

Tg2576 transgenic mice and non-transgenic littermate controls (on a C57B6/SJL background) at ages ranging from 8 to 14 months were tested. Details about Tg2576 mice are provided in section 1.7.2. Mice were taken from a colony bred at Cardiff University. They were housed either individually (males) or in cages of no more than three mice (females). Males required separation due to a high level of aggression towards each other. While progressing through behavioural testing, it turned out that females show higher degree of variability in testing and higher numbers are necessary for statistical analysis (however statistical comparison did not reveal any significant differences between females and males). Thus further testing (Exp C in ibuprofen and DHA study) was performed only in males. When not undergoing behavioural testing, animals received a constant supply of water and food pellets containing either control or treatment (e.g. ibuprofen or DHA; see chapters 3 and 4 for details). Mice were checked regularly for symptoms of illness and weighed every two weeks during the treatment period. The lighting was set to 14 hours of light and 10 hours of darkness. During testing, animals were water deprived for 20 hours prior to running in the T-maze. After finishing testing each day (around 2 hours), mice were given 2 hours of free access to water. The experimenter was blinded to the genotype of the mice throughout the study. All procedures were carried out in strict accordance with the UK Animals (Scientific Procedures) Act, 1986.

Testing apparatus

The dimensions of the T-maze are shown in Figure 2.1. The walls of the T-maze were constructed of clear Perspex and the floor was made of laminate. The maze was mounted on a table, raised 75 cm from the floor, and situated in the middle of a room at roughly equal distance from each wall. There were external cues (posters, cupboards and door) in the room to help mice to navigate. The T-maze consisted of one long (start) arm and two slightly shorter (reward) arms of equal length, put together in a shape of letter T. A removable opaque Perspex door, located 10 cm from the beginning of the longer arm, separated the Start Box from the rest of the maze. The left and the right reward arm of the T-maze were also separated from the longer start arm by removable opaque Perspex doors. Black Perspex drinking wells were placed at the end of both reward arms.

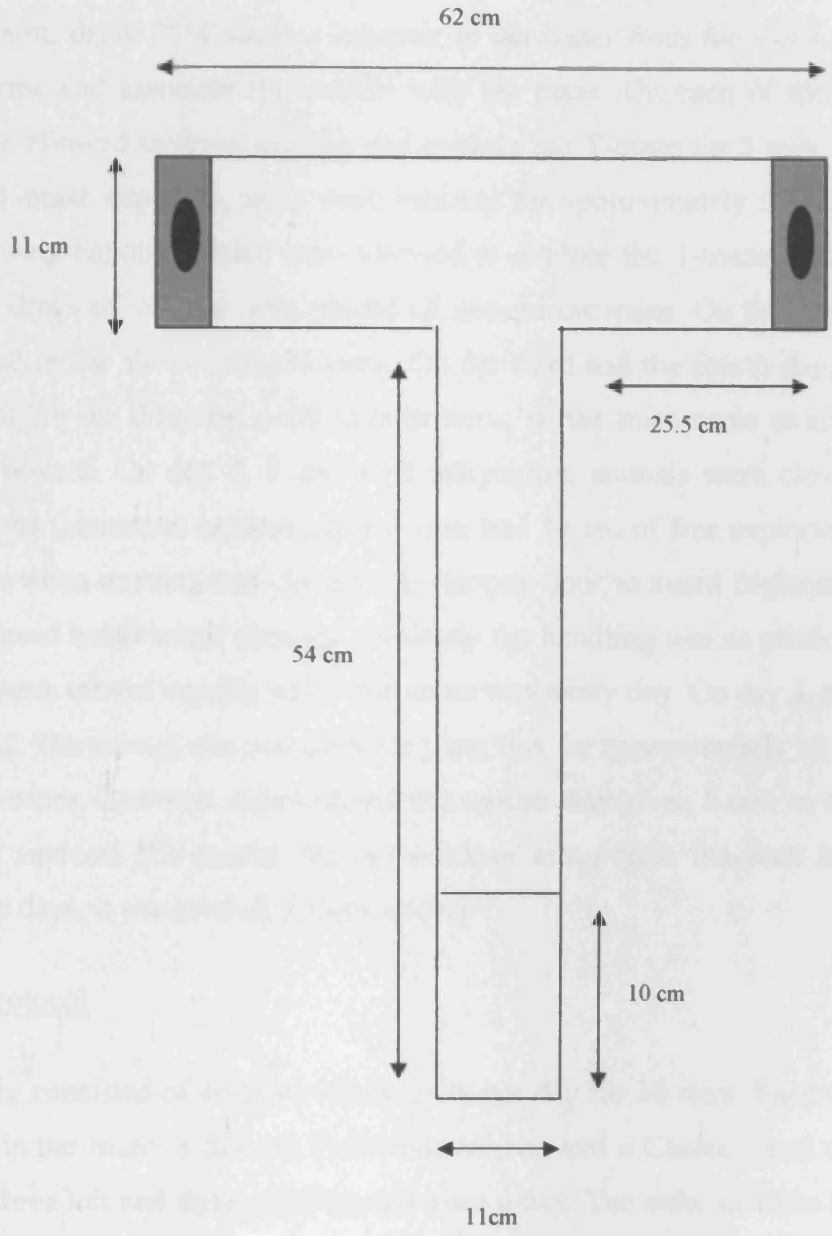


Figure 2.1 Dimensions of the T maze. All the walls are 25cm high.

Testing procedure

Adaptation

Adaptation consisted of 4 days of pre-training during which animals learned to run down the start arm, drink 25% sucrose solution in tap water from the wells at the end of the reward arms and associate the reward with the maze. On each of the adaptation days, mice were allowed to drink sucrose and explore the T-maze for 5 min. One day prior to the first T-maze exposure, mice were handled for approximately 5-10 min. On the first day following handling, mice were allowed to explore the T-maze with all of the doors open and drops of sucrose were placed all around the maze. On the second day, sucrose was placed in the shorter reward arms. On the third and the fourth day, the sucrose was placed only in the drinking wells in both arms, so the mice came to associate the wells with the reward. On day 2, 3 and 4 of adaptation, animals were closed in one of the reward arms (chosen at random) after 4 min and 30 sec of free exploration. Special care was taken when opening and closing the Perspex door to avoid frightening the mice and stress induced behavioural changes. Similarly the handling was as gentle as possible, and the mice were treated equally and in the same way every day. On day 4, the Start Box was introduced. The animal was placed in the Start Box for approximately 10 sec, and then the opaque Perspex door was removed and the mouse was given 5 min to explore the maze and drink sucrose. If a mouse did not drink or move from the Start Box during the 4 adaptation days, it was excluded from testing.

Testing protocol

The testing consisted of sessions of six trials per day for 10 days. Each trial consisted of two runs in the maze: a Sample (information) run and a Choice (test) run. Each mouse received three left and three right Sample runs a day. The order of trials for each day was determined by a pseudo-random schedule with no more than two consecutive left or right trials. The mice were tested in groups of six to eight, resulting in an interval ranging from 10 to 15 min between each trial-pair. Reward consisted of an approximately 50 µl drop (as measured by pipette) of 25% sucrose solution in tap water. On the Sample run, a Perspex door was placed at the entrance of the opposite reward arm forcing the mouse to enter the assigned arm where it found a drop of 25% sucrose in the well. The animal was

given time (approximately 15 sec) to drink the reward and then was placed in the Start Box again. The Test Run started immediately (no delay between Sample Run and Test Run). On the Test run, the Perspex door was removed and the animal was presented with the choice of both goal arms of the maze. To receive a reward the animal had to enter the arm previously un-visited (non-match to place). A choice was considered to be made when the mouse placed both of its back feet across an entrance to a reward arm. After the animal made its choice, the entrance to the chosen arm was blocked off to prevent the mouse from leaving. If the mouse entered the incorrect arm, it was enclosed in the goal arm, without reward, for approximately 30 sec. The testing protocol is illustrated in Figure 2.2. If after 5 min the mouse failed to enter an arm on either run, the trial was not included in the data. If the mouse did not complete more than 50% of the trials for the first 3 days, it was excluded from the study.

Data analysis

Percentage of correct choices was counted for each mouse for each day. The results were averaged over blocks of 2 days and over testing groups. A learning curve was created and differences between groups were calculated using repeated measures ANOVA (with genotype, diet and sessions as factors). The learning criterion was gained if the mouse reached $\geq$80% correct choices for three consecutive days. The percent of mice from each group reaching a learning criterion was counted and the difference between groups was calculated using chi-square test (Fisher's test). Further analysis included measuring the tendency to respond persistently by turning in the same direction within a single test session. This analysis was carried out to determine whether animals that were performing poorly were doing so because they persevered with one response, or because they responded randomly. The perseveration count was calculated for each mouse as the number of consecutive free choices made in the same direction every day (regardless of the direction (left or right) the higher score was chosen). Finally to evaluate if the conditions throughout the testing time were biased, the preference towards either side (Right or Left) was calculated for each mouse by counting the number of trials on which an animal made a right and a left choice, regardless of whether this was the correct response. The statistical significance of differences in the value of Perseveration Count and Right/Left Preference was determined by the two-factor ANOVA, with genotype and

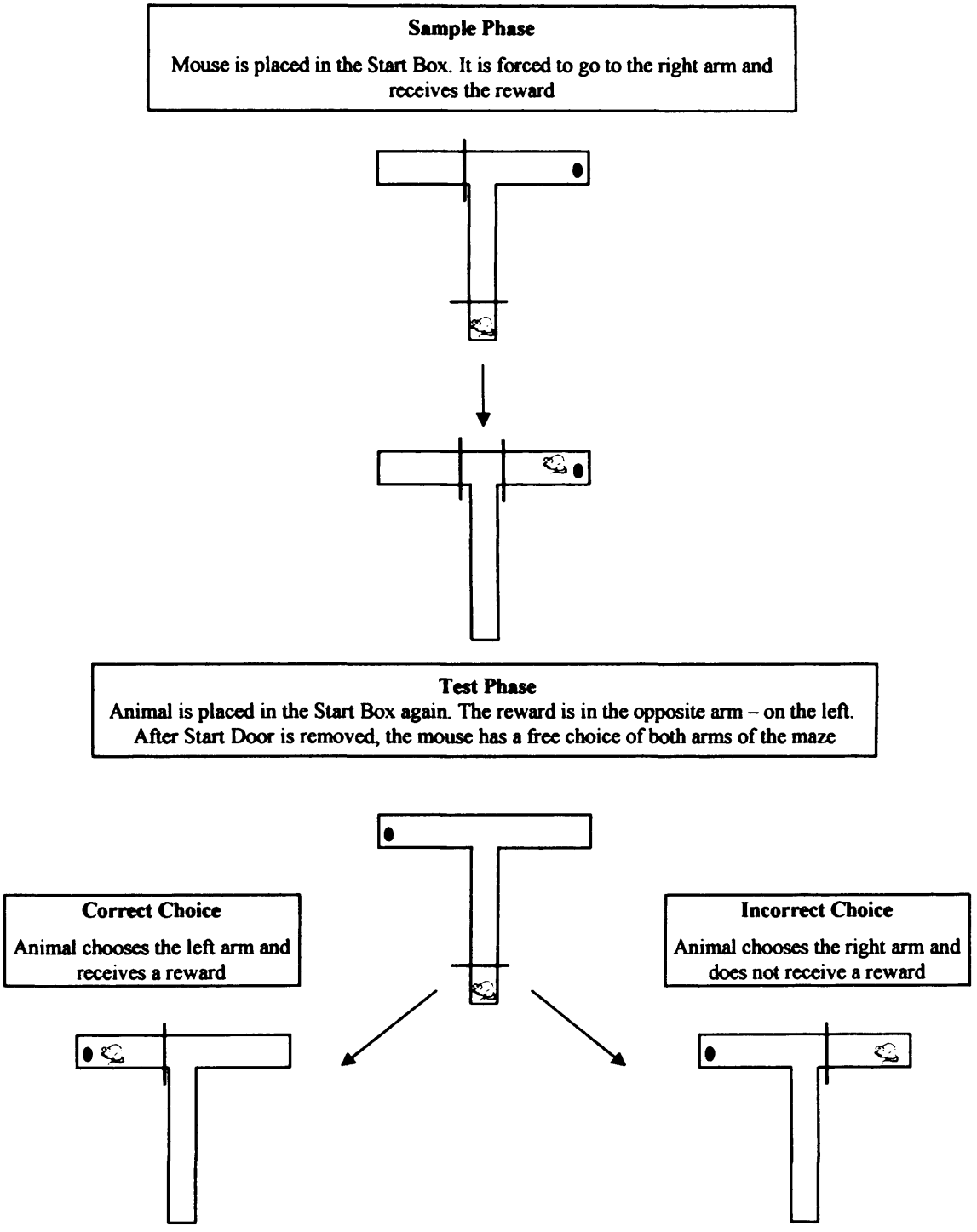


Figure 2.2. Forced Choice Alternation Task protocol. Example with the sample choice to the right arm. ● represents a reward - drop of sucrose.

the diets as factors and Tukey`s pair-wise comparisons post hoc tests to assess differences between groups.

2.1.2. Morris Water Maze, Rat Reference Memory

Theoretical background

The Morris water maze was first described 20 years ago as a device to investigate spatial learning and memory in laboratory rats (Morris et al., 1984). Since then it has become one of the most frequently used laboratory tools in behavioural neuroscience. Many methodological variations of the Morris water maze task have been and are being used by research groups, for many different applications. Lesions in distinct brain regions like hippocampus, striatum, basal forebrain, cerebellum and cerebral cortex were shown to impair water maze performance (reviewed in D'Hooge & De Deyn, 2001). Finally, the Morris water maze task has often been used in the validation of rodent models for neurocognitive disorders and the evaluation of possible treatments (D'Hooge & De Deyn, 2001). Transgenic heterozygous GP56 rats were tested in two kinds of Morris water maze task showing no significant impairment comparing to non-transgenic littermates (reviewed in Marshall, 2002).

Subjects

The animals tested were homozygous transgenic GP56 rats and non-transgenic littermate controls (on a Sprague- Dawley background) at the age of 18 months. Details about GP56 rats are provided in chapter 1.7.3. Rats were taken from a local colony created and bred at Cardiff University. They were housed in cages of no more than three rats. Animals received a constant free supply of water and food pellets, either control or containing high-cholesterol. Rats were checked regularly for symptoms of illness and weighed every week. The room temperature was monitored daily and kept at a constant level and the lighting was set to 14 hours of light and 10 hours of darkness. During testing the experimenter was blinded to the genotype of the rats. All procedures were carried out in strict accordance with the UK Animals (Scientific Procedures) Act, 1986.

Testing apparatus

Water maze experiments were performed in a circular pool (diameter: 200 cm, height: 60 cm) made of fibreglass with the inner surface painted white. The pool was filled to a depth of 35 cm of water (maintained at 26.0±1.0°C), under which a 10 cm square platform was submerged to a level approximately 1.5-2 cm below the surface of the water. The water was made opaque by addition of 500 ml of non-toxic white paint (Opacifier E308, Rohm Haas). The pool was located in the centre of a small room illuminated by four 50W halogen lamps. Extra-maze visual cues such as posters on the wall, a screen (behind which the experimenter stood) and a curtain were present in the room.

Swimming activity of each rat was monitored via a video camera mounted in the ceiling directly above the pool. This was connected to a video recorder with an attached tracking device and software (HVS Image analysis, LTD). The head of each rat was painted black with a marker pen in order to provide adequate contrast for the software to track the path of the rat. A PC computer recorded latency to find the platform, swim speeds and path lengths. The software automatically ended a trial after 120 sec. The experimenter was able to follow the rat's progress during the course of the experiment on a monitor, connected to the video camera.

Testing protocol

The animals were divided into four groups that were counterbalanced for genotype, diet and gender. These groups were assigned a particular platform location (NE, NW, SE or SW) to avoid being biased to any position of the platform. Each rat was given four trials per day for nine consecutive days to locate the hidden platform. For each trial, the rat was placed in the pool facing the wall at one of eight pseudorandomly assigned start positions representing compass points (see Figure 2.3). For each trial, the rat was allowed to swim a maximum of 120 sec in order to find the platform. When successful the rat was allowed 20 sec rest on the platform. If unsuccessful, within the allotted time period, the rat was guided to the platform by the experimenter. It was given a score of 120 sec and then also allowed the 20 sec rest period. In either case the rat was immediately given the next trial

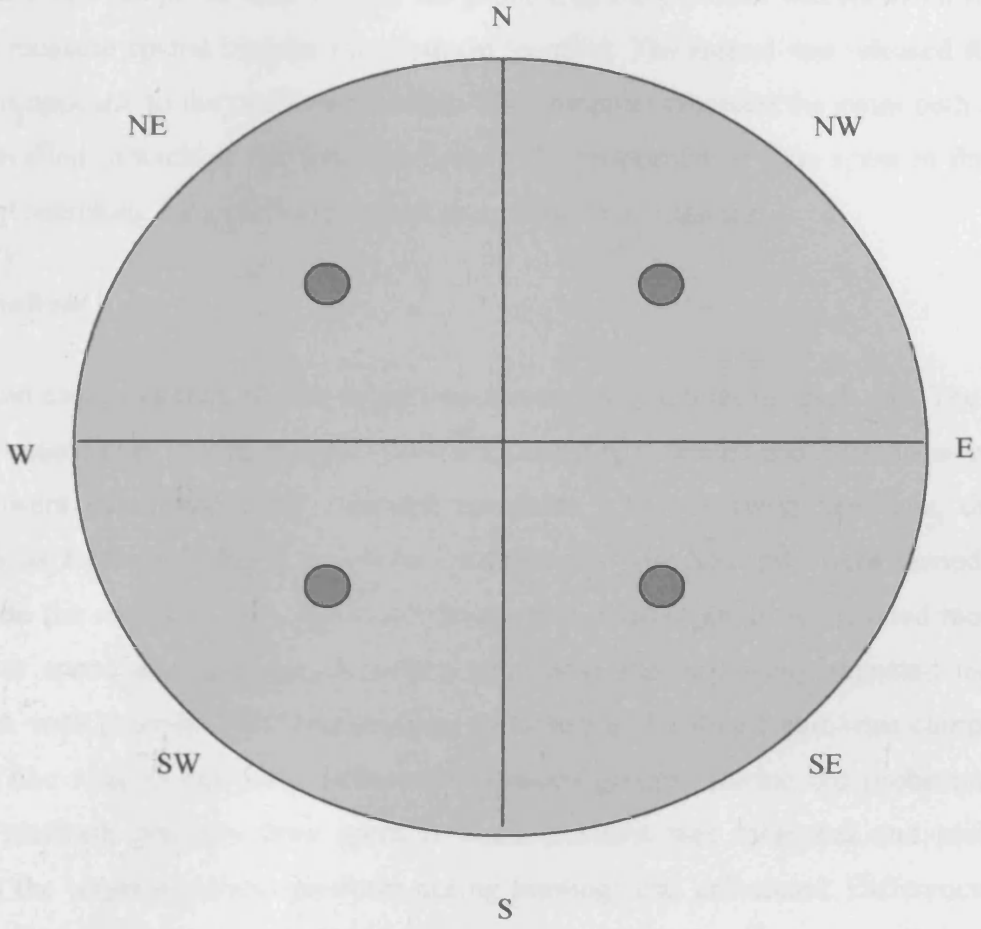


Figure 2.3. Water maze - platform positions, quadrant allocations and start locations

Platform positions and start locations. Small circles represent four platform positions for four rat groups. The letter outside the maze represent 8 different start points. The maze is divided into four equal quadrants. N- North; S-South; E-East; W-West; NE-North-East; NW-North-West;SE- South-East or SW-South-West.

after the rest period. Once all the trials had been completed, the rat was dried with a hand towel and returned to its holding box.

On day 4, 7 and 10, approximately 20 hours after the last training session, the animals received a 120 sec probe trial. During the probe trial the platform was removed from the pool to measure spatial bias for the platform location. The animal was released from the quadrant opposite to the platform location. The computer recorded the swim path and the time travelled in each of the four quadrants. The proportion of time spent in the target quadrant searching for a platform served as a measure of retention.

Data analysis

The mean escape latency of four trials was counted for each rat for each day. The results were averaged over testing groups. A learning curve was created and differences between groups were calculated using repeated measures ANOVA (with genotype, diet and sessions as factors). Tukey's pair-wise comparisons post hoc tests were carried out to determine the source of any significant interaction. Further analysis included measuring the swim speed and path length, which were also analysed using repeated measures ANOVA with genotype, diet and sessions as factors and Tukey's pair-wise comparisons as post hoc tests to calculate differences between groups. During the probe trials (no escape platform present), time spent in each quadrant was measured and preference towards the target quadrant (platform during training) was calculated. Differences were determined by the two-way ANOVA, with group and a diet as factors and Tukey's pair wise comparisons post hoc tests to calculate differences between groups.

2.2. Electrophysiological studies of hippocampus *in vivo*

Theoretical background

The hippocampus is one of the first structures in the brain affected in AD and it also appears to play a crucial role in spatial learning and memory (Kandel & Abel, 1995). The hippocampus is also the structure in which electrophysiological recordings first revealed the phenomenon of long-term potentiation (LTP) (Bliss & Lomo, 1973). LTP is a prolonged increase in the efficiency of synaptic transmission following brief, high frequency stimulation and it is widely considered to represent a molecular correlate of

learning and memory. Hippocampal synaptic transmission and plasticity are disrupted in transgenic mice overexpressing human APP (reviewed in Rowan et al., 2003 and chapter 1.7.2), and in rats after exogenous application of synthetic Aβ in vivo (Walsh et al., 2002).

Subjects

Tg2576 mice from the Ibuprofen study and GP56 rats from the cholesterol study were tested for hippocampal synaptic transmission and plasticity impairments in a paradigm *in vivo*. Tg2576 mice overexpressing human mutated APP and littermate controls weighing 20-30g were obtained from the local colony at Cardiff University. Animals were anaesthetised with urethane (15% saline solution 1.2 - 1.8g/kg body weight i.p. for non-recovery surgery). GP56 rats overexpressing human mutated APP and littermate controls weighing from 300 (females) to 900g (males) were also obtained from the local colony at Cardiff University. Animals were anaesthetised with urethane (30% saline solution 1.2g/kg body weight i.p. for non-recovery surgery).

Anaesthetised animals were placed in a stereotaxic device (ASI Instruments, Bilaney Consultants, Sevenoaks, Kent, UK), the skull was exposed and the animal's body temperature was monitored and regulated at the range of 36-37°C with a heating pad. Animals were chosen blindly to genotype and drug treatment throughout the experiments.

Testing equipment

Extracellular recordings were made with electrodes placed in the dentate gyrus of hippocampus. Inputs to this layer come from the medial perforant path, which arises from the medial entorhinal cortex and terminates mainly on granule cells of dentate gyrus. Field excitatory postsynaptic potentials (fEPSPs) were evoked from the dentate granule cell population in response to electrical stimulation of the medial perforant path.

A concentric bipolar stimulating electrode (250 μm diameter wire insulated with Teflon except for the cut tips) was placed in the medial perforant path. Coordinates with the skull surface flat were for rats - 7.5 mm posterior from bregma, 4.1 mm lateral from the midline, 2.8 mm ventral below dura, and for mice - 4.2 mm posterior from bregma (the level of lambda), 2.8 mm lateral to the midline, 1.8-2.2 mm ventral.

Evoked potentials were recorded extracellularly from the cell body layer of the dentate gyrus using either a stainless steel electrode or carbon-fibre electrode (made in the laboratory). Coordinates with the skull surface flat were, for rats - 3.8 mm posterior from bregma, 2.2 mm lateral to the midline, 2.8 mm ventral, and for mice - 2.0 mm posterior to bregma, 1.8 mm lateral to the midline, 1.7-2.2 mm ventral, below dura. The recording electrode was lowered into the dentate gyrus until the maximal response was observed.

Electrical stimulation was provided by a Digitimer stimulator, amplified and filtered using a Warner DP-301 Differential Amplifier. Stimuli were given every 15-30 sec and the intensity of the test stimuli was chosen to be between 10 and 1000 μA. Evoked fEPSPs were recorded using the LTP analysis program (http://www.ltp-program.com; Anderson & Collingridge, 2001).

Testing protocol

When a satisfactory response from dentate gyrus was found, the experiment started. First the Input/Output curves (I/O) were generated by increasing the stimulus intensity in 100 μA steps from 0 to 1000 μA. Three fEPSPs were recorded at each intensity, and the slope and maximum amplitude of the fEPSP as well as the amplitude of the population spike were measured and averaged. Calculating I/O curves helps to evaluate synaptic potency and also allows selection of the optimal intensity for synaptic plasticity experiments. The chosen intensity was intended to evoke 30-40% maximal slope and/or 1 mV population spike (although this was not always possible; see results chapter 3). The intensity was typically 300-400 μA and was used for paired pulse studies, tetanisation and LTP recording.

To evaluate short-term synaptic interactions, paired-pulse stimuli were delivered to the perforant pathway with inter-stimulus intervals ranging from 30 to 300 ms (performed only for rats). Three consecutive impulses delivered at each interval were averaged. The relative amplitude of the second population spike (P2) to the first one (P1) was calculated and expressed as percent (%P2/P1).

Following collection of input/output data and paired-pulse study, baseline stimulation was performed for at least 20 min by stimulating every 30 sec. LTP was induced by tetanising the perforant pathway with theta-burst parameters consisting of 6 trains of 6 bursts, and

each burst consisted of 6 pulses at 400 Hz, which were delivered every 20 sec over 2 min. Immediately after the end of tetanus, recordings of the evoked potential continued for further 60 min.

Data analysis

The characteristic response of the dentate granule cells to perforant path stimulation consists of a positive-going excitatory postsynaptic potential (fEPSP) with a superimposed negative-going field Population Spike (PS). The fEPSP slope was measured as the maximum slope of the fast rising phase. The PS amplitude was measured by averaging the distance from the negative peak to the midpoint of the preceding and following positive peak. All values were expressed as percentage change relative to the mean control response during the 20 min baseline prior to tetanic stimulation.

Statistical analyses included the repeated measures ANOVA for I/O curves, Paired-Pulse Study and LTP experiments with diet, genotype and either intensity, or interval or time as repeated measures. ANOVA was followed by Tukey's pair-wise comparisons as post hoc tests to assess differences between groups.

2.3. ELISA – Enzyme Linked-Immuno-Sorbent Assay

Theoretical background

The Biosource Signal Select human β-Amyloid 40 and 42 ELISA kits are used for the *in vitro* quantitative determination of human Aβ40/42 in samples. Aβ40 and Aβ42 kits are based on a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal capture antibody specific for the NH_2 terminus of human Aβ has been coated onto the wells of the microtiter strips. Samples, including standards of known human Aβ content and unknowns are pipetted into these wells, followed by the addition of a rabbit polyclonal antibody specific for the 1-40 or 1-42 sequence of human Aβ. The sandwich antibody was then detected using a horseradish peroxidase conjugated mouse anti-rabbit IgG. After removal of excess anti-rabbit antibody, a substrate solution is added, which works with the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of Aβ40 or Aβ42 present in the original specimen.

The brains of Tg2576 mice show increasing concentrations of both detergent-soluble (SDS) and detergent-insoluble (FA) Aβ 40 and 42 with age (Kawarabayashi et al., 2001). GP56 rats have significant, but lower concentrations of both Aβ species, and these concentrations do not increase with age (Marshall, 2002). The insoluble Aβ (FA) was not present in the GP56 rat brains at any age tested.

Brain sample preparation

For the determination of Aβ levels by ELISA, frozen samples of mouse and rat brains were used. The brains for ELISA and immunohistochemistry (see chapter 2.4) were collected after electrophysiological experiments (for DHA and the last Ibuprofen group after the end of behavioural testing). In order to remove the brain, the cranial bone was cut away and the brain was lifted and freed from the vessels and cranial nerves connecting it to the base of the skull. The cerebrum, cerebellum and some of the hindbrain were removed in one piece. The brain was either quick-frozen in isopentane on dry ice for ELISA or immersed in fixative for immunohistochemical staining. For the Ibuprofen study the whole Tg2576 mouse brain was used. For the DHA study, one hemisphere separated along the midline of Tg2576 mouse brain was used. For the cholesterol study with GP56 rats, half of the brain cut along the midline was used.

For the Tg2576 mice, two-step extraction with sodium dodecyl sulfate (SDS) and formic acid (FA) was performed, while for rats only the FA fraction was measured. The SDS fraction represents soluble Aβ, while FA represents insoluble Aβ, possibly from plaques (Kawarabayashi et al., 2001). For rats, only FA extraction was used as it allows determination of total Aβ content, as they do not have insoluble Aβ (Marshall, 2002).

The brain samples were weighed and the volume of 2% SDS or 70% FA (containing Complete protease pellet, Roche), was calculated (75 or 150 mg of brain per 1 ml). The frozen brain samples were then put in 5 ml round-bottom tubes with the appropriate volume of SDS or FA and sonicated. The tube was placed on ice and sonicated in 10 seconds bursts two or three times, until brain structure was dissolved. Care was taken to avoid foaming by keeping the sonicator probe in the solution.

After sonication, 1.4 ml was taken from each brain extract and spun at 100,000 g for 1 hour at 4°C. After centrifugation, the supernatant was transferred to a new tube. For the two-step extraction, it was labelled as SDS extracts and for the one-step extraction with FA, it was labelled as the final extract.

For the two-step extraction further 1.4ml of 70% formic acid in water was added to the pellet from the SDS extraction and sonicated and spun as above. The supernatant was carefully removed from under the lipid layer and put in tubes as FA extract.

All the extracts (SDS and FA) were aliquoted into small tubes (60-80µl) and kept frozen at -80°C.

ELISA protocol summary

ELISA was performed using commercially available human β-Amyloid 40 and 42 kits from Biosource.

Standard peptide (Aβ40 or Aβ42) provided in the kit was resuspended following manufacturer's instructions and serial dilutions (1000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/ml) were prepared. Standards for FA extracts were diluted with Standard Diluent and for SDS extracts, with Standard Diluent with the amount of SDS equal to the amount contained in the samples, e.g. for dilution 1:80 for SDS Aβ40, the concentration of SDS was 0.025%.

Once samples were defrosted, the 2% SDS extracts were first diluted 1:40 in EC buffer (0.02 M phosphate buffer, pH 7, 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace [Dainipponseiyaku, Suita, Osaka, Japan], 0.2% bovine serum albumin, 0.05% CHAPS and 0.05% sodium azide) and further dilutions were performed in Sample Diluent from the kit. The Aβ capture took place in buffer containing 0.025% or 0.02% SDS. FA extracts were neutralized initially by 1:20 dilution into 1 M Tris phosphate buffer, pH 11, and then diluted as necessary with the Sample Diluent buffer from the kit. The dilution factors for samples for both 40 and 42 β-Amyloid in SDS and FA fractions are provided in the table in the table in Figure 2.4.

Table A

	Ab40-SDS	**Ab42-SDS**	**Ab40-FA**	**Ab42-FA**
Tg2576 mice	1:40 with EC buffer, then 1:2 or 1:2.5 with Sample Diluent **1:80/100 final**	1:40 with EC buffer, then 1:2 or 1:2.5 with Sample Diluent **1:80/100 final**	1:20 with TPB, then 1:5 or 1:7 with Sample Diluent **1:100/140 final**	1:20 with TPB, then 1:5 with Sample Diluent **1:100 final**
GP56 rats	x	x	1:20 with TPB, then 1:2 with Sample Diluent **1:40 final**	1:20 with TPB, then 1:2 with Sample Diluent **1:40 final**

Table B

Primary Antibody	**Specificity**	**Dilution**	**Source**
Rabbit Polyclonal Antibody to Glial Fibrillary Acidic Protein (GFAP)	Astrocytes in the brain	1/3000 (1%BSA in PBS)	DAKO
Monoclonal Mouse Antibody Anti- Alzheimer Precursor Protein A4	full-length APP	1/200 (1%BSA in PBS)	Chemicon
Rabbit Polyclonal Antibody anti-amyloid-beta, 37-42	Human Amyloid-beta, 37-42	1/200 (1%BSA in PBS)	Chemicon
Isolectin	Activated Microglia	1/100 (solution containing 1mM Ca)	Sigma
Secondary Antibody	**Specificity**	**Dilution**	**Source**
Goat Anti-Rabbit (GAR)-HRP Conjugate	Rabbit IgG	1/200 (1%BSA in PBS)	Biorad
Goat Anti-Mouse (GAM)-HRP Conjugate	Mouse IgG	1/200 (1%BSA in PBS)	Biorad

Figure 2.4. Table A encloses all the dilutions for FA and SDS extracts for measuring β-amyloid 40 and 42 in the brain. Table B contains the antibodies, both primary and secondary, used for immunohistochemical staining of paraffin-embedded brain sections.

Initially, the plate was washed 4 times with the Wash Buffer provided in the kit. 100 µl of standards dilutions and samples were then pipetted into appropriate wells of the pre-coated microtiter plate. All standards and samples were run in duplicate. The plate was incubated with brain extracts for 2 hours at room temperature while shaking on an orbital plate shaker or overnight at 4°C without shaking.

Unbound amyloid peptide was removed from standards and samples by washing the plate 4 times with the Wash Buffer. 100 µl of diluted detection antibody (rabbit polyclonal anti-Aβ40 or Aβ42) was pipetted into the wells. The plate was incubated for further 2 hours at room temperature while shaking. After this time, excess antibody was removed using Wash Buffer as above. 100 µl of the horseradish peroxidase-conjugated anti-rabbit antibody was then pipetted into the wells and the plate was incubated for additional 2 hours at room temperature with shaking.

After final wash with Wash Buffer (5 times), 100 µl of the chromogen solution was added to each well and the liquid in the wells began to turn blue (due to its reaction with peroxidase). The plate was incubated for 20-25 min at room temperature and protected from light. Horseradish peroxidase reaction was terminated by adding 100 µl of the Stop Solution into each well. The absorbance was measured at 450 nm. The blank well was created for the plate reader by adding 100 µl of both Stabilised Chromogen and Stop Solution. The plate was read within 2 hours after adding the Stop Solution.

Data analysis

After reading absorbance, standard curves were plotted. The curve was drawn from the absorbance of the standards (optical density) against the known standard concentrations. Analysis was performed using Microsoft Excel and the best curve linear fit was used. The Aβ40 or Aβ42 concentrations for unknown samples were read from the standard curve and multiplied by the dilution factor (details in the table A in Figure 2.4). Differences were calculated using two-way ANOVA with genotype and treatment as factors.

As these Biosource kits had not been tested on SDS and FA brain extracts before, I encountered an unexpected problem. For the SDS extracts, the readings of optical density for low concentrations were not linear, but were bending logarithmically towards axis Y.

As a result, some of the low readings for the SDS brain extracts (especially non-transgenic) were below the detectable level. The probable explanation would be that either the presence of SDS in the standards or reaction between SDS and brain in samples were shifting the optical density. To optimise it, standards should have been spiked with a small amount of non-transgenic brain tissue, to keep the conditions more similar. It is also possible that the problem involved antibody reacting to endogenous APP. In that case the proper control would be an APP knockout brain, but it was not available.

2.4. Immunohistochemistry – paraffin wax sections

Theoretical background

Immunohistochemistry techniques are used for staining various tissues with antibody stains. The tissue is fixed, embedded in a wax medium, then sectioned, stained and examined under a microscope. The antibodies recognising epitopes of specific proteins present in the tissue are labelled with tagged secondary antibodies, which are conjugated with coloured markers. For this project, the immunohistochemistry was used to analyse the effect of different modulators (ibuprofen, DHA and cholesterol) on the derivatives of APP and cerebral inflammation, particularly astrocytes and microglia, in the brain of Tg2576 transgenic mice and transgenic GP56 rats. Tg2576 mice start showing β-amyloid plaques and activated astrocytes and microglia after the age of 9 months (Hsiao et al., 1996). There was no data on histopathology of GP56 rats.

Tissue preparation

Brains from 20 mice from the DHA experiment, 20 mice from the last Ibuprofen experiment and 10 rat brains from the cholesterol experiment were processed for immunohistochemistry for APP, β-amyloid, astrocytes and activated microglia. Some of the sections were double-stained to visualise both amyloid plaques and the astrocytes that surround them. Brains were removed as described in section 2.3, and immediately immersed in a fixative (4% paraformaldehyde in water) for at least 24 hours to avoid tissue autolysis and enhance subsequent staining of the tissue. After the fixation period the brain was cut coronally and processed for paraffin wax embedding using automatic tissue processors. The automated processor takes the cassettes through a series of graded

Ethanol baths to dehydrate the tissues (70% Methanol 1 hour, 90% Methanol 1 hour, 95% Methanol 1 hour, 100% Methanol 1 hour, 100% Ethanol 1 hour) and then into xylene (1 hour x 2) Hot paraffin (65°C) can then permeate the tissues. Processed tissue blocks can be stored in the cassettes at room temperature for years.

For sectioning, paraffin blocks were cooled on ice for at least 2 hours and the excess wax was trimmed away. 6 μm sections were cut from the tissue block by means of a Surgipath microtome. At the cutting edge of the knife, the wax shavings accumulated in the form of a continuous ribbon, which was picked up with forceps and floated on the surface of the water bath warmed to 45°C. Individual sections were separated with forceps and placed onto the surface of clean superfrost pre-coated microscope glass slides. The slides with paraffin sections were placed to dry in a 65°C oven for 30 minutes and afterwards in a 37°C oven overnight to bond the tissue to the glass. Slides were stored at room temperature.

Before the staining could be performed, the paraffin had to be removed from the tissue. The sections were first washed with xylene (3 changes of 5 min) and then re-hydrated in 100% ethanol baths (3 changes of 5 min). After de-paraffinisation and re-hydratation, epitopes were exposed by treating the sections with 10 mM citrate buffer, pH 6.0 (2g of citric acid in 1 litre of distilled water, pH adjusted to 6.0 with 5M NaOH) in a microwave for 30 min. As soon as the boiling finished, slides were removed from the microwave and left at room temperature for about 45 min to cool down. Special care was taken not to allow the slides to dry during the procedure.

Staining - peroxidase anti-peroxidase (PAP) technique

Dry slides were washed with phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.5) for 5 min. To reduce background, the endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxidase solution (4ml of 30% H_2O_2 in 400ml of PBS) for 30 min at room temperature.

Afterwards, slides were washed with PBS three times for 5 min. Excess buffer was drained off from slides and brain sections were marked around with Pan Pen (to keep the antibody solution on the section). Non-specific binding was blocked by incubation in 150-

200 µl of 1% Bovine Serum Albumin (BSA) solution in PBS for 30 min at room temperature. Then, sections were incubated with the primary antibody diluted in 1% BSA in PBS overnight in a humidifying chamber at 4°C (150-200 µl per section). Primary negative controls were also included by incubating sections in 1% BSA in PBS alone to control non-specific staining of secondary antibodies. The primary antibodies and dilutions are summarised in the Table B on Figure 2.4.

The next day, sections were removed from the humidifying chamber, adjusted to room temperature and washed for 15 min with PBS (3 changes). The secondary antibody was prepared in 1% BSA in PBS, applied to the sections (150-200 µl per section) and left for 2 hours at room temperature. The secondary antibodies and dilutions are summarised in Table B on Figure 2.4. For isolectin, secondary antibody was not added. Excess antibody was removed by washing in three changes of PBS for 15 minutes.

The next step was 3,3-diaminobenzidine (DAB) development. The sections were incubated in 0.05% DAB, 0.003% H_2O_2 in PBS until the optimal colour intensity had been achieved (up to 5 min). Once the desired signal was achieved, the reaction was stopped by washing the slides in the PBS for 5 min.

If the sections were double stained with another primary antibody, the procedure was repeated by first blocking the peroxidase activity. Then the sections were washed in PBS, incubated with the second primary antibody overnight, washed with PBS and incubated with the appropriate secondary antibody. After that the sections were developed with Vector SG. 5ml of PBS, 3 drops of Chromogen from the kit and 3 drops of Hydrogen Peroxide Solution were mixed and sections were incubated at room temperature until optimal staining developed, usually 5-10 min. Once the desired signal was achieved, the reaction was stopped by washing the slides in the PBS for 5 min.

Finally the sections were washed in running water and counterstained in haematoxylin solution for 30 to 45 sec. After counter-staining, sections were again washed in tap water to blue the nuclei.

After these procedures, the sections were dehydrated in 100% ethanol for 5 min, and dried at room temperature. Finally, the sections were mounted in a drop of mounting

medium DPX. A protective glass cover slip was placed over the section. After drying, the sections were examined under the light microscope.

Data analysis

Sections were examined under a light microscope and pictures were taken with a digital camera (Nikon) attached to the microscope. Analysis was limited to basic plaque and surrounding astrocytes count.

Chapter 3

Can ibuprofen treatment prevent, treat or delay AD-like impairment in transgenic Tg2576 mice?

3. 1. Introduction

Overview

Ibuprofen, one of the NSAIDs, has been implicated in many epidemiological studies in reducing the risk of developing Alzheimer's disease. Transgenic mice modelling this disease show reduced learning ability and Aβ pathology, which develops with aging. This project investigated the effect of dietary administration of ibuprofen on learning ability, synaptic physiology and pathology in Tg2576 Alzheimer's disease model mice at different stages of development of pathology.

Ibuprofen

Boots-affiliated Company first introduced ibuprofen in the UK in 1969 under the trade name Brufen. It belongs to a family of non-steroidal anti-inflammatory drugs described in section 1.5.4. The chemical name of ibuprofen is *2-(4-Isobutyl-phenyl)-propionic acid*

and its chemical formula is $C_{13}H_{18}O_2$. Ibuprofen is a white powder and exists in two enantiomers but only the S-ibuprofen is active in humans (reviewed in Rainsford, 1999).

Ibuprofen is used to relieve the symptoms of a wide range of illnesses such as headaches, backache, period pain, dental pain, neuralgia, rheumatic pain, muscular pain, migraine, cold and flu symptoms and arthritis. Ibuprofen has a low incidence of serious side effects, like gastrointestinal or kidney damage. It is rapidly metabolised in humans, usually leaving the body within 24 hours (reviewed in Rainsford, 1999). Ibuprofen crosses the BBB and acts on the central nervous system. Its concentration in the cerebrospinal fluid peaks in 3 hours after the oral dose (Bannwarth et al., 1995).

Neuroinflammation and animal models

Murine models of AD that overexpress APP and develop Aβ plaques also exhibit proinflammatory activation of microglial cells and astrocytes (Frautschy et al., 1998; Benzing et al., 1999; Stalder et al., 1999; Mehlhorn et al., 2000; Bornemann et al., 2001). Microglial activation around plaques in Tg2576 transgenic mice (Frautschy et al., 1998) is accompanied by the release of a wide range of pro-inflammatory molecules (IL-1β, TNF-α and IL-6) that mediate the auto-activation of these cells and a concomitant astrocytosis (Benzing et al., 1999). These findings provide evidence that Tg2576 mice exhibit features of the inflammatory pathology seen in AD and suggest that this mouse is a useful animal model for studying the role inflammation may play in this disease.

The influence of neuroinflammation on learning ability and synaptic plasticity was also investigated outside of explicit AD models (Hauss-Wegrzyniak et al., 1998; 2000; 2002). Chronic neuroinflammation produced by infusion of lipopolysaccharide (LPS) into the fourth ventricle for 4 weeks resulted in impaired performance of rats in spatial memory spontaneous alternation test in a T-maze (Hauss-Wegrzyniak et al., 1998). The effects of longer infusion of LPS and assessment whether these effects could recover over time were also investigated (Hauss-Wegrzyniak et al., 2000). The results showed that prolonged LPS infusion did not increase the inflammatory reaction and did not produce a significantly greater behavioural impairment. Furthermore waiting for 37 days after the cessation of the LPS infusion did not decrease the density of activated microglia and did not improve performances in the Morris water maze task. The results suggest that inflammation may contribute to the pathogenic mechanisms that underlie the clinical

expression of AD (Hauss-Wegrzyniak et al., 2000). Further studies showed that numerous highly activated microglia were distributed throughout the cingulate gyrus, entorhinal cortex, hippocampus and dentate gyrus (Hauss-Wegrzyniak et al., 2002). In addition, chronic neuroinflammation resulted in the loss of pyramidal cells within the entorhinal cortex and a significant attenuation of LTP within the dentate gyrus (Hauss-Wegrzyniak et al., 2002). All these changes reproduce some features of AD supporting the hypothesis that inflammatory processes play a role in the pathogenesis of AD.

Testing further the LPS model, this same group showed that Flurbiprofen attenuated the neuroinflammatory reaction and reduced the inflammation-induced memory deficit as tested in spatial learning in the Morris water maze (Hauss-Wegrzyniak et al., 1999). Flurbiprofen improved the performance of young rats, but had no effect on older animals (Hauss-Wegrzyniak et al., 1999). The results suggest that NSAID therapies may work better before the onset of AD. Therefore the potential treatments should be initiated in adults before age-associated inflammatory processes within the brain have a chance to develop.

NSAIDs studies in animal models

Epidemiological results showing that long-term treatment with NSAIDs reduces the risk of developing AD (reviewed in chapter 1.5.4) have lead to extensive studies in AD animal models. The aim is to gain further understanding of the mechanism of preventive action of NSAIDs and other anti-inflammatory agents.

Lim and colleagues (2000) first reported that treatment of Tg2576 mice for 6 months (from 10 to 16 months of age) with ibuprofen resulted in a significant reduction of amyloid plaque burden and in total Aβ measured by ELISA. These studies also demonstrated that ibuprofen treatment led to a reduction of plaque-associated microglia and a corresponding reduction in proinflammatory cytokine levels in the brain.

As excessive use of NSAIDs targeting COX-1 can cause gastrointestinal, liver and renal toxicity, Lim and colleagues (2001) also tested curcumin, as an alternative to NSAIDs, in a similar paradigm. Curcumin derives from the curry spice turmeric and has potent antioxidant properties. Treatment with curcumin resulted in reduction of the astrocytic

marker GFAP, insoluble Aβ, soluble Aβ and plaque burden. Microgliosis was also suppressed in neuronal layers but not adjacent to plaques.

To further investigate the effect reported in 2000, Lim and co-workers (2001) attempted to assess the influence of chronically administrated ibuprofen over 3 to 6 months on mouse behaviour in an open field task, that is sensitive to hippocampal and basal ganglia damage, as well as to hind limb dysfunction. Ibuprofen treatments in transgenic females restored values of the open field behaviour to non-transgenic levels. Also, significant reductions of both soluble and insoluble Aβ were observed. Although IL-1β and insoluble Aβ were more effectively reduced with longer treatment, the magnitude of the effect on soluble Aβ was not dependent on treatment duration. However, the direct relationship between the diet-induced changes and learning performance in training tests was not established.

Ferulic acid is an antioxidant and anti-inflammatory agent derived from plants and Yan and colleagues (2001) assessed its potential protective activity against Aβ toxicity in vivo. Mice were given water containing ferulic acid or control and after 4 weeks Aβ42 was administered via intracerebroventricular injection. Injection of control mice with Aβ42 impaired performance on the step-through passive avoidance test, the spontaneous alternation in the Y-maze and the spatial reference water. However mice treated with ferulic acid prior to Aβ42 administration were protected from these changes.

Similar results were obtained in the study using NCX-2216, a nitric oxide (NO)-releasing derivative of the COX-1-preferring NSAID flurbiprofen (Jantzen et al., 2002). Doubly transgenic APP/PS1 mice were treated with NCX-2216, ibuprofen and cyclooxygenase-2-selective NSAID celecoxib between 7 and 12 months of age. The NO-releasing derivative of flurbiprofen dramatically reduced both Aβ loads and Congo red staining. This reduction was associated with a dramatic increase in the number of activated microglia suggesting their role in clearing Aβ deposits. In contrast, ibuprofen caused only modest reductions in Aβ load and celecoxib had no effects on amyloid deposition at all.

Yan et al. (2003) studied the effect of pioglitazone (PPARγ agonist) and ibuprofen administered orally for four months in 11-month-old transgenic Tg2576 mice. They confirmed that ibuprofen reduced amyloid plaque burden, microglial activation and

soluble Aβ42, but in their study pioglitazone only modestly reduced soluble Aβ levels and did not affect amyloid plaque burden or microglia activation. Further studies using APP-expressing cells showed that ibuprofen directly affected APP processing, specifically reducing the production of Aβ42. This could indicate that PPARγ activation is not involved in the Aβ lowering effect, although other studies gave contradictory results (see chapter 1.5.4).

All these studies performed in rodents suggest that ibuprofen and other NSAIDs are important agents that can modify amyloid pathology as well as learning and memory functions in AD animal models. The diversity of results, however, reflect the fact that the mechanism(s) by which NSAIDs may reduce the severity of Alzheimer's disease and stage of the illness at which intervention would be most effective are not yet well understood. The main hypotheses how NSAIDs act to achieve these effects are described in section 1.5.4.

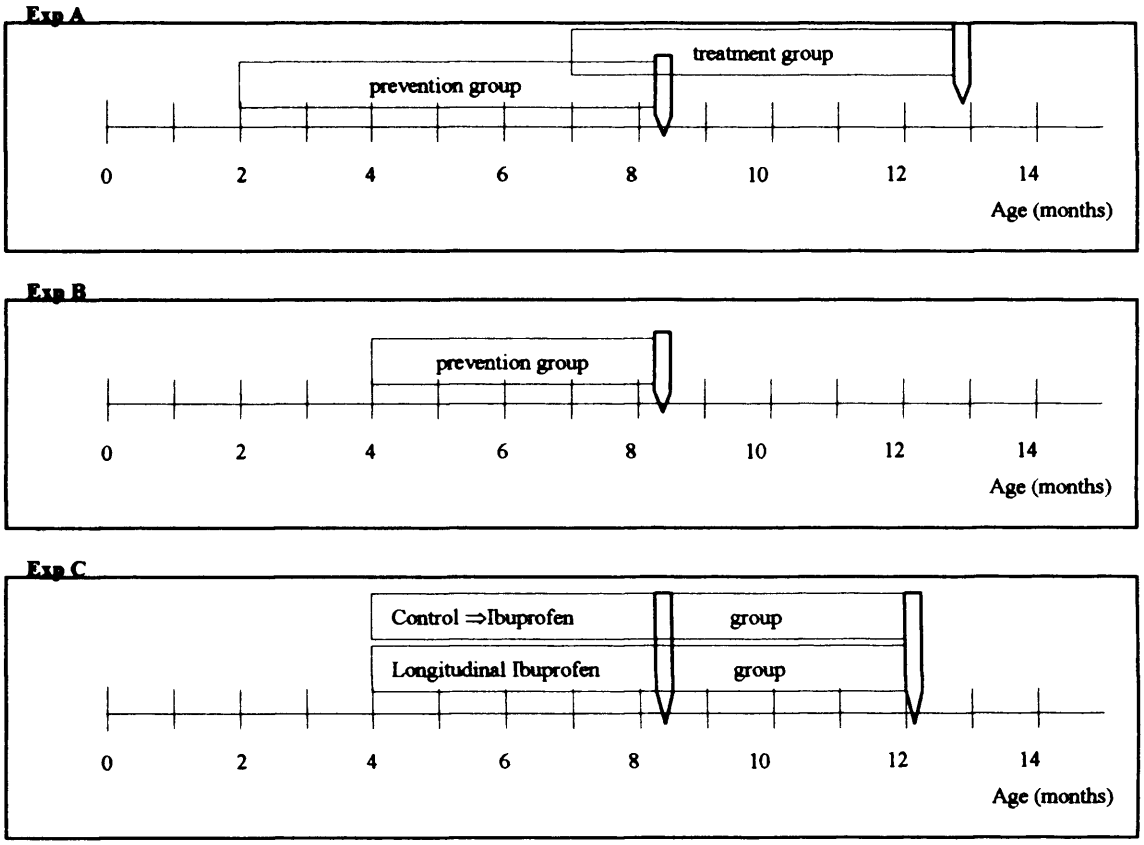
The aim of this study was to test whether ibuprofen can prevent, postpone or treat the development of amyloid pathology, memory impairment and synaptic disturbances in the brains of APP over-expressing Tg2576 transgenic mice. The project was intended to assess the effect of various paradigms of chronic ibuprofen administration on learning and memory impairment in the Forced Choice Alternation task, in synaptic physiology and in the induction of LTP *in vivo*, in the levels of Aβ40 and 42 in the brain and on markers of amyloid pathology in the brain.

3.2. Materials and methods

Animals and diets

Male and female transgenic Tg2576 mice, aged from four to fourteen-months, and their non-transgenic littermates (20-35 g body weight) were used for experimental procedures. The project consisted of three experimental parts, which are described and shown in Figure 3.1. The mice for each type of experiment were randomly divided into four groups: the transgenic/ibuprofen group, the transgenic/control-diet group, the non-transgenic/ibuprofen group and the non-transgenic/control-diet group. Mice were group housed with littermates (with transgenic and non-transgenic housed together), and all

Experimental design



Exp	Group	Electro	ELISA	Immuno-staining	numbers of transgenic mice
A	prevention	yes	yes*	no	Ibuprofen=6 Control=6
	treatment	yes	yes*	no	Ibuprofen=12 Control=12
B	prevention	no	no	no	Ibuprofen=20 Control=20
C	Longitudinal Ibuprofen	no	yes	yes	Ibuprofen longitudinally=24
	Control ⇒Ibuprofen	no	yes	yes	Ibuprofen after control=22

Figure 3.1. Experimental design. Testing was performed in three parts, part A and C were performed at Cardiff University while part B was done at the University of Minnesota in collaboration with Prof Karen Hsiao Ashe. Mice were all tested in the T-maze (indicated by ⊓). In experiment C mice were tested for the second time after 2 months. Group A went through electrophysiology *in vivo*, and the brains were analysed for β-amyloid using ELISA in Prof Steven Younkin's laboratory (indicated by a star (*), results not available). Group B was left in Prof Ashe's laboratory for further analysis.

testing was conducted during the light phase. Transgenic mice were compared to littermate controls to ensure that age and background strains were as equivalent as possible.

The ibuprofen group was put on a special chow prepared by Harlan (US) by incorporating ibuprofen powder into standard rodent chow. The ibuprofen-enriched special diet contained 375ppm of ibuprofen, corresponding to 1g of chow-contained 0.375mg of ibuprofen. Assuming that a healthy mouse eats around 5g of a chow a day, the ibuprofen dose for a mouse was estimated to be 1.875 mg per day. That accounts for a dose of around 62.5 mg/kg per day (assuming the average mouse weighs 30g). This value is similar to the dose typically administered to humans (15-30mg/kg/day; Rainsford, 1999). Also this dose has already been found to be effective at reducing Aβ and used by other groups in Tg2576 mice studies (Lim et al., 2000; 2001).

For the control chow, nothing was added to the standard Harlan rodent chow. The chows were stored at 4°C and fresh supplies were given to the mice every day. The quality of the diet and the ibuprofen content was assessed and provided by Harlan (US).

Experimental Procedures

The full experimental procedure consisted of a series of phases, which were conducted on all animals. These included:

- Maintaining on the special chows (ibuprofen or control) and drinking water *ad libitum* for a various amount of time depending on the experimental group (Figure 3.1). For longitudinal experiments, some mice were kept on ibuprofen while others were transferred from control to ibuprofen for an additional 11 weeks.

- Forced Choice Alternation task as described in Chapter 2.1.1. During testing, mice were put on water deprivation regime (20 hours of no water, 2 hours experiment with 25% sucrose reward and 2 hours of free water access after experiment). Mice were kept on the special chow diets until the end of testing.

- Electrophysiology *in vivo* was performed in only one of the experimental groups (group A, see Figure 3.1). Basic synaptic transmission and induction of LTP was assessed

in vivo by recording from dentate gyrus whereas stimulating the perforant pathway (described in chapter 2.2).

- Mice were killed after behavioural and electrophysiological experiments. The brains were dissected and the hemispheres separated along the midline (excluding cerebellum). The half brains were assigned for β-amyloid ELISA, ibuprofen measurements and immunohistochemistry.

- ELISA was conducted using commercially available kits (Biosource) in SDS and FA brain extracts to assess the amount of β-amyloid 40 and 42 in brain tissue (details in Chapter 2.3). ELISA measurements in mice brains from groups A and B were performed in Prof Steven Younkin's laboratory.

- Immunostaining was undertaken using antibodies recognising β-amyloid, APP, activated astrocytes and microglia recognising antibodies (as described in Chapter 2.4);

- Ibuprofen levels were measured in plasma and in chopped and mixed whole half of the brain (hippocampus was not extracted separately). The measurements were performed by Pablo Morentin-Gutierrez in collaboration with Prof Paul Chapman in the Merck laboratories.

Statistical analysis

All data were expressed as means ± SEM. The statistical significance of differences in the learning parameters in the FCA and the values of electrophysiological analysis were measured by repeated-measures 3-factor ANOVA, with genotype and diet as between subject factors and time or stimulus intensity as within subject factor. Tukey's pair-wise comparisons post hoc tests were carried out to determine the source of a significant main effect or interaction. The statistical significance of differences in the value of Perseveration Count and the β-amyloid levels between the groups was determined by the 2-factor ANOVA, with genotype and diet as bewteen subject factors and Tukey's pair wise comparisons post hoc tests. The proportion of mice reaching learning criterion was compared using pair-wise Fisher's test.

3.3. Results

Body weight, survival assessment and chow quality

There were no significant differences in weight gain over the treatment period between the transgenic and non-transgenic groups, whether on control or ibuprofen-enriched diets (data shown in Figure 3.2.). The weight data are only available from experimental group A and C, as mice in group B were fed and cared for by Research Animal Services at the University of Minnesota, under the supervision of Prof K. Hsiao Ashe's group. Two age groups of mice from experiment A were combined into one weight group as all the mice were on the special diets for the same period of time and were adult. Although the mice on ibuprofen seemed to be fatter, repeated measures 3-factor ANOVA with week as a repeated measure and diet and genotype as between-subject factors revealed no significant differences (all $p>0.05$).

During the time of chronic administration of either ibuprofen or control diets some of the mice died (data shown in Figure 3.2.). The numbers of lost mice were similar for all groups on both types of diet, but show a higher mortality rate for transgenic Tg2576 mice compared to non-transgenic littermates. This finding is consistent with other groups breeding Tg2576 mice (Karen Hsiao Ashe`s or Steven Younkin's laboratories).

The quality of the diet and the ibuprofen content was assessed and provided by Harlan (US). The content of ibuprofen in a rodent chow during storage time (around 3months at 4 °C) was not confirmed. Fresh supplies were bought every 3 months from Harlan (US).

Forced Choice Alternation task in the T-maze

The forced-choice alternation (FCA) task assesses spatial episodic-like memory using the T-maze. Training lasts for 10 days and each mouse receives 6 trials per day. The percent of correct choices for each mouse was calculated daily. The aim of the experiment was to compare the influence of ibuprofen supplementation as prevention (before 8 months of age) and as a treatment (after 8 months of age). Before the age of 7-8 months, Tg2576 mice show no impairments in T-maze FCA, while after that age the deficit in FCA task and others starts to develop (Chapman et al., 1999).

Body weight and survival assessment

	Final weight	Died during treatment period (4-6 months)
Group A		
Transgenic/Ibuprofen	**30.9 ± 1.4g**	**3**
Transgenic/Control	**29.9 ± 1.5g**	**2**
Non-transgenic/Ibuprofen	**33.1 ± 0.9g**	**0**
Non-transgenic/Control	**32.8 ± 1.2g**	**1**
Group C		
Transgenic/Ibuprofen	**30.7 ± 0.7g**	**4**
Transgenic/Control	**29.9 ± 0.5g**	**5**
Non-transgenic/Ibuprofen	**32.1 ± 0.8g**	**2**
Non-transgenic/Control	**32.4 ± 0.9g**	**1**

Figure 3.2. Final weights for mice from experimental groups A (age 9 months) and C (age 11-12 months). Data are expressed as means ± SEM. Repeated measures 3-factor ANOVA with week as a repeated measure and diet and genotype as between-subject factors revealed no significant differences (all $p>0.05$). During the time of chronic administration of either ibuprofen or control diets more transgenic than non-transgenic mice died. The diet did not seem to have influence but the results show a higher mortality rate for transgenic Tg2576 mice.

Experiment A. The aim of the first experiment was to compare the prevention and treatment effects of ibuprofen given to mice at the age of 2-3 months and 6-8 months. During testing none of the mice was excluded from analysis. Graphs A and B on Figure 3.3 show the effect of chronic administration of dietary ibuprofen on episodic-like memory related learning ability in Tg2576 and their non-transgenic littermates at the age of 8 and 12-14 months, respectively.

Figure 3.3A shows learning curves for the prevention group averaged across groups and for 2 days. Repeated measures ANOVA (with day as a repeated factor and genotype and diet as between subject factors) revealed a significant effect of day of training ($F=2.8$, $p=0.018$) confirming overall improvement during training for all the groups. The main effect of genotype was just significant ($F=4.8$, $p=0.043$) indicating that both groups of transgenic mice were impaired in the FCA task compared to non-transgenic mice. The effect of diet and the interaction between genotype and diet did not reach significance (all $p> 0.05$). Post-hoc Tukey's pair-wise comparisons did not confirm any significant differences between groups. Although not significant, there was a visible predisposition of transgenic mice on ibuprofen diet to reach a higher percent correct each day. Also the numbers of mice were low (4-6) making statistical analysis problematic.

Figure 3.3B illustrates learning curves for the older group, testing the effect of ibuprofen as a treatment. Data was averaged across groups and for 2 days. Repeated measures ANOVA (with day as a repeated factor and genotype and diet as between subject factors) revealed a significant effect of day of training ($F=4.4$, $p<0.001$) confirming the overall improvement during training of all the groups. The main effect of genotype was highly significant ($F=57.8$, $p<0.001$) indicating that all the transgenic mice were impaired in the FCA task compared to non-transgenic mice. Also the interaction between day and genotype reached significance ($F=2.2$, $p=0.046$) indicating that transgenic mice reacted in a different way to training in the T-maze, meaning that they did not learn as well as non-transgenic littermates. The effect of diet and the interaction between genotype and diet did not reach significance (all $p> 0.05$) indicating no influence of ibuprofen on performance in the T-maze. Post-hoc Tukey's pair-wise comparisons confirmed that transgenic mice on both diets were significantly different from non-transgenic mice regardless of which diet they were fed. This result confirms the finding that an effect of genotype on performance of Tg2576 mice is very strong.

Learning in Tg2576 and non-transgenic littermates on Ibuprofen/Control chow as a prevention and as a treatment – experiment A

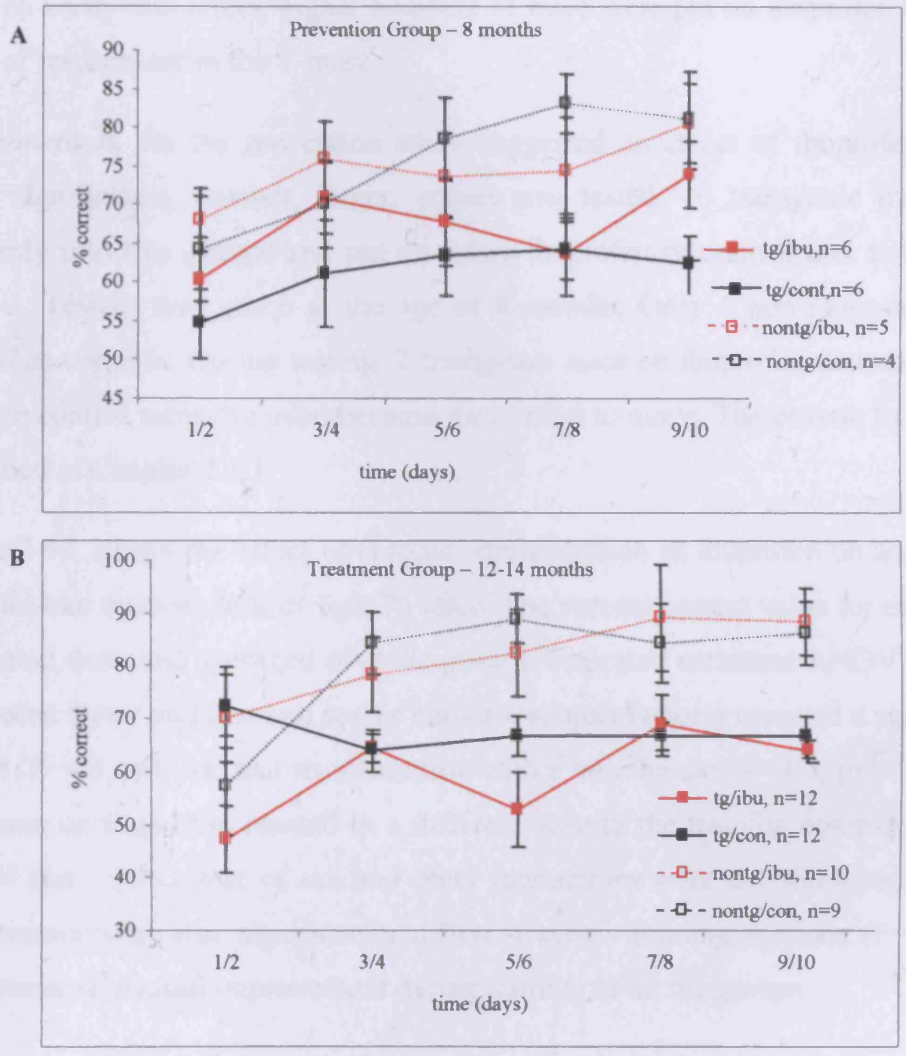


Figure 3.3. Chronic administration of Ibuprofen in Tg2576 mice as a prevention (from 2 to 8 months) and as a treatment (from 6-8 to 12-14 months) of age. Graph A represents the percentage of correct responses averaged across groups and for 2 days in prevention group. Analysis revealed significant main effect of day of training and genotype. The effect of diet and interaction between genotype and diet did not reach significance. Graph B shows learning acquisition curve for transgenic and non-transgenic mice from a treatment group. Data were averaged across groups and for every 2 days. Analysis revealed significant main effects of day of training, genotype and a significant interaction between day and genotype. The effect of diet and interaction between genotype and diet did not reach significance indicating no influence of ibuprofen on performance in the T-maze at this age of mice.

Comparing graphs from the prevention and treatment groups, it seems that 8-month-old transgenic mice were protected to some extent against developing impairment in the FCA task. To verify this effect, higher numbers of mice were put on ibuprofen diet before the onset of impairment in the T-maze.

Experiment B. As the prevention study suggested an effect of ibuprofen, but did not reach significance, another, larger, cohort was tested. 40 transgenic mice were split randomly into two groups and put on either ibuprofen or control diet at the age of 4-5 months. Testing took place at the age of 8 months. Only 4 non-transgenic mice were trained as controls. During testing, 2 transgenic mice on ibuprofen diet and 3 transgenic mice on control were excluded because they failed to move. The criteria for exclusion are described in Chapter 2.1.1.

Figure 3.4A shows the effect of chronic administration of ibuprofen on acquisition of an episodic-like memory task in Tg2576 mice. The percent correct value for each mouse was calculated daily and averaged over the groups. Repeated measures ANOVA (with day as a repeated factor and diet and sex as between subject factors) revealed a significant effect of diet ($F=4.6$, $p=0.04$) and its interaction with a training day ($F=4.5$, $p<0.001$) indicating that mice on ibuprofen reacted in a different way to the training comparing to mice on control diet. The effect of sex and other interactions were not significant (all $p>0.05$). Performance was also significantly different across training sessions ($F=3.8$, $p<0.001$) confirming an overall improvement during training of all the groups.

The percent of mice reaching Learning Criterion (defined as 80% correct or better on at least 3 consecutive days during training) was also affected by ibuprofen pre-administration as shown on Figure 3.4 (B). The paired comparison between groups was performed using Fisher's exact test, two-sided and revealed that a significantly smaller percent of transgenic mice on control diet ($p=0.0032$) reached the Learning Criterion comparing to transgenic littermates on ibuprofen diet, indicating a significant effect of treatment. Finally, the nature of the response strategies in the transgenic animals and the influence of diet were also examined. Figure 3.4C shows the mean Perseveration Count, which is measured as a maximum number of consecutive free choices made in the same direction for each animal per day, averaged over the training days and the groups. A 2-

Learning in Tg2576 transgenic mice on Ibuprofen/Control chow – experiment B

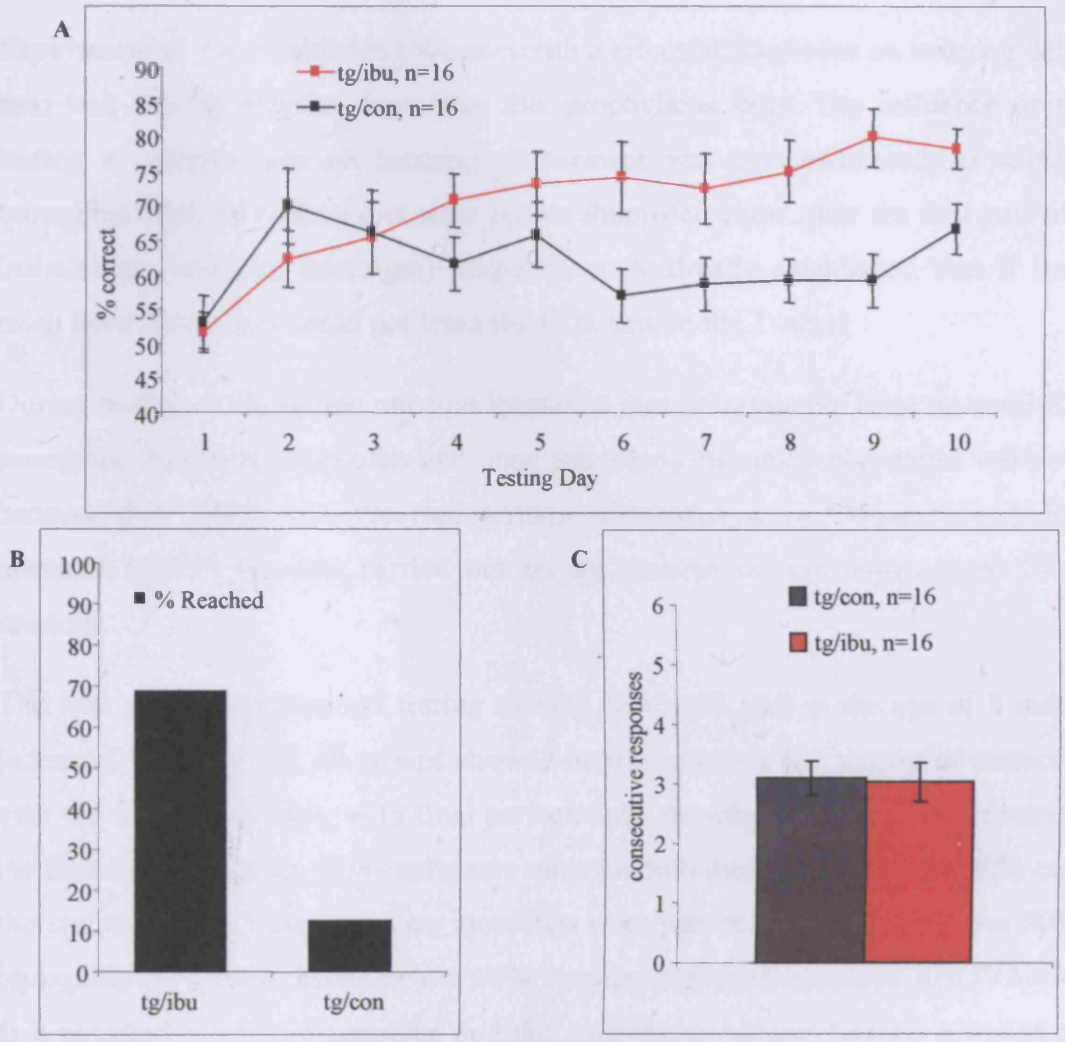


Figure 3.4. Chronic pre-administration of Ibuprofen in Tg2576 mice delays the onset of impairment in spatial episodic –like memory assessed using the T-maze. Graph A represents the percentage of correct responses for each day during 10 days of training for each mouse averaged over the groups. Analysis comparing transgenic mice on control and ibuprofen diets revealed significant effect of diet, training day and their interaction. The effect of sex was not significant. Graph B shows the percentage of mice in each group that reached learning criterion, defined as 80% correct or better on at least 3 consecutive days. The paired comparisons between groups revealed significant difference between transgenic mice on control and ibuprofen diets. Graph C shows Perseveration Count Mean which is measured as a maximum number of consecutive free choices made in the same direction for each animal per day averaged over the days and the groups. A 2-factor ANOVA did not show any significant differences.

factor ANOVA (with diet and sex as between subject factors) did not show any significant differences.

Experiment C. On establishing the preventive effect of ibuprofen on learning deficit, the next task was to measure how long this prophylaxis lasts. The influence of previous testing at younger age on learning impairment was also addressed, as some of the transgenic mice on control diet were put on ibuprofen chow after the first part of testing (mimicking treatment paradigm). Experiment A already established that if transgenic mice were naïve, they could not learn the FCA task in the T-maze.

During testing, 3 transgenic mice on ibuprofen diet, 2 transgenic mice on control, 1 non-transgenic mouse on ibuprofen and none non-transgenic mice on control were excluded because they failed to move (see criteria for exclusion in Chapter 2.1.1). Repeated measures ANOVAs were carried out on the percent correct scores from 10 training sessions.

The first part of longitudinal testing in FCA task took part at the age of 8 months. As indicated in Figure 3.5, all groups showed improvement in the percent of correct choices over the 10 training days, with final performance showing significant improvement from the first day of training. Non-transgenic mice on both diets reached above 90% correct by the end of testing, transgenics on ibuprofen were just below, reaching above 80% while transgenic on control stayed below 60% correct. Repeated measures ANOVA (with day as a repeated factor and genotype and diet as between subject factors) revealed a highly significant effect of day of training ($F=7.3$, $p<0.001$) confirming overall improvement during training of all the groups. The main effect of genotype was significant ($F=31.1$, $p<0.001$) indicating that all the transgenic mice were impaired in the FCA task compared to non-transgenic mice. The interaction between genotype and diet ($F=4.2$, $p=0.045$) reached significance, indicating that ibuprofen influenced performance of Tg2576 mice in FCA task. Also, the interaction between day and diet ($F=2.1$, $p=0.04$) reached significance, pointing to an influence of diet on acquisition of the FCA task. The effect of diet and any other interactions were not significant (all $p>0.05$). As non-transgenic mice in this task are approaching a ceiling effect (more than 90% correct choices on the last day of testing) it was impossible to distinguish between non-transgenic on control or ibuprofen enriched diets. To examine the effect of ibuprofen on non-transgenic animals,

delays between the sample and test trials in the FCA task in the T-maze could have been introduced.

To avoid this confound, transgenic mice were also analysed separately. Repeated measures ANOVA (with training day as a repeated factor and diet as the between subject factor) revealed a significant main effect of training day ($F=4.3$, $p=0.001$), confirming a general improvement during testing. The analysis also showed a significant main effect of diet ($F=9.1$, $p=0.004$) indicating a significant influence of ibuprofen on tested animals. Also the interaction between day and diet remained significant ($F=2.9$, $p=0.009$) pointing to an influence of ibuprofen on acquisition of FCA task in transgenic mice.

Post-hoc Tukey's pair-wise comparisons confirmed that transgenic mice on control diet were significantly worse than non-transgenic on either diet and transgenic mice on ibuprofen diet. However transgenic mice on ibuprofen diet were significantly different from transgenic and non-transgenic on control diets but no different from non-transgenic on ibuprofen diet. The interaction between day and diet might also be explained as both the transgenic and non-transgenic mice on ibuprofen diet started at better percent of correct choices than mice on control diet (Figure 3.5). After accomplishing ten training days, all mice on control diet were switched to ibuprofen diet while those on ibuprofen diet did not change.

The analysis of the first part of testing suggests that transgenic mice on control diet showed impairment in the FCA task and ibuprofen diet made this impairment significantly smaller. Nonetheless, transgenic mice on ibuprofen were still different from non-transgenic, so ibuprofen only minimised deficit in the T-maze.

10 weeks later, the same mice were re-tested (at 11 months of age). During 10 weeks 1 transgenic mouse on control diet and 2 transgenic mice on ibuprofen diet died and no further mice were excluded from testing. As pictured on Figure 3.5, all the non-transgenic mice regardless of diet had maintained the same performance levels demonstrated at 8 months, but all transgenic animals (regardless of diet) showed impaired performance on the task. This interpretation was confirmed by repeated measures ANOVA (with day as a repeated factor and genotype and diet as between subject factors) and revealed a significant main effect of genotype ($F= 45.9$, $p<0.001$) and a significant main effect of day ($F=7.76$, $p<0.001$) indicating that all mice improved during training. A main effect of

Learning in Tg2576 and Non-Transgenic littermates on Ibuprofen/Control chow – experiment C

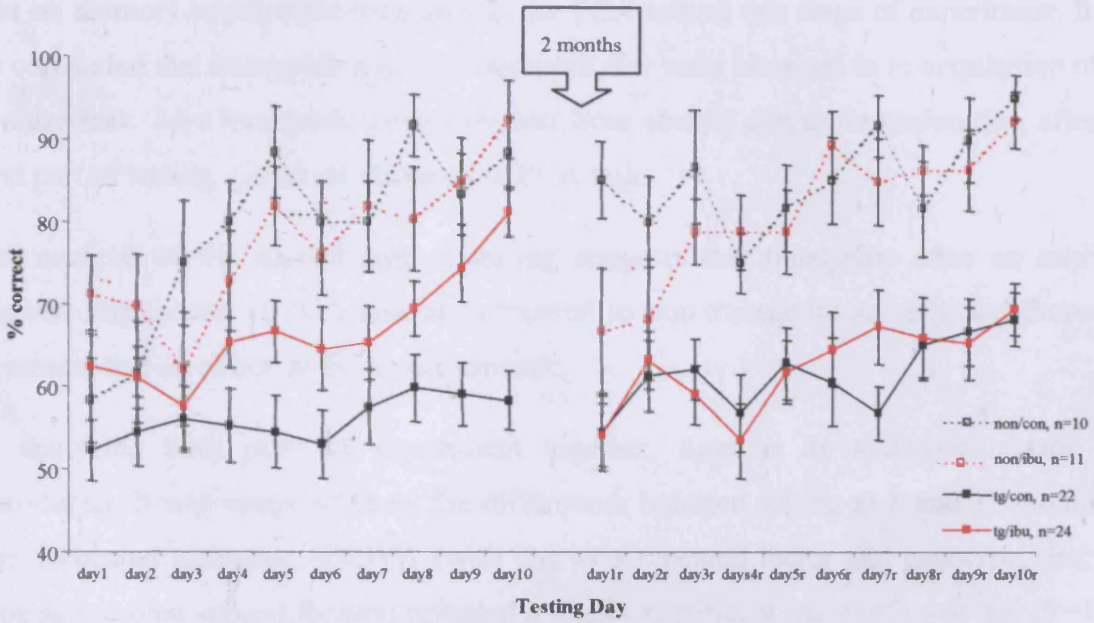


Figure 3.5. Chronic pre-administration of Ibuprofen in Tg2576 mice delays the onset of impairment in spatial episodic –like memory assessed using the T-maze. Data are mean ± SEM of the percentage of correct responses for each day during both parts of training for each mouse averaged over the groups. Repeated Measures ANOVA for the first part revealed significant effect of a training day, genotype and significant interactions of a diet with genotype and of a day and diet. After excluding non-transgenic animals from analysis, the effect of diet reached also significance. For the re-testing, repeated measures ANOVA revealed a highly significant effect of genotype and a training day. The effect of diet was not significant.

diet and interactions between diet, genotype and day were not significant (all $p > 0.05$). Excluding non-transgenic animals from the analysis only confirmed significant main effect of day ($F=3.7$, $p<0.001$) whereas diet and interaction between day and diet remained not significant (all $p>0.05$).

Post-hoc Tukey's pair-wise comparisons confirmed that transgenic mice were significantly worse than non-transgenic mice irrespective of diet, indicating no effect of diet on memory impairment measured in the FCA task at this stage of experiment. It can be concluded that transgenic mice on ibuprofen diet were impaired in re-acquisition of the T-maze task. Also transgenic mice switched from control diet to ibuprofen diet, after the first part of testing, remained impaired in FCA task.

The analysis of the second part of testing suggests that transgenic mice on any diet showed impairment in FCA task as compared to non-transgenic animals and ibuprofen treatment had no effect on FCA performance.

In analysing both parts of experiment together, time as an additional factor was introduced. It was meant to show the differences between testing at 8 and 11 months of age. Repeated measures ANOVA (with day as a repeated factor and genotype, diet and time as between subject factors) revealed a highly significant main effect of day ($F=13.8$, $p<0.001$) and genotype ($F= 76.6$, $p<0.001$). The interaction between diet and genotype was significant ($F=3.9$, $p=0.048$) indicating that ibuprofen influenced Tg2576 mice performance in the T-maze during both stages of testing. Also interaction between diet, day and time was significant ($F=2.1$, $p=0.036$) indicating a different influence of ibuprofen on FCA acquisition of Tg2576 mice at two parts of testing. A main effect of diet and other interactions between diet, genotype, time and day were not significant (all $p> 0.05$). Excluding non-transgenic mice from the analysis (see above for explanation), confirmed the significant effect of day ($F= 76.6$, $p<0.001$) and diet ($F= 76.6$, $p<0.001$). Time and its interaction with either diet or genotype did not reach significance (all $p> 0.05$).

Post-hoc Tukey's pair-wise comparisons confirmed that transgenic mice on either diet during both parts of testing were significantly different from non-transgenic mice regardless of diet. This result confirms the fundamental finding that an effect of genotype

on performance of Tg2576 mice in the T-maze is very strong and that ibuprofen diet had only modest effect.

The analysis of both parts of testing suggests that ibuprofen given as prevention (before the onset of deficit) delays the development of impairment in Tg2576 transgenic mice in FCA task but does not reverse it.

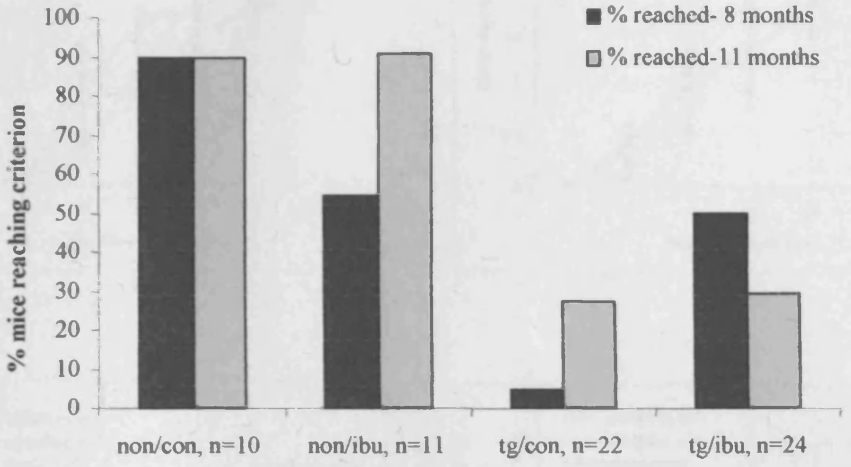
The percent of mice reaching Learning Criterion (defined as 80% correct or better on at least 3 consecutive days during training) was affected by ibuprofen administration at the first part of testing (8 months old) as shown on Figure 3.6. This effect disappeared at the second part of testing (at the age of 11 months); a significantly smaller percentage of transgenic mice reached Learning Criterion comparing to non-transgenic littermates, indicating a significant effect of only genotype and no effect of diet (for analysis see Figure 3.6). Thus, the preventive, but not the treatment, effect of ibuprofen on learning of transgenic mice as measured by percentage of correct choices (Figure 3.5) was confirmed by analysis of Learning Criterion.

Electrophysiology in vivo

To determine whether the behavioural deficits in aged Tg2576 mice were associated with changes in synaptic function and whether ibuprofen affected this, I examined field potentials in the dentate gyrus in response to medial perforant pathway stimulation in Tg2576 mice *in vivo*. Mice from experiment A (see Figure 3.1), after finishing testing in the T-maze, were used. For analysis, changes in the field-excitatory postsynaptic potential (fEPSP) slope and pop spike (PS) amplitude were measured.

First, synaptic function was assessed by using a range of stimulus intensities to generate input-output (I/O) curves to study baseline responses before induction of LTP. I/O curves of the fEPSP slope and PS amplitude were created as a function of stimulus intensity (see Figure 3.7). Although the numbers of mice in the prevention group were quite low (n=4-6), analysis could still be performed. Repeated measures 3-factor ANOVA (diet x genotype, with stimulus intensity as the repeated-measure factor) revealed no significant effects of either genotype or diet for the size of the fEPSP slope or the PS amplitude at any intensity (all $p > 0.05$). An overall effect of stimulus intensity was significant for both the fEPSP slope and the PS amplitude (both $p<0.001$) indicating a normal physiological

Reaching Learning Criterion – results from experiment C

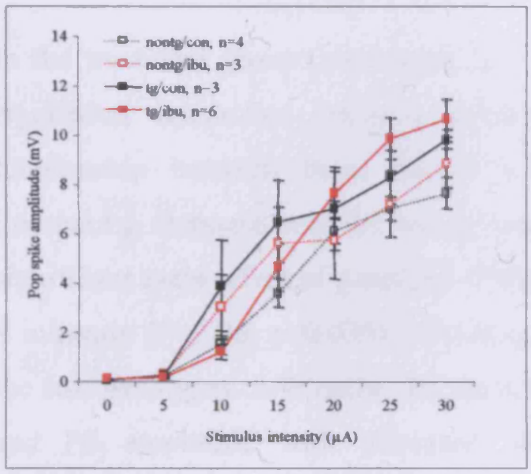


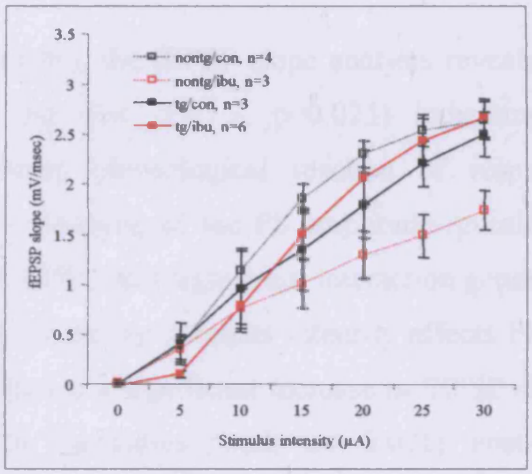
Paired Comparisons Between Groups	Age of 8 months	Age of 11 months
TG/CON – TG/IBU	S, $p<0.001$	NS, $p>0.05$
TG/CON – NONTG/IBU	S, $p=0.0025$	S, $p<0.001$
TG/CON – NONTG/CON	S, $p<0.001$	S, $p=0.0017$
TG/IBU – NONTG/IBU	NS, $p>0.05$	S, $p<0.001$
TG/IBU – NONTG/CON	NS, $p>0.05$	S, $p=0.002$
NONTG/CON – NONTG/IBU	NS, $p>0.05$	NS, $p>0.05$

Figure 3.6. The percentage of mice in each group at two time points that reached learning criterion, defined as 80% correct or better on at least 3 consecutive days. Ibuprofen had significant effect on transgenic Tg2576 at the age of 8 months (transgenic mice on ibuprofen were not different from non-transgenic on any diet and were significantly different from transgenic on control diet). After 11 weeks this effect was abolished and all transgenic were significantly worse than all non-transgenic. Ibuprofen had no effect. The paired comparisons between groups were performed using Fisher's exact test, two-sided.

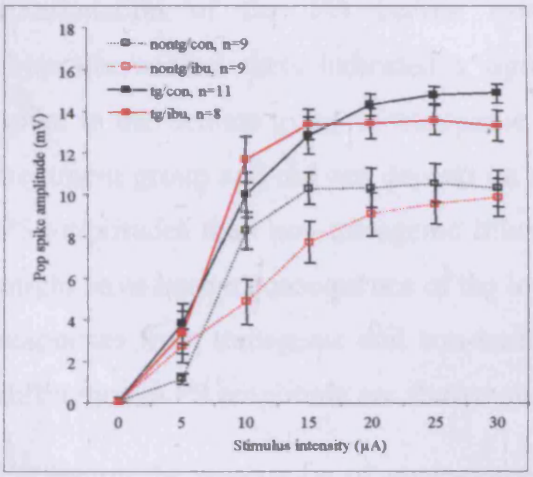
I/O Curves for responses recorded in Dentate Gyrus in transgenic Tg2576 and non-transgenic mice treated with ibuprofen/control diet

A





B



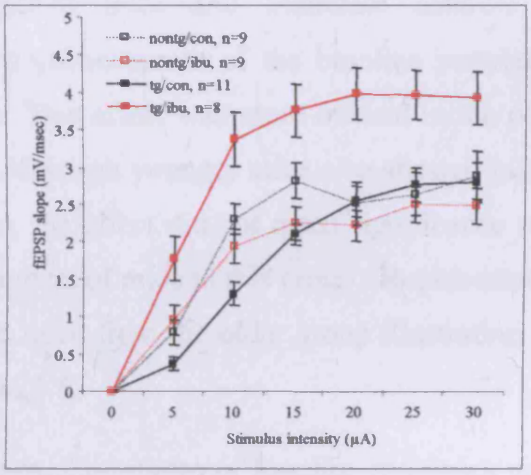


Figure 3.7. I/O curves of the fEPSP slope and pop-spike amplitude as a function of stimulus intensity before induction of LTP for prevention (A) and treatment (B) groups from Experiment A. The y-axis denotes the slope of the fEPSP measured in mV per ms or PS amplitude measured in mV and the x-axis indicates the lowest and highest intensities used in mA. In the prevention group, repeated measures 3-factor ANOVA (diet x genotype, with stimulus intensity as the repeated-measure factor) revealed no significant effects of either genotype or diet for the size of the fEPSP slope or the PS amplitude at any intensities, although an overall effect of stimulus intensity was significant for both groups ($p<.0001$) (A). For the treatment group, analysis revealed significant interaction between genotype and diet for the size of the fEPSP slope and a significant main effect of genotype and significant genotype x intensity interaction for the PS amplitude measurements (B).

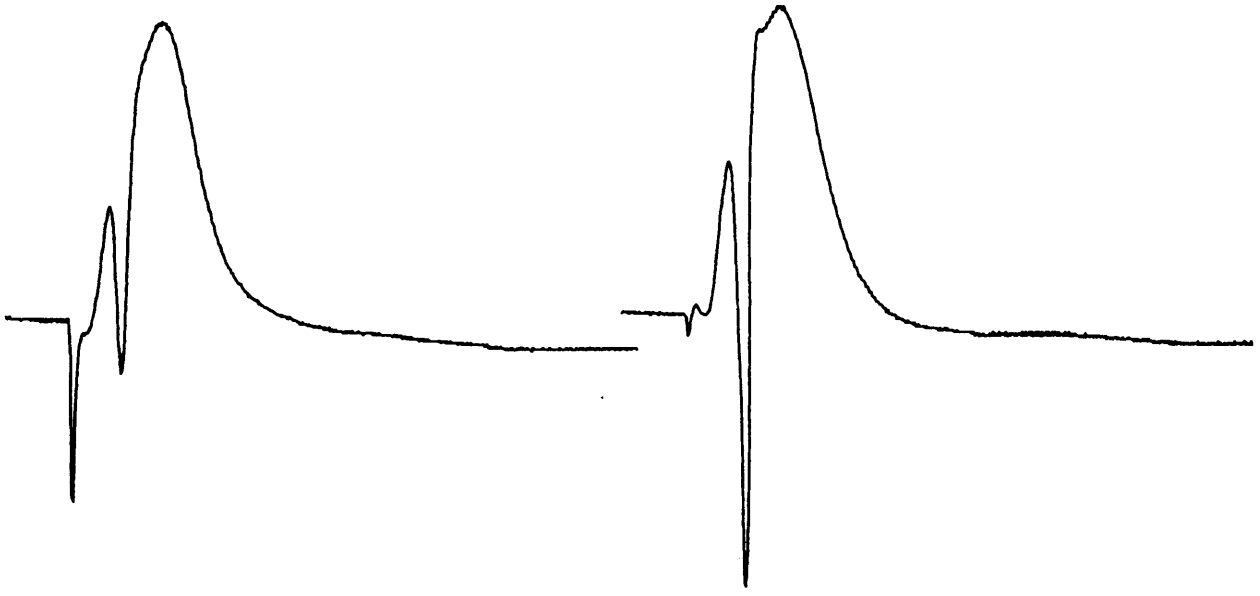
reaction of increasing response to increasing stimulus intensity. Post-hoc comparisons between groups did not find any significant differences (Figure 3.7A).

In the treatment group (mice aged 12-14 months), the fEPSP slope analysis revealed a significant interaction between genotype and diet ($F=5.5$, $p=0.025$) indicating a relationship between these factors influencing physiological reaction of response (increasing response with increasing current). Analysis of the PS amplitude revealed a significant main effect of genotype ($F=8.9$, $p=0.005$) and significant interaction genotype x intensity ($F=3.03$, $p=0.036$) indicating that increasing stimulus intensity affects PS of the two genotypes differently. All the mice showed a significant increase in fEPSP slope and PS amplitude with increased stimulus intensities (both $p< 0.001$) Post-hoc comparisons between groups did not find any significant differences (Figure 3.7B).

Examination of the I/O curves from Tg2576 mice and littermate controls on ibuprofen/control diets indicated a significant enhancement of the baseline population spike in the dentate gyrus of transgenic mice. This effect was more evident in the older treatment group and did not depend on diet. Although younger mice also showed higher PS amplitudes than non-transgenic littermates, the effect did not reach significance (this might have been a consequence of the low numbers of mice in this group). Representative responses from transgenic and non-transgenic mice from the older group illustrating the difference in PS amplitude are shown on Figure 3.8.

Following the generation of input-output curves, 20-minutes of baseline responses were recorded (see methods for details) followed by theta-burst stimulation of the perforant pathway (see chapter 2.2). All data were normalized with respect to the mean fEPSP slope or mean PS amplitude during this 20-min period (Figure 3.9). Although care was taken to ensure the stability of the pre-tetanus baseline, analyses after the completion of the experiments indicated that responses were declining in most subjects prior to theta-burst stimulation. Ultimately, it was therefore impossible to conduct statistical analyses of the fEPSP after LTP. Most of the mice had to be excluded by the end of 20-minute baseline, as their fEPSP response had decreased below 90% of average (Figure 3.9)

Representative baseline responses recorded in dentate gyrus in transgenic Tg2576 and non-transgenic mice treated with ibuprofen/control diet



Non-transgenic mouse
30 μA

Transgenic mouse
10 μA

Figure 3.8. Baseline responses recorded in dentate gyrus after stimulating middle perforant pathway from Tg2576 mice and littermate controls on both diets. Analysis indicated a significant enhancement of the baseline population spike in the dentate gyrus of transgenic mice. This effect was more evident in the older treatment group and did not depend on diet.

LTP induction recorded in dentate gyrus of hippocampus in transgenic Tg2576 and non-transgenic mice treated with ibuprofen/control diet

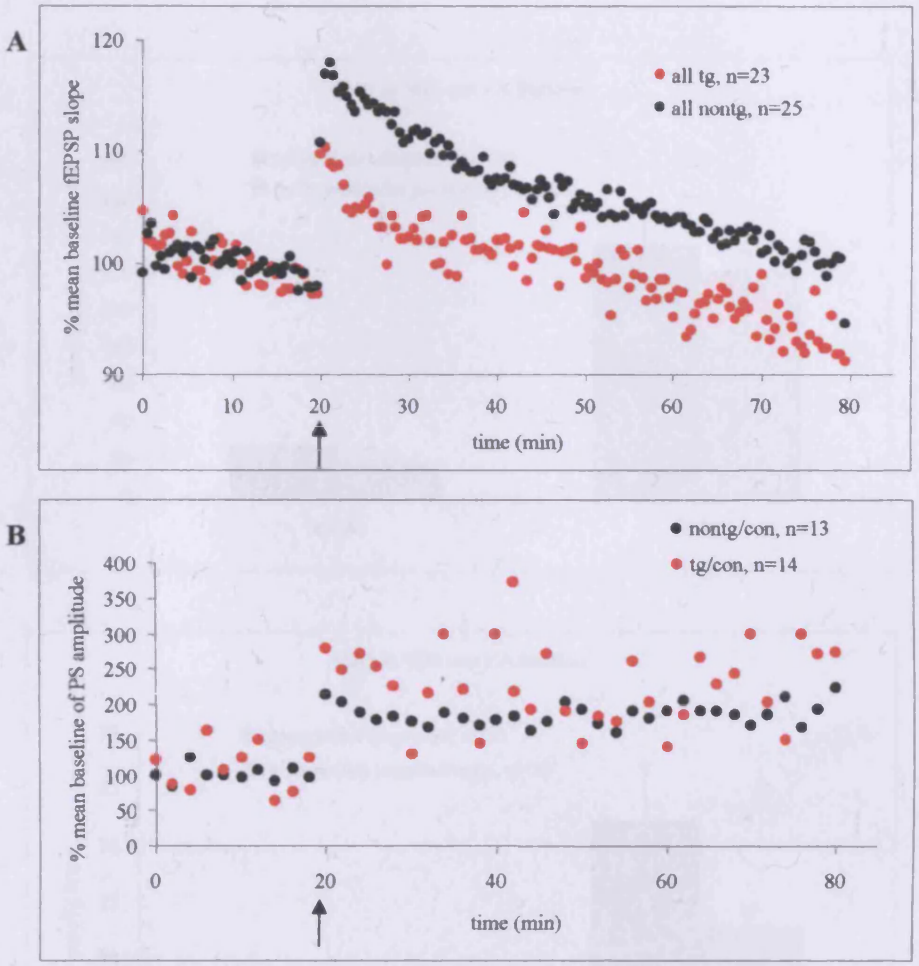


Figure 3.9. LTP in the dentate gyrus recorded for 60 minutes after the induction, of fEPSP slope (A) and PS amplitude (B) in the Tg2576 mice on either ibuprofen or control diet. The y-axis denotes the percentage of the baseline fEPSP slope (A) or PSA (B) after induction of LTP (indicated by arrow), and the x-axis denotes the time of recording. Burst stimulation induced an immediate increase in both EPSP slope and PSA for all groups of mice. Graph A represents averaged data for all the mice regardless of diet and type of experiment (prevention or treatment group) for the measurement of fEPSP slope. Although the LTP seems to be greater in non-transgenic as compared to transgenic, statistical analysis was not performed due to steady decline of recordings. Graph B shows averaged data for PS amplitude measurements just for animals on control diet (data for mice on ibuprofen diet were similar and excluded to simplify the graph). Data are means of every 2 minutes. Although the baseline seems to be more stable, but PS amplitude measurements were very unstable for transgenic mice.

β-amyloid levels in transgenic Tg2576 mice treated longitudinally for 6 months (ibuprofen) or 3 months (control) with ibuprofen

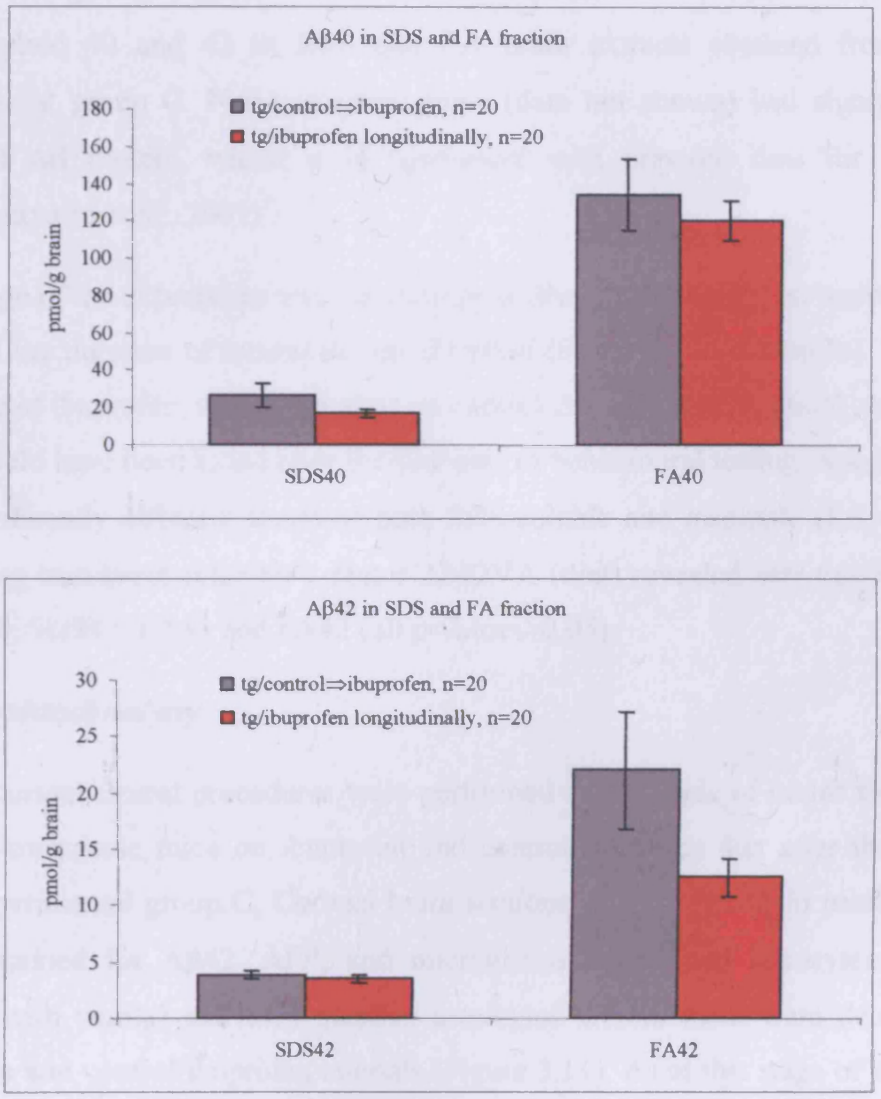


Figure 3.10. The levels of β-amyloid 40 and 42 in transgenic Tg2576 mice treated with either ibuprofen longitudinally or control ⇒ ibuprofen chows were not affected by diet (one-way ANOVA with diet as a factor). Transgenic mice from experiment C were used for β-amyloid 40 and 42 measurements and thus there were no transgenic mice on control diet and the influence of ibuprofen on Tg2576 mice could not have been assessed. Two-step extraction with sodium dodecyl sulfate (SDS) and formic acid (FA) was performed. The SDS fraction represents soluble Aβ, while FA represents insoluble Aβ, possibly from plaques (Kawarabayashi et al., 2001).

β-amyloid brain levels

Ibuprofen treatment had no significant effect on Aβ in the brains of the APP overexpressing mice. Figure 3.10 shows results obtained from Sandwich ELISA analysis of β-amyloid 40 and 42 in SDS and FA brain extracts obtained from mice from experimental group C. Non-transgenic mice (data not shown) had significantly lower levels of Aβ protein, which is in agreement with previous data for Tg2576 mice (Kawabarayashi et al., 2001).

The design of the experiment was not ideal as all the mice at this point were on ibuprofen, although the duration of treatment was different (6 months or 3 months). To assess the influence of ibuprofen, transgenic mice on control diet would be required. Also a group of mice should have been killed after the first part of behavioural testing. None of the groups had significantly different levels of both SDS-soluble and insoluble (FA) Aβ. Analysis comparing transgenic mice by 1-factor ANOVA (diet) revealed non-significant changes in SDS40, SDS42, FA40 and FA42 (all p-values>0.05).

Immunohistochemistry

Immunohistochemical procedures were performed on a sample of brains from transgenic and non-transgenic mice on ibuprofen and control/ibuprofen diet after the final testing from experimental group C. Coronal brain sections of brain halves in paraffin wax were double stained for Aβ42, APP, and microglia and activated astrocytes. Comparable plaques with similar activated swollen astrocytes around them were detected in both ibuprofen and control/ibuprofen animals (Figure 3.11). As at this stage of experiment all the animals were on ibuprofen chow and there were no visible difference in the plaque burden and astrocytosis, detailed statistical analysis was not performed. This result agrees with ELISA data that revealed no significant differences in any fraction of Aβ.

Ibuprofen levels in the plasma and brain tissue

Ibuprofen levels were measured in the brain tissue and the plasma (blood) from 22 transgenic and non-transgenic mice from Experimental group C. At this stage of the experiment all the mice were on ibuprofen diet so no control was available. No significant differences could be observed in the ibuprofen level in either brain or blood of transgenic and non-transgenic animals (ANOVA with genotype as a factor, all p-values>0.05). The

Double Immunostaining for β-amyloid and astrocytes in Tg2576 mice brain sections on Ibuprofen/Control diet.

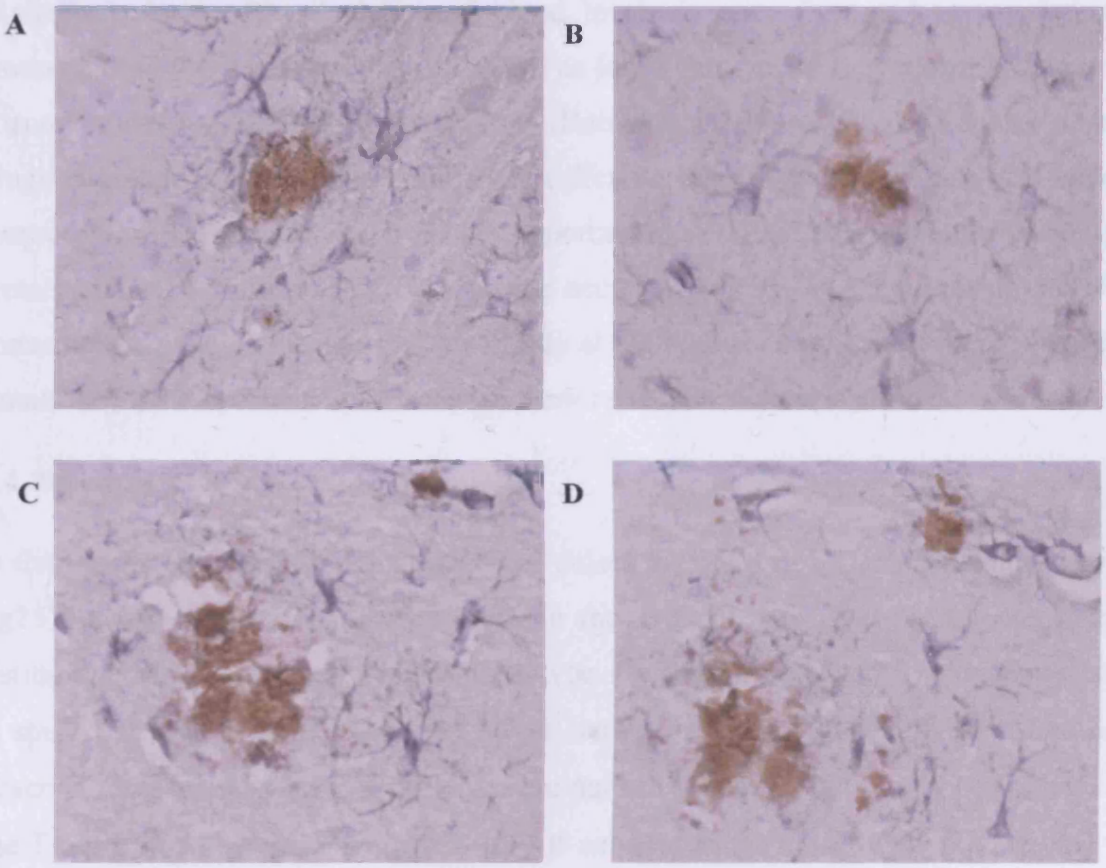


Figure 3.11. Representative examples of a 12-month-old Tg2576 mouse brain section through the cerebral cortex (A,B) and hippocampus (C, D). Sections were subjected to DAB immunohistochemistry for β-amyloid (brown colour) counterstained with Vector SB immunohistochemistry for GFAP (grey-blue colour) to label activated astrocytes surrounding plaques. This demonstrates a prominent β-amyloid-plaque-associated astroglial response. Sections A and C come from mice from Ibuprofen Longitudinally group while sections B and D come from mice from Control⇒Ibuprofen diet. There was no visible difference in the number of plaques and number of astrocytes around plaques.

average plasma concentration for all the mice was 13000±7000 nM and the average brain concentration was 600±400 nM. These numbers are similar to plasma and brain levels measured in Tg2576 mice in other studies (Ericksen et al., 2003). In humans, a single oral dose of 400 mg of ibuprofen induces a rise in plasma concentrations to 20-40 μg/ml (Cmax) within 1-2 hours; concentrations fall back to about 5μg/ml after 6 hours (Rainsford, 1999). Re-calculation of blood levels in mice indicated concentration of around 2.6 μg/ml, which is in similar range as for humans. Also the ratio of brain/plasma concentrations, 0.05, is similar to humans (Rainsford, 1999). Although kinetics of most drugs in human and mouse is completely different, this result confirms the presence of ibuprofen in the mouse brain. It is also important to note that plasma and brain samples were taken in the afternoon (when testing occurred) and dosing, through normal food consumption, would have taken place mostly at night, thus complicating any estimates of Cmax. Thus, the optimum dose for mice cannot easily be determined.

3.4. Discussion

In this study I have reported that ibuprofen delays the onset of behavioural impairment in Tg2576 mice on a spatial learning task in the T-maze, when it is administered early during the evolution of their disease phenotype. By contrast, if ibuprofen supplementation is started at a later point, when Aβ levels start to build up, no significant effect on is observed. Straightforward associations were not fully established, as only performance in the T-maze was improved, while levels of β-amyloid in the brains were not affected and electrophysiological studies were not conclusive.

What effects did ibuprofen administration have on behavioural impairment in FCA in Tg2576 mice?

The results clearly indicate that chronic administration of ibuprofen to young (2-4 month) Tg2576 mice reduced transgene induced impairment in learning and memory when tested at 8 months of age. These transgenic mice begin to show memory impairment at the age of 8 months as measured with the Forced Choice Alternation task in the T-maze (Chapman et al., 1999). When testing was performed later (after 10 months of age) the protective effect of ibuprofen was abolished. Because no other measurements indicated any mechanism of action, it is unclear how ibuprofen produced any effect on the onset of behavioural deficits.

Why did ibuprofen not reverse memory impairments in Tg2576 mice?

As twice confirmed with naïve (experiment A) and pre-tested (experiment C) animals, ibuprofen at a approx. dose of 62.5mg/kg/day did not succeed in reversing memory deficits as tested in the FCA task in the T-maze. A few possible explanations for this situation are as follows:

- Ibuprofen is not capable of reversing learning and memory impairment in Tg2576 mice given after the onset of pathology. The accumulation of Aβ is not reversible, so ibuprofen is only effective in younger mice when it can prevent the creation of harmful species of Aβ (e.g. oligomers), but not remove them once formed. The same argument would hold if the initial effects are due to anti-inflammatory actions of ibuprofen that are insufficient to rescue behaviour in more advanced stages of pathology.

- The measured average plasma and brain concentrations were similar, although a bit on the lower side as compared to other studies (Ericksen et al., 2003). It is possible that this small difference will account for this effect and if the dose were a bit higher, ibuprofen would have reversed the deficit.

- The FCA task in the T-maze is too difficult for Tg2576 mice. Thus as a prevention the effect is just minor, and as a treatment the effect disappears. Confirming this finding with another test, such as the water maze or an object recognition task, would help to answer this question.

- Results from a study using a chronic model of neuroinflammation (Hauss-Wegrzyniak, 1999) showed that Flurbiprofen attenuated the neuroinflammatory reaction and reduced the inflammation-induced memory deficit as tested in spatial learning in Morris water maze. It is interesting that Flurbiprofen improved the performance of only young, but not old rats. This result supports the data from this project that NSAID therapies designed to influence the onset of AD should be initiated before age-associated inflammatory processes within the brain have a chance to develop pathology.

Why did ibuprofen have no effect on β-amyloid levels and plaque deposition in Tg2576 mice?

As the main cause of learning impairment in Tg2576 is thought to be an over-production of human mutated APP and its further metabolic changes (Ashe, 2001), brain concentrations of SDS-soluble and insoluble (FA fraction, mainly contained in plaques) Aβ and β-amyloid plaques were measured in experimental animals.

During these experiments I had no access to the brains after testing mice at the age of 8-9 months, as Aβ measurements at this stage were performed in Prof Steve Younkin's and Prof Karen Hsiao-Ashe' laboratories. Unpublished data from their work did not show any significant differences in any measured fractions of Aβ (Aβ40 and 42 in SDS and FA fractions). This result suggests that either the preventive effect of ibuprofen on the learning and memory deficit is not due to reduction of APP-induced pathology or that the APP metabolic product responsible for memory impairment was not measured. This could support the hypothesis that some form of Aβ (proto-fibrillar or oligomeric), that is transient and difficult to measure, might be associated with the onset of behavioural deficits in mice and humans. It is unlikely that deposited β-amyloid is playing a major role in the onset of the observed memory deficits, since plaques are infrequent at eight months of age (Hsiao et al., 1996).

This finding is different from that reported by Lim et al. (2000). These authors reported a decrease in total concentrations of both SDS-insoluble and soluble Aβ in the cortex and the hippocampus in old Tg2576 mice after 4 month ibuprofen treatment. Two reasons can account for this difference. The measurements from my mice were performed using the entire cerebral hemisphere rather than sub regions of the brain (hippocampus and cortex) and the mice used by this group were older at the time of analysis (14 months of age).

The Aβ ELISA and immunostaining results from Experiment C were uninformative, as all the mice were on ibuprofen and control mice brains were not available. The only confirmation comes that they still had plaques.

Why were the electrophysiological data not conclusive?

The memory deficits in aged Tg2576 mice might reflect a decline in synaptic function and/or plasticity. To address this hypothesis, I recorded field potentials response *in vivo* from the dentate gyrus. The *in vivo* protocol was chosen because of the possibility that extracellular soluble Aβ is important to behavioural and/or synaptic pathology, in which case *in vitro* preparations might underestimate the extent of the deficit. Unfortunately, the experiments were flawed in that I was not able to maintain stable baseline responses, making quantitative analysis of LTP impossible. The only significant result was the increase in PS amplitude as reported previously (Chapman et al., 1999). The population spike, measured at the same time as the fEPSP amplitude, is an indication of the excitability of the population of neurons that are synoptically activated by afferent stimulation. Larger population spikes indicate a greater percentage of the neuronal population reaching action potential threshold, and suggest enhanced excitability that may be related to decrease in resting membrane potential, decreased synaptic inhibition, and changes in the sensitivity of post-synaptic potassium channels, or any combination of these.

The physiological abnormalities detected in the dentate gyrus *in vivo* in Tg2576 mice baseline population spike are also consistent with the hypothesis that behavioural deficits might be caused by relatively subtle physiological abnormalities. This suggests that functional deficits, even in the absence of cell loss or plaque deposition, might contribute significantly to the cognitive deficits of AD.

3.5. Conclusions and future questions

This study supports the clinical and epidemiological data providing evidence that long-term NSAID therapy has a clear effect on the incidence of AD (reviewed in chapter 1.5). It also, unfortunately, mirrors these data in failing to provide a suitable explanation for these effects.

Tg2576 mice show impairment in the FCA task in the T-maze and ibuprofen only postponed the development of the deficit. Re-testing them in the same test after 10 weeks abolished this positive effect. Ibuprofen, even as prevention, did not alter any Aβ fractions (data from collaborative laboratories). This is an important reminder that we do

not fully understand the relationship between observable pathology and memory impairment in mouse and human.

To provide the important and necessary data concerning the role of ibuprofen and other NSAIDs in AD development, future studies will need to focus on establishing a more in-depth understanding of all the mechanism involved in the action of this group of drugs. The simplest explanation for the action of NSAIDS is their ability to inhibit both COX-1 and –2 and therefore, interleukin and prostaglandin formation. This reduces inflammation but also has other features. For example, prostaglandin H2 was shown to markedly accelerate the formation of dimers and higher oligomers of amyloid Aβ42 (Boutaud et al., 2002). Another recognised target of NSAIDs action is PPARγ (Landreth et al., 2001). NSAIDs have also been shown to target directly γ-secretase (Weggen et al., 2001; Eriksen et al., 2003).

So although chronic use of NSAIDs is widely known to reduce the risk of AD, we still know little about the mechanisms involved. More research needs to be undertaken to clarify the mechanism underlying this action but also more questions need to be answered about the direct causes of dementia.

Chapter 4

Can an n-3 fatty acid enriched diet prevent AD-like impairment in transgenic Tg2576 mice?

4.1. Introduction

Overview

Docosahexaenoic acid (DHA; 22:6-3), a major n-3 fatty acid of the brain, has been implicated in restoration and enhancement of memory-related functions. Alzheimer's disease impairs memory and transgenic mice, modelling this disease, show reduced learning ability. In this project we investigated the effect of dietary DHA on learning ability and pathology in Tg2576 Alzheimer's disease model mice.

PUFA and learning and memory in animal models

The fatty acid composition of brain lipids and their biological role has been extensively studied, as reviewed in chapter 1, and numerous animal studies have addressed the relationship between the diet-induced changes in PUFA content and learning performance in behavioural tests.

In 1987 Yamamoto and co-workers performed one of the first studies with spontaneously hypertensive rats, which were fed diets enriched with either safflower oil (rich in n-6) or with perilla oil (rich in n-3). Diet modifications significantly affected the ratio of n-3/n-6 of the rats' brains and the rats on perilla oil were superior in learning ability in a brightness discrimination task.

Enslen (1991) and collaborators studied the effect of dietary restriction of n-3 using Sprague-Dawley rats. They reported lower exploratory activity in a novel environment in rats fed with safflower (n-6) oil comparing to those fed normal or soybean (n-3) diets. Furthermore, inferior performance in the brightness discrimination task of rats bred on n-6 enriched diet was reported to be restored by supplementing with ALA, an n-3 PUFA precursor (see Figure 1.6; Okaniwa et al., 1996). This suggested that learning impairment caused by n-3 deficiency could be a reversible process.

In 1997 Yoshida and co-workers compared two generations of rats fed safflower oil (enriched in n-6 fatty acids) with rats fed perilla oil (rich in n-3 fatty acids). They confirmed that the rats fed safflower oil showed inferior learning ability (as tested in the brightness discrimination test), but also showed a 30% decrease in synaptic vesicle density in the hippocampal CA1 region of tested rats. This effect was only observed following behavioural manipulations and therefore may be linked to the formation of memory.

Gamoh and co-workers investigated the effect of 10-week supplementation of DHA to young (Gamoh et al., 1999) and old (Gamoh et al., 2001) rats, which were kept on an n-3 PUFA deficient diet for three generations. Rats were tested for working and reference learning performance in an eight-arm radial maze. In young rats, DHA significantly reduced the number of reference memory errors, without affecting the number of working memory errors. Further analysis revealed a negative correlation between reference memory errors and a DHA/AA ratio in rat brains. In old rats DHA affected both reference and working memory errors.

Although most of the studies support the hypothesis that long chain PUFAs have a positive influence on learning and memory, Wainwright et al. (1994,1999) did not find any relationship between brain fatty acid composition and performance in a working-memory task in the Morris water maze for either rats or for mice.

In the mice studies, one of the first investigations was performed on specifically inbred senescence-accelerated mice (SAMP8). Umezawa (1995) and co-workers supplemented mice diets with either safflower oil or perilla oil. The proportions of n-3/n-6 in the brain reflected the n-3/n-6 balance of the diets and the group given perilla oil (n-3) showed much greater improvement in learning in the active avoidance task, higher ratio of correct responses in light and dark discrimination task and reduced exploratory behaviour in open field.

In 1998 Suzuki and collaborators studied the effect of 12-month feeding of a fish oil diet (rich in DHA) on maze-learning ability, fatty acid composition and synaptic membrane fluidity in mice. The results showed a positive influence of DHA on learning, on the ratio of DHA/AA in the brain and on the fluidity of the membranes.

More recently, the same authors (Lim & Suzuki, 2000a, 2000b, 2001, 2002a, 2002b) in multiple studies demonstrated that the intake of DHA ethyl ester improved the learning ability as measured by the maze-learning test in old mice rather than in young mice. Additionally old mice, in general, had a poorer learning ability than young mice. They have also shown that old mice had a lower DHA level in brain phosphatidylcholine than young mice and suggested that these lower levels of DHA in brain phosphatidylcholine in old mice were associated with an inferior learning ability and perhaps influenced synaptic membrane fluidity.

All these studies performed in rodents suggest that DHA is an important component for maintaining and improving learning and memory functions in aged animals. However, the mechanism by which diet-induced changes can influence learning performance in training tests has not been established yet.

PUFA and Alzheimer models

The effect of n-3 PUFA has also been investigated in Alzheimer rodent models. Hashimoto (2002) and collaborators studied the effect of dietary DHA on avoidance learning ability in β-amyloid peptide infusion Alzheimer's disease model rat. Pre-administration of DHA had a beneficial effect on the decline in avoidance learning ability and this was associated with an increase in the cortico-hippocampal DHA/AA molar ratio and a decrease in neuronal apoptotic products. Additionally, DHA pre-supplementation

increased cortico-hippocampal glutathione levels and glutathione reductase activity and suppressed the increase in lipid peroxide and reactive oxygen species levels in the rat brain.

PUFA and amyloid pathology

Although the influence of DHA and other PUFA on cognitive functions has been widely investigated, any direct interaction between them and Aβ is still unknown. The product of the oxygenation of AA by cyclooxygenase (COX) activity, prostaglandin H_2 (PGH_2) markedly accelerates the formation of dimers and higher oligomers of Aβ42 in studies *in vitro* (Boutand et al., 2002), providing a basis for linking dietary PUFA and amyloid cascade hypothesis.

Based on these different reports, my study aimed to determine whether there is a relationship between DHA level and learning and memory impairment in Tg2576 mouse Alzheimer model. The study examined the effect of dietary DHA on learning and memory impairment measured in Forced Choice Alternation task, levels of 40 and 42 β-amyloid, markers of amyloid pathology and on brain fatty acid composition.

4.2. Materials and Methods

Animals and diets

Four-month-old male Tg2576 mice and their non-transgenic littermates (25-40 g body weight) were studied. The mice were randomly divided into four groups: the transgenic DHA group ($n = 22$), the transgenic control group ($n = 19$), the non-transgenic DHA group ($n = 16$) and the non-transgenic control group ($n = 15$). Mice were group housed with littermates (with transgenic and non-transgenic housed together), and all testing was conducted during the light phase. Transgenic mice were compared to littermate controls to ensure that age and background strains were equivalent.

The DHA group was put on a special chow prepared by Special Diet Services (SDS, UK) by incorporating 5% of Martek Oil (derived from algae and obtained from Martek, US) into standard rodent chow. Martek oil is expected to contain up to 40% of pure DHA, as provided in the company leaflet. The DHA-enriched special diet produced by SDS contained an average of 4.5 mg of DHA in a gram of a chow, as measured by gas

chromatography in collaboration with Prof J.Harwood. Assuming that a healthy male mouse eats around 5g of chow a day, the DHA dose for mice was estimated to be 22.5mg per day. That accounts for a dose of around 750mg/kg per day (average mouse weighs 30g). Even if the estimation is too high, the range is similar to other projects, where rats were fed orally with an ethyl-ester derivative of DHA at a dose of 300mg/kg/day (Gamoh et al., 1999, 2001).

For the control chow, the added volume of Martek oil was substituted for the same volume of a mixture of palm oil, lard, olive oil and coconut oil in the proportions (3:3:3:1). The oils were chosen in order to substitute n-3 PUFA with more saturated or monounsaturated fats but not to increase the total n-6 PUFA content. DHA content and lipid analysis in a special chow was assessed on arrival and after two months upon arrival. There was no significant reduction in DHA level during the feeding and storage time. The chows were stored at 4 °C and fresh supplies were given to the mice every day. The fatty acid compositions of the diets at two different time points are given in Figure 4.1.

Experimental Procedures

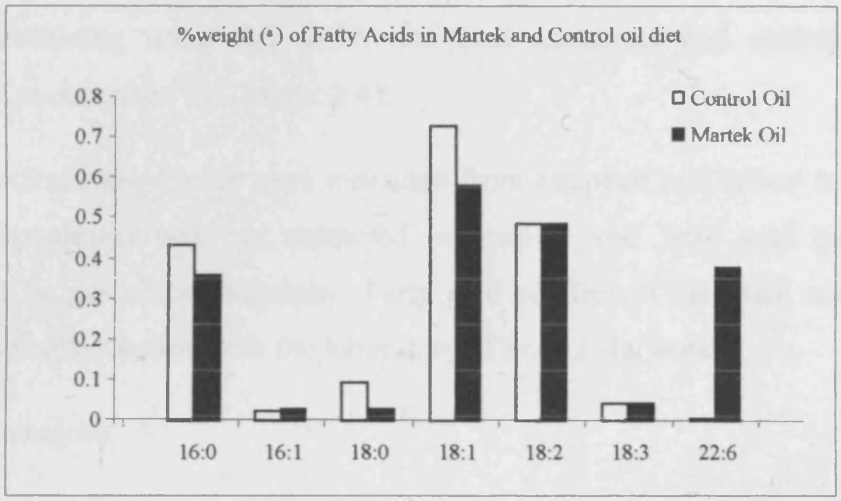
The full experimental procedure consisted of a series of phases, which were conducted on all animals. These included:

- Feeding with special chows (DHA and control) and drinking water *ad libitum* from the age of 4 till 8-8.5 months. Mice were weighed every 2 weeks.

- Forced Choice Alternation task as described in Chapter 2.1.1. The experimental design is pictured in Figure 4.2. During testing, mice were put on water deprivation regime (20 hours of no water, 2 hours experiment with 25% sucrose reward and 2 hours of free water access after experiment). Mice were kept on the special chow diets till the end of testing (9-9.5 months).

- Brain dissections. Mice were killed after the behavioural experiments. The brains were dissected and the hemispheres separated along the midline. Half of the brain was frozen on dry ice for Aβ ELISA analysis. The other hemisphere was either immersed in 4% paraformaldehyde for immunostaining (20 brains) or frozen for fatty acid measurements.

Fatty Acid Composition of Diets



FATTY ACIDS	DHA diet, weight% mean ±sem	CONTROL diet,weight% mean ±sem
16:0	0.38 ± 0.04	0.44 ± 0.03
16:1 n-7	0.03 ± 0.01	0.03 ± 0.01
18:0 *(S)	0.03 ± 0.01	0.09 ± 0.01
18:2 n-6	0.53 ± 0.06	0.49 ± 0.04
18:3n-3 (ALA)	0.05 ± 0.01	0.04 ± 0.01
22:6n-3 (DHA) *(S)	0.43 ± 0.05	0.01 ± 0.01

Figure 4.1 Fatty acid composition of diets (Martek oil and control oil) at the time of arrival to the laboratory. Major fatty acids above 2% of total content are shown. Mean± sem are calculated from triplicates. Diets were analysed at the time of arrival and after 2 months of storage. 2-factor ANOVA analysis with diet and time as factors revealed significant difference in 22:6n-3 (DHA) and 18:0 (Stearic Acid) levels between Martek and Control diet but no effect of storage time and no interaction between storage time and a type of diet. The control diet also contains small amounts of medium-chain fatty acids (10-14C) but, individually, there were less than 2% and have not been included in the table.

(a) % weight was calculated as a percent of certain fatty acids content (mg) in 1 g of dry diet.

*(S) – significant difference in the fatty acid content.

- ELISA measurement using commercially available kit (Biosource) in SDS and FA brain extracts to assess the total amount of Aβ 40 and 42 in brain tissue (details in Chapter 2.3);

- Immunostaining using Aβ, APP, activated astrocytes and microglia recognising antibodies (as described in Chapter 2.4);

- Lipid extractions. Lipids were extracted from chopped and mixed whole half of the brain (hippocampus was not extracted separately) and fatty acid composition was determined by gas chromatography. Fatty acid profiles in the brain and in diets were measured in collaboration with the laboratory of Prof. J.Harwood.

Statistical analysis

All data are presented as the means ± SEM. The statistical significance of differences in the learning and perseveration measured over the training time between the groups was determined by repeated-measures 3-factor ANOVA, with genotype and diet as between-subject factors and training day as a within-subject factor. The statistical significance of differences in the value of Perseveration Count, Right/Left Preference, fatty acids and the Aβ levels between the groups was determined by the 2-factor ANOVA, with genotype and diets as between-subject factors. Tukey's pair wise comparisons post hoc tests were used to determine differences between groups. The % mice reaching learning criterion was compared using pair wise Fisher's test. Relationship between various factors was counted using Regression analysis and Pearson Correlation test. Differences were considered to be significant at $P < 0.05$.

4. 3. Results

Body weight, survival assessment and chow quality

There were no significant differences in body weight gain over the treatment period between the transgenic and non-transgenic groups whether on control or DHA-enriched diets. Final body weight for transgenics on DHA diet was 29.9 ± 0.5g, transgenics on control diet was 30.7 ± 0.7g, non-transgenics on DHA was 32.1 ± 0.8g and non-transgenics on control diet was 32.4 ± 0.9g.

Experimental Design

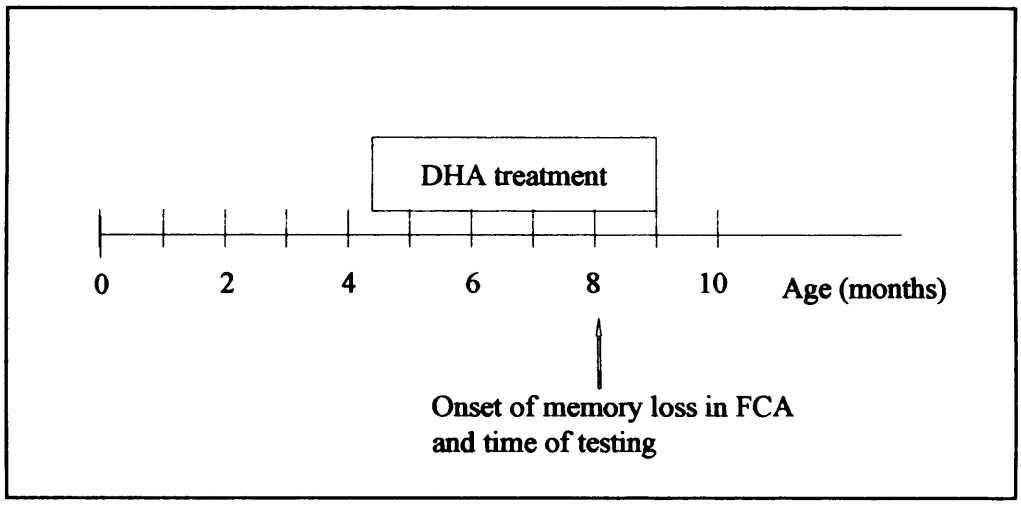


Figure 4.2 Experimental design. The box above the timeline represents the time when mice were kept on DHA/control chow. At the age of 4-4.5 months they were switched from a standard chow and kept on special diets until the end of experiments. The arrow underneath the timeline indicates the initiation of memory loss and the start of behavioral testing in the T-maze (FCA – Forced Choice Alternation task). Numbers of mice tested are: transgenic on DHA diet (tg/DHA) -16, transgenic on control chow (tg/control) -12, non-transgenic on DHA diet (nontg/DHA) -14 and non-transgenic on control chow (nontg/control) -13.

Analysis was performed using repeated measures 3-factor ANOVA with week as a repeated measure and diet and genotype as between-subject factors and revealed no significant differences. The effects of week ($F=0.98$, $p=0.961$), diet ($F=0.82$, $p=0.486$) or genotype ($F=0.23$, $p=0.87$) were not significant. Also none of the interactions were significant.

During the time of chronic administration of either DHA or control diets (from 4 until 8-8.5 months of age) four mice died in the transgenic-DHA group, three in the transgenic-control group and one from non-transgenic-control group. These numbers are similar for groups on both types of diet, but show a trend (not significant, Fisher exact test, $p=0.123$) to a higher mortality rate for transgenic Tg2576 mice compared to non-transgenic littermates. This finding is consistent with other groups breeding Tg2576 mice (Karen Hsiao-Ashe's or Steve Younkin's laboratories).

During the experiment, quality analysis on the specially prepared chows was performed. There was no significant difference between the constitution of the chow at the time of arrival, nor after two months of storage. Martek Oil had consistently significantly more of the n-3 polyunsaturated fatty acid (22:6) DHA and less of the saturated Oleic acid (18:0) compared to control diet. Detailed results of constitution of both Martek Oil and Control Oil enriched animal chows are presented in Figure 4.1.

Forced Choice Alternation task in the T-maze

During testing, 2 transgenic mice on DHA diet, 3 transgenic mice on control, 2 non-transgenic mice on DHA and 1 non-transgenic mouse on control were excluded because they failed to move. The criteria for exclusion are described in Chapter 2.1.1.

Figure 4.3 shows the effect of chronic administration of dietary DHA on episodic-like memory related learning ability in Tg2576 mice and their non-transgenic littermates. Forced Choice Alternation (FCA) task assesses spatial episodic like memory using the T-maze. Training lasts for 10 days and each mouse receives 6 trials a day. The percent correct value for each mouse was calculated daily. Repeated measures 3-factor ANOVA revealed highly significant effect of genotype, indicating that all the transgenic mice were impaired in FCA task. The time of training had a significant effect, confirming overall improvement during training of all the groups. Statistical analysis revealed no main effect of diet, indicating no influence of dietary DHA on performance in the T-maze. However

when the interaction between genotype and diet was considered, a significant result was observed indicating a protecting effect of DHA-enriched diet but only for transgenic mice. No other interactions were significant. Post-hoc Tukey's pair-wise comparisons confirmed that transgenic mice on DHA-enriched diet were significantly better than transgenic mice on the control diet, indicating a protective effect of DHA against memory impairment measured in the FCA task. It is noticeable on the Figure 4.3 that the rate of improvement of the transgenics on DHA was comparable with all the non-transgenics, but that they started from a lower level. If the experiment had been extended, it is possible they would have reached similar level.

The percent of mice reaching Learning Criterion (defined as 80% correct or better on at least 3 consecutive days during training) was not affected by DHA administration as shown on Figure 4.4. Transgenic mice overall in a significantly smaller percent reached Learning Criterion comparing to non-transgenic littermates. It indicates significant effect of only genotype and no effect of a diet (analysis performed using exact Fisher test). Thus, although transgenics fed DHA were clearly able to improve during training (Fig.4.3), most of them did not attain the 80% threshold.

Figure 4.5 shows the effect of chronic administration of DHA on response strategies (perseveration) in Tg2576 mice and their non-transgenic littermates. The analysis was performed to distinguish various response strategies while being tested in the T-maze. Graph A shows the perseveration count for four groups of animals as the mean number of consecutive free choices made in the same direction averaged for each group and over 2 days of training. Repeated-measures two-way ANOVA analysis was performed based on these scores and revealed a significant main effect of genotype and no effect of diet, time or any interaction. Similarly analysis performed on data from graph B revealed only a significant effect of genotype, indicating no effect of DHA on response strategies.

To evaluate if the conditions throughout the testing time were biased, the preference towards any of sides (Right or Left) was calculated with no significant effect of either right or left side (Figure 4.6).

Learning in Tg2576 and Non-Transgenic littermates on DHA/Control chow

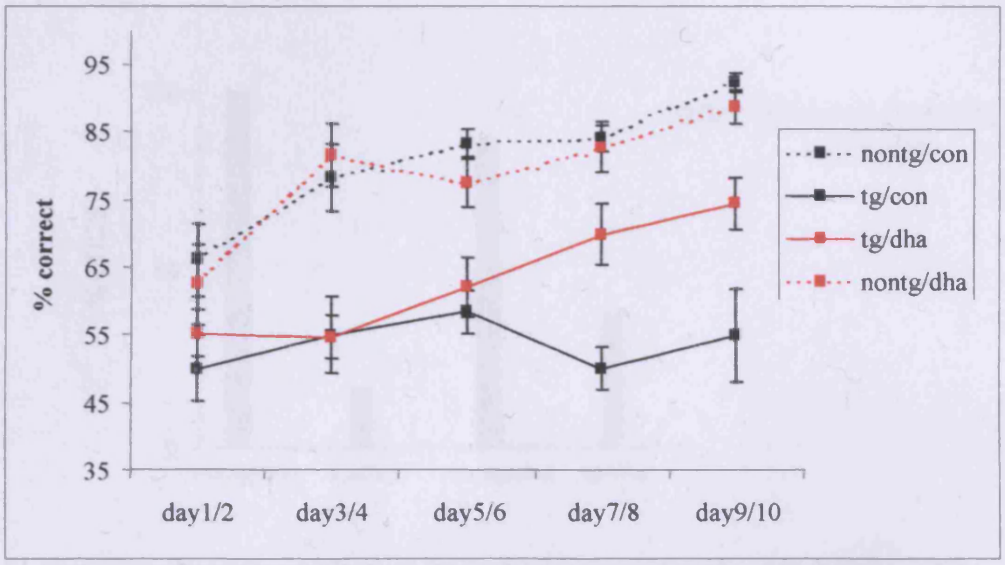
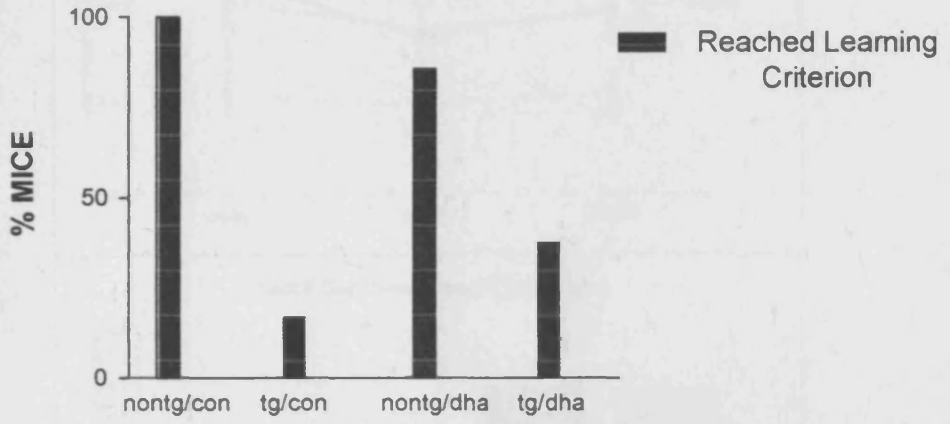


Figure 4.3 Chronic administration of dietary DHA in Tg2576 mice preserves spatial episodic –like memory as assessed using the T-maze. Data are mean ± s.e.m. of the percentage of correct responses averaged over two days for each mouse. Repeated Measures ANOVA, revealed highly significant effect of a genotype ($F=68.6$, $p<.001$), and a significant interaction of a diet with genotype ($F=5.3$, $p= 0.025$), but no significant effect of diet ($F=2.1$, $p=0.154$). The effect of time (days of training) was highly significant for all subjects ($F= 8.91$, $p<.0001$). Interactions of time and diet ($F=2.307$, $p=0.072$), time by genotype by diet ($F=1.820$, $p=0.140$) and time by genotype ($F=2.007$, $p=0.108$) were not significant. Post hoc analysis using Tukey`s pair-wise comparisons revealed that transgenic mice on both DHA and control diets perform significantly less well than either non-transgenic group (all $p<0.001$), but that transgenic mice on DHA were also significantly better compared to transgenic mice on control diet ($p=0.049$). Numbers of mice are tg/DHA=16, tg/control=12, nontg/DHA=14, nontg/control=13.

Reaching Learning Criterion



Paired Comparisons Between Groups	P value	Stat.significance.
TG/CON – TG/DHA	0.4010	NS
TG/CON – NONTG/DHA	0.0011	S
TG/CON – NONTG/CON	<0.0001	S
TG/DHA – NONTG/DHA	0.0106	S
TG/DHA – NONTG/CON	0.0004	S
NONTG/CON – NONTG/DHA	0.4815	NS

Figure 4.4. The percentage of mice in each group that reached learning criterion, defined as 80% correct or better on at least 3 consecutive training days. DHA has no significant effect on either Transgenic Tg2576 or Non-transgenic mice. There is a significant effect of genotype, meaning that significantly fewer mice reached learning criterion among transgenic than non-transgenic subjects. The paired comparisons between groups were performed using Fisher's exact test, two-sided. Numbers of mice are tg/DHA=16, tg/control=12, nontg/DHA=14, nontg/control=13.

Response Strategies - Perseveration Count

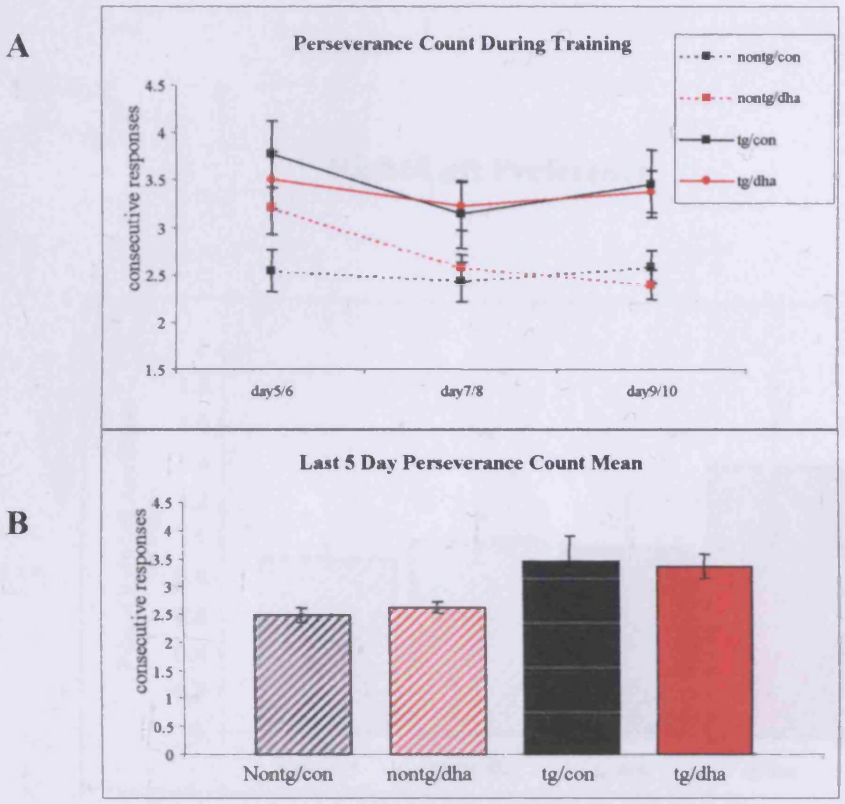


Figure 4.5 Transgenic Tg2576 mice tend to adopt a perseveration strategy significantly more often (as quantified by the Perseverance Count [PC]) than Non-transgenic littermates, whether they have been given DHA-rich diet or control diet. PC is measured as a maximum number of consecutive free choices made in the same direction for each animal per day. Data are mean ± s.e.m. of the PC averaged for two days during training (A) or averaged over the last five days (B) for each mouse. A repeated measures ANOVA performed on data shown in graph A revealed a significant effect of genotype ($F=15.0626$, $p=0.0003$) and no effect of diet ($F=0.0919$, $p=0.7631$), with no interaction of these factors ($F=0.5551$, $p=0.4597$). Time as a within-subjects factor was not significant on its own ($F=4.493$, $p=0.0162$), or in any interaction with diet and genotype. An ANOVA carried out on the data represented in (B) revealed a significant effect of genotype ($F=16.66$, $p < .0001$), and no significant effect of diet ($F=0.04$, $p=0.852$) and no interaction of these factors ($F=0.3$, $p=0.589$). Numbers of mice are tg/DHA=16, tg/control=12, nontg/DHA=14, nontg/control=13.

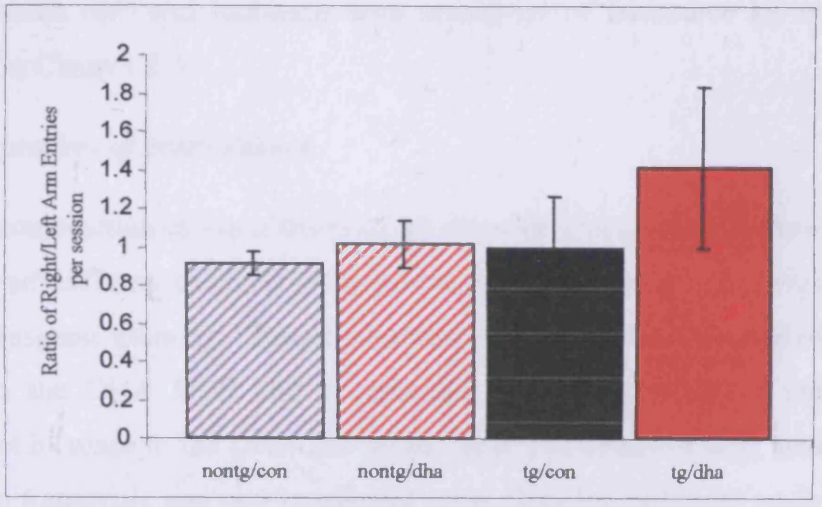


Figure 4.6.. The ratio of Right/Left arm entries was counted over all testing days and averaged for groups, data are means ± s.e.m. Although there was a slight tendency for transgenic mice on DHA diet to turn towards the right arm, there were not statistically significant differences among any of the groups (measured by ANOVA with genotype and diet as factors). Numbers of mice are tg/DHA=16, tg/control=12, nontg/DHA=14, nontg/control=13.

β-amyloid brain levels

Figure 4.7 shows results obtained from Sandwich ELISA analysis of Tg2576 mice of β-amyloid 40 and 42 in SDS and FA extracts. Non-transgenic mice (data in the table only) had significantly lower levels of Aβ-amyloid as compared to Tg2576 (previously reported by Kawabarayashi, 2001). DHA did not affect any fraction of β-amyloid in either transgenic or non-transgenic mice. The non-significant results from analysis of β-amyloid 42 in FA fraction are likely to be due both to the expected low levels (transgenic mice only 9.5 month old) and problems with sensitivity of Biosource kit on brain extracts (described in Chapter 2.3).

Fatty acid profiles of brain tissues

Fatty acid composition of brain tissue of the experimental animals is shown in Figure 4.8. No significant differences could be observed in the fatty acid composition of transgenic and non-transgenic animals. Chronic administration of Martek Oil resulted in a significant increase in the DHA level and a reduction in the AA level in the brain, with a concomitant increase in the DHA/AA molar ratio. The levels of fatty acids were affected similarly in transgenic and non-transgenic mice. Detailed statistical analysis is presented in the table in the Figure 4.8.

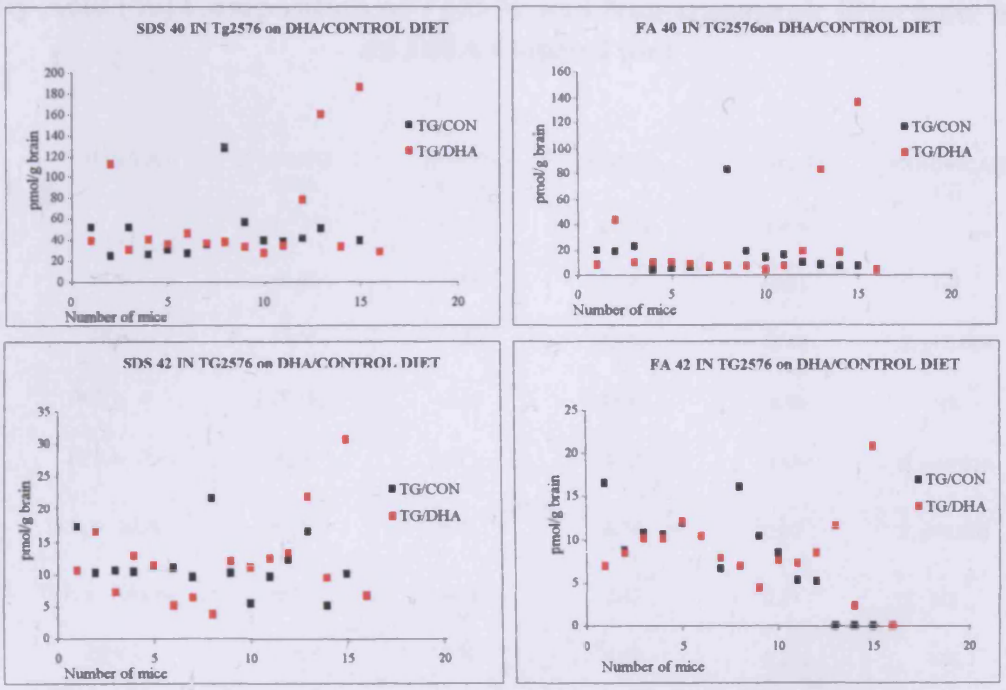
Immunohistochemistry

Coronal sections of half brains in paraffin wax were cut at 6 μm and double stained for Aβ42, APP, microglia and activated astrocytes. A small number of plaques were detected in both DHA and control-treated transgenic animals (Figure 4.9). The numbers of plaques were too low for meaningful statistical analysis. This outcome was expected as Tg2576 mice at the age of 9.5 months show very few diffuse plaques (Hsiao et al., 1996). Also the planned analysis of activated astrocytes and microglia was not useful for the same reason.

Relationship between learning ability, β-amyloid and fatty acids levels

The possible relationships between learning ability (expressed as an average of percent correct during the last 4 days of training), β-amyloid and fatty acid levels in the brain was examined.

β-amyloid levels in transgenic Tg2576 and non-transgenic mice treated with DHA/control diet



	SDS 40	SDS 42	FA 40	FA 42	Total Ab
GROUP	pmoles/g brain ± SEM	pmoles/g brain ± SEM	pmoles/ g brain ± SEM	pmoles/g brain ± SEM	pmoles/g brain
TG/DHA, n=16	59.89 ± 11.9	11.9 ± 1.7	23.65 ± 9.0	9.01 ± 1.1	104.45
TG/CON, n=15	44.84 ± 6.5	11.33 ± 1.0	16.05 ± 5.0	8.05 ± 1.3	80.27
NONTG/DHA, n=5	1.74 + 0.7	5.60 + 1.9	2.29 + 0.5	4.78 ± 1.4	14.42
NONTG/CON, n=4	1.14 ± 0.3	5.10 ± 0.97	1.46 ± 0.5	4.67 ± 2.1	12.38

Figure 4.7 β-amyloid levels in transgenic Tg2576 and non-transgenic mice are not affected by diet. Non transgenic mice had significantly lower levels of both SDS-soluble and insoluble (FA) β-amyloid measured by two-way ANOVA (genotype, diet) after excluding 3 outliers (more than 2 standard deviations). The values are respectively SDS40 ($F=41.22$, $p<0.001$), SDS42 ($F=13.32$, $p=0.001$), FA40 ($F=10.56$, $p=0.003$). For FA42 ($F=3.30$, $p=0.078$) the difference was not statistically significant, probably due to problems with ELISA accuracy (see methods section in Chapter 2.3). The test revealed no effect of diet in any of the fractions.

Fatty Acid (%) Composition of Tg2576 and Non-transgenic littermate brains on DHA/Control diet

FORMULA	NONTG DHA	NONTG CON	TG/DHA	TG/CON	SIGNIFICANCE
	n = 14	n = 9	n = 12	n = 8	
16:0	16.91	17.06	16.76	16.81	NS
18:0	17.16	17.88	16.66	18.44	S, p=0.009
18:1, n 9	17.31	15.97	15.41	15.99	NS
18:1, n 7	3.37	3.47	3.22	3.63	S, p=0.019
18:2, n 6(LA)	0.67	0.51	0.78	0.40	S, p=0.001
18:3, n 3(ALA)	0.12	0.13	0.43	0.13	NS
20:0	0.51	0.30	0.40	0.29	NS
20:1, n 9	2.34	2.18	1.92	1.74	NS
20:3, n 6	0.84[a]	0.53[b]	0.97[a]	0.48[b]	S, p<0.001
20:4, n 6(AA)	4.23[a]	7.54[b]	4.13[a]	7.38[b]	S, p<0.001
22:4, n 6	1.22[a]	2.01[b]	1.08[a]	2.36[b]	S, p<0.001
22:6, n 3(DHA)	15.49[a]	11.94[b]	15.13[a]	12.85[b]	S, p<0.001
DHA/AA MOLAR RATIO	4.0[a]	1.7[b]	4.0[a]	1.9[b]	S, p<0.001

Figure 4.8. Fatty acid composition (major components) of brain lipids in different dietary groups. Amount of each fatty acid was calculated as a % in total fatty acids, using gas chromatography. 2-factorial (diet, genotype) ANOVA revealed no main effect of genotype but a significant effect of diet for some of the fatty acids. No interaction between genotype and diet was detected. Detailed statistical results are presented in the table. Groups that are different ($p<0.05$) are marked with **a** or **b**.

Double Immunostaining for β-amyloid and astrocytes in Tg2576 mice brain sections on DHA/Control diet.

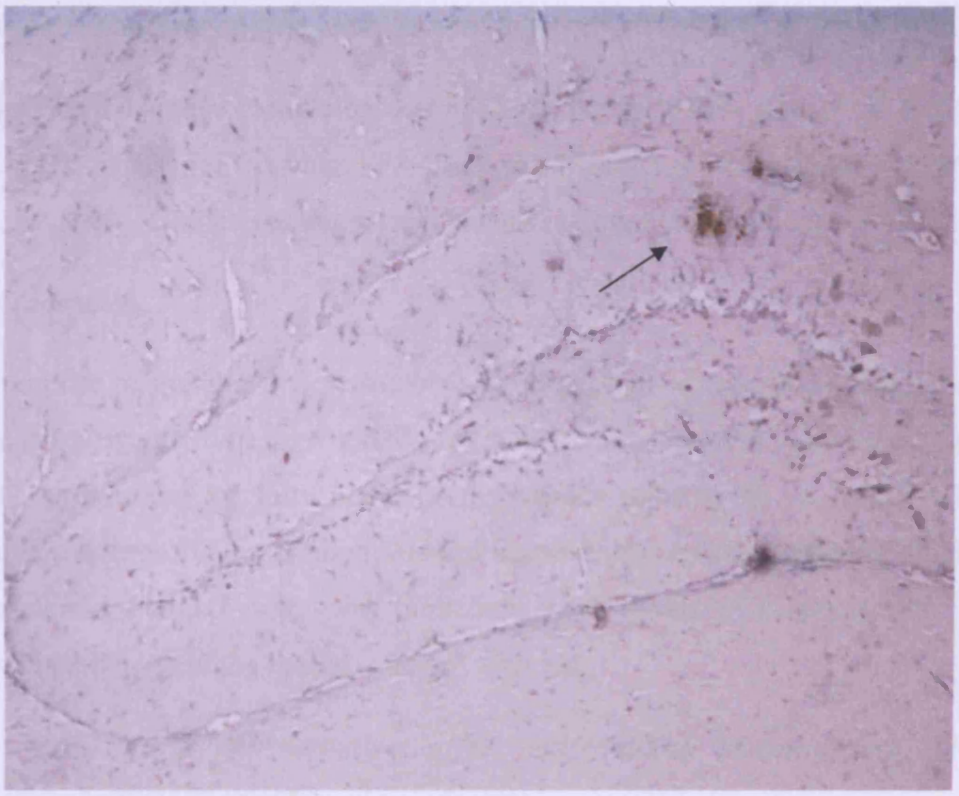


Figure 4.9 A representative example of a 9-month-old Tg2576 mouse brain section through the hippocampus. This section was taken from the mouse on DHA diet. Sections were subjected to immunohistochemistry for β-amyloid (brown colour) counterstained with immunohistochemistry for Glial fibrillary acidic protein (GFAP), (grey-blue colour) to label activated astrocytes. The method revealed a prominent β–amyloid-plaque-associated astroglial response (indicated by an arrow) in mice on both diets, and no differences were detected. The mean number of plaques and a number of astrocytes around plaques were counted, but data were not suitable for statistical analysis due to the small number of plaques (at the age of 9 months, Tg2576 mice have only single plaques, in a preliminary stage). In fact, it has been reported that Tg2576 mice start showing prominent plaque pathology at the age of 10 to 12 months (Hsiao et al. 1996).

Regression analysis revealed a significant positive relationship between the molar ratio of DHA/AA in the brain and learning ability ($r = 0.48$; $P < 0.05$) and a significant negative relationship between the value of AA (as % of total fatty acids) in the brain and learning ability ($r = 0.45$; $P < 0.05$). Graphs and statistical analysis are presented in the Figure 4.10. The % level of DHA in the brain did not correlate with the learning ability. Also, none of the fractions of β-amyloid showed any correlation with the learning ability.

A significant negative correlation was observed between the value of DHA (as % of total fatty acids) in the brain and brain FA fraction of β-amyloid 40 levels in Tg2576 mice ($r = 0.43$, $P < 0.05$). Graphs and statistical analysis is presented in the Figure 4.11.

4.4. Discussion

The purpose of the above experiments was to explore whether administration of dietary DHA to transgenic mice, over-expressing human mutated APP, would alter their learning ability, proportions of fatty acids in their brains and levels of β-amyloid and APP pathology in their brains. Straightforward associations were not fully established, as only performance in the T-maze was improved, while levels of β-amyloid and β-amyloid induced pathology in the brains were not affected.

How did DHA supplementation affect episodic-like learning and APP-induced pathology in Tg2576 mice?

The results clearly indicate that chronic administration of dietary DHA to Tg2576 mice influenced transgene induced impairment in Forced Choice Alternation task in the T-maze. As only interaction of genotype x diet was significant, it cannot be concluded that learning in FCA task is enhanced, but only that some aspect of performance is improved.

These transgenic mice have been shown to start showing memory impairment at the age of 8 months as measured with Forced Choice Alternation task in the T-maze (Chapman et al., 1999). As the main cause of learning impairment in Tg2576 is thought to be an over-production of human mutated APP and its further metabolic changes (Ashe, 2001), experimental animals were checked for changes in SDS-soluble and insoluble (FA fraction, mainly contained in plaques) β-amyloid and β-amyloid plaques.

Relationship between behavioural results and fatty acids levels

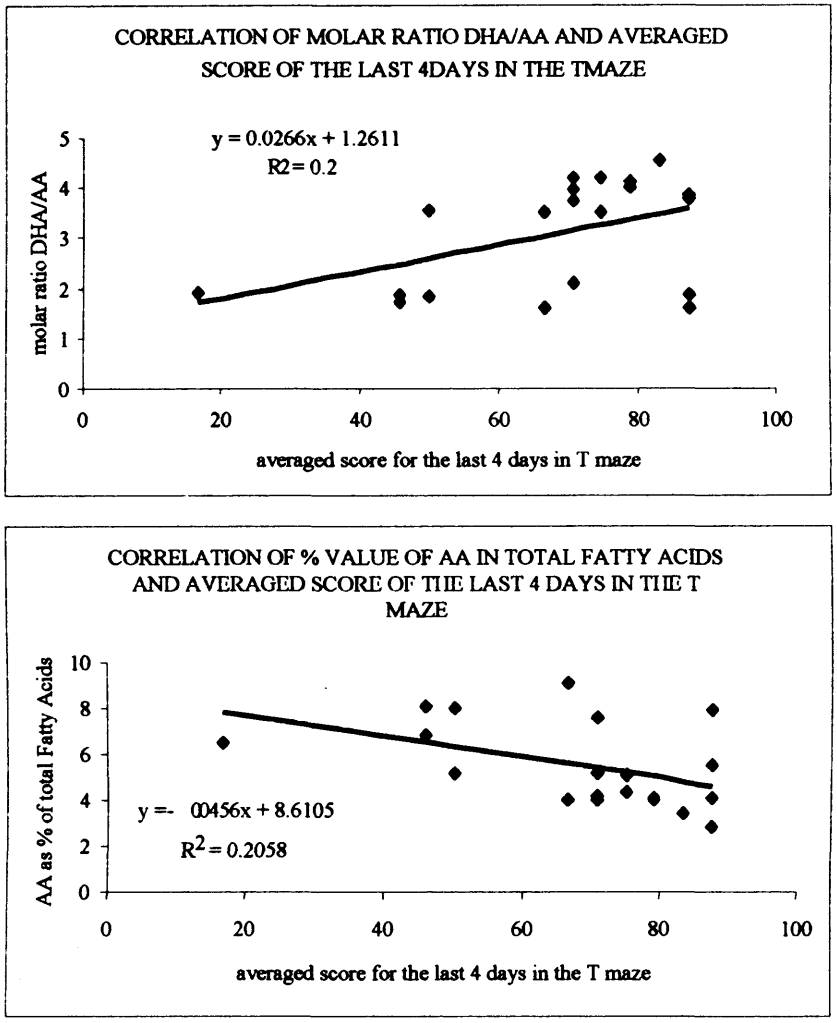


Figure 4.10. Regression analysis between the behavioural results and fatty acids levels revealed a significant positive correlation between averaged learning score for the last 4 days and the ratio of DHA/AA in mice brains (p=0.048). Averaged learning score for the last 4 days showed also a negative correlation with AA amount (as % of total fatty acids) (p=0.045). The correlation graphs were prepared using linear trend line function in Excel (Microsoft Office). Numbers of mice are tg/DHA=7, tg/control=7, nontg/DHA=5, nontg/control=4.

Relationship between β-amyloid measurements and fatty acids level in the brains of Tg2576 mice and non-transgenic littermates.

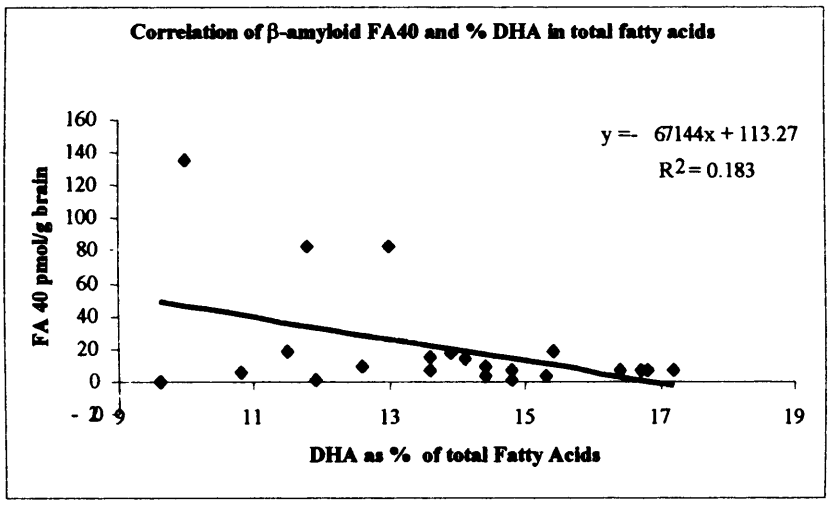


Figure 4.11. Regression analysis revealed a negative correlation between the level of β-amyloid 40 in FA extract and the % DHA in total fatty acids in individual mice brains (p=0.047). The correlation graphs were prepared using linear trend line function in Excel (Microsoft Office). Numbers of mice are tg/DHA=8, tg/control=6, nontg/DHA=5, nontg/control=3.

No changes were detected, suggesting either the preventive effect of DHA on learning and memory deficit is not due to reduction of APP-induced pathology or that the APP metabolic product responsible for memory impairment was not measured.

To support the latter hypothesis, most APP mice models that show increased levels of β-amyloid and impairment in learning and memory, do not show a correlation between learning ability and total β-amyloid levels. More detailed analysis of metabolic APP products (e.g. measuring β-amyloid oligomers like dimers or tetramers) in Tg2576 brains is required to answer this question.

The positive influence of DHA and other n-3 PUFAs on learning and memory in various rodent models, which has been referred to previously (Yamamoto et al., 1987; Yoshida et al., 1997; Lim & Suzuki 2000, 2001, 2002a, 2002b), has not been related to a reduction in APP pathology. Therefore, the preventive effect of DHA on learning and memory impairment in Tg2576 mice might be due to some other functions of DHA, like anti-oxidant properties, increasing membrane fluidity or preventing neuronal damage.

Tg2576 mice on DHA-enriched diets were significantly different from transgenic littermates on control diet, but also significantly different from non-transgenic mice. That means that DHA improved but did not completely reverse memory deficit. Its effect was only minor. This could explain why the effect of DHA was not significant in the numbers of mice that reached the learning criterion in 10 days of training. Similarly DHA had no effect on Tg2576 perseveration.

There were no measurable differences between non-transgenic mice on either DHA or control diets. If non-transgenic mice were affected by DHA-enriched diet in the same way as Tg2576, this could suggest that learning improvement is not due to influencing APP pathology. However, non-transgenic mice reached 85% or better of correct responses. Ceiling effect in non-transgenic mice could mask general enhancement of performance by DHA in all mice. Therefore, to see if DHA feeding could result in an improvement, the task should be made more difficult. One of the ways to do this would be to introduce delays between sample and choice runs of each trial. More difficult tasks might have also helped determine whether DHA influenced APP-pathology or improved memory in a different mechanism.

What effect did DHA administration have on fatty acid composition in mice brains?

In both transgenic and non-transgenic mice with pre-administered DHA, the increase in DHA content in the brain was accompanied by a significant increase in the DHA/AA molar ratio, which positively correlated with learning in FCA task. This finding agrees with the previous studies performed with infusion β-amyloid rat model where the cortico-hippocampal DHA/AA molar ratio correlated positively with acquisition of avoidance learning ability (Hashimoto et al., 2002) and was inversely related to memory errors in Y-maze tasks (Gamoh et al., 1999).

The DHA/AA ratio is recognised as an indicator of the antioxidative action of DHA (Gamoh et al., 1999). It would therefore appear that a decrease in the DHA/AA ratio might contribute to increased level of oxidation (lipid peroxide). Also lipid peroxide level is a recognised factor for neuronal damage. Therefore an increased DHA/AA ratio in the brain may play a protective role against oxidative neuronal damage and learning ability deficit in AD mouse model. The free radical hypothesis of AD suggests that increased production of lipid peroxides causes deterioration of a wide variety of cellular enzymes, ultimately exacerbating the neurodegenerative processes (Yatin et al., 1999). Chronic treatment with antioxidants (e.g. alpha-tocopherol) has been found to improve cognitive functions in aging (Yamada et al., 1999); a chronological process frequently associated with increased oxidative damage and neurodegenerative diseases including AD.

Another plausible reason relates to the anti-inflammatory nature of n-3 as opposed to n-6 PUFAs. This has been documented in a wide variety of tissues and is thought to be relevant to arthritis (Curtis et al., 2001) and cardiovascular disease (Calder et al., 2003). Decreased ratios of n-6/n-3 acids would be expected to lower inflammatory response. Aggravated inflammatory response is a part of Alzheimer pathology, and therefore raised DHA should help to alleviate this activation and might explain the preventive effect seen in transgenic mice on DHA-enriched diet.

4.5. Conclusions and future questions

The results of this study suggest that dietary DHA ameliorates the deterioration of learning ability as tested in FCA task in the T-maze in an AD mouse model. The fact that DHA supplementation improves memory in Tg2576 transgenic mice might help to

understand how learning is impaired in AD patients and may allow a better insight into AD pathology.

This study supports the clinical and epidemiological data suggesting that a high-PUFA diet reduces the risk of developing AD. The application of this knowledge to the treatment and prevention of AD is a major focus of current research. The data has suggest that diet rich in n-3 PUFA can reduce the risk of developing the disease and therefore that drugs which modulate PUFA levels could be considered as potential therapy for AD.

To provide the important and necessary data concerning the role of n-3 PUFA in AD, future studies need to focus on establishing a more in-depth understanding of the function of brain PUFAs and their possible interactions with APP.

Chapter 5

Can high cholesterol diet trigger amyloid cascade-induced pathology in transgenic GP56 rats?

5.1. Introduction

Overview

Recent data suggests that high plasma cholesterol is coincident with susceptibility to Alzheimer's disease. However, no direct evidence has been reported linking cholesterol metabolism and the pathogenesis of AD. The AD model transgenic rats (GP56) overexpress human mutated APP but show no detectable AD-related pathology (described in chapter 1). These animals were used to test the hypothesis that β-amyloid pathology can be modulated by diet-induced hypercholesterolemia. The project investigated the effect of dietary administration of cholesterol-enriched diet on learning ability, synaptic physiology and Aβ pathology in these rats.

Cholesterol in learning and memory studies in animals

While the hypothesis that high cholesterol levels could affect learning and memory can be easily tested, only a few animal studies have been undertaken to clarify this relationship.

In 1985 Kessler and co-workers investigated the effects of learning on the constitution of brain membranes. Rats were trained in a T-maze, and after reaching certain criteria, were sacrificed. Biochemical analysis revealed a marked learning-induced decrease in the level of cholesterol in the hippocampal and cortical regions and increase in the membrane lipid fluidity. In another study, Miller et al. (1994) treated DBA mice with cholesterol pellets for 11 days. This study showed that short treatment with cholesterol enhanced learning performance in DBA mice in the Morris water maze. O'Brien's group investigated the effect of blocking cholesterol synthesis on conditioned eye blink response in rabbits. Chronic inhibition of cholesterol synthesis in just-weaned rats impaired acquisition of the classically conditioned eye blink response (O'Brien et al., 2000). While these studies showed interesting results, other groups have not investigated this subject further and no final conclusion can be drawn from the limited results so far available.

Cholesterol in AD animal models

As described in Chapter 1.6.3, brain cholesterol homeostasis is thought to be largely independent of peripheral fluctuations in cholesterol content (Edmond et al., 1991). Therefore dietary cholesterol should have no effect on brain cholesterol concentrations and should not affect cerebral processing of APP. However, this has not proved to be the case.

Sparks and co-workers provided the first evidence that increased dietary cholesterol affected amyloid production. They demonstrated that a high cholesterol diet for 4 weeks caused increased β-amyloid immunoreactivity in rabbit hippocampal neurons (Sparks et al., 1994). However, other groups could not reproduce these results. Subsequent work by Sparks et al. (2003) revealed that traces of copper in the animal drinking water (from the tap) were essential to induce β-amyloid plaques and learning deficit in the rabbits on a high-cholesterol diet.

Experiments on transgenic mouse models have provided conflicting evidence on the role of cholesterol in APP processing and the production of Aβ. Feeding APP transgenic mice a 5% cholesterol diet for 8 weeks resulted in a significant reduction in brain levels of APP derivatives, Aβ40, and Aβ42 (Howland et al., 1998). Conversely, another study using the double mutant PS/APP transgenic mouse revealed opposite effects following similar

treatment. This second study found that a high cholesterol diet resulted in increased Aβ production and deposition of amyloid plaques (Refolo et al., 2000).

In response to epidemiological data indicating the ability of statins to reduce the risk of AD (Jick et al., 2000; Wolozin et al., 2000), more detailed studies on animals were initiated to investigate their role in more detail. High doses of statins were found to reduce both intracellular and extracellular levels of Aβ40 and Aβ42 peptides in primary cultures of hippocampal neurons, in brain tissue and in cerebrospinal fluid of guinea pigs (Fassbender et al., 2001). Studies on PS/APP transgenic mice using statins have shown that they can significantly reduce the levels of Aβ40 and Aβ42 (Petanceska et al., 2002). Another study by Refolo (2001) and colleagues used a different cholesterol synthesis inhibitor (BM15.766) and found reduced plaque formation in the PS/APP transgenic mice. This is an important finding as it suggests that plaque formation can actually be inhibited by reducing cholesterol levels and that the previously observed effects are not drug-specific or due to the side effects of statin use.

A recently published study by Park et al. (2003) investigated the effect of lovastatin on β-amyloid pathology in Tg2576 mice. This statin was found to reduce plasma and brain cholesterol levels in both female and male mice, but to enhance the amounts of β-amyloid in females only. Although there is evidence that gender differences may play a role in AD development (Molero et al., 2001), this hypothesis requires further research and might be applicable only to this animal model.

Cholesterol, APP processing and amyloid plaque pathology

As reviewed in chapter 1, brain cholesterol metabolism is not well understood. It is known that cholesterol accumulates in senile plaques and tangles of AD patients and in mutant APP transgenic mice (Mori et al., 2001). Recently, a study in PS/APP double transgenic mice showed that Aβ, ApoE, and cholesterol co-localize in the core of fibrillar plaques (Burns et al., 2003). This could indicate a role for both cholesterol and ApoE in plaque deposition. Removal of either of them prevents plaque deposition (Pitas et al., 1987; Refolo et al., 2001). Furthermore, extracellular cholesterol does not bind to soluble Aβ, but associates strongly with aggregated Aβ (Avdulov et al., 1997) and is therefore an

ideal candidate as the "seed" required for deposition of aggregated Aβ. Binding of cholesterol to aggregated Aβ may also prevent clearance of Aβ to the periphery.

Synaptic plasticity and cholesterol

Many factors (age, stress, diet, environment and drugs) can affect neuronal plasticity, including long-term potentiation (LTP). Koudinov et al. (1998, 2001) investigated extensively the relationship of neuronal cholesterol redistribution and synthesis with synaptic plasticity and neurodegeneration. Extracellular recording of field-evoked postsynaptic potentials (fEPSP) showed enhanced paired pulse facilitation (PPF) and impaired LTP in the CA1 region of adult rat hippocampal slices subjected to induced cholesterol efflux. This study suggested that cholesterol is essential for normal synaptic plasticity.

The results of electrophysiological studies performed in transgenic mice overproducing and accumulating Aβ are not very consistent. Chapman et al. (1999) found normal synaptic transmission and impairment in LTP *in vitro* and *in vivo* in both CA1 and dentate gyrus of the hippocampus of Tg2576 mice. Larson (1999) and co-workers found altered synaptic function in PDAPP mice in advance of amyloid plaque formation and normal long-term synaptic plasticity. Furthermore, Giacchino (2000) and his group found normal baseline synaptic function in PDAPP mice and deficits in CA1 LTP maintenance in both young and aged animals. This variation in results might be due to the differences in procedures (e.g. preparation of brain slices in the presence of kynurenate, a broad-spectrum glutamate-receptor antagonist), exposure to learning and memory testing in the past, or differences in transgenic mouse strains.

Results of studies in rats are no clearer. Stephan (2001) and co-workers injected aggregated Aβ into the dorsal dentate gyrus of rats *in vivo*, tested them on several different learning tasks and assessed synaptic transmission and plasticity *in vivo*. Rats were tested after 7-16 weeks after injection and were found to be impaired specifically in working memory type tasks; synaptic transmission and long-term potentiation were also severely impaired. Although these findings suggest that aggregated amyloid induces cognitive deficits similar to those observed in AD, the effect of injection and the time scale make it difficult to draw a final conclusion. But as more interest is currently focused on less aggregated forms of Aβ (reviewed in chapter 1.3.2), Walsh et al. (2002) injected

intracerebroventricularly soluble oligomers and found that they inhibited the late phase of LTP in the rat hippocampus *in vivo*. These experiments attribute an inhibition of hippocampal LTP *in vivo* specifically to oligomers, not monomers or fibrils of Aβ.

GP56 transgenic rats overexpressing the mutated (Swedish) human APP have not been tested for synaptic physiology impairments. Although they have not shown any impairment on behavioural learning and memory tasks in the T-maze or water maze (Marshall, 2002), it is still possible that they manifest deficits in neuronal plasticity.

Although studies employing animal models of AD are inconsistent, they suggest generally that cholesterol modulates APP processing and consequently alters Aβ production and senile plaque deposition. To further explore the interaction between cholesterol and AD, the effects of a high cholesterol (5%) diet on APP pathology, behaviour and synaptic plasticity in GP56 transgenic rat were studied. I chose to study the GP56 rats, rather than Tg2576 mice, because of the likelihood of a floor effect on spatial learning in the mice (which are severely impaired at 8 months of age) and because of the intriguing possibility that increasing cholesterol might reveal deficits in animals that otherwise failed to express a behavioural or physiological phenotype.

5.2. Materials and Methods

Animals and diets

Fifteen-month-old Sprague-Dawley transgenic homozygous GP56 rats and non-transgenic littermate controls were studied. Animals of both sexes weighing 300-500g (females) and 500-800g (males) were used in the study. The rats were randomly divided into four groups: the transgenic cholesterol group (n =12), the transgenic control group (n =8), the non-transgenic cholesterol group (n =6) and the non-transgenic control group (n = 5). Rats were group housed with littermates (transgenic and non-transgenic housed together), and all testing was conducted during the light phase. Transgenic rats were compared to littermate controls to ensure that age and background strains were equivalent.

The cholesterol group was put on a special chow prepared by Special Diet Services (SDS, UK) by incorporating 5% of pure cholesterol into standard rodent chow. Analysis of the quality of the diet and exact amount of cholesterol was provided by SDS (UK). From previous data at Cardiff we know that a healthy male rat (500-800g) eats around 50g (10-

16g/100g weight) of chow a day and a healthy female rat (300-500g) eats around 30g of chow a day. From these figures, the cholesterol dose for rats was estimated to be on average 1.5-2.5 g per day. That accounts for a dose of around 4g/kg per day. The range is similar to other studies, where mice were put on 5% enriched cholesterol diet (Refolo et al., 2000). For the control chow, nothing was added into standard rodent chow, which contains traces of cholesterol (analysis provided by SDS, UK).

Experimental Procedures

The full experimental procedure consisted of a series of phases, which were conducted on all animals. These included:

- Feeding with special cholesterol-enriched or control chow and drinking water *ad libitum* for 9 weeks. Rats were weighed every week;

- Water Maze training to measure spatial learning as described in chapter 2.1.2. Rats were kept on special diets until the end of testing;

- Electrophysiology *in vivo* measured in dentate gyrus for induction of LTP (details in chapter 2.2). Rats were killed after electrophysiological experiments, brains removed, cut into two halves for Aβ measurements and immunostaining;

- ELISA measurement using commercially available kit (Biosource) in formic acid-treated (FA) brain extracts to quantify the total amount of β -amyloid 40 and 42 in brain tissue (details in chapter 2.3);

- Immunostaining using β-amyloid, APP, activated astrocytes and microglia recognising antibodies (as described in chapter 2.4)

Statistical analysis

All data are expressed as means ± SEM. The statistical significance of differences in the learning parameters in the water maze over the training time and the values of electrophysiological analysis measured over time were calculated by repeated-measures 3-factor ANOVA, with genotype and diet as between-subject factors and time as the within subjects factor. The statistical significance of differences in β-amyloid levels between the groups was determined by 2-factor ANOVA, with genotype and diet as

between-subject factors. Tukey's pair wise comparisons post hoc tests were carried out to determine the source of a significant main effect or interaction. Correlations were counted using regression analysis and Pearson Correlation test. Differences were considered to be significant at $p < 0.05$.

5.3. Results

Body weight and animal survival assessment

To examine the effects of dietary cholesterol on GP56 rats, animals were placed on either a high-cholesterol diet or a basic diet containing traces of cholesterol for 9 weeks. There was a significant difference in body weight gain over the treatment period between animals on control or cholesterol-enriched diets regardless of genotype. Repeated measures over the feeding time 3-factor ANOVA, with diet and genotype as between subject factors, revealed a significant effect of diet ($F_{(1,27)}$=6.08, p=0.02) and no effect of genotype or no interaction. Final body weight for transgenic rats on cholesterol diet was 106.7 ±1.4% original weights, transgenic rats on control diet 99.9±2.7%, non-transgenics on cholesterol diet 106±2.4% and non-transgenics on control diet 102±2.0%. During the time of chronic administration of either cholesterol-enriched or control (from 15 till 18-18.5 months of age) none of the rats died.

Reference Memory in the Water Maze

Figure 5.1 shows the effect of chronic administration of cholesterol on spatial reference learning ability in GP56 rats and their non-transgenic littermates fed with cholesterol-enriched or control chow. Nearly all animals swam well in the pool and learned to use the platform as a mean of escape from the water (two transgenic rats (one on each diet) were excluded due to problems with swimming). During spatial training, all animals spent progressively less time at or near the sidewalls and showed a gradual decrease in the latency to escape from the water. A repeated measure ANOVA revealed significant main effect of genotype, indicating that the transgene causes performance impairments (longer latency period to find hidden platform). There was also a main effect of a training day, confirming an overall improvement during training, and diet, indicating an influence of the treatment on rats' performance in the water maze. There were no significant

Escape Latency in the Reference Water maze in transgenic GP56 and non-transgenic rats fed with cholesterol or control diet

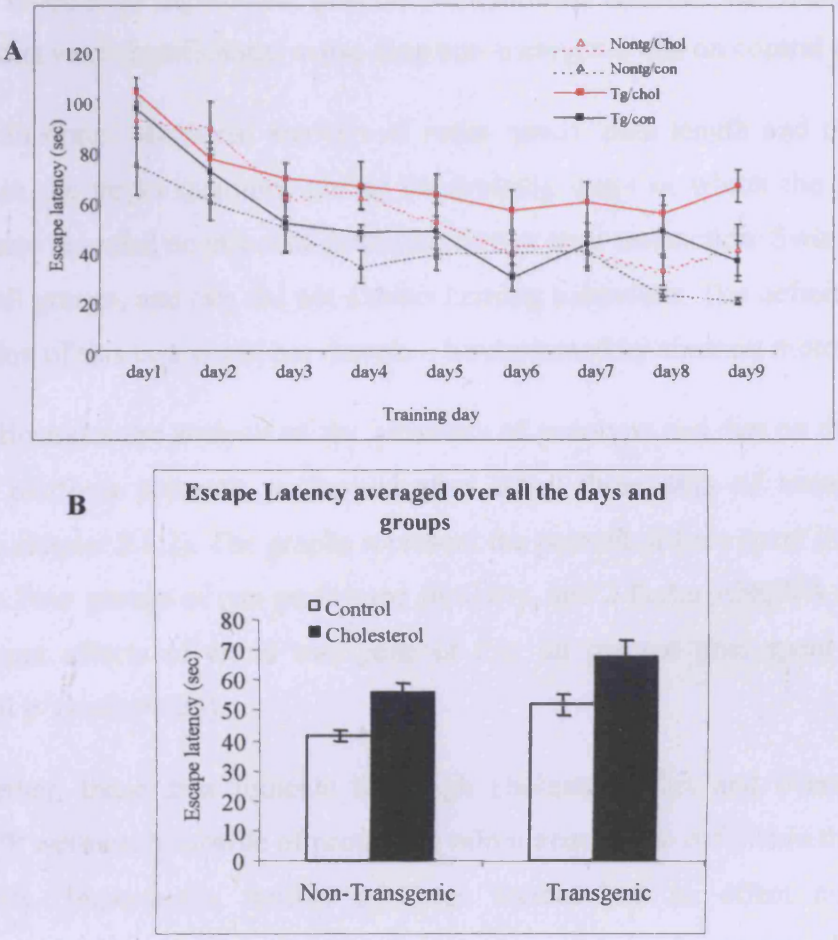


Figure 5.1 Impaired spatial learning in GP56 rats and non-transgenic littermates on either control or cholesterol diet. On the graph A, the time to reach the platform is plotted against training day and demonstrates the latency (mean and standard error) to escape onto the hidden platform. Repeated measure ANOVA for escape latency data (sec) revealed a significant main effect of genotype ($F_{(1,28)}$=5.33, p=0.029), significant effect of diet ($F_{(1,28)}$=10.08, p= 0.004) but no interaction of this factors ($F_{(1,24)}$=0.03, p=0.874). The performance of each group improved similarly across the days (overall day effect $F_{(8,21)}$=13.14, p<.0001), but neither of the factors showed a significant interaction with training day. Although interaction of genotype and diet was not significant, post hoc Tukey`s`s pair wise comparisons revealed a significant difference between non-transgenic rats on control diet and transgenic on cholesterol (p=0.02). Graph B demonstrates column chart of escape latency averaged over all trials and over all training days. Numbers of rats are tg/chol=11, tg/con=7, nontg/chol=6, nontg/con=5.

interactions between any of these factors indicating that the diet did not affect transgenic rats in a different way than non-transgenic, and there was not particular interaction of high cholesterol and APP that could affect learning in the water maze. Although interactions failed to be significant, post hoc comparisons revealed that transgenic rats on cholesterol diet were significantly worse than non-transgenic rats on control diet.

Figure 5.2 illustrates statistical analysis of swim speed, path-length and the percent of time spent in the target quadrant during the training trials in which the platform was present. These revealed no effect of genotype, diet or their interaction. Swim speeds were similar for all groups, and rats did not exhibit floating behaviour. The deficits observed in the acquisition of this task could not therefore be explained by aberrant motor behaviour.

Figure 5.3 illustrates the analysis of the influence of genotype and diet on the probe trials (no escape platform present), performed after every three days of training (protocol described in chapter 2.1.2). The graphs represent the percent of time spent in all quadrants of the maze. Four groups of rats performed similarly, and 2-factor ANOVA did not reveal any significant effects of either transgene or diet on percent time spent in the target quadrant (all p-values>0.05).

Taken together, these data indicate that high cholesterol diet and overexpression of mutated APP were each capable of producing minor acquisition deficits in the water maze independently. Importantly, neither of these factors had an effect on probe trial performance, which was relatively poor even in control animals, compared to published literature. This is likely to be a strain effect, as hooded rats (Lister or Long-Evans) are more commonly used to test reference memory in the water maze, and Sprague-Dawley are known to perform poorly (D'Hooge & De Deyn, 2001).

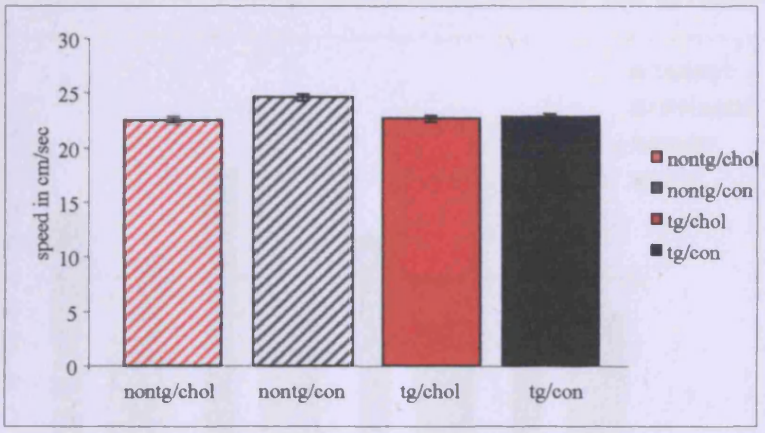
Electrophysiology in vivo

After behavioural testing, electrophysiological analysis was undertaken to verify whether the reference water maze impairment correlated with a deficit in synaptic transmission and plasticity measured in the dentate gyrus. For analysis, changes in the field-excitatory postsynaptic potential (fEPSP) slope and pop spike amplitude (PSA) were measured.

Basal synaptic function was assessed by using a range of stimulus intensities to generate input-output (I/O) curves and to determine the appropriate stimulus intensity for baseline

Swim Speed and % time in a target quadrant during water maze training in transgenic GP56 and non-transgenic rats fed with cholesterol or control diet

A



B

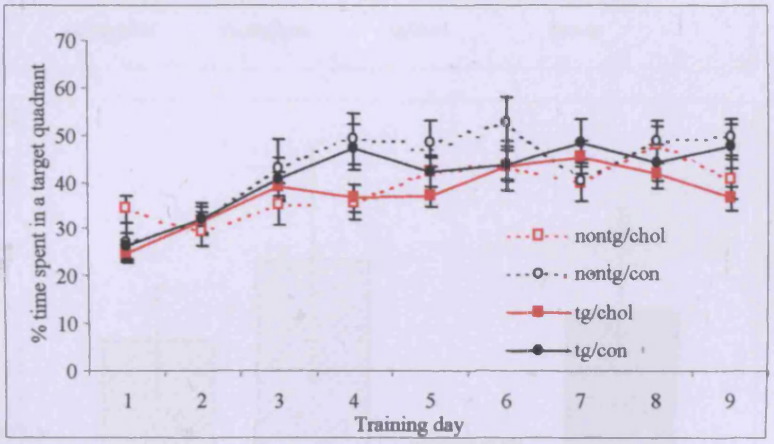


Figure 5.2 There were no significant effects of genotype or diet on swim speed, % time in a target quadrant or path length. Graph A shows swim speed averaged over all trials and training days while graph B represents increasing % time spent in target quadrant during the training days. Repeated measures 2-factor ANOVA for swim speed data revealed no effect of genotype, no effect of diet and no interaction of these factors (all p-values > 0.05). Statistical analysis of path-length (averaged over trials and training days) showed also no effect of genotype, no effect of diet and no interaction (all p-values > 0.05). 3-factor ANOVA performed on % time spent in a target quadrant data revealed also no effect of genotype, no effect of diet and no interaction (all p values > 0.05). Overall, the training day number was significant for % time spent in a target quadrant ($F_{(8,18)}$=8.53, p<. 0001) but did not show any interactions (all p-values >0.05).

% time spent in each quadrant during probe trials in reference water maze in transgenic GP56 and non-transgenic rats on cholesterol or control diets

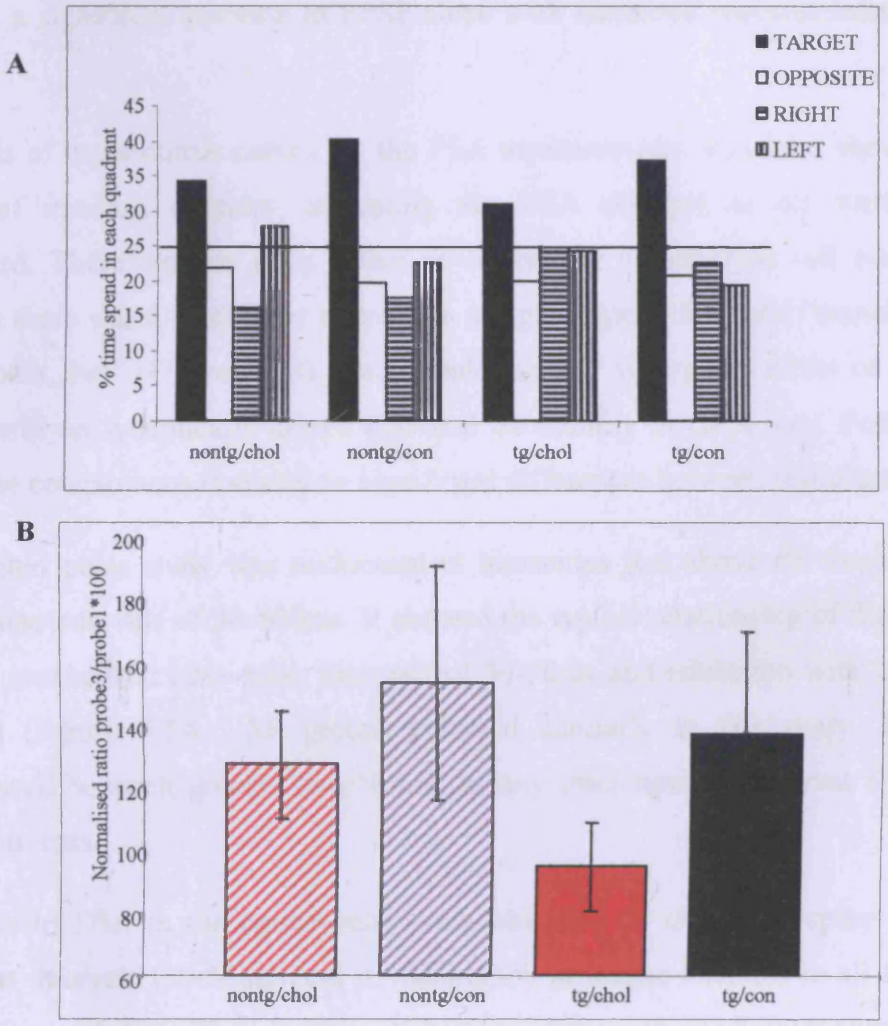


Figure 5.3 Bar graph A demonstrates the time spent in each of the four quadrants of the water maze (target, opposite, right and left) on the third probe. Rats were subjected to three probe trials which followed every three days of training. 2-factor ANOVA (genotype and diet) revealed no significant effect of any of these factors during any of the probes (all p-values> 0.05). To analyse if rats improved during training and spend more time in target quadrant during the 3rd probe compared to the 1st one, normalised ratio was calculated. 2-way ANOVA (genotype and diet) revealed no significant effect or interaction of any of these factors on this ratio (all p-values> 0.05). There were no statistical differences between rats during probe trials.

responses before induction of LTP. There were no statistical differences in fEPSP slope measurements (all p-values > 0.05), as calculated by repeated measure 3-factor ANOVA (diet x genotype, with stimulus intensity as the repeated-measure factor). All the rats showed a significant increase in EPSP slope with increased stimulus intensities (Figure 5.4A).

Analysis of input-output curves for the PSA measurements showed a significant overall effect of stimulus intensity, indicating that PSA changed as the stimulus intensity increased. There was no main effect of either diet or genotype (all p-values > 0.05). Though there was a significant interaction of "genotype x diet" and "stimulus intensity x genotype x diet" (Figure 5.4B) which could suggest synergistic effect of transgene and cholesterol on synaptically driven neuronal excitability in GP56 rats. Post hoc Tukey's pair wise comparisons revealed no significant differences between tested groups.

The paired pulse study was performed at intensities just above PS threshold and with inter-pulse-intervals of 30-300ms. It showed the typical relationship of facilitation of the fEPSP, maximal at inter-pulse intervals of 30-50ms and inhibition with longer stimulus interval (Figure 5.5A). All groups behaved similarly in this study. No significant differences between groups were found at any inter-stimulus interval for slope EPSP measurements.

Analysis of PSA in the paired-pulse study revealed no change of spike at short inter-stimulus intervals (30-50ms) and its facilitation at longer intervals in all four groups of rats. (Figure 5.5B). This outline of first no-change at short inter-stimulus intervals followed by facilitation at longer intervals reflects inhibition and disinhibition processes characteristic for postsynaptic events (Joy et al., 1993). Statistical analysis of PSA values revealed significant effect of inter-pulse interval and significant interaction of "inter-pulse interval x genotype x diet". Although short-term presynaptic plasticity and network excitability in the dentate gyrus seem to be impaired at middle length inter-pulse intervals (50-150ms) and seem to depend on both transgene and cholesterol, being the most impaired for transgenic rats on cholesterol diet, but no significant differences between groups were established (Tukey's pair wise comparisons post hoc tests).

After collecting the paired pulse data, a 20-minute baseline was recorded followed by tetanic stimulation of the perforant path (see chapter 2.2). All data were normalized with

I/O Curves for responses recorded in Dentate Gyrus in transgenic GP56 and non-transgenic rats fed with cholesterol or control diets

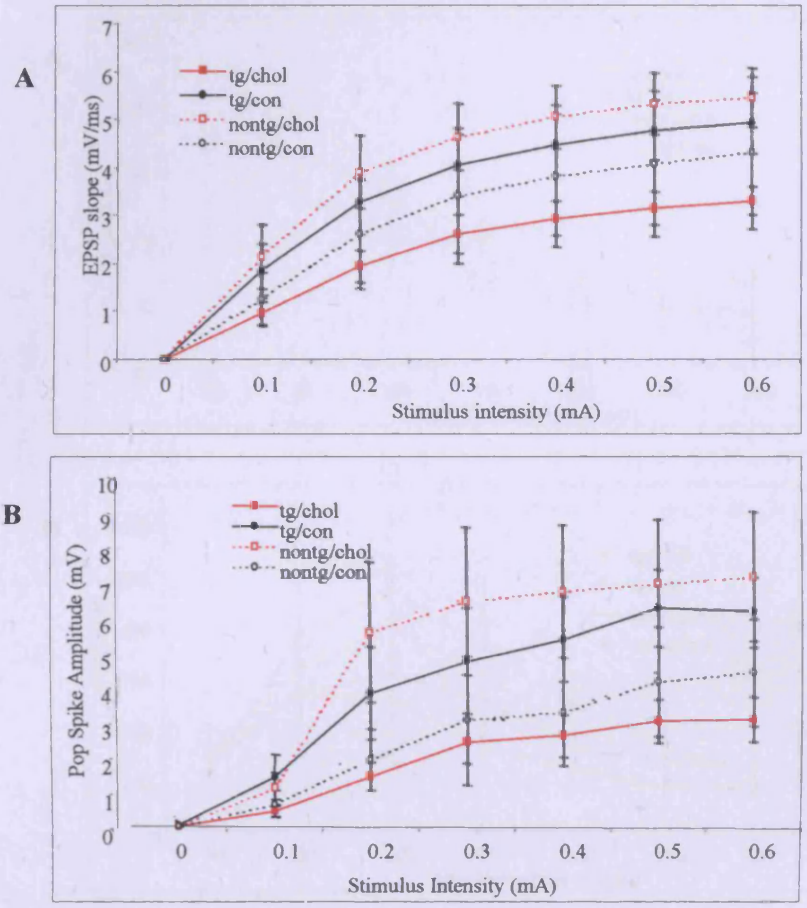


Figure 5.4 I/O curves of the fEPSP slope (A) and PS amplitude (B) as a function of stimulus intensity before induction of LTP. The y-axis denotes the slope of the fEPSP measured in mV per ms (A), or PS amplitude measured in mV (B) and the x-axis indicates the lowest and highest intensities used in mA. Repeated measure 3-factor ANOVA (diet x genotype, with stimulus intensity as the repeated-measure factor) revealed no significant effects of either genotype or diet for the size of the fEPSP slope at all intensities, although an overall effect of stimulus intensity was significant ($F_{(6,16)}$=13.69, p<.0001) (A).

For the PSA measurements, repeated measures 3-factor ANOVA (diet x genotype, with stimulus intensity the repeated-measure factor) revealed no significant main effect of either genotype or diet, but significant interaction between them ($F_{(1,22)}$=5.43, p=0.029). The overall effect of stimulus intensity was also significant ($F_{(6,17)}$=16.17, p<0.001), and interaction genotype x diet x stimulus intensity reached also significant difference ($F_{(6,17)}$=3.04, p=0.033). Post hoc Tukey`s`s pair wise comparisons revealed no differences between tested groups (B).

Paired Pulse Curves for responses recorded in DG of hippocampus in transgenic GP56 and non-transgenic rats fed with cholesterol or control diets

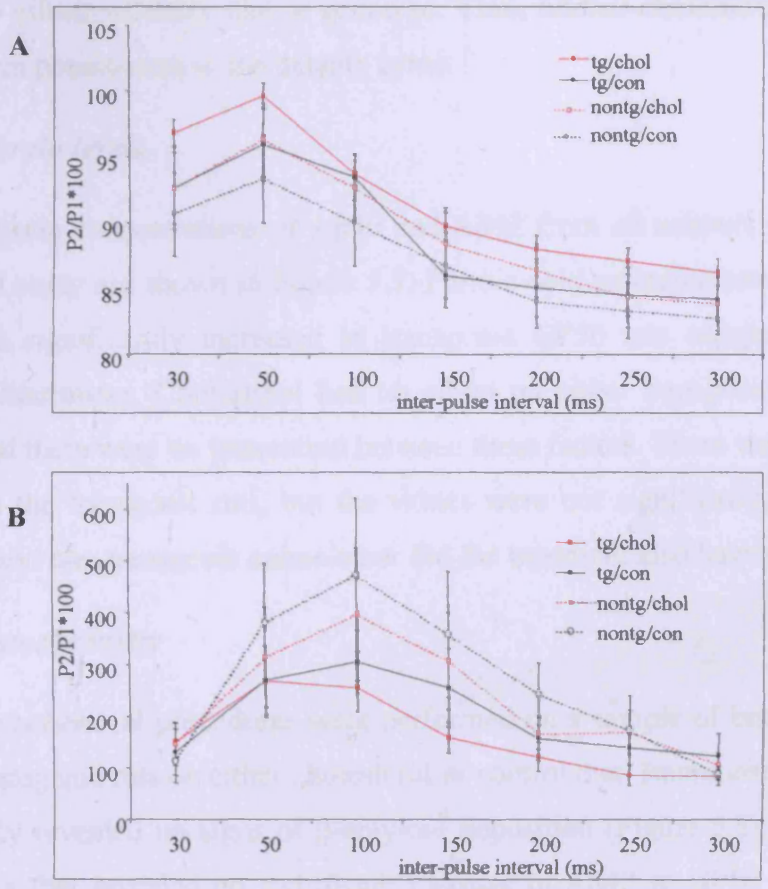


Figure 5.5 PP facilitation curves(values of the relative amplitude in the second potential with respect to the first potential (P2/P1 × 100)) of the EPSP slope (A) and PS amplitude (B) as a function of inter-stimulus interval before induction of LTP. The y-axis denotes the P2/P1 × 100 ratios of slope of the fEPSP (A), or PS amplitude (B) and the x-axis indicates the range of inter-pulse intervals in ms. Repeated 3-factor ANOVA (diet x genotype, with inter-pulse interval as the within subject factor) revealed no significant effects of either genotype or diet for the size of the fEPSP slope at all time intervals, although an overall effect of inter-pulse interval was significant ($F_{(6,16)}$=48.27, p<.0001) (A).

For the PSA measurements, repeated 3-factor ANOVA (diet x genotype, with inter-pulse interval as the within subject factor) revealed no significant main effect of either genotype or diet. The overall effect of pulse interval ($F_{(6,20)}$=8.26, p<.0001) and interaction genotype x diet x inter-pulse interval ($F_{(6,20)}$=3.45, p=0.0167) reached significance (B).

respect to the mean fEPSP slope or mean PSA during this 20-min period (Figure 5.6). 60 minutes following tetanic stimulation, all four groups of rats showed significant and similar potentiation of fEPSP slope ($p< 0.0001$) and PSA ($p<0.0001$). Statistical analysis revealed no effects of either diet or genotype. Thus, neither cholesterol nor APP affected the long-term potentiation in the dentate gyrus.

β-amyloid brain levels

The mean brain concentrations of Aβ40 and Aβ42 from all animals participating in the behavioural study are shown in Figure 5.7. Formic-acid extracted total amounts of Aβ40 levels were significantly increased in transgenic GP56 rats comparing to their non-transgenic littermates. Cholesterol had no effect on either transgenic or non-transgenic animals, and there were no interaction between these factors. There were detectable levels of Aβ42 in the transgenic rats, but the values were not significantly different between transgenic and non-transgenic animals nor did the treatment also have an effect.

Immunohistochemistry

Immunohistochemical procedures were performed on a sample of brains from transgenic and non-transgenic rats on either cholesterol or control diet. Immunostaining with an anti-Aβ antibody revealed no signs of β-amyloid deposition (Figure 5.8), which agrees with ELISA data that revealed no significant increase in Aβ42 in GP56 rats on either diet. Because a common feature of Alzheimer pathology is inflammation (Akiyama et al., 2000), GFAP and OX-42 immunostaining was used to detect signs of astrocytosis and activation of macrophages and microglia. There was a GFAP- and OX-42-immunopositive reaction present in all the rats but there were no obvious differences between any of the groups. Therefore, the conclusion is that neither genotype nor cholesterol was able to induce significant β-amyloid pathology in GP56 rats.

Relationship between learning ability, synaptic physiology and β-amyloid levels

Although rats showed slightly impaired performance in the water maze and most of them exhibited normal dentate LTP, it is possible that some of the rats that displayed less LTP showed a bigger impairment in the behavioural task or had higher levels of β -amyloid.

However, inspection of the data revealed no significant correlation between the mean escape latency averaged over the 9 spatial training trials and the amount of LTP 1 h post-

LTP induction recorded in dentate gyrus of hippocampus in transgenic GP56 and non-transgenic rats fed with cholesterol or control diets

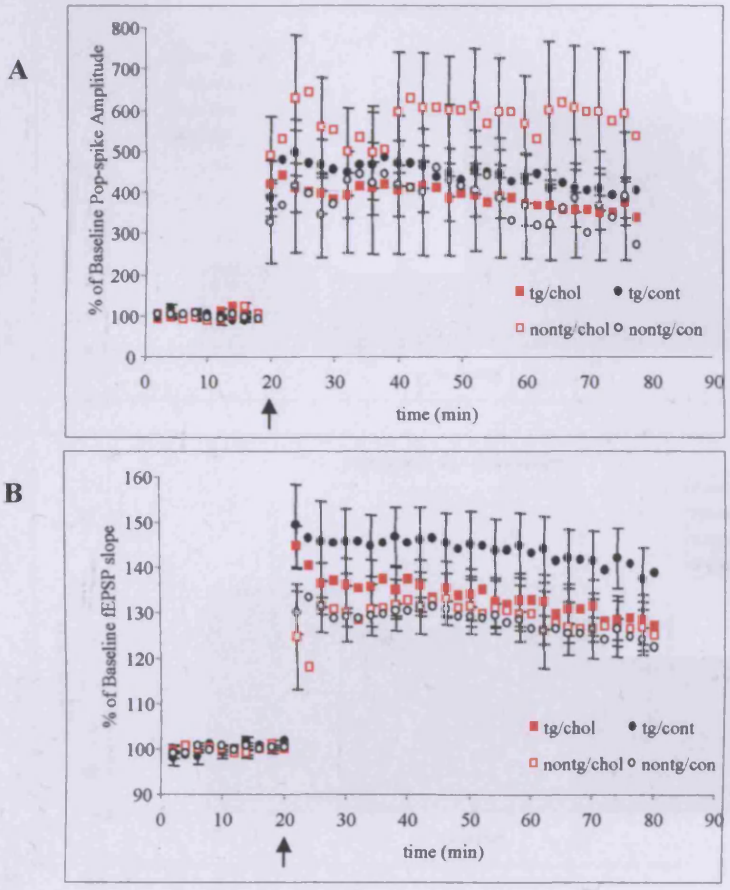


Figure 5.6 LTP in the dentate gyrus recorded for 60 minutes after the induction, of fEPSP slope (A) and PSA (B) in the GP56 rats on either cholesterol or control diet. Tetanic stimulation induced an immediate increase in both EPSP slope and PS amplitude for all groups of rats. Data are means taken every 2 minutes with SEM values included for every 2nd mean response. The y-axis denotes the percentage of the baseline fEPSP slope (A) or PSA (B) after induction of LTP (indicated by arrow), and the x-axis denotes the time of recording. Neither administration of cholesterol and/nor presence of the APP transgene had a significant effect on induction and maintenance of LTP in rats. Overall effect of tetanus was significant for both fEPSP slope ($F_{(7,14)}$=25.08, p<.0001) and for PS amplitude values ($F_{(7,14)}$=11.72, p<.0001).

β-amyloid levels in transgenic GP56 and non-transgenic rats fed with cholesterol or control diets

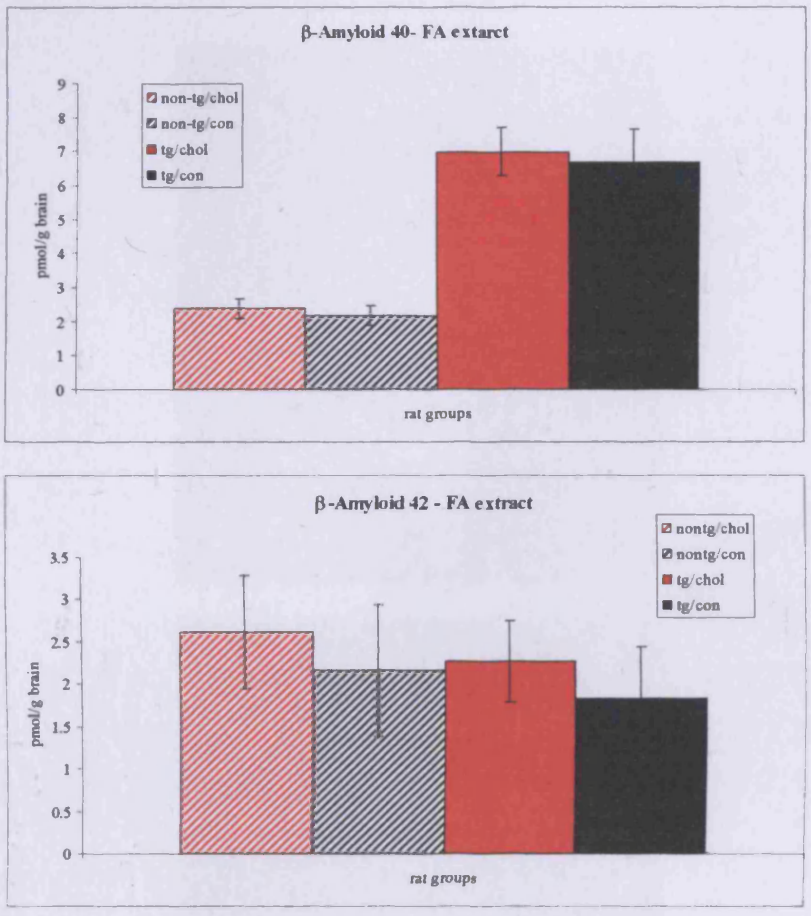


Figure 5.7 β-amyloid 40 and 42 levels in FA extract in transgenic GP56 and non-transgenic rats are not affected by diet. 2-factor ANOVA (genotype, diet) showed that non-transgenic rats had significantly lower levels of Aβ40 in FA extract (FA40), but genotype had no effect on Aβ42 in FA extract (FA42). Analysis showed significant main effect of genotype for FA40 ($F_{(1,20)}$=23.17, p<.0001), and no other factors (diet, interactions or genotype and diet for FA42) were significant (all p-values >0.05).

Immunostaining for β-amyloid and astrocytes in GP56 rat brain sections from rats on cholesterol or control diets.

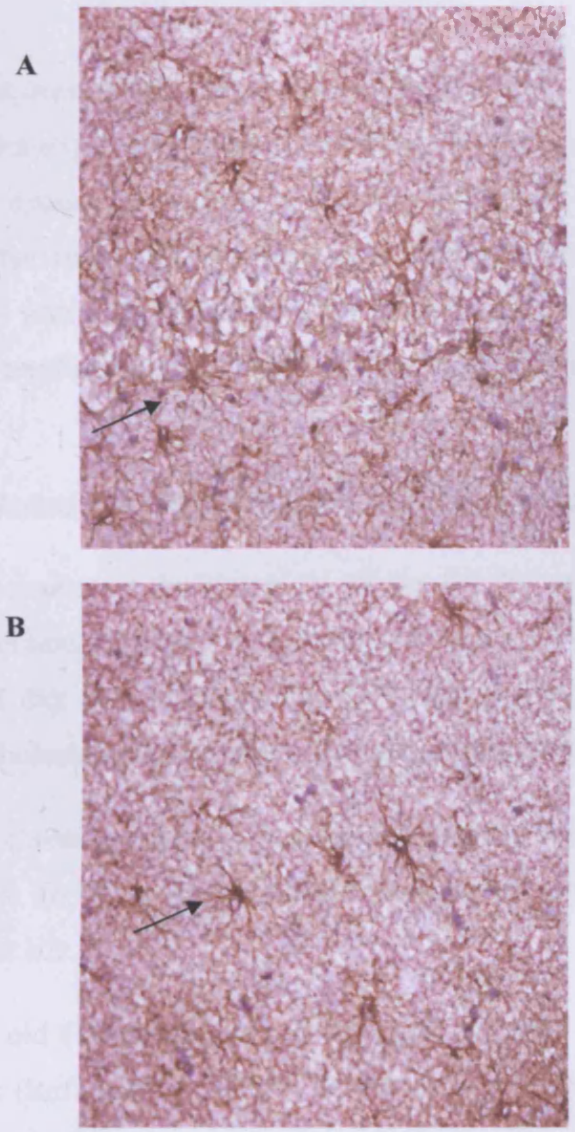


Figure 5.8 Representative examples of 19-month-old GP56 rat brain sections subjected to immunohistochemistry for β-amyloid to label possible amyloid pathology and for GFAP (A, B) to label activated astrcytes (10-20x). No β-amyloid pathology was noticed in transgenic rats on either cholesterol or control diet. Activation of astrocytes (indicated with an arrow) was identical among all transgenic and non-transgenic rats regardless of the diet and is typical for this age of animals. These sections are taken from the hippocampus of GP56 transgenic rats on high-cholesterol (A) and control diets (B).

tetanus measured as an average of the last 10 minutes (p-values> 0.05). Nor was there a significant correlation between the mean escape latency averaged over the 9 spatial training trials and the concentration of β-amyloid 40 and 42 (all p-values> 0.05) in the rat brain.

5.4. Discussion

The purpose of these experiments was to explore whether increases in dietary cholesterol in transgenic rats over-expressing human mutated APP would induce amyloid pathology in their brains, thus causing disruptions in learning ability, synaptic plasticity and levels of β-amyloid in the brain. This association was not really established, as only performance in the water maze was slightly impaired, while synaptic plasticity was normal, levels of β-amyloid were low and no β-amyloid pathology was detected in brain sections.

Why did the high cholesterol diet fail to induce AD-like pathology in GP56 rats?

Results of previous studies on the biological relationship between cholesterol and AD are controversial (see section 5.1) and re-examination of this question in transgenic GP56 rats on high cholesterol diet did not clarify this issue any further. There are a few possible explanations why cholesterol did not induce significant β-amyloid pathology in these rats:

- The amount of cholesterol provided in the diet was too low and/or the administration period was too short. To imitate conditions for humans, rats might have needed to eat this diet for most of their life.

- Rats were too old (18-month old). Looking at previous studies, when mice were younger - 5 weeks (Refolo et al., 2000), cholesterol succeeded in inducing β -amyloid pathology. Conversely it had an opposite action in older mice - 210-408 days old (Howland et al., 1998). As investigated in human studies, higher levels of cholesterol correlate with risk of developing AD but only in midlife (Kivipelto et al., 2001). By comparison, during the progression of the disease in older individuals, the levels of cholesterol seem to be lower than in healthy people (Mason et al., 1992). This could be a possible explanation of the difference.

- The transgene does not have a big enough effect in GP56 rats, when compared to similar transgenic mice models, like Tg2576. The difference might be due to an insertion place of the transgene, strain sensitivity to human APP or different immunological responses. Thus cholesterol could be a trigger factor in inducing AD-like pathology in transgenic mice highly overexpressing APP, but not in GP56 rats.

- Rats might have a (as yet unknown) mechanism (microglia? astrocytes?) that prevents them from accumulating β-amyloid. Although the expression of APP is only 3 times smaller than Tg2576 mice, the levels of produced Aβ are 100 times less! To support this hypothesis, no successful AD transgenic rat model showing β-amyloid pathology has been generated up to now.

- There was a lack of significant co-factor helping cholesterol to induce APP-associated pathology. Normal rabbits on high cholesterol diet show high levels of amyloid and even plaques, but only if there is also copper in their drinking water (Sparks et al., 2003).

What effect did cholesterol feeding have on spatial learning in water maze on GP56 rats?

As previously described by Marshall (2002) heterozygous GP56 rats show no impairment in either episodic-like memory in the T-maze or in spatial learning in the water maze. Homozygous GP56 rats, not previously assessed for behaviour impairments, were used for this project. The results of this study demonstrate a subtle behavioural impairment in escape latency during acquisition with significant effect of only diet and genotype, while no deficit on probe trials was detected. There could be a number of reasons for this observation:

- The protocol used for this study included giving a probe trial to rats after every 3 days of training (averaging 3 probes altogether). It is possible that the choice of this protocol was a mistake. Rats could have been over-probed and learned that every third day was a trial with no escape platform. Consequently they did not search for the platform. Following a standard protocol, with 1 probe trial at the end of testing, may have given a more accurate result.

- Deficits detected during the acquisition could be due to the effects of either cholesterol or genetic modifications on other processes, not directly involved with the learning mechanism. Performance in complex learning tasks, such as the water maze, can be disrupted in a number of different ways which are independent of the learning process per se, such as by effects on sensory-motor or motivational processes necessary for normal task performance (reviewed in D'Hooge & De Deyn, 2001)

- The uncommon pattern of deficit in water maze, meaning impairment in escape latency during training and poor probe performance could be also caused by the rat strain characteristics. There are reported differences in water-maze learning ability between different strains of rats and mice (reviewed in D'Hooge & De Deyn, 2001).

- All rats on high-cholesterol diet gained more weight than the controls on normal diet, which could make it more difficult for them to swim. This is a less likely explanation, since there was no statistical difference in the swim speed among all groups.

What can be concluded from deficits in synaptic plasticity?

The analysis of electrophysiological recordings in dentate gyrus showed slight changes of population spike, but not EPSP slope measurements. Although measurements of fEPSP slope are a standard way of evaluating synaptic plasticity in experiments *in vitro*, recordings *in vivo* are more susceptible to be masked by the dipole of the overlaying population spike. Therefore, calculating the EPSP from these potentials could be inappropriate because it varies strongly with the amplitude of the population spike.

Input-output recordings for PSA revealed a significant interaction of "genotype x diet" and "stimulus intensity x genotype x diet". Although transgenic rats on cholesterol diet seemed to have the smallest population spikes and the difference was the most visible at higher intensities, post hoc comparisons revealed no differences between rat groups. These results could suggest a synergistic effect of transgene and diet limiting synaptically driven neuronal excitability in GP56 rats. There were no significant differences in the stimulus intensity needed to obtain threshold in both transgenic and non-transgenic rats on either diet.

Similarly in the paired-pulse paradigm, differences were observed in PSA, but not in fEPSP slope. Analysis revealed significant interaction of "inter-pulse interval x genotype

x diet". Short-term facilitation of PSA characterises presynaptic function. Although it seems to be impaired at middle length inter-pulse intervals (50-150ms) and seems to depend on equally cholesterol diet and the transgene (being the most impaired for transgenic rats on cholesterol diet), post hoc comparisons between tested groups revealed no differences. Long-term synaptic plasticity was not impaired in any group of the rats.

These data suggest that dysregulation of cholesterol homeostasis combined with mutant APP over-expression could impair both basic neurotransmission and presynaptic functioning, but this effect was very minor. Perhaps higher doses of either APP or cholesterol, or a longer administration period of cholesterol, could amplify this effect.

Why did cholesterol treatment not alter amyloid pathology in GP56 rats?

Although cholesterol seems to have no effect on Aβ levels in this model, subtle disturbances in spatial learning and short-term synaptic plasticity were detected. As described in chapter 2.3, ELISA techniques have many limitations and, as levels of amyloid were low anyway (all values were at the bottom of the standard curve), it is possible that the differences could not be detected. To answer this problem, the Aβ levels could be measured using a more sensitive technique such as mass spectroscopy.

Transgenic mice carrying the same transgene (Tg2576) overexpressing the same mutated human APP show similar types of disturbance in synaptic plasticity and behaviour, but the features are more prominent (reviewed in chapter 1.7). The processes underlying these impairments are not fully understood, but are based on the same underlying genetic manipulation; high over-expression of APP. GP56 rats do not show this level of impairment, regardless of having high levels of APP. As cholesterol feeding in transgenic GP56 rats induced slightly more evident behavioural and physiological deficits (some factors were significant), we might conclude that high dietary cholesterol played some kind of role in inducing Aβ pathology, or induced some other processes that led to observed effects.

Other studies using mice transgenic models also provided conflicting evidence. Refolo et al. (2000) demonstrated that cholesterol feeding resulted in a significant increase in the accumulation of Aβ in PS/APP mice, but an earlier study using APP gene-targeted mice showed, in contrast, that a similar high-cholesterol diet resulted in a significant reduction in Aβ peptides (Howland et al., 1998). These conflicting experimental outcomes may be

attributed to the differences between the genetic models. Refolo et al. (2000) used standard transgenic mice over-expressing many copies of both APP and PS1 genes, while Howland et al. (1998) used a genetically-engineered (knock-in) mouse model in which the mouse APP gene was partly replaced with a human mutated gene. GP56 rats are transgenics overexpressing human mutated APP. Also the age of animals might be important. Younger animals (5 weeks old mice fed for 9 weeks, [Refolo et al., 2000]) could be more susceptible to the effects of high cholesterol diet, because at this age β-amyloid pathology is starting to develop. Older mice (30-50 weeks old fed for 8 weeks on a diet, [Howland et al., 1998]) could be more resistant, as they already have established pathology. Finally the difference in the background strain of the mice used in the two studies could make them more or less sensitive to cholesterol diet causing alterations in APP processing and/or Aβ deposition.

Given the difference apparent between two mouse models, it is perhaps not surprising that we failed to see any effect of cholesterol on GP56 rats, since using a different species than mice would involve even more differences in metabolism and physiology.

5.5. Conclusions and future questions

This study does not really support the clinical and epidemiological data suggesting that a high-cholesterol diet raises the risk of developing AD. GP56 transgenic rats failed to exhibit any Aβ pathology and showed only some disturbances in spatial learning and neurotransmission.. If we assume that the noticed changes could precede development of Aβ pathology, the results of this study support the role of cholesterol as an inducing factor of these changes. This finding suggests that diet could be crucial in modifying the risk of developing various diseases, including Alzheimer's disease.

To provide the important and necessary data concerning the role of cholesterol in AD, studies will need to focus on establishing a more in-depth understanding of brain cholesterol metabolism. It is plausible that specific subcellular fractions, e.g., synaptic membranes or caveolae-like microdomains from specific brain regions have altered levels of cholesterol in AD, and this could provide the basis for cholesterol mediated Aβ pathology. The simplest explanation for the sensitivity of APP processing to cholesterol, though, is chemical; the transmembrane proteins that process APP might simply require a high cholesterol environment to function. A somewhat more biological hypothesis is that

regulation of APP processing can respond to changes in cholesterol membrane dynamics. For instance, synaptic plasticity may need alterations in cholesterol, other lipids and membrane proteins, possibly including those that may control APP processing. The association of APP and Aβ with uptake of cholesterol also raises the possibility that APP or Aβ contributes to cholesterol trafficking. However, although cholesterol clearly influences APP metabolism, data has yet to surface suggesting that APP influences cholesterol metabolism.

Although research from genetics, epidemiology, cell biology and studies in animals suggest that cholesterol plays a central role in the biology of APP and Aβ, these results were not fully confirmed in this project. More research needs to be under-taken to clarify the functional significance underlying the connection between cholesterol and Aβ.

Chapter 6

General Discussion

6.1. Overview

Forgetfulness was, in the past, accepted as a normal consequence of ageing; however this assumption is now known to be untrue. This realisation has facilitated the expanding understanding of dementia (Mattson et al., 2000). Life expectancy is increasing constantly, and the elderly proportion of the population is growing rapidly in relation to the young. Dementia, however, is growing even more rapidly, since incidence increases exponentially after the age of 65.

Significant progress has been made during the last two decades in understanding many aspects of AD, including its neuropathology, the mechanisms of its biochemical and cellular abnormalities and genetic causes and risk factors. Also, more environmental and pharmacological risk factors influencing developing AD have been identified. In parallel, several kinds of non-transgenic and transgenic animal models for AD have been developed. In this PhD project, I aimed to use transgenic animal models of AD to answer questions regarding the influence of dietary (cholesterol and DHA) and pharmacological manipulations (ibuprofen) on AD-like pathology in two such animal models.

6.2. Why transgenic animals are imperfect, but indispensable, models of Alzheimer disease.

A new phase of AD research commenced when the first transgenic mouse model showing deposits of human Aβ protein in brain, was reported (Games et al., 1995). There are significant numbers of researchers who doubt the utility of rodent models of AD (or any human disorders), but an equally large number who recognise the value of developing these models. In either case, their widespread use demands that we find the best way to apply what we can learn from them to the development of both novel therapeutic strategies and an understanding of the disease.

Those who question the applicability of rodent AD models claim that:

- They develop neither neurofibrillary tangles (NFTs) nor neuronal loss, which are essential hallmarks of AD (see chapter 1.7).

- The amyloid peptides generated in APP Tg2576 transgenic mice are physically and chemically distinct from those characteristics of AD (Kalback et al., 2002). Transgenic mouse Aβ peptides lack the N-terminal degradations, post-translational modifications and cross-linkages abundant in the stable Aβ peptide deposits observed in AD

- The Aβ peptides that create the amyloid plaque cores in transgenic mice, in contrast to AD, are soluble in Tris-SDS-EDTA solutions, revealing both monomeric and SDS-stable oligomeric species of Aβ (Kalback et al., 2002).

- The transgenic mouse levels of carboxy-terminal APP fragments are nearly 10-fold greater than those of human brains, and this condition may contribute to the unique pathology observed in these animals (Kalback et al., 2002).

- APP transgenic mice show weak inflammatory responses as compared to AD patients (Schwab et al., 2004). In both elderly transgenic mice (APP23 mice) and AD, similar staining for Aβ has been observed and amyloid deposits were ApoE-positive and surrounded by activated astrocytes. However, mice had only weakly activated microglia, which are gathered around the periphery of the deposits. In contrast, AD lesions had strongly activated microglia, which were associated with the plaque core. Also

immunostaining for complement proteins was weak in transgenic mice but very strong in AD deposits.

- The genetic mouse background influences the transgene-induced pathology in mice very strongly. It can change mortality rates and has a significant effect on the results of behavioural tests and makes the testing incoherent (Carlson et al., 1997).

In spite of all these problems, transgenic rodent models have invaluable advantages:

- Transgenic animal models overexpressing APP with mutations linked to familial AD show Aβ deposits that closely resemble the amyloidosis observed in AD (see chapter 1.7). Therefore, they are useful for assessing the effects of drugs that inhibit Aβ synthesis, fibril formation, and deposition in the brain.

- Analysing anatomy and physiology of transgenic animals overexpressing various AD-linked genes (APP, PS or Tau) allows better understanding of the impact of certain types of AD pathology on specific brain systems (e.g. temporal lobe versus frontal lobe systems) and the physiological processes, which are responsible for the formation of AD neuropathology and dementia. For example in Tg2576 mice, Shie and co-workers (2003) demonstrated intraneuronal Aβ deposition in the somas of hippocampal CA1/subiculum neurons far in advance of the occurrence of extracellular Aβ plaques. Similar to extracellular Aβ deposits, these deposits increased exponentially with age. This finding supports the role of the transgenic mice as a promising model for the study of *in vivo* mechanisms of intraneuronal Aβ deposition and seeding relevant to AD, and for testing measures to prevent or ameliorate intraneuronal Aβ deposition.

- Transgenic models provide an opportunity to evaluate potential therapies on specific aspects/features of AD and cognition. Also the impact of potential risk factors on hippocampal Aβ on neuronal functions, behavioural changes, and amyloid plaque formation can be assessed and investigated in cellular and biochemical details.

Although many controversial theories have been raised about the validity of rodent animal models, and it is true that results from testing them cannot be directly linked to humans, we should not underestimate how much scientist have already learned from them. Moreover, at the moment there is no better alternative. Even the "humble" fruit fly might contribute to the understanding of Alzheimer's disease (Greeve et al, 2004). Thus,

for example, signalling from the Notch receptor is essential for proper cell-fate determinations and tissue patterning and requires a presenilin-dependent transmembrane-cleaving activity that is closely related or identical to the γ-secretase proteolysis of APP involved in AD. A compound inhibiting γ-secretase in transgenic mice induces developmental defects in Drosophila remarkably similar to those caused by genetic reduction of Notch. These results show that genetics and developmental biology can provide new understanding of the function of proteins like Notch receptor and its function in humans (Micchelli et al, 2003).

Consequently, differences in disease progression and biochemistry must be considered when using transgenic animals to evaluate drugs or therapeutic interventions intended to reduce the Aβ burden in Alzheimer's disease. Nevertheless, these animals are widely used for developing and assessing the validity of novel drugs and interventions and also considering the function of suspected risk factors. So, while accepting that that direct transfer of results from mouse to human is not possible, we can learn much from experiments in transgenic models of AD.

6.3. Can we treat AD or only prevent?

A number of drugs are now available to minimise and delay the cognitive and behavioural symptoms of AD, but none of them are disease modifying. In general, however, scientists concentrate more on developing treatments, whereas prevention is relatively neglected. One possible reason for this is that there is no consistent method for an early diagnosis available.

Normally, the degenerative process has already progressed to an advanced stage with massive loss of cell mass before diagnosis can be made. In view of the limited capacity of the central nervous system tissue to repair, early intervention in the degenerative processes to spare as much tissue as possible will be crucial to obtain success. The transition from normal cognitive performance to the AD phenotype is probably gradual and takes years.

The interesting cases are patients with so-called mild cognitive impairment. As various studies have shown, these patients are at a very high risk of developing AD (Jack et al., 2000). Thus patients with mild cognitive impairment are an important group to test

potential preventive therapies. A number of ongoing clinical trials are recruiting such individuals to test whether certain drugs are effective in reducing the rate at which they progress to Alzheimer's disease.

Moreover, a variety of medications have been associated with significant reductions in the risk of developing AD, such as an anti-inflammatory therapy, antioxidant supplementation or statins. Early diagnosis may then help to increase the possibilities for developing and using these therapies.

For the studies reported in this PhD thesis, I investigated the role of the NSAID ibuprofen, as a potential agent for prevention or treatment in transgenic mice models. Ibuprofen and other NSAIDs have been implicated in many epidemiological studies in reducing the risk of developing AD (see chapter 1.5). As transgenic mice modelling this disease show age-related reduction in learning ability and concomitant increase in Aβ pathology, I investigated the effect of dietary administration of ibuprofen on learning ability, synaptic physiology and pathology in Tg2576 Alzheimer's disease model mice at different stages of the development of pathology.

The results from the study indicated that ibuprofen delays the onset of behavioural impairment in Tg2576 mice as tested in the FCA task in the T-maze, when it is administered early during the evolution of their disease phenotype. By contrast, if ibuprofen supplementation is started at a later point, when Aβ levels start to build up, no significant effect on learning and memory is observed. Only performance in the T-maze was improved, while levels of Aβ in the brains were not affected and electrophysiological studies were not conclusive. This study supports the clinical and epidemiological data providing evidence that long-term NSAID therapy has a dramatic effect on the incidence of AD in contrast to little effect as a symptomatic treatment.

6.4. Is modern lifestyle linked to developing Alzheimer's disease?

Researchers have identified a number of factors that may predispose to developing Alzheimer's disease. Some of them, like genes, are very hard to change, but more and more evidence is accumulating around lifestyle factors like unhealthy diet, diabetes, high cholesterol and blood pressure. Studies have shown correlations between high blood pressure, obesity, atherosclerosis, high cholesterol, high levels of homocysteine and the

subsequent development of AD years later. As the first neurodegenerative events in AD may start developing 20 or 30 years before the first symptoms appear, it seems that keeping healthy in younger life is vital to ensure healthy ageing.

High blood cholesterol as a risk factor for developing AD

High blood cholesterol can narrow the arteries and raise the risk of heart disease but may also induce neurodegeneration in the brain in AD. Several lines of evidence support the theory that an elevated blood cholesterol level is related to the development of Alzheimer's disease. In experimental models, animals fed high fat and high-cholesterol diets exhibited impaired learning and memory performance compared with animals on control diets and also demonstrated more Aβ deposition in the brain, greater loss of neurons, and other Alzheimer's disease-related neuropathology (Puglielli et al., 2003; Refolo et al., 2000). One study of 444 Finnish men found that an elevated blood cholesterol level in midlife was associated with 3 times the risk of developing Alzheimer's disease in late life (Notkola et al., 1998).

Additionally two studies of patients who had been prescribed statins found a significantly lower risk of Alzheimer's disease compared with similar patients who were not prescribed these medications (Jick et al., 2000; Wolozin et al., 2000). Statins, which reduce the levels of plasma cholesterol by inhibiting the enzyme HMG-CoA reductase, have been shown to reduce the risk of Alzheimer s disease by up to 70% (Austen et al., 2002).

Based on these data, the purpose of the project with GP56 transgenic rats was to explore whether increases in dietary cholesterol in transgenic rats over-expressing human mutated APP would induce Aβ pathology in their brains, thus causing disruptions in learning ability, synaptic plasticity and levels of Aβ in the brain. Homozygous GP56 rats were used and the results of this study demonstrated no important behavioural deficit. Although both the presence of the mutant transgene and the high cholestertol diets affected rat performance, these effects appeared to act independently and did not interact. There was no effect on Aβ levels in this model.

The relation between dietary cholesterol and development of APP driven pathology in GP56 rats was not established. There are a few possible explanations why cholesterol did not induce significant Aβ pathology in these rats: 1) the amount of cholesterol provided in

the diet was too low, 2) the administration period was too short or 3) the rats were too old. GP56 transgenic rats failed to exhibit any Aβ pathology and showed only slight disturbances in learning and memory and neurotransmission. If we assume that the observed changes could precede development of Aβ pathology, the results of this study support the role of cholesterol as an inducing factor of these changes, a topic that clearly requires further investigation using more sensitive experimental methodology.

DHA supplementation as a factor reducing AD risk

Saturated fatty acids are known to be bad for the heart, but as recent research is pointing out, they may also be bad for the brain. Studying more than 800 people over the age of 65, Morris et al (2003) found that the subjects with the highest intake of saturated fats were at over twice the risk of AD as those with the lowest intake of such fats. High intake of unsaturated, unhydrogenated fats (like PUFA) seemed to offer a slight protection against developing AD (Morris et al., 2003). Another study by the same authors established that older people who eat fish at least once per week reduce their chances of getting the illness four years later by 60 percent (Morris et al., 2003). These authors also examined the risk of disease according to intake of the n-3 fatty acids (see chapter 1.6). Higher total intake of the n-3 fatty acids was significantly associated with a lower risk for AD. DHA (one of the n-3 fatty acids) provided the strongest association, EPA was not associated, and ALA was associated with lower risk only among persons with the ApoE ε4 allele.

I investigated the effect of dietary DHA on learning ability and pathology in Tg2576 Alzheimer's disease model mice. The results indicated that chronic administration of dietary DHA to these mice slightly reduced transgene induced impairment in learning and memory as measured with the forced choice alternation task in the T-maze. These transgenic mice start showing memory impairment at the age of 8 months (Chapman, 1999). Tested at this age, animals on DHA-enriched diet performed significantly better than transgenic littermates on control diet, but also significantly worse than non-transgenic mice. This implies that DHA improved, but did not completely reverse the memory deficit and indicated that its effect was only modest. Also both in transgenic and non-transgenic mice that received DHA supplementation, the increase in DHA content in the brain was accompanied by a significant increase in the DHA/AA molar ratio, which

positively correlated with learning in the FCA task. No changes were detected in Aβ levels or plaque pathology, suggesting either the preventive effect of DHA on learning and memory deficits is not due to reduction of APP-induced pathology or that the APP metabolic product responsible for memory impairment was not measured. This study supports the clinical and epidemiological data suggesting that a high-PUFA (especially DHA) diet during the lifetime reduces the risk of developing AD.

6.5. Future plans and experiments

There are a number of points raised by these results that would merit further investigation.

For the ibuprofen and DHA studies, although the effects of NSAIDs and n-3 fatty acids are known to reduce the risk of AD, there is still little known about the mechanisms underlying their action. More research needs to be undertaken to clarify the mechanism underlying the action of both NSAIDs and n-3 fatty acids but also more questions need to be answered about the direct causes of dementia.

DHA supplementation was only tested in a prevention paradigm and it would be valuable to determine the effect of DHA on transgenic Tg2576 mice after 8 months of age.

Although both ibuprofen and DHA has a preventive effect on the development of memory impairment in the FCA task in the T-maze, it would also be important to confirm this finding using another behavioural task, like water maze.

Additionally more detailed biochemical analysis of Aβ and its subspecies, such as oligomers, should be performed. Also measuring markers of oxidative stress and inflammation in Tg2576 mice brains might provide more insight into action of both NSAIDs and n-3 fatty acids.

And finally comparing various doses and/or treatment periods, especially for cholesterol might help to induce pathology in GP56 rats.

This thesis has demonstrated that animal models are a valuable but limited tool in investigating pathology of Alzheimer's disease.

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PUBLICATIONS

1. Lee JH, Lau KF, Perkinton MS, Standen CL, Rogelj B, **Falinska A**, McLoughlin DM, Miller CC (2004). The neuronal adaptor protein X11beta reduces Abeta levels and amyloid plaque formation in the brains of transgenic mice. J Biol Chem. 2004 Sep 3

2. Chapman PF, **Falinska AM**, Knevett SG, Ramsay MF (2001). Genes, models and Alzheimer's disease. Trends Genet 2001 May; 17(5): 254-61

3. Westerman MA, Kotilinek LA, Lim GP, **Falinska AM**, Younkin LH, Cleary J, Chapman PF, Younkin SG, Frautschy SA, Cole GM, Ashe KH. "Restoration of memory by NSAIDs in a mouse model of Alzheimer's disease". Paper submitted to Neuron (2004).

PRESENTATIONS AT MEETINGS

1. **AM Falinska**; V.J. Marshall; P.F. Chapman "The effects of high-cholesterol diet on behaviour and synaptic physiology in APP transgenic rats". Society for Neuroscience 32nd Annual Meeting in Orlando, November 2 - 7, 2002

2. **AM Falinska**; A.S. Yates; P.F. Chapman "The effects of chronic ibuprofen treatment on learning and memory in APP transgenic mice". Society for Neuroscience's 31st Annual Meeting San Diego, CA November 10 - 15, 2001

3. "NSAIDs as a potential treatment for Alzheimer Disease" a seminar presented at the Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, June 2002

4. "Effects of anti-inflammatory agents on memory in Tg2576 mice" a talk presented at The Biology of Alzheimer's Disease in Transgenic Mice - The Third Meeting, San Diego, California, Nov 2001