EXAMINING OLFACTORY LEARNING AND MEMORY IN THE TRIPLE TRANSGENIC AND FIVE TIMES MOUSE MODELS OF ALZHEIMER'S DISEASE USING AN OPERANT OLFACTOMETER

by

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease that is the leading cause of dementia. Olfactory dysfunction is one of the earliest symptoms of AD, and mice show a remarkable ability to learn olfactory based tasks. This thesis presents three studies that used transgenic mouse models of AD and assessed their performance in operant olfactometers. The first study examined the 3xTg-AD and 5xFAD models of AD on an olfactory detection task at six months of age. The female 3xTg-AD mice showed a decreased ability to detect ethyl acetate at the lowest concentrations presented compared to their wildtype controls, while there was no such deficit found in the male 3xTg-AD mice, nor the 5xFAD mice. The second study examined the 5xFAD model at 12 months of age on an odour detection task, and applied signal detection measures. Odour detection was not impaired in the 5xFAD mice, but learning was, and this learning impairment was worse in the female 5xFAD mice than the males. Female mice also showed a more conservative response bias. The third study assessed 5xFAD mice on an olfactory matching to sample working memory task at six months of age. This was the first study to demonstrate that mice could perform such a task, with all mice able to learn the task with a two second delay, and the best performing mice completing delays up to 30 seconds. The 5xFAD mice showed no working memory deficits on this task, though the female mice performed better than the males. Taken together, these studies highlight the remarkable abilities of mice to perform olfactory based tasks and demonstrate their use in assessing mouse models of AD.

List Of Abbreviations Used

Abbreviation	**Meaning**
3xTg-AD	Triple transgenic Alzheimer's Disease mouse model
5xFAD	Five times familiar Alzheimer's Disease mouse model
Aβ	Amyloid Beta
AICD	Amyloid Precursor Protein Intracellular Domain
AD	Alzheimer's Disease
AIC	Akaike Information Criterion
AICc	Akaike Information Criterion corrected for small sample size
APP	Amyloid Precursor Protein
BACE1	Beta-site Amyloid Precursor Protein Cleaving Enzyme-1
c	Response Bias
CA1	A Region of the Hippocampus
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CI^{95}	95% Confidence Interval
d'	Sensitivity Index
DNA	Deoxyribonucleic Acid
EA	Ethyl Acetate
ILIβ	Interleukin 1 Beta
ILI10	Interleukin 10
ILI4	Interleukin 4

ISD	Inter-Stimulus Delay
ITI	Inter-Trial Interval
MCI	Mild Cognitive Impairment
MMSE	Mini Mental State Exam
MRI	Magnetic Resonance Imaging
NCAM	Neural Cell Adhesion Molecule
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PPM	Parts Per Million
PS1	Presenilin-1
S+	Rewarded Stimulus
S-	Unrewarded Stimulus
sAPPα	Soluble Amyloid Precursor Protein Alpha
sAPPβ	Soluble Amyloid Precursor Protein Beta
TXNIP	Thioredoxin Interacting Protein
WT	Wild Type

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1 Introduction

1.1 Mechanisms of AD

With more than 55 million cases worldwide, Alzheimer's disease (AD) accounts for over 70 percent of all dementia cases (Alzheimer Association, 2021; Gauthier et al., 2021). AD is neuropathologically defined by the accumulation of amyloid beta (Aβ) and hyperphosphorylated tau, synaptic dysfunction, cerebral atrophy, immune disfunction, and gliosis (Lane et al., 2018; S. Li & Selkoe, 2020; Meier-Stephenson et al., 2022; Pei et al., 2020; Pini et al., 2016).

The amyloid hypothesis of AD holds that aggregation of misfolded Aβ is key to precipitating the disease. Amyloid precursor protein (APP) can be processed by two pathways, the non- amyloidogenic α-secretase pathway, and the amyloidogenic β-secretase pathway. When processed by the α-secretase pathway, APP is cleaved by α-secretase into sAPPα and C83. Then C83 is cleaved by γ-secretase, a membrane bound complex including presenilin-1 (PS1) (Duyckaerts et al., 2009), into p3 and APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β-secretase, also referred to as beta-site APP cleaving enzyme-1 (BACE1), into sAPPβ and C99; γ-secretase then cleaves C99 into Aβ and AICD (Finder, 2010)

Various isoforms of Aβ are produced. The most common forms vary from 39- 42 amino acids long with $A\beta_{42}$ the most damaging. $A\beta_{42}$ is more hydrophobic than the shorter peptides due to the additional amino acids on the membrane bound C-terminus, making it more likely to precipitate in an aqueous solution (Duyckaerts et al., 2009). The C terminal fragment of APP has also been implicated in AD pathology and has been demonstrated to accumulate in mitochondria, resulting in their dysfunction (Devi & Ohno, 2012).

Another mechanism involved in AD pathology is the aggregation of tau into neurofibrillary tangles (Bancher et al., 1989). When tau becomes hyperphosphorylated, it oligomizes into helical filaments, which impair axonal transportation, leading to cellular disfunction (Finder, 2010). Hyperphosphorylated tau is found at increased levels in at least 25 other neuropathologies (Spillantini & Goedert, 2013), and some have suggested that the formation of neurofibrillary tangles may be a protective mechanism in response to oxidative stress (Lee et al., 2005). However, the cognitive symptoms of AD correlate better with neurofibrillary tangles than Aβ burden (Nelson et al., 2012). The precise mechanisms by which the hyperphosphorylation of tau begins remains unknown, though the sites that are hyperphosphorylated do differ between tauopathies, with tau in AD patients showing increased phosphorylation at Ser202, Thr231, and Ser235 compared to other tauopathies (Samimi et al., 2021).

Approximately 95 to 99 percent of AD cases are late onset, or spontaneous, AD. This manifestation of AD does not have a clear cause, though environmental and lifestyle factors a thought to play important roles (Scheltens et al., 2021). The remaining proportion of AD cases, known as familial AD, and are caused by heritable mutations to genes such as APP, PS-1, or presenilin-2 (PS-2) (Bateman et al., 2012). Though familial AD comprises only a small portion of AD cases, it is the focus of much of the animal research due to the ability to create transgenic models of AD using mutations identified in familial AD. However, it has been suggested that this approach is a factor contributing to the low success rate (< 1%) of translating pharmaceutical interventions from animal models to humans (Cummings et al., 2014).

1.2 Olfaction In AD

Although the most salient behavioural symptoms of AD are deficits in learning and memory (Scheltens et al., 2021; Toepper, 2017; Weintraub et al., 2012), one of the earliest symptoms of AD is a loss of olfactory function (Alves et al., 2014; Devanand et al., 2015; Son et al., 2021). Reduced olfactory sensitivity is well documented in the elderly population (Murphy et al., 2002; Schubert et al., 2011) and can be the result of age-related reductions in the number of olfactory receptors (Doty et al., 1984), an overall thinning of the olfactory epithelium (Naessen, 1971; Paik et al., 1992), a reduction in olfactory bulb volume (Buschhüter et al., 2008; Thomann et al., 2009) or synaptic dysfunction (Daulatzai, 2015). Olfactory deficits have been observed in more

than 50 percent of the population over the age of 65 (Mobley et al., 2014; Ottaviano et al., 2016) including those with mild cognitive impairment (MCI), AD, and other types of dementia (Attems et al., 2015; de Moraes e Silva et al., 2018; Devanand, 2016; Doty & Kamath, 2014; Kotecha et al., 2018; Zou et al., 2016). Olfactory bulb volume is correlated with Mini Mental State Exam (MMSE) score in patients with MCI and AD (Thomann et al., 2009). However, the relationship between healthy aging, olfactory dysfunction, and dementia is complex and determination of these deficits may depend on the tests used (Doty, 2017; Marin et al., 2018) as olfactory dysfunction may be due to deficits in odour detection, odour identification, or odour memory (de Moraes e Silva et al., 2018; Tzeng et al., 2021). AD may also be due to immune system disfunction (Meier-Stephenson et al., 2022) and there is a close relationship between olfaction and the immune system (Bryche et al., 2021; Lampinen et al., 2022; Strous & Shoenfeld, 2006).

Deficits in both odour detection and odour identification are observed in age-related neurodegenerative disorders such as AD (Alves et al., 2014; Doty, 2017; Doty et al., 1987; Karpa et al., 2010; Koss et al., 1988). Aging individuals with difficulty in odour identification have a higher chance of developing MCI than those with normal olfactory abilities, even though they have intact cognition (Wilson et al., 2007). Olfactory dysfunction has been shown to be a better predictor of AD onset than loss of verbal episodic memory (Devanand et al., 2015) as extensive Aβ plaque deposits occur throughout the olfactory system in AD patients (Duyckaerts et al., 2009), however patients are often unaware of their olfactory deficits (Devanand et al., 2000). Carriers of

the epsilon 4 isoform of apolipoprotein E, which is associated with higher risk of non-familial AD (Holtzman et al., 2012; Y.-W. A. Huang et al., 2017), showed an age-related decline in odour identification ability but not in odour detection threshold, picture identification, or dementia rating scale scores (Calhoun-Haney & Murphy, 2005). Age-related decline in odour identification has also been correlated with a faster rate of cognitive decline, and reduced volume of the hippocampus, entorhinal, fusiform, and middle temporal cortices (Dintica et al., 2019). Although a number of tests for measuring olfactory deficits in AD have been described, there is no standard measure of olfactory dysfunction that can be used as a clinical tool in diagnosing AD (Gros et al., 2017; Quarmley et al., 2017; Velayudhan et al., 2015).

1.3 The Rodent Olfactory System

The rodent olfactory system is a complex system consisting of the main and accessory olfactory pathways (Figure 1-1). The traditional view was that the main olfactory system is responsible for detecting and responding to odourant, while the accessory olfactory system is responsible for responding to pheromones. However, evidence shows that this strict division of the systems is misleading, with much overlap between the systems and both playing roles in the detection of odourants and pheromones (Restrepo et al., 2004).

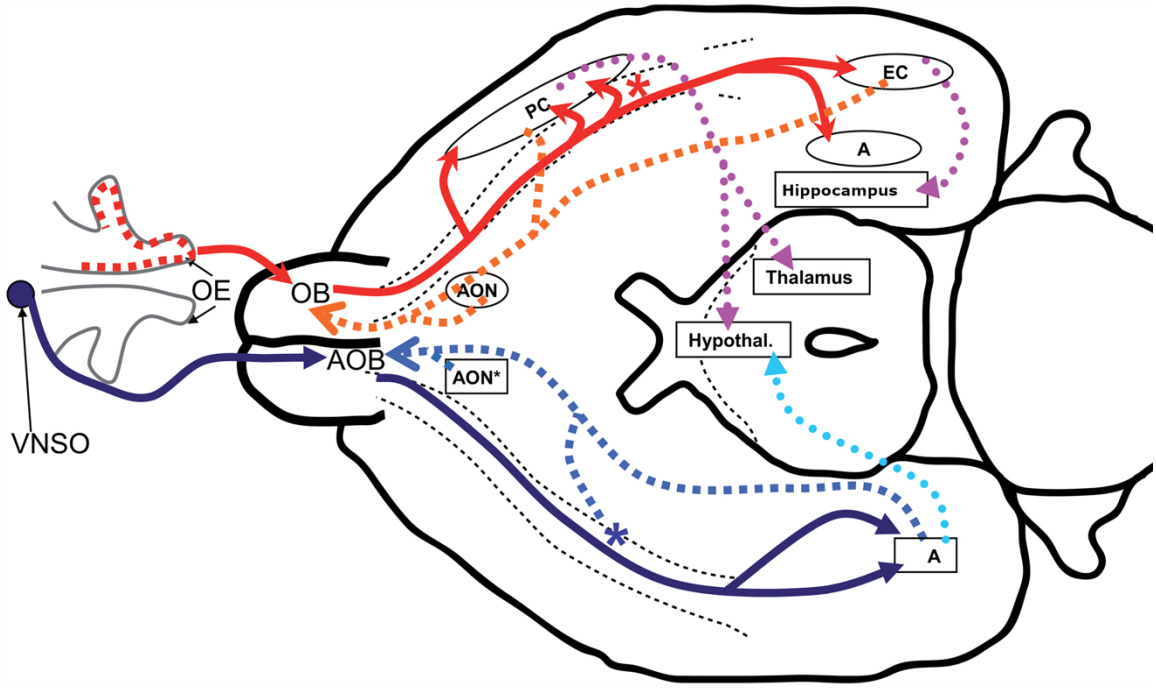

Figure 1-1: Overview of the main and accessory olfactory pathways. The main olfactory pathways (shown in red) go from the olfactory epithelium (OE) to the olfactory bulb (OB). The olfactory bulb projects to the olfactory cortex, which consists of the anterior olfactory nucleus (AON), the olfactory tubercle, the piriform cortex (PC), the amygdala (A), and the entorhinal cortex (EC). These regions project both back to the olfactory bulb, and on to other brain regions such as the hippocampus, thalamus, and hypothalamus. The accessory olfactory system (shown in blue) starts in the vomeronasal organ (VNSO) and project to the accessory olfactory bulb (AOB). The accessory olfactory bulb in turn projects to the amygdala, and onto the hypothalamus. Adapted from de Castro (2009), see appendix A for copywrite permission.

The main olfactory system starts with the olfactory epithelium. Here olfactory receptor neurons each express one of the approximately 1500 olfactory receptors encoded in the

mouse genome, compared to the approximately 900 found in the human genome (Young et al., 2002). These olfactory receptor neurons project to the olfactory bulb, where the axons of different olfactory receptor neurons expressing the same olfactory receptors converge on the same glomeruli (Mombaerts, 2006).

The projections of the olfactory bulb form the lateral olfactory tract, which enervates the olfactory cortex. The olfactory cortex consists of the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex, the amygdala, and the entorhinal cortex (de Castro, 2009). The olfactory cortex has both intrinsic connections within and between the structures of the olfactory cortex, and extrinsic outputs to neocortical and subcortical regions such as the hippocampus, insular, orbital, and perirhinal cortices, and the thalamus and hypothalamus (Ennis et al., 2015).

The accessory olfactory system starts with the vomeronasal organ, located within the nasal septum at the base of the olfactory cavity. The receptor neurons of the vomeronasal organ project to the accessory olfactory bulb, which is embedded in the dorsal section of the olfactory bulb (Mucignat-Caretta, 2010). The main projections of the accessory olfactory bulb are the amygdala, the thalamus and the anterior olfactory nucleus (de Castro, 2009; Ennis et al., 2015).

1.4 Mouse Models Of AD

Starting with the PDAPP mouse which expressed mutant human APP driven by a platelet derived growth factor promoter (Games et al., 1995), nearly 200 different transgenic mouse models of AD have been engineered (*Alzheimer's Disease Research Models | ALZFORUM*, n.d.; Myers & McGonigle, 2019). Most of these models, such as the 5xFAD mouse, involve mutations that result in amyloid pathology in the brain. Others, such as the P301S mouse develop tau pathology. Some, such as the 3xTg-AD mouse, have mutations that result in both amyloid and tau pathology (Mckean et al., 2021; Trujillo-Estrada et al., 2021). My lab uses both the 3xTg-AD and 5xFAD mouse models of AD. The neuropathological changes of these mice are summarized in Table 1-1 and Table 1-2, while their behavioural, sensory, and motor changes are summarized in Table 1-3 and Table 1-4 .

Table 1-1: Summary of neuropathological changes found in the 3xTg-AD mouse model.

Model	Change	Age (months)	Source
3xTg-AD	Myelin abnormalities	2	Falangola et al., 2020
	Increased gamma oscillations in the olfactory bulbs	3-5	M. Chen et al., 2021
	Amyloid deposits in the olfactory bulbs	3	Mitrano et al., 2021
	Reduced cerebrovascular space in the hippocampus	11	Bourasset et al., 2009
	Decreased glucose metabolism in the piriform and insular cortex	11	Adlimoghaddam et al., 2019
	Increased microglia density in the hippocampus	12	Rodríguez et al., 2010, 2015

Table 1-2: Summary of neuropathological changes found in the 5xFAD mouse model.

Model	Change	Age (months)	Source
5xFAD	Soluble $A\beta_{40}$ and $A\beta_{42}$	0.5	Boza-Serrano et al.. 2018
	Intraneuronal Aβ	1.5	Eimer & Vassar, 2013
	Amyloid deposits	2	Eimer & Vassar, 2013: Oblak et
	Decreased myelination	2	Gu et al., 2018
	Changes in gene expression	2	Bundy et al., 2019
	Increased activation of microglia	2	Chithanathan et al.. 2022
	Upregulation of IL1β and TNF in olfactory bulbs	2	Chithanathan et al.. 2022
	Decreased glucose metabolism in olfactory bulbs	6	Xiao et al., 2015
	Decreased dendritic spine density	3	Aytan et al., 2018
	Decreased neurogenesis in the dentate gyrus	3	Aytan et al., 2018
	Decreased glucose metabolism in the hippocampus and cortex	6	Xiao et al., 2015
	Neuronal loss	9	Eimer & Vassar, 2013
	Increased astrocyte densities in the hippocampus	12	Forner et al., 2021
	Decreased whole brain glucose metabolism	13	Macdonald et al.. 2014
	Increased astrocyte densities in cortex	18	Forner et al., 2021

Table 1-3: Behavioural and sensory changes in the 3xTg-AD mouse model.

Model	Change	Age (months)	Source
3xTg-AD	Impaired working and reference memory on the 8 arm radial maze	2	Stevens & Brown, 2015
	Retention deficits in the MWM	4	Billings et al., 2005
	Impaired olfactory sensitivity	6	Roddick et al., 2016
	Impaired short and long term contextual fear	6	Billings et al., 2005
	Impaired reversal on a Barns maze	7	Fertan, Rodrigues, et al., 2019
	Impaired on MWM	9	Baazaoui & Iqbal, 2017

Table 1-4: Behavioural, sensory, and motor changes in the 5xFAD model.

Model	Change	Age (months)	Source
5xFAD	Hearing dysfunction	3-4	O'Leary et al., 2017
	Impaired on hippocampal dependant tube maze	4	Girard et al., 2014
	Decreased long term fear conditioning	4	Kimura & Ohno, 2009
	Reduced rearing	6	Oblak et al., 2021
	Hyperactivity	6	Oblak et al., 2021
	Decreased short term fear conditioning	6	Kimura & Ohno, 2009
	Impairments in visuo-spatial learning in the MWM	6	O'Leary & Brown, 2022
	Impaired rotarod performance	9	O'Leary, Mantolino, et al., 2020
	Reduced body weight	9	O'Leary, Mantolino, et al., 2020
	Impaired olfactory learning	12	Roddick et al., 2022

1.4.1 The 3xTg-AD Mouse

The 3×Tg-AD mice have three mutations, the Swedish (K670N/ M671L) mutation to amyloid precursor protein (APP), a mutation to presenilin-1 (PS1) (M146V), and a tau

mutation (P301L) (Oddo et al., 2003). As early as one month of age, the 3xTg-AD mice show texture differences in their retinas (Ferreira et al., 2020).

At two months of age, diffusion magnetic resonance imaging (MRI) is able to detect myelin abnormalities in the 3xTg-AD mice (Falangola et al., 2020), possibly due to increased levels of apoptosis in oligodendrocyte seen in this model (Desai et al., 2010). Local field potential recordings of the olfactory bulbs of 3-5 month old 3xTg-AD mice showed increased gamma oscillations (M. Chen et al., 2021), and amyloid deposits were found in the olfactory bulbs of 3xTg-AD mice as early as 13 weeks of age, appearing first in the granule layer, then the external plexiform layer (Mitrano et al., 2021).

Female 3xTg-AD mice show more amyloid plaques and neurofibrillary tangles than male 3xTg-AD mice, as well as increased activation of microglia and astrocytes (J.-T. Yang et al., 2018). There is reduced cerebrovascular space in the hippocampus of 3xTg-AD mice at 11 months of age (Bourasset et al., 2009). At 11 months old there is decreased glucose metabolism in the piriform and insular cortex of the 3xTg-AD mouse (Adlimoghaddam et al., 2019), regions involved in olfaction (Zatorre et al., 1992). The 3xTg-AD mice also show increased microglia density in the hippocampus by 12 months old (Rodríguez et al., 2010, 2015).

Behaviourally, 3xTg-AD mice show impaired working and reference memory as early as 2 months old in a eight arm radial maze (Stevens & Brown, 2015). They show retention

deficits in the Morris Water Maze at 4 months old, showing impaired performance on the first trial of a testing day compared to the last trial of the previous day (Billings et al., 2005). By 6 months old the 3xTg-AD mice also show impaired short and long term retention of contextual fear conditioning (Billings et al., 2005), and by 7 months show impaired reversal and less improvement in performance across days in a Barnes maze (Fertan, Rodrigues, et al., 2019). By 9 months of age performance in the Morris Water Maze is impaired in 3xTg-AD mice (Baazaoui & Iqbal, 2017).

In terms of motor function, the 3xTg-AD mice show a complex phenotype, doing better than wildtype mice on some tasks, such as the rotarod, but poorer on others, such as grip strength (Stover et al., 2015).

The 3xTg-AD mice have larger spleens than wildtype mice, and the spleens contained Aβ, however, the spleens have lower levels of CD4 and CD8 T cells than the wildtype mice (Fertan, Rodrigues, et al., 2019). The 3xTg-AD mice also showed increased levels of cerebral thioredoxin interacting protein (TXNIP) which increases oxidative stress (Fertan, Rodrigues, et al., 2019).

1.4.2 The 5xFAD Mouse

The 5xFAD mouse model of AD carries three human APP mutations (Sweden, London, and Florida) and two presenilin 1 (PS1) mutations (M146L and L286V), collectively

causing accelerated $A\beta_{42}$ accumulation (Oakley *et al.* 2006). Unlike the 3xTg-AD mouse model, which shows tau and Aβ pathology, the 5xFAD model shows only Aβ pathology. Soluble $A\beta_{40}$ and $A\beta_{42}$ is detectable as early as two weeks of age in 5xFAD mice and increases with age (Boza-Serrano et al., 2018), and intraneuronal Aβ begins to form in the 5xFAD mice as early as 1.5 months old (Eimer & Vassar, 2013), with amyloid deposits forming at two months old (Eimer & Vassar, 2013; Oblak et al., 2021), and neuronal loss starting at nine months old (Eimer & Vassar, 2013).

Decreased dendritic spine density is reported as early as 3 months of age (Aytan et al., 2018), along with decreased neurogenesis in the dentate gyrus at 3 (Aytan et al., 2018) and 7 (Fiol-deRoque et al., 2013) months old. Decreased myelination in the CA1 of the hippocampus is found as early as 2 months old in 5xFAD mice (Gu et al., 2018). By 12 months of age 5xFAD mice show increased astrocyte densities in the hippocampus, spreading to the cortex by 18 months (Forner et al., 2021).

Changes in gene expression in the hippocampus are detectable at 2 months old, and increase substantially by 4 months old (Bundy et al., 2019). These changes in gene expression were greater in the female 5xFAD mice than the male 5xFAD mice (Bundy et al., 2019). The 5xFAD mice also differ from wildtype mice in cytokine levels. The anti-inflammatory IL4 is lower, while the pro-inflammatory IL1β is upregulated in the 5xFAD mice compared to wildtype mice (Boza-Serrano et al., 2018). Interestingly, the anti-inflammatory IL10 is also upregulated in the 5xFAD mice (Boza-Serrano et al., 2018).

Indicators of inflammatory activation in the microglia cells of 5xFAD mice have been detected before amyloid plaques form (Boza-Serrano et al., 2018). Upregulation of the proinflammatory cytokines IL1β and tumor necrosis factor occurs in the olfactory bulbs of 5xFAD mice as early as two months old, and is accompanied by increased activation of microglia (Chithanathan et al., 2022).

At 6 months old, 5xFAD mice show decreased glucose metabolism in the hippocampus, cortex, and olfactory bulbs. Interestingly, this study also found reduced glucose metabolism in the olfactory bulbs at 3 months old, but no differences in the hippocampus or cortex at this age (Xiao et al., 2015). This suggests the metabolic changes happen earlier in the olfactory bulbs. Whole brain glucose metabolism in decreased at 13 months of age (Macdonald et al., 2014)

Behaviourally, 5xFAD show deficits in contextual fear conditioning. When tested 1 day after fear conditioning, 5xFAD mice show decreased freezing at 6, but not 4 months of age. However, when tested 30 days after conditioning, the deficits do appear in the 4 month old 5xFAD mice (Kimura & Ohno, 2009). Impairments in visuo-spatial learning, as measured by the Morris Water Maze, show in the 5xFAD model at 6 months of age (O'Leary & Brown, 2022).

In an olfactory tubing maze, a hippocampal dependent task, 4 and 6 month old 5xFAD mice show learning deficits when trained with 12 trials per session, but not 20 trials per

session, indicating that their ability to detect the odours were intact, and the deficits are due to hippocampal dysfunction (Girard et al., 2014). Female 5xFAD mice show an age dependent reduction in investigation of social odours and lower levels of social behaviours (Kosel et al., 2019). Hearing dysfunction is present as early as 3 to 4 months old in the 5xFAD mice (O'Leary et al., 2017).

In addition to cognitive deficits, 5xFAD mice also show impaired motor behaviour. Beginning at nine months, 5xFAD show impaired performance on a rotarod compared to wildtype littermates, as well as reduced body weight (O'Leary, Mantolino, et al., 2020). Reduced rearing in an open field is also reported as early as six (Oblak et al., 2021) and nine months of age (O'Leary, Mantolino, et al., 2020).

Hyperactivity, both in an open field and in home cage wheel running, is reported at 6 and 12 months of age (Oblak et al., 2021). No differences in spontaneous alternation in a Y maze were found at 6 or 12 months of age (Oblak et al., 2021).

1.5 Olfaction In AD Mice

There are several methods for measuring olfactory processes in mice, including habituation-dishabituation, Pavlovian, and instrumental conditioning (Schellinck, 2018; Slotnick & Restrepo, 2005), and olfactory deficits have been shown in a number of different mouse models of AD (Tzeng et al., 2021). For example, both the Tg2576 and

the APP/PS1 mice have olfactory deficits accompanied by Aβ pathology in the olfactory pathways (Wesson et al., 2010; Yao et al., 2017). In the APP/PS1 mice, olfactory dysfunction precedes visuo-spatial learning deficits (W. Li et al., 2019). Olfactory deficits have also been reported the Tα1-3RT tau transgenic mice (Macknin et al., 2004) and in the 3xTg-AD mouse, which has both Aβ and tau transgenes (Mitrano et al., 2021; Roddick et al., 2016). Following intraventricular injection of $A\beta_{42}$, female C57BL/6 mice show deficits in the buried food and habituation/dishabituation tasks and have increased neuropathology in the olfactory bulbs and hippocampi (Raj et al., 2019). However, different mouse models of AD differ in the degree of neuropathology in the olfactory system. The APP/PS1 and Tg2576 mice show Aβ plaques in the olfactory system, but hAPP-J20 mice do not (Whitesell et al., 2019). The 3xTg-AD mice, particularly females, show deficits in odour detection (Roddick et al., 2016) and in finding buried food (Mitrano et al., 2021), and also show amyloid deposits in the olfactory pathways, which are more severe in females than males (Oh et al., 2010). On the other hand, the 5xFAD mice show no deficits in odour detection or olfactory working memory in go/no-go or delayed matching-to-sample tasks in the operant olfactometer at six months of age (Roberts et al., 2020; Roddick et al., 2014, 2016) and, despite significant Aβ pathology in the olfactory bulb, hippocampus, amygdala, and piriform cortex, 5xFAD mice between nine and 15-months of age did not show age-related deficits in olfactory memory in a classical conditioned odour preference task (O'Leary, Stover, et al., 2020). An olfactory working memory capacity test, using a non-matching to sample digging paradigm, did however show diminished performance in three month

old male 5xFAD mice (Jiang et al., 2022). A hippocampal based olfactory maze test found impaired performance in four and six month old 5xFAD mice, but not two month old mice (Girard et al., 2014). Interestingly, this study found better performance among the 5xFAD mice than the wildtype mice on a reversal trial. This reversal was performed after a two week break from testing, and could indicate that the wildtype mice remembered the original training better than the 5xFAD mice.

In a habituation-dishabituation test, 3xTg-AD mice showed no differences from control using either social nor non-social odours at six months old (Nguyen et al., 2020). Twelve month old 3xTg-AD mice showed no impairment in a buried food test, interestingly, this study also found that the male 3xTg-AD mice were quicker to find the buried food than the female 3xTg-AD mice at 26, 39, and 52 weeks, but not 13 weeks (Mitrano et al., 2021).

In a social transmission of food preference test, 3xTg-AD mice at 18 months old show decreased social transfer of food preference, but no decrease in neophobia (Cassano et al., 2011). In an olfactory cross-habituation test, eight month old, but not four month old 5xFAD mice showed deficits (Mariani et al., 2017). Both APP/PS1 mice and 3xTg-AD mice show more errors in an olfactory based cookie finding test in an eight arm radial maze, and greater latency in a buried food test at three to five months of age (M. Chen et al., 2021).

P301S tau mice, expressing mutant human tau (Allen et al., 2002), showed decreased olfactory sensitivity in an ascending odour investigation task, at three months old for non-social odours, and four months old for social odours (S. Yang et al., 2016). P301L tau mice also show olfactory deficits at six months old in a buried cookie test and an odour habituation test (Hu et al., 2016). The P301L mice also show decreased levels of nitric oxide in their olfactory bulbs (Hu et al., 2016), and the nitric oxide signaling pathway has been linked to olfactory memory formation (Okere et al., 1996). Mice expressing human ApoE4 showed decreased olfactory habituation compared to mice expressing human ApoE3 at six months old, but did not show a difference at 12 months old (Peng et al., 2017). Female 5xFAD mice spend less time investigating social, but not non-social, odours in a habituation-dishabituation test at 12 months old (Kosel et al., 2019).

The 5xFAD mice showed increased latency to find a food pellet at six, but not three, months old in two olfactory based tasks, a buried food task and an olfactory maze task (Xiao et al., 2015). Meanwhile, the Tg-SwDI mouse, with the Swedish, Dutch, and Iowa APP mutations, showed no deficit in the buried food test up to 12 months old (Setti et al., 2022).

Phillips et al. (2011) tested both the APPswe/PSEN1ΔE9 amyloid and the htau tau models of AD mice, expressing Aβ and tau pathology respectively, from six to 18 months of age on a variety of tasks in an operant olfactometer. There was no olfactory sensitivity deficit in either model; both were able to easily detect 0.01ppm n-hexanal up

to 15 months of age, but not at 18 months, when the wildtype mice also failed to detect the hexanal. On a variety of two odour discrimination tasks and learning transfer tasks, there were no differences between the performance of either model and the wildtype mice, however, both models showed slower learning on a reversal task from seven to 15 months of age (Phillips et al., 2011).

APPswe/PSEN1ΔE9 mice at 12-13 months of age show lower responsivity than the wildtype mice in the local field potentials of the olfactory bulbs in response to odours, and a decrease in the coherence of the spontaneous activity of the two bulbs (Liu et al., 2013). AβPPswe/PS1E9 show soluble Aβ in the olfactory epithelium at one to two months of age, and in the olfactory bulbs by three to four months of age. This is accompanied by decreasing levels of olfactory marker protein in the olfactory epithelium and degeneration of olfactory sensory neurons, a decline in olfactory ability, as assessed with a habituation-dishabituation test, starting at three months old, and increased amyloid load in olfactory cortices, such as the anterior olfactory nucleus and piriform cortex, by three to four months of age (Wu et al., 2013b).

Tg2576 mice at 5.5-6 months old showed habituation on an olfactory habituation task when the odours were presented with a five minute intertrial interval, but not when the intertrial interval was 15 minutes (Guérin et al., 2009). However, another study found that while Tg2576 mice were impaired on an olfactory habituation task, they had normal performance on an odour discrimination task, and showed normal electrophysiological

responses to odours in the piriform cortex from three to 12 months old (Xu et al., 2014).

1.6 Measures Of Olfactory Learning And Memory

Olfaction plays a pivotal role in directing the behaviour of rodents, with mice having up to 1500 genes that encode olfactory receptors (Young & Trask, 2002). Rats are able to learn complex olfactory tasks, showing learning to learn (Slotnick & Katz, 1974) and will preferentially attend to an olfactory stimuli over an auditory or visual stimulus (Nigrosh et al., 1975).

1.6.1 Discrimination Learning

Discrimination learning involves training the animal to discriminate between two stimuli. Responding can be done as either a forced choice task, where the animals must choose one of two or more responses when presented with a stimulus, or as was the case in the studies included in this thesis, as a go / no-go task, where the animal must learn to respond to one stimulus, and inhibit responding to another (Figure 1-2). In a go / no-go task one odour will be the rewarded stimulus (S+), and the other will be the unrewarded stimulus (S-). Animals are commonly trained either until they reach a performance criterion, such as 85% accuracy, or for a set number of trials. In the

operant olfactometer, two odour discrimination tasks are often used to measure learning and memory (Slotnick & Restrepo, 2005).

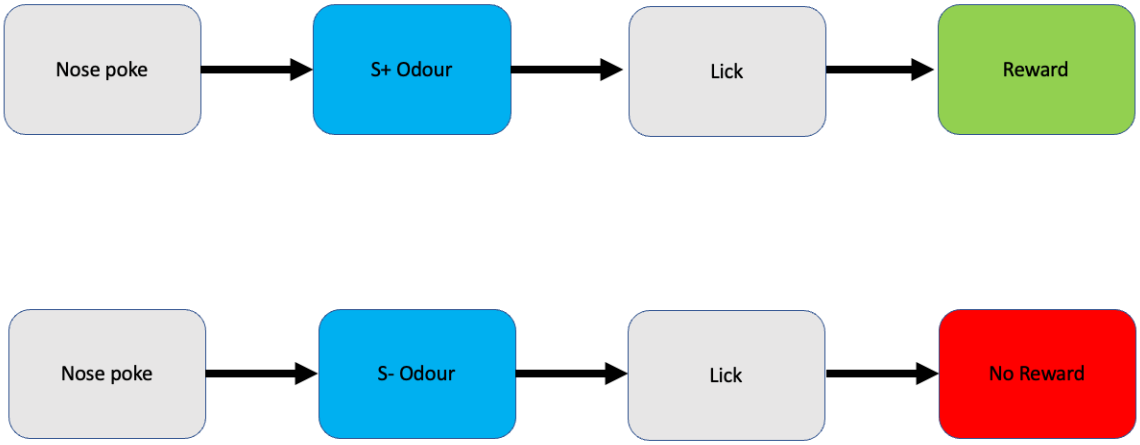

Figure 1-2: Design of a go / no-go two odour discrimination task. The trial is initiated by a nose poke from the mouse, which leads to the presentation of one of two odours, followed by a response window. When the odour presented is the S+, licking during the response window leads to a reward. When the odour presented is the S-, licking during the response window does not lead to a reward.

Examining the effects of age on olfactory performance, Patel and Larson (2009) found that 24 month old C57BL/6J mice were slower to learn an olfactory discrimination task than four month old mice, making approximately 70% more errors before reaching criterion.

1.6.2 Reversal Learning

Reversal tasks can be performed in a variety of apparati, from the Morris Water Maze (Vorhees & Williams, 2006), to visual discrimination tasks (Chudasama & Robbins, 2003), as well as on olfactory guided digging tasks (Mihalick, Langlois, Krienke, et al., 2000). There is some evidence of impaired reversal learning in AD mouse models. Tg2576 mice, which over-express human APP with the Swedish familiar AD mutation, show impaired reversal learning at six months of age (Zhuo et al., 2007, 2008). Interestingly, this study found no difference between Tg2576 mice and wildtype mice at 14 months old as the performance of both genotypes had deteriorated greatly, indicating an age dependent decline in reversal learning (Zhuo et al., 2007). $APP^{NL\text{-}F/NL\text{-}F}$ mice, which develop an increased ratio of $A\beta_{42}$:$A\beta_{40}$, also showed impaired reversal learning on the Morris Water maze (Shah et al., 2018), while APPPS1-21 mice show impaired reversal in a visual discrimination task (Van den Broeck et al., 2019). Juvenile mice have also been shown to be better at an odour guided digging reversal task than adult mice (Johnson & Wilbrecht, 2011).

Reversal learning in the operant olfactometer involves changing the values of stimuli the mouse has previously learned. Thus, after learning that stimulus A was a rewarded stimulus and stimulus B was an unrewarded stimulus in a two odour discrimination task, the mouse is presented with the reversal task when stimulus A is now unrewarded while stimulus B is now the rewarded stimulus.

1.6.3 Working Memory And Delayed Matching To Sample Tasks

Working memory tasks involve requiring the animal to store a memory of a stimulus or stimuli and use that memory to determine how to respond at a later timepoint. The three main classifications of working memory tasks used with animals are i) goal maintenance, ii) memory capacity, and iii) interference control (Dudchenko et al., 2013). Goal maintenance type tasks include delayed matching and non-matching to sample tasks, as well as delayed win-shift tasks. In these types of tasks, the animal is presented with a stimulus and must remember it over a delay period before making a response that depends upon the stimulus. These tasks can vary the length of the delay period to change the demands on the animal's working memory.

Memory capacity tests involve the animal retaining a memory of multiple stimuli and using it to determine how to respond. An example of this type of task is the olfactory working memory capacity task (G.-D. Huang et al., 2020). In this task the animal is presented with a variable number of odours before being presented with the same odours plus a novel odour and must dig in the novel odour to get a reward. This type of task is able to control for the demand on the animals working memory by varying the number of stimuli initially presented.

Interference control tasks include n-back tasks, where the animal is presented with a series of stimuli and responds when the presented stimuli matches the stimulus presented a number of steps earlier in the series (Ko & Evenden, 2009). These tasks require the animal to continually update the stimuli it is remembering and can increase working memory demands by increasing the number of steps back the animal must respond to.

A commonly used task to assess rodent working memory is spontaneous alternation in either a Y, T, or cross maze. When placed in any of these mazes mice will spontaneously alternate their entries into the arms, going into the arm which they have entered least recently (Lalonde, 2002). This task has the advantage that it relies on the animals natural behaviour and doesn't require the animal to learn how to perform a task. However, both spatial memory (Lalonde, 2002) and anxiety (Bats et al., 2001) have been show to affect spontaneous alternation, and an animal that always goes in one direction (side preference) when entering an arm in the maze would score prefect alternations. In the operant olfactometer, rats are able to perform a go / no-go delayed matching to sample task with a delay of up to 10 seconds (Lu et al., 1993), however, prior to the paper included in this thesis (Roddick et al., 2014) no one had shown that mice were also capable of performing this task. A go / no-go delayed matching to sample task involves presenting the animal with one odour, and following an inter-stimulus delay, a second odour. If the odours are the same the animal will be rewarded for licking, and if the two odours are different the animal will not be rewarded for licking (Figure 1-3).

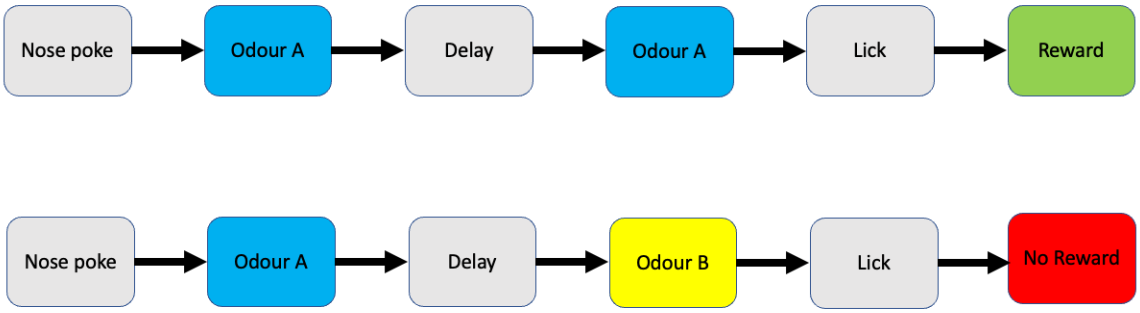

Figure 1-3: Design of a go / no-go olfactory delayed matching to sample task. The trial is initiated by a nose poke from the mouse, which leads to the presentation of the first odours, followed by an inter-stimulus delay then a second odour presentation and a response window. When the two odours presented are the same, licking during the response window leads to a reward. When the two odours presented are different, licking during the response window does not lead to a reward.

1.6.4 Olfactory Sensitivity

Olfactory sensitivity has been examined in mice using operant olfactometers. Using a modification of a two odour discrimination task. One stimulus is the odour on which sensitivity is being assessed, and the other is an odourless substance or clean air. Simple organic compounds are typically used in sensitivity tasks, such as ethyl acetate (Bodyak & Slotnick, 1999), octyl aldehyde (Slotnick & Restrepo, 2005), or n-hexanal (Phillips et al., 2011), with varying thresholds for the different compounds.

A study of the olfactory detection thresholds of CD-1 mice to aldehydes with four to nine carbon chains found that the thresholds varied, but there was no relationship between the number of carbons and the detection thresholds (Laska et al., 2006). Similarly, no relationship was found between the number of carbons on alcohols and the olfactory detection threshold of C57BL/6J mice (Williams & Dewan, 2020).

Olfactory sensitivity can also be affected by normal aging. Twenty-four month old C57BL/6J mice showed a higher detection threshold for ethyl acetate than 4 month old mice on a sensitivity test (Patel & Larson, 2009). Similarly, while testing two strains of AD mice, Phillips et al (2011) found that the wildtype, C57Bl/6J, mice showed a higher detection threshold for n-hexanal at 18 months old compared to 15 month old and younger mice.

1.7 This Thesis

This thesis presents a series of papers using the operant olfactometer to evaluate the learning, memory, and olfactory function of two mouse models of AD. As olfactory dysfunction is an early symptom of AD, and mice show a remarkable ability to learn olfactory tasks compared to tasks which rely on other sensory modalities, it is important to assess rodent AD models on olfactory based tasks. The experiments in these papers consider three variables, the genotypes, the sexes, and the ages of the mice.

The first paper presented is an assessment of the olfactory sensitivity of the 5xFAD and 3xTg-AD models of AD at six months of age. The second paper is an assessment of the olfactory sensitivity of 5xFAD mice at 12 months of age. The final paper presented is an evaluation of the working memory of 5xFAD mice using an olfactory delayed matching to sample task

2 Sex And Genotype Differences In Odor Detection In The 3×Tg-AD And 5XFAD Mouse Models Of Alzheimer's Disease At 6 Months Of Age

While reports are mixed, olfactory detection deficits have been reported in human AD patients (Djordjevic et al., 2008; Doty et al., 1987; Murphy et al., 1990). The aim of this study was to evaluate the 3xTg-AD and 5xFAD mouse models of AD on their ability to detect an odour at a low concentrations, and assess whether there are any differences between the transgenic mice and their wildtype controls. As both models show cognitive deficits as early as four months of age (Girard et al., 2013; Webster et al., 2014), it was hypothesised that deficits would be seen in both models. While deficits were found in the female 3xTg-AD mice, no deficits were found in the male 3xTg-AD mice, nor the 5xFAD mice.

The manuscript below was published in *Chemical Senses*, see Appendix B for the copyright permission letter from the publisher. I collaborated in the design of the study, collected much of the data, analysed the results, wrote the initial draft of the paper, incorporated feedback from the coauthors, and responded to the peer review upon submission of the paper.

Roddick, K. M., Roberts, A. D., Schellinck, H. M., & Brown, R. E. (2016). Sex and Genotype Differences in Odor Detection in the 3×Tg-AD and 5XFAD Mouse Models of Alzheimer's Disease at 6 Months of Age. *Chemical Senses*, *41*(5), 433–440. https://doi.org/10.1093/chemse/bjw018

2.1 Abstract

Deficits in odor identification and detection are early symptoms of Alzheimer's disease. Two transgenic mouse models of Alzheimer's disease, the 5XFAD and the 3xTg-AD mice and their wildtype controls, were assessed for olfactory detection with decreasing concentrations of ethyl acetate in a go no-go operant olfactometer task at six months of age. For both the 5XFAD and their B6SJLF1 wildtype littermates, females made fewer errors in detecting the ethyl acetate than males on all but the lowest odor concentrations. Female 5XFAD mice performed slightly better than their female wildtype littermates on the higher odor concentrations, though not at the lowest concentration. The 3xTg-AD females showed decreased olfactory detection compared to their wildtype B6129S1 controls, while there was no difference in the males. Therefore, while the 5XFAD mice showed no olfactory detection deficits, female 3xTg-AD mice had impaired olfactory detection at low odor concentrations but males did not. This difference in odor detection should be considered in studies of olfactory learning and memory, as differences in performance may be due to sensory rather than cognitive factors, though detection seems unimpaired at high odor concentrations.

2.2 Introduction

Olfactory dysfunction is an early symptom of Alzheimer's Disease (AD) and other neurodegenerative diseases and it has been proposed that olfactory deficits could be used as an early test for diagnosing AD (Schofield et al., 2012; Stamps et al., 2013). However, these deficits could arise from impairments in two different aspects of olfactory function: odor identification and odor detection. While odor identification deficits have been widely reported in AD patients (Rahayel et al., 2012; Ruan et al., 2012; Schofield et al., 2012), there are fewer studies on olfactory detection deficits in AD. Stamps et al. (2013) reported that AD patients had impaired odor detection in their left nostril compared to their right nostril, however Doty et al. (2014) were unable to replicate this finding. Testing for odor detection is important because the failure of odor perception provides information about the integrity of the olfactory pathways (Masurkar & Devanand, 2014). Patients with AD and/or mild cognitive impairment display atrophy of the olfactory bulb and olfactory tract as detected by MRI (Thomann et al., 2009). AD patients also show different fMRI patterns to odor concentrations than age matched controls (Wang et al., 2010).

An ever-increasing number of genetically modified mouse models of AD are being developed, with the Jackson Laboratory listing over 90 mouse models of AD in their database (www.jax.org). While no mouse model exactly replicates the symptoms of

human AD, some models have higher validity than others (Webster et al., 2014). A number of olfactory related-deficits have been reported in mouse models of AD (Masurkar & Devanand, 2014). Deficits in olfactory learning and memory have been shown in Tg2576, APP/PS1, 3xTg-AD, and 5XFAD mouse models of AD (Masurkar & Devanand, 2014). The APP/PS1 (Wu et al., 2013a) and Tg2576 (Wesson et al., 2011, 2013) mice show reduced odor detection in a habituation-dishabituation task, while Tg2576 mice show normal odor detection in a buried food task (Deacon et al., 2009). Because the buried food and habituation-dishabituation tasks provide only crude measures of odor detection ability, and failure to perform in these tests does not necessarily indicate failure to detect odors, operant olfactometer methods have been used to provide more reliable and valid procedures for determining odor sensitivity (see Schellinck et al., 1991; Schellinck & Brown, 1999). Using an automated olfactometer, the sensitivity of a number of non-transgenic mouse strains for odor detection has been assessed. Adult male C57BL/6J mice detected 0.01 ppm n-hexanal (Phillips et al., 2011); adult male CF-1 mice detected 0.00005 % ethyl acetate (Bodyak & Slotnick, 1999); and 2 to 8 month old male and female mice of unstated strain(s) detected 0.00001 % n-octanal (Slotnick & Restrepo, 2005). C57BL/6J mice up to 15 months old could detect 0.01 ppm n-hexanal, but 18 month old mice could not (Slotnick & Restrepo, 2005).

While the 3xTg-AD mouse develops both amyloid and tau pathology, the 5XFAD mouse develops only amyloid pathology. If olfactory dysfunction is a feature of AD, then one might expect to see age-related olfactory dysfunction in these mouse models; however,

the data from the published studies is difficult to interpret because it cannot be determined whether the deficits in olfactory-guided behavior are related to olfactory perception or cognitive performance. In a social transmission of food preference test, 18 month old 3xTg-AD mice displayed reduced food preference compared to control mice following social interactions, but the 3xTg-AD mice did not differ from controls in their ability to detect buried food, suggesting that they had no olfactory deficit (Cassano et al., 2011). However, female 3xTg-AD mice could discriminate food-related odors at 4 to 5 months of age, but not at 10 to 18 months of age in a habituation-dishabituation task (Coronas-Sámano et al., 2014), suggesting that they did have an olfactory deficit. At 4 and 6 months of age, 5XFAD mice had poorer performance on an olfactory H maze task than wildtype littermates (Girard et al., 2013), suggesting an olfactory deficit, but with an increased number of training trials in a different olfactory tubing maze task, 2 to 6 month old 5XFAD mice showed no such deficits (Girard et al., 2014), indicating that the 5XFAD mice were able to detect the odors and that the impaired performance was due to a cognitive deficit.

The 3xTg-AD and 5XFAD mouse models of AD have not been tested for odor detection, thus the purpose of the present study was to use automated olfactometers to evaluate the ability of male and female 3xTg-AD and 5XFAD mice and their wildtype controls to detect decreasing concentrations of ethyl acetate. We hypothesized that, if the transgenic mice had a deficit in odor perception, they would show impaired odor detection and their performance would decrease at low concentrations. Because we

previously found that female 5XFAD mice performed better than males on olfactory delayed-matching-to-sample learning (Roddick et al., 2014), we also hypothesized that there would be a sex difference in odor detection. As it has been reported that cognitive deficits begin at 4 to 6 months of age in both the 5XFAD (Girard et al., 2013, 2014) and 3x-TgAD mice (Webster et al., 2014), we tested our mice at 6 months of age.

2.3 Materials And Methods

2.3.1 Animals

All mice were obtained from a colony bred at Dalhousie University from mice purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were weaned at 22 days of age, separated into groups of 2–4 same sex littermates and housed in 30 × 18 × 12 cm polycarbonate cages with wire tops and *ad lib* access to food (Purina Rodent laboratory chow #5001).

The 3xTg-AD mice have three mutations, the Swedish (K670N/M671L) mutation to APP, a mutation to PS1 (M146V), and a tau mutation (P301L) (Oddo et al., 2003). Unlike the 5XFAD mice, where wildtype littermates were used as controls, the 3xTg-AD mice and their B6129S/F2 controls were bred separately (Blaney et al., 2013). The 5XFAD mice have five mutations; three on the APP gene, the Swedish (K670N/M671L), Florida

(I716V) and London (V717I) mutations, and two mutations to PS1 (M146L and L286V) (Oakley et al., 2006).

We tested 3xTg-AD (B6;129-Psen1^{tm1Mpm} Tg(APPSwe,tauP301L)1Lfa/Mmjax; JAX stock number 004807) (7 females and 5 males from 6 litters) and B6129SF2/J (JAX stock number 101045) control mice (8 females and 6 males from 5 litters) and 5XFAD mice (B6SJL-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas/Mmjax; JAX stock number 006554) (5 females, 6 males from 6 litters) and their wildtype (B6SJLF1/J stock number 100012) littermates (4 females and 9 males from 6 litters) when they reached 6 months of age.

Ten days prior to the start of testing, the mice were individually housed, water deprived and fed with a mash of powdered rodent chow mixed with a measured amount of water. While on water restriction, the mice were weighed daily and the amount of water given in their mash adjusted to maintain their body weight at 80–85% of free feeding weight. As mice learned to respond in the olfactometer and received increasing amounts of water reward, the level of water restriction was decreased by gradually increasing the amount of water in their mash. All animal protocols adhered to the Canadian Council on Animal Care guidelines and were approved by the University Committee on Laboratory Animals (protocol #s 11-033 and 13-044).

2.3.2 Apparatus

Two computer-controlled eight-channel liquid dilution olfactometers (Knosys Olfactometers Inc., Lutz, FL), based on those described by (Slotnick & Restrepo, 2005), were used. Air from a compressor was sent through a filter after which it was split into two pathways, one of which flowed through rubber tubing into a glass manifold as clean air, and the other flowed through a second glass manifold, which controlled the air flow through odor saturation bottles and into a glass T-junction, where clean and odorized air flows converged. The two outflows of the T-junction were controlled by a final valve, which directed the airflow to the odor sampling port, or to the exhaust. The odor sampling port, which opened into the animal chamber, contained a reinforcement tube delivering the water reward, and a sensor that detected when the mice were licking this tube.

2.3.3 Odors

Ethyl acetate was used as the odorant as it has commonly been used in olfactory detection tasks in rodents (Bodyak & Slotnick, 1999; Kraemer & Apfelbach, 2004; Larson et al., 2003; Patel & Larson, 2010; Slotnick & Restrepo, 2005). The concentrations of ethyl acetate used in the odor solutions were 6.3×10^{-6}, 5.6×10^{-7}, 4.9×10^{-8}, 4.4×10^{-9}, 3.9×10^{-10}, and 3.4×10^{-11}M. These concentrations result in vapor concentrations of ethyl

acetate in the head spaces of the odorant bottles of 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 ppm respectively. The vapor concentration presented to the mice in the odor sampling port was approximately 5% of the concentration in the head space of the odorant bottles (Slotnick & Restrepo, 2005). All odors were diluted with heavy mineral oil.

2.3.4 Behavioral Testing

The mice were first trained on a simple odor discrimination program to learn the procedure for receiving water reward from the olfactometer. They were initially rewarded for simply licking the reinforcement tube and were then required to keep their head inside the odor sampling port for 1 sec before being rewarded. During the testing phase the mice were presented with a stimulus odor when they inserted their head into the odor sampling port, either a rewarded stimulus (S+) consisting of air pumped through the ethyl acetate odorant bottle, or an unrewarded stimulus (S-) of air pumped through an odorant bottle containing mineral oil. When the mice were presented with the S+, they were rewarded with water for licking the reinforcement tube; no reward was provided for licking when the S- was presented. Four types of responses were possible: hits, false alarms, correct rejections, and misses. The mice were presented first with the highest concentration of ethyl acetate (1ppm) as the S+ and given 5 blocks of 20 trials, each consisting of 10 S+ trials and 10 S- trials presented in a random order, for a total of 100 trials at this concentration. They were then tested on

the remaining concentrations in descending order. They received 100 trials over 5 blocks on each odor concentration except for the lowest concentration (0.00001ppm) on which they received 200 trials over 10 blocks. The result of each trial the mice completed was individually recorded for analysis. The criterion for learning the discrimination was 85% correct on one or more of the five blocks of 20 trials.

2.3.5 Statistical Analyses

All statistical analyses were performed using the statistical program R (www.R-project.com). The data were treated as binomial data with the result of each individual trial, scored as either correct or incorrect, analyzed. Backward stepwise regression of generalized linear models with model selection based on Akaike Information Criterion (AIC) and bootstrapped confidence intervals were used to examine the results. Separate analyses were done for the 3xTg-AD and 5XFAD mice, using genotype, sex, ethyl acetate concentration, and testing block as fixed effects and mouse as a random effect (to account for individual differences). Models were also run examining only the lowest ethyl acetate concentration, using genotype, sex, and testing block as fixed effects and mouse as a random effect. Bootstrapped confidence intervals of the difference in mean score between groups of mice were used to examine effects found in the models. Mean accuracy scores for each odor concentration for each group of animals were calculated (number of correct trials / number of total trials * 100%) and confidence intervals of the means were bootstrapped for use in figures.

2.4 Results

2.4.1 Odor Detection Criterion

Figure 2-1 shows the percentage of correct responses for each mouse on the block with their highest performance for each odor concentration, and Figure 2-2 shows the percent of mice in each group that reached criterion on at least one block for each odor concentration. Differences in the percent of mice that reached the criterion of 85% correct on at least one block of trials for each odor concentration were analyzed with backward stepwise regression of generalized linear models.

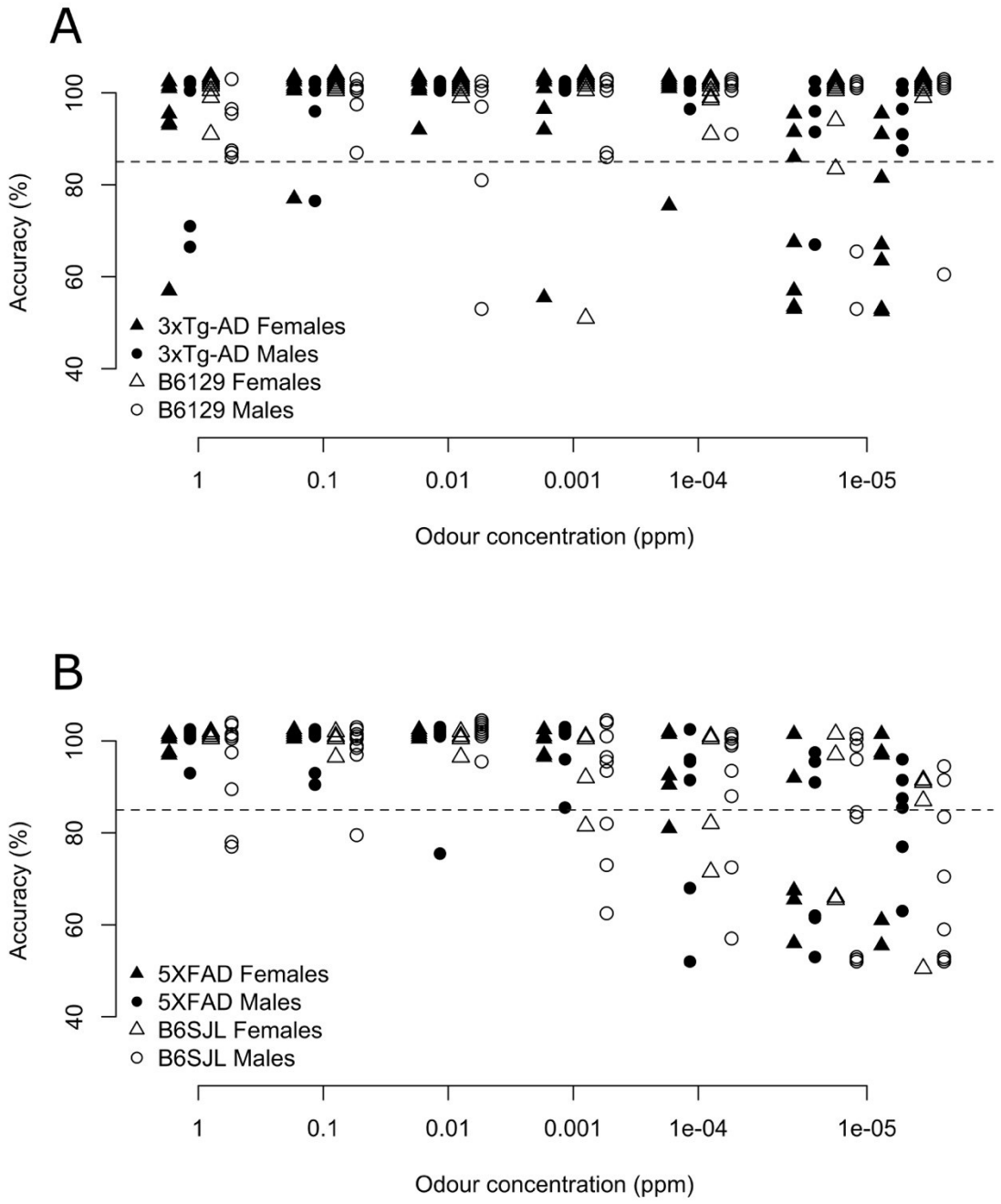

Figure 2-1: The accuracy (percent correct) for each individual female and male 3xTg-AD mouse (A) and 5XFAD mouse (B) and their wildtype controls on their highest scoring block of 20 trials for each concentration of ethyl acetate tested.

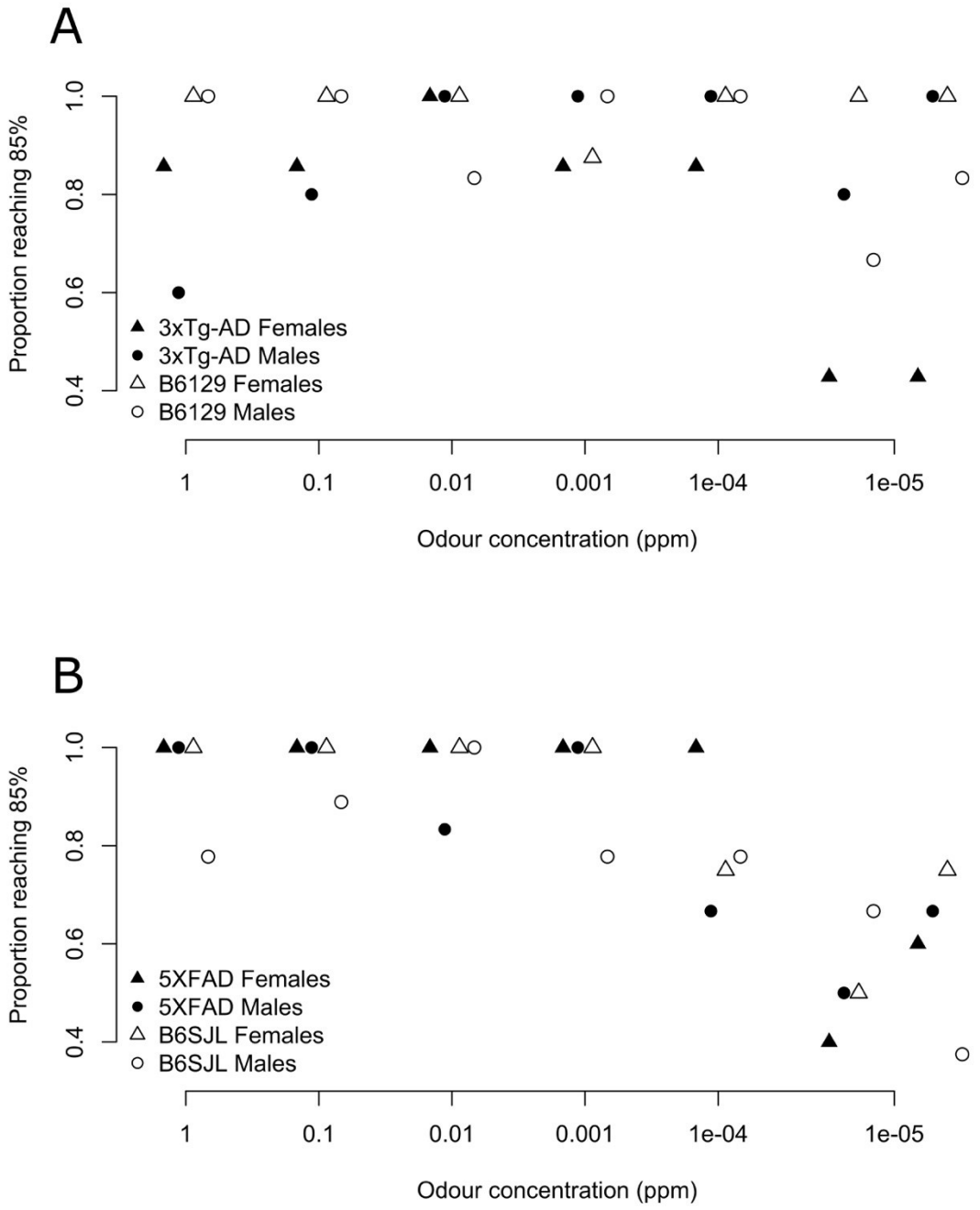

Figure 2-2: The proportion of female and male 3xTg-AD mouse (A) and 5XFAD mouse (B) and their wildtype controls in each group which reached the threshold of 85% correct on their highest scoring block on each concentration of ethyl acetate tested.

For the 3xTg-AD mice (Figure 2-2A) the best fitting model included fixed effects for sex, genotype, and ethyl acetate concentration, and sex by genotype, sex by ethyl acetate concentration, and genotype by ethyl acetate concentration interactions, with mouse as a random variable (AIC = 136.4). This model was significantly different from the null model of a random effect of mouse (χ^2_8 = 20.11, p = 0.00265). There were significant effects of genotype (p = 0.0133), sex (p = 0.0278), and significant sex by genotype (p = 0.0089) and sex by ethyl acetate concentration (p = 0.0438) interactions.

Overall, wildtype mice (92%) reach criterion significantly more often than 3xTg-AD mice (79%) (p = 0.0133), and male mice (88%) reach criterion significantly more often than female mice (86%) (p = 0.0438). However, as indicated by the sex by genotype interaction, while the wildtype females performed significantly better than 3xTg-AD females (CI^{95} of the difference: 10.5% to 36.7%); there was no genotype effect in males (CI^{95} of the difference: -14.8% to 13.8%). Additionally, while the male mice reached criterion more often than the female mice on the lowest three lowest odor concentrations, the females reached criterion more often than males on the three highest odor concentrations, explaining the sex by concentration interaction (p = 0.0438).

For the 5XFAD mice (Figure 2-2B), the best fitting model included fixed effects for sex, genotype, ethyl acetate concentration, and sex by ethyl acetate concentration, and

genotype by ethyl acetate concentration interactions, with mouse as a random variable (AIC = 162.35). This model was significantly different from the null model of a random effect of mouse (χ^2_7 = 29.77, p < 0.00002). There was a significant effect of ethyl acetate concentration (p < 0.00001) and a significant interaction between sex and ethyl acetate concentration (p < 0.00001). Virtually all 5XFAD and their wildtype control mice were above the criterion of 85% correct at the four highest odor concentrations (1, 0.1, 0.01, and 0.001 ppm). However, the proportion of the 5XFAD mice reaching criterion did not differ from that of the wildtype mice at any odor concentration. As the concentration of ethyl acetate decreased, significantly fewer mice reached criterion (p < 0.00001), but significantly more females than males reached criterion at some concentrations (p < 0.00001) (Figure 2-1B).

2.4.2 Trials To Learn The Discrimination At Each Odor Concentration

For the 3xTg-AD mice, the best model had fixed effects for sex, genotype, ethyl acetate concentration, and trial block, as well as sex by genotype, sex by ethyl acetate concentration, sex by trial block, genotype by trial block, ethyl acetate concentration by trial block, sex by genotype by trial block, and sex by ethyl acetate concentration by trial block interactions, with mouse as a random effect (AIC = 14601). This model differed significantly from the null model of a random effect of mouse (χ^2_{11} = 574.96, p < 0.00001). There were significant effects of ethyl acetate concentration (p < 0.00001), trial block (p < 0.00001), sex by trial block interaction (p < 0.00001), genotype by trial

block interaction ($p < 0.00001$), odor concentration by trial block interaction ($p < 0.00001$), sex by genotype by trial block interaction ($p < 0.00001$), and sex by odor concentration by trial block interaction ($p < 0.0035$).

The 3x-TgAD mice had within-session learning curves, shown by the effect of trial block, and also between-session learning curves, shown by the effect of ethyl acetate concentration (Figure 2-2A). The odor concentration by trial block interaction indicates that the within-session learning curves differed across odor concentrations, while the sex by trial block and genotype by trial block interactions show that male and female mice, and transgenic and wildtype mice had different within-session learning curves. On the highest four ethyl acetate concentrations presented, female wildtype mice were significantly more accurate than male wildtype mice (CI^{95} of the difference: 5.2% to 11.7%). There was no sex difference in the performance of the 3xTg-AD mice (CI^{95} of the difference: -6.0% to 1.8%).

Examining performance of the 3xTg-AD mice on the lowest ethyl acetate concentration found that the wildtype mice were more accurate than the 3xTg-AD mice ($p = 0.00036$), and that there were significant sex by genotype ($p = 0.031$), sex by trial block ($p = 0.00064$), and genotype by block ($p = 0.00038$) interactions. At the lowest concentration the transgenic males were significantly more accurate than the transgenic females (CI^{95} of the difference: 16.7% to 27.1%), and the wildtype females were significantly more accurate than the wildtype males (CI^{95} of the difference: 2.7% to 14.5%). While the

wildtype males were no more accurate than the transgenic males at the lowest concentration (CI^{95} of the difference: -6.3% to 2.7%), the female wildtype mice were more accurate than the transgenic females (CI^{95} of the difference 15.6% to 24.3%) (Figure 2-3A).

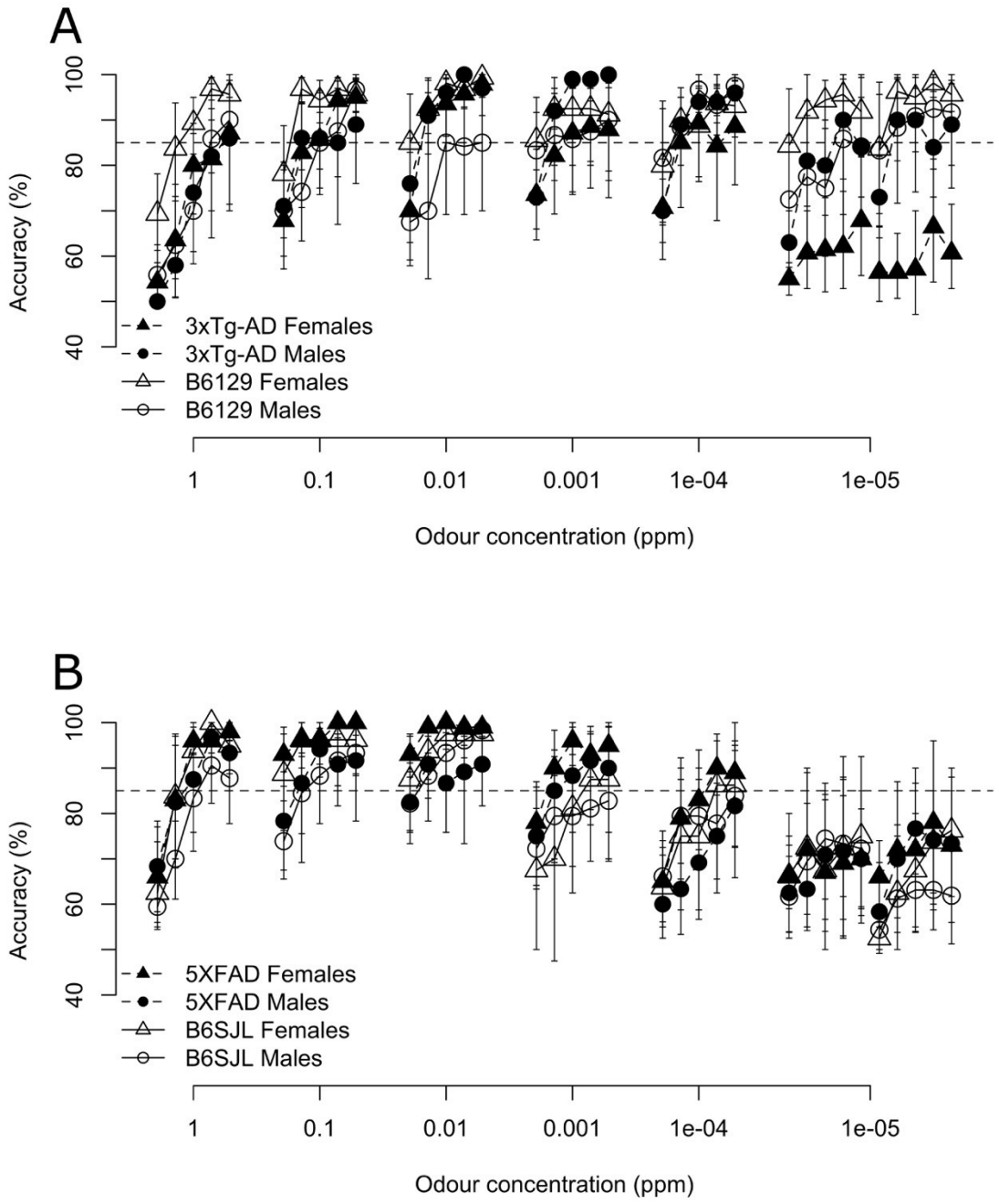

Figure 2-3: Mean accuracy (± 95% CI) of the female and male 3xTg-AD mice (A) and 5XFAD mice (B) and their wildtype controls on each concentration of ethyl acetate

tested. The mice received 5 blocks of 20 trials on each concentration except for the lowest, 10-5 ppm, on which they received 10 blocks of 20 trials.

For the 5XFAD mice, the best model of the data had fixed effects for genotype, sex, ethyl acetate concentration, and trial block, and genotype by sex, genotype by concentration, genotype by trial block, sex by odor concentration, sex by trial block, concentration by trial block, genotype by sex by odor concentration, genotype by sex by block, and sex by odor concentration by block interactions, and with mouse as a random effect (AIC = 15871). This model was significantly different from the null model of a random effect of mouse (χ^2_{13} = 347.57, $p < 0.00001$). The effects of sex ($p = 0.018$), ethyl acetate concentration ($p < 0.00001$), trial block ($p = 0.00087$), sex by odor concentration ($p = 0.000054$), sex by trial block ($p = 0.012$), odor concentration by trial block ($p < 0.00001$), genotype by sex by odor concentration ($p = 0.0018$), genotype by sex by block ($p = 0.020$), and sex by odor concentration by block ($p = 0.000046$) were significant.

The 5XFAD mice showed within-session learning curves, as indicated by the effect of trial block; and between-session learning curves, as indicated by the effect of ethyl acetate concentration (Figure 2-3B). The odor concentration by trial block interaction indicates that the within-session learning curve changed across odor concentrations, while the sex by trial block interaction suggests that male and female mice showed different within-session learning curves. On the highest four ethyl acetate concentrations, females were significantly more accurate than males in both the 5XFAD

(CI^{95} of the difference: 3.8% to 10.8%) and wildtype mice (CI^{95} of the difference: 0.2% to 7.8%). However, this sex difference was not present on the lowest concentration of ethyl acetate (CI^{95} of the difference: -2.0% to 7.1%). The 5XFAD female mice were significantly more accurate than wildtype females on the highest four ethyl acetate concentrations (CI^{95} of the difference: 0.5% to 8.3%), but not on the lowest concentration (CI^{95} of the difference: -5.3% to 9.2%). There was no difference between male 5XFAD and wildtype mice on either the highest four ethyl acetate concentrations (CI^{95} of the difference: -2.5% to 4.4%), or the lowest concentration (CI^{95} of the difference: -2.1% to 8.6%).

2.5 Discussion

The 3xTg-AD mice, both males and females, reached criterion at the highest five odor concentrations and performed as well as their wildtype controls (Figure 2-1A and Figure 2-2A). However, at the lowest concentration fewer female 3xTg-AD mice were able to reach criterion than wildtype females, indicating that they may have some olfactory dysfunction. The learning curves (Figure 2-2A) indicate that the 3xTg-AD and wildtype control mice could learn the task. While the 3xTg-AD mice did not differ in accuracy from their wildtype controls when performance on the highest odor concentrations was analyzed, the female 3xTg-AD mice showed an impairment on the lowest concentration of ethyl acetate.

The majority of the 5XFAD mice reached criterion on the highest concentration of ethyl acetate and performance declined on the lowest two concentrations, even though some individual mice, both 5XFAD and wildtype, did reach criterion on the lowest odor concentration (Figure 2-1B). These results indicate that the 5XFAD mice have no olfactory deficits compared to their wildtype controls. The learning curves (Figure 2-3B) show that both the transgenic and wildtype 5XFAD females performed better than their male counterparts at high odor concentrations but this difference was not present at the lowest odor concentration. This suggests that this sex difference in performance is not associated with differences in sensitivity, but with other factors such as learning, attention, or strategies used to complete the task. The lack of any significant difference between the transgenic and wildtype 5XFAD mice suggests that this model of AD does not display olfactory detection deficits at this age.

The results of the present experiment show that both the 3xTg-AD and 5XFAD mouse models of AD and their wildtype controls can detect 0.0001 ppm ethyl acetate. Male 3xTg-AD mice can detect 0.00001 ppm, but females cannot. Some 5XFAD mice of both sexes, but not all, can detect 0.00001 ppm ethyl acetate. Based on our previous results (Roddick et al., 2014) we predicted that 5XFAD females would have better olfactory detection than males, but no such sex difference was found in this study. A sex difference was, however found in 3xTg-AD mice in which females had poorer olfactory detection than males at the lowest odor concentration.

Although our data show that neither the 3xTg-AD nor the 5XFAD mice have deficits in olfactory detection at higher odor concentrations, the 3xTg-AD mice appear to have a better olfactory ability than the 5XFAD mice at the lowest concentrations used. Whereas the male 3xTg-AD mice and their male and female wildtype controls (Figure 2-3B) reached criterion at the lowest odor concentration, the 3xTg-AD females failed to reach criterion. Both the 5XFAD mice and their wildtype controls failed to reach criterion at the lowest odor concentration. The mice displayed both within and between session learning curves, indicating that this task involves both short term working memory within days and long term memory between days. All of the mice showed an increase in accuracy as they progressed through the trial blocks at each different odor concentration, but showed decreasing ability to detect ethyl acetate as the concentration decreased. These findings indicate that the task became increasingly difficult for the mice as the concentration of ethyl acetate decreased, and that when presented with a new concentration they needed to relearn to either respond to, or attend to this new odor concentration, even though they had already learned to respond at the prior, higher, concentration.

As all groups of mice were able to reach at least 85% accuracy on the highest four odor concentrations, it appears that, at least at these concentrations, any deficits seen in studies of learning and memory are cognitive and not sensory deficits. This is in agreement with research on human AD patients, which indicates that there are cognitive deficits in tasks such as odor naming but no deficits in olfactory detection

(Doty et al., 2014; Rahayel et al., 2012; Ruan et al., 2012; Schofield et al., 2012). The finding of olfactory deficits in the 3xTg-AD mouse model but not the 5XFAD mouse model is in agreement with prior research. Coronas-Sámano et al. (2014) found evidence of olfactory deficits in the 3xTg-AD mouse, though not until 8 months of age, while Girard et al. (2014) showed that deficits in the 5XFAD model on an olfactory task were cognitive and not detection deficits. There are several differences between the 3xTg-AD and the 5XFAD mouse models. While both models develop amyloid pathology, only the 3xTg-AD mouse develops tau pathology. The 3xTg-AD mouse also displays symptoms of autoimmune inflammation (Marchese et al., 2014). Additionally, 3xTg-AD mice show a large sex difference in terms of mortality, with the transgenic males dying earlier than the transgenic females (Rae & Brown, 2015). However, none of these deficits explain the sex by genotype differences in olfactory detection in these mice. Studies of olfactory performance in other mouse models of AD also have confounds between sensory and cognitive deficits. In the olfactory habituation – dishabituation task, AβPPswe/PS1E9 mice were unable to discriminate vanilla or ethyl acetate odors at 3 months of age, while wildtype controls showed discrimination of these odors until 6 months old (Wu et al., 2013b) suggesting an olfactory deficit in this mouse model. Tg2576 mice also show impaired habituation at 6-7 months and 16 months, as well as abnormal neural activity in the olfactory system as early as 3-4 months (Wesson et al., 2011, 2013). Phillips et al. (2011) tested B6.Cg-Tg(APPswe,P-SEN1dE9)85Dbo/J and B6.Cg-MAPT$^{tm1(EGFP)Klt}$Tg(-MAPT)8cPdav/J mice, which have amyloid and tau pathology, respectively, from 6 to 18 months of age on olfactory and visuospatial tasks and, while

they found visuospatial deficits in both strains, neither strain displayed olfactory deficits. Martel et al. (2015) tested male THY-Tau22 mice, a model of tau pathology, at 4, 8, and 12 months of age on spontaneous odor exploration and discrimination tasks and on a number of operant olfactometer tasks. Performance decreased with age on both tasks, but the transgenic mice did not show impaired odor discrimination, performing better than the wildtype controls on some tasks. The THY-Tau22 mice did, however, show an increased delay between withdrawing their nose from the odor sampling port and making contact with the water reinforcement tube, suggesting that they had either a motor impairment or reduced motivation in this task.

In summary, the data from this study show that odor detection deficits were only present at low concentrations of ethyl acetate, and then only in the female 3xTg-AD mice, not in male 3xTg-AD mice. Both male and female 5XFAD mice showed a decline in odor detection at 10^{-5} ppm but did not differ from wildtype controls. In order to examine olfactory deficits in other mouse models of AD, the influence of cognitive deficits must be dissociated from the sensory deficits in tests of odor-related behavior.

2.6 Acknowledgments

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada [A7441] to HMS and REB.

3 A Signal Detection Analysis Of Olfactory Learning In 12-Month-Old 5xFAD Mice

While deficits were found in the female 3xTg-AD mice in the first study, no deficits were seen in the 5xFAD model at six months of age. The aim of this study was to evaluate the 5xFAD model at a later age point to see if olfactory sensitivity deficits would emerge as they aged. In this paper additional signal detection measures were calculated to distinguish sensitivity deficits from learning and memory deficits. It was hypothesised that the 5xFAD mice would show both sensory and learning and memory deficits at this advanced age. While learning deficits were found, no sensitivity deficits were found at this advanced age.

The manuscript below was published in the *Journal of Alzheimer's Disease*, see Appendix C for the copyright permission letter from the publisher. I collaborated in the design of the study, collected much of the data, analysed the results, collaborated on the initial draft of the paper, wrote the second draft of the paper, incorporated feedback from the coauthors, and responded to the peer review upon submission of the paper.

Roddick, K. M., Fertan, E., Schellinck, H. M., & Brown, R. E. (2022). A Signal Detection Analysis of Olfactory Learning in 12-Month-Old 5xFAD Mice. *Journal of Alzheimer's Disease*, *88*(1), 37–44. https://doi.org/10.3233/JAD-220049

3.1 Abstract

Although Alzheimer's disease is most often studied in terms of memory impairments, olfactory dysfunction begins in the early stages. We tested olfactory learning, sensitivity and response bias using signal detection methods in 12-month-old male and female 5xFAD mice and their wildtype controls in the operant olfactometer. Odour detection was not reduced in the 5xFAD mice, but learning was, which was worse in female 5xFAD mice than in males. Female mice were more conservative in their response strategy. Signal detection analysis allows us to discriminate between cognitive and sensory deficits of male and female mouse models of AD.

3.2 Introduction

Although the most salient cognitive symptoms of Alzheimer's disease (AD) are learning and memory deficits (Scheltens et al., 2021; Toepper, 2017; Weintraub et al., 2012), one of the earliest symptoms is a decline in olfactory function (Alves et al., 2014; Devanand et al., 2015; Son et al., 2021), which has been shown to be a better predictor of AD onset than loss of verbal episodic memory (Devanand et al., 2015). This predictive property of olfactory dysfunction makes the olfactory pathway a worthy target for diagnosing AD and studying its progression. However, the distinction between healthy aging and AD, in terms of olfactory deficits, is complex (Attems et al., 2015; Devanand,

2016; Doty & Kamath, 2014). Olfactory deficits have been observed in more than half of the population over 65 (Mobley et al., 2014; Ottaviano et al., 2016) including those with mild cognitive impairment, AD, and other types of dementia, along with cognitively healthy individuals (Attems et al., 2015; de Moraes e Silva et al., 2018; Devanand, 2016; Doty & Kamath, 2014; Zou et al., 2016). Age-related decline in odour identification has been correlated with a faster rate of cognitive decline, and reduced volume of the hippocampus, entorhinal, fusiform, and middle temporal cortices (Dintica et al., 2019). Thus, the presence of olfactory learning and identification deficits may be an important feature differentiating AD phenotypes from healthy aging.

Investigating olfactory deficits in mouse models of AD can help us understand the effects of AD related mutations in these mice (Tzeng et al., 2021). Both the Tg2576 and the APP/PS1 mice have olfactory deficits accompanied by amyloid-β (Aβ) pathology in the olfactory pathways (Wesson et al., 2010; Yao et al., 2017). Olfactory deficits have also been reported in tau transgenic mice (Macknin et al., 2004) and in the 3xTg-AD mouse, which carries both Aβ and tau pathology related transgenes (Mitrano et al., 2021; Roddick et al., 2016). On the other hand, the 5xFAD mice showed no deficits in odour detection or olfactory working memory in the operant olfactometer (Roberts et al., 2020; Roddick et al., 2014, 2016) and, despite significant Aβ pathology in the olfactory bulb, 5xFAD mice did not show age-related deficits in olfactory memory in a Pavlovian conditioned odour preference task (O'Leary, Stover, et al., 2020). While the conditioned odour preference task is valuable for testing long-term odour memory

(Wong & Brown, 2007) it does not provide a measure of olfactory learning as is obtained from the operant olfactometer (Roddick et al., 2014, 2016). Therefore, the aim of the present study was to examine olfactory sensitivity and learning in the operant olfactometer in 12-month-old 5xFAD mice. This timepoint was selected as the 5xFAD mice show abnormal whisking behaviour (Flanigan et al., 2014; Grant et al., 2020), impaired hearing (O'Leary et al., 2017), cognitive deficits (Albrecht et al., 2014; Gür, Fertan, Alkins, et al., 2019; O'Leary & Brown, 2022), motor deficits (Jawhar et al., 2012; O'Leary, Mantolino, et al., 2020), and metabolic deficits at this age, leading to increased frailty scores and early death (Gendron et al., 2021; Rae & Brown, 2015).

3.3 Methods

3.3.1 Subjects

Male 5xFAD mice (B6SJLT-Tg (APPSwFlLon, PSEN1*M146L*L286V) 6799Vas/Mmjax) were purchased from Jackson labs (Bar Harbour, Maine; stock #034840) and bred with female B6SJLF1/J mice (Jackson labs; stock #100012), and the offspring were tested. The 5xFAD mouse carries three human APP mutations (Sweden, London, Florida) and two presenilin 1 mutations (M146L and L286V), causing accelerated $A\beta_{42}$ accumulation (Oakley et al., 2006). Five male and 8 female WT, and 5 male and 9 female 5xFAD mice at 12 months of age were tested. Mice were naive to behavioural testing. Pups were weaned at 21 days of age and housed in same sex groups of 2-4 in transparent

polyethylene cages (35 × 12 × 12 cm) with *ad libitum* food (Purina Rodent Chow #5001) and tap water. Housing cages contained pine chip bedding and a polyvinyl chloride tube (5 cm diameter, 8 cm long) for enrichment. The housing room was on a 12:12 hour reversed light/dark cycle. Mice were genotyped for the APP and PS1 transgenes using polymerase chain reaction by Dr. Chris Sinal (Department of Pharmacology, Dalhousie University). All test procedures were approved by the Dalhousie Committee on Animal Care (Protocol #14-059).

3.3.2 Apparatus

Liquid dilution olfactometers (Knosys Olfactometers Inc.) previously described (Roddick et al., 2016; Slotnick & Restrepo, 2005) were used. Air was sent through a filter after which it was split into two pathways, one as clean air, and the other flowed through a manifold which controlled the air flow through saturation bottles and into a T-junction, where clean and odourized air flows converged. The odour sampling port contained an infrared beam to detect nose-pokes, a reinforcement tube delivering the reward, and a sensor that detected when the mice were licking the tube.

3.3.3 Odors

Ethyl acetate was used as the odourant as it has commonly been used in olfactory detection tasks in mice (Bodyak & Slotnick, 1999; Patel & Larson, 2009; Roddick et al., 2016). Vapor concentrations in the odourant bottles used were 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001ppm. The vapor concentration presented to the mice in the odour sampling port was approximately 5% of the concentration in the odourant bottles (Slotnick & Restrepo, 2005). All odours were diluted with heavy mineral oil.

3.3.4 Water Restriction

Ten days prior to the start of testing mice were individually housed and placed on water restriction. Mice were weighed daily and given measured amounts of mash (powdered food pellets mixed with water) to maintain their weight at approximately 85% of free feeding weight.

3.3.5 Behavioral Testing

All behavioural testing was done during the dark phase of the light/dark cycle. The mice were initially trained for 20 trials to lick the reinforcement tube to receive a water reward and were rewarded for simply licking the reinforcement tube. The inter-trial interval increased from 0.1 sec to 12 seconds over the 20 trials. During the next stage of training an odour was introduced and the mice were required to keep their head in the odour sampling port while the final valve diverted the odour into the port. The amount

of time the mice were required to keep their head in the port increased from 0.1 sec to 1.1 sec over 120 trials. This training was completed when the mice performed 20 trials with the final valve on for 1.1 sec. During the testing phase the mice were presented with a stimulus odour when they inserted their head into the odour sampling port, either a rewarded stimulus (S+) consisting of air pumped through the ethyl acetate odourant bottle, or an unrewarded stimulus (S−) of air pumped through an odourant bottle containing mineral oil. When the mice were presented with the S+, they were rewarded for licking the reinforcement tube. Trials were initiated by the mice poking their nose into the odour sampling port, with a minimum inter trial interval of 4s. The mice were presented first with the highest concentration of ethyl acetate (1ppm) and given 5 blocks of 20 trials, each consisting of 10 S+ trials and 10 S− trials presented in a random order. They were then tested on the remaining concentrations in descending order. They received 100 trials over 5 blocks on each odour concentration except for the lowest concentration (0.00001 ppm) on which they received 200 trials over 10 blocks. The mice received 100 trials per day, with the lowest concentration tested over two days. These extra blocks on the lowest concentration provide a period of stable performance for the signal detection analysis.

3.3.6 Statistical Analyses

R version 4.1.1 (R Core Team, 2021) was used for all analyses, using the “nlme” (Pinheiro et al., 2022) and “MuMIn” (Bartoń, 2020) packages. To determine if there were

differences in odour sensitivity, the sensitivity index (*d'*) for each mouse on the last five blocks of the lowest odour concentration were calculated and compared with linear models. This measures the difference between the distributions of the signal and noise, with a larger *d'* indicating the signal was more readily detected. The *d'* value was calculated by subtracting the z score that corresponded to the false-alarm rate from the z score that corresponded to the hit rate (Stanislaw & Todorov, 1999) To examine differences in response strategy, the response bias (*c*) for each mouse on the last 5 blocks of the lowest odour concentration were calculated and compared with linear models. This measures the general tendency to respond on any given trial and was calculated by taking the negative mean of the z score that corresponded to the hit rate and the z score that corresponded to the false-alarm rate (Stanislaw & Todorov, 1999). The last 5 blocks were used as these signal detection measures assume stable performance. Overall performance and learning were investigated by comparing the accuracy of the mice with linear mixed effects models. Models were compared using Akaike Information Criterion corrected for small sample size (AICc) (Hurvich & Tsai, 1989). All possible valid models, including null models without any effects, were calculated and the model with the lowest AICc was reported. The use of AICc allows for different models to be compared, and the model which best fits the data to be selected.

3.4 Results

3.4.1 5xFAD And WT Mice Do Not Differ In Olfactory Sensitivity

To determine if there were differences in olfactory sensitivity, the sensitivity index (*d'*) on the last five blocks of the lowest odour concentration were compared. The null model (AICc = 80.7, weight = 0.459) best explained the data, indicating no differences in the ability of the mice to detect this odour (Figure 3-1).

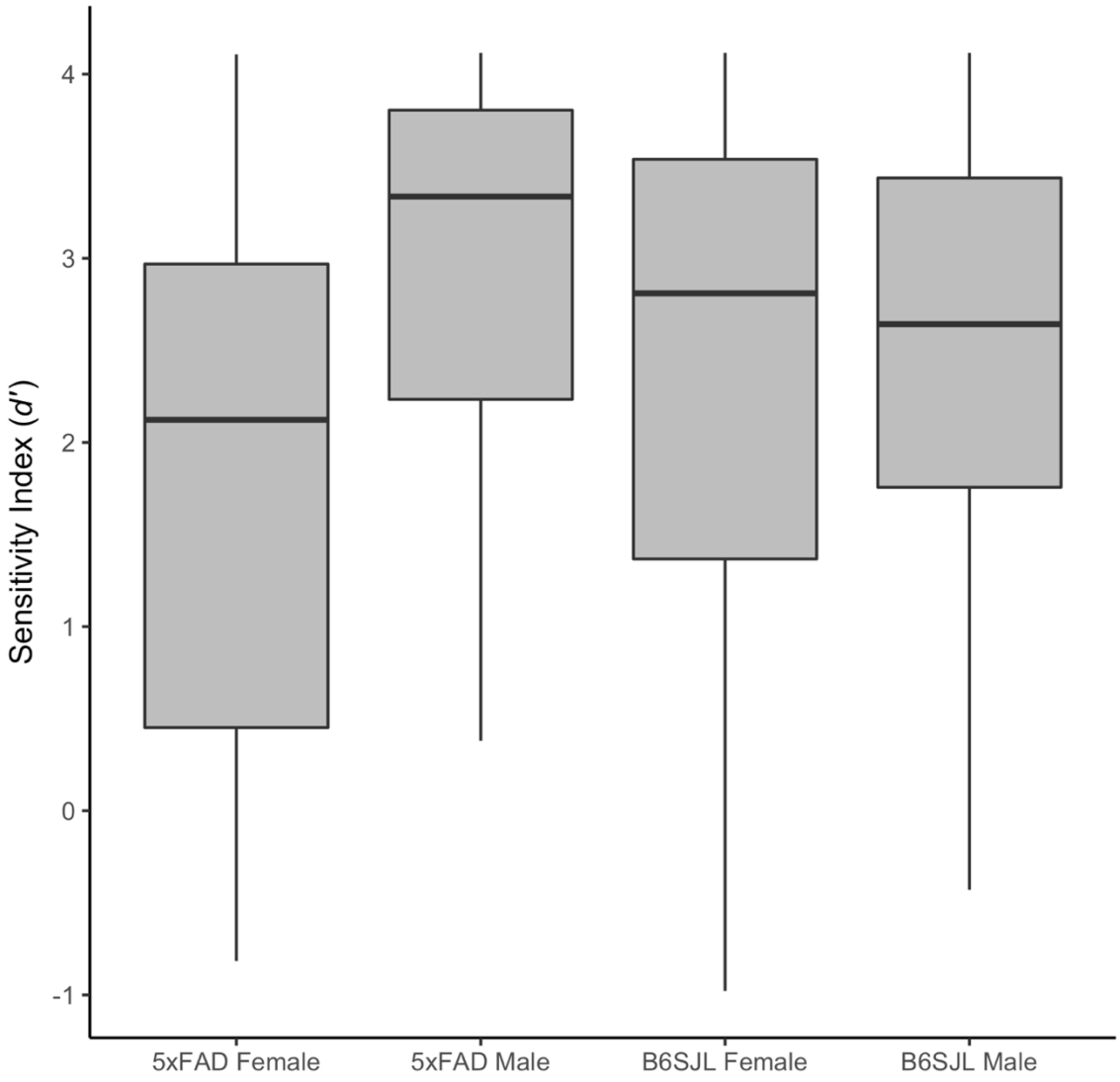

Figure 3-1: Boxplots, showing the median, 25th, and 75th percentiles, with whiskers extending to the furthest points within 1.5 interquartile ranges, and outliers shown as dots, for the sensitivity index (d') on the last five block of trials for male and female 5xFAD and WT (B6SJL) mice on the lowest odour concentration (0.00001 ppm ethyl acetate). There were no significant differences between groups.

3.4.2 Female Mice Are More Conservative In Their Responses Than Males

To determine if there were differences in response strategies used by the mice, their response bias (*c*) on the last five blocks of the lowest odour concentration were compared. The model with a main effect of sex (AICc = 55.7, weight = 0.685) best explained the data, with the female mice (c = - 0.285 $\pm$ 790) having a more conservative bias than the male mice (c = -0.468 $\pm$ 0.407; Figure 3-2). This model differed significantly from the null model (AICc = 60.3, weight = 0.069, p = 0.011).

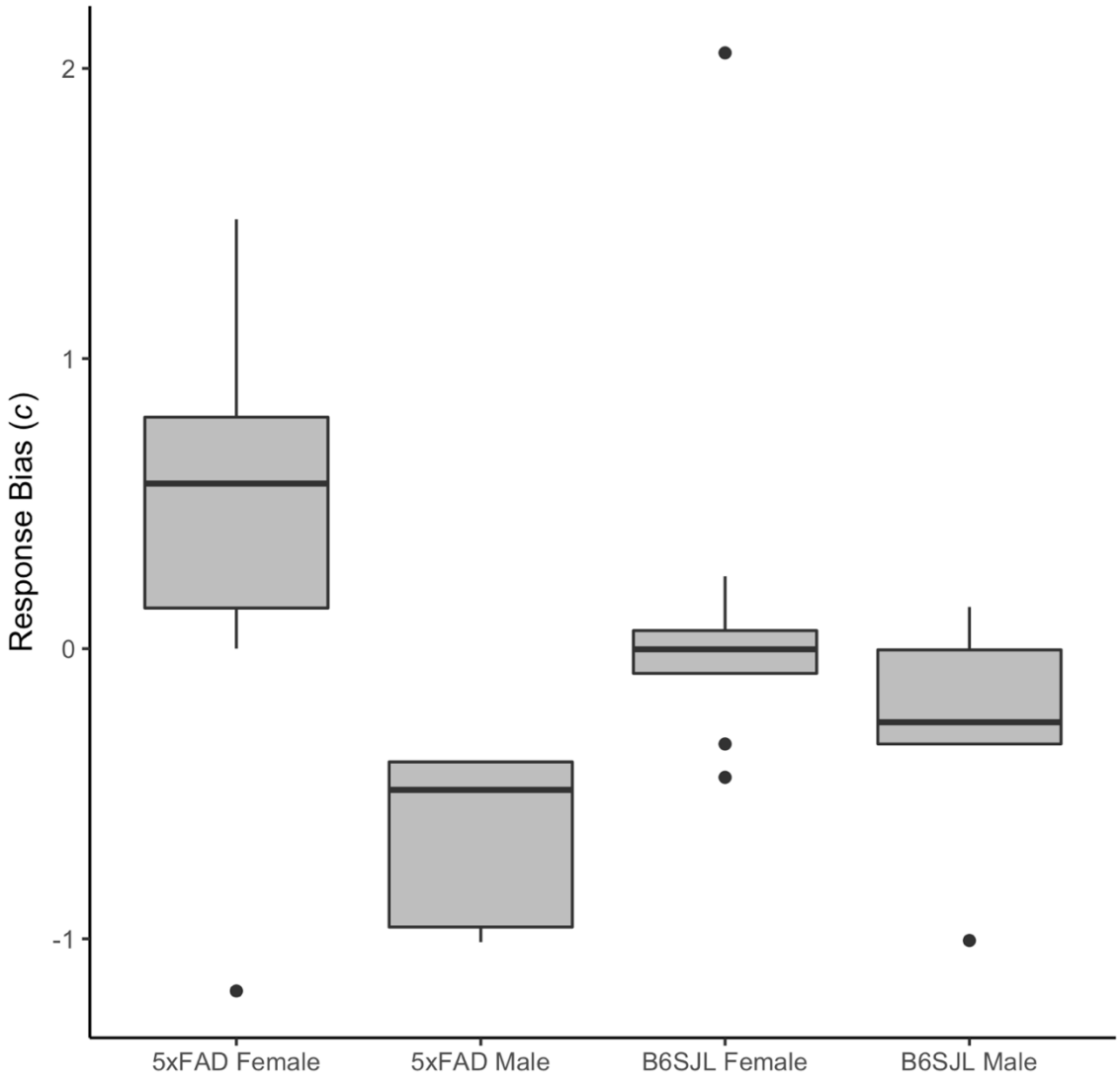

Figure 3-2: Boxplots, showing the median, 25th, and 75th percentiles, with whiskers extending to the furthest points within 1.5 interquartile ranges, and outliers shown as dots, for response bias (c) on the last five block of trials for male and female 5xFAD and WT (B6SJL) mice on the lowest odour concentration (0.00001 ppm ethyl acetate). The female mice had a more conservative response bias than the male mice ($p = 0.011$) and there was no effect of genotype.

3.4.3 5xFAD Mice Show Olfactory Learning Deficits

To determine if there were differences in learning rate, the accuracy levels across all blocks were compared. The model with main effects of genotype, sex, odour concentration, and block, as well as genotype by sex, genotype by block, sex by block, sex by odour concentration, and block by odour concentration interactions (AICc = -613.2, weight = 0.141) best explained the data and differed significantly from the null model (AICc = -358.0, weight < 0.001, $p < 0.0001$). The overall trend is that the WT mice had higher accuracy (0.83 ± 0.20) than the 5xFAD mice (0.81 ± 0.21), and male mice had higher accuracy (0.85 ± 0.18) than female mice (0.79 ± 0.22). When splitting the data by genotype, there was a main effect of sex in the 5xFAD mice, with the males having higher accuracy (0.88 ± 0.16) than the females (0.76 ± 0.22), but no such difference in the WT mice. Additionally, the main effect of block was significant for the WT ($p = 0.03$) but not for the 5xFAD mice ($p = 0.45$), suggesting a lower effect of learning, or relearning, the task across blocks in the 5xFAD mice. Accuracy increased as the mice progressed through blocks (Figure 3-3), and this increase was most pronounced on the first odour concentration (1ppm) with accuracy on the first block (0.52 ± 0.09) being much lower than the final block (0.83 ± 0.15), and less pronounced on the final odour concentration (0.00001ppm) going from 0.77 (± 0.21) on the first block, to 0.87 (± 0.20) on the second block, to 0.88 (± 0.16) on the final block. The males (0.56 ± 0.14) had lower accuracy than the females (0.76 ± 0.19) on the first odour concentration (1ppm),

but males had higher accuracy on all other concentrations. There is a trend where on the first blocks of each odour concentration the difference in accuracy between the males (0.75 ± 0.19) and females (0.66 ± 0.21) was greater than the difference between the males (0.89 ± 0.15) and females (0.86 ± 0.19) on the fifth blocks. This larger difference between performance on the first and fifth blocks suggests the females were slower than the males to relearn the task with each new odour concentration.

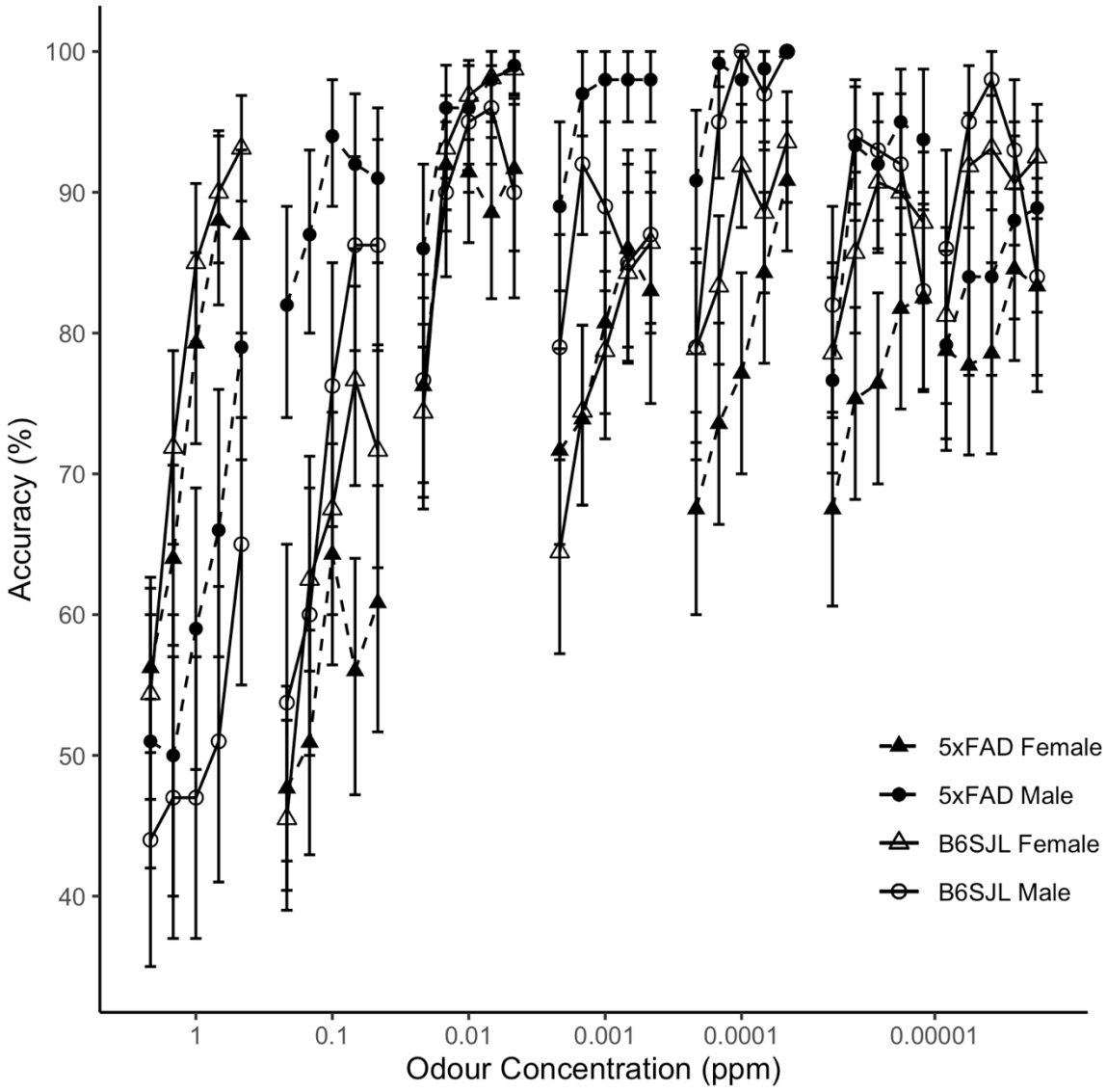

Figure 3-3: Mean (± 95% confidence interval) accuracy of female and male 5xFAD and WT (B6SJL) mice on each block of 20 trials for each of the six odour concentrations. Overall, the wildtype mice had higher mean accuracy (0.83 ± 0.20) than the transgenic mice (0.81 ± 0.21), and male mice had higher mean accuracy (0.85 ± 0.18) than female mice (0.79 ± 0.22). Wildtype mice had a greater effect of block than transgenic mice, and accuracy was greatest overall (0.91 ± 0.13) on the third odour concentration (0.01 ppm ethyl acetate).

3.5 Discussion

We studied odour detection and olfactory learning in 12-month-old 5xFAD mice. Our results indicated that: the 5xFAD mice showed deficits in olfactory learning at 12 months of age and this deficit is greater in female mice than male mice; the 5xFAD mice did not have deficits in olfactory sensitivity; and the 5xFAD mice did not use a different strategy than WT mice to learn the odour detection task, but the female mice of both genotypes were more conservative with their responses than males. The operant olfactometer does not rely on auditory or visuospatial cues, nor on motor performance, which makes it an ideal task to measure learning and memory in the 5xFAD mice, which show age related sensory (Gür, Fertan, Alkins, et al., 2019), motor (O'Leary, Mantolino, et al., 2020), and cognitive (O'Leary & Brown, 2022) deficits. While deficits in odour detection and olfactory learning in humans with AD may be confounded by loss in olfactory sensitivity (Velayudhan, 2015), our results show that the 5xFAD mice do not have decreased olfactory sensitivity at 12 months of age. This agrees with our previous findings in 5xFAD mice at 6 months of age (O'Leary, Stover, et al., 2020; Roddick et al., 2016), hence the operant olfactometer can be reliably used to study learning deficits in the 5xFAD mice. We had fewer male mice than female mice in this study. This is often an issue with studies of aged mice due to differences in mortality rates in AD mouse models (Rae & Brown, 2015).

Our results also showed an impairment in olfactory learning in the 5xFAD mice at 12 months of age, which agrees with (Girard et al., 2014) who showed olfactory learning deficits are present as early as 4 months of age in this model. Performance of the mice was highest on the third odour concentration tested (0.01ppm). We propose this is because when tested at this concentration the mice had learned the task well from the prior testing, and where being presented with an odour concentration which was easily detected.

However, O'Leary and Brown (2022) did not show any deficits in olfactory memory using the conditioned odour preference task. This may be due to the nature of the tests, as the conditioned odour preference task relies on Pavlovian conditioning (Schellinck et al., 2001), and the olfactometer uses operant conditioning (Slotnick & Restrepo, 2005). Indeed, intact Pavlovian learning in the fear conditioning task has been shown in the 5xFAD mice (Bhattacharya et al., 2014), while operant conditioning is disturbed (Roddick et al., 2014). Similarly, an intact eye blink reflex has been shown in AD patients (Solomon et al., 1995), yet patients show deficits in operant conditioning (Spira & Edelstein, 2007). The female 5xFAD mice appeared to have a greater impairment in learning compared to the males, which is in line with findings of greater pathology in female 5xFAD mice compared to males (Manji et al., 2019; Sil et al., 2022).

We also showed that female mice were more conservative in their responses. Sex differences in mouse decision making have been reported (C. S. Chen, Ebitz, et al., 2021;

C. S. Chen, Knep, et al., 2021; Orsini & Setlow, 2017), and we have previously reported sex differences in AD mouse models (Fertan, Wong, et al., 2019; Grant et al., 2020; Gür, Fertan, Kosel, et al., 2019; O'Leary & Brown, 2022; Roddick et al., 2014, 2016). This further demonstrates the importance of examining sex differences when working with mouse models of AD.

Our results show the value of analysing olfactory learning and memory using the olfactometer, as it is not confounded by other behavioural or sensory dysfunctions seen in the 5xFAD mice. Thus, it can be reliably used to study disease mechanisms and test novel therapeutics. The value of a signal detection analysis is that it examines the cognitive strategies used by the mice, and allows the for the discrimination between cognitive and sensory deficits in mouse models of AD.

3.6 Author Contributions

K.M.R.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. E.F.: Manuscript writing, final approval of manuscript. H.M.S.: Conception and design, final approval of manuscript. R.E.B: Conception and design, manuscript writing, final approval of manuscript.

3.7 Acknowledgments

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3.8 Conflict of Interest

The authors have no conflict of interest to report.

3.9 Data Availability

The data set used in this study is available at https://doi.org/10.5683/SP3/1OSVQO

4 Olfactory Delayed Matching To Sample Performance In Mice: Sex Differences In The 5XFAD Mouse Model Of Alzheimer's Disease

Rats are able to complete an olfactory delayed matching to sample task (Lu et al., 1993), but no one had tested mice on such a task. This study was designed to test if mice could complete such a complex task and evaluate the 5xFAD model for working memory deficits. It was hypothesised that the mice would be able to complete this task, and that the 5xFAD mice would show impaired working memory, shown by not being able to complete the task at longer delays. This was the first paper to show that mice were capable of performing an olfactory delayed matching to sample task. No deficits in working memory were found in the 5xFAD mice, though the female mice performed better than the males.

The manuscript below was published in *Behavioural Brain Research*, see Appendix D for the copyright permission letter from the publisher. I collaborated in the design of the study, collected much of the data, analysed the results, wrote the initial draft of the paper, incorporated feedback from the coauthors, and responded to the peer review upon submission of the paper.

Roddick, K. M., Schellinck, H. M., & Brown, R. E. (2014). Olfactory delayed matching to sample performance in mice: Sex differences in the 5XFAD mouse model of Alzheimer's disease. *Behavioural Brain Research*, *270*, 165–170.

https://doi.org/10.1016/j.bbr.2014.04.038

4.1 Abstract

While olfactory delayed matching-to-sample tasks have been used to assess working memory in rats, no such tasks have been tested in mice. Olfactory delayed matching-to-sample learning was assessed in male and female 5XFAD mice, a model of Alzheimer's disease, and their wildtype (B6SJL F1) littermates at 6 -7 months of age using an operant olfactometer. All 5XFAD and wildtype mice were able to learn the delayed olfactory matching-to-sample task at 2 and 5 second delays. Fewer mice learned with a 10 second delay and only one mouse learned with a 30 second delay. Female mice showed higher levels of performance on the delayed matching-to-sample task than males, indicative of better working memory. These results demonstrate for the first time that mice are able to learn an olfactory delayed matching to sample task.

4.2 Introduction

Rodents perform remarkably well on olfactory learning tasks. Rats show "learning to learn" when serially presented with olfactory discrimination problems, and can achieve

near errorless learning, which was previously thought only to occur in primates (Slotnick, 1993, 1994, 2001; Slotnick et al., 1991; Slotnick & Katz, 1974). Rats show considerably faster learning on olfactory discrimination tasks than on visual and auditory discrimination tasks (Nigrosh et al., 1975) and rats are able to perform a matching-to-sample task, with delays of up to 10 seconds between the sample and comparison odour (Lu et al., 1993). The olfactory sensitivity of mice is similar to that of rats and, while mice take longer to complete initial training, spend more time between trials unengaged in the task, and made more errors during acquisition of a task, they were able to reach a level of performance comparable to rats on olfactory discrimination learning tasks, and showed retention of the olfactory memories after 32 days (Bodyak & Slotnick, 1999). Mice also rapidly learn a Pavlovian conditioned odour preference task and retain the conditioned odour preference for at least 60 days after testing (Schellinck et al., 2001).

As a result of recent advances in genetic engineering techniques, an ever-increasing number of genetically modified mouse models of AD have been developed (Bales, 2012; Chin, 2011; Hall & Roberson, 2012). Many of these mouse models of AD have learning and memory deficits on visual spatial tasks such as the Morris water maze (Stover & Brown, 2012) and the Barnes maze (O'Leary & Brown, 2009), but no studies of olfactory learning and memory have been done on these mice. An advantage of using an olfactometer to study learning and memory in mice is that both olfactory discrimination learning and working memory can be examined. While olfactory matching-to-sample

tasks have been used to evaluate working memory in rats (April et al., 2011; Lu et al., 1993; Peña et al., 2006), mice have not been tested on olfactory matching-to-sample tasks. Working memory in mice is commonly examined with spontaneous alternation in Y mazes (Kimura et al., 2010; Oakley et al., 2006; Ohno et al., 2007) or cross mazes (Hillmann et al., 2012; Jawhar et al., 2012). When placed in either of these mazes mice will spontaneously alternate their entries into the arms, going into the arm which they have entered least recently (Lalonde, 2002). The problem with these tests is that while they require working memory for the animals to remember the arms last entered, they rely on the concept of innate exploration of novel stimuli, and there are many other factors which could influence performance. If an animal were to simply turn the same direction every time they went to enter another arm they would display perfect alternation. Additionally, both anxiety (Bats et al., 2001) and spatial memory (Lalonde, 2002) have been shown to affect spontaneous alternation. Because tests of spontaneous alternation can be confounded in this way, goal directed tasks involving discrete stimulus presentations may better assess working memory, and are thus more valid tests of working memory (Dudchenko et al., 2013).

The present study is the first to test mice on an olfactory matching-to-sample task. Male and female 5XFAD mice and their wildtype littermates were tested on an olfactory delayed matching-to-sample working memory task at 6 to 7 months of age. Mice have not previously been evaluated on an olfactory delayed matching-to-sample task, but because both 5XFAD mice (Devi & Ohno, 2012; Jawhar et al., 2012; Kimura et al., 2010)

and AD patients (Belleville et al., 1996; Gagnon & Belleville, 2011) have deficits in working memory, we hypothesized that the 5XFAD mice would show deficits on the delayed matching-to-sample task. Relative to other transgenic mouse models of AD, the 5XFAD mouse shows an early onset of AD pathology, with Aβ plaques detectable at 2 months of age, as well as high levels of $A\beta_{40}$ and $A\beta_{42}$ in the brain, and low levels of complement factor H, an immune suppressor, decreasing levels of which has been linked to inflammatory neuropathology in AD (Alexandrov et al., 2011; Oakley et al., 2006).

4.3 Materials And Methods

All animal protocols adhered to the Canadian Council on Animal Care guidelines and were approved by the University Committee on Laboratory Animals (protocol # 11-033).

4.3.1 Animals

We tested 6 - 7 month old 5XFAD mice (5 females, 6 males) and their wildtype (B6SJL) littermates (4 females, 9 males). The 5XFAD mouse model of AD has five mutations found in familial AD; three to the APP gene, the Swedish (K670N/M671L), Florida (I716V) and London (V717I) mutations, and two mutations to presenilin 1 (M146L and L286V) (Oakley et al., 2006). The mice were obtained from an in-house colony bred at Dalhousie University from mice purchased from The Jackson Laboratory (Bar Harbor, ME; strain

numbers 006554 and 100012). The mice were weaned at 22 days of age and separated into same sex groups of 2-4 siblings housed in 30×18×12cm polycarbonate cages with wire tops and *ad lib* access to food (Purina Rodent laboratory chow #5001). Genotypes were determined using PCR with DNA from ear punches, and mice testing positive for retinal degeneration ($Pde6b^{rd1}$ gene mutation) were not used. Ten days prior to the start of testing, the mice were individually housed, water deprived and fed with a mash of powdered rodent chow mixed with a measured amount of water. While on water restriction mice were weighed daily and the amount of water given in their mash adjusted to maintain their body weight at 80-85% of free feeding weight. As mice learned to respond in the olfactometer and received increasing amounts of water reward, the level of water restriction was decreased by gradually increasing the amount of water in their mash.

4.3.2 Olfactometers

Two computer controlled eight-channel liquid diffusion olfactometers (Knosys Olfactometers Inc., Lutz, Fl) based on those described by Slotnick & Restrepo (2005) were used (Figure 4-1). In the olfactometers, filtered air from a compressor was pumped through bottles containing the odour solutions into a final valve, which directed the odour-laden air to an odour sampling port or an exhaust tube. The odour sampling port contained a reinforcement tube delivering water as a reward, and a sensor which detected when the animals were licking the water tube. Odour solutions were made by

mixing commercially available odourants with mineral oil. The odours used, cardamom, lavender, dillweed, and patchouli (Aldrich Chemical Company Inc. Milwaukee, WI) were not found to be aversive to the mice in pilot studies, and mice were observed when initially presented with the odours for signs of aversion such as withdrawing their head from the odours sampling port.

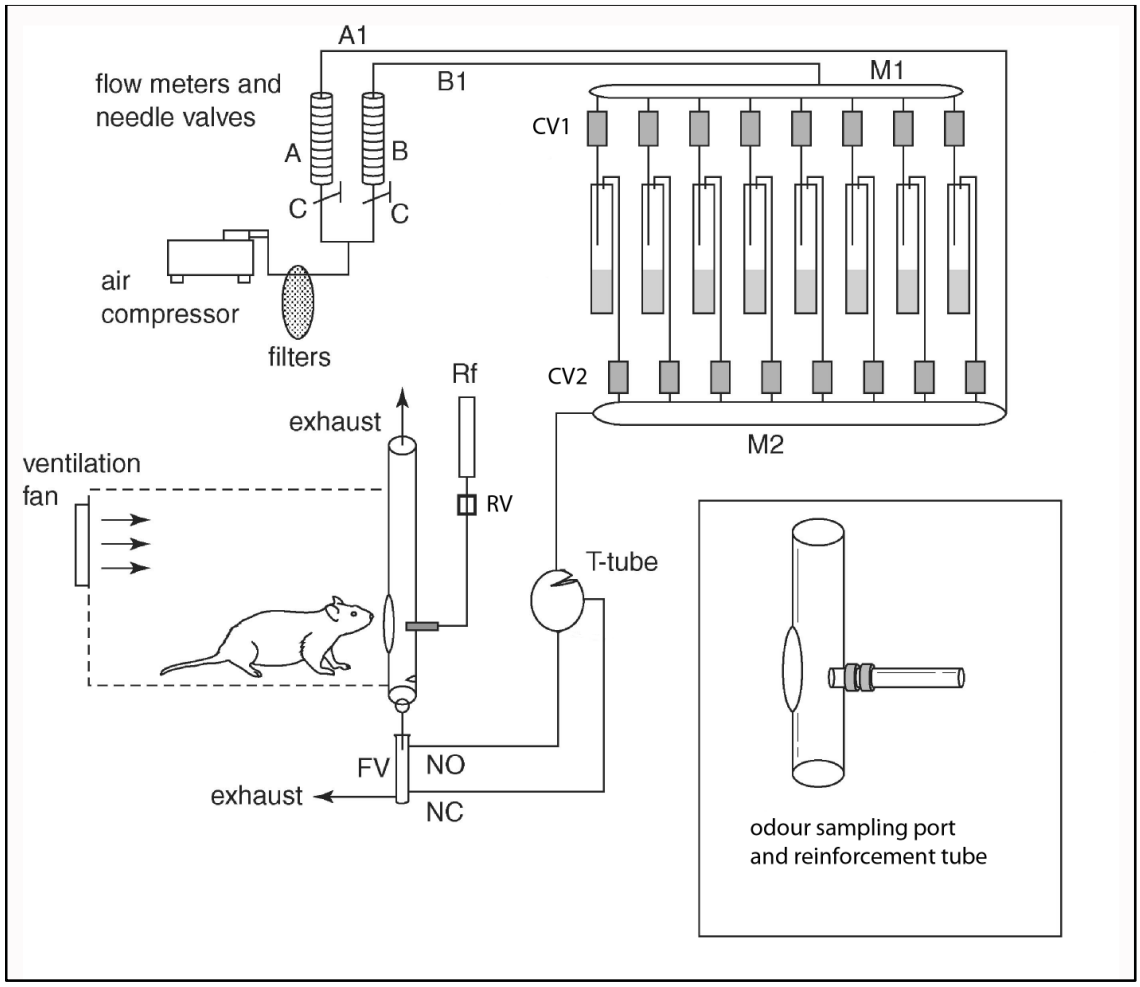

Figure 4-1: Diagram of the olfactometer. Air from the compressor is first sent through a filter after which it is spilt into two pathways. The first pathway flows through a needle valve (C), which controls the rate of airflow, and a flow meter (A), which measures the

airflow. The air then flows through tubing (A1) into a glass manifold (M2) as clean air. The second pathway, which supplies odourized air, flows through a different needle valve and flow meter (B) into a different glass manifold (M1). Pairs of control valves (CV1 and CV2), which are normally closed, control the flow of air along the second pathway from M1, through the odour saturation bottles, and into M2, where the clean and odourized air flows converge. A glass T-tube, with a push-in to create turbulence and mix the airflow from the two pathways, has two outflows controlled by the final valve (FV) which directs airflow either to the odour sampling port via the normally open (NO) port, or to the exhaust via the normally closed (NC) port. The odour sampling port opens to the animal chamber and contains the reinforcement tube connected to the water storage (Rf). The reinforcement valve (RV) controls the flow of water to the reinforcement tube. Adapted from Slotnick and Restrepo (Slotnick & Restrepo, 2005).

4.3.3 Behavioural Procedure

The mice were initially trained on a two odour discrimination and an odour reversal task so that they could learn the procedure for receiving water reward in the olfactometer. The matching-to-sample task started with two days of matching trials. During these trials, mice were presented with a sample odour (A) and then, after a 2 second inter-stimulus delay (ISD), the same odour (A) was presented as a comparison. The mice were rewarded with water for licking the reinforcement tube and there was a 5 second inter-trial interval (ITI). After two days of A-A matching trials, mice were given one day with

both matching (A-A) and non-matching (A-B) trials. Non-matching trials were introduced after the mice had been presented with 10 matching trials. During non-matching trials the comparison odour was different than the sample odour and the mice were not rewarded for licking the reinforcement tube. The mice next received two days of B-B matching trials, in which they were rewarded for licking on each trial, followed by one day of mixed B-B matching and B-A non-matching trials, on which they were rewarded for licking only on B-B trials. Mice were trained for one hour or until they completed 100 matching trials. Mice were then presented with all four types of trials, A-A, B-B, A-B, and B-A, with the same odours used during matching to sample training. Trials were divided into blocks of 20, with 5 of each of the 4 types of trials in each block. Mice were considered to have reached criterion, and advanced to the test phase, when they correctly responded to 80% of each of the 4 types of trials in one block.

In the test phase, mice were presented with a new pair of odours (C and D), using the same 2 sec ISD and 5 sec ITI. After criterion (80% of each of the 4 types of trials) was reached on the 2 sec ISD, the ISD was increased to 5 sec, and then 10, and 30 sec after they reached the 80% criterion at each ISD. The ITIs were 1.1 times the length of the ISD. Prior to advancing from one ISD to the next, the mice were presented with a series of all matching trials with ISDs incrementally increasing from the ISD of the stage previously completed to the next stage. For example, when the ISD was to be increased from 2 to 5 sec, mice would first be presented with 2.5, 3, 3.5, 4, and 4.5 sec ISDs. This was to ensure the mice would learn to continue to perform the task at the longer delay.

In order to facilitate responding at longer ITIs fifteen of the mice (7 tg, 8 wt) were run with a slight variation on this task. This variation provided the mice with small reinforcements during the ISD to encourage them to continue attending to the task during the delay period. Small reinforcements were given every 5 sec during the ISD up to 10 seconds prior to the end of the delay. Additionally, the ITIs were different. Up to a 10 sec ISD, the ITIs were 6 sec, above that ITIs were half the length of the ISD.

The mice were tested for a maximum of 10 blocks of 20 trials per day. The test session was ended before 10 blocks were complete if the mice stopped performing the task. At the 2 sec delay mice commonly completed 10 blocks, but as the delays increased, and the amount of time required for the mice to complete 10 blocks increased; mice completed progressively fewer block of trials before they stopped performing the task.

4.3.4 Statistical Analyses

All statistical analyses were performed with the statistical program R (www.R-project.org). The number of errors to reach criterion during the matching to sample task was analyzed with linear regression models. The training phase and test phase were analysed separately. Model selection was based upon Akaike's Information Criterion (AIC), the evidence ratios and Akaike weight of the models were also calculated. AIC is used to compare the quality of statistical models of data, it takes into account the

complexity of the models and how well the model fits the data (Akaike, 1974; Burnham & Anderson, 2004). F tests were used to compare the best fitting models to the appropriate null model, and confidence intervals (95%) of the effects were calculated. At each delay of the test phase, only data from mice that reached criterion were included. A Cox proportional hazards regression was also used to analyze the likelihood of mice failing to reach criterion on each delay in the test phase.

4.4 Results

One wildtype male mouse died before completing the matching to sample task, and data from this mouse are not included in this analysis. During the training phase of the matching-to-sample task wildtype male mice made more errors than 5XFAD males, wildtype females, and 5XFAD females (Figure 4-2). Linear regression models of the number of errors made in the task were compared using AIC. The model using sex, genotype, and sex by genotype interaction was found to be the best model (AIC = 284.02, weight = 0.717) (Table 4-1). This model differed significantly from the null model ($R^2 = 0.505$, $F_{3,19} = 6.454$, $p = 0.0034$). Confidence intervals (95%) of the coefficients showed little evidence of a difference between 5XFAD and wildtype mice (-124.0 to 164.9 errors) or between female and male mice (-132.4 to 128.4 errors), but considerable evidence of increased errors by the wildtype males (-1.7 to 369.2 errors).

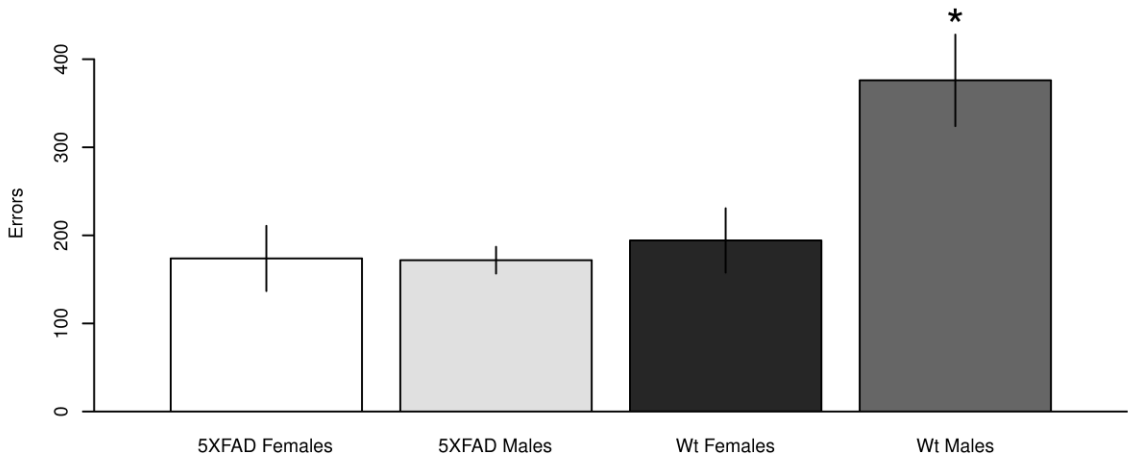

Figure 4-2: Errors on delayed matching to sample training. Mean (± SEM) number of errors made by wildtype (Wt) and 5XFAD male and female mice on the training phase of the delayed matching to sample task. The wildtype males made more errors than all other groups.

Table 4-1: Akaike's Information Criterion model comparison of training phase data.

Model	*df*	AIC	Δ AIC	ER	w_i
errors ~ genotype * sex	5	284.02	0	1	0.717
errors ~ genotype + sex	4	286.71	2.69	3.84	0.187
errors ~ genotype	3	288.38	4.36	8.83	0.081
errors ~ sex	3	292.43	8.41	67.00	0.011
errors ~ 1	2	294.18	10.16	160.80	0.005

The Cox proportional hazards regression analysis of the proportion of mice failing to reach criterion on each delay length of the test phase of the matching to sample task found that females performed better than males (z = 2.32, p = 0.020) (Figure 4-3). Similarly, linear regression modeling of the number of errors made found the model using sex and delay, with mouse as a random effect was found to be the best fit (AIC = 722.79, weight = 0.454) (

Table 4-2). This model differed significantly from the null model of delay with mouse as a random effect ($R^2 = 0.142$, $F_{2,50} = 5.094$, $p = 0.0284$). Confidence intervals (95%) of the coefficients showed evidence of more errors made by male than female mice (11.3 to 194.3). The model using genotype, sex, and delay, with mouse as a random effect (AIC = 724.77, weight = 0.170) did not differ significantly from the null model ($R^2 = 0.142$, $F_{2,49} = 2.508$, $p = 0.0919$). The confidence intervals (95%) of this model showed similar evidence of more errors made by male than female mice (10.3 to 197.2 errors), and little evidence of more errors by the 5XFAD mice than the wildtype mice (-85.5 to 98.8 errors) (Figure 4-4). Individual learning curves are shown for all mice that reached criterion at the 2 sec, 5 sec, 10 sec, and 30 sec delays (Supplemental Figures 1, 2, 3, 4 in Appendix E).

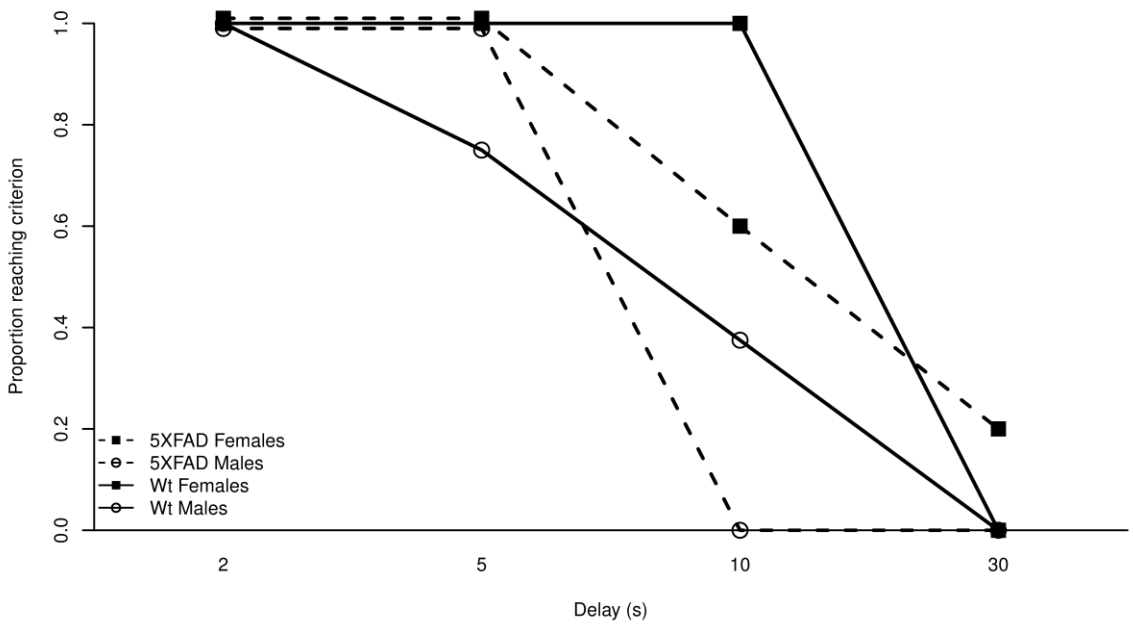

Figure 4-3: Proportion of mice reaching criterion at each delay in the delayed matching to sample task. Female mice were more likely to reach criterion on a delay than male mice ($p = 0.020$).

Table 4-2: Akaike's Information Criterion model comparison of test phase data.

Model	*df*	AIC	Δ AIC	ER	w_i
errors ~ sex + delay + (1 | mouse)	6	722.79	0	1	0.454
errors ~ genotype + sex + delay + (1 | mouse)	7	724.77	1.98	2.69	0.169
errors ~ sex * delay + (1 | mouse)	8	725.47	2.68	3.81	0.119
errors ~ delay + (1 | mouse)	5	726.13	3.34	5.30	0.086
errors ~ genotype + sex + genotype:sex + delay + (1 | mouse)	8	726.14	3.35	5.33	0.085
errors ~ genotype * delay + (1 | mouse)	8	727.88	5.09	12.73	0.036
errors ~ genotype + delay + (1 | mouse)	6	728.10	5.30	14.17	0.032
errors ~ genotype * sex * delay + (1 | mouse)	13	729.22	6.42	24.79	0.018

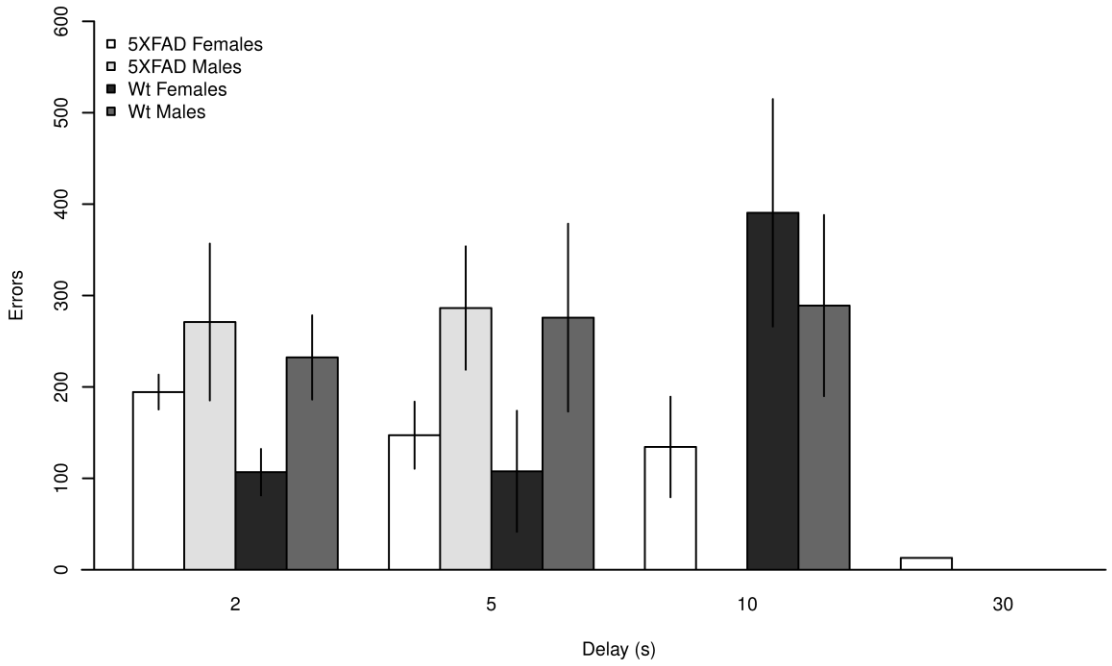

Figure 4-4: Errors on delayed matching to sample. Mean (± SEM) number of errors made by wildtype (Wt) and 5XFAD male and female mice at each delay prior to reaching criterion. At each delay only the data from mice which successfully reached criterion are included. At the 2 second delay n = 5 5XFAD females, 6 5XFAD males, 4 Wt females, 8 Wt males; at the 5 second delay n = 5 5XFAD females, 6 5XFAD males, 4 Wt females, 6 Wt males; at the 10 second delay n = 3 5XFAD females, 0 5XFAD males, 4 Wt females, 3 Wt males; and at the 30 second delay n = 1 5XFAD females. Overall, female mice made fewer errors than male mice.

4.5 Discussion

While previous studies have shown that rats are able to learn olfactory delayed matching-to-sample tasks (April et al., 2011; Lu et al., 1993; Otto & Eichenbaum, 1992), this is the first study to demonstrate that mice can learn an olfactory delayed matching-to -sample task. While the mice were able to learn the task, it took many trials and was difficult for them to complete. To complete the matching-to-sample task, mice took a mean of 2304 ± 826 trials (70+ days) as they made hundreds of errors prior to reaching criterion on both the training and test phases of the matching-to-sample task. Learning to inhibit responding on the non-matching trials was the most difficult aspect of the learning process and the majority of the mice had difficulty generalizing this learned inhibition as they made approximately the same number of errors prior to reaching criterion for each subsequent delay. There were only two instances where mice improved their performance. One wildtype female mouse, upon reaching criterion on the 2 sec delay, made only 6 errors prior to reaching criterion on the 5 sec delay, but made 700 errors before reaching criterion on the 10 sec delay. The only mouse to reach criterion at the 30 sec delay, a 5XFAD female, made 73 errors at the 10 sec delay and then only 13 errors at the 30 sec delay.

Female mice performed better than males, making fewer errors and reaching longer delays in the matching to sample task. This suggests that females have better olfactory working memory than males. Sex differences are seldom examined in studies of olfactory learning and, when assessed, the results are mixed. Slotnick and Restrepo (2005), found no sex differences in mice of Swiss-Webster, CF-1, C57BJ, FVB, mixed FVB

and C57BL backgrounds, and mice derived from the 129/Sv strain on olfactometer based odour discrimination and detection tasks, and Schellinck, Arnold, and Rafuse (2004) found no sex differences in NCAM deficient mice in a conditioned odour discrimination task. Mihalick, Langlois, and Krienke (2000), on the other hand, reported better performance by male C57BL/6J and DBA/2J mice than females on an olfactory reversal task, but the difference was subtle, and only significant at later stages in a series of reversals. Sex differences in learning and memory studies with 5XFAD mice have not been examined, with many studies not reporting the sex of the mice used in the experiments (Devi & Ohno, 2010a, 2010b, 2012; Kimura et al., 2010; Kimura & Ohno, 2009; Oakley et al., 2006). Because genetically modified mice often show sex differences in brain and behaviour, indicating an interaction between genetic manipulation and sex, it is important to test both male and female transgenic mice (Schellinck et al., 2010).

The present study demonstrated for the first time that mice are able to learn an olfactory delayed matching-to-sample task with delays up to 10 seconds. Strong evidence was found for better working memory in female mice than in males on the matching-to-sample task. However, there was little evidence of olfactory working memory deficits on the delayed matching-to-sample tasks in the 5XFAD mice compared to the wildtype mice. Using delayed alternation tasks in an olfactory H maze, Girard et al. (2013) found that 4 and 6 month old 5XFAD mice had poorer working memory performance than littermate controls, but the sex of the mice was not reported, the task required locomotion, and had no fixed delay intervals, thus it is difficult to compare

their results with those of the present study. Motor deficits have been found in 5XFAD mice (Jawhar et al., 2012), and could confound performance on the olfactory H maze task.

There are many benefits of using operant-olfactometer-based tasks when testing mouse models of neurological disorders. There is the possibility of confounding motor or visual deficits in these mice, and tasks using the operant-olfactometer avoid these by requiring very little motor activity and no reliance on visual stimuli; in order to make a response the mice only need to lick a spout. Additionally, the ability of rodents to perform olfactory tasks with greater ease than tasks using other sensory modalities, makes the olfactometer an ideal testing apparatus (Nigrosh et al., 1975). Given how difficult it was for the mice to perform on this olfactory matching-to-sample task, it seems doubtful that mice would be able complete a similar matching to sample task which required them to rely on visual or auditory stimuli.

4.6 Acknowledgments

The authors would like to thank Dr Chris Sinal, Pharmacology Department, Dalhousie University for genotyping the mice.

5 Discussion

5.1 What Was Found?

5.1.1 Female, But Not Male, 3xTg-AD Mice Show A Deficit In Olfactory Sensitivity At Six Months Of Age

In the olfactory sensitivity test at six months old, the female 3xTg-AD mice showed impaired performance on the lowest odour concentration tested compared to the female wildtype mice, despite performing well on the higher odour concentrations, while there was no such impairment in the male 3xTg-AD mice.

This is not the only study to find olfactory deficits in the 3xTg-AD mouse model. Mitrano et al. (2021) found that 3xTg-AD mice performed worse than wildtype mice in a buried food test, and similar to my study, found greater deficits in the female than the male 3xTg-AD mice.

Furthermore, Coronas-Sámano et al. (2014) tested female 3xTg-AD mice on a variety of social odour guided tasks and found reduced responses to the social odours in the 3xTg-AD mice compared to wildtype controls. They also tested the mice on a habituation-dishabituation task using food odours. In this experiment, 16-18 month old female 3xTg-

AD mice, but not younger mice, showed a decreased dishabituation to a novel odour (Coronas-Sámano et al., 2014).

Female 3xTg-AD mice have a greater Aβ burden than male 3xTg-AD mice (Carroll et al., 2010; Creighton et al., 2019; Stimmell et al., 2019), which may explain why this deficit was found in female, but not male, mice. Additionally, female 3xTg-AD mice and their wildtype controls have lower motivational levels than their male counterparts (Fertan, Wong, et al., 2019; Gür, Fertan, Kosel, et al., 2019), which could also contribute to the poorer performance in females when the task became more difficult for them to complete.

In human patients, females with MCI or AD show a faster cognitive decline following diagnosis than males (Ferretti et al., 2018). While there is evidence that females may be a greater risk of developing AD, at least a significant portion of this increased risk is accounted for by the increased longevity of females and age being such an important risk factor for AD (Zhu et al., 2021).

Findings of sex differences in the 3xTg-AD model are inconsistent (Dennison et al., 2021). Studies have found more severe symptoms in male 3xTg-AD mice, with greater deficits in reference and working memory (Stevens & Brown, 2015), and increased frailty (Kane et al., 2018). While others have found transiently worse performance, which disappeared with age, in females on stressful tasks (Clinton et al., 2007).

In this study (Roddick et al., 2016), the best performance of the individual mice on any of the blocks of 20 trials at each odour concentration was shown in Figure 2-1. The benefit of this approach to displaying the data is that by eliminating the learning curves and examining only the peak performance of the mice, it allows one to differentiate between the learning and sensory aspects of the task.

5.1.2 5xFAD Mice Do Not Show An Olfactory Sensitivity Deficit At Six Nor 12 Months Of Age

The 5xFAD mice did not show an olfactory sensitivity deficit when tested at either 6 or 12 months old. These findings are in agreement with other studies that have found no deficits 5xFAD mice from 3 to 15 months old on a conditioned odour preference test (O'Leary, Stover, et al., 2020).

While studies using olfactory tubing mazes have found deficits in 5xFAD mice as early as 4 months old on a hippocampal (Girard et al., 2014), and frontal cortex (Girard et al., 2013) dependant tasks, these tasks used easily detectable odour concentrations, so would not be a measure of olfactory sensitivity. Similarly, 5xFAD mice show deficits in odour cross habituation (Wesson et al., 2010) and altered performance on an odour preference test (Roberts et al., 2020), however neither of these test olfactory sensitivity.

To date, the two studies presented in this thesis (Roddick et al., 2016, 2022) are the only ones to directly measure olfactory sensitivity in the 5xFAD model.

This finding differs from the olfactory sensitivity deficit found in the female 3xTg-AD mice at six months of age. While both are models of AD, there are differences between these mice. The most obvious one being that, of these two, only the 3xTg-AD model has a tau mutation, resulting in neurofibrillary tangles (Oakley et al., 2006; Oddo et al., 2003). It is possible that this tau mutation is the cause of the olfactory sensitivity deficits in the female 3xTg-AD mice, as tau burden is better correlated with cognitive decline in humans than Aβ burden (Nelson et al., 2012).

The olfactory system is affected early in AD, with the olfactory bulb and tract showing particularly early pathology in autopsies (Christen-Zaech et al., 2003; Kovács et al., 2001). However, the precise nature of the olfactory deficits resulting from this pathology is less clear. While it has been suggested that there is decreased olfactory sensitivity in AD (Murphy et al., 1990), other studies have suggested the deficits are ones of olfactory memory and recognition (Nordin & Murphy, 1998), or olfactory identification, but not sensitivity (Kareken et al., 2001; Larsson et al., 1999). When AD patients, MCI patients, and healthy controls were compared on olfactory sensitivity, discrimination, and identification, a slight impairment in sensitivity was found in the AD and MCI patients, but severe deficits in discrimination and identification (Djordjevic et al., 2008).

A meta-analysis of studies examining olfactory deficits in AD and Parkinson's Disease (PD) patients found that olfactory deficits were more severe in AD than PD, with the deficits detected in AD being primarily deficits of odour identification and recognition (Rahayel et al., 2012). Interestingly, though olfactory deficits were overall less apparent in PD, deficits in olfactory sensitivity were more apparent in PD than AD. This suggests that that while olfactory deficits may be a common symptom of various neurodegenerative diseases, it may be possible to differentiate between the different diseases based on the precise nature of the olfactory deficits.

Olfactory disfunction is a predictor of progression from normal cognition to MCI (Wilson et al., 2007), and from MCI to AD (Conti et al., 2013; Devanand et al., 2008; Lojkowska et al., 2011). In AD, the severity of olfactory dysfunction is related to the severity of other cognitive symptoms. AD patients with olfactory dysfunction have more severe deficits in global cognition, memory, attention, visuospatial ability, apathy, and activities of daily living than AD patients without olfactory dysfunction (Yu et al., 2018).

5.1.3 5xFAD Mice Show Learning Deficits At 12 Months Old

While the 5xFAD mice did not show olfactory sensitivity deficits, they did show learning deficits at 12 months of age, as shown by lower overall accuracy on the olfactory sensitivity test, including at the easily detectable odour concentrations. While other

papers have reported intact learning and memory in 5xFAD mice at this age (O'Leary, Stover, et al., 2020), that study used a conditioned odour preference test with four days of training, when mice can learn the task after only two days of training (Schellinck et al., 2001). In the conditioned odour preference test, mice are trained with a sugar reward in association with odour A and no sugar reward with odour B, and then tested for their odour preference memory one or more days later. Odour memory is inferred from the percentage of time spent digging in odour A in the memory probe trial. There is no measure of odour learning per se, just a measure of their memory of the odour associations on the test day. On the other hand, the operant olfactometer procedure produces detailed learning curves, allowing for the evaluation of the of the subject's performance as it learns the task.

The conditioned odour preference task also relies on a more natural behaviour of the mouse (digging for food) than the operant olfactometer, and takes less time for the mice to learn (Schellinck et al., 2001; Slotnick & Restrepo, 2005). Thus, it is likely that the conditioned odour preference task is easier for the mice to complete than the operant olfactometer tasks, and this may be the reason for the lack of olfactory memory deficits reported in the 5xFAD mice (O'Leary, Stover, et al., 2020). This would appear to be supported by the results of that study, as the mice showed greater than 85% preference score when tested both one and 90 days after training (O'Leary, Stover, et al., 2020).

5.1.4 Mice Can Perform An Olfactory Delayed Matching To Sample Task

The study presented here was the first to show that mice were able to perform an olfactory delayed matching to sample task. Rats have previously been shown to be able to perform such tasks (Lu et al., 1993), but there were no studies showing mice could perform them.

Experiments using olfactory non-matching to sample tasks have since been published (G.-D. Huang et al., 2020; Jiang et al., 2022). These studies did not use an olfactometer, but used odour pots and the mice responding by digging in the correct odour pot to uncover a reward. This is a similar response from the mice as the conditioned odour preference test. While this test has the advantage of relying on a more natural behaviour to respond, it would take much longer between trials than the operant olfactometer, allowing studies using the operant olfactometer to collect more data in a similar time.

5.1.5 Female 5xFAD And Wildtype Mice Perform Better Than Males On The Olfactory Delayed Matching To Sample Task

The female mice performed better than the male mice on the olfactory delayed matching to sample task, making fewer errors and reaching longer delays. This suggests that the female mice have better working memory than the males, at least when tested

on goal maintenance type tasks. Reports of sex differences in the working memory of mice have been mixed. Fertan et al. (2019) found that female mice had poorer working memory than males in a Hebb-Williams maze, and a meta-analysis of working memory in the radial arm maze found that males performed better than females (Jonasson, 2005). On the other hand, Bimonte et al. (2000) tested both mice and rats in water radial arm mazes and found better working memory in the females of both species.

Spontaneous alternation is a commonly used measure of working memory. Carroll et al. (2010) found poorer spontaneous alternation in a Y maze in female mice, while other studies found no sex differences (Carreira et al., 2017; Stevanovic et al., 2022).

Unlike these other tests, the operant olfactometer does not require locomotion nor involve the mouse navigating the testing apparatus. This leads to the possibility that the matching to samples task in the operant olfactometer is less of a hippocampal-dependant task than the other studies, which could explain some of the differences in the sex differences found in these studies.

Female 5xFAD mice show more severe increases in inflammation and Aβ deposits than male 5xFAD mice (Manji et al., 2019; Sil et al., 2022), which could lead one to predict any impairment in working memory would be more likely to be observed in the female than the male 5xFAD mice.

More conservative responding was found in the female mice on the olfactory sensitivity task and, while the same signal detection measures could not be applied to the delayed matching to sample task, it is possible that the female and male mice were also using different response strategies on the matching to sample task as well.

5.1.6 5xFAD Mice Are Not Impaired On An Olfactory Delayed Matching To Sample Task

The 5xFAD mice performed as well as the wildtype mice in the olfactory delayed matching to sample task. This suggests the at the 5xFAD mice do not have a working memory deficit at six to seven months of age. 5xFAD mice have been tested in a delayed reaction task in an olfactory H maze and showed deficits as early as four months old (Girard et al., 2013). Similar to working memory (Funahashi, 2017; Jones, 2002), this olfactory H maze task has been shown to depend on frontal cortex function (Del'Guidice et al., 2009). While they do not claim that the task is a working memory task, successfully completing the task does require the mouse to remember the side of the first chamber it was rewarded in, and maintain this memory for the amount of time required to reach the opposite test chamber, which would meet the definition off a goal maintenance working memory task (Dudchenko et al., 2013). While there is no explicit delay period in the design of this task, there is an inherent delay given the time it takes for the mouse to travel from one test chamber to the opposite chamber. However, this delay could be variable and influenced by any motor deficits, such as those show to occur in 5xFAD mice (O'Leary, Mantolino, et al., 2020).

The working memory of 5xFAD mice was explicitly tested in olfactory non matching to sample tasks (G.-D. Huang et al., 2020; Jiang et al., 2022). Both studies tested 5xFAD mice and, similar to the study presented here, found no deficits in the 5xFAD mice in the non-matching to sample test, but when they performed a capacity test, increasing the number of odour pots the mice needed to remember, they did find a deficit in working memory capacity in the 5xFAD mice (G.-D. Huang et al., 2020; Jiang et al., 2022). These findings suggest that while there may be no deficit in a goal-maintenance-type working memory task, there is a deficit in memory-capacity-type working memory tests.

In studies of working memory in human patients with AD mixed results have also been found (Huntley & Howard, 2010). It appears that any working memory deficits in AD are very specific and will only apparent in specific tasks.

Investigations of the impact of AD on working memory have produced conflicting results. While some studies find impaired working memory, others find no such impairments (Huntley & Howard, 2010). Using 0, 1, and 2 - back working memory tasks, Fraga et al., (2018) found that AD patients had impaired working memory compared to healthy controls. While on a letter - number sequencing task, MCI patients show a mild impairment (Johns et al., 2012), and performance on an n - back task was similar between MCI patients whose condition progressed, and those whose condition was

stable, though there were differences in the theta brain wave activity while performing the task (Deiber et al., 2009).

It may be that these different findings regarding working memory in AD is due to the nature of the different tasks used and how specific to working memory the task is. Huntley and Howard (2010) found that working memory impairments are more likely to be found in tasks which also require the central executive system, suggesting it may be impairments of the central executive that are being detected in these test, and not working memory.

Interestingly, impairments in some working memory tests in AD may be due specifically to misbinding errors, where the patients swap features of objects being held in their memory causing them to misremember the features of presented objects (Zokaei & Husain, 2019). This is in contrast to PD, where errors appear to be due to random guessing.

5.2 What More Needs To Be Done?

The female 3xTg-AD mice showed olfactory sensitivity deficits at six months of age. At what age this deficit first appears remains to be found. Testing of female 3xTg-AD at younger ages would allow for the comparison of when this deficit appears in relation to the other cognitive symptoms and neuropathologies. This information could reveal

whether the sensitivity deficits proceed the appearance of cognitive symptoms and which neuropathologies, such as Aβ, tau, and inflammation, are correlated with the sensitivity deficits.

The 5xFAD mice did not show working memory deficits at 6 months of age. It would be interesting to test these mice at older ages to see if deficits appear later in their life. Other studies have found olfactory working memory deficits in 5xFAD mice as early as three months of age, however, these deficits were only found in working memory capacity tests, not in accuracy tests (G.-D. Huang et al., 2020; Jiang et al., 2022). The delayed matching to sample task increases the difficulty of the task by increasing the delay period the initial odour must be remembered, rather than increasing the number of odours that needs to be remembered, as in the capacity tests.

The olfactory matching to sample task also introduced large jumps in the delay the mice needed to remember the first odour stimulus, going through 2, 5, 10, and 30 sec. The size of these jumps may have made it difficult for the mice to learn the next delay. The relatively small proportion of mice which were able to complete the 10 and 30 second delays also reduced the power of the design to detect deficits at these longer delays. Using smaller increases in the delay may allow for a more fine grained analysis of working memory in these mice.

5.3 Summary And Conclusions

Together, these studies show the remarkable abilities of mice when performing olfactory based tasks, from their ability to detect very low concentrations of odours, to their ability to perform a complex matching to sample task.

In both AD models tested the mice showed no issues with olfactory detection at the higher odour concentrations used, and any deficits did not appear until very low concentrations of the odours were tested. Thus, while olfactory dysfunction in human patients with AD could lead to the conclusion that one should use caution when testing mouse AD models on olfactory based tasks, these results indicate that as long has easily detectable odour concentrations are used, such tasks are appropriate, and could even be preferred due the abilities of mice on olfactory based tasks.

Sex differences were found in all three studies, highlighting the importance of examining sex differences when working with mouse models of diseases.

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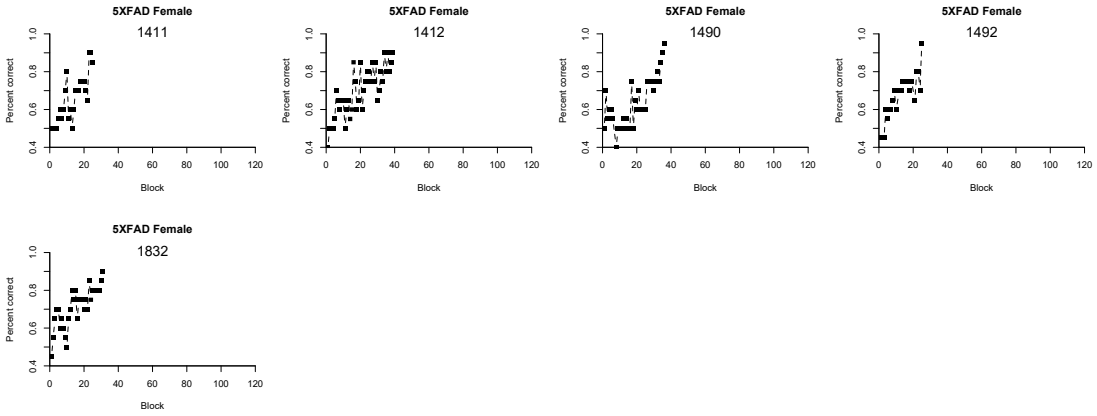
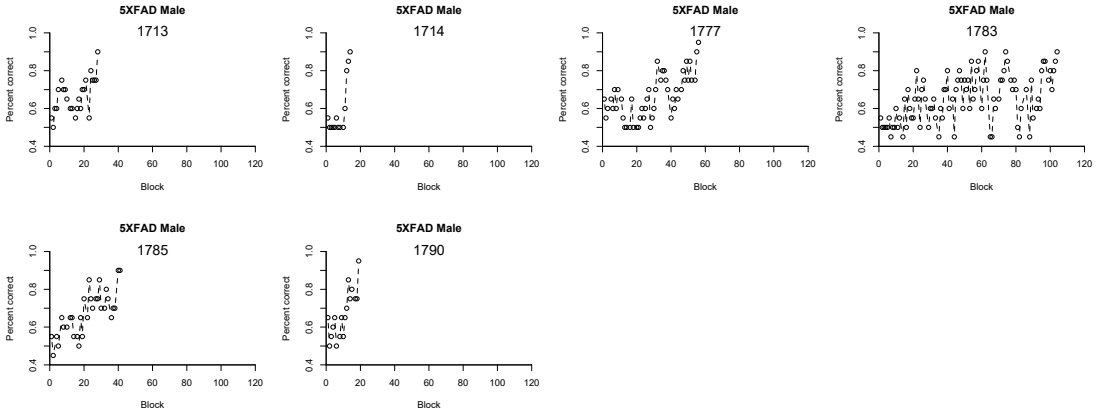


Supplementary Figure 1B



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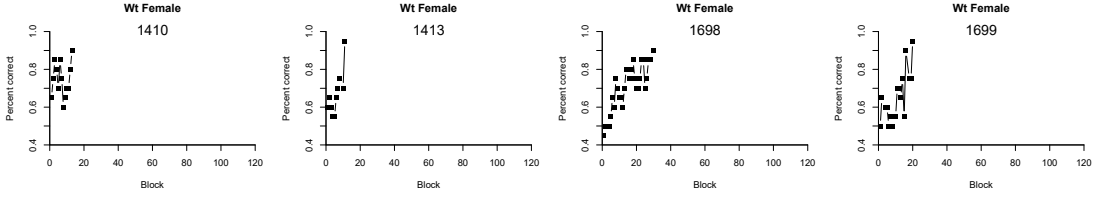
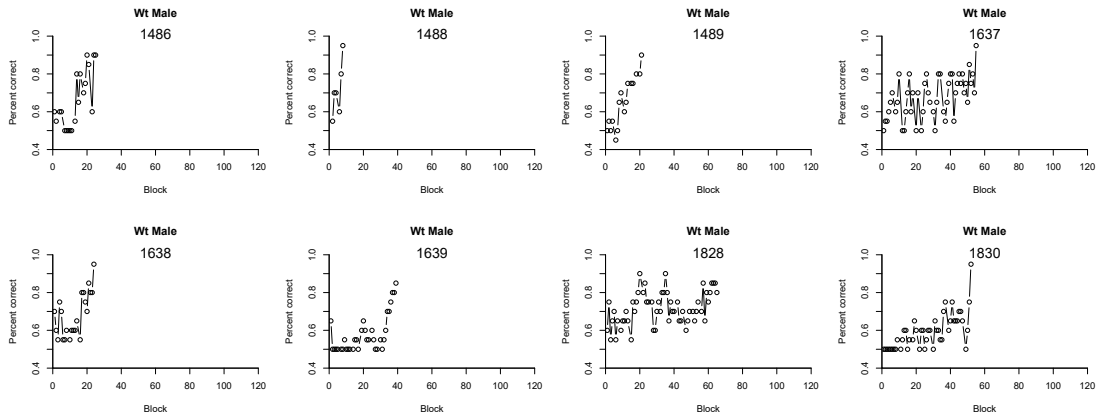
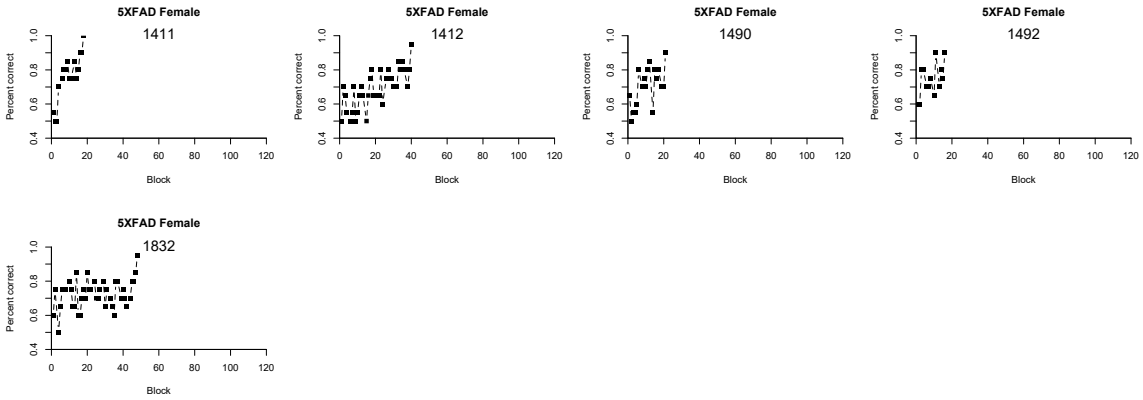


Supplementary Figure 1D



Supplemental Figure 1: Individual learning curves of the A) 5xFAD females, B) 5xFAD males, C) WT females and D) WT males on the 2 second delayed matching to sample task. Plots show the percent correct responses in each block of 20 trials the mouse completed prior to finishing the task at this delay. The four digit numbers are the subject identifiers.

Supplementary Figure 2A



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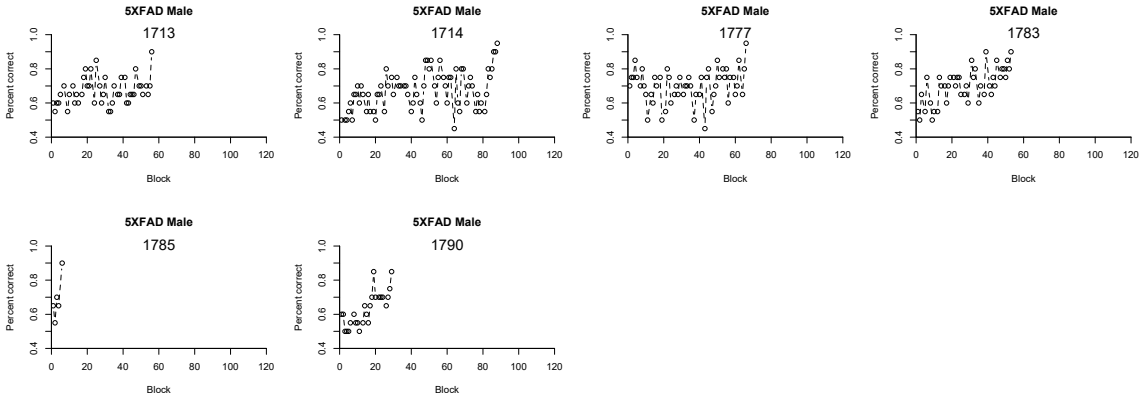

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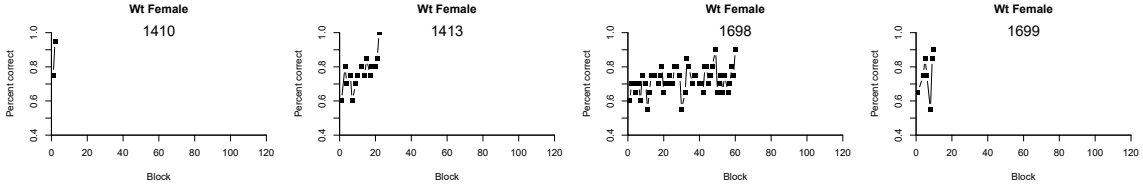

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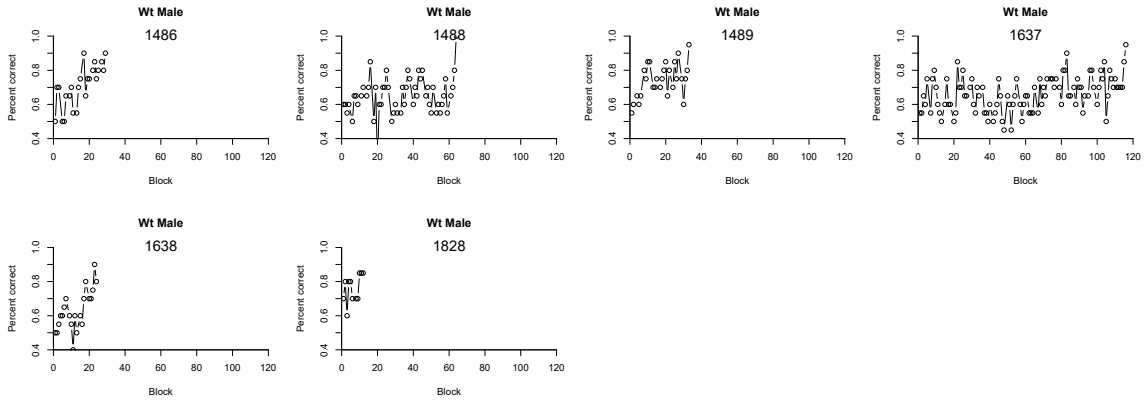
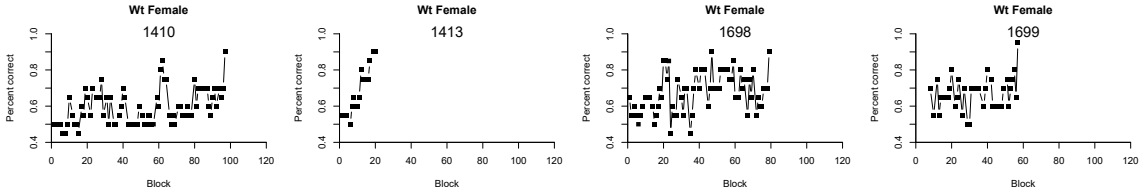


Supplemental Figure 2: Individual learning curves of the A) 5xFAD females, B) 5xFAD males, C) WT females and D) WT males on the 5 second delayed matching to sample task. Only mice which completed the task are shown. Plots show the percent correct responses in each block of 20 trials the mouse completed prior to finishing the task at this delay. The four digit numbers are the subject identifiers.

Supplementary Figure 3A

Supplementary Figure 3C



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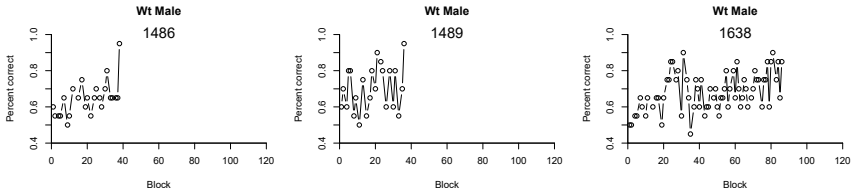
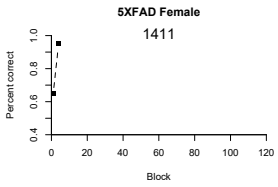


Supplemental Figure 3: Individual learning curves of the A) 5xFAD females, C) WT females and D) WT males on the 10 second delayed matching to sample task. Only mice which completed the task are shown. Plots show the percent correct responses in each block of 20 trials the mouse completed prior to finishing the task at this delay. The four digit numbers are the subject identifiers.

Supplementary Figure 4



Supplemental Figure 4: Individual learning curve of the 5xFAD female mouse which completed the task on the 30 second delayed matching to sample task. Plot shows the percent correct responses in each block of 20 trials the mouse completed prior to finishing the task at this delay. The four digit number is the subject identifier.