THE EFFECTS OF REPEATED MILD STRESS ON A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease (AD), the most common form of dementia, is a devastating age-related neurodegenerative disorder. There is a growing body of evidence suggesting that leading a stressful lifestyle is associated with a heightened risk of developing AD. This is supported by preclinical evidence using transgenic mice over-expressing genetic mutations leading to overt β-amyloid protein production, a pathological marker of AD; stress in such mice has been capable of exacerbating AD-associated pathologies, including accelerating memory impairments and elevating β-amyloid levels. In contrast, a recent study from our group demonstrated that a repeated mild stress procedure, novel cage stress, improved a short-term memory deficit and reduced the normal age-related increase in β-amyloid levels. This thesis aimed to further characterise the beneficial effects of novel cage stress on AD-associated pathology in the TASTPM mouse model (double transgenic hAPP695swe x PS-1.M146V) which exhibits overt, age-related β-amyloid pathology. First, age-related changes in AD-associated pathology, with or without exposure to novel cage stress, were assessed using a multidisciplinary approach incorporating measures of cognitive performance, *in vivo* magnetic resonance imaging and post-mortem analysis of β-amyloid levels. The aim was to detect an age where we observe the most robust effect of stress; this time window was subsequently targeted to investigate a potential underlying mechanism, namely signalling though the glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAr). Studies described throughout this thesis, alongside previously published data, indicate novel cage stress appears to improve AD-associated pathology in TASTPM mice, but independently of AMPAr function. It is likely that novel cage stress is insufficiently severe to induce detrimental effects, but, rather, subsequent repeated stimulation and physical activity may improve pathological status. A better understanding of lifestyle risk factors of AD, such as stress, will aid in identifying those at risk of developing the disorder. Moreover, discovering the underlying mechanisms linking stress with AD may open novel therapeutic avenues to treat the disorder.

ABSTRACTS

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. Alzheimer's disease

1.1.1. The impact on society

Alzheimer's disease (AD), a devastating age-related neurodegenerative disorder, was first described by, and later named after the German psychiatrist Alois Alzheimer in 1907, and documented in a paper translated into English to read "About a peculiar disease of the cerebral cortex" (Alzheimer., 1907). The prevalence of AD is increasing due to the aging of the human population, likely a result of improved healthcare and living standards, alongside better recognition of the disorder. In the USA, it is estimated that there are 5.2 million sufferers of AD, 13% of those over 65 years old have AD (Alzheimer's Association, 2008). In England and Wales it is estimated that 39,400 patients will be diagnosed with AD each year (Copeland et al., 1999).

AD does not only affect the sufferer directly, but also causes severe emotional strain to family members and carers; it is estimated that 42% of the UK population is affected by the disease either directly, or indirectly (Alzheimer's Research Trust website, www.alzheimers-research.org.uk/info/statistics/). Indeed, the negative emotional impact of caring for a sufferer of AD, professionally or personally, is well recognised and has been described (Grossberg, 2008; Nichols et al., 2008; Roepke et al., 2008; Vellone et al., 2008). For instance, caregiver anxiety and stress have been shown to be directly proportional to the degree of AD clinical severity (Ferrara et al., 2008; Tun et al., 2008).

This high prevalence is coupled with a severe economic burden with an estimated annual cost of over £14 billion in the UK during 2000. Despite this expense, investment into researching AD is comparatively lower than other major disorders including cancer and heart disease (Lowin et al., 2001). There is currently no cure for AD; pharmacological treatments are often aimed at alleviating the symptoms of AD rather than preventing, or slowing the disease process (current therapeutic strategies to combat AD are discussed further in Section 1.1.3).

1.1.2. The neuropathology of AD

Clinically, AD manifests first as subtle memory impairments, followed by a gradual cognitive decline ultimately leading to a total loss of cognitive capacity and, ultimately, death within approximately 5 to 6 years following disease recognition (Molsa et al., 1986). Only identification of known pathological markers, commonly post-mortem, can confirm the variant of dementia as AD. Brain atrophy is coupled with the presence of two forms of inclusion in the brain: first, senile plaques composed of the insoluble form of the β-amyloid peptide and second, neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, a microtubule-associated protein.

Unlike these neuropathological inclusions, severe brain atrophy can be routinely monitored throughout the disease process to assess pathological status, using magnetic resonance imaging (MRI) a tool allowing for the non-invasive imaging of brain tissue in a living subject. Such studies have

confirmed a correlation between a decline in cognitive performance and brain atrophy (Di Paola et al., 2007), and have demonstrated regional tissue loss in the brain associated with specific symptoms of AD including delusions and apathy (Bruen et al., 2008). The direct cause of the loss of brain tissue in AD-affected brain is not fully understood. One theory is that the excessive release of excitatory amino acids, such as glutamate, is a key contributing factor leading to excitotoxicity and ultimately cell death (Walton and Dodd, 2007). Moreover, the development of the neuropathological inclusions associated with AD, including senile plaques and NFTs, are believed to play a key role in the neurodegenerative processes of AD.

Amyloid as a potential marker of pathology in AD was first documented in 1984 (Glenner and Wong, 1984), and later confirmed as the main component of senile plaques (Masters et al., 1985); it is now known that senile plaques are composed of a highly insoluble form of β-amyloid protein. The processing of β-amyloid, (altered due to various genetic mutations described later), formation of plaques and subsequent detrimental effects on brain function was collectively referred to as the "amyloid cascade hypothesis" (Hardy and Allsop, 1991).

Under normal, non-pathological conditions, amyloid precursor protein (APP), a membrane-bound protein, is cleaved by the enzyme α-secretase to release C83, which is subsequently cleaved by γ-secretase releasing soluble peptide fragments which are non-pathological. However, in the pathological state of AD, APP is cleaved by β-secretase to release the peptide C99, which is

subsequently cleaved by γ-secretase leading to the eventual aggregation of neurotoxic β-amyloid peptide which is fibrillogenic and forms β-amyloid plaques. β-amyloid can vary in length but is commonly 40 ($A\beta_{1-40}$) or 42 ($A\beta_{1-42}$) amino acid residues in length, figure 1.1 (Nordberg, 2004). Typically, $A\beta_{1-40}$ is the more common variant, unlike the rarer $A\beta_{1-42}$ residue which aggregates more readily to form β-amyloidal deposits associated with the disease.

Although there is evidence demonstrating that β-amyloid has a detrimental effect on neurons *in vitro* (Yankner et al., 1990; Malouf, 1992; Pike et al., 1993), possibly through enhancing vulnerability to insults (for review, (Cotman et al., 1992)), the role of β-amyloid in the neurodegenerative processes observed *in vivo,* during states of pathology, is unclear. One proposed mechanism is that β-amyloid can modulate cellular calcium homeostasis by affecting the function of synaptic ion channels. Indeed, calcium dysregulation is a clinical feature of AD and can possibly lead to synaptic loss (for review, (Green et al., 2007)). Under normal conditions, APP cleavage products are secreted during neuronal function (Nitsch et al., 1993), but such products may act to suppress neuronal activity given application of β-amyloid to cultured rat hippocampal cells reduced long-term potentiation (Lambert et al., 1998); therefore, β-amyloid secretion following synaptic activity might act via negative-feedback thus attenuating synaptic activity. Synaptic deregulation due to APP cleavage products, and subsequent impaired memory formation, may be due to the ability of soluble APP to activate potassium channels, which would further suppress synaptic transmission (Furukawa et al., 1996). Some reports have indicated a direct correlation between β-amyloid levels and brain

atrophy levels (Archer et al., 2006) and, with cognitive performance in AD sufferers (Naslund et al., 2000), these findings are not consistent, given that β-amyloid levels have been shown not to be correlated to brain atrophy (Josephs et al., 2008). It is worthy of note that β-amyloidal deposits are not exclusive to AD; for instance, inclusions have been identified in Parkinson's disease (Braak and Braak, 1990). It remains unclear, however, whether β-amyloid in AD, and other neurological disorders, acts as a cause, or a result of the brain atrophy.

Under normal conditions tau proteins stabilise microtubules within neurons. During certain pathological states tau can become hyper-phosphorylated forming the intracellular inclusions NFTs. Tauopathies, a term used to describe neurological disorders associated with the formation of NFTs, include AD (Iqbal et al., 2005), progressive supranuclear palsy (Webb et al., 2008) and Downs syndrome (Hanger et al., 1991). The role that NFT formation plays in the pathogenesis of these disorders is not fully understood. Interestingly, NFTs are not expressed during states of disease only, but occur during normal aging (Geschwind, 2003), as are β-amyloid plaques (Coria et al., 1987), which, together, suggest that disorders like AD may represent an accelerated form of normal aging.

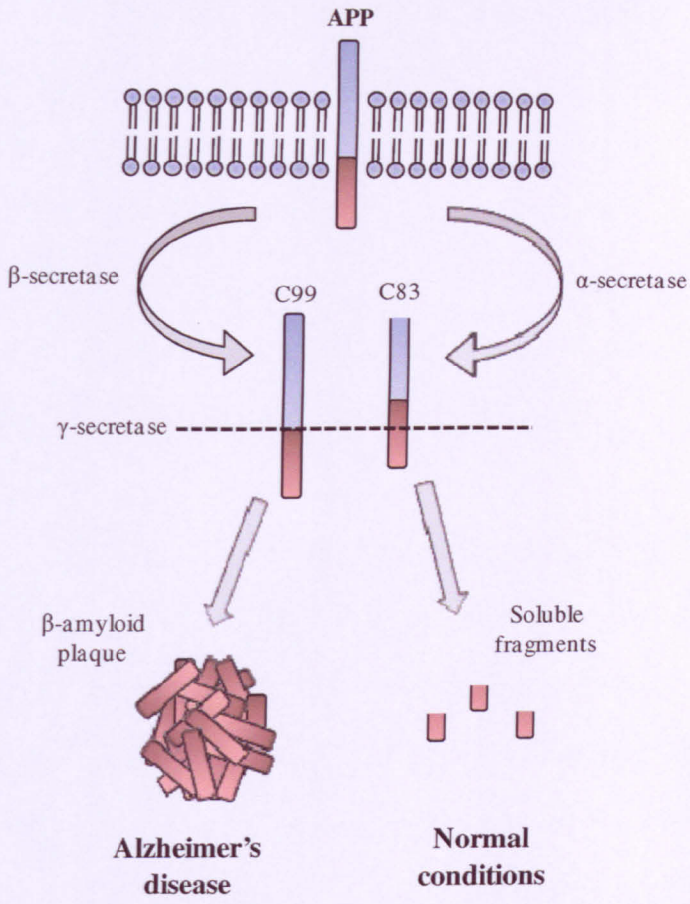


Figure 1.1. Processing of APP to release neurotoxic β-amyloid. Membrane-bounded APP can be cleaved by either β- or α-secretase, releasing peptide fragments C99 and C83 respectively. These are subsequently cleaved by γ-secretase to release β-amyloid and soluble peptide fragments from C99 and C83 respectively. β-amyloid aggregates forming neurotoxic β-amyloid plaques, a characteristic pathological marker of AD.

The significance of the roles played by β-amyloid and tau in AD is a matter of constant debate and a traditional division of opinion lead to two beliefs, first, that β-amyloid was principally responsible (those in support of this hypothesis were termed "bap-tists"), or second, that tau is the main cause of the disease

(those in support of this hypothesis were termed "tau-ists") (Mudher and Lovestone, 2002). Despite β-amyloid and tau receiving much attention from researchers, other pathological marker proteins have been associated with AD; for instance the protein ubiquitin has been found associated with NFTs in AD (Lennox et al., 1988), the pre-synaptic protein α-synuclein has been observed in an aggregated state in AD (Arai et al., 2001) and the nuclear protein p62 has been found co-localised with NFTs (Kuusisto et al., 2002).

Having introduced the major pathological markers found in AD sufferers and the hypothesis that the deposition of classical inclusions occurs in an accelerated aging-like manner, it is now important to establish the endogenous difference between those unaffected and those who suffer from AD. We know that some genetic factors can make individuals prone to developing AD and the relevance of these factors will be discussed in the following sections.

1.1.3. Genetic basis of AD

There are, principally, two forms of AD classified by the age of disease onset. The more rare variant comprising 5-10% of AD cases, named "early-onset", is typically developed under the age 65 years old. "Late-onset" or "sporadic" AD is observed in sufferers over 65 years old, and is the more common form of AD, comprising approximately > 90% of cases (Strobel G., 2005; Alzforum, http://www.alzforum.org/eFAD/overview/essay2/default.asp).

Around half of patients suffering from early-onset AD possess a hereditary form of AD known as "familial" AD. Several genes which are mutated in familial AD have been identified; the first description of a missense mutation on chromosome 21, adjacent to the APP gene, possibly being linked with AD was in 1991 (Goate et al., 1991). On the APP gene itself, there are mutations at positions 692/693 leading to decreased α-secretase cleavage, positions 670/671 causing enhanced β-secretase cleavage, and 717 resulting in enhanced γ-secretase cleavage, all resulting in enhanced production of pathological β-amyloid (Rubinsztein, 1997). Presenilins, proteins which are part of the complex forming γ-secretase, also pose genetic risk factors for developing AD, first described in 1996 (Scheuner et al., 1996). Mutations on chromosome 14 and chromosome 1 have been linked with mutated presenilin 1 and 2 (PS-1 and PS-2) respectively (Rubinsztein, 1997).

Until recently, there was only one known genetic risk factor for the "late-onset" AD. Apolipoprotein (apoE), involved in lipid metabolism, has three splice variants, apoE2, apoE3 and apoE4. Those expressing the apoE4 allele are at a higher risk of developing AD, as first described in 1993 (Corder et al., 1993; Saunders et al., 1993), and have higher β-amyloid levels (Riemenschneider et al., 2000). Interestingly, those with apoE2 have a lowered risk suggesting this allele provides some protective effect (Corder et al., 1994). It has been suggested that the negative effects of possessing apoE4 are actually due to not possessing the more protective apoE3 or E2 alleles (Esler et al., 2002). More recent studies, however, have revealed novel genetic risk factors which are likely to be associated with sporadic AD, including CLU (the gene

which encodes clusterin), PICALM and the complement component receptor 1 (CR1) (Harold et al., 2009; Lambert et al., 2009).

A better understanding of this area of AD genetics may aid identification of those at risk of developing AD in later life, and such improved diagnosis will in-turn lead to earlier treatment of the disorder. Much research has been put into improving the diagnostic tools used to detect AD at the early stages to allow for early pharmacological intervention. These methods of diagnosing AD, and the current status of treatments are now discussed.

1.1.4. Diagnosis and treatment

The clinical stages of AD are believed to be preceded, in many cases, by mild cognitive impairment (MCI), but a comparably lower level of cognitive decline, affecting 5-6% of the aged population. Other strategies are aimed at recognising and identifying MCI due to the damage that occurs to the brain at this stage before progression to AD, and the high transfer rate; one study showed 41% of MCI sufferers progress to AD (Geslani et al., 2005). One of the major problems is the lack of, or misdiagnosis of AD, given that complete confirmation of AD at present, requires post-mortem identification of the pathological markers described in Section 1.1.2. Current diagnosis of AD does not rely on one single test, instead a battery of tests is commonly used to determine pathological status.

One of the first clinical manifestations of AD is a disturbance in episodic memory performance (the ability to recall specific details, such as events or facts), (Backman et al., 2001), followed by a gradual decline in all cognitive domains. Therefore, one of the first stages involves assessment of cognitive performance through memory tests including the Clinical Dementia Rating, Mini Mental State Examination and the Functional Assessment Staging, the outcomes of which have been proven to correlate with AD-associated pathological changes (for review, (Thind and Sabbagh, 2007)). Despite this reasonably robust correlation between cognitive performance and disease status, more specific tests for direct, quantifiable measures of pathology, for instance, measuring levels of biomarkers in both the periphery and CNS, would present a useful avenue to aid diagnosis (for review, (Borroni et al., 2006)).

Measuring β-amyloid, and APP forms, in both the blood plasma (Sundelof et al., 2008), and platelets (Borroni et al., 2003), respectively, has been shown to be capable of predicting the development of AD. Alterations in CSF β-amyloid have been identified; a lower $A\beta_{1-42}$ level was found in AD sufferers (Riemenschneider et al., 2000), possibly due to an ineffective clearance of the peptide from the brain. Potential biomarkers are not exclusive to β-amyloid-related systems, but the total levels of tau protein in the CSF of sufferers of MCI may act as a predictor of AD (Maruyama et al., 2001). Despite evidence for such biomarker-aided diagnosis of AD, these approaches are still at the evaluation stage. As mentioned above, diagnosis often requires a variety of tests, and imaging of the brain has been used to identify early changes as markers of the disease state.

Two commonly used, and extensively researched, imaging methods to study AD pathology include positive emission tomography (PET) and magnetic resonance imaging (MRI). The principle of the former, PET, involves the introduction of a tracer that binds and allows the identification of the location of the protein of interest. Several compounds have been produced which bind to brain β-amyloid, and these have been tested in AD sufferers. The aim of these is, therefore, to quantify β-amyloid in a living subject (for review,(Nordberg, 2004)). MRI is a powerful tool capable of identifying early, non-invasive biomarkers of AD, and also allowing for monitoring disease progression. Given that it is recognised that reductions in particular brain region volumes occur in AD patients, structural MRI may predict the progression from MCI to AD (Karas et al., 2008). Further studies into morphological changes in AD and MCI sufferers may provide a better knowledge of surrogate markers of disease, thus aiding disease diagnosis (for review, (Kantarci and Jack, 2004)). A detailed description of MRI, and its use as a tool for studying AD-associated pathologies can be found in Section 2.3.

As mentioned above, research is put into improving the diagnosis of AD in order to provide appropriate therapeutic intervention as early as possible. At present there is no cure for AD; however, agents designed to improve symptoms of AD are currently used. The classes of treatment available, and currently being researched, are described below (Helmuth, 2002). The most common therapy at present targets neurotransmitter systems which are affected in AD. For example, cholinesterase inhibitors, such as donepezil, slow the breakdown of the neurotransmitter acetylcholine, levels of which are decreased

in AD (Garcia-Alloza et al., 2005). Pharmacological routes to target neurotransmitter systems are not exclusive to the cholinergic pathways, drugs which block the receptors for glutamate, such as memantine, aim to prevent the detrimental effects of excitotoxicity due to elevated activity of these receptors in states of AD (Wenk, 2006). Treatment with donepezil and memantine have been shown to improve the symptoms of AD (Evans et al., 2000; Reisberg et al., 2003). Another potential therapy involves compounds which can inhibit the β and γ-secretases, thus lowering the production of pathological β-amyloid. Such secretases modulators are in clinical trials (Wolfe, 2008). Furthermore, metal ions such as copper and zinc in the CNS are thought to exacerbate AD through interactions with β-amyloid and chelators of these ions, reducing free ion concentrations, could provide a novel therapeutic strategy (for review ((Cuajungco et al., 2000)). There is, also, evidence to suggest that high levels of cholesterol are a risk factor for AD; statins, drugs which lower cholesterol, could potentially lower the risk of AD. However, there is insufficient evidence of any therapeutic value of statins at present (Eckert et al., 2005). Another promising area of treatment is through immunisation against β-amyloid. Immunotherapy can be achieved actively, or passively. Active immunisation involves inducing the body's immune system to target β-amyloid reducing its deposition, whereas passive immunotherapy involves constant exposure to an exogenous antibody to target β-amyloid and lower its aggregation (Wisniewski and Konietzko, 2008). Unfortunately there were problems associated with early clinical studies of active immunotherapy with the unexpected development of meningoencephalitis in a small proportion of the subjects; this, however, has

not impeded more recent progress as both passive and active immunotherapies are currently in clinical trials (Brody and Holtzman, 2008).

Research into effective diagnosis and therapies has been dependant largely on gaining a better understanding of the mechanisms underlying AD pathology. Much of what we know about such mechanisms relies upon the various animal models developed. These models provide the means to test novel therapeutic strategies, as mentioned above, pre-clinically prior to taking compounds into the clinic. The various animal models that are currently available to study AD-associated pathology will be discussed in the next section. The extent to which the models recapitulate features of AD, and how the models can be used to test disease-modifying agents, will also be addressed.

1.2. Animal Models of AD

To study the disease pre-clinically pathological features of AD must be replicated. Animal models of AD expressing one or more of these features have been developed for this purpose. As one would expect, normal laboratory animals exhibit age-related cognitive deficits (Kadar et al., 1990; Shukitt-Hale et al., 2004; von Bohlen und Halbach et al., 2006), but they do not exhibit AD-associated pathologies, such as β-amyloid or tau-based inclusions, even very late in life (Dayan, 1971). Therefore normally aging animals do not directly model this disease state. It is, therefore, necessary to turn to animals which have had some form of manipulation to induce a disease-like phenotype.

Below, the range of animal models currently used to study AD-like pathology is discussed; these have been divided into three groups:

1) Animals which have site-specific neurotoxic brain lesions to induce cognitive deficits.

2) Models in which β-amyloid has been exogenously introduced into the brain.

3) Genetically manipulated animals which express AD-associated pathologies endogenously.

The validity of these three approaches have been summarised in Table 1.1.

1.2.1. Brain lesion animal models

Selective lesions to particular brain regions can induce cognitive impairments; for instance, bilateral lesions to rat entorhinal cortices with the excitatory amino acid ibotenate impaired spatial memory performance in the Morris Water Maze (Eijkenboom et al., 2000) and excitotoxic lesions to rat hippocampus result in conditional learning deficits and partial impairment in visual discrimination learning at the Y-maze paradigm (Murray and Ridley, 1999). Interestingly, interactions have been observed when lesioning areas with cholinergic system involvement, which had subsequent effects on the levels of APP production. Selective lesions to the neocortex and hippocampus induced elevated expression of APP in these areas which provides evidence of possible links between neurodegeneration, cholinergic systems and APP expression in

AD (Leanza, 1998). Despite this subtle link between lesions and APP levels, this technique does not directly result in the production of the main pathological markers of AD, β-amyloid plaques and NFTs, and only mimics neuronal loss that occurs in AD. In this respect, the model is of little value when attempting to study or manipulate the pathological mechanisms underlying AD, although donepezil partially reversed cognitive deficits induced by brain lesions in the rat demonstrating some predictive validity (Spowart-Manning and van der Staay, 2005).

Another issue which needs to be addressed is the speed at which the brain damage occurs. During lesioning, the destruction of brain tissue occurs acutely, that is, shortly after the manipulation has taken place, which is not modelling the gradual long-tem loss of brain tissue that occurs in AD. Brain lesions therefore provide the means to study the effects of loss of brain material, although studies with this model appear to be modelling brain damage rather than AD *per se*.

	Face validity	Predictive validity	Construct Validity
Lesion-based animal models	• Cell death • Cognitive deficits[1]	• Neurotransmitter system-targeting drugs improve cognition[2]	• Model cell loss that occurs in AD • Not due to AD-specific mechanisms
β-amyloid-infusion animal models	• Enhanced Aβ presence in the brain[3] • Cognitive deficits[4] • Mild cell death, no gross neurodegeneration[3]	• Neurotransmitter system-targeting drugs improve cognition[5]	• Presence of Aβ in the brain, but often not a gradual deposition • Aβ -induced cell death and cognitive impairments
β-amyloid over-expressing transgenic mouse models	• Aβ plaque formation[6] • Progressive accumulation of Aβ[7] • Cognitive deficits[8] • Mild cell death, no gross neurodegeneration[9] • Characteristic markers of pathology also present, such as tau associated with Aβ[10]	• Neurotransmitters system-targeting drugs improve cognition[11] • Secretase-inhibitors lower Aβ[12] • Immunotherapy ameliorates phenotype[13]	• Gradual deposition of Aβ in the brain • Aβ -induced cell death and cognitive impairments

Table.1.1. An evaluation of existing animal models of AD. Lesion-based, β-amyloid-infused and β-amyloid over-expressing transgenic animal models are compared with regard to three factors assessing their validity. Criteria of validity used is described in (Willner and Mitchell, 2002). First, face validity, the way the model expresses symptomology similar to AD. Second, predictive validity, how known drugs to treat AD affect pathology and the ability to predict clinical effectiveness of novel therapeutic agents. Third, construct validity, what is the theoretical link with AD. References within the table: [1](Pepeu et al., 1986), [2](Santucci et al., 1991), [3](Frautschy et al., 1991), [4](Alvarez et al., 1997), [5](Yamada et al., 2005), [6](Quon et al., 1991), [7](Shoji et al., 2000), [8](Moran et al., 1995), [9](Games et al., 1995), [10](Sturchler-Pierrat et al., 1997), [11](Van Dam and De Deyn, 2006), [12](Chang et al., 2004), [13](Morgan et al., 2000).

1.2.2. β-amyloid-infusion animal models

The toxicity of β-amyloid protein has been demonstrated by its direct introduction to the brain via a single intracerebroventricular (icv) injection, or though continuous infusion using an osmotic mini-pump, to induce memory impairments in rodents (Nakamura et al., 2001; Stepanichev et al., 2004). An early investigation demonstrated that continuous infusion of β-amyloid into the cerebral ventricles induced deficits in spatial memory in the Morris Water Maze test (Nitta et al., 1994). Also, administration of β-amyloid peptide into rodent brains has been shown to cause damage to neural tissue. Moreover, it has been shown that a bilateral icv administration of aggregated β-amyloid induced decreased neuronal density, specifically in the CA1 region of the hippocampus, which was coupled with working and reference memory impairments in the eight-arm radial maze (Stepanichev et al., 2004). Moreover, rats exposed to icv-infused β-amyloid peptides showed a higher level of cognitive impairment 80 days after administration, which suggests that this model exhibits a slower, progressive decline in various behavioural paradigms (Nakamura et al., 2001).

Rats infused with β-amyloid, then treated with either donepezil or memantine, demonstrated improvements in deficits in the delayed-matching-to-position paradigm (Yamada et al., 2005). Despite the benefits of using β-amyloid to replicate this disorder in animals it only models one aspect of AD, for instance, hyperphosphorylated tau, and thus NFTs, are absent. It is certainly arguable that, in this model, a continuous infusion of β-amyloid provides a more

accurate reflection of AD compared to a single injection (Nitta et al., 1994). However, even this style of β-amyloid administration does not replicate the gradual, increasing deposition that starts from a relatively early age in clinical AD, and the effects of the rapid, sudden appearance of β-amyloid in the brain is likely to produce adverse effects which do not occur in AD.

1.2.3. Genetically manipulated animal models

As described in Section 1.1.3, genes have been identified which are implicated in the development of early-onset, familial AD. Knowledge of the genetics of AD has been used to model AD-like pathology in transgenic mice. The preclinical study of β-amyloid-associated pathologies was revolutionised in 1991 when the first genetically engineered animals were developed to endogenously over-express mutated human APP and, thus, exhibit extensive β-amyloid pathology in the form of β-amyloid plaque formation (Quon et al., 1991). One of the earlier investigations in this area reported that these "β-amyloid overexpressing" transgenic mice developed deficits in alternation and spatial memory in the Y-maze and Morris Water Maze tests respectively, which correlated with β-amyloid plaque pathology (Hsiao et al., 1996). Interestingly, spatial memory deficits in the Morris Water Maze have been shown to precede β-amyloid deposition in transgenic mice (Van Dam et al., 2003). A distinct advantage that transgenic mice have over β-amyloid-infused animal models is the slower progressive deposition of β-amyloid, which does not require the animals to undergo surgery.

Transgenic mouse models of AD over-expressing β-amyloid are most commonly either singly transgenic, over-expressing mutated human APP (Quon et al., 1991), or double transgenic, achieved by crossing transgenic mice expressing APP with mice over-expressing mutated PS-1 (Borchelt et al., 1997). Triple transgenic mice have been developed which are positive for APP and PS-1 and tau mutations; these mice exhibit abnormalities in unconditioned behaviours such as altered startle responses and hyperactivity (Pietropaolo et al., 2008). Studies carried out by Borchelt et al. used the double mutant APP x PS-1 transgenic mice to demonstrate an elevation of β-amyloid plaques. 9 month old APP x PS-1 were comparable to singly transgenic APP at 18 months of age (Borchelt et al., 1997). These double, or triple, transgenic mice with two, or more, traits have more face validity (a measure of how well the disease is mimicked) given that they express more of the characteristics of the disease. In addition, multiple transgenic mice are useful to researchers given their higher level of β-amyloid pathology, making studies aimed at reducing β-amyloid levels easier. This also reduces the age of β-amyloid pathology onset, and thus reducing the length of studies and making them more ethically sound and economically attractive.

Such β-amyloid over-expressing mice provide effective tools in aiding research into improved diagnosis. For instance, they are used for optimising MRI as a diagnostic tool. Much work has been done to resolve individual plaques *in vivo* (Jack et al., 2005), as described in more detail in Section 2.3. β-amyloid-over-expressing transgenic mice have also proved to be useful when screening for therapeutic agents and appear to demonstrate a degree of predictive validity;

for instance, β-secretase inhibitors lower β-amyloid levels in transgenic mice (Chang et al., 2004). One such β-secretase inhibitor is currently in clinical trials (Ghosh et al., 2008). Moreover, β-amyloid immunotherapy improved cognition and lowered β-amyloid pathology in an β-amyloid-over-expressing mouse model (Wilcock et al., 2004). Other evidence supporting the predictive validity of the transgenic mouse model exists. Memantine and donepezil, drugs used to treat AD which antagonise the glutamatergic and agonise the cholinergic system respectively, improved performance at the Morris Water Maze memory test in a β-amyloid-over-expressing mouse line (Van Dam and De Deyn, 2006; Van Dam et al., 2008).

Given that AD is a disease which expresses several pathological features, it is beneficial if an animal model expresses more than one pathological marker. Transgenic mouse models over-expressing β-amyloid have been found to have tau-positive cells which surround β-amyloid plaques in doubly transgenic mice (Samura et al., 2006). Also, some studies have demonstrated cell loss in double transgenic mouse models over-expressing β-amyloid (Casas et al., 2004; Howlett et al., 2008; Liu et al., 2008). However, it has been argued that β-amyloid expressing transgenic mouse models of AD are incomplete given that they lack key aspects of the disease, such as NFT formation (Schwab et al., 2004) and neurodegeneration (Stein and Johnson, 2002). Another issue that is worthy of note is that transgenic mice currently available which over-express mutated APP and PS-1 model the rarer familial form of AD, whereas the vast majority of human cases are of the sporadic variety. Despite this, the closest model available to researchers at this time is the double, or triple, transgenic

mouse model, expressing two or more known genetic risk factors for AD and providing information on β-amyloid-associated cognitive dysfunction. These models have improved our understanding of the pathological mechanisms underlying AD and have provided the means to screen potential therapeutic agents pre-clinically.

The principles of AD, its pathologies, how it is diagnosed and current therapies to combat its symptoms have been described above. A variety of animal models have been introduced and their relevance in modelling AD has been discussed. The conclusion was then drawn that the most effective model of β-amyloid-based pathology seen in AD, in terms of validity, was the transgenic mouse model over-expressing β-amyloid. This thesis deals with interactions between environmental and pharmacological interventions, and β-amyloid pathology in the β-amyloid over-expressing mouse line, TASTPM. The next section provides a description of what is currently known about TASTPM mice.

1.2.4. The TASTPM mouse line

This mouse line was created by crossing TAS10 mice, positive for human mutated APP (hAPP695swe), with TPM mice, over-expressing a PS-1 mutation (M146V); the resulting mouse line is the double transgenic (APP x PS-1) TASTPM mice (Howlett et al., 2004). Cognitive impairments have been observed in TASTPM mice from 6 months of age which correlates with increasing brain β-amyloid pathology. Co-expression of PS-1 mutation with

APP accelerates the onset of the pathology as levels of β-amyloid pathology between 6 and 8 months of age were comparable to those observed in 16 month old singly transgenic TAS10 mice (Howlett et al., 2004). Disturbances in unconditioned behaviours have also been described in the TASTPM mouse including heightened aggression, lowered body weight and hyperactivity, which are reminiscent of abnormalities observed in clinical AD (Pugh et al., 2007). Further investigation, using microdissection, into the composition and localised effects of β-amyloid plaques revealed phosphorylated tau and localised cell loss surrounding the inclusions (Howlett et al., 2008).

The TASTPM mouse recapitulates β-amyloid-based pathologies seen in AD and bears a resemblance to other psychological factors associated with the disorder, and is, therefore, an appropriate model for the studies described in later chapters. Given that this thesis deals with the issues of the effects of environmental manipulations on β-amyloid-associated pathologies in a transgenic mouse model of AD it is important, firstly, to introduce and describe interventions such as exposure to a stressful or an enriched environment.

1.3. The stress response and environmental enrichment

1.3.1. The stress response

The definition of stress is the strain placed on an individual resulting in subsequent adaptive physiological changes, or responses, which can be either beneficial (resulting in enhanced performance), or harmful potentially leading

to the development of psychiatric disorders (for review, (Ehlert and Straub, 1998)). Stressors in the laboratory rodent commonly include environmental manipulations, such as restraint, forced-swim, exposures to temperature outside of the normal range and exposure to novelty. Below, the stress response is introduced and the beneficial and detrimental effects of stress reviewed.

During states of stress the hypothalamus releases corticotrophin releasing hormone (CRH) which acts upon the pituitary gland. which subsequently releases adrencorticortropic hormone (ACTH) into the blood. This activates the adrenal glands which secrete the corticosteroid cortisol in humans or corticosterone in laboratory rodents – these corticosteroids belong to the glucocorticoid (GC) family (Papadimitriou and Priftis, 2009). Corticosterone is responsible for mediating many of the physiological responses to stress; for example, increasing core body temperature and normal weight gain in rodents (Bhatnagar et al., 2006), as well as providing negative feedback to the pituitary and hypothalamus to "switch-off" the pathway. Collectively this pathway is known as the hypothalamic-pituitary-adrenal (HPA) axis, figure 1.2.

As described above, acute stressors induce a transient rise in GCs which is rapidly reversed after the stressor has been removed; this can actually be beneficial in humans by improving memory performance (Duncko et al., 2007; Smeets et al., 2007). This effect of acute stress has also been observed in the rat (Bangasser et al., 2005), and the mouse (Weiss et al., 2005). Long-term inappropriate or uncontrollable stress, such as a stressful working life, is linked with the development of affective disorders such as depression (Pittenger and

Duman, 2008), and has been shown to impair memory performance in humans (Lupien et al., 1998), the rat (Conrad et al., 1996; Mizoguchi et al., 2000) and the mouse (Song et al., 2006).

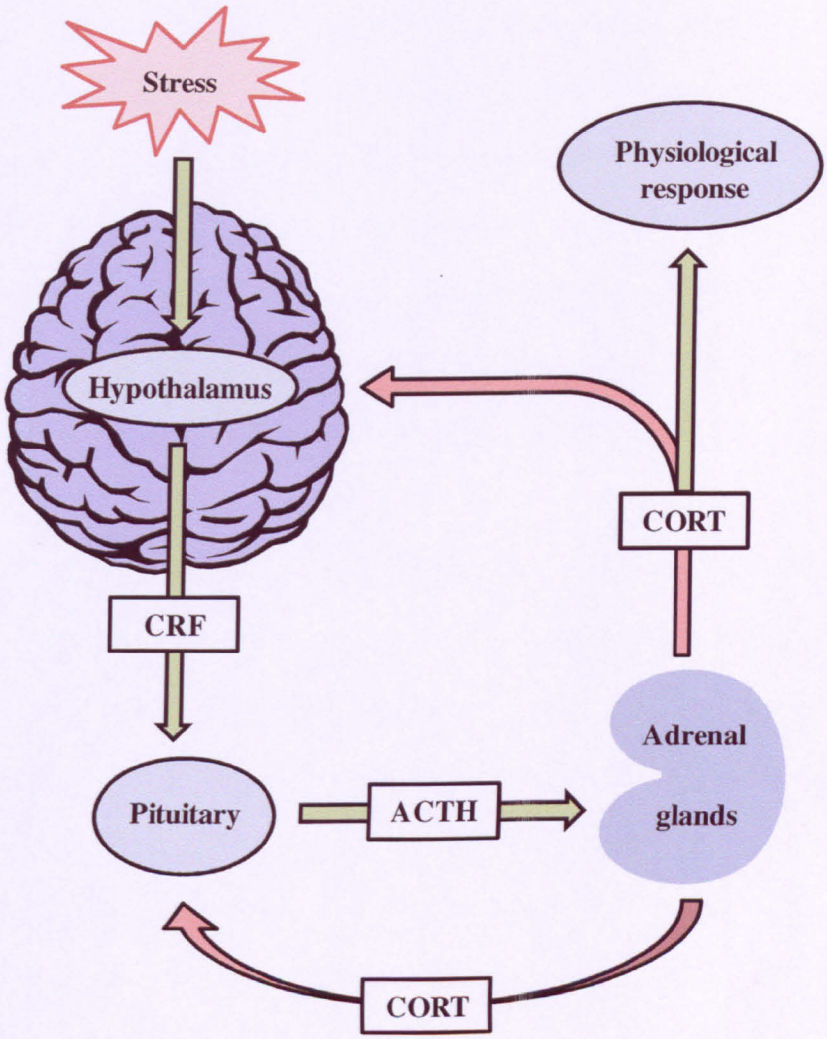


Figure 1.2. HPA axis. The hypothalamus releases corticotrophin-releasing hormone (CRH) in response to emotional or environmental stressors. Upon activation by CRH, the pituitary gland secretes adrenocorticotropin hormone (ACTH) into the blood which acts on the adrenal glands on the kidneys. These adrenal glands release cortisol or corticosterone (CORT) which induces various physiological responses to stress and acts to inhibit activity of the hypothalamus and pituitary in a negative-feedback loop. Green arrows represent activatory pathways, red arrows indicate inhibitory pathways.

The effects of stress, and thus GCs, on memory performance are largely dependent on its impact on the hippocampus. The hippocampus provides negative regulatory control over the HPA axis and, due to its large concentration of GC receptors, is sensitive to the presence of cortisol/corticosterone (for review, (Kim and Diamond, 2002)). One suggestion is that a brief elevation of corticosterone, following acute stress provides stimulation to the hippocampus, thereby enhancing performance in hippocampal-dependant memory tasks (Beylin and Shors, 2003). However, long-term exposure to GCs can cause hippocampal atrophy possibly leading to further dysregulation of the HPA axis (for review, (Raber, 1998)).

Interestingly, stress appears to have a negative impact on normal aging (for review (Pardon and Rattray, 2008)). Moreover, changes in the function of the HPA axis occur during aging (for review (Meaney et al., 1995)). Much evidence in this field focuses on the effects of early-life/post-natal stress on cognitive impairments which occur as part of the normal aging process (see (Pardon and Rattray, 2008) and references therein). However, evidence to suggest that mid-life stress can exacerbate age-related cognitive impairments is sparse; one particular study demonstrated that chronic unpredictable stress, applied during adulthood in the rat, worsened the age-related decline in spatial cognition that occurred later in life (Sandi and Touyarot, 2006). It is clear from such existing studies that stress can interact negatively with the formation of age-related cognitive impairments.

An important factor to consider when looking at the positive versus the negative effects of stress is the intensity of the stressor. Severe or traumatic stressors are thought to have more detrimental effects to cognitive function such as amnesia (for review, (Joseph, 1999)), whereas mild stressors are believed to be beneficial in aging (for review, (Minois, 2000)), probably by providing the stimulation needed for enhanced attention. The boundaries dividing a mild stressor, and thereby enriched environment, (a manipulation which is generally beneficial in various states of pathology (for review see, (Laviola et al., 2008)), is sometimes poorly defined. There appears to be, at least, some overlap between mild stressors and environmental enrichment which is thought to be beneficial in disease states – the principles of these beneficial effects of enrichment will now be briefly described.

1.3.2. Environmental enrichment

A large body of evidence exists to indicate a positive effect of an active life-style, including physical exercise cognitive function in normal aging (Anstey and Christensen, 2000; Newson and Kemps, 2005; Angevaren et al., 2008). Moreover, exercise has been proved to improve symptoms in sufferers of psychiatric disorders such as depression (for review, Environmental enrichment has been shown to improve cognitive deficits induced by brain damage in the rat (Passineau et al., 2001), and age-related cognitive impairments in the mouse (Frick et al., 2003).

Several lifestyle risk factors are thought to modulate the risk of developing AD including diet, exercise and mental activity (for review, (Weih et al., 2007)). Stress and environmental enrichment both interact with psychiatric disorders and memory performance, as described above, and the interactions of these environmental manipulations with pathologies associated with AD, both clinically and pre-clinically, are reviewed in the next section.

1.4. Environmental manipulations and AD-related pathology

1.4.1. Stress and AD-associated pathologies

There is emerging evidence supporting a role of stress as a risk factor in the development of age-related neurodegenerative disorders, including AD. As described above, a wealth of evidence exists associating stress with, for instance, hippocampal dysfunction and associated memory loss, supporting the hypothesis of a role of stress in the development of dementia. Indeed, individuals likely to experience stress are at over double the risk of developing AD, and experience a more rapid decline in cognitive performance and worse episodic memory deficits during AD (Wilson et al., 2003; Wilson et al., 2004; Wilson et al., 2005; Wilson et al., 2006). Proneness to suffering distress throughout life was also found to be associated with increased incidence of developing MCI (Wilson et al., 2007). Moreover, a study revealed that aged, non-demented subjects who experience stress, and harbour the apoE4 allele, perform worse at certain memory tests (Peavy et al., 2007), although expression of apoE4 was not found to be associated with distress proneness

(Wilson et al., 2007). This association between a stressful life and increased risk of AD does not appear to correlate directly with characteristic pathological markers such as β-amyloid plaques and NFTs (Wilson et al., 2003; Wilson et al., 2006; Wilson et al., 2007).

This relationship is also supported by studies examining the effects of emotional and environmental stressors on AD-associated pathologies in animal models of the disorder, these are summarised in Table 1.2. Chronic mild psychological stress was found to accelerate the age-dependent accumulation of cortical and hippocampal β-amyloid plaques, soluble β-amyloid in hippocampal interstitial (ISF) fluid, and memory deterioration in β-amyloid over-expressing transgenic mice (Dong et al., 2004; Kang et al., 2007). Moreover, repeated stress enhanced localised tau accumulation, β-amyloid plaques and degenerated neurons in the hippocampus of β-amyloid over-expressing transgenic mice (Jeong et al., 2006). Treatment with the synthetic GC dexamethasone in APP x PS-1 x tau triple transgenic mice enhanced β-amyloid levels and elevated tau accumulation (Green et al., 2006). In addition, treatment with the synthetic GC prednisolone, elevated β-amyloid levels in the cortex of the β-amyloid-infused rat brain, indicating a reduced clearance of β-amyloid (Harris-White et al., 2001).

Although, as mentioned in Section 1.2, normal rodents do not form AD-like pathology with physiological aging, the effects of stress on normal animals have provided information linked to changes observed in AD. For instance, severe acute and repeated stress enhanced localised APP mRNA production in

normal rat brain (Rosa et al., 2005). Unlike most rodents, non-human primates develop β-amyloid plaques as part of normal aging. Cortisol administration to macaque monkeys resulted in an increased $A\beta_{(1-42)}$:$A\beta_{(1-40)}$ ratio in the brain, and a reduction in plasma $A\beta_{(1-42)}$, changes similar to those observed in AD. However no effect on β-amyloid plaque deposition was observed (Kulstad et al., 2005). Changes with stress relevant to AD are not restricted to β-amyloid; acute and repeated stress in normal mice induced phosphorylation of tau protein, a precursor stage to NFT formation (Okawa et al., 2003; Feng et al., 2005; Ikeda et al., 2007), which appears to be mediated by CRF receptor signalling (Rissman et al., 2007). Despite such findings not being directly relevant to AD given the lack of associated pathology, they aid understanding of the mechanisms connecting stress and the formation of pathological markers similar to those in AD.

As described in Section 1.1.3, mutations in APP and PS are risk factors for the rare familial AD variant, but a known genetic risk factor in development of the common, sporadic late-onset AD is the possession of apoE4 allele. Variations in CSF cortisol levels in AD sufferers have been reported to be dependent on apoE genetic status, with subjects possessing the apoE4 allele presenting the highest CSF cortisol levels, while aopE2 expressers had the lowest cortisol levels (Peskind et al., 2001). Although links between the apoE genotype and effects of stress remain to be understood, it has been speculated that different variants of apoE may enhance the susceptibility to stress-related insults (Peavy et al., 2007). Despite transgenic mice expressing human apoE4 being available, no studies have looked into effects of stress on these animals to date.

Model / Age / Sex	Stressor	Behavioural end-point	AD-like pathological markers	Ref
Tg APP vs. Littermates, 6m, M & F.	Social isolation from weaning.	↓ contextual fear memory in stressed APP mice. = performance at cued fear conditioning.	↓ hippocampal cell proliferation, ↑ cortical and hippocampal Aβ plaque burden.	1
Tg APP, 11m.	Immobilisation 6h/day, 4 days/week, 8 months.	↓ passive avoidance and social transfer of food preference.	↑ hippocampal Aβ plaque burden, ↑ intraneuronal Aβ & tau phosphorylation & neurodegeneration in the hippocampus, entorhinal cortex and piriform cortex.	2
Tg APP vs. littermates, 3 - 4m, M & F	1) Isolation (3m). 2) Restraint (3h). 3) CRH infusion into hippocampus. 4) CRH antagonist into hippocampus + restraint. 5) TTX infusion into hippocampus, + restraint. 6) CORT 50mg/kg.	NR	1, 2, 3) ↑ Aβ levels in hippocampal ISF. 4) ↓ stress-induced rise in ISF Aβ. 5) ↓ hippocampal ISF Aβ with TTX, = with TTX + stress. 6) = Aβ in hippocampal ISF. 1) = $A\beta_{1-42}$:$A\beta_{1-40}$ ratio. 1, 2) = full-length APP. 1) = CRH level. 2) ↑ CRH level.	3
Triple tg (APP x PS-1 x tau), 4 & 13m, M.	1) Dexamethasone, 1 mg/kg/day i.p. for 7 days. 2) Dexamethasone, 5 mg/kg/day i.p. for 7 days.	NR	**4 months old:** 1) ↑ soluble $A\beta_{1-42}$, = insoluble Aβ. 2) ↑ soluble $A\beta_{1-40}$, soluble and insoluble $A\beta_{1-42}$, ↑ intracellular Aβ, ↑ Tau levels, = Tau phosphorylation . **13 months old:** 2) ↑ insoluble Aβ42 & Aβ40, ↑ tau cortical accumulation, = tau hippocampal accumulation, = tau hyperphosphorylation	4

Table 1.2. Summary of preclinical studies into stress and GCs on transgenic mouse models of AD. This table summarises the links between emotional stressors, or models of emotional stressors by introduction of exogenous GCs, and AD-associated pathology. ↑= increases, ↓= decreases, (=)= no effect, WT= wild-type, m= months, M= male, F= female, w= weeks, h= hours, CRH= corticotrophin releasing-hormone, tg = transgenic, TTX= tetrodotoxin, CORT= corticosterone, ISF= interstitial fluid, NR= not reported. [1](Dong et al., 2004), [2](Jeong et al., 2006), [3](Kang et al., 2007), [4](Green et al., 2006).

Dysfunctional allostasis of the HPA-axis is a widely accepted clinical feature of AD (for review, (Pomara et al., 2003)). Although there are conflicting findings as to whether transgenic mice expressing β-amyloid exhibit HPA-axis dysregulation some studies have reported enhanced measures of basal corticosterone levels in APP over-expressing mice (Touma et al., 2004). This, however, is not a consistent observation (Gil-Bea et al., 2007). Elevated basal plasma corticosterone levels in APP x PS-1 x tau triple transgenic mice were observed from 9 months of age (Green et al., 2006). An altered stress response in APP over-expressing mice has been reported, appearing prior to the onset of plaque development, which is indicative of a detrimental role of soluble β-amyloid in HPA-axis dysregulation (Pedersen et al., 1999). HPA-axis hyperactivity and subsequent sensitivity in transgenic mice for AD may, therefore, be a modulating factor in the severity of the response to stress.

The limited number of pre-clinical and clinical studies into stress effects on AD does not yet allow for a good understanding of how stress can impact upon AD. Interestingly, stress effects on AD appear to occur apparently independently of neuropathological markers in humans, but enhance similar markers modelled in animals. There is an emerging link between stress and the production/degradation of β-amyloid; it is important to understand how GC responses to stress affect the β-amyloid cascade, along with other underlying mechanisms linking stress with AD since this may provide novel avenues for therapeutic intervention. For example, through targeting the GC stress response systems it might be possible to slow or prevent neurodegenerative diseases, an issue that has been recently discussed (Dhikav and Anand, 2007). An ongoing study of this type investigating the effects of the GC-antagonist mifepristone

could provide information on the therapeutic potential of targeting this system to enhance cognitive performance in AD sufferers (Belanoff et al., 2002).

As described in Section 1.3.1, stress can have a negative effect on cognitive function and, as described above, appears to exacerbate AD-associated pathology. Another environmental manipulation, enrichment can improve cognitive decline associated with normal aging, its effects on AD-like pathology will therefore be described briefly in the next section.

1.4.2. Environmental enrichment and AD-associated pathologies

Literature exists indicating that a lifestyle which involves more exercise, social and leisure activities lowers the risk of developing dementia or AD (Fabrigoule, 2002; Fratiglioni et al., 2004; Perez and Cancela Carral, 2008). Furthermore, heightened education levels were seen to be negatively correlated with AD severity (Roe et al., 2008). The notion of living in an "enriched environment" lowering the risk of AD is supported by studies with β-amyloid over-expressing transgenic AD mouse models. It has been reported that environmental enrichment, such as access to several play items, attenuated cognitive impairments (Jankowsky et al., 2005) and reduced β-amyloid pathology (Lazarov et al., 2005) in a double transgenic mouse model of AD. In addition, one study using β-amyloid over-expressing single transgenic mice showed that exposure to home cages enriched with items to encourage exercise and stimulation improved anxiety-related behaviour in the elevated plus maze test (Gortz et al., 2008). Although this connection is not fully understood, one likely explanation for these effects is the associated increased level of

enhanced exercise which has been shown to lower brain β-amyloid in transgenic mice (Adlard et al., 2005), and to improve performance in a variety of behavioural tests in normal mice (Clark et al., 2008). Interestingly, corticosterone was elevated in mice as an acute response to exercise (Girard and Garland, 2002). As discussed above in Section 1.3.1, mild or acute stressors can improve brain function. Additionally, the evidence cited above supports a potential link between HPA-axis function and environmental enrichment, ultimately providing benefit to cognitive function.

There is significant evidence, summarised above, to suggest that changing lifestyle appears to be capable of modulating the pathologies associated with AD. Being subject to inappropriate / uncontrollable or living a stressful lifestyle appears to worsen AD-associated pathologies, whereas an active lifestyle with an appropriately stimulating environment seems to provide some benefits in relation to the disorder. Improving our understanding of the mechanism underlying such associations will not only guide us to improve our lifestyles and therefore lower the risk/burden of AD, but may also open novel therapeutic avenues to combat the disorder.

1.5. Aims and objectives

The overall aim of this thesis is to investigate further the interactions between stress and AD-associated pathology in the TASTPM double transgenic mouse model over-expressing β-amyloid. This will be achieved using a multidisciplinary approach incorporating *in vivo* measurements of cognitive

performance, and pathological status using *in vivo* MRI, alongside *ex vivo* determination of β-amyloid burden using a variety of analytical techniques. The specific objectives of the studies are listed below:

1. To characterise the main behavioural paradigm used in this thesis to assess cognitive functioning in mice, and to set-up small animal *in vivo* MRI acquisition, and to optimise the MRI and immunohistochemistry image analysis tools used.
2. Once these experimental procedures have been established they will be used to describe the TASTPM mouse model. More specifically, the effects of a repeated mild stress procedure on AD-associated pathologies in TASTPM mice at a variety of ages during early-to-moderate stages of pathology will be investigated. From these findings, a time-window in TASTPM mouse pathology when we see the largest effects of stress will be identified.
3. This time window will then be used to study a potential underlying mechanism responsible for the relationship between stress and the AD-like pathologies in TASTPM mice, by targeting the glutamatergic system pharmacologically.

Overall, the studies should provide a profile of early AD-like pathologies in TASTPM mice, furthering our understanding of interactions with lifestyle risk factors, such as stress, and will clarify the mechanisms through which these effects are exerted.

CHAPTER 2

OPTIMISATION OF METHODS

2.1. Introduction

Studies into the effects of stress on AD-like pathology in β-amyloid overexpressing mice often rely on changes in the behavioural end-point of an animal as an indicator of modulation of AD-associated pathology by environmental or pharmacological intervention *in vivo*. Throughout studies reported in this thesis, a behavioural test was used consistently to identify changes to mouse memory performance with such challenges. This behavioural measure was used in combination with *in vivo* magnetic resonance imaging (MRI) applied, in this instance, to study changes in brain region structure and morphology, as well as providing an indicator of changes in the properties of brain tissue in the early stages of β-amyloid deposition. These *in vivo* markers of pathological status of the TASTPM mouse were supported by post-mortem quantification of β-amyloid; one method used for this purpose was immunohistochemistry to identify the protein.

The purpose of this chapter is to introduce various methods which were used throughout this thesis; suitability and optimisation of the methods implemented are described with particular reference to the following:

1. The optimisation of a behavioural paradigm to assess memory performance in mice.
2. The development of MRI image acquisition protocols, and a semi-automated image analysis tool to extract data from these scans.

3. The formulation of a semi-automated image algorithm to quantify β-amyloid burden from immunohistochemistry sections.

2.2. Memory performance test optimisation

2.2.1. Introduction

Contextual fear conditioning (CFC) is a tool capable of studying the memory performance of laboratory rodents. The test relies upon the development of a classical Pavlovian association between a conditioned stimulus (CS) and an unconditioned stimulus (US). Typically, the CS is a memory cue, such as a sensory stimulus or novel context, and the US is commonly a series of footshocks administered as a negative associated cue (for review (Maren, 2001)). When the US is presented in the presence of the CS the animal develops a CS-US association and exhibits a conditioned response (CR); when the US is a footshock, the CR is often an emotional fear-like response, exhibited in rodents as immobility (Maren, 2001). This represents a training stage when the memory is acquired, and is referred to as "memory acquisition" in the following studies. The intensity of the CR can be tested at a later time point by presenting the CS in the absence of the US; the strength of the CR is indicative of memory retained and thus immobility of the animal is a measure of "memory retention". During the memory retention test, the animal is exposed to the CS without the US (footshocks), therefore the CS-US association becomes weaker, the animal anticipates the footshock less with continued exposure to the CS, and the CR declines. This process is known as

"memory extinction" and represents a form of re-learning that the CS is no longer aversive, which can be tested multiple times following the memory retention trial, figure 2.1.

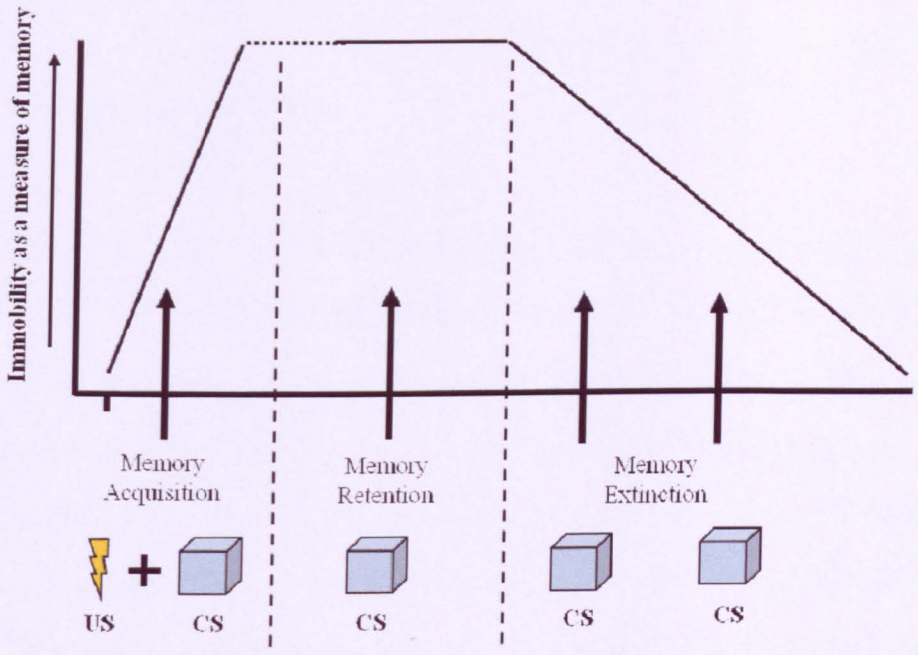


Figure 2.1. Contextual memory acquisition, retention and extinction. During CFC conditioning learning is expressed as immobility exhibited by the mouse during memory acquisition. The level of immobility increases with repeated application of the footshocks (US) in the presence of the CS, a novel context. If the animal has learnt, and can successfully remember the CS-US association, during the memory retention test the mouse will anticipate the US and exhibit a high level of immobility when presented with the CS alone. During the memory extinction trial(s), if the mouse learnt to no longer associate the CS with the US during the memory retention trial, the immobility levels decline.

Similar paradigms have been well described in the literature and have been used to study memory deficits in β-amyloid-overexpressing transgenic mice for AD (Comery et al., 2005; Saura et al., 2005; Jacobsen et al., 2006; Quinn et al., 2007; Riddell et al., 2007). For example, the test has proven to be sufficiently sensitive to identify an accelerated onset of memory deficit with chronic stress, and attenuated cognitive impairments with pharmacological intervention in such mice (Dong et al., 2004).

Unlike many behavioural paradigms one can use to study memory systems in rodents, CFC conditioning provides the potential to test both short, and long-term memory retention and extinction. Briefly, the nature of these two forms of memory, and how they are tested, is important when specifically studying AD-associated pathology. Clinical symptomology of AD show an age-related profile of memory loss; for instance, remote memory, those memories formed and retained over relative longer periods of time, appears to be affected later in the disease process (Sartori et al., 2004).

CFC memory tests provide a robust measure of cognitive performance, which is essential when studying potential loss of memory in a mouse model for AD. Therefore, CFC in mice was optimised, as described below, for the first-time at the University of Nottingham.

2.2.2. Methods

Animals

Male C57Bl/6 mice (Charles River Laboratories, UK) were used. Mice were divided into two main groups, shocked and non-shocked; non-shocked mice were used in this study to confirm the negative CR expressed during this test was due to the shocks, and not an unconditioned response to the novel operant chamber. Mice were then further divided into two subgroups depending on the form of memory tested (either short-term or long-term memory); short-term memory shocked mice (n=4), short-term memory non-shocked mice (n=2), long-term memory shocked mice (n=4), long-term memory non-shocked mice (n=3). Animals were individually housed with food and water available *ad libitum*. Animals were kept in a constant environment, on a 12h light:dark cycle, temperature and humidity were controlled automatically. All procedures were carried out according to the Animals (Scientific Procedures) Act 1987, under license PPL 40/2715 granted to Professor Charles Marsden.

Memory testing

There is variation of CFC protocols used between different groups. The CFC protocol used in the following studies was adapted from a previous study (Frankland et al., 2004). In that study the authors used CFC to identify regions of the mouse brain involved in both short and long-term memory retention. The protocol involved 5 footshocks (0.75 mA, 2 seconds, once per minute) as the US, and memory retention was tested 24h later for short-term memory, and 36 days later for long-term memory.

In this optimisation, the protocol described above was adapted to suit our requirements, the US involved 10 footshocks (0.4mA, 1 second, once per minute) delivered by a shock generator (Campden Instruments, Loughborough, UK); the mouse was placed into the "Skinner box" operant chamber (CS) and the first shock was administered 60 sec later. The operant chamber measured 25cm x 25cm x 38cm. Footshocks were administered through alternate steel bars, spaced 1 cm apart, at the base of the chamber. The CFC box was thoroughly cleaned with 20% ethanol solution between sessions to remove any scent markers which may act as confounding associative olfactory cues. Mice were placed individually into the operant chamber during all trials and their behaviour was video-taped, by a camera suspended above the experimental apparatus, and subsequently scored using Ethovision (Noldus, Wageningen, Netherlands), figure 2.2. This software allows for the simultaneous recording of manually scored immobility as well as activity (distance moved by the mouse); for a large cohort of 3 month old mice (both WT and TASTPM, total n=29), activity (cm) and immobility (sec) exhibited by the mice during CFC memory acquisition were found to be highly correlated (R^2=0.96, figure 2.3), therefore, automatically recorded activity was used as a general indicator of fear memory acquisition throughout the remainder of this thesis.

In this optimisation, short- and long-term memory retention was assessed 24 h and 33 days later respectively, which involved re-exposure for 3 mins to the CS (operant chamber) in the absence of the US; immobility was manually scored. Although several memory extinction trials can be performed during a CFC

experiment, only one extinction trial (identical to the memory retention trial) was performed for both the short- and long-term memory tests 48 h following the memory retention test.

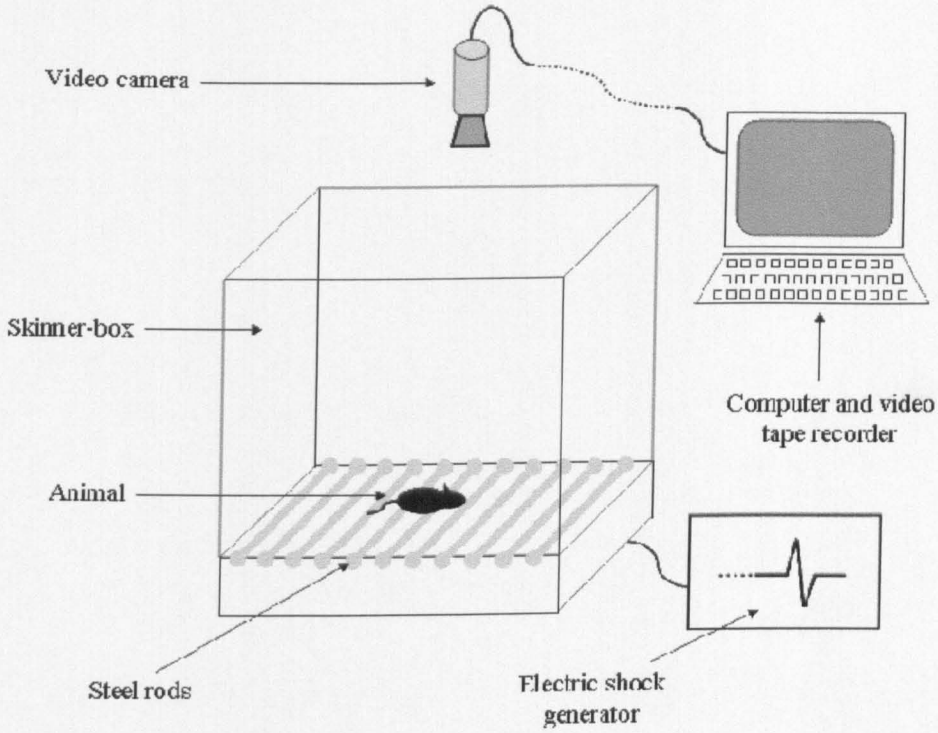


Figure 2.2. CFC apparatus. Diagram representation of CFC experimental apparatus. Mice were placed individually in the "Skinner-box" operant chamber. Footshocks were administered from an electric shock generator via the steel rod floor. The mouse's immobility was recorded via a video camera and scored manually.

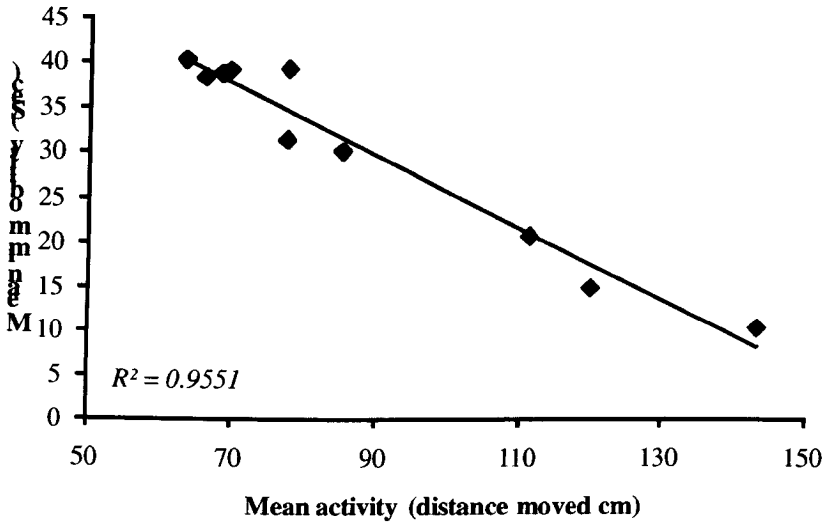


Figure 2.3. Correlation between activity and immobility as measures of fear memory acquisition. Mean activity (distance moved, cm) and mean immobility (sec) for each of the 10 minutes/footshocks during the CFC memory acquisition trial exhibited by a mixed population of 3 month old mice (both WT and TASTPM, n=29). The two measures of this conditioned fear response are highly correlated ($R^2 = 0.09551$).

All CFC conditioning results were analysed using the statistical software package SPSS 14.0, with a repeated measures ANOVA and Tukey's post-hoc. For "memory acquisition" Time (total distance moved, cm per minute over the 10 minute trial) was the within-subject factor, and Shock (shocked vs. no shocked) was the between-subject factor. To analyse performance during the memory retention and extinction tests, Trial (performance at the memory retention test vs. extinction test) was the within-subject factor, Shock and Memory (short-term vs. long-term memory), were the between-subject factors. Values are expressed as mean immobility (sec) ± standard error of mean (SEM).

2.2.3. Results

During contextual fear memory acquisition both groups showed a change in activity over the 10 minute trial ($P = 0.026$), a Time X Shock interaction did not quite reach statistical significance ($P = 0.05$). Mice exposed to footshocks showed an overall lower level of activity ($P < 0.0001$), figure 2.4.

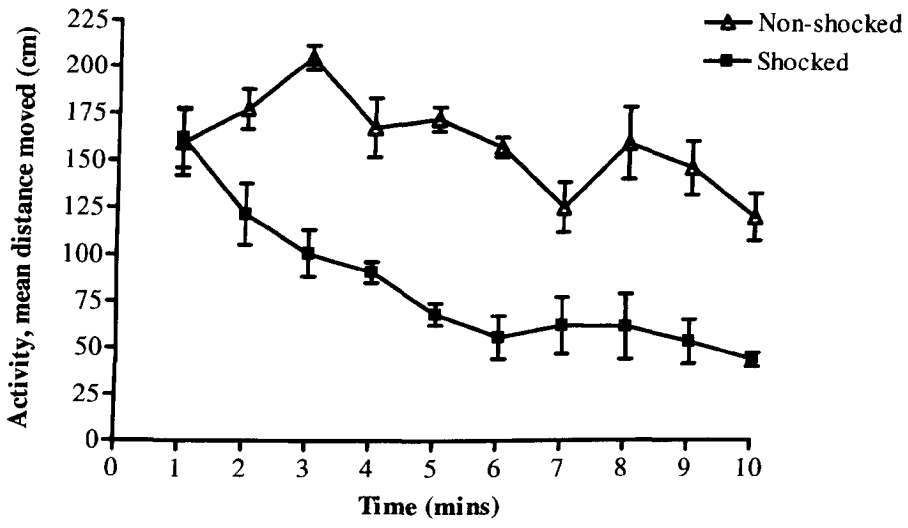


Figure 2.4. CFC memory acquisition. Mean (± SEM) activity (distance, cm) of mice exposed to either no footshocks (n=5) or footshocks (n=8) over the 10 min training period. Both shocked and non-shocked mice showed a decline in activity as a function of time ($P = 0.026$); mice exposed to repeated footshocks show a progressive decrease in activity as a function of time, as compared to non-shocked mice, which did not quite reach statistical significance ($P = 0.05$). Non-shocked mice had, overall, a higher level of activity compared to shocked mice throughout the trial ($P < 0.0001$).

During the memory retention tests, there was a difference between non-shocked and shocked immobility for both the short- and long-term memory

performance ($P < 0.0001$); shocked mice in both the long and short-term memory groups exhibited high levels of immobility, indicative of a conditioned fear response, figure 2.5. Reduced immobility between the memory retention and extinction trials was observed in the shocked short-term memory group only ($P = 0.004$), but did not quite reach statistical significance in the long-term memory group ($P = 0.083$). During both the memory retention and extinction trials, there were no differences between the short- and long-term memory groups, figure 2.5.

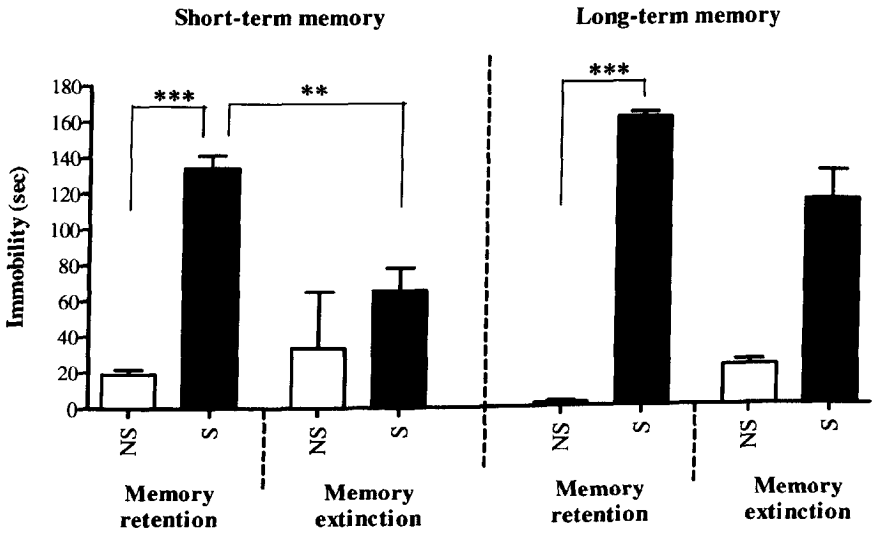


Figure 2.5. Short- (24 h) and long-term (33 days) memory retention and extinction. Mean (± SEM) level of immobility expressed by mice in the short-term memory group (left, non-shocked (NS, open bars, n=2) shocked (S, closed bars, n=4)) and long-term memory (right, non-shocked (NS, open bars, n=3) and shocked (S, closed bar, n=4)). During the memory retention trial non-shocked mice expressed lower immobility (***$P < 0.0001$) compared to shocked mice in both the short and long-term memory tests. A significantly (**$P = 0.004$) lower level of immobility from the memory retention to the extinction trial was exhibited by shocked mice in the short-term memory studies. This, however, did not quite reach statistical significance for the long-term memory group ($P = 0.083$).

2.2.4. Discussion

CFC was optimised to allow for the study of both short and long-term memory performance in mice. Mice successfully acquired and retained aversive contextual memory for up to 33 days, and appear to have successfully exhibited memory extinction.

Mice acquired an aversive contextual memory with repeated footshock exposure in a novel context as demonstrated by the suppression of activity in the shocked mice, this is consistent with the well described classical conditioning behaviour (Maren, 2001). Collectively, both groups showed a change in activity over time; however, while the non-shocked mice remained relatively active throughout the 10 min trial, shocked mice showed a constant reduction upon repeated shock, indicative of enhanced immobility behaviour. Retention of this aversive contextual memory was intact at similar intensity both 24h and 36 days following training, similar to findings described previously (Frankland et al., 2004).

Many studies have been aimed at identifying the brain regions involved in processing contextual memories. A molecular imaging study revealed involvement of the anterior cingulate cortex in the consolidation of long-term contextual memory in mice (Frankland et al., 2004). However, the majority of studies have been aimed at identifying brain areas responsible for expression of conditioned fear responses by localised damage to brain structures. For instance, damage to the basolateral amygdala, hippocampus and prefrontal

cortex in rats have disrupted conditioned memory performance in the rat (Kjelstrup et al., 2002; Berlau and McGaugh, 2003; Sierra-Mercado et al., 2006). Such studies provide an indication of the brain anatomy involved in memory retention expression, which is dependent on the type of memory involved, be it short or long-term memory, (for review, (Wiltgen et al., 2004)).

During this optimisation the short-term memory group expressed significant memory extinction, whereas the long-term memory group only showed a general trend towards extinction. A higher number of subjects in the long-term memory study may have provided higher statistical power to reach a significant level. Alternatively, another theory is that this may represent a known difference between short and long-term memory. Molecular pathways underlying short-term memory are thought to be less robust and believed not to involve structural changes in brain architecture, whereas long-term memory relies upon semi-permanent structural protein changes (for review, (Wiltgen et al., 2004)). Such robustness may lead to elevated resistance to the process of memory extinction. Similar studies to those described above have revealed the brain circuitry involved in fear memory extinction. Similar to the memory retention circuitry, extinction appears to involve interactions between amygdala and prefrontal cortex, while the hippocampus appears to play a more regulatory role (for review, (Ji and Maren, 2007)).

In this experiment, the optimisation of CFC was successful as the aversive cue, footshock, was sufficient to induce a conditioned memory (CR) which was evident for at least one month following training. Further to this, mice were

capable of extinguishing this fearful contextual memory upon re-exposure to the CS in the absence of the US. This protocol was therefore used throughout the following studies as a measure of cognitive function and memory performance in TASTPM mice, along with wild-type controls, and to assess the effects of various treatments on cognitive performance.

2.3. Magnetic resonance imaging (MRI)

2.3.1. Introduction

A behavioural endpoint such as CFC provides a tool to study memory performance in transgenic mouse model of AD; this information can be correlated with *in vivo* imaging methods to provide a more complete picture of pathological status. MRI is a tool which allows the non-invasive imaging of the brain in a living subject.

The basic principal allowing for the imaging of tissue by MRI relies upon the magnetic properties of hydrogen protons. Protons spin along an axis, and these axes of multiple protons are randomly aligned in tissue. When placed inside a strong magnetic field, for instance within an MRI magnet, hydrogen ions, and the precession of these ions, align either in a high-energy, or low-energy state. A radiofrequency (RF) pulse is administered and the axes, and their precession, flip to a new angle, termed the "flip-angle". The flip-angle is commonly 90^{o}. As the axes and precession relax back to the original spin state and direction, aligned with the magnetic field, a signal is released called T1 relaxation. The

signal released by the precession of the ions during the relaxation is called T2 relaxation, see figure 2.6. T1 and T2 relaxation reflect tissue composition, for instance the rate of relaxation can be dependent on tissue water content, as described in more detail below. Relaxation can then be received using a specially designed coil, transferring the signal to data in order to create an image. High resolution images, from such scans, are routinely used by clinicians to study changes in brain characteristics, such as morphology, associated with degenerative diseases observed in AD.

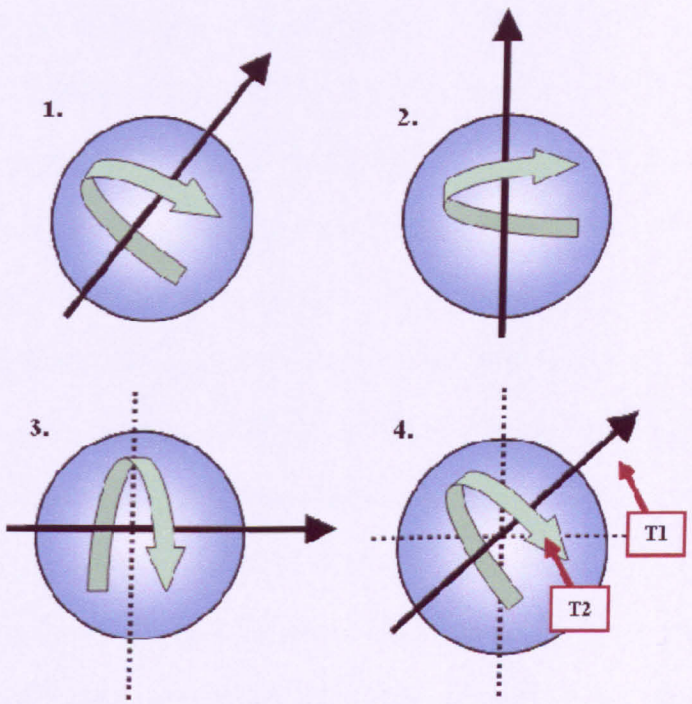


Figure 2.6. Diagrammatic representation of the principles of MRI. 1. Hydrogen protons (blue circles) are randomly aligned and spin on their axis (precession, green arrow) in tissue. **2.** When placed in magnetic field, the axis, and precession align. **3.** A radiofrequency pulse causes all ions, and their precession, to align to a new flip-angle (commonly 90°). **3.** Relaxation of the proton axis, and its precession, from this aligned state, back to the original spin-states, provides signal (T1 and T2 relaxation respectively). Signals can be received by a coil and subsequently converted into an image.

Thus, *in vivo* MRI offers a potential diagnostic tool to aid the identification of AD at the early stages, during disease development, to allow for earlier therapeutic intervention. Preclinical research using β-amyloid-overexpressing transgenic mice for AD have shown that MRI can be an appropriate tool to monitor changes associated disease progression through a variety of methods (see below, and references therein).

MRI clinical studies have shown changes in specific brain region volumes, such as the hippocampus and entorhinal cortex (Fox et al., 1996), have been associated with an increased risk of conversion from MCI to AD (Apostolova et al., 2006). Monitoring specific brain area volume loss through MRI during the early stages of AD can, therefore, provide the potential to predict the conversion of AD from MCI (for review, (Chetelat and Baron, 2003)), which may allow for early pharmacological treatment. Similarly, this tool has been exploited to study alterations to brain morphology in β-amyloid-overexpressing transgenic mouse models for AD. Histological examination of 3.5 month old PDAPP mouse line showed substantial reductions in corpus callosum and hippocampal volumes (Gonzalez-Lima et al., 2001); this finding has been supported by *ex vivo* MRI on cadavers of the same mouse line at a similar age where hippocampal volume was reduced prior to β-amyloid deposition, but no significant differences were found for whole brain volume (Redwine et al., 2003). *In vivo* MRI has been used to identify changes in brain region volumes in transgenic mice over-expressing β-amyloid (Weiss et al., 2002; Van Broeck et al., 2008).

Another tool available to monitor disease progression is T2 relaxometry. This is calculated by repeated measurements following the RF pulse, at a number of time points during the ion precession relaxation stage, figure 2.6(4). T2 relaxation times are reflective of tissue composition, changes of which are associated with certain disease states. Elongation of T2 relaxation time has been associated with clinically diagnosed AD, and has been shown to be correlated with clinical severity (Laakso et al., 1996) and declines in cognitive performance (Kirsch et al., 1992). This, however, is not universally observed; a similar study found no change in T2 relaxation in the hippocampus of subjects suspected to have AD (Campeau et al., 1997). In contrast to prolonged T2 relaxation in clinical AD, shortened T2 relaxation times have been observed in various transgenic mouse models of AD overexpressing β-amyloid (Helpern et al., 2004; Falangola et al., 2005b; El Tannir El Tayara et al., 2006; El Tayara Nel et al., 2007; Falangola et al., 2007). The reason for the fundamental difference in T2 relaxation times clinically and preclinically is unknown. T2 relaxometry, nevertheless, offers a non-invasive *in vivo* approach to monitor pathological changes when studying AD, clinically or preclinically, and is sufficiently sensitive to detect early changes before overt onset of pathology.

Another tool made available by MRI to aid accurate diagnosis of AD is the imaging of individual β-amyloid plaques *in vivo*. Work has been done to achieve this by application of an exogenous contrast agent, for instance Pittsburg compound B, and applying positive emission topography (PET), as discussed in Section 1.1.3. The non-invasive identification of β-amyloid plaques through high-resolution MRI has not, as yet, been accomplished in

human AD sufferers. However, a wealth of evidence showing that this can be achieved in β-amyloid-overexpressing transgenic mice is available. Early studies involved the application of an exogenous contrast agent to aid the visualisation of β-amyloidal deposits, which has proven successful (Poduslo et al., 2002; Wadghiri et al., 2003; Higuchi et al., 2005). Subsequently, β-amyloid plaques have been successfully imaged in various transgenic mouse brains in the absence of an exogenous contrast agent both *ex vivo* (Lee et al., 2004; Zhang et al., 2004), and *in vivo* (Jack et al., 2004; Jack et al., 2005; Vanhoutte et al., 2005; Borthakur et al., 2006; Braakman et al., 2006; Dhenain et al., 2007; Faber et al., 2007; Wengenack et al., 2008). It is clear that resolving, and thus identifying, β-amyloid plaques presents a potential method of assessing pathological burden in these mice and, ultimately, clinical AD. This method may be less appropriate, however, when studying the early stages of β-amyloid deposition given its inability to identify inclusions prior to 5 months of age in double transgenic mice known to exhibit deposits as early as 2.5 months old (Dhenain et al., 2007).

MRI-based studies aimed at assessing early pathological changes which occur in β-amyloid-overexpressing mice may lead to improved identification of more sensitive and subtle neurological changes that could potentially be transferred to the clinic. In the following studies *in vivo* MRI was used to study regional brain volumes (volumetry) with the aim of detecting changes in TASTPM mice. Given changes in T2 relaxation times can be observed in transgenic mice at earlier stages, as compared to imaging individual plaques, regional T2 relaxometry was used as a measure of pathological status. Here, the

optimisation and acquisition of the *in vivo* MRI scans (volumetry and relaxometry) in mice, and the development of an analytical data-extraction method, were carried out for the first time on a 7T magnet (Bruker Biospin, Germany) at the University of Nottingham.

2.3.2. Acquisition of images

The optimisation of acquiring high-resolution (T2-weighted) images for studying volumetry, and T2 relaxometry scans of the mouse brain was performed in collaboration with Prof. Dorothee Auer, Dr. Mirjam Schubert, School of Medical and Surgical Sciences, and Dr. Malcolm Prior, Brain and Body Centre, University of Nottingham. Scanning was performed on a 7T magnet, with a 31cm bore diameter for rodent studies.

To develop the T2-weighted rapidly acquired refocused spin echo (RARE) image to study volumetry, preliminary scans were carried out on a mouse brain *ex vivo.* The mouse brain was placed into the magnet, and a fast-gradient echo (FLASH) sequence was applied to determine the anatomical position of the T2-weighted slices. A variety of multi-slice, multi-echo T2-weighted spin-echo sequences were applied to the *ex vivo* mouse brain. The image with the greatest clarity and contrast, determined visually, was chosen. For T2 relaxometry scans, again, the slices were established using a FLASH sequence, and multi-slice, multi-echo T2-weighted spin-echo sequences were performed, although with different TEs, as described below. The final protocol for acquisition of T2-weighted volumetric images, to study volumetry, was as follows: echo

time (TE) = 9.266 ms; effective TE = 29.54 ms; repetition time (TR) = 5.259 s; RARE factor = 8; FOV = 16.64 mm; in plane resolution 0.13 mm; slice thickness = 0.5 mm, 16 repetitions (manually realigned and averaged post-acquisition), 15 coronal slices were taken; for an example image see figure 2.7A. The protocol for the acquisition of T2 relaxometry scans, with the aim of creating a map of T2 relaxation values for the whole brain, was as follows: TE = 10.187 ms; effective TE = 21.65, 62.40, 103.14, 143.81, 184.64 and 225.39 ms; TR = 19.743 s; RARE factor = 4; FOV= 14.976 x14.976 mm; in plane resolution = 0.234 mm; 4 averages, slice thickness of 1mm (15 coronal slices taken).

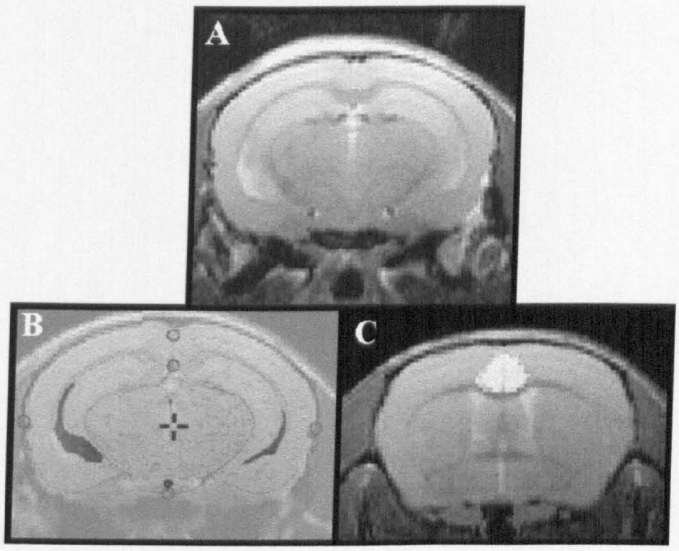
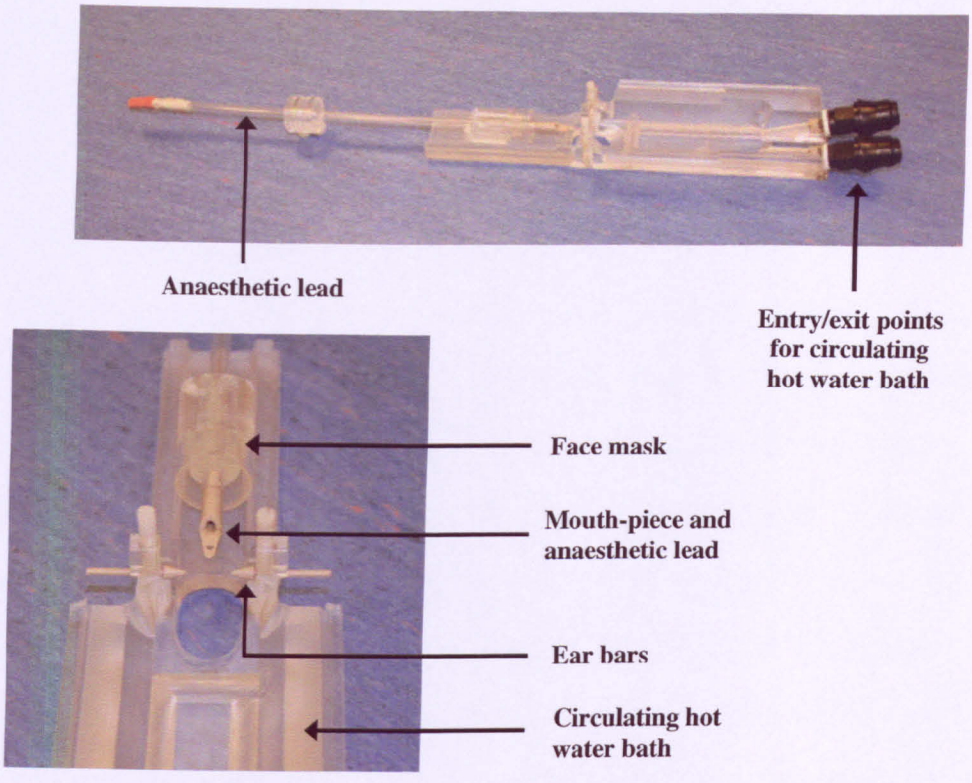


Figure 2.7. Representative T2-weighted volumetry images, and semi-automated image analysis tool. A: *In vivo* T2-weighted volumetric coronal scan of 5.5 month old TASTPM mouse. **B:** Registered brain atlas with example MRI scan following neuroanatomical-landmarking; the digital mouse brain atlas was superimposed over the scan. **C:** Following image registration the ROI (anterior cingulate cortex) was delineated onto the mouse brain atlas, and then projected back onto the MRI scan to demonstrate the accurate positioning of the ROI.

In vivo scanning time took, in total, approximately 2 hours 30 mins per mouse, during which time the mouse was under gaseous anaesthesia using a purpose-built mouse holder, figure 2.8. For *in vivo* scans mice were initially anaesthetised in an anaesthetic-induction chamber under a mixture of 5% isoflurane and oxygen (0.7-0.9 litre/min). When fully anaesthetised mice were transferred to a purpose-built plastic mouse holder, figure 2.8. A tooth-bar and ear-bars were used to position the mouse in front of the anaesthetic lead. During the scanning mice were maintained under anaesthesia with a mixture of ~1.5-2% isoflurane, and oxygen (0.7-0.9 litre/min). Levels of anaesthesia were assessed by monitoring breathing rate via an automated system calculating breaths per minute; anaesthesia dose was adjusted when necessary to correct for changes in respiration. Core-body temperature was monitored through a rectal thermometer, and adjusted using the circulating hot water bath which warms the plastic holder and the body of the mouse. Core-body temperature was kept at ~37.5°C (Lauda Ecoline Staredition 003, Harvard Apparatus), and respiration was monitored automatically with the depth of anaesthesia modulated to regulate breathing-rate (SA Instruments Inc., New York). To perform the scans the mouse was placed in a volume coil, from which the RF pulse signals were transmitted, and a receive-only head coil was placed closely over the skull to receive the signal (all scanning equipment from Bruker Biospin, Germany). This apparatus was then placed into the horizontal bore of the magnet and the scanning procedure started. Specific details of anaesthetic and scanning protocols will be described in greater detail in Chapter 3.



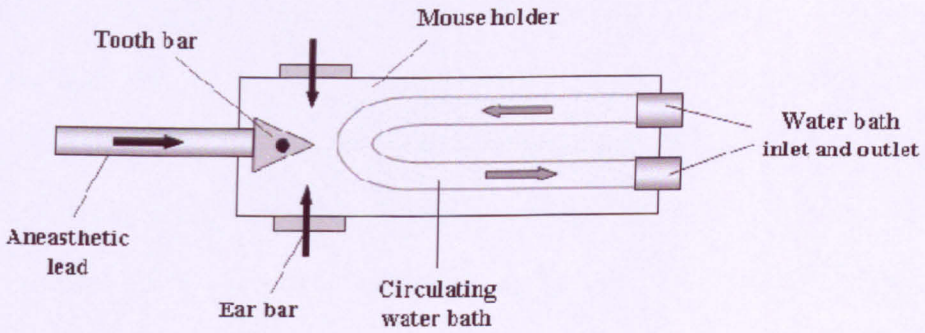


Figure 2.8. Mouse holder for MRI scans. Gaseous anaesthetic was administered through the anaesthetic lead, and warm water circulated through the holder by the entry-points to warm the mouse. A tooth-bar held the mouse's nose over the anaesthetic lead, and ear-bars held the head in place to reduce any movement artefact. A plastic cuff was fixed to the animal's head to further reduce any respiration-related movement.

2.3.3. Image analysis tool

For the image analysis, a semi-automated method was developed in collaboration with Dr. Alain Pitiot, Brain & Body Centre, University of Nottingham. First, the volumetric scans were manually co-registered with a digital mouse brain atlas (Paxinos G, Franklin KBJ, The Mouse Brain in Stereotaxic Coordinates, Deluxe Edition of the Atlas Second Edition, Academic Press, San Diego, California, 2001). Registration was conducted using the Register program (Montreal Neurological Institute, Canada) and involved identification of, for each MR scan, a number of pairs of neuroanatomical land-marks on both the scans and mouse brain atlas. Land-marks were distributed throughout the brain to allow for precise registration. Once land-marking was complete, the program computed a flexible transformation to match these pairs of points using thin-plate-splines (a morphological tool) as a transformation model, ensuring both flexibility and a precise match of the two images. Registration was performed for each MRI brain slice. Once registration had been completed, the MRI scans were super-imposed onto the atlas, allowing one to assess the accuracy of the image correspondence, figure 2.7B. Next, the regions of interest (ROIs) that were studied in the brain were manually delineated onto the mouse brain atlas using the Display program (Montreal Neurological Institute, Canada). Delineations were then projected from the atlas onto the MRI image, figure 2.7C, using the transformation computed above, this was checked manually for all mice to ensure we obtained robust, accurate results. This method of ROI delineation, directly onto the mouse brain atlas, allows one to be confident that the results

are reproducible, as ROI delineation is consistent between mice. Each ROI was converted from a total voxel count and expressed as a volume in mm^2. The volume for each ROI was then calculated as a percentage of the whole brain volume.

To study brain volumetry and regional T2 relaxometry, ROIs were delineated and classified using the mouse brain atlas (Paxinos & Franklin, 2001); the inclusion criteria are described below. For the purpose of the investigations described in this thesis, the delineations were designed to allow for robust, consistent results which are easily reproducible. Anterior cingulate cortex (ACC) was taken from Bregma 2.34mm to Bregma -0.22mm. Retrosplenial cortex was taken from Bregma -0.34mm to Bregma -3.88mm. Thalamus was taken from Bregma -0.22mm to -3.88mm; from -0.22mm to -2.30mm a horizontal line was drawn from the most ventral part of the caudate putamen (striatum), any thalamus lying below this line was excluded (figure 2.9C); from -2.46mm to -3.16mm a horizontal line was drawn from the most ventral part of the fasciculus retroflexus, thalamus above this was included. Corpus callosum was taken from Bregma 1.10mm to Bregma -2.54mm; from Bregma 1.10mm to Bregma -0.34mm, the ROI included up to the lateral edge of the cingulum (figure 2.9B); from Bregma -0.46mm to Bregma -1.06mm the corpus callosum was characterised by a vertical line drawn at the lateral edge of the fibria; from Bregma -1.22mm to Bregma -2.54mm the corpus callosum was characterised by a vertical line taken at the most lateral edge of the thalamus, bisecting the ROI (figure 2.9C). The caudate putamen was taken from Bregma 1.94mm to Bregma -2.30mm; from Bregma 1.54mm to Bregma 0.62mm, the ROI was

taken above a horizontal line drawn at the most ventral part of the anterior commisure, anterior part (figure 2.9D); from Bregma -0.7mm to Bregma -1.70mm a horizontal line was drawn at the most dorsal pat of the basal lateral amygdaloid nucleus, anterior part, caudate putamen above this line was included. The hippocampus was taken from Bregma -0.94mm to Bregma -3.88mm. Subiculum was taken from Bregma -2.46mm to Bregma -3.88mm.

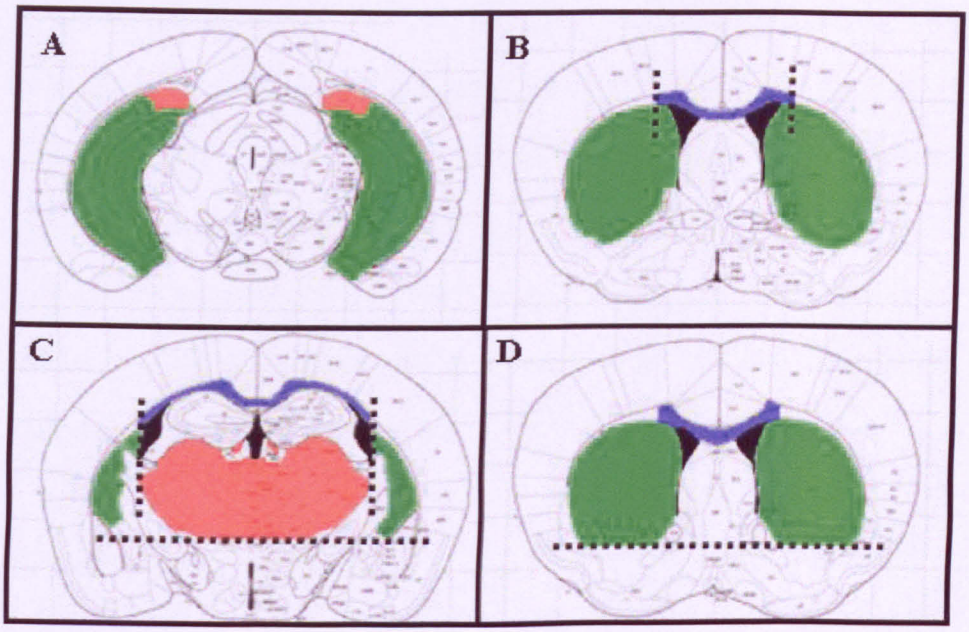


Figure 2.9. Example ROI delineations onto a digital mouse brain atlas to study volumetry and relaxometry. A. Bregma -3.28mm, hippocampus in green, and subiculum in red. **B.** Bregma 0.26mm, caudate putamen in green and corpus callosum in blue; corpus callosum within horizontal lines taken at the most lateral edge of the cingulum was included. **C.** Bregma -1.34mm, thalamus in red, corpus callosum in blue and caudate putamen in green; corpus callosum was included within horizontal lines drawn at the most lateral edge of the thalamus; thalamus was included above a vertical line drawn at the most ventral part of the caudate putamen. **D.** Bregma 0.62mm, caudate putamen in green, and corpus callosum in blue; caudate putamen was included above a horizontal line drawn at the most ventral part of the anterior commisure, anterior part.

The MRI acquisition protocols for both the volumetry and T2 relaxometry studies were optimised to provide sufficient resolution, and intensity, in a realistic scanning time-frame required for *in vivo* studies, and were considered appropriate to determine the pathological status of TASTPM mice. The semi-automated image analysis tool was set up to produce consistent, robust results with a high level of accuracy, appropriate to study changes in the parameters described above. Moreover, T2 relaxation times acquired through the use of the semi-automated method were in a similar range to those achieved by manual ROI segmentation, further confirming the reliability of the analysis method. These MRI studies provide an assessment of pathological status *in vivo;* it is, however, important to correlate such findings, and findings from behavioural studies, with direct post-mortem quantification of a known pathological marker, such as detection of β-amyloid through immunohistochemistry.

2.4. Immunohistochemistry

2.4.1. Introduction

TASTPM mice start to deposit β-amyloid in the form of extracellular plaques from as early as 3 months old, the occurrence of which increases with age (Howlett et al., 2004). Quantification of plaques, identified through immunohistological staining, is frequently used as a method of detecting pathological changes in such mice. Many studies rely on manual counting of plaques. However, this type of analysis can be problematic when the applied

pharmacologic and/or environmental interventions may result in subtle changes to β-amyloid pathology. This can prove difficult if an inaccurate manual quantification is adopted, possibly leading to false-negative results. One option is to accurately assess β-amyloid burden through the use of a semi-automated method of quantification. Here, a robust method to accurately and reproducibly measure TASTPM mouse β-amyloid plaques was developed to support data acquired through the MRI studies and behavioural endpoints mentioned above.

2.4.2. Immunohistochemistry methodologies

This method has been developed to assess levels of β-amyloid pathology. Alterations to staining procedures specific to the antigen studied are described in each experimental chapter where appropriate. Brain sections were processed under standard procedures. The processing, sectioning and staining of tissue, described below, was principally performed by the laboratory of Prof. James Lowe, Department of Histopathology, Queen's Medical Centre, University of Nottingham.

Brain tissue suspended in 4% PFA was refrigerated prior to processing. Tissue was subjected to paraffin wax infusion processing using a LEICA TP 1050 apparatus, (Leica Microsystems, Milton Keynes, UK), and was paraffin wax block embedded using a LEICA EG 160 apparatus. Blocks were then sectioned using a LEICA RM 2135 rotary microtome. Variations in sectioning protocols will be described for each study, where appropriate. Sections for determining anatomical position acting as location markers within the brain were stained

using standard haematoxylin and eosin stain. Sections for immunostaining were de-waxed in xylene, which was removed by subsequent washing in alcohol (industrial methylated spirits (IMS)). Sections were then rinsed in tap water, followed by deionised water. For β-amyloid plaque staining, sections were subjected to a concentrated (90-100%) formic acid pre-treatment (~5sec exposure), then washed thoroughly with tap water. The immunostaining procedure was conducted automatically using Dako Techmate 500 Plus (Ely, Cambridgeshire, UK), using a labelled streptavidin biotin technique. Immunostaining process worked through capillary action between two closely positioned sections, in which solution is drawn up between them. To ensure consistent staining, the slides were immersed in 0.1% Tween 20 for 20 mins prior to staining. Immunostaining was conducted according to standard procedures in the Department of Histopathology. Between all steps listed below, sections were washed with buffer (Dako REAL wash buffer 1). Briefly, primary antibody (Ab), (for β-amyloid, βA4 mouse mono-clonal primary Ab raised in QMC, commercially available from Leica Vision Biosystems), was diluted in Dako REAL Ab diluent (product S2022) at 1:3000 concentration for 60 mins. Antigen detection was achieved by 30 min exposure to dual link secondary Ab (Dako REAL link biotinilated secondary Ab, anti-mouse and anti-rabbit), followed by 30 min exposure to horseradish peroxidise (Dako REAL streptavidin peroxidise), horseradish peroxidise was stained for 10 min with chromogen diaminobenzadine exposure (reagents part of DAKO kit K5001). Sections were counter-stained with Gills3 haematoxylin for 30 sec, dehydrated with alcohol (IMS) methcol, and cleared with xylene. Slides were mounted with DPX glue.

2.4.3. Semi-automated image analysis

In order to study subtle changes in regional β-amyloid plaque pathology with various treatments, a semi-automated method was developed to allow for a robust and reproducible quantification of β-amyloid. Technical assistance was provided by Mr. Trevor Grey, Department of Histopathology, Queen's Medical Centre, University of Nottingham.

Brain sections chosen to present equal expression of ROIs were thoroughly cleaned prior to analysis. Sections were scanned by the Nanozoomer Digital Pathology slide scanner (Hamamatsu, Japan), at x20 magnification. ROIs were then individually captured at x5 magnification. The files were transported to AnalySIS Pro 3.1 Soft Imaging System (Munich, Germany), where they were analysed. First, preliminary studies were conducted to set-up colour thresholds to automatically detect, and detect the dark-stained β-amyloid deposits against the background tissue. The detection of β-amyloid deposits was further optimised by the development of inclusion criteria aimed to discriminate antigen from non-specific background staining. For this purpose, example β-amyloidal deposits and non-β-amyloid artefactual inclusions were manually identified and criteria including object sphericity, elongation, convexity and shape factor were recorded. For these factors, exclusion criteria were established with the aim of excluding non-β-amyloidal stain. For each section, ROIs were manually delineated onto the images.

Novel ROI exclusion criteria were established to ensure calculation of accurate assessment of β-amyloid load. ROI delineation for IHC analysis was similar to that described for MRI analysis (Section 2.3). The following ROI delineations were used in experimental Chapter; half the brain was available for analysis. ROIs studied included the hippocampus, retrosplenial cortex, caudate putamen, thalamus and corpus callosum. The hippocampus was taken from Bregma -0.94mm to Bregma -3.88mm (figure 2.10A). The retrosplenial cortex was taken from Bregma -0.34mm to -3.88mm (figure 2.10A & B). The caudate putamen was taken from Bregma 1.10mm to -2.18mm (figure 2.10A & B); from Bregma 1.10mm to -0.82mm a vertical line was drawn from the most ventral part of the lateral vertical, all caudate putamen above this was included (figure 2.10B). The thalamus was taken from Bregma -0.22mm to Bregma -3.88mm (figure 2.10A & B); from Bregma -0.94mm to -2.30mm a horizontal line was drawn from the most ventral part of the caudate putamen, all thalamus above this was included (figure 2.10A). The corpus callosum was delineated from Bregma 1.10mm to -2.54mm where the ROI splits; from Bregma 1.10mm to -0.82mm corpus callosum was included up to the edge of the lateral ventricle (figure 2.10B); from Bregma -0.94mm to -2.54mm a vertical line was drawn at the most lateral edge of the thalamus, corpus callosum within this was included (figure 2.10A).

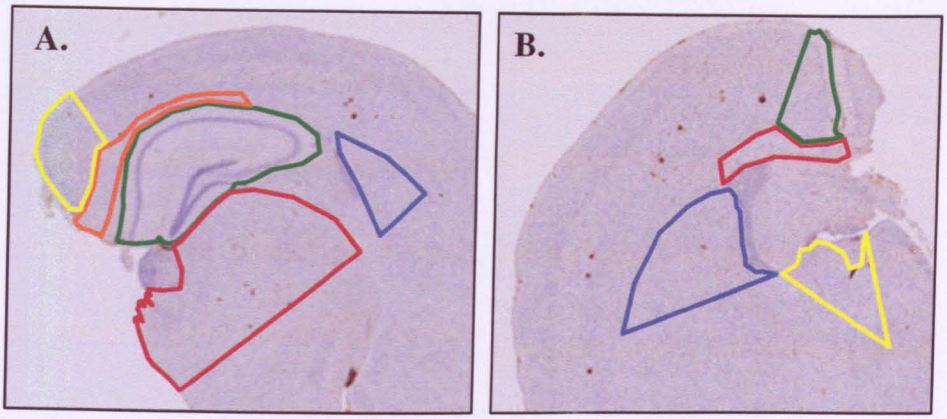


Figure 2.10. IHC analysis, representative ROI delineations. A. Bregma -1.94mm; retrosplenial cortex (yellow), corpus callosum (orange, taken from the lateral edge of the thalamus), hippocampus (green), thalamus (red, taken from the most ventral part of the caudate putamen), caudate putamen (blue). **B.** Bregma -0.58mm; retrosplenial cortex (green), corpus callosum (red, taken up to the lateral edge of the lateral ventricle), caudate putamen (blue, taken from the most ventral part of the lateral ventricle), thalamus (yellow).

Immunohistochemical analysis of β-amyloid burden was also analysed in independent groups of TASTPM mice in Chapter 3. This was performed between Bregma -0.94mm to -3.88mm, the whole brain was available for analysis and subsequently only the retrosplenial cortex, thalamus and hippocampus were studied using the delineation criteria detailed above. In one study the whole brain was available for analysis which led to potential difficulties in ROI delineation. For example, if a vertical line is drawn down the midline of the brain section, splitting it into left and right hemispheres, the position of the section was not always the same on both hemispheres, i.e. the left side can be more anterior than right. Some ROIs bisect, and were delineated across the left and right of the brain; using the thalamus as an example, where the thalamus was one whole unit across the brain the mean corresponding atlas plate number of the left and right side of the brain was

taken, and the thalamus was delineated as described on that plate number. Alternatively, when a ROI splits into two separate entities, for example thalamus splits at Bregma -2.18mm, and is expressed independently on both the left and right side of the brain, the ROI was treated as two independent units and delineated depending on the location on that specific side of the brain. Specific details, such as sections available for analysis for all studies, in the following experimental Chapters are described therein.

Level of β-amyloid, calculated as the percentage of ROI covered by the protein, for all ROIs were then collated; an overall mean percentage area of ROI covered by β-amyloid was calculated allowing for direct comparison between treatment groups. To ensure reproducibility of the ROI delineation, and resulting β-amyloid quantification data, example brains were re-scored at random and ROI area and percentage ROI covered in β-amyloid were compared with the original data set, where an acceptable level of confidence of reproducibility was achieved. Example reproducibility (% confidence) of % amyloid burden for particular ROIs are follows; hippocampus = 98%, retrosplenial cortex = 95%, thalamus = 92% and corpus callosum = 99%. Example reproducibility of ROI delineation are as follows; hippocampus = 99%, retrosplenial cortex = 91%, thalamus = 89%, caudate putamen = 74% and corpus callosum = 93%. This method has been designed to accurately quantify β-amyloid. Abiding by clear rules in delineating ROI, and maintaining strict self-assessment, ensures this image analysis algorithm was robust and reproducible. This will be applied throughout this thesis to assess, or aid the assessment of, β-amyloid pathology in TASTPM mice with various treatments.

CHAPTER 3

EFFECTS OF REPEATED MILD STRESS ON EARLY-TO-MODERATE STAGES OF PATHOLOGY IN TASTPM MICE

3. General Introduction

A connection between emotional stressors and AD-associated pathology in β-amyloid-overexpressing transgenic mice was discussed in detail in Section 1.4.1. In the main, the evidence indicates that repeated and acute severe stressors have been shown to enhance β-amyloid levels as well as accelerate memory deficits in such mice modelling AD.

Contrary to these investigations, a recent study from our group demonstrated that a repeated mild stress procedure, called "novel cage stress", involving daily exposure to a novel environment for 5 weeks, applied from 4 months of age, had the opposite effects; first, it lowered brain β-amyloid levels, and second, prevented the onset of a short-term contextual memory deficit in 5.5 month old TASTPM mice, as assessed, using the contextual fear conditioning (CFC) paradigm described in Section 2.2 (Pardon et al., 2009). This study also revealed that TASTPM mice exhibited deficits in short-term contextual memory extinction from 4 months of age (Pardon et al., 2009), when the short-term memory retention was still intact. We do not know, however, whether stress, applied earlier, is capable of modulating milder states of AD-associated pathology exhibited by TASTPM mice. We also do not know whether stress can interfere with long-term memory performance.

This chapter aims to further describe the age-related Alzheimer's-like pathology that occurs in the TASTPM mice, and to determine the effects of novel cage stress on these age-related pathological changes. For this purpose

these studies are described below in two parts grouped depending on the pathological status of the animals tested:

1. The first part deals with time periods corresponding to mild pathological changes in TASTPM mice, between the ages 3 and 4.5 months, (Section 3.1).

2. The second deals with TASTPM mice exhibiting mild-to-moderate levels of pathology from the ages of 5.5 to 6.5 months, (Section 3.2).

Understanding the progression of pathology in TASTPM mice, and how this is affected by stress, at a variety of time-points will identify a critical window in which the effects of stress on AD-related pathology are most abundant. This time-window will then be subsequently used in a later chapter to investigate a potential underlying mechanism connecting the pathological changes in TASTPM mice with repeated mild stress.

3.1. Effects of stress during early stages of pathology in TASTPM mice

3.1.1. Introduction

There is a body of clinical evidence suggesting that stress throughout adult life poses as a life-style risk factor for the development of AD (see Section 1.4.1., and references therein). This evidence indicates that stress, either prior to pathological onset, or during the early stages, may enhance the risk or severity of AD. As described above the repeated mild stressor, novel cage stress, applied from 4 months of age in TASTPM mice, a time-point associated with

mild β-amyloid burden, improved the AD-like pathology (Pardon et al., 2009). Interestingly, a deficit in contextual memory extinction was observed in TASTPM mice at 4 months of age, which remained unaffected by subsequent novel cage stress exposure when studied in 5.5 month old TASTPM mice.

As demonstrated in Section 2.2., CFC can be used as an appropriate tool to test contextual fear memory performance in mice, and was used in the study described above as the behavioural endpoint. However, in order to determine pathological changes to unconditioned mouse behaviours not associated with a cue, learning paradigm or aversive environment, a suitable test is required. Spontaneous alternation, a behavioural test performed in a standard T-maze can allow one to study a form of working memory in a freely moving animal outside of a fearful environment. Spontaneous alternation in the T-maze has proven to be sufficiently sensitive to detect memory deficits in transgenic mice over-expressing a calcium-binding protein implicated in AD, named S100β, which exhibit neurological dysfunction (Gerlai et al., 1994)

Here, we tested the hypothesis that the beneficial effects of novel cage stress may be dependent on the pathological status in TASTPM mice, by investigating the earliest stages of β-amyloid deposition. We, therefore, aimed to establish whether the short-term contextual fear extinction deficit can be detected in TASTPM mice aged 3 months, corresponding to the first signs of insoluble β-amyloid deposit formation (Howlett et al., 2004), and if repeated novel cage stress applied from 3 months onwards can prevent, or reverse, a deficit in extinction of contextual fear in 4.5 month old TASTPM mice. For this purpose we, first, compared 3 month old TASTPM and wild-type (WT)

mice for the acquisition, retention and extinction of contextual fear memory. These memory tests were supported with assessments of spontaneous alternation performance, a measure of unconditioned working memory performance. We, secondly, assessed the impact of 5 weeks exposure to novel cage stress, applied from 3 months of age, on age-related changes in short-term contextual fear behaviour, spontaneous alternation, and regional brain levels of soluble and insoluble β-amyloid in TASTPM mice at 4.5 months of age.

3.1.2. Methods

Animals

Male TASTPM (Transgenic Unit, Biomedical Services Unit, University of Nottingham) and control WT, C57Bl/6 mice (Charles River Laboratories, UK) were used. Mice were grouped for experimentation purposes depending on the age at which they were studied; either 3 months old, or 4.5 months old. Mice in the 4.5 month old group were singly housed from 3 months of age due to the development of age-related aggression observed in the TASTPM mouse line (Pugh et al., 2007). Food and water were available *ad libitum*, a cardboard play-tube and bedding were available as part of standard environmental enrichment. Animals were kept under standard conditions in a constant environment, on a 12h light:dark cycle. Temperature and humidity were controlled automatically. All procedures were carried out according to the Animals (Scientific Procedures) Act 1987, under license PPL 40/2715 granted to Professor Charles Marsden.

Group & Experimental Design

In the 3 month group, base-line measures of contextual memory performance, unconditioned exploration in the T-maze in 3 month old TASTPM (n=8) compared with WT mice (n=9); following behavioural assessments, mice were humanely killed and TASTPM brain β-amyloid was quantified. An independent group of TASTPM mice was individually housed at 3 months, and half were exposed to novel cage stress from 3 months of age for 5 weeks (n=7 for each stress condition). At 4.5 months of age short-term contextual memory performance, unconditioned exploration and β-amyloid levels were assessed in these mice. At both 3 and 4.5 months short-term memory performance and exploration were tested using the CFC test and spontaneous alternation test respectively. Locomotor activity was tested in the 4.5 month old mice to determine any effects of 5 weeks repeated stress exposure on activity and reaction to a novel environment. For a simplified experimental design see figure 3.1.

Novel cage stress

In order to induce a state of repeated mild stress, novel cage stress was applied as previously described, (Pardon et al., 2004; Pardon et al., 2005; Pardon et al., 2009), being performed once daily, four times per week over a 5 week period. Stress was applied on days 1 to 4 of the working week and weekly home cage changes took place on day 5.

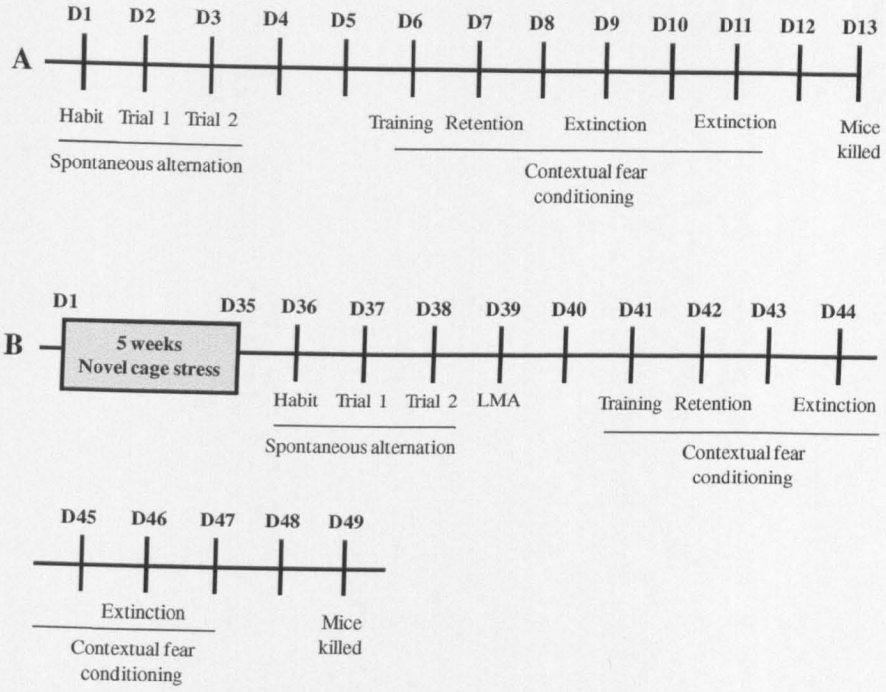


Figure 3.1. Experimental Design. A: 3 month groups were tested for spontaneous alternation, a full description of the protocol can be found in Section 3.1.2, followed by contextual fear conditioning. Mice were then humanely killed. **B:** Half of the 4.5 month old group were stressed from 3 months of age for 35 days and tested for spontaneous alternation, locomotor activity (LMA) then performance in the contextual fear conditioning test. Mice were humanely killed 48h following T4. D = Experimental day number.

The procedure involved mice being taken from their home cage and placed into a clean novel cage for 1h. The novel cages were half the size of the home cage measuring 15 x 16 x 15 cm. To achieve this a normal cage was divided in the centre by a transparent perforated plastic divider. The novel cages had a thin layer of sawdust on the floor, but were devoid of all normal environmental enrichment stimuli (cardboard play-tube and bedding); food and water were not provided throughout the stress session. Mice were lifted in and out of the novel cages by the tail; to control for handling effects control non-stressed TASTPM

mice were similarly manipulated and lifted from the home cage by the tail, and replaced immediately back into the home cage at times corresponding to the start and the end of the stress procedure. Stressed mice were removed from the novel cage after 1h exposure and placed back into their home cage. Between each stress session, sawdust was discarded and the novel cages were thoroughly washed with disinfectant wash to remove olfactory scent markers.

Physiological measures

Novel cage stress has been previously shown to reduce normal weight gain in mice (Pardon et al., 2004). To determine the effects of 5 weeks of novel cage stress on TASTPM mouse body weight, measures were taken both prior to, and after the stress period. Body weight changes over time were analysed using a repeated measures ANOVA with Stress as the between-subject factor.

Faecal boli were counted for mice exposed to novel cage stress at the end of the 1h stress session to provide an indicator of emotional reactivity to the novel environment, as previously described (Pardon et al., 2004; Pardon et al., 2005; Pardon et al., 2009). Changes in stress reactivity with repeated novel cage exposure were analysed using a repeated measures ANOVA with Stress Session as the within-subject factor. A post-hoc test was used comparing weekly mean defecation counts, using a series of paired samples t-tests comparing each weekly mean score with the score from the previous week (for example, week 1 vs. week 2, and week 2 vs. week 3). As seen previously (Pardon et al., 2004; Pardon et al., 2005), the handling procedure, which is very brief, rarely induced defecations; therefore, this analysis was solely performed on mice subjected to novel cage stress.

For all results described below, values are quoted as mean ± SEM, and Tukey's post-hoc test was used for multiple comparisons in the analysis where appropriate.

Locomotor activity

Locomotor activity was measured following the spontaneous alternation trials in the 4.5 months old TASTPM mice only (figure. 3.1) and was used to determine any differences in the activity of 4.5 month old non-stressed and stressed TASTPM mice. Mice were individually placed in one of 8 identical Plexiglas chambers measuring 13 x 17 x 20 cm, for 30 mins. Locomotor activity (distance moved, cm) was recorded and video-taped via a camera positioned above the boxes; the level of activity was recorded automatically using Ethovision software (Noldus, Wageningen, Netherlands).

Resulting data was expressed, first, as total distance travelled throughout the 30 min trial analysed using an independent samples t-test to determine any effects of 5 weeks of novel cage stress on total ambulation. Second, to study habituation to the novel environment, total distance travelled over 5 min time intervals (6 intervals in total) was analysed using a repeated measures ANOVA with Time as the within-subject factor, and Stress as the between-subject factor.

Spontaneous alternation

Spontaneous alternation was tested in all mice as a measure of spatial working memory performance. A standard T-maze was built in-house, (top horizontal arm 94cm x 8cm x 15cm, bottom vertical arm 40.5cm x 8cm x 16cm). The

mouse was placed in the vertical arm starting chamber, and held by a sliding plastic door for ~10 sec; the door to the holding chamber was then removed. The mouse was free to explore the maze; at the T-junction the mouse then chose to explore either the left or right horizontal arm. Once the mouse had made this decision, and explored one arm, the opposite arm was closed off using a plastic sliding door thus preventing the mouse from exploring the opposite arm. The mouse was then returned to the starting chamber, held there for ~10 sec, again released and allowed to explore the maze, figure 3.2. Mice were exposed to two spontaneous alternation trials, separated by 24h, the results from each trial were averaged for each mouse. During each trial, the mouse had the opportunity to undertake the decision making process, and explore, up to 6 times within 10 mins. Performance of the mice during this task was assessed by the number of successful alternations in arm directions between each attempt at exploration. As 6 alternations/trials were permitted per session the mouse could perform a maximum of 5 successful alternations during the session; the result was expressed as a percentage of successful alternations during the trial; for example, if the mouse alternated successfully 4 out of the five possible times, it showed 80% alternation. This test was run similarly to that used previously by our group on NMRI mice (Pardon et al., 2004), and was subsequently used to study age-related changes in spatial working memory in TASTPM mice during the PhD studies of Gillian Scullion (Scullion., 2008). Due to unexpected aversive responses to the apparatus exhibited by TASTPM mice, non-performance at this task, (i.e. the mouse not exploring the maze due to an anxiety-like behaviour), was quantified during the test. Mice that did not complete 6 decisions, and explorations, within both 10

min sessions were recorded as non-performers and were excluded from the analysis. Mice which made 6 alternations on one trial-day, only, were recorded as partial non-performers and the single value was subsequently used for analysis. Mice were habituated to the T-maze, and exposed to the plastic sliding doors for 10 mins, 24 h prior to the first trial (figure 3.1). T-maze equipment was thoroughly cleaned with 20% ethanol between all tests to reduce olfactory cues.

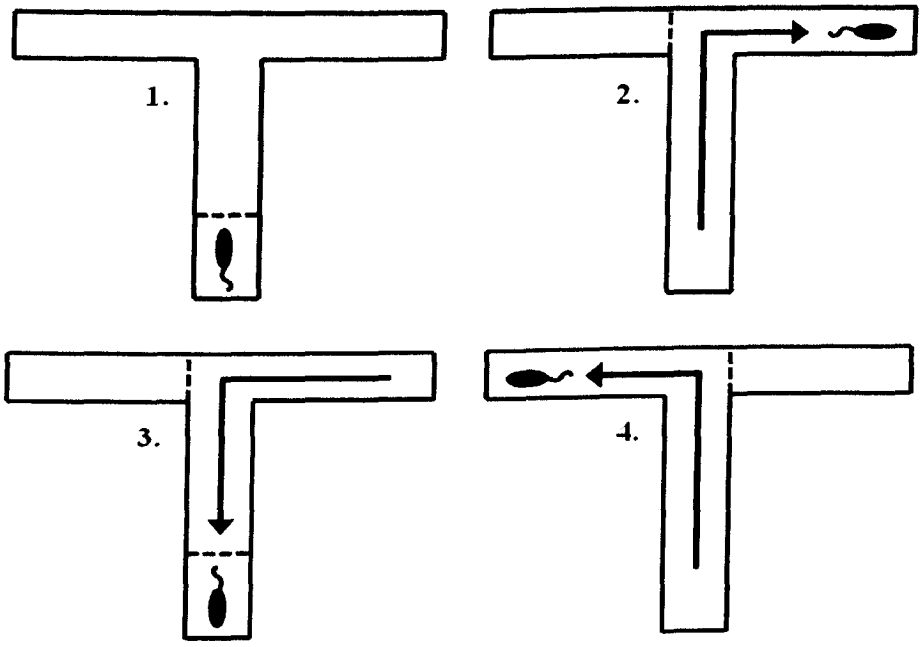


Figure 3.2. Spontaneous alternation trial protocol. 1. Mouse placed in the starting chamber. **2.** Mouse released from starting chamber and explored either left or right arm, the opposite arm was closed off. **3.** Mouse was returned to the starting chamber. **4.** Mouse was, again, free to explore either the left or the right arm, the opposite arm was closed off (as in step 2). This protocol was repeated up to 6 times within a 10 min period.

To determine any age-related changes in performance of TASTPM mice a paired samples t-test was used to compare TASTPM performance at 3 and 4.5

months of age, Age was the between-subject factor. An independent samples t-test was used to determine the effects of stress in 4.5 month old TASTPM mice, with Stress as the between-subject factor.

Short-term memory tests

CFC was used here to study short-term contextual memory performance; the protocol was conducted as described in detail in Section 2.2. In brief, during memory acquisition mice were exposed to 10 footshocks (1 sec duration, 0.4 mA) in the CFC operant chamber over 10 mins (figure 3.1) 24 h later mice were returned to the operant chamber for 3 mins in the absence of shocks and immobility was scored as a measure of contextual memory retention (figure 3.1); at two 48 h intervals after the memory retention test, mice were, again, exposed to the context for 3 mins and immobility was scored to study extinction (figure 3.1). Similar to the CFC optimisation (described in Section 2.2) behaviour of the mice was video recorded and subsequently scored. For the 3 min memory retention and extinction trials behaviour was scored three times by the same experimenter who was blind to the treatment groups; the two values closest together were taken for an average value. On occasion, when the two values closest together were > 20 sec apart, this was considered unacceptable variation and the behaviour was rescored until two values were < 20 sec different. This method of scoring was adopted for all further experiments described in this thesis to maintain consistency between studies.

Activity (distance moved, cm) during the memory acquisition trial was compared using a repeated measures ANOVA with Time as the within-subject factor, and Strain or Group (comparing 3 month old, 4.5 month old non-

stressed and 4.5 month old stressed TASTPM mice) as the between-subject factor. Memory performance was analysed using two methods. First, mean immobility (sec) between groups was compared using a repeated measures ANOVA with Trial as the within-subject factor, and Strain or Group as the between-subject factor. Tukey's post-hoc test was then used for multiple comparisons where appropriate. The second analytical method to study contextual memory extinction involved the production of extinction indexes. Three extinction indexes for each group mice were created by calculating differences in immobility between the CFC trials. "Extinction" was calculated by subtracting performance (total immobility, sec) during the final extinction trial by that during the memory retention trial; "Extinction 1" was the first extinction subtracted by the memory retention trial; for "Extinction 2", the final extinction trial was subtracted by the first extinction trial. Negative scores arising from these indexes reflected a drop in immobility between trials, and therefore extinction, and data were compared to 0 (representing no extinction) using a one-sample t-test.

Quantification of brain β-amyloid levels.

Brain β-amyloid was quantified to assess age-related changes in the pathological status of TASTPM mice and determine the effects of 5 weeks of novel cage stress on this parameter. For both the 3 month and 4.5 month old groups, mice were killed 24 or 48h following the final CFC extinction trial respectively. The brains were removed from the skull and the frontal cortex (defined as brain tissue above the corpus callosum near the anterior of the brain), hippocampus and cortex were dissected and immediately frozen using

dry ice and stored at -80°C. Upon tissue analysis, brain was defrosted and weighed to allow expression as pg β-amyloid per milligram (mg) of wet tissue. Brain samples were kept on ice, added phosphate buffered saline (PBS) containing protease inhibitor cocktail solution, homogenised using a hand homogeniser, and then frozen at -80°C until analysis. Lysate was defrosted and centrifuged at 4°C, 14,000 rpm (13.5cm rotor, Sigma 3-18K) for 20 mins, 50µl of the supernatant was used to quantify $A\beta_{1-40}$ in the soluble fraction. The pellet was re-suspended in 100µl PBS and protease inhibitor cocktail solution, vortexed and spun at 4°C, 9000 rpm (13.5 cm rotor, Sigma 3-18K) for 20 mins; the supernatant was removed and the pellets frozen at -80°C. To quantify the levels of insoluble $A\beta_{1-42}$ associated with deposited β-amyloid plaques, the pellet was re-suspended in 100µl of 5M guanidine HCl solution. Soluble $A\beta_{1-40}$ and insoluble $A\beta_{1-42}$ levels were assessed using a solid phase sandwich enzyme-linked immunosorbant assay (ELISA) kit (Bioscience International, Inc., California, USA). Assays were performed following standard instructions included with the kit and have been successfully used previously in our group to monitor brain changes in β-amyloid in TASTPM mice (Pardon et al., 2009). Briefly, wells of a 96-well plate were coated in a monoclonal antibody recognising the NH_2-terminus of the β-amyloid protein. 50 µl of sample, either the soluble or insoluble fraction of β-amyloid, was added to the well, along with the detection antibody (Rabbit ant-Hu $A\beta_{1-40}$ / $A\beta_{1-42}$). The resulting complex was recognised by a third antibody labelled with alkaline phosphatase. A fluorescent substrate solution was added to the wells, and fluorescence was quantified using a plate reader. All brain samples were run in duplicates and values were averaged during analysis. Resulting data were

calculated using a standard curve run with the sample and expressed as pg $A\beta_{(1-40)}$ or $A\beta_{(1-42)}$ per mg of wet tissue. Previous optimisation of ELISAs has shown no detectable levels of human β-amyloid in WT mice (Pardon et al., unpublished); therefore, analysis was only conducted on the TASTPM mouse brains.

Levels of β-amyloid in the ROIs studied were compared between 3 month old TASTPM and 4.5 month old non-stressed and stressed TASTPM mice using a one-way ANOVA with Group as the between subject factor.

3.1.3. Results

Physiological measures

Overall, there was a significant Time effect ($P < 0.0001$) indicating increased body weight for both the non-stressed and stressed TASTPM mice before and after the novel cage stress period with age (figure 3.3A); however, there was no effect of repeated novel cage stress.

Stressed TASTPM mice demonstrated a significant change in weekly defecation count over the 5 week stress period ($P = 0.014$) as revealed by a repeated measures ANOVA. Weekly defecation number during the 5 weeks of the novel cage stress period was then compared with the score from the previous; there was a significant increase in weekly defecation counts from week 2 to week 3 only ($P < 0.0001$); all other weekly comparisons were non-significant, figure 3.3B.

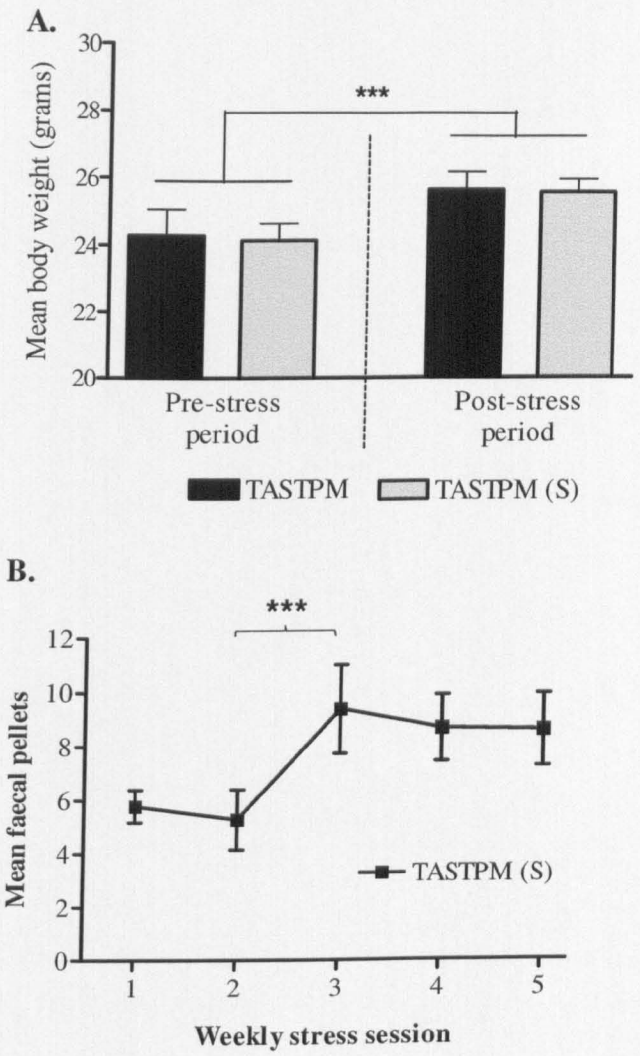


Figure 3.3. Physiological measures. A: Changes in mean (± SEM) body weight between (grams) non-stressed TASTPM (TASTPM, n = 7) and stressed TASTPM mice (TASTPM (S), n=7) between 3 to 4.5 months of age (prior to, and following the stress period respectively). Overall, both groups displayed a significant increase in body weight over time (***P < 0.0001), but stress had no effect on this weight gain. **B:** weekly mean defecation count (± SEM) for TASTPM stressed mice (n=7) (defecation count during the 1h stress session could not be quantified in non-stressed mice residing in their home cages), over the 5 week stress period. There was a significant (P = 0.014) change in defecation over time, but only one significant difference between weekly defecation counts, an increase in defecation from week 2 to week 3 (***$P < 0.0001$).

Locomotor activity performance

Throughout the locomotor activity trial, (distance moved, cm) was separated into six 5 min time intervals to determine habituation to a novel arena, there was an overall Time effect (P = 0.002) as activity of all mice declined over time, and a Time X Stress interaction (P = 0.013), figure 3.4. However, when analysing total activity (distance moved) throughout the 30 min trial there was no significant difference between non-stressed (4999.9 ± 332.0cm) and stressed (4983.0 ± 214.3cm) TASTPM mice.

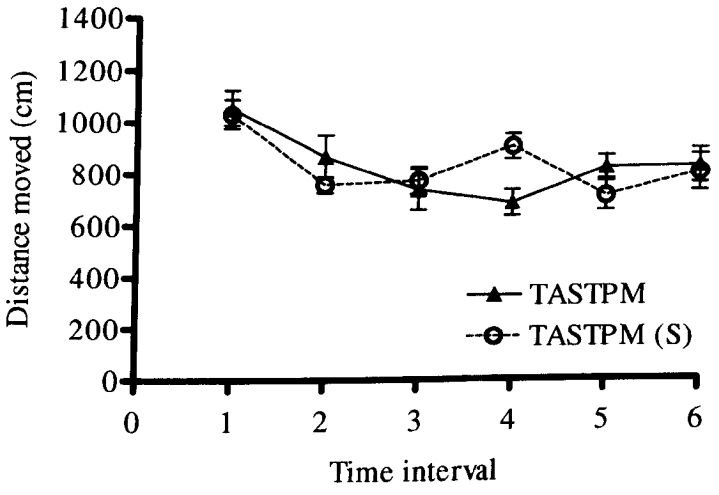


Figure 3.4. Locomotor activity performance: Mean (± SEM) locomotor activity (distance moved, cm) over a 30 min trial expressed as 5 min time intervals for 4.5 month old non-stressed (TASTPM, n=7), and stressed TASTPM mice (TASTPM (S), n=7). Both groups displayed a decline in activity as a function of time (P = 0.002), and there was a difference between the two groups with time (P = 0.013), but no overall difference in total activity throughout the trial.

Spontaneous alternation performance

At 3 months of age there was no difference between WT and TASTPM mice for spontaneous alternation performance. There were no significant age-related changes in performance of TASTPM mice from 3 to 4.5 months of age. There was no difference between non-stressed and stressed 4.5 month old TASTPM mice, although stressed TASTPM mice showed a trend towards a reduction in correct alternations compared to non-stressed TASTPM mice ($P = 0.083$), table 3.1.

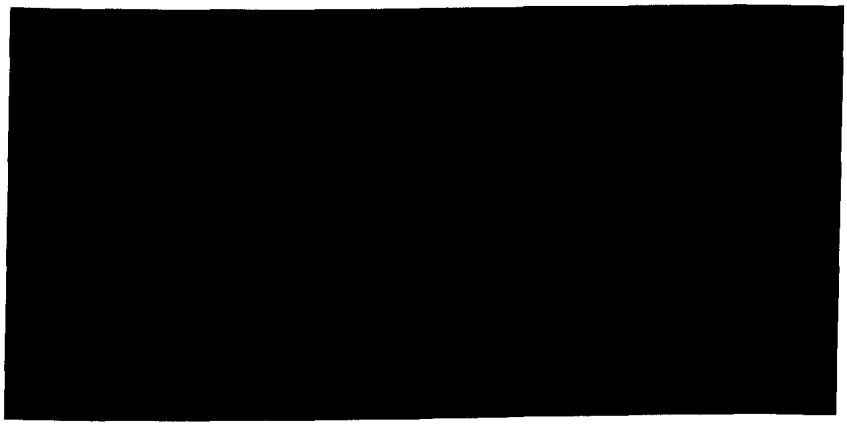


Table 3.1. Spontaneous alternation performance. Mean (± SEM) performance (percentage correct alternation) of WT (n = 9), TASTPM non-stressed (n = 6) and stressed (n = 7) at two ages, 3 and 4.5 months old. There was no difference in spontaneous alternation performance between 3 month old WT and TASTPM mice, or any age-related difference between 3 and 4.5 month old TASTPM mice. At 4.5 months TASTPM stressed mice appeared to display a lower performance at this task compared to non-stressed TASTPM mice, but this was not statistically significant ($P = 0.083$).

In this task, non-performers were classed as mice which did not complete 6 arm explorations within both two 10 min trials, and partial non-performers were mice which only performed 6 arm explorations in one of the two trials. It

is worthy of note that there was 1 non-performer, and three partial non-performers (completed 6 explorations on one test day only) in the 4.5 month non-stressed TASTPM mice. All WT and stressed TASTPM mice studied here performed this test fully; this is the reason for the variable number of subjects in the above analysis.

Short-term memory tests

An overall significant decline in activity (distance moved, cm) over time during the memory acquisition trial for mice in both 3 month and 4.5 month old groups was observed ($P < 0.0001$) indicating successful acquisition of conditioned fear. At 3 months of age a Time X Strain interaction did not quite reach statistical significance ($P = 0.05$), figure 3.5A. At 4.5 months of age, stress had no effect on the decline in activity during training, figure 3.5B.

During the memory retention tests, immobility (sec) was similar in WT and TASTPM mice at 3 months of age. At 3 months of age, WT mice exhibited a reduced immobility from the memory retention to the last extinction trial ($P = 0.025$), and had a significantly lower level of immobility during the final extinction trial compared to TASTPM mice ($P = 0.004$), figure 3.6A. There was no age-related change in TASTPM mouse immobility levels from 3 months to 4.5 months of age during the memory retention test, and stress from 3 months of age had no effect. At 4.5 months of age, TASTPM mice did not exhibit changes in immobility from the memory retention to the extinction trials, and there was no difference when compared to the performance of 3 month old TASTPM mice. Pre-exposure to 5 weeks of novel cage stress had no

effect on 4.5 month old TASTPM mouse immobility levels across the extinction trials, figure 3.6B.

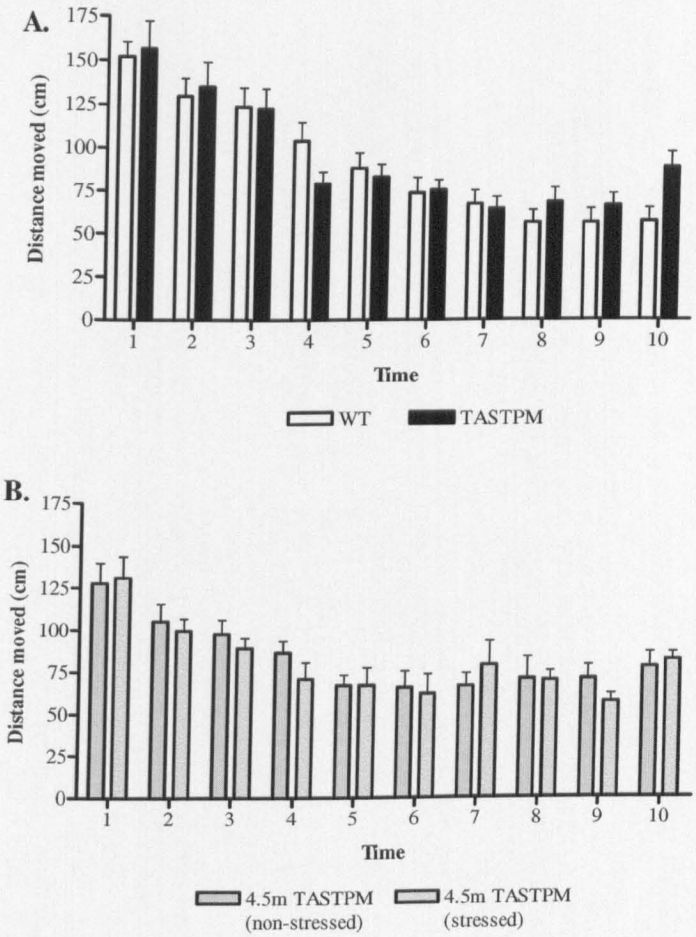


Figure 3.5. CFC memory acquisition performance. A: Mean (± SEM) activity (distance moved, cm) over 10 min memory acquisition trial, 3 month old WT (n=9) and TASTPM (n=8) both exhibited reduced activity upon repeated footshock exposure (once per minute). **B:** Mean (± SEM) activity (distance moved, cm) over 10 min memory acquisition, 4.5 month non-stressed (n=7) and stressed TASTPM mice (n=7) mice both displayed similar reductions in activity over the 10 min training period.

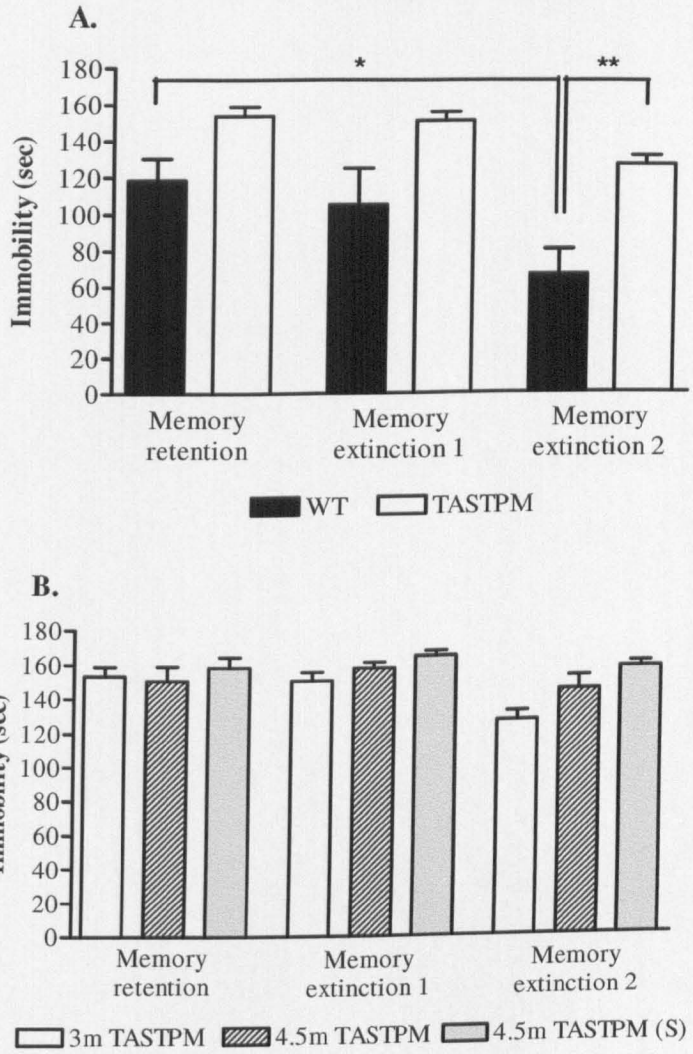


Figure 3.6. CFC short-term memory retention and extinction. A: Mean (± SEM) immobility (sec) of 3 month old WT (n=7) and TASTPM mice (n=8), immobility is similar between WT and TASTPM mice during the memory retention trial. WT mice demonstrated a significant reduction in immobility from the memory retention to the final extinction trial (*P = 0.025). During the final extinction trial WT mice had a significantly lower level of immobility compared to TASTPM mice (**P = 0.004). **B:** Mean (± SEM) immobility (sec) of 3 month old TASTPM (3 month, n=8), and 4.5 month old TASTPM mice non-stressed (4.5 month NS, n=7) and stressed (4.5 month (S), n=7) mice. There was no age-related change in immobility exhibited by TASTPM mice during the trials.

Three extinction indexes for each group mice were created based on the differences in immobility between the CFC trials. "Extinction" refers to the difference between the memory retention and final extinction trial, "Extinction 1" refers to the difference between the memory retention and first extinction trial, and "Extinction 2" is the difference between the first and final extinction trial; results were compared to 0 using a one samples t-test. Significant differences in "Extinction were observed in 3 month old WT ($P = 0.005$) and TASTPM ($P = 0.001$) mice only. During "Extinction 1" no significant differences were observed in any group. Significant differences were observed during "Extinction 2" in all groups ($P < 0.05$), with the exception of 3 month old WT mice where there was no statistical significance ($P = 0.063$), figure 3.7.

Brain β-amyloid levels in TASTPM mice

Regional levels of $A\beta_{(1-40)}$ were compared between 3 month old, and 4.5 month old non-stressed and stressed TASTPM mice. There were significant Group effects in the hippocampus ($P = 0.001$) and frontal cortex ($P = 0.026$), but not the cortex. There was no significant difference between 3 and 4.5 month old $A\beta_{(1-40)}$ levels, but stressed 4.5 month old TASTPM mice had significantly higher levels compared to non-stressed mice in the hippocampus only ($P = 0.005$), table 3.2.

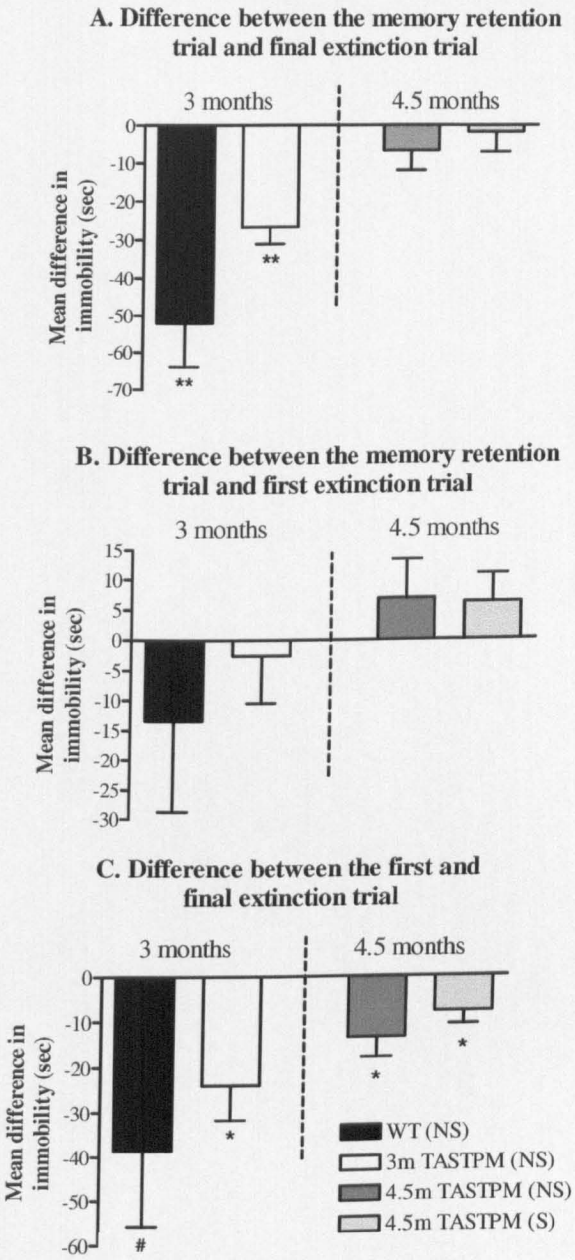


Figure 3.7. Extinction indexes of 3 and 4.5 month old mice. Mean (± SEM) extinction indexes were calculated for 3 month old WT mice (3m WT, n = 7), 3 month old TASTPM mice (3m TASTPM, n = 8), 4.5 month old TASTPM non-stressed mice (4.5m TASTPM, n = 7) and 4.5 month old TASTPM stressed mice (4.5m TASTPM (S), n = 7). A. There was a significant difference between the memory retention and final extinction trial in 3 month old WT and TASTPM mice only (**$P < 0.01$). B. No groups showed any difference between the memory retention and first extinction trial. C. Between the first and last extinction trial all mice exhibited significant differences (*$P < 0.05$), except 3 month old WT mice which did not reach statistical significance ($^{\#}P = 0.063$).

		TASTPM			
		3 month old	4.5 month old (non-stressed)	4.5 month old (stressed)	
$A\beta_{(1-40)}$	*Hippocampus*	3.49 (0.32)	3.80 (0.20)	5.72 (0.46)	**
	Frontal cortex	0.04 (0.01)	0.11 (0.02)	0.12 (0.03)	*
	Cortex	1.20 (0.18)	1.75 (0.27)	1.97 (0.40)	
$A\beta_{(1-42)}$	*Hippocampus*	12.28 (1.11)	14.45 (3.13)	19.69 (2.11)	#
	Frontal cortex	12.72 (1.18)	17.28 (2.28)	20.75 (1.21)	**
	Cortex	12.30 (0.72)	17.72 (2.07)	21.78 (3.42)	*

Table 3.2. Brain β-amyloid levels in TASTPM mice. Mean (±SEM) levels of $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ (pg/mg wet tissue) in the hippocampus, frontal cortex and cortex of 3 month old TASTPM mice (n=8), 4.5 month non-stressed (n=6-7) and stressed (n=7) TASTPM mice. $A\beta_{(1-40)/42}$ levels were compared across the three groups using a one-way ANOVA with Group as the within group difference, Tukey's post hoc test was used for multiple comparisons. For $A\beta_{(1-40)}$ levels, there was a significant Group effect in the hippocampus ($^{**}P < 0.01$) and frontal cortex ($^{*}P < 0.05$); there were no significant age-related changes, but stress elevated levels in the hippocampus of 4.5 month old mice. For $A\beta_{(1-42)}$ levels, there were significant Group effects in the frontal cortex ($^{**}P < 0.01$) and cortex ($^{*}P < 0.05$), but did not reach statistical significance in the hippocampus ($^{\#}P = 0.05$); however, there were no significant age or stress-related changes in levels between the three groups.

When comparing $A\beta_{(1-42)}$ levels between the three groups, there was a Group effect in the frontal cortex ($P = 0.007$), cortex ($P = 0.023$), but not quite in the hippocampus ($P = 0.05$). There were no age-related changes between 3 and 4.5

month old TASTPM mice in $A\beta_{(1-42)}$ levels in any brain region, nor any effect of stress on 4.5 month old TASTPM mice.

3.1.4. Discussion

The studies described herein were aimed, first, to determine base-line short-term memory retention and extinction of TASTPM mice at a pre-pathological age. Second, to assess the effects of 5 weeks of novel cage stress, applied from 3 months of age, on age-related changes in memory retention and extinction between 3 and 4.5 month old TASTPM mice. The contextual memory behavioural endpoint was supported with assessments of working memory and brain β-amyloid levels.

Short-term memory, and spontaneous exploration activity, remained intact in 3 month old TASTPM compared to WT mice. Although TASTPM mice expressed short-term contextual memory extinction at 3 months of age, it appeared to be impaired when compared to age-matched WT mice. Stress from 3 months of age had no effect on short-term contextual memory retention, memory extinction or spontaneous alternation performance, but increased $A\beta_{(1-40)}$ in the frontal cortex of 4.5 month old TASTPM mice, only.

Our group has shown, in a similar previous study, that TASTPM mice develop a short-term contextual memory retention deficit between 4 and 5.5 months of age; consistent with this, we report here intact short-term memory retention in 3 month old TASTPM mice. In the studies described above, we have shown that there was no age-related change in short-term memory retention from 3

months to 4.5 months of age in TASTPM mice, indicating that the short-term memory deficit described previously, (Pardon et al., 2009), likely occurs between 4.5 and 5.5 months of age. As demonstrated previously, 5 weeks of novel cage stress applied from 4 months of age reversed the memory deficit present in 5.5 month old TASTPM; an identical stress regimen applied from one month earlier (3 months of age) had no effect on short-term memory retention in 4.5 month old TASTPM mice. These results, combined, imply that effects of stress on short-term memory retention of TASTPM mice are age-dependant; this may implicate brain β-amyloid, known to increase with age in TASTPM mice (Howlett et al., 2004), playing a role in this effect. Indeed, previously, a stress-induced reduction in brain β-amyloid levels coincided with the reversal of short-term memory deficit (Pardon et al., 2009). In addition to evidence described in the current study, a body of research appears to support this hypothesis by linking environmental stressors with changes in brain β-amyloid levels in similar β-amyloid over-expressing transgenic mouse lines (Kang et al., 2007; Dong et al., 2008), which have also been linked with changes in behaviour (Dong et al., 2004; Jeong et al., 2006).

We have previously demonstrated that TASTPM mice exhibit a short-term contextual memory extinction deficit from 4 months of age, prior to developing a short-term memory retention deficit (Pardon et al., 2009). It is worthy of note that two methods of analysing the CFC extinction performance were used, the first, referred to here as Analysis 1, was a repeated measures ANOVA assessing the change of immobility scores as a factor of time (repeated exposure to the context). The second, Analysis 2, involved a series of subtractions of scores between trials providing an extinction index, extinction

was represented by a negative score, and this was compared to 0. These two types of analysis allow for some different interpretations of the data.

Here we demonstrated, using Analysis 1, that TASTPM mice at 3 months of age exhibited a memory extinction deficit when compared to age-matched WT mice. There was also no age-related change in TASTPM mice as a memory extinction deficit was also evident in 4.5 month old TASTPM mice, plus there was no effect of 5 weeks of repeated mild stress on memory extinction. The absence of a stress effect on TASTPM memory extinction deficit is consistent with a similar finding when studied in mice 1 month older, i.e. stress from 4 months of age had no effect on extinction performance in 5.5 month old TASTPM mice (Pardon et al., 2009). Together, these findings suggest that, during periods corresponding to early-to-moderate pathological status in TASTPM mice, the deficit in short-term memory extinction is resistant to the effects of repeated mild stress, and can be observed from as early as 3 months of age in TASTPM mice. This extinction deficit was described for the first time by our group (Pardon et al., 2009), and may reflect a degree of cognitive inflexibility in the TASTPM mice, given their inability to extinguish a previously acquired memory, which may be independent of brain β-amyloid deposits given its presence at the pre-pathological age of 3 months.

The second method of analysis, Analysis 2 using extinction indices, revealed no extinction in any group between the memory retention and first extinction trial, but all groups demonstrated extinction between the first extinction trial and final extinction trial. Interestingly, only 3 month old WT and TASTPM mice exhibited full extinction from the memory retention trial to the final

extinction trial. These findings indicate that only 3 month WT and TASTPM mice exhibit overall extinction over three trials, whereas 4.5 month old TASTPM non-stressed and stressed mice posses a partially impaired contextual memory extinction.

The major differences between the two analytical methods are that, first, using Analysis 2, 3 month TASTPM mice appear to exhibit normal contextual memory extinction similar to that observed in age-matched WT mice. Second, using Analysis 2, both non-stressed and stressed 4.5 month TASTPM mice, although not exhibiting overall extinction, do display extinction in the later trials only. Both analyses indicate a decline in contextual memory extinction with age in TASTPM mice between 3 and 4.5 months, and reveal that stress has no effect on extinction, but findings from Analysis 2 indicate that a pathological change occurs between the ages 3 and 4.5 months in TASTPM mice which is responsible for the development of the partial impairment in memory extinction. Although not reaching statistical significance, there was a trend towards an increase in β-amyloid in all brain regions studied between 3 and 4.5 months of age, implicating the presence of β-amyloid being, at least, partially responsible for the development of the extinction deficit in TASTPM mice.

A body of evidence suggests β-amyloid levels directly correlate with the development of memory deficits in transgenic mice over-expressing the protein, (for review, (Morgan, 2003)). Conversely, evidence also suggests that memory deficits in similar mouse lines can occur prior to overt β-amyloid deposition (Holcomb et al., 1999; Dodart et al., 2000), indicating the cognitive

deficits maybe due to an β-amyloid-independent mechanism such as brain atrophy or changes in synaptic density. Depending on whether the results from the study described here are to be interpreted as 3 month old TASTPM mice possessing a short-term memory extinction deficit, or not, in-turn dictates whether this likely occurs via a β-amyloid-independent, or β-amyloid-dependent manner respectively, and the literature cited above can support either argument. Analysis 1 indicated that the 3 month TASTPM mouse extinction deficit was evident when compared to age-matched WT mice; it is likely that extinction is still evident, but not detectable when compared to robust extinction observed in the control WT group. In summary, these data indicate a worsening of short-term memory extinction between 3 and 4.5 months of age in TASTPM, as revealed by both analytical methods, but stress had no effect on this process.

Several studies into the mouse neuroanatomy of contextual fear memory extinction implicate the hippocampus as a key structure (Fischer et al., 2004; Fischer et al., 2007; Lattal et al., 2007; Sananbenesi et al., 2007), and transgenic mice exhibiting hippocampal reduction are deficient in contextual memory extinction (Schimanski et al., 2002). Furthermore, evidence suggests a role for the prefrontal cortex (Herry and Garcia, 2002; Barrett et al., 2003; Sgobio et al., 2008) and the amygdala (Herry and Mons, 2004) in extinction of fear memory associated with a stimulatory cue. A model for the expression and regulation of memory extinction which involves communication between these three areas has been proposed; simply, hippocampal activity may feedback to the prefrontal cortex, which subsequently provides a regulatory input to the amygdala, overall affecting expression of memory extinction (for review, (Ji

and Maren, 2007)). One or more of these brain regions are likely to be affected as part of the early pathological changes that occur in the TASTPM mouse between 3 and 4.5 months of age; certainly β-amyloid levels were seen to increase (although not statistically significant) in the hippocampus and frontal cortex of TASTPM mice between these two ages.

Working memory in TASTPM mice, as assessed by spontaneous alternation performance, remained intact at 3 months of age when compared to age-matched WT mice. There was no age-related change in TASTPM mouse performance at this task between 3 and 4.5 months. Repeated mild stress from 3 months of age showed a trend towards a reduced performance at this task in TASTPM mice, suggesting a detrimental role of stress on working memory performance; indeed mild stress has been shown to impair performance at this task in WT mice in a previous study (Bats et al., 2001). Mice which did not complete one or more trials at this test were recorded as non-performers. Non-performers at this task were within the 4.5 month old non-stressed TASTPM group only; all stressed 4.5 month old TASTPM successfully completed all trials, as did 3 month old WT mice. A similar study demonstrated that the number of TASTPM mice unable to perform at this task increased with age (Scullion et al., unpublished), suggesting this poor performance may be associated with pathological severity. The non-performers throughout this task appeared to demonstrate an aversive response to the experimental apparatus and environmental surroundings, which likely led to the reduced exploration observed. This theory is supported by the known age-related increasing aggression and anxiety-like behaviours in TASTPM mice described previously (Pugh et al., 2007). Interestingly, pre-exposure to stress in the 4.5 month old

TASTPM mice appears to have reduced this fearful phenotype in the T-maze despite nearly impairing performance. A more detailed account of unconditioned aversive behaviours of these groups, such as time periods spent immobile during T-maze exposure, are needed before any conclusions can be drawn.

As mentioned above, there were non-significant increases in β-amyloid in the hippocampus, frontal cortex and cortex of TASTPM mice with age from 3 to 4.5 months, as one would expect. Repeated mild stress from 3 months of age had little effect on β-amyloid levels, with the exception of significantly increasing $A\beta_{(1\text{-}40)}$ in the frontal cortex. Contrary to this, a previous study saw novel cage stress applied from one month later (4 months) being capable of attenuating the age-related increase in β-amyloid levels of 5.5 month old TASTPM mice. It is, therefore, possible that the effects of stress on β-amyloid levels depend on the age of testing; stress might only have a beneficial effect on β-amyloid when a certain threshold level has been reached. This, in turn would reflect the behavioural findings of that previous investigation given stress improved memory performance at a later age of 5.5 months in TASTPM mice only. However, the reason as to why novel cage stress should increase β-amyloid levels in the current study is unknown, but remains partly consistent with several findings that describe a variety of stressors being capable of increasing levels of this pathological marker in similar mouse models (Dong et al., 2004; Jeong et al., 2006; Kang et al., 2007; Dong et al., 2008)

In summary, reported above is the presence of a short-term contextual memory extinction deficit which developed between 3 and 4.5 months of age in

TASTPM mice. However, short-term memory retention remained intact at 3 months old, and was unaffected by age in TASTPM mice. Pre-exposure to repeated novel cage stress had no effect on either memory retention or extinction expressed by 4.5 month old TASTPM mice. Brain β-amyloid levels appeared to increase with age but largely remained unaffected by novel cage stress. It is clear from this study, and the previous study from our group, that a short-term memory deficit developed in TASTPM mice between 4 and 5.5 months of age, and a short-term memory extinction deficit develops around 1 month earlier. Novel cage stress had a positive effect on both memory and β-amyloid levels between 4 and 5.5 months of age, but had little effect when applied one month earlier and, in fact, elevated levels in one brain region. It is, therefore, possible that these positive effects are only detectable when studied at a time-point associated with a higher level of pathological severity.

3.2. Effects of stress on early-to-moderate stages of β-amyloid pathology in TASTPM mice

3.2.1. Introduction

The previous section (Section 3.1) dealt with characterising the behaviour of TASTPM mice during early stages of β-amyloid pathology, between 3 and 4.5 months of age. These studies, alongside those conducted previously by our group, (Pardon et al., 2009), indicated that TASTPM mice develop a short-term contextual memory deficit between 4.5 and 5.5 months of age. Repeated novel

cage stress was capable of reversing this short-term contextual memory deficit as well as attenuating the increased brain β-amyloid levels in 5.5 month old TASTPM mice, but had no effect in mice one month younger. We also observed a short-term contextual memory extinction deficit which developed between 3 and 4.5 months of age in TASTPM mice, and repeated stress exposure had no effect on this apparently robust impairment at all ages investigated.

As described in Section 1.1.2, clinical AD is associated with memory loss, initially, as an inability to form and recall recent/episodic (short-term) memories (Backman et al., 2001; Ringman, 2005; Gauthier et al., 2006); this eventually leading to loss of remote (longer-term) memories later in the disease progression (Sartori et al., 2004). Interestingly, a similar pattern of deficits has been observed in an APP x PS-1 double transgenic mouse line; short-term memory was impaired during a radial-arm-water-maze memory paradigm (dependant on intact hippocampal function) in young (3 month old) mice, a time-point corresponding to mild pathology, but longer-term memory remained intact in these mice until 6 months of age (Trinchese et al., 2004), a time-point associated with more moderate pathological burden. The short-term memory deficit found during the earlier stages of β-amyloid pathology in TASTPM mice supports these findings; however, we do not, as yet, know whether long-term memory performance and other AD-associated pathologies are affected in older TASTPM mice corresponding to stages of more moderate pathological severity. Moreover, the effects of novel cage stress exposure on the various pathologies exhibited by older TASTPM mice are currently unknown. Therefore, these issues are addressed herein by investigating the performance

of stressed and non-stressed TASTPM mice, between 5.5 and 6.5 months of age.

The importance of MRI as a tool for monitoring pathological progression, both clinically and preclinically, is described in detail in Section 2.3. This tool is therefore used in the following study to determine pathological status of TASTPM mice, and to assess whether the effects of repeated mild stress can be detected.

It has been suggested that the positive effects of repeated novel cage stress on TASTPM mice may be, in part, due to enhanced levels of environmental stimulation and exercise, discussed in Section 3.1.4. Although novel cage stress has been shown to elevate plasma corticosterone levels upon both acute and repeated exposure (Pardon et al., 2004; Pardon et al., 2005), it is possible that this is insufficient to induce a significant detrimental effect on TASTPM mice. In order to assess any detrimental effects of corticosterone, an independent group of TASTPM mice were administered high levels of corticosterone, behavioural phenotype, and pathological status was subsequently assessed and compared to that of TASTPM mice exposed to novel cage stress.

This section is, therefore, separated into two experiments:

1. Aimed to assess long-term memory performance of TASTPM mice at 5.5 months of age, and determine whether pre-exposure to novel cage stress can affect the long-term memory performance at the contextual

fear conditioning (CFC) test. *In vivo* MRI was then used to study AD-associated pathologies in TASTPM mice, and whether they were modulated by stress. These studies were then supported with post-mortem quantification of β-amyloid using immunohistochemistry.

2. Aimed to assess long-term memory performance of TASTPM mice one month older than those in Experiment 1, at 6.5 months of age, and determine whether pre-exposure to novel cage stress, or corticosterone at a high dose, can affect long-term contextual memory and spontaneous alternation activity. Post-mortem studies, using both ELISA and immunohistochemistry, were then performed to assess brain β-amyloid levels to compare between the various treatment groups.

Long-term memory performance of mice in Experiment 1 and Experiment 2 was also compared to determine any age-related changes or whether stress had a different effect at the two ages studied.

3.2.2. Methods

Experiment 1

Animals

Male WT (n=19) (C57Bl/6) mice (Charles River Laboratory, UK) and TASTPM (n=18) (Transgenic Unit, Biomedical Services Unit, University of Nottingham) were used. All mice were group housed until 3 months of age when they were individually housed for welfare purposes, as described in

Section 3.1.2. Mice were kept under standard conditions, food and water available *ad libitum*, cardboard play-tube and bedding were available as part of standard environmental enrichment, temperature and humidity were controlled automatically and mice were kept on a 12h light:dark cycle. All procedures were carried out according to the Animals (Scientific Procedures) Act 1987, under license PPL 40/2715 granted to Professor Charles Marsden.

Experimental design

The aim of this experiment was to determine effects of stress during early-to-moderate stages of pathology in 5.5 month old TASTPM mice. WT and TASTPM mice were divided into two subgroups, non-stressed and stressed mice (WT non-stressed, n=10; WT stressed, n=8; TASTPM non-stressed, n= 9; TASTPM stressed, n=9); structural MRI revealed one stressed WT mouse had an enlarged, atrophied hippocampus; this mouse was removed from all analysis. Mice in the stressed groups were subjected to 5 weeks of repeated novel cage stress from 4 months of age until 5.5 months of age, at which point all mice were tested for long-term contextual memory performance at the CFC test (memory acquisition occurred prior to the stress period, at 4 months of age). Pathological status was determined by *in vivo* MRI, a terminal procedure. Post-mortem immunohistochemistry was then used to assess β-amyloid burden; for experimental protocol see figure 3.8.

Novel cage stress

Novel cage stress was performed as described in Section 3.1.2. Briefly, mice were placed in a novel clean cage, half the size of their home cages, for 1h daily, four times a week (days 1-4 of the working week, weekly cage changes

took place on day 5). Novel cages were thoroughly cleaned between stress sessions with standard detergent to remove olfactory cues between animals. Novel cage stress was conducted for a total of 5 weeks. Non-stressed mice, both non-stressed WT and TASTPM, were manipulated to control for handling effects at times corresponding to the start and end of the 1h stress session.

Physiological measures

Repeated novel cage stress has been previously shown to reduce normal weight gain in mice (Pardon et al., 2004), therefore body weight was recorded, prior to novel cage stress at 4 months of age, and following stress at 5.5 months of age, for all mice. Body weight changes were analysed using a repeated measures ANOVA with Time as within-subject factors, and Strain and Stress as the between-subject factors. For all results described below values are quoted as mean ± SEM, and Tukey's post-hoc test was used for multiple comparisons in the analysis where appropriate.

Fecal boli were counted following each stress session for WT and TASTPM mice exposed to novel cage stress. This was used as an indicator of emotional reactivity to the novel environment. To determine reactivity to stress over 5 weeks, a repeated measures ANOVA was used with the repeated Stress Session as the within-subject factor, and Strain as the between-subject factor.

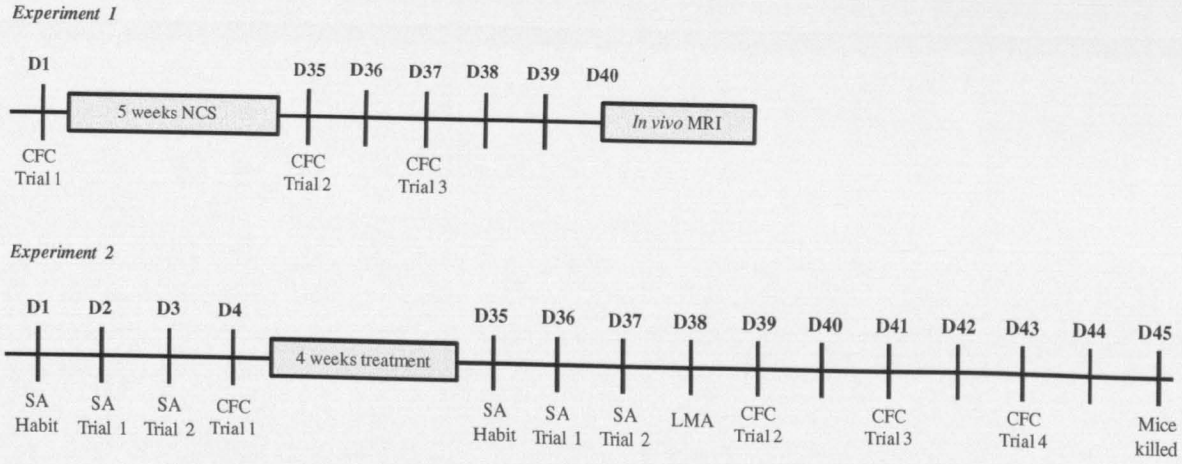


Figure 3.8. Experimental time lines. Experiment 1: all mice were subject to CFC memory acquisition (Trial 1) at 5.5 months of age, prior to 5 weeks of novel cage stress (NCS) period, and were then tested for long-term memory retention (Trial 2) and extinction (Trial 3), 35 and 37 days later respectively. All mice were then exposed to *in vivo* MRI from day 40, a terminal procedure. **Experiment 2:** all mice were tested in the spontaneous alternation (SA) test (involving two trials), following habituation to the apparatus, and prior to CFC conditioning. Similar to Experiment 1, all mice were exposed to CFC training before the "treatment period", from 5.5 months of age. Throughout the treatment period, mice were either exposed to 4 weeks novel cage stress, or 3 weeks corticosterone treatment followed by a week wash-out period, depending on their group. Following this, mice were then re-tested for SA performance, then a locomotor activity test (LMA), long-term memory retention (Trial 2, 35 days following Trial 1) and finally two extinction trials (Trials 3 and 4, 37 and 39 days following Trial 1 respectively). Mice were humanely killed two days after the final extinction trial.

Long-term memory tests

CFC was used as a tool to study the long-term memory performance of all mice. The protocol was similar to that described in Section 2.2. Mice were first exposed to the contextual memory acquisition trial prior to the stress period, at 4 months of age, figure 3.8. This involved individual placement in the CFC operant chamber for 10 min in which footshocks were administered (10 shocks, 1 sec duration administered once per minute, 0.4mA intensity). Mice were returned to the operant chamber 35 days after memory acquisition to test for long-term contextual memory retention; novel cage stress was applied between the memory acquisition trial and memory retention trial, figure 3.8. During the memory retention trial, mice were placed in the chamber without footshocks for 3 min and immobility was scored manually. Mice were returned to the conditioning chamber 48h after the memory retention test to assess long-term contextual memory extinction. This trial was identical to the memory retention trial (3 min context exposure in the absence of foot shocks, immobility scored manually). Given this was the first study, chronologically, which applied this memory test to TASTPM mice during this PhD, only one extinction trial was performed; this is unlike the remainder of the studies described throughout this thesis where two trials were performed in order to investigate the unexpected extinction deficit previously described in Section 3.1.3.

Contextual memory acquisition was measured automatically as activity (total distance moved, cm) over the 10 min trial. Activity was analysed using a repeated measures ANOVA with Time as the within-subject factor, and Strain as the between-subject factor. As with previous experiments, CFC memory

retention and extinction behaviour was recorded, and manually scored as immobility (sec) for each trial using the scoring criteria detailed in Section 3.1.2. Memory retention and extinction were analysed using the two methods detailed and described in Section 3.1.2. First, memory performance between the retention and extinction trials was analysed using a repeated measures ANOVA with the different Trial as the within-subject factor, and Strain and Stress as the between-subject factors.

The second method of analysis used involved calculating an extinction index. "Extinction" was calculated by subtracting performance during the memory extinction trial, by the memory retention trial. Negative values, therefore, represent extinction, i.e. a decrease in immobility from the memory retention to the extinction trial. Mean values for all groups were then compared to 0 using a one-sample t-test.

In vivo MRI

Scanning followed the CFC long-term memory extinction trial, figure 3.8. The MRI scanning protocol and procedures were described in Section 2.3. Briefly, mice were anaesthetised (gaseous anaesthesia ~5% isoflurane, ~0.7-0.9 litre/min O_2 flow rate) and placed in a plastic mouse holder throughout the scanning procedure, anaesthetic was delivered at ~1.5-2% isoflurane, 0.7-0.9 litre/min O_2. Core body temperature and respiration rate were monitored, and adjusted with a circulating hot-water bath and anaesthetic modulation respectively.

All scanning was conducted using a 7T magnet (Bruker Biospin, Germany) in the Brain & Body Centre, University of Nottingham. Mice were positioned

inside the magnet and scanned for volumetry and T2 relaxometry, both performed in a coronal direction. Scanning time was approximately 20 mins per mouse for each scan performed. Following scanning, mice were administered a lethal dose of pentobarbitone (i.p.) and perfusion-fixed with saline followed by 4% paraformaldehyde (PFA). Brains were removed from the skull and stored in 4% PFA at 4°C for immunohistochemistry.

Image analysis involved a semi-automated registration method (detailed in Section 2.3.3). Regions of interest (ROIs) studied by MRI included hippocampus, subiculum, anterior cingulated cortex (ACC), retrosplenial cortex, thalamus, corpus callosum and caudate putamen. Using the semi-automated image analysis tool (described in Section 2.3.3) volumtery scans produced a voxel count for each ROI in all mice which was converted to volume in mm^2 and subsequently calculated as a percentage of whole brain volume. T2 relaxation times (msec) were calculated and averaged for each ROI. Volumetry and T2 relaxometry data for each ROI, in all groups, were analysed using a two-way ANOVA with Strain and Stress as the between-subject factors.

In order to assess the level of accuracy obtained using the semi-automated image analysis tool, T2 relaxometry data were also calculated by manually overlaying identified ROIs directly onto the images using Paravision software (Bruker Biospin, Germany). Relaxation times were acquired directly via this method for all ROIs, similar to a method described previously (El Tannir El Tayara et al., 2006). Due to the slice thickness of the T2 relaxometry scans, representative examples of ROIs were limited, hippocampus (4 examples),

subiculum (2 examples), ACC (1 example), retrosplenial cortex (3 examples), thalamus (2 examples), corpus callosum (3 examples) and caudate putamen (4 examples). All manually calculated T2 relaxation times available for each ROI were averaged and compared using a two-way ANOVA with Strain and Stress as the between-subject factors. Due to unexpected deaths during the scanning procedure, and poor image quality in some mice, the number of subjects for the MRI study was as follows: non-stressed WT (n=8-9), stressed WT (n=7), non-stressed TASTPM (n=9) and stressed TASTPM mice (n=8).

Immunohistochemistry

Following MRI, mice were perfusion-fixed with 4% PFA and whole brains were removed, stored in 4% PFA at 4°C and processed for immunohistochemical detection of β-amyloid protein deposits. All sections were prepared and stained under standard conditions by staff in the Department of Histopathology, Queen's Medical Centre, Nottingham. The process of tissue embedding, sectioning and staining are described in Section 2.4.2. Briefly, brains were paraffin wax-embedded using a LEICA EG 160, sectioned on a LEICA RM 2135 rotary microtome, and immunostained for β-amyloid using a Dako Techmate 500 Plus. Full brains were serially sectioned (40μm thickness), and the ROIs studied were the hippocampus, retrosplenial cortex and thalamus; these were selected for study on the basis of having a sufficient number of representative examples of each; hippocampus (7-8 examples), retrosplenial cortex (6-7 examples) and thalamus (5-8 examples).

Sections were taken for β-amyloid quantification as described in Section 2.4.3., and were scanned using a Nanozoomer Digital Pathology slide scanner

(Hamamatsu, Japan) at 20x magnification. ROIs were manually delineated directly on the image and % ROI covered in β-amyloid was calculated using AnalySIS Pro 3.1 Soft Imaging System (Munich, Germany). Details on ROI delineation and β-amyloid quantification are described in Section 2.3. Amount of β-amyloid for all ROIs, across the sections, were averaged giving a mean score of β-amyloid cover per ROI for each mouse. Mean % β-amyloid per ROI in non-stressed and stressed TASTPM mice was compared using an independent samples t-test with Stress as the between-subject factor.

In addition to β-amyloid load quantification, numbers of "dense core" plaques (for the purpose of these investigations these were classified as plaques with a clear centre indicating a dense core of β-amyloid, figure 3.9A) and numbers of "diffuse" plaques (plaques without a dense-core indicating an earlier state of plaque maturity figure 3.9B) were manually counted in the retrosplenial cortex, hippocampus and thalamus. Mean values between stressed and non-stressed TASTPM mice were compared using an independent samples t-test for all ROIs.

Experiment 2

Animals

Male TASTPM (n=26, Transgenic Unit, Biomedical Services Unit, University of Nottingham) were used. Mice were housed under identical conditions to those described above in Experiment 1.

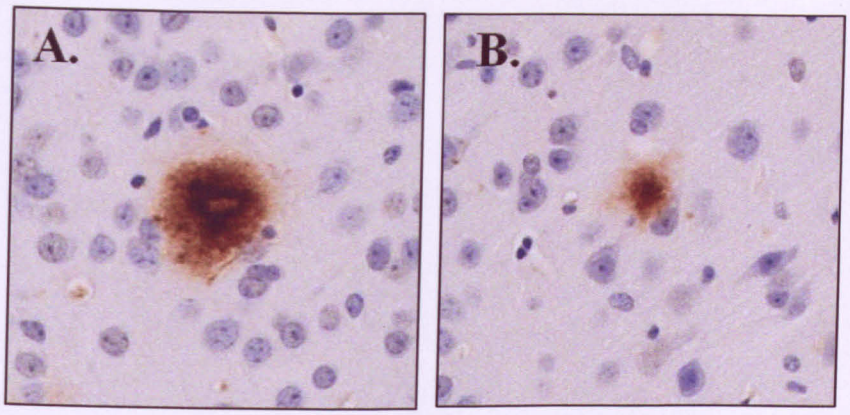


Figure 3.9. β-amyloid plaque morphology classification. 5.5 month old TASTPM mouse brain, x20 magnification **A.** Dense-cored plaque, defined as having a clear central core composed of dense β-amyloid. **B.** Diffuse plaque, defined as having no clear central core.

Experimental design

The aim of this experiment was to determine effects of stress or corticosterone treatment during moderate stages of pathology in 6.5 month old TASTPM mice. There were no WT mice in Experiment 2 a group of 5.5 month old TASTPM mice were tested for base-line working memory performance in the spontaneous alternation test. TASTPM mice were independently grouped depending upon the treatment given. Control non-stressed TASTPM (n=8), stressed TASTPM (n=9) and corticosterone-treated mice (n=7); two mice (one non-stressed and one in the corticosterone-treated group) died during the experiment and were subsequently removed from all analysis. Stressed TASTPM mice were exposed to 4 weeks of repeated novel cage stress and corticosterone-treated TASTPM mice were subjected to 3 weeks of chronic corticosterone treatment (followed by a 1 week wash-out period) both these treatments were administered from 5.5 months of age. After the treatment

period, TASTPM mice (then at 6.5 months of age) were tested for working memory, followed by assessments of locomotor activity. Mice were then tested for long-term contextual memory performance (memory acquired prior to treatment period, at 5.5 months of age). 48h following the final memory extinction test, all mice were humanely killed and brains removed for assessment of β-amyloid levels. Half of each brain was used for immunohistochemistry and the other half for ELISA; see figure 3.8 for experimental protocol.

Novel cage stress

Novel cage stress was performed similar to that described above in Experiment 1; conducted for 1h once daily, four times per week, for a total of 4 weeks. TASTPM mice which were not in the stressed group, both control non-stressed and those treated with corticosterone, were manipulated to control for handling effects at times corresponding to the start, and end, of the 1h stress session.

Physiological measures

As with Experiment 1, body weights of all mice were monitored at 5.5 months, prior to both the initial spontaneous alternation testing and the treatment period, and again following the treatment period at 6.5 months of age. Body weight changes were analysed using a repeated measures ANOVA with the two time-points (Time) as within-subject factors and Group as the between-subject factor.

Fecal boli were counted following each stress session for stressed TASTPM mice. Weekly mean defecation counts of stressed TASTPM mice were

compared using paired samples t-tests comparing mean score of each week, with the mean score from the previous week (as used and described in Section 3.1.2).

Corticosterone preparation and treatment

As described above, one group of TASTPM mice were subjected to 3 weeks of chronic high dose corticosterone treatment administered orally in the drinking water. Corticosterone treatment was in parallel with the novel cage stress-treated group, figure 3.8. 500mg of corticosterone (Sigma-Aldrich, Dorset, UK) was dissolved in 20ml ethanol, using a sonic probe, and was made up in tap water to 1% stock ethanol solution, to deliver corticosterone at a concentration of 25mg/ml. For the treated TASTPM mice normal drinking water was replaced with the 25mg/ml corticosterone solution for 4 days (full 24h periods) until day 5 when it is was then replaced with normal tap water for 3 days (full 24h periods), until the treatment regimen started again the following week; non-stressed and stressed TASTPM mice water bottles were replaced with 0.1% ethanol solution during this treatment period for control purposes. This weekly routine continued for three weeks and was followed by a one week wash-out period prior to behavioural testing to ensure that any changes measured were a result of long-term effects of corticosterone treatment, rather than acute effects of high systematic levels of corticosterone. Water bottles were weighed daily for all mice (TASTPM non-stressed, stressed and corticosterone-treated mice), and mean fluid consumption per day over the 3 week treatment period was calculated and analysed using a one-way ANOVA with Group as the between-subject factor.

Spontaneous alternation

Spontaneous alternation was used as a measure of working memory performance. Performance in this test was assessed prior to the treatment period, at 5.5 months of age, and following the treatment period at 6.5 months of age, figure 3.8. Spontaneous alternation was performed as detailed in Section 3.1.2. In brief, mice were subjected to 10 min habituation to the T-maze 24h before the test trials. Test trials involved 10 min exposure once daily to the T-maze, for two days, in which mouse could explore either the left or right arms a total of 5 times; a correct alternation was defined as alternating from left-to-right between explorations. Results were expressed as % correct alternations, out of possible 5 decisions, throughout the trials. This value was then averaged between the two trials. Mice which did not complete 5 decisions in one of the two trials were recorded as partial non-performers (the single value was used for analysis) and mice which did not complete either trial were recorded as complete non-performers, and excluded from the analysis. To assess age-related changes in spontaneous alternation performance, the performance of non-stressed TASTPM mice at 5.5 months was compared to the performance of the same animals at 6.5 months of age using a paired-samples t-test. To determine the effects of various treatments on exploration, performance of 6.5 month old non-stressed, stressed and corticosterone-treated mice were compared using a one-way ANOVA, with Group as the between-subject factor.

Locomotor activity

This was assessed following the treatment/stress at 6.5 months of age and was used to determine any effects of repeated novel cage stress, or chronic corticosterone exposure on mouse ambulation or habituation to a novel arena. Locomotor activity was tested as described in Section 3.1.2; briefly, mice were individually placed into the test chambers for 30 min and activity (distance moved, cm) was tracked automatically using Ethovision software. Data were expressed as either activity during 5 min time-intervals, to determine habituation to the novel arena, and analysed using a repeated measures ANOVA with Time-interval as the within-subject factor and Group as the between-subject factor. In addition, total activity over 30 min, assessing overall locomotor activity, was analysed using a one-way ANOVA with Group as the between-subject factor.

Long-term memory tests

As with Experiment 1, CFC was used as a tool to study the long-term memory performance. The protocol was identical to that described above; memory acquisition occurred prior to stress/treatment period (at 5.5 months of age); 35 days later memory retention was tested (at 6.5 months of age) and, 48h following this, memory extinction was assessed. There was one modification from the protocol used in Experiment 1. An extra extinction trial (3 min exposure to the context in the absence of foot shocks, see figure 3.8) 48h following the first extinction trial.

Activity (distance moved, cm) during contextual memory acquisition was analysed using a repeated measures ANOVA with Time as the within-subject

factor and Group as the between-subject factor, to ensure there was no difference between the groups before the treatment period. Memory performance between the retention and extinction trials was analysed using a repeated measures ANOVA with the different Trial as the within-subject factor and Group as the between-subject factor.

A second method of analysing these data from the memory tests involved calculating a series of extinction indices, introduced in Section 3.1.2. "Extinction" was calculated by subtracting performance during the final extinction trial by the memory retention trial; "Extinction 1" was calculated by subtracting the first memory extinction trial by the memory retention trial; "Extinction 2" was calculated by subtracting performance during the final extinction trial by the first memory extinction trial. All values for all groups in these extinction indices were compared to 0 using a one-sample t-test.

To determine changes in memory performance with age, and whether stress had different effects at the two ages studied, non-stressed and stressed TASTPM mice from Experiment 1 and Experiment 2 were compared. Performance across the memory retention and first extinction trial was analysed using a repeated measure ANOVA with Trial as the within-subject factor and Age and Stress as the between-subject factors.

Immunohistochemistry quantification of β-amyloid

Following the final CFC memory extinction trial, mice were humanely killed and half brains were taken, stored in 4% PFA at 4°C, and processed for immunohistochemical detection of β-amyloid protein deposition. All sections were prepared and stained identically to that described above in Experiment 1.

On these brain sections the ROIs studied were hippocampus (4 examples), retrosplenial cortex (4 examples) and thalamus (4 examples), the relatively low number of representative sections for ROIs in Experiment 2 is attributable to only half a brain being available for analysis.

Image analysis of brain sections, and detection of β-amyloid, was performed as described for Experiment 1 β-amyloid was compared between the three groups (TASTPM non-stressed, stressed and corticosterone-treated) using a one-way ANOVA with Group as the between-subject factor. The morphology of β-amyloid plaques alters as the disease progression occurs with "diffuse plaques", characterised by a constant mass of β-amyloid representing early plaque maturity, and "dense cored" plaques with a core of densely aggregated β-amyloid representing later stages of plaque maturation (Harigaya et al., 2006). Numbers of "dense core" plaques, and "diffuse" plaques, were manually counted in the retrosplenial cortex only and mean values between the three groups were compared using a one-way ANOVA with Group as the between-subject factor.

ELISA quantification of β-amyloid

To quantify β-amyloid levels in the non-stressed and stressed TASTPM mice only, half brains were dissected into three brain regions, hippocampus, cortex and frontal cortex. Within these regions, soluble $A\beta_{(1\text{-}40)}$ and insoluble $A\beta_{(1\text{-}42)}$ β-amyloid were assessed using a solid-phase sandwich ELISA using kits from Biosource International, Inc., California, USA. The process of preparing the samples and performing the assay are described in detail in Section 3.1.2. In both instances 50 µl of sample was added to wells lined with monoclonal

antibody (specific to β-amyloid peptides). A secondary antibody was added to bind this complex, followed by a fluorescent substrate solution. Fluorescence was analysed using a plate reader. All brain samples were analysed in duplicate. Concentration of β-amyloid was calculated, using a standard curve run with the sample, and expressed as pg $A\beta_{(1-40)}$ or $A\beta_{(1-42)}$ per mg of wet tissue. Results were analysed using an independent samples t-test with Stress as the between-subject factor to compare between non-stressed and stressed 6.5 month old TASTPM mice.

3.2.3. Results

Experiment 1

Physiological measures

All mice demonstrated an increase in body weight from the start to the end of the stress period ($P < 0.0001$); there was no difference in weight gained between WT and TASTPM mice, or with stress, and there was no Strain X Stress interaction. Overall, WT mice had a significantly higher body weight compared to TASTPM mice ($P < 0.0001$), figure 3.10A.

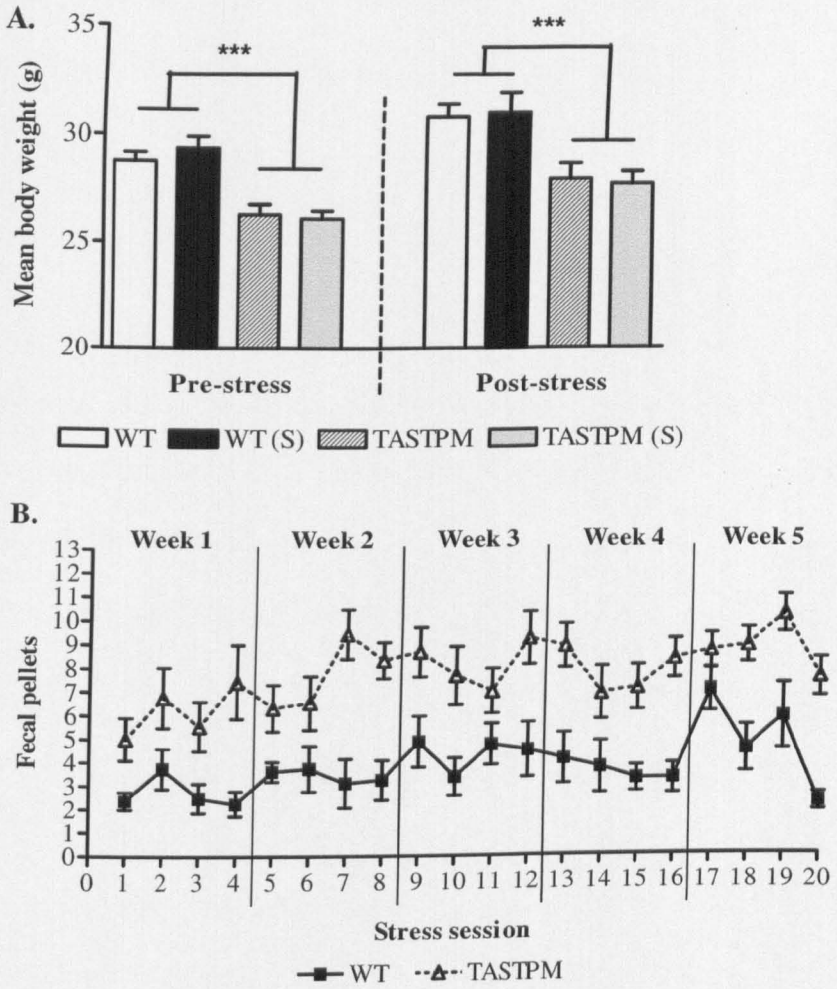


Figure 3.10. Physiological measures, Experiment 1. A: Mean (± SEM) body weight (grams), prior to (4 months of age), and following the novel cage stress period (5.5 months of age) for WT non-stressed (WT, n=10) WT stressed (WT (S), n=8), TASTPM non-stressed (TASTPM, n=9) and TASTPM stressed (TASTPM (S) n=9). All mice demonstrated an increase in body weight with time ($P < 0.0001$), TASTPM mice had consistently lower body weight compared to WT mice ($***P < 0.0001$). Stress had no effect on the weight of either strain. **B**: Mean (± SEM) novel cage stress defecation count for stressed WT (n=8) and TASTPM (n=9) mice. TASTPM mice had a consistently higher level of defecation compared to WT mice ($P < 0.0001$).

Throughout the novel cage stress sessions, stressed TASTPM mice had a higher level of defecation compared to stressed WT mice ($P < 0.0001$), figure 3.10B.

Memory performance

Mice were subjected to CFC memory acquisition prior to 5 weeks of novel cage stress, at the age of 4 months; all mice demonstrated a decline in activity over the 10 min trial ($P < 0.0001$) in response to repeated footshocks. There was a Strain effect ($P = 0.017$) as WT mice, overall, had a lower level of activity during training ($P = 0.044$), as compared to TASTPM mice, figure 3.11A.

At 5.5 months old, TASTPM mice did not exhibit a long-term memory retention deficit, stress had no effect on memory retention in either WT or TASTPM mice and neither group exhibited extinction (a reduction in immobility between the memory retention and extinction trials). Overall, there was a significant difference in immobility between WT and TASTPM between the CFC trials ($P = 0.019$), but there were no significant differences revealed by a multiple comparisons test, (figure 3.11B).

An extinction index revealed that none of the four groups exhibited significant extinction, although TASTPM non-stressed mice did exhibit a significant increase in immobility between these trials ($P = 0.003$, figure 3.11C).

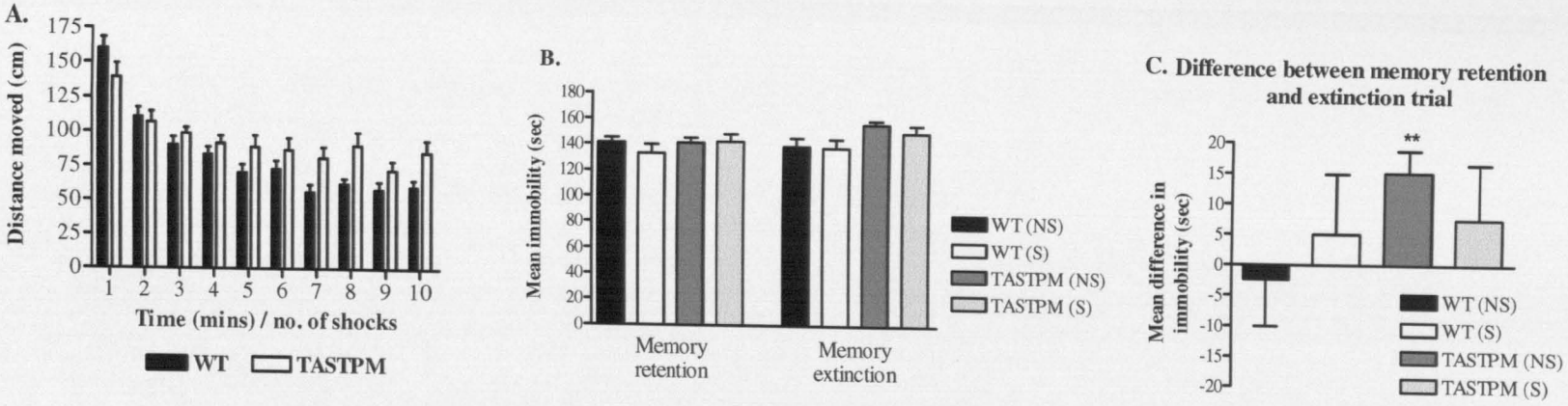


Figure 3.11. CFC long-term memory retention and extinction performance, Experiment 1. A: Mean (± SEM) activity during contextual memory acquisition for 4 month old WT (n=18) and TASTPM mice (n=18). All mice demonstrated a decline in activity as a factor of time ($P < 0.0001$), WT had an overall lower level of activity ($P = 0.044$). **B:** Mean (± SEM) immobility (sec) during the memory retention and extinction trial for WT non-stressed (WT, n=10), WT stressed (WT (S), n=8), TASTPM non-stressed (TASTPM, n=9), and TASTPM stressed (TASTPM (S), n=9) mice. TASTPM mice had intact memory retention, and stress had no effect on either strain. No group displayed significant memory extinction, but there was an overall difference between WT and TASTPM mice across the memory tests ($P = 0.019$). **C:** Mean (± SEM) difference in immobility between the memory retention and extinction trial for WT and TASTPM non-stressed and stressed mice. TASTPM non-stressed mice exhibited an increase in immobility from the memory retention to the extinction trial (**$P = 0.003$).

MRI measures

Volumetry and T2 relaxometry scans were performed on WT and TASTPM non-stressed and stressed mice at 5.5 months of age to provide an *in vivo* measure of pathological status in the ROIs investigated. WT mice had a lower caudate putamen volume overall compared to TASTPM mice which failed to reach statistical significance ($P = 0.05$). There were no differences with Strain, Stress, or a Stress X Strain interaction for the remaining ROI volumes studied (table 3.3). In addition, there were no differences in whole brain volume between WT non-stressed ($462.63mm^3 \pm 12.24$), WT stressed ($455.14mm^3 \pm 9.79$), TASTPM non-stressed ($460.4mm^3 \pm 6.73$), or TASTPM stressed mice ($464.26mm^3 \pm 10.58$).

Manual assessments of T2 relaxation times revealed non-significant Strain X Stress effects in the subiculum ($P = 0.05$), hippocampus ($P = 0.05$). T2 relaxation times, calculated using the semi-automated tool, revealed significant Stress effects in the thalamus ($P = 0.046$) and subiculum ($P = 0.029$). A Strain effect did not quite reach statistical significance for the caudate putamen T2 relaxation ($P = 0.05$). There was a significant Strain X Stress interaction in the thalamus ($P = 0.041$) and retrosplenial cortex ($P = 0.025$), table 3.4. A multiple comparisons test on those ROIs listed above revealed a significant difference between stressed WT and TASTPM mice in the thalamus ($P = 0.048$), and significant differences between non-stressed and stressed TASTPM mice in the thalamus ($P = 0.022$), retrosplenial cortex ($P = 0.030$) and subiculum ($P = 0.025$).

	WT		TASTPM		Strain
	Non-stressed	Stressed	Non-stressed	Stressed	Effect
Anterior cingulate cortex	0.85 (0.02)	0.85 (0.05)	0.86 (0.03)	0.80 (0.04)	
Retrosplenial cortex	1.47 (0.03)	1.47 (0.03)	1.45 (0.04)	1.50 (0.05)	
Thalamus	3.89 (0.09)	3.89 (0.07)	3.85 (0.07)	3.84 (0.08)	
Caudate putamen	4.67 (0.07)	4.88 (0.11)	4.61 (0.07)	4.61 (0.09)	$*P = 0.05$
Corpus callosum	0.62 (0.02)	0.64 (0.02)	0.62 (0.02)	0.62 (0.02)	
Subiculum	0.37 (0.06)	0.45 (0.04)	0.41 (0.07)	0.46 (0.06)	
Hippocampus	4.67 (0.14)	4.89 (0.07)	4.73 (0.08)	4.86 (0.07)	

Table 3.3. MRI volumetry results, Experiment 1. ROIs expressed as mean (± SEM) percentage of whole brain volume (± SEM) for 5.5 month old non-stressed WT (n=8), stressed WT (n=7), non-stressed TASTPM (n=9) and stressed TASTPM (n=8). There was no difference in whole brain volume between TASTPM and WT mice, stressed or non-stressed. There appeared to be a Strain effect on the caudate putamen volume which did not reach statistical significance ($*P = 0.05$).

Immunohistochemistry quantification of β-amyloid

Levels of β-amyloid plaque pathology were quantified using a semi-automated method to analyse immunohistochemistry sections stained for the protein. There was no difference in mean percentage of ROI covered in β-amyloid between non-stressed and stressed TASTPM mice in the retrosplenial cortex (0.031 ± 0.01 vs. 0.018 ± 0.004, $P = 0.199$), hippocampus (0.059 ± 0.019 vs. 0.050 ± 0.010, $P = 0.675$), and the thalamus (0.047 ± 0.016 vs. 0.041 ± 0.009, $P = 0.757$).

	Manually defined					Semi-automated method						
	WT		TASTPM		Stress X Strain	WT		TASTPM		Strain	Stress	tress x Strain
	Non-stressed	Stressed	Non-stressed	Stressed	interaction	Non-stressed	Stressed	Non-stressed	Stressed	effect	effect	interaction
ACC	49.01 (0.23)	48.42 (0.10)	48.77 (0.23)	49.78 (0.81)		47.48 (0.35)	47.15 (0.47)	47.30 (0.24)	47.18 (0.87)			
Retrosplenial cortex	47.51 (0.13)	47.65 (0.22)	47.41 (0.20)	48.44 (0.70)		44.98 (0.27)	44.65 (0.31)	44.41 (0.46)	46.35 (0.70)			*P = 0.025
Hippocampus	50.23 (0.22)	49.91 (0.20)	49.58 (0.17)	50.98 (0.80)	*P = 0.05	47.74 (0.44)	48.54 (0.69)	47.18 (0.36)	48.26 (0.57)			
Subiculum	45.86 (0.43)	45.12 (0.29)	45.69 (0.20)	46.88 (0.81)	*P = 0.05	44.75 (0.45)	44.99 (0.45)	44.13 (0.51)	46.37 (0.69)		*P = 0.029	
Corpus callosum	45.51 (0.35)	44.66 (0.41)	45.39 (0.47)	45.90 (0.55)		46.83 (1.31)	48.83 (1.49)	48.34 (1.75)	48.23 (1.10)			
Caudate putamen	47.32 (0.25)	47.34 (0.20)	47.33 (0.23)	48.23 (0.54)		48.11 (0.51)	47.61 (0.49)	47.18 (0.26)	46.23 (0.90)	*P = 0.05		
Thalamus	45.88 (0.37)	45.78 (0.13)	46.02 (0.40)	46.87 (0.56)		44.21 (0.22)	44.19 (0.30)	44.10 (0.34)	45.72 (0.56)		*P = 0.046	*P = 0.041

Table 3.4. T2 relaxometry, Experiment 1. Mean (± SEM) T_2 relaxation time (msec) (± SEM) for all manually defined ROIs (left) and ROIs delineated using a semi-automated registration method (right), studied in WT non-stressed (n=9-8), WT stressed mice (n=7), non-stressed TASTPM mice (n=9) and stressed TASTPM mice (n=8). Using the manual method, a multiple comparisons test revealed that there was no Strain or Stress effect on relaxation times, but there was a trend towards a Strain X Stress interaction for the subiculum and hippocampus, but this was not statistically significant *(P = 0.05). Using the semi-automated method, there was a non-significant Strain effect in the caudate putamen (*P = 0.05), a Stress effect in the thalamus (P = 0.046) and subiculum (P = 0.029) and a Strain X Stress interaction in the thalamus (P = 0.041) and retrosplenial cortex (P = 0.025).

Next we assessed differences in levels of morphology of β-amyloid plaques between non-stressed (n=6-7) and stressed (n=9) TASTPM mice, the lower number of subjects in the non-stressed group is due to a lower number of example ROIs available; mice with insufficient expression of ROI examples were excluded from the analysis. There was a trend towards a reduction in dense core plaques with stress in the retrosplenial cortex only (P = 0.088). There was no difference in diffuse plaque number in this ROI, and no significant differences found in the other ROIs for either plaque type, table 3.5.

		TASTPM		
		Non-stressed		**Stressed**
Hippocampus	Dense-core	1.63 (0.57)		1.58 (0.41)
	Diffuse	4.39 (1.10)		3.90 (0.78)
Retrosplenial cortex	Dense-core	0.24 (0.10)	#	0.06 (0.04)
	Diffuse	1.17 (0.35)		1.06 (0.23)
Thalamus	Dense-core	1.21 (0.45)		1.13 (0.29)
	Diffuse	2.49 (0.72)		1.75 (0.37)

Table 3.5. Plaque morphology, Experiment 1. Mean (± SEM) dense-core and diffuse plaque number in non-stressed (n = 6-7) and stressed (n = 9) 5.5 month old TASTPM mice in the hippocampus, retrosplenial cortex and thalamus. There was a trend towards reduction in dense-core plaques with stress but this failed to reach statistical significance ($^{\#}P = 0.088$).

Experiment 2

Physiological measures

From 5.5 to 6.5 months of age, all mice exhibited an increase in body weight as a factor of time ($P < 0.0001$); overall, there was a difference in body weight gain between control, stressed and corticosterone-treated mice ($P = 0.002$), although there was no significant difference between these groups from the multiple comparisons test, figure 3.12A. To determine changes in TASTPM mouse defecation during the novel cage stress exposure over the four week period, weekly mean defecation counts were compared to the score of the previous week. The only significant difference was an increase in defecation count between week 3 and week 4 ($P = 0.025$), figure 3.12B.

Given that corticosterone was administered through the drinking water, the amounts drunk daily by each mouse averaged over the 3 week period was compared between all conditions (control, stressed and corticosterone-treated TASTPM mice). There was, overall, a difference in volume consumed between the three groups ($P < 0.0001$), and a multiple comparisons test revealed corticosterone-treated mice drank significantly more than control non-stressed TASTPM mice (9.54ml ± 1.43 vs. 3.37ml ± 0.06, $P < 0.0001$) and stressed TASTPM mice (9.45ml ± 1.43 vs. 4.33ml ± 0.24, $P < 0.0001$). Average daily intake of corticosterone over the three weeks was 23.9 mg, equating to approximately 873mg/kg per day.

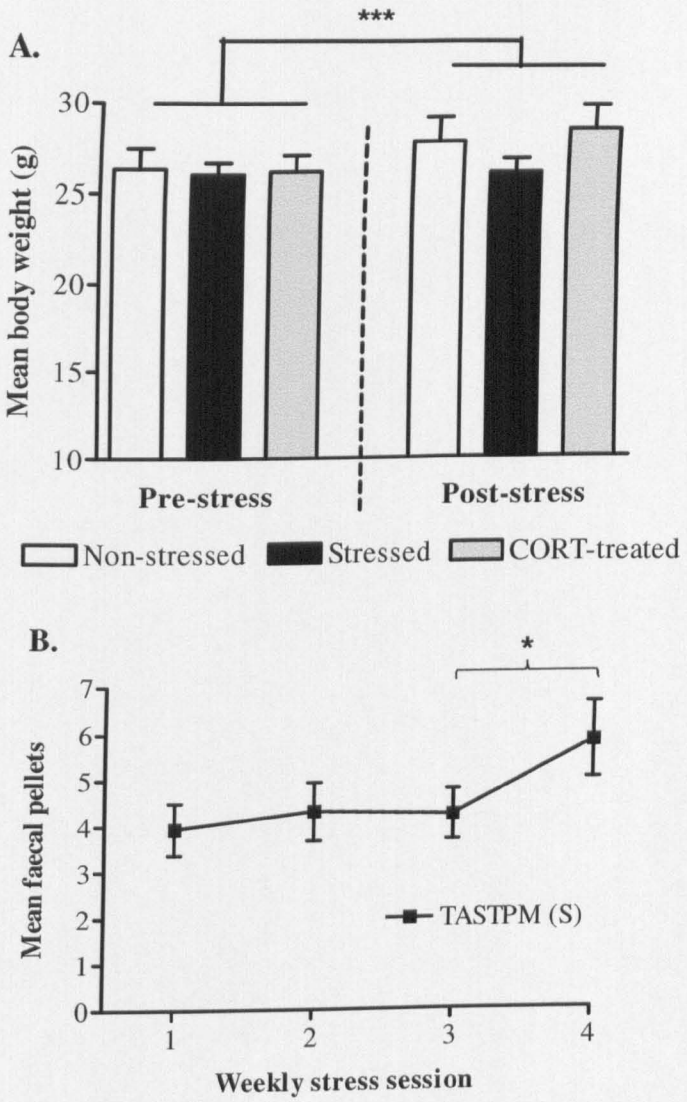


Figure 3.12. Physiological measures, Experiment 2. A: Mean (± SEM) body weight (grams) prior to the stress/treatment period at 5.5 months of age and following the stress/treatment period at 6.5 months of age for TASTPM non-stressed (n=8), TASTPM stressed (TASTPM (S), n=9) and TASTPM corticosterone (TASTPM CORT, n=7). All mice demonstrated an increase in body weight over time (***$P < 0.0001$). **B:** Weekly mean defecation counts for stressed TASTPM mice. The only significant difference between weeks was between week 3 and 4 (*$P = 0.025$).

Locomotor activity

At 6.5 months of age, all mice tested demonstrated a decline in activity (distance moved, cm) over time (P = 0.001) throughout the trial; however, there was no difference in habituation to the novel arena between control, stressed and corticosterone-treated mice. In addition, there was no difference in total activity over the 30 min trial between the three groups.

Spontaneous alternation performance

Spontaneous alternation performance (mean % correct alternations throughout trials) was assessed in TASTPM mice at 5.5 months of age, prior to the treatment period, and at 6.5 months of age following the treatment period. First, comparing age-related performance of TASTPM mice from 5 months (n=3) to 6.5 months (n=3) there was no change in performance (66.67% ± 6.67 vs. 60% ± 0) assessed using a paired-samples T-test. A one-way ANOVA comparing the performance of 6.5 month old non-stressed (n=4), stressed (n=7) and corticosterone-treated (n=6) TASTPM mice revealed no differences between the three groups (65% ± 5 vs. 51.43% ± 5.08 vs. 65% ± 8.06).

The reason for the low group numbers in the analysis of the spontaneous alternation tests is attributable to the number of non-performers in this task. TASTPM mice at 5.5 months of age had 4 full non-performers (did not perform in either trial). At 6.5 months old, non-stressed TASTPM had 1 partial non-performer (performed on only one trial, single value was used for analysis) and 4 full non-performers, stressed TASTPM mice had 2 full non-performers, and corticosterone-treated TASTPM mice had 2 full non-performers and 1 partial non-performer.

Long-term memory test

TASTPM mice were exposed to CFC memory acquisition at 5.5 months of age. Overall, all groups demonstrated a significant decline in activity as a factor of time, indicating successful conditioning (P = 0.008), and there was no difference between the three groups (non-stressed, stressed and corticosterone-treated TASTPM mice), figure 3.13.

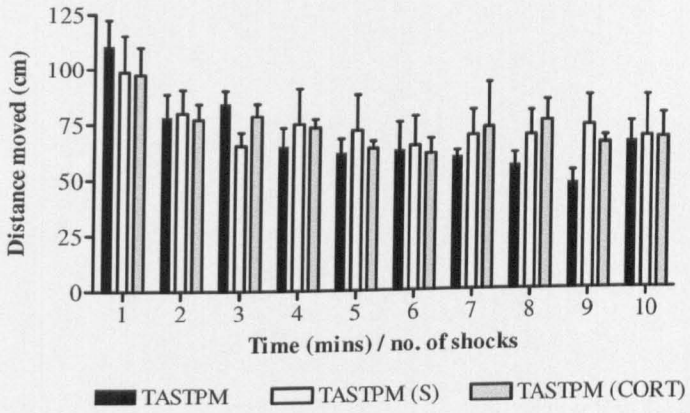


Figure 3.13. CFC memory acquisition, Experiment 2. Mean (± SEM) activity (distance moved, cm) over time (10 mins) for the three groups non-stressed (TASTPM, n=8), stressed (TASTPM (S), n=9) and corticosterone-treated TASTPM mice (TASTPM (CORT), n=7) prior to the treatment period at 5.5 months of age. Overall, TASTPM demonstrated a decline in activity over time (P = 0.008), but there was no difference between the three groups.

There was no difference between non-stressed, stressed or corticosterone-treated TASTPM mouse long-term memory retention. All three groups of mice demonstrated changes in immobility across the memory retention and extinction trials (P = 0.023). There was no Trial X Group interaction, although a multiple comparisons test revealed a significant difference, over the trials,

between non-stressed and stressed TASTPM mice only (P = 0.014). Stressed TASTPM mice had lower immobility levels compared to non-stressed TASTPM during the first extinction trial only (P = 0.033), figure 3.14.

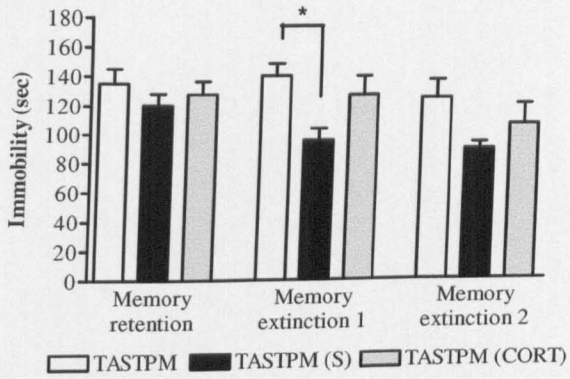


Figure 3.14. CFC memory retention and extinction, Experiment 2. Mean (± SEM) immobility during the memory retention test and both extinction tests for non-stressed (TASTPM, n=8), stressed (TASTPM (S), n=9) and corticosterone-treated TASTPM mice (TASTPM (CORT), n=7). Overall, all groups of TASTPM mice showed a change in immobility over the three trials (P = 0.023) and there was a difference between TASTPM non-stressed and stressed mice (P = 0.014). A multiple comparisons test showed a significant difference between non-stressed and stressed TASTPM mice during the first memory extinction trial only (*P = 0.033).

A series of extinction indices were calculated to quantify changes in immobility levels between trials. Non-stressed TASTPM mice did not exhibit extinction between the memory retention and final extinction trial (Extinction), but demonstrated a trend towards a decrease in immobility between the first and final extinction trial (Extinction 2, P = 0.083). Stressed TASTPM mice exhibited a significant drop in immobility from the memory retention and final extinction trial (Extinction, P = 0.018), and between the memory retention and

first extinction trial (Extinction 1, $P = 0.028$), but not between the first and last extinction trial (Extinction 2). Corticosterone-treated mice did not demonstrate a significant change in immobility between any of the trials, figure 3.15.

Age-related changes in memory performance

The performance of TASTPM mice from Experiment 1, at 5.5 months of age, and Experiment 2, at 6.5 months of age, were compared to determine any age-related changes in memory performance with or without stress; therefore in these analyses, Age and Stress are the between-subject factors. Given that in Experiment 1 there was only one memory extinction trial along with the memory retention trial, the analysis was only conducted on performance during these trials for both Experiments 1 and 2.

During memory acquisition (data acquired 36 days prior to long-term memory tests), there was an overall decline in activity throughout the trial ($P < 0.0001$) exhibited by all mice, and there was an overall difference between the two ages (4 months vs. 5.5 months, $P = 0.002$); mice in the older group had consistently lower levels of activity throughout the trial, figure 3.16.

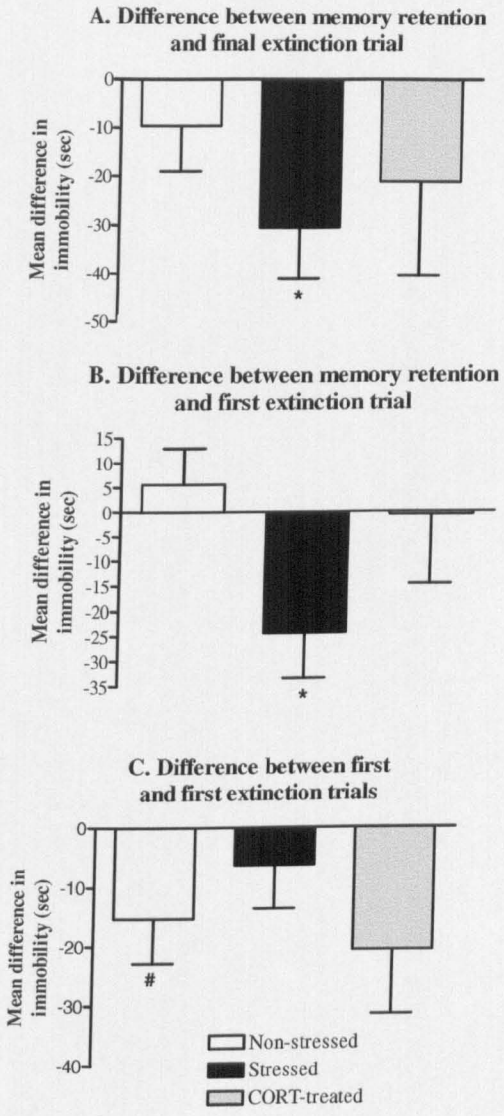


Figure 3.15. Extinction indices of TASTPM mice, Experiment 2. Mean (± SEM) immobility (sec) for each extinction index of 6.5 month old non-stressed TASTPM (non-stressed, n=8), stressed (stressed, n=9), and corticosterone-treated mice (CORT-treated, n=7). Stressed TASTPM mice exhibited significant differences between the memory retention and final extinction trial, "Extinction" (A), and memory retention and first extinction trial "Extinction 1" (B) ($^{*}P < 0.05$). There was a trend towards extinction occurring between the first and last extinction trial, "Extinction 2" (C), exhibited by non-stressed TASTPM mice , but this did not reach statistical significance ($^{\#}P = 0.083$). Corticosterone-treated TASTPM mice did not show any changes in immobility between any Trials compared.

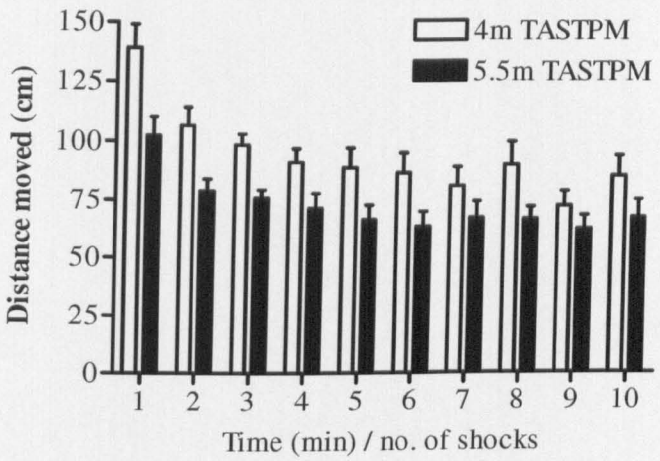


Figure 3.16. Age-related changes in TASTPM mouse memory acquisition. Mean (± SEM) activity (distance moved, cm) exhibited by 4 month old (n=18) and 5.5 month old TASTPM mice (n=24). All mice demonstrated a decline in activity over the 10 minute trial ($P < 0.0001$), and 5.5 month old TASTPM mice had an overall lower level of activity throughout the trial ($P = 0.002$).

During the memory retention and extinction trials there was an overall effect of Stress ($P = 0.008$), Age ($P < 0.0001$), and a Stress X Age interaction ($P = 0.028$). There were no age-related differences during the long-term memory retention trial in either stressed or non-stressed TASTPM mice and no groups demonstrated a change in immobility between memory retention and extinction. During the memory extinction trial there was no age-related change in non-stressed TASTPM mice, but stressed 6.5 month old TASTPM showed lower immobility compared to age-matched non-stressed TASTPM mice ($P = 0.001$), figure 3.17.

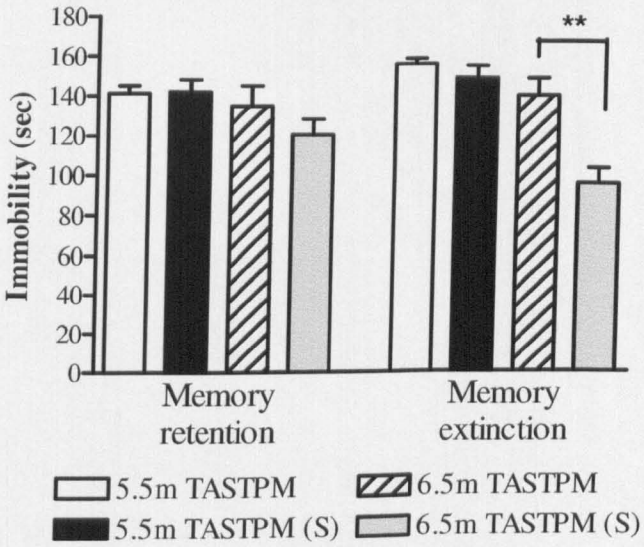


Figure 3.17. Age-related changes in TASTPM mouse memory impairment. Mean (± SEM) immobility (sec) of TASTPM mice at 5.5 months of age non-stressed (5.5m TASTPM, n=9), and stressed mice (5.5m TASTPM (S), n=9), and 6.5 months of age non-stressed (6.5m TASTPM, n=8), and stressed mice (6.5m TASTPM, n=9). Immobility during the memory retention test was unaffected by age or stress. During the memory extinction trial 6.5 month old stressed TASTPM mice displayed a lower level of immobility compared to 6.5 month old non-stressed mice (**$P = 0.001$).

β-amyloid quantification

In Experiment 2, brain β-amyloid levels were quantified in 6.5 month old non-stressed and stressed TASTPM mice frontal cortex, cortex and hippocampus using an ELISA. This was supported by assessments of β-amyloid in the hippocampus, retrosplenial cortex and thalamus of non-stressed, stressed and corticosterone-treated TASTPM mice using immunohistochemistry.

An ELISA assessing levels of $A\beta_{(1-40)}$ revealed a trend towards a decrease with stress in the frontal cortex of 6.5 month old TASTPM mice which did not reach statistical significance (P = 0.062, 0. 74pg/mg ± 0.15 vs. 0.44pg/mg ± 0.05); there was, however, no difference in $A\beta_{(1-40)}$ in the hippocampus or cortex with stress. In addition, there was no difference in $A\beta_{(1-42)}$ with stress in 6.5 month old mice in any brain region studied.

Immunohistochemical detection of β-amyloid pathology, to assess the mean percentage of ROI covered in β-amyloid in 6.5 month old non-stressed (n =6-5), stressed (n=9) and corticosterone-treated (n=6) TASTPM mice, was not found to be different for the hippocampus (0.29% ± 0.9 vs. 0.42% ± 0.06 vs. 0.46% ± 0.07), retrosplenial cortex (0.19% ± 0.06 vs. 0.28% ± 0.07 vs. 0.24% ± 0.09) or the thalamus (0.31% ± 0.1 vs. 0.43% ± 0.1 vs. 0.41% ± 0.09).

Due to a trend towards a change in dense core plaque number found in only the retrosplenial cortex of TASTPM mice with stress in Experiment 1, differences in the number of dense-core and diffuse plaques was assessed in this ROI for 6.5 month old non-stressed (n=6), stressed (n=9) and corticosterone-treated (n=6) TASTPM mice. There was no significant difference in dense core plaques (0.67 ± 0.12 vs. 0.75 ± 0.23 vs. 0.96 ± 0.28) or diffuse plaques (2.04 ± 0.25 vs. 2.78 ± 0.48 vs. 3.79 ± 1.15) in the retrosplenial cortex.

3.2.4. Discussion

The aim of the experiments described above was to assess the effects of repeated mild novel cage stress on the pathologies exhibited by the TASTPM mouse model of AD. Specifically, the question of how repeated mild stress might affect these mice during the early-to-moderate stages of β-amyloid pathology, at both 5.5 and 6.5 months of age, was addressed.

The major findings of these studies were as follows:

1. TASTPM mice have an intact long-term contextual memory retention up to the age of 5.5 months. An absence of long-term contextual memory extinction is evident at all ages.
2. Stress had no affect on memory performance at 5.5 months of age, but appears to have enhanced long-term contextual memory extinction in the oldest (6.5 month old) TASTPM mice only.
3. *In vivo* MRI assessing volumetric changes and changes in brain tissue T2 relaxometry, appear to be capable of detecting AD-associated pathologies and effects of stress exposure in TASTPM mice.
4. In general, there were no detectable effects of stress on brain β-amyloid levels in the TASTPM mice at two ages studied, although pre-exposure to stress almost lowered $A\beta_{(1\text{-}40)}$ in the frontal cortex of 6.5 month old TASTPM mice.

In the current study we found 5.5 month old TASTPM mice had an intact long-term memory retention, whereas we have previously shown that TASTPM mice at the same age have a short-term memory deficit (Pardon et al., 2009). Contrary to this, a similar study demonstrated impaired remote memory (30 days) expression of conditioned fear in a β-amyloid overexpressing mouse model under 4 months of age (Kimura and Ohno, 2009). The difference between the nature of short- and long-term memory consolidation and storage should be addressed. Molecular pathways underlying long-term memories are thought to be robust and held in a consolidated state as compared to shorter-term memories, for instance, application of an amnesic agent directly after memory acquisition affects subsequent retention of that memory, whereas treatment with an amnesic agent several days later has no effect on retention as the memory has been consolidated into a more "permanent" state (for review, (Nadel and Land, 2000)). This robust nature of longer-term memories may be, in part, the reason why it is thought to be preserved until the later stages of clinical AD (Sartori et al., 2004). Moreover, clinical data are supported by preclinical evidence as longer-term, reference memory was intact until 6 to 8 months of age in double transgenic β-amyloid-overexpressing mice (Trinchese et al., 2004), a time point when, presumably, the brain regions responsible for storage of longer-term memories were compromised. Studies show long-term memories are stored in higher cortical areas (for review, (Wiltgen et al., 2004)); for instance, one study demonstrated an important role for the anterior cingulate cortex in expression of long-term memory in mice at a similar paradigm to that described in the current study (Frankland et al., 2004). It is, therefore possible that higher cortical areas, such as the cingulate cortex, are

preserved until later stages of pathology in β-amyloid overexpressing mice. Indeed, our *in vivo* MRI studies demonstrated no pathological changes occurred in the anterior cingulate cortex of these mice. Unlike its robust, beneficial effect on short-term memory, novel cage stress had no effect on long-term memory retention of TASTPM mice. Whether or not the beneficial effect of stress requires the pre-existence of a memory deficit remains to be determined.

Our previous studies have shown that TASTPM mice develop a short-term memory extinction deficit between 3 and 4.5 months of age, which was resistant to novel cage stress, however, studies using another β-amyloid overexpressing mouse line showed an intact short-term memory extinction in mice under 4 months of age (Kimura and Ohno, 2009). Consistent with our previous observations, in the current study we detected a lack of long-term memory extinction in TASTPM mice at 5.5 months of age. Interestingly, control WT mice also exhibited a lack of memory extinction during the long-term memory tests, suggesting that the lack of extinction by the TASTPM mice was not a deficit of the model *per se*, given it was observed in normal mice. As we do not have evidence as to whether this deficit also occurs in younger WT mice we cannot rule out the possibility that a long-term memory extinction deficit may represent a normal age-related change that occurs in mice. Indeed, it is believed that longer-term memories are more resistant to extinction (Lopez et al., 2008) possibly due to robust neurochemical pathways underlying such memories.

Novel cage stress had no effect on the long-term memory retention at either 5.5 or 6.5 months of age. It is possible that the absence of a stress effect on long-term memory is due to the nature of memory being studied; short-term memory maybe more susceptible to change compared to longer-term memories; given the known differences between the two memory systems described above this is not unlikely. It is worthy of note that a fundamental difference between the current study and our previous study looking into short-term memory, is that here footshocks were administered prior to the stress procedure. It is, therefore, possible that pre-exposure to the severe stressor of footshocks may alter the stress-response to novel cage stress. An unpublished study by our group supports this hypothesis; pre-exposure to a footshock prior to novel cage stress elevated defecation level and unconditioned aversive behaviours during the stress sessions compared to non-shocked mice. This indicated pre-exposure to footshocks may change the perceived intensity of novel cage stress.

Although stress had no effect on long-term memory retention, it did enhance extinction-like behaviour in 6.5 month old TASTPM mice. As discussed above, it is difficult to interpret whether this as a positive or a negative change. Extinction, the ability to re-learn that a stimulus is no longer aversive, is a natural response to deal with life trauma. When this is deficit it represents a lack of cognitive flexibility and, generally, a factor which induces a deficit in extinction is considered as a negative change to a normal memory processes. Novel cage stress appears to be inducing changes in the predicted direction, i.e. enhancing memory extinction, which supports the notion of a beneficial effect of this stressor. However, at 5.5 months WT mice did not exhibit long-term

contextual memory extinction, neither did TASTPM mice. Thus, enhancing extinction-like behaviour may, in fact, represent a negative change in memory performance. Novel cage stress is capable of affecting memory extinction in older TASTPM mice only, which suggests that a disease-related change evident in older mice only may underlie the effect of stress here. For instance, it may be that β-amyloid levels, known to increase with age in this mouse line (Howlett et al., 2004), need to reach a certain "threshold" level before stress has a significant effect. Indeed, stress almost reduced brain β-amyloid levels in the oldest mice studied only, however, the effects of stress on β-amyloid were not as robust as previously reported (Pardon et al., 2009), making these findings unclear. What is clear, however, is that stress was capable of affecting the long-term contextual memory extinction of 6.5 month old TASTPM mice.

A high dose of corticosterone had no detectable effect on memory performance in TASTPM mice, it is therefore likely that any effect stress may be having on memory performance occurs through a corticosterone-independent mechanism. Corticosterone administration has been shown to be capable of facilitating memory performance when administered at an acute low dose of 250μm/kg (Brinks et al., 2009). However, corticosterone delivered at a high concentration of 26.8mg/kg, chronically for 3 weeks, has been shown to impair long-term memory performance (Coburn-Litvak et al., 2003). It is therefore surprising that high levels of exposure to corticosterone for 3 weeks in the current study had no effect on long-term contextual memory. Interestingly, corticosterone-treated mice consumed a significantly higher volume of fluid as compared to all other groups. This effect was unexpected. A separate study conducted by

our group to investigate this phenomenon further demonstrated that WT mice, free to drink corticosterone in solution, drank to excess. Such a high level of fluid consumption was not due to the ethanol in which the drug was solubilised, and these high doses of corticosterone had no effect on cognition (Pardon et al., unpublished). Interestingly, another study demonstrated that a lower dose of corticosterone was incapable of inducing a conditioned place preference in rats (Dietz et al., 2007), thus, corticosterone may only be rewarding when delivered at high doses. Indeed, chronic high dose of corticosterone appears to have enhanced the rewarding properties of brain stimulation in the rat (Barr et al., 2000), which supports this hypothesis. The reason underlying this apparent, addictive or rewarding property of high corticosterone administration is not known and would require further investigation.

In Experiment 1, *in vivo* MRI was used to detect AD-associated changes in 5.5 month old TASTPM mice, compared to age-matched WT mice with or without prior novel cage stress exposure. The results presented above indicate that, using a semi-automated tool, measurements of brain region volumetry revealed a smaller caudate putamen volume in TASTPM mice. To my knowledge, there have been no reports of caudate putamen volume changes in β-amyloid over-expressing transgenic mice. However, previous studies have shown the striatum to be susceptible to developing β-amyloid-based pathologies in similar mouse strains (Radde et al., 2006). Moreover, in clinical AD the striatum is subject to volume loss, determined by MRI (de Jong et al., 2008), providing further support of the face validity of this model. The reason for these volume

changes observed pre-clinically is not, as yet, clear given a general criticism of such transgenic AD mouse models is the apparent absence of gross neurodegeneration (Stein and Johnson, 2002).

Tissue T2 relaxometry was used as an indirect method of assessing β-amyloid-associated changes in tissue composition. Here, we were able to detect differences in T2 relaxation times between the two genotypes; although not reaching statistical significance, TASTPM mice appear to have shortened T2 relaxation times compared to WT mice, which is consistent with previous findings in similar mouse models (El Tannir El Tayara et al., 2006; El Tayara Nel et al., 2007; Falangola et al., 2007). Stress elongated T2 relaxation times in TASTPM mice; to our knowledge there are no studies that have directly examined the effects of stress on brain tissue T2 relaxation times, although in humans an elongated T2 relaxation time has been associated with exposure to previous early-life extremely stressful events (Anderson et al., 2002). Despite the cause of the elongation of T2 relaxation with stress being unclear, one possibility is the heightened brain tissue water content that occurs following stress exposure (Duric and McCarson, 2005), which is known to elongate T2 relaxation time (Laule et al., 2007). The ability to detect differences with stress, in addition to our observation of Stress X Strain interactions, supports the contention that stress can modulate AD-associated pathologies in TASTPM mice and that T2 relaxometry is an appropriate tool to assess such changes.

The mechanism underlying the change in T2 relaxation times in AD sufferers and transgenic mouse models is not yet fully understood. Changes in relaxometry have also been found during normal aging (Chang et al., 1996),

and was thought to correlate to brain density and brain water content. T2 relaxation is also sensitive to tissue iron levels; iron has been found to be co-localised with β-amyloid plaques in transgenic mice (Falangola et al., 2005a), and, therefore, may play a role in the shortening of T2 relaxation observed in similar mice (El Tannir El Tayara et al., 2006; El Tayara Nel et al., 2007; Falangola et al., 2007). However, changes in T2 relaxation have been observed prior to deposition of the iron associated with β-amyloid deposits (El Tayara Nel et al., 2007). Moreover, one group found changes in T2 relaxation in a β-amyloid over-expressing transgenic mouse model prior to β-amyloid deposition, and changes in relaxation times have been observed in PS transgenic mice devoid of β-amyloid pathology (Falangola et al., 2007), indicating a factor independent of β-amyloid can play a role. This is, in part, consistent with our findings given that repeated mild stress had no effect on β-amyloid levels, but managed to alter T2 relaxation times in TASTPM mice. Moreover, our study confirmed that application of a semi-automated method to study T2 relaxometry in TASTPM mice heightened sensitivity as evidenced by harvesting more significant between-group differences compared to the values manually determined, consistent with a similar study (Falangola et al., 2005b),

Contrary to our previous study, repeated mild stress, in the main, had no detectable effects on β-amyloid levels in TASTPM mice at either 5.5 months or 6.5 months of age. Interestingly, there was an trend towards a decrease of $A\beta_{(1-40)}$ in the frontal cortex of stressed 6.5 month old TASTPM mice (as determined by an ELISA) which was consistent with a previous study where we observed that novel cage stress reduced brain β-amyloid levels in 5.5 month

old TASTPM mice (Pardon et al., 2009). However, the absence of a stress-effect at 5.5 months of age in the current study opposes these findings. Whether this lack of change with stress at 5.5 months of age, like the absence of behavioural effects, was due to the stress procedure being applied after pre-exposure to the stressful footshock (part of the CFC memory acquisition protocol) is not known. Immunohistochemistry, as a tool to determine pathological status, relies on the presence of β-amyloid plaques; as these are a phenomena associated more with the later stages in pathological progression, it may be a tool better suited to studying later stages of pathology, when higher levels of β-amyloid plaques are found. The semi-automated tool used to analyse the immunohistochemistry appears to be sensitive enough to detect age-related increases in brain β-amyloid deposition; for example, the hippocampus of 5.5 month old TASTPM mice is 0.059% covered in β-amyloid, whereas it is 0.29% covered in β-amyloid in 6.5 month old TASTPM. However, the technique was incapable of detecting any changes associated with stress, or high corticosterone exposure in 6.5 month old TASTPM mice. Whether this is an issue associated with the level of resolution of the technique, or simply due to an absence of effect caused by the manipulations, is yet to be determined. However, the absence of any change in β-amyloid using immunohistochemistry was in agreement with a similar, general lack of effect found by employing an ELISA.

Immunohistochemistry did, however, allow us to detect a marginally lower number of "dense-core" plaques with stress in Experiment 1 (although not reaching statistical significance). Given that this form of plaque represents later

stages of pathology (Harigaya et al., 2006), these results indicate that stress maybe capable of slowing down the pathological progress. Indeed, there was no detectable change in diffuse plaque number with stress, suggesting that stress interacts with plaque maturity rather than formation. Although literature on this area is sparse, one study demonstrated that rats administered β-amyloid along with the GC prednisolone had a higher level of dense-core plaques compared to diffuse (Harris-White et al., 2001). These findings may, therefore, suggest that mild stress exposure can be beneficial by lowering dense-core plaque number, whereas higher levels of GCs can increase the number of this type of plaque

The method by which stress can modulate the morphology of β-amyloid plaques, appears to affect $A\beta_{(1\text{-}40)}$ in the frontal cortex of the oldest TASTPM mice, and how stress was able to reduce β-amyloid in our previous studies is still not known. Chronic high exposure to corticosterone had no effect on memory performance or β-amyloid levels in TASTPM mice. Indeed, it has been previously shown that exposure to corticosterone does not mimic stress-induced elevations in hippocampal interstitial fluid in a β-amyloid-over-expressing transgenic mouse line (Kang et al., 2007). It is, therefore, possible that a corticosterone-independent system is responsible for the various stress effects observed on brain β-amyloid in transgenic mouse models (see Section 1.4.1).

In summary, the evidence above demonstrates that long-term memory remained intact in TASTPM mice up to 5.5 months of age and was unaffected

by repeated mild stress up to 6.5 months of age. *In vivo* MRI provided a means to study the early pathological markers of β-amyloid-associated pathology in TASTPM mice and was sensitive enough to pick up effects of stress; lengthening T2 relaxation times in TASTPM mice. In the main, stress was incapable of modulating β-amyloid levels in these animals, but may be capable of altering plaque morphology. The key findings, together with those describing the pathologies, and effects of stress on TASTPM mice during early stages of pathology (Section 3.1) are summarised below, figure 3.18.

The aim of these studies was to determine a key point in the early-to-moderate pathology of TASTPM mice where we see the maximum effect of stress. This time-point appears to be at 5.5 months of age when stress reversed a short-term memory deficit and lowered brain β-amyloid levels (Pardon et al., 2009). This time-window will therefore be used in the next section to explore a potential mechanism underlying this beneficial effect of stress on TASTPM mouse short-term memory performance and β-amyloid pathology.

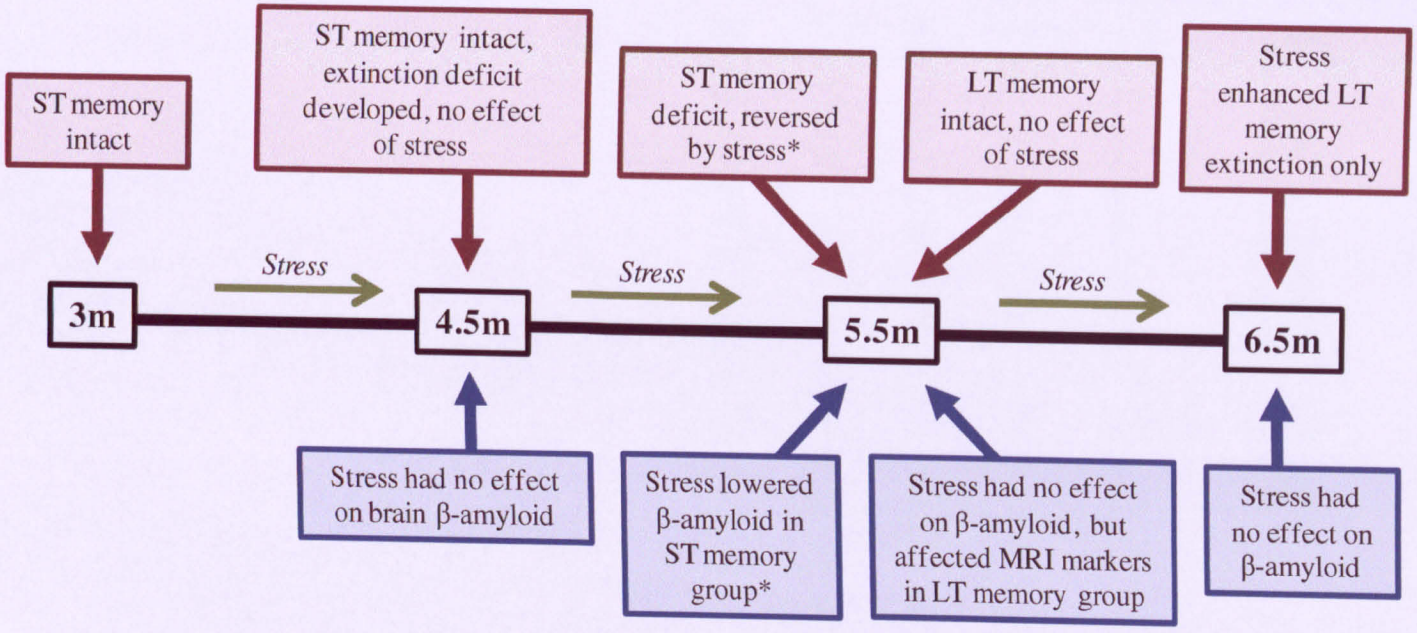


Figure 3.18. Summary of key findings from Section 3. Short-term (ST) memory remained intact in TASTPM mice until 5.5 months of age where it was deficit, pre-exposure to novel cage stress reversed the development of this impairment. Long-term (LT) memory remained intact up to 6.5 months of age in TASTPM mice, pre-exposure to stress at any age studied had no effect on LT memory retention, but enhanced extinction in the oldest group of TASTPM only. Pre-exposure to stress had no effect on brain β-amyloid until 5.5 months of age where it was capable of attenuating normal age-related increases in β-amyloid. Stress was incapable, however, of reducing β-amyloid in mice exposed to the long-term memory tests. *(Pardon et al., 2009).

CHAPTER 4

THE ROLE OF GLUTAMATE RECEPTOR SIGNALLING IN AD-ASSOCIATED PATHOLOGY, AND ITS INTERACTIONS WITH STRESS IN TASTPM MICE

4.1. Introduction

Throughout Chapter 3 interactions between a repeated mild environmental manipulation, novel cage stress, and AD-related pathology in TASTPM mice were described. Effects of this stressor were investigated during both mild and moderate stages of β-amyloid pathology. From these studies it was evident that TASTPM mice developed a short-term memory extinction deficit between 3 and 4.5 months of age, but stress had no effect on memory performance or β-amyloid levels between these ages. A previous study by our group demonstrated that stress between 4 and 5.5 months of age prevented the development of a short-term memory retention deficit and attenuated brain β-amyloid accumulation (Pardon et al., 2009), but had no effect on memory extinction. A separate study showed an intact long-term memory in 5.5 month old TASTPM mice and pre-exposure to stress had little effect. Furthermore, stress between 5 and 6.5 months of age had no effect on long-term memory retention, but enhanced memory extinction. For a summary of these major findings see figure 3.18.

The findings summarised above are, in part, consistent with the existing literature (see Section 1.4.1, and references therein) in that an environmental stressor was capable of modulating β-amyloid levels in β-amyloid over-expressing transgenic mouse models of AD. Unlike other findings in which stress exacerbated AD-like pathologies, our results indicated a beneficial role of repeated mild stress. The mechanisms through which this positive modulation of pathology occurs, remain unknown. In order to develop an

understanding of processes underlying this positive interaction, we must first take a time-point in TASTPM mouse pathology when we see the most significant effect of novel cage stress. In figure 3.18 the effects of stress on pathologies in TASTPM mice with increasing age are summarised. Novel cage stress applied between 4 and 5.5 months of age on short-term memory was the only occasion when a positive modulatory effect of stress was observed on both behaviour and β-amyloid levels. Due to the robust, quantifiable change with stress at this time-point, it will be used to study the mechanisms linking repeated mild stress with the various pathological markers expressed in this model. To study a potential mechanism one must find an element that links processes occurring during progression of AD-like pathology with events which are known to be involved in the stress response.

Levels of the excitatory amino acid (EAA) neurotransmitter, glutamate, have been correlated with cognitive impairment in normal aging (Zahr et al., 2008), and depletion of brain glutamate levels is a feature of clinical AD (Hyman et al., 1987). Glutamate levels have also been shown to decrease with age in double transgenic mice overexpressing β-amyloid (Marjanska et al., 2005; von Kienlin et al., 2005; Oberg et al., 2008) and this neurotransmitter system is known to be involved in the processes of contextual fear memory learning (Lu and Wehner, 1997; Smith and Wehner, 2002) and extinction (Zushida et al., 2007). Moreover, the glutamatergic system is known to play a key role in the stress response (for review, (Moghaddam, 2002)). Given its involvement in learning and memory, if glutamate levels decline with age in TASTPM mice, as reported in similar mouse strains, this may play a role in the development of

the short-term memory deficit between 4 and 5.5 months of age, and memory extinction deficit between 3 and 4.5 months, exhibited by TASTPM mice.

Glutamate is the most abundant neurotransmitter in the brain and acts through EAA receptors, one of which is the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAr). AMPAr are tetrameric, comprised of four protein subunits ($GluR_{1-4}$), each of these subunits can be expressed as one of two splice variants termed "flip" or "flop", referring to an altered state of functioning, resulting in different receptor properties. For example, the expression of the "flip" variant has been linked to prolonged AMPAr desensitisation (Mosbacher et al., 1994), thus enhancing activity of these receptors with constant agonist exposure. AMPAr functioning has been previously shown to be important in conditioned memory performance (Woolley et al., 2009), for instance, a potentiator of AMPAr enhanced contextual memory extinction in mice (Zushida et al., 2007). AMPAr are also important in the stress response as blocking these receptors during immobilisation stress prevented increases in ACTH (Kusakawa et al., 2007), part of the HPA axis pathway (figure 1.2). Furthermore, complicated links have been demonstrated between AMPAr expression and corticosterone (Groc et al., 2008). An association between AMPAr and AD-related pathology also exist; a reduction in AMPAr-mediated signalling was observed in APP x PS-1 double transgenic mice (Chang et al., 2006) and receptor subunit expression was reduced in vulnerable regions of the hippocampus of AD sufferers, an effect which correlated with the severity of pathological markers (Ikonomovic et al., 1995). The findings summarised above suggest glutamate signalling through

AMPAr as a likely candidate for studying the underlying mechanism linking stressors with AD-related pathology in the TASTPM mice. Therefore, one hypothesis is that an age-related decline in glutamate leads to the development of behavioural abnormalities, which are reversed by repeated mild stress through providing daily stimulation via enhanced glutamatergic signalling through AMPAr. Indeed, elevated activity of AMPAr has been observed following an environmental enrichment paradigm (Gagne et al., 1998).

A further connection between stress and glutamatergic signalling exists through the cellular splicing factor SC35. SC35, which is elevated during states of stress (Meshorer et al., 2005), can affect AMPAr function (Crovato and Egebjerg, 2005) and lead to the expression of a variant of acetylcholine esterase (AChE) (an enzyme responsible for the breakdown of the neurotransmitter acetylcholine), believed to be protective against β-amyloid-related insults (Berson et al., 2008).

Here we aimed to investigate the connection between stress and AD-associated pathology in TASTPM mice in relation to function through AMPAr and SC35 protein expression. To achieve this, two experiments were conducted. Experiment 1 aimed to correlate changes in memory performance with changes in brain glutamate levels between 4 and 5.5 months of age, early stages of β-amyloid pathology of TASTPM mice.

Experiment 2 investigated the involvement of glutamate signalling through AMPAr in the effects of repeated mild stress on TASTPM mice. A

pharmacological approach was taken to determine whether it was possible to mimic the effects of stress on TASTPM mice through daily administration of aniracetam. Aniracetam is a cognitive-enhancing, nootropic modulator which elevates AMPAr conductance, and has been previously reported to improve memory performance at a conditioning test in mice (Lu and Wehner, 1997; Smith and Wehner, 2002). This study was supported with a separate group of TASTPM mice being exposed to novel cage stress and administered the AMPAr antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), known to disrupt conditioned memory in mice (Mead et al., 1999). Mice were treated with NBQX prior to each stress session in order to lower AMPAr signalling during the stress procedure with an aim of blocking the positive effects of novel cage stress.

4.2. Methods

Experiment 1

Animals

Male WT (C57Bl/6, n=15) (Charles River Laboratories, UK) and TASTPM mice (Biomedical Services Unit, Transgenic Unit, Nottingham, n=16) were used. As with previous experiments mice were group housed until the age of 3 months, after which they were singly housed. Food and water were available *ad libitum*, a cardboard play-tube and bedding were also available as part of standard environmental enrichment. Animals were kept under controlled conditions in a constant environment on a 12h light:dark cycle, temperature and humidity were maintained automatically. All procedures were carried out

according to the Animals (Scientific Procedures) Act 1987, under licence 40/2715 granted to Professor Charles A. Marsden.

Experimental design

WT and TASTPM mice were divided into two subgroups depending on the age of behavioural testing: 4 months of age (n=8 per strain) and 5.5 months of age (n=7 WT and n=8 TASTPM mice). Short-term memory was assessed using contextual fear conditioning (CFC) at both ages for both strains. Mice were humanely killed by decapitation 48h following the final CFC extinction trial, their brains removed and half were stored in 4% PFA at 4°C until immunohistochemical analysis was performed for β-amyloid in 5.5 month old TASTPM mice only. The other halves were dissected and the hippocampus and frontal cortex were used for glutamate quantification using HPLC to determine any age-related changes, or differences between the two strains, for the experimental protocol see figure 4.1.

Short-term memory test

CFC (contextual fear conditioning) was used to measure short-term memory performance as described in Section 2.2.2. Briefly, mice underwent CFC training (memory acquisition), receiving 10 foot shocks (0.4mA), once per minute for 10 mins. To test short-term memory retention, mice were returned to the CFC operant chamber 24h later, in the absence of shocks, for 3 mins during which behaviour was video-taped and immobility later scored manually. Two extinction trials were conducted 48h apart; again, mice were returned to

the operant chamber for 3 mins and immobility was scored. The experimental design is summarised in figure 4.1.

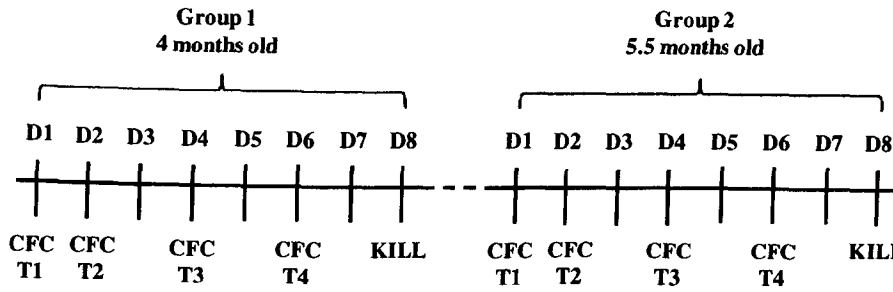


Figure 4.1. Experimental design, Experiment 1. Contextual fear conditioning (CFC) was tested identically for WT and TASTPM mice in both age-groups, 4 and 5.5 months of age. CFC memory acquisition (T1) was conducted on day 1 (D1), memory retention (T2) was conducted on day 2 (D2), the first memory extinction trial (T3), was conducted on day 4 (D4) and the last extinction trial (T4), was conducted on day 6 (D6). At both 4 and 5.5 months of age, mice were humanely killed 48h following T4, on day 8 (D8).

Acquisition of conditioned fear was analysed using a two-way repeated measures ANOVA with Time as the within-subject factor, and Age and Strain as the between-subject factors. Performance across the memory retention and extinction trials was assessed in both experiments using a repeated measures ANOVA with the Trial as the within-subject factor, and Age and Strain as the between-subject factors. For all results described below, values are quoted as mean ± SEM, and Tukey's post-hoc test was used for multiple comparisons in the analysis where appropriate.

As with previous studies, extinction of conditioned fear throughout the test was assessed using a series of extinction indexes, as described in Section 3.1.2.

Briefly, "Extinction" was calculated by subtracting immobility scores during the memory retention trial from the final extinction trial; "Extinction 1" was calculated by subtracting performance during the memory retention trial from that during the first extinction trial; "Extinction 2" was calculated by subtracting immobility in the first extinction trial from the final extinction trial. Extinction is therefore represented by a negative score, and this was compared to 0 using a one-sample t-test for each group.

Brain glutamate levels

Reverse-phase HPLC was used to quantify brain regional glutamate levels in WT and TASTPM mice at 4 and 5.5 months of age. 48h following the final memory extinction trial, mice were killed and brains were removed. The hippocampus and frontal cortex were dissected out, snap-frozen on dry ice and stored at -80°C until they were analysed. Brain samples were weighed, hand homogenised in 500µl methanol, left on ice for 10 mins, then centrifuged at 4°C, 10,000rpm (13.5 cm rotor, Eppendorf 5417R) for 10 mins. The pellets were discarded, and supernatant used for HPLC analysis. Samples were run at x200 concentration (sample diluted in methanol), 100 µl of the sample used for analysis was added to a derivitisation reagent, and held at room temperature for 20 mins prior to injection. Before analysing experimental samples a standard curve was run to prove linearity between glutamate concentration and chromatogram peak height, which proved to be highly correlated (data not shown, R^2 = 0.98). Alongside each daily batch of samples, a glutamate standard was run at the beginning, and at the end of each day and this was subsequently used for analysis. Results were calculated as nmoles glutamate

per mg wet tissue and were compared between groups using a two-way ANOVA with Strain and Age as the between-subject factors.

Immunohistochemistry

Quantification of β-amyloid in 5.5 month old TASTPM mice was performed using immunohistochemistry similar to that described in Section 3.2.2. All mice were killed 48h following the final memory extinction trial. Half brains were removed, fixed in 4% PFA and kept at 4°C for subsequent immunohistochemistry processing. The processing of brain samples was conducted as described previously in Section 2.4.2. In brief, brain tissue was embedded in paraffin wax. Blocks were sectioned using a LEICA RM 2135 rotary microtome. Sections were then immunostained for β-amyloid using the βA4 antibody with a concentrated formic acid pre-treatment. Representative sections were taken between Bregma 1.10mm and Bregma -3.88mm allowing sufficient expression of regions of interest (ROIs): hippocampus (5-7 examples), thalamus (5-8 examples), retrosplenial cortex (5-8 examples), corpus callosum (5-10 examples) and caudate putamen (5-9 examples). Sections were then scanned using the Nanozoomer Digital Pathology slide scanner at x20 magnification. ROIs were delineated using the AnalySIS Pro 3.1 Soft Imaging System. Deposited β-amyloid was automatically detected and results were averaged for each ROI to give a final value as a percentage of ROI covered in β-amyloid. Levels of β-amyloid in 5.5 month old TASTPM were compared to groups of mice in Experiment 2; the reason for this comparison and the type of analysis used is described later.

Experiment 2

Animals

A total of 15 WT mice were used, separated into two subgroups: non-stressed vehicle-treated (non-stressed WT, n=7), stressed vehicle-treated (stressed WT, n=8). A total of 32 TASTPM mice were used and separated into four subgroups: non-stressed vehicle-treated (non-stressed TASTPM, n=8), stressed vehicle-treated (stressed TASTPM, n=8), non-stressed aniracetam-treated (aniracetam-treated TASTPM, n=8), and stressed NBQX-treated mice (NBQX-treated TASTPM, n=8). As described above, all mice were individually housed at 3 months of age and kept under standard housing conditions.

Experimental design

The aim of Experiment 2 was to determine effects of various treatments on memory performance and β-amyloid pathology in the groups of TASTPM mice detailed above. Further details on the drug preparation, dosage and stress procedure are described in detail below. The various treatments were applied between the ages 4 and 5.5 months in WT and TASTPM mice. Following the stress and treatment period, at 5.5 months of age, all mice were tested for locomotor activity and short-term memory performance in the CFC paradigm. 48h following the final CFC memory extinction trial, mice were killed and their brains removed to quantify β-amyloid levels. For experimental design, see figure 4.2.

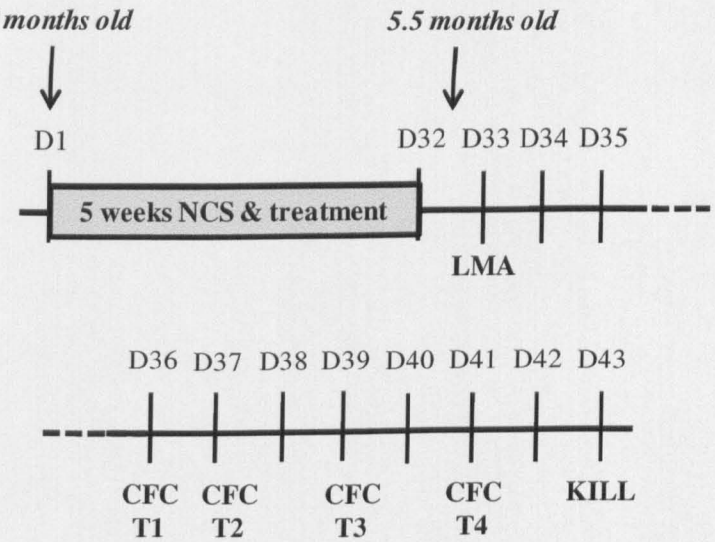


Figure 4.2. Experimental design, Experiment 2. Various treatments (drug administration and/or stress) were started on experimental day 1 (D1) at 4 months of age. After the 5 week "treatment" period, locomotor activity was tested on day 33 (D33). Memory acquisition during the CFC test (T1) was conducted on experimental day 36 (D36), memory retention (T2) was tested 24h later on day 37 (D37). Two 48h intervals later extinction trials (T3 and T4) were conducted on days 39 and 41 (D39 and D41) respectively. 48h following the final extinction trial (T4), mice were humanely killed and brains removed for analysis on day 43 (D43).

Drug preparation and administration

Both aniracetam (NIMH, Bethesda, USA & Tocris Bioscience, Bristol, UK) and NBQX (Ascent Scientific, Western-Super-Mare, UK) were solubilised in sterile vehicle (10% hydroxypropyl-β-cylcodextrin), all injections were made at 0.25ml volume and delivered intraperintoneally. Aniracetam was administered at 100mg/kg (i.p.), a dose previously reported capable of disrupting performance in a similar fear conditioning paradigm in mice (Lu and Wehner, 1997; Smith and Wehner, 2002). Aniracetam was solubilised by sonication in a sonic water bath. NBQX was delivered at 15mg/kg (i.p.), a dose which has been shown previously to disrupt expression of a conditioned

response in mice (Mead et al., 1999), NBQX was directly solubilised in vehicle by vortex mixing. Dosage times were based on those described in the studies cited above: aniracetam-treated mice were dosed 30 mins prior to the start of the stress session, whereas NBQX was injected 15 mins prior to the start. All other non-treated groups of WT and TASTPM mice were vehicle-injected prior to the novel cage stress session.

Novel cage stress

Novel cage stress was applied to WT and TASTPM mice, and NBQX-treated TASTPM mice. This was performed as described in Section 3.1.2. In brief, mice were individually placed for 1h in novel clean cages, half the size of the home cage. Food, water, bedding and play tube were not available. Non-stressed mice were handled for control purposes; they were lifted by the tail from the home cage and replaced into the home cage at corresponding times to the start and finish of the 1h stress session. At the end of the session, stressed mice were replaced in their home cages, the number of faecal boli was recorded, and the novel cages were thoroughly cleaned with standard disinfectant to remove olfactory scent markers.

To determine emotional reactivity, unconditioned behaviours were manually scored during the first and final stress sessions. These included rearing (defined as total elevation of body rearing on hind legs without support of the forelimbs against the cage wall), grooming (defined as a single episode of grooming) and tail-rattles, an emotional aversive response exhibited by mice (defined as the tail being vigorously shaken). These unconditioned behaviours have been used

in previous investigations to assess stress reactivity in mice (Riittinen et al., 1986; Gasparotto et al., 2007). Given an absence of activity in non-stressed mice in their home cages at times corresponding to the stress procedure, unconditioned behaviours were not scored in non-stressed mice. Unconditioned behaviours were analysed using a repeated measures ANOVA, with Stress Session as the within-subject factor (to compare performance during the first and last stress session) and Group as the between-subject factor.

Physiological measures

As with previous studies described earlier, body weight of all mice was recorded at two time points. First, prior to the stress/treatment period at 4 months of age and, second, following the stress/treatment period at 5.5 months of age. Body weight results were compared using a repeated measures ANOVA with Age as the within-subject factor and Group as the between-subject factor. As mentioned above, defecation number was counted at the end of each stress session throughout the treatment period for all mice in the stressed groups. Results were analysed using a repeated measures ANOVA with repeated Stress Session as the within-subject factor, and Group as the between-subject factor.

Locomotor activity

To determine whether the drug/stress treatments had any effects on the locomotor activity of WT or TASTPM mice, performance of all mice was tested 24h following the final treatment and/or stress session, see figure 4.2. The test was conducted as described in Section 3.1.2; briefly, mice were

individually placed in one of eight identical test chambers (13 x 17 x 20 cm) for 30 mins and activity (distance moved in cm) was videotaped and subsequently measured automatically by Ethovision software. Locomotor activity performance was assessed two ways. First, as activity over six 5 min time intervals to determine habituation to the novel environment, analysed using a repeated measures ANOVA with Time as the within-subject factor and Group as the between-subject factor. Second, overall total ambulation over the 30 mins trial was tested by comparing total activity using a one-way ANOVA with Group as the between-subject factor.

Short-term memory test

Short-term memory performance was assessed using CFC identically to that described above in Experiment 1; details on the timing of the trials are summarised in figure 4.2. During the 10 min memory acquisition trial activity (distance moved, cm per minute) of all mice was compared using a repeated measures ANOVA with the Time as the within-subject factor and Group as the between-subject factor. Performance across the memory retention and extinction trials was assessed using a repeated measures ANOVA with the Trial as the within-subject factor and Group as the between subject factor. Extinction indexes were calculated, as described above in Experiment 1, and values for all groups were compared to 0 using a one-samples t-test.

To determine any effects of repeated injection on cognition in WT and TASTPM mice, memory performance of 5.5 month non-injected mice from Experiment 1 and 5.5 month old vehicle-injected mice from Experiment 2 was

compared. Activity during the memory acquisition trial was analysed using a repeated measures ANOVA with Minutes as the within-subject factor and Strain and Injection as the between-subject factors. In addition, performance throughout the memory retention and extinction trials was compared using a repeated measures ANOVA with Trial as the within-subject factor and Strain and Injection as the between-subject factors.

Immunohistochemistry

48h following the final CFC extinction trial, mice were killed and their brains were removed. Half brains were used to quantify levels of β-amyloid in the four groups of TASTPM mice (non-stressed, stressed, aniracetam-treated and NBQX-treated TASTPM mice). The method of section preparation and analysis for Experiment 2 was identical to that described above in Experiment 1. The number of representative examples for each ROI in Experiment 2 were as follows: hippocampus (6-9 examples), thalamus (7-10 examples), retrosplenial cortex (6-10 examples), corpus callosum (6-10 examples) and caudate putamen (6-10 examples). The mean percentage of each ROI covered in β-amyloid was compared between the four TASTPM groups using a one-way ANOVA with Group as the between-subject factor. In addition, to determine effects of 5 weeks of vehicle injection on β-amyloid levels, levels in 5.5 month old TASTPM mice in Experiment 1 were compared to that of 5.5 month old vehicle-treated non-stressed control TASTPM mice using an independent samples t-test. Morphology of β-amyloid plaques in the retrosplenial cortex was manually assessed (as described in Section 3.2.2), levels of dense-core and diffuse plaques were compared between the four

groups of TASTPM mice from Experiment 2 using a one-way ANOVA with Group as the between subject factor.

Levels of SC35 protein expression were quantified in all mice in Experiment 2 (non-stressed and stressed WT and TASTPM, aniracetam- and NBQX-treated TASTPM mice). Brains were sectioned identically to that used for β-amyloid assessment described above, and stained following a previously described protocol (Meshorer et al., 2005). Stained sections were scanned using the Nanozoomer Digital Pathology slide scanner at x40 magnification. To assess levels of SC35 expressed representative portions of ROIs were delineated directly onto the images using the NDP Digital Slide Viewer (Hamamatsu, Japan). To ensure consistency, all effort was made to ensure the representative portions were taken in the same place in the brain between animals. Details of the portions taken for analysis are listed below. Hippocampal functional regions, CA1, CA3 and dentate gyrus were studied; each ROI was delineated as 5000μm^2. For the retrosplenial cortex all portions were 0.1mm^2 (figure 4.3A), until the corpus callosum splits at Bregma -2.7mm, after which portions were 0.05mm^2 adjacent to the corpus callosum. For the caudate putamen, a 0.1mm^2 portion was taken in the centre of the ROI between Bregma 1.10mm and -0.82mm (figure 4.3C), and between Bregma -0.94 to -3.88mm, 0.05mm^2 portions were taken in the centre of the ROI. For the corpus callosum a 0.013mm^2 portion was taken until this ROI splits at Bregma -2.70mm^2 (figure 4.3A & C). For the thalamus, a 0.05mm^2 portion was taken between Bregma -0.22mm and -0.82mm below the ventricles (figure 4.3C), a 0.1mm^2 portion was taken between Bregma -0.94mm and -2.06mm beneath the hippocampus

(figure 4.3A), a 0.05mm^2 portion was taken adjacent to the hippocampus between Bregma -2.18mm and -3.28mm (figure 4.3B) and a 0.015mm^2 portion was taken between Bregma -3.40mm and -3.88mm next to the hippocampus. Example portions available and analysed for each ROI were as follows: retrosplenial cortex (5-10 portions), corpus callosum (6-11 portions), caudate putamen (5-10 portions), thalamus (5-11 portions), dentate gyrus (5-8 portions), CA1 (5-8 portions) and CA3 (5-8 portions).

Within the portions delineated the intensity of the cellular stain was manually scored as one of three levels of intensity: high expression scored 3 (figure 4.4A), medium expression scored 2 (figure 4.4B) and low expression scored 1 (figure 4.4C). Mean intensity scores in all ROIs, for each mouse, was calculated to provide an "intensity read-out". The intensity read-out was compared between the six groups of mice using a one-way ANOVA with Group as the between-subject factor.

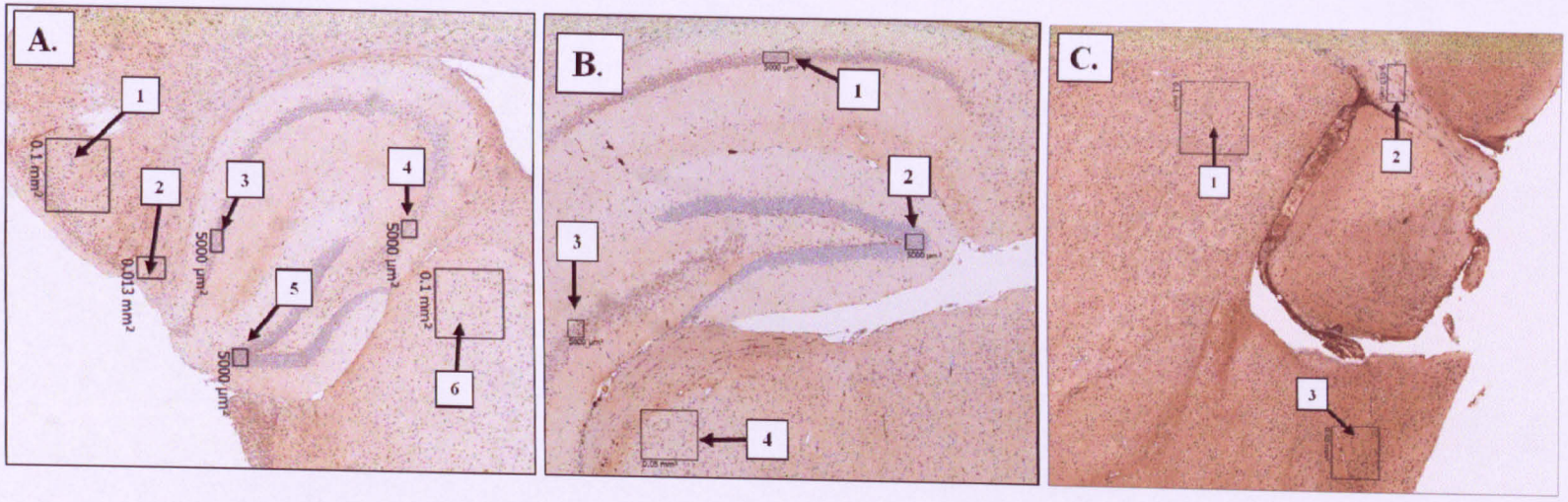


Figure 4.3. Brain sections immunostained for SC35, portions of ROIs delineated. Images captured at x2.5 magnification. **A.** Bregma -1.46mm: 1 = retrosplenial cortex ($0.1mm^2$), 2 = corpus callosum ($0.013mm^2$), 3 = CA1 ($5000\mu m^2$), 4 = CA3 ($5000\mu m^2$), 5 = dentate gyrus ($5000\mu m^2$), 6 = thalamus ($0.1mm^2$) **B.** Bregma -3.16mm: 1 = CA1 ($5000\mu m^2$), 2 = dentate gyrus ($5000\mu m^2$), 3 = CA3 ($5000\mu m^2$), 4= thalamus ($0.05mm^2$) **C.** Bregma -0.22mm: 1 = caudate putamen ($0.1mm^2$), 2 = corpus callosum ($0.013mm^2$), 3 = thalamus ($0.05mm^2$). All efforts were made to ensure portions of ROIs were delineated in a similar location for each ROI.

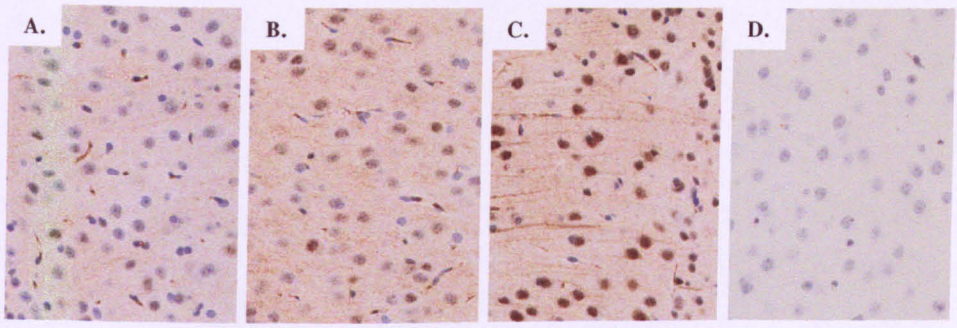


Figure 4.4. SC35 expression intensity criteria for scoring. Images captured in the mouse retrosplenial cortex at x40 magnification to demonstrate SC35 stain intensity. **A.** Low intensity if SC35, nuclei are fully transparent, some evidence of cellular expression, receiving an intensity score of 1.**B.** Medium intensity of staining, most nuclei are partially transparent, with evidence of strong cellular SC35 immunoreactivity, receiving an intensity score of 2. **C.** High expression of SC35 with heavy nuclear staining, almost non-transparent, receiving an intensity score of 3. **D.** Negative control section, no evidence of specific SC35 immunoreactivity.

As with the β-amyloid studies described above, SC35 expression from 5.5 month old TASTPM mice in Experiment 1 was compared to that of 5.5 month old vehicle-treated non-stressed control TASTPM mice from Experiment 2; this was to determine any injection-related changes in SC35 expression using an independent samples t-test. Due to either loss of tissue when sectioning or insufficient portions of each ROI available for analysis, number of subjects in each group was as follows: non-stressed WT (n=6-7), stressed WT (n=8), non-stressed TASTPM (n=7-8), stressed TASTPM (n=7-8), aniracetam-treated TASTPM (n=7-8), NBQX-treated TASTPM (n=6-8) and non-injected, non-stressed TASTPM mice from Experiment 1 (n=6).

4.3. Results

Experiment 1

Short-term memory performance

Age and strain-dependant changes in performance in the CFC test were compared in WT and TASTPM mice at 4 and 5.5 months of age. During the memory acquisition trial all mice, at both ages studied, demonstrated a decline in activity throughout the trial ($P < 0.0001$) indicating successful memory acquisition. There was an overall difference between WT and TASTPM mice ($P = 0.009$) and a trend towards a difference between the two age groups, 4 and 5.5 months old, but this did not reach statistical significance ($P = 0.05$). There was, however, no Strain X Age interaction, figure 4.5A&B.

When analysing performance during the memory retention and extinction tests of WT and TASTPM mice, between the two ages, there was an overall significant effect of repeated trial ($P = 0.006$) and an overall difference between the two strains ($P < 0.0001$), but there were no global differences between the two age groups. There was, however, nearly a Trial X Strain X Age interaction ($P = 0.05$). A multiple comparisons test revealed no differences in memory retention in WT and TASTPM at any age and no groups demonstrated extinction, except 5.5 month old WT mice where there was a trend towards a decline in immobility from the memory retention to the final extinction trial ($P = 0.066$). In addition, 5.5 month old WT mice had a significantly lower level of immobility than age-matched TASTPM during the final extinction trial ($P = 0.041$), a difference which was not detected in mice one month younger, at 4 months of age. Also, there was a trend towards an

age-related difference in immobility levels when comparing 4 and 5.5 month old WT performance during the final extinction trial ($P = 0.08$), figure 4.6A.

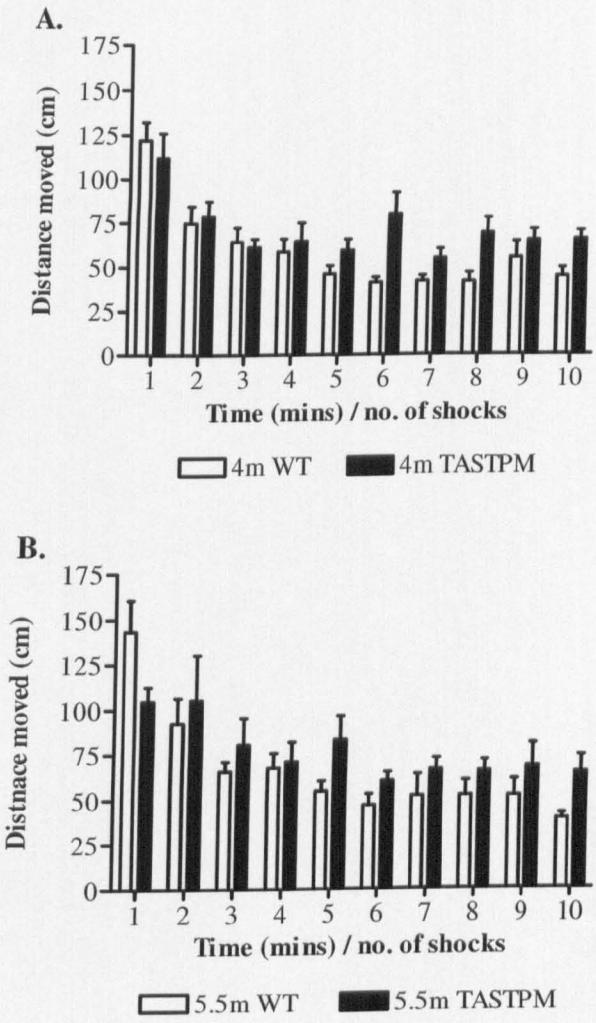


Figure 4.5. Contextual memory acquisition, Experiment 1. A. Mean (± SEM) activity (distance moved, cm) over the 10 min trial for 4 month old WT (4m WT, n=8) and TASTPM mice (4m TASTPM, n=8). **B.** Mean (± SEM) activity over 10 min trial for 5.5 month old WT (5.5m WT, n=7) and TASTPM mice (5.5m TASTPM, n=8). All mice demonstrated a decline in activity throughout the trial ($P < 0.0001$) indicating successful conditioning. Overall there was a significant difference between WT and TASTPM mice ($P = 0.009$) and non-significant trend towards a difference between the two ages studied ($P = 0.05$), but no Age X Strain interaction

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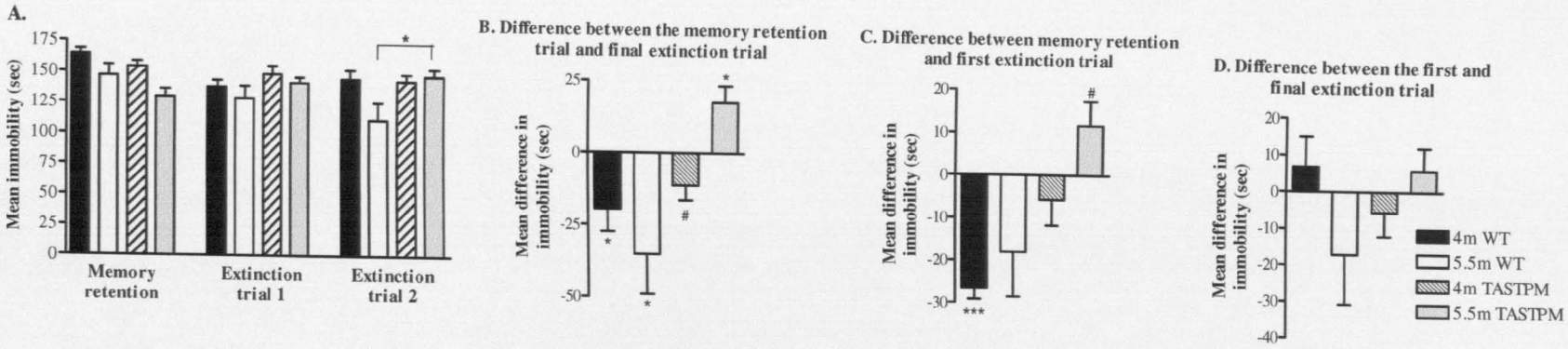

Figure 4.6. Short-term memory performance, Experiment 1. A. Mean (± SEM) immobility (sec) during the memory retention, and the memory extinction trials, for 4 month old WT (4m WT, n=8) and TASTPM mice (4m TASTPM, n=8), and 5.5 month old WT (5.5m WT, n=7) and TASTPM mice (5.5m TASTPM, n=8). Overall there was a difference between the two strains ($P < 0.0001$). Immobility of this group during the final extinction trial was significantly lower than that of age-matched TASTPM mice ($^{*}P = 0.041$). **B.** Mean (± SEM) extinction indices; 4 and 5.5 month old WT mice exhibit extinction between the memory retention and final extinction trial, "Extinction" ($^{*}P < 0.05$), 4 month old TASTPM showed a trend towards extinction ($^{\#}P < 0.1$), but 5.5 month old TASTPM exhibited increased immobility ($^{*}P < 0.05$). **C.** The only group demonstrating significant extinction between the memory retention and first extinction trial, "Extinction 1", was 4 month old WT mice ($^{***}P < 0.0001$), again, 5.5 month old TASTPM demonstrated an trend towards an increase in immobility ($^{\#}P < 0.1$). **D.** No groups demonstrated significant changes in immobility between the first and final extinction trial, "Extinction 2".

Extinction indexes were calculated to determine differences in immobility levels between memory test trials for each group. Both 4 and 5.5 month old WT mice exhibited significant ($P < 0.05$) extinction between the memory retention and final extinction test (Extinction), 4 month old TASTPM showed a trend towards significance ($P = 0.062$), but 5.5 month old TASTPM mice did not show any extinction. Instead, 5.5 month old TASTPM mice exhibited a significant increase in immobility ($P = 0.018$). WT mice at 4 months of age were the only group to exhibit extinction between the memory retention trial and first memory extinction trial (Extinction 1, $P < 0.0001$). None of the groups demonstrated extinction between the first and final extinction trial (Extinction 2), figure 4.6B.

Brain glutamate levels

Glutamate levels from the frontal cortex and hippocampus of WT and TASTPM mice were compared at two ages, 4 and 5.5 months of age. There was no significant difference with age, or between the two strains, and no Strain X Age interaction for either brain region, table 4.1.

Experiment 2

Physiological measures

Body weight for all mice in Experiment 2 was recorded prior to (at 4 months of age), and following the stress/treatment period (at 5.5 months of age) for the six groups, non-stressed WT, stressed WT, non-stressed TASTPM, stressed

TASTPM, aniracetam-treated TASTPM and NBQX-treated TASTPM mice. All mice demonstrated a change in body-weight over time ($P < 0.0001$) and there was an overall difference between the six groups ($P = 0.001$). There appeared to be a difference in weight-gain between non-stressed WT and TASTPM mice but this did not reach statistical significance ($P = 0.05$) and there was no observed effect of repeated stress, or drug-treatments.

	WT		TASTPM	
	4 months	**5.5 months**	**4 months**	**5.5 months**
Frontal cortex	10.60 (1.39)	11.76 (0.82)	11.26 (0.66)	12.47 (0.64)
Hippocampus	11.41 (0.86)	11.04 (0.44)	11.06 (1.11)	10.45 (0.62)

Table 4.1. Brain glutamate levels, Experiment 1. Mean (± SEM) glutamate (nmoles per mg tissue) in frontal cortex and hippocampus of 4 month old WT (n=8) and TASTPM (n=8), and 5.5 month old WT (n=7) and TASTPM (n=8). There was no effect of Age, Strain, or a Strain X Age interaction for either frontal cortex or hippocampal glutamate levels.

Defecation was counted at the end of each novel cage stress session for the three stressed groups: stressed WT and TASTPM, and NBQX-treated TASTPM mice. Changes in defecation levels over the stress period for all mice did not change over time, but there was an overall difference between the three groups ($P < 0.0001$). A multiple comparisons test revealed a significant difference between stressed WT and TASTPM mice ($P < 0.0001$) and between stressed TASTPM and NBQX-treated TASTPM mice ($P = 0.044$), figure 4.7.

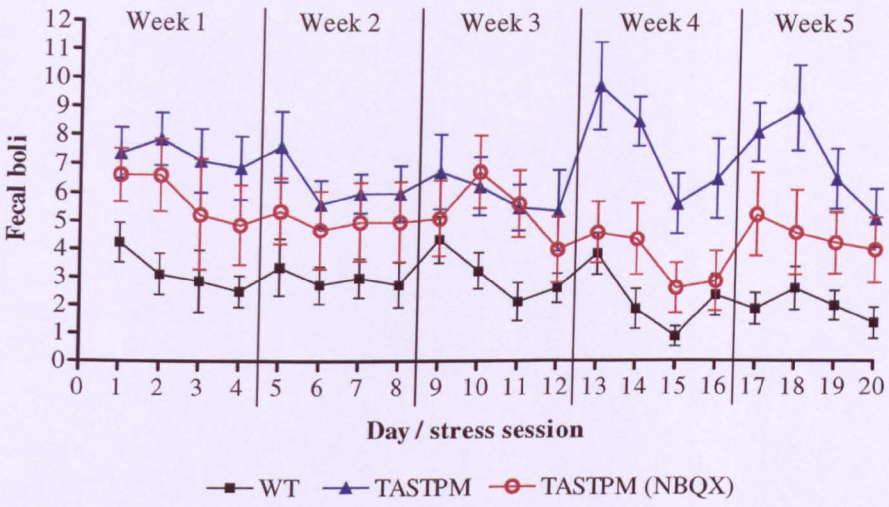

Figure 4.7. Novel cage stress, Experiment 2. Mean (± SEM) defecation count for stressed WT (n=8), TASTPM (n=8) and NBQX-treated TASTPM mice (TASTPM (NBQX), n=8) over the 20 novel cage stress sessions. There was a difference in defecation counts between stressed WT and TASTPM mice ($P < 0.0001$) and stressed TASTPM and NBQX-treated TASTPM mice ($P = 0.044$).

Unconditioned behaviours during stress exposure

To determine changes in unconditioned behaviours between the three stressed groups (stressed WT and TASTPM, and NBQX-treated TASTPM mice) in response to novel cage stress, rearing, grooming and tail-rattles were counted during the first and last stress exposure. Overall, there was a difference in tail-rattles and rearing comparing performance during the first session with last stress session ($P = 0.034$ and $P = 0.025$ respectively), but there was no difference in grooming over time. There was no difference between the three groups in unconditioned behaviours, table 4.2.

	First stress session			Final stress session		
	Tail	**Groom**	**Rear**	**Tail**	**Groom**	**Rear**
WT	0.14	7.43	22.43	0.00	7.14	30.00
	(0.14)	(0.65)	(5.48)	(0.00)	(0.74)	(8.80)
TASTPM	9.71	5.43	11.00	0.00	5.43	24.57
	(5.68)	(0.97)	(4.10)	(0.00)	(1.09)	(11.56)
TASTPM	12.25	9.00	22.25	0.00	6.75	39.88
(NBQX)	(7.30)	(2.83)	(4.88)	(0.00)	(1.78)	(10.37)

Table 4.2. Unconditioned behaviours throughout the novel cage stress, Experiment 2. Mean (± SEM) tail-rattles (Tail), grooming sessions (Groom) and rearings (Rear) for the first and last novel cage stress expressed by 5.5 month old stressed WT (WT, n=7), stressed TASTPM (TASTPM, n=7) and NBQX-treated TASTPM (TASTPM (NBQX), n=8). Tail-rattles and rearings changed over time ($P < 0.05$), whereas grooming sessions did not. There was no difference in unconditioned behaviours between the groups tested.

Locomotor activity

Locomotor activity was assessed following the novel cage stress/treatment period to determine any effects of these manipulations on habituation to a novel environment and total activity over 30 mins. Overall, all mice demonstrated habituation ($P < 0.0001$) and a difference between all groups nearly reached statistical significance ($P = 0.079$). However, a multiple comparisons test did not reveal any between-group differences and there was no difference in total activity throughout the 30 mins, table 4.3.

	LMA habituation						LMA
	0-5mins	5-10mins	10-15mins	15-20mins	20-25mins	25-30mins	Total activity
WT	869.98	736.38	738.07	729.51	692.95	657.53	4424.42
(Non-stressed)	(83.23)	(46.06)	(75.88)	(72.31)	(49.77)	(80.81)	(353.72)
WT	1015.09	761.16	670.97	793.02	659.79	678.33	4578.36
(Stressed)	(76.30)	(70.91)	(50.79)	(51.40)	(75.58)	(83.22)	(355.41)
TASTPM	751.98	566.21	617.34	514.19	560.33	483.47	3493.52
(Non-stressed)	(71.75)	(43.29)	(48.52)	(38.64)	(42.36)	(58.98)	(250.62)
TASTPM	916.43	727.66	675.03	632.24	654.01	613.90	4219.27
(Stressed)	(57.12)	(91.19)	(57.83)	(59.25)	(75.33)	(79.82)	(374.46)
TASTPM	917.54	685.75	732.75	672.91	723.26	720.12	4452.33
(Aniracetam)	(63.79)	(59.08)	(83.75)	(89.40)	(66.92)	(74.55)	(392.93)
TASTPM	918.91	742.27	733.25	722.44	634.10	739.17	4490.13
(NBQX)	(66.60)	(57.84)	(77.41)	(65.85)	(57.79)	(59.84)	(337.13)

Table 4.3. Locomotor activity, Experiment 2. Mean (± SEM) habituation to the locomotor activity (LMA) chamber across six 5 min time intervals, and mean (± SEM) total activity (distance moved, cm) over the 30 min trial, for non-stressed WT (n=7), stressed WT (n=8), non-stressed TASTPM (n=8), stressed TASTPM (n=8), aniracetam-treated TASTPM (n=8), and NBQX-treated TASTPM (n=8). All mice exhibited a decline in activity throughout the trial ($P < 0.0001$), but there was no significant difference between groups in habituation, or total activity throughout the trial.

Short-tem memory tests

All groups of mice were subjected to memory acquisition of CFC following the stress/treatment period at 5.5 months of age. All mice, demonstrated a decline in overall activity (distance moved, cm) ($P < 0.0001$) indicating successful acquisition of contextual fear. There were no between-group differences. Moreover, there was no difference in activity throughout this trial when comparing memory acquisition of non-injected and injected, WT and TASTPM mice from Experiments 1 and 2.

When assessing changes in immobility across the memory retention and extinction trials, there was an overall effect of repeated Trial in all mice (P = 0.038), but no significant difference between the six groups (P = 0.073), figure 4.8A. A multiple comparisons test revealed a trend towards a difference between non-stressed WT and NBQX-treated TASTPM mice only, but this did not reach statistical significance (P = 0.05). As with memory acquisition, there was no difference between WT and TASTPM non-injected and injected mice with regard to performance across the memory retention and extinction trials.

Extinction between memory trials was assessed in each group by the calculation of extinction indices. "Extinction", the difference between the final memory extinction trial and memory retention was significant in the stressed WT mice only (P = 0.031). "Extinction 1", calculated by subtracting performance during the memory retention trial from the first extinction trial, showed no statistical significance in any group studied, although, non-stressed TASTPM mice demonstrated an non-significant trend towards an increase in immobility (P = 0.05). Finally, "Extinction 2", the difference between the first and final extinction trial, was significant in stressed WT mice only (P = 0.049), but did not quite reach statistical significance for non-stressed TASTPM mice (P = 0.05) figure 4.8B.

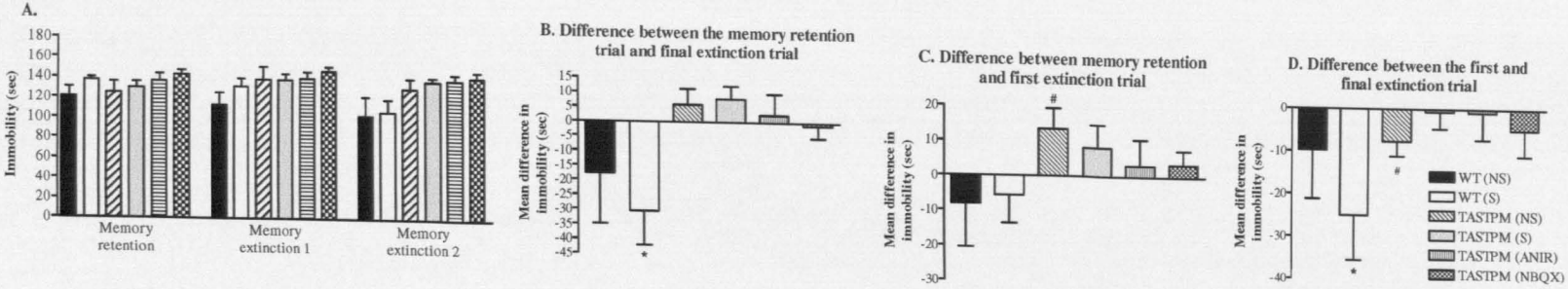

Figure 4.8. Short-term memory performance, Experiment 2. A. Mean (± SEM) immobility (sec) during memory retention and extinction trials exhibited by non-stressed WT (WT (NS), n=7), stressed WT (WT (S), n=8), non-stressed TASTPM (TASTPM (NS), n=8), stressed TASTPM (TASTPM (S), n=8), aniracetam-treated TASTPM (TASTPM (ANIR), n=8), and NBQX-treated TASTPM mice (TASTPM (NBQX), n=8). Overall, all mice demonstrated a decline in immobility over the three trials ($P = 0.038$), but no significant difference between the six groups ($P = 0.073$). **B.** Extinction indices for all mice. Only stressed WT mice demonstrated a significant level ($^{*}P < 0.05$) of "Extinction" between the memory retention and final extinction trial. **C:** Non-stressed TASTPM mice exhibited a non-significant trend towards an increase in immobility from the memory retention to the first memory extinction trial ($^{\#}P < 0.1$), "Extinction 1". **D.** Stressed WT was the only group to show significant drop in immobility between the first and final memory extinction trial ($^{*}P < 0.05$) "Extinction 2", non-stressed TASTPM mice showed a non-significant trend towards extinction at this stage ($^{\#}P < 0.1$).

Immunohistochemical quantification of β-amyloid

Levels of β-amyloid were quantified using immunohistochemistry in the retrosplenial cortex, hippocampus, corpus callosum, caudate putamen and the thalamus. There was no difference in mean percentage of area covered in β-amyloid between the four groups of TASTPM mice, in any of the ROIs studied. To assess any effects of five-weeks of repeated vehicle injection on TASTPM β-amyloid levels, vehicle-treated non-stressed TASTPM from Experiment 2 were compared to age-matched non-injected TASTPM mice from Experiment 1. Injection had no effect on brain β-amyloid levels in the ROIs studied, table 4.4. Furthermore, there was no difference in numbers of dense-core or diffuse plaques in the retrosplenial cortex of the four groups of TASTPM mice.

	TASTPM mice				
	Experiment 1	Experiment 2			
	Non-stressed	Non-stressed	Stressed	Aniracetam	NBQX
Corpus callosum	0.087 (0.030)	0.048 (0.019)	0.069 (0.020)	0.060 (0.015)	0.047 (0.014)
Caudate putamen	0.005 (0.002)	0.004 (0.001)	0.006 (0.001)	0.006 (0.002)	0.009 (0.003)
Hippocampus	0.125 (0.007)	0.077 (0.023)	0.113 (0.027)	0.096 (0.012)	0.141 (0.041)
Retrosplenial cortex	0.191 (0.032)	0.114 (0.047)	0.142 (0.055)	0.132 (0.052)	0.156 (0.047)
Thalamus	0.151 (0.026)	0.087 (0.026)	0.111 (0.032)	0.078 (0.027)	0.098 (0.040)

Table 4.4. β-amyloid pathology, Experiment 2. Mean (± SEM) percentage area covered in β-amyloid for each ROI studied in non-stressed 5.5 month old TASTPM mice from Experiment 1 (n=6-8) and non-stressed (n=7-8), stressed (n=7-8), aniracetam-treated (n=7-8) and NBQX-treated (n=7-8) TASTPM mice from Experiment 2. There was no difference in β-amyloid levels between the four treatment groups, or any effects of repeated injection in non-stressed TASTPM mice when comparing non-stressed TASTPM mice from Experiments 1 and 2.

SC35 expression

Levels of expression of SC35 protein was manually quantified in all groups of mice, both WT and TASTPM, results are expressed as a mean "intensity read-out" as described above in Section 4.2, where high expression scores 3, medium scores 2 and low scores 1. There was an overall difference between all groups in the caudate putamen ($P < 0.0001$), thalamus ($P = 0.035$), hippocampal dentate gyrus ($P < 0.0001$), CA1 ($P < 0.0001$) and CA3 ($P = 0.001$) but not in the retrosplenial cortex ($P = 0.05$). There was no detectable effect of 5 weeks of novel cage stress on SC35 expression in the ROIs studied in either WT or TASTPM mice. Non-stressed TASTPM mice showed significantly lower SC35 expression compared to non-stressed WT mice in the caudate putamen ($P = 0.042$), dentate gyrus ($P = 0.003$), CA1 ($P = 0.002$) and CA3 ($P = 0.003$). Moreover, stressed TASTPM had a lower SC35 expression compared to stressed WT mice in the caudate putamen ($P = 0.030$) and dentate gyrus ($P = 0.022$), table 4.5. There were no differences when comparing the expression of SC35 in all ROIs, between non-stressed and aniracetam-treated TASTPM mice, or between stressed and NBQX-treated TASTPM mice.

As with the β-amyloid studies, expression of SC35 in 5.5 month TASTPM mice from Experiment 1 were compared to age-matched non-stressed TASTPM mice in Experiment 2 to determine any effects of repeated injection; there was a significant difference between the two groups in the corpus callosum only ($P = 0.012$), where injected mice had lower SC-35 expression.

	WT			TASTPM				
	Non-stressed	Stressed		Non-stressed	Stressed	Aniracetam	NBQX	Non-injected
Retroplenial cortex	2.70 (0.08)	2.75 (0.06)		2.30 (0.24)	2.17 (0.16)	2.08 (0.23)	2.22 (0.25)	2.20 (0.07)
Corpus callosum	2.00 (0.19)	1.94 (0.10)		1.53 (0.19)	1.72 (0.18)	1.65 (0.16)	1.70 (0.17)	$ 2.23 (0.11)
Caudate putamen	2.27 (0.14)	2.27 (0.11)	*	1.61 (0.20)	# 1.60 (0.14)	1.54 (0.17)	1.48 (0.12)	1.34 (0.08)
Thalamus	2.37 (0.08)	2.40 (0.07)		1.74 (0.19)	1.96 (0.16)	1.95 (0.22)	1.87 (0.19)	1.59 (0.05)
Dentate gyrus	2.63 (0.11)	2.48 (0.10)	**	1.73 (0.18)	# 1.76 (0.11)	1.76 (0.20)	1.82 (0.19)	1.92 (0.11)
CA1	2.74 (0.08)	2.60 (0.07)	**	1.76 (0.19)	2.04 (0.16)	1.96 (0.21)	1.98 (0.20)	2.03 (0.03)
CA3	2.73 (0.08)	2.58 (0.08)	**	1.88 (0.17)	2.03 (0.09)	2.07 (0.17)	2.13 (0.22)	2.01 (0.06)

Table 4.5. SC35 expression, Experiment 2. Mean (± SEM) intensity score (± SEM) for expression of SC35 in all ROIs studied for non-stressed WT (n=6-7), stressed WT (n=8), non-stressed TASTPM (n=7-8), stressed TASTPM (n=7-8), aniracetam-treated TASTPM (n=7-8), NBQX-treated TASTPM mice (n=6-8) form Experiment 2, and non-stressed, non-injected TASTPM mice from Experiment 1 (n=6). SC-35 expression was significantly lower in non-stressed TASTPM, compared to non-stressed WT mice ($^{*}P < 0.05$, $^{**}P < 0.01$) and in stressed TASTPM compared to stressed WT ($^{\#}P < 0.05$). There was a significant reduction in SC-35 expression in the corpus callosum of injected non-stressed TASTPM mice compared to non-injected TASTPM mice only ($^{\$}P = 0.012$).

4.4. Discussion

A previous study by our group demonstrated how the TASTPM mouse developed a short-term memory deficit at 5.5 months of age, which was prevented with pre-exposure to repeated mild stress (Pardon et al., 2009). The

aim of the studies described above were to, first, further investigate age-related changes in memory performance in TASTPM mice and how these correlate with changes in levels of the excitatory amino acid glutamate. Second, given its interaction with stress, memory systems and AD, AMPAr signalling was targeted pharmacologically to either prevent or mimic the beneficial effects of repeated mild stress on AD-associated pathologies exhibited by TASTPM mice.

No detectable changes in short-term memory retention were found between 4 and 5.5 months of age in TASTPM mice. However, consistent with other studies described previously in this thesis, a short-term memory extinction deficit appears to develop in TASTPM mice between 4 and 5.5 months of age - a time point known to correlate with increasing brain β-amyloid levels (Pardon et al., 2009), but we found no age-related changes in brain glutamate levels. In the investigations described above, stress was incapable of affecting AD-associated pathologies in TASTPM mice, and manipulations of the AMPAr signalling had little effect on these factors.

The first experiment dealt with investigating changes in short-term memory performance that occur between 4 and 5.5 months of age in TASTPM mice. Although, here, we were not able to replicate the age-related short-term memory retention deficit in TASTPM mice as observed in our previous study, 5.5 month old TASTPM mice did have a lower level of memory retention as compared to younger 4 month old TASTPM mice (immobility, sec, 129.66 ± 6.20 vs. 154.13 ± 4.49). Despite this difference not reaching statistical

significance it may indicate a decline in memory performance over time, as demonstrated previously (Pardon et al., 2009). In addition, a short-term memory extinction deficit appeared to develop between the ages of 4 and 5.5 months of age in TASTPM mice which is consistent with findings described in Chapter 3. Given we know of an age-related increase in brain β-amyloid levels in TASTPM mice between these two ages, these changes in cognitive function add support to the hypothesis that β-amyloid can influence memory performance in such mice (for review, (Morgan, 2003)). The method by which β-amyloid can modulate memory performance in not fully known, but cell loss due to its local neurotoxic effects is one possibility (see Section 1.1.2. and references therein). Indeed, cell loss has been observed as a later-stage pathology in β-amyloid over-expressing mouse models (see Section 1.2.3 and references therein), which may be at least partly responsible for memory deficits observed in such mice. Evidence exists demonstrating lower neurotransmitter levels, possibly a downstream event of cell loss, in β-amyloid overexpressing transgenic mice, including the main excitatory amino acid transmitter, glutamate (Marjanska et al., 2005; von Kienlin et al., 2005; Oberg et al., 2008), findings which support the known lowered brain glutamate level observed in clinical AD (Hyman et al., 1987). Contrary to these observations, TASTPM mouse brain glutamate levels were no different to those of WT mice and did not change with age. One possible explanation for this discrepancy may be that changes in glutamate are strain-dependant and that different mutations expressed in various transgenic mouse models may alter the aggression, or form of the pathology (for instance, leading to varying degrees of neuronal cell loss). Another possibility is that between 4 and 5.5 months of

age is still considered an early stage of β-amyloid pathology, and it is likely that if a drop in glutamate levels was to occur in the TASTPM mouse brain, it would be easier to detect during the later stages of the pathology. The lack of changes in glutamate levels in young TASTPM mice indicate that the changes observed in the functioning of memory systems, for instance the development of a memory extinction deficit, do not necessarily correlate directly with levels of this neurotransmitter. It is worthy of note that our study employed HPLC to measure glutamate - a technique which allows only the determination of tissue glutamate content, as opposed to extracellular glutamate levels which can be measured by microdialysis. Using microdialysis could, therefore, be a potential follow-up study given it can provide more information on the functional neurochemistry of TASTPM mice brains.

Experiment 2 aimed to determine to what extent repeated mild novel cage stress exerts its effects on TASTPM mice through glutamatergic signalling. Studies have shown that exposure to stressors enhance glutamatergic transmission (Moghaddam, 2002; Quinton and Yamamoto, 2007) and blocking such transmission prevented the effects of stress on physiological responses, such as hyperthermia (Iijima et al., 2007). More direct links between the stressor we use, and glutamatergic function exists; for instance, one hypothesis is that novel cage stress affects mice through enhancing exercise and elevating stimulation through enrichment, both requiring glutamatergic function (Leung et al., 2006; Segovia et al., 2006). Despite the connection between brain glutamate levels and memory performance being shown not to correlate in the current study, there is evidence in the literature demonstrating that blocking

glutamatergic transmission can interfere with performance in memory-based paradigms, such as fear conditioning (Schulz et al., 2001). Given that stress is widely believed to interact with memory performance in rodents (see Section 1.3.1 and references therein), it is possible that glutamate transmission is, at least, partially responsible for underlying stress and memory interactions. As described in Section 4.1, glutamate signalling through AMPAr receptors appears to be a plausible candidate. It is important to note at this point, however, that although we targeted AMPAr, which are believed to be involved in memory systems and stress responses (see Section 4.1) other glutamate receptors such as the N-methyl-D-aspartic acid (NMDAr) are still active. Like the AMPAr, NMDAr have been shown to be involved in conditioned memory, as receptor antagonists impair memory performance (Mathur et al., 2009) and are believed to play a role in the stress response (Schasfoort et al., 1988). Therefore, although we targeted a key glutamate receptor in these studies, glutamate signalling could continue through alternative pathways such as the NMDAr.

We exposed a group of TASTPM mice to novel cage stress, the hypothesis being that exposure to novelty regularly elevated glutamate transmission, thus providing a beneficial stimulus to protect memory systems against decline. Half of these stressed TASTPM mice were pre-treated with the AMPAr antagonist NBQX to reduce AMPAr signalling due to elevated glutamate release during the stress session. Consistent with previous findings, defecation level throughout the stress procedure, indicative of emotional reactivity to novelty, was higher in TASTPM than WT mice (see Section 3.2). Interestingly,

pre-treatment with NBQX appears to have lowered the defecation number, indicative of a lowered emotional reactivity in this group. If this was indeed the case we would have expected some changes with unconditioned behaviours exhibited during stress exposure, however, there was a lack of any significant effect of NBQX-treatment on such behaviours. It is possible that a lack of effect may be due to weak statistical power given the large variability within groups; during the first stress session, mean body rears was over double for NBQX-treated TASTPM mice compared to control TASTPM mice, but this was not statistically significant.

In terms of the long-term effects of stress, with or without NBQX exposure, on short-term memory function, there was no difference as neither group demonstrated a memory retention deficit, and both groups exhibited the memory extinction deficit. The absence of an effect of NBQX on memory performance is not consistent with a previous study demonstrating the drugs ability to disrupt conditioned memory behaviours in mice at the same dose (Mead et al., 1999). One major difference between these two studies is that the authors administered the drug 15 mins prior to behavioural testing, whereas there was a two day wash-out period in our study, which was designed so as to investigate the downstream behavioural effects of stress exposure. NBQX may only, therefore, affect memory performance during the period in which it was present in glutamatergic synapses. Re-addressing the hypothesis with the evidence described and presented above it seems clear that elevated signalling through AMPAr during the novel cage stress exposure is unlikely to be

important in the mechanism linking the stressor with the previously described beneficial effects.

A separate group of non-stressed TASTPM mice were assigned to two different treatment groups; one was exposed to repeated administration of the AMPAr positive modulator aniracetam. Despite not being statistically significant, there appeared to be a slight increase in locomotor activity in the aniracetam-treated group compared to the controls. Although there was no gross effect of aniracetam treatment on memory retention, control mice demonstrated increased memory from the memory retention to the first extinction test and a drop in memory expression between the first and the final extinction test, neither of which was demonstrated by the aniracetam-treated TASTPM mice. The reason for these differences between the two groups is not clear; previous studies using the same dose showed that the drug was capable of improving memory performance in mice (Lu and Wehner, 1997; Smith and Wehner, 2002). However, as with the NBQX group, the wash-out period between the two studies was different, but the results presented above do indicate that aniracetam, although incapable of affecting short-term memory retention, may affect the complex processes underlying extinction of memory in TASTPM mice. Expression of memory extinction represents an active form of relearning (for review, (Cammarota et al., 2007)) and is therefore different from memory retention, which is simply expression of a conditioned response. Aniracetam may be capable of disrupting the systems which deal with extinction only, indeed, disruptions in glutamate transmission have been linked to changes in the expression of memory extinction (Kim et al., 2007). It is

possible that daily increases in glutamate transmission, induced by repeated aniracetam exposure, are capable of affecting memory extinction performance of TASTPM mice (a process we know to be deficient from an early age) but had no effect on memory retention performance. Interestingly, it has been previously shown that normal AMPAr function is required for memory extinction, but not memory acquisition (Dalton et al., 2008). The absence of a stress effect on extinction, or memory retention, suggests that the links between glutamate and the stress response are weak, as was found with the NBQX study described above. An interesting point arising from Experiment 2 is that although stress had no effect on memory retention or extinction in TASTPM mice, it appears to have facilitated normal extinction behaviour in WT mice (used as a control group in the above experiments).

Consistent with findings from Chapter 3, stress was incapable of changing β-amyloid pathology in 5.5 month old TASTPM mice as determined by immunohistochemistry and neither NBQX nor aniracetam was capable of changing β-amyloid levels. Interestingly, although clinically available drugs targeting the glutamatergic system to prevent excitotoxicity were not originally aimed at lowering β-amyloid pathology, some evidence exists to show that the NMDAr antagonist memantine may be capable of lowering β-amyloid levels (for review, (Rogawski and Wenk, 2003)), although to our knowledge no such evidence exists linking AMPAr and β-amyloid production. As aniracetam enhances signalling through glutamatergic pathways and NBQX reduces signalling, it is surprising that altering the levels of neuronal firing, over a 5 week period had no effect on β-amyloid levels given neuronal activity is

thought to be positively correlated to β-amyloid secretion (Kamenetz et al., 2003). This evidence is contradictory, however, to the notion that environmental enrichment can attenuate brain β-amyloid levels (Lazarov et al., 2005), a treatment which likely leads to elevated brain activity.

SC35 is a serine-arginine rich splicing factor, the expression of which is elevated during states of stress (Meshorer et al., 2005). Stress-induced elevation of SC-35 leads to the alternative splicing of AChE to a variant expressed in stress states (Meshorer et al., 2005). This alternatively spliced read-through AChE (AChE-R) is thought to be neuroprotective against β-amyloid and is under-expressed in the brains of AD sufferers (Berson et al., 2008). SC35 is also capable of changing the flop:flip ratio of AMPAr (Crovato and Egebjerg, 2005), to a formation associated with reduced signalling following activation by glutamate. This, overall, would suggest that stress-induced SC35 may reduce glutamate transmission through this system, but may also provide neuroprotection through enhanced expression of AChE-R. There were differences between WT and TASTPM mouse base-line expression of SC35; in all cases TASTPM mice had lower SC35 expression. Interestingly, AChE-R has been shown to be lower in the AD-affected brain, indicating a possible link existing between lower SC35 expression in the presence of β-amyloid (Berson et al., 2008). The absence of a stress effect on SC35 in either strain was unexpected; this may have been due to the length of time following the last stress session to death (11 days), although studies have demonstrated a long-term, rather than just acute, elevation of SC35 following stress (Meshorer et al., 2005); it is worthy of note that the current study and this cited study used

two different stressors, the later being the more severe cold water stress. To our knowledge there have been no studies investigating the effects of mild stressors on SC35 expression. There was no detectable effect of the glutamatergic treatments on SC35 expression in TASTPM mice, suggesting that AMPAr function does not directly feedback to the expression of SC35.

Potential confounding factors should be addressed in relation to studying SC35 in these mice, including the stressful nature of the behaviour tests used. For instance, the intensity of the learning trial during the contextual fear conditioning memory test; all mice, including non-stressed mice, were exposed to footshocks known to induce a classic stressful response (Baez et al., 1996), also, mice were exposed to novelty during the locomotor activity tests. These manipulations after the treatment period may have dampened any subtle changes to SC35 expression which may have changed with the various treatments. Another possible factor is that all mice were exposed to a daily injection, which likely elicits a stress response and, therefore, may lead to unwanted changes in SC35 expression in this study. However, only one injection-associated change was observed between non-injected and injected TASTPM mice in the above studies, a decrease in corpus callosum SC35 expression in injected mice. This drop in SC35 expression was contrary to what we expected, which would have been an increase in expression in injected mice that would have likely experienced this daily stressor. In general, injected mice had little change in SC35 levels, which was consistent with the finding that repeated injection had no detectable effect on either memory performance in WT and TASTPM mice, or β-amyloid levels in the brains of TASTPM mice;

this indicates SC35 expression, memory systems and β-amyloid pathology are no directly correlated in this study.

These studies aimed to investigate the role of glutamate signalling, first on age-related changes in memory performance and, second, on the stress-induced changes in AD-associated pathologies in TASTPM mice. It appeared that behavioural changes and increases in brain β-amyloid that occur in young TASTPM mice did not correlate with changes in brain glutamate levels. Furthermore, both enhancing glutamatergic signalling through AMPAr to mimic repeated stress exposure, or decreasing signalling during stress exposure, were incapable of modulating gross AD-associated pathologies. Taken together, these studies indicate that glutamate levels and signalling through AMPAr, despite being clinical targets for AD therapy, had little influence on early pathological changes in TASTPM mice and appeared not to underlie the changes that occur with exposure to repeated mild stress.

CHAPTER 5

GENERAL DISCUSSION

5.1. Reviewing the aims and hypothesis

As described in detail in Chapter 1, a variety of tools are available to study AD-like pathology preclinically, one of which is the β-amyloid over-expressing transgenic mouse model. A growing body of clinical and preclinical evidence indicates that a link exists between emotional stressors and AD-associated pathologies. While most preclinical studies imply that relatively severe stressors exacerbate β-amyloid-associated pathology in transgenic mouse models, a study by our group found that a repeated mild stressor improved AD-associated pathology in double transgenic TASTPM mice (Pardon et al., 2009). This thesis aimed to, first, optimise a behavioural paradigm to determine memory performance in TASTPM mice. Once this, along with other tests to assess AD-like pathology in TASTPM mice were set up, they were employed to describe the progression of pathology in the mild-to-moderate stages of β-amyloid pathology in TASTPM mice and how such pathologies are affected by novel cage stress. With these studies, the time-point at which optimal effects of repeated mild stress on TASTPM mice were observed was investigated further in order to identify underlying mechanisms, specifically, the involvement of signalling through the AMPAr - a major component of the glutamatergic neurotransmitter system.

5.2. Summary of key findings

Contextual fear conditioning was a suitable test to study both short- and long-term memory in mice. Using this test, along with various other assessments of

pathological burden, our studies indicated that novel cage stress was beneficial with regard to AD-associated pathologies in TASTPM mice. The most robust effect was observed between 4 and 5.5 months of age when TASTPM mice developed a short-term memory deficit during a time corresponding to rapidly increasing brain β-amyloid levels. Novel cage stress prevented this memory impairment and lowered β-amyloid load. This time-point was subsequently used to investigate the role of glutamate signalling via the AMPAr in this stress effect. It appears, from the studies detailed in this thesis, that AMPAr activity was not directly involved in this link between repeated mild stress and AD-like pathology in TASTPM mice, described in more detail below.

5.3. Early AD-like pathology in TASTPM mice, and effects of novel cage stress

As discussed in detail in Chapter 3 a short-term memory extinction deficit developed in TASTPM mice between 3 and 4.5 months of age. Short-term memory retention remained intact in TASTPM mice until 5.5 months of age, whereas long-term memory retention remained intact in TASTPM mice at all ages studied. Repeated mild stress reversed the short-term memory retention deficit, but had no effect on long-term memory retention. Interestingly, stress had no effect on the seemingly robust short-term memory extinction deficit, but appeared to enhance long-term memory extinction in the oldest group of TASTPM mice only, at 6.5 months of age. It is likely that these differences in short- and long-term memory performance, with or without stress, in TASTPM mice, arises from the nature of the memories involved which are thought to be

held in different brain areas (Wiltgen et al., 2004), as discussed in Section 3.2.4.

In vivo measures of pathological burden through MRI revealed that stress was capable of modulating pathological markers, confirming this technique as a potential method to study disease status in mice. Pre-exposure to repeated mild stress lowered brain β-amyloid levels in 5.5 month old TASTPM mice, which correlated with the improved short-term memory performance. Collectively, the results from the above studies confirm that environmental manipulations are capable of modulating early pathological changes in β-amyloid-overexpressing transgenic mice, but unlike the majority of previous studies looking at exposure to more severe stressors (see Section 1.4) a milder stress procedure appears to attenuate, rather than potentiate, AD-like pathology.

Mild stressors are generally thought to be beneficial in normal aging through activation of adaptive response pathways leading to anti-aging effects, a process called hormesis (for review, (Rattan, 2004)); it is therefore possible that in the studies described throughout this thesis, repeated novel cage stress provided sufficient hormesis, which was protective against the pathological insults in TASTPM mice. Alternatively, these positive effects detected may simply be due to elevated levels of physical exercise known to occur during the novel cage stress exposure (Pardon et al., 2004; Pardon et al., 2005). Indeed, as described in Section 1.4.2., enhanced exercise is believed to be beneficial to AD-like pathology in both clinical (Fratiglioni et al., 2004) and preclinical

studies (Adlard et al., 2005), as well as general aging (Castillo-Garzon et al., 2006).

5.4. The role of glutamate signalling

As described in Section 4.1, neuronal signalling through AMPAr is important in the stress response, plus AMPAr activity is lower in β-amyloid overexpressing mice (Chang et al., 2006). Such evidence presented altered AMPAr function as a likely candidate linking stress and AD-like pathology in TASTPM mice. A further link emerged from the literature, i.e. that the splicing factor SC35 elevated during states of stress is capable of lowering AMPAr signalling, and inducing the expression of a variant of the acetylcholinesterase enzyme, thought to be protective against β-amyloid related insults. We, therefore, investigated whether a pharmacological attempt to increase AMPAr signalling was capable of mimicking stress, and if blocking AMPAr function during stress could prevent its positive effects, whilst also looking at whether these treatments, or stress itself, was capable of affecting SC35 expression. Neither treatment gave rise to significant changes in memory performance, or brain β-amyloid burden. It appears from these studies that AMPAr activity plays little role in the beneficial effects of novel cage stress on TASTPM mice, it is possible, however, that AMPAr signalling could modulate pathological markers which were not tested here. Other potential mechanisms underlying this link are discussed in Section 5.6

5.5. Subjective scoring of rodent behavioural studies

Some of the key findings in this thesis came from behavioural paradigms which had subjective endpoints. It has been recognised that "manual" scoring of animal behaviour data, for instance immobility associated with a fear conditioning paradigm, can vary between investigators and this can lead to variations in the outcomes of studies (Pham et al., 2009). The nature of behavioural scoring, for example, what an investigator considers to be an immobile animal, will inevitably vary between laboratories, thus making comparisons in behavioural results across groups difficult. In the investigations described in this thesis, all behavioural experiments involving subjective scoring of immobility behaviour were scored blind by the same investigator using consistent scoring criteria. To ensure internal consistency existed, the performance during one behavioural experiment (comparing CFC memory retention in non-stressed and stressed TASTPM mice) was re-scored using the same criteria after a gap of over one year and with several studies intervening between scorings. Results were compared using a two-way ANOVA with Stress and Scoring (first vs. second scorings) as the between-subject factors, using Tukey's post-hoc test for multiple comparisons. There was no effect of Stress, and no difference between the two scores, for either group, (see table 5.1). This provides evidence that all studies conducted within this thesis are consistent for the same observer, and therefore, effectively comparable to each other.

	TASTPM mice	
	Non-stressed	**Stressed**
First scoring	150.90 (8.14)	158.53 (6.08)
Second scoring	140.77 (7.30)	152.37 (5.28)

Table 5.1. Within-experimenter consistency in behavioural scoring. Mean immobility (sec, ± SEM) during a CFC memory retention trial for non-stressed (n=7) and stressed (n=7) TASTPM mice. There was no difference in immobility between non-stressed and stressed mice during either score, and there was no overall difference between the two scores.

However, behavioural data from such experiments scored by a different investigator could give rise to different values in such experiments, as described previously (Pham et al., 2009). Such differences likely arise from subtle variations in the subjective criteria of behavioural scoring; for instance, a total lack of mobility maybe easily scored equally by all investigators, but smaller, more subtle body movements require a decision to be made by the investigator as to whether or not this is to be considered "immobility". In order to test this using a behavioural study described in this thesis, immobility scored by myself for a CFC memory retention study (comparing immobility of WT and TASTPM mice), was compared to that of another experienced investigator. To compare the subjective outcomes from these different observations I re-scored the immobility for this experiment, strictly abiding by the second investigator's scoring criteria. All data were analysed using a two-way ANOVA with Strain and Scoring as the between-subject factors, and Tukey's

post-hoc test was used for multiple comparisons. The key findings of this analysis were as follows: first, there were significant differences between my original scores and the second investigator's scores ($P < 0.0001$); second, adopting the scoring criteria of the second investigator gave data that were not significantly different between the two scorers (table 5.2). Overall, this limited investigation into the nature of immobility scoring demonstrates the well known subjective nature of behavioural analysis (Pham et al., 2009), and that through adopting another investigator's criteria, differences in the dataset can be apparent.

		WT	TASTPM
Experimentor 1	*Score 1*	118.40	153.39
		(11.66)	(5.41)
	Score 2	62.37	102.08
		(15.93)	(6.02)
	Experimentor 2	58.97	102.33
		(17.15)	(4.70)
	Locomotor activity	219.22	100.38
		(42.74)	(11.52)

Table 5.2. Comparing behavioural scoring between two investigators. Mean immobility (sec, ± SEM) and locomotor activity (distance moved, cm) of WT (n=7) and TASTPM mice (n=8) during the CFC memory retention trial. Experimenter 1 used their original scoring criteria (score 1) and adopted that of Experimenter 2 (score 2). There was no difference in immobility between WT and TASTPM mice using any scoring criteria. There was a significant difference between Experimenter 1's Score 1 and Score 2 ($P < 0.0001$) and Experimenter 1's Score 1 and Experimenter 2's scores ($P < 0.0001$). There was, however, no difference in scores between Experimenter 1 score 2 and scores by Experimenter 2.

On comparison of the scoring criteria employed, it appeared that the difference between the two investigator's scores was largely due to the exclusion of minor head movements as part of the immobility behaviour in the original scorings. The fairly low intensity footshocks (0.4mA) administered throughout the behavioural studies described in this thesis lead to a behaviour expressed as immobility rather than a more clear-cut freezing, which would be easier to quantify. Thus, small movements, such as head twitches, can lead to different end-scores. Locomotor activity can be used as a "back-up" measure to support immobility scores as one expects an inverse relationship between the two measures. Indeed, in the study outlined above locomotor activity (total distance moved during the trial) does match the outcome of the immobility scores; generally there was higher ambulation in the TASTPM compared to the WT mice, although this did not reach statistical significance, table 2. This is not, however, an ideal measurement of conditioned fear; for example, it scores a complete 360^{o} rotation of the mouse on the spot, as inactivity, which is clearly not the case. It has been recommended that to standardise behavioural measurements, such as those described above, one could rely on automated analysis software capable of discriminating immobility from mobility in laboratory rodents (Pham et al., 2009), this may present a solution to this well known issue.

Indeed, efforts were made by our group to attempt to set-up an automated measure of fear-related immobility in the mouse, but the results were not fully convincing, and problems were likely due to similar issues associated with automated measures of locomotor activity during these tests, described above.

Whether or not one includes small behaviours, such as minor head twitches, in their scoring of mouse immobility, it is clear that the measure of immobility, used consistently throughout this thesis, was a fear-response which relied on the memory of prior footshock administration, as demonstrated in the initial method optimisation (described in Section 2.1).

Together the comparisons of scorings summarised above highlight the importance of every experimenter within a research group adopting the same rigid set of criteria for behavioural scoring and if all studies are scored consistently by the same experimenter, then results should remain accurate and comparable, ensuring the same behavioural process is measured. Standardising behavioural scoring between laboratories to allow for cross-laboratory comparisons is largely unrealistic; although using an automated analysis tool may help.

Indeed, with the benefit of hindsight, modifications to the execution of other methodologies described within this thesis could be made to improve the output. For instance, problems arising from the nature of brain sectioning, part of the immunohistochemistry procedure, lead to different numbers of representative sections for analysis being available between studies; this, therefore, made direct comparisons of β-amyloid pathology between studies impossible. Another potential issue that is worthy of note was the intensity of the CFC memory test. Exposure to footshocks is itself a stressor (Baez et al., 1996), and such shocks may impact on the effects of pre-exposure to 5 weeks of repeated novel cage stress. Lowering the number of footshocks administered

during the CFC training trial, for instance, may reduce stress experienced by the mouse, therefore making the test more appropriate for these experimental conditions. It is important to reflect on such potential issues surrounding this body of work, and work similar to this, to improve experimental rigor for future studies.

5.6. Future directions

To expand on the studies detailed in this thesis, it is important to establish the changes which occur in the mouse with exposure to novel cage stress. An *in vivo* approach, such as microdialysis, to measure extracellular levels of various neurotransmitters during novel cage exposure could provide a clearer picture of how stress mediates its effects on TASTPM mice. For instance, using this technique, acetylcholine has been shown to be elevated during emotional arousal (Imperato et al., 1991). If, indeed, acetylcholine levels are found to be elevated during novel cage stress exposure this system would be a potential target as an underlying mechanism. However, information on neurotransmitter levels would need to be coupled with knowledge of the state of the receptor and signal transduction systems; studies into protein expression using techniques such as Western blotting to determine receptor expression would provide a more complete picture of the changes which occur following novel cage stress exposure.

We know from previous studies that novel cage stress induces elevated levels of activity in mice (Pardon et al., 2004), which provides an indication that this

acts as an enriching stimulus. Brain-derived neurotrophic factor (BDNF), a protein which supports cell survival, is known to be elevated following exercise (for review (Cotman and Berchtold, 2002), and is believed to protect neurons against the neurotoxic effects of β-amyloid-overexpression in transgenic mouse models of AD (Um et al., 2008). It is possible that increases in activity during novel cage stress lead to elevated BDNF expression, and subsequent heightened neuronal survival, therefore, giving rise to a positive end-point. As it is generally accepted that BDNF is elevated during states of environmental enrichment (Adlard et al., 2004) and is protective against AD-associated insults (Um et al., 2008), this would be an appropriate target to study the beneficial effects of repeated mild, novel cage stress.

Many research groups have recently focused on effects of corticotrophin releasing factor (CRF), a component of the stress response pathway and HPA axis, which appears to play a key role in the modulation of AD-like pathology with stress. For example, CRF administered to β-amyloid-overexpressing transgenic mice mimicked the negative effect of acute severe stress (Kang et al., 2007) and evidence suggests that CRF receptors mediated the phosphorylation of tau protein following acute stress (Rissman et al., 2007). CRF signalling thus appears the appropriate target if one was to study the negative effects of a severe stressor. It is unlikely, however, that the beneficial effects of the repeated mild stress procedure described in this thesis would have the same neurochemical consequences as acute severe stress which adversely affects AD pathology.

There are, clearly, several possible mechanisms which may underlie the beneficial effects of novel cage stress on AD-like pathology in TASTPM mice; whether these effects are due to altered neurotransmitter signalling, improved cell survival due to up-regulation of neuroprotective agents, or due to an event directly part of the stress response, is yet to be determined.

5.7. Conclusion

A better understanding of the effects of lifestyle factors on the development of neurodegenerative disorders, such as AD, will improve our recognition of those at risk of developing the disorder. Moreover, discovering pathways underlying such links may open novel therapeutic avenues. In the studies described throughout this thesis, repeated mild stress seems to be beneficial to AD-like pathology in β-amyloid-overexpressing mice. This effect appears to be dependent on the pathological status of the animal, the type of memory tested and the methods by which they are studied; therefore such considerations are important when planning future studies into this area. Signalling through AMPAr appears to not play a key role in this beneficial effect, but further studies, as described above, should elucidate the underlying mechanisms.

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