The Influence of β-amyloid Pathology on Emotional Learning and Memory in the APPswe Model of Alzheimer's Disease

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Dedicated to my father, David Lelos.
You would have been so proud.

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Contents

Thesis Summary

Previous studies have shown that both the amygdala and frontal cortex contribute to emotional and motivational processes in rodents and humans. These regions show extensive amyloid pathology in humans with Alzheimer's disease (AD) and in mouse models of AD. However, the impact of amyloid production on emotional and motivational processes in mouse models of AD has not been systematically examined. The presence of pathology within key regions linked to emotional processes led to the hypothesis that Tg2576 mice, which express a human genetic mutation associated with early onset AD, would show age-related deficits in emotional reactivity and incentive and aversive learning and memory processes. Biochemical and immunohistochemical analysis confirmed the presence of extensive amyloid pathology in cortical, amygdala and medial temporal lobe structures in Tg2576 mice. Behavioural studies established impairments in anxiety, behavioural disinhibition and fear conditioning in Tg2576 mice. In contrast, other experiments showed that appetitive instrumental and Pavlovian conditioning remained goal-directed in aged Tg2576 mice. However, context-outcome associations were insensitive to post-conditioning changes in the value of the outcome in aged but not in young Tg2576 mice. In order to gain insight into how Aβ pathology influenced hippocampal-amygdala system activity during emotional learning, the final set of experiments assessed changes in the expression of the immediate early gene product c-Fos following acquisition and retrieval of fear memories. The findings from this thesis indicate that Tg2576 mice display relatively circumscribed changes in emotional reactivity and emotional memory processes that may reflect age-related alterations in amygdalo-hippocampal network interactions.

Abbreviations

Aß	ß-amyloid
ACH	amyloid cascade hypothesis
AD	Alzheimer's disease
ANOVA	analysis of variance
APOE	apolipoprotein E
APP	amyloid precursor protein
BLA	basolateral amygdala
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CeN	central nucleus of the amygdala
CR	conditioned response
CS	conditioned stimulus
DG	dentate gyrus
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
FAD	familial Alzheimer's disease
GABA	gamma-aminobutyric acid
hAPP	human amyloid precursor protein
IEG	immediate early gene
IL	infralimbic cortex
IR-nuclei	immunoreactive nuclei
ITI	inter-trial interval
mPFC	medial prefrontal cortex
NMDA	N-methyl-D-aspartate
NFT	neurofibrillary tangles
OFC	orbitofrontal cortex
LA	lateral amygdala
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIT	Pavlovian-to-instrumental transfer
PL	prelimbic cortex
PS1	presenilin-1
PS2	presenilin-2
SEM	structural equation modelling
S.E.M.	standard error of the mean
TBS	tris-buffered saline
TG	transgenic
US	unconditioned stimulus
WT	wild-type

Chapter 1: Introduction

1.0 Overview of the general introduction

The most deleterious symptom of Alzheimer's disease (AD) is the loss of cognitive function, which is most often characterized by severe memory impairments, particularly during the early stages of the disease. These cognitive abnormalities are accompanied by marked neuropathological changes, including the aggregation of β-amyloid and tau proteins and eventual cell loss. The precise mechanisms by which amyloid and tau production disrupt the functioning of neural circuits is still a matter of great debate. One major step towards answering this question has been the development of transgenic animal models of AD-related pathology. These models have provided valuable insights into disease mechanisms and their influence on cognitive systems. This introduction will provide an overview of the biological, behavioural and cognitive features of Alzheimer's disease, with a specific focus on the research that has been conducted on learning and memory processes in both human patients and animal models. It is argued that disease-related changes in emotion and emotional memory represent a significant component of the behavioural deficits exhibited by patients with AD. Nevertheless, these aspects of cognition and memory have been largely overlooked in mouse models of amyloid pathology, which has, understandably, focused on hippocampus-dependent spatial memory functions. Very few studies have investigated the effect of ß-amyloid pathology on brain systems contributing to emotional information processing, such as the amygdala. Thus, the following overview will summarise our current knowledge of the cognitive and behavioural impairments shown by AD patients and mouse models of this disorder. The review will then consider the impact that amyloid production has on emotional and motivational processes and conclude by hypothesising that ß-amyloid pathology present in the amygdala and frontal cortical regions in a mouse model of amyloid pathology (Tg2576 mice) will disrupt emotional information processing.

1.1 Alzheimer's disease: Prevalence and characteristics

Alzheimer's disease is a neurodegenerative disorder, which is characterized by severe deficits in cognitive function and progressive neurological impairment. The disorder was first recognised by the eponymous German psychiatrist, Alöis Alzheimer, in 1901, whilst treating 50-year old patient Auguste Deter. Before her death in 1906, Auguste D. displayed a host of symptoms, including severe loss of memory and comprehension, progressive cognitive decline, aphasia, paranoia, auditory hallucinations, acute psychosocial incompetence and even transient vegetative states (Maurer, Volk & Gerbaldo, 1997). Post mortem analysis of her brain tissue revealed evidence of neurological anomalies that were later characterized as neuritic plaques, neurofibrillary tangles and arteriosclerotic damage (Maurer, Volk & Gerbaldo, 1997). In 1912, Kraepelin suggested that the disorder discovered by Alzheimer be given his name and Auguste D. was subsequently recognised as the first person to be diagnosed with 'Alzheimer's disease' (Berrios, 1990).

1.1.1 Prevalence of AD

Alzheimer's disease is currently recognised as the leading cause of dementia, affecting an estimated 26.6 million people across the world in 2006 (Alzheimer's Association, 2007). An estimate of 700,000 sufferers in the UK has been suggested, with almost 163,000 new cases being reported each year (Alzheimer's Association, 2007). Approximately 5 million people currently have a diagnosis of AD in America (Alzheimer's Association, 2007). Over 4.9 million of the AD sufferers in America are aged 65 or over, while an estimated 200,000-500,000 AD patients are aged under 65. The latter are characterised as the less common early-onset form of the disease (Alzheimer's Association, 2007). It is estimated that 1 in 10 people over the age of 65 is diagnosed with probable Alzheimer's disease, with figures rising to 1 in 2 by the age of 85 (ADEAR, 2008). The prevalence of the disease in women is thought to be higher than in men, at a ratio of almost 3 to 1, while other forms of dementia, such as vascular dementias, tend to affect the sexes equally (Andersen et al., 1999).

1.1.2 Diagnosis of AD

While a definitive diagnosis of AD can only be made post-mortem, probable AD is diagnosed through a variety of cognitive tests and brain scans. The cognitive tests assess problem-solving abilities, attention, visuomotor coordination and abstract thinking and include the Mini-Mental State Exam, the Dementia of the Alzheimer Type Inventory or the Blessed Dementia Scale (McDougall, 1990). Physical examinations, neurological assessments and the use of both computerised tomography (CT) and functional magnetic resonance imaging (fMRI) scans also aid in the diagnosis of the disease. Such assessments provide information regarding extent of memory loss and neural atrophy exhibited by each patient. The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR, APA, 2000) provides a standardized framework of criteria to facilitate the diagnosis of AD. According to the DSM-IV-TR, the primary hallmark of probable AD is an impairment in memory, which can manifest itself either as difficulty in recalling previously learnt information or as an inability to remember new pieces of information. Memory impairment must occur in the presence of one or more other cognitive deficit(s), which could include aphasia, apraxia, agnosia and problems with executive function (DSM-IV-TR, 2000). It has also been shown that AD patients demonstrate a particular impairment in memory for emotional events (Mori et al., 1999). The processing of emotional stimuli is thought to be affected early in the course of the disorder, affecting patients' ability to discriminate emotional facial expressions (Kohler et al., 2005; Hargrave, Maddock & Stone, 2002), although a correlation is often noted with concurrent decreases in spatial or verbal cognitive function (Luzzi, Piccirilli & Provinciali, 2007; Cadieux & Greve, 1997).

Although impairments in memory and executive function are considered to be the primary features of AD, many non-cognitive neuropsychiatric symptoms also occur in the majority of AD suffers, including displays of apathy (Apostolova et al., 2007; Starkstein et al., 2001) paranoia, hallucinations/delusions (Perez-Madriñan et al., 2004), aggression/agitation, decreased inhibition (Burns, Jacoby & Levy, 1990a, b, c, d) and motor impairments (Camarda et al., 2007). Apathy,

which manifests itself as a loss of motivation and acute emotional blunting, is thought to be present in up to 70% of patients in the mild to moderate stages of the disease (Landes et al., 2001). In this context, apathy is defined as a motivational disorder comprising the following three core features: reduced goal-directed behaviour, reduced goal-directed cognitive activity, and diminished emotions (Robert et al., 2009). Functionally, apathy has been linked to hypometabolism in the basal ganglia and prefrontal circuitry, which results in the impairments in the execution of voluntary, goal-directed behaviours (Levy & Dubois, 2006; D'Antona et al., 1985).

1.1.3 Neurodegeneration

Post mortem analysis of brain tissue from AD patients has revealed the presence of distinct neuropathological changes at both the cellular and anatomical levels. One of the most distinctive hallmarks of the disorder is the devastating neuronal loss observed throughout cortical and subcortical structures. Progressive neurodegeneration has been measured using a variety of imaging techniques, with some estimates of the reduction in volume of left and right frontal lobes at 13% and 16%, respectively, of matched controls in the early stages of the disorder (Laakso et al., 1995). Severe atrophy occurs throughout medial temporal lobe regions, with an estimated decrease in the volume of the hippocampus of 38%, and 14% and 18% of the left and right amygdalae, respectively (Laakso et al., 1995). Similarly, an MRI study of amygdala atrophy estimated a 39.7% decrease in amygdala volume in AD patients in the early stages as compared to patients suffering from other forms of dementia. A 41.4% decrease in amygdala volume was observed between AD patients and healthy controls (Maunoury et al., 1996).

1.1.4 Neurobiology of AD

Primary histopathological hallmarks of AD include the presence of extracellular senile plaques, which contain aggregated amyloid peptides, and the intracellular accumulation of hyperphosphorylated tau protein. Amyloid, or senile, plaques consist of insoluble fibrillar proteins with a high content of ß-pleated secondary structure. ß-amyloid (Aß), the 39-43 amino acid peptide of 4KDa, is derived

through abnormal sequential proteolytic cleavage of the amyloid precursor protein (APP) by ß- and γ-secretases (Czech et al., 1997; Evin et al., 2003; Citron et al., 1992). The mechanisms underlying the pathological accumulation of Aß have not yet been fully elucidated, although the increase in plaque formation is known to occur as a result of the aggregation of monomeric Aß into oligomers, protofibrils and eventually to amyloid fibrils (Ward et al., 2000). The increase in Aß concentration in the brain is thought to either result from an increase in Aß production and/or from a reduction in Aß degradation combined with decreased clearance from the brain (Hardy & Higgins, 1992; Rosenberg, 2000).

The accumulation of intracellular hyperphosphorylated tau protein results in altered microtubule function, deficits in axonal transport and the formation of neurofibrillary tangles (NFT; Brion et al., 1991). Other histopathological features of AD-affected tissue include plaque associated and non-associated neuritic dystrophy (Larner, 1995; Dickson & Vickers, 2001; Einstein, Buranosky & Crain, 1994), microglia activation, oxidative stress, neuroinflammation (Shie & Woltjer, 2007; Streit, 2004), loss of synapses and probable apoptosis (Honig & Rosenberg, 2000). Although the complex interplay between ß-amyloid and tau pathology has yet to be clearly defined, there is evidence that subjecting hippocampal neurons to amyloid fragments results in an increase in tau protein phosphorylation, suggesting that amyloid pathology may precede and influence tau pathology, consistent with the amyloid cascade hypothesis (Pavia et al., 1998; see section 1.1.7 for a discussion of the amyloid cascade hypothesis). Furthermore, mutations on the gene encoding the tau protein result in excessive NFT deposition, without any evidence of amyloid (Hardy et al., 1998). Even when mutant mice express both APP and tau mutations, levels of amyloid remain unaffected, despite increases in NFT formation (Lewis et al., 2001).

1.1.5 Progression of amyloid deposition throughout the brain

German researchers, Heiko and Eva Braak defined six stages of AD (Braak & Braak, 1991; Braak, Braak, & Bohl, 1993) based on the progression of pathological changes throughout the demented brain. Their primary method of classification focused on changes in the development of NFTs and neuropil

threads, with the stages I-II classified as transentorhinal, stages III-IV as the limbic stages and stages V-VI as the isocortical stages. As a secondary method of classification, Braak and Braak (1991, 1993) grouped the stages of amyloid deposition into three broad categories. In Stage A, tissue showed low levels of amyloid deposition in the cortex, with the hippocampal formation as yet unaffected; Stage B tissue demonstrated more extensive amyloid deposition throughout most isocortical layers, with the hippocampus only showing mild pathology; Stage C tissue exhibited greater pathology throughout the isocortical layers, still only mild pathology throughout the hippocampal formation, and mild but consistent amyloid deposition throughout the nuclei of the thalamus and hypothalamus.

A decade later, Heiko Braak in conjunction with Dietmar Thal and colleagues (Thal et al., 2002), identified five distinct phases in which ß-amyloidosis occurs throughout the brain, with the neocortex being affected in Phase 1 and regions of the MTL, including the CA1 area, entorhinal region and the amygdala, being implicated in Phase 2. Neuritic plaques were found to be consistently present in high densities within the occipital and temporal lobes. Within the amygdala complex specifically, the accessory basal and cortical nuclei, as well as the periamygdaloid cortex were found to be the areas with the most extensive levels of senile plaques and NFTs (Arnold, et al., 1991; Kromer Vogt et al., 1990). In Phase 3, amyloid deposition extended to further subcortical regions, including the caudate nucleus, striatum, thalamus, hypothalamus and white matter. Phases 4 and 5 are characterized by more profound subcortical amyloid deposition, extending into a host of regions including the substantia nigra, reticular formation of the pons and cellebellar molecular and granuale cell layers (Thal et al., 2002). Furthermore, it is worth noting that the progression of neuropathogenesis has been show to correlate well with the expression of cognitive changes (Thal et al., 2002; Thind & Sabbagh, 2007).

1.1.6 Genetic influences

The majority of cases of Alzheimer's disease occur in people aged over 65 years and are categorised as the late-onset or sporadic form of the disorder. Only 1-5%

of cases are classified as the early-onset (<65 years) form of the disorder (McMurtray et al., 2006). Although the origins of sporadic and early onset AD remain unclear, a number of risk factors have been identified, including age, lifestyle factors and genetic influences. Four genes in particular have been linked to the development of sporadic and familial forms of the disorder. In particular, increased risk of developing the late-onset form of AD has been linked to the inheritance of the apolipoprotein E (APOE) gene (Corder et al., 1993). The APOE gene, found on chromosome 19, has three alleles, APOE -ϵ2, -ϵ3, -ϵ4. While APOE ϵ2 is thought to be potentially neuroprotective against AD, expression of APOE ϵ4 has been shown to correlate with increased risk of developing the disease and earlier age of onset (Corder et al., 1993). The increase in risk of developing AD correlates with an increased number of alleles (Corder et al., 1994; Chartier-Harilni et al., 1993). The protein encoded by APOE ϵ4, apoE ϵ4, has also been shown to be immunoreactive in senile plaques and NFTs (Namba et al., 1991). In vitro, apoE ϵ4 has been shown to increase phosphorylation of tau, and in vivo, overexpression of human apoE ϵ4 or ϵ3 in mutant mice led to increased plaque formation in the presence of the human amyloid precursor protein (hAPP; Huang et al., 2004). The apoE ϵ4 protein has also been associated with a decrease in presynaptic terminals, an increase in tau phosphorylation and in gliosis surrounding Abeta plaques (Huang et al., 2004).

Early-onset, or familial, AD (FAD) has been linked to the inheritance of a number of autosomal dominant mutations. Three genes in particular, APP, presenilin-1 (PS1) and presenilin-2 (PS2), have been identified (Citron et al., 1997). Nearly 200 genetic mutations have been documented on the PS1 and PS2 genes, on chromosomes 14 and 1, respectively, which normally encode transmembrane proteins in the γ-secretase protease complex (Bentahir et al., 2006). An important substrate of γ-secretase is amyloid precursor protein (APP) which, when cleaved by ß- and γ-secretases, produces ß-amyloid, the primary component of senile plaques.

A number of missense mutations have been identified on the APP gene, found on chromosome 21, that are tightly linked to FAD. This suggests that APP may play a primary pathogenic role in the development of the autosomal dominant form of the

disorder (Chapman et al., 2001; Thomas & Fenech, 2007). APP is an integral membrane protein whose normal function has not been fully elucidated, although there is a suggestion that it plays a role as a regulator of synapse formation and in synaptic plasticity (Priller et al., 2006; Turner et al., 2003). APP can be cleaved by three proteolytic enzymes, a- ß- and γ- secretases. When it is cleaved by a-secretase, it releases its extracellular domain, leading to a non-amyloidgenic processing pathway. In contrast, sequential proteolytic processing of APP by ß- and γ- secretases within the transmembrane domain produces ß-amyloid (Aß), a 39- to 42- amino acid peptide. In its fibrillar form, this peptide forms the primary component of senile plaques.

1.1.7 The Amyloid Cascade Hypothesis

The most widely regarded theory of AD pathogenesis to date is the amyloid cascade hypothesis (ACH; Hardy & Higgins, 1992). The main tenet of this theory holds that AD is driven by the accumulation of amyloid in the brain. It is argued that faulty cleavage of APP leads to an increase in Aß peptides, particularly a rise in the 42 residue peptide, resulting in neurodegeneration (Hardy & Higgins, 1992). All other pathological changes (tau pathology and cell loss) are thought to be downstream consequences of increased deposition and/or decreased clearance of Aß. The ACH was based on several lines of evidence, including the discovery that Aß is the primary component of senile plaques (Levy, Prelli & Frangione, 2006), evidence that people with trisomy 21 inevitably develop AD (Otson & Shaw, 1969), and the discovery that genetic mutations around the APP cleavage site favour proteolytic processing by ß- and γ- secretases, which ultimately leads to an increased production of Aß and its aggregation into amyloid fibrils (Citron et al., 1992). Multiple lines of evidence have emerged in the last decade in support of the ACH, including, firstly, the discovery that mutations on the presenilin genes (PS1, PS2) alter Aß metabolism, leading to an increase in Aß production (Hardy & Selkoe, 2002). Secondly, mutations of the gene encoding the tau protein, even in the most severe cases of NFT deposition, do not lead to an increase in amyloid deposition (Hardy et al., 1998). Thirdly, transgenic mice expressing both mutant human APP mutations and tau mutations demonstrate an increase in NFT formation, while amyloid deposition remains unaffected (Lewis et al., 2001).

These lines of evidence suggest that NFT formation is likely to occur downstream from changes in Aß processing, and consequently rule out the likelihood that amyloid deposition occurs as a consequence of NFT formation. Next, the discovery that crossing apoE knockout mice with APP transgenic mice markedly reduced amyloid deposition supported the link between the apoE gene and Aß metabolism (Bales et al., 1997). Lastly, there is a growing body of evidence suggesting a link between the development of AD in humans and genetic influences on Aß metabolism and clearance, such as those potentially produced by specific loci on chromosome 10 (Myers et al., 2000; Bertram et al., 2000). Hence, there exist several lines of evidence in support of the amyloid hypothesis and the primary role of amyloid metabolism in the pathogenesis of AD.

Although the amyloid hypothesis offers a tractable and testable theory of the cascade of events underlying AD, it has not succeeded in providing a detailed explanation of the origin of the disorder, nor an understanding of the precise mechanism(s) underlying the effects of altered Aß metabolism. Critics of the theory have argued that it cannot account for many aspects of AD pathogenesis. Importantly, it has been suggested that the degree of Aß deposition does not correlate well with cognitive decline and neuropsychiatric measures of dementia (Arriagada et al., 1992; Roses, 1994). Proponents of the amyloid cascade hypothesis, however, argue that the methods of analysis differs significantly between studies and research based on the more precise biochemical assays, especially of the 1-40 residue peptide, do show correlations with cognitive decline (Näslund et al., 2000; Cummings et al., 1996; Lue et al., 1999). Another argument concerns the discovery that the impact of APP, PS1 and PS2 mutations on *in vitro* cell culture models can often differ from the impact of the mutations *in vivo,* when expressed in the brains of transgenic mouse models. For example, Citron and colleagues (1997) reported, but are unable to account for, an increase in total Aß peptide concentration in brain tissue of double transgenic mice expressing APP and PS1 mutations. This increase was not observed in PS1-transfected 293 cells. Competing theories even question the neurotoxic role of ß-amyloid deposits, suggesting that the deposition of the peptide may protect against metal toxicity by limiting the effects of redox-active ions, and, thereby, aiding in protective antioxidant events (Rao et al., 2006; Bishop & Robinson, 2004; Savory, Ghribi &

Herman, 2002; Robinson & Bishop, 2002). Consistent with this suggestion, studies in which Aß is effectively cleared from the brains of AD patients, via immunization, have reported neither a decrease in the progression of neurodegeneration, nor an increase in, nor a restoration of, cognitive abilities (Holmes et al., 2008). Such controversy in the literature suggests that while the amyloid hypothesis can be viewed as a valuable delineation of a potential cascade of events that occur during disease progression, much of what occurs at the genetic and molecular levels is not currently well defined or understood. Consequently, many of the neurological mechanisms that underpin the impairment in cognition and behaviour in this disorder remain to be elucidated. The use of animal models of AD, therefore, represents a valuable means of investigating the impact of ß-amyloid (and other AD-related pathological characteristics) in the brain at both the molecular and behavioural levels.

1.2 Emotional expression and perception in AD

Although memory deficits are synonymous with AD, many patients also show changes in emotional processes. Neuropathological changes occur early in the development of the AD and these can lead to severe disruptions in the patients' mood and cognitive responses to emotional stimuli. Such changes include unprovoked displays of apathy, loss of motivation and goal-directed behaviours, paranoia, inappropriate sexual advances, bursts of hostility and anxiety (Grossberg, 2003). Cognitive changes are also manifest as deficits in emotion recognition and memory for emotionally-valenced stimuli. The following section will provide a brief review of the primary disturbances in emotional expression and perception in AD and their putative neural substrates.

1.2.1 Emotional disturbances in AD

Alzheimer's patients demonstrate changes in emotional expression that manifest themselves as a host of neuropsychiatric symptoms, including apathy, aggression, disinhibition and anxiety (Hollingworth et al., 2006; Lesser & Hughes, 2006). Anxiety has been shown to be increased in up to 70% of AD patients (Feretti, et al., 2001; Teri et al., 1999) and is diagnosed by assessing neuropsychiatric symptoms of excessive worry, restlessness, irritability, muscle tension, fear behaviour, and respiratory symptoms (Starkstein et al., 2007). Cognitive and behavioural changes in anxiety-related or disinhibitory behaviour are typically diagnosed in clinical settings using self-report tests or assessment instruments such as the Behavioural Syndromes Scale for Dementia (Devanand et al., 1992) or the Hayling Sentence Completion Test (Nash et al., 2007). Behavioural dysfunction in AD patients presents as cognitive disinhibition on tasks of attention and memory (Amieva et al., 2004; Nash et al., 2007) and behavioural disinhibition, in the form of aggression, hyperactivity, socially inappropriate behaviour, self-destructive behaviour and sexual disinhibition (Lesser & Hughes, 2006; Devanand et al., 1992). A recent study of AD patients revealed a distinct pattern in which symptoms of anxiety were more prevalent early on in the course of cognitive decline rather than the later stages of the disease. Bierman and colleagues (2005, 2007), report a correlation between increased anxiety and higher cognitive

function, followed by a progressive pattern of decreased anxiety in the presence of decreased cognitive function.

Apathy, a symptom of executive cognitive dysfunction, is one of the most prevalent neuropsychiatric syndromes seen in AD patients (Landes et al., 2001). Teng et al. (2007) suggest that the emergence and expression of apathy can be considered a predictor of AD in patients with mild cognitive impairment. Apathy manifests itself as a loss of initiation, a decrease in motivation and maladaptive changes in goal-directed behaviours (Boyle & Malloy, 2004; Brown & Pluck, 2000). Goal-directed behaviours can either be self-initiated or environment-stimulated. These behaviours include activities such as executing appropriate responses to environmental stimuli and planning (Robert et al, 2009). In addition, AD–associated changes in motivational behaviours are not thought to be associated with altered consciousness, cognitive decline, emotional angst or stress (Marin et al., 1991).

The neurological basis of apathy in AD is thought to reflect the interaction between cholinergic deficiency and frontal lobe impairment, as well as pathology within medial temporal lobe structures (Boyle & Malloy, 2004; Liu et al., 2004). Other neuropsychiatric symptoms, such as increased agitation, have been correlated with a higher burden of neurofibrillary tangles in the amygdala and frontal cortex (Cummings & McPherson, 2001; Liu et al., 2004), while apathy, depression and loss of motivation have been correlated with reduced frontal lobe and amygdala metabolism in late-stage AD patients (Cummings & McPherson, 2001; Senanarong et al., 2004; Liu et al., 2004). Emotional impairments, anxiety and agitation are also primary symptoms of a pre-senile form of dementia, which is characterized by localized amygdala degeneration (Shibuya-Tayoshi et al., 2005; Truitt et al., 2009). Furthermore, the severity of symptoms of irritability and agitation correlate with amygdala activation in fMRI assessments of AD patients (Wright et al, 2007).

1.2.2 Perceptual deficits in emotion recognition

Multiple studies have confirmed the development of impaired recognition of emotion in facial expressions in the early stages of AD, which is dissociable from the ability to recognize non-emotional features of a face (Hargrave et al., 2002; Albert, Cohen & Koff, 1991; Spoletini et al., 2008). Impairments in the production, repetition and comprehension of emotional prosody (the communication of non-verbal affective components of language) have also been reported in marginally and mildly impaired AD patients (Roberts, Ingram, Lamar, & Green, 1996). Research into the neural correlates of emotional processing in neurodegenerative disorders, such as AD, have demonstrated a loss of empathy and dysfunctional emotion processing, which is thought to correlate with degeneration in the right frontotemporal networks in the brain (Rankin et al., 2006). Several lines of evidence also consistently implicate the amygdala in face and emotion processing, particularly for the recognition of sad, angry and fearful facial expressions (Guyer et al., 2008; Fitzgerald et al., 2006). Such deficits in emotion recognition and production represent perceptual impairments that are distinct from the cognitive dysfunction assessed via emotional memory tasks.

1.2.3 Cognitive deficits in emotional memory

AD impairs the patients' ability to process fearful stimuli (Spoletini et al., 2008) and impairs the phenomenon of enhanced emotional memory (Hamann et al., 2000). In non-demented individuals, emotional arousal is typically correlated with enhanced memory for an emotional stimulus. A study of emotional memory and emotional expression in an early-stage AD patients found that even when the patients demonstrated normal emotional responses to stimuli, they displayed a deficit in memory enhancement for emotionally-laden stimuli (Hamann et al., 2000). More specifically, AD patients experienced emotion-related memory enhancement when recalling positively-valenced stimuli that was comparable to non-demented individuals. In contrast, negatively-valenced stimuli failed to enhance memory in AD patients. Hamann et al., (2000) suggested that the neural basis of the emotional memory impairment was linked to neurological dysfunction within the amygdala circuitry.

In summary, the previous sections outlined some of the key pathological, and psychological changes presented by patients with AD and highlighted the impact of Alzheimer's pathology on cognition, including memory and emotion processes. The following sections will summarise research on AD-related pathology and cognition in transgenic mouse models of AD.

1.3 Transgenic models of Alzheimer's disease

1.3.1 Transgenic models

The development of transgenic animal models has provided an invaluable method of studying the effect of genetic mutations on brain neurobiology and behaviour. The genome of a species can be altered in a number of ways to create genetically modified mice, including transgenic or knockout mice. Transgenic models are created via site-directed mutagenesis of a specific gene, by homologous recombination of a selected DNA sequence and the original chromosome, in embryonic stem cells (Capecchi, 1989). Gene targeting allows for the successful integration and expression of a human transgene in the genome of different mammalian species. The species of choice for genetic manipulation is often the mouse, *Mus musculus*, because of the extensive mapping of the mouse genome and the discovery that the mouse shares a large percentage of genes, 99%, in common with *homo sapiens* (Mouse Genome Sequencing Consortium, 2002). As a result, transgenic mouse models have become a powerful tool for studying pathogenesis and cognitive dysfunction in genetically inherited human diseases, such as the familial forms of AD. A large number of mouse models of AD exist (see, for example, Table 1) and therefore this section will focus on the description and characterisation of one model, Tg2576, which is the main focus of this thesis.

1.3.2 APP models and the Tg2576 mouse

Transgenic mice expressing human AD-related mutations develop pathological features that mimic many aspects of AD. To date, however, no one animal model based on a single mutation has successfully mimicked every aspect of the human

disorder, including amyloid deposition, tangle formation and cell loss. The molecular processes underlying the lack of neurodegeneration remain unclear, although an increase in transthyretin, a protein thought to be neuroprotective against amyloid fibril formation, and increased expression of genes involved in cell survival have been suggested as possible mechanisms of neuronal preservation (Stein & Johnson, 2002, but see Sousa et al., 2007). A means of circumventing this issue has been to create double, or even triple, transgenic models, which harbour multiple transgene insertions. For example, insertion of APP and/or PS1 mutations into mutant tau mice has, depending upon precise genetic loci, been shown to express multiple aspects of AD pathology, including amyloid deposition and tangle pathology (Pérez et al., 2005). Such methods succeed in demonstrating multiple features of human AD in mice, while attracting the obvious criticism that creating such genetic modifications is of limited applicability since they do not occur in human AD sufferers.

Since the discovery of familial AD mutations and susceptibility genes, the majority of AD-like transgenic models have been created with familial APP and PS1 mutations, which result in the overproduction of ß-amyloid and age-dependent cognitive deficits in mice. A recent review by Eriksen and Janus (2007) summarizes the current status of several transgenic models used in AD research (see also Duff & Suleman, 2004; Hock & Lamb, 2001). Table 1 (taken from Eriksen & Janus, 2007), presents an outline of a variety of single, double and triple transgenic APP mouse models, their transgene mutations and known cognitive deficits. A review of each transgenic model of AD is beyond the scope of this chapter, and thus on the grounds of parsimony the focus instead will be on the model used in this thesis, the Tg2576 APP transgenic mouse.

Tg line name	Transgene(s) mutation	Promoter	Cognitive deficits	Age of onset of neuropathology
PDAPP	APP mini gene, Indiana V717F	*PDGFβ*	Impaired: reference and working memory, OR	6–8 months: Aß plaques, dystrophic neurites, gliosis, loss of synaptic densities, hippocampal atrophy
Tg2576	APP Swedish, 695.K670N-M671L	Hamster *PrP*	Impaired reference and working memory, OR, CFC (trace and delayed paradigms)	9–11 months: Aß plaques, astrogliosis, microgliosis, increased oxidative stress, dystrophic neurites
TgAPP22	APP Swedish, 695.K670N-M671L, and Indiana V717F	Murine *Thy-1*	Not reported	18 months: Aß deposits
TgAPP23	APP Swedish, 695.K670N-M671L	Murine *Thy-1*	Impaired: reference memory, passive avoidance. Abnormal reflexes and stereotypic behaviour, seizures	6 months: Aß plaques, neuronal loss in CA1 region of HP
TgCRND8	APP Swedish, 695.K670N-M671L, and Indiana V717F	Syrian hamster *PrP*	Impaired reference and working memory. Accelerated extinction of taste aversion, reduced PPI, susceptibility to seizures	3 months: Aß plaques, increased inflammatory response
TgAPP/Ld/2	APP London V642I	Murine *Thy-1*	Impaired reference memory. Neophobia, "freezing"	13–18 months: Aß diffuse deposits, astrocytosis, microgliosis, neuronal degeneration
TgAPP/Sw/1	APP Swedish, 695.K670N-M671L	Murine *Thy-1*	Neophobia	18–25 months: Aß diffuse deposits, astrocytosis, microgliosis
TgAPP/Fl TgAPP/Du	APP Flemish, APP/A692G, APP Dutch, APP/E693Q	Murine *Thy-1*	Increased aggression, spontaneous seizures	No amyloid pathology in parenchyma or vasculature
J20	APP Swedish, 695.K670N-M671L and Indiana V717F	*PDGFβ*	Impaired reference memory	5–7 months: diffuse Aß deposits in DG and cortex. 8–10 months: Aß plaques, gliosis
TgAPPSweArc Arc6, Arc48	APP Swedish, 695.K670N-M671L, Indiana V717F, and Arctic E693K (E22G)	*PDGFβ*	Not reported	2–3 months: Aß deposits, neuritic plaques
Tg(M146L)1Tg(M146L)76Tg(L286V)198	PSEN1, M146L, L286V	Syrian hamster *PrP*	No behavioural abnormalities	No abnormal pathology up to 22 months
TgAPP/PS1	APP Swedish, 695.K670N-M671L (Tg2576); PSEN1, M146L	*PDGFβ + PrP*	Impaired: reference and working memory	6 months: accelerated Aß deposition, gliosis
TgAPP/PS1	APP Swedish, 695.K670N-M671L (Tg2576); PSEN1, A246E (Tg(PSEN1)5)	*PDGFβ + PrP*	Impaired: reference memory, trace fear conditioning	9 months: Aß plaques, gliosis, dystrophic neurites

Table 1: Overview of the main APP-based transgenic mouse models of AD, taken from Eriksen & Janus, 2007.

1.3.3 The APPswe mutation

Most AD-linked mutations on the amyloid precursor protein occur in or around the Aß cleavage site (Figure 1). Typically, mutations occurring around the transmembrane domain of APP have the effect of altering the cleavage pathway of γ-secretase, which leads to a higher yield of toxic 1-42 Aß fragments, rather than the soluble 1-40 Aß fragments (Suzuki et al., 1994). Normal processing of the APP protein involves a-secretase cleaving within the Aß domain, resulting in fragments of secretary APP (sAPPa) and a p3 C-terminal fragment. The p3 fragment is then cleaved by γ-secretase, releasing short p3-40 and p3-42 peptides. The "Swedish" mutation (APPswe), named after a large family in Sweden carrying an autosomally dominant early-onset form of AD, favours a pathway in which APP is metabolised by ß-secretase. The ß-secretase site resides in the more N terminal region of the Aß domain and cleavage here results in secretary APPß (sAPPß) and A4CT. When A4CT is cleaved by γ-secretase in the Aß region, it produces Aß 1-40 and Aß 1-42 fragments.

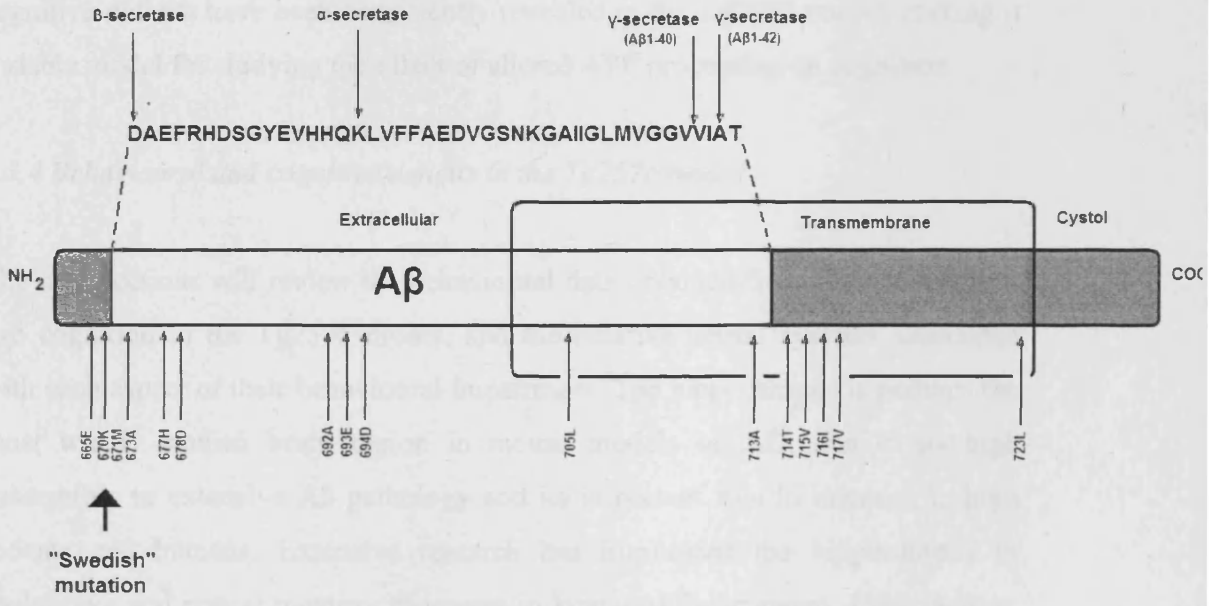


Figure 1: Segment of the amyloid precursor protein with Aß region and cleavage sites of the a-, ß- and γ-secretases. Amino acid substitution sites in identified FAD mutations, with the Swedish mutation labelled.

The Tg2576 mouse model is one of the most widely used APP models and overexpresses the Swedish 695-amino acid (K670N-M671L) double mutation in a hybrid background strain of C57B1/6J x SJL, under control of a hamster prion protein gene promoter (Hsiao et al., 1995, 1996; Mullan et al., 1992). Tg2576 mice display multiple features of amyloid-related neuropathology, including age-dependent deposition of soluble and insoluble Aß 1-40 and Aß 1-42 throughout cortical and subcortical brain regions. Aß levels increase at about six months of age, and congophilic plaque formation begins between nine and twelve months (Kawarabayashi et al., 2001). At this age, mice demonstrate a five-fold increase in the concentration of Aß 1-40 and a fourteen-fold increase in the concentration of Aß 1-42, as compared to young Tg2576 mice (Hsiao et al., 1996). Overt amyloid pathology occurs in conjunction with the development of astrogliosis (Irizarry et al., 1997), microgliosis (Frautschy et al., 1998), increased oxidative stress (Pappolla et al., 1998; Smith et al., 1998), and dystrophic neurites (Irizarry et al., 1997; Sasaki et al., 2002). Although this model mimics multiple aspects of amyloid-related pathology, it lacks a number of important characteristics of AD, including overt neurodegeneration and extensive tau hyperphosphorylated neurofibrillary tangle pathology. Despite this, age-dependent behavioural and cognitive deficits have been consistently revealed in the Tg2576 model, making it a viable model for studying the effect of altered APP processing on cognition.

1.3.4 Behavioural and cognitive deficits in the Tg2576 model

The next sections will review the behavioural data obtained from tests of memory and cognition in the Tg2576 model, and the putative neural systems associated with each aspect of their behavioural impairment. The hippocampus is perhaps the most widely studied brain region in mouse models of AD, due to its high susceptibly to extensive Aß pathology and its important role in memory in both rodents and humans. Extensive research has implicated the hippocampus in declarative and spatial memory processes in humans (Eichenbaum, 1999; Squire, Stark & Clark, 2004) and spatial memory processes in rodents (Jarrard, 1993). Standard spatial memory tasks for rodents include the T-maze non-matching to sample task (Blodgett, McCutchan & Mathews, 1949), a test of rewarded alternation, and the Morris water maze (Morris, 1984), a test of spatial reference

memory. Contextual fear conditioning has also often been employed as a means of assessing the impact of amyloid pathology on contextual and cued emotional memory in rodents (e.g., Maren & Holt, 2000). These deficits will be considered in more detail below.

While considerable evidence has amassed indicating amyloid-induced deficits in hippocampal function in the Tg2576 model, relatively little is known about the functional integrity of other neuronal systems that also display extensive amyloid pathology. It is vital to consider the impact of the APPswe mutation and altered β-amyloid metabolism on other neural systems to understand how amyloid production influences brain function more generally. In particular, the amygdala nuclei have been implicated in many fundamental aspects of learning and memory and have been implicated in cognitive and perceptual deficits in AD (as described previously). The following section will summarise our current understanding of the effect of β-amyloid pathology on emotion and memory functions attributable to the amygdala-cortical network in APP mice. It will become apparent that, other than superficial investigations, little is known about how the APPswe mutation impacts on emotional circuits in the brain.

1.3.5 Spatial memory in Tg2576 mice

Before considering emotional memory systems in more detail, the major effects of amyloid production on memory processes in Tg2576 mice will be summarised.

The majority of behavioural tasks used to study cognition in Tg2576 mice have focused on spatial memory tasks. Multiple studies have demonstrated impairments in Tg2576 mice as young as 9 months in spontaneous alternation in the Y-maze task (Hsiao et al., 1996; Ognibene et al., 2005; King & Arendash, 2002) and in the T-maze rewarded alternation task (Barnes, Hale, & Good, 2004; Chapman et al., 1999; Corcoran et al., 2002; but see also Bizon, Prescott & Nicolle, 2007). It has been consistently shown that hippocampal lesioned rats demonstrate a marked decrease in spontaneous and rewarded alternation in these paradigms (Stevens & Cowey, 1973; Means, Leander & Isaacson, 1971; Aggleton, Hunt, & Rawlins,

1986). Interestingly, however, deficits have also been reported on these tasks after lesions of the prelimbic and medial prefrontal cortex (Delatour & Gisquet-Verrier, 1996; Divac et al., 1975).

Perhaps the best known and most widely studied test of spatial memory in rodents is the Morris water maze task. This procedure requires animals to learn the position of a hidden escape platform using extra-maze cues to encode the platform position. The hidden platform version of the water maze task is considered to be particularly sensitive to hippocampal lesions in rodents (Morris, 1984; Morris et al., 1982; Cho, Friedman & Silva, 1999). Consistent with evidence of abnormal hippocampal physiology, Tg2576 mice show impaired learning in this task as early as 6 months of age (Hsiao et al., 1996; Westerman et al., 2002; Kotilinek et al., 2002; Stackman et al., 2003; Lesné et al., 2006; Quinn et al., 2007). Indeed, Tg2576 mice show robust deficits in several other commonly used spatial memory tasks, including the 8-arm radial arm-maze task (Pedersen et al., 2006; Watanabe et al., 2009), a water-based version of the radial arm maze (Morgan et al., 2000; Arendash et al., 2001) and the T-maze forced choice alternation task (Chapman et al., 1999).

Tg2576 mice also show spatial memory deficits in tasks that do not require explicit navigation to a location. For example, Tg2576 mice are capable of detecting object novelty and demonstrate appropriate responses to objects encountered more or less recently, but are impaired at detecting changes in the spatial arrangement of objects in an environment (Hale & Good, 2005; Ognibene et al., 2005). Furthermore, Good, Hale and Staal (2007) reported a core deficit in visuo-spatial memory that may underpin deficits in 'episodic-like' memory processes in aged Tg2576 mice. At an anatomical level, the impairment in the ability of animals to integrate item and spatial information is likely to be the result of damage to the hippocampal formation. Indeed, Knierim, Lee & Hargreaves (2006) have proposed that the spatiotemporal representations, characteristic of object-place associations, are formed in the dentate gyrus-CA3 hippocampal network.

Historically, impaired contextual fear conditioning has been associated with abnormal hippocampal function (e.g. Phillips & LeDoux, 1992; Maren &

Fanselow, 1997). Consistent with other evidence of hippocampal dysfunction , there is a body of data showing that contextual fear conditioning is impaired in Tg2576 mice, even as young as five months of age (Davidson, Putnam & Larson, 2000; Dong et al., 2005; Quinn et al., 2007; Comery et al., 2005; Dineley et al, 2002; Ohno et al., 2006). More specifically, age-dependent deficits in freezing elicited by contextual cues are evident in middle-aged (16-18 month) and older Tg2576 mice, but not in young (<5 months) Tg2576 mice (Corcoran et al., 2002). However, ascribing fear conditioning deficits to abnormal hippocampal function must be made with caution in the light of more recent evidence that questions the ability of cued and context conditioning paradigms to segregate the role of the hippocampus in learning and memory (Maren, 2008)

Interestingly, although contextual fear conditioning has typically been considered to be dependent upon the formation of a hippocampal-dependent conjunctive or configural representation (e.g., Rudy & Sutherland, 1989), more recent anatomical theories postulate a role for the basolateral amygdala in this form of learning. It has been argued that the basolateral amygdala is involved in the formation of feature representations that can influence contextual fear processing either independently or via hippocampal-amygdala interactions (Rudy, Huff & Matus-Amat, 2004; see sections 1.4.3 and 1.4.11 for a more extensive discussion of the neural bases of fear conditioning). Furthermore, although deficits in spatial learning/memory are commonly ascribed to abnormal hippocampal function, it is worth noting that lesions of the medial prefrontal cortex in rats have also been shown to impair acquisition in the water maze task (Mogensen et al., 1995). Thus, similarly to the watermaze, T- and Y-mazes, deficits in freezing to the context in Tg2576 mice may be the result of hippocampal damage or damage to closely linked neural systems.

Perhaps some of the clearest evidence for APP-related deficits in hippocampal function comes from assessments of hippocampal physiology in Tg2576 mice. Cacucci et al. (2001) recorded place cells in aged (16 months) and young (3 months) Tg2576 mice and found degraded environmental representations in the aged, but not the young, cohort. They also show that the degraded place cell performance correlated well with impairments in the acquisition of the T-maze

spatial memory task. In the Tg2576 mouse model, Jacobsen and colleagues (2006) have revealed correlations between early-onset deficits in hippocampal neurotransmission and long-term potentiation (LTP), reduced dendritic spine density in the dentate gyrus and deficits in a contextual fear conditioning task. Similarly, while revealing evidence of intact fast synaptic transmission and short term plasticity in the hippocampal formation, Chapman and colleagues (1999, although see Fitzjohn et al., 2001) established a correlation between spatial memory deficits in the T-maze and aberrant *in vivo* and *in vitro* LTP in the CA1 and dentate gyrus regions in Tg2576 mice. It has been suggested that Aß 1-42 may disrupt the mechanisms underlying LTP via activation of Aß and metabotropic glutamate receptors, followed by subsequent induction of c-Jun N-terminal kinase (JNK), cyclin-dependent kinase 5 (Cdk5) and p38 MAP kinase (MAPK) (Wang et al., 2004). Furthermore, *in vivo* injection of Aß 1-40 has been shown to result in a delayed reduction in N-methyl-D-aspartate (NMDA) receptor dependent baseline synaptic transmission in the CA1 region (Cullen et al., 1996). Hence, there is solid evidence suggesting that APPswe pathogenesis induces deficits in hippocampal LTP and impairs hippocampal pyramidal cells, which may underpin impaired memory encoding at both cellular and behavioural levels in Tg2576 mice.

1.3.6 Non-hippocampal memory systems

Comparatively little is know about other neural systems underlying learning and memory in the Tg2576 mouse model. Middei et al. (2004) showed that plasticity in fronto-striatal circuitry was intact in Tg2576 mice and that they were capable of superior procedural learning. Here, the authors hypothesised that pathological disruption of the hippocampal system allowed for enhancement of fronto-striatal contributions to learning in the form of improved motor habit learning (Middei et al., 2004). Interestingly, frontal lobe lesions that increase habit learning in rats have been shown to have a profound effect on motivational, goal-directed aspects of instrumental learning, such as contingency learning and incentive learning (Balleine & Dickinson, 1998; Coutureau & Killcross, 2003; Killcross & Coutureau, 2003). This aspect of learning has yet to be investigated in APP models.

One of the functions associated with components of the frontal cortex in rodents is executive function and goal-directed behaviour. Executive function can be defined as the ability to plan, initiate appropriate actions, shift set, acquire rules, inhibit actions and monitor complex goal-directed behaviour planning and select relevant sensory information (Tanji & Hoshi, 2008). The ability to reverse a learned stimulus-reward association, known as discrimination reversal learning, represents one form of executive function and has been shown to be dependent on the prefrontal cortex (Schoenbaum et al., 2002; McAlonan & Brown, 2003). Consistent with functional alterations in frontal lobe activity in Tg2576 mice, Zhuo and colleagues (2007) recently reported a deficit in reversal learning in 6-month old female Tg2576 mice, but also reported that this deficit was not present in the same cohort of animals at 14 months of age. Zhuo et al. (2008) again reported a similar deficit in reversal learning at 6 months of age in female Tg2576 mice, which is not present at 3 months of age, and that these deficits correlated with increased Abeta load in the prefrontal cortex. On a water-based T-maze reversal task, however, 11 month old mice have been shown to demonstrate intact reversal learning (Dong et al., 2005). The precise role of frontal amyloid pathology in the behavioural deficits in Tg2576 remains controversial. This research clearly highlights the importance of considering the effects of Aβ pathology on neural systems outside the hippocampus.

1.3.7 Emotion and motivation in the Tg2576 model

It is widely acknowledged that the amygdala plays an important a role in the recognition of emotion and emotional memory, as well as being a key structure in circuitry involving the consolidation, expression and extinction of fear. To date, the only form of emotional memory investigated in Tg2576 mice has been fear conditioning, and the results here have been variable (e.g. Corcoran et al., 2002; Barnes & Good, 2005). The following two sections outline the studies reporting impairments in fear conditioning and aberrant responses to unconditioned aversive stimuli in APP transgenic mice.

One of the few aspects of emotion-related behaviour assessed in APP models is the acquisition and expression of fear-related memories. The neural systems involved in processing emotional or fear-related events include the hippocampal formation, but perhaps the most well-studied are the amygdala and frontal cortical regions (Sotres-Bayon, Cain & LeDoux, 2006; Kim & Jung, 2006; Quirk & Mueller, 2008). Several studies have reported deficits in the context-based version of the fear conditioning paradigm in Tg2576 mice (Jacobsen et al., 2006; Dong et al., 2005; Quinn et al., 2007; Comery et al, 2005; Dineley et al., 2002; Ohno et al., 2006). However, the findings are less consistent when evaluating fear elicited by a conditioned Pavlovian cue. Maren and colleagues (Corcoran et al., 2002; see also Dong et al., 2005) demonstrated an impairment in contextual fear conditioning in Tg2576 mice only when the salience of the context was reduced, but no impairment in freezing to a conditioned auditory stimulus. Barnes and Good (2005), however, demonstrated normal freezing to the conditioned context, but an impairment in freezing to a Pavlovian conditioned auditory cue. Furthermore, Dong et al., (2005) reported impaired freezing in 11 month old Tg2576 mice to both contextual and auditory cues. Tg2576 deficits in contextual and cued fear conditioning implicate the amygdala-hippocampal circuitry as the sites of impairment (Phillips & LeDoux, 1992; see section 1.4.3 and 1.4.11 for discussions on the neural correlates of fear conditioning). However, while similar paradigms were employed in these studies, slight differences in experimental procedure (see section 3.5 for a comprehensive comparison) means that the precise psychological nature of these deficits and their neural substrates remains to be clarified.

1.3.8 Anxiety and behavioural disinhibition in the Tg2576 model

The Tg2576 model shows increased activity in exposed areas of an open-field arena and decreased avoidance of the open arm on the elevated plus maze (EPM) paradigm (Ognibene et al., 2005; Lalonde et al., 2003; King et al., 1999; Gil-Bea et al., 2007) consistent with a reduction in anxiety. An overall increase in locomotor activity has been suggested as the main mechanism of disinhibitory behaviour that is evident in the plus maze task (Gil-Bea et al., 2007). Behavioural disinhibition in rodents can be defined as the inability to withhold inappropriate responding (Paine & Olmstead, 2004). However, other groups have reported no

differences in locomotor activity *per se* during open-field and EPM testing, and thus suggested Tg2576 mice show a genuine decrease in anxiety (Ognibene et al., 2005). Furthermore, another APP transgenic mouse model expressing the APPswe mutation, in conjunction with the Indiana mutation, displayed increased anxiety as measured by the plus-maze task, reduced expression of calbindin and c-Fos in the hippocampus and significantly up- and down-regulated anxiety-related gene expression in the amygdala (Lee et al., 2004). Interestingly, Adriani et al. (2006) reported a deficit in motor impulsivity in Tg2576 mice, which manifested itself as an inability to inhibit nose-poke responding at short delays in an operant paradigm. Adriani et al. correlated this behavioural deficit with increased serotonin turnover in the frontal cortex. Thus, while the precise influence of the APP mutation on anxiety and emotional reactivity remains controversial, the evidence suggests that the APPswe mutations alter the functional properties of the brains systems supporting emotional perception, learning and memory.

Before outlining the nature of the neural systems that support emotional learning and memory, it is worth discussing two important caveats that must be acknowledged in developing and assessing mouse models of human neurodegenerative processes: the influence of sex and background strain.

1.3.9 Important caveats: Sex effects in the Tg2576 model

The majority of published research on Tg2576 mice uses male transgenic mice, and little comparative information is available concerning the effect of the APPswe mutation on female mice. An investigation of the effects of sex on APP pathogenesis in mice is of interest as women appear more susceptible to AD than their male counterparts. This observation is not well-understood, although oestrogens have been implicated in mediating this increased risk (Czlonkowska, Ciesielska & Joniec, 2003; Candore et al., 2006). Oestrogens are thought to play neuromodulatory roles in the brain through neuroprotective actions against, for example, increases in oxidative stress and excitatory neurotoxicity (Goodman et al., 1996). They have also been implicated in processes that modulate synaptic plasticity and reduce ß-amyloid formation (Foy et al., 2000). It has been postulated that age-dependent changes in oestrogen production may lead to increased levels

of Aß and Aß deposition (Petanceska et al., 2000). Consistent with this hypothesis, female hAPP mice demonstrate greater amyloid pathology than age-matched males (Callahan et al., 2001). Furthermore, Callahan et al., (2001) reported that 11-15 month-old Tg2576 female mice showed a higher senile plaque load, as well as higher levels of Aß 1-40 and Aß 1-42, relative to male mice.

Sex-related differences in behaviour have been reported on several tasks in Tg2576 mice. King et al. (1999) report that male Tg2576 mice were impaired relative to their female counterparts on the open field activity task and on the number of arm entries in a y-maze task. Female Tg2576 mice were impaired relative to male mice on a measure of passive avoidance and in a circular platform task, a task of spatial memory and learning (King et al., 1999). Deacon et al. (2008) reported that aged female Tg2576 mice showed deficits on a novel paddling Y-maze task and age-independent impairments in nest construction and burrowing behaviour. These cohorts of mice, however, did not show any impairments on a variety of other tasks, including the T-maze and Y-maze spontaneous alternation tasks and the T-maze reference memory task that are sensitive to the APP mutation in male Tg2576 mice (Deacon et al., 2008). Interpretation of this pattern of results is further hampered by the absence of male cohorts to permit a direct sex comparison. Overall, however, there is preliminary evidence to suggest that male and female Tg2576 mice may show parallel behavioural changes on some but not all behavioural tasks. To date, a systematic correlational investigation of the consequence of age- and sex- dependent effects of ß-amyloid on cognition has not been reported.

1.3.10 Important caveats: Background strain and the Swedish mutation

It is important to recognise the effect of the background strain of the mouse model on the phenotype created by the insertion of the Swedish double mutation. As with any transgenic model, it is not necessarily always the case that the expressed phenotype represents a direct reflection of the genetic alteration (Linder, 2006). Wolfer and Lipp (2000) argue that genetic variation and environmental influences could account for essentially all of the variability obtained using behavioural indices of learning and memory. For example, in comparison to the Tg2576 model,

C57BL/6 inbred mice expressing the APPswe and V717F mutations present not only a different behavioural profile (increased fear and anxiety instead of increased disinhibition), they also do not display plaque deposition up to 18 months (Lee et al., 2004). Hence, it is vital that any genetic mutation is expressed and analyzed in multiple background strains to determine the precise influence of the transgene insertion.

The genetic background for the APPswe mutation is the C57BL/6J x SJL hybrid and the Tg2576 model is maintained by repeated backcrossing into this strain (Hsiao et al., 1996). It has been suggested that the best method of maintaining genetic mutations is to perform 3 repeated backcrosses to at least 2 different inbred strains, then use the F1 hybrids from crossing the 2 congenic lines to analyse the phenotype (Branbury Conference, 1997). The APPswe mutation, however, has been shown to be deleterious, even lethal, to inbred strains (Hsiao et al., 1995; Carlson et al., 1997). Hsiao et al. (1995) reported that insertion of the APPswe mutation into FVB/N mice resulted in premature death and detrimental changes in central nervous system function, despite no overt extracellular amyloid deposition. The APPswe mutation was also shown to be lethal after 3-4 backcrossings when expressed in C57BL/6 mice (Carlson et al., 1997). Hence, this mutation has been found to be best maintained using the outbred C57BL/6 x SJL hybrid. Similarly, overexpression of V717F mutant human APP has also been shown to be best maintained on a mixture of Swiss, DBA/2 and C57BL/6 outbred mice (Games et al., 1995).

As a means of demonstrating the effect of different genetic backgrounds on phenotypic expression of the APPswe mutation, Lassalle and colleagues (2008) altered genetic heterogeneity (homozygous versus heterozygous) and the strain of origin (inbred C57BL6, inbred CBA, F1 C57BL/6 x SJL) and tested them using standard behavioural assays. All results were compared to an isogenic control group and were conducted on an F1 colony. Their results confirmed that the cognitive phenotype was most consistent between the isogenic controls and wild-type mice when the APPswe mutation was expressed in the C57BL/6 x SJL background (Lassalle et al., 2008). This study not only confirmed the rationale for

using this outbred strain, it also highlighted the need to always thoroughly assess the effect of genetic background on phenotype expression.

In sum, the above sections outlined the principles underpinning the use of transgenic models of disease, as well as summarising the current status of behavioural research using the Tg2576 model. The next section will consider the neural correlates of emotion.

1.4 Neural correlates of emotional memory

The use of neuroimaging techniques and lesion studies has allowed for the identification of several functionally important neural networks involved in emotional learning and memory. Based on the pattern of pathology evident in the APPswe mouse model discussed above, this section will briefly review the contribution of three key structures involved in emotional information processing and their anatomical connectivity: the amygdala nuclei, hippocampal formation and frontal cortical regions. An understanding of their basic anatomy and connectivity is relevant to discussion of their role in memory and emotion, particularly in the light of the work presented in Chapter 5. Table 2 (provided at the end of this section) summarises the contribution of these regions to a variety of appetitive and aversive procedures, each of which will be discussed in more detail below.

1.4.1 The amygdala complex: Connectivity

Historically, the amygdala formation has the strongest association with emotional memories and the perception of emotional stimuli (LeDoux, 2000). The amygdala is a multinuclear, almond-shaped structure that is situated on the medial edge of the temporal lobe. Subregions have been identified based on patterns of cytoarchitectonics, histochemistry, connectivity, functionality and distribution of neurotransmitters (Krettek & Price, 1978; Swanson & Petrovich, 1998; Pitkänen, 2000). Pitkänen (2000) identified three functionally distinct nuclear groups in the rat amygdala complex: the basolateral nuclei, the centromedial group and the superficial nuclei. The basolateral complex (BLA) comprises the basal nucleus, the lateral nucleus and the basomedial networks. The centromedial complex comprises the central nucleus (CeN) and the medial nuclei. The superficial nuclei include the cortical nuclei and the nucleus of the lateral olfactory tract (Pitkänen, 2000). The amygdala complex supports profuse efferent and afferent connections, both extrinsically and intrinsically among the individual amygdala nuclei. Heavy connections exist between the basal and lateral nuclei. Furthermore, the central nucleus receives moderate to heavy connections from the lateral and basal nuclei (Pitkänen, 2000).

Each of the amygdala nuclei supports a distinct pattern of connectivity with extra-amygdala regions. Efferent connections from the somatosensory cortex target the lateral, basal and CeN, while auditory and visual information innervate the amygdala via cortical and thalamic regions (Shi & Davis, 2001; LeDoux, Farb & Ruggiero, 1990). Multi-modal information reaches the amygdala via efferent projections from the hippocampal formation, perirhinal cortex and prefrontal cortex (Pitkänen, 2000).

The basal nucleus supports reciprocal projections with the medial temporal lobe memory system, including the CA1, CA3, subiculum and parasubiculum regions of the hippocampal formation, as well as the prefrontal cortex (Pitkänen, 2000). The CeN projects to multiple regions, including the hypothalamus and brainstem, while receiving some inputs from the prefrontal cortex, thalamus, medial temporal lobe system and hypothalamus (Pitkänen, 2000). The lateral nucleus projects to the prefrontal cortex and medial temporal lobe region, while also receiving inputs from the prefrontal cortex, the medial temporal lobe region and the hypothalamus (Pitkänen, 2000). Although the above does not constitute a comprehensive review of the dense connectivity of the amygdala nuclei, it is clear that the amygdala supports numerous reciprocal connections between cortical, hypothalamic, hippocampal and brain stem regions.

1.4.2 The amygdala complex: Unconditioned fear and anxiety

The amygdala complex has been implicated in the expression of unconditioned fear and anxiety-related responses (Gray & McNaughton, 2003; Truitt et al., 2009). Basolateral, central, lateral and medial nuclei of the amygdala regulate distinct aspects of unconditioned fear (Chen, Shemyakin & Wiedenmayer, 2006; Davis & Shi, 1999; Müller & Fendt, 2006). For example, a discrete subpopulation of interneurons in the basolateral amygdala has been identified that modulate anxiety-like behaviours (Truitt et al., 2009). Furthermore, injection of anxiolytics into the basolateral/medial nuclei of the amygdala in rats reduces anxiety-related behaviours in the elevated plus maze, a standard task of anxiety (Nunes-de-Souza et al., 2000; Zangrossi & Graeff, 1994). Injection of both anxiogenic and anxiolytic drugs has also been shown to alter immediate early gene expression in

the CeN (Thompson & Rosen, 2006) and exposure to the elevated plus maze increases c-Fos expression in the basolateral and central amygdala nuclei of rats (Albrechet-Souza, Borelli & Brandão, 2008).

1.4.3 The amygdala complex: Conditioned fear

Pairing(s) of a conditioned stimulus (e.g., a Tone, CS) and unconditioned stimulus (i.e., foot shock, US) endows the CS with the ability to evoke a representation of the US, resulting in a conditioned fear response (CR) – in rats this is typically a freezing response that reflects the specific sensory properties of the shock. Extensive evidence indicates that the amygdala is involved in learning and expression of conditioned fear responses (e.g. Kim & Jung, 2006; Paré, Quirk & LeDoux, 2004). For example, pre-training neurotoxic and electrolytic lesions of the basolateral amygdala have been shown to produce deficits in the acquisition and expression of conditioned fear to both contextual cues and discrete auditory (or visual) CSs (Campeau & Davis, 1995; Fanselow & LeDoux, 1999; Nader, Majidishad, Amorapanth & LeDoux, 2001; LeDoux, Cicchetti, Xagoraris & Romanski, 1990; Vazdarjanova, Cahill & McGaugh, 2001; Phillips & LeDoux, 1992).

Despite a large body of data indicating a crucial role for the BLA in fear conditioning, there is evidence that fear conditioning can occur after lesions of this structure. For example, extensive overtraining of the CS-footshock association can enhance freezing to the context in pre-training BLA lesioned rats. The neural basis of this BLA-independent fear conditioning is thought to be the CeN (Maren, 1999) or midbrain periaqueductal gray (Bandler & Shipley, 1994). Overtraining of the CS-US association in post-training lesioned rats, however, does not reinstate conditioned freezing, supporting the role of the BLA as a region necessary for the expression of Pavlovian CS-US fear associations (Maren, 1999).

Because the CeN provides heavy projections to, and modulatory influences upon, hypothalamic and brainstem nuclei, it has often been viewed as the region responsible for mediating various autonomic and somatomotor reactions characteristic of fearful behaviors (Davis, 1992; Campeau & Davis, 1995). It has

been suggested that the CeN can be influenced by both conditioned and unconditioned nociceptive stimuli, resulting in fear-defensive autonomic and somatomotor reactions (Davis, 1992). In accordance with this, neurotoxic and electrolytic lesions of the CeN in rats (as well as the lateral and basal nuclei) abolished freezing behaviour to both an auditory and context CS (Goosens & Maren, 2001). However, Koo, Han and Kim (2004) demonstrated that while electrolytic lesions of the CeN abolished freezing behaviour to the auditory CS and the context, neurotoxic lesions, which spared the fibres of passage, resulted in conditioned fear responses to the context and only a mild impairment in conditioned freezing to the auditory CS. It is worth noting, therefore, that evaluating electrolytic damage to the CeN may lead to a distorted view of the function of the CeN. Furthermore, it is worth noting that, although BLA projections to the CeN are thought to be important for conditioning, the CeN is also the target of afferent connections from the parabrachial nucleus, the parietal insular cortex and the posterior intralaminar nucleus of the thalamus, which relay nociceptive inputs and aversive sensory information that are also necessary for fear conditioning (Shi & Davis, 1999). Thus, while the BLA and the CeN are generally thought to support distinct aspects of fear conditioning, there appears to also be overlap in their contributions to the expression of conditioned fear. In accordance with this, Koo et al. (2004) have suggested that the BLA and the CeN may function in parallel during conditioned fear expression, via their efferent projections to downstream output structures. That is, conditioned fear expression involves parallel pathways, one of which courses through the CeN, via the BLA, while the other does not make active use of the CeN. Specifically, they argue that multi-modal CS information, such as context processing, involves a pathway that projects from the BLA to the CeN. For simpler modality-specific CS information, such as auditory tone processing, however, two pathways are engaged, one which projects from the BLA to the CeN and the other which supports projections from the BLA through the CeN but does not actively make use of the latter structure (Koo et al., 2004). Thus, either of these pathways may be capable of maintaining conditioned fear expression to the tone CS if the other is destructed.

The amygdala also plays a pivotal role in memory consolidation processes (Packard, Cahill & McGaugh, 1994). For example, Huff and Rudy (2004)

demonstrated that amygdala inactivation by muscimol, either before or after pre-exposure to a conditioning context reduced its ability to enhance fear memory in a contextual fear conditioning paradigm. Furthermore, injection of a protein synthesis inhibitor into the amygdala did not affect the consolidation of the fear memory, but post-conditioning injection caused an impairment in both contextual and auditory cued fear conditioning. Thus, Huff and Rudy (2004) suggest that the amygdala plays a role in regulating memory consolidation in the hippocampal region during the formation of conditioned fear memories.

1.4.4 The amygdala complex: Incentive learning

In contrast to aversive conditioning, incentive learning tasks involve the conditioning of appetitively motivated behaviours. Although the representation of affect (i.e. fear versus expectation of a food reward) differs between the paradigms, the formation of motivationally driven CS-US associations that specifies the sensory-emotional attributes of the US is consistent across paradigms. Furthermore, it will become clear in the discussion below that marked parallels are seen between the functions of individual amygdala nuclei in each form of learning. Considerable research has determined that aspects of both instrumental and Pavlovian appetitive incentive learning are dependent upon BLA and CeN function (Blundell et al., 2001; Cardinal et al., 2003; Everitt et al., 2000).

In appetitive Pavlovian, or "classical", conditioning procedures, a contingency is arranged between an initially neutral stimulus, such as a tone, and a biologically relevant US, such as a food reward (Pavlov, 1927). As a result of the tone-food pairings, the CS becomes capable of evoking conditioned responses (CR) based on the animals' representation of the CS-US association. Evidence has accumulated showing that the associative representations formed by such pairings result in a representation not only of the sensory properties of the US but also the motivational value of the US. For example, Hatfield and colleagues (1996) used a Pavlovian reinforcer devaluation procedure in which following light (CS) - food (US) pairings, the motivational value of the appetitive reward was devalued by injecting lithium chloride intraperitoneally following consumption of the US. While normal rats showed a reduction in conditioned behaviours elicited by the

CS, rats with pre-training BLA-lesions were insensitive to the post-conditioning changes in the incentive/motivational value of the reinforcer. In contrast, rats with CeN lesions, like normal rats, demonstrated an intact ability to spontaneously adjust their conditioned responses to the current value of the reinforcer (Hatfield et al., 1996). Pickens and colleagues (2003) demonstrated that BLA lesions made *post-conditioning* did not impair the ability of animals to spontaneously alter their responses to the conditioned light stimulus following a change in the incentive value of the reward. Hence, the authors concluded that the BLA is involved in forming representations of the association between CSs and the incentive motivational properties of a reward, but it is not essential for maintaining or updating these representations in memory, nor for expressing them in behaviour (Pickens et al., 2003).

Although the evidence presented thus far does not implicate the CeN in associative processes representing rewards, this structure, via its connections to hypothalamic and brain-stem structures, is involved in regulating behavioural and autonomic responses (Everitt et al., 2003). There is also evidence that the CeN, but not the BLA, is involved in certain aspects of Pavlovian conditioning procedures, such as autoshaping (Parkinson, Everitt, Robbins, 2000). Autoshaping occurs when an animal, presented with one reinforced and one non-reinforced CS, selectively approaches the CS that has been associated with food. The acquisition of this form of Pavlovian responding has been shown to be sensitive to CeN lesions, which suggests that the CeN is important for the development of a Pavlovian approach response (Parkinson, Everitt & Robbins, 2000). This preparatory approach behaviour is considered to be a form of sign tracking, which is under the control of Pavlovian mechanisms, as opposed to more flexible goal-directed instrumental behaviours (Parkinson, Everitt & Robbins, 2000; Williams & Williams, 1969).

While Pavlovian conditioning procedures result in an association between a CS and a US, instrumental conditioning paradigms are designed to arrange a contingency between an animals' behaviour and a US. A representation of the contingency between the action and the motivational properties of the outcome indicates that the animals' behaviour is goal-directed. Using an instrumental conditioning paradigm, in which rats were trained to lever press or pull a chain for

a food reward, Balleine and colleagues (2003) demonstrated that BLA-lesioned rats were impaired relative to sham-lesioned controls when the incentive value of the outcome was diminished by sensory-specific satiety. The lesion did not have an effect on the acquisition of the instrumental response, but did significantly decrease the impact of a satiety-specific devaluation treatment on responding for a reward, when tested both in extinction and with the reward available (Balleine et al., 2003).

Pavlovian CS-outcome associations have also been shown to be capable of exerting an influence over instrumental responding. A paradigm designed to assess this component of motivational learning, the Pavlovian-to-instrumental (PIT) transfer task, involves separately training an animal to perform an instrumental response to receive a food reward, while also conditioning the animal to associate a Pavlovian CS with the delivery of a food reward in alternate sessions. The transfer test then assesses the influence of the Pavlovian CS on behaviour, with the expectation that the presentation of the CS would enhance instrumental responding. It has been suggested that the Pavlovian stimulus becomes capable of facilitating instrumental responding when the Pavlovian CS and the instrumental action have been associated with the same outcome because this allows for the transfer of motivational learning (Corbit & Balleine, 2005). The PIT transfer effect, therefore, is thought to reflect the effect of learned motivational influence of the CS over the instrumental response because the CS evokes a representation of the motivational aspects of the action-US association. Considerable evidence points to the amygdala as a primary component of the neural system underlying this form of transfer (Dayan & Balleine, 2002). Furthermore, it has been suggested that the BLA and the CeN play different but complementary roles in this transfer phenomenon. That is, the BLA is critical for motivational processing of the outcome-specific incentive value of the US, while the CeN is involved in processing the more general motivational properties of the US (Corbit & Balleine, 2005). Indeed consistent with this view, Corbit and Balleine (2005) reported a double dissociation in the functions of the BLA and CeN. They designed a PIT task that allowed for outcome-specific and general transfer effect to be assessed simultaneously. Specifically, they conducted Pavlovian conditioning sessions with three stimuli each paired with a different outcome. However, only two of

outcomes were also associated with an instrumental response. This way, they were able to assess a general transfer effect elicited by the stimulus that was not associated with an outcome used in instrumental training. Their results showed that BLA lesioned rats were impaired on outcome-specific transfer, while CeN lesioned animals demonstrated an impairment only in the general transfer test (Corbit & Balleine, 2005). Corbit & Balleine (2005) concluded, therefore, that the BLA was necessary for a conditioned stimulus to gain access to the current sensory-specific incentive value of an associated US, while the CeN was implicated in the formation of an association that incorporates the general motivational properties of the US (see also Everitt et al., 2003).

Overall, the evidence reviewed here suggests that motivational learning processes are largely dependent upon the amygdala complex. Moreover, it is clear that the amygdala nuclei play a role in both aversive and appetitive conditioning paradigms – insofar as both rely upon accessing a representation of the (sensory-specific) motivational properties of the US. The BLA is, therefore, necessary for a CS to retrieve the motivational value of the US with which it is associated. The ability of the BLA to update the current incentive value of the US is consistent for CS-footshock, CS-reward and action-reward associations. The CeN, although not necessarily a site of CS-US association, is required for the maintenance of the more general motivational attributes of a CS and is involved in regulating behavioural and autonomic responses to CS presentations.

1.4.5 The frontal lobes: Connectivity

The prefrontal cortex comprises numerous subregions, including the orbitofrontal and ventromedial areas, the dorsolateral prefrontal cortex and the anterior and ventral cingulate cortex. Amongst others, the infralimbic (IL), prelimbic (PL) and orbitofrontal (OFC) regions support projections to the basal and lateral nuclei of the amygdala (Pitkänen, 2000). The lateral amygdala nucleus projects to the IL and PL cortices, while the basal nucleus supports efferent projections to the IL, PL, orbitolateral and medial OFC regions (Pitkänen, 2000). Although the CeN receives inputs from the infralimbic and prelimbic cortical regions, amongst

others, it does not support any projections to the prefrontal cortex (Pitkänen, 2000).

The frontal cortex also supports dense projections to the basal ganglia, via several distinct cortico-striatal loops (McGeorge & Faull, 1989). Projections from the PL cortex innervate the dorsomedial striatum, while the OFC projects predominantly to the dorsolateral striatum (McGeorge & Faull, 1989). Broadly speaking, the prefrontal cortex is involved in numerous higher order functions, including attention, working memory, decision making and goal-directed behaviour (Heidbreder & Groenewegen, 2003; Vertes, 2006). The functions of distinct prefrontal networks will be discussed below in terms of their involvement in anxiety/fear and motivational learning processes.

1.4.6 The frontal lobes: Unconditioned fear and anxiety

In humans, the amygdala-prefrontal circuitry has been consistently implicated in the development of anxiety disorders (Guyer et al., 2008; Monk et al., 2008). In rats and primates, however, there are minimal studies implicating the OFC in anxiety-related behaviours (Rempel-Clower, 2007). For example, OFC lesions in rodents affect behaviour in a hyponeophagia task, an unconditioned fear-inducing test. However, in the same study, OFC lesions had no effect on the successive alleys task, a standard anxiety test (Rudebeck et al., 2007). Thus, through its connections with the basolateral, basomedial and lateral nuclei of the amygdala, the OFC may play a role in unconditioned anxiety-related behaviours, although the amygdala may be considered the primary output region of such behavioural responses.

Similar to the OFC, few studies have reported evidence that the medial prefrontal cortex (mPFC) cortex is involved in unconditioned fear/anxiety. It has, however, been shown that exposure to the elevated plus maze increases c-Fos expression in the mPFC in rats (Albrechet-Souza, Borelli & Brandão, 2008). Furthermore, injection of anxiolytic drugs into, or lesions of, the mPFC have also been shown to reduce anxiety behaviours in the elevated plus maze (Shah & Treit, 2003, 2004). Thus, the frontal cortex may be involved in the expression of anxiety-related

behaviours, although greater investigation into the precise role of each subregion is required.

1.4.7 The frontal lobes: Conditioned fear

The prefrontal cortex is often considered to be involved in fear extinction processes (e.g. Milad & Quirk, 2002; Akirav & Maroun, 2007), rather than the acquisition or expression of fear. For example, lesions of the ventromedial prefrontal cortex block extinction learning in fear conditioned rats (Quirk, et al., 2000). It has been suggested that the prefrontal cortex influences amygdala function during extinction by inhibiting amygdala-dependent fear-related processes, although the mechanisms by which this occurs are currently unknown (Akirav & Maroun, 2007; Rosenkranz & Grace, 2002; Sotres-Bayon, Bush & LeDoux, 2004).

The prefrontal cortex is not typically associated with deficits in the acquisition or the expression of conditioned fear. In fact, damage to the prefrontal cortex has been shown to enhance freezing to a conditioned tone (Morgan & LeDoux, 1995). Furthermore, cellular imaging of the plasticity-associated gene zif268 revealed no change in expression in the ventromedial prefrontal cortex or ventral and lateral regions of the OFC upon retrieval of either contextual or cued fear memory (Thomas, Hall & Everitt, 2002). It has been shown, however, that lesions of the ventrolateral prefrontal cortex reduce fear reactivity to aversively conditioned contextual stimuli, while having no impact on CS acquisition or response extinction. Thus, it is suggested that the ventrolateral region may specifically play a role in processing contextual information rather than being implicated in fear extinction processes. Overall, destruction of the ventrolateral prefrontal cortex not only maintains freezing responses to conditioned stimuli, but it can, in some cases, increase the conditioned fear response.

1.4.8 The frontal lobes: Incentive learning

The frontal cortical circuitry is involved in the executive control of higher-order goal directed behaviours (Royall et al., 2002; Baxter et al., 2000). Interestingly,

lesions of the orbitomedial cortex have been implicated in the development of apathetic behaviours in humans and primates (Levy & Dubois, 2006). In this context, apathy is defined as a reduction in goal-directed behaviours that can be observed voluntarily and measured quantitatively. Furthermore, Levy & Dubois, (2006) suggested that the deficit reflects disruption of processing emotional-affective information, which is necessary for associating emotional-affective signals with goal-directed behaviours (Levy & Dubois, 2006).

In rats, the OFC is implicated in the encoding of outcome expectancy, an aspect of goal-directed behaviour (Furuyashiki, Holland & Gallagher, 2008). Different populations of neurons in the lateral OFC regions encode not only rewards and the environmental cues that predict them, but they also demonstrate response-selective firing that is specifically linked to the behavioural action, in this case nose poking behaviour (Furuyashiki, Holland & Gallagher, 2008). It has been reported that lesions of the OFC do not disrupt the acquisition of Pavlovian CS-outcome associations, but they do impair the ability of the animal to respond to changes in the current incentive value of the outcome (Gallagher, McMahan & Schoenbaum, 1999). Furthermore, Ostlund and Balleine (2007) reported that lesions of the orbitofrontal cortex abolished the transfer effect in a PIT task but did not disrupt instrumental learning or devaluation performance.

The medial frontal cortex is also thought to be an important component of the neural circuitry involved in the selection of voluntary actions (Rushworth, 2008). Specifically, this cortical region has been implicated in representing the value of an action by updating representations of both the reward itself and the effort/costs associated with executing the action (Rushworth, 2008). Furthermore, the mPFC, which supports substantial reciprocal connections with the amygdala complex, has been shown to be necessary for the acquisition of action-outcome associations in instrumental conditioning (Ostlund & Balleine, 2005).

The ventral portions of the mPFC, PL and IL regions, have been implicated in limbic system function. For example, while the IL influences autonomic activity, including heart rate and blood pressure (Neafsey, 1990), the PL is involved in more cognitive tasks, such as delayed response paradigms (Dalley, Cardinal & Robbins, 2004). Through their interactions with the basal ganglia, the PL cortex

and the sensorimotor cortex have been found to be involved in learning goal-directed behaviours and stimulus-bound habitual behaviours, respectively (Balleine, Liljeholm & Ostlund, 2009). Specifically, pre-training, but not post-training, lesions of the PL cortex disrupt performance on an outcome devaluation task, a test of incentive learning (Ostlund & Balleine, 2005). This suggests that although the PL cortex is not necessarily the site of action-outcome associations, it is involved in goal-directed learning. Thus, the medial and orbitofrontal cortices have been implicated in distinct aspects of incentive learning and motivationally influenced, goal-directed behaviours.

1.4.9 The hippocampal formation: Connectivity

The hippocampal formation consists of the hippocampus proper (CA1, CA2, CA3 subregions), the dentate gyrus, the subicular complex and the entorhinal cortex. The hippocampus comprises an integral part of the temporal lobe memory system. Projections from the hippocampal formation, with the exception of the dentate gyrus, innervate multiple amygdala nuclei, including the CeN, basal and lateral nuclei (McDonald, 1998; Maren, 1999; Pitkänen, 2000). The lateral and basal nuclei support moderate to heavy projections to the CA1 region, subiculum and parasubiculum. The basal nucleus also projects to the CA3 region of the hippocampus. The CeN does not provide any know projections to the hippocampus, although it receives inputs from the subiculum (Pitkänen, 2000).

1.4.10 The hippocampal formation: Unconditioned fear and anxiety

The ventral hippocampus, unlike the dorsal region, has often been associated with the expression of unconditioned fear and anxiety (Bannerman et al., 2003). Lesions or inactivation of the ventral hippocampus reduces anxiety-related behaviour on multiple anxiety tests, including hyponeophagia, black/white 2-compartment box test, a successive alleys test, a social interaction test and the elevated plus maze task (McHugh et al., 2004; Bertoglio, Joca & Guimarães, 2006). Intra-CA1 injection of cannabinoids significantly altered fear responses in

the elevated plus maze task (Roohbakhsh et al., 2007) and increased c-Fos expression has been reported in the CA3 region of the hippocampus after exposure to the elevated plus maze (Linden et al., 2004; but see also Albrechet-Souza et al., 2008).

1.4.11 The hippocampal formation: Conditioned fear

Historically, investigations of the role of the hippocampus in fear conditioning have supported the idea that damage to this region results in an anterograde amnesia for contextual stimuli, as well as a time-dependent retrograde amnesia that is specific to contextual memory (Anagnostaras, Gale & Fanselow, 2001). Fanselow and colleagues have argued that the hippocampus is vital for acquiring, consolidating and temporarily maintaining a configural/multimodal representation of contextual stimuli. In contrast, simple elements or stimuli, such as an auditory cue and simple features of the context, are able to be processed effectively in the absence of the hippocampus (Young et al., 1994; Anagnostaras et al., 1999, 2001; Anderson et al., 2006). It has also been shown that the dentate gyrus specifically is essential for establishing a valid representation of a context to which emotional experiences, either hedonic or aversive, can be associated (Hernández-Rabaza et al., 2008; Lopez-Fernandez et al., 2007). For example, Hernández-Rabaza et al. (2008) showed that pre-training lesions of the dentate gyrus that spared other hippocampal subregions resulted in impaired contextual fear conditioning. Furthermore, these rats were impaired relative to controls on a place conditioning procedure in which the context was paired with a cocaine reward. Thus, it is suggested that the dentate gyrus plays a specific role in establishing a representation of the context in which positive or negative stimuli are associated.

Although a role of the hippocampus in processing contextual stimuli is fairly well-accepted, the specific involvement of this region in contextual fear conditioning is somewhat more controversial (see Maren, 2008). While a number of lesion studies in rats have supported the role of the hippocampus in contextual fear conditioning (e.g. Phillips & LeDoux, 1992; Maren & Fanselow, 1997), several studies have reported successful fear conditioning to the context after lesions or inactivation of this structure (e.g. Maren et al., 1997; Richmond et al., 1999). Interestingly, Maren

et al. (1997) showed that while hippocampal lesions made at a number of time points post-training resulted in a temporally-graded amnesia for the context, lesions made 1 week prior to training did not abolish freezing behaviour to the context. Importantly, however, deficits in freezing to the auditory CS were observed at all time points in pre- and post-training lesioned animals. Furthermore, Wiltgen et al., (2006) reported that even when pre-training hippocampal lesioned rats demonstrated deficits in freezing to the context, this impairment could be rescued by exposing the animals to extensive CS-US pairings. These results suggest that conditioning to the context can occur in the absence of the hippocampus, presumably via an alternative neural network which is capable of conditioning to the discrete or elemental contextual cues. Maren (2008) suggests that the involvement of the hippocampus in contextual fear conditioning may be dependent on a variety of factors, including the nature of the contextual representation (i.e. configural versus elemental), number of CS-US pairings and the intensity of the unconditioned stimulus. Thus, although the hippocampus clearly plays a role in conditioning to the context (in some circumstances), a consensus view on the specific nature of its contribution remains to be achieved.

A contributing factor to the lack of consensus regarding the hippocampal contribution to fear conditioning is the fact that the dorsal and ventral components of this structure appear to make distinct contributions to this form of learning (Richmond et al., 1999). Specifically, Richmond et al. (1999) report deficits in contextually conditioned freezing after excitotoxic lesions of the complete or ventral hippocampus, but not after lesions of the dorsal hippocampus. The authors, however, account for the impairment by suggesting an increase in locomotor activity in situations of stress following complete and ventral hippocampal lesions. Bast, Zhang & Feldon (2003), however, reported that the blockade of NMDA-receptors in the dorsal hippocampus (both pre- and post-conditioning) resulted in a deficit in contextual, but not tone conditioning. Although generally it is accepted that the dorsal hippocampus is involved primarily in contextual fear, Ji and Maren (2005) have demonstrated that pre-training electrolytic lesions of this region can, in fact, disrupt freezing to an auditory CS. In the case of the ventral hippocampus, lesions, NMDA-receptor blockade and muscimol inactivation of this region have been shown to disrupt both contextual and cued fear conditioning (Esclassan et al.,

2008; Maren & Holt, 2004; Sutherland, O'Brien & Lehmann, 2008; Zhang, Bast & Feldon, 2001). Furthermore, ventral hippocampal infusion of Tetrodotoxin, which blocks neuronal activity, prevented fear conditioning to both tone and context (Bast, Zhang & Feldon, 2001). This pattern of findings has led several authors (e.g., Esclassan et al., 2008) to conclude that the dorsal hippocampus is involved in temporal and contextual aspects of event representation, while the ventral hippocampus is involved in fear and anxiety processes.

In addition to the dorsal-ventral dissociation, there is also evidence for a functional dissociation between subregions of the hippocampal circuitry. Hunsaker & Kesner (2008) revealed a dissociation between CA1 and CA3 lesions across the dorsal-ventral axis. Their results revealed that the dorsal CA3 and dorsal CA1 regions mediated contextual fear conditioning, while the ventral CA3 and ventral CA1 regions were involved in the retrieval of contextually conditioned fear. Furthermore, lesions of the ventral CA3 region resulted in impaired retrieval of auditory fear associations when tested both 24 and 48 hours after conditioning (Hunsaker & Kesner, 2008).

There is clearly some degree of overlap between the effects of amygdala and hippocampal lesions on contextual and cued fear conditioning. Maren and Fanselow (1995) have previously suggested a model to explain the potential interactions between the hippocampus and amygdala during fear conditioning. They proposed that the formation of contextual representations in the dorsal hippocampus influences the development of CS-US representations in the amygdala via the ventral hippocampus. Specifically, they suggested that the dense connections between the ventral hippocampus and the amygdala may be particularly important for allowing contextual representations formed in the dorsal hippocampus to come into association with US representations in the amygdala (Maren, 2001). Hence, it has been argued that although the ventral hippocampus may not be directly involved in establishing contextual representations, it may be required to convey these associations to the amygdala for association with footshock (Maren, 2001). Interestingly, however, Maren and Holt (2004) have demonstrated that pre-training electrolytic lesions and muscimol inactivation of the ventral hippocampus can result in an impairment in the acquisition of auditory

conditioned fear, while not affecting contextual fear. Post-training lesions of the ventral hippocampus, however, were shown to disrupt both contextual and auditory fear expression. These results suggest that the ventral hippocampus independently plays a role in the acquisition of auditory conditioned fear, as well as affecting the expression of both cued and contextually conditioned fear responses (Maren & Holt, 2004).

Thus, overall, it becomes clear that while the role of the hippocampus in context/spatial processing is fairly well-established, its involvement in fear conditioning is more controversial. Broadly speaking, the dorsal hippocampus does appear to engage in the temporal and contextual aspects of event representation, while the ventral region, perhaps via its connections to the amygdala, appears to process anxiety-related and cue-related features of an aversive event. Notably, however, both forms of fear learning can occur without the involvement of the hippocampus. Therefore, the presence of an intact hippocampus might bias the animal towards developing a configural representation of the event and may support learning about cued stimuli, but the loss of either of these processes may not necessarily reflect hippocampal dysfunction *per se.* As Maren (2008) suggests, the involvement of the hippocampus in fear conditioning is likely to be highly dependent upon the experimental design and the specific parameters employed. Thus, the degree of involvement of the hippocampus in any given experiment would need to be thoroughly assessed, as would the form of the disruption, be it fear learning, consolidation or expression.

1.4.12 The hippocampal formation: Incentive learning

The available evidence suggests that unlike the amygdala, lesions of the hippocampus do not impair incentive motivational learning processes. For example, Corbit & Balleine (2000) reported that rats with hippocampal lesions showed normal incentive learning processes (outcome devaluation effects) but impaired reaction to changes in the causal relationship between an action and its consequences (i.e. they were insensitive to contingency degradation). These authors suggested that this deficit may reflect the important role that the background context cues play in evaluating the predictive accuracy of a CS on an

action. They proposed that if hippocampal lesions impaired the formation of context-outcome associations it would make these animals less sensitive to contingency degradation. The application of this analysis to hippocampal function was subsequently drawn into question, however, with the publication of a second paper from the Balleine group that revealed the impairment in contingency degradation was likely mediated by damage to fibres of passage from the entorhinal cortex (Corbit, Ostlund & Balleine, 2002). Thus, the contribution of the hippocampus to the representation of causal relationships in instrumental learning remains to be clarified.

Ito, Everitt and Robbins (2005) hypothesized that the hippocampus is involved in inhibitory processes during appetitive conditioning, a concept that was based upon evidence that hippocampal lesions facilitated the conditioning of anticipatory locomotor activity for food and facilitated the acquisition of autoshaping behavior (Ito, Everitt & Robbins, 2005). The authors admit that theoretical interpretation of this facilitation was speculative but (similar to Corbit & Balleine, 2000) suggested that the impairment may reflect specific contextual-mediated inhibitory influences on Pavlovian approach behaviours. Thus, it is clear that hippocampal damage does not impact upon goal directed behaviours in rats. The available evidence suggests that the hippocampus may contribute to context-mediated representational processes contributing to appetitive associative learning but, at best, the conditions under which these processes are manifest are ill-defined at present.

In summary, Table 2 provides an overview of a series of behavioural paradigms that focus on aversive and appetitive motivation and learning, and the involvement of amygdala, hippocampal and cortical subregions.

		Unconditioned Fear/Anxiety: EPM	Contextual Fear Conditioning	Cued Fear Conditioning	Instrumental Outcome Devaluation	PIT-Outcome Specific
Amygdala	BLA	√	√	√	√	√
	CeN	√	√	√	×	×
Hippocampus	CA1	√	√	√	×	×
	CA3	√	√	√	×	×
	DG	×	√	×	×	×
	Dorsal	×	√	√	×	×
	Ventral	√	√	√	×	×
Frontal Cortex	mPFC	√	×	×	√	×
	OFC	×	×	×	×	√

Table 2: Summary of the brain regions implicated in each test of emotional memory. Inclusion is based upon lesion, inactivation and cellular imaging studies (see text). √ = evidence suggests this region is implicated in the given task. × = no evidence this brain region is implicated in the given task.

1.5 Thesis Aims

Despite considerable focus on the effect of AD pathology on emotion regulation and motivation in humans, very few studies have attempted to examine the impact of ß-amyloid pathology on emotional and goal-directed learning processes in animal models of AD. It is argued that such an analysis is vital in order to understand how the pathological processes of AD influence emotional/goal-directed learning processes that appear impaired during disease progression.

The aim of the present research, therefore, was to investigate the impact of ß-amyloid pathology on key emotional and motivation processes associated with amygdala, hippocampal and frontal cortex functions in Tg2576 mice. It was hypothesized that the accumulation of ß-amyloid in these regions would impact upon both emotional and incentive (goal-directed) learning processes. It was predicted, therefore, that Tg2576 mice would show age-related impairments in tests of unconditioned/conditioned fear and anxiety and appetitive goal-directed learning. Given the lack of consensus surrounding the contribution of the hippocampus to contextual and cued forms of aversive learning, an analysis of region specific changes in neuronal activity was also carried out using immediate early gene imaging following fear conditioning. The analysis of gene expression was carried out in conjunction with a statistical modelling technique to determine whether and how the APPswe mutation altered the pattern of network activity within the hippocampal-amygdala axis.

Chapter 2: Colony Preparation and Biochemical Analyses

2.0 Introduction

Prior to presenting the results of the behavioural studies, this chapter summarises the methods employed for both the generation of the Tg2576 colony, as well as the biochemical, immunohistochemical and anatomical analyses performed on the APPswe brain tissue. After generation of the colony, genotypic analysis was performed to identify mice harbouring the HuAPP695 mutation. Immunohistochemical analysis of aged tissue was performed to visualise transgene-dependent changes in amyloid protein expression in specific brain regions of Tg2576 mice. Protein quantitation, however, was achieved using enzyme-linked immunosorbent assays to provide a precise measurement of soluble and insoluble forms of the 1-40 and 1-42 species of amyloid in multiple brain regions. Finally, a retrograde tracer was surgically introduced into the BLA to examine the general features of amygdala connectivity in wild-type and Tg2576 mice.

2.1 Generation of the Tg2576 colony

Tg(HuAPP695SWE)2576 mice were maintained in a hybrid background of C57Bl/6J x SJL. The Tg2576 line was originally founded by a C57Bl/6J x SJL F3, which was then twice crossed back into C57Bl/6J (Chapman et al., 1999). In order to maintain the genetic contributions of each background and ensure overexpression of the hAPP transgene, subsequent breeding of the colony has involved crossing Tg2576 into a C57Bl/6J x SJL F1 line. In practice, the breeding of further generations has involved pairing a male Tg2576 mouse with a female C57Bl/6J x SJL F1. As a result, the contribution of C57Bl/6J has been estimated to range between 59% to 88% and the contribution of SJL to be between 12-41% (Chapman et al., 1999).

At approximately 6 weeks of age, mice were weaned and placed in single-sex cages consisting of 2-4 mixed-genotype littermates. Mice were immediately ear marked where necessary and tail tips of approximately 2mm were taken for genotyping.

2.2 Genotyping of the Tg2576 cohort: Polymerase chain reaction

2.1.1 Introduction

Mice were genotyped using polymerase chain reactions (PCR) to determine whether they harboured the APPswe mutation. PCR is a molecular biology technique that utilizes a DNA polymerase to amplify DNA by enzymatic replication. Amplification reactions require a DNA sample to be combined in a solution containing multiple reagents, including a thermostable DNA polymerase, oligonucleotide primers and deoxynucleoside triphosphates (dNTPs). The solution is placed in a thermal cycler, in which each heated cycle denatures the DNA helix and synthesizes a copy of each strand of the template DNA, ultimately increasing the template sequence exponentially. The amplified nucleic acid can then be analysed by gel electrophoresis to identify the presence of the Swedish mutation.

2.1.2 Method

Extraction of DNA

Tail tips (>1mm) were removed at 4 weeks of age using ethyl chloride and stored at -20°C until use. Tissue was digested by incubating in 600 µl of TES buffer (1M Tris HCl, 0.5M EDTA, 10% SDS) at 55°C for 20 minutes. The solution was incubated overnight at 55°C after the addition of 1.2 µl proteinase K. DNA extraction commenced the following day by adding 0.2 ml of 5M ammonium acetate and centrifuging at 13,000 rpm for 10 minutes to pellet the proteins. 650 µl of supernatant was then removed and placed in an Eppendorph containing 600 µl isopropanol. This solution was centrifuged at 13,000 rpm for 2 minutes to pellet the DNA-containing fraction. Supernatant was extracted, 150 µl of cold ethanol added and the Eppendorph was centrifuged for 2 minutes at 13,000 rpm. This step was repeated three times before the samples were placed in an oven dryer for 15 minutes. DNA was re-suspended in 60 µl of TE buffer (1M Tris HCl, 0.5M EDTA) and incubated overnight at 55°C.

PCR Analysis of Genomic DNA

For each DNA sample, master mix was prepared using 2.5 µl 10x buffer (supplied in BIOTAQ Red DNA Polymerase, Bioline), 1.25 µl 50mM $MgCl_2$, 1.25 µl 10mM dNTPs, 0.25 µl each of Primer 1501 (50 pmol), Primer 1502 (10 pmol) and Primer 1503b (10 pmol), 1.5 µl Taq Red (1 µg/µl), 16.75 µl RNase-free H_2O and 1 µl DNA. This solution was vortexed and centrifuged at 4000 rpm for 4 seconds before being placed in the PCR cycler. Oligomer primers used for detecting the APPswe transgene were 1503b (5'-CTGACCACTCGACCAGGTTCTGGGT-3') and 1502 (5'-GTGGATAACCCCTCCCCCAGCCTAGACCA-3'), while a probe for the endogenous prion protein required Primers 1502 and 1501 (5'-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3'). PCR amplification was carried through 30 cycles as follows: 1) denature at 94°C for 45 seconds, 2) anneal at 60°C for 30 seconds, 3) incubate at 72°C for 1 min 30 seconds. Samples were incubated for a further 5 minutes at 72°C before the reaction was terminated at 4°C. The amplification products were then separated by 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by staining with ethidium bromide. Samples were run along side a 100bp DNA ladder and known transgenic and wild-type samples. A lane containing master mix and water, in place of DNA, was also included as a means of detecting potential contamination. Figure 2 depicts a representative photograph showing PCR amplification of genomic DNA. The ~300-bp fragment represents the HuAPP695 region with the K670N, M671L APP transgene. The ~600-bp fragment represents the endogenous prion protein, which is present in both wild-type and transgenic Tg2576 mice.

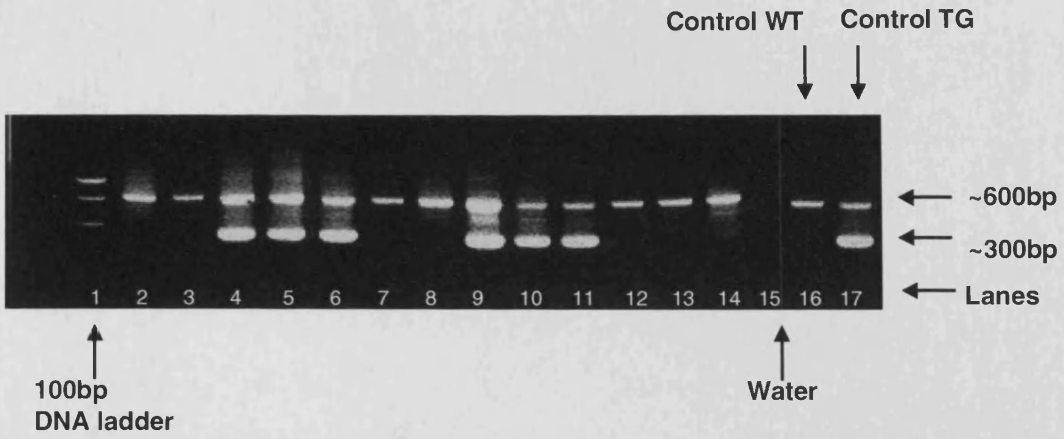


Figure 2. Representative photograph showing PCR amplification of genomic DNA from transgenic Tg2576 and wild-type mice. Lane 1 = 100bp DNA ladder; Lanes 2, 3, 7, 8, 12-14 = ~600-bp fragment represents the endogenous prion protein, which is present in wild-type mice; Lanes 4-6, 9-11: ~600-bp fragment of prion protein and ~300-bp fragment, which represents the HuAPP695 region with the K670N/M671L APP transgene; Lane 15 = H_2O control lane; Lane 16 = known WT genomic DNA sample; Lane 17= known TG genomic DNA sample.

2.3 Immunohistochemical analysis

2.3.1 Introduction

Immunohistochemistry is a common immunological and biochemical method of localizing proteins in a section of tissue. This process is based on the principle that monocolonal and polycolonal antibodies interact with specific antigens in biological tissues, allowing the distribution of the antigen to be visualised.

Indirect immunohistochemistry involves the binding of an unlabelled primary antibody with a specific antigen, followed by the addition of a conjugated secondary antibody, which must be against the immunoglobulins (IgG) of the species in which the primary antibody was raised (Miller, 2001). The biotinylated secondary antibody is then conjugated with streptavidin-horseradish peroxidase. This complex combines streptavidin, a biotin binding protein, with a peroxidase enzyme, to catalyse a reaction with 3,3'-Diaminobenzidine (DAB) to produce a brown-coloured stain (Hayat, 2002). This stain can be further enhanced with nickel to produce a purple-grey stain. This method allows the identification of the precise location of proteins in tissue.

Amyloid burden has been identified in the Tg2576 model by Congo red staining of neuritic plaques in the cortex and temporal lobe structures (Hsiao et al., 1996). Age-dependent immunohistochemical analysis of the 1-40 and 1-42 species of amyloid has also been performed in the Tg2576 model. Results revealed minimal deposition as early as 8-10 months of age, with substantial ß-amyloid deposition by 23 months (Kawarabayashi et al., 2001). Although the amyloid deposits revealed in the 23 month old cohort in this study appeared to be comparable to those seen in AD patients (Kawarabayashi et al., 2001), Sasaki et al. (2002) report the presence of both large (>50 micron) and giant (>75 micron) congophilic plaques in aged mice that are larger than those typically seen after post-mortem analysis of human AD tissue. The formation of the plaques, however, has been found to be qualitatively similar to those seen in human AD patients in terms of physical characteristics, including the localization of ß-amyloid 1-40 and 1-42 fragments (Terai et al., 2001). Thus, the distribution of amyloid was examined in

aged Tg2576 mice used in this thesis to confirm the presence of pathology in theoretically relevant brain regions.

Immunohistochemical analysis of Tg2576 tissue was performed as a means of visualising the distribution and localisation of amyloid overexpression. Specifically, it was of interest to determine whether expression of the 1-40 and 1-42 species of amyloid was detectable in the amygdala complex, as well as confirming the presence of pathology throughout hippocampal and frontal lobe regions.

2.3.2 Method

Twenty-two month old and five month old mice, five of each genotype at each time point, were deeply anaesthetised via intra-peritoneal injection of 0.2ml of the pentobarbital Euthatal. The mice were then exsanguinated, via insertion of a cannula into the left cardiac ventricle, with 80ml of 0.01M PBS, pH 7.4. This was followed immediately by perfusion with 100ml of cold 4% paraformaldehyde in 0.01M PBS (PFA). The brain tissue was extracted and fixed for a further 8 hours in 4% PFA at 4°C, before being transferred to a solution of 30% reagent grade sucrose in dH_2O for 48 hours at 4°C. Tissue was mounted on a freezing microtome at -20°C and cut into 30µm coronal sections. Sections were stored at -20°C in ethylene glycol-based cryoprotectant until they were used for immunohistochemistry.

Immunohistochemical staining of the tissue for the 1-40 and 1-42 species of amyloid commenced with an antigen retrieval stage, designed to reduce the effect of the PFA fixative and enhance staining of the antigen. This involved immersing the tissue in 85% formic acid in dH_2O for 10 minutes at 25°C, then rinsing with 0.1M TBS (Tris-buffered saline, pH 7.4). Endogenous peroxidise activity was blocked by incubating the issue in a solution of 10% methanol, 10% H_2O_2 in dH_2O for 5 minutes. After washing 4 x 10 minutes with 0.1M TBS, non-specific binding was blocked by immersing tissue in 3% normal goat serum (NGS) in 0.1% Triton X-100 in 0.1M TBS (TXTBS). Tissue was then transferred to the primary antibody (Rabbit anti ß-amyloid 1-42, Catalog # AB5078P or Rabbit anti ß-amyloid 1-40,

Catalog # AB5074P, Chemicon) diluted 1:1000 with 1% NGS in TXTBS and incubated overnight at room temperature on a stirrer. Tissue was then washed in TBS 4 x 10 minutes, and incubated in goat anti-rabbit biotinylated IgG secondary antibody (Vectastain Elite ABC kit, Catalog # PK-6101, Vector Laboratories), diluted 1:200 in TBS with 1% normal goat serum (NGS), for 2 hours. After washing thoroughly, tissue was immersed in an avidin-biotinylated enzyme complex (Vectastain Elite ABC kit, Catalog # PK-6101, Vector Laboratories), consisting of 1% reagent A and 1% reagent B in TBS for 2 hours. Tissue was washed with TBS, followed by 0.05M Tris buffer, and stained with the peroxidase substrate 3,3'-diaminobenzidine (DAB) with nickel (Ni^{2+}) for 1-3 minutes (DAB Substrate Kit, Vector Laboratories). The reaction was stopped with cold 0.1M PBS and mounted onto gelatin-coated slides. After dehydrating in ascending series of alcohols and clearing in xylene, the slides were coverslipped with DPX (Di-n-butyl Phthalate in Xylene) mounting medium.

2.3.3 Results

Figure 3 depicts staining of the 1-42 species of ß-amyloid with DAB plus nickel in 22 month old transgenic Tg2576 mice. Figure 4 depicts staining of the 1-40 species of amyloid in aged transgenic Tg2576 mice. Tissue from wild-type mice (data not shown) was devoid of any staining, regardless of whether it was treated with the antibody against the 1-40 or 1-42 fragment. Similarly, tissue from 5 month old mice (data not shown) demonstrated no staining of either the 1-40 or 1-42 species of ß-amyloid.

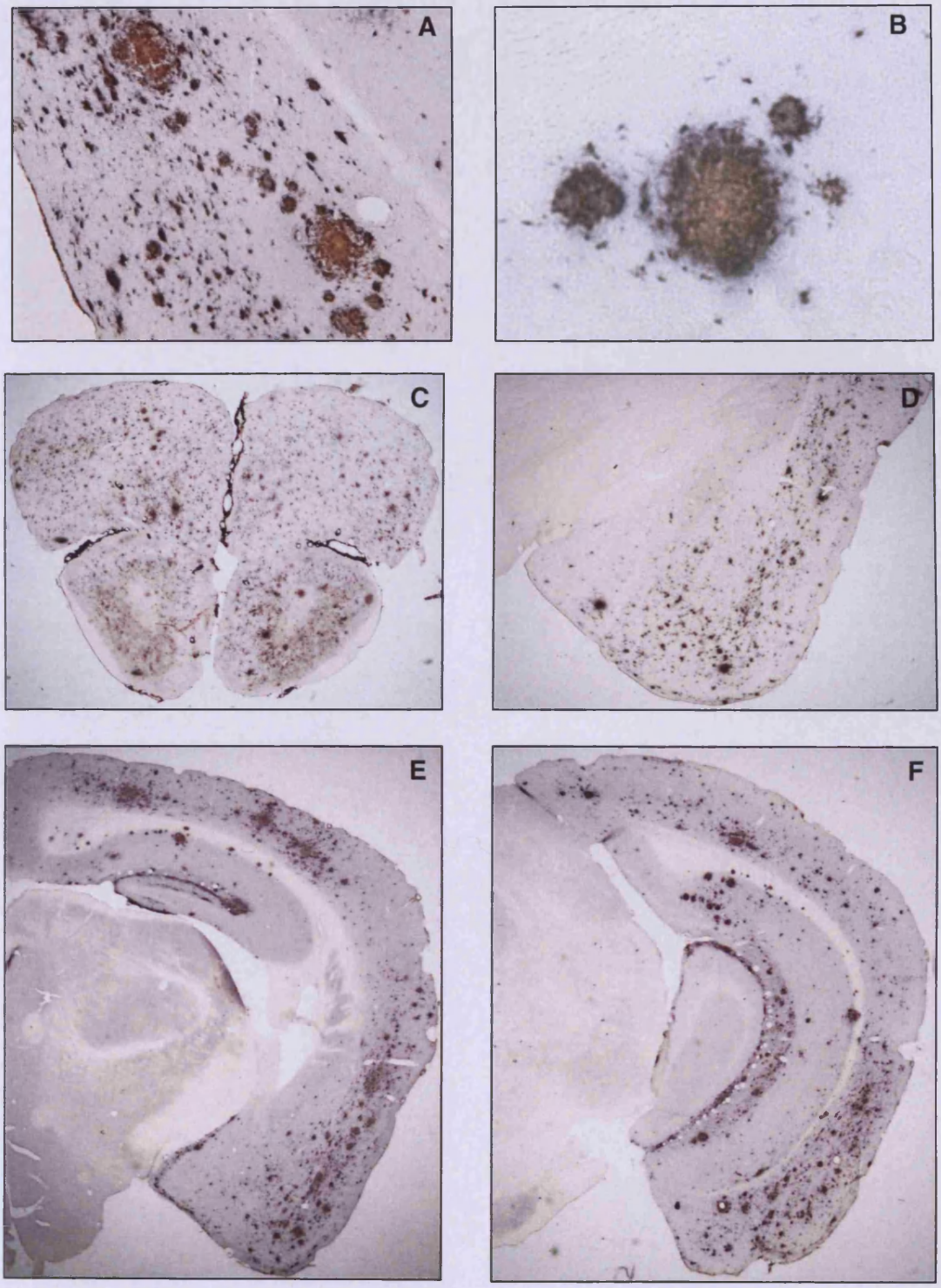


Figure 3: Immunohistochemical staining of the 1-42 fragment of β-amyloid in aged transgenic Tg2576 mice. A= 5x magnification of β-amyloid 1-42 deposits; B = 10x magnification of β-amyloid 1-42 deposits; C = distribution of β-amyloid 1-42 in the frontal cortex; D = β-amyloid 1-42 in the amygdala; E = β-amyloid 1-42 in the dorsal hippocampus; F = β-amyloid 1-42 in the ventral hippocampus.

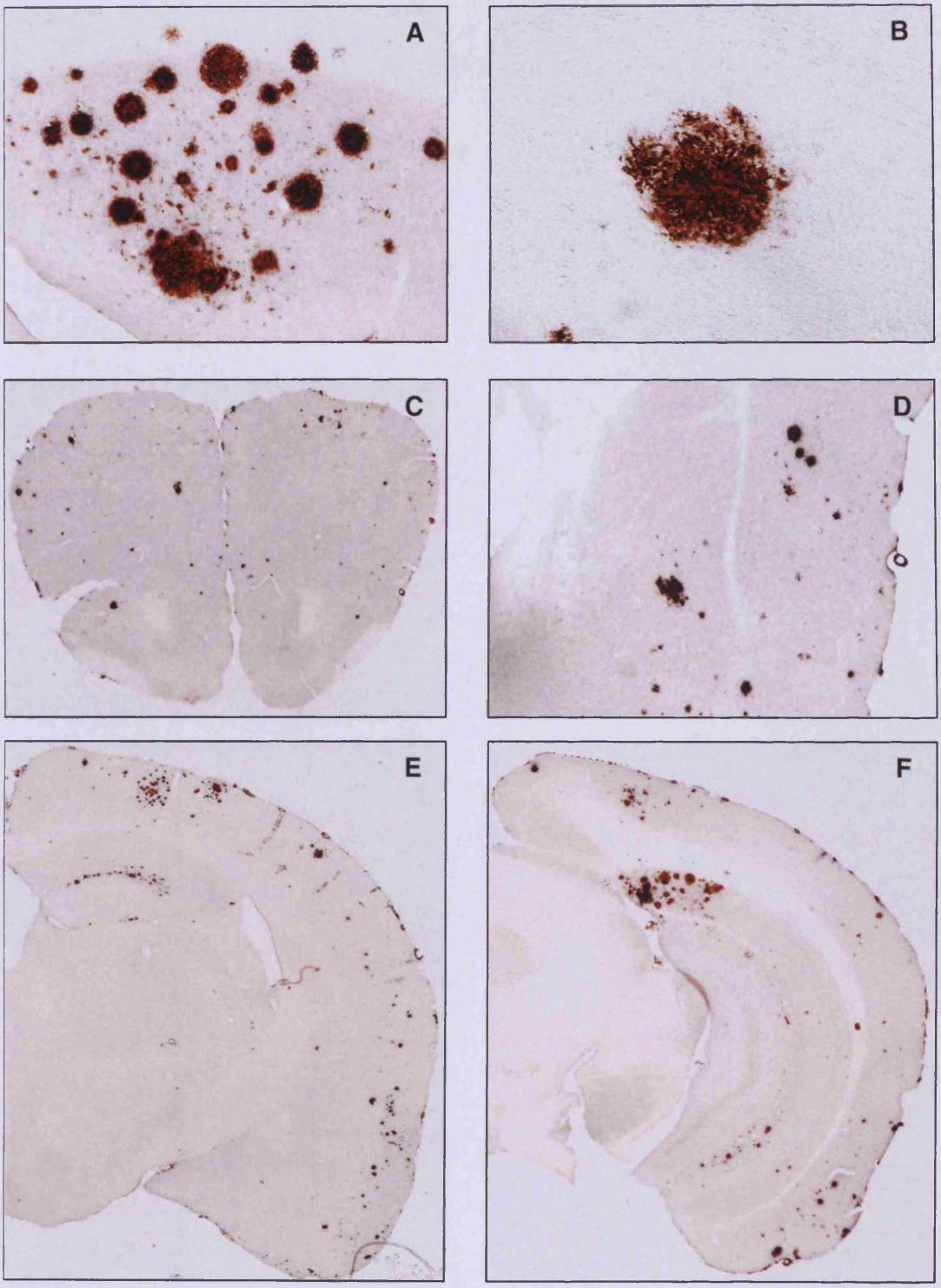


Figure 4: Immunohistochemical staining of the 1-40 fragment of β-amyloid in aged transgenic Tg2576 mice. A= 5x magnification of β-amyloid 1-40 deposits; B = 10x magnification of β-amyloid 1-40 deposits; C = distribution of β-amyloid 1-40 in the frontal cortex; D = β-amyloid 1-40 in the amygdala; E = β-amyloid 1-40 in the dorsal hippocampus; F = β-amyloid 1-40 in the ventral hippocampus.

2.3.4 Discussion

The immunohistochemical application of antibodies against ß-amyloid 1-40 and 1-42 on tissue from aged Tg2576 mice revealed extensive pathology throughout cortical and medial temporal lobe structures. The presence of ß-amyloid pathology, as a result of hAPP overexpression, was observed in the hippocampus, frontal cortex and amygdala complex. Furthermore, protein deposition can be seen in the prelimbic and infralimbic regions of the cortex, and in other cortical areas, including the perirhinal and piriform cortex, and the subiculum. The finding that tissue from young (5 month old) transgenic Tg2576 mice and wild-type mice of both ages was completely devoid of staining confirms that the pathology is both age-dependent and specific to mice overexpressing the hAPP transgene.

2.4 Enzyme-linked immunosorbent assays

2.4.1 Introduction

Enzyme-linked immunosorbent assay (ELISA) is a biochemical method used for the quantitation of proteins in biological tissues. This immunological technique is based on the principle that an antigen-antibody complex can be linked with an enzyme, which, following further chemical reactions, emits a chromogenic signal that can be measured. Sandwich ELISAs, which constitute one form of this technique, can detect minute levels of an antigen in a sample. In a sandwich ELISA, a plate is coated with a capture antibody and the biological sample is added, allowing the antigen in the sample to interact with the antibody. A detection antibody is added, which couples with the antigen, and an enzyme-linked secondary antibody is included, which attaches to detection antibody. Finally, a chromogenic substrate is introduced, which is converted by the enzyme into a colour that can be measured by the level of absorbance.

Protein quantitation by ELISA has been performed previously in the Tg2576 model (e.g. Chauhan & Siegel, 2007; Lim et al., 2005). Results consistently demonstrate higher levels of Aß 1-40 than Aß 1-42 in the soluble fraction (Sigurdsson et al., 2001) and the insoluble fraction (McGowan et al., 2005). Sandwich ELISAs were used a means of measuring the quantity of soluble and insoluble forms of the 1-40 and 1-42 species of ß-amyloid in the Tg2576 model. In order to compare regional differences in amyloid pathology, cortical, hippocampal and amygdala regions were dissected bilaterally from the brain tissue of 22 month old mice. This age was selected in order to assess the optimal extent of pathology present at the terminal stage of a mouse life span.

2.4.2 Method

Protein Extraction

Four twenty–two month old Tg2576 and wild-type mice were culled via cervical dislocation and brain tissue was immediately extracted. Tissue was maintained on

ice while the hippocampus, amygdala and the entire cortex were dissected from each hemisphere. For each region, the dissected portions of the two hemispheres were pooled together and snap frozen in liquid nitrogen and stored at -70°C until ready to be processed.

During the protein extraction phase, tissue was weighed and maintained on ice. Extraction buffer (2% SDS in dH_20 with 1% Inhibitor Cocktail (Calbiochem, catalogue #539134)) was added at a rate of 1ml/75mg wet tissue. Tissue was homogenised and rotated overnight at 4°C, before being centrifuged at 100,000g (28,300rpm) for 1 hour at 4°C. Supernatant containing the soluble amyloid was removed, diluted 1:40 in phosphate buffer (20 mM sodium phosphate, 0.2 mM EDTA, 0.4 M NaCl, 0.05% CHAPS, 0.05% sodium azide, pH 7) and stored at -20°C. 70% formic acid in dH_20 was added to the tissue residue at a rate of 1ml/150mg of starting tissue wet weight. Tissue was homogenized and centrifuged at 100,000g for 1 hour at 4°C. Supernatant containing insoluble amyloid was removed, taking care not to include the lipid layer, and neutralized with 1M Tris phosphate buffer, pH11, at a 1:20 dilution.

Protein Assay

The total volume of protein in each region was determined using the bicinchoninic acid method (BCA, Pierce). Protein standards ranged from 1 mg/ml of bovine serum albumin (BSA) to 0.01 mg/ml. BCA working reagent (WR) was prepared by mixing 50 parts BCA Reagent A (Pierce, Cat. 23223) with 1 part BCA Reagent B (Pierce, Cat. 23224). After 25µl of each sample and standard were added in duplicate to the 96-well plate, 200µl of WR was applied and the plate was mixed thoroughly for 30 seconds. The plate was then incubated at 37°C for 30 minutes, cooled at RT for 5 minutes, and the absorbance was read at 540nm. Data are presented below as pg of ß-amyloid per µg of total protein, where total protein refers to total soluble protein, since levels of insoluble protein were negligible.

ELISA

To quantify soluble Aß 1-40, the capture antibody, 6e10 (Chemicon), was used at a concentration of 5 µg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) and absorbed onto a 96-well plate (Greiner). The antibody solution was incubated overnight at 4°C, the solution was then aspirated and the plate washed 4 x with PBST (13.7 mM NaCL; 0.25 mM KCL; 0.8 mM Na_2HPO_4; 0.15 mM KH_2PO_4; 0.05% Tween; pH 7.2). Unbound sites on the plastic were blocked with 1% (w/v) non-fat milk in PBST for 1 hour at room temperature. The blocking solution was aspirated and the plate washed thoroughly before the addition of standards and samples. The ß-amyloid 1-40 peptide standards were prepared in PBST and used at a range of 50ng/ml to 0.39ng/ml, with two PBST control samples included. The samples were diluted a further 1:60 in PBST. Standards and samples were incubated for 1.5 hours. The solutions were then aspirated and the plate washed 4 x in PBST. The detection antibody, BAM401AP (Autogen Bioclear), was diluted 1:2200 in PBST and incubated for 1.5 hours. After aspiration and washing, the plate was blocked with 1% non-fat milk in PBST for 30 minutes. The plate was again washed and enzyme-labelled anti-rabbit IgG (HRP, Vector Laboratories) was incubated for 1 hour at a 1:3000 dilution in PBST. After washing, enzyme substrate o-phenylenediamine (OPD, in 0.1M citrate-phosphate buffer, pH 5.0) was incubated for approximately 20 minutes while the colour developed, then the reaction was stopped with 2.5M H_2SO_4. The absorbance was determined at 492nm and data were analyzed using GraphPad Prism 4.0.

The method differed for quantifying Aß 1-40 in the insoluble fraction only in that the samples were further diluted 1:80 and the amyloid beta 1-40 standards ranged from 75ng/ml to 0.039ng/ml.

In the protocol for the ß-amyloid 1-42 ELISA for the soluble fraction, the samples were further diluted 1:10 and the standards ranged from 50ng/ml to 0.039ng/ml. The detection antibody anti-amyloid-ß 1-42 (Chemicon) was used at a dilution of 1:3000 in PBST for both 1-42 ELISAs.

For the insoluble fraction of the 1-42 Aß ELISA, the samples were diluted a further 1:40 after the original dilution and the standards ranged from 400ng/ml to 0.039ng/ml. Given the dilution of the samples, it was necessary to spike the standards with formic acid to account for the impact of the acid on the samples. (Pilot data revealed that the impact of the formic acid was not detectable at dilutions of 1:80 or greater, making it unnecessary to spike the standards for the ß-amyloid 1-40 insoluble fraction.)

2.4.3 Results

Figure 5 depicts the results of the ß-amyloid 1-40 and 1-42 ELISA. Although ELISAs were run on dissected brain regions of four transgenic and four wild-type mice, data are only presented for the Tg2576 mice since the level of absorbance was negligible for wild-type tissue and no plaques were present in any of the WT tissue.

Data were analysed by ANOVA, with region (cortex, hippocampus and amygdala) as the dependent factor. Analysis of the soluble Aß 1-40 revealed a non-significant difference between the regions [$F_{(2,6)} = 1.588$, $p>0.05$]. Analysis of soluble Aß 1-42 also revealed no statistical difference between regions [$F_{(2,6)} = 0.599$, $p>0.05$], as did insoluble Aß 1-42 [$F_{(2,6)} = 0.693$, $p>0.05$]. There was, however, a significant main effect of region for insoluble Aß 1-40 [$F_{(2,6)} = 33.102$, $p>0.05$]. This is indicative of higher insoluble Aß 1-40 in the hippocampus and amygdala than the cortex.

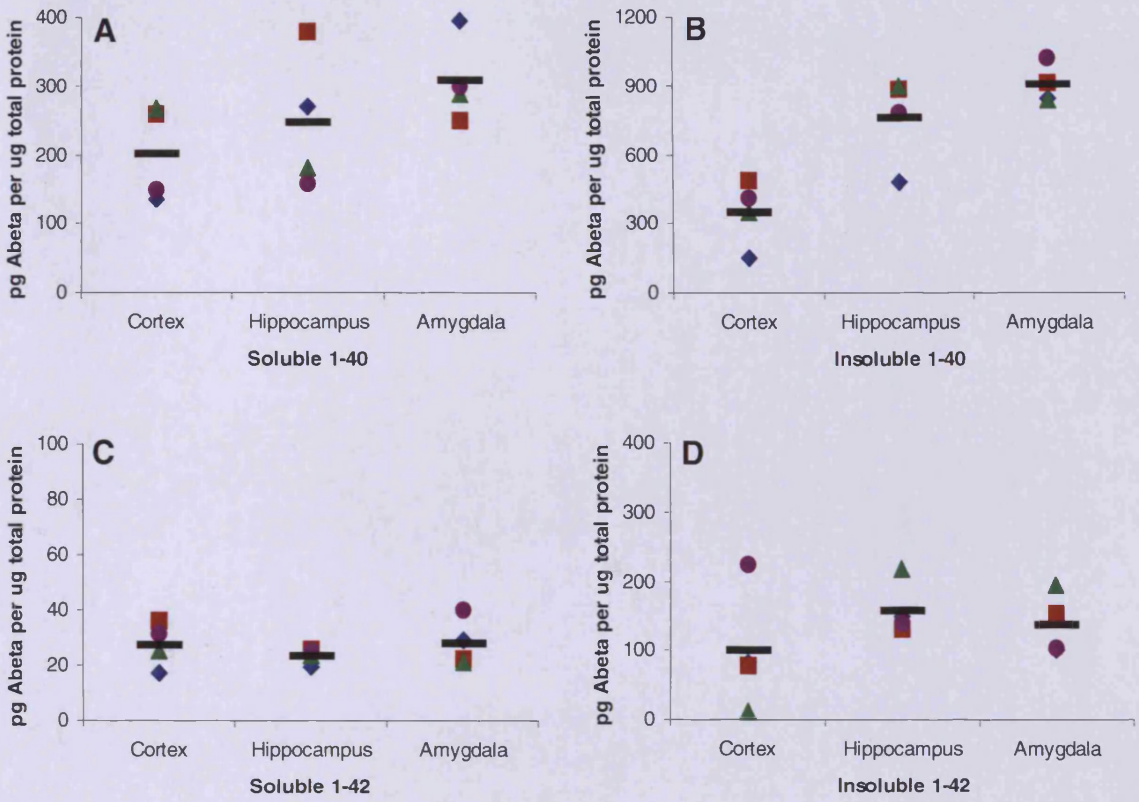


Figure 5: ELISAs for β-amyloid 1-40 and 1-42 in soluble and insoluble fractions. Data are presented as pg of β-amyloid per μg of total protein. A= levels of soluble 1-40; B= levels of insoluble 1-40; C= levels of soluble 1-42; D = levels of insoluble 1-42. Black bar represents the mean.

2.4.4 Discussion

The aim of this experiment was to simply quantify amyloid proteins in the cortex, hippocampus and amygdala as a means of establishing and comparing levels of amyloid deposition between the regions. Results revealed comparable levels of soluble β-amyloid 1-40 and 1-42 between the three regions, as well as comparable levels of insoluble β-amyloid 1-42. Levels of insoluble β-amyloid 1-40 were lower in the cortex, but were similar between the hippocampus and amygdala. These results confirm that amyloid pathology in the amygdala is comparable to, or even potentially greater than, that seen in other affected regions in the Tg2576 brain.

2.5 Fluorogold tracing of anatomical connections

2.5.1 Introduction

Prior to examining the potential effect of the APPswe mutation on emotion and motivation, it was important to determine whether the gross anatomical connectivity of the amygdala was influenced by the APPswe mutation. Retrograde fluorescent tracers, such as Fluorogold (Fluorochrome, LLC), provide a well-established method for examining the distribution of neuronal inputs into a chosen region. This analysis was intended as a preliminary study to evaluate gross connectivity of the amygdala in wild-type and transgenic Tg2576 mice.

Afferent connectivity of the BLA

The anatomical connections of the amygdala have been outlined previously and the main reciprocal connections are summarised in Figure 6. In summary, the entorhinal and perirhinal cortices provide substantial inputs into the lateral and basal nuclei, as does the subicular region of the hippocampal formation (Pitkänen et al., 2001). The CA1 region of the hippocampus projects to the basal nucleus (Pitkänen et al., 2001). Projections from the frontal cortex to the basal and lateral nuclei include afferent ventral orbital area, ventrolateral orbital area, lateral orbital area, and agranular insular area (Rempel-Clower, 2007). Weak projections exist from the infralimbic prefrontal cortex to the basal nucleus, while the prelimbic cortex supports dense projections to the basal and lateral nuclei (Vertes, 2004). It is worth noting that, although Fluorogold is primarily a retrograde tracer, there is evidence that it can travel bi-directionally (Schmued & Fallon, 1986).

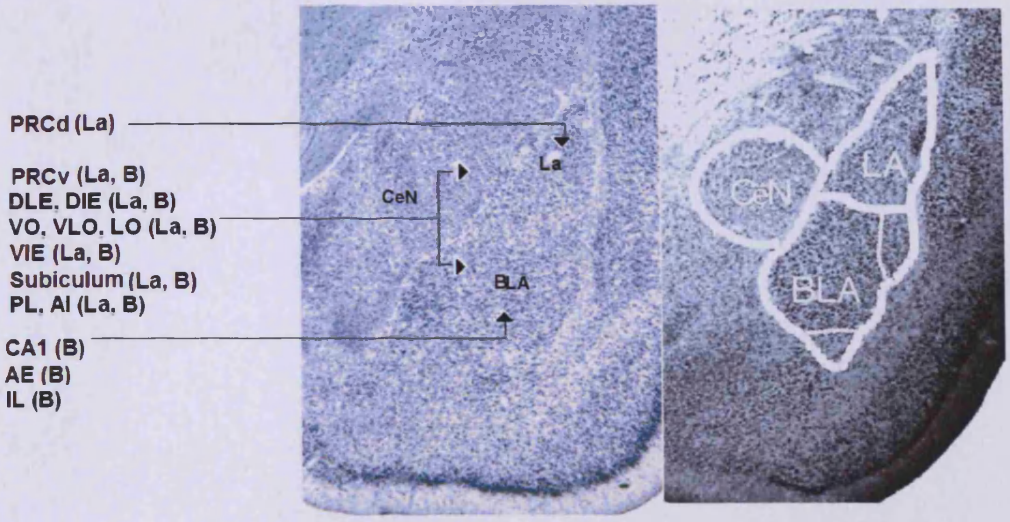


Figure 6: Afferent connections of the lateral and basolateral amygdala nuclei from the hippocampal formation and frontal cortices. Abbreviations: La = lateral nucleus, B = basal nucleus; AE = entorhinal cortex, amygdalo-entorhinal transitional subfield; AI = agranular insular area of the prefrontal cortex CA1 = CA1 field of the hippocampus; DIE = entorhinal cortex, dorsal intermediate entorhinal subfield; DLE = entorhinal cortex, dorsal lateral entorhinal subfield; IL = infralimbic region of the prefrontal cortex; LO = lateral orbital area of the prefrontal cortex; PRCd = perirhinal cortex, dorsal portion; PL= prelimbic region of the prefrontal cortex; PRCv = perirhinal cortex, ventral portion; VIE = entorhinal cortex, ventral intermediate entorhinal subfield; VLO = ventrolateral orbital area of the prefrontal cortex; VO = ventral orbital area of the prefrontal cortex. Images adapted from Corbit & Balleine, 2005.

2.5.2 Method

Bilateral injections of Fluorogold were made into the BLA of three aged (22-23 month old) wild-type and three Tg2576 mice, weighing 20-25g. Fluorogold was dissolved to a 4% solution in 0.2 M phosphate buffer, pH 7.4, and maintained in the dark at 4°C. Given the nature of this study, and the lack of definitive information concerning optimal quantities for use in the mouse brain, Fluorogold was injected in volumes ranging from 0.6 µl – 0.2 µl. Mice were anesthetized with 4% isoflurane and maintained in mouse stereotaxic frame throughout the duration of the surgery on 2.5% isoflurane. Holes were drilled bilaterally through the skull and tracer injections were made through a 30 gauge cannulae attached via plastic tubing to a 1 µl Hamilton syringe. After a survival time of 6-7 days, mice were deeply anesthetized via intraperitoneal injection of Euthatal and perfused transcardially with phosphate buffered saline (0.1M PBS, pH 7.4, 60-70 ml per animal), followed by an ice-cold fixative (4% paraformaldehyde in 0.1M PBS, pH

7.4, 150-200ml per animal). The extracted brain was then immersed in the same fixative for 2 hours at 4°C, before being transferred to a 30% sucrose solution (in dH_20) for 24 hours at room temperature. 40μm coronal sections were sliced on a freezing microtome at -20°C, and every third section was serially mounted onto double-subbed slides, air-dried overnight, dehydrated in an ascending series of alcohols and coverslipped in DPX. Sections were visualized and analysed with a Leica DMRB microscope, equipped with a fluorescent bulb and an Olympus DP70 camera, and AnalysisD imaging software (Soft-Imaging Systems).

2.5.3 Results and Discussion

Figure 7 depicts samples of Fluorogold labelling in the amygdala, frontal cortical and ventral hippocampal region in wild-type and Tg2576 mice. Visual inspection of the patterns of neuronal labelling indicated that there were no overt differences between the wild-type and transgenic mice in the viability of afferent amygdala inputs. Differences in intensity of the label reflect differences in the quantity of Fluorogold injected, rather than a quantitative difference dependent on transgene status. Thus, quantification of neurons in each region is not viable due to the slight differences in the quantity of tracer, but broad inspection of the connectivity can be assessed.

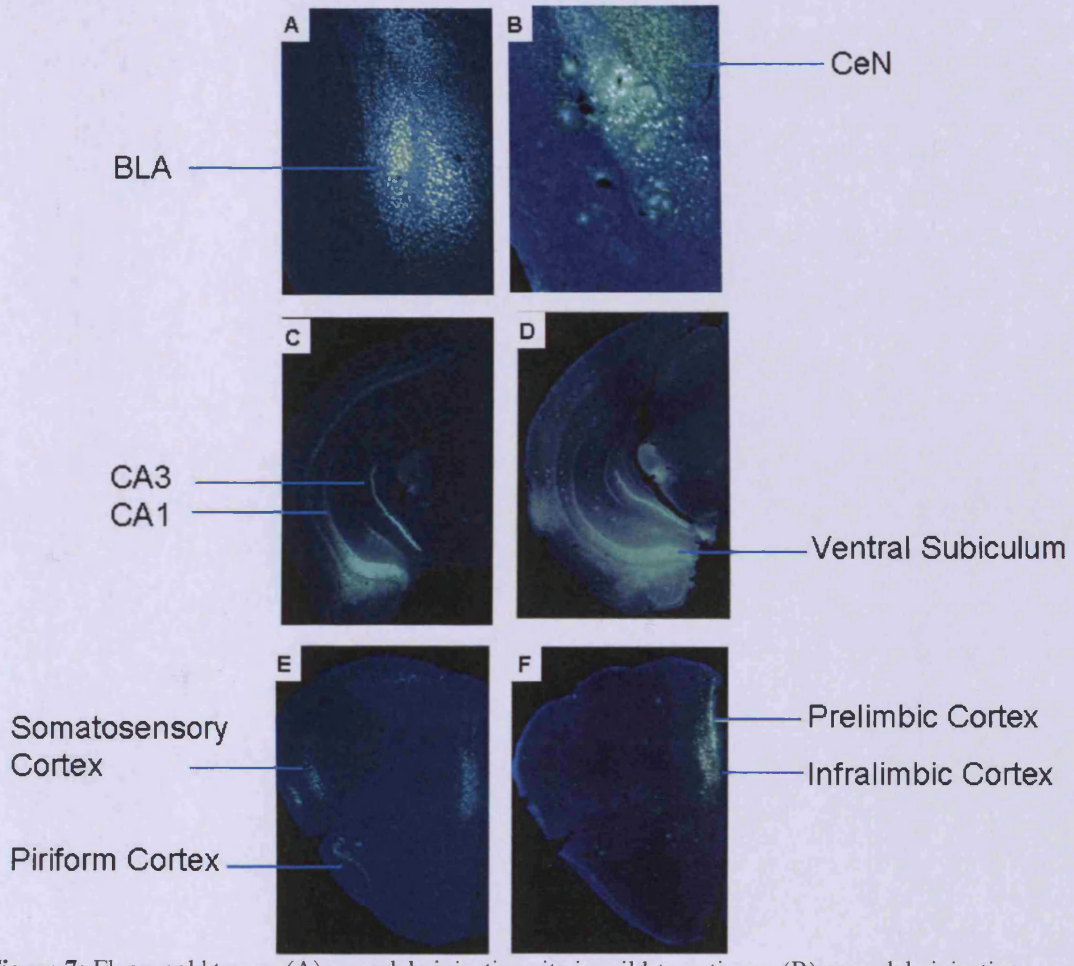


Figure 7: Fluorogold tracer: (A) amygdala injection site in wild-type tissue, (B) amygdala injection in Tg2576 tissue, (C) wild-type ventral hippocampus, (D) Tg2576 ventral hippocampus, (E) wild-type frontal cortex, (F) Tg2576 frontal cortex.

Visual inspection of Figure 7 suggests that the overall pattern of Fluorogold labelling in hippocampus and frontal cortex is comparable between wild-type and Tg2576 mice. In both genotypes, regions of the ventral hippocampus show considerable staining, particularly in the subiculum and CA1 and CA3 regions of the hippocampus. In the frontal lobes, staining of the prelimbic and infralimbic cortices is comparable between the genotypes. In the wild-type tissue, staining of the dorsal and ventral agranular insular cortices and the piriform cortex is evident, while less staining of this region is evident in the Tg2576 tissue. Although some evidence of cellular staining can be found at a higher magnification, the difference in saturation may be due to differences in the quantity of Fluorogold injected, or may reflect a change in the viability of this connection. For the purpose of the present study, however, the circuitries most relevent to an investigation of

emotional memory are those connecting the BLA with the prelimbic and infralimbic cortices and with the CA1 and CA3 regions of the ventral hippocampus. Furthermore, it is worth noting that retraction of the cannula resulted in Fluorogold deposition in the CeN of many of the animals (e.g. Figure 6B). This led to increased staining of the ventral hippocampus, particularly the CA1 and subiculum regions. Nevertheless, this preliminary study confirmed that the gross anatomical connectivity of the amygdala remained intact in Tg2576. However, for future research, a thorough evaluation of the patterns of connectivity throughout the Tg2576 mouse brain and quantification of labelled neuronal populations in each region may yield subtle differences between the Tg2576 and wild-type mice.

In summary, the use of Fluorogold retrograde tracer established that Tg2576 mice showed comparable patterns of connectivity from the frontal cortex and hippocampus to the amygdala. These data cannot, however, address the issue of whether transgenic mice display altered synaptic function, nor can they confirm functional viability of these pathways. The behavioural tasks in the following chapters aim to assess the functional characteristics of the amygdala and closely related cortical structures in emotion information processing.

2.6 Chapter Discussion

One aim of this chapter was to summarise the procedures used for breeding and identifying the APPswe mutant used for experimental testing. A second aim of this chapter was to establish the pattern of ß-amyloid pathology in the Tg2576 model biochemically, as a means of comparing pathology in the amygdala with that of the hippocampus and cortex. This was achieved using both immunohistochemistry to visualize the pattern of deposition and protein quantitation by ELISA to measure the level of ß-amyloid burden in dissected brain regions. Lastly, the pattern of gross anatomical connectivity of the amygdala, cortex and hippocampus was examined to confirm that the basic anatomical pathways were not grossly altered by expression of the APPswe mutation. In summary, the results of these analyses revealed extensive amyloid deposition throughout hippocampal, cortical and amygdala regions in aged Tg2576 mice. Nevertheless the connectivity of these regions did not appear to be disrupted. The aim of the following chapters was to examine the functional integrity of amygdala-cortical circuits with specific reference to emotion and incentive learning processes.

Chapter 3: Unconditioned and Conditioned fear in Tg2576 mice

3.1 Introduction

The aim of the experiments presented in this chapter was to test the hypothesis that the APPswe mutation would disrupt both unconditioned and conditioned emotional behaviour in Tg2576 mice. Experiments 1 and 2 assessed the performance of wild-type and Tg2576 mice in an elevated plus maze and marble burying task, respectively. Experiment 3 examined whether Tg2576 mice display impaired fear conditioning to a context and/or a Pavlovian auditory stimulus.

3.2 Experiment 1: Elevated Plus Maze

3.2.1 Introduction

The elevated plus maze is constructed from a cross-shaped platform that is raised above floor level. Two arms are 'closed' with high walls along each side, and two arms are 'open', with no enclosed sides. This task is considered to be an ethologically valid measure of anxiety in mice because it uses 'natural' stimuli, such as height and lack of protection, that are capable of inducing anxiety in many species, including rodents (McHugh, Deacon, Rawlins & Bannerman, 2004; Silveira, Sandner & Graeff, 1993). Normal animals typically spend less than 25% of their time exploring the more anxiety-provoking 'open' arms than they do exploring the 'closed' arm (Dawson & Tricklebank, 1995). Anxiolytic drugs have been shown to alter patterns of exploratory activity in rodents by increasing both the number of entries into and the amount of time spent in the open arms (e.g. Peng et al., 2004; Wada & Fukuda, 1991).

Based on between study comparisons, previous research suggests that there is an age-dependent change in elevated plus-maze behaviour in Tg2576 mice. Ognibene et al., (2005) reported that 7-12 month old wild-type and Tg2576 mice crossed into the open arms a comparable number of times during a nine minute session. However, the same Tg2576 mice also showed a tendency (which did not meet conventional levels of significance) to spend more time in the open arms than wild-type controls (Ognibene et al, 2005). In contrast, Lalonde et al. (2003) showed that 17-month old Tg2576 mice spent significantly more time in the open arms than wild-type mice using a cohort of approximately 70% male and 30% female mice. Of course, by using a mixed-sex cohort, it is impossible to determine whether there might be an interaction between amyloid deposition and sex on this task (see section 1.3.9 for a discussion of sex differences in the Tg2576 mouse model). Thus, while evidence suggests that Tg2576 mice may show an age-dependent deficit in the elevated plus maze task, a within study assessment of the effects of age on the mutation limits the conclusions that may be drawn from such an analysis. Thus, the main aim of this study was to systematically evaluate the

effects of age and examine the impact of sex on elevated plus-maze activity in Tg2576 mice.

In the present study, the effects of the APPswe mutation were compared in male and female mice at approximately 3, 10 and 15 months of age. Assuming that changes in behaviour are related to amyloid production, it was expected that young Tg2576 mice, which show no amyloid deposits and normal synaptic function, would not demonstrate genotypic differences on this task. Ten month-old mice show relatively few amyloid deposits but nevertheless show elevated levels of amyloid, synaptic abnormalities and robust deficits in spatial learning (e.g. Hsiao, 1996). Fifteen-month old mice show extensive amyloid deposition through out the amygdala, hippocampus and cortex. Given that damage to the medial temporal lobes, particularly the amygdala, is associated with decreased expressions of fear and anxiety (Lilly et al., 1983; Meunier et al., 1999), we anticipated that aged Tg2576 mice would show a deficit in the elevated plus maze task. Finally, in order to assess whether sex differences were evident on this task in aged mice, a cohort of aged (17-month old) female Tg2576 and wild-type mice were also tested on the elevated plus maze.

3.2.2 Methods

Design

Three cohorts of male mice were evaluated in the EPM. Initially, a 15 month old cohort was assessed. Having detected transgene-dependent changes at this age, two further cohorts, aged 10 months and 3 months, were examined as a means of gauging whether the genotypic differences observed in the 15 month cohort were age-dependent or age-independent. Finally, a cohort of female mice, aged 17 months was compared to the aged male cohort to determine whether the impairments in the aged cohort were sex -dependent or -independent.

Subjects

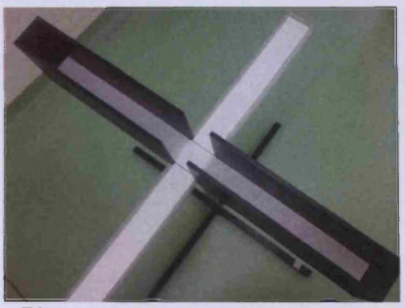
Tg(HuAPP$_{695}$SWE)2576 mice, which express the Swedish (K670N/M671L) double mutation in a hybrid background strain of C57B1/6J x SJL, under control of a hamster prion protein gene promoter (Hsiao et al., 1996) were used. Generation and maintenance of the colony are detailed in Section 2.1. Mice were housed in a temperature-controlled room (temperature 22 ± 2 °C, relative humidity 60 ± 10%, lights on from 08:00 a.m. to 08:00 p.m.) with *ad libitum* access to water and food. Where possible, mice were housed in littermate groups of 2-4 mice per cage. All behavioural testing was performed during the light phase of the circadian cycle.

Experiment 1A: The 3-month old cohort consisted of fifteen male Tg2576 mice and fifteen male wild-type littermate controls. The 10-month old cohort consisted of ten male Tg2576 mice and ten wild-type littermate controls. The 15-month cohort consisted of twenty-one Tg2576 transgenic mice and fifteen littermate controls, aged 15 months. Although the number of mice assigned to each group was constrained by practical issues, it is worth noting that in order to achieve a power lever of 0.8, it would be ideal to have approximately 18 mice of each genotype at each time point.

Experiment 1B: Female Tg2576 mice and wild-type littermates aged 17 months were used. The cohort consisted of twelve Tg2576 transgenic mice and ten wild-type controls.

Apparatus

The EPM was constructed from two open arms, measuring 8cm x 50cm, which ran from north to south and the two enclosed arms, measuring 8cm x 50cm x 10cm, which ran from west to east (see Picture 1). The platform was elevated 90cm from the floor and had a white floor. The walls forming the enclosed arms were painted black. The maze was situated inside a testing room with multiple environmental cues, including shelves and a computer system. A camera was mounted on the ceiling and connected to a DVD player and monitor (VM-904K, Shiba Electrics Ltd.) to ensure all sessions were recorded.



Picture 1: Elevated Plus Maze

Method

Mice were transported to the test room in individual home cages. Prior to the start of each trial, the EPM was cleaned thoroughly with 70% alcohol in distilled water (dH_2O) and the video recording equipment was activated. The mouse was placed in the centre platform of the EPM, facing a corner between open and closed arms. The starting position was counterbalanced across genotypes. The experimenter moved to the edge of the room and observed the behaviour of the mouse on the monitor. It was necessary to remain in the room given the potential for the mouse to fall from the open arms. In the rare situation in which this occurred, the animal was retrieved from the floor and placed back onto the centre platform of the EPM. Each animal was given one session of five minutes duration, after which the animal was removed from the maze, placed back into the home cage and returned to the holding room.

Scoring and Data Analysis

The videos were later scored for three measures: (i) the latency to enter the open arms; (ii) the time spent in the open and the closed arms; (iii) the total number of entries into the open and the closed arms. The animal was considered to have entered the arm when all four paws had crossed the boundary between the centre platform and the arm. Standard hand-held timers were used for measuring durations.

Data were excluded from analysis if the animal did not move from its start position on the central platform for the first four minutes of a five minute trial. Based on

this criterion, three mice were excluded from the 10 month old male cohort (one wild-type, two transgenics), one transgenic 15 month old male and one wild-type animal from the female cohort. Furthermore, the data from two Tg2576 mice in the 10 month old cohort were removed from the analysis because durations of zero seconds were recorded in both the open and closed arms (because they remained in the central platform), making the calculation of discrimination ratios difficult.

The raw data were analysed by ANOVA, using SPSS 12.0.1 software. As a means of eliminating the variability introduced by individual response rates and increase sensitivity to group differences, discrimination ratios were also calculated for each cohort on the frequency and duration measures. Ratios for the mean frequency to enter into the open arms were calculated using the formula: [Frequency to enter into the open arm / Total entries into open and closed arms]. Ratios for the duration of time spent in the open arms were calculated using the formula: [Mean time spent in open arms / Total time spent in open and closed arms]. Data were analysed using between-subjects t-test to investigate the effect of transgene and one-sample t-test to compare the performance of each genotype to chance (0.5).

3.2.3 Results: Experiment 1A

Frequency to enter open and closed arms

The mean frequency with which each cohort entered the open and closed arms is presented in Figure 8.

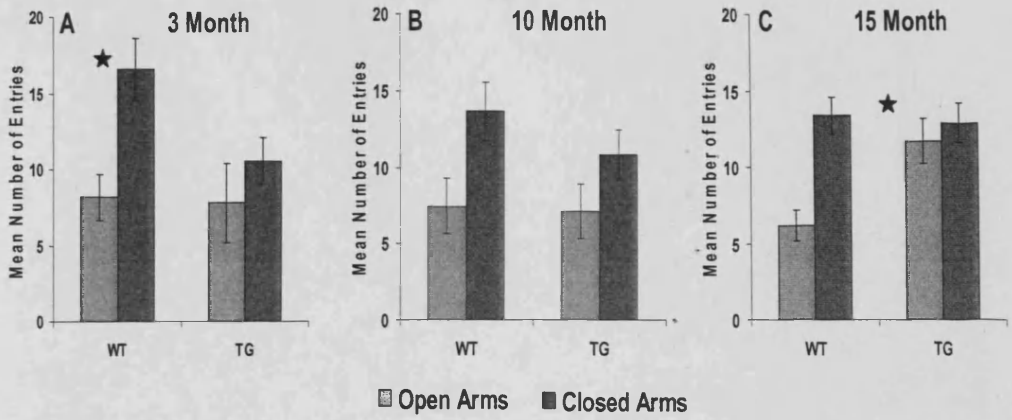


Figure 8. Mean frequency of entry into open and closed arms in the EPM during the 5 minute test trial. (A) 3 month male cohort; (B) 10 month male cohort; (C) 15 month male cohort. TG = Tg2576 transgenic mice; WT = wild-type control mice. Light grey bars = Open arms; Dark grey bars = Closed arms. Star = $p<0.05$, stars indicate data from interactions only, not main effects. Error bars = standard error of the mean (+/- S.E.M.).

For the 3 month male Tg2576 cohort, visual inspection of Figure 8A suggests that wild-type mice entered the closed arm more frequently, although this effect was reduced in Tg2576 mice. Statistical analysis revealed a non-significant effect of genotype [$F_{(1,26)} = 0.581$, $p>0.05$], a significant effect of arm type [$F_{(1,26)} = 22.781$, $p<0.001$] and a significant genotype by arm type interaction [$F_{(1,26)} = 5.556$, $p<0.03$]. Tests of simple effects revealed a significant effect of arms type for wild-type [$F_{(1,26)} = 27.374$, $p<0.001$] but not Tg2576 [$F_{(1,26)} = 2.724$, $p>0.05$] mice, they also revealed a non-significant effect of genotype for both the open and closed arms [$F_{(1,26)} = 0.065$ and 3.536, p's >0.05, respectively]. Thus, while wild-type mice showed a preference for entering the closed arms of the maze, this preference was not present in Tg2576 mice.

For the 10 month old cohort, both wild-type and transgenic Tg2576 mice entered the closed arm more frequently. Statistical analysis revealed a non-significant effect of genotype [$F_{(1,15)} = 1.918$, $p<0.05$], a significant effect of arm type [$F_{(1,15)} = 27.571$, $p<0.001$] and a non-significant genotype by arm type interaction [$F_{(1,15)} = 0.475$, $p>0.05$]. Thus, all animals made more entries into closed arms, regardless of transgene status at 10 months of age.

For the 15 month cohort, the wild-type mice entered into the closed arms more frequently than the open arms. The Tg2576 mice entered into both arm types a comparable number of times. This impression was confirmed by ANOVA, which revealed a non-significant main effect of genotype [$F_{(1,33)} = 2.460$, $p>0.05$], a significant effect of arm type [$F_{(1,33)} = 17.606$, $p<0.001$] and a significant genotype by arm type interaction [$F_{(1,33)} = 8.983$, $p=0.005$]. Tests of simple effects revealed a significant effect of arm type for wild-type mice [$F_{(1,33)} = 22.637$, $p<0.001$], but not for Tg2576 mice [$F_{(1,33)} = 0.838$, $p>0.05$]. Furthermore, the analysis revealed a significant effect of genotype for the open arms [$F_{(1,33)} = 7.978$, $p<0.008$], but not for the closed arms [$F_{(1,33)} = 0.076$, $p>0.05$].

Duration of time spent in open and closed arms

The duration of time spent in the open and closed arms for each cohort is depicted in Figure 9.

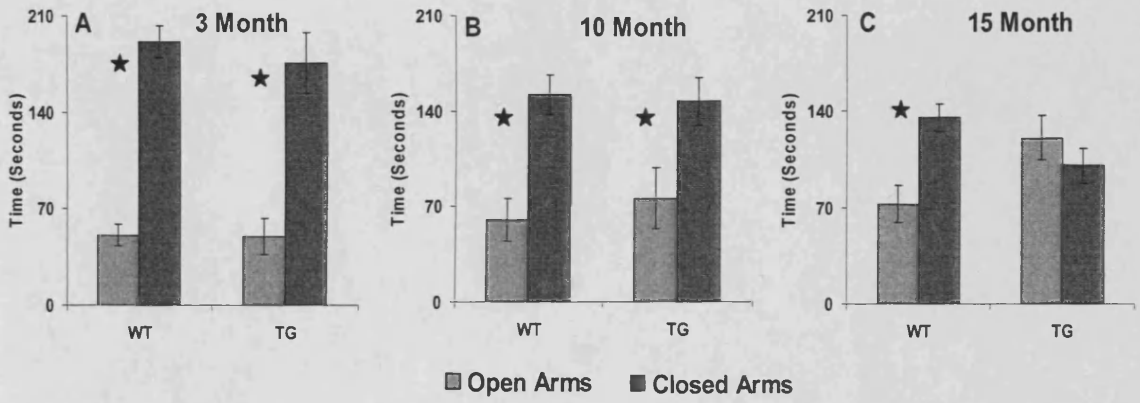


Figure 9. Mean time spent in open and closed arms in the EPM during the 5 minute test trial. (A) 3 month male cohort; (B) 10 month male cohort; (C) 15 month male cohort. TG = Tg2576 transgenic mice; WT = wild-type control mice. Light grey bars = Open arms; Dark grey bars = Closed arms. Star = $p<0.05$, stars indicate data from interactions only, not main effects. Error bars = +/- S.E.M.

Separate analyses of the 3 and 10 month male cohorts, revealed non-significant main effects of genotype [$F_{(1,26)} = 3.613$; $F_{(1,15)} = 0.685$; respectively, p's>0.05], significant main effects of arm [$F_{(1,26)} = 78.246$; $F_{(1,15)} = 11.445$; respectively, p's<0.005] and non-significant genotype by arm interactions [$F_{(1,26)} = 0.020$; $F_{(1,15)}$

= 0.182; respectively, p's>0.05]. In contrast, the analysis of the 15 month male cohort revealed a non-significant main effect of genotype [$F_{(1,33)}$ = 1.234, p>0.05], a significant effect of arm type [$F_{(1,33)}$ = 1.312, p>0.05] and significant genotype by arm interaction [$F_{(1,33)}$ = 4.950, p<0.04]. Tests of simple effects revealed a significant effect of genotype for both the open and closed arms [$F_{(1,33)}$ = 4.809 and 4.222, p's <0.05, respectively]. The analysis also revealed a significant effect of arm type for wild-type [$F_{(1,33)}$ = 4.970, p<0.04], but not Tg2576 [$F_{(1,33)}$ = 0.416, p>0.05] mice. Thus, a robust deficit in the elevated plus maze in terms of both durations and arm entries emerged in 15 month old, but not younger, Tg2576 mice.

Latency to enter open arms

The latency to first enter an open arm was also analysed to determine whether this measure was more sensitive to genotypic differences in younger animals.

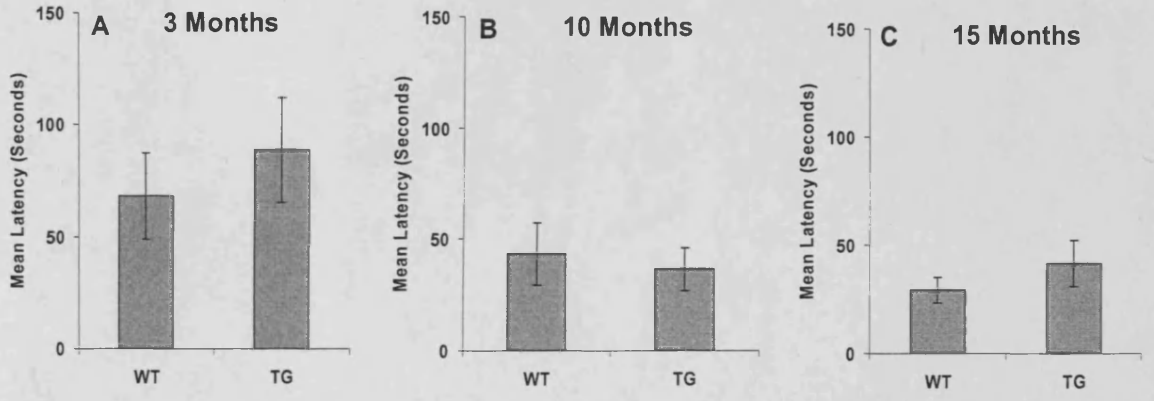


Figure 10. Mean latency (in seconds) to enter the open arms in the EPM. (A) 3 month male cohort; (B) 10 month male cohort; (C) 15 month male cohort. TG = Tg2576 transgenic mice; WT = wild-type control mice. Light grey bars = Open arms; Dark grey bars = Closed arms. Error bars = +/- S.E.M.

Data for the 3, 10 and 15 month old males are presented in Figure 10A, B and C, respectively. Results confirmed a non-significant effect of genotype for each male cohort [3 months old: t(26) -0.689, p>0.05; 10 months old: t(15) = 0.383, p>0.05; 15 months old: t(33) = -0.914, p>0.05].

Discrimination ratios

Discrimination ratios for the frequency to enter into the open arms are displayed in Table 3.

		Wild-type				Tg2576		
Age	**Sex**	**Mean Discrimination Ratio**	**St.Dev**	**S.E.M.**		**Mean Discrimination Ratio**	**St.Dev**	**S.E.M.**
3 Month	Male	0.30	0.12	0.03		0.31	0.20	0.05
10 Month	Male	0.31	0.17	0.06		0.36	0.18	0.06
15 Month	Male	0.31	0.14	0.04		0.45 **	0.16	0.04
17 Month	Female	0.36	0.21	0.07		0.36	0.21	0.06

Table 3: Discrimination ratios depicting the proportion of total arm entrances that were into the open arms. St.Dev = standard deviation, S.E.M. = standard error of the mean ** = $p<0.05$.

For the 3 and 10 month old male cohorts, independent samples t-test revealed a non-significant effect of genotype [$t(26) = -0.922$; $t(15) = -0.554$, respectively, p's>0.05]. For the 15 month male cohort, however, an independent samples t-test revealed a significant main effect of genotype [$t(33) = -2.879$, $p=0.007$].

For wild-type mice, one-sample t-tests revealed a significant difference between response rates and 0.5 at every age [$t(14) = -6.570$; $t(8) = -3.351$; $t(14) = -5.541$; 3, 10 and 15 month cohorts respectively, p's=0.01]. A significant difference between response ratios and chance was evident at 3 months of age for Tg2576 mice [$t(12) = -3.008$, $p<0.02$], but not at 10 or 15 moths of age [$t(11) = -2.199$; $t(19) = -1.368$; respectively, p's>0.05].

Discrimination ratios for the proportion of time spent in the open arms are displayed in Table 4.

		Wild-type				Tg2576		
Age	**Sex**	**Mean Discrimination Ratio**	**St.Dev**	**S.E.M.**		**Mean Discrimination Ratio**	**St.Dev**	**S.E.M.**
3 Month	Male	0.21	0.14	0.04		0.22	0.19	0.05
10 Month	Male	0.28	0.20	0.07		0.33	0.25	0.09
15 Month	Male	0.33	0.19	0.05		0.53 **	0.26	0.06
17 Month	Female	0.32	0.30	0.10		0.30	0.29	0.08

Table 4: Discrimination ratios depicting the proportion of time that was spent in the open arms. St.Dev = standard deviation, S.E.M. = standard error of the mean ** = $p<0.05$.

For the 3 and 10 month old male cohorts, independent samples t-tests revealed a non-significant effect of genotype [$t(26) = -0.205$; $t(15) = -0.419$, respectively, p's>0.05]. For the 15 month male cohort, however, an independent samples t-test revealed a significant main effect of genotype [$t(33) = -2.478$, $p<0.02$].

One sample t-tests revealed a significant difference between response rates and 0.5 for wild-type mice at 3, 10 and 15 months of age [$t(14) = -8.166$; $t(8) = -3.269$; $t(14) = -3.395$, respectively, p's<0.02]. For Tg2576 mice, the difference between the response ratio and chance was significant at 3 months [$t(12) = -5.273$, $p<0.001$], but non-significant at 10 and 15 months [$t(7) = 1.977$; $t(19) = 0.492$, respectively, p's>0.05].

3.2.4 Results: Experiment 1B

Frequency to enter open and closed arms

Experiment 1B examined the influence of the APPswe mutation on indices of anxiety in aged female Tg2576 mice. Although visual inspection of Figure 11A

suggests that 17 month old female wild-type mice entered into the closed arms more frequently than the open arms, statistical analysis via ANOVA revealed non-significant effects of genotype [$F_{(1,20)} = 0.320$, $p>0.05$], arm type [$F_{(1,20)} = 2.492$, $p>0.05$] and a non-significant interaction between these factors [$F_{(1,20)} = 0.944$, $p>0.05$]. These results suggest that no differences were evident in the number of arm entries exhibited by each genotype.

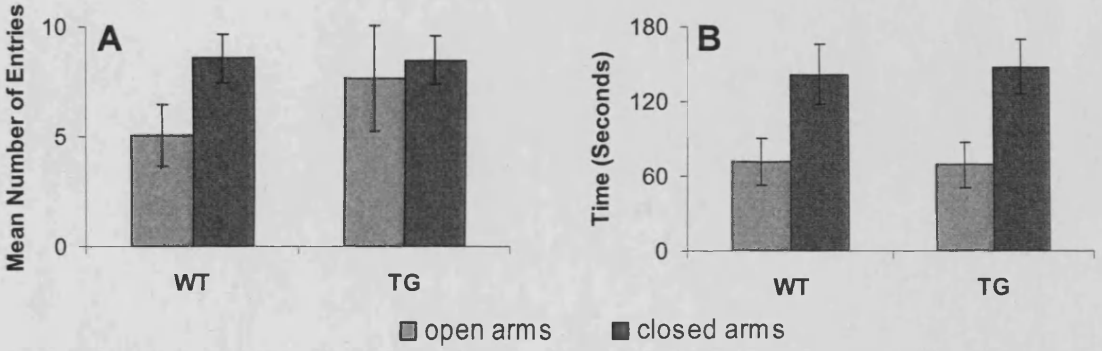


Figure 11. (A) Mean frequency of entry into open and closed arms in the EPM during the 5 minute test trial for 17 month female cohort. (B) Mean duration of time spent in open and closed arms. TG = Tg2576 transgenic mice; WT = wild-type control mice. Light grey bars = Open arms; Dark grey bars = Closed arms. Error bars = +/- S.E.M.

Duration of time spent in open and closed arms

Figure 11B depicts the mean time the female mice spent in the open and closed arms for each genotype. Statistical analysis revealed non-significant main effects of genotype [$F_{(1,20)} = 0.040$, $p>0.05$], a significant effect of arm type [$F_{(1,20)} = 6.608$, $p<0.02$], and a non-significant interaction between these factors [$F_{(1,20)} = 0.024$, $p>0.05$].

Latency to enter open arms

The latency to enter the open arms of the arms was also examined and the results are presented in Figure 12. This figure suggests the Tg2576 mice entered the open arms of the maze more slowly than the wild-type mice. However, statistical analysis revealed a non-significant effect of genotype [$t(20)= -2.048$, $p>0.05$].

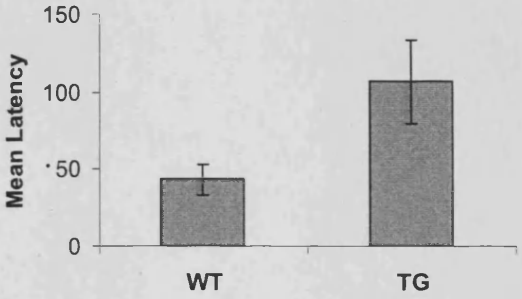


Figure 12. Mean latency to enter into open and closed arms. TG = Tg2576 transgenic mice; WT = wild-type control mice. Light grey bars = Open arms; Dark grey bars = Closed arms. Error bars = +/- S.E.M.

Discrimination ratios

Table 3 shows the discrimination ratios for the proportion of total arm entries made into the open arms. An independent samples t-test revealed a non-significant effect of genotype [$t(20) = 0.004$, $p>0.05$]. One sample t-tests revealed a non-significant difference between response rates and chance (0.5) for wild-type mice [$t(9) = -2.138$, $p=0.06$], but a significant difference for Tg2576 mice [$t(11) = -2.382$, $p=0.036$].

Table 4 depicts the discrimination ratios for the proportion of total time spent in the open arms. An independent samples t-test revealed a non-significant effect of genotype [$t(20) = -0.138$, $p>0.05$]. One sample t-tests revealed a non-significant difference between response rates and chance (0.5) for wild-type mice [$t(9) = 1.907$, $p=0.089$], but a significant difference for Tg2576 mice [$t(11) = 2.403$, $p=0.035$].

3.2.5 Discussion

The results of this study show that at 3, 10 and 15 months of age the wild-type mice crossed into the open arms more frequently than the closed arms and spent more time in the closed arms during the test. This pattern of behaviour differed however in the Tg2576 mice. At 3 months of age, Tg2576 mice spent more time in the closed versus the open arms. However, there the mice did not show a

difference in the number of times they entered into the open and closed arms. At 10 months of age, the Tg2576 showed comparable performance to the wild-type mice on both of the main measures. In contrast, at 15 months of age, the male Tg2576 mice showed a clear deficit on both measures and, unlike wild-type mice, entered the open arms and spent more time in this area of the maze than the wild-type mice. These results suggest that aged male Tg2576 mice show reduced anxiety. Furthermore, it is unlikely that the genotypic difference is a reflection of increased locomotor activity because no effect of genotype was evident in the number of arm entries and latency to enter the open arm made by the mutant and wild-type mice, suggesting there were no changes in gross locomotor activity.

Previous studies that have incorporated the elevated plus maze in to a test battery have revealed a tendency for aged Tg2576 mice to display disinhibited behaviour and reduced anxiety, as evidenced by an increased amount of time spent in the exposed arms of the maze (Ognibene et al., 2005; Lalonde et al., 2003). The results of the experiments presented here corroborate those of previous studies and also further our understanding by showing that the deficit appears to be age-dependent in male Tg2576 mice.

Interestingly, when aged female Tg2576 mice were tested on the same task, the mutant mice appeared to show greater levels of anxiety relative to the wild-type mice.

The results revealed that, unlike 15 month old male Tg2576 mice, 17 month old female Tg2576 mice displayed a preference for the closed arms of the maze. This suggests that the APPswe mutation did not affect performance on the elevated plus maze task in female mice in the same way as aged male mice.

Although the nature of the sexdifference is not clear, several possible explanations can be offered. Despite the higher levels of A-beta expressed in female Tg2576 mice (Callahan et al., 2001; see section 1.3.9), female mice also undergo hormonal cycles that could affect performance on the EPM. The oestrous cycle in female mice is known to be irregular around 14 months of age, with persistent anoestrus setting in soon after (Maekawa & Maita, 1996). Hence, this cohort of female mice was at a point in their life cycle when changes in oestrogen levels occur. There is a

well-documented literature examining the interaction of oestrogen with anxiety behaviours in rodents (e.g. Schneider & Popik, 2007; Walf & Frye, 2006). It remains possible that changes in the oestrogen cycle promoted anxiety-related behaviours in the Tg2576 female mice. Unfortunately, younger mice were not available at the time of testing to determine whether EPM behaviour was different in younger female Tg2576 mice.

Interestingly, the effect of housing on anxiety must also be acknowledged. Where possible, mice were housed in littermate groups, but if necessary, due to aggressive behaviour, mice were re-housed singly. This procedure was adopted more frequently in the case of male mice than female mice, which may have impacted upon subsequent social behaviours in male and female mice. However, given that it was not varied systematically between the sexes or age-cohorts, the precise effect of this housing practice can not be identified.

It is also worth noting that the female Tg2576 mice showed longer latencies to enter into the open arms of the maze. It may be, therefore, that this significant delay in entering the open arms led to a biased view of greater time in the closed arms. Had the test been conducted over 10 or 15 minutes, or had data analysis commenced from the point at which each arm type had been explored a different pattern of exploratory activity may have emerged. Nevertheless, the longer latency to visit the open arms is consistent with an increase in anxiety in aged female Tg2576 mice.

In summary, the APPswe mutation has an age- and sex-dependent effect on performance in the elevated plus maze task. These former findings parallel other studies with mice expressing the Swedish mutation. The hAPP/PS1 transgenic mouse model, which expresses the Swedish (K670N/M671L) double mutation crossed with PS1 (M146V) mutation under control of the Thy-1 promoter, demonstrates a similar pattern of reduced anxiety in the elevated plus maze (Pugh et al., 2007). It is, however, interesting to note that other APP mouse models have demonstrated increased levels of anxiety in the elevated plus maze relative to wild-type controls. The Tg-ßCTF99/B6 model, which expresses ß-secretase cleaved Aß-bearing carboxyl-terminal fragments in an inbred C57BL/6 background strain,

has been shown to display age-related increases in anxiety (Lee et al., 2006), as has the Tg-APP (Sw, V717F)/B6 mouse model, which expresses the Swedish double mutation and the V717F Indiana mutation in inbred C57BL/6 mice (Lee et al., 2004). See section 1.3.10 for a discussion of the effect of background strain on the expression of APP mutations. Thus, there is a body of evidence showing changes in anxiety in mouse models expressing human APP mutations, although the precise nature of these changes appears to be influenced by several factors.

3.3 Experiment 2: Marble Burying

3.3.1 Introduction

The aim of experiment 2 was to extend the analysis of anxiety in this model to a task that has not previously been reported in the APPswe model. Marble burying is a popular test of neophobia and is often used with mouse models of anxiety (Archer et al., 1987). This task taps into the species-typical defensive reaction of spontaneously burying unfamiliar stimuli, making this task another putatively ethologically valid measure of anxiety (Poling, Cleary & Monaghan, 1981). Marble burying is sensitive to the administration of anxiolytic and antipsychotic drugs, which are normally used to treat anxiety disorders or obsessive-compulsive disorder. Administration of such drugs results in a reduction in burying behaviour in mice (e.g. Nicolas, Kolb & Prinssen, 2006; Matsushita et al., 2005), thus suggesting that this task is sensitive to the neural processes mediating anxiety. Given the impact of the APPswe mutation on the EPM and the presence of amyloid pathology in neural pathways shown to be involved in stress and anxiety, it was expected that aged Tg2576 mice would demonstrate a decrease in neophobia compared to wild-type or young Tg2576 mice, which would manifest itself as a tendency to bury fewer marbles.

3.3.2 Method

Subjects

The mice used in this experiment were the same as those used in the EPM task. The marble burying task was run one week after the EPM, hence all mice were of approximately the same age as previously reported. Experiment 2A refers to data from the 3 month, 10 month and 15 month old male cohorts, while Experiment 2B refers to data from the 17 month old female cohort.

Apparatus

The test was conducted in a hard plastic box, measuring 30cm by 50cm by 15cm, which was filled with 5cm of non-allergenic bedding sawdust. Twenty marbles were arranged in five rows of four marbles each (see Picture 2). The marbles were constructed of glass and had a 15 mm diameter. A variety of colours and patterns were present in each cage, but every cage received exactly the same arrangement of colours and patterns to ensure no differences between each testing station. The marbles were equidistant from neighbouring marbles, at a space of 5 cm. A metal-framed cage top was placed on the plastic box to ensure the animal could not escape.

Picture 2: Marble Burying Apparatus

Method

Mice were transported to the testing room in their home cages. Seven test stations were arranged, allowing for seven mice to be analysed simultaneously. Mice were placed in the plastic box, the metal cage top was secured and the mice were left for 30 minutes in the test room without the presence of the experimenter. After the time elapsed, the mice were removed from the boxes and returned to their home cages. Photographic evidence of each test box was obtained immediately to ensure a record of the results. These images were then used to calculate the number of marbles left unburied.

Scoring

A marble was considered to have been buried if the marble was at least 2/3 covered in sawdust, as reported in previous studies (e.g. Njung'e & Handley, 1991).

3.3.3 Results: Experiment 2A

Figure 13 presents the results of the marble burying task for each of the three male cohorts. Visual inspection of these graphs suggests that wild-type mice consistently buried more marbles in the test period than transgenic Tg2576 mice. This impression was confirmed using the Mann-Whitney U statistical test, which revealed a significant effect of genotype for the 3 month, 10 month and 15 month cohorts [U = 52.0, 13.0, 72.5, respectively, p's=0.02].

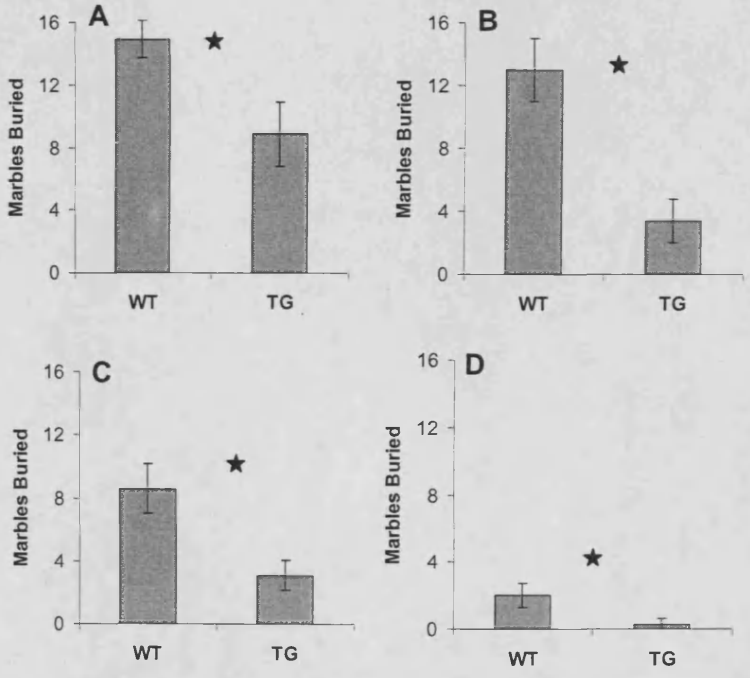


Figure 13: The mean number of marbles buried by wild-type controls and transgenic Tg2576 mice during 30 minute marble burying task. (A) 3 month male cohort; (B) 10 month male cohort; (C) 15 month male cohort; (D) 17 month old female cohort. Error bars = +/- S.E.M.

3.3.4 Results: Experiment 2B

Data for the 17 month old female cohort are presented in Figure 13D. Despite an overall lower rate of burying, the pattern of behaviour depicted in the graph appears comparable to that displayed by the male cohorts, with wild-type mice burying more marbles than transgenic Tg2576 mice. The Mann-Whitney U test confirmed this impression by revealing a significant effect of genotype [$U = 161.5$, $p=0.01$] on marble burying behaviour in the female cohort.

3.3.5 Discussion

The results of the marble burying task revealed a consistent pattern in which transgenic Tg2576 mice buried fewer marbles than wild-type controls. The discovery that mice as young as 3 months show a difference in marble-burying behaviour indicates that the effect of the mutation is age-independent. Thus, one possible conclusion from this study is that the difference in burying behaviour in Tg2576 mice is a result of non-specific changes induced by the APPswe transgene. It is worth noting, however, the youngest cohort may be at an age that is already displaying age-dependent changes in neuronal viability. It has been reported that Tg2576 mice aged 4 months demonstrate a decrease in spine density in the outer molecular layer of the dentate gyrus region of the hippocampus (Jacobsen et al., 2006). Thus, it may nevertheless remain the case that the deficit in the marble burying task may be age-dependent – unfortunately younger mice were not available to test this hypothesis. Perhaps the most parsimonious explanation, however, is that behaviour in the marble burying task is dependent upon a neuronal change that occurs intrinsically in the Tg2576 model as a result of the transgene insertion/expression.

It has been suggested that increases in locomotor activity may form the basis of behavioural disinhibition (e.g., decreased levels of anxiety) in the Tg2576 model (Gil-Bea et al., 2007). Lalonde and colleagues (2003) reported increased locomotor activity in Tg2576 during tests of anxiety and neophobia. However, when they divided their mice into 3, 9, 14, and 19 month old cohorts, they report

that only the three month old mice exhibited more locomotor activity than their wild-type littermates (Lalonde et al., 2003). Furthermore, in the EPM, no genotypic differences in the frequency to enter into the arms were observed, which suggests that overall the genotypes showed a comparable level of locomotor activity (see also Experiment 1). It could be argued, therefore, that it is unlikely that differences in locomotor activity across the age ranges could explain the genotypic changes in burying behaviour in Experiment 2.

One line of evidence supporting the use of the marble burying task as a measure of anxiety is the finding that the administration of anxiolytic compounds results in decreased marble burying behaviours. However, Thomas and colleagues (2009) recently examined multiple aspects of marble burying behaviour in mice, including the influences of genetic background strain and object novelty. They also correlated behaviour in the marble burying task with two other anxiety tasks, namely the open-field and light-dark test. They concluded that rather than representing a measure of anxiety, this task may represent a genetically driven tendency to perform a repetitive digging behaviour, which is independent of object novelty. Interestingly, this view accords well with the data presented here. The aged female Tg2576 showed no deficit in the EPM task yet showed a robust impairment in the marble-burying task. This would suggest that the two tasks are measuring different properties of the animals' reaction to novelty and spontaneous anxiety. Taken together with the evidence that the deficit in marble-burying in the Tg2576 mice was age-independent, it seems plausible that the behavioural differences between wild-type and Tg2576 mice on this task reflect a transgene insertion and not Aß-related pathogenesis linked to changes in anxiety.

In summary, marble burying behaviour has been thought to index naturally anxious and neophobic behaviours elicited in animals when presented with aversive or unfamiliar stimuli. It is possible, however, that behaviour in this task is more genetically driven and independent of levels of anxiety. In the present study, the decreased level of marble burying in Tg2576 mice was shown to be both sex- and age-independent. These results suggest that the genotypic change in behaviour represents an intrinsic, non-developmental effect of the APPswe transgene insertion on the tendency to perform a repetitive digging behaviour. The exact

basis of this very robust age-independent difference between Tg2576 and wild-type mice remains unclear.

3.4 Experiment 3: Fear Conditioning

3.4.1 Introduction

The results of Experiments 1 and, to a limited extent, those of Experiment 2 provide provisional evidence for age-related (and age-independent) changes in measures of spontaneous fear/anxiety. In the present study, the effect of the APPswe mutation on conditioned emotional behaviours was investigated.

Fear conditioning occurs when a motivationally neutral CS is paired with an aversive, biologically significant US, resulting in a conditioned fear response (CR). Pavlovian fear conditioning paradigms typically involve the pairing of an auditory CS, such as a tone, with an aversive footshock and result in fear behaviours such as freezing, analgesia (Fanselow & Helmstetter, 1988; Finn et al., 2006), changes in autonomic responses and 20-40 kHz ultrasonic vocalisations (Finn et al., 2006). The neural circuitry underlying fear conditioning has been studied extensively and is known to involve both the amygdala (e.g. Kim & Jung, 2006; Paré, Quirk & LeDoux, 2004) and hippocampus (e.g. Anagnostaras, Gale & Fanselow, 2001). The presence of ß-amyloid pathology in the hippocampal formation and the amygdala in APPswe mice, would clearly lead to the prediction that these mice would show a deficit in fear conditioning. However, a review of the literature indicates that there is a lack of agreement regarding the effect of the APPswe mutation on fear conditioning. Several authors have shown that Tg2576 mice are impaired in freezing to a context (Davidson, Putnam & Larson, 2000; Dong et al., 2005; Quinn et al., 2007; Comery et al., 2005; Dineley et al, 2002; Ohno et al., 2006). The results obtained using a Pavlovian cued fear conditioning paradigm have been somewhat more controversial. Corcoran et al. (2002) revealed that when the conditioning context was highly distinctive, both young (2-4 month old) and aged (16-18 month old) Tg2576 mice demonstrated normal conditioned freezing behaviour to the auditory cue and to the context CS. When the salience of the context was decreased, however, a context-specific deficit in conditioned freezing became evident in aged Tg2576 mice (Corcoran et al., 2002). Barnes and Good (2005) also demonstrated appropriate conditioned fear responses to the context and tone cues in young 4 month old Tg2576 mice. However, they reported

normal expression of conditioned freezing to the contextual CS, but reduced freezing behaviour to the auditory cue stimulus in aged 16 month old mice. They also reported a transgene-specific deficit in post-shock freezing behaviour in aged Tg2576 mice. In addition, Dong et al. (2002) reported that Tg2576 mice aged 9-10 months showed a deficit in freezing to both cued and context conditioned stimuli.

Given the controversy summarised above, the main aim of the present experiment was to assess the effect of the APPswe mutation on the acquisition and expression of conditioned fear in Tg2576 mice bred in the Cardiff laboratory. Given the presence of amyloid pathology within hippocampal and amygdala regions it was hypothesized that aged Tg2576 mice would show deficits in cued and/or contextual fear conditioning.

3.4.2 Method

Subjects

Twelve female Tg2576 mice and 10 wild-type littermate controls, aged seventeen months, were used in this experiment. Mice were housed in groups of 2-4 per cage and maintained on a 12 hour light-dark schedule. All testing took place during the light phase, between 09:00 and 18:00. Mice were allowed *ad-libitum* access to food and water throughout the duration of the experiment. Mice were maintained, and all procedures were conducted, in accordance with all Home Office (United Kingdom) regulations.

Apparatus

The conditioning apparatus consisted of two Coulbourn conditioning chambers (Coulbourn Instruments, Allentown, PA, USA), made from aluminium panels on the left and right walls and clear Perspex on the front and back of the chambers. The chambers measured 18 cm wide by 17 cm deep by 21 cm high and supported a speaker on the right wall and house light on the left wall. The front Perspex panel folded downwards to allow access and a wall-mounted camera recorded each session through the back Perspex panel.

The roof of the chamber held an infrared activity monitor (Coulbourn Instruments, Model H24-61MC), set to measure changes in movement via sensitivity to changes in a mouse's infrared body heat signature. The auditory stimulus consisted of a 5 kHz tone, which was set at a volume of 75 dB (A scale) in each conditioning chamber. The chamber was illuminated by the houselight on the left wall and the modular shock floor consisted of 5 mm steel bars placed 5 mm apart (Coulbourn Instruments). The floor grid was connected to a Coulbourn precision-regulated shock unit (model number H13–16), which had the shock amplitude set at 0.4 mA for a duration of 2 seconds. The conditioning chambers were housed in sound-attenuating boxes made of white melamine, measuring 70 cm wide by 50 cm deep by 50 cm high. A fan mounted on the right hand wall ventilated the sound attenuating boxes and provided background noise measuring 60 dB. The conditioning chambers were connected to a computer via a Coulbourn Habitest (Universal Linc), which both controlled the presentation of stimuli within the chambers, and recorded locomotor activity measurements. The software used to control the chambers, record and process the data was written using Coulbourn Graphic State Notation.

During conditioning and the context test, the chambers were presented as described above and cleaned with 70% alcohol between each animal. During the tone test, however, the novel context was constructed from inserts depicting a black and white checkerboard pattern. This patterned lined the left, right and front panels and a Perspex panel with the same pattern covered the floor grid. The floor panel was then covered with light tracing of fresh sawdust and the chamber cleaned with a solution of Hibiscrub (AstraZeneca) diluted 1:10 in distilled water between each subject. The conditioning session and the context test occurred in the same box for each animal, while the tone test was conducted in a novel chamber. The assignment of mice to each chamber was fully counterbalanced across genotypes.

Procedure

Day 1 consisted of a 13.5 min conditioning session, which entailed a habituation period of 360 seconds, followed by three 30 second tone presentations, each of which co-terminated with a two second footshock. An inter-trial interval period of 120 seconds was set between each tone-shock pairing and the mice remained in the chamber for 120 seconds after the final tone-shock presentation before being returned to the homecage. The floor grid and walls were wiped with 70% alcohol wipes between each animal.

On Day 2, the mice received both a context test and a tone test to evaluate fear elicited by the context and the auditory CS. The order of presentation of the tone and context tests was fully counterbalanced across wild-type and Tg2576 mice and the tests were separated by an interval of 4 hours. During the context extinction test, the mice were placed in the conditioning chamber used on day 1, and freezing behaviour was monitored for 8 minutes. The chamber was cleaned in between each animal with a 70% alcohol wipe. The tone test consisted of placing the mouse in the novel context and monitoring freezing behaviour for 6 minutes, at which point a continuous tone was presented for a further 6 minutes. The chamber was cleaned between each mouse with a diluted concentration of Hibiscrub to remove olfactory contaminants and debris left during the test.

Scoring of freezing

Freezing behaviour was defined as a complete suppression of locomotor activity and of all movements except those necessary for respiration. The behaviour of the mice was recorded using a camera and video recorder (Panasonic, Model Number NV-MV20) during both the conditioning and test sessions. Freezing was scored by the experimenter by using an automated program to record responses and assess every 2 seconds whether the animal was showing signs of immobility or freezing. A second observer, who was blind with respect to the genotype of the mice, also scored a randomly selected set of mice from each group. The Pearson's correlation coefficient for the comparison between the scores was $r = 0.945$.

The infrared monitor embedded in the ceiling of the chamber was capable of measuring both lateral and vertical movements of the animal and would usually be used to provide an independently-analyzed measure of locomotor activity.

Unfortunately, there was a failure in this equipment during one phase of testing, which resulted in inaccurate measures of locomotor activity for a subset of mice. As a result this measure could not be used for all animals and thus only the freezing data are reported in this study.

3.4.3 Results

The mean percentages of time spent freezing during the conditioning phase and test phases are presented in Figure 14. The left column Figure 14A-C depicts conditioning data, while the right column Figure 14D-E depicts the results of the context and tone tests. Data are presented in blocks depicting mean percentage of freezing during 1st and 2nd half of each phase. This reduced the variability while maintaining sensitivity to the changes in behaviour that occurred at the start and the end of each stage. Statistical analysis was performed using ANOVA with genotype as a between subject variable and block (1st versus 2nd half of phase) as a within-subjects variable.

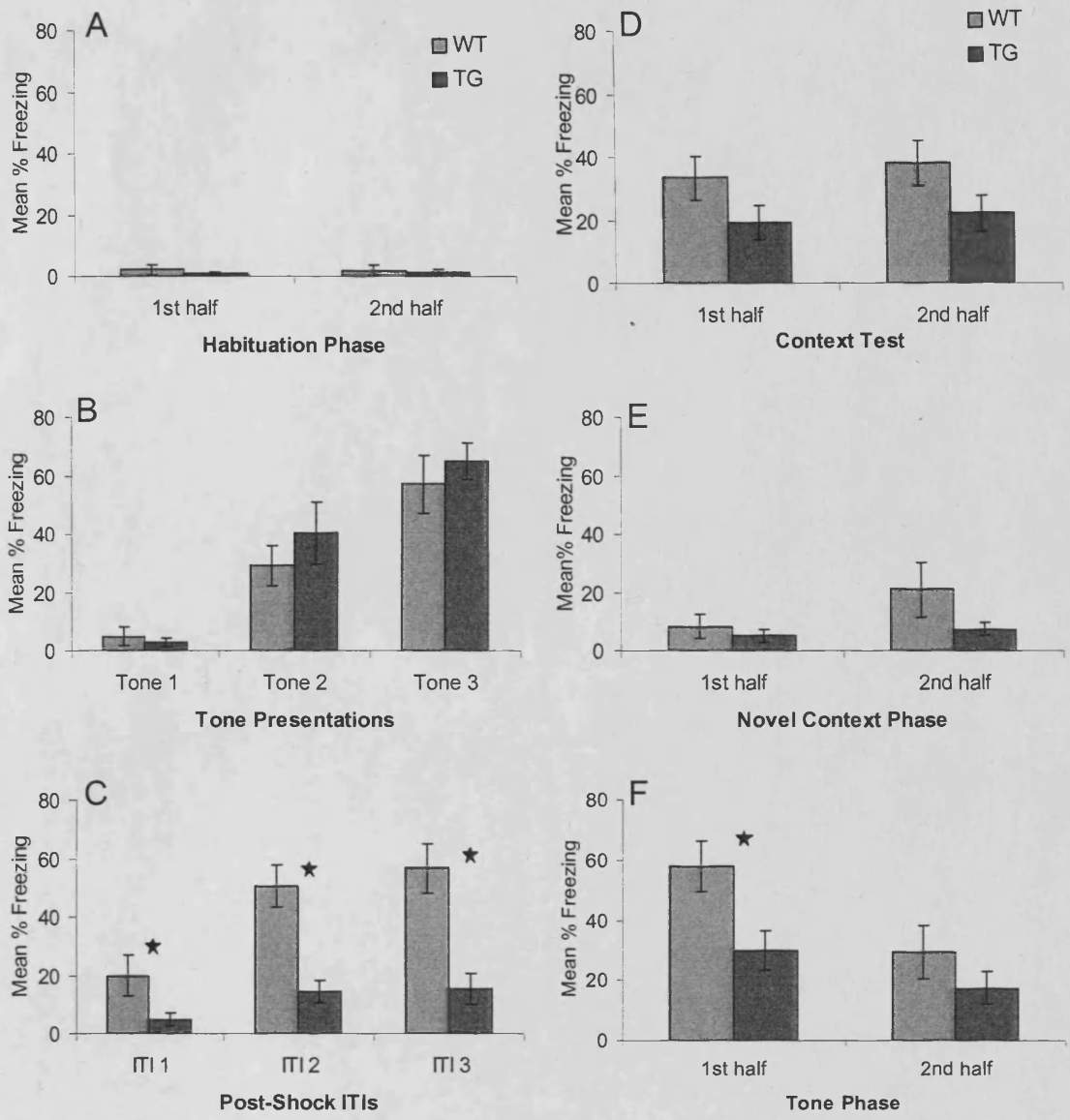


Figure 14: Mean percent of time spent freezing during conditioning and test phases for Tg2576 mice and wild-type controls. Freezing during (A) baseline, acclimatization period of conditioning session; (B) tone presentations; (C) post-shock it is; (D) context test; (E) novel context phase of tone test; (F) tone phase of tone test. Light grey bars = wild-type; Dark grey bars = Tg2576. Star = $p<0.05$. Error bars = +/- S.E.M.

Conditioning Phase: Habituation Phase

The data from the initial 6 minutes of the conditioning phase, in which mice were allowed to acclimatise to the conditioning chambers, constituted one measure of baseline freezing behaviour (see Figure 14A). Statistical analysis of the first and second halves of this period revealed a non-significant effect of genotype [$F_{(1,20)}$ = 0.226, $p>0.05$], a non-significant effect of block [$F_{(1,20)}$ = 1.172, $p>0.05$] and a

non-significant block by genotype interaction [$F_{(1,20)} = 4.043$, $p>0.05$]. Hence, no genotypic differences in baseline levels of freezing/activity were evident.

Conditioning Phase: Tone Presentations

Analysis of freezing behaviour during successive tone presentations (see Figure 14B) revealed a non-significant effect of genotype [$F_{(1,20)} = 0.662$, $p>0.05$], but a significant effect of tone [$F_{(2,40)} = 37.901$, $p<0.001$]. The interaction between these factors was non-significant [$F_{(2,40)} = 0.546$, $p>0.05$]. This suggests that all animals, regardless of transgene-status, increased freezing behaviour during successive tone-shock presentations.

Conditioning Phase: Post-shock ITIs

Analysis of the first 30 seconds of the ITI (Figure 14C), the period immediately following a footshock presentation, revealed a significant effect of genotype [$F_{(1,20)} = 22.235$, $p<0.001$], a significant effect of ITI [$F_{(2,40)} = 18.105$, $p<0.001$] and a significant interaction between these factors [$F_{(2,40)} = 5.381$, $p<0.01$]. Furthermore, tests of simple main effects revealed a significant effect of genotype for each ITI [minimum: $F_{(1,20)} = 5.009$, $p<0.04$] and a significant effect of ITI for wild-type [$F_{(2,19)} = 25.146$, $p<0.001$], but not for Tg2576 mice [$F_{(2,19)} = 2.768$, $p>0.05$].

Analysis of the last 30 seconds of the ITI period (see Table 5) revealed a non-significant effect of genotype [$F_{(1,20)} = 0.108$, $p>0.05$], a significant effect of ITI [$F_{(2,40)} = 14.781$, $p<0.001$] and a non-significant interaction between these factors [$F_{(2,40)} = 1.345$, $p>0.05$]. Overall, these results indicate a transgene-dependent difference in freezing behaviour immediately following the presentation of a footshock but not towards the end of the ITI.

	Tone 1		Tone 2		Tone 3	
	Freezing	S.E.M.	Freezing	S.E.M.	Freezing	S.E.M.
Wild-type	15.3	7.5	36.6	11.8	15.3	10.5
Tg2576	23.3	6.9	49.4	8.5	23.3	10.3

Table 5: Mean percentage of freezing behaviour elicited during last 30 seconds of tone period for wild-type and Tg2576 mice. S.E.M. = standard error of the mean.

Tone test: Novel context phase

Statistical analysis of the baseline data from the novel context phase of the tone test (Figure 14E) revealed a significant effect of block [$F_{(1,20)} = 8.193$, $p<0.05$], a non-significant effect of genotype [$F_{(1,20)} = 1.666$, $p<0.05$] and a non-significant block by genotype interaction [$F_{(1,20)} = 4.155$, $p>0.05$]. Hence, there were no statistical differences in levels of freezing between wild-type and transgenic mice during this baseline phase.

Tone test: Tone phase

Analysis of the data from the tone presentation stage (Figure 14F) revealed a significant main effect of block [$F_{(1,20)} = 74.893$, $p<0.001$], a non-significant main effect of genotype [$F_{(1,20)} = 4.026$, $p>0.05$], and a significant block by genotype interaction [$F_{(1,20)} = 11.639$, $p=0.003$]. Tests of simple effects revealed a significant effect of block for both wild-type and Tg2576 mice [$F_{(1,20)} = 66.724$ and 15.116, $p=0.001$, respectively]. More importantly, however, a significant effect of genotype was revealed in the first block [$F_{(1,20)} = 7.230$, $p<0.02$], but not the second [$F_{(1,20)} = 1.417$, $p>0.05$]. Hence, transgenic mice displayed an initial but transient deficit in freezing to the tone in the first half of the test session.

Context Test

Analysis of conditioned fear during the context test (Figure 14D) revealed a non-significant effect of block [$F_{(1,20)} = 1.358$, $p>0.05$], a non-significant effect of genotype [$F_{(1,20)} = 3.283$, $p=0.085$] and a non-significant interaction between these factors [$F_{(1,20)} = 0.067$, $p>0.05$]. Hence, there were no differences in levels of conditioned fear between wild-type and Tg2576 mice during the context test.

3.4.4 Discussion

Previous studies (e.g. Barnes & Good, 2005; Corcoran et al., 2002) have suggested that aged Tg2576 mice display deficits in freezing to contextual and/or auditory CSs in a fear conditioning task. The results of the present experiment support a subset of these claims, and indicate that the freezing deficit in aged Tg2576 mice is

specific to the auditory CS. In contrast, there was a non-significant effect of freezing during the context test, although there was a numerical trend for greater freezing in the wild-type mice. It is worth noting that power analysis recommends approximately 15 mice of each genotype to be used to achieve a power level of 0.8. Thus, although it is possible that lack of power has obscured this effect, this interpretation must be considered with caution without further empirical evidence.

The present study supports the findings of Barnes and Good (2005; see also Corcoran et al., 2002), who reported both a deficit in cued fear and an impairment in post-shock freezing behaviour in Tg2576 mice. It is worth noting that the cue freezing deficit in the Tg2576 cohort is unlikely to be the result of impaired sensory perception of the tone. The increase in freezing with each subsequent tone presentation during conditioning suggests that Tg2576 and wild-type mice were both capable of perceiving the auditory stimulus. Nor is it likely to reflect a deficit in performance of the freezing response *per se*, as the Tg2576 mice showed comparable levels of freezing to wild-type mice during conditioning and the context test. The present study therefore replicated the pattern of impaired freezing elicited by a punctate cue reported by Barnes & Good (2005). The deficit in freezing elicited by a tone CS is consistent with a deficit in neural systems supporting freezing i.e., the amygdala. This interpretation will be considered in more detail in the Chapter Discussion.

3.5 Chapter Discussion

The aim of this chapter was to assess anxiety, unconditioned and conditioned fear in the Tg2576 mouse model as a means of assessing the impact of amyloid pathology on aspects of emotionality. The EPM succeeded in establishing the phenotype of decreased anxiety in transgenic mice. Furthermore, the EPM (and marble burying) task extended previous knowledge by providing data for a series of age groups and comparing the aged male cohort with a cohort of aged female Tg2576 mice. The results clearly show that male Tg2576 show an age-dependent profile of deficits on the EPM task. In contrast, aged female Tg2576 mice do not show aberrant behaviour on the EPM task. The limited availability of young female mice precluded assessment of EPM anxiety at earlier time points. Nevertheless, the results suggest a sexdifference in EPM behaviour in aged Tg2576 mice. In contrast to the EPM, the marble burying task revealed an age-independent deficit in both male and female Tg2576 mice. This seemingly contradictory finding can be reconciled by considering a current theory of marble burying behaviour, which suggests that it is guided fundamentally by genetic variations in burying behaviour *per se*, rather than neophobic reactions. One conclusion from this study therefore is that marble burying is insensitive to age-related changes in anxiety, unlike the EPM task. The results indicate, however, that Tg2576 mice show an age-related reduction in anxiety. The final experiment in this chapter examined the effects of the APPswe mutation on conditioned fear in aged Tg2576 mice. The results replicated those reported by Barnes & Good (2005) in showing that aged Tg2576 mice display a deficit in cued fear conditioning but not context conditioning.

Previous reports of conditioned fear in the Tg2576 model have suggested that the deficit in conditioned freezing is specific to contextual stimuli (Corcoran et al., 2002), while others report impaired freezing to the Pavlovian CS (Barnes & Good, 2005). An obvious discrepancy between many of these studies is the different paradigms employed to examine conditioned fear. That is, studies that used a contextual fear conditioning design, without any auditory stimuli, overwhelmingly report deficits in freezing to the context (e.g. Comery et al., 2005; Dineley et al,

2002). Studies using an auditory cued paradigm, however, have reported transgene specific deficits in freezing to a tone and/or contextual CS (e.g. Dong et al., 2005).

Barnes and Good (2005) suggest that the inconsistency in behavioural results obtained using different conditioning paradigms may be resolved by considering the effect of cue competition on the strength of the CS-US association. That is, the contextual CS was attributed greater associative strength than the tone CS due to its greater salience, and therefore elicited higher levels of freezing. Hence, this greater salience allowed the contextual stimuli to support conditioning better than the auditory stimulus, leading to an overshadowing effect and an impairment with the less salient cue. This interpretation is supported conceptually by data from Corcoran et al. (2002) which demonstrated that the salience of the context CS had a profound effect on its ability to support high levels of conditioning, such that a deficit in context conditioning became apparent only when the level of freezing supported by the tone was greater than that supported by the context. When the salience of the context was increased, however, no impairments were observed. In the present study, however, the percentage of freezing observed during exposure to the tone was comparable to that seen during exposure to the context, if not initially higher. The same theory, therefore, of cue competition cannot be easily applied to these data. However, the trend towards significance seen for the context test may in part reflect the relatively similar associative strengths of the conditioned stimuli.

While it is suggested that impairments in the EPM, marble burying and fear conditioning tasks may be related to changes in anxiety, it is worth considering whether they could also reflect changes in locomotor activity. For example, an animal that is hyperactive may enter the open arms more frequently simply by virtue of its increased activity. This explanation for the genotypic differences is unlikely for two reasons. Firstly, in the EPM, the rate of arm entry was comparable between the Tg2576 and wild-type mice at all ages. Secondly, the latency to enter an open arm was also comparable between the wild-type and Tg2576 mice at all ages. Lastly, levels of baseline freezing observed in the acclimatisation and novel context phases of the fear conditioning task revealed no differences between the genotypes. These observations indicate that gross levels of locomotor activity were

comparable between wild-type and Tg2576 mice and that locomotor changes did not underlie the deficits observed in Tg2576 mice.

It is worth noting that mice used in the fear conditioning task are the same female mice that do not show an impairment on the EPM. If the deficit in fear observed in the Tg2576 mice reflected a change in the neural systems supporting anxiety, then no deficit in fear conditioning would be expected. Although there appears to be a discrepancy here, in fact there is evidence to suggest that the neural systems supporting anxiety and fear can be dissociated to some extent. For example, Veening et al. (2009) suggest that the septohippocampal system may be implicated more in anxiety, while the CeN is primarily involved in fear. Thus, further exploration of motivational processing in Tg2576 mice is warranted to probe the basis of the fear deficit observed in Tg2576 mice and the neural system supporting this impairment.

The results of Experiment 3 and Experiment 1 provide indirect evidence that the neural systems supporting emotion are impaired in Tg2576 mice. One candidate region that may provide the anatomical locus for these behavioural abnormalities is the amygdala. Several lines of evidence support this suggestion. For example, a recent study, using immunohistochemistry and factor analysis revealed a correlation between behaviour in the EPM and activation of the amygdala and the medial prefrontal cortex (Albrechet-Souza, Borelli & Brandão, 2008). Extensive evidence also indicates that the amygdala is involved in learning and expression of conditioned fear responses, particularly to auditory stimuli (e.g. Kim & Jung, 2006; Paré, Quirk & Ledoux, 2004; Selden et al., 1991). Selden and colleagues (1991) demonstrated that lesions of the BLA can prevent conditioned fear-related responses to an auditory CS, while leaving aversive learning about contextual cues intact. However, electrolytic and neurotoxic lesions of the CeN in rats have also been shown to abolish freezing behaviour to both an auditory CS and the context (Goosens & Maren, 2001). Despite these parallels, it is also clear that similar patterns of deficits may emerge following lesions of the hippocampal formation. Thus, at present, it remains unclear as to the nature of the neural systems that may underlie the deficits observed in aged Tg2576 mice.

There are some features of the fear conditioning deficit observed in Tg2576 that are consistent with a functional impairment in amygdala function. For example, it has been suggested that subregions within the amygdala complex make distinct contribution to the representational processes underlying conditioned responding. The immediate reaction to a footshock is increased locomotor activity and agitation, and this is considered to represent the unconditioned reaction to the US. Following the initial burst of locomotor activity, the behaviour changes and freezing behaviour becomes evident. The development of freezing is thought to represent a US-specific conditioned response that reflects the sensory specific components of the US. In Experiment 3, it was noted that Tg2576 mice showed a marked deficit in the development of post-shock freezing (see also Barnes & Good, 2005). The impairment in post-US freezing may reflect either an impairment in the formation of an association between the contextual CS and the affective features of the US or perhaps an inability to access a representation of the affective properties of the US (Fanselow, 1990; Lattal & Abel, 2001). Interestingly, impairments in post-shock freezing have been observed after electrolytic lesions of the lateral amygdala (Wallace & Rosen, 2001). In contrast, excitotoxic lesions of the LA alone did not result in a post-shock freezing impairment. Nevertheless, lesions of the LA that extended beyond and included damage to the basal nucleus, amygdalostriatal transition region, caudate putamen and piriform cortex, did result in a deficit in post-shock freezing. These data suggests that the LA is necessary for learning and memory of fear conditioning, whereas the fibers of passage through the LA and amygdalostriatal transition region play a role in the expression of freezing behaviour, as do the regions beyond the LA. Indeed, post-shock freezing deficits have also been observed in rats with hippocampal damage. Lesions of the ventral hippocampus have been found to reduce freezing behaviour after the delivery of an unsignalled footshock (Bannerman et al., 2003; Kjelstrup et al., 2002; Richmond et al., 1999). In contrast, lesions of the dorsal hippocampus have no effect upon freezing behaviour to unsignalled footshock (Richmond et al., 1999).

Several theorists have suggested that subregions within the amygdala make distinct contributions to representational processes supporting learning about outcomes and thus conditioned responding. More specifically, it has been

suggested that the BLA mediates the associative processes that support conditioning of the sensory features of the US and is responsible for the flinching and freezing components of the conditioned response. In contrast, the CeN is postulated to support conditioning of the motivational affective properties, including changes in heart rate, blood pressure and withdrawal behaviours (Paré, Quirk & Ledoux, 2004, Balleine & Killcoss, 2006; Maren, 2005). Hence, the BLA and CeN both play a role in regulating conditioned fear responses to auditory cued stimuli via processing of the sensory-specific and motivational properties of the outcomes.

In conclusion, the results from Experiments 1 and 3 provide evidence for changes in emotional learning and memory processes in Tg2576 mice. However, the results provide no clear behavioural evidence for a candidate neural system that may be responsible for these abnormalities (e.g., hippocampus and/or amygdala). The main aim of the experiments reported in Chapter 4 is to provide a detailed assessment of emotional/motivational processes that are more directly attributable (theoretically) to impairments in amygdala function using appetitive Pavlovian and instrumental learning tasks.

Chapter 4: Motivational Processing in Tg2576 mice

4.1 Introduction

In Experiment 3, Tg2576 mice displayed aberrant formation of a CS-US representation in Pavlovian fear conditioning (see chapter 3.4). One potential explanation for this deficit is that Tg2576 mice failed to form an association between the CS and the sensory-specific motivational attributes of the US that appear to underpin the development of the freezing conditioned response. Anatomically, this type of deficit could reflect amyloid-induced changes in amygdala function. One prediction that follows from this analysis, therefore, is that Tg2576 mice will be impaired on tasks that assess the formation of sensory–specific reward associations during incentive learning. Incentive value refers to the affective and motivationally relevant properties of an appetitive reward. In animals, it is possible to manipulate the value of a reward by using outcome devaluation procedures. Outcome devaluation paradigms involve reducing the incentive value of an outcome via either reward-specific satiety or by injecting lithium chloride following ingestion of a specific reward. Dickinson and Balleine (1994) revealed that post-training shifts from hunger to satiety only have an effect on instrumental performance when the shift in the incentive value of the appetitive outcome is made explicit through the animals' consummatory experience (which permits incentive learning about the change in the incentive value of the reward). Specific satiety, therefore, reduces instrumental performance, not because it reduces overall drive or motivation, but because it reduces the incentive value linked to the specific sensory feature(s) of an appetitive outcome (Balleine and Dickinson, 1994). Furthermore, the anatomical substrates of this form of learning are relatively well understood.

Several studies have shown a functional dissociation between the BLA and the CeN in motivational processes. Specifically, the BLA is necessary for a conditioned stimulus to gain access to the current incentive value of an associated US, while the CeN is necessary for forming a representation of the general affective motivational properties of a reward. These properties of a reward can enter into associations with Pavlovian cues or instrumental actions (See chapter 1; Corbit & Balleine, 2005; Everitt et al., 2003). In addition to the amygdala, frontal cortical regions have also been implicated in goal-directed aspects of incentive

learning processes (e.g. Furuyashiki & Gallagher, 2007). It has been theorized that the orbitofrontal cortex is involved in encoding information about the sensory-specific properties of an outcome and the incentive value of behavioural goals, thereby influencing action selection during devaluation procedures (Delamater, 2007). Ostlund and Balleine (2007), however, suggest that this region plays a specific role in influencing goal-directed actions. They argue that the orbitofrontal cortex is necessary for allowing Pavlovian cues to facilitate instrumental performance during CS-outcome learning, but is not involved in influencing behaviour when the incentive value of an outcome is reduced during outcome devaluation procedures (Ostlund & Balleine, 2007).

The presence of amyloid pathology in the amygdala complex and frontal cortical fields associated with goal-directed learning, together with behavioural deficits in tasks linked to anxiety and fear processes, suggests that these learning systems may be compromised in aged Tg2576 mice. However, the effect of the APPswe mutation on motivational processing has not been extensively investigated in Tg2576 mice. The principal line of evidence indicating a deficit in emotional/motivational memory in Tg2576 mice comes from evidence of fear conditioning deficits. Deficits in freezing elicited by a CS are suggestive of an impairment in the ability of the CS to gain access to a representation of the sensory-specific features of the US (Balleine & Killcross, 2006). In order to test this hypothesis directly, further examination of the effects of the APPswe mutation on incentive learning processes are required. In this chapter, instrumental and Pavlovian incentive learning tasks were used to test the hypothesis that the APPswe mutation impairs incentive motivational learning processes.

4.2 Experiment 4a and b: Instrumental Devaluation in Aged Tg2576 mice

4.2.1 Introduction

The basolateral nucleus of the amygdala plays a role in goal-directed learning during instrumental conditioning and shows extensive amyloid pathology in

Tg2576 mice. The functional integrity of this system with respect to incentive learning has not been assessed in Tg2576 mice. In rats, damage to the BLA results in an impairment in the animal's ability to form a representation of the sensory aspects of a motivationally significant event (Blundell, Hall & Killcross, 2001). This impairment is manifest as an inability to diminish behavioural responding for a reward after the incentive value has been reduced. In Experiment 4 (a & b), aged male and female Tg2576 mice were assessed in their ability to form an instrumental action-outcome association and to change their behaviour appropriately after the outcome was devalued using a sensory-specific satiety manipulation. That is, they were trained to nose poke into two manipulanda, each resulting in the presentation of a different reward. The incentive value of one of the rewards was then reduced by sating the animal with that outcome. The mice were then tested in extinction to assess instrumental responding on the two manipulanda. It was predicted that aged mice harbouring the APPswe mutation would demonstrate an inability to use a representation of the action-outcome association to change their instrumental behaviour according to the current incentive value of the outcome. Furthermore, given that mild sexdifferences were observed between male and female Tg2576 mice on the EPM, it became of interest to determine whether sexmight impact upon on any transgene-driven impairments in incentive processing. Thus, aged male and female mice (18 and 15 months, respectively) were used in the instrumental devaluation task.

4.2.2 Method

Experiment 4a: Subjects: Tg2576 male mice expressing the Swedish double mutation on the APP gene ($HuAPP_{695}SWE$) were used in this study. A total of 19 mice were used, 8 heterozygous transgenic mice and 11 wild-type controls, aged approximately 18 months. All mice were housed in mixed genotype cages of 2-4 mice. Mice were maintained in a 12 hour light/dark cycle and were tested between 9:00 and 20:00.

Experiment 4b: Subjects: Tg2576 female mice expressing the Swedish double mutation on the APP gene ($HuAPP_{695}SWE$) were used in this study. A total of 22

mice were used, 12 heterozygous transgenic mice and 10 wild-type, aged approximately 15 months. Mice were maintained, and all procedures were conducted, in an identical manner to those of the operant outcome devaluation task conducted with male Tg2576 mice, with the exception of the duration of training (discussed below).

All mice were food deprived to 85% of their *ad libitum* baseline weights and water was removed one hour prior to testing. All mice were weighed and health checked daily to ensure that food and water restrictions had no aversive affect on the animals' health. Mice were maintained, and all procedures were conducted, in accordance with all Home Office (United Kingdom) regulations.

Apparatus

Behavioural testing was conducted using 8 identical operant conditioning chambers (Med Associates, St. Albans, VT), which were housed in sound attenuating boxes. Each chamber measured 12 cm high by 15 cm wide by 14 cm deep and the front, back and ceiling panels were constructed of clear Perspex. The grid floor was fabricated from steel rods measuring 5mm wide, situated 5mm apart. The Perspex panel on the front wall opened out to allow entry into the chamber. The side walls were constructed of metal inserts and contained the input and output manipulanda. One wall housed a magazine for dispensing food pellets and sucrose solution, as well as two nose pokes on either side of the magazine. Each nose poke was illuminated by a yellow light recessed within the centre, and nose poke entries were recorded via an infrared beam spanning the diameter of the nose poke horizontally. Nose pokes into the magazine were also recorded via an infrared beam situated horizontally across the entrance in the magazine bay. The other side wall held a house light in the centre, as well as tone generator and a clicker that were situated on the outer panel of the wall. The 3 kHz tone generator was designed to emit a 65dB tone, whereas the clicker emitted a 10Hz train of clicks at 65dB. A computer equipped with a Med-PC program (Med Associates) controlled the operant chambers and recorded activity. The food rewards consisted

of 20mg food pellets (TestDiet, MLab Rodent Tablet, Sandown Scientific, IN, USA) and 0.1ml measures of 20% sucrose solution in distilled H_2O.

Behavioural training and testing

Magazine training/Habituation: Behavioural conditioning commenced with two days of magazine training and habituation to the operant chambers. This consisted of two x 20-minute sessions per day, one in the morning and one in the afternoon, with a break of approximately four hours in between. In each session the mice were trained to enter the magazine to collect food rewards. The food pellets or sucrose solution was delivered to the magazine on a computer-generated random time 30-second schedule (RT-30). If the animal received magazine training with food pellets in the morning session, then sucrose solution would be delivered in the afternoon session and the opposite pattern would occur on counterbalanced days, following the pattern ABBA. The order in which the food rewards were delivered was counterbalanced across sessions, days and genotypes.

***Instrumental Training: Experiment 4a*:** Instrumental conditioning of action-outcome assignments began on the third day. Each day consisted of two x 30-minute sessions, one in the morning and one in the afternoon, with a break of approximately four hours in between sessions. Mice were trained to nose poke into the left or right manipulandum to receive a food reward (either food pellets or sucrose solution). Each animal received two daily conditioning sessions, one of each action-outcome contingency. The action-outcome assignments and order of training sessions were fully counterbalanced across sessions, days and genotypes. Within each session, only the manipulandum that was part of the action-outcome assignment being trained was illuminated and actively delivered rewards when entered, although nose poke entries into both the illuminated ('correct') and the un-illuminated ('incorrect') manipulandum were recorded. Throughout all conditioning sessions the house light remained off in order to increase the salience of the illuminated nose poke manipulandum. On the first day only, each manipulandum was smeared with a small sample of the food reward that was being delivered in that session in order to encourage initial responding.

Mice first received 3 days (6 sessions) of instrumental training on a continuous reinforcement schedule (CRF; i.e. every action was rewarded with an immediate outcome), followed by 3 days (6 sessions) on a Fixed Ratio (FR)-5 schedule (i.e. every five actions were rewarded with an outcome). Mice then received 1 day each (2 sessions) on an FR-10, an FR-15, an FR-2 and an FR-30 schedule.

Instrumental Training: Experiment 4b: Behavioural conditioning was conducted in a similar manner to that described above for male Tg2576 mice. The sole difference in procedure concerned the length of the instrumental conditioning phase. Given that female Tg2576 mice demonstrated slower acquisition, as well as lower rates of responding, at each stage of training, female mice were given additional sessions of conditioning in comparison to male mice. Specifically, females received 16 days (32 sessions) of instrumental conditioning, whereas males received 10 days (20 sessions) of instrumental conditioning.

The satiety specific devaluation treatment, extinction test, rewarded test and consumption test were all conducted in an identical fashion for male and female Tg2576 mice.

Satiety Specific Devaluation Procedure and Extinction Test: After the last day of instrumental training, the incentive value of one of the food rewards was decreased by using a satiety specific devaluation procedure, whereby mice were allowed *ad libitum* access to either pellets or sucrose solution in their cages for 120 minutes. Pellets were placed in a small weighing boat and placed at one end of the cage, while sucrose solution was presented in a bottle at the other end of the cage. Both containers were weighed before and after the devaluation treatment to determine the quantity consumed. The allocation of devalued food reward was fully counterbalanced across genotype and action-outcome assignment. After the satiety specific devaluation treatment, mice were tested in the operant chambers in extinction for 30 minutes. As before, the house light remained off throughout the session. During the extinction test, however, both manipulanda were illuminated, but no rewards were delivered. Entries into both nose poke manipulanda and the magazine were recorded.

Rewarded Test: After the extinction test, mice were given one day (2 sessions) of re-training to reinstate responding on the manipulanda. The day after re-training, mice again received a satiety specific devaluation treatment. During this treatment, mice were sated with the opposite food reward to that which they received in the extinction test. Again the satiation period lasted for 120 minutes, after which the mice were tested in the operant chambers with access to both illuminated manipulanda for 30 minutes. In this test phase, unlike the extinction test, rewards associated with both manipulanda were available and were delivered on a FR-30 schedule.

Consumption test of specific satiety: After allowing one day for weights to re-stabilize, mice were given a consumption test of specific satiety to ensure that all mice were able to discriminate between the two food rewards. The consumption test consisted of a third satiety specific devaluation treatment, in which mice received *ad libitum* access to the opposite food reward to that which they received in the rewarded test. The satiation period occurred exactly as described above and afterwards the mice were left for 30 minutes with no access to either food reward. They were then presented with a choice of both food pellets and sucrose solution. A cup containing 3 grams of food pellets was placed at the back of the animal's holding cage, while a drinking bottle containing the sucrose solution was placed at the other end of the cage. The weight of the drinking bottle was recorded prior to the start of the consumption test. Mice were allowed 30 minutes to consume the two rewards. Rates of consumption were measured by determining the difference in weight of each food type before and after the consumption test.

4.2.3 Results

Instrumental conditioning

Figure 15A/B depicts the mean responses per minute in the correct and incorrect nose poke manipulandum across conditioning for male and female mice, respectively. Due to the nature of the instrumental training procedure, the results of the two daily sessions were averaged across each day. Visual inspection of Figure 15 suggests that both genotypes and both sexes demonstrated an increase in

instrumental response on the correct manipulandum during this acquisition phase. Female mice received more conditioning sessions due to the considerably lower rate of response they were demonstrating relative to male mice. Given that male and female mice received different amounts of instrumental training, data for each sexwere analysed independently.

Male Tg2576 and wild-type mice: A mixed ANOVA, with day (1-10) and response (correct versus incorrect) as the within-subjects factors and genotype as the between-subjects factor, revealed no effect of genotype [$F_{(1,17)}$ = 0.003, $p>0.05$], a significant effect of training day [$F_{(9,153)}$ = 27.414, $p<0.001$] and a significant effect of response [$F_{(1,17)}$ = 34.481, $p<0.001$]. This confirms that all mice, regardless of transgene status, demonstrated an increase in correct responding over the conditioning sessions. Results also revealed a significant day by response interaction [$F_{(9,153)}$ = 24.437, $p<0.001$], while all other interactions were non-significant ($Fs<1$). Tests of simple effects revealed a significant effect of day on both correct responses [$F_{(9,153)}$ = 26.591, $p<0.001$] and incorrect responses [$F_{(9,153)}$ = 7.249, $p<0.001$], as well as a significant effect of response on all 10 conditioning days [minimum value: $F_{(1,17)}$ = 9.347, $p<0.010$].

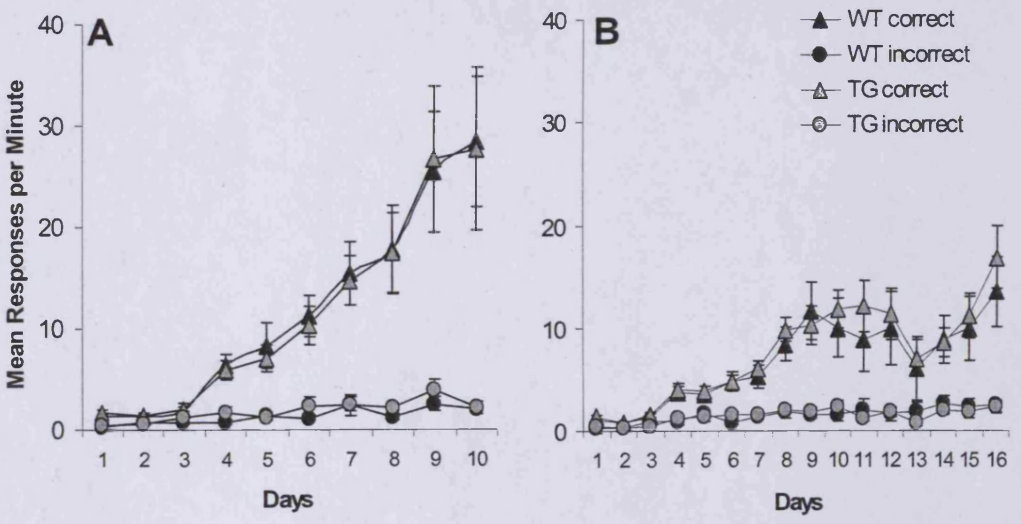


Figure 15: Mean nose pokes per minute on correct and incorrect manipulanda for Tg2576 and wild-type control mice across instrumental training days. A = Response rates for male mice; B = Response rates for female mice. Triangles denote 'Correct' responses; Circles denotes 'Incorrect' responses. Black symbols denote responses by wild-type control mice; Grey symbols denote responses by Tg2576 transgenic mice.

Female Tg2576 and wild-type mice: A mixed ANOVA, with day (1-16) and response (correct versus incorrect) as the within-subjects factors and genotype as the between-subjects factor, revealed no effect of genotype [$F_{(1,20)} = 0.144$, $p>0.05$], a significant effect of training day [$F_{(15,300)} = 22.920$, $p<0.001$] and a significant effect of response [$F_{(1,29)} = 35.614$, $p<0.001$]. This confirms that all mice, regardless of transgene status, demonstrated an increase in correct responding over the conditioning sessions. Results also revealed a significant day by response interaction [$F_{(15,300)} = 14.887$, $p<0.001$], while all other interactions were non-significant ($Fs<1$). Tests of simple effects on the day by response interaction revealed a significant effect of day for both correct [$F_{(15,300)} = 20.321$, $p<0.001$] and incorrect [$F_{(15,300)} = 5.141$, $p<0.001$] responses and a significant effect of response on all 16 conditioning days [minimum value: $F_{(1,20)} = 11.330$, $p=0.003$]. These results confirm that both genotypes and sexes showed a comparable increase in correct instrumental response throughout the instrumental acquisition stage.

Devaluation Extinction Test

The mean responses per minute during the 30 minute devaluation extinction test are presented in Figure 16A/B for male and female mice, respectively. 'Devalued' refers to the responses made on the manipulandum associated with the reward that was devalued by the satiation treatment; 'non-devalued' refers to responses made on the manipulandum associated with the reward that was not devalued during the satiation treatment. Visual inspection of Figure 16 suggests that both male and female wild-type control and Tg2576 mice made fewer responses on the manipulandum associated with the devalued reward than the manipulandum associated with the non-devalued reward. This impression was confirmed via statistical analysis using an ANOVA with genotype and response (devalued versus non-devalued) as factors. The analysis of the data from male mice revealed a significant effect of response [$F_{(1,17)} = 12.961$, $p<0.005$], no effect of genotype [$F_{(1,17)} = 0.044$, $p>0.05$] and no response by genotype interaction [$F_{(1,17)} = 0.044$, $p>0.05$]. For female data, a mixed two-way ANOVA, with genotype and outcome

as factors, revealed a similar pattern of results. The analysis confirmed a significant effect of outcome [$F_{(1,20)}$ = 43.472, $p<0.001$], no effect of genotype [$F_{(1,20)}$ = 0.315, $p>0.05$] and no response by genotype interaction [$F_{(1,20)}$ = 0.293, $p>0.05$]. Hence, both aged male and female Tg2576 mice demonstrated more instrumental responding on the manipulandum associated with the non-devalued food reward and comparably less responding on the manipulandum associated with the devalued reward.

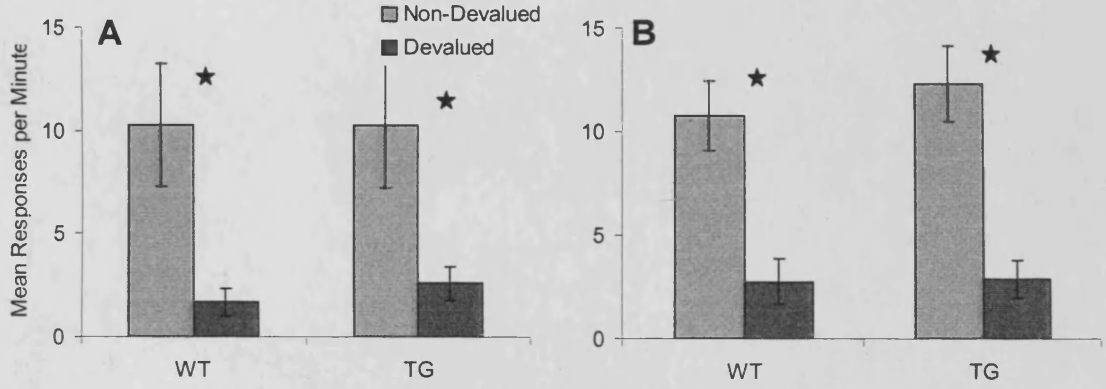


Figure 16: Mean nose pokes per minute on the manipulandum associated with the devalued versus the non-devalued food reward during 30 minute devaluation extinction test. Light grey bars denote responses on 'non-devalued' manipulanda; dark grey bars denote responses on 'devalued' manipulanda. Graph A depicts data from male mice and graph B depicts data from female mice. WT= wild-type control mice; TG= Tg2576 transgenic mice.

Devaluation Rewarded Test

The mean responses per minute during the 30 minute rewarded test are presented in Table 6. As in the extinction test, inspection of these figures suggests that both genotypes again demonstrated a higher rate of response on the manipulandum associated with the non-devalued, as compared to the devalued, food reward. Statistical analysis of data from male mice revealed a significant effect of outcome [$F_{(1,17)}$ = 17.087, $p=0.001$], no effect of genotype [$F_{(1,17)}$ = 0.034, $p>0.05$] and no outcome by genotype interaction [$F_{(1,17)}$ = 0.011, $p>0.05$]. Statistical analysis of data from female mice also revealed an effect of outcome [$F_{(1,20)}$ = 12.956, $p<0.005$], no effect of genotype [$F_{(1,20)}$ = 0.623, $p>0.05$] and no outcome by genotype interaction [$F_{(1,20)}$ = 0.656, $p>0.05$]. Hence, mice responded at a lower rate on the manipulandum delivering the devalued reward, regardless of transgene status.

	Males				Females			
	Non-Devalued Manipulanda		Devalued Manipulanda		Non-Devalued Manipulanda		Devalued Manipulanda	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type mice	32.1	8.0	2.1	1.3	24.7	10.9	0.6	0.4
Tg2576 mice	27.5	9.4	2.5	1.9	16	3.9	0.6	0.8

Table 6: Mean nose pokes per minute on the manipulandum associated with the devalued versus the non-devalued food reward during the 30 minute devaluation rewarded test.

Consumption test of specific satiety

The data for the consumption test are presented in Table 7. Inspection of these figures suggests that after the specific satiety treatment, all mice consumed less of the reward they had been exposed to during the satiation period (the devalued reward) and more of the non-devalued reward. Data for male and female mice were analysed separately via ANOVA, with genotype and outcome (non-devalued versus devalued) as factors. For male mice, results revealed a significant effect of outcome [$F_{(1,17)} = 31.089$, $p<0.001$], no effect of genotype [$F_{(1,16)} = 0.562$, $p>0.05$] and no outcome by genotype interaction [$F_{(1,17)} = 0.562$, $p>0.05$]. Statistical analysis of data from female mice also revealed an effect of outcome [$F_{(1,20)} = 148.355$, $p<0.001$], no effect of genotype ($F_{(1,20)} = 0.039$, $p>0.05$) and no outcome by genotype interaction [$F_{(1,20)} = 0.625$, $p>0.05$]. Hence, regardless of transgene status, all mice consumed less of the food reward with which they were sated.

	Males				Females			
	Non-Devalued Reward		Devalued Reward		Non-Devalued Reward		Devalued Reward	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type mice	1.8	0.4	0.2	0.1	1.9	0.2	0.4	0.2
Tg2576 mice	1.9	0.2	0.4	0.1	1.8	0.3	0.5	0.1

Table 7: Mean consumption (in grams) of devalued versus non-devalued food reward in 30 minutes after a satiety specific devaluation treatment.

4.2.4 Discussion

The results of the instrumental outcome devaluation task demonstrated that, despite displaying amyloid pathology throughout the amygdala complex, aged Tg2576 mice altered their instrumental responding in accordance with the current incentive value of an outcome. Not only was the behaviour of the transgenic mice comparable to that of wild-type mice, their instrumental actions were goal directed and they showed no sign of an impairment on any of the three (extinction, rewarded, consumption) tests. This implies that Tg2576 and wild-type mice were capable of forming an action-outcome representation and using this representation to access information regarding the current incentive value of the outcome and thereby altering their behavioural response. These results indicate that ß-amyloid pathology did not compromise the neural circuitry underlying instrumental outcome devaluation in aged Tg2576 mice.

4.3 Experiment 5a & b: Pavlovian - Instrumental Transfer and CS Devaluation Task

4.3.1 Introduction

The results of Experiment 4a,b show that instrumental action-outcome associations remain sensitive to changes in the incentive motivational value of the outcomes in aged Tg2576 mice. The main aim of the present experiment was to establish the effects of the APPswe on Pavlovian CS-US associations. Pavlovian conditioning results in the formation of associations between a cue and the stimulus-specific sensory properties of an outcome as well as the general motivational properties of a reward (Corbit, Janak & Balleine, 2007). The Pavlovian-instrumental transfer (PIT) task can be used to assess the influence of either the sensory-specific or the general excitatory effects of a reward-related cue on instrumental actions associated with the same appetitive rewards (Blundell, Hall & Killcross, 2001; Hall et al., 2001).

Considerable evidence points to the amygdala as a primary component of the neural system underlying motivational learning (Dayan & Balleine, 2002). The locus of this influence within the amygdala, however, is somewhat controversial. Using a PIT paradigm, Blundell and colleagues (2001) demonstrated that lesions of the BLA disrupted the sensory-specific influence of the conditioned stimuli on instrumental actions. Others, however, have reported that while BLA lesions had no impact on the Pavlovian-to-instrumental transfer effect, lesions of the CeN successfully abolished this form of motivational learning (Hall et al., 2001; Holland and Gallagher, 2003). It is worth noting, however, that the PIT designs differed between these studies, which likely influenced the nature of the transfer effect demonstrated in each paradigm. Specifically, Hall et al. (2001) and Holland and Gallagher (2003) trained their experimental rats on one instrumental response and conditioned one CS-outcome association. According to Balleine (1994), this design likely assessed transfer based upon the more general motivational/affective influence of the Pavlovian CS. Blundell and colleagues (2001), however, conditioned two distinct action-outcome associations and employed two Pavlovian auditory CSs. Their paradigm was designed to assess the effects of devaluation and

transfer manipulations on instrumental responses influenced by the sensory-specific features of the rewards associated with the Pavlovian CSs and instrumental actions (Balleine, 1994). Consistent with this approach, Corbit and Balleine (2005) used a more elaborate PIT paradigm, with three CS-US pairings and two action-outcome contingencies, to reveal a double dissociation between lesions of the BLA and CeN on general and outcome-specific transfer. More specifically, they reported that lesions of the CeN abolished general transfer but spared the sensory specific influence of Pavlovian cues on performance, whereas lesions of the BLA had the opposite effect. The authors conclude that the BLA is crucial for the motivational processing of outcome-specific incentives, while the CeN is involved in processing the general motivational aspects of incentive learning processes (Corbit & Balleine, 2005).

Similar to the instrumental outcome devaluation procedure, Pavlovian outcome devaluation tasks have also been shown to be sensitive to amygdala dysfunction. Pre-training neurotoxic lesions of the BLA disrupted the effect of lithium chloride induced reinforcer devaluation on magazine approach responses after rats were trained to associate a light CS with an appetitive reward (Hatfield et al., 1996). BLA lesions did not, however, disrupt the acquisition of conditioned responding to the CS. Rats with neurotoxic lesions of the CeN, however, demonstrated an appropriate change in approach behaviour in the presence of the visual CS after a change in the incentive value of the appetitive reward (Hatfield et al., 1996). Furthermore, Pickens and colleagues (2003) demonstrate that, unlike pre-training lesions of the BLA, post-training lesions do not disrupt the reinforcer devaluation effect. The authors suggest, therefore, that the BLA is necessary for forming the association between the incentive properties of the appetitive reward and the Pavlovian CS, but is not crucial for the behavioural expression or maintenance of these representations in memory.

The aim of Experiment 5a & b was to assess the effect of the APPswe mutation on Pavlovian incentive learning procedures. In Experiment 3 (fear conditioning), it was suggested that the deficit in freezing elicited by a Pavlovian cue (a tone) may be the result of the aberrant processing of the sensory-specific, but not general motivational, aspects of a US representation. The results of Experiment 4, which

used an instrumental paradigm, contradicted this hypothesis. The main aim of the present set experiments was to determine whether the APPswe mutation specifically affected the ability of Pavlovian cues to access a sensory-specific representation of the outcome. The first stage of the experiment assessed the influence of Pavlovian CSs on goal-directed behaviour using an outcome-specific PIT task, while the second stage assessed the ability of animals to alter their goal-directed behaviours according to the current incentive value of a Pavlovian US using an outcome devaluation procedure. It was expected that impairments in the PIT and devaluation tasks would be revealed if ß-amyloid pathology disrupted Pavlovian incentive learning processes in the aged Tg2576 mice.

4.3.2 Method

Experiment 5a: Pavlovian-Instrumental Transfer

Subjects

Fourteen female wild-type mice and eighteen female Tg2576 mice, aged approximately 22 months, were used in this study. No male mice of an appropriate age were available at the time of testing to provide a sexcomparison (see Experiment 4). Mice were house in mixed genotype litters of 2-4 mice and maintained on a 12 hour light:dark cycle. All testing occurred during the light phase. Mice were food deprived to 85-90% of their baseline weights and were weighed and health checked daily to ensure the food restriction had no averse affects on the animals' health. All mice were maintained, and all procedures conducted, in accordance with Home Office (U.K.) regulations.

Apparatus

Behavioural testing was conducted in the same 8 Med Associates (St. Albans, VT) operant boxes used in Experiment 4. During the instrumental conditioning session, house lights were extinguished in order to enhance the salience of the nose poke illumination. During the Pavlovian conditioning sessions, nose pokes were blocked with circular metallic inserts to prevent nose poke responses. Auditory stimuli in

the chambers consisted of a 2000 Hz tone set at 90 dB and a 90 dB buzzer with a frequency 100 Hz. Food rewards consisted of 20mg food pellets and 20mg sucrose pellets (TestDiet, MLab Rodent Tablet, Sandown Scientific, IN, USA).

Behavioural training and testing

Conditioning: Mice received two consecutive days of magazine training and habituation to the chambers, twice daily. During the twenty minute habituation sessions, the food rewards (either food or sucrose pellets) were delivered on a random time 30 second schedule (RT-30). Mice received one session of exposure to each reward daily. Conditioning commenced on day 3 and consisted of two daily twenty minute sessions, separated by 5 hours.

Ten days of instrumental conditioning sessions were conducted on alternate days. During instrumental sessions, the mice were trained to respond in the nose poke manipulanda as a means of obtaining food rewards and to aid the mice in learning specific action-outcome contingencies. During this phase of conditioning, both nose poke manipulanda were exposed and recorded entries, but only the active nose poke was illuminated and resulted in the presentation of a food reward. On days 3 and 5, the mice received continuous reinforcement when they responded in the active manipulandum and on day 3 the manipulanda were initially smeared with a small quantity of food to encourage responding. On days 7 and 9, mice received food rewards on a fixed-ratio 5 schedule (FR-5), such that they received one pellet for every 5 nose poke responses. Day 11 utilized an FR-10 schedule, while mice received rewards on an FR-15 schedule on days 13 and 15. On days 17, 19 and 21, rewards were delivered on an FR-20 schedule. Each day mice received two sessions, each presenting one action-outcome contingency, which were fully counterbalanced across transgene status and time of day.

Ten days of Pavlovian conditioning sessions were conducted on alternate days. The aim of this conditioning phase was to condition specific auditory CS-reward associations. Mice were presented with five 1-minute presentations of an auditory CS, either a tone or a buzzer stimulus, separated by variable inter-trial intervals (ITI), with a mean duration of three minutes. During the CS presentation, either

food pellets or sucrose pellets were delivered on a RT-30 second schedule. During this phase, magazine entries were recorded, while the nose poke manipulanda were covered. Each day mice received two sessions, each presenting one CS-US contingency, which were fully counterbalance across transgene status and time of day.

Pavlovian-Instrumental Transfer Test: The Pavlovian to instrumental transfer (PIT) test consisted of ten one-minute CS presentations in extinction, each separated by a one minute ITI. Half of the CS presentations were of the tone stimulus, while the other half were of the buzzer stimulus, presented in a pseudo-random order with no more than two simultaneous presentations of the same CS. Nose pokes were accessible and illuminated. Entries into both the magazine and both nose poke manipulanda were recorded.

Experiment 5b: CS Devaluation Task

Subjects and Apparatus

The mice and conditioning equipment used in this stage of the experiment were the same as those used in Experiment 5a.

Behavioural Training

Conditioning: Six days after the PIT test, the animals received two days of re-training using the same Pavlovian conditioning procedure as in Experiment 5a. Mice received two twenty minute sessions per day, which re-conditioned the same CS-outcome contingencies as in the first stage of the experiment.

Satiety Specific Devaluation Procedure and Extinction Test: After the last day of Pavlovian re-training, the incentive value of one of the food rewards was decreased by using a satiety specific devaluation procedure, whereby mice were allowed *ad libitum* access to either food or sucrose pellets in their cages for 120 minutes. The allocation of devalued food reward was fully counterbalanced across genotype and Pavlovian-outcome assignment. After the satiety specific

devaluation treatment, mice were tested in two stages in the operant chambers in extinction. The extinction tests consisted of a 3 minute ITI period, followed by a 1 minute presentation of a CS (either tone or buzz), then two further 1 minute ITI-1 minute CS periods. Hence, in each test the same CS was presented 3 times in total. The 8 minute extinction tests were separated by a 10 minute break and magazine entries were recorded during the ITI and CS presentations. Each animal received one extinction test in which the tone acted as the auditory CS and one in which the buzz was the CS. The order of the extinction tests was fully counterbalanced.

Consumption Test: Mice then underwent a consumption test in their home cages to ensure that they could distinguish perceptually between the food and sucrose pellets. The consumption test consisted of sating the animal for 120 minutes with one of the two food rewards, then after a 30 minute break, mice were presented with both food rewards and allowed a further 30 minutes to consume as much of each as desired. The containers containing the food and sucrose pellets were weighed before and after the consumption test to determine the quantity consumed.

4.3.3 Results

Experiment 5a: Pavlovian Instrumental Transfer Task

Conditioning Phase

Raw data: The data from the instrumental and Pavlovian conditioning phases are presented in Figure 17. Visual inspection suggests that during instrumental conditioning both genotypes increased responding on the correct nose poke across the conditioning session, with wild-type mice increasing more rapidly. Inspection of the Pavlovian conditioning phase suggests that both genotypes reduced magazine responding during the ITI as compared to the CS presentations, with wild-type mice responding at a greater rate. Data were analysed by ANOVA with genotype, session and nose poke (correct versus incorrect) as factors. Results of the instrumental conditioning phase revealed significant main effects of day [$F_{(9,270)} = 68.609$, $p<0.001$], nose poke [$F_{(1,30)} = 97.714$, $p<0.001$] and genotype

[$F_{(1,30)}$ =10.999, p=0.002]. Furthermore, there was a significant day by genotype interaction [$F_{(9,270)}$ = 10.849, p<0.001], significant nose poke by genotype interaction [$F_{(1,30)}$ = 8.033, p=0.008] and significant day by nose poke interaction [$F_{(9,270)}$ = 63.725, p<0.001]. The three way interaction was also significant [$F_{(9,270)}$ = 9.983, p<0.001].

The genotype by day interaction was analysed using tests of simple effects, which revealed a significant effect of genotype on days 4-10 [minimum: $F_{(1,30)}$ = 5.279, p<0.03] and a significant effect of day for both wild-type and Tg2576 mice [$F_{(9,22)}$ = 17.270 and 11.881, p<0.001, respectively]. Further analysis of the genotype by nose poke interaction revealed a significant effect of nose poke for both wild-type and Tg2576 mice [$F_{(1,30)}$ = 71.903 and 28.407, p<0.001, respectively], but only a significant effect of genotype for the 'correct' nose poke [$F_{(1,30)}$ = 9.482, p<0.005], not the 'incorrect' nose poke [$F_{(1,30)}$ = 3.655, p>0.05].

The day by nose poke interaction was analysed using tests of simple effects, which revealed a significant effect of nose poke on days 1-10 [minimum: $F_{(1,30)}$ = 57.733, p<0.001], but a significant effect of day for the 'correct' nose poke only [$F_{(9,22)}$ = 22.210, p<0.001], not the 'incorrect' nose poke [$F_{(9,22)}$ = 1.191, p>0.05]. Further analysis of the three way interaction revealed a significant effect of genotype on the 'correct' nose poke only on days 6-10 [minimum: $F_{(1,30)}$ = 9.094, p<0.006].

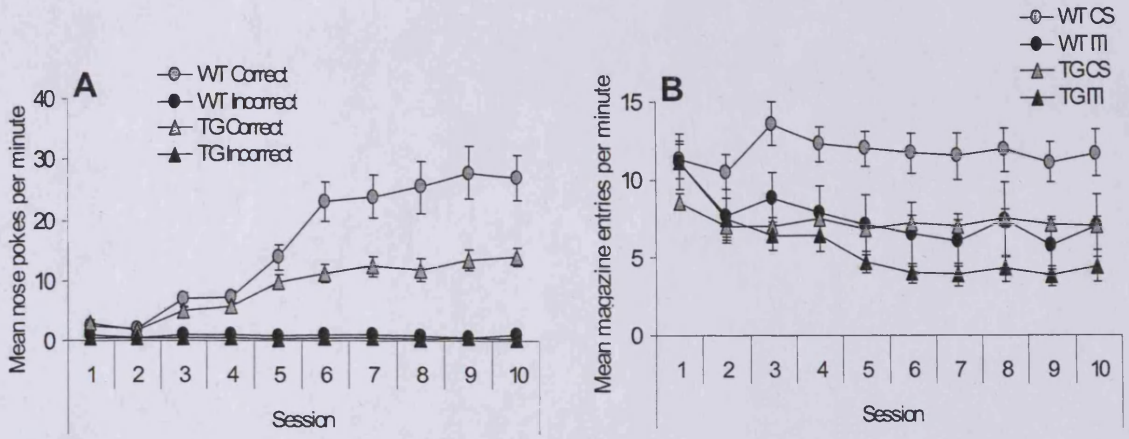


Figure 17: Mean responses per minute during (A) the instrumental and (B) the Pavlovian conditioning phases. In graph A, grey symbols indicate responses on the correct manipulandum, while black symbols indicate responses on the incorrect manipulandum. Circles denote wild-type mice; triangles denote Tg2576 mice. In graph B, grey symbols indicate responses during the presentation of the CS, while black symbols indicate responses during the ITI period. Circles denote wild-type mice; squares denote Tg2576 mice.

Data from the Pavlovian conditioning phase were analysed by ANOVA with genotype, session and phase (CS versus ITI) as factors. Results revealed significant main effects of day [$F_{(9,270)} = 9.340$, $p<0.001$], phase [$F_{(1,30)} = 26.465$, $p<0.001$] and genotype [$F_{(1,30)} = 7.015$, $p<0.02$]. Furthermore, there was a significant day by genotype interaction [$F_{(9,270)} = 2.156$, $p<0.03$], a significant phase by genotype interaction [$F_{(1,30)} = 4.884$, $p<0.04$] and a significant day by phase interaction [$F_{(9,270)} = 17.041$, $p<0.001$]. The three way interaction was non-significant [$F_{(9,270)} = 0.891$, $p>0.05$].

The genotype by day interaction was analysed by tests of simple effects, which revealed a significant effect of genotype on days 3-10 [minimum: $F_{(1,30)} = 4.687$, $p<0.04$] and a significant effect of day for both wild-type and Tg2576 mice [$F_{(9,22)} = 3.965$ and 4.432, p's <0.005, respectively].

Analysis of the phase by genotype interaction revealed a significant effect of phase for both genotypes [$F_{(1,30)} = 24.039$ and 4.920, p's <0.04, respectively], but a significant effect of genotype only for the CS phase [$F_{(1,30)} = 20.139$, $p<0.001$]. The effect of genotype for the ITI phase was non-significant [$F_{(1,30)} = 1.441$, $p>0.05$].

Analysis of the phase by day interaction revealed a significant effect of phase for days 2-10 [minimum: $F_{(1,30)} = 7.181$, $p<0.02$]. Furthermore, results revealed a non-significant effect of day for the CS phase [$F_{(9,22)} = 1.255$, $p>0.05$], but a significant effect of day for the ITI phase [$F_{(9,22)} = 8.628$, $p<0.001$].

Discrimination ratios: As a means of eliminating the contribution of differences in response rates to the assessment of conditioning in each stage, discrimination ratios were calculated according to the formula: [correct response/(correct + incorrect responses)]. In the instrumental phase, correct responses refer to responses made on the active manipulandum and in correct responses refer to responses made on the inactive manipulandum; in the Pavlovian phase, correct responses refer to responses made during the CS presentations and incorrect responses were those made during the ITI period. The results, which are presented in Figure 18, were analysed by ANOVA with day and genotype as factors. Visual inspection suggests that during both phases of conditioning, correct responses increased with increased conditioning and there appear to be no differences between the genotypes. Analysis of the instrumental conditioning phase confirmed this impression by revealing a significant main effect of day [$F_{(9,270)} = 12.729$, $p<0.001$], a non-significant effect of genotype [$F_{(1,30)} = 1.246$, $p>0.05$] and a non-significant interaction [$F_{(9,270)} = 1.002$, $p>0.05$]. One sample t-tests revealed a significant difference from 0.5 for wild-type mice on each of the 10 days [minimum: $t(13) = 5.996$, $p<0.001$] and for Tg2576 mice on each of the 10 days [minimum: $t(17) = 7.388$, $p<0.001$].

Analysis of the Pavlovian conditioning phase also confirmed this impression by revealing a significant effect of day [$F_{(9,270)} = 25.192$, $p<0.001$], a non-significant effect of genotype [$F_{(1,30)} = 1.471$, $p>0.05$] and a non-significant interaction [$F_{(9,270)} = 0.991$, $p>0.05$]. One sample t-tests revealed a significant difference from 0.5 for wild-type mice on days 2-10 [minimum: $t(13) = 3.693$, $p=0.003$] and for Tg2576 mice on each of the 10 days, except day 2 [minimum: $t(17) = 2.167$, $p<0.05$].

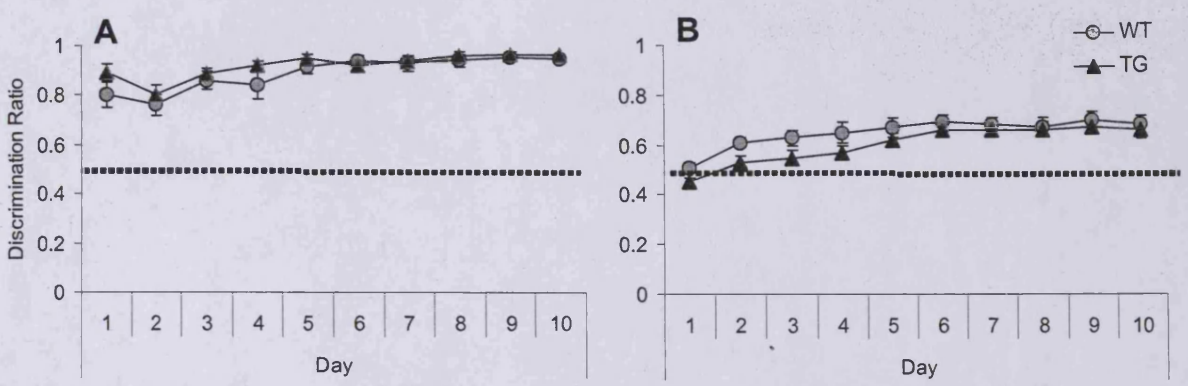


Figure 18: Discrimination ratios for the proportion of responses (A) on the correct nose poke during the instrumental conditioning phase and (B) during the CS presentations in the Pavlovian conditioning stage. Grey circles denote wild-type control mice; black triangles denote transgenic Tg2576 mice.

Pavlovian-to-Instrumental Transfer Test

Raw data: The results of the PIT test are displayed in Figure 19. Unfortunately, due to low rates of responding overall during the PIT test, it was necessary to remove mice that failed to emit a response during CS presentations from the analysis. Two Tg2576 mice were therefore removed from the analysis, leaving a total of 16 mice in this condition. Data were analysed to determine whether responding on the nose poke manipulandum during the CS presentation showed outcome specificity. An ANOVA, with CS (correct vs. incorrect) and genotype as factors, revealed a significant main effect of CS [$F_{(1,28)} = 7.579$, $p=0.010$], a non-significant effect of genotype [$F_{(1,28)} = 0.598$, $p>0.05$] and a non-significant interaction [$F_{(1,28)} = 0.317$, $p>0.05$]. This suggests that both wild-type and Tg2576 mice responded more frequently on the nose poke that was conditioned with the same outcome as the CS.

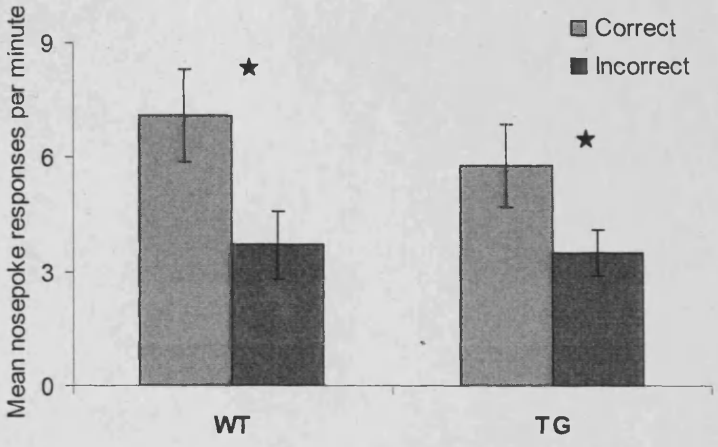


Figure 19. Mean nose poke responses during the Pavlovian to instrumental transfer (PIT) test. Light grey bars denote responses made during the presentation of the CS associated with same reward, while dark grey bars denote responses made during the presentation of the CS associated with a different reward. WT = wild-type; TG = transgenic Tg2576. Error bars = +/- S.E.M. * = $p<0.05$.

Discrimination Ratios: Discrimination ratios were calculated as means of eliminating variability in response rates (see Figure 20). The ratios were calculated according to the formula [Correct CS/(Correct CS + Incorrect CS)] for the outcome-specific PIT effect. An independent samples t-test comparing wild-type and Tg2576 mice revealed a non-significant difference between the genotypes [$t(28) = 0.664$, $p>0.05$]. A one-sample t-test comparing the performance of wild-type mice during the CS to 0.5 revealed that responding was significantly different to chance [$t(13) = 2.334$, $p<0.05$], while Tg2576 mice did not differ significantly from chance [$t(15) = 1.008$, $p>0.05$]. These results only partially support the results of the ANOVA performed on the raw data. Interestingly, a one-sample t-test comparing the combined cohort of wild-type and transgenic Tg2576 mice to chance revealed a significant difference between the proportion of CS responses and 0.5 [$t(29) = 2.187$, $p<0.05$]. These results suggest that the main effect of CS may perhaps have been driven more strongly by the data from the wild-type mice. However, the absence of a significant interaction in this analysis urges caution with accepting this interpretation and one must acknowledge that the lack of a statistical difference in the Tg2576 group may reflect a lack of power. Indeed, power analysis revealed the need for approximately 54 Tg2576 mice for a power level of 0.8 to be achieved in this cohort. Nevertheless, the results from the one-sample t-test, combined with a non-significant interaction and a non-significant

main effect of genotype, suggest that the main effect of CS, albeit a relatively weak effect, can be considered to be evident in both wild-type and Tg2576 mice. Furthermore, the more general motivational impact of the CS on instrumental response was assessed by comparing the overall responses made during the CS presentations and the ITI periods. The results (data not shown) indicated task was not sensitive to this aspect of behaviour in either wild-type or Tg2576 mice. This, however, was unsurprising given that the task was designed to assess the sensory-specific and not the general motivational enhancement elicited by the CS (see Corbit et al., 2007).

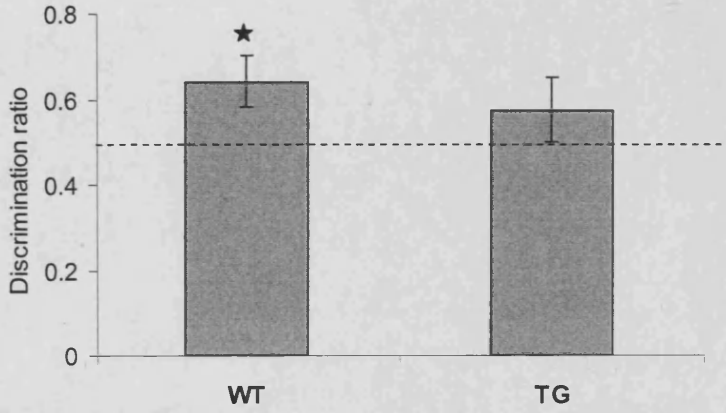


Figure 20: Discrimination ratios for the outcome-specific PIT effect. Bars represent the ratio of responses occurring during the associated CS, as compared to the non-associated CS. Dotted lines represent chance levels of responding (0.5). WT = wild-type; TG = Tg2576. Error bars = +/- S.E.M. * = $p<0.05$.

Overall, the results of the PIT task suggest that, regardless of transgene status, all mice were capable of demonstrating outcome-specific transfer. That is, the presence of ß-amyloid pathology in the amygdala was not sufficient to completely eliminate the selective excitatory effect of reward-related CSs on instrumental responding consistent with the results of Experiment 4.

Experiment 5b: CS Devaluation Task

Re-training of Pavlovian conditioning

Magazine entries made during the two days of re-training (Table 8) were analysed by ANOVA, in which day, genotype and phase (CS vs. ITI) were factors. Results revealed a significant main effect of day [$F_{(1,30)} = 21.668$, $p<0.001$], a significant main effect of phase [$F_{(1,30)} = 78.184$, $p<0.001$] and a non-significant main effect of genotype [$F_{(1,30)} = 2.687$, $p>0.05$]. There was a significant phase by genotype interaction [$F_{(1,30)} = 8.912$, $p=0.006$] and a non-significant day by genotype interaction [$F_{(1,30)} = 2.260$, $p>0.05$]. All other interactions were non-significant ($Fs<1$). The phase by genotype interaction was analysed using tests of simple main effects, which revealed a significant effect of phase for both wild-type and Tg2576 mice [$F_{(1,30)} = 62.173$ and 19.602, $p<0.001$, respectively]. Furthermore, results revealed a significant effect of genotype for the CS phase [$F_{(1,30)} = 9.954$, $p=0.004$], but not the ITI phase [$F_{(1,30)} = 0.018$, $p>0.05$].

	Day 1				Day 2			
	CS		ITI		CS		ITI	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type mice	15.5	1.5	4.3	0.6	20.1	2.0	8.2	1.6
Tg2576 mice	11.1	1.1	5.5	1.7	13.4	0.9	7.5	1.5

Table 8: Mean magazine entries per minute during two days of Pavlovian re-training.

Extinction Test

Data from the extinction tests are presented in Figure 21. Data were removed from analyses if no responses were made during either devaluation task, which resulted in the removal of data for a further one wild-type and two transgenic mice. Thus, 13 wild-type and 16 Tg2576 mice were used for the analysis. Visual inspection of Figure 21 suggests that both genotypes made more magazine entries during the presentation of the non-devalued CS. This impression was confirmed by statistical analysis using an ANOVA with genotype and CS (devalued vs. non-devalued) as

factors. Results revealed a significant main effect of CS [$F_{(1,27)} = 4.874$, $p<0.05$], a non-significant main effect of genotype [$F_{(1,27)} = 0.451$, $p>0.05$] and a non-significant interaction [$F_{(1,27)} = 0.510$, $p>0.05$]. Hence, all mice reduced responding in the magazine during the presentation of the auditory CS associated with the devalued food reward.

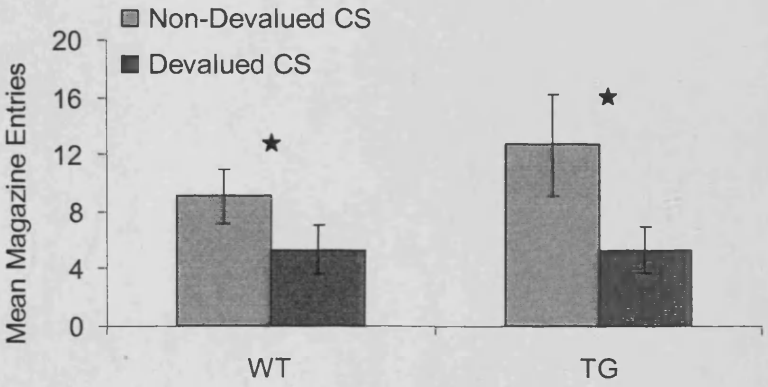


Figure 21: Mean magazine entries during the CS devaluation extinction tests. Grey bars denote responses made during the presentation of the auditory CS associated with the non-devalued food reward; striped bars denote responses made during the presentation of the auditory CS associated with the devalued food reward. WT = wild-type; TG = transgenic Tg2576. Error bars = +/-S.E.M.

Discrimination ratios [Non-devalued CS/Total CS responses] were also calculated and are presented in Figure 22. An independent samples t-test revealed a non-significant difference between the genotypes [$t(27) = -0.029$, $p>0.05$]. One-sample t-tests revealed non-significant differences between response rates and chance in wild-type [$t(12) = 1.547$, $p>0.05$] and Tg2576 [$t(15) = 1.613$, $p>0.05$] mice. Interestingly, however, a one-sample t-test combining the wild-type and transgenic Tg2576 mice revealed a significant difference between proportion of responses during the non-devalued CS did differ significantly from chance [$t(28) = 2.269$, $p<0.05$]. These results suggest that the lack of significant effect seen in the separate one-sample t-tests may be attributable to a lack of power. Indeed, power analysis recommends employing 24 mice of each genotype to achieve a power level of 0.8. Nevertheless, these results corroborate the claim that the Pavlovian devaluation effect is unaffected by the APPswe mutation in aged Tg2576 mice.

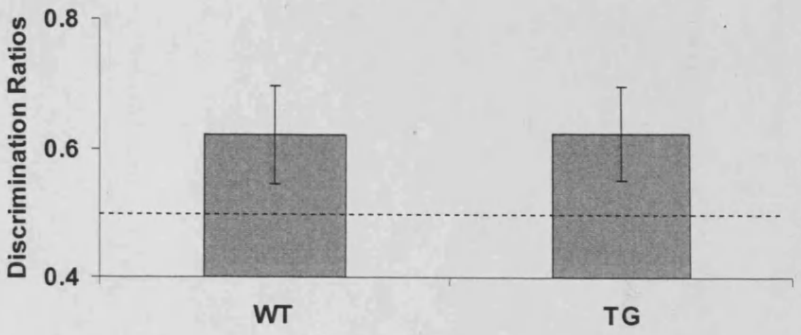


Figure 22: Discrimination ratios for responding during the non-devalued CS, as compared to the devalued CS. Dotted line denotes chance responding (0.5). WT = wild-type; TG = transgenic Tg2567. Error bars = +/- S.E.M.

Consumption test of specific satiety

The data for the consumption test are presented in Table 9. Inspection of these figures suggests that after the specific satiety treatment, all mice consumed less of the reward they had been exposed to during the satiation period (the devalued reward) and more of the non-devalued reward. Data were analysed via ANOVA, with genotype and outcome (non-devalued versus devalued) as factors. Results revealed a significant effect of outcome [$F_{(1,30)} = 61.394$, $p<0.001$], no effect of genotype [$F_{(1,30)} = 3.089$, $p>0.05$] and no outcome by genotype interaction [$F_{(1,30)} = 0.215$, $p>0.05$]. Hence, regardless of transgene status, all mice consumed less of the food reward with which they were sated.

	Non-Devalued Reward		Devalued Reward	
	Mean	SEM	Mean	SEM
Wild-type mice	1.6	0.2	0.4	0.1
Tg2576 mice	1.3	0.1	0.3	0.1

Table 9: Mean consumption (in grams) of devalued versus non-devalued food reward in 30 minutes after a satiety specific devaluation treatment.

4.3.4 Discussion

The PIT task was designed to assess the ability of the CS to influence instrumental responding. Despite overall differences in response rates during the conditioning stage, the proportions of correct responses performed during the instrumental and Pavlovian conditioning sessions were comparable between Tg2576 and wild-type mice. Hence, both genotypes demonstrated an ability to acquire the instrumental and Pavlovian approach behaviour. The PIT test assessed the selective excitatory influence of auditory CSs on instrumental responding. The results revealed that wild-type and Tg2576 mice increased responding on the manipulandum that was associated with the same reward as the CS. This confirms that both genotypes were able to form a representation of the association between the sensory-specific properties of the reinforcer and an auditory CS, and were able to use this representation to guide instrumental behaviours. The lack of a behavioural impairment in Tg2576 mice suggests that the presence of ß-amyloid pathology in the BLA was not sufficient to disrupt sensory-specific transfer in this task. See Chapter Discussion (Section 4.6) for a more thorough consideration of Pavlovian-instrumental transfer behaviour in Tg2576 mice. The CS devaluation task was designed to assess the influence of incentive processing on Pavlovian responding. The results of this test revealed that all mice, regardless of transgene, were capable of forming a representation of the CS-outcome association and using this to access information regarding the current incentive value of the outcome. This conclusion is based on the finding that both Tg2576 and wild-type mice preferentially decreased responding on the manipulandum associated with the devalued reward. Hence, the APPswe mutation does not disrupt either outcome specific Pavlovian-instrumental transfer or outcome-specific Pavlovian incentive learning using discrete conditioned stimuli.

4.4 Experiment 6: Context-Outcome Learning: Aged Male and Female Tg2576 mice

4.4.1 Introduction

Results from Experiments 4 and 5 revealed that wild-type and Tg2576 mice are capable of forming goal-directed representations of action-outcome and CS-outcome associations. Furthermore, they can use instrumental or Pavlovian cues to assess information regarding the current incentive value of the reinforcer. These results suggest that despite the presence of β-amyloid in structures critical for incentive learning (i.e., the amygdala) these functions are not disrupted in aged Tg2576.

The foregoing analysis suggests that the neural systems supporting incentive learning processes involving discrete punctate cues and actions remains intact in ageing Tg2576 mice. This contrasts with extensive evidence for ß-amyloid induced deficits in hippocampal synaptic density, plasticity and spatial navigation (Chapman et al., 1999; Hsiao et al., 1996; Jacobsen et al., 2006; Ognibene et al., 2005). One task that has often been linked to impairments in hippocampal function is context-based fear conditioning. In this simple procedure the animal receives unsignalled presentations of a footshock in a distinctive context. As a result of these presentations, wild-type mice show fear (freezing) when placed in the conditioning context (Jacobsen et al., 2006; Radulovic et al., 1998). Rats and mice with hippocampal lesions, however, often show deficits in contextual freezing (Richmond et al., 1999; Gerlai, 1998; but see Maren, 2008). Previous studies have also shown that mice overexpressing APP mutations are impaired on contextual fear conditioning (Comery et al., 2005; Corcoran et al., 2002; Dineley et al, 2002; Dong et al., 2005; Maren & Holt, 2000; Ohno et al., 2006; Quinn et al., 2007), consistent with the hypothesis that hippocampal function is impaired by β-amyloid production. Although deficits in contextual fear conditioning are often thought to reflect a deficit in forming a representation of the context (Fanselow, 2000), an alternative interpretation is that the APPswe mutation may specifically impair the ability of a contextual CS to gain access to a representation of the sensory-specific properties of a US. Despite the absence of a context-freezing deficit in Tg2576

mice (Experiment 3), the present study examined whether the APPswe mutation would influence context-based sensory specific outcome associations. More specifically, Experiment 6a examined the effects of the APPswe mutation on an appetitive context-outcome devaluation task. In addition, Experiment 6b examined whether the Tg2576 mutation impaired the formation of a context representation by training the mice on a Pavlovian context-discrimination task (Experiment 6b). If the APPswe mutation impaired the formation of a context representation then one might predict deficits on both tasks. In contrast, if the mutation impaired associations involving the specific sensory (but not the general affective) properties of a US with a context representation, one would predict a deficit in the former but not the latter procedure.

4.4.2 Method

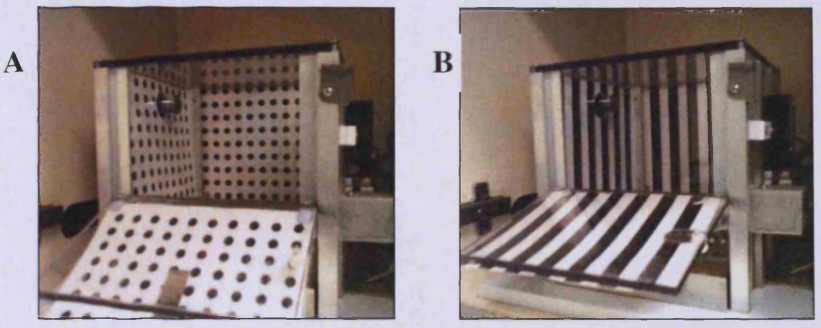
Subjects

The male mice used in this experiment were a subset of those used in Experiment 4, the remaining mice died prior to the start of the current experiment. Initially, 7 male Tg2576 mice and 8 male wild-type controls, aged 20 months, were used. Mice were food deprived to 85% of *ad libitum* baseline weight and water was removed one hour prior to the start of testing. In a separate replication, 8 female Tg2576 mice and 8 female wild-type littermate controls that had survived to 22 months of age were used. Male and female mice were maintained, trained and tested in an identical manner throughout the experiment.

Apparatus

The operant chambers used were the same as those used in Experiments 4 and 5. For this experiment, however, the walls and ceiling of the chambers were altered such that they denoted two distinct contexts, one displaying a pattern of spots and the other a pattern of stripes (See Picture 3). The spot pattern consisted of 6mm diameter black spots, situated 7mm apart on a white background; the stripe pattern consisted of alternating 11cm black and white stripes, situated in a vertical direction on the chamber walls. Throughout the behavioural training sessions, the

house light was illuminated to ensure that the mice could detect the different contexts.



Picture 3: Pattern of spots (A) and stripes (B) used in operant chambers to denote distinct contexts.

Behavioural Training

Experiment 6a: Context conditioning and outcome devaluation

Context Conditioning: The context conditioning phase consisted of two x 10 minute sessions per day, for four days. Both conditioning sessions occurred in the afternoon and were separated by approximately 15 minutes. During this training phase, mice were placed into one context (e.g. spotted context) where a food reward (e.g. pellets) was delivered on a random time thirty second schedule (RT-30). Mice were then placed back into their home cages for 15 minutes, before being placed into the second context and receiving the other food reward. Behaviour was measured in terms of rate of entry into the magazine. Training was fully counterbalanced cross genotype, order of context and context-reward assignment.

Satiety specific devaluation treatment: The day after training concluded, all mice underwent a satiety specific devaluation treatment. This consisted of allowing each mouse free access to one of the two food rewards in the home cages for 120 minutes.

Context Extinction Test: After the devaluation treatment, mice were tested in the operant chambers in extinction. This consisted of placing a mouse in one context

and measuring the mouse's rate of magazine entry for 10 minutes, in the absence of rewards. The mouse was placed back into its home cage for 15 minutes before being tested in the second context.

Experiment 6b: Context Discrimination

Baseline conditioning: As a means of re-instating responses in the operant chambers after the extinction test, mice were given two days (four sessions) of context conditioning, which proceeded in an identical manner to that given during the context devaluation task.

Context Discrimination: In this task, context conditioning was similar to that of the context devaluation task except that now one of the two contexts was no longer reinforced. For half the mice, the context in which food pellets were delivered was now no longer reinforced, and for the other half of the mice, the context in which sucrose was delivered was now no longer reinforced. This effect was fully counterbalanced across context and genotype. Mice again received two 10 minute sessions each afternoon, separated by approximately 15 minutes, for eight days.

Context Extinction Test: The day after training concluded, mice were tested in the operant chambers in extinction. This consisted of placing an animal in a context and measuring the animal's rate of magazine entry for 10 minutes in the absence of rewards. The animal was again placed back into its home cage for 15 minutes before being tested in the second context.

4.4.3 Results

Experiment 6a: Context Devaluation Task: Aged Tg2576 mice.

In order to ensure that sensitivity to genotypic differences was maintained, and since all mice underwent identical training and testing procedures throughout all stages of the experiment, it was decided to collapse the data for male and female mice and to analyse them collectively. To ensure that results were not skewed by

combining data for male and female mice, sex was included as a factor during statistical analyses and data are presented separately in graphs.

Context Conditioning

Figure 23 depicts the mean magazine entries per minute made by wild-type and transgenic Tg2576 mice during the context conditioning phase. Raw data for each sex are depicted independently in each figure. Visual inspection of graphs A and B suggests that both sexes and both genotypes showed an increase in rate of magazine entry over the four day acquisition phase. Data were analysed using a mixed ANOVA with genotype, e and day as factors. Results revealed a non-significant main effect of genotype [$F_{(1,27)} = 0.763$, $p>0.05$], a non-significant main effect of sex [$F_{(1,27)} = 0.223$, $p>0.05$] and a significant main effect of day [$F_{(3,81)} = 7.346$, $p<0.001$]. Furthermore, statistical analysis demonstrated a significant day by sex interaction [$F_{(3,81)} = 3.719$, $p<0.02$], while all other interactions were non-significant [maximum: [$F_{(1,27)} = 1.475$, $p>0.05$]. Follow-up tests of simple effects indicated that there was a significant effect of sex on day one only [$F_{(1,71)} = 4.872$, $p<0.001$], a non-significant effect of day for male mice [$F_{(3,81)} = 1.188$, $p>0.05$] and a significant effect of day for female mice [$F_{(3,81)} = 10.193$, $p<0.001$]. Overall the results indicate that there was no effect of genotype on magazine response during the context conditioning phase.

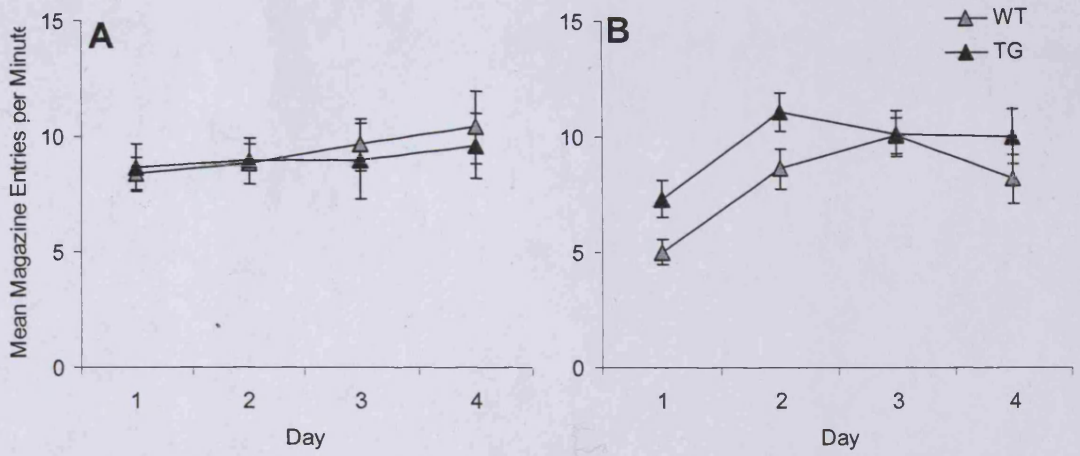


Figure 23: Mean magazine entries per minute during context conditioning phase. Graph A depicts data from male mice; graph B depicts data from female mice. Light grey triangles denote responses from wild-type mice; dark grey triangles denote responses from Tg2576 mice. Error bars = +/- S.E.M.

Devaluation extinction test

Raw data: Results of the devaluation extinction test are depicted in Figure 24. Visual inspection of this figure suggests that wild-type control mice showed a higher rate of magazine entry into the context that was associated with the non-devalued food reward relative to the context that was associated with the devalued food reward. Furthermore, regardless of sex, Tg2576 mice showed a comparable amount of magazine activity in both contexts. Statistical analysis of the data was conducted using a mixed ANOVA with genotype and sex as the between subjects factors and context (non-devalued vs. devalued) as the within subjects factor.

Results indicated that there was a significant main effect of context [$F_{(1,27)}$ = 18.146, $p<0.005$], a non-significant main effect of genotype [$F_{(1,27)}$ = 2.453,$p<0.05$] and a significant effect of sex [$F_{(1,27)} = 5.875$, $p<0.05$]. Furthermore, statistical analyses revealed a significant context by genotype interaction [$F_{(1,27)}$ = 7.132, $p<0.02$], while all other interactions were non-significant (all $Fs<1$). Tests of simple effects revealed a significant effect of context for wild-type control mice [$F_{(1,27)} = 24.873$, $p<0.001$] and a non-significant effect of context for Tg2576 mice [$F_{(1,27)} = 1.226$, $p>0.05$]. Furthermore, tests of simple main effects revealed a non-

significant effect of genotype in the non-devalued context [$F_{(1,51)} = 0.184$, $p>0.05$], but a significant effect of genotype in the devalued context [$F_{(1,51)} = 8.325$, $p<0.001$]. This reveals that Tg2576 mice responded at a significantly higher rate in the devalued context than did wild-type mice. This indicates that while wild-type mice demonstrated preferential responding in the non-devalued context, this effect was impaired in Tg2576 mice.

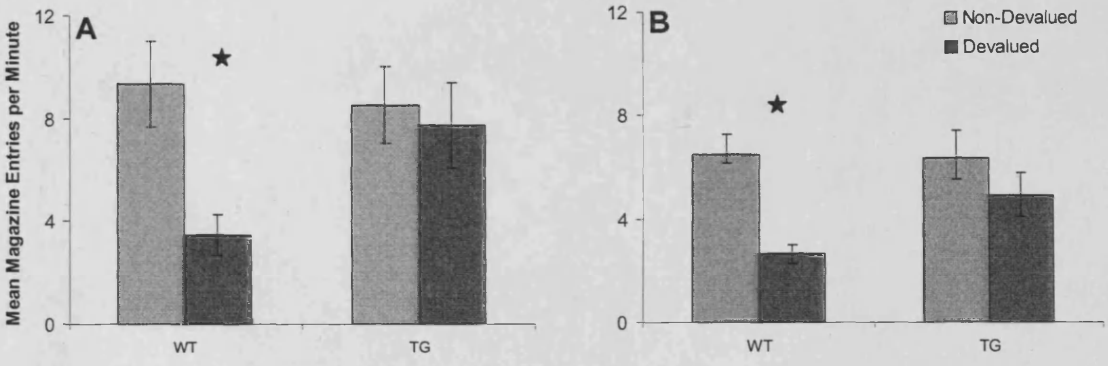


Figure 24: Mean magazine entries per minute during 10 minute extinction test. 'Non-devalued' refers to the context associated with the non-devalued food reward; 'devalued' refers to the context associated with the devalued food reward. Graph A = male data; Graph = female data. WT=wild-type; TG=transgenic. Error bars = +/- S.E.M.

Discrimination Ratios: In order to determine whether individual variation in response rates within each group obscured an effect of devaluation, discrimination ratios were calculated such that the number of magazine entries in the devalued context was divided by the total number of magazine entries in both contexts [response in devalued context / (response in non-devalued context + response in devalued context)]. Results are shown in Figure 25.

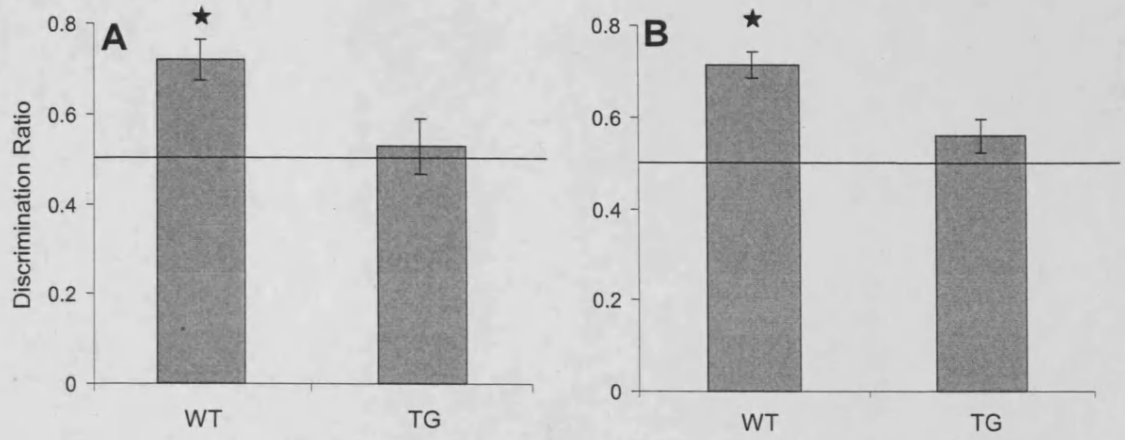


Figure 25: Discrimination ratios depicting proportion of magazine entries into the context associated with the devalued food reward. Graph A = male data; Graph B = female data. WT=wild-type; TG=transgenic. Lines = 0.5, chance. Error bars = +/- S.E.M.

Visual inspection of Figure 25 suggests that wild-type mice performed a smaller portion of their total responses in the devalued context, as compared to the non-devalued context, whereas transgenic mice performed approximately half their total responses in the devalued context. Data were analysed by ANOVA with genotype and sex as factors. Results revealed a significant main effect of genotype [$F_{(1,27)}$ = 15.295, p=0.001], a non-significant main effect of sex [$F_{(1,27)}$ = 0.098, p>0.05] and a non-significant interaction [$F_{(1,27)}$ = 0.181, p>0.05]. This confirms that Tg2576 mice differed significantly from wild-type mice. To ensure each genotype differed significantly from chance, one-sample t-tests were conducted on wild-type and transgenic data. Results indicated that the response ratios for wild-type mice differed significantly from 0.5 [t(15)= -8.227, p<0.001], while the response ratios for Tg2576 mice did not differ significantly from chance [t(14)= -1.314, p>0.05]. These data, therefore, further confirm that Tg2576 mice demonstrate an aberrant context-outcome devaluation response.

Experiment 6b: Context Discrimination Task: Aged Tg2576 mice.

Context conditioning

Mice underwent a discrimination task to determine whether Tg2576 mice were capable of distinguishing between the two contexts. Mice first underwent two days of context conditioning identical to that they had received previously, to re-

establish magazine responding in both contexts. Data (see Table 10) were analysed using a mixed ANOVA with genotype and sex and day as the between subjects factors and context (that which was later reinforced versus that which was later non-reinforced) as the within subjects factor. This analysis was conducted as a means of ensuring that both contexts supported comparable levels of responding before any manipulation was introduced. Results indicated that there was a non-significant effect of day, no difference between response rates in the two contexts, no effect of genotype or sex and no significant interactions [maximum: $F(1,27) = 1.536$, $p>0.05$].

		Day 1				Day 2			
		Context A		Context B		Context A		Context B	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Males	Wild-type mice	105.0	34.4	74.4	16.1	94.8	30.9	85.4	23.3
	Tg2576 mice	71.7	14.4	63.9	17.4	83.9	22.3	78.9	22.2
Females	Wild-type mice	65.6	10.1	60.8	25.0	67.4	7.5	74.6	20.2
	Tg2576 mice	77.0	13.0	67.0	25.4	77.9	9.0	73.9	13.0

Table 10: Mean magazine entries during two days of context re-conditioning for male and female mice of each genotype. SEM = standard error of the mean.

Figure 26 depicts the mean rate of response per minute in the magazine during the acquisition stage, in which one context continued to be reinforced and the other was now no longer reinforced. Visual inspection of this figure suggests that over the first few days of conditioning both wild-type and transgenic mice showed a decrease in responding in the context that was no longer reinforced. Statistical analysis of the data was conducted using a mixed ANOVA with genotype, sex, day and context (reinforced versus non-reinforced) as factors. Results indicated a significant effect of day [$F_{(7,189)} = 7.139$, $p<0.001$], a significant effect of context [$F_{(1,27)} = 25.736$, $p<0.001$], a non-significant effect of genotype [$F_{(1,27)} = 0.032$, $p>0.05$] and a non-significant effect of sex [$F_{(1,27)} = 3.616$, $p>0.05$]. Results also revealed a significant day by sex interaction [$F_{(7,189)} = 4.009$, $p<0.001$] and a

significant day by context interaction [$F_{(7,189)}$ = 2.222, $p<0.05$]. All other interactions were non-significant.

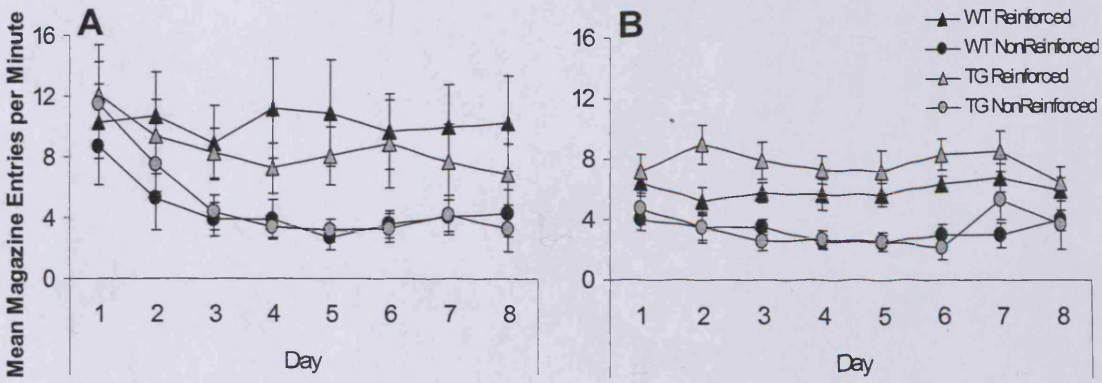


Figure 26: Mean rate of magazine entry per minute in reinforced and non-reinforced contexts during the conditioning phase. Graph A = male mice; Graph B = female mice. Triangles = Reinforced contexts; Circles = Non-Reinforced contexts. Black symbols = wild-type mice; Grey symbols = Tg2576 mice. Error bars = +/- S.E.M.

Tests of simple effects revealed a significant effect of sex on days one and two only [$F_{(1,50)}$ = 16.406, $p<0.001$; F(1,50) = 5.388, $p<0.05$] and a non-significant effect of sex on conditioning days three to eight. They also revealed a significant effect of day for male mice [$F_{(7,189)}$ = 4.937, $p<0.001$] but not female mice [$F_{(7,189)}$ = 0.507, $p>0.05$]. Furthermore, test of simple effects showed there to be a non-significant effect of context on day one [$F_{(1,27)}$ = 2.103, $p<0.05$], but a significant effect of context on days two to eight. Lastly, there was no significant effect of day for the reinforced context [$F_{(7,189)}$ = 0.737, $p>0.05$], but a significant effect of day was found for the non-reinforced context [$F_{(7,189)}$ = 11.497, $p<0.001$]. These findings imply that both wild-type and transgenic Tg2576 mice demonstrated a comparable decrease in magazine responding in the non-reinforced context over the eight day conditioning stage.

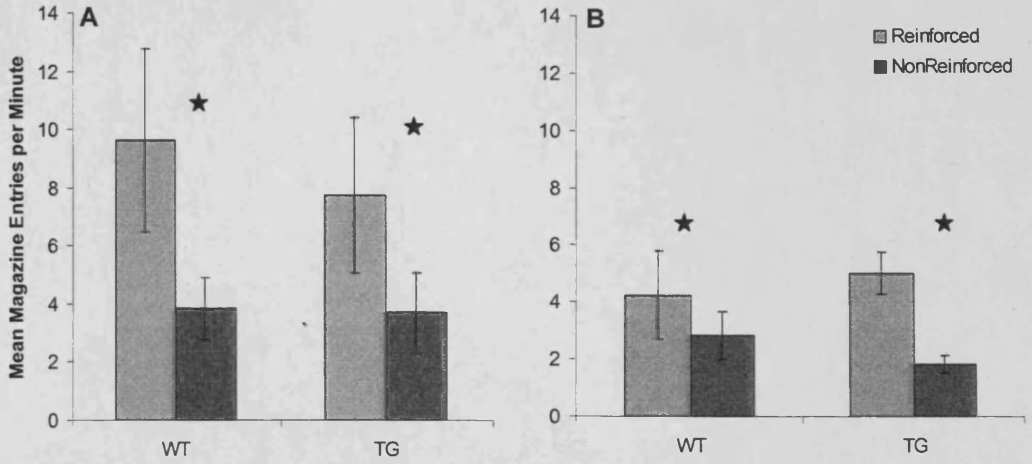


Figure 27: Mean rate of magazine entry per minute in reinforced and non-reinforced contexts during 10 minute discrimination extinction test. WT = wild-type, TG = transgenic Tg2576. Light grey bars denote response rates in the reinforced context; dark grey bars denote response rates in the non-reinforced context. Graph A = male data; Graph B = female data. * = $p<0.05$.

Extinction test

Raw data: Figure 27 shows the mean rate of magazine entry in the reinforced and non-reinforced contexts during the ten minute discrimination test for male (Graph A) and female (Graph B) mice. Visual inspection of the figure suggests that both wild-type and Tg2576 mice demonstrated higher levels of magazine responding in the context that continued to be reinforced, as compared to the context that had not been reinforced. This impression was confirmed by statistical analysis, whereby data were evaluated using a mixed ANOVA with genotype, sex and context (reinforced versus non-reinforced) as factors. Results revealed a significant effect of context [$F_{(1,27)}$ =11.449, p=0.002], a significant effect of sex [$F_{(1,27)}$ = 4.256, p=0.049] and a non-significant effect of genotype [$F_{(1,27)}$ = 0.183, p>0.05]. Furthermore, results indicated that there was a non-significant context by sex interaction [$F_{(1,27)}$ = 1.514, p>0.05] and all other interactions were non-significant (all Fs<1). This indicates that overall females responded at a lower rate than male mice, and both genotypes demonstrated a higher rate of response in the reinforced, as compared to the non-reinforced context.

Discrimination Ratios: The data were also analysed in the form of a discrimination ratio to assess the level of discrimination independent of individual

response rates. Results are presented in Figure 28, which suggests that the non-reinforced context elicited a smaller proportion of responses than the reinforced context for both wild-type control mice and transgenic mice. Data were analysed by ANOVA with genotype and sex as factors. Results revealed a non-significant main effect of genotype [$F_{(1,27)} = 0.154$, $p>0.05$], a non-significant main effect of sex [$F_{(1,27)} = 0.078$, $p>0.05$] and a non-significant interaction [$F_{(1,27)} = 1.126$, $p>0.05$]. This confirms that the performance of transgenic Tg2576 mice did not differ from that of wild-type mice. Furthermore, both genotypes appear to respond more than chance (0.5) in the reinforced context. This impression was confirmed by statistical analysis using one sample t-tests to compare the performance of wild-type and transgenic mice to 0.5. One sample t-tests revealed a significant difference between the performance of wild-type mice [$t(15)= 2.841$, $p<0.02$] and Tg2576 mice relative to 0.5 [$t(14)= 3.921$, $p=0.002$]. This confirms that both genotypes responded more frequently in the reinforced context.

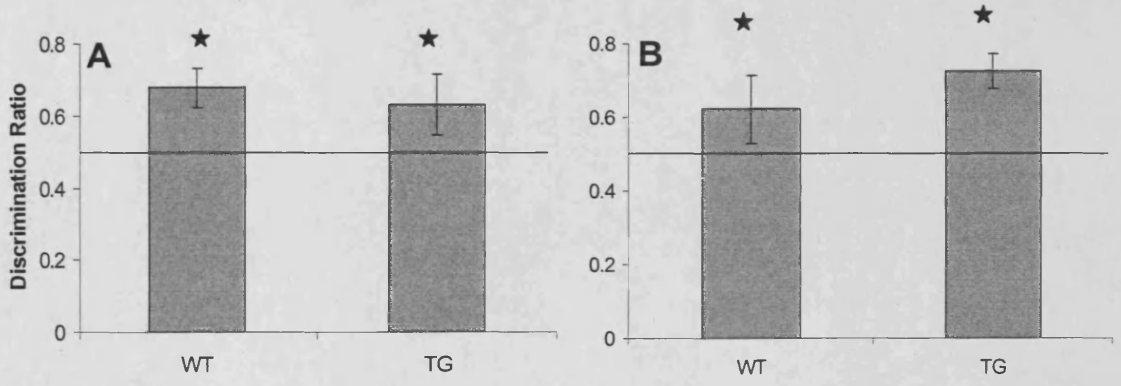


Figure 28: Discrimination ratios depicting the proportion of total magazine responses occurring in the reinforced context. Graph A = male data; Graph B = female data. WT = wild-type control; TG= transgenic Tg2576. Lines denote chance (0.5). Error bars = +/- S.E.M.

4.4.4 Discussion

Experiment 6a was designed to assess the ability of aged transgenic Tg2576 mice to use contextual cues to access a representation of the current incentive value of an outcome. The results from the conditioning stage demonstrated that Tg2576 and wild-type mice performed comparable levels of magazine approach during conditioning. In contrast, the extinction test following outcome devaluation

revealed that Tg2576 mice failed to selectively adjust their magazine approach behaviour. Experiment 6b showed that both groups acquired the context discrimination and displayed preferential magazine responding in the previously reinforced context during an extinction test. The latter result suggests that Tg2576 mice were not impaired in the ability to discriminate between the contextual stimuli and that the deficit in incentive learning in Experiment 6a was a not a result of a generalised impairment in withholding responding during an extinction test.

Typically, an impairment in motivational learning or an inability to alter approach behaviour according to the current incentive value of a reward would be considered either a deficit in managing goal-directed behaviours or an impairment in assigning emotional value to a CS. Hence, one possible explanation of the context devaluation experiment is that the APPswe mutation impaired processes supporting motivational learning. However, the fact that Tg2576 mice showed normal Pavlovian CS and instrumental devaluation together with normal Pavlovian instrumental transfer suggests that the cognitive impairment in incentive learning is specific to the processing of contextual stimuli. This finding supports a subset of evidence from fear conditioning tasks that suggest that transgenic Tg2576 mice are impaired at contextual fear conditioning (e.g. Corcoran et al., 2000). A full discussion of the theoretical implications of these results will be reserved until the general discussion. Before this, the results of experiments that examine the age-dependent nature of this deficit are presented. See the Chapter Discussion (see section 4.6) for a thorough consideration of and possible explanations for the deficit observed in this task.

4.5 Experiment 7a and b: Context-Outcome Learning in Young and Middle-Aged Tg2576 mice

4.5.1 Introduction

As a means of ascertaining whether the impairment observed in Experiment 6 represents an age-dependent effect of the APPswe mutation, Experiments 7a and b assessed Pavlovian incentive learning using the contextual outcome devaluation paradigm in young (3-4 months) and middle aged (~14 month old) wild-type and Tg2576 mice. The aim of these studies was to determine the age of onset of the impairment in context-outcome devaluation. Changes in hippocampal dendritic spine density, together with impairments in the induction of LTP and fear conditioning have been reported in Tg2576 mice as young as 4-5 months of age, which precedes the emergence of amyloid pathology in this model (Jacobsen et al., 2005). The first group that was analysed, therefore, was a cohort of 3-4 month old wild-type and Tg2576 mice. Amyloid deposition typically becomes manifest around 12 months of age in Tg2576 mice (Hsiao et al., 1996), therefore to determine whether a context-outcome devaluation impairment coincided with an age at which amyloid deposition is manifest, the second study assessed context-outcome devaluation in a cohort of 14 month old wild-type and Tg2576 mice.

4.5.2 Method

Experiment 7a: Subjects: Mice used in this task consisted of 16 male Tg2576 mice and 16 male wild-type littermate controls, aged 3 months at the start of testing. In addition, a cohort of 16 female Tg2576 transgenic mice and 16 female wild-type littermate controls, aged 4 months, were tested. Mice were food deprived to 85% of *ad libitum* baseline weight. Male and female mice were maintained in an identical manner throughout the context devaluation task.

Experiment 7b: Subjects: Mice used in this task consisted of 17 male Tg2576 mice and 15 male wild-type littermate controls, aged 14 months at the start of testing. In addition, a cohort of 8 female Tg2576 transgenic mice and 10 female wild-type littermate controls, aged 14 months, were tested. Mice were food

deprived to 85% of *ad libitum* baseline weight and water was removed one hour prior to the start of testing. Male and female mice were maintained in an identical manner throughout the context devaluation task.

Apparatus

The operant chambers used were the same as those used in Experiment 6.

Behavioural Training

The context conditioning, satiety specific devaluation treatment and the context extinction test were conducted in exactly the same manner as with the aged cohort in Experiment 6. To ensure that mice in these younger cohorts were able to distinguish between food rewards, all mice underwent a consumption test of specific satiety, which was conducted identically to that reported above for the aged cohort of Tg2576 and wild-type mice in Experiment 5.

4.5.3 Results

Experiment 7a: Context Devaluation Task: Young Tg2576 mice.

Context Conditioning

Figure 29 shows the mean rate of magazine entry per minute for young wild-type and Tg2576 mice during the context conditioning phase. Visual inspection of the figure suggests that both genotypes demonstrated an increase in magazine responding across training days. This impression was confirmed by statistical analysis, using a mixed ANOVA with genotype, sex and day as factors. Results revealed a significant effect of day [$F_{(3,180)}$ =35.015, $p<0.001$], a non-significant effect of genotype [$F_{(1,60)}$ =1.762, $p>0.05$] and a significant main effect of sex [$F_{(1,60)}$ =28.666, $p<0.001$]. Furthermore, results revealed a significant day by sex interaction [$F_{(3,180)}$ =3.214, $p<0.05$], a non-significant day by genotype interaction [$F_{(3,180)}$ = 0.591, $p>0.05$], a non-significant genotype by sex interaction [$F_{(1,60)}$ =

1.981, $p>0.05$] and a significant day by genotype by sex interaction [$F_{(3,180)}$ =6.996, $p<0.001$].

Tests of simple effects, to investigate the day by sex interaction, revealed a significant effect of day for both male and female mice [$F_{(3,180)}$ = 23.721 and 14.577, p's<0.001, respectively] and a significant effect of sex on each of the four conditioning days [minimum: $F_{(1,140)}$ = 8.816, p=0.004]. Thus, female mice demonstrated lower rates of response during conditioning than male mice.

Furthermore, an analysis of the three way interaction was conducted using a mixed ANOVA with genotype as the between subjects factor and day as the within subjects factor, for male and female data independently. Results for male data revealed a significant effect of day [$F_{(3,90)}$ = 17.576, $p<0.001$], a non-significant effect of genotype [$F_{(1,30)}$ = 3.210, $p>0.05$] and a significant day by genotype interaction [$F_{(3,90)}$ = 4.215, $p<0.01$]. Tests of simple effects revealed a significant effect of day for both genotypes [$F_{(3,90)}$ = 17.783 and 4.019, p's <0.02, respectively] and a significant effect of genotype on day 2 only [$F_{(1,76)}$ = 7.611, p=0.007].

For female data, statistical analysis revealed a significant effect of day [$F_{(3,90)}$ = 22.414, $p<0.001$], a non-significant effect of genotype [$F_{(1,30)}$ = 0.004, $p>0.05$] and a significant day by genotype interaction [$F_{(3,90)}$ = 2.919, $p<0.05$]. Tests of simple main effects revealed a significant effect of day for both genotypes [$F_{(3,90)}$ = 4.714 and 20.620, p's <0.005, respectively], but no effect of genotype on any of the days [maximum: $F_{(1,61)}$ = 2.607, $p>0.05$].

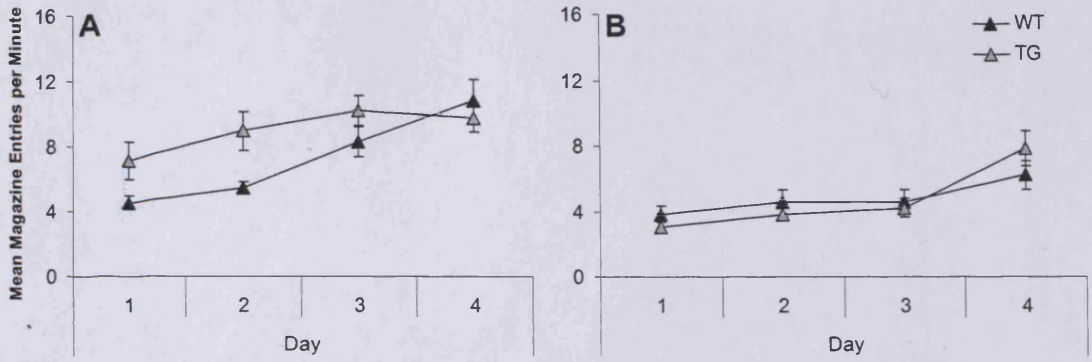


Figure 29: Mean rate of magazine entry per minute in reinforced and non-reinforced contexts during the conditioning phase. Graph A = male mice; Graph B = female mice. Triangles = Reinforced contexts; Circles = Non-Reinforced contexts. Black symbols = wild-type mice; Grey symbols = Tg2576 mice. Error bars = +/- S.E.M.

Devaluation extinction test

Raw data: Results of the ten minute devaluation extinction test are shown in Figure 30. Visual inspection of the figure suggests that both genotypes demonstrated higher rates of magazine responding in the non-devalued context as compared to the devalued context. This impression was confirmed by ANOVA with genotype, sex and context (non-devalued versus devalued) as factors. Results revealed a significant effect of context [$F_{(1,60)}$ = 12.439, p=0.001] and a non-significant effect of genotype [$F_{(1,60)}$ = 0.141, p>0.05]. All other effects and interactions were non-significant (all Fs<1). These results confirm that all mice, regardless of transgene status, responded at a higher rate in the non-devalued context.

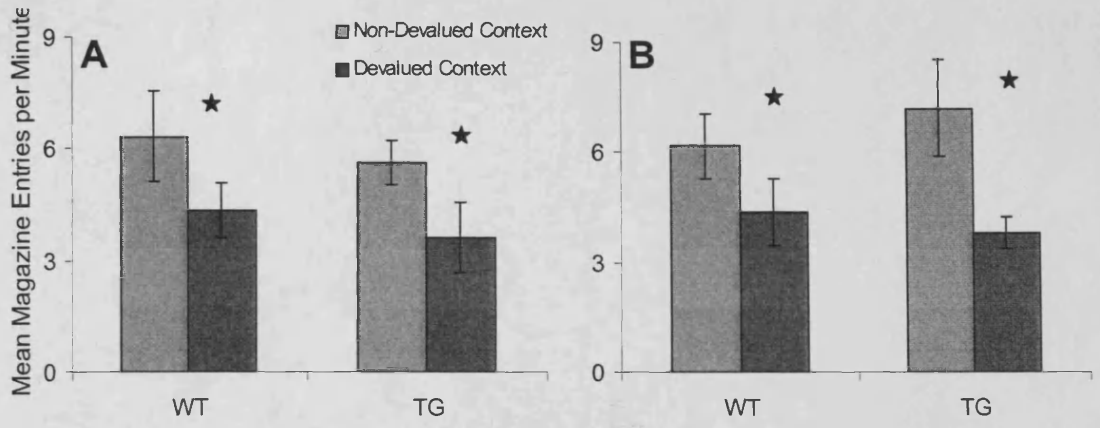


Figure 30: Mean magazine entries per minute during 10 minute extinction test. 'Non-devalued' refers to the context associated with the non-devalued food reward, and is denoted by the light grey bars; 'devalued' refers to the context associated with the devalued food reward, and is denoted by the dark grey bars. WT = wild-type; TG = Tg2576. Error bars = +/- S.E.M.

Discrimination Ratios: As a means of eliminating the individual differences in response rates, the raw data were converted into discrimination ratios for the proportion of total responses occurring in the non-devalued context [response in non-devalued context / total responses]. Results are depicted in Figure 31. Visual inspection of this figure suggests that for both genotypes, the proportion of total responses occurring in the non-devalued context was greater than chance. Data were analysed by ANOVA with genotype and sex as factors. Results revealed a non-significant main effect of genotype [$F_{(1,60)} = 0.756$, $p>0.05$], a non-significant main effect of sex [$F_{(1,60)} = 0.009$, $p>0.05$] and a non-significant interaction [$F_{(1,60)} = 0.792$, $p>0.05$]. These results confirm that the performance of Tg2576 mice did not differ from that of wild-type mice. One sample t-tests were used to compare discrimination ratios to chance (0.5) and they confirmed that the performance of both wild-type and transgenic mice differed significantly from 0.5 [$t(31)= -2.382$ and -3.385 , p's <0.05, respectively].

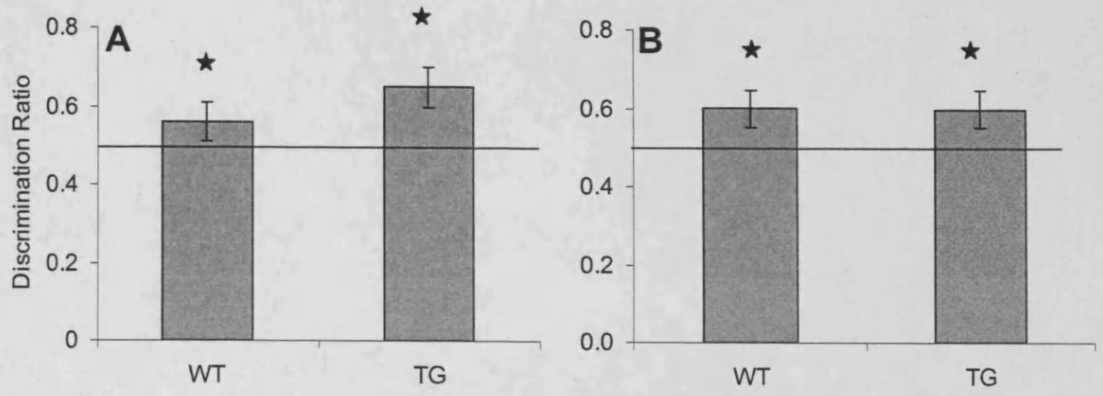


Figure 31: Discrimination ratios for proportion of total responses occurring in the non-devalued context. Graph A = male data; Graph B = female data. WT= wild-type control mice; TG= transgenic tg2576 mice. Lines denote chance responding (0.5). Error bars = +/- S.E.M.

Consumption Test: **All mice were given a consumption test of specific satiety to ensure that mice could distinguish between the food rewards. Results of the consumption test are shown in Table 11. Visual inspection of the figure suggests that both genotypes consumed more of the non-devalued food reward as compared to the devalued food reward, at test. This impression was confirmed by ANOVA with genotype, sex and reward type (devalued versus non-devalued) as factors. Results confirmed a significant main effect of reward [$F_{(1,60)}$ = 39.584, $p<0.001$], a non-significant effect of genotype [$F_{(1,60)} = 1.486$, $p>0.05$], a non-significant effect of sex [$F_{(1,60)} = 0.277$, $p>0.05$] and a non-significant reward by genotype interaction [$F_{(1,60)} = 1.139$, $p>0.05$]. All other interactions were non-significant (all Fs<1).**

	Males				Females			
	Non-Devalued Reward		Devalued Reward		Non-Devalued Reward		Devalued Reward	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type mice	1.3	0.2	0.4	0.1	1.4	0.2	0.4	0.1
Tg2576 mice	1.1	0.2	0.4	0.1	1.1	0.2	0.5	0.1

Table 11: Mean consumption (in grams) of non-devalued and devalued food rewards during satiety specific devaluation test.

Experiment 7b: Context Devaluation Task: 14 month Tg2576 and wild-type mice.

Context Conditioning

Figure 32 depicts the mean rate of magazine entry per minute for 14-month old wild-type and Tg2576 mice during the context conditioning phase. Visual inspection of the figure suggests that both genotypes demonstrated an increase in magazine responding across training days. This impression was confirmed by statistical analysis, using a mixed ANOVA with genotype, sex and day as factors. Results revealed a significant effect of day [$F_{(3,138)}$ =37.732, $p<0.001$], a non-significant effect of genotype [$F_{(1,46)}$ =0.139, $p>0.05$] and a non-significant main effect of sex [$F_{(1,46)}$ =0.481, $p>0.05$]. Furthermore, results revealed a non-significant day by sex interaction [$F_{(3,138)}$ =2.455, $p<0.05$] and a non-significant genotype by sex interaction [$F_{(3,138)}$ = 2.275, $p<0.05$]. All other interactions were non-significant (Fs<1). These data suggest that wild-type and transgenic Tg2576 mice demonstrated a comparable increase in responding over the conditioning period.

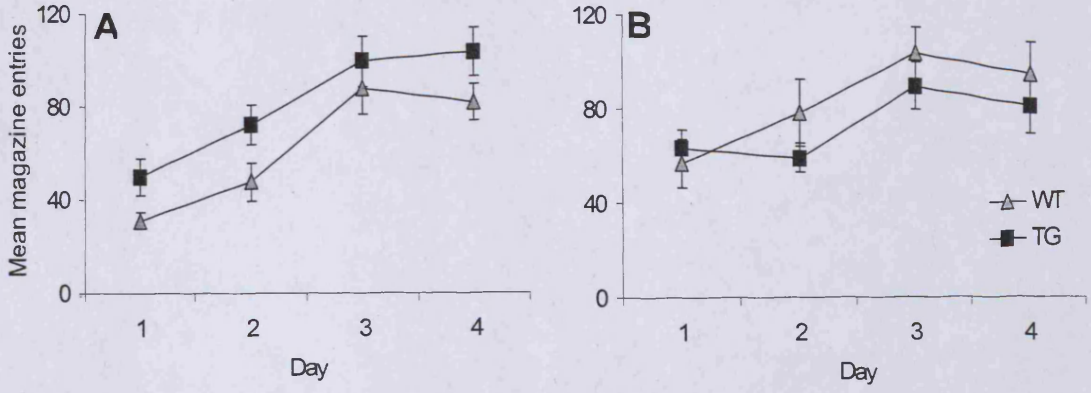


Figure 32: Mean magazine entries for wild-type control and Tg2576 mice during context conditioning phase. Graph A depicts data from male mice and Graph B depicts data from female mice. Grey triangles = wild-type; Black squares = Tg2576. Error bars = +/- S.E.M.

Devaluation extinction test

Raw data: Results of the ten minute devaluation extinction test are depicted in Figure 33. Visual inspection of the figure suggests that both genotypes demonstrated higher rates of magazine responding in the non-devalued context as compared to the devalued context. This impression was confirmed using a mixed ANOVA with genotype, sex and context (non-devalued versus devalued) as factors. Results revealed a significant effect of context [$F_{(1,46)} = 11.737$, $p=0.001$], but non-significant effects of genotype [$F_{(1,46)} = 1.462$, $p>0.05$] and sex [$F_{(1,46)} = 2.600$, $p>0.05$]. Furthermore, all interactions were non-significant [maximum: $F_{(1,46)} = 2.240$, $p>0.05$].These results confirm that transgenic Tg2576 mice responded comparably to their littermate controls, and both genotypes responded at a higher rate in the non-devalued context.

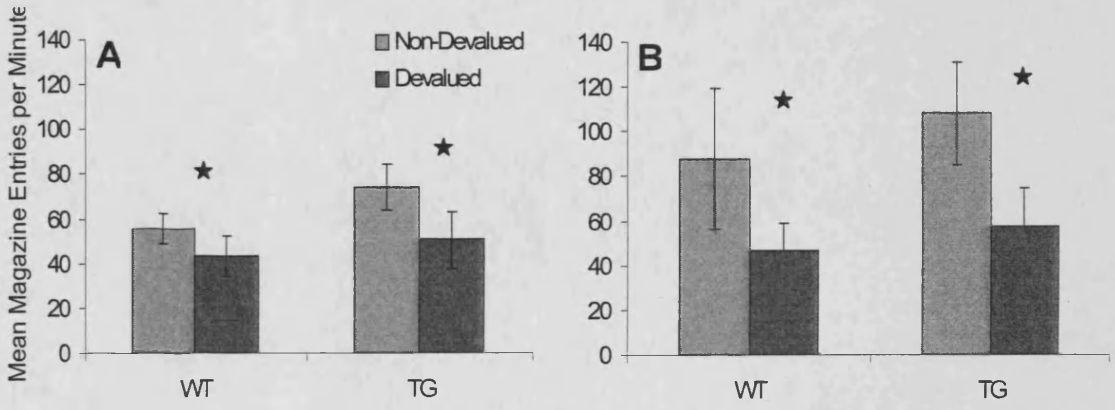


Figure 33: Mean magazine entries during 10 minute extinction test. 'Non-devalued' refers to the context associated with the non-devalued food reward; 'devalued' refers to the context associated with the devalued food reward. WT=wild-type; TG=transgenic. Graph A = male data; Graph B = female data. Error bars = +/- S.E.M.

Discrimination ratios: As a means of eliminating the effect of variable rates of response between mice, raw data were converted into discrimination ratios for the proportion of total responses occurring in the non-devalued context [response in non-devalued context / total responses]. Results are depicted in Figure 34. Visual inspection of this figure suggests that for both genotypes, the proportion of total responses occurring in the devalued context was less than that occurring in the

non-devalued context. Data were analysed by ANOVA with genotype and sex as between-subjects factors. Results revealed a non-significant main effect of genotype [$F_{(1,46)}$ = 0.509, p>0.05], a non-significant main effect of sex [$F_{(1,46)}$ = 0.503, p>0.05] and a non-significant interaction [$F_{(1,46)}$ = 0.091, p>0.05]. This confirms that the performance of transgenic Tg2576 mice did not differ from that of wild-type mice. One sample t-tests confirmed that the performance of both wild-type [t(22)= 3.394, p=0.003] and transgenic mice [t(26)= 3.540, p=0.002] differed significantly from 0.5. These results confirm that all mice responded more frequently in the non-devalued context, regardless of sex or transgene status.

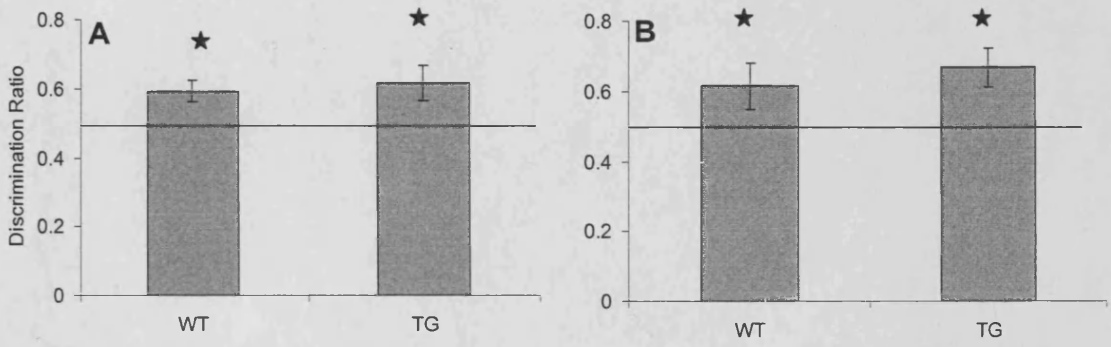


Figure 34: Discrimination ratios for proportion of total responses occurring in the non-devalued context. WT= wild-type control mice; TG= transgenic tg2576 mice. Lines denote chance responding (0.5). Graph A = male mice; Graph B = female mice. Error bars = +/- S.E.M.

Consumption Test

All mice were given a consumption test of specific satiety to ensure that mice could distinguish between the food rewards. Results of the consumption test are shown in Table 12. Visual inspection of the figures suggests that both genotypes consumed more of the non-devalued food reward as compared to the devalued food reward. This impression was confirmed via ANOVA with genotype, sex and reward type (devalued versus non-devalued) as factors. Results confirmed a significant main effect of reward [$F_{(1,46)}$ = 63.880, p<0.001], a non-significant effect of genotype [$F_{(1,46)}$ = 0.009, p>0.05], a non-significant effect of sex [$F_{(1,46)}$ = 1.547, p>0.05] and a significant three way interaction between these factors [$F_{(1,46)}$ = 5.086, p<0.03]. All other interactions were non-significant [maximum: $F_{(1,46)}$ =

1.235, p>0.05]. Tests of simple effects revealed a significant effect of sex on the devalued reward for Tg2576 mice [$F_{(1,46)} = 5.284$, $p<0.03$], but not wild-type mice [$F_{(1,46)} = 0.038$, $p>0.05$]. Thus, female Tg2576 mice consumed more of the devalued reward than their male counterparts. However, consumption of the non-devalued reward was significantly greater than the devalued reward for both genotypes and sexes.

	Males				Females			
	Non-Devalued Reward		Devalued Reward		Non-Devalued Reward		Non-Devalued Reward	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type mice	1.1	0.3	0.2	0.1	1.7	0.3	0.3	0.2
Tg2576 mice	1.5	0.1	0.1	0.1	1.2	0.3	0.5	0.2

Table 12: Mean consumption (in grams) of non-devalued and devalued food rewards during satiety specific devaluation test.

4.5.4 Discussion

The aim of Experiment 7a and b was to determine whether the impairment in context-based incentive learning was evident at earlier stages of development. The results reveal an intact ability to form a representation of a context-US association, and to use this to access information regarding the current incentive value of an outcome, in both young and middle-aged Tg2576 mice. Such results support the suggestion that the impairment reported in Experiment 6 represents an age-dependent effect of the APPswe mutation.

4.6 Chapter Discussion

The aim of this chapter was to investigate motivational processing in Tg2576 mice. Although substantial ß-amyloid deposition is present in the amygdala nuclei and frontal cortex in aged Tg2576 mice, impairments in incentive learning were not evident in an instrumental incentive learning task (Experiment 4), a Pavlovian-instrumental transfer task or an auditory CS devaluation task (Experiment 5). These results led to the conclusion that the functions of the BLA, CeN and prefrontal cortex were not sufficiently impaired to disrupt either action-outcome or CS-outcome associations or incentive motivational processes. Thus, both wild-type and Tg2576 mice were capable of using these instrumental and Pavlovian cues to access a representation of the current sensory-specific incentive motivational properties of the associated outcomes and to alter their behaviour in response to these changes in outcome value.

Interestingly, the only area in which aged Tg2576 mice were found to be impaired was in using contextual cues to access a representation of the current incentive value of an outcome (Experiment 6). This deficit was only apparent in aged (20-22 months) Tg2576 mice, and not young (3-4 months) or middle-aged (14 months) mice (Experiment 7). Thus, this impairment represents an age-dependent effect of the APPswe mutation.

Before discussing possible explanations for this pattern of results, it is worth placing this set of experiments in the context of the literature and noting statistical considerations that became apparent during data analysis. In terms of statistical issues, it must be acknowledged that in some analyses main effects of the outcome manipulation were noted across all groups but subsequent discrimination ratio analyses indicated weak effects in one or other groups. This suggests that power may have been an issue in this set of experiments. In the future it would be worth increasing the size of each cohort to account for this and for the fact that response rates were low, necessitating the removal of unresponsive mice. Nevertheless, the direction of performance in both the wild-type and Tg2576 mice was comparable in each experiment and it is likely that additional studies will confirm the absence of a deficit in Tg2576 mice.

Previous studies have reported an impairment in context-US processing in the Tg2576 model using fear conditioning paradigms (e.g. Corcoran et al., 2002; Dong et al., 2005; Jacobsen et al., 2006). An impairment in auditory CS-US processing has also been observed in this model after fear conditioning (Barnes & Good, 2005; Experiment 3). However, no previous studies of appetitive incentive learning have been reported in APP mouse models. Indeed, there have only been a handful of studies even investigating motivational processing in mice. Johnson et al., (2007) demonstrated that neuronal activity regulated pentraxin (Narp), a substance enriched in the brain that is known to cluster AMPA receptors and to regulate excitatory synaptogenesis, is necessary for processing information in the instrumental outcome-specific devaluation task. Deletion of the AMPA receptor glutamate receptor 1 subunit similarly has also been shown to disrupt incentive learning (Johnson et al., 2005) and fear conditioning (Humeau et al., 2007). Furthermore, using homologous recombination techniques, Crombag et al. (2008) showed that simultaneous phosphorylation of the Ser831 and the Ser845 sites on the AMPA-GluR1 receptor impaired outcome-specific transfer in a PIT task. Given that Tg2576 mice do not show deficits on these aspects of appetitive motivational learning, it is likely that in the Tg2576 model neither Narp nor GluR1 receptors are significantly disrupted.

The PIT test assessed the selective excitatory influence of reward-related auditory CSs on instrumental responding. This task further established that the APPswe mutation had no effect on the conditioning of action-outcome or CS-outcome associations. Furthermore, the results revealed no effect of the transgene on the facilitation of instrumental response during the presentation of the outcome-specific CS. It has been argued that the BLA is required for the CS to facilitate the sensory-specific excitatory effect on instrumental responding (Corbit & Balleine, 2005; Everitt et al., 2003). Interestingly, Parkinson et al. (2000) also report that lesions of the BLA and CeN can have distinct effects on Pavlovian autoshaping. Specifically, it was found that, while BLA lesioned rats showed no deficit in approach behaviour, CeN lesioned rats did not preferentially approach the magazine during the presentation of the CS+ in the conditioning phase (Parkinson, Robbins & Everitt, 2000). A similar result was obtained in Experiment 6, whereby

female Tg2576 mice made significantly fewer magazine entries during the presentation of the CS than wild-type mice. It is worth noting, however, that Tg2576 mice also demonstrated fewer instrumental responses than wild-type mice during conditioning. Furthermore, the discrimination ratios from the Pavlovian phase revealed that Tg2576 and wild-type mice performed a similar proportion of correct behavioural responses during both the instrumental and the Pavlovian sessions. It is suggested, therefore, that future research may aim to evaluate the general motivational forms of incentive learning using the PIT design in Tg2576 mice (see Corbit & Balleine, 2005). Such an experiment would shed light on the functional integrity of the CeN in this model. Typically, a design that incorporates both general and outcome-specific forms of transfer is more complex and demanding (e.g. Corbit & Balleine, 2005) and, therefore, establishing appropriate parameters in an outcome-specific design first may be warranted. In conclusion, the absence of a behavioural impairment in Tg2576 mice on instrumental and punctate CS incentive learning procedures suggests that the presence of ß-amyloid pathology in the BLA was not sufficient to disrupt processing or associations based on the sensory-specific attributes of reward.

Acceptance of this conclusion has to be qualified in the light of evidence that the APPswe mutation impaired the effects of post-conditioning changes in outcome value on context-mediated conditioned responding. In aged animals, the context devaluation task revealed an impairment of Tg2576 mice to use a representation of the context-outcome association to adjust their magazine approach behaviour according to the current incentive value of the outcome. One interpretation of this deficit is that the Tg2576 mice were unable to discriminate between the contexts. A simple version of this interpretation is challenged by the finding that the same Tg2576 mice were able to acquire a context discrimination at the same rate as control mice. This would indicate, therefore, that the context devaluation deficit was not a product of gross sensory deficits in Tg2576 mice.

If one accepts this conclusion, then it is of interest to examine why the APPswe mutation influenced context- but not CS-based devaluation paradigms. One plausible explanation is that the deficit may not reflect the nature of the Pavlovian cue (auditory CS versus Context CS), but rather an influence of the strength of

conditioning on sensitivity to the devaluation manipulation. Outcome devaluation in the auditory CS paradigm followed after 10 conditioning sessions, the PIT test and two further conditioning sessions. In contrast the animals received only 4 sessions of context conditioning in Experiment 6. The latter procedure therefore involved less extensive conditioning than the former study and may have led to a weaker association between the sensory qualities of the US and the context in aged Tg2576 mice. Had less extensive auditory CS-US training been carried out in Experiment 5, Tg2576 mice may also have been less sensitive to the CS devaluation manipulation. Although it is not possible to counter this argument without further empirical tests, it is worth noting that the level of conditioned responding in Tg2576 in Experiment 5 was generally lower than the wild-type mice. To the extent that this might reflect weaker associative strength (all else remaining the same), then the Tg2576 mice, nevertheless, still showed a comparable outcome devaluation effect. Clearly, however, further experiments are required to resolve this issue and if confirmed would suggest that aged Tg2576 are slower to represent the unique sensory qualities of the reinforcer or slower to form associations based on these features with age.

An alternative (although related) account for the above dissociation is that the outcome devaluation deficit in Tg2576 may reflect the salience of the influence of context. Although a functional hippocampus appears necessary for context conditioning (Aguado, Hall, Harrington & Symonds, 1998), the extent to which the reinforcement contingencies require the context to be processed (or the extent to which it influences performance) can influence the sensitivity of a task to hippocampal damage. For example, Thibaudeau et al. (2007) reported that hippocampal lesions had no effect on an appetitive trace conditioning task, but impaired trace conditioning in an aversive paradigm. The authors suggested that the reinforcement contingencies necessitated greater context processing in the aversive paradigm than the appetitive paradigm because the aversive task required the animal to discriminate between two contexts (home cage and conditioning chamber), while in the appetitive task the US (water) was available in both contexts, which negated the need to actively process contextual information. Good, de Hoz and Morris (1998) offered a similar analysis of the effects of hippocampal damage in rats on context processing. These authors suggested that a task is

"contingent" upon the processing of contextual cues if the task necessitates the discrimination of two contexts for successful completion of the task. They refer to contextual processing as "incidental" if conditioning occurs in, but is not actively reliant upon, a particular context. Furthermore, they reported an effect of hippocampal lesions on a test of context specificity of conditioned responding in which context processing was incidental, but no effect of hippocampal damage on a complex biconditional discrimination task in which processing of contextual cues was necessary for conditioning.

One could apply a similar analysis to the pattern of deficits in Tg2576 mice reported in Experiment 5 and 6. For example, during the appetitive context conditioning task, rewards were initially delivered in both conditioning contexts. Therefore, not only did both contexts equally represent nourishment, the delivery of the reward was not contingent upon any programmed behavioural response, nor was the reward preceded by a salient phasic cue. According to the analysis offered by Thibaudeau et al., (2007; see also Good et al., 1998), this situation may result in incidental processing of contextual cues by the hippocampus. If the APPswe mutation impaired this incidental level of context processing, representations of each context may have become less distinctive together with their associative relationship with the unique sensory properties of each reinforcer. This analysis is consistent with the finding that in the context discrimination task, in which only one context signalled the delivery of a food reward, Tg2576 mice were capable of successfully acquiring the context discrimination – a situation that presumably reflects contingent processing of the context. This interpretation also receives support from a study conducted by Corcoran et al. (2002). In this study, aged Tg2576 mice showed normal tone and context fear memory when the context supported high levels of freezing. In contrast, when the salience of the context was reduced, Tg2576 mice showed a marked deficit in conditioned freezing to the context. Corcoran and colleagues suggest that the weaker context-CS association was more readily disrupted by hippocampal pathology present in Tg2576 mice.

Another potential explanation for this pattern of results is that there is a specific deficit in the pathway linking the hippocampal representation of the context to the sensory specific representation of the outcome. That is, the hippocampal context

representation may still have access to the general motivational qualities of the outcome, while the sensory specific features are not processed sufficiently, at least under conditions that favour weak associative relationships. Although each of the amygdala nuclei may function sufficiently in tasks utilizing only punctuate CSs, it may be that an interaction between the hippocampal formation and amygdala is necessary to form the integrated context and sensory-specific outcome representation. This interaction may be impaired in aged Tg2576 mice. This analysis could explain the APPswe deficit in context-outcome devaluation, but intact discrimination learning.

Although the specific nature of the deficit in aged Tg2576 mice remains to be explored further, it is clear from the results of Experiment 7 that 3 and 14 month old transgenic mice were able to perform the context devaluation task appropriately. This would suggest that the devaluation deficit is age and, presumably, amyloid-level dependent. Given other evidence that 3 month old Tg2576 mice are able to acquire spatial information normally, the normal pattern of responding in young Tg2576 was anticipated. Perhaps most surprising, however, was the fact that 14-month old Tg256 performed as well as control mice on the context devaluation task, despite the fact that Tg2576 mice at 12 months of age and above frequently show deficits in spatial navigation and fear conditioning tasks (e.g. Corcoran et al., 2002; Hsiao et al., 1996). This literature suggests that at 14-months of age Aβ-related deficits in hippocampal function should have been sufficient to impair the formation of spatial/contextual memories.

One obvious explanation for the absence of a deficit at 14 months of age in the context-devaluation task is that the threshold for disruption of hippocampal circuitry sufficient to disrupt performance on this task was not achieved. Much of the literature implicating the hippocampus in context processing is based on complete lesions of the hippocampal formation, or gross damage to at least half of the structure (Good, de Hoz & Morris, 1998; Kim & Fanselow, 1992; Penick & Solomon, 1991; Phillips & LeDoux, 1992). The context devaluation task may not be sensitive to early stage hippocampal synaptic pathology that nevertheless may be sufficient to disrupt spatial memory processes required for navigation.

Interestingly, Good & Hale (2007) suggested that middle aged Tg2576 mice had access to a representation of the environment that specified gross spatial attributes such as the position of items in a spatial array. This is also consistent with other evidence that place cells in the hippocampus of Tg2576 mice are not absent but show quantitative differences in the spatial information encoded by these cells (Cacucci et al., 2008). These observations suggest that Tg2576 mice may have access to a degraded representation of the context at 14 months of age sufficient to support, for example, incidental processing of the context. Unfortunately there are no published studies on the nature of place learning or place cell activity in Tg2576 mice aged over 20 months to determine whether the spatial representations alter dramatically in aged Tg2576 mice. The results of these experiments would lead one to predict that place cells would show further age-dependent deterioration.

In conclusion, the pattern of results presented in this chapter indicates that Tg2576 mice do not show gross impairments in instrumental or Pavlovian appetitive incentive learning. This contradicts the initial hypothesis that amyloid pathology present in cortical and amygdala networks would be sufficient to disrupt incentive motivational processes in Tg2576 mice. Nevertheless, there was evidence that aged Tg2576 mice were insensitive to outcome devaluation manipulations when these representations were accessed by contextual cues. The context-US devaluation deficit observed in Experiment 6 can be explained in terms of a deficit in incidental context processing/memory and or an age-related deficit in the pathways integrating the hippocampal representation of context with sensory-specific incentive attributes of reward. The finding that this deficit interacts with the age of the transgenic mice suggests that it may reflect Aβ-mediated alterations in hippocampal-amygdala interactions.

Chapter 5: Functional Neuroimaging of Fear Conditioning and Memory

5.1 Introduction

The pattern of behavioural results observed in Experiments 1-7 is suggestive of a cognitive deficit in motivational/emotional learning, which may be a result of the manifestation of ß-amyloid pathology in the amygdala and/or hippocampal formation. These regions are implicated in the cognitive processes investigated in Experiment 3, which examined conditioned fear, and Experiment 6, which examined incentive learning processes using contextual stimuli. Tg2576 mice demonstrated impaired memory formation in these tasks, but learning was unimpaired in incentive motivation tasks thought to be supported by frontal-amygdala circuitry (Experiments 4 & 5). One interpretation offered for this pattern of results is that the APPswe mutation may disrupt interactions between the amygdala and hippocampus in supporting emotional and incentive memory. In the present chapter, this hypothesis was explored further. The monitoring of neural activity across structures using sophisticated electrophysiological unit recording procedures remains a challenge in mouse preparations, as does the development of functional magnetic resonance imaging procedures for small animals. However, the impact of learning and memory on the pattern of neural activation in structures such as the hippocampus and amygdala can be traced readily using an analysis of immediate early gene expression. The present set of experiments used the fear-conditioning paradigm to explore hippocampal and amygdala activation patterns during learning and memory retrieval. The choice of task was based on the extensive evidence showing learning-related changes in IEG expression in rodent hippocampus and amygdala, evidence of changes in emotional conditioning in Tg2576 mice and the relatively rapid and robust changes in performance, which provide adequate temporal constraints to link brain changes in c-Fos activation and behaviour.

5.1.1 c-Fos expression during fear acquisition and retrieval

The formation of long-term memory is thought to depend on changes in synaptic efficacy and the reprogramming of gene expression. Cellular stimulation leads to memory consolidation and plasticity by inducing a signalling cascade that results in the production of transcription factor proteins. Transcription factors, in turn,

regulate the expression of genes (Herdegen & Leah, 1998). Following the presentation of plasticity-inducing stimuli, the first genes to be expressed are those that do not require de novo protein synthesis, namely the immediate early genes (IEGs). Activation of constitutive transcription factors leads to the expression of IEGs, which encode inducible transcription factors (ITFs) and synthesize protein products. For example, the *c-fos* IEG encodes the mRNA that synthesizes the c-Fos protein (Tischmeyer & Grimm, 1999). Hence, the transient changes that occur in the expression of ITF-encoding IEGs can be examined by measuring the synthesis of the protein product. Furthermore, analysis of the expression of IEG products, such as c-Fos, can provide a measure of stimulus-induced cellular activity in distinct cytoarchitectonic subregions.

The synthesis of c-Fos protein has often been used to identify activation of specific brain regions and neuronal populations during the acquisition of fear (e.g. Milanovic et al., 1998), as well as during fear memory retrieval (e.g. Strekalova et al., 2003). Differences in the quantity of c-Fos immunoreactive nuclei (IR-nuclei) have been found in cytoarchitectonic subregions of the amygdala complex and hippocampus after unconditioned and conditioned fear. In one study, Milanovic et al. (1998) subjected a cohort of C57BL/6N mice to a Pavlovian fear conditioning procedure (CS/US), while control groups received either exposure to the context only or immediate exposure to the shock. Their results reveal higher levels of c-Fos IR-nuclei in the CA1 region of the hippocampus and the medial nucleus of the amygdala in fear-conditioned mice. Furthermore, the two cohorts exposed to the footshock only demonstrated increased c-Fos expression in the CeN, as compared to the context-only control group (Milanovic et al., 1998). Multiple other studies report increased c-fos expression in the lateral and basolateral nuclei of the amygdala (Radwanska et al., 2002; Majak & Pitkanen, 2003; Perez-Villalba et al., 2005) following the acquisition of cued fear. The involvement of the CeN in cued fear conditioning is more controversial, with evidence for increased c-Fos protein expression (Milanovic et al., 1998; Radulovic et al., 1998), but also reports of no changes in c-Fos in this region (Perez-Villalba et al., 2005). Furthermore, in other studies, cellular imaging of IEGs has revealed increases in CA1 and CA3 expression after fear acquisition in contextual and cued fear paradigms (Kubik,

Miyashita & Guzowski, 2007; Perez-Villalba et al., 2005). Overall, therefore, the acquisition of fear conditioning in normal rodents is associated with changes in IEG expression in the hippocampus and the amygdala.

As well as acquisition, IEG analysis has been used to index neural activity associated with the retrieval of fear memories. Campeau et al., (1991) reported substantially elevated levels of c-fos mRNA after the retrieval of both conditioned and unconditioned (i.e. footshock presentation only) fear memories. When fear memory is tested in extinction, protein synthesis-dependent reconsolidation occurs. Reconsolidation refers to the retrieval of a memory trace, which becomes unstable and requires an active process involving the synthesis of proteins to stabilize the memory (Tronson & Taylor, 2007). This component of long-term memory has been shown to be dependent on the synthesis of c-Fos protein (Miller & Marshall, 2005; Inda, Delgado-García & Carrión, 2005). During the retrieval of a context-associated fear memory, increases in c-Fos protein have been observed in the central, basolateral and basomedial nuclei of the amygdala, as well as in the dentate gyrus and CA1 region of the hippocampus (Beck & Fibiger, 1995), in comparison to 'context but no-shock' and 'shock-no context' control groups. During the retrieval of an auditory cued fear memory, increased c-Fos expression has been reported in the basal nucleus and CeN of the conditioned groups, while no differences were observed in subregions of the hippocampus (Hall, Thomas & Everitt, 2001). Interestingly, Conejo et al. (2007) report significantly decreased expression of c-Fos protein in the BLA and medial nucleus of the amygdala after retrieval of an auditory cued fear memory. Taken in combination, these results implicate the subregions of the amygdala and hippocampus in the retrieval of contextual and cued fear memories.

5.1.2 c-Fos expression and APP models

Few studies have investigated changes in IEG expression in transgenic mice that demonstrate amyloid pathology. Decreases in c-Fos protein have been reported in the dentate gyrus of transgenic mice expressing human amyloid precursor proteins (hAPP), which correlate with deficits in the Morris water maze (Palop et al., 2003) and cross maze learning (delpolyi et al., 2008). Interestingly, the Tg-APP(Sw,

V717F)/B6 mouse model, which expresses both the Swedish and Indiana mutations, shows reduced baseline levels (i.e. without behavioural manipulation) of c-Fos expression in the CA1, CA3 and DG regions of the hippocampus (Lee et al., 2004).

Differences in c-Fos expression after the conditioning of cued fear have been reported in APP[V717I] mice. Dewachter et al. (2009) found decreased c-Fos expression in the CA1 region of the hippocampus and the lateral nucleus of the amygdala in mutant mice, as compared to wild-types, after fear acquisition. They did not, however, report the age of the mice nor did they compare the training condition to appropriate control conditions in which the mice were presented with the stimuli independently. Furthermore, despite having conducted a probe test immediately before culling the mice, no behavioural data were presented to indicate that the training had successfully conditioned fear. Hence, although this study presented preliminary evidence for altered conditioning-dependent c-Fos expression in an APP model, it was not sufficiently well designed to control for changes in c-Fos resulting from novel exposure to a context or other stimuli nor did it confirm the acquisition of conditioned fear. It therefore remains unclear how the APP mutation influences neural activity related to IEG expression and behaviour.

The main aim of this experimental chapter is to evaluate learning-induced changes in c-Fos expression to establish the impact of the APPswe mutation on the neural substrates of fear conditioning, with an emphasis on hippocampal and amygdala activity. The first study examined the effects of the mutation on IEG expression immediately after fear conditioning (Experiment 8) and the second study (Experiment 9) examined IEG activity following the retrieval of fear memory in Tg2576 and wild-type mice (Experiment 9). It is expected that the presence of amyloid deposits may disrupt the molecular mechanisms associated with memory formation and retrieval, such as the activation of transcription factors and the expression of IEGs. Thus, since Tg2576 mice are impaired in fear conditioning paradigms, it is likely that regions implicated in the learning and expression of fear, such as the amygdala and hippocampus, will demonstrate transgene-dependent changes in c-Fos expression.

5.2 Experiment 8: c-Fos expression during fear acquisition

5.2.1 Introduction

Experiment 3 revealed deficits in post-shock freezing after the presentation of the tone-shock pairings during the conditioning of cued fear in aged transgenic Tg2576 mice. This pattern of results suggests that the APPswe mutation may disrupt processes engaged during the acquisition of conditioned fear associations. In the present experiment, therefore, the expression of the *c-fos* IEG product, c-Fos protein, was used to map neural activity in the amygdala and hippocampus immediately following fear conditioning. Although one previous study has reported IEG changes as a function of conditioning in the hippocampus and amygdala of APP mice (Dewachter et al., 2009), this study failed to control for non-specific activation. Experiment 8a, therefore, compared a fear-conditioned cohort with a control condition that received exposure to the same stimuli, with the exception of the footshock. Furthermore, Experiment 8b was conducted to assess the impact of the shock alone on IEG expression. This experiment consisted of immediately exposing a subset of animals to three consecutive shocks, with no context or tone exposure. IEG counts were then obtained from sub-regions of the hippocampus and amygdala. The dorsal and ventral regions of the hippocampus were also examined. It was expected that conditioning related changes would be evident in the dorsal hippocampus (Bast, Zhang & Feldon, 2003). However, given the involvement of the ventral hippocampus in anxiety-related processes (Bannerman et al., 2003) and in cued fear conditioning specifically (Maren & Holt, 2004), it was expected that transgene-dependent changes in IEG expression might be most evident in the ventral hippocampus in Tg2576 mice. Similarly, it was hypothesised that c-Fos expression might be disrupted in the basolateral or central nuclei of the amygdala due to their involvement in acquisition and expression of fear (Campeau & Davis, 1995; Davis, 1992).

5.2.2 Method

Subjects

Experiment 8a: Fifteen male Tg2576 mice and eighteen male wild-type littermate controls, aged 19 months, were used in this experiment.

Experiment 8b: Six male Tg2576 mice and six male wild-type littermate controls, aged 19 months, were used.

All mice were housed in groups of 1-2 per cage and maintained on a 12 hour light-dark schedule. All testing took place during the light phase, between 09:00 and 18:00. Mice were allowed *ad libitum* access to food and water for the duration of the experiment.

Apparatus

The apparatus was identical to that used in Experiment 3. However, only one conditioning chamber was used throughout conditioning. This was done for logistical reasons because the brains were extracted exactly 90 minutes after conditioning, making it impossible to condition two mice simultaneously.

Procedure

Experiment 8a: Mice were randomly assigned to one of two conditions. The CS/US group, which consisted of 10 Tg2576 and 13 wild-type mice, received identical fear conditioning to that reported in Experiment 3. Briefly, after a habituation period, the mice received three presentations of the tone CS, which co-terminated with the footshock US. The second group was the 'CS Only' condition, which consisted of 5 mice of each genotype and received similar training to the CS/US group, but without the presentation of the footshock.

Experiment 8b: The 'US only' group, which consisted of 6 mice of each genotype, was exposed only to footshock. Mice were placed into the conditioning chamber, where they immediately received three consecutive footshocks for 2 seconds each, separated by 2 seconds intervals. They were then immediately removed from the chamber, and returned to their home cages. The conditioning session lasted 14 seconds.

During the three days prior to training, all mice were habituated to a dark room for 4 hours each day. On the training day, mice were placed in the dark room for two hours before the experiment and placed back in the room for a further 90 minutes after conditioning, at which point the mice were culled and the brains extracted for IEG analysis. This served to reduce the expression of IEG products to any stimuli other than those presented during the training procedure.

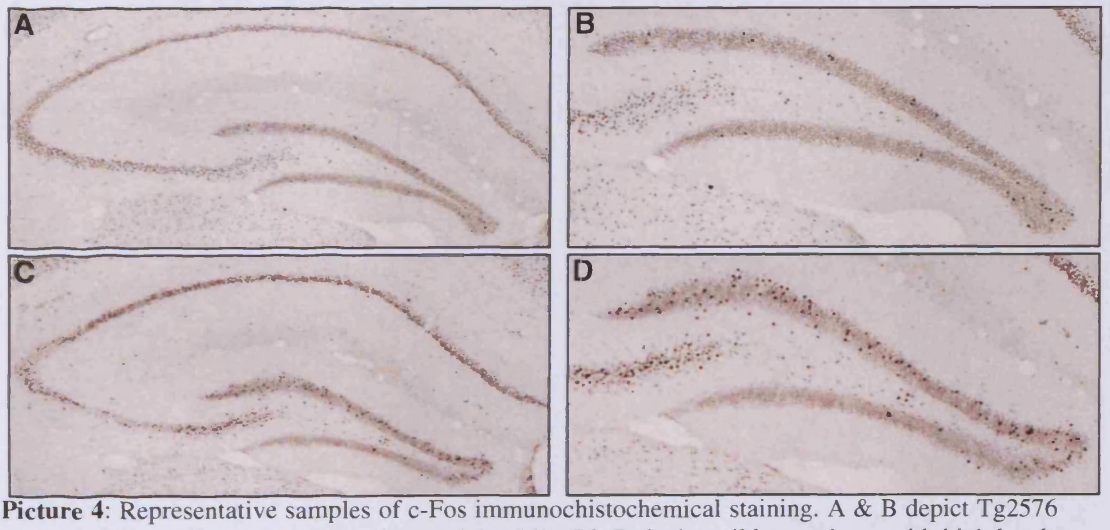
As in Experiment 3, levels of freezing are presented as an index of behavioural changes. In addition to this, locomotor activity changes were recorded using an infrared locomotor activity monitor. This measure is presented as an objective confirmation of basic behavioural changes that occurred during conditioning and testing.

Immunohistochemistry

Ninety minutes after the training session commenced, the mice were injected via the intra-peritoneal cavity, with 0.2ml Euthatal, a pentobarbital. The mice were then exsanguinated, via insertion of a cannula into the left cardiac ventricle, with 80ml of 0.1M PBS, pH 7.4. This was followed immediately by perfusion with 100ml of cold 4% PFA in 0.1M PBS. The brain tissue was extracted and fixed for a further 8 hours in 4% PFA in 0.1M PBS at 4C, before being transferred to a solution of 30% reagent grade sucrose in dH_2O for 48 hours at 4C. Tissue was mounted on a freezing microtome at -20C and cut into 30μm coronal sections. Sections were stored at -20C in ethylene-glycol based cryoprotectant until they were used for immunohistochemistry.

Immunohistochemical staining of the tissue for c-Fos protein commenced with an

antigen retrieval stage, designed to reduce the effect of the PFA fixative and enhance staining of the antigen. This involved immersing the tissue in 10mM citrate buffer and incubating in a water bath for 30 minutes at 70C. Endogenous peroxidise activity was then blocked by incubating the issue in 0.3% hydrogen peroxidase, 0.2% TritonX-100 in 0.1M PBS (PBST; pH 7.4) for 10 minutes. After washing 4 x 10 minutes with 0.1M PBST, tissue was incubated in the primary antibody (anti-Fos, Ab-5 Rabbit Polyclonal, Calbiochem) diluted 1:3000 in PBST, for 48 hours at 4C. Tissue was then washed in PBST 4 x 10 minutes, and incubated in goat anti-rabbit biotinylated IgG, diluted 1:200 in PBST with 1.5% normal goat serum (NGS), for 2 hours. After washing thoroughly, tissue was immersed in an avidin-biotinylated enzyme complex (Vectastain Elite ABC, Vector Laboratories), consisting of 1% reagent A and 1% reagent B in PBST for 1 hour. Tissue was washed with PBS, followed by 0.05M Tris buffer, and stained with the peroxidase substrate 3,3'-diaminobenzidine (DAB) with nickel (Ni^{2+}) for 1-3 minutes (DAB Substrate Kit, Vector Laboratories). The reaction was stopped with cold 0.1M PBS and mounted onto gelatin-coated slides. After dehydrating in ascending series of alcohols and clearing in xylene, the slides were coverslipped with DPX (Di-n-butyl Phthalate in Xylene) mounting medium. See Picture 4 for representative samples of c-Fos immunohistochemical staining.



Picture 4: Representative samples of c-Fos immunochistochemical staining. A & B depict Tg2576 tissue with few c-Fos immunoreactive nuclei, while C& D depict wild-type tissue with high levels of c-Fos immunoreactivity. A & C: septal pole of the dorsal hippocampus; B & D: dentate gyrus.

Regions of interest

Cytoarchitectonic subfields within the amygdala and hippocampal formation were identified using every third coronal section, based on the nomenclature of Paxinos & Franklin (2003). The primary auditory cortex was used as a control area. The regions of interest and the neuroanatomical coordinates used for cell counting are displayed in Table 13. Analyses of the dorsal and ventral regions of the hippocampus were performed on the same plane, while subregions of the hippocampus were counted in the septal pole due to the clear differentiation of the cytoarchitectonics subregions in this area in the mouse brain.

Region	**Subregion**	**From Bregma**	**To Bregma**
Amygdala	Basolateral Nucleus	-1.22mm	-1.82mm
	Central Nucleus	-1.06mm	-1.58mm
Hippocampus	CA1 region	-1.94mm	-2.30mm
	CA3 region	-1.94mm	-2.30mm
	Dentate Gyrus	-1.94mm	-2.30mm
	Dorsal Hippocampus	-2.92mm	-3.16mm
	Ventral Hippocampus	-2.92mm	-3.16mm
Cortex (Control)	Primary Auditory	-2.18mm	-2.80mm

Table 13: Cytoarchitectonic subfields within the amygdala, hippocampal formation and cortex used for counting c-Fos IR-nuclei. Coordinates represent the area closest to and furthest from bregma used for cell counting based on the coronal sections portrayed in Paxinos & Franklin (2001).

Image Analysis

Sections were captured using a 5x objective on a Leica DMRB microscope equipped with an Olympus DP70 camera. The microscope was connected to a standard computer, where images were captured using AnalysisD (Soft-Imaging Systems) software. After image processing, counts of the Fos-IR nuclei were performed using Image J (version 1.38e, National Institutes of Health, U.S.A.). Cell counting always occurred without knowledge of group allocation. For all brain areas analyzed, counts were taken from at least six consecutive sections across both hemispheres, and were averaged to produce a mean IR measure.

Cell counts

Eight wild-type and eight Tg2576 mice were chosen from Experiment 9. These data are presented here as baseline cell counts in the regions later targeted for immunohistochemical analysis of c-Fos expression. Half of the mice chosen underwent CS/US conditioning, while the other half underwent CS Only presentation. Furthermore, half of the mice had tissue extracted for IEG analysis after the tone test, and half after the context test. Tissue was extracted as described above (section 5.2.2) stored in ethylene-glycol based cryoprotectant at -20°C until use. For histochemical staining, tissue was washed 5 times in 0.1M PBS, mounted onto gelatine-coated slides and left to airdry overnight. Cresyl violet staining consisted of immersing slides in xylene (2 x 3 minutes), 100% ethyl alcohol (ETOH, 1 x 2 minutes), 90% ETOH (1 x 2 minutes), 70% ETOH (1 x 2 minutes), 50% ETOH (1 x 2 minutes), deionized H_2O (1 x 2 minutes), 0.5% cresyl violet (1 x 3 minutes), deionized H_2O (1 x 30 seconds), 50% ETOH (1 x 2 minutes), 70% ETOH (1 x 2 minutes), 90% ETOH (1 x 2 minutes), 100% ETOH (1 x 2 minutes), xylene (2 x 3 minutes). Slides were then coverslipped with DPX and allowed to air-dry overnight.

Images were captured and analysed as described in section 5.2.2. The BLA, CeN, DG and auditory cortex, all regions known to be susceptible to amyloid pathology, were analysed. Consistent with the c-Fos analyses, cell counts were obtained using a 5x objective for the BLA, CeN and auditory cortex. For the dentate gyrus, however, the density of the cells was so great that an accurate cell count was not possible at this magnification. For this region, therefore, images were taken at 40x objective and all cells in a $50um^2$ section were hand-counted using ImageJ software. Data were analysed for each region using a one-way ANOVA with genotype as the between subjects variable.

5.2.3 Results: Experiment 8a: Behaviour

Behavioural data are presented first as measures of gross locomotor activity changes, then measures of freezing behaviour are reported for each phase of conditioning.

Infrared locomotor activity scores

Habituation Phase: One group of mice received CS/US conditioning while the other experienced CS Only presentations. Infrared locomotor activity measures for each stage of conditioning are depicted in Figure 35. For the habituation stage (Figure 35A & B), the data were analysed by ANOVA, with genotype, condition (CS/US and CS Only), block (first and second halves) as factors. Results revealed a significant effect of block [$F_{(1,29)} = 4.401$, $p<0.05$], but no other significant main effects or interactions. The main effects of genotype [$F_{(1,29)} = 0.025$, $p>0.05$] and condition [$F_{(1,29)} = 0.188$, $p>0.05$] were non-significant, as were all the interactions [maximum: $F_{(1,29)} = 3.319$, $p>0.05$]. Hence locomotor activity increased overall in the second half of the habituation phase, but no effect of transgene was present on this baseline measure of locomotor activity.

Tone Presentations: Data from the tone presentations were analysed by ANOVA, with genotype, condition and tones (1-3) as variables (Figure 35C & D). Results revealed a non-significant effect of genotype [$F_{(1,29)} = 0.041$, $p>0.05$] and condition [$F_{(1,29)} = 0.347$, $p>0.05$], and a trend towards but non-significant main effect of tone [$F_{(2,58)} = 2.976$, $p=0.059$]. The tone by condition interaction was significant [$F_{(2,58)} = 8.173$, $p<0.001$]. All other interactions between these factors were non-significant [maximum: $F_{(1,29)} = 1.768$, $p>0.05$].

To investigate the tone by condition interaction, tests of simple main effects were performed. Results revealed a significant effect of tone presentations for the CS/US condition [$F_{(2,28)} = 15.889$, $p<0.001$], but not for the CS Only condition [$F_{(2,28)} = 0.466$, $p>0.05$]. The effect of condition was significant for tone 3 [$F_{(1,29)} = 8.045$, $p<0.01$], but not for tones 1 and 2 [$F_{(1,29)} = 0.510$ and 0.279, respectively, p's>0.05]. Hence, locomotor activity was significantly decreased during the course of CS-US presentations.

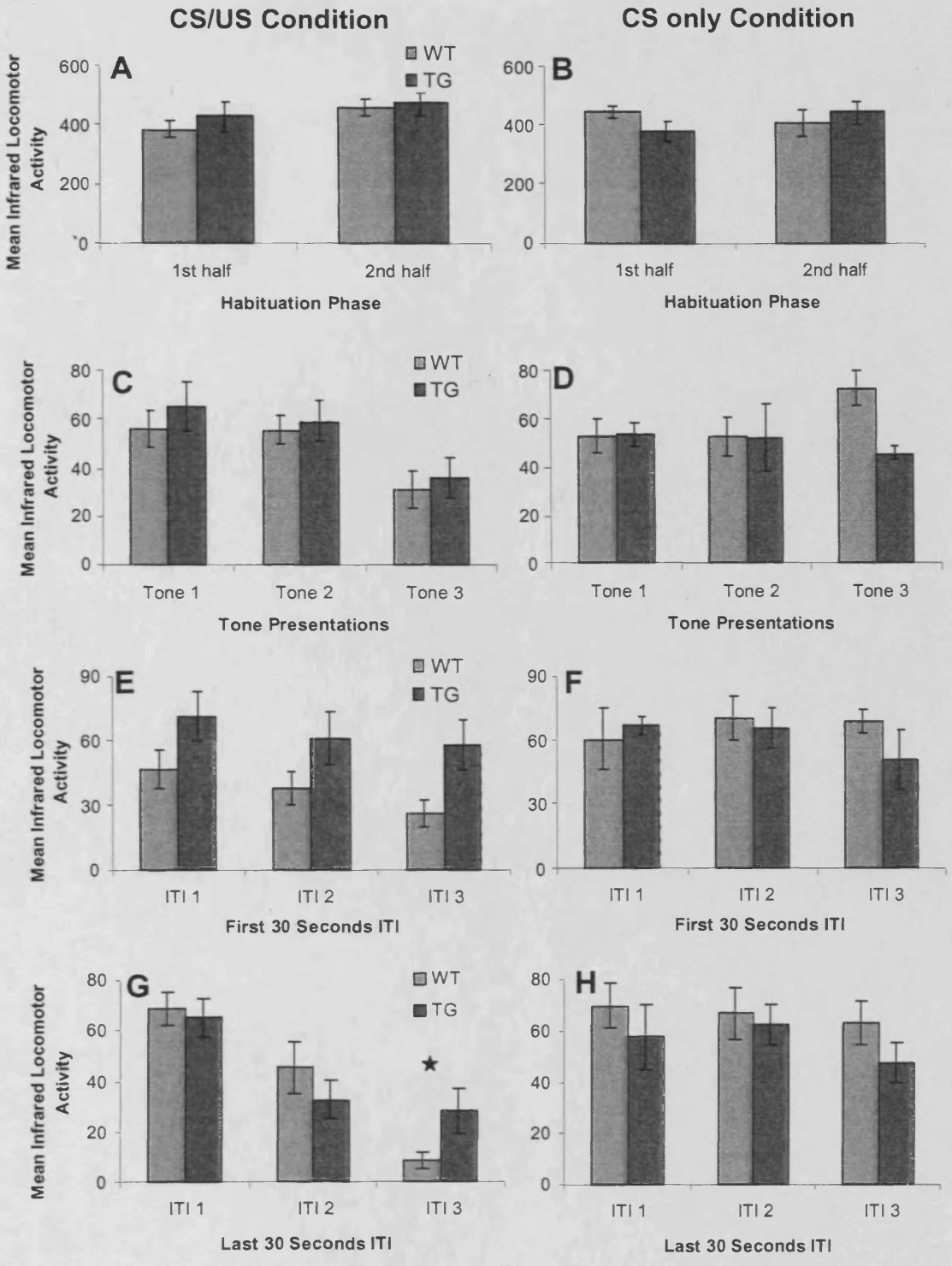


Figure 35: Mean infrared locomotor activity measures during CS/US conditioning (Left column) and CS Only conditioning (Right column). A & B = activity measures during the first and second half of the 6 minutes habituation period. C & D = activity measures during the 28 second tone presentations. E & F = activity measures during the first 30 seconds of the post-shock ITI periods. G & H = activity measures during the last 30 seconds of the post-shock ITI periods. Light grey bars = wild-type mice; Dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

ITI periods: Data from the first 30 seconds of the ITI periods following the CS presentation, which represents the post-shock response, were analyzed by ANOVA with genotype, condition and ITI (1-3) as variables. Results are depicted in Figure 35E & F. Statistical analysis revealed non-significant effects of genotype [$F_{(1,29)}$ = 1.271, $p>0.05$], condition [$F_{(1,29)} = 2.101$, $p>0.05$] and ITI [$F_{(2,58)} = 2.019$, $p>0.05$]. All interactions between these factors were non-significant [maximum: $F_{(1,29)}$ = 2.821, $p>0.05$]. Hence, locomotor activity scores did not change significantly as a function of condition or genotype in the first 30 seconds of the ITI period.

Data from the last 30 seconds of the ITI period were similarly analysed (Figure 35G & H). Results revealed a non-significant effect of genotype [$F_{(1,29)} = 0.451$, $p>0.05$], but a significant main effect of ITI [$F_{(2,58)} = 15.040$, $p<0.001$] and condition [$F_{(1,29)} = 7.631$, $p<0.01$]. The ITI by condition interaction was also significant [$F_{(2,58)} = 7.888$, $p<0.001$]. All other interactions were non-significant [maximum: $F_{(2,58)} = 2.209$, $p>0.05$]. Tests of simple effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,28)} = 43.648$, $p<0.001$], but not when the CS was presented alone [$F_{(2,28)} = 0.502$, $p>0.05$]. The effect of condition was significant for the second and third ITIs [$F_{(1,29)} = 5.297$ and 24.509, respectively, p's<0.05], but not for the first ITI [$F_{(1,29)} = 0.723$, $p>0.05$]. Hence, locomotor activity decreased during the end of the last two ITIs in the CS/US condition, but not when the CS was presented without the footshock. This effect was equally apparent in wild-type and Tg2576 mice.

Freezing Behaviour

Habituation Phase: Each phase of conditioning was also analysed to determine the mean percentage of time spent freezing. Results are depicted in Figure 36. For the habituation stage (Figure 36A & B), data were analysed with genotype, condition and block as factors. Results revealed non-significant main effects of genotype [$F_{(1,29)} = 0.002$, $p>0.05$], condition [$F_{(1,29)} = 0.693$, $p>0.05$] and block [$F_{(1,29)} = 0.116$, $p>0.05$]. All interactions between these factors were non-significant [maximum: $F_{(1,29)} = 0.045$, $p>0.05$]. Hence, no genotypic differences emerged in baseline freezing behaviour.

Tone Presentations: Analysis of the data from the tone presentations (Figure 36C & D) revealed a non-significant main effect of genotype [$F_{(1,29)} = 0.195$, $p>0.05$], a significant effect of tone [$F_{(2,58)} = 12.172$ $p<0.001$] and a significant effect of condition [$F_{(1,29)} = 17.682$, $p<0.001$]. The tone by condition interaction was significant [$F_{(2,58)} = 15.718$, $p<0.001$]. All other interactions were non-significant [maximum: $F_{(2,58)} = 0.270$, $p>0.05$]. Tests of simple effects revealed a significant effect of tone for the CS/US condition [$F_{(2,28)} = 41.258$, $p<0.001$], but not for the CS Only presentations [$F_{(2,28)} = 0.085$, $p<0.001$]. The effect of condition was significant for tones 2 and 3 [$F_{(1,29)} = 8.715$ and 23.071, respectively, p's<0.007], but not for tone 1 [$F_{(1,29)} = 0.022$, $p>0.05$]. Thus, freezing behaviour increased with successive tone presentations during CS/US conditioning equally in both genotypes.

ITI periods: Data from the first 30 seconds of the ITI periods were analyzed by ANOVA (Figure 36E & F). Statistical analysis revealed a significant effect of genotype [$F_{(1,29)} = 9.167$, $p<0.006$], a significant effect of ITI [$F_{(2,58)} = 9.896$, $p<0.001$] and a significant effect of condition [$F_{(1,29)} = 18.833$, $p<0.001$]. Furthermore, significant interactions were revealed between ITI and genotype [$F_{(2,58)} = 4.474$, $p<0.02$], ITI and condition [$F_{(2,58)} = 7.704$, $p<0.002$], genotype and condition [$F_{(1,29)} = 5.834$, $p<0.03$] and ITI, genotype and condition [$F_{(2,58)} = 3.732$, $p<0.03$].

Tests of simple main effects for the ITI and genotype interaction revealed a significant effect of ITI for wild-type [$F_{(2,28)} = 10.806$, $p<0.001$], but not Tg2576 mice [$F_{(2,28)} = 0.583$, $p>0.05$]. Furthermore, the effect of genotype was significant during the second and third ITI [$F_{(1,29)} = 4.864$ and 10.589, respectively, p's<0.04], but not for the first ITI [$F_{(1,29)} = 1.214$, $p>0.05$]. Thus, wild-type mice showed increased freezing during the successive ITIs, a pattern not apparent in Tg2576 mice.

For the ITI by condition interaction, tests of simple main effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,28)} = 21.456$, $p<0.001$], but not when the tone was presented alone [$F_{(2,28)} = 0.163$, $p>0.05$]. A significant effect of condition was apparent during the second and third ITIs [$F_{(1,29)} = 11.064$

and 19.514, respectively, $p<0.003$], but not during the first ITI [$F_{(1,29)} = 3.181$, $p>0.05$]. Thus, freezing behaviour differed as a function of experience. Freezing increased with successive ITI periods in the CS/US condition, but not when the CS was presented alone.

For the genotype by condition interaction, tests of simple main effects revealed a significant effect of conditioning for wild-type [$F_{(1,29)} = 23.766$, $p<0.001$], but not Tg2576 mice [$F_{(1,29)} = 1.780$, $p>0.05$]. Furthermore, a significant effect of genotype was apparent during CS/US conditioning [$F_{(1,29)} = 24.152$, $p<0.001$], but not during CS Only presentation [$F_{(1,29)} = 0.135$, $p>0.05$].

For the three-way interaction, the effect of genotype was not statistically significant for the first ITI during the CS/US condition [$F_{(1,29)} = 3.958$, $p=0.056$], but reached significance for the second and third ITIs [$F_{(1,29)} = 9.856$ and 30.090, respectively, p's<0.005]. No effect of genotype was apparent during any of the three ITIs when the CS was presented alone [$F_{(1,29)} = 0.000$, 0.315 and 0.040, respectively, $p>0.05$].

Data from the last 30 seconds of the ITI period were similarly analysed (Figure 36G & H). Results revealed a non-significant effect of genotype [$F_{(1,29)} = 0.187$, $p>0.05$], but a significant main effect of ITI [$F_{(2,58)} = 12.450$, $p<0.001$] and condition [$F_{(1,29)} = 20.117$, $p<0.001$]. The ITI by condition interaction was significant [$F_{(2,58)} = 13.214$, $p<0.001$]. All other interactions were non-significant [maximum: $F_{(2,58)} = 0.635$, $p>0.05$]. Tests of simple effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,28)} = 39.971$, $p<0.001$], but not for CS Only presentation [$F_{(2,28)} = 0.022$, $p>0.05$]. Furthermore, a significant effect of condition was evident for the second and third ITI [$F_{(1,29)} = 10.993$ and 26.526, respectively, p's<0.003], but not the first ITI [$F_{(1,29)} = 1.133$, $p>0.05$]. Thus, at the end of the ITI period, more freezing was evident in mice that underwent CS/US presentations, but this did not differ as a function of transgene status.

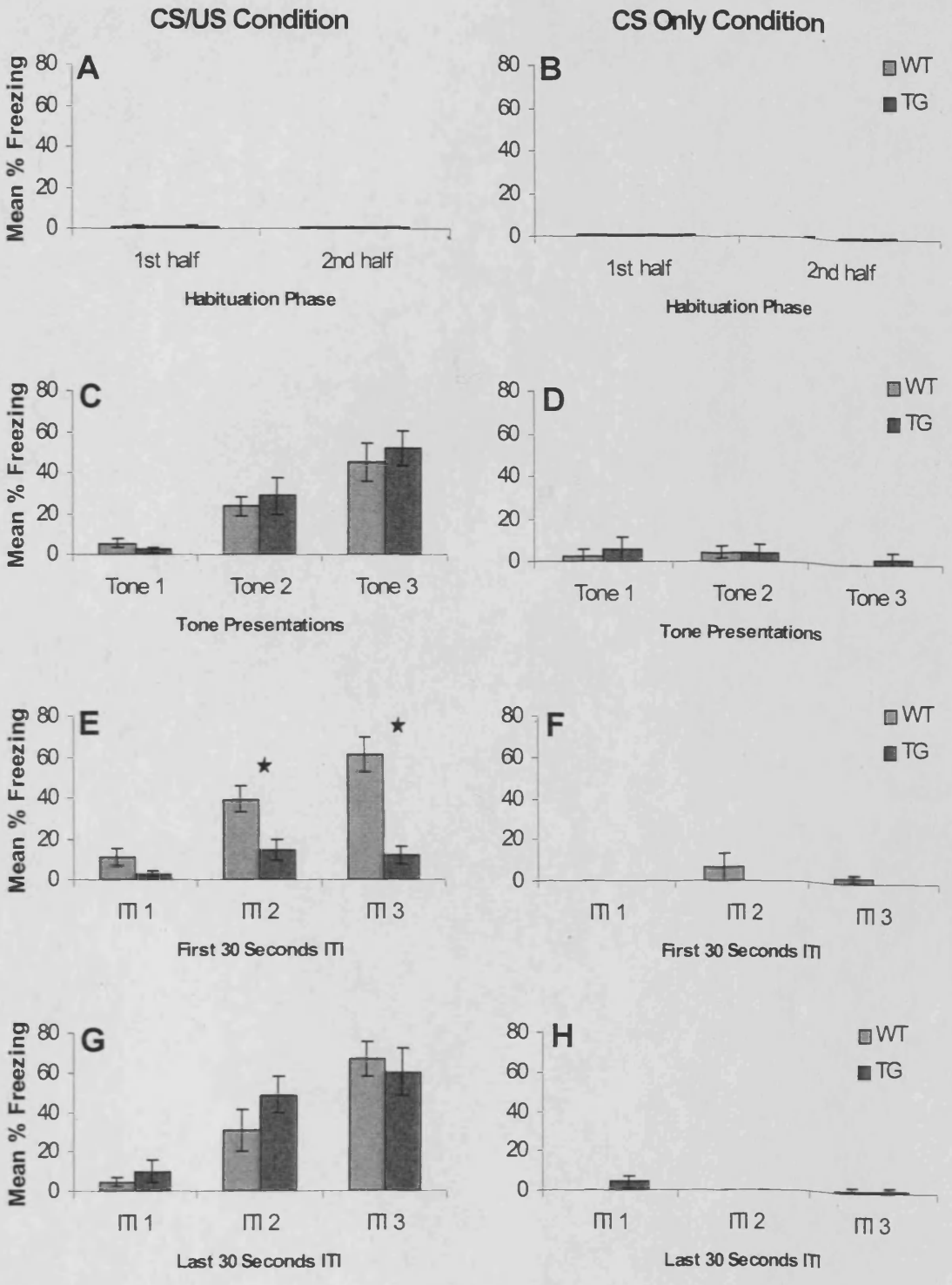


Figure 36: Mean percentage of freezing during CS/US conditioning (Left column) and CS Only conditioning (Right column). A & B = freezing measures during the first and second half of the 6 minutes habituation period. C & D = freezing measures during the 28 second tone presentations. E & F = freezing measures during the first 30 seconds of the post-shock ITI periods. G & H = freezing measures during the last 30 seconds of the post-shock ITI periods. Light grey bars = wild-type mice; Dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M. * = $p<0.05$.

5.2.4 Results: Experiment 8b: Behaviour

US Only Condition

Infrared locomotor activity measures were obtained for the US only condition. Given the brevity of each phase (2 seconds per shock and ITI), it was not feasible to measure freezing behaviour by hand. Therefore, the computer-generated infrared measure of activity served as the primary measure of behaviour for this condition.

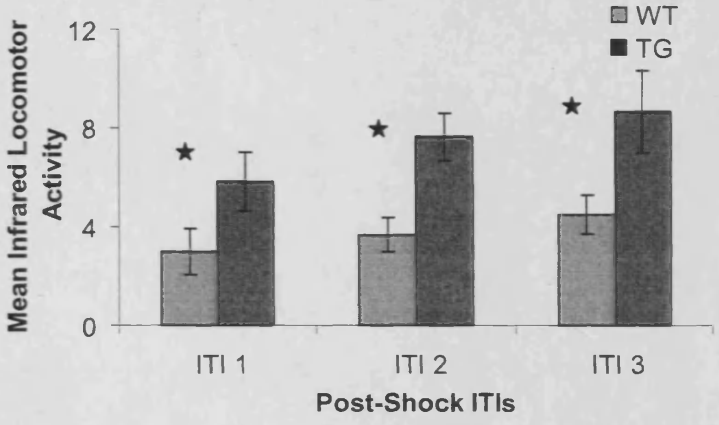


Figure 37: Mean infrared locomotor activity data from the US only condition. ITIs represent the 2 second intervals between the footshocks. Light grey bars = wild-type controls; Dark grey bars = transgenic Tg2576 mice. Error bars = +/-S.E.M. * = $p<0.05$.

Infrared locomotor activity scores

Data from the US only condition are presented in Figure 37. Data were analysed by ANOVA, with genotype and ITI as factors. Results revealed a significant effect of ITI [$F(2,20) = 4.068$, $p<0.05$], a significant effect of genotype [$F(1,10) = 8.370$, $p<0.02$] and a non-significant ITI by genotype interaction [$F(2,20) = 0.454$, $p>0.05$]. These results resemble those from the CS/US condition. Thus, transgenic Tg2576 mice showed higher levels of locomotor activity after presentations of the footshock.

Thus, overall, measures of locomotor activity and freezing behaviour revealed no evidence of transgene-related differences in baseline activity during the habituation phase, nor any genotypic differences during the tone presentations. The analysis of freezing behaviour revealed a deficit in post-shock freezing in Tg2576 mice in the 30 seconds immediately following footshock, but not in the final 30 seconds of the ITI period. Finally, Experiment 8b revealed significantly increased locomotor activity immediately following footshock in Tg2576 mice as compared to their wild-type controls. Thus, these data reveal a specific deficit in post-shock activity in Tg2576 mice that is not evident during other phases of conditioning.

5.2.5 Results: Experiment 8a: Cell counts and c-Fos Immunohistochemistry

Cell counts

The Tg2576 model represents a model of amyloid pathology rather than a model of neurodegeneration. In accordance with this, the introduction of the model was followed closely by multiple reports of intact cell survival in regions susceptible to neurodegeneration in humans (Irizarry, et al., 1997; Stein & Johnson, 2002). The presence or absence of cell loss has been analysed in Tg2576 at mice between 11 months of age (Irizarry, et al., 1997) and 16 months of age (Stein & Johnson, 2002). In Experiments 8 and 9, cell counts of c-Fos immunoreactive nuclei were used to analyse neural activation in 19 month old mice. In order to ensure that baseline cell counts were comparable between wild-type and Tg2576 mice and overt cell loss was not present in this aged cohort, tissue from a subset of mice (wt: n=8; tg: n=8) in Experiment 9 was Nissl stained and cell counts were taken from cortical, hippocampal and amygdala regions. These data are presented here as baseline data regarding mean cell counts in regions targeted for c-Fos expression in Experiments 8 & 9. Nissl stain uses cresyl violet to stain nissl bodies, which contain RNA and polyribosomes, in neuronal cells. Hence, the number of cells in each region can be quantified to determine whether transgene-dependent differences exist in this aged cohort.

Figure 38 depicts the mean cell counts for the auditory cortex, BLA, CeN and dentate gyrus in 19 month old wild-type and Tg2576 mice. Statistical analysis of cell counts in the auditory cortex revealed a non-significant effect of genotype [$F_{(1,15)} = 0.024$, $p>0.05$]. A non-significant effect of genotype was also revealed in the BLA and CeN subregions of the amygdala [$F_{(1,15)} = 0.459$ and 2.251, p's >0.05, respectively]. Furthermore, no effect of genotype was revealed in the dentate gyrus [$F_{(1,14)} = 3.734$, $p>0.05$].

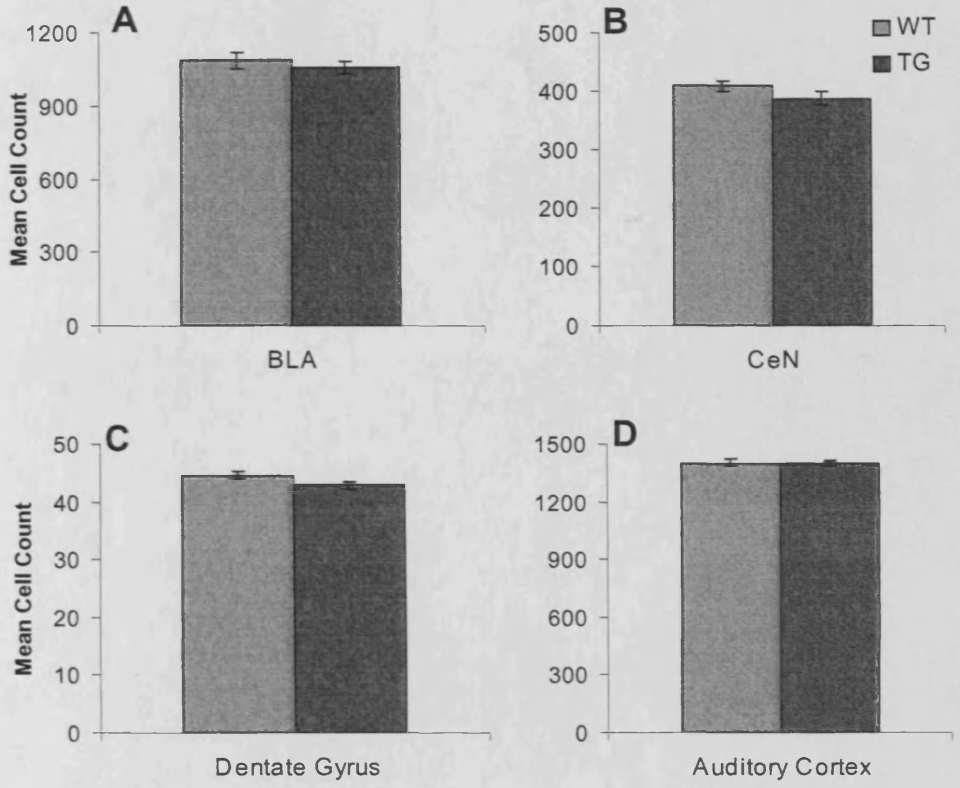


Figure 38: Mean cell counts for the (A) BLA, (B) CeN, (C) dentate gyrus and (D) auditory cortex in 19 month old mice. Light grey bars = wild-type mice; dark grey bars = Tg2576 mice. Error bars = +/- S.E.M.

In summary, no genotypic differences in cell counts emerged in any of the regions examined. Hence, the present data further corroborate those reported in previous studies (e.g. Irizarry, et al., 1997; Stein & Johnson, 2002), and extend the age at which no cell loss is reported to 19 months. These results allow one to draw comparisons between c-fos expression in Tg2576 and wild-type mice without the added complication of baseline differences in viable cells.

c-Fos Immunoreactivity

Hippocampus: Subregions

Raw data: Figure 39 depicts the mean c-Fos immunoreactive counts in each sub-region of the hippocampus. To determine whether genotype had an impact on hippocampal c-Fos expression as a function of experience, the raw data were analysed using an ANOVA with genotype, condition (CS/US, CS Only) and region (CA1, CA3, DG) as factors. Results revealed a non-significant main effect of genotype [$F_{(1,29)} = 0.0004$, $p>0.05$], a non-significant effect of condition [$F_{(1,29)} = 0.389$, $p<0.05$], but a significant main effect of region [$F_{(2,58)} = 72.928$, $p<0.001$]. Paired samples t-tests revealed a significant difference between all three regions for both genotypes (all p's<0.001). All interactions were non-significant [maximum: $F_{(1,29)} = 1.743$, $p>0.05$].

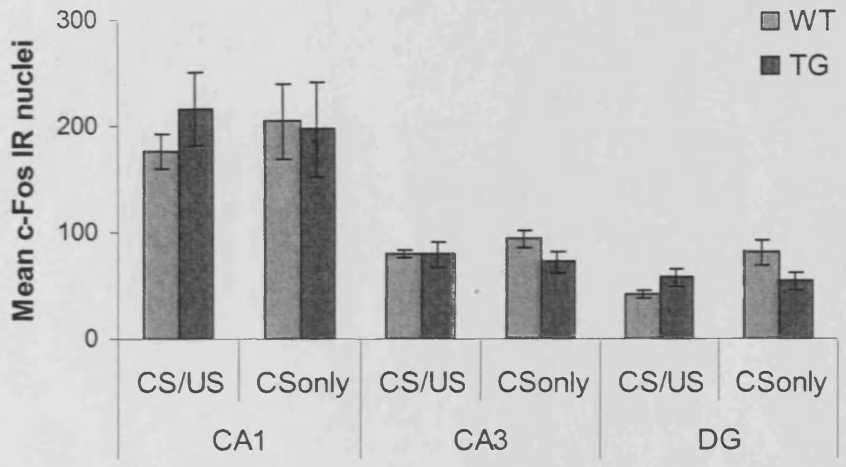


Figure 39: Mean c-Fos immunoreactive nuclei in the CA1, CA3 and dentate gyrus subregions of the hippocampus. Raw data are presented for the CS/US and CS Only conditions for wild-type and Tg2576 mice. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/-S.E.M.

Suppression Ratios. To determine how the pattern of c-Fos expression was influenced by CS/US conditioning relative to CS Only presentation, a ratio was calculated using the formula: [CS/US c-Fos count/(CS/US c-Fos count plus the average c-Fos count for the CS Only condition)] for each brain region. The results

are presented in Figure 40. An ANOVA revealed a significant effect of genotype [$F_{(1,21)} = 5.315$, $p<0.04$], a significant effect of region [$F_{(2,42)} = 5.165$, $p<0.01$] and a significant interaction between these factors [$F_{(2,42)} = 6.020$, $p=0.005$]. Tests of simple main effects revealed a significant effect of genotype for DG [$F_{(1,21)} = 22.766$, $p<0.001$], but not for the CA1 or CA3 regions [F's $_{(1,21)} = 0.470$ and 1.250, $p>0.05$, respectively].

Furthermore, analysis of the suppression ratios revealed a significant effect of region for wild-type mice [$F_{(2,20)} = 11.828$, $p<0.001$] but not for Tg2576 mice [$F_{(2,20)} = 0.101$, $p>0.05$]. Post hoc comparisons revealed a significant difference between the DG and the other hippocampal regions (p's <0.002) in wild-type mice. These data suggest that CS/US conditioning, relative to CS Only presentation, resulted in a suppression of c-Fos protein in the dentate gyrus of wild-type mice. However, this pattern of c-Fos expression was not demonstrated in transgenic Tg2576 mice. Nevertheless, c-Fos expression in the remaining hippocampal subregions did not differ as a function of transgene.

The level of c-Fos expression elicited by CS/US presentation differed significantly from 0.5 for wild-type mice in the CA3 and DG regions [$t(12) = -3.213$ and -10.262, p's <0.01, respectively], but not in the CA1 region [$t(12) = -2.096$, $p>0.05$]. For Tg2576 mice, suppression ratios did not differ from 0.5 in any of the three hippocampal subregions [maximum $t(9) = -0.285$, $p>0.05$]. These results suggest that, while wild-type mice demonstrated a change in c-Fos expression in the CA3 and DG as a result of CS/US conditioning, Tg2576 mice did not display any differential c-Fos expression as a function of experience in any of the three hippocampal subregions.

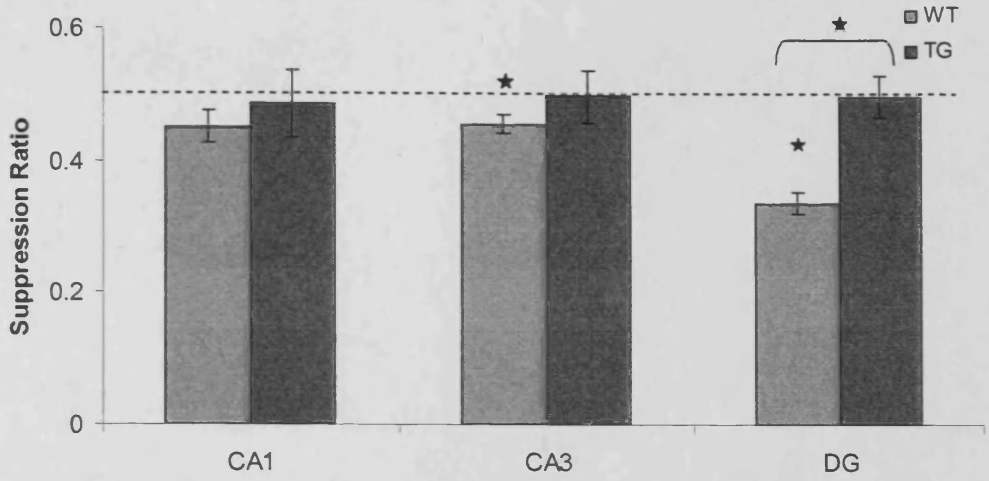


Figure 40: Mean suppression ratios for each hippocampal subregion. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

In sum, when CS/US conditioning was directly compared to the CS Only control condition, a genotypic difference in c-Fos expression was observed in the dentate gyrus, but not in other hippocampal subregions. Furthermore, while wild-type mice displayed differential c-Fos expression after CS/US presentation, relative to CS Only presentation, in the CA3 and DG regions, no differential c-Fos expression was observed in any of the subregions in Tg2576 mice.

Hippocampus: Dorsal vs. Ventral

Raw data: Figure 41 depicts c-Fos counts in the dorsal and ventral regions of the hippocampus. To determine whether genotype had an impact on c-Fos expression in the dorsal or ventral region of the hippocampus as a function of experience, raw data were analysed using an ANOVA with genotype, condition (CS/US, CS Only) and region (dorsal vs. ventral) as factors. Results revealed a non-significant effect of genotype [$F_{(1,29)} = 0.020$, $p>0.05$], a non-significant effect of condition [$F_{(1,29)} = 0.110$, $p>0.05$], but a significant main effect of region [$F_{(1,29)} = 41.949$, $p<0.001$]. This indicates that c-Fos expression was higher overall in the ventral region of the hippocampus than the dorsal region. The three-way interaction was not significant [$F_{(1,29)} = 3.423$, $p=0.075$]. All other interactions were non-significant [maximum: $F_{(1,29)} = 2.844$, $p>0.05$].

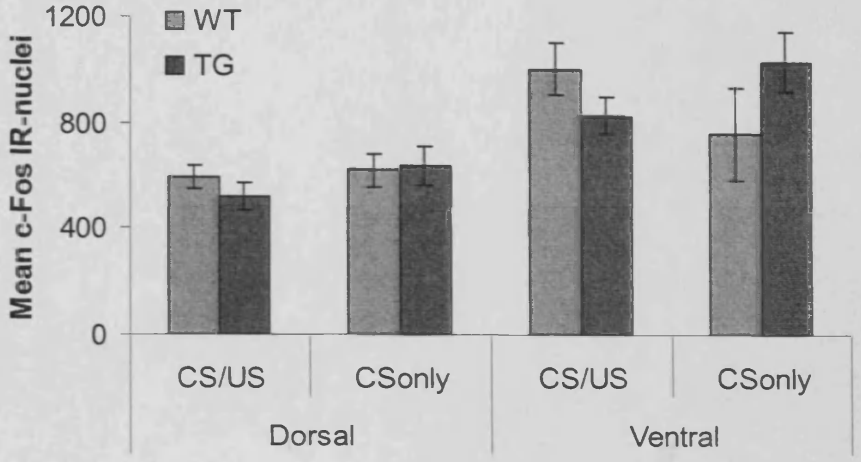


Figure 41: Mean c-Fos immunoreactive nuclei in the dorsal and ventral regions of the hippocampus. Raw data are presented for the CS/US, CS Only conditions for wild-type and Tg2576 mice. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Suppression Ratios. A suppression ratio was calculated to determine how the pattern of c-Fos expression was influenced by CS/US presentation relative to the CS Only presentation. The results are presented in Figure 42. Statistical analysis revealed a significant main effect of genotype [$F_{(1,21)}$ = 7.483, $p<0.02$], which confirms that Tg2576 mice demonstrated greater overall suppression of c-Fos than wild-type mice in the dorsal and ventral hippocampus. Results also revealed a non-significant effect of region [$F_{(1,21)}$ = 3.898, $p>0.05$] and a significant region by genotype interaction [$F_{(1,21)}$ = 4.496, $p<0.05$]. Tests of simple effects revealed a significant effect of region for wild-type mice [$F_{(1,21)}$ = 9.641, $p=0.005$], but not for Tg2576 mice [$F_{(1,21)}$ = 0.009, $p>0.05$]. Furthermore, results revealed a non-significant effect of genotype for the dorsal region [$F_{(1,21)}$ = 1.878, $p>0.05$], but a significant effect of genotype for the ventral region [$F_{(1,21)}$ = 10.014, $p<0.005$]. These results suggest that, as a result of CS/US conditioning, wild-type mice showed greater c-Fos activation in the ventral hippocampus than the dorsal region, a pattern that is not present in Tg2576 mice.

The level of c-Fos expression elicited by the CS/US condition did not differ significantly from 0.5 for wild-type mice in either the dorsal or ventral regions of

the hippocampus [t(12) = -1.019 and 1.923, p's >0.05, respectively]. For Tg2576 mice, however, c-Fos expression differed significantly from 0.5 in both regions [t(9) = -2.451 and -2.893, p's<0.04, respectively].

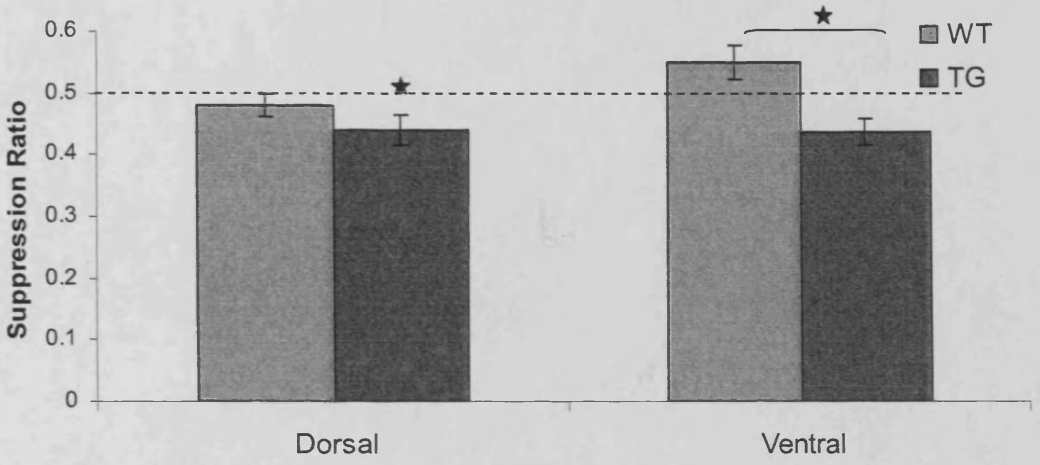


Figure 42: Mean suppression ratios for dorsal and ventral regions of the hippocampus. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M. * = p<0.05.

In summary, these results suggest that c-Fos expression was higher in the ventral region of the hippocampus overall after CS/US presentation for both genotypes. As a result of CS/US presentation, however, wild-type mice demonstrated greater c-Fos activation in the ventral hippocampus than the dorsal region, while Tg2576 mice displayed no differential c-Fos activation as a function of CS/US presentation. Furthermore, a suppression in c-Fos expression was observed in Tg2576 mice after CS/US, relative to CS Only, presentation, a pattern that was not observed in wild-type mice.

Amygdala

Raw data: Figure 43 depicts c-Fos counts in the BLA and CeN regions of the amygdala. To determine whether genotype had an impact on c-Fos expression in the amygdala as a function of experience, raw data were analysed using an ANOVA with genotype, condition (CS/US, CS Only) and region (BLA and CeN) as factors.

Results revealed a non-significant main effect of genotype [$F_{(1,29)}$ = 0.432, p>0.05], a non-significant main effect of condition [$F_{(1,29)}$ = 0.287, p>0.05], but a

significant main effect of region [$F_{(1,29)}$ = 198.053, p<0.001]. All interactions were non-significant [maximum: $F_{(1,29)}$ = 1.140, p>0.05].

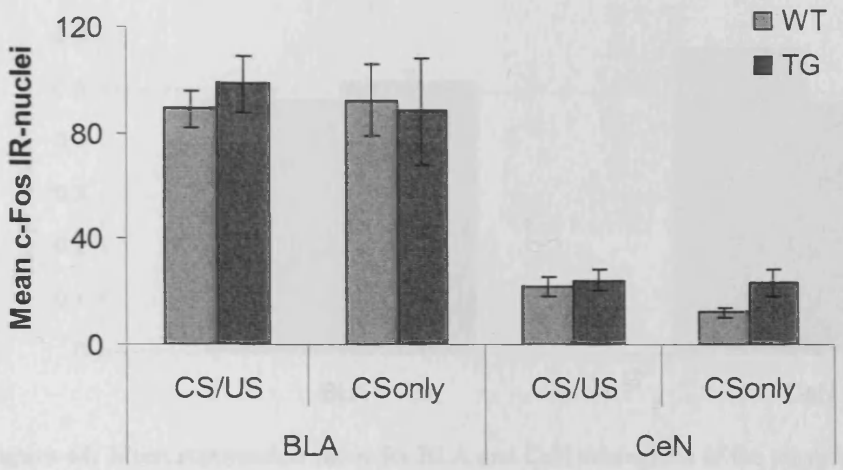


Figure 43: Mean c-Fos immunoreactive nuclei in subregions of the amygdala. Raw data are presented for the CS/US, CS Only and US Only conditions for wild-type and Tg2576 mice. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Suppression Ratios. A suppression ratio was calculated to determine how the pattern of c-Fos expression was influenced by the CS/US conditioning relative to the CS Only presentation. The results are presented in Figure 44. Statistical analysis revealed a non-significant main effect of both genotype [$F_{(1,21)}$ = 0.764, p>0.05] and region [$F_{(1,21)}$ = 1.820, p>0.05], but a significant interaction between these factors [$F_{(1,21)}$ = 8.046, p<0.01]. Tests of simple effects revealed a non-significant effect of genotype for either the BLA [$F_{(1,21)}$ = 1.171, p>0.05] or the CeN [$F_{(1,21)}$ = 3.206, p>0.05]. Results did, however, reveal a significant effect of region for wild-type mice [$F_{(1,21)}$ = 10.074, p>0.05], a pattern that was not evident in Tg2576 mice [$F_{(1,21)}$ = 0.979, p>0.05]. This indicates that c-Fos expression in CeN increased following CS/US presentations for wild-type mice but not Tg2576 mice.

One-sample t-tests demonstrated that the level of c-Fos expression elicited by the CS/US presentation did not differ significantly from 0.5 for wild-type mice in either the BLA or CeN subregions of the amygdala [t(12) = -0.952 and 2.050,

p's>0.05, respectively], nor did it differ from 0.5 for Tg2576 mice [t(9) = 0.606 and -0.527, p's>0.05, respectively].

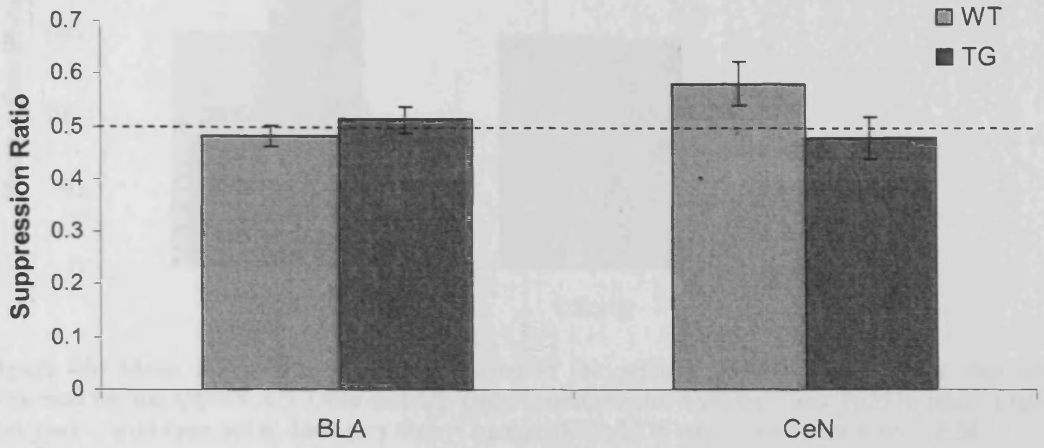


Figure 44: Mean suppression ratios for BLA and CeN subregions of the amygdala. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Overall, wild-type mice displayed a relative increase in c-Fos expression in the CeN, but not the BLA, after CS/US conditioning. This pattern of c-Fos expression was not observed in the in the Tg2576 mice. However, no genotypic differences in expression were revealed in the amygdala as a result of CS/US or CS Only presentation.

Primary Auditory Cortex

Raw data: Figure 45 depicts c-Fos counts in the dorsal and ventral regions of the hippocampus. To determine whether genotype had an impact on c-Fos expression in the primary auditory cortex as a function of experience, data were analysed using an ANOVA with genotype and condition (CS/US, CS Only) as factors. Statistical analysis revealed a non-significant main effect of genotype [$F_{(1,29)}$ = 0.294, p>0.05], a non-significant effect of condition [$F_{(1,29)}$ = 0.462, p<0.001] and a non-significant interaction between these factors [$F_{(1,29)}$ = 0.425, p>0.05].

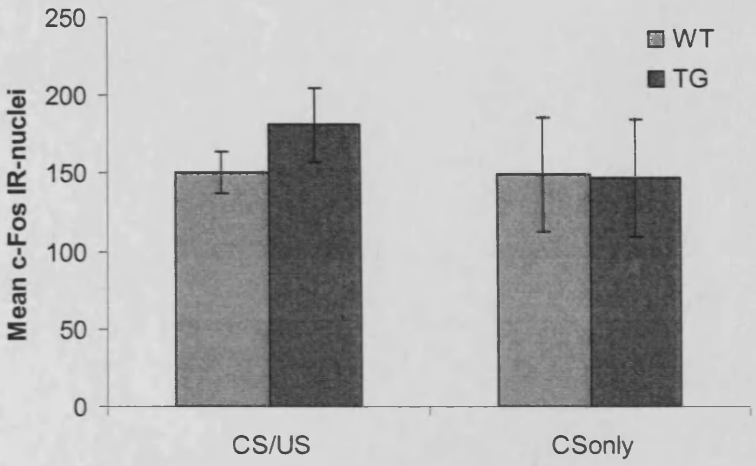


Figure 45: Mean c-Fos immunoreactive nuclei in the primary auditory cortex. Raw data are presented for the CS/US, CS Only and US Only conditions for wild-type and Tg2576 mice. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Suppression Ratios. A suppression ratio was calculated to determine how the pattern of c-Fos expression was influenced by CS/US experience relative to the CS Only condition. The results are presented in Figure 46. Statistical analysis, via one-way ANOVA with genotype as a factor, revealed a non-significant main effect of genotype [$F_{(1,22)} = 1.538$, $p>0.05$]. Furthermore, levels of c-Fos expression did not differ significantly from 0.5 for either wild-type [$t(12) = -0.463$, $p>0.05$] or Tg2576 mice [$t(9) = 1.244$, $p>0.05$].

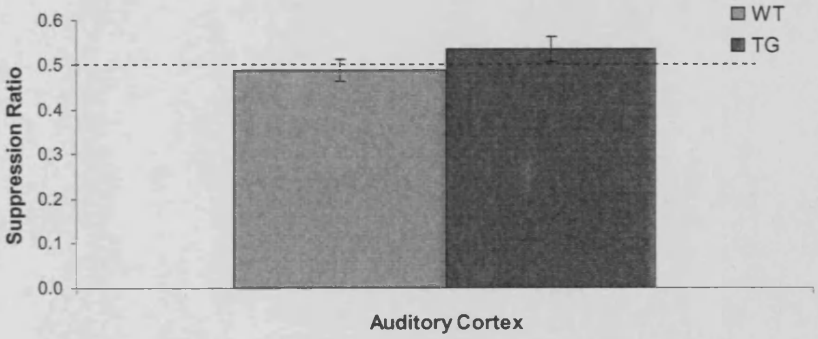


Figure 46: Mean suppression ratios for the primary auditory cortex. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Overall, these results demonstrate that c-Fos expression in the primary auditory cortex was increased equally in both the CS/US and CS Only conditions and no differential expression was observed between wild-type and Tg2576 mice. See

Experiment 8b for a discussion of c-Fos activation in the primary cortex after CS/US and CS Only presentation, as compared to exposure to the footshock only.

5.2.6 Results: Experiment 8b: c-Fos Immunocytochemistry

Hippocampus: Subregions

Figure 47A depicts the mean c-Fos counts in each sub-region of the hippocampus after presentations of the US only. To determine whether genotype had an impact on hippocampal c-Fos expression as a function of footshock experience, the data were analysed using an ANOVA with genotype and region (CA1, CA3, DG) as factors. Results revealed a significant main effect of genotype [$F_{(1,10)} = 8.725$, $p<0.02$], a significant main effect of region [$F_{(2,20)} = 25.710$, $p<0.001$] and a significant interaction between genotype and region [$F_{(2,20)} = 6.216$, $p<0.008$]. Tests of simple effects revealed a significant effect of region for both wild-type and Tg2576 mice [$F_{(2,9)} = 4.341$ and 25.4778, p's<0.05, respectively]. Furthermore, results revealed a significant effect of genotype for the CA1 region [$F_{(1,10)} = 9.310$, $p<0.02$], but not for the CA3 or DG regions [$F_{(1,10)} = 2.029$ and 2.285, p's>0.05, respectively].

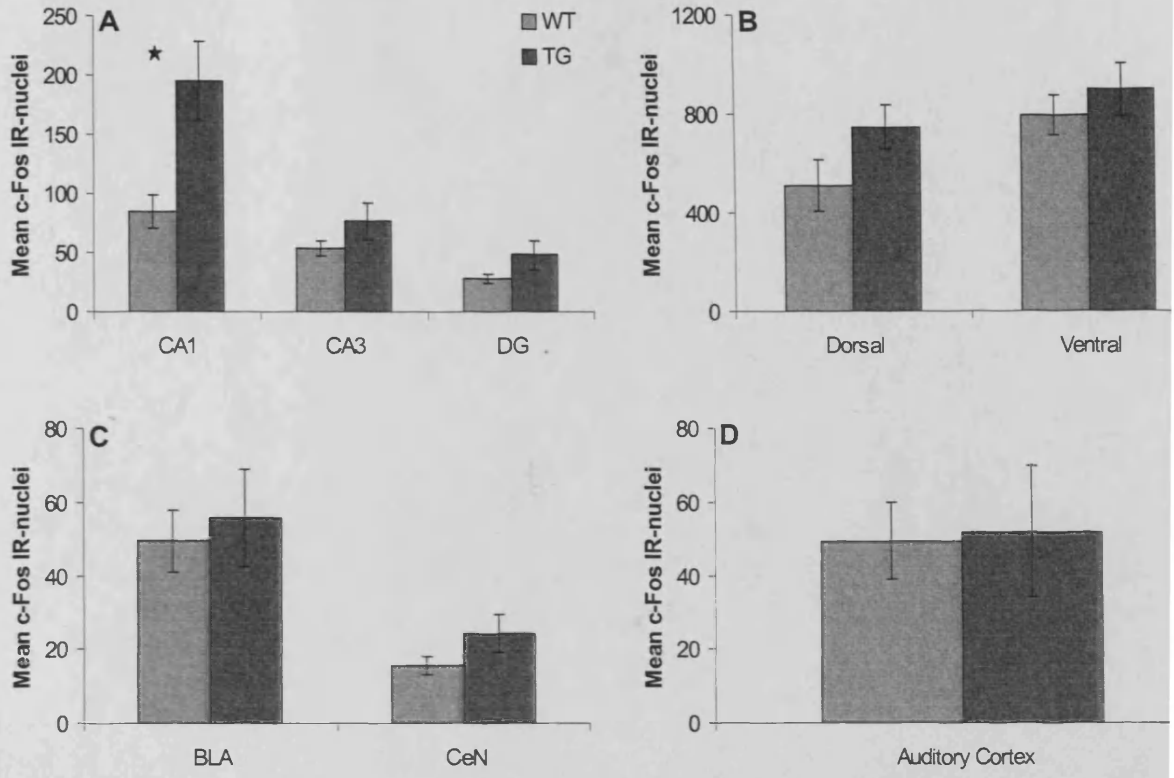


Figure 47: Mean c-Fos IR nuclei in subregions of the hippocampus after shock-only exposure. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M. * = $p<0.05$.

Hippocampus: Dorsal vs. Ventral

Figure 47B depicts the c-Fos counts in the dorsal and ventral regions of the hippocampus after US Only presentations. Statistical analysis revealed a significant main effect of region [$F_{(1,10)} = 11.581$, $p<0.007$], a non-significant main effect of genotype [$F_{(1,10)} = 1.999$, $p>0.05$] and a non-significant interaction between these factors [$F_{(1,10)} = 1.042$, $p>0.05$].

Amygdala: Subregions

Figure 47C depicts the c-Fos counts in the subregions of the amygdala. Statistical analysis revealed a significant main effect of region [$F_{(1,10)} = 28.369$, $p<0.001$], a non-significant main effect of genotype [$F_{(1,10)} = 0.550$, $p>0.05$] and a non-significant interaction between these factors [$F_{(1,10)} = 0.042$, $p>0.05$].

Primary auditory cortex

Figure 47D depicts the c-Fos counts in the primary auditory cortex. An ANOVA with genotype as a between-subjects factor revealed a non-significant effect of genotype [$F_{(1,11)} = 0.008$, $p>0.05$]. It is interesting to note that when compared to the CS/US and CS Only presentations (see Figure 43), it is clear the c-Fos expression was considerably lower in the shock-only condition (mean: 50.8, S.E.M.: 9.9), and high after CS/US and CS Only presentations (mean: 163.6, S.E.M.: 12.7 and mean: 148.2, S.E.M.: 24.6, respectively). This confirms the sensitivity of the auditory cortex to the presentation of the tone. Furthermore, activation of this region was found to be comparable in both wild-type and Tg2576 mice (see Figures 46 & 47).

Overall, no transgene-dependent changes in c-Fos expression were observed in the amygdala, the primary auditory cortex or the dorsal/ventral regions of the hippocampus after shock-only exposure. Tg2576 mice showed greater c-Fos expression overall in the hippocampus and this reflected higher levels of c-Fos expression in the CA1 region.

5.2.7 Discussion

The results of the behavioural analysis revealed a number of transgene-dependent differences in behaviour. Firstly, it is important to mention that both locomotor activity measures and freezing data revealed no transgene-dependent difference in baseline activity levels during the acclimatisation phase. This ensures that any subsequent genotypic differences in behaviour can be attributed to learning rather than baseline performance.

Significant decreases in locomotor activity and increases in freezing behaviour were observed with successive tone presentations in the CS/US condition for both genotypes. This confirms that both genotypes were capable of both detecting the auditory stimulus and of demonstrating conditioned changes in behaviour following pairing of the CS with footshock. In the CS/US condition, locomotor activity decreased with successive ITIs, during the last 30 seconds, regardless of

genotype. The freezing data, however, revealed a significant effect of genotype during the first 30 seconds of the post-shock periods in the CS/US condition. The impact of transgene-status was not evident, however, during the last 30 seconds of these periods. Why such a stark difference in behaviour is observed between the two measures is not immediately clear. However, visual inspection of Figure 35E suggests a genotypic difference in locomotor activity during the first 30 seconds of the post-shock period. If these data were analysed separately by ANOVA, without including "condition" as a factor, the analysis reveals a significant effect of ITI [$F_{(2,42)} = 5.154$, $p=0.01$] and, more importantly, a significant main effect of genotype [$F_{(1,21)} = 4.915$, $p<0.04$]. The interaction was non-significant ($F<1$). Thus, the similar levels of locomotor activity observed in the control condition served to obscure both the progressive decrease in locomotor activity observed with each successive ITI, as well as the significantly higher level of locomotor activity elicited by Tg2576 mice, compared to wild-type mice.

The finding that post-shock freezing behaviour was significantly lower in Tg2576 mice replicates the findings of Barnes & Good, (2005). However, it is interesting to also note the same pattern of behaviour was evident in the US only condition. That is, locomotor activity measures revealed significantly higher levels of activity in Tg2576 mice than wild-type controls in the 2 seconds immediately following presentation of the footshock. The precise implications of this phenomenon will be discussed fully in the chapter discussion.

The analysis of IEG expression following CS/US presentation revealed a number of transgene dependent changes in neural activation. Several conclusions can be drawn from the observed levels of c-Fos expression. Firstly, while CS/US conditioning produced a *suppression* of c-Fos protein in the dentate gyrus of wild-type mice, this was not demonstrated in transgenic mice. c-Fos expression in the other hippocampal subregions, however, did not differ as a function of transgene and indicates a transgene specific effect on dentate gyrus activity. Secondly, after CS/US conditioning, Tg2576 mice demonstrated greater suppression of c-Fos activity relative to the control condition in the hippocampus (particularly in the ventral hippocampal region). Lastly, in the footshock only condition, Tg2576 mice

demonstrated significantly higher c-Fos expression in the CA1 region of the hippocampus than wild-type mice.

CS/US conditioning evoked genotypic differences in IEG activation in the dentate gyrus and the ventral region of the hippocampus. More specifically, the proportion of c-Fos expression in sub-regions of the hippocampus did not differ significantly between the CS Only and CS/US for Tg2576 mice, in contrast to wild-type mice. Neither the wild-type nor the Tg2576 mice showed significant changes in c-Fos expression in the amygdala relative to the control condition. Interestingly, the region that demonstrated the most pronounced changes in c-Fos expression following fear conditioning and non-contingent shock presentation was the hippocampus. In the former case, the most notable change was in the dentate gyrus and, in the latter, the CA1 region. The differences in c-Fos expression in the dentate gyrus are consistent with evidence that this region is compromised at the molecular, cellular and behavioural levels in Tg2576 mice (e.g. Jacobsen et al., 2006). The functional significance of these changes remains open to further research. However, it has been suggested that the DG plays a specific role in the spatiotemporal separation of novel and complex cues. This pattern separation process is thought to allow for the disambiguation of complex stimuli and for the encoding of individual patterns of information from an environment (Kesner et al., 2004). In keeping with this, disruption of the projections from the DG to the CA3 region disrupts encoding of spatial information, although it is not necessarily vital for the retrieval of information (Lee & Kesner, 2004). Thus, it is possible that the behavioural deficits observed in Tg2576 mice during tone conditioning and testing (Experiment 3) were the manifestation of aberrant encoding of conditioned stimuli and the US. This proposal will be discussed in more detail in the chapter discussion.

One unexpected difference between the Tg2576 and wild-type mice was in the pattern of c-Fos expression present following massed foot-shock alone trials reported in Experiment 8b. There were lower numbers of c-Fos immunoreactive nuclei in the CA1 region in wild-type mice relative to Tg2576 mice. This difference was not evident in Experiment 8a following conditioning. There are several possible explanations for this difference between control and Tg2576 mice

in Experiment 8b. For example, it is possible that Tg2576 mice differ in their reaction to novelty and/or stress and this results in greater activation of the hippocampus. Unfortunately, in the present experiment it is not possible to determine whether this increase in c-Fos expression reflects a reaction to short-term exposure to a novel context or exposure to footshock *per se*. The behaviour of Tg2576 mice in the elevated plus maze (Experiment 1) revealed changes in reaction to anxiety-provoking stimuli. This latter observation suggests changes in the processing of aversive stimuli in Tg2576 mice. Nevertheless, no differences were observed in c-Fos expression in the amygdala, which clearly contributes to processing emotionally significant events. The possibility remains, therefore, that the higher levels of c-Fos activation in Tg2576 mice might reflect processing related to a novel environment. Although IEG data cannot provide a definitive picture of neural activity at a given time-point, the data are consistent with evidence that CA1 neurons show impaired encoding of contextual information in Tg2576 mice (Caccuci et al., 2008). In contrast, no gross differences were evident in c-Fos activation in the amygdala. This latter conclusion must be qualified by the fact that the wild-type, but not the Tg2576 mice, showed relatively more c-Fos expression in the CeN relative to the BLA. Although speculative, this may reflect the fact that the wild-type mice showed more freezing behaviour at least immediately following shock presentation than Tg2576 mice.

In summary, these data confirm that Tg2576 mice demonstrate aberrant hippocampal neural activity following CS/US presentation in a fear conditioning task. There was evidence for subtle alterations in the pattern of c-Fos activation in the amygdala nuclei, although the clearest pattern of changes in Tg2576 mice was evident in the hippocampus. The next experiment aimed to evaluate whether the expression of c-Fos in the hippocampus and amygdala was influenced by the APPswe mutation following the retrieval of context and tone fear memories.

5.3 Experiment 9: c-Fos expression following memory retrieval

5.3.1 Introduction

Experiment 8a,b revealed deficits in post-shock freezing during the conditioning and changes in the pattern of c-Fos expression, primarily in the hippocampus, of Tg2576 mice. In the present experiment, the expression of c-Fos protein was used to identify regions of neural activity following the retrieval of fear memories. Despite evidence of aberrant freezing behaviour in transgenic Tg2576 mice during the retrieval of contextual fear (e.g. Corcoran, Lu, Turner & Maren, 2002) and cued fear (Barnes & Good, 2005), there has been no attempt to identify the neural correlates of this behavioural impairment.

The lateral, basal and central nuclei of the amygdala play a role in the acquisition and expression of conditioned fear to an auditory CS (Anglada-Figueroa & Quirk, 2005). When tested in extinction, impairments in freezing to a tone have been revealed after lesions to both the central and basolateral nuclei (Koo, Han & Kim, 2004). Furthermore, c-Fos expression has been shown to increase in the basal and central nuclei of the amygdala after the retrieval of an auditory-cued fear memory, while no increase in IEG expression was found in the hippocampus (Hall, Thomas, Everitt, 2001). The current literature suggests a major contribution of the amygdala to the retrieval of fear memories elicited by a punctate auditory CS. Furthermore, c-Fos expression has been shown to be increased in the hippocampus during contextual fear conditioning and after context re-exposure when tested in extinction (Milanovic et al., 1998). This procedure should, therefore, be sensitive to APPswe-induced changes in amygdala and/or hippocampal function during fear memory retrieval. Given evidence of impaired contextual fear in Tg2576 mice (e.g. Comery et al., 2005; Dineley et al., 2002), as well as impairments in auditory cued fear in this mutant strain (Barnes & Good, 2005), it is likely that altered c-Fos expression will be evident in both the hippocampus and amygdala. The animals were conditioned and tested as described in Experiment 3. However, in order to assess the effects of context and cue recall on IEG expression in isolation, half the

animals were sacrificed after completing the cued test and the remainder after completing the context test.

5.3.2 Method

Subjects

Twenty-four female Tg2576 mice and twenty-four female wild-type littermate controls, aged 19 months, were used in this experiment. Mice were housed in groups of 2-4 per cage and maintained on a 12 hour light schedule. Mice were allowed *ad libitum* access to food and water throughout the duration of the experiment.

Apparatus

The apparatus was identical to that used in Experiment 8. All mice were conditioned in the same operant chamber, which was subsequently used to test contextual fear memory. Memory for auditory cued fear was tested in a second chamber, which had distinctly different contextual cues, including black and white checked walls, sawdust on the floor to a depth of 1mm and the cleaning solution, 10% Hibiscrub, provided a unique odour. The infrared locomotor activity monitor was set to record mouse micro-movements.

Procedure

Mice were randomly assigned to either the conditioning (CS/US) or control (CS Only) condition. The CS/US group, which consisted of 14 Tg2576 mice and 14 wild-type mice, received identical fear conditioning to that administered in Experiments 3 and 8. Briefly, after a habituation period, the mice received three presentations of the tone CS, which co-terminated with the aversive shock US, separated by an ITI of 120 seconds. The CS Only control group, which consisted of 10 Tg2576 mice and 10 wild-type mice, received exposure to the chamber and the auditory CS in the same manner as the CS/US group, except that no footshock was presented.

All mice were tested for fear responses elicited by both the context and the auditory CS. They received one test 24 hours after conditioning and the second test 24 hours later. Order of testing was fully counterbalanced across genotypes. Prior to testing, animals were placed in a dark quiet room for 2 hours. After testing, animals were returned to their home cages and placed back in the dark room. Ninety minutes after the start of the second behavioural test, mice were culled via intraperitoneal injection of pentobarbital and brain tissue was extracted for IEG analysis. Therefore, brain tissue for half the mice in each group was analysed for IEG expression after the context test and the other half for expression after the tone test.

Immunohistochemistry and Image Analysis

Immunohistochemical staining and image analysis were performed exactly as described in Experiment 8. The regions of interest chosen for cell counting were also identical.

5.3.3 Results: Behaviour

Infrared Locomotor Activity Scores

Acquisition

Habituation Phase: For the habituation stage (Figure 48A & B), the data were analysed by ANOVA, with genotype, condition (CS/US versus CS Only) and block (first versus second) as factors. Results revealed a non-significant effect of block [$F_{(1,44)} = 0.000$, $p>0.05$] and a non-significant effect of condition [$F_{(1,44)} = 3.399$, $p>0.05$]. The effect of genotype failed to reach conventional levels of statistical significance [$F_{(1,44)} = 4.053$, $p=0.0503$], although the analysis suggests that the APPswe mice were (numerically) more active than their wild-type controls. All interactions were non-significant [maximum: $F_{(1,44)} = 0.8869$, $p>0.05$]. Thus, Tg2576 mice demonstrated a numerical increase in locomotor activity during the acclimatization phase relative to wild-type controls.

Tone Presentations: Data from the tone presentations were analysed by ANOVA, with genotype, condition and tones (1-3) as variables. Results are depicted in Figure 48C & D. Statistical analysis revealed significant main effects of tone [$F_{(2,88)} = 19.648$, $p<0.001$], genotype [$F_{(1,44)} = 4.258$, $p=0.045$] and condition [$F_{(1,44)} = 16.832$, $p<0.001$]. The tone by condition interaction was also significant [$F_{(2,88)} = 13.725$, $p<0.001$]. All other interactions were non-significant [maximum: $F_{(1,44)} = 2.956$, $p>0.05$]. Tests of simple effects revealed a significant effect of tone for the CS/US condition [$F_{(2,43)} = 37.300$, $p<0.001$], but not for the CS Only condition [$F_{(1,44)} = 4.258$, $p=0.045$]. Furthermore, a significant effect of tone was evident for tones two and three [$F_{(1,44)} = 11.139$ and 34.931, respectively, p's<0.002], but not tone one [$F_{(1,44)} = 0.002$, $p>0.05$]. These results indicate that, although wild-type mice displayed lower locomotor activity overall, both genotypes displayed decreased locomotor activity with successive tone presentations. Furthermore, a change in locomotor activity during tone presentation occurred as a function of conditioning.

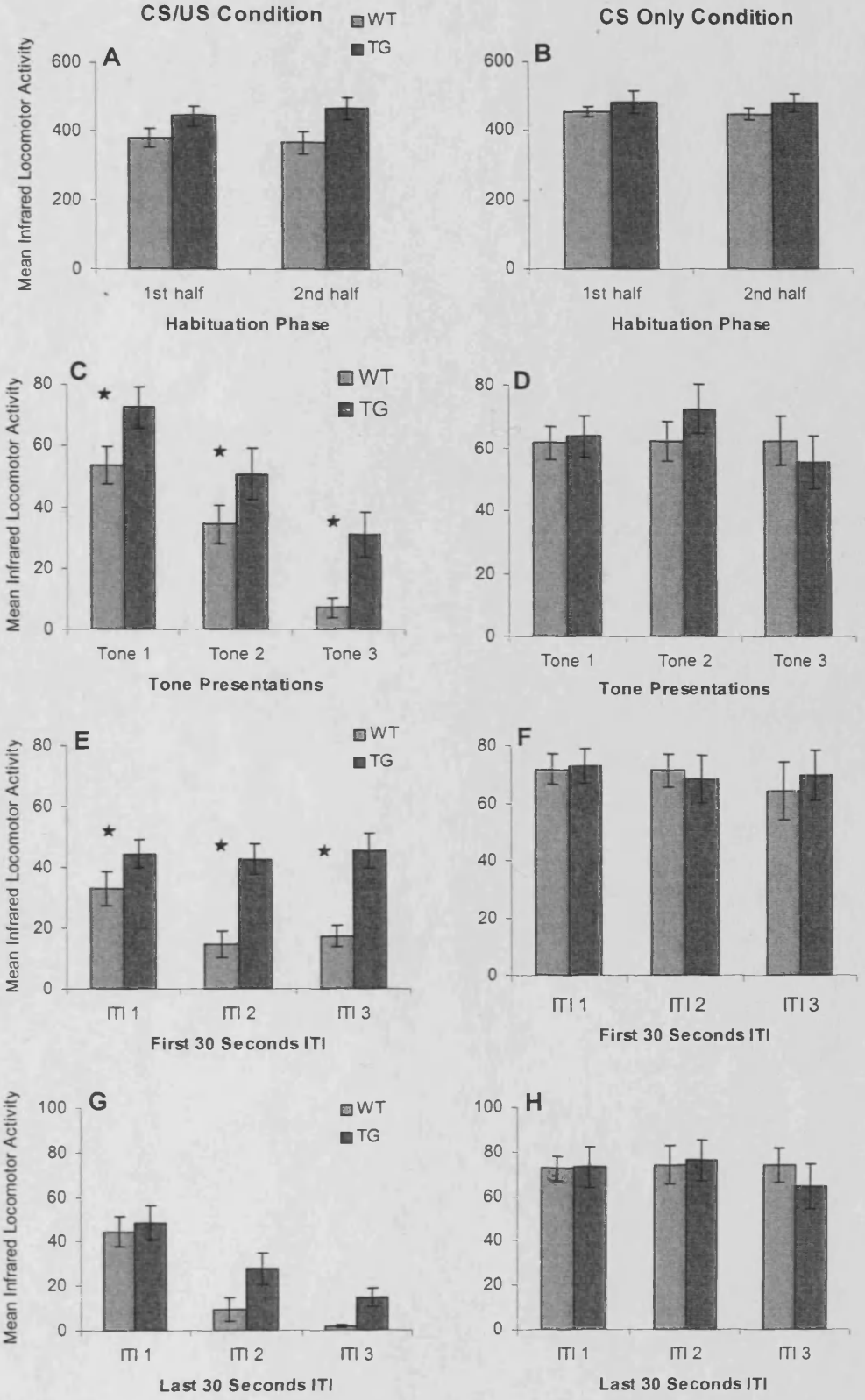


Figure 48: Mean infrared locomotor activity measures during CS/US conditioning (Left column) and CS Only presentation (Right column). A & B = activity measures during the first and second half of the 6 minutes habituation period. C & D = activity measures during the 28 second tone presentations. E & F = activity measures during the first 30 seconds of the post-shock ITI periods. G & H = activity measures during the last 30 seconds of the post-shock ITI periods. Light grey bars = wild-type mice; Dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M. * = $p<0.05$.

ITI Periods: Data from the first 30 seconds of the ITI periods, which represents the post-shock locomotor activity, were analyzed by ANOVA with genotype, condition and ITI (1-3) as variables. Results are depicted in Figure 48E & F. Statistical analysis revealed a significant effect of genotype [$F_{(1,44)}$ = 5.858, $p<0.02$], a significant effect of ITI [$F_{(2,88)}$ = 7.759, $p<0.001$] and a significant effect of condition [$F_{(1,44)}$ = 13.248, $p<0.001$]. Furthermore, significant interactions were revealed between ITI and condition [$F_{(2,88)}$ = 3.432, $p<0.04$] and genotype and condition [$F_{(1,44)}$ = 4.895, $p<0.04$].

For the ITI by condition interaction, tests of simple main effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,43)}$ = 13.251, $p<0.001$], but not for the tone only condition [$F_{(2,43)}$ = 0.507, $p>0.05$]. A significant effect of condition was apparent during the second and third ITIs [$F_{(1,44)}$ = 17.023 and 11.718, respectively, $p=0.001$], but not during the first ITI [$F_{(1,44)}$ = 3.091, $p>0.05$]. Thus, activity differed as a function of experience. Freezing increased with successive ITI periods in the CS/US condition, but not in the CS Only condition.

For the genotype by condition interaction, tests of simple main effects revealed a significant effect of condition for wild-type [$F_{(1,44)}$ = 17.125, $p<0.001$], but not Tg2576 mice [$F_{(1,44)}$ = 1.019, $p>0.05$]. Furthermore, a significant effect of genotype was apparent during CS/US conditioning [$F_{(1,44)}$ = 12.878, $p<0.001$], but not during CS Only presentation [$F_{(1,44)}$ = 0.018, $p>0.05$].

Data from the last 30 seconds of the ITI period were similarly analysed (Figure 48G & H). Results revealed a non-significant effect of genotype [$F_{(1,44)}$ = 0.697, $p>0.05$], but a significant main effect of ITI [$F_{(2,88)}$ = 18.265, $p<0.001$] and condition [$F_{(1,44)}$ = 72.192, $p<0.001$]. The ITI by condition interaction was significant [$F_{(2,88)}$ = 14.518, $p<0.001$]. All other interactions were non-significant [maximum: $F_{(1,44)}$ = 1.558, $p>0.05$]. Tests of simple effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,43)}$ = 34.295, $p<0.001$], but not for CS Only presentation [$F_{(2,43)}$ = 0.795, $p>0.05$]. Furthermore, a significant effect of condition was evident for the first, second and third ITIs [$F_{(1,44)}$ = 12.423, 58.319

and 108.900, respectively, p's=0.001]. Thus, at the end of the ITI period, more freezing was evident in mice that underwent CS/US presentations, but this did not differ as a function of transgene status.

Freezing Measures

Habituation Phase: For the habituation stage (Figure 49A & B), the data were analysed by ANOVA, with genotype, condition and block (first versus second) as factors. Results revealed a non-significant effect of block [$F_{(1,44)} = 0.547$, $p>0.05$], a non-significant effect of genotype [$F_{(1,44)} = 0.074$, $p>0.05$], and a non-significant effect of condition [$F_{(1,44)} = 0.223$, $p>0.05$]. All interactions were also non-significant [maximum: $F_{(1,44)} = 0.547$, $p>0.05$] This confirms that no genotypic differences in freezing emerged during the habituation phase.

Tone Presentations: Data from the tone presentations were analysed by ANOVA, with genotype and tones (1-3) as variables. Results are depicted in Figure 49C & D. Statistical analysis revealed significant main effects of genotype [$F_{(1,44)} = 7.608$, $p<0.001$], tone [$F_{(2,88)} = 46.964$, $p<0.001$] and condition [$F_{(1,44)} = 46.843$, $p<0.001$]. The tone by condition interaction was significant [$F_{(2,88)} = 44.243$, $p<0.001$], as was the tone by genotype interaction [$F_{(2,88)} = 6.652$, $p<0.003$]. Furthermore, the genotype by condition interaction was significant [$F_{(1,44)} = 8.019$, $p<0.008$], as was the three-way interaction [$F_{(2,88)} = 6.699$, $p<0.002$].

For the tone by condition interaction, tests of simple effects revealed a significant effect of tone for the CS/US condition [$F_{(2,43)} = 92.560$, $p<0.001$], but not for the CS Only presentations [$F_{(2,43)} = 0.022$, $p>0.05$]. The effect of condition was significant for tones 2 and 3 [$F_{(1,44)} = 18.131$ and 73.846, respectively, p's<0.001], but not for tone 1 [$F_{(1,44)} = 0.699$, $p>0.05$]. Thus, freezing behaviour increased with successive tone presentations during CS/US conditioning.

For the tone by genotype interaction, tests of simple effects revealed a significant effect of tone for both wild-type and Tg2576 mice [$F_{(2,43)} = 37.574$ and 7.810, p's<0.001, respectively]. The effect of genotype was significant for tone 3 [$F_{(1,44)} = 11.329$], but not for tones 1 and 2 [$F_{(1,44)} = 0.047$ and 3.175, p's>0.05,

respectively]. Thus, an effect of genotype was apparent during the third tone only, but both genotypes demonstrated increased freezing behaviour with successive tone presentations.

For the genotype by condition interaction, tests of simple effects revealed a significant effect of genotype during the CS/US condition [$F_{(1,44)}$ = 18.751, $p<0.001$], but not the CS Only condition [$F_{(1,44)}$ = 0.002, $p>0.05$]. The effect of condition was significant for both wild-type and Tg2576 mice [$F_{(1,44)}$ = 46.812 and 8.050, p's=0.008, respectively]. Thus, both genotypes demonstrated more freezing behaviour during the CS/US condition.

For the three-way interaction, statistical analysis revealed a significant effect of tone for wild-type mice in the CS/US condition [$F_{(2,43)}$ = 88.463, $p<0.001$], but not the CS Only condition [$F_{(2,43)}$ = 0.010, $p>0.05$]. Similarly, a significant effect of tone was apparent for Tg2576 mice in the CS/US condition [$F_{(2,43)}$ = 17.644, $p<0.001$], but not the CS Only condition [$F_{(2,43)}$ = 0.040, $p>0.05$].

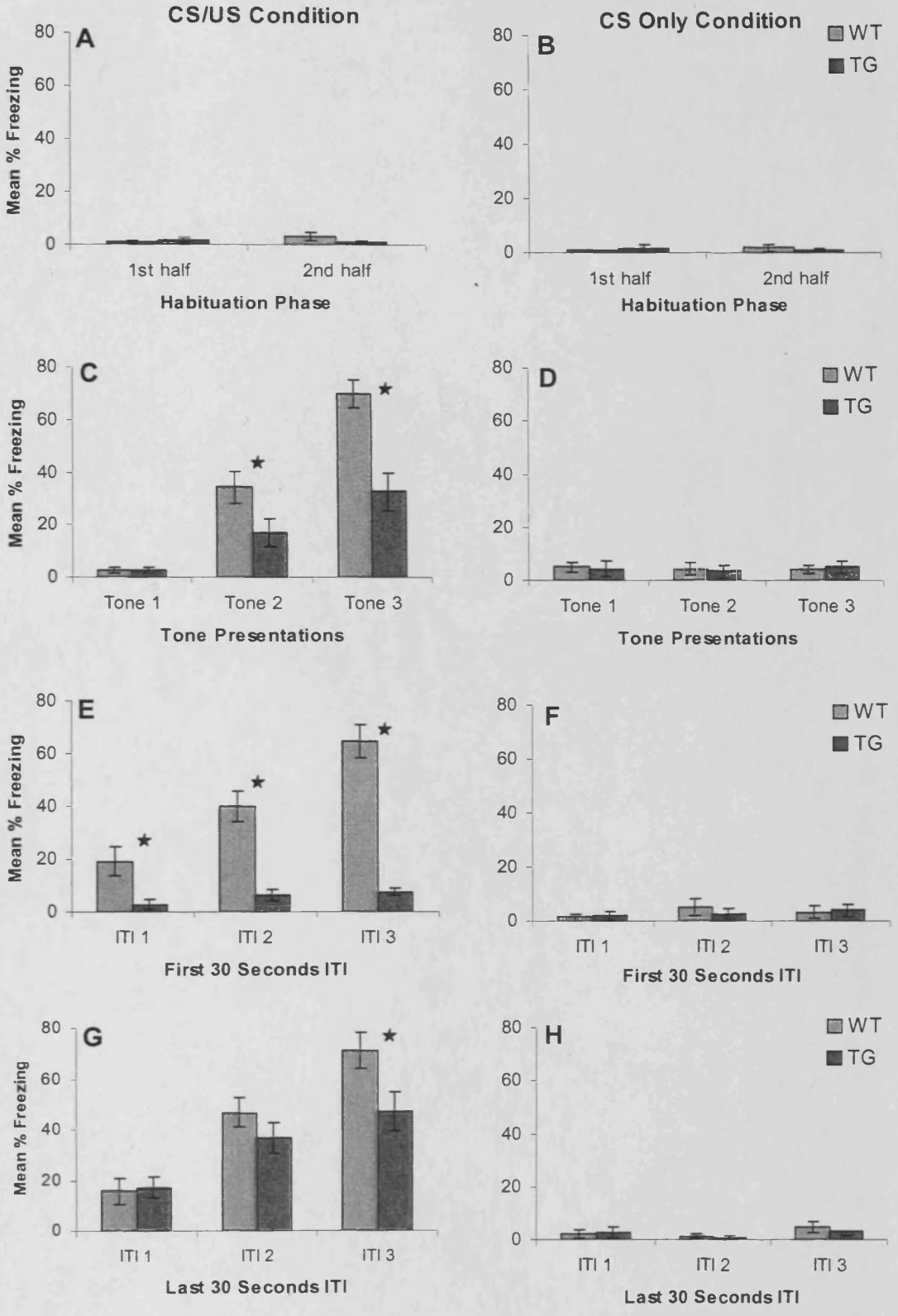


Figure 49: Mean percentage of freezing during CS/US conditioning (Left column) and CS Only presentation (Right column). A & B = freezing measures during the first and second half of the 6 minutes habituation period. C & D = freezing measures during the 28 second tone presentations. E & F = freezing measures during the first 30 seconds of the post-shock ITI periods. G & H = freezing measures during the last 30 seconds of the post-shock ITI periods. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

ITI periods: Data from the first 30 seconds of the ITI periods were analyzed by ANOVA (Figure 49E & F). Statistical analysis revealed significant main effects of genotype [$F_{(1,44)} = 34.689$, $p<0.001$], ITI [$F_{(2,88)} = 21.373$, $p<0.001$] and condition [$F_{(1,44)} = 43.739$, $p<0.001$]. Significant interactions were revealed between ITI and condition [$F_{(2,88)} = 15.661$, $p<0.001$] and genotype and condition [$F_{(1,44)} = 33.003$, $p<0.001$]. Furthermore, the ITI by genotype interaction was significant [$F_{(2,88)} = 12.064$, $p<0.001$], as was the three-way interaction [$F_{(2,88)} = 12.430$, $p<0.001$].

For the ITI by condition interaction, tests of simple main effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,43)} = 37.182$, $p<0.001$], but not for the tone only condition [$F_{(2,43)} = 0.271$, $p>0.05$]. Furthermore, a significant effect of condition was apparent during each of the three ITIs [$F_{(1,44)} = 6.602$, 22.773 and 63.809, respectively, $p<0.02$]. Thus, freezing behaviour differed as a function of experience. Freezing increased with successive ITI periods in the CS/US condition, but not in the tone only condition.

For the genotype by condition interaction, tests of simple main effects revealed a significant effect of conditioning for wild-type [$F_{(1,44)} = 76.364$, $p<0.001$], but not Tg2576 mice [$F_{(1,44)} = 0.377$, $p>0.05$]. Furthermore, a significant effect of genotype was apparent during CS/US conditioning [$F_{(1,44)} = 81.216$, $p<0.001$], but not during CS Only presentation [$F_{(1,44)} = 0.009$, $p>0.05$].

For the ITI by genotype interaction, further analysis revealed a significant effect of ITI for wild-type [$F_{(2,43)} = 27.328$, $p<0.001$], but not Tg2576 mice [$F_{(2,43)} = 0.545$, $p>0.05$]. Furthermore, the effect of genotype was apparent for all three ITIs [$F_{(1,44)} = 4.613$, 20.775 and 48.100, p's<0.04, respectively]. Thus, during the first 30 seconds of every ITI period, Tg2576 mice froze less than wild-type mice, and freezing behaviour changed as a function of ITI for wild-type, but not Tg2576 mice.

Analysis of the three-way interaction revealed a significant effect of genotype during each of the three ITI periods in the CS/US condition [$F_{(1,44)} = 12.043$,

42.836 and 118.182, p's=0.001, respectively]. There were no effects of genotype during any of the ITI periods in the CS Only condition [all F's < 1].

Data from the last 30 seconds of the ITI period were similarly analysed (Figure 49G & H). Results revealed a non-significant effect of genotype [$F_{(1,44)} = 1.851$, $p>0.05$], but significant main effects of ITI [$F_{(2,88)} = 32.356$, $p<0.001$] and condition [$F_{(1,44)} = 78.537$, $p<0.001$]. The ITI by condition interaction was significant [$F_{(2,88)} = 28.426$, $p<0.001$]. The genotype by ITI interaction did not reach conventional levels of statistical significance [$F_{(2,88)} = 3.058$, $p=0.052$]. All other interactions were non-significant [next maximum: $F_{(2,88)} = 2.235$, $p>0.05$]. Tests of simple effects revealed a significant effect of ITI for the CS/US condition [$F_{(2,43)} = 53.026$, $p<0.001$], but not for the CS Only condition [$F_{(2,43)} = 0.326$, $p>0.05$]. Furthermore, a significant effect of condition was evident for all three ITIs [$F_{(1,44)} = 12.132$, 64.098 and 76.965, p's<0.002, respectively]. Thus, at the end of the ITI period, more freezing was evident in mice that underwent CS/US presentations, but this did not differ as a function of transgene status.

Overall, the results of the locomotor activity scores and freezing measures revealed greater activity in Tg2576 mice during the tone presentations. Aberrant behaviour was also evident during the post-shock period in Tg2576 mice, but was not present in the last 30 seconds of the ITI period. Interestingly, the transgene-driven change in behaviour during the tone presentations activity was not evident in either Experiment 3 or 8. Importantly, however, despite the observed genotypic differences, both Tg2576 and wild-type mice demonstrated a progressive change in freezing and locomotor activity with successive tone presentations, indicating an intact ability to perceive the auditory stimulus.

Test Phases

Tone Test: The tone test consisted of two phases. In the first phase, animals were placed in a novel context and, in the second phase, they were exposed to a continuous presentation of the tone CS. Figure 50A & B depicts infrared locomotor activity measures from the novel context phase of the tone test. Data were analysed by ANOVA with block (1st versus 2nd half of the phase), condition

and genotype as factors. Statistical analysis of the novel context phase revealed non-significant main effects of block [$F_{(1,44)} = 0.423$, $p>0.05$] and genotype [$F_{(1,44)} = 0.178$, $p>0.05$], but a significant main effect of conditioning [$F_{(1,44)} = 14.560$, $p>0.05$]. All interactions were non-significant [maximum: $F_{(1,44)} = 1.031$, $p>0.05$].

Figure 50C & D shows the locomotor activity data from the CS phase of the tone test. Analysis of the locomotor levels during the tone presentation revealed a non-significant main effect of block [$F_{(1,44)} = 0.012$, $p>0.05$], but significant main effects of both genotype [$F_{(1,44)} = 4.096$, $p<0.05$] and condition [$F_{(1,44)} = 21.703$, $p<0.001$]. The genotype by condition interaction failed to reach conventional levels of statistical significance, [$F_{(1,44)} = 3.349$, $p=0.074$]. All other interactions were non-significant [maximum: $F_{(1,44)} = 1.078$, $p>0.05$]. These results indicate that transgenic Tg2576 mice demonstrated significantly higher levels of locomotor activity overall during the tone presentation, as compared to their wild-type littermates. Furthermore, locomotor activity differed significantly between the CS/US and CS Only conditions.

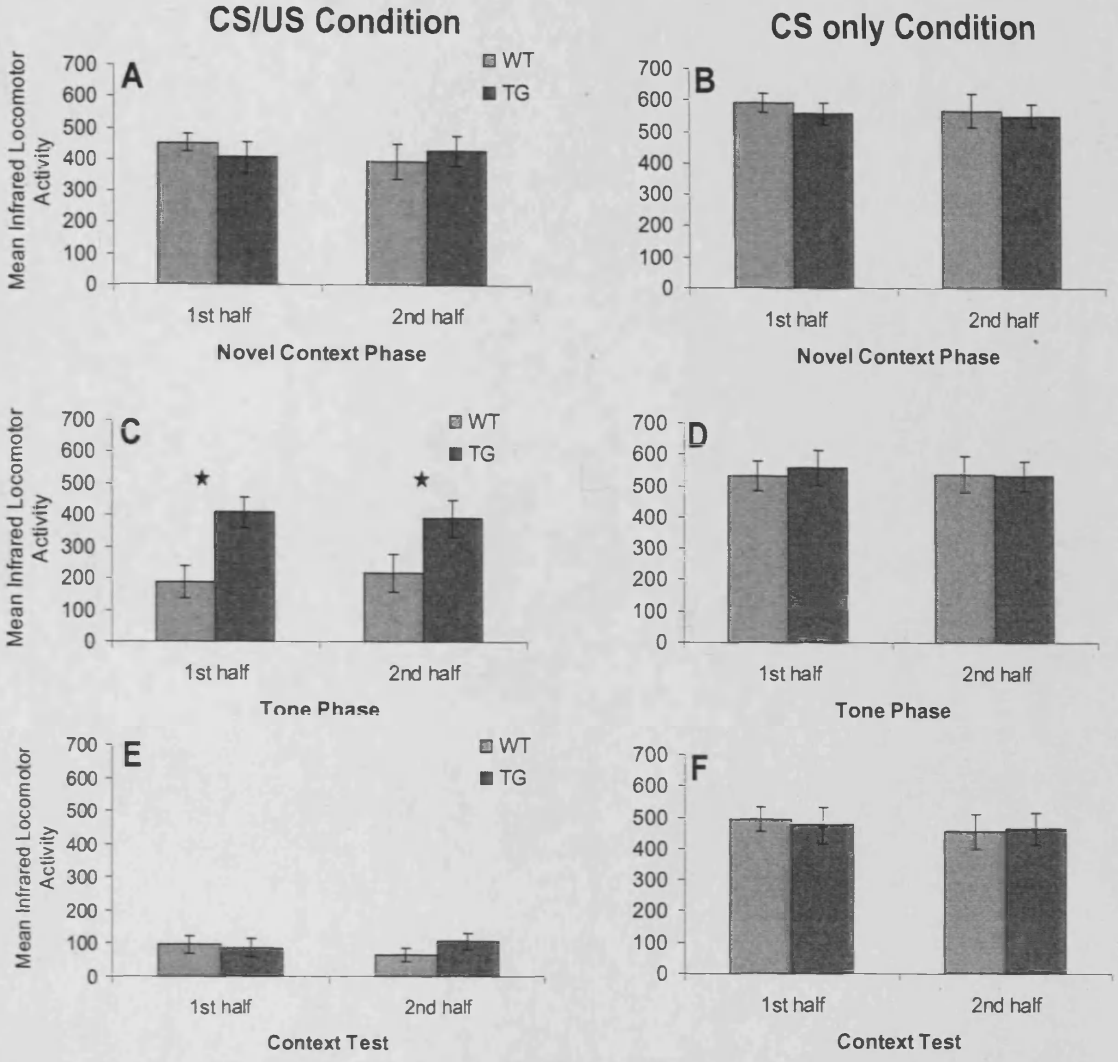


Figure 50: Mean infrared locomotor activity measures during test phases in mice that underwent CS/US conditioning (Left column) and CS Only presentation (Right column). A & B = activity measures during the first and second half of the novel context phase of the tone test. C & D = activity measures during the two halves of the CS presentation during the tone test. E & F = activity measures during the context test, split into 1st and 2nd halves. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Context Test: Data from the context test were analysed by ANOVA, with block (1st versus 2nd half), condition and genotype as variables. Figure 50E &F depicts data from the context text. Results revealed non-significant main effects of block [$F_{(1,44)} = 1.871$, $p>0.05$] and genotype [$F_{(1,44)} = 0.022$, $p>0.05$], but a significant effect of condition [$F_{(1,44)} = 124.942$, $p<0.001$]. All interactions were non-significant [maximum: $F_{(1,44)} = 0.543$, $p>0.05$].

Freezing Measures

Tone Test

Novel Context Phase: Figure 51A & B depicts freezing data from the novel context phase of the tone test. Statistical analysis revealed significant main effects of block [$F_{(1,44)}$ = 5.146, $p<0.03$], genotype [$F_{(1,44)}$ = 4.271 $p<0.05$] and conditioning [$F_{(1,44)}$ = 7.009, $p<0.02$]. The block by condition interaction was significant [$F_{(1,44)}$ = 4.688, $p<0.04$], as was the condition by genotype interaction [$F_{(1,44)}$ = 7.846, $p<0.008$].

Tests of simple effects for the block by condition interaction revealed a significant effect of block for the CS/US condition [$F_{(1,44)}$ = 11.794, $p<0.002$], but not for the CS Only condition [$F_{(1,44)}$ = 0.005, $p>0.05$]. Furthermore, the effect of condition was significant for the second block [$F_{(1,44)}$ = 6.294, $p<0.02$], but not for the first block [$F_{(1,44)}$ = 2.324, $p>0.05$]. Thus, freezing increased during the second half of the novel context phase in the CS/US condition, but not for mice in the CS Only condition.

Statistical analysis of the genotype by condition interaction revealed a significant effect of genotype for the CS/US condition [$F_{(1,44)}$ = 14.217, $p<0.001$], but not when the CS was presented alone [$F_{(1,44)}$ = 0.231, $p>0.05$]. The effect of condition was also shown to be significant for wild-type [$F_{(1,44)}$ = 14.844, $p<0.001$], but not Tg2576 mice [$F_{(1,44)}$ = 0.012, $p<0.05$].

Thus, the results show that wild-type mice in the CS/US condition showed an increase in freezing during the context habituation phase of the test. This may reflect generalisation of fear between the conditioning and test context. Nevertheless, this pattern was not evident in the Tg257676 mice, who showed low levels of freezing to the context similar to that shown by Tg576 mice in the CS alone condition.

Tone Phase: Levels of freezing elicited by the tone presentation are depicted in Figure 51C & D. Analysis of the data revealed a non-significant main effect of

block [$F_{(1,44)}$ = 0.010, $p>0.05$], a significant main effect of genotype [$F_{(1,44)}$ = 11.038, $p<0.002$] and a significant effect of condition [$F_{(1,44)}$ = 16.476, $p<0.001$]. The condition by genotype interaction was also significant [$F_{(1,44)}$ = 11.827, $p<0.002$]. All other interactions were non-significant [maximum: $F_{(1,44)}$ = 0.537, $p>0.05$].

Statistical analysis of the interaction revealed a significant effect of condition for wild-type mice [$F_{(1,44)}$ = 28.111, $p<0.001$], but not Tg2576 mice [$F_{(1,44)}$ = 0.192, $p>0.05$]. Furthermore, a significant effect of genotype was evident after CS/US conditioning [$F_{(1,44)}$ = 27.430, $p<0.001$], but not in the CS Only condition [$F_{(1,44)}$ = 0.006, $p>0.05$]. Thus, Tg2576 mice that underwent CS/US conditioning froze significantly less during the tone presentations than wild-type mice.

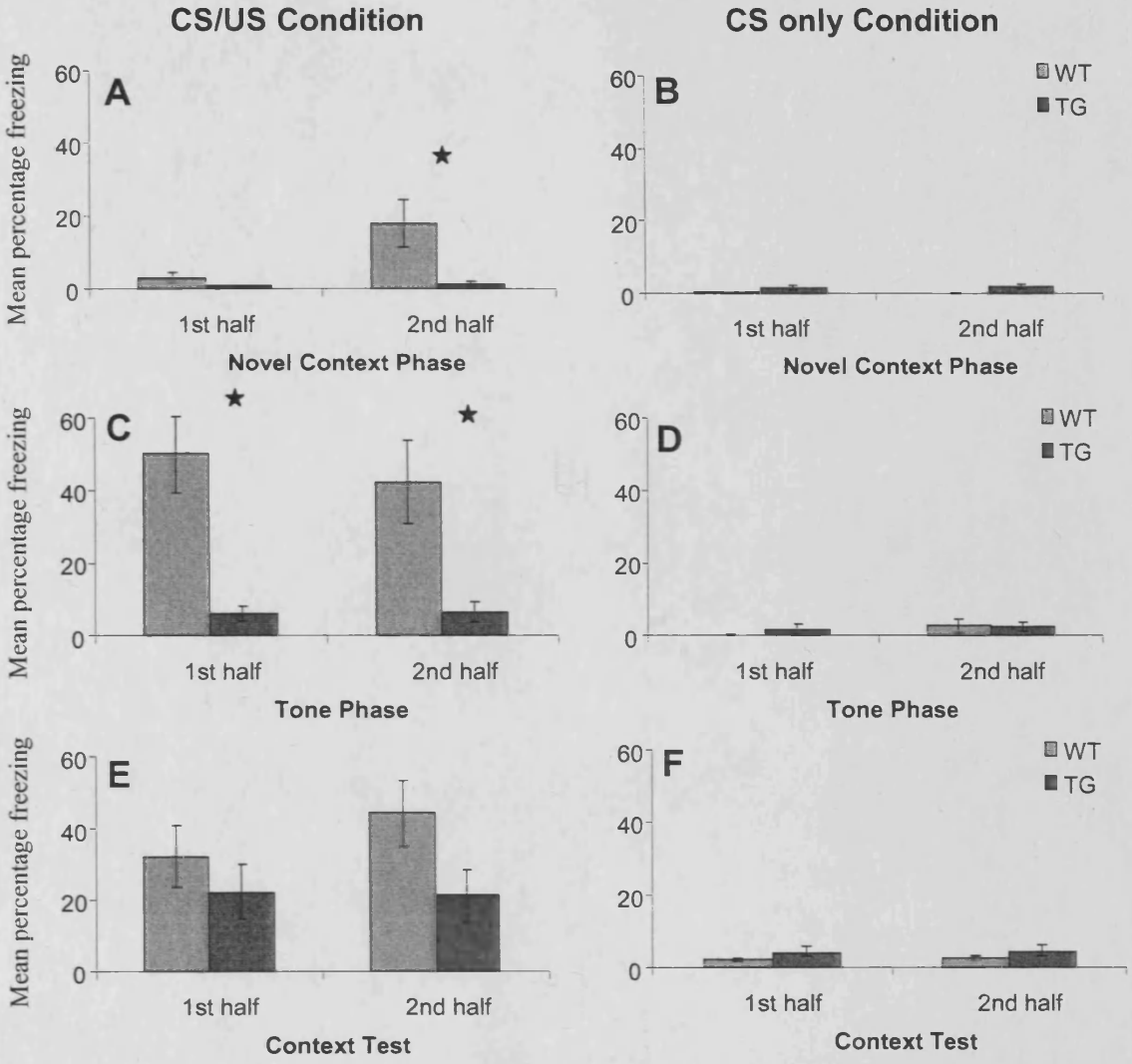


Figure 51: Mean percentage of freezing behaviour revealed during test phases for mice that underwent CS/US conditioning (Left column) and CS Only presentation (Right column). A & B = freezing measures during the novel context phase of the tone test. C & D = freezing measures during the CS presentation phase of the tone test. E & F = freezing measures during the context test. Grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/-S.E.M.

Context Test

Figure 51E & F depicts freezing data from the context test. Statistical analysis revealed non-significant effects of block [$F_{(1,44)}$ = 1.928, $p>0.05$] and genotype [$F_{(1,44)}$ = 1.176, $p>0.05$]. The main effect of condition was significant [$F_{(1,44)}$ = 16.441, $p<0.001$]. All interactions were non-significant [maximum: $F_{(1,44)}$ = 2.515, $p>0.05$]. Thus, no genotypic differences were revealed in the levels of freezing elicited by the context, although significantly more freezing occurred in mice that underwent CS/US conditioning.

Thus, overall, the infrared locomotor activity data and the freezing measures confirm a genotypic difference in behaviour during the presentation of the auditory stimulus during the tone test in CS/US conditioned mice. Furthermore, no differences in behaviour were observed during the context test.

5.3.4 Results: c-Fos Immunocytochemistry

Hippocampus: Subregions

Figure 52 depicts the mean c-Fos counts in each subregion of the hippocampus. To determine whether genotype or condition had an impact on IEG expression, c-Fos counts were analysed following either the Tone or the Context test.

Tone Test

An ANOVA with genotype, condition and region (CA1, CA3, DG) as factors revealed a main effect of genotype [$F_{(1,20)} = 5.629$, $p<0.03$] and a main effect of region [$F_{(2,40)} = 54.647$, $p<0.001$]. The effect of condition and all interactions were non-significant [maximum: $F_{(1,20)} = 2.807$, $p>0.05$]. Thus, higher counts were obtained in the CA1 region overall, but, more interestingly, Tg2576 mice showed a lower levels of c-Fos expression overall in the hippocampus compared to wild-type mice irrespective of condition.

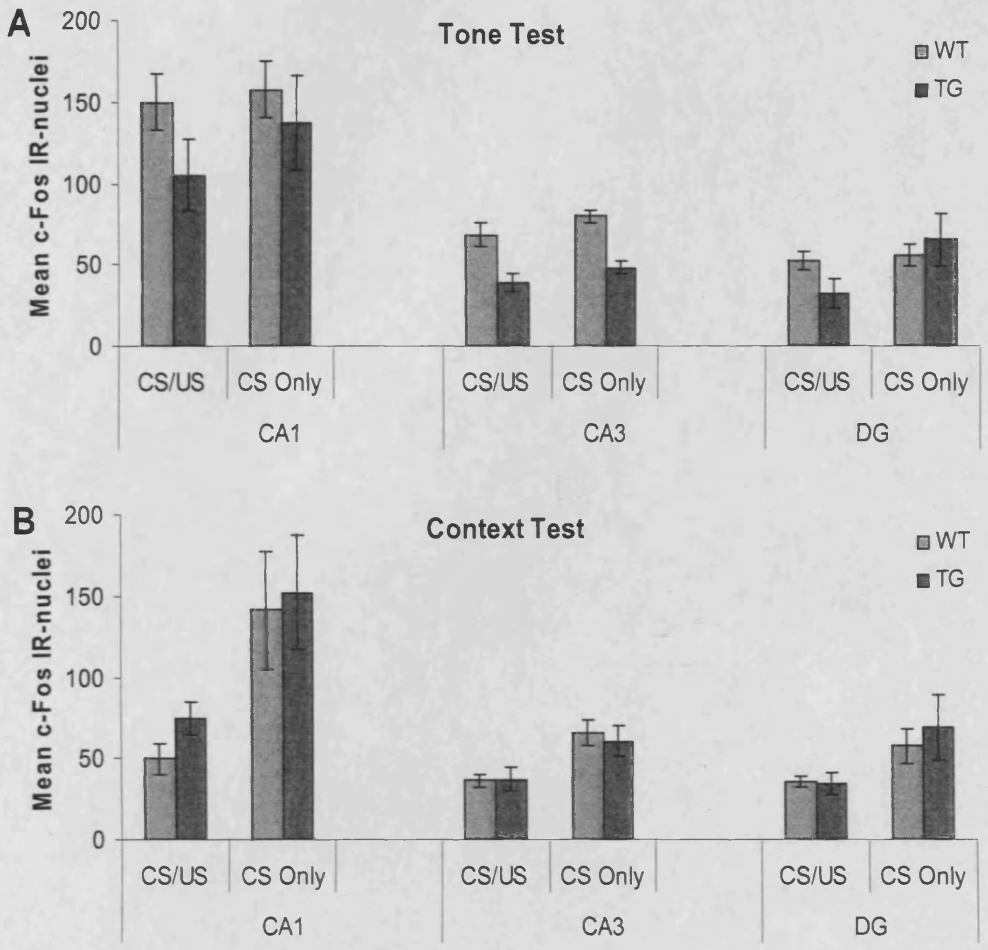


Figure 52: Mean c-Fos immunoreactive nuclei in the CA1, CA3 and dentate gyrus subregions of the hippocampus. Data for the CS/US and CS Only conditions are presented according to c-Fos expression during (A) the Tone and (B) the Context tests. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Context Test

Statistical analysis of the data from the context test revealed a main effect of condition [$F_{(1,20)} = 19.504$, $p<0.001$] and a main effect of region [$F_{(2,40)} = 23.983$, $p<0.001$]. The effect of genotype was non-significant [$F_{(1,20)} = 0.430$, $p>0.05$], while the region by condition interaction was significant [$F_{(2,40)} = 6.400$, $p<0.004$]. All other interactions were non-significant [maximum: $F_{(2,40)} = 0.612$, $p>0.05$].

Investigation of the region by condition interaction revealed a significant effect of condition for the CA1, CA3 and DG regions of the hippocampus [$F_{(1,20)} = 14.022$, 14.033 and 7.266, p's<0.02, respectively]. Furthermore, a significant effect of

region was revealed for the CS Only condition [$F_{(2,19)} = 16.993$, $p<0.001$], but not the CS/US condition [$F_{(2,19)} = 2.272$, $p>0.05$]. Thus, each region of the hippocampus demonstrated a decrease in c-Fos expression during the context test, but, unlike the tone test, no effect of genotype was evident.

Suppression Ratios. To determine how the pattern of c-Fos expression was influenced by the CS/US presentations relative to the CS Only condition, a ratio was calculated using the formula: CS/US c-Fos count/(CS/US c-Fos count plus the average c-Fos count for the CS Only condition) for each brain region. These ratios are shown in Figure 53. An ANOVA was conducted with region and genotype as factors for each test phase.

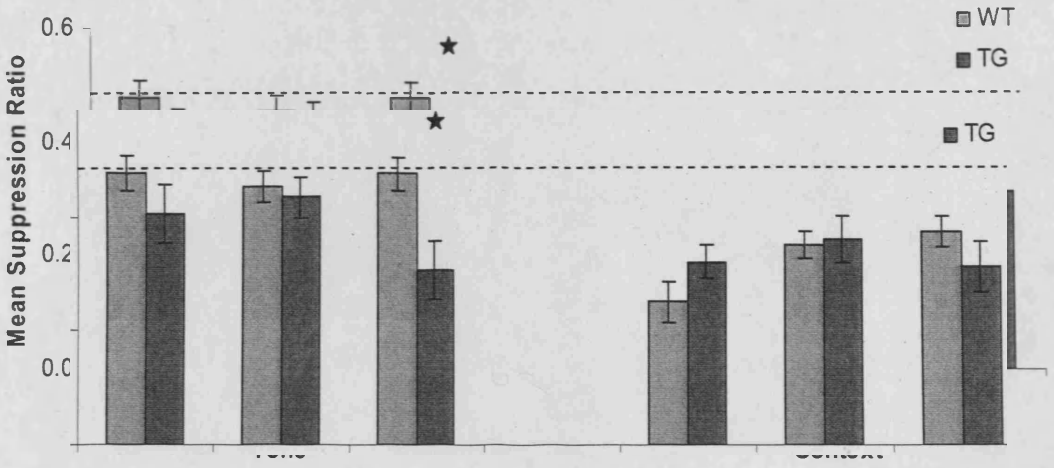


Figure 53: Mean suppression ratios for each hippocampal subregion. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M. * = $p<0.05$.

For the tone test, the main effect of region was non-significant [$F_{(2,24)} = 2.390$, $p>0.05$], the main effect of genotype failed to reach conventional levels of statistical significance [$F_{(1,12)} = 3.667$, $p=0.080$], but the interaction between these factors was significant [$F_{(2,24)} = 3.830$, $p>0.05$]. Tests of simple effects revealed a significant effect of hippocampal region for Tg2576 mice [$F_{(2,11)} = 6.679$, $p<0.02$], but not wild-type mice [$F_{(2,11)} = 0.428$, $p>0.05$]. More interestingly, the effect of genotype was non-significant for the CA1 and CA3 regions [$F_{(1,12)} = 1.338$ and 0.171, p's>0.05, respectively], but it was significant for the DG region [$F_{(2,24)} = 8.246$, $p<0.02$]. Furthermore, for wild-type mice, suppression ratios did not differ

significantly from chance (0.5) for any of the hippocampal subregions [t(6) = -0.722, -1.697 and -0.830, p's>0.05]. For Tg2576 mice, the CA1 and CA3 regions did not differ significantly from chance [t(6) = -1.814 and -1.813, p's>0.05, respectively], but the DG did differ significantly [t(6) = -3.781, p<0.01].

For the context test, the main effect of region was not significant [$F_{(2,24)}$ = 2.895, p=0.075]. The effect of genotype was also non-significant [$F_{(1,12)}$ = 0.051, p>0.05], as was the interaction between these factors [$F_{(2,24)}$ = 2.220, p>0.05]. Furthermore, suppression ratios for all three brain regions differed significantly from chance for both wild-type [t(6) = -6.844, -6.469, -4.804, p's<0.005] and Tg2576 mice [t(6) = -5.860, -3.484 and -4.175, p's<0.02]. Thus, relative to the CS Only condition, Tg2576 mice demonstrated significantly greater c-Fos suppression in the dentate gyrus than wild-type mice during the tone test. In contrast no differences in c-Fos expression were evident following the context extinction test.

In summary, analysis of the raw data revealed that the tone test resulted in an overall lower level of c-Fos expression in the hippocampus of Tg2576 mice that was independent of condition. However, suppression ratios revealed that Tg2576 mice showed a conditioning related reduction in c-Fos expression in the DG region following the tone test. In contrast, wild-type mice showed no such reduction. The context test revealed no gross genotypic differences in hippocampal c-Fos expression, and that each sub-region showed conditioning-related changes in IEG levels.

Hippocampus: *Dorsal vs. Ventral*

Figure 54 depicts the mean c-Fos counts in the dorsal and ventral hippocampus.

Tone Test

An ANOVA with genotype, condition and region (dorsal versus ventral) as factors revealed non-significant main effects of genotype [$F_{(1,20)}$ = 0.068, p>0.05] and region [$F_{(1,20)}$ = 0.585, p>0.05]. The effect of condition was not statistically significant [$F_{(1,20)}$ = 4.010, p=0.059]. All interactions were non-significant

[maximum: $F_{(1,20)}$ = 0.729, p>0.05]. Thus, no difference in IEG expression was revealed between the dorsal and ventral regions of the hippocampus as a result of the tone test.

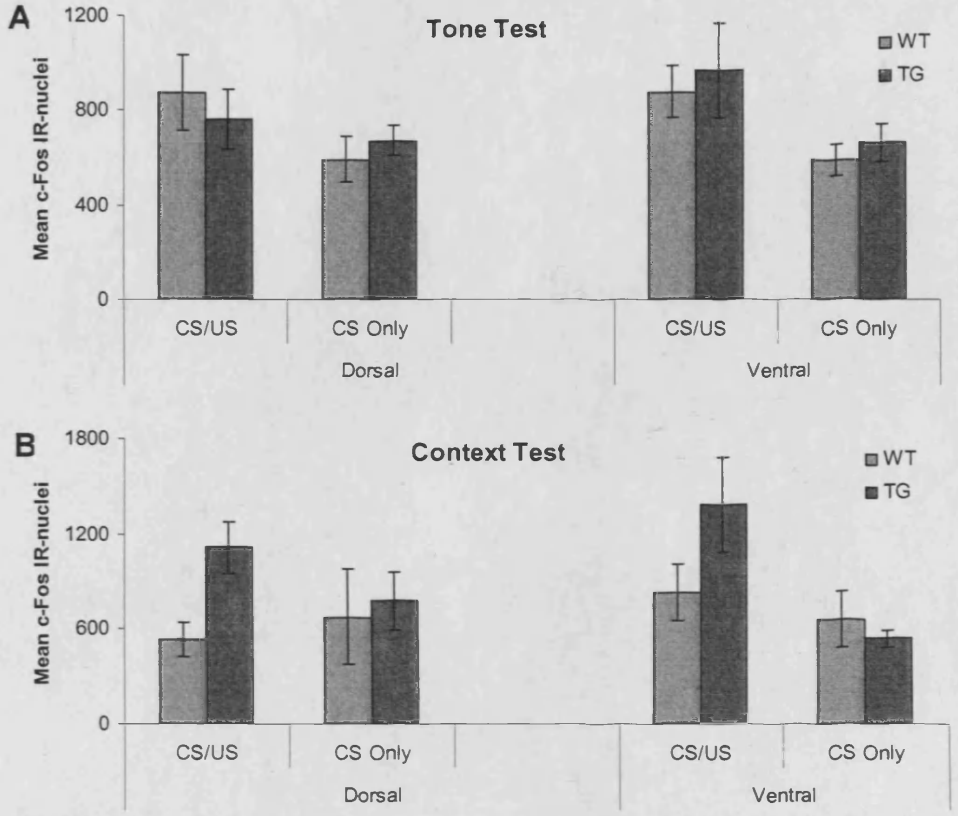


Figure 54: Mean c-Fos immunoreactive nuclei in the dorsal and ventral regions of the hippocampus. Data for the CS/US and CS Only conditions are presented as c-Fos expression during (A) the Tone and (B) the Context tests. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Context Test

Statistical analysis of the data from the context test revealed non-significant effects of condition, genotype and region [$F_{(1,20)}$ = 2.707, 2.335, 0.732, p's>0.05, respectively]. The region by condition interaction was significant [$F_{(1,20)}$ = 4.827, p<0.04]. All other interactions were non-significant [maximum: $F_{(1,20)}$ = 2.707, p>0.05]. Tests of simple effects revealed a significant effect of conditioning for the ventral hippocampus [$F_{(1,20)}$ = 5.238, p<0.04], but not for the dorsal hippocampus [$F_{(1,20)}$ = 0.261, p>0.05]. Furthermore, the effect of region was significant for the CS/US condition [$F_{(1,20)}$ = 5.590, p<0.03], but not in the CS Only condition [$F_{(1,20)}$ = 0.772, p>0.05]. Thus, conditioning related changes in IEG expression were

evident in the ventral hippocampus in both genotypes, although numerically the effect of conditioning appears larger in Tg2576 mice.

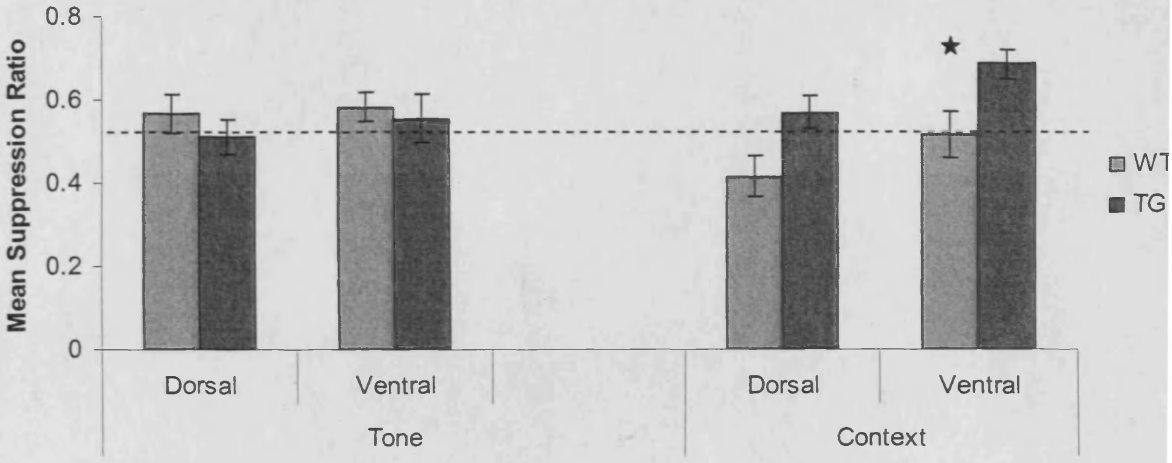


Figure 55: Mean suppression ratios for each dorsal and ventral regions of the hippocampus. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Suppression Ratios. An ANOVA with genotype and region as factors was conducted on the suppression ratio scores from each test phase (see Figure 55). Statistical analysis of the tone test data revealed non-significant main effects of genotype and region, and a non-significant interaction [maximum: $F_{(1,12)} = 1.648$, $p>0.05$]. Furthermore, the suppression ratios for the dorsal hippocampus did not differ significantly from chance for either wild-type or Tg2576 mice [$t(6) = 1.440$ and 0.250, p's>0.05, respectively]. For the ventral hippocampus, the ratio for wild-type and Tg2576 mice also did not differ significantly from chance [$t(6) = 2.391$ and 0.980, p's>0.05].

An analysis of the suppression ration in animals receiving the context test revealed significant main effects of region [$F_{(1,12)} = 33.394$, $p<0.001$] and genotype [$F_{(1,12)} = 6.870$, $p<0.03$], but a non-significant interaction between these factors [$F_{(1,12)} = 0.210$, $p>0.05$]. Furthermore, the suppression ratios for the dorsal hippocampus did not differ significantly from chance for either wild-type or Tg2576 mice [$t(6) = -1.724$ and 1.704, p's>0.05, respectively]. For the ventral hippocampus, the ratio for wild-type mice did not differ significantly from chance [$t(6) = 0.302$, $p>0.05$], but for Tg2576 mice it did differ significantly from chance [$t(6) = 5.230$, $p=0.002$].

In summary, analysis of the raw data revealed an overall increase in c-Fos expression in the ventral hippocampus after the context test in the CS/US condition. Furthermore, the suppression ratio analysis demonstrated that, relative to the CS Only cohort, the CS/US group demonstrated greater IEG expression in the ventral hippocampus after the context test. A more detailed analysis revealed that, Tg2576 mice demonstrated an increase c-Fos activity relative to the control group, whereas this failed to reach significance in wild-type mice after the context test.

Amygdala

Figure 56 shows the mean number of c-Fos immunoreactive nuclei for Tg2576 and wild-type mice in the BLA and CeN subregions of the amygdala for the CS/US and CS Only conditions, after tone or context tests.

Tone Test

Statistical analysis revealed that the main effects of condition, genotype and region were all non-significant [$F_{(1,20)}$ = 2.993, 0.697 and 1.578, p's>0.05, respectively]. The region by genotype interaction was significant [$F_{(1,20)}$ = 10.361, p<0.004]. All other interactions were non-significant [maximum: $F_{(1,20)}$ = 3.392, p>0.05]. Tests of simple effects revealed a significant effect of region for wild-type [$F_{(1,20)}$ = 10.013, p<0.005], but not Tg2576 mice [$F_{(1,20)}$ = 1.926, p>0.05]. Furthermore, results revealed a significant effect of genotype for the BLA [$F_{(1,20)}$ = 4.819, p=0.04], but not the CeN [$F_{(1,20)}$ = 0.556, p>0.05]. Thus, c-Fos expression was significantly lower overall in the BLA for all Tg2576 mice after the tone test.

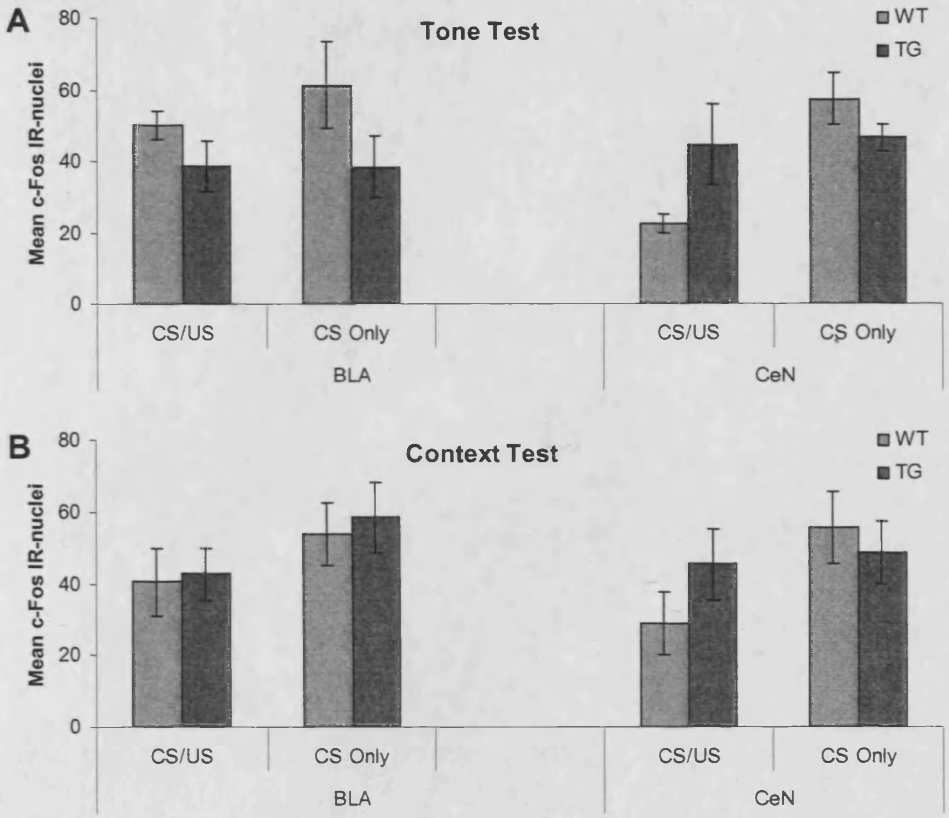


Figure 56: Mean c-Fos immunoreactive nuclei in the BLA and CeN subregions of the amygdala. Raw data for the CS/US and CS Only conditions are presented as c-Fos expression during (A) the Tone and (B) the Context tests. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Context Test

Statistical analysis revealed no main effects of condition, genotype or region [$F_{(1,20)}$ = 2.911, 0.216 and 1.470, p's>0.05, respectively], nor any significant interactions between these factors [maximum: $F_{(1,20)}$ = 3.109, p>0.05].

Suppression Ratios. Statistical analysis of the suppression ratio data from the tone test (Figure 57) revealed a non-significant effect of genotype [$F_{(1,12)}$ = 3.200, p>0.05], but a significant effect of region [$F_{(1,12)}$ = 20.023, p<0.001] and a significant interaction between these factors [$F_{(1,12)}$ = 9.357, p<0.05]. Tests of simple effects revealed a significant effect of region for wild-type [$F_{(1,12)}$ = 28.377, p<0.001], but not Tg2576 mice [$F_{(1,12)}$ = 1.002, p>0.05]. Furthermore, a significant effect of genotype was evident in the CeN [$F_{(1,12)}$ = 6.421, p<0.03], but not the BLA [$F_{(1,12)}$ = 0.341, p>0.05]. One-sample t-tests also revealed significant differences between suppression ratios and 0.5 for wild-type mice in the BLA and

CeN [t(6) = -2.907 and -9.520, p's<0.03, respectively], but not for Tg2576 mice [t(6) = -0.467 and -0.884, p's>0.05, respectively]. Thus, Tg2576 mice demonstrated significantly less c-Fos suppression in the CeN than wild-type mice after the tone test.

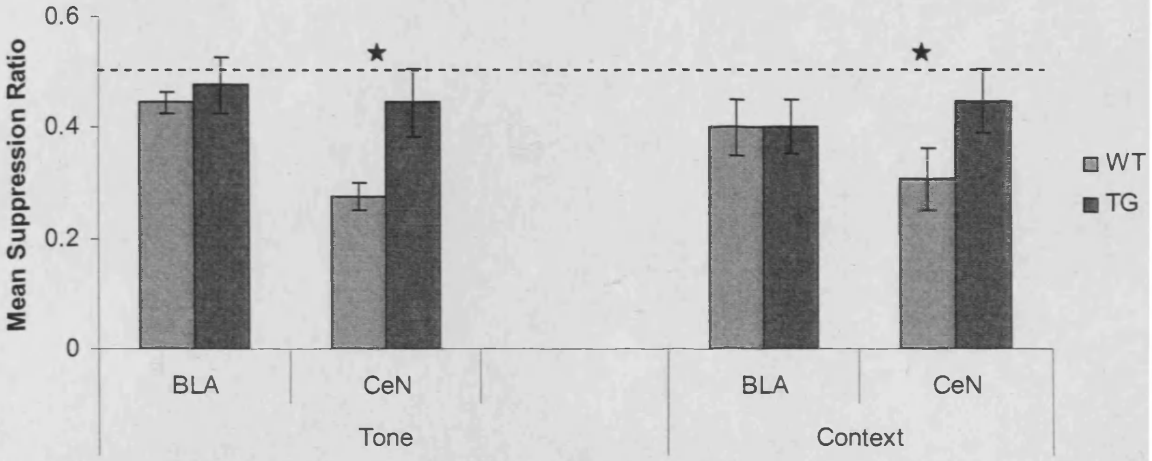


Figure 57: Mean suppression ratios for each subregion of the amygdala. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

For the context test, statistical analysis revealed non-significant main effects of genotype and region [$F_{(1,12)}$ = 0.945 and 1.249, p's>0.05, respectively], but a significant interaction between these factors [$F_{(1,12)}$ = 11.613, p<0.01]. Tests of simple effects revealed a significant effect of region for wild-type mice [$F_{(1,12)}$ = 10.238, p<0.008], but not Tg2576 mice [$F_{(1,12)}$ = 2.623, p>0.05]. The effect of genotype was non-significant for both the BLA and CeN [$F_{(1,12)}$ = 0.0005 and 2.944, p's>0.05, respectively]. Furthermore, suppression ratios did not differ from chance for either wild-type or Tg2576 mice in the BLA [t(6) = -2.002 and -2.059, p's>0.05, respectively]. In the CeN, a significant difference was observed for wild-type [t(6) = -3.418, p<0.02], but not Tg2576 mice [t(6) = -0.873, p>0.05]. Thus, wild-type mice demonstrated differential suppression of IEG expression in the CeN of the amygdala after the context test, a pattern not evident in Tg2576 mice.

In summary, analysis of the raw data revealed an overall decrease in c-Fos expression in the BLA after the tone test in all Tg2576 mice. Furthermore, the

suppression ratios revealed that Tg2576 mice showed less IEG suppression in the CeN than wild-type mice.

Primary Auditory Cortex

Figure 58 shows the mean number of c-Fos immunoreactive nuclei for Tg2576 and wild-type mice in the primary auditory cortex for the CS/US and CS Only conditions after tone or context tests. This region was used as a control region to further ensure baseline levels of c-Fos expression do not differ as a function of transgene, and to confirm that this auditory region functions comparably in both genotypes.

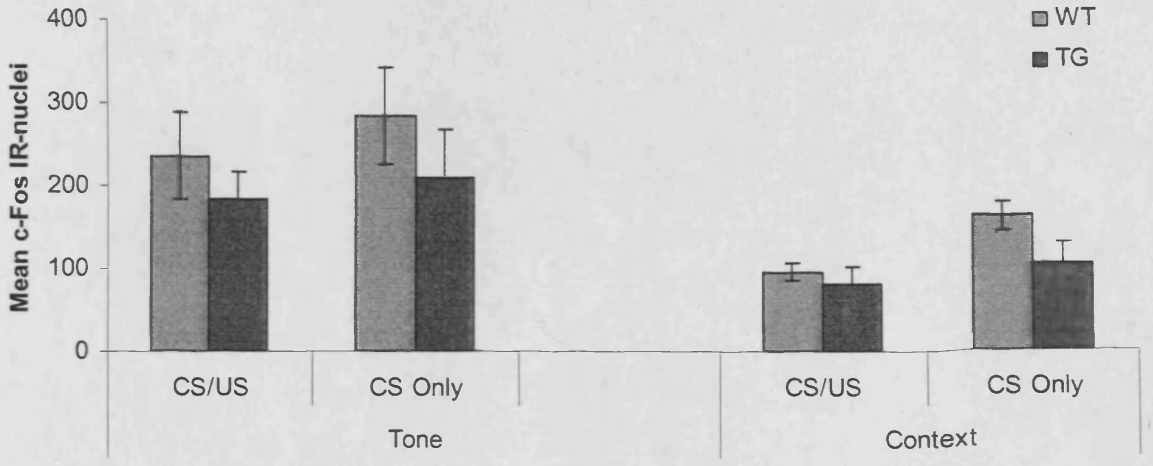


Figure 58: Mean number of c-Fos immunoreactive nuclei in the primary auditory cortex. Raw data for the CS/US and CS Only conditions are presented as c-Fos expression during the Tone and Context tests. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/- S.E.M.

Tone Test

Statistical analysis revealed non-significant main effects of genotype or condition [$F_{(1,20)} = 1.604$ and 0.560, p's>0.05, respectively], nor an interaction between these factors [$F_{(1,20)} = 0.050$, p>0.05].

Context Test

Statistical analysis revealed a non-significant main effects of genotype [$F_{(1,20)}$ = 3.535, $p>0.05$], a significant effect of condition [$F_{(1,20)}$ = 5.475, $p<0.03$], and a non-significant interaction between these factors [$F_{(1,20)}$ = 1.349, $p>0.05$].

Tone versus Context Test

It was expected that greater c-Fos expression would be evident in the primary auditory context after exposure to the tone stimulus. Equally, fewer IR-nuclei were expected after the context test. Thus, an ANOVA was conducted with Test type as a factor to evaluate activity in this control region. Results revealed a significant effect of test [$F_{(1,40)}$ = 19.226, $p<0.001$]. No other main effect or interaction reached significance [maximum: $F_{(1,40)}$ = 3.433, $p>0.05$]. Thus, c-Fos was significantly increased in the auditory cortex after exposure to the tone stimulus.

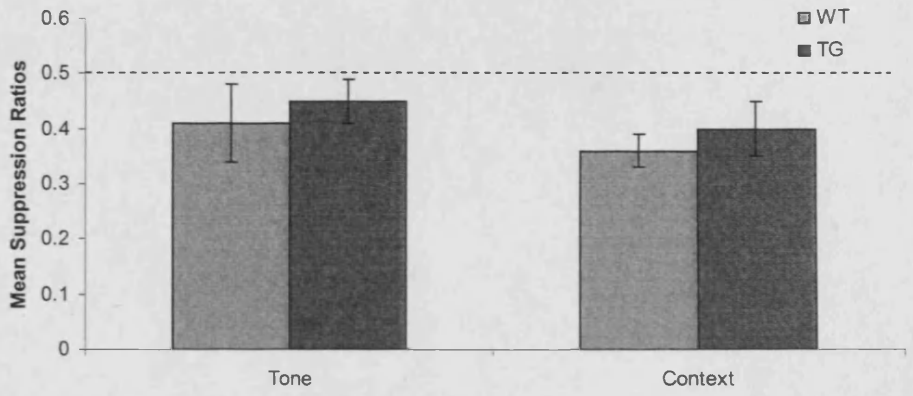


Figure 59: Mean suppression ratios for the primary auditory cortex. Light grey bars = wild-type mice; dark grey bars = transgenic Tg2576 mice. Error bars = +/-S.E.M.

Suppression Ratios. An ANOVA, with genotype as a factor, was conducted on the suppression ratios for each test (Figure 59). For the tone test, results revealed a non-significant effect of genotype [$F_{(1,13)}$ = 0.202, $p>0.05$]. Similarly, the effect of genotype was non-significant for the context test data [$F_{(1,13)}$ = 0.434, $p>0.05$]. Furthermore, suppression ratios did not differ from chance in the tone test cohort for either wild-type or Tg2576 mice [t(6) = -1.322 and -1.327, p's>0.05,

respectively]. After the context test, suppression ratios differed significantly from 0.5 for wild-type [$t(6) = -5.111$, $p<0.005$], but not Tg2576 mice [$t(6) = -1.766$, $p>0.05$]. Thus, overall, activation of the auditory cortex was substantially increased after exposure to the tone stimulus.

5.3.5 Discussion

The aim of this experiment was to evaluate the impact of the APPswe mutation on two forms of memory representation and to use IEG expression as a means of identifying the patterns of neural activation in normal and mutant mice.

From a behaviour perspective, the profile of impairments revealed in this experiment was similar to those obtained in Experiments 3 and 8. Tg2576 mice consistently demonstrated a post-shock freezing deficit. Thus, this pattern of behaviour, which was originally reported in Good & Barnes (2005), has been replicated three more times, confirming the robustness of the behavioural impairment across sexes and a narrow range of ages (17-19 months).

Unlike the previous replications, in the present experiment Tg2576 mice demonstrated increased activity and decreased freezing during the tone presentations. It is possible that this may reflect an overall difference in activity levels between the genotypes, although this is unlikely given that no differences in locomotor activity or freezing were evident in the CS Only condition. Nor is it likely to reflect a sex difference since the present experiment and Experiment 3 used female mice, while Experiment 8 used male mice. Most importantly, however, this is unlikely to be a reflection of the mutant animal's ability to perceive the auditory stimulus since a conditioned suppression of locomotor activity and conditioned increase in freezing were still evident in Tg2576 mice with successive tone presentations. Thus, one explanation for this difference might be an impairment in the relative strength of the association between the tone CS and the sensory-specific motivational qualities of the US. A weaker association may encourage less pronounced freezing behaviour in transgenic mice.

The results of the tone and context test closely parallel those seen in Experiment 3 and Barnes & Good (2005). That is, an impairment in freezing and locomotor activity were evident during the CS presentation of the tone test, but not during the context test. The main aim of this experiment was to evaluate the pattern of neural activation in mutant and control mice following the tone and context tests.

After the tone test, an overall reduction in c-Fos expression was observed in the hippocampus of Tg2576 mice, as compared to wild-type controls. Interestingly, however, compared to the CS Only condition, suppression ratios revealed that Tg2576 mice showed a comparable level of IEG expression in each subregion, except the dentate gyrus, a pattern not observed in wild-type mice. Thus, unlike wild-type mice, the Tg2576 mice showed a reduction in c-Fos expression in the dentate gyrus following the tone test. In addition, Tg2576 mice demonstrated an overall decrease in c-Fos expression in the BLA and less suppression, relative to the CS Only condition, in the CeN than wild-type mice.

In the context test, conditioning related changes were evident in the ventral hippocampus and hippocampal subregions. However, Tg2576 mice demonstrated less c-Fos suppression overall in the hippocampus than wild-type mice after the context test. Interestingly, the majority of conditioning-related changes in IEG expression after the context test were evident in the hippocampus, and most changes in the amygdala were seen after the tone test. The implications of this pattern of IEG expression will be discussed further in the chapter discussion.

5.4 Structural Equation Modelling of Neural Activation

5.4.1 Introduction

By generating data reflecting changes in c-Fos protein expression during conditioning or memory retrieval, it is possible not only to analyse genotypic alterations in IEG expression levels in any given brain region, but also to use these data to develop and compare theoretical models of neural activity within and between networks. The use of structural equation modelling (SEM) allows both the examination of individual relationships between anatomically connected regions and the evaluation of the network dynamic as a whole (McIntosh & Gonzalez-Lima, 1991; Kelloway, 1998). Hence, the analysis of anatomically-valid and theoretically-driven models allows an interpretation of the relationship between two or more brain regions to be evaluated in the context of a greater dynamic neural network. One caveat to note, however, is that although SEM provides an interesting method to propose interactions between brain regions, by virtue of being a mathematical modelling technique it can only be used to speculate about patterns of neural activation and cannot be considered a definitive delineation of neural processing. Nevertheless, the information gained from such an analysis may inform theory regarding the nature of brain system changes in APPswe mice.

Using the c-Fos data presented above, the aim is to generate theoretical models to assess the relationship between subregions of the hippocampus and the amygdala. SEM has been applied to neuroscientific data previously to propose models of activation after specific activities have been performed or memories activated. Jenkins et al. (2003), for example, applied SEM to c-Fos data to reveal different patterns of hippocampal activity after two spatial memory tasks in rats. Furthermore, SEM is often applied to MRI imaging data to infer functional interactions between brain regions (e.g. Caclin & Fonlupt, 2006; Rowe et al., 2005). Thus, the selection of regions used in the present experiment is based upon known anatomical connectivity patterns (Pitkänen et al., 2000).

The aim of this analysis was to test anatomically plausible models of neural activation following fear conditioning and memory retrieval based on c-Fos expression in the hippocampus and amygdala. One goal of this computational modelling technique was to ascertain whether genotypic differences might emerge in either the broad pattern of network dynamics, or within particular regions of connectivity. Although it is expected that different models of neural activation will be appropriate for each stage of fear conditioning or testing, how the APPswe mutation influences these models has not been investigated. It is hypothesised that wild-type and Tg2576 mice will demonstrate different patterns of activation after CS/US conditioning (Experiment 8) and after the tone test (Experiment 9), but that neural models may be comparable between the genotypes following the context retrieval test.

5.4.2 Method

Path analyses of the neural network models were conducted using the statistical software Amos 6.0 (SPSS, Chicago). With SEM, the number of regions included in each model is statistically limited by the number of animals in each condition and, in turn, by the restriction imposed by the degrees of freedom (Kelloway, 1998). In the present study, computational path analyses were based on the maximum likelihood estimation (MLE), the most widely used and most consistent method of statistical estimation (Kelloway, 1998). The MLE estimates all parameters in a model simultaneously. One consequence of this method is that if one aspect of the model is poorly specified, this error will be reflected in all estimated parameters (Kelloway, 1998), making it an appropriately conservative method of estimation.

In order to determine how well an anatomically- or theoretically-derived model fits the data, a number of fit indices are considered. Firstly, the χ^2 value is used to assess the discrepancy between the matrix of covariances derived from elements in the original sample data and the covariance matrix derived by the model specifications. Hence, a non-significant result provides an indication that the null hypothesis, the prediction that there is no difference between the covariance matrices derived from the sample data and the model specifications, is correct. A

limitation of this method of estimation, beyond providing only a dichotomous accept or reject decision, is its reliance upon sample size. Since the power of the test increases with increased sample size, a study with few *n* is likely to be more lenient and may fail to detect subtle differences between the covariance matrices derived from the sample data and the specified model (Fan, Wang & Thompson, 1996). Hence, along with the χ^2 value, three other fit indices are commonly used to determine the goodness of fit of a given model.

The three additional fit indices used to assess the suitability of the model are the goodness-of-fit index (GFI), the comparative fit index (CFI) and the root mean square error of approximation (RMSEA). These indices are particularly suitable to cases in which there is a small *n*, as in the present study. The GFI and CFI indices have a range between 0 and 1, where 0 indicates a total lack of fit and 1 indicates a perfect fit. The GFI is based on the discrepancies between the implied covariances obtained from the model and the observed covariances obtained from the data. Hence, it takes into account the proportion of variance accounted for by the model (Blalock & Blalock, 1985). Furthermore, the GFI, and to a lesser extent the CFI, tends to show a downward bias with smaller sample sizes, which makes it a fairly conservative fit index (Fan, Wang, and Thompson, 1996). The CFI measures the extent to which the null hypothesis is false by taking into account the comparative fit of models ranging from unrestricted to saturated. It has been recommended to use the CFI index with small sample sizes (Fan, Wang and Thompson, 1996; Hu and Bentler, 1998). The RMSEA is used to determine how well a given model approximates the true model by calculating the error of approximation between the models. It is based on an analysis of the residuals, rather than path strength, with smaller values indicating a better fit. In this case, the approximation is considered to be very good when the RMSEA value is less than 0.05, and it is considered to be outstanding at values less than 0.01 (Steiger, 1990).

In sum, a model can be considered a good fit and, in essence, to represent a plausible network of neural activation, when the GFI = 0.9, the CFI = 0.90 – 0.95, $\chi^2 > 0.05$ and the RMSEA = 0.05 (Tabachnik & Fidell, 1996). Although both the GFI and CFI were chosen to take into account the effect of the small sample size on the goodness of fit of the model, it should be mentioned that the estimates of

path strengths between brain regions remain accurate even with small sample sizes (Boucard, Marchand & Noguès, 2007).

Initially, a baseline model, based on anatomical connections was tested. It was primarily based on the known projections connecting the subregions of the amygdala with either the subregions of the hippocampus, or the broader dorsal/ventral regions of the hippocampus. After the initial estimate of fit, the model was adjusted according to the path strengths to determine the best possible fit, while always remaining anatomically viable. Computational analyses were performed for each theoretical model using data from Experiment 8 (fear training) and Experiment 9 (retrieval of fear memory during context and tone tests). Data were used from the CS/US conditioned groups only since the small sizes of the control groups made these data inappropriate for SEM. In each circumstance, the best fit model was initially determined for the wild-type control group and, if this model failed to fit the data from the Tg2576 animals, a separate model of network dynamics was considered for the transgenic mice. Where possible, each best-fit model was compared, using multi-group analysis, to both genotypes. This allowed an overall comparison of the fit of the model across the genotypes. This analysis was based on the χ^2 difference test which, because it is highly dependent on sample size, is not sensitive to subtle group differences in sample sizes as small as those used here. Hence, any genotypic differences revealed by the χ^2 test can be assumed to be robust. Furthermore, the multiple-group analysis approach allowed the comparison of individual path strengths between the genotypes. This made it possible to reveal significant transgene-dependent changes in the strength of distinct neural pathways.

5.4.3 Results: Fear Conditioning data

Table 14 depicts the Pearson's product-moment coefficients for wild-type (standard print) and transgenic Tg2576 (bold print) data from the fear conditioning task (Experiment 8). In contrast to wild-type data, numerous correlations, significant to either the 0.05 or 0.01 level, can be observed between brain regions in Tg2576 mice. Particular correlations are evident between the CA1 and CA3 regions of the hippocampus and the BLA and CeN regions of the amygdala in

Tg2576 mice. Fewer correlations are observed between brain regions in wild-type mice. With increased correlation between brain regions comes increased power for structural equation modeling to test a model. This increased power allows for statistical rejection of the null hypothesis, which predicts that there will be no difference between the covariance matrices derived from the sample data and those derived from the model specifications. High correlations may also be indicative of multicollinearity between brain regions, which, in itself, has no bearing on the predictive value of a model.

It is interesting to note, however, that for both wild-type and Tg2576 mice, significant correlations can be seen between locomotor activity and freezing behaviour, further supporting the robust nature of these variables as behavioural indices.

	Measure	DG	CA1	CA3	BLA	CeN	dHP	vHP	Locomotor	Freezing
DG	r p		.246 .418	-.050 .872	.587(*) .035	.669(*) .012	-.058 .851	.135 .661	-.097 .752	-.051 .869
CA1	r p	**.311** **.382**		.339 .257	.410 .164	.543 .055	-.078 .800	-.300 .320	-.358 .230	.497 .084
CA3	r p	**.566** **.088**	**.751(*)** **.012**		.188 .538	.388 .190	.066 .830	.090 .770	.146 .635	-.162 .538
BLA	r p	**.240** **.505**	**.767(**)** **.010**	**.834(**)** **.003**		.592(*) .033	-.410 .165	-.041 .894	-.162 .598	.198 .516
CeN	r p	**.546** **.102**	**.664(*)** **.036**	**.899(**)** **.000**	**.690(*)** **.027**		-.252 .407	.070 .820	-.363 .222	.246 .418
dHP	r p	**.634(*)** **.049**	**.690(*)** **.027**	**.848(**)** **.002**	**.548** **.101**	**.848(**)** **.002**		.690(**) .009	.481 .096	-.278 .358
vHP	r p	**.277** **.438**	**.193** **.594**	**.070** **.849**	**.064** **.860**	**.176** **.628**	**.451** **.191**		.295 .327	-.135 .661
Locomotor	r p	**.506** **.136**	**.180** **.619**	**.320** **.368**	**.229** **.525**	**.452** **.190**	**.508** **.134**	**.629** **.051**		-.876(** .000
Freezing	r p	**-.569** **.086**	**-.475** **.166**	**-.566** **.088**	**-.536** **.110**	**-.549** **.101**	**-.457** **.185**	**-.272** **.447**	**-.732(*)** **.016**	

Table 14: Pearson's Correlations. Standard print = Pearson's product-moment coefficient for wild-type data, n=13. Bold print/Shaded = Pearson's product-moment coefficient for transgenic Tg2576 data, n=10. r = Pearson's product-moment coefficient; p = probability. (*)Correlation is significant at the 0.05 level (2-tailed). (**) Correlation is significant at the 0.01 level (2-tailed).

SEM of wild-type fear conditioning data

Figure 60A presents an optimal theoretical model of neural activation in wild-type animals based solely on c-Fos expression data from the CS/US training condition. Figure 60B tests the same model with data from Tg2576 mice. Along with estimates of path strength between each brain region, the squared multiple correlation (R^2) is also depicted for all dependent brain regions in the model. This value represents the percent variance that can be explained in that variable or brain region. The four fit indices suggest that the activation pattern depicted in this system represents a very good model of neural activity for wild-type mice during CS/US conditioning. This model reveals correlations between the CA1 and CA3 subregions of the hippocampus and BLA and CeN subregions of the amygdala, with the CA1 influencing the amygdala regions and BLA projecting outputs to the CA3 region and receiving inputs from the CeN. Interestingly, during the development of this model, it became apparent that the dentate gyrus – CA3 connection was not directly involved in this system of neural activation. When this model was applied to Tg2576 data, however, the fit was less than optimal, suggesting a moderating effect of the manipulation on the pattern of neural activity.

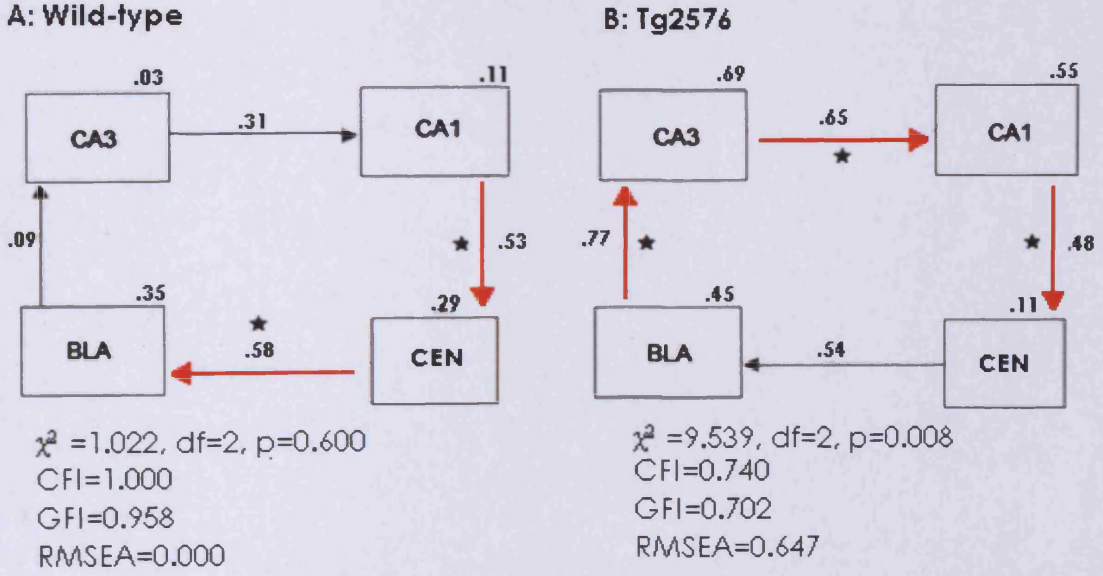


Figure 60: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during CS/US conditioning. Optimal model for wild-type data. Red arrows and * = $p<0.05$.

Although Figure 60A represents an optimal model for wild-type mice during conditioning, it is worth noting that the two path strengths that reach statistical significance are the connections between the CeN and both the CA1 and BLA regions. Interestingly, it is the CeN region that is thought to be the output region for behavioural responses, such as freezing (Davis, 1992). The other path coefficients did not reach significance. This is often an indication that the overall level of cohesion between the variables is high, and a lot of the shared variance is explained, which results in minimal remaining contributions from individual paths. So, the model will represent a good fit because overall much of the shared variance is accounted for, but each path contributes only minimally in terms of its direct effect. This could potentially be due to the greater influence of other anatomically related regions that are not present in this model, or even to a greater psychological construct that has greater influence over the pattern of activation in these regions.

The use of multiple group analysis allows the comparison of path coefficients between the genotypes, and can be an indication of transgene-dependent changes in effective connectivity between brain regions. Stacking the two models depicted in Figure 60 revealed a significant transgene-dependent difference between the overall structural weights of the models (χ^2 = 10.668, df = 4, p = 0.030). Furthermore, it also revealed a significant genotypic difference between the weight of the BLA – CA3 path (χ^2 = 8.076, df = 2, p = 0.018). This might suggest that this particular connection is dysfunctional in the Tg2576 model. Furthermore, as noted above, this is likely to be a robust statistical difference since this statistical measure is known to be relatively insensitive to group differences with the number of subjects used in the present experiment.

SEM of Tg2576 fear conditioning data

Given the poor fit of the above model with data from Tg2576 animals, it became of interest to determine the optimal model of neural activation for animals overexpressing the APPswe mutation. Figure 61B depicts the optimal model determined for Tg2576 mice during CS/US conditioning, while Figure 61A shows this model applied to wild-type data.

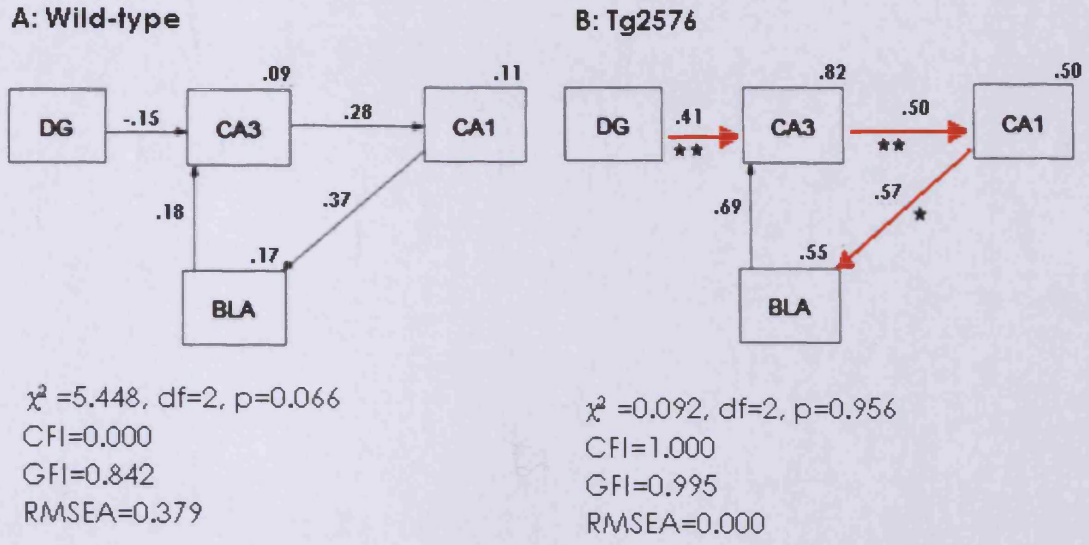


Figure 61: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during CS/US conditioning. Optimal model for Tg2576 data. Red arrows and * = p<0.05, ** = p<0.01.

The four fit indices suggest that this model is a very good fit with the data for Tg2576 mice. Furthermore, significant path coefficients are evident for the DG to CA3, CA3 to CA1 and CA1 to BLA paths. This suggests that the effective connectivity, the directional influence of one region on the next, is accounted for by this model. When applied to the wild-type data, however, the model is a poor fit, as evidenced particularly by the χ^2 value and the CFI of 0. When these models were stacked, the results revealed a significant difference in model fit between the genotypes (χ^2 = 12.534, df = 4, p = 0.014), although there were no significant changes in individual path coefficients. Interestingly, however, the only path that tended towards significance was the BLA – CA3 path (χ^2 = 4.910, df = 2, p = 0.086). Given the insensitivity of the χ^2 value to group differences with small cohorts, and evidence of a difference in this path in the model presented in Figure 60, along with a trend towards significance in the current model, it is suggested that this amygdala – hippocampal connection may be a particular site of amyloid-pathology driven dysfunction. The important role of each region is evidenced by findings that the CA3 region has been shown to be vital for the immediate formation of a unified memory representation after only one exposure to fear

conditioning (Cravens et al., 2006) and the BLA is necessary for the acquisition of conditioned fear (Maren et al., 1996).

It is interesting to see that similar patterns of neural activation are evident for both genotypes. The influence of the CA3 – CA1 – BLA system is consistent, suggesting a central role for this processing pathway in the formation of conditioned fear. However, modelling of neural activity in wild-type mice suggests a central role for the CeN in this system. In contrast, modelling of the Tg2576 c-Fos activity highlighted an important influence of DG. Thus in the transgenic mice, the DG appears to strongly influence activation in the CA3, while the CeN becomes dissociated from the model. When the DG was included in the optimal wild-type model, the fit of the model was considerably reduced. The same held true for inclusion of the CeN in the optimal Tg2576 model.

The CeN has been shown to be vital to fear conditioning. Lesions of the CeN result in profound deficits in both the acquisition and expression of conditional fear (Iwata et al., 1986; Young & Leaton, 1996). Furthermore, pharmacological interventions suggest that this is due to impairment in the performance of conditioned fear responses, rather than an associative deficit (Fanselow & Kim, 1994; Goosens et al., 2000). It is possible, therefore, that dissociation of the CeN from the amygdala – hippocampal system in Tg2576 mice during fear conditioning could result in behavioural deficits both during conditioning and when the fear memory is retrieved. Furthermore, the additional influence of the DG, an area known to be highly compromised by APPswe expression, on the hippocampal system could impact upon memory formation. Hence, although core regions appear to interact in a similar manner in wild-type and Tg2576 mice, the SEM analysis suggests that the mutation leads to irregular association and dissociation of vital components of the neural network during the acquisition of conditioned fear.

5.4.4 Results: Fear memory retrieval: Context Test

Table 15 depicts the Pearson's product-moment coefficient for wild-type (standard print) and transgenic Tg2576 (bold print) data for the Context Test. Although the

pattern of collinearity is not striking for each genotype, a few subregional correlations are evident and the correlation between freezing behaviour and locomotor activity approaches significance for the wild-type cohort. The fact that IEG expression in any given region does not correlate as frequently with other regions as it did during the conditioning phase suggests that the power may be sub-optimal for structural equation modeling. Indeed, power analysis revealed the need for fifty-two animals per genotype to achieve a power level of 0.8 when the effect size was relatively small.

	Measure	DG	CA1	CA3	BLA	CeN	dHP	vHP	Locomotor	Freezing
DG	r		.332	.374	.538	.303	.189	.293	.083	-.284
	p		.467	.409	.213	.509	.684	.523	.860	.536
CA1	r	**-.386**		.235	.170	.138	.354	.272	-.529	-.070
	p	**.393**		.613	.715	.767	.436	.555	.222	.881
CA3	r	**-.063**	**.769(*)**		.714	.765(*)	.090	.361	-.140	.339
	p	**.894**	**.043**		.071	.045	.848	.427	.764	.457
BLA	r	**.199**	**.058**	**.170**		.951(**)	.439	.746	-.084	.163
	p	**.669**	**.901**	**.715**		.001	.325	.054	.857	.727
CeN	r	**.258**	**.592**	**.851(*)**	**.583**		.371	.704	-.183	.369
	p	**.571**	**.161**	**.015**	**.170**		.413	.078	.695	.415
dHP	r	**-.897**	**.102**	**.017**	**-.251**	**-.328**		.916(**)	-.700	.343
	p	**.006**	**.828**	**.972**	**.586**	**.472**		.004	.080	.451
vHP	r	**-.271**	**.252**	**.532**	**.282**	**.426**	**.495**		-.574	.403
	p	**.556**	**.586**	**.219**	**.540**	**.341**	**.259**		.178	.370
Locomotor	r	**.090**	**.070**	**.472**	**.538**	**.490**	**.100**	**.709**		-.718
	p	**.848**	**.881**	**.285**	**.213**	**.264**	**.830**	**.075**		.069
Freezing	r	**-.464**	**-.183**	**-.294**	**-.436**	**-.425**	**.494**	**-.177**	**-.658**	
	p	**.294**	**.695**	**.522**	**.328**	**.342**	**.260**	**.703**	**.108**	

Table 15: Pearson's Correlations for Context Test. Standard print = Pearson's product-moment coefficient for wild-type data, n=7. Bold print/Shaded = Pearson's product-moment coefficient for transgenic Tg2576 data, n=7. r = Pearson's product-moment coefficient; p = probability. (*)Correlation is significant at the 0.05 level (2-tailed). (**) Correlation is significant at the 0.01 level (2-tailed).

Figure 62A presents an optimal theoretical model of neural activation in wild-type animals based on c-Fos expression data from the cohort that underwent Context testing. Figure 62B tests the same model with data from Tg2576 mice. The four fit indices suggest that the flow of information depicted in this system represents a very good model of neural activation for wild-type mice during exposure to the

contextual stimuli. In this model, the BLA communicates with the hippocampal subregions and the flow proceeds from the BLA to the CeN. Interestingly, and similarly to the CS/US conditioned cohort, it became apparent that the dentate gyrus – CA3 connection was not directly involved in this system of neural activation. When this model was applied to Tg2576 data the fit was not appropriate (Figure 62B).

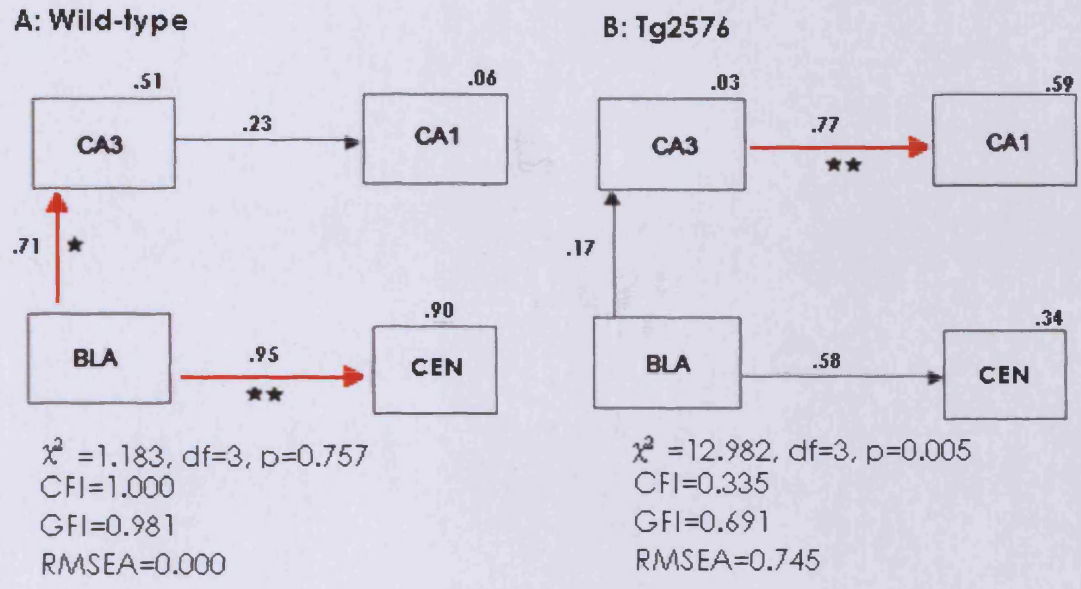


Figure 62: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during Context Test in CS/US conditioned cohort. Optimal model for wild-type data. Red arrows and * = $p<0.05$, ** = $p<0.01$.

A better fitting model of neural activation was subsequently developed for the Tg2576 data (see Figure 63B). It is interesting to note that, as with the CS/US conditioned cohort, the pattern of activation from the BLA to the CA1 region, via the CA3 region, is consistent between the two optimal models. Thus, this neural pathway has been consistent across four cohorts of animals, during both conditioning and memory retrieval. However, in comparison to the wild-type model, the optimal model for Tg2576 mice shows involvement of the dentate gyrus, and de-coupling of the CeN. Strikingly, the optimal model for Tg2576 mice during contextual memory retrieval is identical to that developed for the CS/US conditioned cohort of Tg2576 mice.

This finding suggests that the APPswe mutation may impact on the pattern of neural activity observed in transgenic mice, particularly in the dentate gyrus, in a similar and consistent manner during both conditioning and memory retrieval. Furthermore, not only is there consistency in the neural patterns of Tg2576 mice undergoing different aspects of fear conditioning, there is also a similar alteration in neural activation between the genotypes during conditioning and memory retrieval. That is, the dentate gyrus and CeN become similarly coupled and de-coupled between the genotypes in each condition.

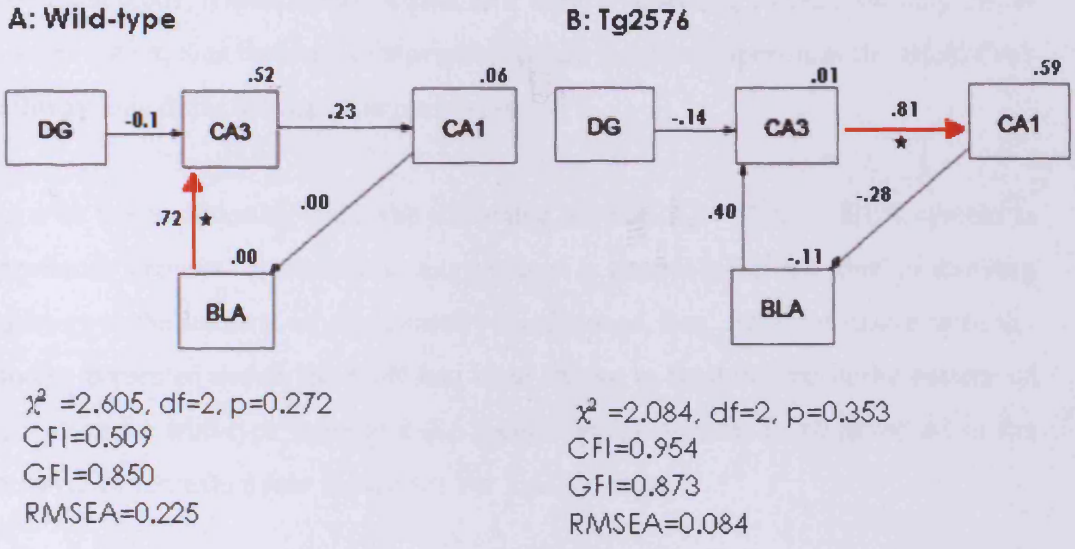


Figure 63: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during the Context Test in CS/US conditioned cohorts. Optimal model for Tg2576 data. Red arrows and * = $p<0.05$, ** = $p<0.01$.

The use of multiple group analysis allows the comparison of path coefficients between the genotypes, and can be an indication of transgene-dependent changes in effective connectivity between brain regions. Stacking the two models depicted in Figure 62A (optimal for wild-type data) revealed a significant transgene-dependent difference between the overall structural weights of the models (χ^2 = 14.165, df = 6, $p<0.03$). Furthermore, it also revealed that the difference in the weight of the BLA – CA3 and BLA-CeN paths approached significance (p's = 0.059 and 0.053, respectively). This might suggest that this particular connection is disrupted in the Tg2576 model. As noted above, this statistical measure is relatively insensitive to group differences with as few subjects as used in the present experiment, which suggests that a larger *n* may have revealed significant

differences in these path strengths between the genotypes. Nevertheless, one must be cautious in accepting such interpretations without further validation.

Multiple group analysis was also performed on the optimal Tg2576 model and corresponding wild-type model. Stacking the models revealed a non-significant overall difference between the fit of the models (χ^2 = 4.689, df = 4, p>0.05). Comparison of the individual path strengths revealed a trend towards a significant transgene-dependent difference between the DG-CA3 and BLA-CA3 paths (p's = 0.060 and 0.061, respectively). Again, if it were a reflection of the relatively low *n* in each cohort, then these results might provide further support that the BLA-CA3 pathway may differ between the genotypes.

As with the conditioning data, the influence of the CA3 – CA1 – BLA system is consistent between the models, suggesting a central role for this processing pathway in the retrieval of contextually conditioned fear. Also consistent with the models presented above, the CeN has been shown to be involved in the pattern of activation for wild-type mice and the dentate gyrus appears to be involved in the retrieval of contextual fear memories for Tg2576 mice.

5.4.5 Results: Fear memory retrieval: Tone Test

Table 16 depicts the Pearson's product-moment coefficient for wild-type (standard print) and transgenic Tg2576 (bold print) data for the Tone Test. Although the pattern of collinearity is not extensive for either genotype, a limited set of subregional correlations are evident for each genotype and the correlation between freezing behaviour and locomotor activity is significance for the Tg2576 cohort.

	Measure	DG	CA1	CA3	BLA	CeN	dHP	vHP	Locomotor	Freezing
DG	r		.700	.384	.023	-.406	.368	.139	-.399	-.367
	p		.080	.395	.962	.366	.417	.767	.375	.418
CA1	r	**.214**		.883(**)	.192	.035	.208	.340	.201	-.545
	p	**.645**		.008	.680	.941	.654	.456	.665	.206
CA3	r	**.563**	**.525**		.092	.336	.197	.425	.576	-.677
	p	**.188**	**.266**		.844	.462	.672	.342	.176	.095
BLA	r	**.121**	**.831(*)**	**.538**		.167	-.129	.147	.204	-.124
	p	**.795**	**.021**	**.213**		.720	.783	.753	.661	.791
CeN	r	**-.064**	**.609**	**.755(*)**	**.744**		.471	.341	.855(*)	-.218
	p	**.892**	**.147**	**.050**	**.055**		.286	.455	.014	.639
dHP	r	**-.281**	**.435**	**-.144**	**.728**	**.270**		.589	.099	-.039
	p	**.541**	**.329**	**.758**	**.064**	**.558**		.164	.833	.934
vHP	r	**.005**	**.351**	**.224**	**.776(*)**	**.429**	**.834(*)**		.188	.127
	p	**.992**	**.440**	**.629**	**.040**	**.336**	**.020**		.686	.786
Locomotor	r	**.457**	**.330**	**.007**	**.192**	**-.295**	**.247**	**.268**		-.543
	p	**.303**	**.470**	**.987**	**.680**	**.520**	**.593**	**.562**		.208
Freezing	r	**-.220**	**-.310**	**.069**	**-.504**	**.092**	**-.736**	**-.749**	**-.786(*)**	
	p	**.635**	**.499**	**.883**	**.249**	**.845**	**.059**	**.053**	**.036**	

Table 16: Pearson's Correlations for Tone Test. Standard print = Pearson's product-moment coefficient for wild-type data, n=7. Bold print/Shaded = Pearson's product-moment coefficient for transgenic Tg2576 data, n=7. r = Pearson's product-moment coefficient; p = probability. (*)Correlation is significant at the 0.05 level (2-tailed). (**) Correlation is significant at the 0.01 level (2-tailed).

Figure 64A presents an optimal theoretical model of neural activation in wild-type animals based on c-Fos expression data from the cohort that underwent Tone testing. Figure 64B tests the same model with data from Tg2576 mice. The four fit

indices suggest that the pattern of activation depicted in this system represents a reasonable, but not excellent, model of neural activation for wild-type mice during exposure to the tone stimulus. It is, however, the best fit achieved with data from wild-type mice.

Interestingly, this model was a better fit with the Tg2576 data, but not the best fit that can be achieved with Tg2576 data. Most notably, the only path that is of a significant weight is the CA3 to CA1 connection for the wild-type, while the CA1 to BLA and BLA to CeN paths are significant in the Tg2576 data set. This suggests that even though the model can be applied to data sets for both genotypes, it is sensitive to differences in path strengths, which may be an indication of transgene-driven alterations in the overall dynamics of the neural network.

When the optimal wild-type model was stacked to compare path coefficients for the two genotypes, the multiple-group analysis revealed a non-significant difference between the overall fit of the models (χ^2 = 7.677, df = 6, p>0.05). However, a significant difference between the BLA-CeN path was revealed (p<0.02).

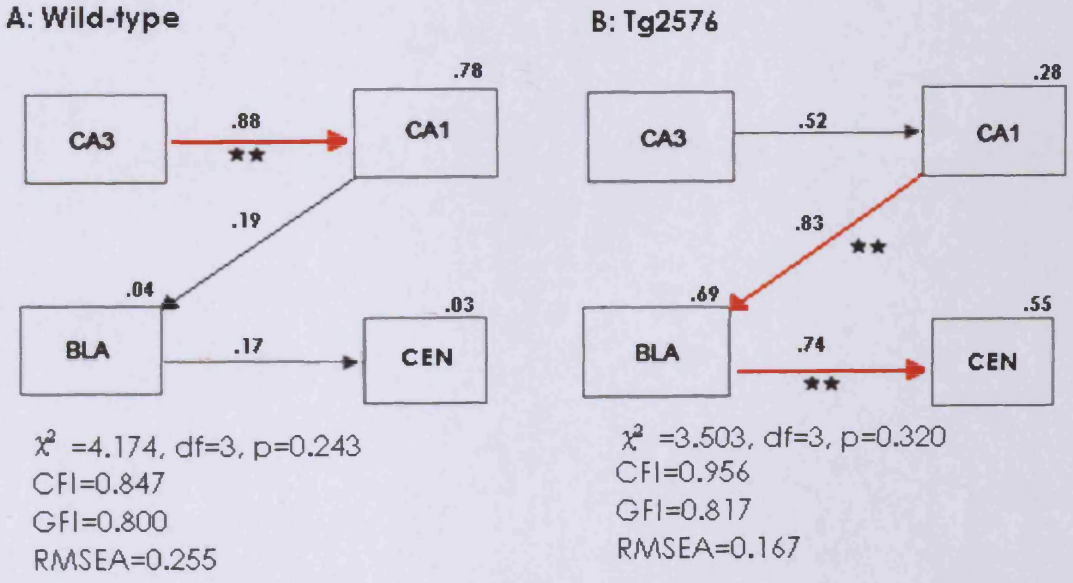


Figure 64: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during Tone Test in CS/US conditioned cohort. Optimal model for wild-type data. Red arrows and * = p<0.05, ** = p<0.01.

Figure 65B depicts the optimal model that fits the Tg2576 data from the Tone test. Strikingly, the most appropriate model is the same as that generated for the CS/US conditioning and Context test data sets. Furthermore, the lack of fit of wild-type data is also consistent with this model. Although the implications of this will be discussed further below, it is interesting to note that three c-Fos data sets from three independent cohorts of Tg2576 mice have resulted in the generation of an identical model of neural network dynamics. The excellent fit of this model to the Tone test data suggests a prominent role for the interaction of these brain regions in this task.

When the optimal Tg2576 model was stacked to compare genotypic differences in the path coefficients, results showed that the difference in the overall fit of the model approached significance (χ^2 = 8.401, df = 4, p=0.078), while none of the individual path strengths were significantly different.

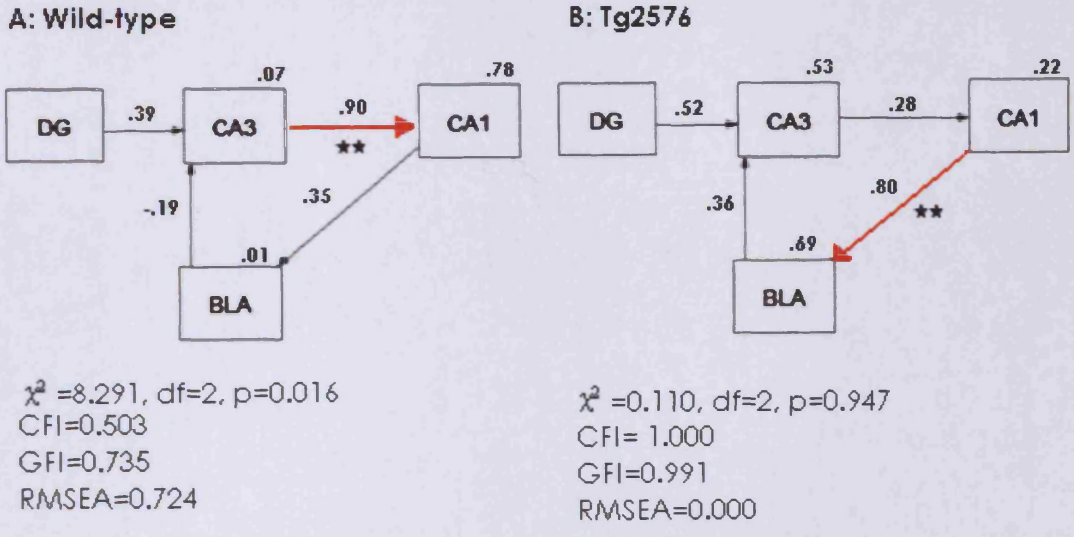


Figure 65: Structural equation modelling of data from wild-type (A) and Tg2576 (B) mice during the Tone Test in CS/US conditioned cohorts. Optimal model for Tg2576 data. Red arrows and * = p<0.05, ** = p<0.01.

5.4.6 Discussion

The aim of the present study was to apply SEM to the c-Fos data generated from Experiments 8 and 9, as a means of informing theory about transgene-driven alterations in the dynamics of the neural networks underpinning fear conditioning. Firstly, the results revealed a series of models that best suited the data for wild-type mice, depending upon whether the animals underwent CS/US conditioning, Context testing or Tone testing. Firstly, it is worth noting that in all wild-type models, the CA1, CA3, BLA and CeN combined to generate the best fitting model. The DG, however, was always dissociated from the wild-type models and significantly lowered the fit when it was included in the network. This suggests that in control animals, the DG is not integrated into the network comprising the remaining hippocampal and amygdala subregions. Of course, it is always possible that the DG is actively interacting within other systems or at other time points (not those optimal for c-Fos expression), which necessitates the caveat that the generation of neural models does not preclude the involvement of this region in fear conditioning or retrieval in wild-type mice.

Given that none of the models that were generated based on the wild-type data provided a particularly good fit for Tg2576 data, separate models were developed for these data sets. Not only were the DG, CA3, CA1 and BLA consistently inter-related in these models, it was striking to find that the same model provided the best fit for the data in every condition. This finding has multiple implications. Firstly, the CeN was consistently dissociated from the system and including it in the model reduced the fit considerably for every data set. This suggests that activity within the CeN is differentially regulated in Tg2576 mice. Perhaps the most striking feature of the SEM models, however, was the fact that the DG was integrated into the model in Tg2576 mice. This region being the initial region in the proposed network would be expected to have an impact upon all other regions. This is interesting not only because this region was dissociated from the network within the wild-type models, but also because LTP in the DG has been shown to be compromised in Tg2576 mice (Chapman et al., 1999). Not only was this impairment shown to be correlated with impaired spatial memory (Chapman et al., 1999), Experiments 8 and 9 of the present study consistently reported genotypic

differences in DG c-Fos expression in Tg2576 mice. To obtain the same outcome with three independent cohorts of animals suggests that the interplay between these regions is reliable and c-Fos expression occurs in similar patterns throughout this system in each condition. Furthermore, the incompatability of this model to wild-type data lends support to the idea that transgene-driven differences in the neural network dynamics may underlie behaviour differences.

On the other hand, it would have been expected that different conditions (e.g. training versus memory retrieval) would necessitate different patterns of activation. It would also be expected that different networks might underlie memory retrieval of the Tone versus the Context, especially given the behavioural difference seen between the groups. Thus, it is possible that system dynamics are less flexible in Tg2576 mice than wild-type mice. That is, wild-type mice may be capable of more complex neural interactions that allow them to adapt to each condition, while Tg2576 mice may be more rigid in their ability to process input or adapt their output due to changes in the discrete function of a specific region or impairment in the system as a whole.

The impact of behavioural output on SEM

The SEM analysis reported above was conducted without behavioural variables incorporated into the models. This was done to ensure that any transgene-dependent differences in the patterns of neural activation were not influenced by the behavioural output, which has been shown to differ between the genotypes during conditioning and testing. However, the insertion of behavioural variables allows for the development of models that specifically examine the impact of neural activation on behaviour, or allows for current models to be tested in light of this behavioural output. As shown in Tables 14 and 16, freezing behaviour and locomotor activity have been shown to be significantly correlated in both genotypes. This aspect of SEM will not be discussed in depth here because the incorporation of the response variables as outputs of a model is inappropriate in the light of significant behavioural differences. Nevertheless freezing and locomotor activity measures were considered as potential outputs from any of the regions depicted in each optimal model presented above. Interestingly, the results

revealed striking consistencies in the impact of the two forms of behaviour on the fit of the model. Furthermore, there were remarkable consistencies in the brain regions that were most strongly correlated with both forms of response. For example, for the CS/US conditioning data, for both optimal models (optimal for wild-type data and optimal for Tg2576 data) and for both behavioural indices (freezing and locomotor activity), the region most highly correlated with behaviour for the wild-type animals was always the CA1 region. For Tg2576 mice, the dentate gyrus and CeN regions always correlated best with the two behavioural indices. Furthermore, there was very little, if any, detriment to the fit of the models when these variables were incorporated. For the optimal models for the testing conditions, the results can be summarised as follows: for wild-type mice, the CA3 region best correlated with freezing behaviour during both fear tests, while locomotor activity correlated best with the CA1 region during the context test and the CeN during the tone test; for Tg2576 mice the BLA correlated best with both behavioural indices during the context test and the BLA and DG correlated best with freezing behaviour and locomotor activity, respectively, in the tone test. Such results, however, must be interpreted with caution since including the behavioural indices in the models presented above might not represent the optimal model for this combination of variables. Furthermore, defining links between behaviour and a discrete region at the start of a model will impact upon the fit of the model downstream, whereas correlating behaviour with the CeN, for example, will impact less upon the subsequent fit of the model. However, despite the caveats, it is worth noting the ability of the models to support the behavioural variables and the consistency in the findings for each genotype.

In sum, SEM provides an interesting method of addressing the question of whether system dynamics linked to learning and memory were altered by expression of the APPswe mutation. Remarkable consistencies in the SEM models supported the robustness of the method and revealed distinct and reliable patterns of activation for each genotype. Several caveats must, however, be mentioned since SEM cannot be considered a definitive method of evaluating network dynamics. As it is a mathematical modelling technique, it provides only a theoretical framework for examining collinearity between inter-related regions. It is highly dependent upon multiple factors, including the *n* in each condition, the number of brain regions

sampled and the constraints of the anatomical projections. Thus, it is interesting to probe the IEG data for distinct transgene-dependent patterns of interconnectivity using SEM, while simultaneously recognising the need to validate the findings with other *in vivo* and *ex vivo* techniques.

5.5 Chapter Discussion

The aim of this chapter was to identify transgene-dependent changes in neural activation after fear conditioning and fear memory retrieval in the Tg2576 model. The set of experiments and analyses presented here demonstrated consistent impairments in freezing to a tone CS and post-shock changes in activity, as well as a distinctly altered pattern of conditioning-dependent c-Fos expression in aged Tg2576 mice.

Firstly, it is worth noting the sex- and age-differences of the cohorts of mice used in each of the three fear conditioning experiments presented in this thesis. Experiments 3 (standard Pavlovian fear conditioning paradigm) and 9 (c-Fos fear memory retrieval) used female mice, while male mice were used in Experiment 8 (c-Fos fear training). Furthermore, the cohort of mice in Experiment 3 were aged 17 months, while in both Experiment 8 and 9, the mice were 19 months old. Thus, while the sex or age differences may account for minor behavioural differences observed between the experiments, the use of female mice in Experiments 3 and 9 ensured that sex was not a confounding factor in the two cohorts that underwent tone and context testing.

Overall, the behavioural data not only largely resembled those presented in Experiment 3, but also extended the previous data set by including an objective infrared locomotor activity measurement. Furthermore, the data presented in Experiments 8 and 9 demonstrated interesting similarities in the regions displaying aberrant c-Fos expression in Tg2576 mice. One of the most striking findings is that both conditioning and fear memory retrieval revealed a transgene-dependent difference in c-Fos expression in the dentate gyrus and ventral hippocampal regions. Importantly, these differences were not present in the unconditioned control group(s).

An interesting finding from the fear conditioning task (Experiment 8) was that the proportion of c-Fos expression exhibited by Tg2576 did not change between CS Only and CS/US presentation. Wild-type mice demonstrated either a suppression (DG) or enhancement (ventral hippocampus, CeN) in the proportion of c-Fos IR-

nuclei expressed during CS/US conditioning. Thus, genotypic differences in c-Fos expression as a function of conditioning were evident in the dentate gyrus and ventral hippocampus. When tested in extinction (Experiment 9), transgene-dependent changes in c-Fos expression were evident in the dentate gyrus as a result of the tone test, in the ventral hippocampus after the context test, and in the CeN after both tests. A mild change in IEG expression was observed in the BLA after tone conditioning.

These results add to previous evidence of impaired synaptic function in the dentate gyrus of Tg2576 mice. King and Arendash (2002) report prolonged synaptophysin expression in the dentate gyrus of aged transgenic mice, which they suggest represented pathological synaptic processing and which also correlates with impaired cognitive function. Dong et al. (2007) reported decreased synapse density and decreased synaptophysin-positive bouton density in the dentate gyrus of aged Tg2576 mice in the vicinity of amyloid deposition. Furthermore, Jacobsen et al. (2006) report decreased spine density in the dentate gyrus as early as four months of age, and decreased LTP in the DG and impaired contextual fear conditioning in Tg2576 mice. Palop et al. (2007) reported aberrant excitatory activity in the dentate gyrus of hAPP mice, which they suggest triggers compensatory mechanisms, such as increased synaptic inhibition and the remodelling of inhibitory circuits. These changes are associated with aberrant GABAergic sprouting of interneurons and result in impaired synaptic plasticity in the dentate gyrus (Palop et al., 20007). These data support the current findings, which suggest aberrant neural activity in the dentate gyrus after fear conditioning and memory retrieval in aged Tg2576 mice.

It has been suggested that the dentate gyrus plays a specific role in conjunctively encoding sensory inputs to formulate a representation of the spatial environment (Kesner, Lee & Gilbert, 2004). Furthermore, via its interaction with the CA3 region, the DG has also been implicated in the process of pattern separation (Kesner, 2007). Thus, amyloid-induced disruption to the DG may prevent, or at least disrupt, the integration of sensory inputs (such as an auditory stimulus) into the representation of the environment or context. That is, a functional CA3 region may allow the environmental stimuli to be integrated into a representation of the

fear conditioning context, while a dysfunctional DG may only support weak encoding of the auditory inputs. This is further supported by the finding that memory consolidation after emotionally-charged events, such as fear conditioning, involves the activation of the amygdala, which influences and enhances LTP in the DG (Pape & Stork, 2003). Although the DG and amygdala nuclei are not directly linked, connections exist between the BLA and the DG via the CA3 and CA1 regions of the hippocampus, the subiculum and the entorhinal cortex (Amaral & Witter, 1995; Pitkänen, 2000). Pape & Stork (2003) suggest that the molecular mechanisms by which the amygdala may enhance hippocampal LTP include the involvement of muscarinic and ß-adrenergic receptors, *de novo* protein biosynthesis and temporally correlated electrical activity, such as theta waves, in the two regions.

Impairments in ventral hippocampal c-Fos expression was evident both during conditioning and during context testing in Tg2576 mice. Interestingly, however, Maren and Holt (2004) report that pre-training electrolytic lesions and muscimol inactivation of the ventral hippocampus can result in impaired acquisition of auditory cued conditioned fear, while not affecting contextual fear. Furthermore, the ventral hippocampus has been shown to be one of the primary regions involved in anxiety related responses in the elevated plus maze in rats (Zarrindast et al., 2008). For example, NMDA-receptor antagonists injected into the ventral, but not dorsal, hippocampus reduced fear and anxiety in the EPM in rats (Nascimento Häckl & Carobrez, 2007). Hence, transgene-dependent alterations in neural activation in this region may underlie the impairment in anxiety-related behaviours observed in both the fear conditioning and EPM paradigms in Tg2576 mice.

Interestingly, altered c-Fos expression was observed in the CeN in Tg2576 mice during fear memory retrieval in the tone and context tests. Altered neural activity in this region is possibly related to aberrant freezing responses since the CeN supports heavy projections to, and modulatory influences upon, hypothalamic and brainstem nuclei. Thus, the CeN is often viewed as the region responsible for mediating various autonomic and somatomotor reactions characteristic of fearful behaviors (Davis, 1992; Campeau & Davis, 1995). Furthermore, lesions of the

CeN in rats have been shown to abolish freezing behaviour to both an auditory CS and the context (Goosens & Maren, 2001).

The fact that CeN c-Fos expression is disrupted in transgenic animals during both the tone and context tests is less surprising upon replication of the fear conditioning deficit. Although the genotypic difference in freezing to the context never reached significance, there was clearly a numerical trend in that direction. Hence, a change in neural activation in the CeN likely represents mild pathology in this region, which may have a bearing on the impaired freezing behaviour elicited by Tg2576 mice.

Although the conditioning related changes in c-Fos expression observed in the current experiments broadly supports previous literature, it is worth noting that increased c-Fos activation is often reported after fear conditioning and testing (e.g. Hall, Thomas & Everitt, 2001; Radulovic, Kammermeier & Spiess, 1998). For example, Hall, Thomas and Everitt (2001) report increased c-Fos expression in the amygdala after retrieval of a tone cued fear memory. In the present experiments, however, c-Fos increased after CS/US conditioning or memory retrieval in the ventral hippocampus, but was more frequently suppressed in comparison to the CS Only control condition. Although the reason for this discrepancy is not immediately obvious, it may lie in the experimental parameters employed in different laboratories. For example, Hall, Thomas and Everitt (2001) had a control condition in which the unpaired cohort received five footshock and tone presentations at pseudorandom times. Upon cued memory retrieval, c-Fos was enhanced in the basal nucleus in the experimental, relative to the control, condition. Interestingly, however, Conejo et al. (2007) used a similar control group to the present experiment, in which animals either received tone-shock pairings, or CS Only presentations. Consistent with the experiments presented here, they too reported suppressed c-Fos expression during cued memory retrieval in the cohort that received CS/US conditioning. One potential explanation for this change in c-Fos expression after different control conditions could be the effect of the shock stimulus on levels of c-Fos. It may be that repeated exposure to aversive stimuli results in dampening of IEG expression in the medial temporal lobes, perhaps as a compensatory or self-protective mechanism, to allow more crucial brain regions to

respond to danger in the environment. Another possible explanation could be that different levels of c-Fos expression at test stage in part reflect the process of extinction, a form of new learning about the cued stimulus.

The freezing and locomotor activity data obtained during training and testing are necessary to establish the effectiveness of the fear conditioning procedure, as well as identify transgene-dependent impairments in unconditioned and conditioned behavioural responses. Holahan and White (2004) argue that c-Fos activation in the amygdala does not correlate fully with freezing behaviour and is, therefore, more likely to reflect neural activation in response to either the presentation of unconditioned stimuli or conditioned stimuli. They do suggest, however, that while the CeN and LA were activated by unconditioned stimuli and the BLA, LA and CeN by conditioned stimuli in their experiments, the overall pattern of activation is likely to contribute to the affective experience of fear and, in turn, affect the expression of fear responses.

In an attempt to identify changes in the overall pattern of neural activation, not just at the level of individual regions, SEM analyses were conducted using the c-Fos data. One of the most striking findings from these analysis was the consistent pattern of activation observed in Tg2576 mice. This analysis revealed recurring correlations between hippocampal and amygdala IEG expression in Tg2576 mice that were not observed in wild-type mice. These correlations likely reflect not only the activation of a discrete region but the interaction between regions and the simultaneous integration of multiple neural processes occurring within the system. Thus, while wild-type mice demonstrated flexible and diverse interactions between hippocampal and amygdala subregions depending upon the condition to which they were exposed, subregions of the Tg2576 brain appeared to interact in a consistent, inflexible manner. Thus, it may be that gross differences in neural activity do not exist in a discrete region of the pathological Tg2576 brain, but rather subtle differences exist in the interaction between regions and the broad dynamics of the system are mildly disrupted.

Interestingly, however, the other pattern observed in the SEM analyses was the dissociation of the dentate gyrus and CeN from the optimal wild-type and Tg2576

models, respectively. Not only were these regions uncorrelated with other regions at every stage (conditioning, context test, tone test), but these were also regions consistently identified by the raw c-Fos data as being subtly different between the genotypes during CS/US conditioning. As discussed above, these results also support the literature insofar as deficits in the dentate gyrus have previously been reported in the Tg2576 model. Here, however, the SEM analyses have provided a means of forming a theoretical framework about how mild pathology in these discrete regions might impact upon subregional interactions and the greater system dynamics.

The primary aim of this chapter was to analyse changes in IEG expression in the Tg2576 brain to CS/US conditioning (Experiment 8) and during memory retrieval in response to the conditioned auditory and contextual stimuli (Experiment 9), as a means of identifying transgene-driven alterations in neural processing. Overall, this set of experiments has succeeded in both replicating previous results (Experiment 3; Barnes & Good, 2005), and identifying regions of aberrant neural activation in aged Tg2576 mice. Consistent changes were identified in the dentate gyrus and ventral hippocampus, with mild impairments detected in the BLA and CeN. Thus, given that the hippocampus and amygdala act synergistically to form fear-related memories, disruptions to these regions may result in the weakened integration of the tone into the representation of the environment, resulting in impaired conditioning and subsequent memory retrieval. Furthermore, these data suggest that, although changes in neural activation were evident in discrete regions, it is likely that the overall dynamics of the brain system are disrupted by the presence of amyloid pathology in the Tg2576 model.

Chapter 6: General Discussion

6.1 Overview

The main aim of this thesis was to investigate the impact of amyloid pathology on emotional and motivational learning and memory processes in the APPswe mouse model of amyloid pathology. Given the extensive deposition of ß-amyloid found throughout the amygdala and frontal cortical regions in aged Tg2576 mice, it was hypothesized that functional impairments to these regions would result in altered expression of anxiety and impaired motivational processing. While a more detailed summary is provided below, the overriding conclusions that can be drawn from this series of experiments are as follows: (1) age-dependent changes in anxiety and disinhibition are evident in this model and the mutation leads to subtle age-independent changes; (2) despite extensive pathology in amygdala and cortical regions, the APPswe mutation does not cause gross impairments in appetitive motivational processing and goal-directed performance; Pavlovian fear conditioning, however, is consistently disrupted in APPswe mice; (3) the observed deficits in CS-US processing likely reflect an inability to form an association between a Pavlovian cue (context or tone) and the sensory-specific features of the US (appetitive or aversive), which may be dependent upon the salience and/or influence of the conditioned stimulus. Interestingly, this impairment is robust during aversive conditioning, but is only evident during appetitive conditioning when the CS is the context and the Tg2576 mice are exceptionally old; (4) ß-amyloid pathology causes changes in the expression of the IEG c-Fos within the hippocampus and amygdala during both conditioning and retrieval of fear memories; (5) alterations in the pattern of c-Fos activation were mostly evident in the hippocampal formation, and relatively subtle changes in amygdala activation were present in aged Tg2576 mice. These findings clearly indicate that β-amyloid pathology caused cognitive deficits in tasks supported by the hippocampus (and to a limited extent the amygdala). However, in appetitive tasks that are thought to require a functional amygdala, the APPswe mutation resulted in either no or relatively mild deficits.

6.2 Summary of findings

A review of the literature indicated that there have been few published studies concerning the effects of amyloid production in APP mutant mice on emotional learning or motivational processes. Nevertheless, patients with AD display deficits in emotional processing and goal-directed behaviour and this may reflect changes in the functional characteristics of the hippocampus, amygdala or the (frontal) cortex. Given that Tg2576 mice also show marked elevation of amyloid with age in these areas, it was hypothesised that the APPswe mutation would cause deficits in emotional processing and memory and goal-directed behaviour with age. Thus, it was predicted that aged Tg2576 mice would be impaired on tasks assessing anxiety and appetitive and aversive motivational processes. The results of this thesis only lend partial support to this hypothesis as will be summarised below.

Initially, the presence of pathology in Tg2576 mice was confirmed using a biochemical analysis of the quantity and distribution of ß-amyloid deposits. The results indicated that both Aß 1-40 and Aß 1-42 fragments were present in the hippocampus, amygdala and frontal cortex of aged mutant mice. The levels of amyloid deposition were both extensive (in aged Tg2576 mice) and comparable between these regions (i.e., the amygdala and cortex contained roughly the same amount of amyloid as the hippocampus). Despite this, no cell loss and no gross changes in connectivity were evident in and between these regions.

The experiments presented in Chapter 3 assessed the impact of the amyloid pathology on anxiety behaviours and conditioned fear. The results confirmed earlier reports of both aberrant anxiety-related behaviours and impaired fear conditioning in aged Tg2576 mice. More specifically, Tg2576 mice demonstrated increased exploration of the open arms of an elevated plus maze and impaired freezing during the initial post-shock periods and during the tone test in a fear conditioning paradigm. Thus, it was suggested that these fear conditioning impairments reflected either an impairment in the formation of an association between a CS and the affective features of the US or an inability to access a representation of the sensory-specific affective properties of the US. In the subsequent experiments, this hypothesis was assessed using tractable appetitive conditioning procedures.

In Chapter 4, the experiments assessed the proposition that aged Tg2576 mice may be impaired in goal-directed learning. That is, Tg2576 mice may be unable to form or access a representation of the US that specifies its sensory-specific incentive motivational properties. Instrumental and Pavlovian devaluation tasks and a Pavlovian-instrumental transfer task were used to assess the animals' ability to form action-outcome and CS-outcome associations and to use these to guide behaviour. Results indicated intact formation of these representations in aged Tg2576 mice in all tasks except one utilising the context as the CS. It is argued that this context-US devaluation deficit can be explained in terms of either an impairment in incidental context processing and/or an age-related deficit in the pathways relating the hippocampal representation of context with sensory-specific incentive attributes of reward processed by the amygdala nuclei.

To further probe whether the deficits reported thus far reflect Aβ-mediated alterations in hippocampal-amygdala interactions, c-Fos protein was examined in these regions after fear conditioning and testing as a means of functionally imaging neural activity during these forms of learning and memory retrieval. These data were also analysed using structural equation modeling as a method of generating anatomically-valid models to hypothesize the functional interactions between hippocampal and amygdala regions during these tasks. The results indicated subtle changes in c-Fos expression in hippocampal and amygdala nuclei during all phases of fear learning and memory. Most strikingly, however, the data revealed changes in the dentate gyrus and ventral hippocampus of Tg2576 mice relative to wild-type mice. Furthermore, SEM indicated both broad differences in systemic neural activation between the genotypes, but also a specific coupling and un-coupling of the dentate gyrus in Tg2576 and wild-type models, respectively. Thus, it is argued that because the hippocampus and amygdala work together to form fear-related memories, the altered neural processing observed in Tg2576 mice may result in impaired formation of associative representations of the CS and emotional features of the US that result in impaired conditioning and subsequent memory retrieval. However, this impairment appears to be restricted to tasks in which, arguably, there is a contribution from the hippocampus.

Table 17 provides an overview of the known involvement of amygdala, hippocampal and cortical subregions on aversive and appetitive behavioural tasks. In addition, the effects of the APPswe mutations reported in this thesis are also summarised. It is clear that the majority of deficits revealed in the Tg2576 model are in tasks that previous research has shown to be sensitive to lesions of the hippocampus. Tasks in which the amygdala is exclusively implicated, however, are not overtly sensitive to the APPswe mutation. It would seem reasonable to conclude therefore that the neural locus of the behavioural deficits in Tg2576 mice reported in this thesis lie either with the hippocampal formation or in task specific interactions between this region and the amygdala.

		Unconditioned Fear/Anxiety: EPM	Contextual Fear Conditioning	Cued Fear Conditioning	Instrumental Outcome Devaluation	Pavlovian Outcome Devaluation	PIT-Outcome Specific
TG2576		√	√/×	√	×	× (√ context CS only)	×
Amygdala	BLA	√	√	√	√	√	√
	CeN	√	√	√	×	×	×
Hippocampus	CA1	√	√	√	×	×	×
	CA3	√	√	√	×	×	×
	DG	×	√	×	×	×	×
	Dorsal	×	√	√	×	×	×
	Ventral	√	√	√	×	×	×
Frontal Cortex	mPFC	√	×	×	√	×	×
	OFC	×	×	×	×	×	√

Table 17: Summary of the brain regions implicated in each test of emotional memory. Inclusion is based upon lesion, inactivation and cellular imaging studies. Furthermore, the impact of amyloid pathology on these forms of learning and memory is summarised in the row 'Tg2576'. √ = pathology or lesion causes deficit in the given task. × = no evidence that pathology or lesion causes deficit in the given task.

6.2 Theoretical implications and future directions

Relation to the human condition

The Tg2576 model represents a model of ß-amyloid pathology, which is one of the primary hallmarks of Alzheimer's disease. Thus, it is of interest to first consider the deficits observed in this model in the context of the human condition. Similar to AD patients, Tg2576 mice demonstrate altered expression of anxiety, increased disinhibition and deficits in fear conditioning. Furthermore, the brain regions susceptible to ß-amyloid aggregation in mutant mice closely parallel those affected in AD patients. Although impairments in memory have been revealed in Tg2576 mice, the current set of experiments reveal clear differences (perhaps unsurprisingly) between the human condition and mice that express the APPswe mutation. For example, where Alzheimer's patients show altered expression of apathy and deficits in goal-directed behaviours, Tg2576 mice demonstrate few changes on tasks of motivational processing and goal-directed performance. There are several reasons why the APPswe mice may not show the pattern of motivational/emotional deficits observed in patients with AD. For one, this mouse model expresses one hallmark of the human condition, but other forms of neural damage are not exhibited in the Tg2576 brain, including tau hyperphosphorylation and cell loss. The impact of tauopathy and other aspects of neural damage needs to be assessed both independently and in the context of amyloid pathology, to reveal distinct and combined contributions of these changes in neural integrity to behaviour and cognition. Furthermore, assessing changes in neuropsychiatric symptoms, cognition and memory in humans and in rodents is achieved in a vastly different manner, given the limitations of working with mice. Thus, while theoretically valid conclusions can be drawn from studies with mutant mice, it must be acknowledged that limitations exist in the ability to design comparable experiments to human studies and evaluate high-level, language-dependent cognition. Despite these caveats, however, the relevance of this work to the human condition can be seen in the parallel changes that occur in the neuropsychiatric symptoms of anxiety and the cognitive processing of negative stimuli (i.e. EPM and fear conditioning in mutant mice versus the processing of negative facial emotions and impaired fear conditioning in AD patients). Thus, this mouse model,

which expresses a human transgene, can to a large extent be used for understanding the impact of amyloid pathology on cognition and behaviour.

Age-independent effects of the APPswe mutation

In terms of the Tg2576 mouse model, the findings presented in this thesis have implications both for the current understanding of the impact of ß-amyloid pathology on behaviour, and for potential future research. Firstly, the anxiety tasks presented in Chapter 3 reinforce the need to dissociate between age-dependent and age-independent effects of the mutation, as well as to determine differential deficits of sex in Tg2576 mice. The fact that changes in behaviour may occur in this model in an age-independent fashion contradicts the generally accepted notion that APP strains represent progressive models of amyloid pathology. Given evidence that synaptic deficits may emerge early during development (Jacobsen et al., 2006) in Tg2576 mice, a thorough characterization of the behavioural changes that occur during the early stages of development is required in order to assess the utility/limitations of the model. In a related manner, there is a need to address the impact of strain differences on the manifestation of the APPswe mutation, as well as thoroughly investigate the differential impact of the mutation in male and female mice.

As discussed previously, the age-independent impairment in marble burying likely reflects the influence of the background strain on the genetic manipulation, resulting in an intrinsic, non-developmental effect of the APPswe transgene insertion on the tendency to perform a repetitive digging behaviour. It is of interest to explore the impact of this behavioural phenotype on other aspects of behaviour, including its relation to other tasks that putatively assess impulsivity. For example, the 5-choice serial reaction time (5CSRT) task. Such tests would allow multiple aspects of cognition/executive function to be evaluated, including attention, and perseveration.

Anxiety and the APPswe mutation

Data from the elevated plus maze indicate an age-dependent change in anxiety and disinhibition in APPswe mutant mice. Although these data lend further support to previous research (e.g. Lalonde et al., 2003), it has previously been suggested that this impairment is driven by increased locomotor activity (Gil-Bea et al., 2007). The data presented in this thesis do not support this interpretation of the anxiety deficit observed in aged mutant mice. Not only was no effect of genotype evident in the overall number of arm entries made in the EPM, infrared locomotor activity levels were also comparable between the genotypes during baseline phases in the fear conditioning tasks. Thus, it could be argued that baseline differences in locomotion are unlikely to account for changes in anxiety or disinhibition observed in this model.

The neural basis of the Tg2576 impairment in anxiety may be assumed to be related to abnormal amygdala function. For example, injection of anxiolytics into the basolateral/medial nuclei of the amygdala in rats reduces anxiety-related behaviours in the EPM (Nunes-de-Souza et al., 2000; Zangrossi & Graeff, 1994). However, it is also clear from the work of Bannerman and colleagues that a deficit in anxiety behaviours could be the result of hippocampal perturbation. Not only has the ventral hippocampus been associated with the expression of unconditioned anxiety (Bannerman et al., 2003), lesions or inactivation of this region have also been shown to reduce anxiety-related behaviour on multiple anxiety tests, including hyponeophagia, black/white 2-compartment box test, a successive alleys test, a social interaction test and the elevated plus maze task (McHugh et al., 2004; Bertoglio, Joca & Guimarães, 2006). Furthermore, intra-CA1 injection of cannabinoids has been shown to significantly alter fear responses in the elevated plus maze task (Roohbakhsh et al., 2007). Thus, it could be argued that the changes in anxiety observed in aged Tg2576 mice result from a deficit in amygdala function (which given the results of the outcome devaluation tasks seems unlikely), a deficit in (ventral) hippocampal function or abnormalities in the interaction between these two systems. Although it is difficult to distinguish between the effects of amygdala and ventral hippocampal damage on the EPM, Hugh et al. (2004) found distinct effects of each lesion type on a series of tasks of unconditioned anxiety. Although both lesions caused a reduction in the latency to cross into the more anxiety-arousing section of the two-compartment box test,

excitotoxic lesions of the ventral hippocampus increased the proportion of time spent in the anxiogenic section of the successive alleys apparatus (a modified version of the EPM), while amygdala lesions had no effect in this test. Furthermore, Hugh et al., (2004) report that ventral hippocampal lesions reduced the latency to initiate eating during a hyponeophagia test and increased social interaction, while amygdala lesions increased latency to initiate eating during the hyponeophagia test and had no effect on social interaction. Thus, the results of the EPM align more closely with the impact of ventral hippocampal lesions (insofar as the successive alleys task represents a version of the EPM). It would be of interest however to evaluate the performance of aged Tg2576 mice on tests of hyponeophagia and social interaction to determine whether the pattern of performance consistently resembles that seen after excitotoxic lesions of the ventral hippocampus.

Context and cue processing in Tg2576 mice

The results of the context outcome devaluation task reveal an impairment in 22 month old mice that was not evident in 14 month old mice. Furthermore, this deficit was independent of an inability to discriminate between contexts. Moreover, the impairment is restricted to contextual, and not auditory, conditioned stimuli, which indicates that impairments are only evident when cue processing is directed towards the context and thus potentially requires an intact hippocampus. Based on work by Good et al. (1998), it is hypothesised that the influence of the contextual cue on the behavioural response (i.e. whether the cue was incidental or contingent) might impact upon CS processing and the subsequent strength of the conditioned CS-US association. That is, the nature of the task demands, in terms of context processing, might impact upon the strength and formation of context representations, which are subsequently used to guide behaviour. Thus, it is important to determine whether Tg2576 mice are more sensitive to incidental versus intentional (or contingent) processing of the context. Future experiments might aim to ensure that mice are required to distinguish between responding in a series of distinct contexts (perhaps via the use of aversive stimuli or omission of reward versus positively rewarded contexts), then employ satiety-specific devaluation procedures and extinction tests to assess responding in the two (or

more) positively rewarded contexts. It would be expected that the shift to contingent processing of the contexts would result in recovery of the context devaluation impairment. These experiments would help clarify the precise nature of the deficits observed in the context devaluation task and thus shed light on the precise aspect of cognition that is sensitive to amyloid pathology.

Given the use of contextual cues in this incentive learning task, the neural basis of the observed impairment is likely to involve the hippocampal formation or the pathways linking the hippocampus and amygdala nuclei. Identification of this neural basis might involvement an evaluation of subregions of the hippocampus by comparing the dorsal versus ventral regions, or dissociating hippocampal subregions, such as the dentate gyrus and CA1 areas. Furthermore, it is of interest to determine whether a threshold of damage is necessary to sufficiently disrupt performance on this task, below which performance will remain normal. This can then be compared with the progressive deposition of amyloid and associated cellular disruptions that occur in aged Tg2576 mice to determine the sensitivity of the task to neural pathology and clarify the involvement of the hippocampal formation in a context devaluation task. This would further help clarify whether the impairment observed in aged Tg2576 mice resulted simply from hippocampal dysfunction or whether a more complex interaction between the amygdala and hippocampus is required for cue processing in this task. For this, a disconnection study, in which unilateral hippocampal and amygdala lesions are created with one in each hemisphere, would shed light on the whether the integrity of this neural system is crucial to forming an association between the context and the incentive value of the outcome.

In terms of motivational learning, it is worth considering that incentive learning paradigms focus on the effects of motivational variables on performance by controlling the acquisition of incentive value by the appetitive reward. Although this was not shown to be overtly disrupted in Tg2576 mice, it has not been determined whether contingency degradation tasks may be more sensitive to the effect of the mutation. Contingency learning also involves the formation of a representation of the relationship between an instrumental action and a reward, but it is sensitive to both the contiguity between action and reward, as well as their

causal association. Thus, manipulating this association by degrading the instrumental contingency by the presentation of unpaired rewards will result in decreased responding. Performance on this task has been shown to be sensitive to hippocampal damage (Corbit & Balleine, 2000; Corbit, Ostlund & Balleine, 2002) and, thus, may be sensitive to the impact of the APPswe mutation.

Fear conditioning in Tg2576 mice

Although previous studies (e.g. Corcoran et al., 2002) report an impairment in freezing to contextual stimuli in aged Tg2576 mice, the data presented in this thesis support Barnes & Good (2005) who report deficits in freezing both post-shock and during the tone test in aged mutant mice. As discussed previously (see section 3.5), Barnes and Good (2005) suggest that the inconsistency in behavioural results may be resolved by considering the effect of cue competition on the strength of the CS-US association. That is, the contextual CS was attributed greater associative strength than the tone CS due to its greater salience, and therefore elicited higher levels of freezing. Hence, this greater salience allowed the contextual stimuli to support conditioning better than the auditory stimulus, leading to an overshadowing effect and an impairment with the less salient cue. This effect can differ between studies due slight differences in experimental parameters, including differences in tone frequency and duration. Although this interpretation is supported conceptually by data from Corcoran et al. (2002), in the present set of experiments, the percentage of freezing observed during exposure to the tone was comparable to that seen during exposure to the context, if not initially higher. However, the trend towards significance seen for the context test may in part reflect the relatively similar associative strengths of the conditioned stimuli. Thus, to investigate the potential impact of task parameters on cue salience and subsequent strength of conditioning, it would be of interest to systematically manipulate the salience of the context and tone CSs to determine whether differential impairments in conditioning can be observed in Tg2576 mice. It is plausible to hypothesis that aged mutant mice would be impaired at contextual fear conditioning in the Cardiff laboratory if the salience of the context was decreased (perhaps by pre-exposing mice to the context, as well as ensuring as few distinct

environmental cues as possible) and the salience of the tone increased (by employing a different pitch, volume or duration, for example).

The results of the fear conditioning experiments provide indirect evidence that the neural systems supporting aversive conditioning are impaired in aged Tg2576 mice. The primary candidate regions that may provide the anatomical locus for these behavioural abnormalities are the amygdala and the hippocampus. For example, impairments in post-shock freezing have been observed after electrolytic lesions of the lateral amygdala, although excitotoxic lesions restricted to the LA do not result in a post-shock freezing impairment (Wallace & Rosen, 2001). Thus, although there is evidence that the LA is necessary for learning and memory of fear conditioning, it may be the fibers of passage through the LA and amygdalostriatal transition region that play a role in the expression of freezing behaviour. Furthermore, post-shock freezing deficits have also been observed in rats with hippocampal damage. Lesions of the ventral hippocampus reduce freezing behaviour after the delivery of an unsignalled footshock (Bannerman et al., 2003; Kjelstrup et al., 2002; Richmond et al., 1999) while lesions of the dorsal hippocampus have no effect upon this freezing behaviour (Richmond et al., 1999). Furthermore, although pre-training lesions of the basolateral amygdala have been shown to produce deficits in the acquisition and expression of conditioned fear to discrete auditory CSs (e.g. Nader, Majidishad, Amorapanth & LeDoux, 2001; Phillips & LeDoux, 1992), lesions or inactivation of the ventral hippocampus have also been shown to disrupt cued fear conditioning (Maren & Holt, 2004; Zhang, Bast & Feldon, 2001). Interestingly, Hunsaker & Kesner (2008) even report a dissociation between CA1 and CA3 lesions across the dorsal-ventral axis. Most importantly, they found that lesions of the ventral CA3 region resulted in impaired retrieval of auditory fear associations when tested both 24 and 48 hours after conditioning (Hunsaker & Kesner, 2008). Thus, the impairments observed in aged Tg2576 mice may reflect either amygdala or hippocampal dysfunction. It is suggested, however, that the neural basis is most likely the hippocampal formation since previous studies (i.e. Wallace & Rosen, 2001) suggest that significant destruction of the fibers of passage through the LA would be necessary to cause a deficit in post-shock freezing.

Consistent with this, IEG analysis of patterns of activation during conditioning and retrieval of fear memories identified changes in the dentate gyrus and ventral hippocampus of Tg2576 mice, with only mild impairments detected in the BLA and CeN. Thus, given that the hippocampus and amygdala act synergistically to form fear memories, it is likely that disruptions to these regions result in the weaken integration of the tone into the representation of the environment, resulting in impaired conditioning and subsequent memory retrieval. Furthermore, although changes in neural activation were evident in discrete regions, it is likely that the overall dynamics of the brain system, and possibly the amygdala-hippocampal processing pathways in particular, are disrupted by the presence of amyloid pathology in Tg2576 mice.

Impact of amyloid pathology on the hippocampus versus amygdala

The extant literature, including the results of the present series of experiments, shows that ß-amyloid pathology compromises hippocampal function in Tg2576 mice. Nevertheless, given the pervasive nature of amyloid production and deposition in this and other APP models it is of interest to address the question: how does amyloid production influence other neural/cognitive systems and their integration in mice? The results of this thesis indicate that amygdala function remains largely intact in Tg2576 mice – at least in the context of appetitive goal directed behaviour. The c-Fos studies indicate robust changes in hippocampal IEG expression, even in a task that should theoretically elicit activity changes in the amygdala. The results clearly show that the hippocampal formation is more sensitive to amyloid pathology than the amygdala, and perhaps frontal cortical regions. This raises the question of why the hippocampus should be more sensitive to β-amyloid.

There is abundant evidence to suggest that the cholinergic system is disrupted in AD, and this dysfunction is correlated with the manifestation of cognitive and neuropsychiatric impairments (Davies & Maloney, 1976; Wilcock et al., 1982). Disruption to this system is intimately linked with the glutamatergic hypoactivity observed in AD (Francis, 2003). The importance of glutamate and glutamate

receptors to the mechanisms of synaptic plasticity (LTP and long-term depression, LTD) are well-recognised and are considered to play a role in learning and memory (Baudry & Lynch, 2001; Scannevin and Huganir, 2000). However, since LTP is most commonly investigated within the hippocampal formation, it has not been definitively established whether the neural mechanisms underlying LTP in the amygdala are identical to those underlying hippocampal LTP. In fact, there is some evidence to suggest that the underlying mechanisms may differ between these brain regions. For example, it has been shown that the properties and subunit composition of the NMDA receptor, a glutamate receptor, differ between the lateral nucleus of the amygdala and the CA1 region (Miwa et al., 2008). Not only has the ratio of NR2A and NR2B subunits been shown to differ between the CA1 region and the LA, but Miwa and colleagues (2008) suggest that the differential regulation of these subunits by tyrosine phosphorylation may result in qualitatively different modification of synaptic functions between these brain regions.

Recent evidence suggests that the NR2B subunit is important in the regulation of LTP in the LA (Miwa et al., 2008), while classically the NR2A subunit has been implicated in hippocampal LTP (MacDonald, Jackson & Beazely, 2006). Interestingly, investigation of the effect of Aß 1-40 on NMDA receptors expressing either predominantly NR1/NR2A or NR1/NR2B subunits revealed a difference in the toxicity of ß-amyloid on each subunit. Specifically, Aß 1-40 impaired plasma membrane integrity and decreased intracellular ATP in cells expressing the NR2A, but not the NR2B, subunit (Domingues et al., 2007). Thus, Aß 1-40 is particularly toxic to the NMDA receptor subunit that is implicated in hippocampal LTP, which may account in part for the greater susceptibility of the hippocampal formation to amyloid pathology.

In sum, further investigation of the fear/anxiety, motivational and contextual impairments observed in the Tg2576 mouse would shed light on the precise involvement of hippocampal (and perhaps amygdala/frontal cortical) subregions and the differential impact of amyloid pathology in each of these regions.

6.3 Summary

Ultimately, the Tg2576 mouse model provides a useful means of evaluating the impact of amyloid pathology, one of the primary symptoms of Alzheimer's disease, on behaviour and cognition. The work presented in this thesis extends current understanding of the impact of amyloid pathology to emotional and motivational learning paradigms. Both unconditioned and conditioned aspects of fear and anxiety were assessed and appetitive incentive learning was evaluated in a series of tasks known to be dependent on the integrity of the cortex and medial temporal lobes. Interestingly, the majority of impairments observed in the Tg2576 model were on tasks that involved the extended hippocampal system. In conjunction with the c-Fos and S.E.M. data, it is suggested, therefore, that amyloid pathology results in significant disruption to the hippocampus, particularly the dentate gyrus, as well as causing a more subtle global impairment to the dynamics of the neural system as a whole. This can lead to impaired learning, and subsequent memory retrieval, of associative representations of the sensory-specific features of aversive and appetitive stimuli. These findings are important not only for what they reveal about the impact of ß-amyloid pathology on learning and memory, but they also have more broad implications for studies evaluating the reparative or preventive efficacy of potential AD treatments.

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ADEAR: Alzheimer's Disease Education and Referral Center, National Institute of Aging. National Institute of Health, U.S.A.

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