Behavioural and neuropathological features of the AppNL-F and AppNL-G-F mouse models of Alzheimer's disease

DENISE FORESTEIRE

Behavioural and Neuropathological Features of the App^{NL-F} and App^{NL-G-F} Mouse Models of Alzheimer's Disease

Denise Marie Foresteire

Durham University

Department of Psychology

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Abstract

Anxiety and social withdrawal often emerge during Alzheimer's disease (AD); investigating their neurobiological underpinnings is thus important. To this end, we examined social and anxiety-related behaviours in male and female App^{NL-F} and App^{NL-G-F} mice aged 8 and 15-months, and characterized the microstructural integrity of neural tissue using *ex vivo* diffusion tensor imaging. These novel APP knock-in mice have translational advantages given that they model Aβ pathology without the overexpression of APP. The App^{NL-G-F} mice exhibit a threefold-faster Aβ deposition compared to the App^{NL-F} mice: this provides the added opportunity to explore the differential effects of soluble Aβ oligomers and insoluble fibrillar Aβ on behaviour and its neural substrates. Using the Crawley 3-chamber protocol, preference for sociability in the 8 and 15-month old App^{NL-F} and App^{NL-G-F} mice was intact. A mild impairment in preference for social novelty in the 8-month old App^{NL-F} and App^{NL-G-F} mice, as well as a mild social olfaction deficit in the 8-month old female App^{NL-G-F} mice and the 15-month old App^{NL-F} and App^{NL-G-F} mice, was observed. Regarding the anxiety assessing tasks, both the 8 and 15-month old App^{NL-F} mice displayed unaltered behaviour in the open field and elevated plus-maze; in contrast, the 8-month old App^{NL-G-F} mice combined an ostensibly anxiogenic open field profile with an ostensibly anxiolytic plus-maze profile. This ostensibly anxiolytic plus-maze profile persisted in the 15-month old App^{NL-G-F} mice. Together, these results suggest that the App^{NL-G-F} mouse may enable modeling of the neurobiological links between emergent disinhibition-type behaviour and AD. Consistent with this suggestion, notable microstructural alterations were observed in the orbitofrontal and anterior cingulate cortices of both the younger and older App^{NL-G-F} mice. Microstructural alterations did not emerge in the App^{NL-F} mice until the later age point. Insoluble Aβ likely contributes to the behavioural and neuropathological characteristics seen in the App^{NL-G-F}, but not the App^{NL-F}, mice.

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Abbreviations

Aβ	Amyloid Beta
AD	Alzheimer's Disease
aMCI	Amnestic Mild Cognitive Impairment
APP	Amyloid Precursor Protein
AxD	Axial Diffusivity
BSA	Bovine Serum Albumin
DAB	3,3'-diaminobenzidine
DT	Diffusion Tensor
DTI	Diffusion Tensor Imaging
FA	Fractional Anisotropy
FAD	Familial Alzheimer's Disease
hAPP	Human Amyloid Precursor Protein
h	hour(s)
NL-F	Swedish (K670N/M671L) & Beyreuther/Iberian (I716F) mutation
NL-G-F	Swedish (K670N/M671L), Arctic (E693G) & Beyreuther/Iberian (I716F) mutation
NPS	Neuropsychiatric Symptom(s)
MBI	Mild Behavioural Impairment
MCI	Mild Cognitive Impairment
MD	Mean Diffusivity
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
OCT	Optimal Cutting Temperature Compound
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PN	Product Number
PSEN1	Presenilin-1
PSEN2	Presenilin-2
RD	Radial Diffusivity
ROI	Region(s) of Interest
Tx-PBS	Triton-X 100 in Phosphate Buffered Saline

Candidate's declaration

I, Denise Marie Foresteire, do hereby certify that this thesis, submitted for the degree of M.Sc. by Research, has been written by me, and that it is the record of work carried out principally by myself in collaboration with Dr James Dachtler, and that it has not been submitted in any previous application for any degree. The work presented herein was supported in part by an Alzheimer's Society (United Kingdom) Fellowship (AS-JF-15-008) awarded to Dr Dachtler.

Dr Dachtler carried out the behavioural testing of the 8-month old cohort of mice, and all transcardial perfusions. Dr Dachtler and Dr Eleftheria Pervolaraki performed the MR imaging. The author was responsible for conducting all of the remaining procedures that are described herein including the behavioural testing of the older cohort of mice, all statistical analyses related to the behavioural tests, post-processing and statistical analyses of the diffusion imaging data, and all immunohistochemistry.

Publications

Work conducted as part of this thesis has formed the basis of the following peer reviewed publication:

Pervolaraki, E., Hall, S.P, Foresteire, D., Saito, T., Saido, T.C, Whittington, M.A., Lever, C., Dachtler, J. (2019). Insoluble Aβ overexpression in an *App* knock-in mouse model alters microstructure and gamma oscillations in the prefrontal cortex, and impacts on anxiety-related behaviours. *Disease Models and Mechanisms*, *12*(9), 1-12. DOI: 10.1242/dmm.040550

Statement of Copyright

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CHAPTER - 1

INTRODUCTION

1.1 Alzheimer's disease: prevalence and impact

Alzheimer's disease (AD) is the leading cause of dementia in the elderly (≥ 65 years), and a significant international health-care burden. Approximately two-thirds of the estimated 48-million dementia cases worldwide are attributed to AD (Prince et al., 2015), and by 2050 the number of people living with AD is projected to triple due mainly to the increase in global life expectancy (Livingston et al., 2017). Despite intensive efforts by the scientific community to establish therapeutics that are capable of altering the course of this chronic and progressive neurodegenerative illness, there are no forthcoming treatments that can cure or prevent the disease from developing (Mehta et al., 2017; Cummings et al., 2016). The insidious onset of neurodegeneration that occurs in AD, initially within the medial temporal structures and ultimately throughout the cerebral cortex (Delacourte et al., 1999; Braak & Braak, 1991; Hyman et al., 1984), leaves patients unable to care for themselves, and the effect of this on the patients, caregivers and societies-at-large cannot be overstated (Winblad et al., 2016). It is predicted that by 2060 'AD and other dementias' will be the third leading cause of death globally and the most common cause of death in high-income nations (World Health Organization, 2018). In response to these issues, AD has been declared one of the great health-care challenges of the century (Nichols et al., 2019; Scheltens et al., 2016) as researchers strive toward new ways of identifying individuals who are at-risk of developing AD with the aim to improve quality of life and importantly, establish therapeutics that can act prior to any substantial neurological change.

1.2 Neuropsychiatric symptoms as an early sign of emerging Alzheimer's disease

AD has long been characterized clinically as a disorder of cognition resulting in symptoms such as episodic memory loss, executive dysfunction, visuospatial deficits and topographical disorientation yet these cognitive changes represent only one aspect of the behavioural alterations that are known to manifest in the disease. Neuropsychiatric symptoms (NPS) are nearly universal in AD (McKhann et al., 2011),

and are known to occur more frequently in people with the disease than in the general population (Lyketsos et al., 2011). NPS can be delineated into five categories (Ismail et al., 2016) to describe changes in affect (e.g., anxiety and depression), motivation (e.g., apathy and indifference), impulse control (e.g., disinhibition and agitation), perception (e.g., delusions and hallucinations) and social appropriateness (e.g., loss of tact). According to Steinberg et al. (2008), Lyketsos et al. (2002) and Mega et al. (1996) an estimated 80-97% of AD patients will experience at least one NPS over the duration of the disease. Furthermore, the presence of NPS in AD has been associated with a range of adverse effects, such as greater levels of patient-family dysfunction and caregiver distress (Storti et al., 2016; Fischer, Ismail & Schweizer, 2012; Allegri et al., 2006), impaired quality of life for patients and their caregivers (Hongisto et al, 2018; Shin et al., 2005), hastened functional decline (You et al., 2015; Mortimer et al., 1992) and higher rates of placement in residential care (Voisin et al., 2010; Gilley et al., 2004). As a result, investigating the occurrence of NPS in mild cognitive impairment (MCI) is relevant. MCI confers a risk of AD, and is recognized as the earliest symptomatic stage of the disease. It is a heterogeneous entity whereby 5-15% of cases will evolve to identifiable AD (Albert et al., 2011).

There is substantial evidence to demonstrate that NPS are highly prevalent in MCI. For example, an early informative study by Hwang et al. (2004) found that there were significant differences in the prevalence of NPS between individuals with MCI and matched controls. Apathy (39%) and anxiety (25%) were amongst the two most common disturbances reported by people with MCI, with apathy being particular common in amnestic MCI (aMCI), the most empirically validated precursor to AD. Similar results have been reported in large population-based studies. For instance, Geda et al. (2008) found that individuals with MCI were significantly more likely to report having one or more NPS (51%) compared to people with normal cognitive ageing (27%). Taking into account the odds ratio (OR) and frequency of a symptom, the authors identified apathy (OR, 4.53; $p < 0.001$) and anxiety (OR, 3.00; $p < 0.001$) as the first and third most distinctive features (respectively) between the two groups. Moreover, apathy was higher in aMCI than non-amnestic MCI. Similarly, in a population-based study by Peters et al. (2012), participants with MCI were twice

as likely (31%) to report at least one NPS compared to the participants who were cognitively normal (15.1%), yet less likely compared to the participants with AD dementia (61%). Apathy and anxiety were amongst the most frequently reported symptoms in MCI, as well as aMCI and AD dementia; both were associated with a significant upward trend in prevalence (cognitively normal ≤ MCI or aMCI ≤ AD). As such, researchers have examined whether comorbid presentation of NPS and MCI confers a greater risk for developing AD.

There is evidence that the presence of NPS in MCI increases the risk of AD dementia. In a longitudinal study of MCI patients, Rosenberg et al. (2013) found that the co-morbid presentation of NPS with MCI resulted in a clinically significant risk of progression from MCI to AD; however, whether NPS were a prodrome of the neuropathological process of AD is unclear without biomarker evidence. Similarly, Peters et al. (2013) followed 230 MCI patients over a 3-year period and found that NPS constituted a significant risk of transition to AD dementia. The perspective of NPS as a harbinger of AD has existed for at least twenty years (e.g., Chung & Cummings, 2000; Berger et al., 1999). However, attention to NPS in AD is growing as researchers become more aware of the need to identify individuals at-risk for AD prior to any substantial cognitive decline (Dubois et al., 2016). As a result, Ismail et al. (2016) proposed the diagnostic construct of mild behavioural impairment (MBI) to describe the late-life emergence (≥ 50 years) of sustained and impactful affective and behavioural change as a prodromal feature of AD. MBI is conceptualized to occur prior to or in tandem with the initial signs of AD-related cognitive decline.

Most recently Wise et al. (2019) demonstrated that the late-life emergence of NPS could signal impending AD, and that NPS can manifest prior to AD-related cognitive symptoms. In this prospective cohort study of cognitively normal individuals (≥ 60 years) with no prior history of psychiatric disorder, 59% of the 1,998 participants who developed a cognitive disorder during the course of the study reported the occurrence of NPS prior to a cognitive diagnosis. In contrast, of the 3,124 participants who remained free from a cognitive diagnosis only 24.5% reported an occurrence of NPS. With regard to the 392 participants who received a specific diagnosis of AD, NPS

preceded the diagnosis of MCI in 30% of the participants, and an additional 42% of the participants developed NPS between the diagnosis of MCI and AD dementia; 12% reported no NPS and 17% reported NPS after the diagnosis of AD dementia. Additionally, there were 1,032 participants who were diagnosed with aMCI during the course of the study, of which 54% developed NPS before the aMCI diagnosis and a further 24% developed NPS either after the aMCI diagnosis or before dementia (for those who progress from normal to dementia). This is a key study that supports the MBI construct, and demonstrates that emergent NPS are indeed associated with future AD-related cognitive decline as opposed to ageing per se. It also indicates a rationale for investigating anxiety and social withdrawal as early emergent symptoms of AD. Anxiety was amongst the most common NPS to emerge prior to MCI, and also between the diagnosis of MCI and dementia onset. It was also common for apathy to manifest before and after dementia, albeit somewhat less common to manifest prior to MCI.

1.3 Increased anxiety and reduced social engagement in Alzheimer's disease

In reiteration of the previously cited literature, anxiety is a common feature of MCI (Apostolova & Cummings, 2008). In some studies up to 71% of AD patients reported anxiety concerns (Ferretti et al., 2001). Furthermore, the co-occurrence of anxiety with MCI has been associated with an increased risk of AD dementia (Mah et al., 2015). Moreover, in a study by Palmer et al. (2007), of the MCI patients who also displayed anxiety symptoms, 83.3% developed AD over the 3-year time point as opposed to 40.9% who had MCI without anxiety. Furthermore, Pietrzak et al. (2015) studied healthy older adults with elevated Aβ levels and found that those who also had anxiety symptoms experienced greater cognitive decline. Thus, anxiety appears to be an early feature of AD that has a mediating effect on clinical outcomes.

Early AD-related neurodegenerative changes may explain the increase in anxiety. Donovan et al. (2018) and Hanseeuw et al. (2018) investigated the relationship between brain Aβ plaque burden, as determined by PiB-PET (Pittsburgh Compound B Positron Emission Tomography), and anxiety in cognitively normal community dwelling individuals aged 62 to 90 years. Donovan et al. (2018) assessed participants (n=270) longitudinally and found that higher baseline Aβ burden was significantly associated

with worsening anxiety symptoms over time. Hanseew et al. (2018) investigated participants (*n*=188) at a single time point and found that higher subcortical Aβ burden was significantly associated with greater self-report measures of anxiety; there was no association when assessing cortical Aβ burden which precedes subcortical Aβ deposition. Moreover, anxiety was highest in APOEε4 carriers (individuals at risk of AD) with subcortical amyloidosis. Donovan et al. (2018) and Hanseeuw et al. (2018) replicated the findings in Holmes et al. (2016). Based on the participants' increased levels of Aβ they were likely to have tau accumulation and neurodegeneration that may have mediated the relationship to anxiety. Ramakers et al. (2013) found that the presence of anxiety in MCI patients was associated with abnormal cerebrospinal fluid (CSF) $A\beta_{42}$ [OR 2.3, 95% CI 1.6–3.3] and CSF total tau [OR 2.6, 95% CI 1.9–3.6], and an abnormal ratio of $A\beta_{42}$ and total tau [OR 3.1, 95% CI 2.0–4.7]. These are biomarkers of AD that signal neurodegeneration and predict the progression from MCI to AD.

Social withdrawal is an equally common and debilitating behavioural change in AD (Feldman et al., 2004; Doody et al., 1995). It refers to an individual's withdrawal from their social networks as well as a loss of interest in previously enjoyed social activities. Social withdrawal as a clearly defined phenotype of AD has yet to be well studied; however, the previously cited literature on apathy is relevant to an extent based on shared behavioural features such as a reduced motivation to engage. There is evidence to suggest that social withdrawal may signal the onset of AD. In a retrospective review of medical records of 100 randomly selected autopsy-confirmed AD patients, Jost and Grossberg (1996) found that social withdrawal was present in 40% of patients as well as it being the earliest recognizable NPS to be reported based on patient and caregiver accounts. In some instances social withdrawal was present up to five years prior to a diagnosis of cognitive disorder. Similarly, a retrospective review by Barnes et al. (2015) of the National Alzheimer's Coordinating Center (NACC) dataset (Beekly et al., 2007) found that 'apathy/social withdrawal' was the first and second most commonly reported initial behavioural symptom depending on the age of the person at the time of diagnosis; ≥ 70 years (5,894; 26%) and ≤ 69 years (1,921; 25%), respectively. Furthermore, social withdrawal may exacerbate the risk of developing dementia.

Remaining socially active in later life has been identified as a potential protective factor against dementia (Fratiglioni, Paillard-Borg & Windblad, 2004). In a longitudinal study of community dwelling residents aged 65 years and older, Scarmeas et al. (2001) found that those with higher levels of social interaction had a significantly reduced risk of cognitive decline and dementia. This finding was supported in a meta-analysis of longitudinal cohort studies (Kuiper et al., 2015); however, Brown et al. (2012) found inconclusive evidence in a smaller, but similar systematic review. In a recent Lancet Commission Report, social isolation was found to constitute 2.3% of the total risk for developing AD (Livingston et al., 2017). The size of one's social network may also influence cognitive health. For example, having a larger social network was associated with lower risk of dementia over a 4-year follow up of cognitively normal, community-dwelling elderly women (Crooks, et al., 2008), and Bennett et al. (2016) found that AD patients with larger social networks experienced slower cognitive decline compared to AD patients with small social networks. Moreover, elderly people who identify as lonely have nearly double the risk of developing AD (Wilson et al., 2017); however, subjective feelings of loneliness are not necessarily the same as objective measures of social withdrawal (Cacioppo et al., 2015). Together, the evidence suggests that social withdrawal may be a harbinger of AD and/or impact on the progression of disease. For these reasons, understanding social withdrawal in the context of AD has become a matter of priority. For example, the PRISM consortium (Cuthbert et al., 2019) is a recently established European Union funded research initiative dedicated to investigating the neurobiology of social withdrawal in neurological disorders such as AD.

Anxiety and reduced social engagement in AD has traditionally been placed within the framework of psychiatric nosology, yet it is unclear whether the underlying neurobiology of these symptoms differs when in the context of the AD brain (Lanctôt et al., 2017; Rosenberg, Nowrangi & Lyketsos, 2015). From the perspective that these symptoms are a consequence of AD pathogenesis, it is reasonable to suggest that they are based in unique biological pathways which may in part explain why therapeutics that are typically prescribed to treat psychiatric illness are ineffective in the AD patient (Lanctôt et al., 2017; Rosenberg, Ismail et al., 2016; Nowrangi & Lyketsos, 2015; Geda et

al., 2013). Given the prevalence of these symptoms in AD and the range of adverse effects associated with their presence in AD, the need to develop effective treatments is a matter of urgency. Moreover, given the growing consensus that the late-life emergence of anxiety and social withdrawal may constitute harbingers of AD and impact on disease progression, treatment of these symptoms may provide potential connections to early prevention of AD dementia (Katona et al., 2007). To that end, a main objective of this thesis is to probe the microstructural integrity of the neural tissue of a new generation of AD model mice using *ex vivo* diffusion tensor imaging (DTI). Indications of neural alteration provide a start point in exploring associated underlying altered mechanisms, and in turn, elucidating altered mechanisms is a key stage in the drug discovery process. AD-related neural regions that are relevant to changes in anxiety and social behaviours remain unclear; however, candidate regions can be identified based on the current literature. The following section briefly outlines the rationale behind our chosen candidate regions of interest.

1.4 Neuroanatomical correlates of anxiety and social behaviour

Our candidate regions of interest for DTI were the orbitofrontal cortex, anterior cingulate cortex, the amygdala and the dorsal and ventral hippocampus. There is consistent evidence to suggest that these neuroanatomical regions mediate anxiety behaviours and underlie normal social functioning in rodents as well as humans, and in many instances contribute to both anxiety and social behaviours. The role of the rodent amygdala in anxiety-like behaviour (a response to perceived threat), and more specifically fear (a response to real threat), is well evidenced across laboratories using a variety of experimental techniques (Davis, Rainnie & Cassell, 1994). For example, stimulation of the amygdala elicits a pattern of anxiogenic behaviour whereas amygdalar lesions produce an anxiolytic effect in tests of unconditioned fear (Davis, 1992). The fear-related neurocircuitry identified in rodents provides a basis for understanding the neurocircuitry of anxiety disorder in humans. Its basic elements appear well preserved across species and are likely to support similar functions in humans. In fact a number of *in vivo* neuroimaging studies have demonstrated an association between anxiety and heightened amygadalar activation in humans (Shin & Liberzon, 2010). Adolphs (2010) assigned a slightly broader function to the amygdala

as a component in a neural network, including the orbitofrontal cortex, which processes the saliency and relevance of stimuli, particularly stimuli that may signal unpredictability or potential threat. Thus, the amygdala is additionally viewed as having functional implications in social behaviour. Similarly, Davidson (2002) highlights evidence across species for the role of the amygdala and orbitofrontal cortex (and prefrontal cortex in general) as part of a neural network that mediates affective processing and emotional regulation, and governs different aspects of anxiety. Moreover, Poulin et al. (2011) and Liu et al. (2010) found neurodegenerative changes in the amygdala in MCI patients using volumetric MR imaging, and these changes in volumetric MR measurements predicted conversion to AD.

McHugh et al. (2004) demonstrated a specific role for the ventral hippocampus in response to anxiogenic stimuli. Rats with cytotoxic ventral hippocampus lesions showed reduced anxiety-like behaviour across a number of unconditioned tests of anxiety, including a modified version of the elevated plus-maze. This study extended the findings of Bannerman et al. (2003) by demonstrating that the anxiolytic effects of ventral hippocampus lesions were distinct from the effects of dorsal hippocampus and amygdala cytotoxic lesions in unconditioned tests of anxiety. Thus, McHugh et al. (2004) propose only a limited role for the amygdala in rodent anxiety and instead link the amygdala more specifically to regulating fear responses. The hippocampus has also been implicated in social behaviour. For example, in McHugh et al. (2004), the rats with ventral hippocampus lesions displayed an increase in social behaviour when allowed to freely explore a novel conspecific; this was presumably due to their reduced anxiety. Hitti and Siegelbaum (2014) demonstrated that the silencing of mouse dorsal CA2 pyramidal neurons resulted in a pronounced deficit in preference for social novelty, but not sociability, in the Crawley 3-chamber test, and this was presumably due to a disruption in sociocognitive memory processing. Okuyama et al. (2016) evidenced a role of the ventral CA1 hippocampal region in ability of mice to discriminate a novel conspecific from a previously encountered conspecific. The authors concluded that vCA1 and dCA2 have a shared, albeit undefined role in sociocognitive processing. Moreover, pathology in the hippocampus occurs early in AD, and predicts conversion from MCI to AD (Liu et al., 2010; Devanand et al., 2007).

Etkin, Egner and Kalisch (2011) review a wealth of data across species that implicates the anterior cingulate cortex and medial prefrontal cortex (including orbitofrontal cortex) in the processing of anxiety and fear. These authors further highlight the role of these regions in an array of socio-emotional processes. Apathy, which is characterized in part by social withdrawal (Marshall et al., 2013), is likely to be mediated by a frontal-subcortical circuit, with particular association to the anterior cingulate cortex (Moretti & Signori, 2016; Cummings, 1993). A role for the anterior cingulate cortex and the orbitofrontal cortex in apathy in mild to moderate AD was evidenced using functional MR imaging (Marshall et al., 2013) as well as structural MR imaging (Bruen et al., 2008). Interestingly, in Okello et al. (2009), the anterior cingulate cortex had the highest Aβ burden in MCI patients relative to controls, and a higher anterior cingulate Aβ load predicted faster conversion from MCI to AD.

As stated previously, an objective of identifying a link between brain anatomy and neurodegenerative change is that it provides a basis for investigating the associated functional and biochemical alterations that drive anxiety and social withdrawal symptoms in AD. A more substantive understanding of these early-altered mechanisms may contribute to the future development and testing of novel therapeutics. Thus, we aim to capture and characterize a putative neural signature associated with anxiety-like behaviours and altered social behaviour in AD mice using DTI, which may also address the need of identifying a sensitive imaging marker that could help facilitate the detection of MBI cases with an underlying neurodegenerative process (Canevelli et al., 2016). Animal models form a crucial component in AD research. They allow for the ability to probe more precisely the neuronal circuits involved in behavioural change (Calhoon & Tye, 2015; Cryan & Holmes, 2005), and record more accurately the spatiotemporal pattern between disease processes and pathology. The following section outlines the features of the mouse models that are used in this thesis.

1.5 App^{NL-F} and App^{NL-G-F} knock-in mice: improved models of Alzheimer's disease

Model organisms have been instrumental in AD research, with the most common model organism being the laboratory mouse (*Mus musculus*). The first AD model

mouse was generated in 1995 and since that time over 100 genetically engineered mouse models have been developed for AD research (Hall & Roberson, 2012). AD appears to be a uniquely human disorder. No condition that faithfully recapitulates the key clinicopathological aspects of AD has ever been identified in a nonhuman species (Platt, Reeves & Murphy, 2013). Some mammals do accumulate Aβ in the brain as they age, however, neurofibrillary tangles are rare in these species and the downstream effects of aberrant Aβ accumulation (*e.g.*, neuronal loss, dementia-like syndrome) are practically non-existent (Gerhauser et al., 2012). The ability to generate AD model organisms through genetic manipulation was made possible by the discovery of rare hereditary mutations that result in pathologically and clinically proven cases of the disease.

Autosomal dominant familial AD (FAD) is a rare form of early onset AD (EOAD) (≤ 65 years) caused by a hereditary mutation in one of at least three genes, which code for Amyloid Precursor Protein (*APP)*, Presenilin-1 (*PSEN1)* or Presenilin-2 (*PSEN2)*, all of which affect the processing of amyloid-β (Huang & Mucke, 2012; Bateman et al., 2011). Sporadic late-onset (≥ 65 years) AD has an unclear aetiology and accounts for more than 90% of all cases. Although FAD is responsible for only 1% of the diagnosed occurrences of AD, it shares many of the same molecular and clinical features with sporadic AD. As a result, mice that are transgenic for FAD mutations have been instrumental in identifying pathways implicated in the more common, sporadic form of the disease (Elder, Gama Sosa & De Gasperi, 2010). Despite the positive impact these models have had on AD research, the conventional technology used to generate them has been associated with disadvantages.

The majority of mice used in AD research model Aβ pathology by overexpressing APP. The overexpression paradigm has been shown to cause artifacts that may confound data interpretation (Saito et al., 2014). To overcome these confounds Saito et al. (2014) generated the App^{NL-F} and App^{NL-G-F} mice. These mice have humanized Aβ sequences with familial mutations in the endogenous APP mouse gene. Mice carrying the Swedish (K670N/M671L) and Beyreuther/Iberian (I716F) mutations, termed App^{NL-F}, exhibit an increase in total Aβ levels with an elevated ratio of $Aβ_{42}$ to $Aβ_{40}$. Mice

carrying the additional Arctic (E693G) mutation, referred to as App^{NL-G-F}, exhibit a more oligomerization-prone Aβ peptide that results in a threefold faster, more aggressive Aβ deposition compared to the App^{NL-F} mice (Saito et al., 2014). Both models typify the pathology seen in preclinical AD without APP overexpression or the interruption of other mouse genes. However, these models do not develop tauopathy. These models are anticipated to have greater translational value, and are also suitable for this thesis as they model the initial, preclinical aspects of AD.

1.6 Thesis Aims and Objectives

There are relatively few studies to date that characterize the behaviour of the App^{NL-F} and App^{NL-G-F} mice. The first aim was to extend the behavioural profile of these next generation APP knock-in mouse lines to investigate for phenotypes analogous to the anxiety and social withdrawal symptoms commonly observed in AD. With limited insight into the neurobiology underpinning these affective and behavioural anomalies in AD patients, it is necessary to find AD mice that reliably model these symptoms, particularly mice that more authentically recapitulate the AD pathology expressed in humans. Should these model mice have a robustly relevant phenotype they could serve as a valuable translational tool in discovering the mechanisms that drive anxiety and social withdrawal in people with AD. In addition to promoting basic research on a topic that has not yet been fully understood, a practical reason is to assist in the translation of new medical treatments aimed at alleviating these symptoms. Since mechanisms of action and target engagement are likely to differ in the context of the AD brain versus the non-AD brain (Lanctôt et al., 2017; Rosenberg, Nowrangi & Lyketsos, 2015; Insel et al., 2010), identifying appropriate AD mice for the development and testing of novel therapeutics is an additional necessity. Our first objective was to measure the behavioural performance of male and female App^{NL-F} and App^{NL-G-F} mice using commonly employed tests of rodent emotionality and social interaction, and to compare their performance to age-matched APP wild-type control mice. Chapter 2 provides further details of the behavioural approach.

The second aim was to explore the microstructure of behaviourally relevant brain regions using diffusion tensor imaging (DTI), marking the first study to apply DTI to

the App^{NL-F} and App^{NL-G-F} mice. In order to capture and characterize any subtle instances of neurodegenerative change in the APP knock-in mice, the diffusion tensor model was fitted to *ex vivo* MR images, and diffusion indices were extracted from manually drawn *a posteriori* regions of interest. Given that our wider research initiative was to identify putative biological mechanisms driving anxiety and altered social behaviour in AD, DTI was used primarily as a means to identify which of the targeted brains regions warranted investigating for altered oscillatory activity and gene expression. Herein, the DTI approach served to identify microstructural alterations as a sort of proof-of-concept study to address a key idea put forth by Canevelli et al. (2016) in response to the introduction of the MBI construct by Ismail et al. (2016). Canevelli et al (2016) argues for the need of a sensitive imaging marker that could facilitate the detection of MBI cases with an underlying neurodegenerative process, for the critical reason of preventing false positive diagnoses in preclinical AD trials. DTI may also prove useful in the preclinical evaluation of disease-modifying treatments developed to act specifically in early AD stages (Wiener et al., 2017). For this latter reason, DTI was applied irrespective of the behavioural performance of mice, which is justified given that the subtle microstructural alterations captured by DTI can precede overt behavioural change (Alexander et al., 2007). Details of the DTI approach are outlined in chapter 3.

Building upon these primary aims was the decision to evaluate behaviour and its neuroanatomical correlates in both the App^{NL-F} and App^{NL-G-F} models. This study is the first to compare the behaviour and neural alterations of the two models beyond the original Saito et al. (2014) paper. Explicitly comparing the App^{NL-F} and App^{NL-G-F} mice will help identify to what extent their differing pathologies may or may not account for behavioural or neurological change. Specifically, a comparative analysis of these APP knock-in mouse lines allows us to consider the effect and temporal relationship of different amyloid-β assemblies on the manifestation and underlying neuropathy of neuropsychiatric symptoms (Mucke & Selkoe, 2012). This aim was realized by testing two discrete sets of mice with an approximate mean age of 8 and 15-months. The younger cohort of mice offered a unique opportunity to study whether there were any differential effects of soluble Aβ and plaque-based Aβ on behaviour or neuronal tissue.

At the 8-month age point these models express equivalent amounts of soluble Aβ yet the App^{NL-F} mice have little extracellular Aβ plaques whilst the App^{NL-G-F} mice are nearly plaque saturated (Saito et al., 2014). Testing of the older cohort of mice allowed us to investigate any behavioural or neuronal change that may have gone undetected in the younger mouse cohort. Both models provide the additive benefits of being able to isolate the effects of β-amyloidosis in the absence of tauopathy, and to establish phenotypes without the artifacts associated with APP overexpression.

To summarize, the present study was designed in order to better understand the neurobiology of social withdrawal and anxiety symptoms in AD by performing a targeted characterization of two novel APP knock-in mouse models of the disease. We argue that the App^{NL-F} and App^{NL-G-F} mice, which typify the initial stages of AD (albeit without tauopathy) in a more physiologically relevant way compared with earlier transgenic mice, are a practical approach for the *in vivo* screening of symptoms recently linked to preclinical AD in humans. Specifically, we assessed the sociability of the mice using the Crawley three-chamber protocol, and anxiety-like behaviours using the open field and elevated plus-maze. *Ex vivo* DTI was then performed to evaluate the microstructural integrity of the tissue within the orbitofrontal and anterior cingulate cortices, the anterior part of the amygdala, and the dorsal and ventral hippocampus, brain regions that are historically associated with social and anxiety-related behaviour. Finally, an immunohistochemical analysis investigating the extent of Aβ deposition in these regions was carried out on a subset of mice from the 8-month old cohort using the monoclonal anti-Aβ 6E10. Aβ deposition was qualitatively assessed and compared between tissue samples from the wild-type, App^{NL-F} and App^{NL-G-F} mice.

CHAPTER - 2

ANXIETY-RELATED & SOCIAL BEHAVIOURS OF THE App^{NL-F} and App^{NL-G-F} MICE

A major aim of animal models in science is to elucidate the underlying mechanisms of disease, its related behaviours and the neurobiological links between the two, and to assess pre-clinically the viability of novel pharmacological treatments. To this end, AD model mice have traditionally been used to gain insight on the relationship between AD mediated pathology and cognitive symptoms, such as memory loss and executive dysfunction. Cognitive symptoms are indeed a key behavioural feature of AD, yet symptoms such as anxiety and social withdrawal are equally as common and debilitating for the AD patient as well as being a main source of caregiver distress. Moreover, the late-life onset of impactful and sustained psychiatric illness as a potential harbinger of AD is increasingly being recognized, and it is well evidenced that the presence of anxiety and social withdrawal in MCI is associated with a faster progression to dementia. Thus, understanding the neurobiological basis of anxiety and social withdrawal in the context of the AD brain is a topical initiative, and one that requires an appropriate AD model mouse. The App^{NL-F} and App^{NL-G-F} mice represent a new generation of AD mice based on a knock-in approach that enables the modeling of Aβ pathology without the overexpression of APP. Consequently, experimental results derived from these mice are less likely to be confounded by the artifacts associated with earlier transgenic mice. The behavioural phenotypes of the App^{NL-F} and App^{NL-G-F} mice are yet to be extensively characterized, and of the handful of published studies that are currently available, references to social and anxiety-related behaviours are minimal. Thus, the primary aim of this chapter is to extend the behavioural phenotype of the App^{NL-F} and App^{NL-G-F} mice and investigate for behaviours that are reminiscent of the anxiety and social withdrawal symptoms commonly observed in AD. Our main intent is to identify whether these mice enable the modeling of the neurobiological links between anxiety and social withdrawal and AD, and secondarily, to engage in a comparative analysis of the two models.

2.2 Materials and Methods

All experimental procedures were performed under a UK Home Office Project License and Personal Licenses subject to the restrictions and provisions contained in the Animals (Scientific Procedures) Act 1986, and approved by Durham University's Animal Welfare Ethical Review Board.

2.2.1 Animals

2.2.1.1 Subject Mice

APP knock-in mice (Saito et al., 2014) were sourced from the RIKEN BioResource Research Centre (Japan) via Prof. Michel Goedert of Cambridge University's (UK) MRC Laboratory of Molecular Biology. These mice have humanized Aβ sequences with familial mutations in the endogenous APP mouse gene. Mice carrying the Swedish (KM670/671NL) and Beyreuther/Iberian (I716F) mutations, termed $App^{NL\text{-}F}$, exhibit an increase in total Aβ levels with an elevated ratio of $A\beta_{42}$ to $A\beta_{40}$. Mice carrying the additional Arctic (E693G) mutation, referred to as $App^{NL\text{-}G\text{-}F}$, exhibit a more oligomerization-prone Aβ peptide that results in a threefold faster, more aggressive Aβ deposition compared to the $App^{NL\text{-}F}$ mice (Saito et al., 2014). Both models typify the pathology seen in preclinical AD without APP overexpression or the interruption of other mouse genes. However, these models do not develop tauopathy. Further specifications of the model mice are found in Chapter 1.

Upon arrival at Durham University, mice were backcrossed once to the C57BL/6J line (Charles River, UK), which is the same background strain as the previous institutions where they were bred. We then bred heterozygote pairs (*e.g.*, $App^{NL\text{-}F\ (+/-)}$ x $App^{NL\text{-}F\ (+/-)}$) and selected male and female $App^{NL\text{-}F\ (+/+)}$, $App^{NL\text{-}G\text{-}F\ (+/+)}$ and wild-$type^{(-/-)}$ littermate mice for the study. An approximately equal amount of wild-type control mice were selected from the $App^{NL\text{-}F}$ and the $App^{NL\text{-}G\text{-}F}$ litters. Genotyping was performed by the Principal Investigator (PI) and was determined by polymerase chain reaction using an ear biopsy method. The genotyping protocols are reported in Saito et al. (2014).

Behavioural tests were conducted on two discrete sets of experimentally naïve mice. Set 1 (n=58) had a M_{age} of 7.9 months (±.08). Set 2 (n=60) had a M_{age} of 15.4 months (±.04). Age groups were balanced by genotype and in turn balanced by sex; Table 2.1.

Table 2.1 Descriptive statistics per age group with age reported as number of days postnatal (±SEM).

	~ 8 months				~ 15 months			
	Male		*Female*		*Male*		*Female*	
	No.	*Age*	*No.*	*Age*	*No.*	*Age*	*No.*	*Age*
App^{NL-F}	10	238 (±4.41)	9	246 (±1.23)	10	465 (±5.37)	10	468 (±3.15)
App^{NL-G-F}	10	241 (±5.25)	9	234 (±11.96)	10	464 (±1.38)	10	464 (±2.69)
Wild-Type	9	249 (±3.76)	11	246 (±1.23)	10	468 (±0.77)	10	475 (±3.32)

2.2.1.2 Husbandry

Mice were weaned at postnatal day 21 and group housed (3-5 mice/cage) with same-sex littermates in individually ventilated cages (W17.5 x L31 x H14.5 cm). Cage bedding included shavings, hay and paper wool, and each cage had cardboard tubes and either toy domes or hammocks. Mice were maintained in a temperature (18-22° Celsius) and humidity (45-65%) controlled room under a 12:12 light/dark cycle (lights on at 08:00). Water and autoclaved pellet food (Special Diets Services, UK; PN RM1AP) were available *ad libitum*. Mice were handled by animal technicians at minimum every fortnight for basic husbandry needs.

2.2.2 Apparatus & Procedures

2.2.2.1 General Procedures

The author observed in part the PI's testing of the 8-month old cohort of mice then independently tested the 15-month old cohort using the same procedures. Testing was carried out during light cycle hours in a dedicated laboratory (W2.26 x L3.43 x H2.39 m) that was illuminated by standard fluorescent ceiling lights. The mice were acclimatized by transferring the home cages to the laboratory for a minimum of thirty minutes prior to testing. Handling tubes were then used to transport mice between their home cages and the test apparatus. A tripod-supported webcam (Logitech 720p) was positioned 70 cm above the apparatus and ANY-maze™ video tracking software (Stoelting Co., Wood Dale, IL, USA) was used to capture and record an animal's movement and location parameters; recordings were initiated immediately after placing an animal in an apparatus and continued for the duration of the trial. Apparatus were cleaned between subjects with 70% v/v isopropyl alcohol (AZOWIPE™; PN 81103) and were left to air dry for 5 minutes.

The experimenter was present in the laboratory during testing and out of view, except during the elevated plus maze and buried food test when behaviour was live scored. All mice completed the test battery in the following order with at least one days rest between tests: (1) the open field, (2) the elevated plus-maze, (3) social approach and preference for social novelty, (4) social olfaction, and if applicable (5) the buried food test.

2.2.2.2 Open Field

The apparatus (see Figure 2.1) was an empty, open-top box (45 cm^3) constructed entirely of white opaque acrylic. The internal arena floor (44 cm^2) was partitioned into three virtual zones using the ANY-maze™ software. The center zone was set at 17.5 cm^2. The area within 8 cm of the wall was considered the outer zone, and the intermediate zone was the remaining area between the center and outer zones. Mice were released along the back wall of the arena and were allowed to move freely and undisturbed for 30 minutes. ANY-maze™ auto-capture feature recorded the total distance moved in addition to the number of entries into, and the time spent in, each of the three zones. An entry into a zone was counted when an animal's centre point crossed a virtual boundary.

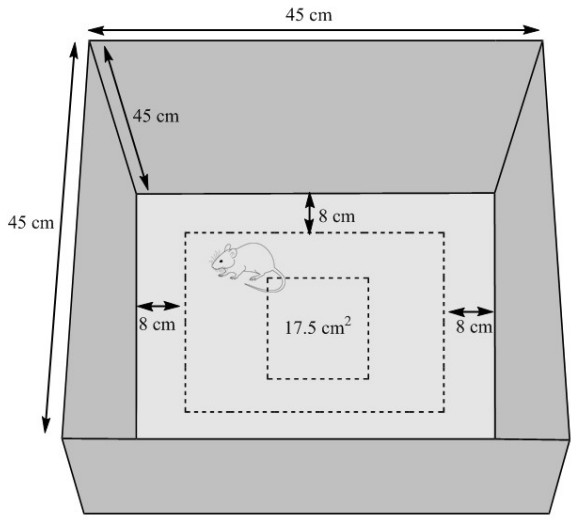


Figure 2.1. **Schematic illustration of the Open Field apparatus.** The open field paradigm is a common measure of exploratory behaviour and general activity in rodents. A mouse freely explores an empty apparatus and anxiety-like levels are principally inferred by the time spent in the centre zone. Less time in the centre zone relative to the wild-type control mice is traditionally regarded as an indication of an anxiogenic-like open field profile.

2.2.2.3 Elevated Plus-Maze

The maze (see Figure 2.2) was constructed entirely of white opaque acrylic and was elevated 38 cm from the floor on a transparent acrylic stand. There were four equally spaced arms (L33 x W5 cm each) extending from a central square resembling the shape of a plus sign. There were two opposing open arms (with no ledges), and two opposing walled arms. The junction of the four arms was 5 cm^2.

Mice were released in the centre of the maze facing away from the experimenter toward an open arm; the same arm in all instances. The mice were allowed to move freely and undisturbed for 5 minutes. ANY-maze™ recorded the number of entries onto, and the time spent on, the open arms, closed arms and the centre square. An entry was counted when all four paws crossed the entrance line of an arm or the centre square. In addition to the conventional spatiotemporal parameters captured by ANY-maze™, the experimenter sat silently 2.5 m from the maze and recorded the number of head-dips by pressing a keyboard button each time a mouse looked over the edge of an open arm towards the floor; ANY-maze™ then tallied the number of key presses. The 'protected' head-dips that occurred from the centre square were not differentiated from the 'unprotected' head-dips that occurred from the open arms.

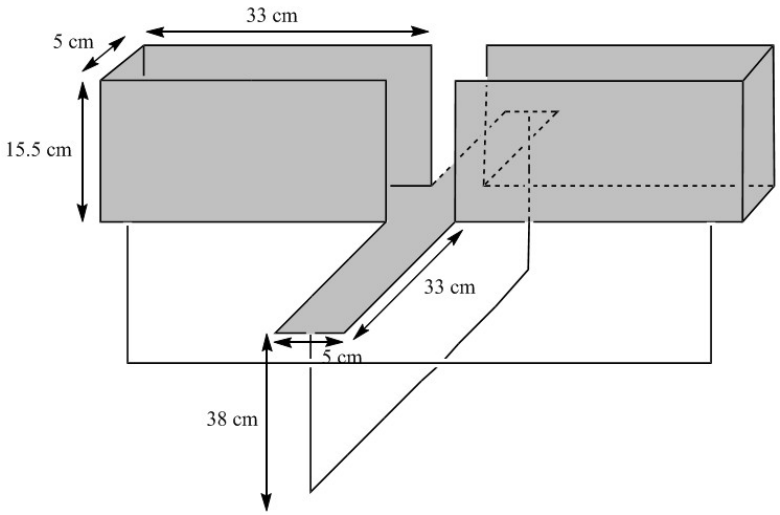


***Figure 2.2*. Schematic illustration of the Elevated Plus-Maze.** The plus-maze is the most common test of rodent anxiety-like behaviour. Open arm activity is the main determinant that correlates with anxiety. Fewer entries onto the open arms and less time on the open arms relative to wild-type mice indicates high anxiety-like levels.

The choice of parameter measures used to assess behaviour in the plus-maze was guided by the selection criteria in Holmes and Rodgers (1998), Rodgers and Dalvi (1997) and Fernandes and File (1996). The primary indices of anxiety are the number of entries into the open arms and the time spent on the open arms; fewer entries and less time reflect anxiety-like behaviour. To correct for activity-induced artifacts, open arm data is expressed as a percentage of the total number of arm entries (open + closed), and a percentage of the total time spent in each arm (open + closed). Central square activity is not scored as part of either the open or closed arms, based on the factor analysis study by Fernandes and File (1996) that indicated it loaded separately from arm activity. The total number of closed arm entries best describes general exploratory behaviour, and the total number of arm transitions is used to quantify general locomotion. These latter two parameters help to interpret open arm activity.

2.2.2.4 Social Approach and Preference for Social Novelty

The apparatus was an open-top, white opaque acrylic box divided into three equal sized chambers (W20 x L40 x H23 cm) by two transparent acrylic walls (W5 x L40 x H23 cm). Dividing walls had a centrally placed doorway (W7.2 x H8 cm) with a transparent, removable guillotine door used to control access to side chambers.

Social approach and preference for social novelty were measured separately in this two-stage test (see Figure 2.3). Testing was immediately preceded by a fifteen-minute habituation period during which time the subject mouse freely explored the empty apparatus, starting from the centre chamber. Once the habituation period ended the doors were replaced and the subject mouse was confined to the centre chamber. An inverted wire pencil cup (Spectrum Diversified; SKU 31570) was then placed in the middle of each side chamber, 6 cm from the back wall. The wire cups (DIA10.2 x H10.8 cm) were made up of vertical stainless steel bars that were spaced 9 mm apart, and were weighted with a water-filled, 250 mL glass laboratory bottle (DURAN®; PN 21.801.36.5). An adult wild-type mouse that was sex-matched and wholly unfamiliar to the subject mouse was placed underneath one of the wire cups; left and right placement was counterbalanced across groups. To test social approach, the subject mouse was given access to the side chambers and was allowed to freely explore for 10

minutes. When the social approach test ended, the doors were replaced and the subject mouse was confined to the centre chamber. To test preference for social novelty, a second stranger mouse (S2) with the same characteristics as stranger mouse 1 (S1) was placed underneath the remaining wire cup. The subject mouse was again given access to the side chambers and allowed to freely explore for 10 minutes. At each stage of testing the guillotine doors were removed simultaneously to avoid influencing an animal's movement. The same four male and four female stimulus mice were used throughout the study, and their identity was counterbalanced across groups; mouse A = S1 and mouse B = S2, then mouse B = S1 and mouse A = S2, etc.

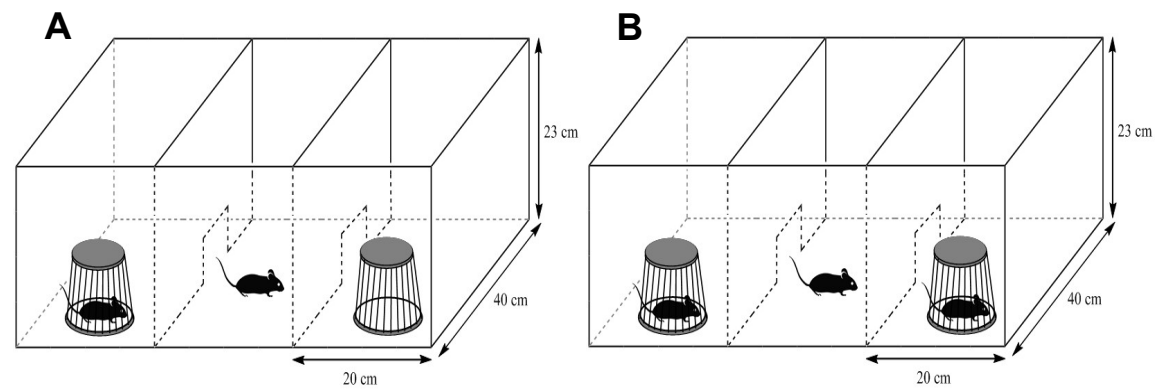


Figure 2.3. **Schematic illustration of the Crawley three-chamber test. A) Social Approach.** The subject mouse has a choice whether to explore the unfamiliar, sex-matched mouse or the empty cage. Preferential exploration of the stranger mouse indicates social approach behaviour. **B) Preference for Social Novelty**. The subject mouse has the choice whether to explore a newly introduced stranger mouse or the original stranger mouse. Preferential exploration of the novel stranger mouse shows a preference for social novelty.

The main parameter was the time spent exploring in proximity to the wire cups. The author collected this data retrospectively by manually scoring session recordings. Videos were replayed offline using ANY-Maze™ software. A virtual zone was overlaid on to the recorded images to create a gap of 2 cm between the base of the wire cups and the perimeter boundary of the circular zone. When a subject mouse's nose fell on or within one of these virtual zones a keyboard button was pressed, then released when the animal's nose exited the zone (separate keys were assigned for left and right). Exploration was not scored if an animal used a cup to rear upward with its nose facing

toward the ceiling, or if an animal's nose fell briefly within a zone for the purpose of moving to another area of the arena. The author scored the entire dataset blind to genotype and sex. The author then re-scored 40% of trials from each age group to assess intra-scorer reliability and found a significant correlation between scores: r = 0.89, p = .004 (8-months); r = .091, p < .001 (15-months).

Performance in the (1) social approach and (2) preference for social novelty test was calculated as a D2 discrimination ratio (Ennaceur & Delacour, 1988) in order to accurately assess the preference for a cue by accounting for the variability in raw exploration times. These 'preference scores' were calculated as: (1) [(the time spent exploring S1 – the empty cup) / (the time spent exploring S1 + the empty cup)], and (2) [(the time spent exploring S2 – S1) / (the time spent exploring S2 + S1)]. Scores can vary between +1 and -1. Using the social approach test as an example, a score greater than zero indicates more time spent exploring S1 than the empty cup, vice versa for a score below zero. A score of zero indicates an equal amount of time spent exploring S1 and the empty cup, or no preferential exploration of either S1 or the empty cup.

2.2.2.5 Social Olfaction

The Crawley sociability and preference for social novelty protocol was adapted to test social olfaction (Dachtler et al., 2014). The same three-chamber apparatus and general procedure was used for this one-stage test. The subject mouse was confined to the centre chamber following the fifteen-minute habituation period. The inverted wire pencil cups were placed in the side chambers and an open petri dish (DIA33 x H10 mm) was positioned centrally underneath each cup. One dish was filled with new bedding whereas the other dish was filled with soiled bedding from a cage of mice (minimum 3) that were sex-matched to the subject mouse and not used as stimulus mice in the social approach and preference for social novelty tests. The subject mouse was then given access to the side chambers and was allowed to freely explore for 10 minutes. The placement of the soiled bedding was left and right counterbalanced across groups.

Social olfaction was quantified by applying the same method of manual scoring described in the social approach and preference for social novelty tests. The author

performed all manual scoring, and was blind to genotype and sex. Intra-scorer reliability was significant (40% trials) at 8-months (r = .90, p < .001) and 15-months (r = .88, p < .011). Performance was calculated as a discrimination ratio (d2) defined as: [(time spent exploring the soiled bedding – time spent exploring the clean bedding) / (time spent exploring the soiled bedding + fresh bedding)]. A score greater than zero (maximum of 1) indicates more time spent exploring the soiled bedding than the clean bedding, vice versa for a score below zero (minimum of -1). A score of zero indicates a null preference, or an equal amount of time exploring both beddings.

2.2.2.6 Buried Food Test

We evaluated mouse ability to smell volatile odours using Yang and Crawley's (2009) buried food test. The motive was to assess anosmia as a potential confounding factor in the tests of social behaviour. Thirty-two of the 39 mice were from the original test battery: 4 male App^{NL-F} (M=476.50 ±8.37 days), 21 App^{NL-G-F} (M=471.57 ±4.26 days; 11 male) and 14 wild-type (M=441.14 ±14.64 days; 10 male).

The main parameter here is the latency to uncover food hidden beneath a layer of clean cage bedding. Weetos™, a crunchy, chocolate-flavoured wheat cereal, were placed in home cages for two consecutive days prior to testing; one piece per mouse, per day, broken into quarters and scattered throughout the cage. The cages were checked each morning to ensure the cereal was consumed and therefore palatable to the mice. The mice were then put on an overnight fast. The chow pellets were removed for approximately nineteen hours prior to testing. The test began by acclimatising the mouse for five minutes to a standard polycarbonate cage (W17.5 x L31 x H14.5 cm) containing clean bedding 3 cm deep. The mouse was then transferred to an empty cage. A Weetos™ piece was then placed in a randomly chosen area 1 cm beneath the cage bedding. The mouse was then re-introduced to the cage and the cage lid replaced. The experimenter retreated approximately 2 m from the cage and recorded with a stopwatch the animal's latency to find the cereal piece (up to a maximum limit of 15 minutes). A mouse was considered to have uncovered the food once it began to eat. Cages were cleaned between subjects with 70% v/v isopropyl alcohol and new bedding was used.

2.2.3 Data Analysis

Data was analysed using two-way independent ANOVA with *genotype* and *sex* as the between-subjects factors. Where data violated the assumption of homoscedasticity and/or normality the research team decided collectively to proceed with analysis given the general robustness of ANOVA and given that there are no viable non-parametric alternatives to two-way ANOVA. Data from the social behaviour assays were analysed using a repeated measures two-way ANOVA with *time* as the within-subjects factor (*e.g.,* time spent exploring stranger mouse 1 vs. time spent exploring the empty cup). One-sample *t*-tests were performed on the discrimination ratios (d2) to assess whether the mean d2 scores differed from zero. If data was not normally distributed a one-sample Wilcoxon signed rank test was run. As this yielded similar results in each instance, only the parametric test results are reported.

Pair-wise comparisons between genotypes were adjusted using the Bonferroni correction, which was chosen in order to mitigate the increased risk of finding a false positive due to heteroscedasticity. The less conservative Tukey correction was also applied as a matter of interest, and was found to yield similar results in all instances (data not included). The variable *sex* was primarily of interest as an interaction variable rather than a main effect. Therefore, main effects of sex are reported, but with limited interpretation. Interactions were investigated using simple main effects analysis. For simple main effect analysis by one-way ANOVA, where Levene's test for equality of variances indicated significant differences the Welch correction is reported with Games-Howell pairwise comparisons. All simple main effects analyses by *t*-test met the assumption of homoscedasticity. Non-parametric equivalents were run if data for the simple main effects analyses were not normally distributed. Since results did not differ, only the parametric test results are reported.

All statistical analyses were performed by the author, using IBM™ SPSS© v.22.0. The critical α level was set to $p \leq 0.05$. Graphs were made using Chart.js v.2.8.0, and were designed by the author to illustrate the underlying distribution of data. Statistical significance within figures is illustrated as: *($p \leq 0.05$), **($p < 0.01$) and ***($p < 0.001$). All data are expressed as mean ± SEM.

2.3 Results

2.3.1 Anxiety-Related Measures

Human studies have recently shown that anxiety may act as an early indicator of AD (*e.g.*, Donovan et al., 2018; Hanseeuw et al., 2018). To investigate this further, and to identify if the APP knock-in mice display an analogous phenotype, we evaluated anxiety-related behaviours in the App^{NL-F} and App^{NL-G-F} mice using two of the most relied upon tests of rodent emotionality: the open field and elevated plus-maze.

2.3.1.1 Results at the 8-month age point

2.3.1.1.1 Open Field

8-month old App^{NL-G-F} mice exhibit heightened avoidance of the open field centre zone

An ANOVA of the mean time spent in the centre zone of the open field by the 8-month old mice revealed an effect of genotype, $F_{(2,52)} = 7.49$, $p = 0.001$ (Fig. 2.4), no effect of sex $F_{(1,52)} = 3.53$, $p = 0.066$, and no interaction between genotype and sex, $F_{(2,52)} = 0.51$, $p = 0.603$. Over the course of the 30-minute open field trial the App^{NL-G-F} mice spent significantly less time exploring the central area of the apparatus compared with the wild-type mice ($p = 0.003$) and the App^{NL-F} mice ($p = 0.006$); there was no difference between the App^{NL-F} mice and the wild-type mice ($p = 1.000$).

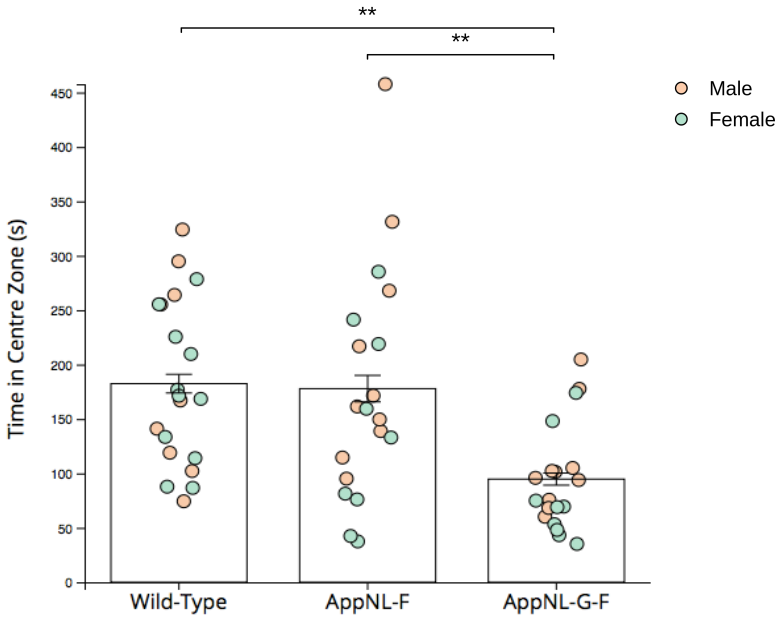


Figure 2.4. **Mean (±SEM) time spent in the centre zone of the open field at 8-months.** The App^{NL-G-F} mice spent significantly less time exploring the central area of the arena.

8-month old App^{NL-G-F} mice are thigmotactic compared to wild-type and App^{NL-F} mice

To establish whether the 8-month old APP knock-in mice were more thigmotactic compared with the wild-type control mice, an ANOVA was carried out on the mean amount of time spent in the outer zone of the open field. Due to the intermediate zone, the time spent in the outer zone nearer to the walls of the apparatus is not an inverse measure of the time spent in the centre zone. The ANOVA revealed an effect of genotype, $F_{(2,52)} = 10.34$, $p < 0.001$ (Fig. 2.5), an effect of sex, $F_{(1,52)} = 4.73$, $p = 0.034$ (M > F), and no interaction between genotype and sex, $F_{(2,52)} = 0.76$, $p = 0.475$. Pairwise comparisons showed that the App^{NL-G-F} mice spent significantly more time in the periphery areas of the apparatus compared with the wild-type mice ($p = 0.001$) and the App^{NL-F} mice ($p = 0.001$); the App^{NL-F} mice and the wild-type mice did not differ in the amount of time spent in the outer zone ($p = 1.000$). The 8-month old App^{NL-G-F} mice display an ostensibly-anxiogenic profile in the open field. They spend less time in the central area of the apparatus, and more time nearer to the walls of the apparatus, relative to the age-matched wild-type control mice and the App^{NL-F} mice.

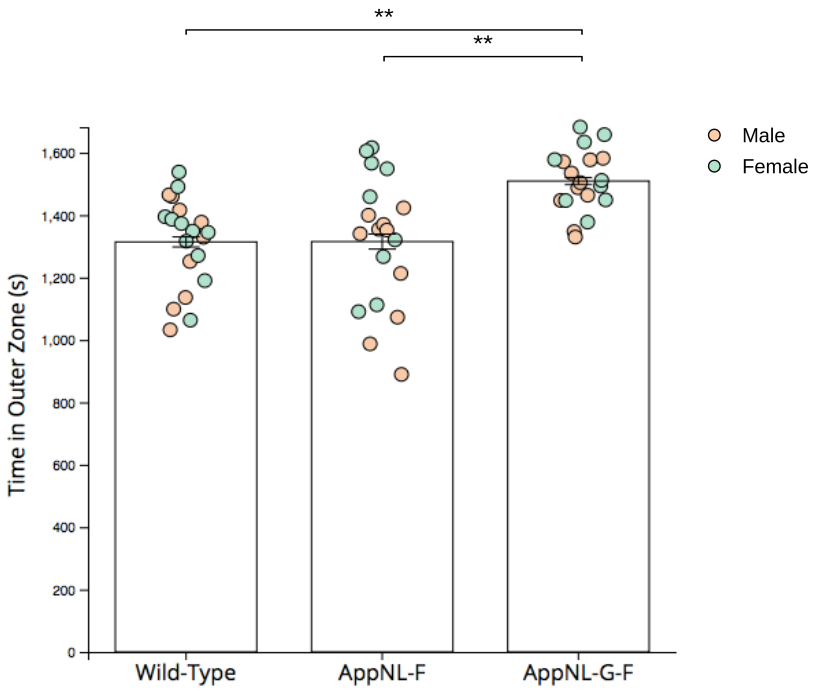


Figure 2.5. **Mean (±SEM) time spent in the outer zone of the open field at 8-months.** The App^{NL-G-F} mice exhibited an increased level of thigmotaxis. They spent significantly more time nearer to the walls of the apparatus compared to App^{NL-F} and wild-type mice.

8-month old App^{NL-G-F} mice enter the centre zone less frequently than wild-type mice

This ostensibly-anxiogenic open field profile observed in the 8-month old App^{NL-G-F} mice was further investigated by assessing the frequency of centre zone entries, which was measured as a ratio of the total number of entries into all three zones. A reduced percentage of centre zone entries relative to wild-type control mice are interpreted as evidence of heighted anxiety-like behaviour (Prut & Belzung, 2003). The ANOVA yielded an effect of genotype, $F_{(2,52)} = 4.23$, $p = 0.020$ (Fig. 2.6), with no effect of sex $F_{(1,52)} = 0.81$, $p = 0.370$, and no interaction between genotype and sex, $F_{(2,52)} = 1.02$, $p = 0.360$. The frequency of centre zone entries was significantly reduced in the App^{NL-G-F} mice (M=15.52 ±.30%) compared with the wild-type mice (M=17.62 ±.51%; $p = 0.015$). The frequency of centre zone entries did not differ between the App^{NL-G-F} and App^{NL-F} mice (M=16.68 ±.63%; $p = 0.362$), or the App^{NL-F} and wild-type mice ($p = 0.567$). This result corroborates our initial finding that the 8-month old App^{NL-G-F} mice have an ostensibly-anxiogenic profile in the open field.

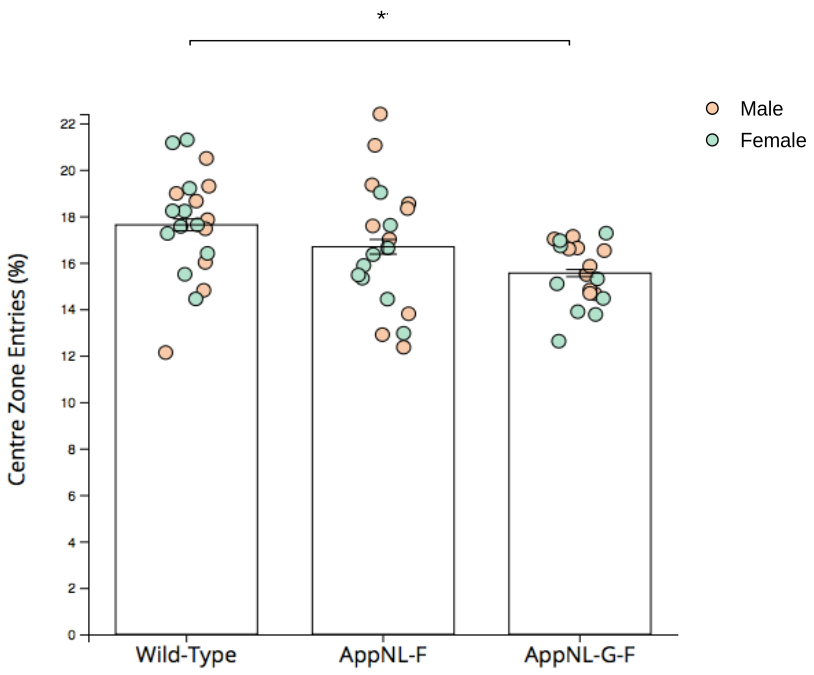


Figure 2.6. **Mean (±SEM) percentage of entries into the open field centre zone at 8-months.** The App^{NL-G-F} mice displayed a reduced percentage of centre zone entries compared with the wild-type mice which supports a finding of an ostensibly-anxiogenic open field profile.

8-month old APP knock-in mice display normal locomotor activity in the open field

To investigate whether altered locomotor activity was driving the anxiogenic-like open field behaviour in the 8-month old App^{NL-G-F} mice, we analysed the mean distance traversed in 5-minute time blocks using repeated measures ANOVA. Over the course of the 30-minute trial, the App^{NL-G-F} mice traversed a similar distance (M=85.02 ±3.9) compared with the wild-type mice (M=74.45 ±6.5) and the App^{NL-F} mice (M=69.88 ±4.3), [RM ANOVA: genotype, $F_{(2,52)} = 2.09$, $p = 0.133$ (Fig. 2.7); sex, $F_{(1,52)} = 1.52$, $p = 0.224$; interaction between genotype and sex, $F_{(2,52)} = 0.26$, $p = 0.766$)]. The effect of time-block was significant, $F_{(5,260)} = 19.47$, $p < 0.001$, and also anticipated, given the well-established result of habituation activity in the open field test (Bailey, Rustay & Crawley, 2006). There was no interaction between time block, genotype and sex, $F_{(2,10)} = 0.31$, $p = 0.978$, however time-block and genotype interacted, $F_{(10,260)} = 16.42$, $p = 0.018$. Using simple main effects analysis by one-way ANOVA, we found that the App^{NL-G-F} mice traversed a significantly greater distance compared to the App^{NL-F} mice ($p = 0.042$) during the fourth time-block [$F_{(2,57)} = 5.68$, $p = 0.021$].

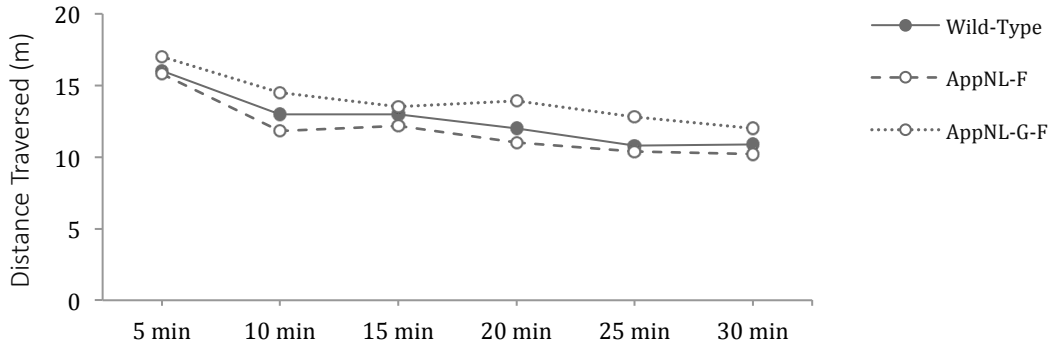


Figure 2.7. **Mean (±SEM) distance traversed in the open field at 8-months of age.** APP knock-in mice displayed intact motor function, traversing similar distances over the course of the 30-minute trial compared with the wild-type mice.

As a second and final measure of general locomotion, an ANOVA was conducted on the mean number of total zone entries over the course of the 30-minute open field trial. There was an effect of sex, $F_{(1,52)} = 5.33$, $p = 0.025$ (M > F), no effect of genotype, $F_{(2,52)} = 1.19$, $p = 0.313$ (Fig. 2.8), and no interaction between genotype and sex, $F_{(2,52)} = 0.13$, $p = 0.879$. Taken together, these results indicate that the ambulatory ability of the 8-month old APP knock-in mice was unaltered, which in turn suggests that the behavioural performance of the 8-month old $App^{NL\text{-}G\text{-}F}$ mice, or more specifically, the avoidance of the central area of the apparatus and an increased tendency for wall-hugging, was likely due to altered anxiety-like levels as opposed to hypo-activity.

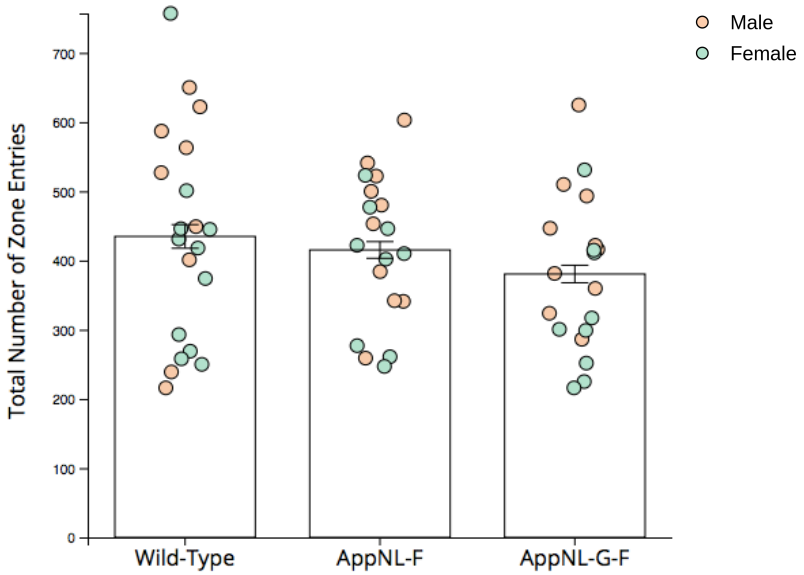


Figure 2.8. **Mean (±SEM) number of total zone entries in the open field at 8-months.** APP knock-in mice displayed unaltered ambulation. They made a similar number of overall entries into the open field zones compared with the wild-type mice.

2.3.1.1.2 Elevated Plus-Maze

8-month old App^{NL-G-F} mice exhibit an increase in open arm activity in the plus-maze

Open arm activity is the critical determinant of anxiety-like behaviour in the elevated plus-maze. Less time spent on the open arms of the maze and fewer open arm entries are indications of an anxiogenic-like profile. An ANOVA of the mean percentage of time spent by the 8-month old mice in the open arms of the maze yielded an effect of genotype, $F_{(2,52)} = 5.68$, $p = 0.006$ (Fig. 2.9), no effect of sex, $F_{(1,52)} = 3.59$, $p = 0.064$, and no interaction between genotype and sex, $F_{(2,52)} = 0.02$, $p = 0.981$. Pairwise comparisons confirmed that over the course of the 5-minute trial the App^{NL-G-F} mice spent significantly more time (M=18.14 ±3.52%) on the open arms of the maze compared with the wild-type mice (M=7.68 ±1.84%; $p = 0.016$) and the App^{NL-F} mice (M=7.84 ±2.10%; $p = 0.020$); the percentage of time spent on the open arms did not differ between the App^{NL-F} mice and the wild-type mice ($p = 1.000$).

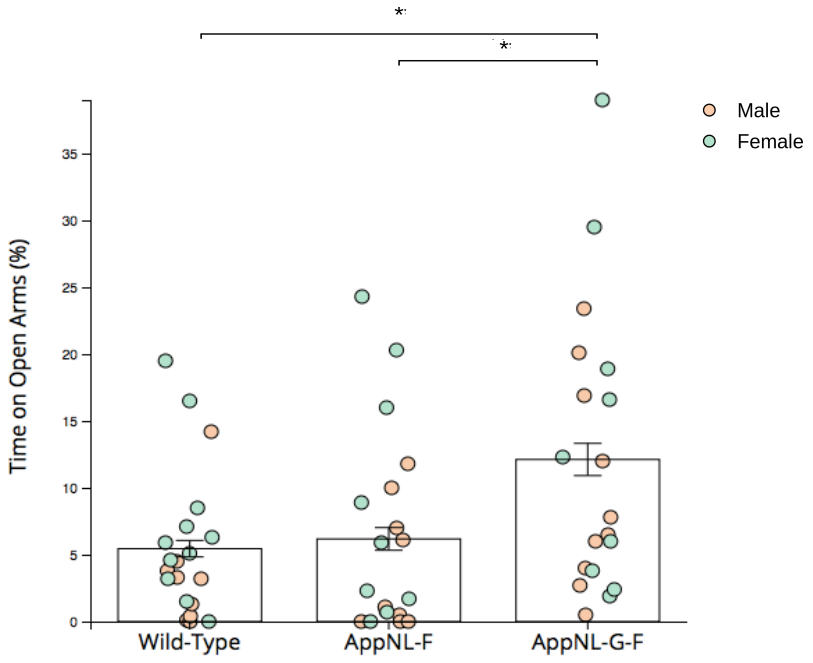


***Figure 2.9.* Mean (±SEM) percentage of time spent on the open arms at 8-months.** The App^{NL-G-F} mice show an increase in open arm activity by spending significantly more time on the open arms of the maze relative to the wild-type and App^{NL-F} mice.

Open arm activity was further analysed by assessing the percentage frequency of open arm entries over the 5-minute trial. The ANOVA revealed an effect of genotype, $F_{(2,52)} = 5.58$, $p = 0.005$ (Fig. 2.10), and an effect of sex, $F_{(1,52)} = 13.12$, $p = 0.001$ (M < F), with no interaction between genotype and sex, $F_{(2,52)} = 0.81$, $p = 0.450$. Pairwise comparisons confirmed that the App^{NL-G-F} mice had a significantly higher frequency of open arm entries (M=32.04 ±3.29%) compared with the wild-type mice (M=18.87 ±2.84%, $p = 0.013$) and the App^{NL-F} mice (M=19.40 ±4.16%, $p = 0.020$); there was no difference between the App^{NL-F} mice and wild-type mice ($p = 1.000$) in the percentage of open arm entries. Together these results indicate a marked increase in open arm activity in the 8-month old App^{NL-G-F} mice, which is evidence of an ostensibly-anxiolytic plus-maze profile. An intriguingly contradictory behavioural pattern has emerged whereby the 8-month old App^{NL-G-F} mice exhibit an ostensibly-anxiogenic profile in the open field yet an ostensibly-anxiolytic profile in the elevated plus maze.

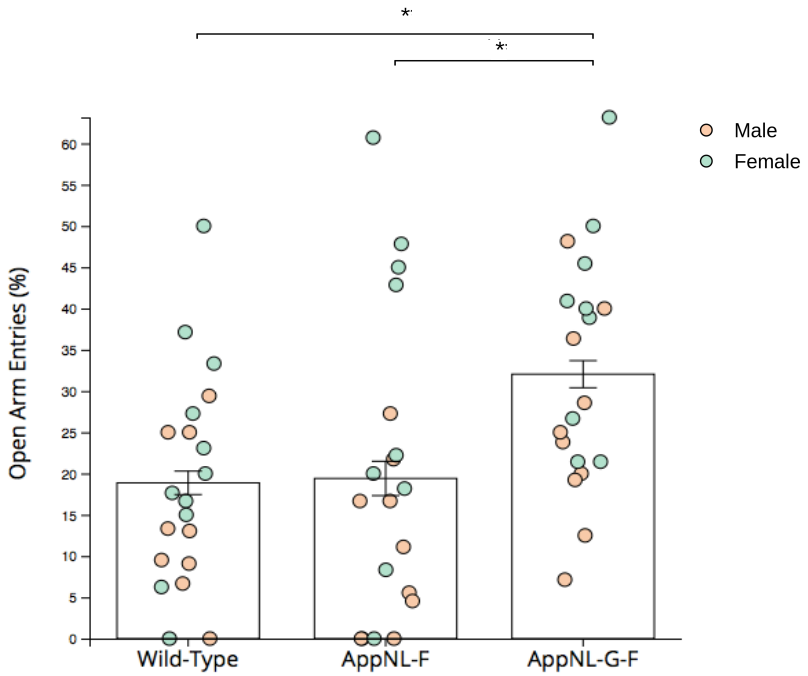


Figure 2.10. **Mean (±SEM) percentage of open arm entries at 8-months.** The App^{NL-G-F} mice display a significantly greater percentage of open arm entries compared with the wild-type mice and the App^{NL-F} mice.

8-month old APP knock-in mice show typical exploratory activity in the plus-maze

The extensive open arm activity observed in the 8-month old $App^{NL\text{-}G\text{-}F}$ mice may reflect low levels of exploratory activity as opposed to anxiolytic-like behaviour. For instance, the first arm a mouse transitions into, whether it be closed or open, could be where the mouse remains (Bailey & Crawley, 2009). To address this potential confound we assessed the number of closed arm entries and the number of total arm transitions. An analysis of variance of the mean number of closed arm entries yielded an effect of sex, $F_{(1, 52)} = 11.79$, $p = 0.001$ (M > F), no effect of genotype, $F_{(2,52)} = 0.65$, $p = 0.524$ (Fig. 2.11-A), and no interaction between genotype and sex, $F_{(2,52)} = 2.28$, $p = 0.112$. An analysis of variance of the mean number of arm transitions revealed no effect of genotype, $F_{(2,52)} = 0.48$, $p = 0.623$ (Fig. 2.11-B), or sex, $F_{(1,52)} = 0.73$, $p = 0.397$, and no interaction between genotype and sex, $F_{(2,52)} = 0.72$, $p = 0.492$. Together these results indicate that the increased open arm activity observed in the 8-month old $App^{NL\text{-}G\text{-}F}$ mice is not based on low exploration of the maze or altered locomotion.

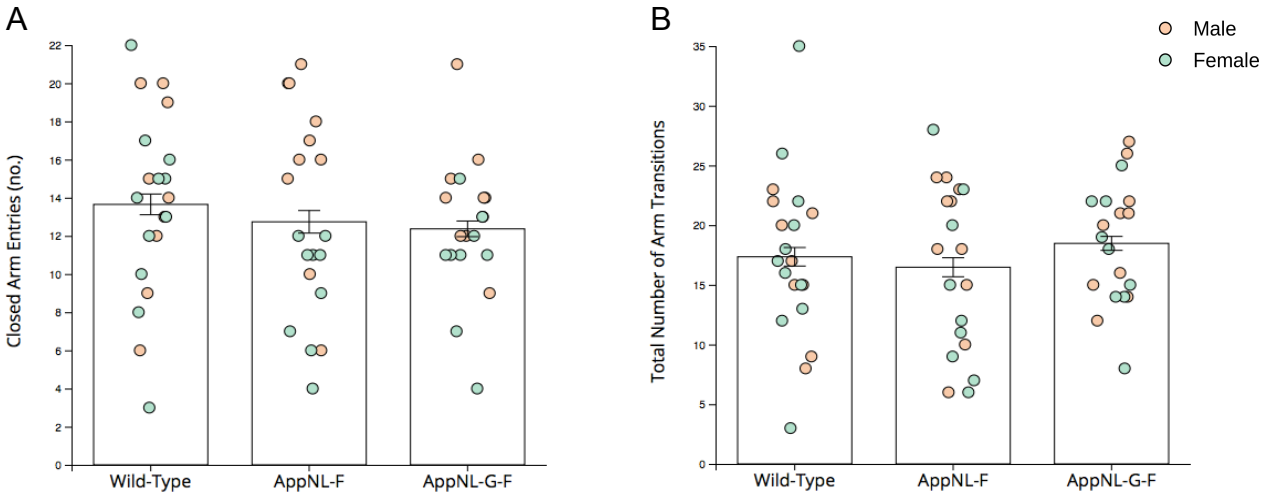


Figure 2.11. **General exploratory and locomotor activity in the plus-maze at 8-months.**

(A) Mean (±SEM) entries into closed arms. **(B)** Mean (±SEM) number of arm transitions.

8-month old APP knock-in mice show equivalent head-dips to wild-type mice

As a final measure of plus-maze behaviour we assessed the number of head-dips mice made during the 5-minute trial. The ANOVA revealed an effect of genotype, $F_{(2,52)}$ = 3.39, p = 0.041 (Fig. 2.12), no effect of sex, $F_{(1,52)}$ = 2.43, p = 0.125, and no interaction between genotype and sex, $F_{(2,52)}$ = 0.19, p = 0.826. Despite the effect of genotype, the pairwise comparisons between genotypes did not reach statistical significance. However, the App^{NL-G-F} mice made more head-dips (M=26.13 ±2.77) compared with the wild-type mice (M=17.80 ±2.67; p = 0.086) and the App^{NL-F} mice (M=18.05 ±2.37; p = 0.108).

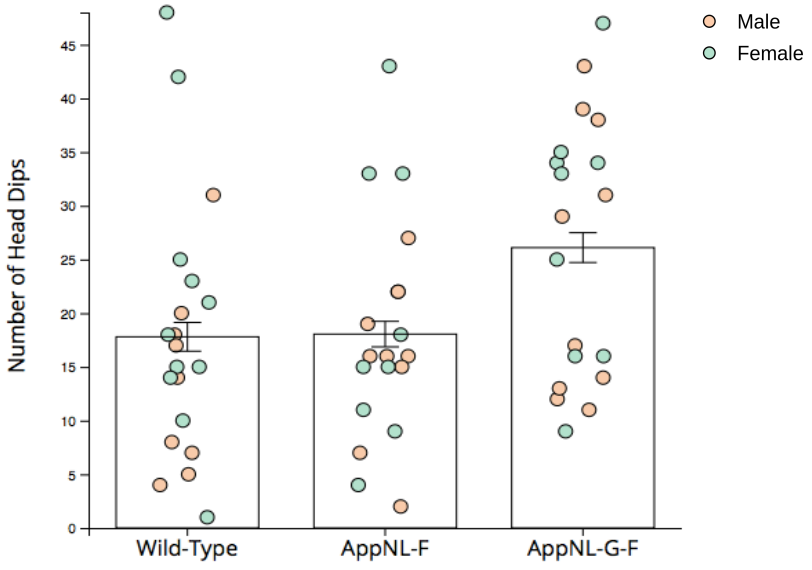


Figure 2.12. **Mean (±SEM) number of head-dips in the plus-maze at 8-months.**

A brief summary of the results indicates that at the 8-month age-point, there were no interactions between genotype and sex on any of the open field or elevated plus-maze variables assessed, and the 8-month old App^{NL-F} mice behaved statistically similar to the age-matched wild-type control mice across all anxiety-related measures. Contrarily, the 8-month old App^{NL-G-F} mice did display altered behaviour compared with the wild-type mice, and the App^{NL-F} mice. The App^{NL-G-F} mice displayed an ostensibly-anxiogenic profile in the open field yet an ostensibly-anxiolytic profile in the elevated plus maze. We now turn to the analyses of anxiety-related behaviour in the 15-month old cohort of mice.

2.3.1.2 Results at the 15-month age point

2.3.1.2.1 Open Field

15-month old APP knock-in mice show unaltered anxiety-like levels in the open field

An ANOVA conducted on the mean amount of time spent in the centre zone of the open field yielded no effect of genotype, $F_{(2,54)} = 0.06$, $p = 0.941$ (Fig. 2.13), no effect of sex, $F_{(1,54)} = 3.29$, $p = 0.075$, and no interaction between genotype and sex, $F_{(2,54)} = 0.29$, $p = 0.744$. Furthermore, an ANOVA conducted on the mean amount of time spent in the outer zone of the open field yielded no effect of genotype, $F_{(2,54)} = 2.47$, $p = 0.094$ (Fig. 2.14), an effect of sex, $F_{(1,54)} = 4.14$, $p = 0.047$ (M < F), and no interaction between genotype and sex, $F_{(2,54)} = 0.38$, $p = 0.688$. As a third and final measure of anxiety-related behaviour in the open field we assessed the percentage of centre zone entries. There was no difference between genotypes, $F_{(2,54)} = 0.22$, $p = 0.801$ (Fig. 2.15), no difference between males and females, $F_{(1,54)} = 0.85$, $p = 0.362$, and no interaction between genotype and sex, $F_{(2,54)} = 0.09$, $p = 0.912$. These results demonstrate that the 15-month old App^{NL-F} and App^{NL-G-F} knock-in mice did not exhibit anxiety-like behaviours when moving freely and undisturbed for 30 minutes in the open field.

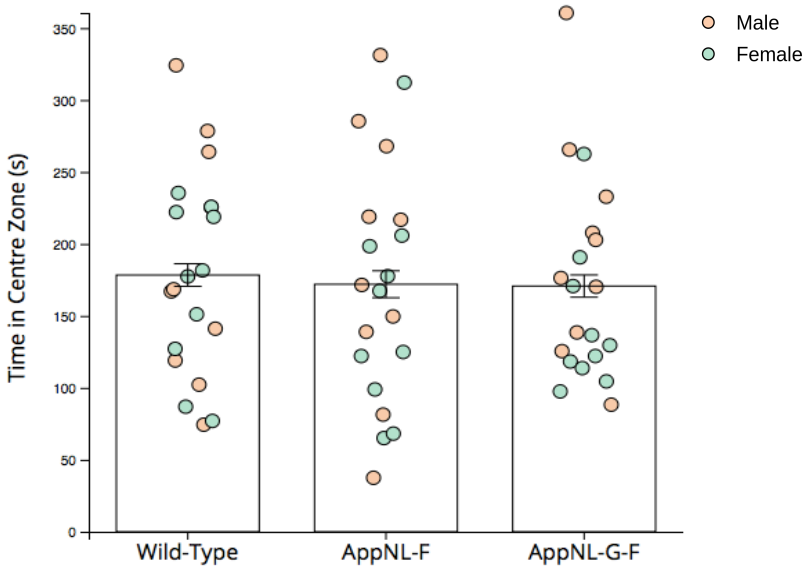


Figure 2.13. **Mean (±SEM) time spent in the centre zone of the open field at 15-months.**
There were no differences between genotypes in the amount of time spent in the centre zone.

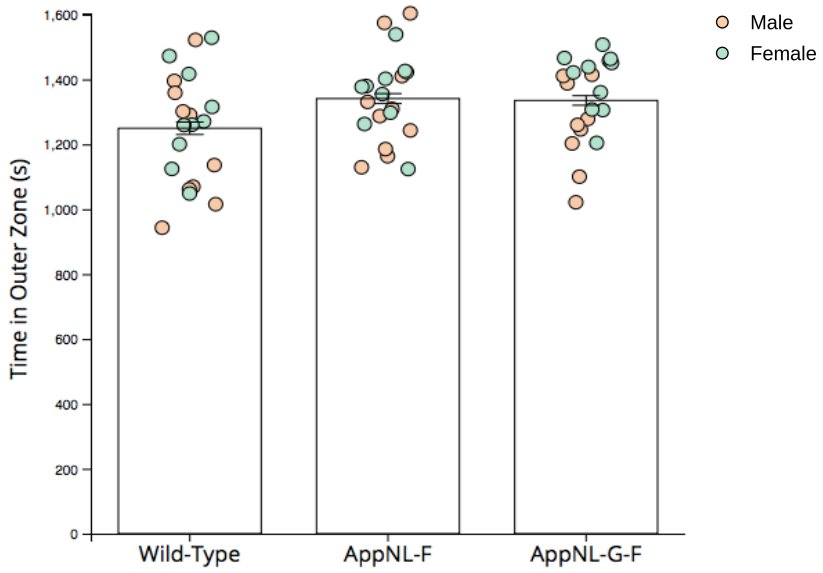


Figure 2.14. **Mean (±SEM) time spent in the outer zone of the open field at 15-months.**

There were no differences between genotypes in the amount of time spent in the outer zone.

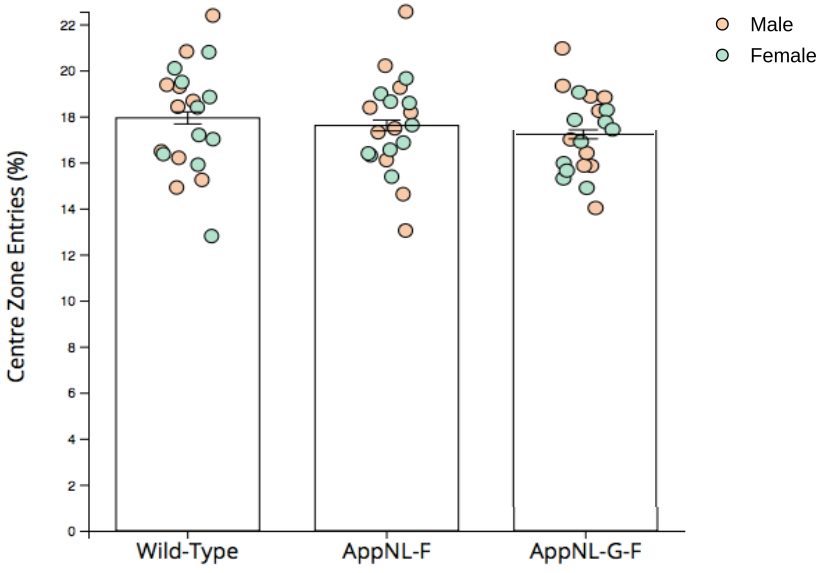


Figure 2.15. **Mean (±SEM) percentage of entries into the center zone at 15-months.**

There were no differences between genotypes in the percentage of centre zone entries.

15-month old App^{NL-G-F} mice exhibit some evidence of hyperactivity in the open field

Measures of general ambulation in the open field remain informative regardless of null anxiety-related findings, and serve as standard protocol when establishing the phenotype of novel mouse models. To assess locomotor activity in the 15-month old APP knock-in mice, a repeated measures ANOVA was run on the mean distances traversed over the course of the 30-minute open field trial. There was a significant effect of genotype, $F_{(2,54)} = 12.74$, $p < 0.001$ (Fig. 2.16). Pairwise comparisons confirmed that the App^{NL-G-F} mice traversed a significantly greater distance in total (M=106.68 ±5.42) compared with the wild-type mice (M=74.46 ±4.75; $p < 0.001$) and the App^{NL-F} mice (M=86.82 ±4.75; $p = 0.010$). There was no effect of sex, $F_{(1,54)} = 0.38$, $p = 0.539$, and no interaction between genotype and sex, $F_{(2,54)} = 2.83$, $p = 0.067$, on the overall distance traversed. There was a significant effect of time block, $F_{(5,270)} = 24.14 = p < 0.001$, which does not warrant a simple-main effects analysis as previously implied. There were no interactions to indicate any differences between groups in the pattern of change across time-blocks: [RM ANOVA: time-block x genotype x sex, $F_{(2,10)} = 1.06$, $p = 0.344$; time-block x genotype, $F_{(10,270)} = 5.67 = p = 0.231$].

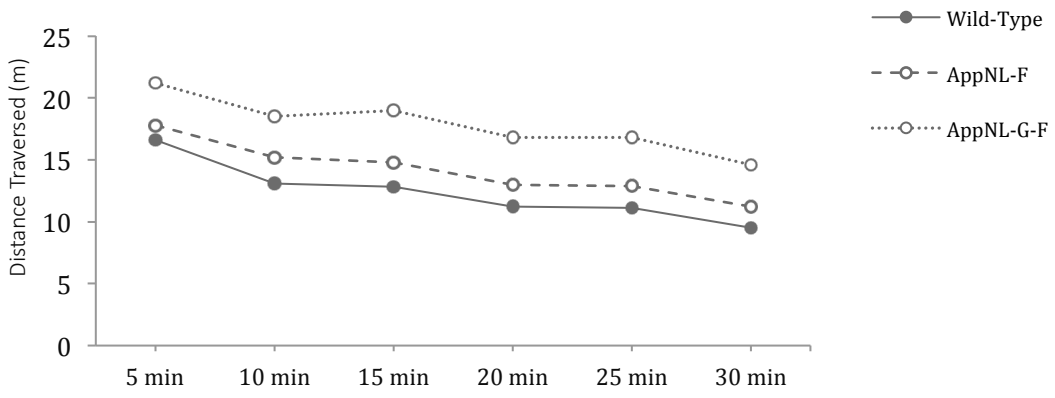


Figure 2.16. **Mean (±SEM) distances traversed in the open field at 15 months.** App^{NL-G-F} mice traversed a significantly greater distance overall compared with the wild-type mice and the App^{NL-F} mice. There were no interactions to indicate differences in the pattern of change over the course of the 30-minute trial.

As a second and final measure of locomotor activity in the open field, an ANOVA was conducted on the total number of zone entries made over the course of the 30-minute trial. There was no effect of genotype, $F_{(2,54)} = 2.13$, $p = 0.129$ (Fig. 2.17), and no effect of sex, $F_{(1,54)} = 0.96$, $p = 0.331$. There was an interaction between genotype and sex, $F_{(2,52)} = 3.35$, $p = 0.042$. Simple main effects analysis by *t*-tests yielded a marginally significant difference between male and female wild-type mice, $t_{(18)} = 2.06$, $p = 0.054$, with no difference between male and female App^{NL-F} mice, $t_{(18)} = -1.44$, $p = 0.167$, or male and female App^{NL-G-F} mice, $t_{(18)} = 1.17$, $p = 0.255$. Simple main effects analysis by one-way ANOVA for each sex was also included for completeness. There was no difference among the male mice in the overall number of zone entries, $F_{(2, 29)} = 2.38$, $p = 0.112$. Although the one-way ANOVA among female mice was significant, $F_{(2,29)} = 3.45$, $p = 0.046$, the pairwise comparisons did not reach statistical significance; wild-type to App^{NL-F} ($p = 0.076$), wild-type to App^{NL-G-F} ($p = 0.116$) and App^{NL-F} to App^{NL-G-F} ($p = 1.000$). Together these results demonstrate that the 15-month old App^{NL-G-F} mice are mildly hyperactive in the open field. They traversed a greater total distance compared with the wild-type mice and the App^{NL-F} mice, yet they did not display any difference in the number of overall zone entries.

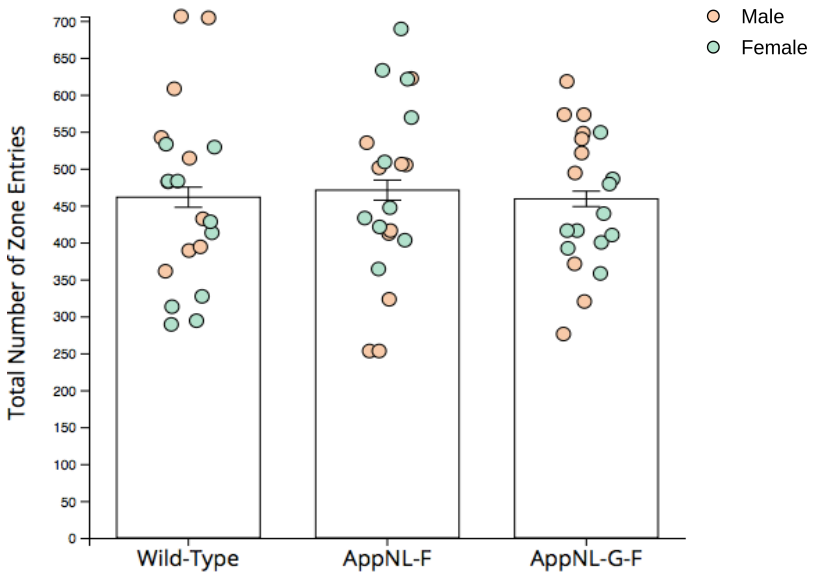


Figure 2.17. **Mean (±SEM) number of total zone entries in the open field at 15 months.** There was no difference between genotypes in the overall number of zone entries. There was a marginally significant difference between male and female wild-type mice.

2.3.1.2.2 Elevated Plus-Maze

15-month old App^{NL-G-F} mice show an increase in open arm activity in the plus- maze

The primary index of anxiety-like behaviour on the plus-maze is open arm activity, which, as previously mentioned, is inferred by the percentage of time spent on the open arms and the percentage of open arm entries, with each variable being a function of the overall arm activity (open + closed). An analysis of variance of the average percentage of time spent on the open arms by the 15-month old mice revealed an effect of genotype, $F_{(2,54)} = 19.19$, $p < 0.001$ (Fig. 2.18-A), no effect of sex, $F_{(1,54)} = 0.002$, $p = 0.966$, and no interaction between genotype and sex, $F_{(2,54)} = 0.34$, $p = 0.712$. Pairwise comparisons confirmed that the App^{NL-G-F} mice spent a significantly greater percentage of time (M=39.17 ±4.90%) on the open arms compared with the wild-type mice (M=9.30 1.77%; $p < 0.001$) and the App^{NL-F} mice (M=16.35 ±3.06%; $p < 0.001$); there was no difference in the percentage of open arm time between the App^{NL-F} mice and the wild-type mice ($p = 0.503$). An analysis of variance of the mean percentage frequency of open arm entries yielded an effect of genotype, $F_{(2,54)} = 13.01$, $p < 0.001$ (Fig. 2.18-B), no effect of sex, $F_{(1,54)} = 1.37$, $p = 0.247$, and no interaction between genotype and sex, $F_{(2,54)} = 0.32$, $p = 0.730$. Pairwise comparisons showed that the App^{NL-G-F} mice made a significantly greater percentage of open arm entries (M=45.56 ±4.21%) compared with the wild-type mice (M=20.86 ±2.70%, $p < 0.001$) and the App^{NL-F} mice (M=27.36 ±3.47%, $p = 0.002$); there was no difference in the percentage of open arm entries between the App^{NL-F} mice and the wild-type mice ($p = 0.603$). Together these results indicate that the 15-month old App^{NL-G-F} mice, but not the 15-month old App^{NL-F} mice, have an ostensibly-anxiolytic plus-maze profile, when allowed to move freely and undisturbed over the course of a 5-minute trial. This finding replicates the ostensibly-anxiolytic plus-maze profile observed in the 8-month old App^{NL-G-F} mice.

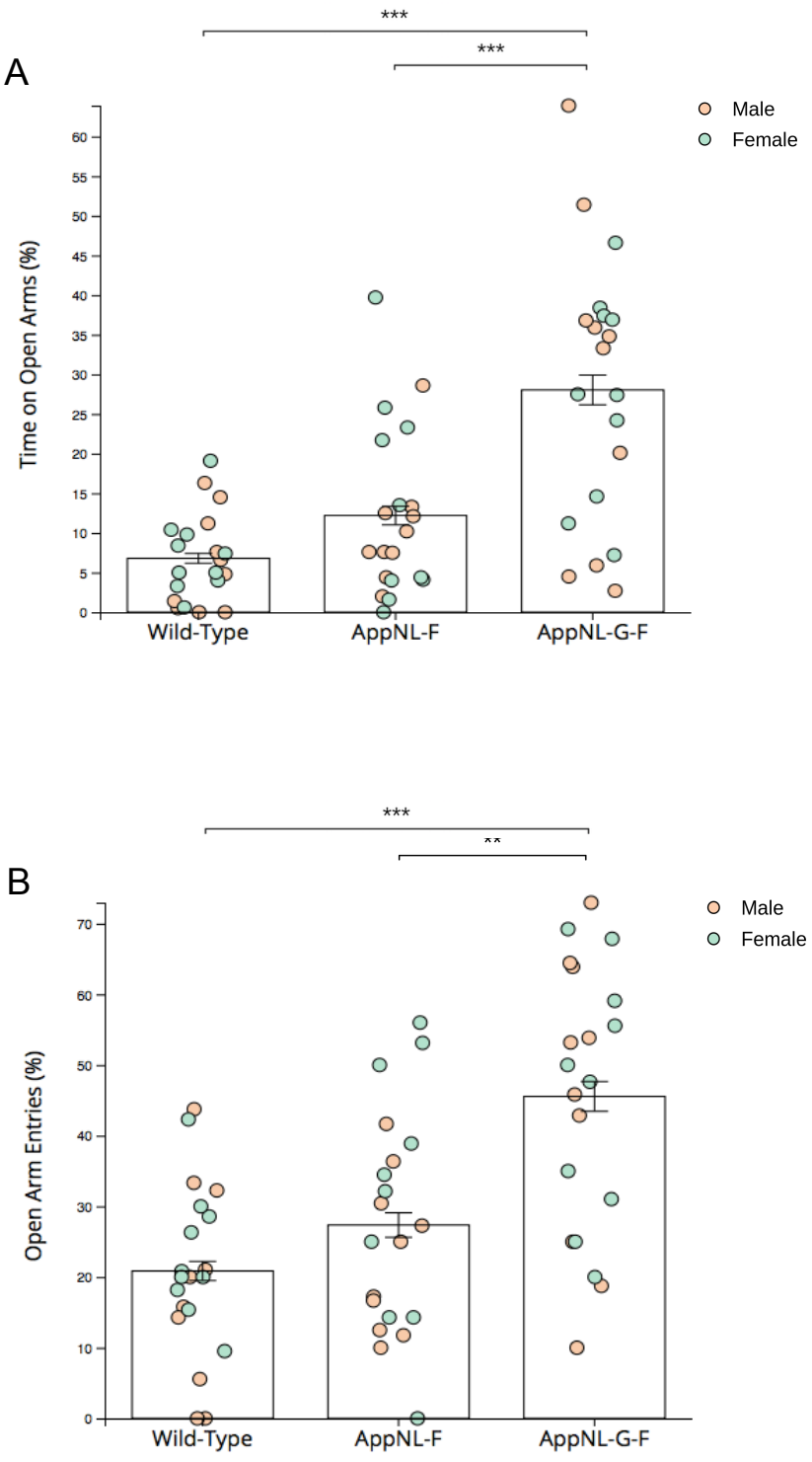


Figure 2.18. **Open arm activity in the elevated plus-maze at 15-months.** App^{NL-G-F} mice exhibited an increase in open arm activity compared with the wild-type mice and the App^{NL-F} mice, which indicates and ostensibly-anxiolytic plus-maze profile. **(A)** Mean (±SEM) percentage of time on the open arms. **(B)** Mean (±SEM) percentage of open arm entries.

15-month old APP knock-in mice have normal exploratory activity in the plus-maze

Increased open arm activity may reflect low levels of exploratory activity or altered ambulation. To assess exploratory activity an ANOVA was conducted on the average number of entries made into the closed arms over the 5-minute trial. There was a marginally significant effect of genotype, $F_{(2,54)} = 2.99$, $p = 0.058$ (Fig. 2.19-A), with no effect of sex, $F_{(1,54)} = 1.56$, $p = 0.217$, and no interaction between genotype and sex, $F_{(2,54)} = 0.22$, $p = 0.801$. On average the $App^{NL\text{-}G\text{-}F}$ mice made 11.4 (±.94) closed arm entries compared to the wild-type mice (M=14.6 ±.87; $p = 0.071$) and the $App^{NL\text{-}F}$ mice (M=13.9 ±1.05; $p = 0.223$). Locomotor activity was assessed by an ANOVA of the mean number of overall arm transitions. We found no effect of genotype, $F_{(2,54)} = 1.62$, $p = 0.208$ (Fig. 2.19-B), no effect of sex, $F_{(1,54)} = 0.61$, $p = 0.440$, and no interaction between genotype and sex, $F_{(2,54)} = 1.02$, $p = 0.366$. The rate of arm transitions was only slightly higher in the $App^{NL\text{-}G\text{-}F}$ mice (M=23.30 ±2.45) compared with the wild-type mice (M=18.65 ±1.11) and $App^{NL\text{-}F}$ mice (M=18.65 ±1.11). Together these results show that the ostensibly-anxiolytic plus-maze profile of the 15-month old $App^{NL\text{-}G\text{-}F}$ mice is not based on a change in exploratory activity or general locomotion.

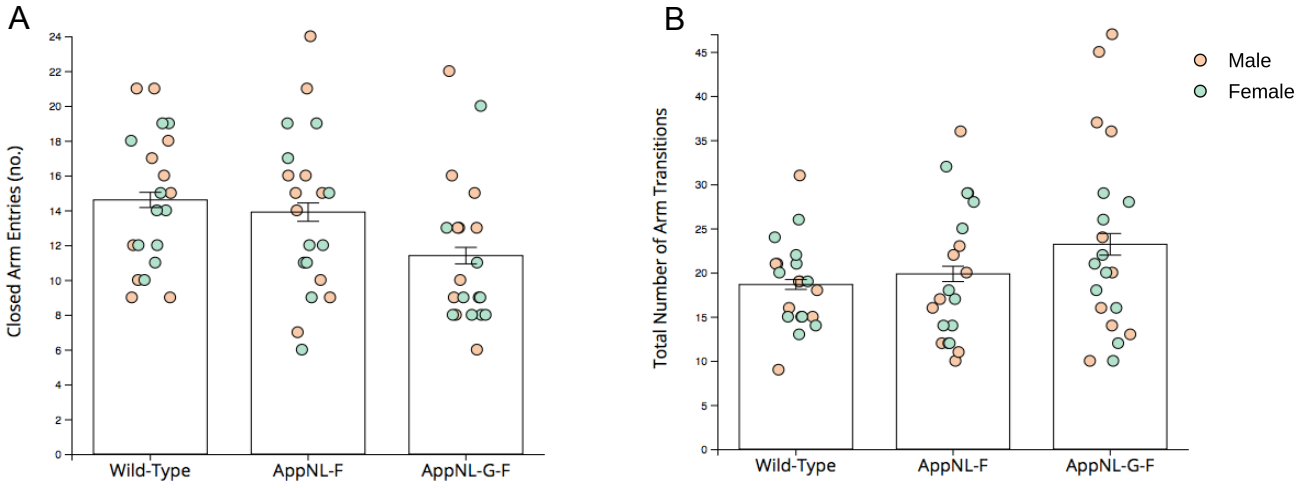


Figure 2.19. **General exploratory and locomotor activity in the plus-maze at 15-months.**

(A) Mean (±SEM) entries into closed arms. **(B)** Mean (±SEM) number of arm transitions.

15-month old App^{NL-G-F} mice make significantly more head-dips in the plus-maze

There was an effect of genotype, $F_{(2,54)} = 11.66$, $p < 0.001$ (Fig. 2.20), no effect of sex, $F_{(1,54)} = 1.19$, $p = 0.280$, and no interaction between genotype and sex, $F_{(2,54)} = 0.44$, $p = 0.645$, on the number of head-dips mice made during the plus-maze trial. Pairwise comparisons confirmed that the App^{NL-G-F} mice made a significantly greater number of head-dips (M=32.65 ±3.92) compared with the wild-type mice (M=12.60 ±1.84; $p < 0.001$) and the App^{NL-F} mice (M=19.20 ±2.75; $p = 0.007$); there was no difference in the number of head-dips between the wild-type mice and the App^{NL-F} mice ($p = 0.374$). In general, more head-dipping is linked with anxiolytic-like behaviour, yet caution is required given that we did not distinguish 'protected' and 'unprotected' head-dips. 'Protected' head-dips, which occur from a closed arm or the central square, may represent risk assessment as opposed to exploratory behaviour (Blanchard et al., 1990).

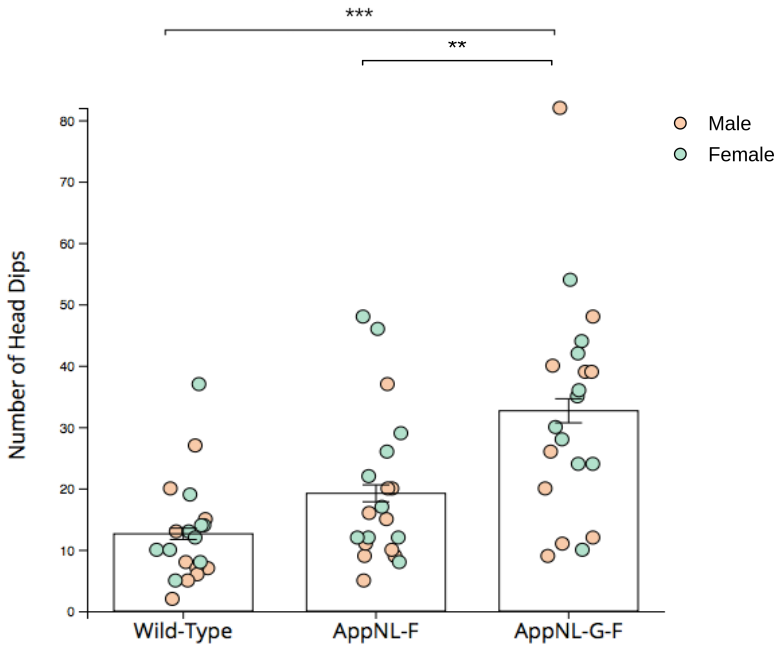


Figure 2.20. **Mean (±SEM) number of head-dips in the plus-maze at 15 months.**

At 15-months both the App^{NL-F} and App^{NL-G-F} mice exhibit unaltered anxiety-like levels in the open field; App^{NL-G-F} showed a mild degree of open-field hyperactivity. The ostensibly anxiolytic plus-maze profile in the 15-month old App^{NL-G-F} mice replicates the results observed at 8-months; this was not driven by low exploratory behaviour or reduced locomotion. We now turn to the analyses of social behaviour.

2.3.2 Measures of Social Behaviour

Taking into account the reports of social withdrawal as an early symptom of AD (*e.g.*, Cuthbert et al., 2019; Jost & Grossberg, 1996), we assessed the willingness of App^{NL-F} and App^{NL-G-F} mice to approach novel conspecifics using the Crawley three-chamber protocol (Moy et al., 2004). To test sociability, mice were given the choice of whether to spend time exploring a cage (*i.e.*, the inverted wire cup) containing an unfamiliar stimulus mouse or an identical empty cage. To test preference for social novelty, mice were given the choice of whether to explore a novel stranger mouse placed underneath the previously empty cage or the original stranger mouse. The protocol was then adapted to assess whether the APP knock-in mice preferred to approach a social odour cue (soiled bedding from a cage of unfamiliar, sex-matched mice) versus a non-social odour cue (clean bedding). This social olfaction test is an extension of the original assay of sociability, but controls for the potential confounding factor of anxiety caused by the presence of a novel conspecific. To briefly review, exploration was defined as the subject mouse directing the nose at a distance of ≤ 2 cm to a cage and/or touching a cage with the nose, and the discrimination ratios were calculated as [(A-B)/(A+B)] to compensate for the variability in individual exploration times.

2.3.2.1 Social Approach & Preference for Social Novelty

2.3.2.1.1 Results at the 8-month age point

8-month old APP knock-in mice prefer exploring a stranger mouse to an empty cage

A repeated-measures ANOVA of mean time spent exploring in proximity to the cages showed no effect of genotype, $F_{(2,52)} = 1.71$, $p = 0.190$ (Fig. 2.21-A), no effect of sex, $F_{(2,52)} = 1.95$, $p = 0.168$, and no interaction between genotype and sex, $F_{(2,52)} = 1.97$, $p = 0.149$. This indicates that there was no difference between groups in their overall tendency to explore the stimuli. There was an effect of cage, $F_{(1,52)} = 145.21$, $p < 0.001$. Significantly more time was spent exploring the cage with the unfamiliar stimulus mouse as opposed to the empty cage (overall $MD = 80.19 \pm 6.59$), and this pattern was similar across groups. There were no interactions between cage and genotype, $F_{(2,52)} = 0.55$, $p = 0.576$, cage and sex, $F_{(1,52)} = 0.15$, $p = 0.697$, or cage, genotype and sex, $F_{(2,52)} = 1.01$, $p = 0.371$. To analyse further, an ANOVA of the mean discrimination ratios revealed no effect of genotype, $F_{(2,52)} = 1.33$, $p = 0.273$ (Fig. 2.21-B), no effect of sex, $F_{(1,52)} = 0.82$, $p =$

0.368, and no interaction between genotype and sex, $F_{(2,52)} = 0.79$, $p = 0.482$. Thus, the groups did not differ in their degree of preferential exploration of the stranger mouse over the empty cage. Finally, a series of one-sample *t*-tests revealed that each of the mean discrimination scores was significantly above zero: wild-type mice ($p < 0.001$), App^{NL-F} mice ($p = 0.001$) and App^{NL-G-F} mice ($p < 0.001$). Together these results demonstrate robust sociability in the 8-month old APP knock-in mice.

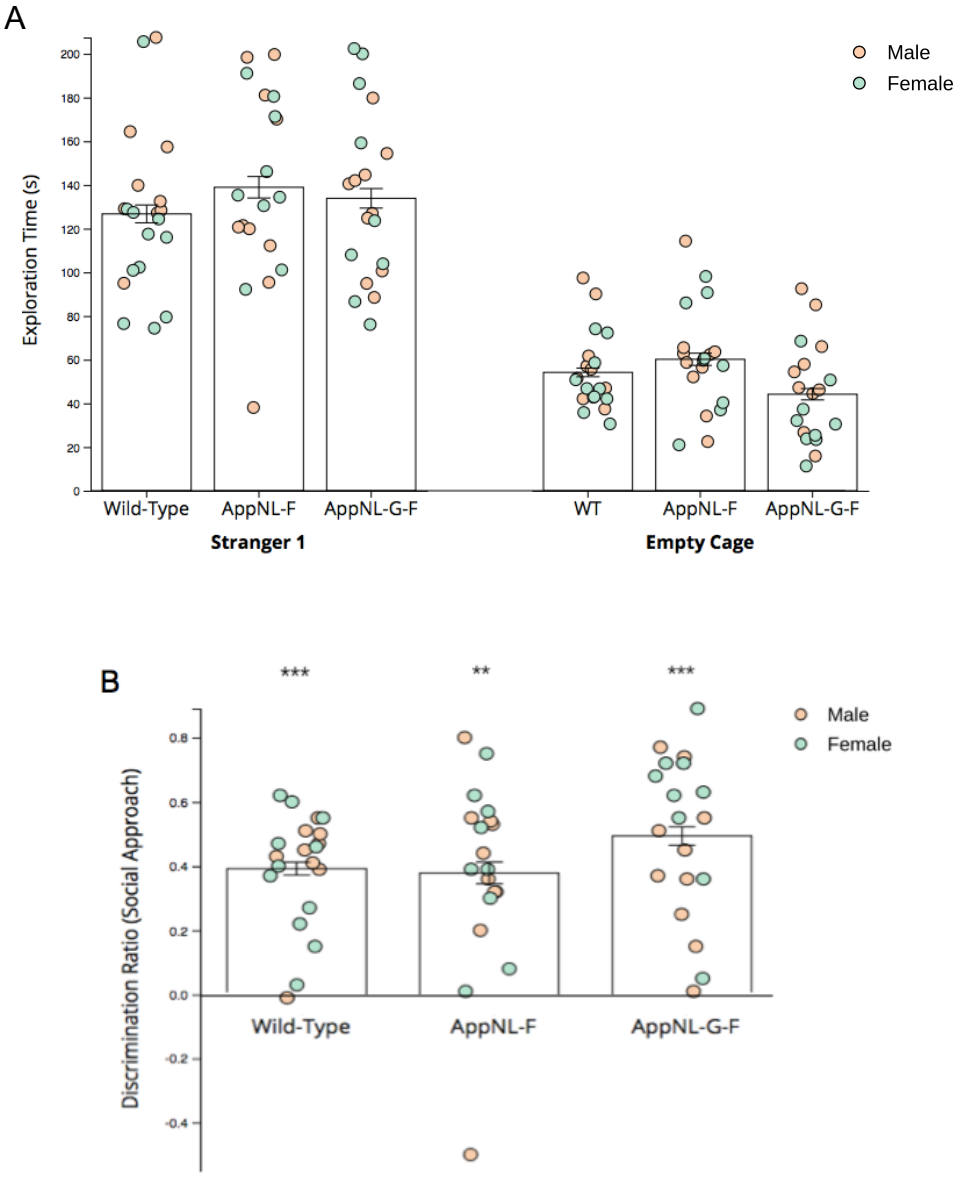


Figure 2.21. **Social approach behaviour in the Crawley 3-chamber test at 8-months.**

(A) Mean (±SEM) exploration time of each cage. **(B)** Mean (±SEM) discrimination ratios.

8-month old APP knock-in mice are mildly impaired in preference for social novelty

Preference for social novelty was assessed by repeated-measures ANOVA of the mean times spent exploring the cages containing the stimulus mice (Fig. 2.22-A). Genotype and sex interacted on the overall time spent exploring in proximity to the cages, $F_{(2,52)} = 3.39$, $p = 0.041$. Simple main effects analysis by *t*-test revealed differences between the male and female wild-type mice, $t_{(18)} = 2.46$, $p = 0.024$ ($MD = 47.95 \pm 19.49$), and the male and female $App^{NL\text{-}G\text{-}F}$ mice, $t_{(17)} = 2.41$, $p = 0.028$ ($MD = 49.56 \pm 20.59$). In each instance, the female mice spent significantly less time exploring the stimuli compared to the male mice. Nevertheless, there was an effect of cage, $F_{(1,52)} = 12.21$, $p = 0.001$. Significantly more time was spent exploring the novel stranger mouse as opposed to the original stranger mouse (overall $MD = 19.26 \pm 5.41$), and this effect of cage was similar across groups. There were no interactions between cage and genotype, $F_{(2,52)} = 1.17$, $p = 0.320$, cage and sex, $F_{(1,52)} = 2.37$, $p = 0.130$, or cage, genotype and sex, $F_{(2,25)} = 0.11$, $p = 0.893$.

This observed preferential exploration of the novel stranger mouse was further investigated for group differences as a discrimination ratio. The ANOVA yielded no effect of genotype, $F_{(2,52)} = 2.12$ $p = 0.130$ (Fig. 2.22-B), no effect of sex $F_{(1,52)} = 1.76$, $p = 0.191$, and no interaction between genotype and sex, $F_{(2,52)} = .440$, $p = 0.647$. Each group preferentially explored the novel stranger mouse over the original stranger mouse, with mean discrimination ratios that were statistically similar across groups. However, the wild-type mice were the only group to have a discrimination score that differed to zero ($p = 0.001$). The discrimination score did not reach statistical significance in either the $App^{NL\text{-}F}$ mice ($p = 0.068$) or the $App^{NL\text{-}G\text{-}F}$ mice ($p = 0.425$). Taken together, these results indicate that the 8-month old APP knock-in mice have a mild impairment in preference for social novelty. Although the APP knock-in mice preferentially explored the novel stranger mouse over the original stranger mouse, with a discrimination ratio that was statistically similar to the wild-type mice, their mean discrimination ratios did not statistically differ to the null discrimination score of zero.

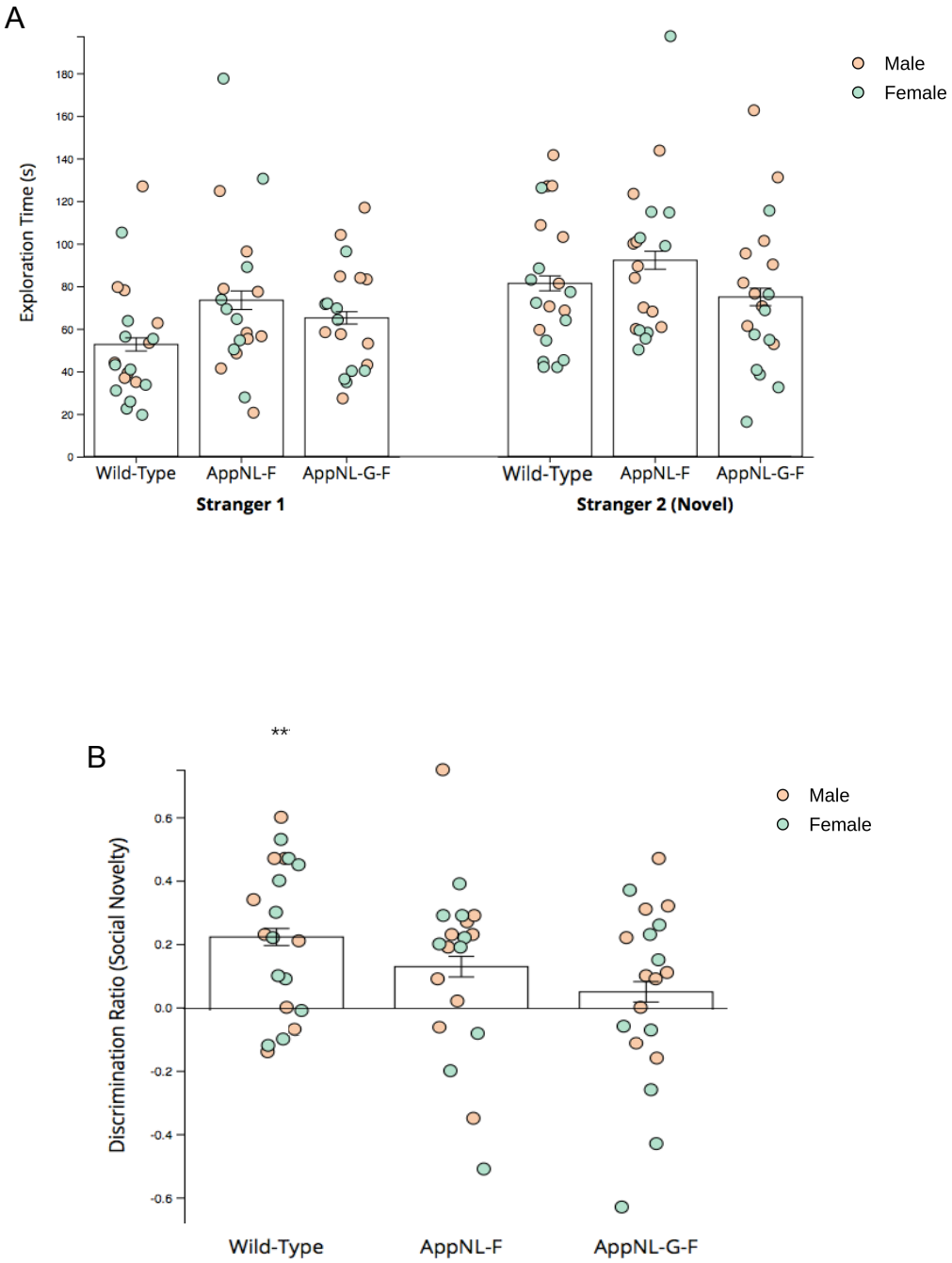


Figure 2.22. **Preference for social novelty in the Crawley 3-chamber test at 8 months.** **(A)** Mean (±SEM) exploration time of stranger mouse 1 and the novel stranger mouse 2. **(B)** Mean (±SEM) preference ratios. The preference ratios did not differ between groups. However, the wild-type mice were the only group to display a ratio significantly above zero. Thus, the 8-month old APP knock-in mice are mildly impaired in social novelty.

2.3.2.1.2 Results at the 15-month age point

15-month old APP knock-in mice prefer exploring a stranger mouse to an empty cage

The sociability of the 15-month old mice was assessed using a repeated measures ANOVA of the mean times spent exploring the stranger mouse and the empty cage (Fig. 2.23-A). There was an effect of sex, $F_{(1,54)} = 6.22$, p = 0.016 (M > F), with no effect of genotype, $F_{(2,54)} = 1.37$, $p = 0.264$, and no interaction between genotype and sex, $F_{(2,54)} = 1.095$, $p = 0.342$). On average, the male mice spent more time exploring in proximity to the stimuli compared with the female mice, however, as noted, there were no differences between the genotypes in their overall exploration time. There was an effect of cage, $F_{(1,54)} = 200.34$, $p < 0.001$. Significantly more time was spent exploring the stranger mouse as opposed to the empty cage (overall $MD = 79.92 \pm 5.55$). This effect of cage was similar across groups. There was no interaction between cage and genotype, $F_{(2,54)} = 0.27$, $p = 0.767$, or cage and sex, $F_{(1,54)} = 1.08$, $p = 0.303$, and no interaction between cage, genotype and sex, $F_{(2,54)} = 0.76$, $p = 0.473$.

To further evaluate whether this preferential exploration of the stranger mouse was similar between groups, an analysis of variance of the mean discrimination ratios was carried out. There was no effect of genotype, $F_{(2,54)} = 1.20$, $p = 0.310$ (Fig. 2.23-B), no effect of sex, $F_{(1,54)} = 0.01$, $p = 0.928$, and no interaction between genotype and sex, $F_{(2,54)} = .214$, $p = 0.808$. Thus, the mean discrimination ratios were similar across groups. Furthermore, a series of one-sample *t*-tests confirmed that the mean discrimination score for each of the three genotypes was significantly greater than the null discrimination score of zero: wild-type mice ($p < 0.001$), App^{NL-F} mice ($p < 0.001$) and App^{NL-G-F} mice ($p < 0.001$). Taken together, these results demonstrate robust sociability in the 15-month old APP knock-in mice. Similar to the 8-month old APP knock-in mice, the 15-month old APP knock-in mice preferred to approach a novel conspecific to an innate object. Furthermore, the mean discrimination ratios were statistically similar to the discrimination ratio displayed by the wild-type mice, and each was significantly greater than zero.

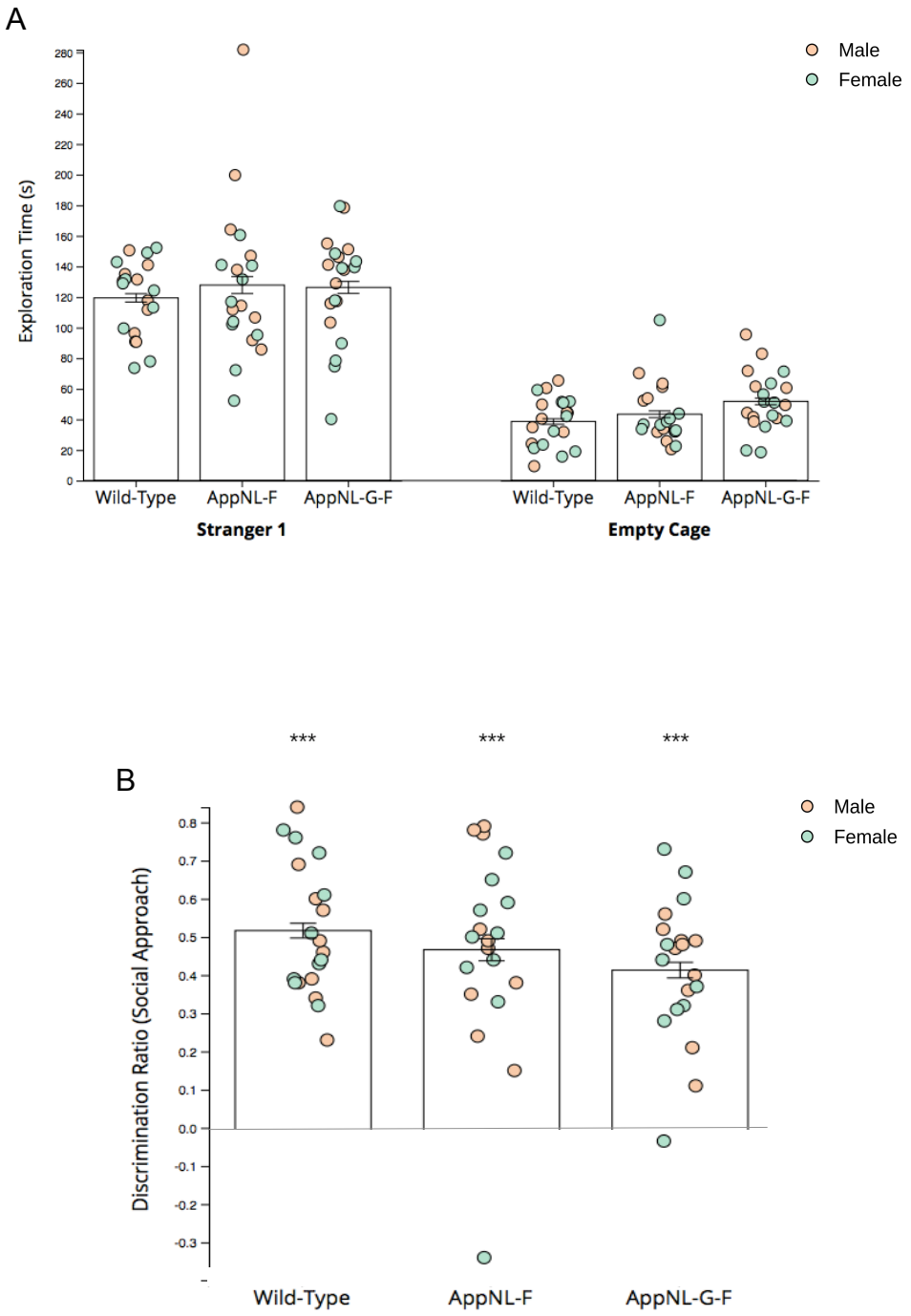


Figure 2.23. **Social approach behaviour in the Crawley 3-chamber test at 15-months.** **(A)** Mean (±SEM) exploration times of the stranger mouse and the identical empty cage. **(B)** Mean (±SEM) preference ratios. Each group demonstrated a robust preference for the stranger mouse over the empty cage

15-month old APP knock-in mice prefer exploring a novel mouse to a familiar mouse

To assess whether the 15-month old mice displayed a preference for social novelty, a repeated measures ANOVA was carried out on the mean times spent exploring in proximity to the cages containing the novel stranger mouse and the original stranger mouse (Fig. 2.24-A). There was an effect of sex, $F_{(1,54)} = 4.86$, $p = 0.032$ (M > F), no effect of genotype, $F_{(2,54)} = 1.67$, $p = 0.197$, and no interaction between genotype and sex, $F_{(2,54)} = 2.09$, $p = 0.133$. Similar to the sociability test, the male mice spent significantly more time exploring in proximity to the stimuli compared with the female mice, with no difference between genotypes in overall exploration time. There was an effect of cage, $F_{(2,54)} = 27.77$, $p < 0.001$. More time was spent exploring the novel stranger mouse as opposed to the original stranger mouse (overall $MD = 22.44 \pm 4.33$). The effect of cage was similar across groups. There was no interaction between cage and genotype, $F_{(2,54)} = 1.26$, $p = 0.292$, cage and sex, $F_{(1,54)} = 0.54$, $p = 0.464$, or cage, genotype and sex, $F_{(2,54)} = 2.01$, $p = 0.144$.

This observed preferential exploration of the novel stranger mouse was further assessed by an analysis of variances of the mean discrimination ratios. The ANOVA yielded no effect of genotype, $F_{(2,54)} = 1.51$, $p = 0.230$ (Fig. 2.24-B), no effect of sex, $F_{(1,54)} = 1.16$, $p = 0.286$, and no interaction between genotype and sex, $F_{(2,54)} = 1.82$, $p = 0.171$. All groups displayed a similar preference for the novel stranger mouse. Furthermore, each of the mean discrimination ratios was significantly above the null discrimination score of zero: wild-type mice ($p = 0.001$), App^{NL-F} mice ($p = 0.035$) and App^{NL-G-F} mice ($p = 0.001$). Taken together, these results demonstrate that the 15-month old APP knock-in mice prefer social novelty. They choose to explore a novel stranger mouse over the previously explored unfamiliar mouse, and when quantified as a discrimination ratio, there was no difference between the APP knock-in mice and the wild-type mice. The 15-month old APP knock-in mice displayed a robust preference for social novelty.

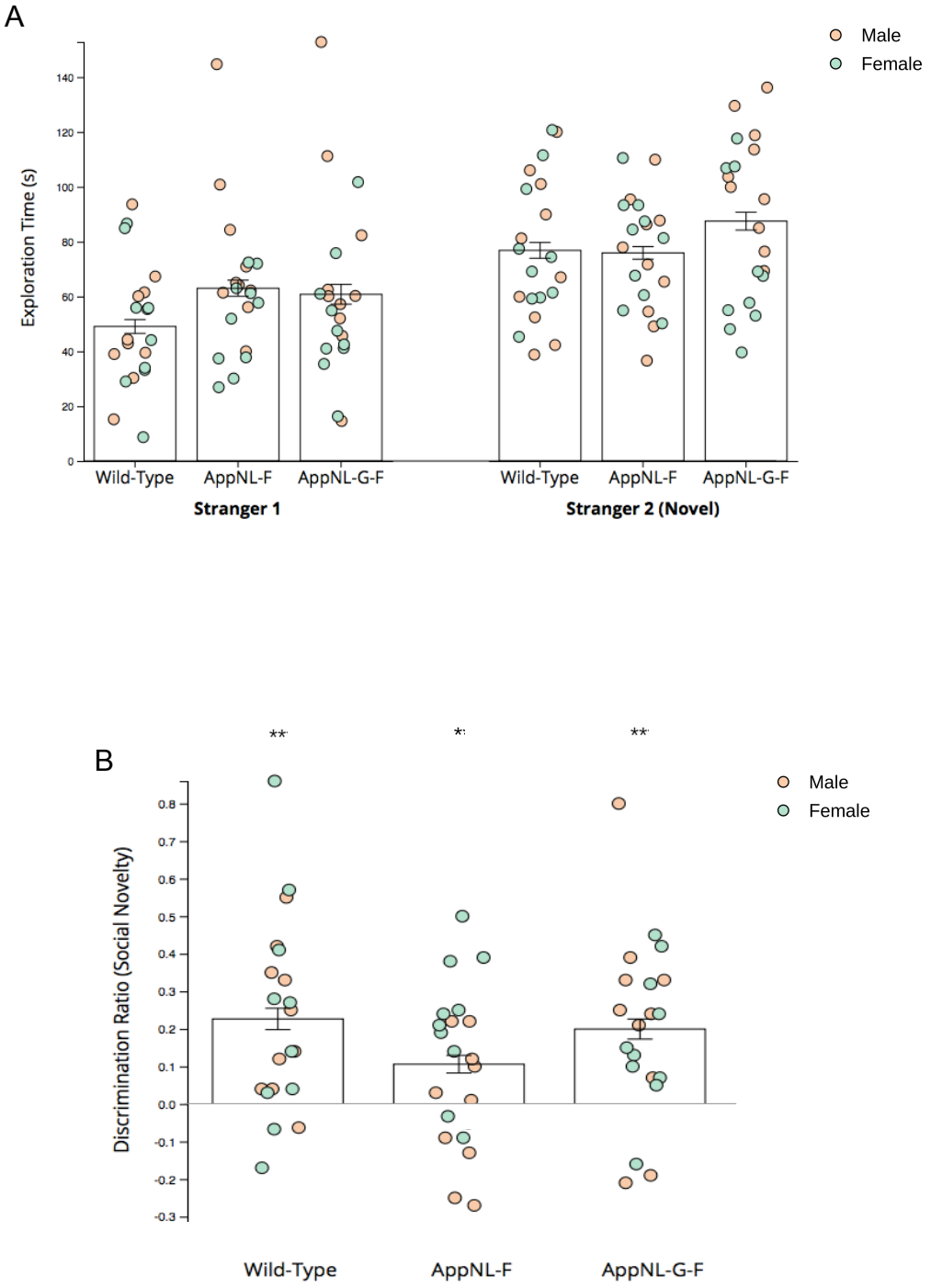


Figure 2.24. **Preference for social novelty in the Crawley 3-chamber test at 15-months.** **(A)** Mean (±SEM) exploration time of stranger mouse 1 and the novel, stranger mouse 2. **(B)** Mean (±SEM) preference ratios. The preference ratios were similar between groups, and each ratio was significantly above the null score of zero.

2.3.2.3 Social Olfaction

2.3.2.3.1 Results at the 8-month age point

8-month old female App^{NL-G-F} mice exhibit a weaker preference for a social odour cue

Social olfaction was assessed using a repeated-measures ANOVA on the mean times spent exploring in proximity to the cage containing the soiled bedding and the cage containing the clean bedding (Fig. 2.25-A). With regard to overall exploration time, there was no effect of genotype, $F_{(2,52)} = 1.02$, $p = 0.369$, no effect of sex, $F_{(1,52)} = 0.99$, $p = 0.324$, and no interaction between genotype and sex, $F_{(2,52)} = .774$, $p = 0.466$. All groups spent a similar amount of time exploring in proximity to the stimuli. There was an effect of bedding, $F_{(1,52)} = 111.60$, $p < .001$. More time was spent exploring the soiled bedding than the clean bedding (overall $MD = 22.44 \pm 4.33$). However, this effect of bedding differed between groups, as was indicated by the interaction between bedding, genotype and sex, $F_{(2,52)} = 4.31$, $p = 0.019$. To investigate this source of difference, a discrimination ratio was calculated for all six groups and a Welch one-way ANOVA was carried out. The ANOVA revealed significant group differences, $F_{(5,23.12)} = 2.72$, $p = 0.045$ (Fig. 2.25-B). Games-Howell pairwise comparisons confirmed a difference in discrimination ratios between the female and male App^{NL-G-F} mice ($p = 0.047$), and the female App^{NL-G-F} mice and female wild-type mice ($p = 0.050$). On average, the female App^{NL-G-F} mice explored the soiled bedding more than the clean bedding. However, their mean discrimination ratio was significantly reduced compared with the discrimination ratio of the male App^{NL-G-F} mice and the female wild-type mice. Furthermore, except for the female App^{NL-G-F} mice ($p = 0.209$), all groups had a discrimination ratio that was significantly above zero: male wild-type ($p = 0.001$); female wild-type ($p < 0.001$); male App^{NL-F} ($p < 0.001$); female App^{NL-F} ($p = 0.015$); male App^{NL-G-F} ($p < 0.001$). Together these results indicate that the 8-month old female App^{NL-G-F} mice have a mild social olfaction deficit.

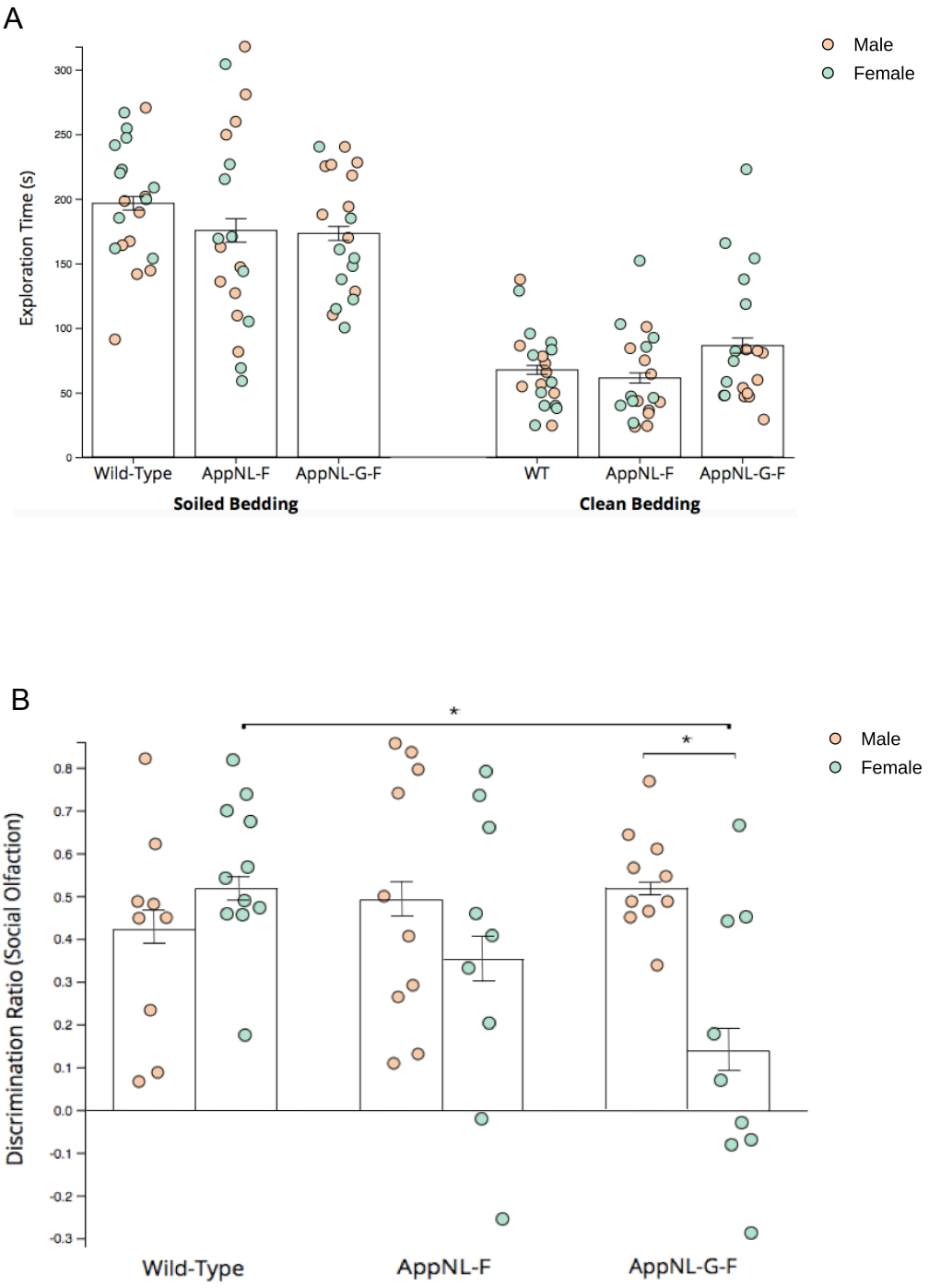


***Figure* 2.25. Social olfaction at 8-months of age. (A)** Mean (±SEM) times spent exploring the bedding stimuli. **(B)** Mean (±SEM) preference ratios. Female App^{NL-G-F} mice displayed a mild social olfaction deficit. They exhibited preferential exploration of the soiled bedding, however, the preference ratio was significantly reduced compared to female wild-type mice, and did not differ to then null preference score of zero.

2.3.2.3.2 Results at the 15-month age point

15-month old APP knock-in mice exhibit a weaker preference for a social odour cue

Social olfaction was assessed at the 15-month age point using a repeated measures ANOVA of the mean times spent exploring in proximity to the bedding stimuli (Fig. 2.26-A). In regard to the overall exploration time, there was an effect of sex, $F_{(1,54)} = 5.36$, $p = 0.024$ (M > F), with no effect of genotype, $F_{(2,54)} = 0.87$, $p = 0.426$, and no interaction between genotype and sex, $F_{(2,54)} = 0.39$, $p = 0.678$. There was an effect of bedding, $F_{(1,54)} = 12.12$, $p = 0.001$. More time was spent exploring in proximity to the soiled bedding than the clean bedding (overall *MD* = 109.84 ±10.91). However, there was an interaction between bedding and sex, $F_{(1,54)} = 9.09$, $p = 0.004$. The female mice did not preferentially explore the soiled bedding (overall *MD* = -0.002 ±0.05). There was no interaction between bedding and genotype, $F_{(2,54)} = 0.71$, $p = 0.499$, or bedding, genotype and sex, $F_{(2,54)} = 0.53$, $p = 0.591$. When comparing the discrimination ratios using a two-way ANOVA, there was an effect of sex, $F_{(1,54)} = 9.09$, $p = 0.004$ (M > F), with no effect of genotype, $F_{(2,54)} = 1.57$, $p = 0.217$ (Fig. 2.26-B), and no interaction between genotype and sex, $F_{(2,54)} = 0.63$, $p = 0.537$. Lastly, the discrimination ratios were tested against the null discrimination score of zero using one-sample *t*-tests. The wild-type mice displayed a discrimination ratio that was significantly above zero ($p = 0.003$) whereas the App^{NL-F} mice ($p = 0.074$) and the App^{NL-G-F} mice ($p = 0.708$) did not. Taken together, these results demonstrate that the 15-month old APP knock-in mice are mildly impaired in social olfaction. The APP knock-in mice preferentially explored the soiled bedding over the clean bedding, and their discrimination ratio was similar to that of the wild-type mice. However, neither the App^{NL-F} mice nor the App^{NL-G-F} mice displayed a discrimination ratio that was significantly different to the null discrimination score of zero. Although genotype and sex did not interact when assessing the discrimination ratios, an inspection of the corresponding graph (Fig. 2.26-B) indicates that the female APP knock-in mice likely contributed to these weaker discrimination ratios.

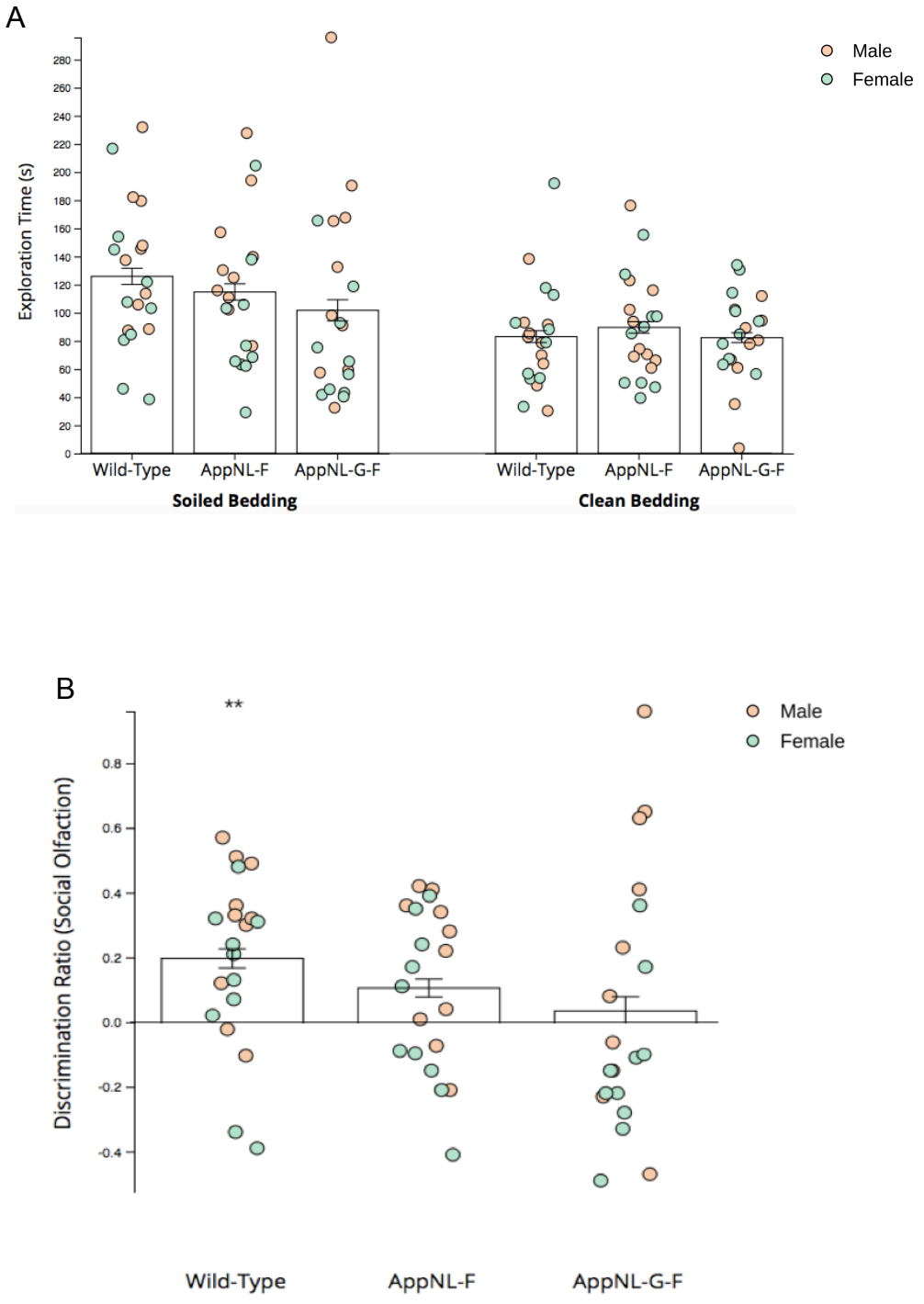


Figure 2.26. **Social olfaction at 15-months of age. (A)** Mean (±SEM) times spent exploring the soiled bedding and the clean bedding. **(B)** Mean (±SEM) preference ratios. There were no differences between genotypes in their mean preference ratios. However, only the wild-mice had a preference ratio significantly above zero.

2.3.2.4 General Olfactory Ability

APP knock-in mice exhibit intact olfaction for an odour unrelated to a social cue

Intact olfaction is critically required to elicit normal social behaviours in mice (Ryan et al., 2008). To test the general olfactory ability of mice we measured the latency to find a chocolate flavoured cereal piece buried underneath clean cage bedding following an approximate nineteen hour period of food deprivation. A Welch one-way ANOVA revealed no differences between genotypes in the latency to uncover the food, $F_{(2,7.00)} = 0.541$, $p = 0.605$ (Fig. 2.27-A). Due to the exceptionally small App^{NL-F} sample size (n=4) the analysis was also conducted as an independent t-test between the wild-type mice and the App^{NL-G-F} mice. The latency to uncover the food was again similar between groups, $t_{(33)} = 0.739$, $p = 0.465$ (Fig. 2.27-B). We can therefore conclude that the mild impairment in preference for social novelty displayed by the 8-month old APP knock-in mice, and the mild social olfaction deficit found in the 8-month old female App^{NL-G-F} mice and the 15-month old APP knock-in mice, was not due to a deficit in general olfactory abilities.

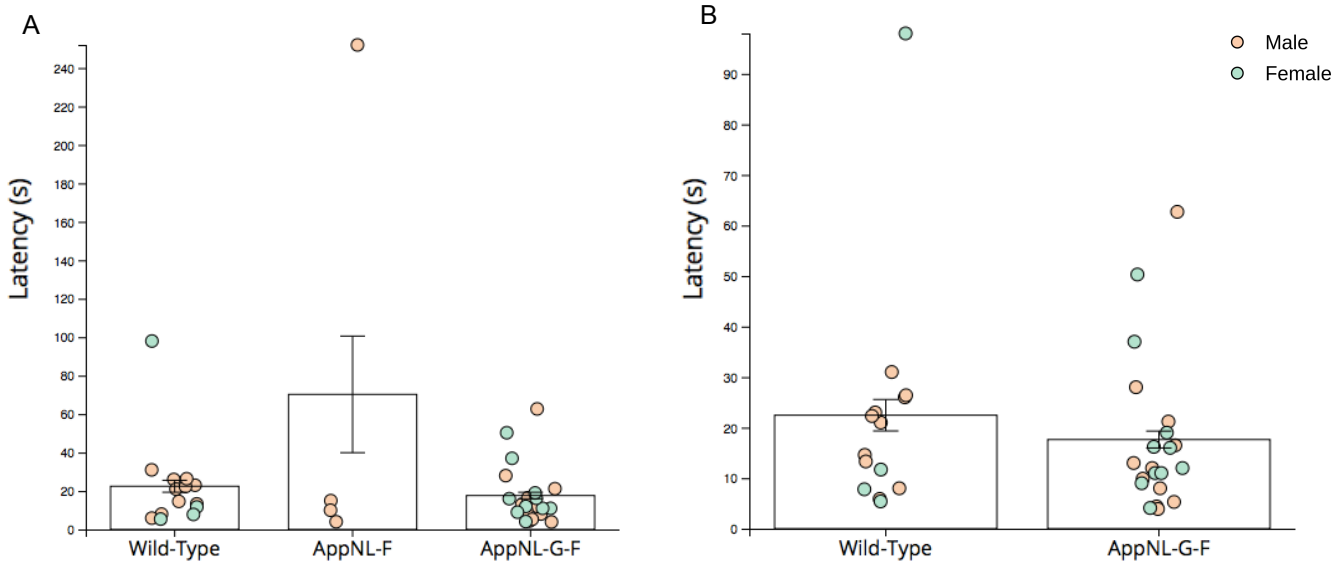


Figure 2.27. **General olfactory ability as assessed in the buried food test.** There were no group differences in the latency to uncover a hidden cereal piece. **(A)** Analysis by one-way ANOVA. **(B)** Analysis by independent t-test.

2.4 Discussion

Anxiety and social withdrawal are clinically significant symptoms of AD; they are highly prevalent in the disease and are associated with a range of adverse effects. Furthermore, late-life emergence of anxiety and social withdrawal is increasingly being recognized as a possible harbinger of AD (Ismail et al., 2016; Lyketsos et al., 2011). Despite this, the neurobiological basis of these symptoms in the context of the AD brain remains unclear. Identifying AD mice that enable the modeling of the neurobiological links between anxiety and social withdrawal and AD is thus important. To this end, we examined social and anxiety-related behaviours in male and female App^{NL-F} and App^{NL-G-F} mice aged 8 and 15-months.

We assessed social behaviour in the App^{NL-F} and App^{NL-G-F} mice using the Crawley 3-chamber protocol (Moy et al., 2004). It is understood that wild-type mice display a propensity for sociability, and also for social novelty whereby a novel conspecific is preferentially explored over a previously encountered mouse (Silverman, 2010). In the context of the Crawley 3-chamber protocol, an animal is viewed as demonstrating sociability and preference for social novelty respectively if under free-choice conditions they preferentially explore an unfamiliar sex-matched mouse as opposed to an innate object, and then preferentially explore a novel, unfamiliar mouse (sex-matched) over the original stranger mouse. It is worthy of note that per the Crawley 3-chamber protocol the original stimulus mouse remains in the same location throughout the test, and thus, the novel stimulus mouse is introduced in to what may be a less explored area of the apparatus. Our results showed that sociability in the 8 and 15-month old App^{NL-F} and App^{NL-G-F} mice was intact, and there was no difference in sociability between the two APP knock-in strains. However, the 8-month old App^{NL-F} and App^{NL-G-F} mice displayed a mild impairment in preference for social novelty that was not explained by a general olfaction deficit, whereas preference for social novelty was intact at 15 months; preference for social novelty did not differ between the two APP knock-in strains. The 8-month old female wild-type and female App^{NL-G-F} mice spent less time overall exploring the stimulus mice during the preference for social novelty stage, and the 15-month old female mice spent less time overall exploring the stimulus mice in each stage of the test; this indicates a minor sex difference (not based on

genotype) in the willingness to engage in social interaction. We describe the behaviour in the 8-month old App^{NL-F} and App^{NL-G-F} mice as only mildly impaired as they did preferentially explore the novel, unfamiliar mouse, and the associated discrimination ratios were statistically similar to the wild-type mice. Yet in contrast to the wild-type mice, the discrimination ratios were not statistically different to chance, or a null preference.

There is currently only one other study that has measured social behaviour in the APP knock-in mice. Latif-Hernandez et al. (2017) used the Crawley 3-chamber protocol to assess social behaviour in 3, 6 and 10-month old female App^{NL-G-F} mice, and compared the behaviour to the App^{NL} control mice generated by Saito et al. (2014). The findings in Latif-Hernandez et al. (2017) were in line with our study. Sociability was intact in the App^{NL-G-F} mice. Preference for social novelty was intact, but with a mild impairment observed at the 10-month age point (i.e., discrimination between the novel stimulus mouse and the original stimulus mouse was at chance), which is similar to the mild impairment we found in the 8-month old App^{NL-G-F} mice (and also App^{NL-F}). As a comprehensive study of social behaviours in the APP knock-in mice has not yet been performed, more studies are needed in order to confirm the social behaviours of the APP knock-in mice. Future investigations would also benefit from including a wider array of social interaction tests in order to more precisely describe the social behaviours of the APP knock-in mice. These preliminary results suggest that the APP knock-in mice do not model the social withdrawal symptoms reminiscent in AD; however, the age-dependent change in preference for social novelty observed at 8 and 10-months of age in the App^{NL-G-F} mice may be of interest.

It is also of interest to compare our results to commonly employed AD mice genetically modified to overexpress Aβ. Filali, Lalonde and Rivest (2011) tested 6-month old male APPswe/PSEN1 mice in the Crawley 3-chamber protocol and found that the animals' willingness to engage in social interaction was intact during each stage of the test; however, sociability was at chance and preference for social novelty was significantly reduced. Similarly, 6-month old male Thy1-$hAPP^{Lond/Swe+}$ mice displayed intact sociability but a significantly reduced preference for social novelty (Faizi et al., 2012).

Kosel et al. (2019) tested female 5xFAD mice aged 3, 6, 9 and 12 months and found that sociability and preference for social novelty was intact relative to the control mice, yet the 5xFAD mice did display an age dependent reduction in the willingness (i.e., amount to time) to engage with the stranger mouse during the sociability test. In all three studies social interaction was measured as the time spent sniffing the stimulus mice. Using a paradigm that permitted contact between subject and control mice, Deacon et al. (2009) found no effect of AD-pathology on the sociability of 23 month-old female Tg2576 mice, and Bories et al. (2012) found no effect of AD-pathology on the sociability of 12 and 18-month old male 3xTg-AD mice or 12-month old female 3xTg-AD mice. However, 18-month old female 3xTg-AD mice showed a reduction in social interaction; this is a time point at which tau pathology has developed in these mice in addition to the Aβ. Different methods of measuring social interaction seemingly generate different results. Our results were in line with Kosel et al. (2019) suggesting that there are similarities between the APP knock-in mice and mice that achieve Aβ by APP overexpression, yet the more pronounced preference for social novelty deficit in Filali, Lalonde and Rivest (2011) and Faizi et al. (2012) suggests the possibility of a behavioural artifact of APP overexpression; however, this statement would require further consideration.

We pre-emptively investigated the potential impact of anxiety on social interaction by testing the animals' willingness to approach a social odour cue in the absence of a novel conspecific. The Crawley 3-chamber protocol was modified by replacing the novel conspecific with soiled bedding from a cage of sex-matched unfamiliar mice. We measured whether an animal preferentially explored soiled bedding over clean bedding. The 8-month old female App^{NL-G-F} mice displayed a social olfaction deficit relative to the male App^{NL-G-F} mice and the female wild-type mice; however, they spent a similar amount of time overall exploring the stimuli. The 15-month old App^{NL-F} and App^{NL-G-F} mice displayed a mild social olfaction deficit. Their preferential exploration of the soiled bedding was at chance, and this was likely due to the fact that the female mice (including wild-type mice) showed a reduced motivation to explore either of the stimuli, and the female mice (including the wild-type mice) did not display a preference for the soiled bedding. The buried food test showed that these deficits were

unlikely to have been caused by anosmia given that the 15-month old App^{NL-F} and App^{NL-G-F} mice found a cereal piece hidden underneath clean cage bedding as easily as the wild-type control mice; however, the author notes a lack of data for female App^{NL-F} mice and a sample size that precluded us from investigating an interaction between genotype and sex (visual inspection of the accompanying graph indicated that female mice were not impaired in the buried food test). Similarly, Kosel et al. (2019) found that female 5xFAD mice overexpressing APP displayed an age-related decrease in sniffing durations in response to social odours from 3 to 12 months of age. Our results indicate that a reduced motivation of female AD mice to explore a social odour is not an artifact of the APP overexpression paradigm; however, additional studies would need to be reviewed in order to corroborate this finding. It is unclear why we would observe changes in motivation to explore a social odour cue, but not a novel conspecific. The author notes that this requires further consideration.

Our most intriguing finding came from the 'anxiety' assessing tasks. First, the 8 and 15-month old App^{NL-F} mice displayed unaltered locomotion and no difference in anxiety-like behaviour in the open field and the elevated plus-maze relative to the wild-type control mice. This is currently the first study to report on open field and elevated plus-maze behaviours in the App^{NL-F} mice. Further studies are necessary in order to determine whether this result replicates across laboratories. In contrast, the 8-month old App^{NL-G-F} mice combined an ostensibly anxiogenic open field profile with an ostensibly anxiolytic plus-maze profile. This ostensibly anxiolytic plus-maze profile persisted in the 15-month old App^{NL-G-F} mice. We confirmed that these findings were not based on changes in locomotion (albeit there was a small degree of hyperactivity in the open field at 15-months) or general exploratory behaviour. Furthermore, in each instance, the significant change in behaviour observed in the App^{NL-G-F} mice was, in addition, relative to the App^{NL-F} mice.

Three studies have investigated open field locomotion and anxiety in the App^{NL-G-F} mice. In each study methods were broadly consistent but not identical to our study; inter-laboratory differences in open field method are indeed common. Whyte et al. (2018) observed in 6-month old male App^{NL-G-F} mice significantly fewer zone entries and

significantly less distance travelled compared to wild-type control mice, whereas Mehla et al. (2019) observed no difference in these behaviours between the male App^{NL-G-F} mice aged 3, 6, 9 and 12 months and wild-type control mice. Latif-Hernandez et al. (2017) also found no difference in distance travelled between female App^{NL-G-F} mice aged 3, 6 and 10 months and age-matched App^{NL} mice. Whyte et al. (2018) measured open field locomotion for only 5 minutes, which may explain the discrepancy between studies; however, we did not observe in our study any difference in the distances travelled within the first 5-minute time block. Together, these studies indicate that the App^{NL-G-F} mice have unaltered locomotion in the open field from 3 to 6 months, some evidence of hypo-activity at 6 months, normal locomotion from 8 to 10 months, and a degree of hyperactivity at 15-months. With regard to open field anxiety as measured by the number of entries and time in the centre of the apparatus, there appears to be some evidence that the App^{NL-G-F} mice display an ostensibly anxiogenic open field profile that is restricted to the 6 and 8-month age point. Mehla et al. (2019) found no evidence of altered anxiety-like levels in App^{NL-G-F} mice aged 3, 6, 9 or 12 months, nor did Whyte et al. (2018) at 6 months. Latif-Hernandez et al. (2017) did observe anxiety-like behaviour in the App^{NL-G-F} mice at 6 months, but not 3 or 10 months. Our results indicate an ostensibly anxiogenic open field profile at 8 months, but not 15 months. Thus, there is some evidence for the presence of an anxiogenic-like open field profile in the App^{NL-G-F} mice between the ages of 6 and 8 months. However, open field behaviour is reportedly variable and viewed as a less reliable measure of rodent anxiety compared to the elevated plus- maze (Prut & Belzung, 2003; Carola et al., 2002; Walsh & Cummins, 1976).

We observed an ostensibly anxiolytic plus-maze profile in the 8 and 15-month old App^{NL-G-F} mice as described by an increased amount of time on and number of entries into the open arms; this behaviour was not explained by altered locomotion or low levels of exploratory behaviour. We anticipated to some degree an altered plus-maze profile given that the hippocampus and amygdala are affected early in people with AD pathogenesis (Poulin et al., 2011) and play a prominent role in rodents' response to anxiogenic stimuli (McHugh et al., 2004; Davis, 1992). Several other AD mouse models exhibit increased open arm activity in the plus-maze (Webster et al., 2014; Lalonde,

Fukuchi & Strazielle, 2012), and our results are broadly similar to the two studies that have reported elevated plus-maze behaviour in the App^{NL-G-F} mice. Latif-Hernandez et al. (2017) observed ostensibly anxiolytic behaviour in the plus-maze in 3 and 6-month old female App^{NL-G-F} mice, but not 10-month old App^{NL-G-F} female mice. Latif-Hernandez et al. (2017), citing Shoji et al. (2016), attributed this latter finding to the aging of the control mice (App^{NL}) that are on a C57BL/6 background. Sakakibara et al. (2018) reported a non-significant anxiolytic-like plus-maze profile in male App^{NL-G-F} mice aged 6-9 months and 15-18 months, and this was relative to App^{NL} and wild-type control mice; the difference was more pronounced relative to the App^{NL} mice. Interestingly, when the App^{NL-G-F} mice in Sakakibara et al. (2018) were re-exposed to the plus-maze they displayed a significant ostensibly anxiolytic profile that the authors noted as highly unusual (Schnieder et al., 2011; File 1993); this result was more pronounced relative to the wild-type control mice. Given that an ostensibly anxiolytic plus-maze profile is commonly observed in AD mice that overexpress APP and exhibit Aβ plaques (Lalonde, Fukuchi & Strazielle, 2012), and that we observed anxiolytic-like behaviour in the plus-maze in the App^{NL-G-F} mice only and not the App^{NL-F} mice (which have very few Aβ plaques), it suggests that this behavioural finding may indeed be associated with the aberrant accumulation of Aβ plaques as opposed to the artifacts associated with APP overexpression.

It is possible that the increased open arm activity observed in the 8 and 15-month old App^{NL-G-F} mice reflects a disinhibtion-like phenotype, which has been suggested by Latif-Hernandez et al. (2017), and also by Ognibene et al. (2005) in a study on Tg2576 mice. Disinhibition is a well-known and equally distressing behavioural feature of AD (Zhao et al., 2016; Steinberg et al., 2008; Lesser & Hughes, 2006; Chung & Cummings, 2000; Mega et al., 1996). It could be manifested within the current study as a failure to inhibit the choice to enter the open arms. Similarly, it may indicate a behavourial phenotype of impulsivity (Kishikawa et al., 2014; Pawlak et al., 2012; Langen & Dost, 2011; Ueno et al., 2002). Masuda et al. (2016) reports impulsive-like behaviour in the App^{NL-G-F} mice using different paradigms. Given the lack of specific data in our study to speak to a disinhibition hypothesis, further conclusions cannot be drawn. However, this is a key finding of this chapter that we hope will facilitate future research

initiatives. Furthermore, we encourage the use of a wider array of behavioural tests in order to more precisely characterize social and anxiety-like behaviours in the APP knock-in mice, in addition to engaging in enhanced analyses of behaviour in the paradigms reported herein. For example, measuring behaviour such as rearing in the open field or stretch-attend postures in the elevated plus-maze may enhance behavioural characterizations (Carobrez & Bertoglio, 2005; Choleris et al., 2001).

To summarize, in this chapter we extended the behavioural profile of male and female homozygous App^{NL-F} and App^{NL-G-F} mice in an age-dependent manner (8 and 15-months) and explored behaviours that are reminiscent of the anxiety and social withdrawal symptoms observed in AD patients. The App^{NL-F} and App^{NL-G-F} mice displayed intact sociability with a minor impairment in preference for social novelty at the 8-month age point that was not explained by a reduced willingness to engage in social exploration. Interest in a social odour was significantly reduced in the 8-month old female App^{NL-G-F} mice, and mildly reduced in the 15-month old App^{NL-F} and App^{NL-G-F} mice, and this was not explained by a general olfaction deficit. Why for example the 15-month old APP knock-in mice had intact sociability and preference for social novelty but a minor deficit in social olfaction is unclear and deserves further attention. The most salient behavioural feature to emerge from this study was the disinhibitory tendencies observed in the 8 and 15-month old App^{NL-G-F} mice in the elevated plus-maze. This finding replicates previous studies on the App^{NL-G-F} mice, and indicates that App^{NL-G-F} mice may enable the modeling of the neurobiological links between disinhibition-type behaviour and AD. Future studies may benefit by directly testing this hypothesis. We now turn to our DTI analysis of the *ex vivo* App^{NL-F} and App^{NL-G-F} mouse brain.

CHAPTER - 3

DIFFUSION TENSOR IMAGING OF THE App^{NL-F} and App^{NL-G-F} MOUSE BRAIN

Diffusion-weighted MR imaging provides neuroscientific and clinical imaging data by sensitizing magnetic field gradients applied in multiple directions to the diffusion properties of tissue water (Alexander et al., 2007; Le Bihan et al., 2001). Brownian motion can describe the random movement of water molecules in neural tissue. When water is unconstrained, the direction of motion in a given molecule is random and is said to be isotropic. In tissue water, the Brownian motion of water is impeded by cell membranes, and is said to be anistropic. Information from the diffusion-weighted images can be mathematically modeled to extract the diffusion tensor, which provides quantitative measures of the orientation and magnitude of water diffusion at each voxel. The modeling of this displacement of water molecules in a multi-dimensional feature space provides insight into the microstructural properties of neural tissue, and has increased potential to distinguish differences in neural architecture within and between subjects (Le Bihan et al., 2015; Basser et al., 1994).

DTI is increasingly being applied in AD research based on its enhanced sensitivity to capture early neurodegenerative related changes that predate the macrostructural changes observable with conventional volumetric techniques (Weston et al., 2015). AD has a long latency period, during which time there is progressive accumulation of molecular pathology followed by irreversible neuronal damage (Jack et al., 2018). Detection of this insidious, pre-symptomatic neurodegenerative change may prove useful as a biomarker of AD (Weiner et al., 2017; Mattson et al. 2015; Nir et al., 2013). Thus, DTI may be a promising tool in identifying individuals at risk for developing AD. For example, Douaud et al. (2013) and Kantarci et al. (2005) demonstrated that increased MD in grey matter predicted conversion from MCI to AD.

The aim of this chapter is to identify whether the App^{NL-F} and/or App^{NL-G-F} mice enable modeling of the neurobiological links between emergent anxiety and social withdrawal in AD by characterizing DTI-derived neuropathology in brain regions thought to underlie these symptoms. As demonstrated by our behavioural results and other

published data, neither the App^{NL-F} nor the App^{NL-G-F} mouse exhibit any notable increase in anxiety-like behaviour, and therefore may not enable the modeling of the neurobiological basis of anxiety in AD. Alterations in social behaviour were mild and restricted to non-significant changes in preference for social novelty that was not explained by a reduced willingness to approach a conspecific. These results were also in line with other published data on the App^{NL-G-F} mice, and indicate that the App^{NL-F} and App^{NL-G-F} mice are not suitable for modeling the neurobiological links associated with social withdrawal in AD. Nevertheless, the original neural regions of interest remain relevant to our diffusion tensor study.

The orbitofrontal and anterior cingulate cortices are thought to underlie decision-making processes (Dias, Robbins & Roberts, 1996), and thus, neuropathology in these regions may relate to the anxiolytic-like behaviour displayed by the App^{NL-G-F} mice in the elevated plus-maze and help identify whether these mice enable the modeling of the neurobiological links between disinhibition-type behaviour and AD. Secondarily, Bannerman et al. (2004) evidenced a role of the ventral hippocampus in mediating behaviour in the elevated plus-maze; lesions to the ventral hippocampus reduced anxiety-like behaviour and promoted exploration of the open arms of the maze. Thirdly, and irrespective of the behavioural results, by applying DTI to the APP knock-in mice we can characterize early neurodegenerative changes in the absence of potential artifacts associated with earlier transgenic mice. Moreover, comparative analysis of the App^{NL-F} and App^{NL-G-F} mice may offer insight into the differential effects of soluble Aβ (as in the App^{NL-F}) and insoluble Aβ (as in the App^{NL-G-F}) on the microstructural integrity of neural tissue. To summarize, the three aims of this chapter are (1) to characterize in the APP knock-in mice the microstructural properties of the neural tissue within brain regions commonly affected in AD, which are the orbitofrontal frontal and anterior cingulate cortices, the amygdala and the ventral and dorsal hippocampus; (2) identify the differential effects, if any, of soluble Aβ and insoluble Aβ on tissue diffusion properties; and (3) identify whether the App^{NL-G-F} mice enable the modeling of the neurobiological links between disinhibition-type behaviour and AD.

3.2 Materials and Methods

3.2.1 Animals

Following behavioural testing, mice were placed into a surgical plane of anaesthesia using an intraperitoneal injection of sodium pentobarbital, and then transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by a solution of 4% paraformaldehyde (PFA) / 0.1 M PBS. The brains were rapidly removed from the skull and post-fixed in 4% PFA / 0.1 M PBS for a minimum of 48 h prior to imaging. Forty-eight whole-brains were imaged in total to include 8 brains (4 male/4 female) per genotype (wild-type / App^{NL-F} / App^{NL-G-F}), per age group. The mean ages of these mice at the time of euthanasia were 9.3 months (±0.45) and 16.9 months (±0.32).

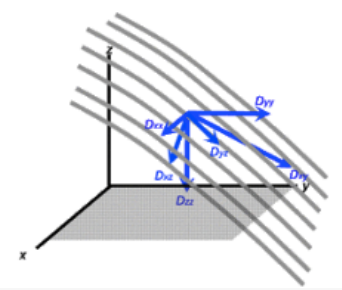
3.2.2 MRI Data Acquisition

MRI data were acquired at the University of Leeds (UK) on a vertical 9.4 Tesla spectrometer (Bruker AVANCE™ II NMR; Ettlingen, Germany) equipped with an 89 mm wide bore, 3 radio frequency channels with digital broadband frequency synthesis (6-620 MHz), and an imaging coil with a diameter of 25 mm for hydrogen (^1H). Each brain was placed into a 15 ml conical tube with a custom designed holding platform and immersed in Fomblin Y-1800 (Sigma-Aldrich, UK); a proton-free fluid used to mitigate magnetic susceptibility distortions that also prevents dehydration (Miller et al., 2011). For each brain, three-dimensional diffusion-weighted images were acquired using a DT-MRI protocol with an echo time (TE) of 35 ms, repetition time (TR) of 700 ms, and 1 signal average. The field of view was set at 168 x 128 x 96, with an achieved cubic resolution of 62.5 μm/pixel and a diffusion-weighting factor of b = 1625 s/mm^2. In each scan, the diffusion-sensitizing gradients were applied in 30 non-collinear, non-coplanar directions, baseline volumes (b=0 images) were collected, and the total imaging time was 20 h.

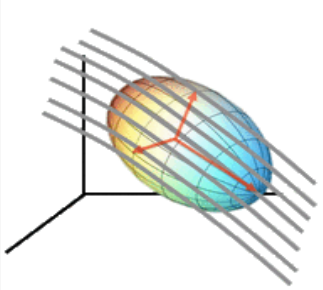
3.2.3. MRI Data Processing

The diffusion-weighted images were reconstructed from the Bruker 2dseq file on a personal computer using DSI Studio (http://dsi-studio.labsolver.org), an open-source software tool for diffusion MR image analysis. Parsing of the raw data was semi-automated and unwanted background, setting a threshold, smoothing of the data and

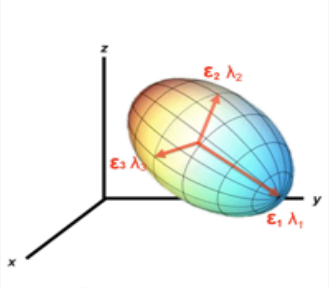
definition of tissue boundaries were completed prior to calculating the tensor. The diffusion-weighted images were then reconstructed by selecting the DTI reconstruction method, a model-based algorithm introduced by Basser, Mattiello and Le Bihan (1994), which assumes the velocity of water diffusion as a three-dimensional Gaussian distribution, and the corresponding diffusion tensor as a 3 x 3 covariance matrix estimated at each voxel in the brain (O'Donnell & Westin, 2011; Le Bihan et al., 2001). The DTI reconstruction method performs eigenanalysis on the calculated tensor, decomposing the matrix into its eigenvalues and eigenvectors (Figure 3.1). The eigenvalues provide information on the rate of diffusion whereas the eigenvectors describe the orientation of diffusion (Jiang et al., 2006). Various mathematical formulas as a function of the eigenvalues and vectors form the basis of quantitative DTI derived scalar indices.



Diffusion along a group of fiber tracts. In the laboratory frame of reference (x-y-z) diffusion coefficients are measured in a minimum of six unique directional combinations described by the diffusion tensor, $\mathcal{D}$.



The simpler frame of reference is to centre the coordinate system within each voxel with the axes parallel and tangent to the principal directions of diffusion. This is represented as an ellipsoid; the off-diagonal elements of the tensor disappear.



The diffusion ellipsoid has three unit vectors (ε_1, ε_2, ε_3), called eigenvectors with corresponding lengths (λ_1, λ_2, λ_3) called eigenvalues.

$$\mathcal{D} = \begin{vmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zx} & D_{zz} \end{vmatrix} \qquad \Lambda = \begin{vmatrix} \lambda_1 & 0 & 0 \\ 0 & \lambda_2 & 0 \\ 0 & 0 & \lambda_3 \end{vmatrix}$$

Figure 3.1. **The diffusion tensor model.** The model summarizes diffusion-weighted imaging data collected in several directions. The rate and orientation of tissue water diffusion within each voxel is the integration of these measurements. The diffusion tensor is a symmetric 3 x 3 matrix, and it can be described by its eigenvectors (ε_1, ε_2, ε_3) and eigenvalues (λ_1, λ_2, λ_3). A diffusion tensor is visualized using an ellipsoid, with the size, shape and orientation of each ellipsoid dictated by the eigenvalues and eigenvectors of the corresponding diffusion tensor. The three orthogonal axes of the tensor coincide with the three eigenvectors, and the length of the three axes is proportional to the three eigenvalues (Soares et al., 2013). Pictorial representation and captions were sourced from www.mriquestions.com.

3.2.4 Quantitative DTI Scalar Indices

We characterized the microstructural integrity of neural tissue using scalar measures derived from the calculated tensor. These scalar indices included the axial diffusivity (AxD), radial diffusivity (RD), mean diffusivity (MD) and fractional anisotropy (FA). The mathematical calculations are shown below. AxD, which is denoted by λ_1, quantifies how fast the water diffuses along the principal axis of diffusion, and RD, which is the average of the secondary (λ_2) and tertiary (λ_3) eigenvalues, quantifies how fast the water diffuses perpendicular to λ_1. MD provides an average of the three eigenvalues to describe the overall diffusion rate of tissue water. Note that diffusivity in DSI Studio has a unit of 10^{-3} mm^2/s. FA quantifies the fraction of diffusion that is anisotropic by comparing the relative difference between the largest eigenvalue (λ_1) to λ_2 and λ_3. It describes in part the shape of diffusion, is rotationally invariant and ranges from a value of 0 to 1. Zero indicates perfect isotropic diffusion (equal in all directions) and is represented as a sphere ($\lambda_1=\lambda_2=\lambda_3$), whereas total anisotropic diffusion would have a value of 1 indicating that there was no diffusivity perpendicular to the principal axis of diffusion. Virtually all tissue will demonstrate some degree of anisotropy, given that physical barriers such as cellular membranes will naturally restrict the diffusivity of tissue water (Basser & Pierpaoli, 1996).

$$AxD = \lambda_1 \quad (1)$$

$$RD = \frac{(\lambda_2 + \lambda_3)}{2} \quad (2)$$

$$MD = \frac{(\lambda_1 + \lambda_2 + \lambda_3)}{3} \quad (3)$$

$$FA = \frac{\sqrt{3}}{\sqrt{2}} \frac{\sqrt{(\lambda_1 - \lambda)^2 + (\lambda_2 - \lambda)^2 + (\lambda_3 - \lambda)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}} \quad (4)$$

where

$$\lambda = (\lambda_1 + \lambda_2 + \lambda_3)/ 3$$

3.2.5 Region of Interest Identification and Segmentation

Our approach was to undertake an *a posteriori* analysis of the microstructural integrity of neural tissue within the orbitofrontal and anterior cingulate cortices, the amygdala, and the dorsal and ventral hippocampus, brain regions that have been identified by previous literature as being relevant to anxiety and social behaviour (see chapter 1). To perform statistical analysis, we extracted the MD and the mean AxD, RD, and FA from five manually drawn regions of interest (ROI) for each reconstructed brain (Figure 3.3). The DTI reconstruction allows for an interactive 2D visualization of grayscale image contrasts of the various scalar indices. Using the Paxinos and Franklin (2012) mouse brain atlas as a reference, the FA map was looped through in orthogonal views of the coronal plane until a target coronal slice was identified. The ROI was then manually drawn on the target slice using the atlas as reference (Figure 3.4). The summary measures were then calculated and extracted semi-automatically from 300 µm of tissue; this included the slice on which the ROI was drawn, plus the slice immediately anterior and posterior to the segmented slice.

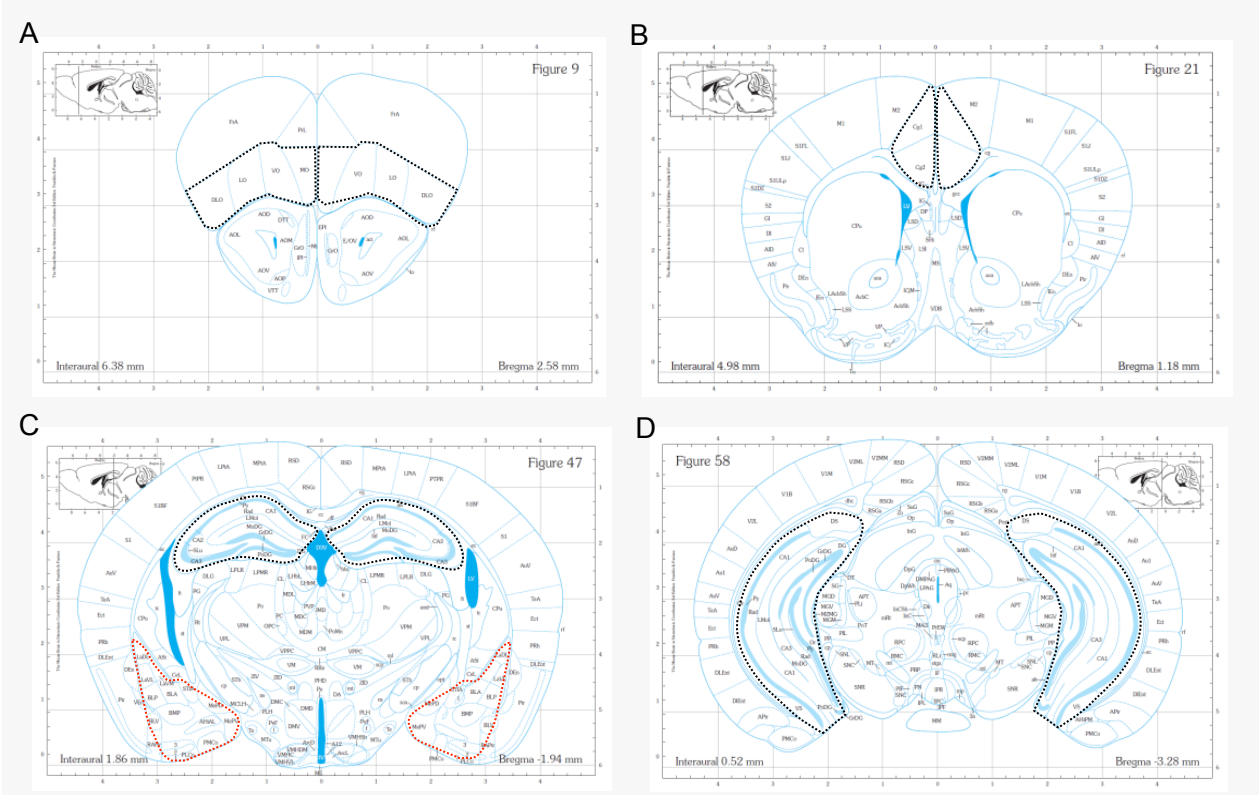


***Figure 3.3.* ROI for diffusion tensor analysis.** Images from the Paxinos and Franklin (2012) mouse brain atlas. The dotted line represents the intended boundary for the manually drawn ROI within **(A)** Orbitofrontal Cortex **(B)** Anterior Cingulate Cortex **(C)** Dorsal Hippocampus (black), Amygdala (red) and **(D)** Ventral Hippocampus.

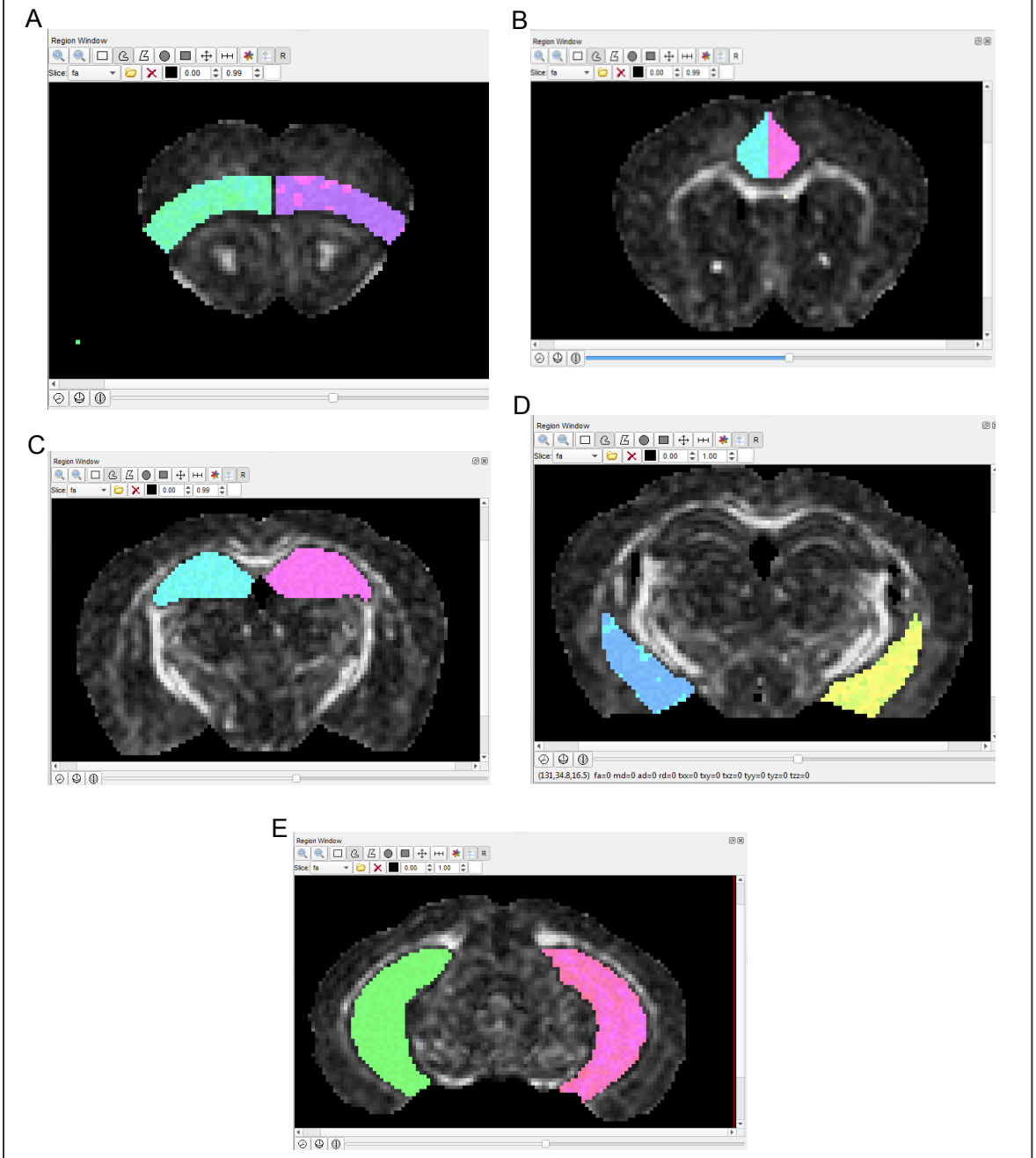


Figure 3.4. **Manually drawn ROI.** Representative coronal sections of a DTI-scanned brain as displayed in DSI Studio. Reconstructed brains were viewed as an FA map. With the aid of the Paxinos and Franklin (2012) mouse brain atlas, the FA map was looped through in orthogonal views of the coronal plane until a target slice was identified. The author then carefully delineated the ROI within each hemisphere. Highlighted areas exemplify a manually drawn ROI for **(A)** Orbitofrontal Cortex (~ Bregma 2.58 mm), **(B)** Anterior Cingulate Cortex (~ Bregma 1.18 mm), **(C)** Dorsal Hippocampus (~ Bregma -1.94 mm), **(D)** Anterior Amygdala (~ Bregma -1.94mm), and **(E)** Ventral Hippocampus (~ Bregma -3.28 mm).

3.2.6 Data Analysis

Data sets were initially analyzed using a mixed model repeated-measures ANOVA with *hemisphere* as the within-subjects factor and *genotype* as the between-subjects factor. Simple main effects analyses are reported where hemisphere and genotype interact, using either paired *t*-test to assess differences between hemispheres within genotype, or one-way ANOVA to assess differences between genotypes within the left and right hemispheres. Non-significant effects of hemisphere and non-significant interactions between hemisphere and genotype are reported in Table 3.1. In the absence of an interaction, the data was collapsed across hemispheres and a one-way ANOVA was used to assess the differences between genotypes. A one-way ANOVA was used in lieu of the between-subjects output in the repeated-measures ANOVA in order to accurately assess homoscedasticity, and subsequently, to select the appropriate correction for pairwise comparisons. A Welch one-way ANOVA with Games-Howell comparisons is reported where data violated homoscedasticity, otherwise the Tukey pairwise comparisons are reported. We did not investigate for interactions between *genotype* and *sex* due to the small sample sizes. The author performed all statistical analyses using IBM™ SPSS© v.22.0 with the critical α level set to $p \leq 0.05$. The author designed the graphs to illustrate the underlying distribution of data, and used GraphPad Prism™ v.8.2.1. Statistical significance within figures is illustrated as *($p \leq 0.05$), **($p < 0.01$) and ***($p < 0.001$). All data are expressed as mean ± SEM.

3.3 Results

3.3.1 9-month age point

3.3.1.1 Orbitofrontal Cortex (9 months)

Results are illustrated in Figure 3.5A-D. There were no effects of hemisphere or interactions between hemisphere and genotype on the DTI derived indices for the orbitofrontal cortex. There was an effect of genotype on all four diffusion properties: MD ($F_{(2,21)} = 6.67$, $p = 0.006$), FA ($F_{(2,21)} = 3.05$, $p = 0.049$), AxD ($F_{(2,21)} = 7.13$, $p = 0.004$) and RD ($F_{(2,21)} = 5.87$, $p = 0.009$). The MD, AxD and RD were significantly increased in the $App^{NL\text{-}G\text{-}F}$ mice compared with the wild-type ($p = 0.015$, 0.020, 0.017) and the $App^{NL\text{-}F}$ mice ($p = 0.013$, 0.006, 0.022), respectively. The FA was significantly increased in the $App^{NL\text{-}G\text{-}F}$ mice compared to the $App^{NL\text{-}F}$ mice ($p = 0.049$), but not the wild-type mice ($p = 0.831$). There were no differences in tissue diffusion properties between the $App^{NL\text{-}F}$ mice and wild-type mice.

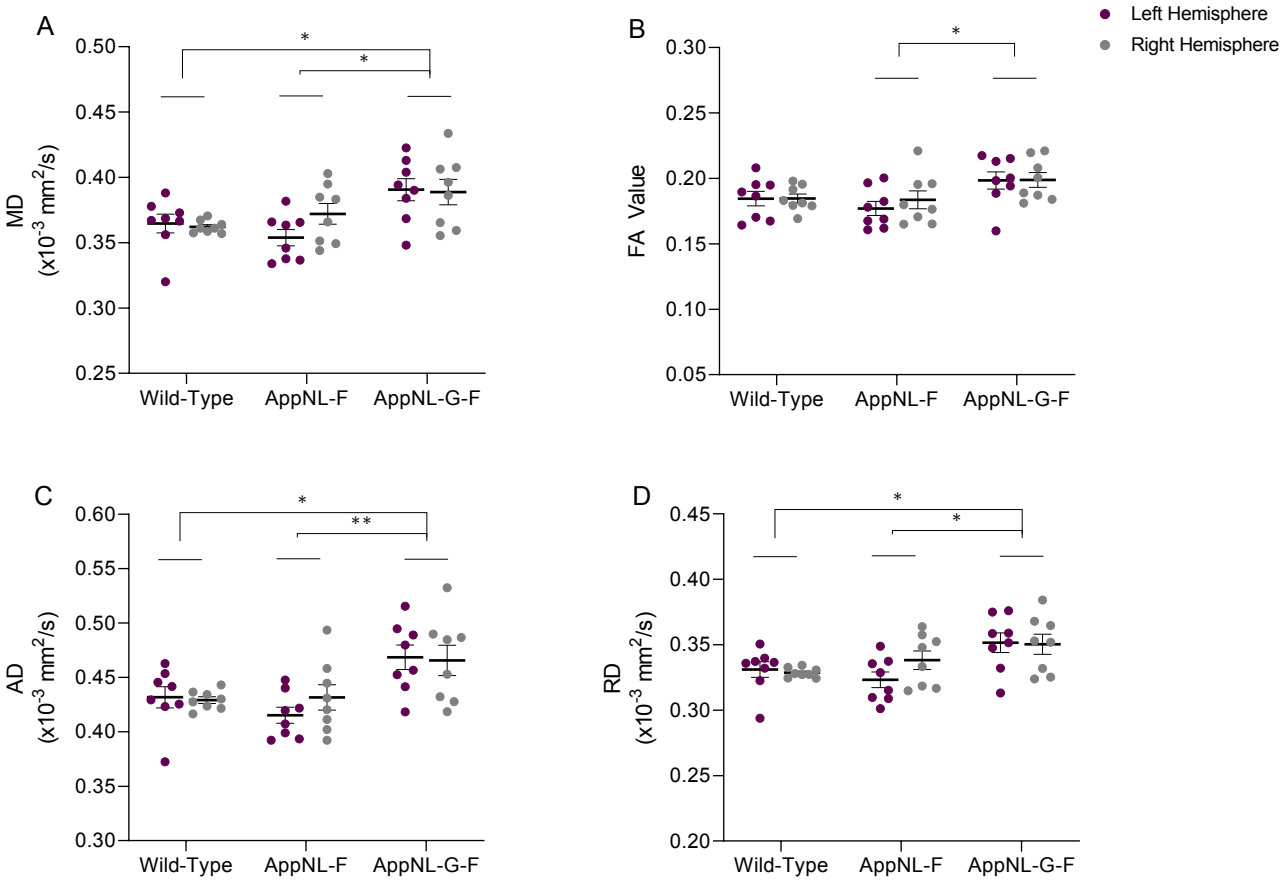


Figure 3.5. **Tissue diffusion properties within the orbitofrontal cortex at 9-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.1.2 Anterior Cingulate Cortex (9 months)

Results are illustrated in Figure 3.6A-D. The FA within the anterior cingulate cortex was similar between genotypes ($F_{(2,21)}$ = 0.87, p = 0.433). An effect of genotype was indicated for the MD ($F_{(2,21)}$ = 4.28, p = 0.028), the AxD ($F_{(2,21)}$ = 4.90, p = 0.018) and the RD ($F_{(2,21)}$ = 3.67, p = 0.043). Pairwise comparisons confirmed a marginal increase in MD within the $App^{NL\text{-}G\text{-}F}$ mice compared with the wild-type mice (p = 0.051), and a significantly higher AxD in the $App^{NL\text{-}G\text{-}F}$ mice compared with the wild-type mice (p = 0.043). For RD, the pairwise comparisons did not reach statistical significance. The $App^{NL\text{-}G\text{-}F}$ mice also had a significantly higher MD (p = 0.047) and AxD (p = 0.027) compared with the $App^{NL\text{-}F}$ mice. There were no differences between the $App^{NL\text{-}F}$ mice and the wild-type mice on any of the tissue diffusion properties. Note that the hemispheres were not separately segmented for the anterior cingulate cortex.

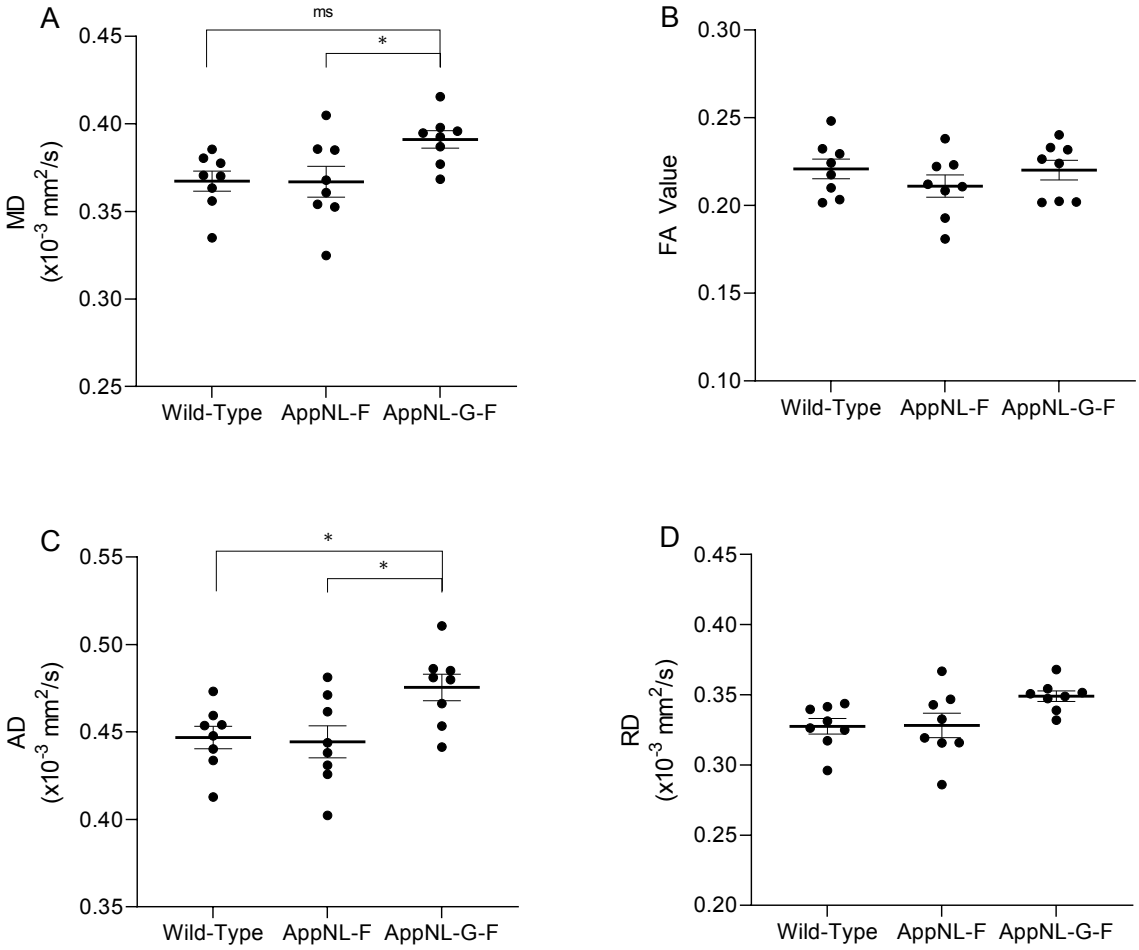


Figure 3.6. **Tissue diffusion properties within the anterior cingulate cortex at 9-months.**
(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.1.3 Amygdala (9 months)

Results are illustrated in Figure 3.7A-D. Hemisphere and genotype interacted on the FA ($F_{(2,21)}$ = 5.66, p = 0.011), and RD ($F_{(2,21)}$ = 4.52, p = 0.023). There was a significant difference in FA between the left and right hemisphere within the wild-type mice ($t_{(7)}$ = 2.67, p = 0.032). The simple main effects analyses for RD were all non-significant. The tissue diffusion properties within the amygdala were similar across genotypes: MD ($F_{(2,21)}$ = 0.33, p = 0.721), FA ($F_{(2,21)}$ = 0.12, p = 0.888), AxD ($F_{(2,21)}$ = 0.43, p = 0.656) and RD ($F_{(2,21)}$ = 0.27, p = 0.768).

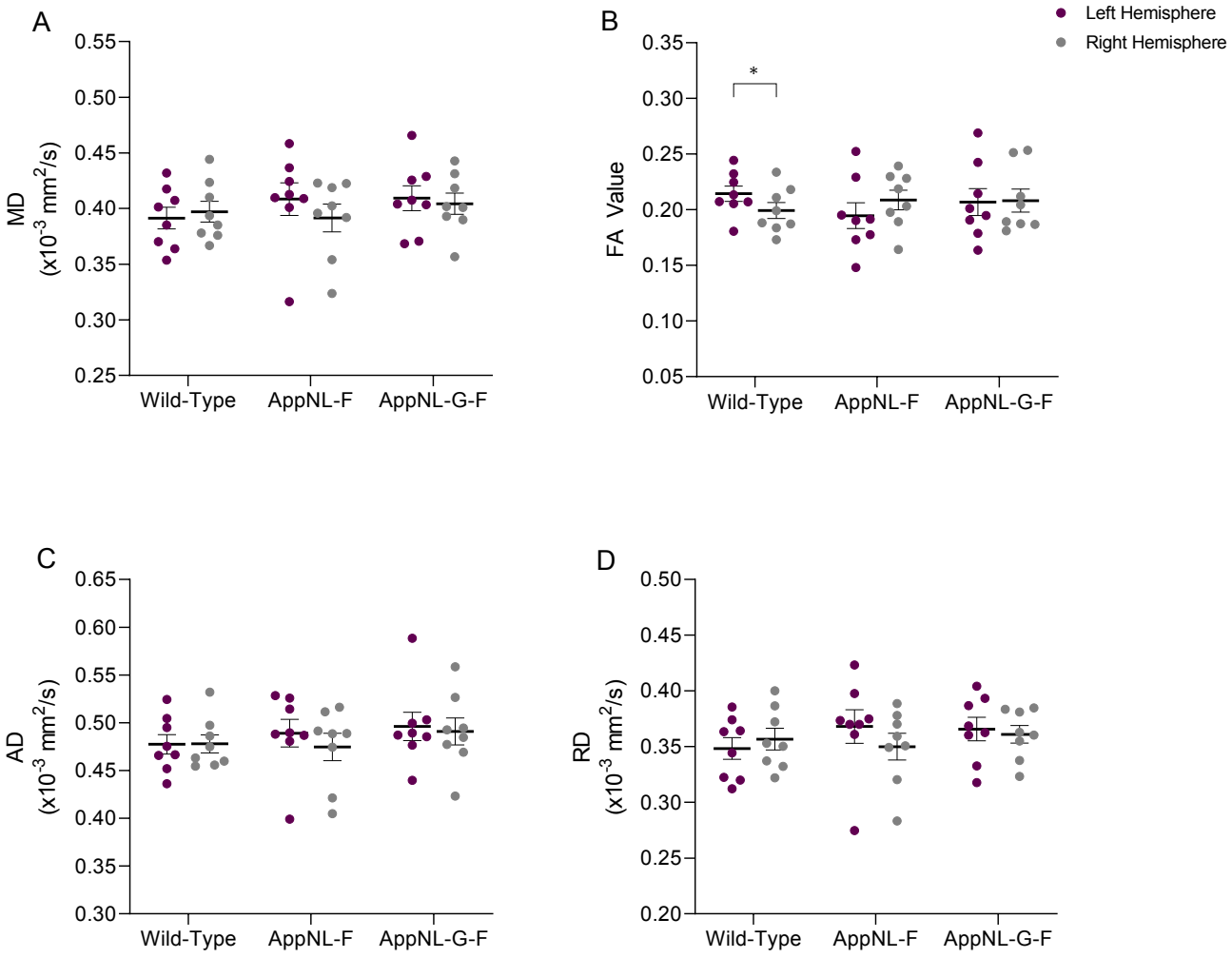


Figure 3.7. **Tissue diffusion properties within the amygdala at 9-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.1.4 Dorsal Hippocampus (9 months)

Results are illustrated in Figure 3.8A-D. There were no effects of hemisphere or interactions between hemisphere and genotype on the DTI derived indices for the dorsal hippocampus. Neither the MD ($F_{(2,21)}$ = 1.29, p = 0.294), the FA ($F_{(2,21)}$ = 0.27, p = 0.792) nor the RD ($F_{(2,21)}$ = 0.70, p = 0.507) differed between genotypes. There was an effect of genotype on AxD ($F_{(2,21)}$ = 3.88, p = 0.037). This was due to a significantly higher AxD in the $App^{NL\text{-}G\text{-}F}$ mice compared with the wild-type mice (p = 0.030). The AxD did not differ between the $App^{NL\text{-}G\text{-}F}$ and $App^{NL\text{-}F}$ mice (p = 0.238), nor the wild-type and $App^{NL\text{-}F}$ mice (p = 0.529).

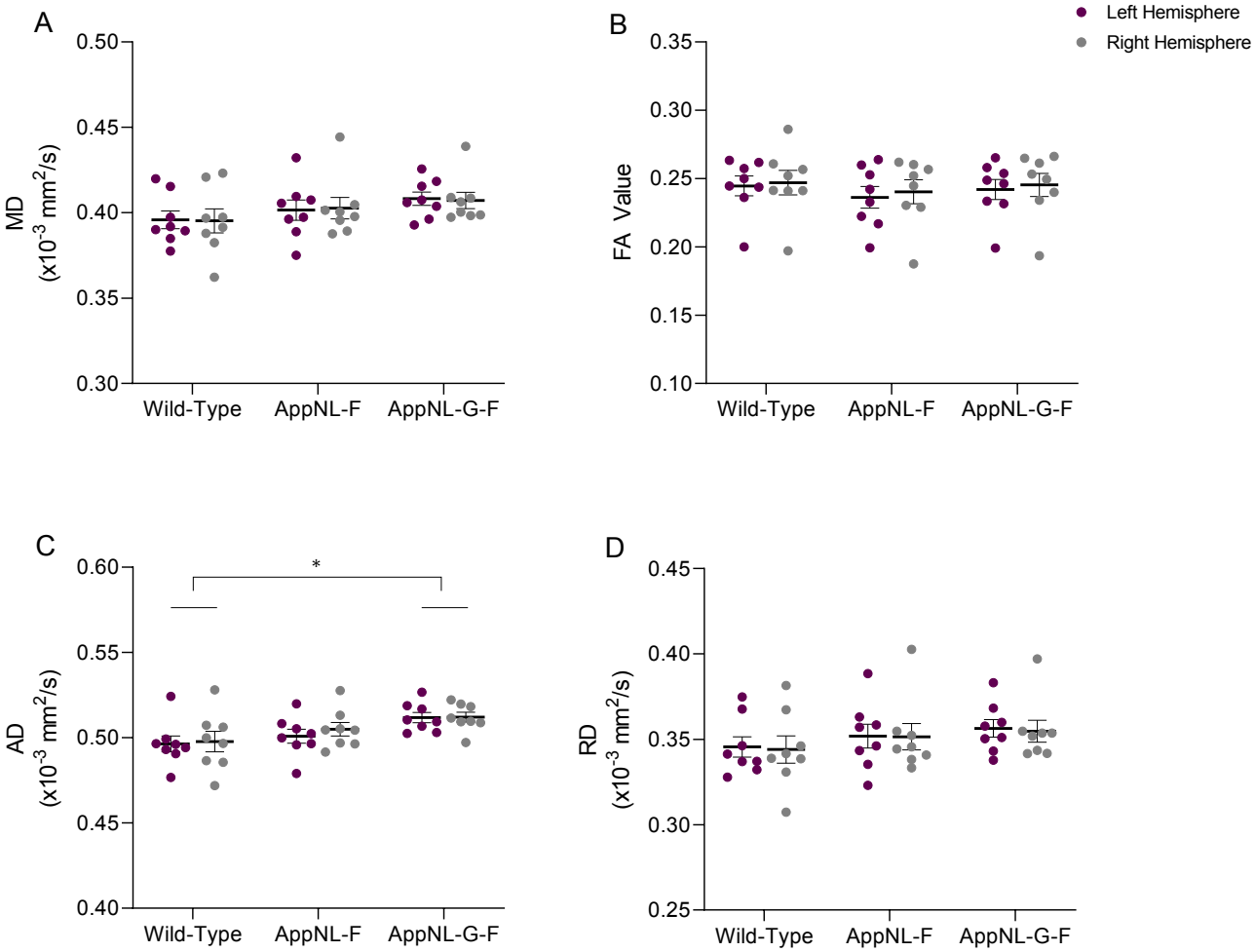


Figure 3.8. **Tissue diffusion properties within the dorsal hippocampus at 9-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.1.5 Ventral Hippocampus (9 months)

Results are summarized in Figure 3.9A-D. The FA ($F_{(2,21)}$ = 2.07, p = 0.151) and AxD ($F_{(2,21)}$ = 2.29, p = 0.125) was similar between genotypes. The MD was significantly increased ($F_{(2,21)}$ = 5.64, p = 0.011) in the $App^{NL\text{-}G\text{-}F}$ mice compared to the wild-type mice (p = 0.015) and the $App^{NL\text{-}F}$ mice (p = 0.035); wild-type vs. $App^{NL\text{-}F}$ mice (p = 0.914). The RD was significantly higher in the left vs. the right hemisphere ($F_{(1,21)}$ = 4.35, p = 0.049; *MD* = 0.004 ±0.002), and the RD was significantly increased ($F_{(2,21)}$ = 8.17, p = 0.002) in the $App^{NL\text{-}G\text{-}F}$ mice compared with the wild-type mice (p = 0.008) and the $App^{NL\text{-}F}$ mice (p = 0.005); wild-type vs. $App^{NL\text{-}F}$ mice (p = 0.973).

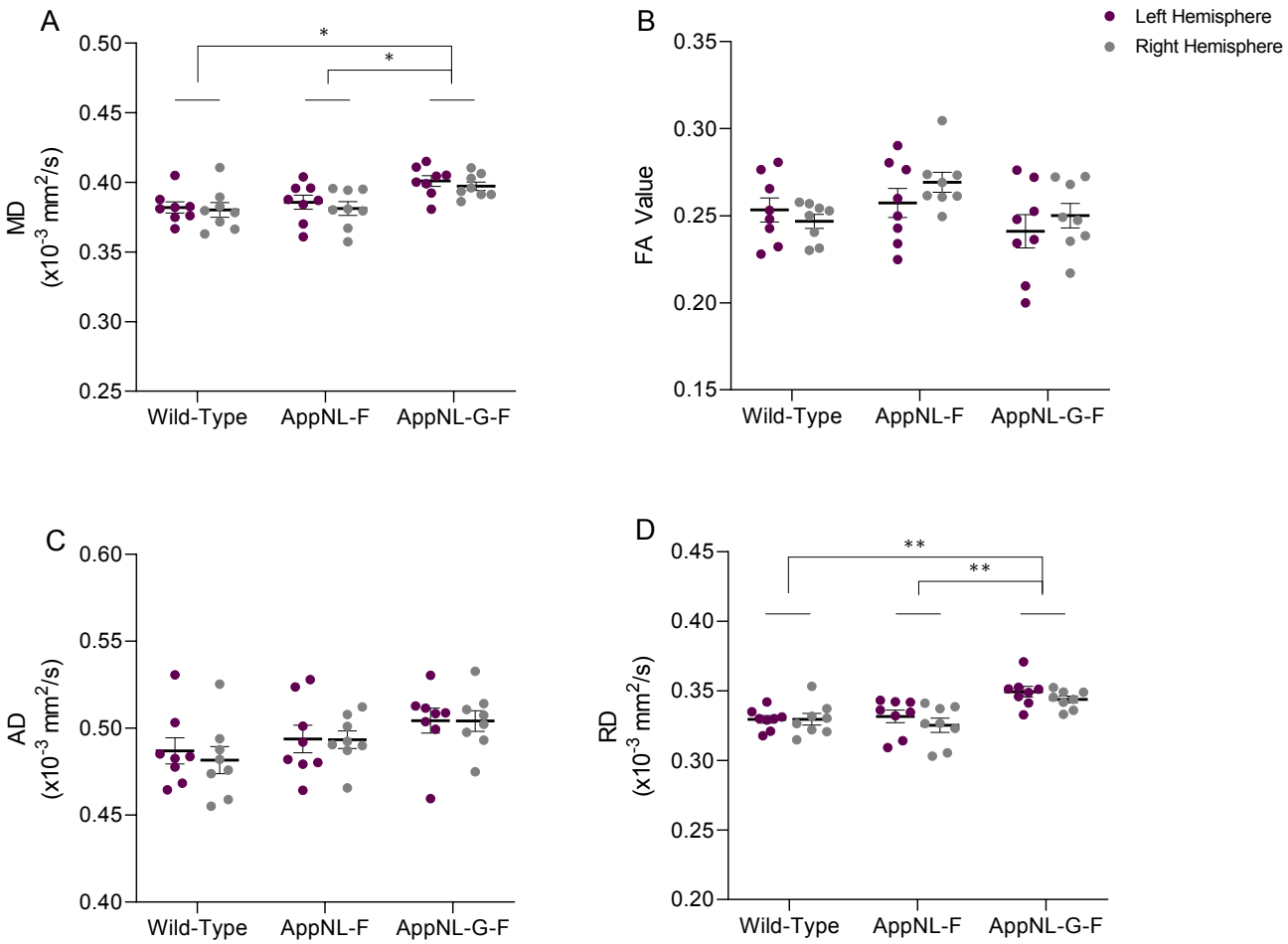


***Figure 3.9.* Tissue diffusion properties within the ventral hippocampus at 9-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.2 17-month age point

3.3.2.1 Orbitofrontal Cortex (17 months)

Results are illustrated in Figure 3.10A-D. There were no effects of hemisphere or interactions between hemisphere and genotype. FA was similar across genotypes ($F_{(2,21)}$ = 0.18, p = 0.838), and AxD was marginally significant ($F_{(2,12.30)}$ = 3.80, p = 0.052; App^{NL-G-F} > wild-type, p = 0.051). There was an effect of genotype on MD ($F_{(2,13.44)}$ = 4.27, p = 0.037) and RD ($F_{(2,13.06)}$ = 3.87, p = 0.048). Compared with the wild-type mice, MD was significantly higher in the App^{NL-F} (p = 0.049) and App^{NL-G-F} mice (p = 0.038) but did not differ between the two (p =1.000), and RD was significantly higher in the App^{NL-G-F} mice compared with the wild-type mice (p = 0.047).

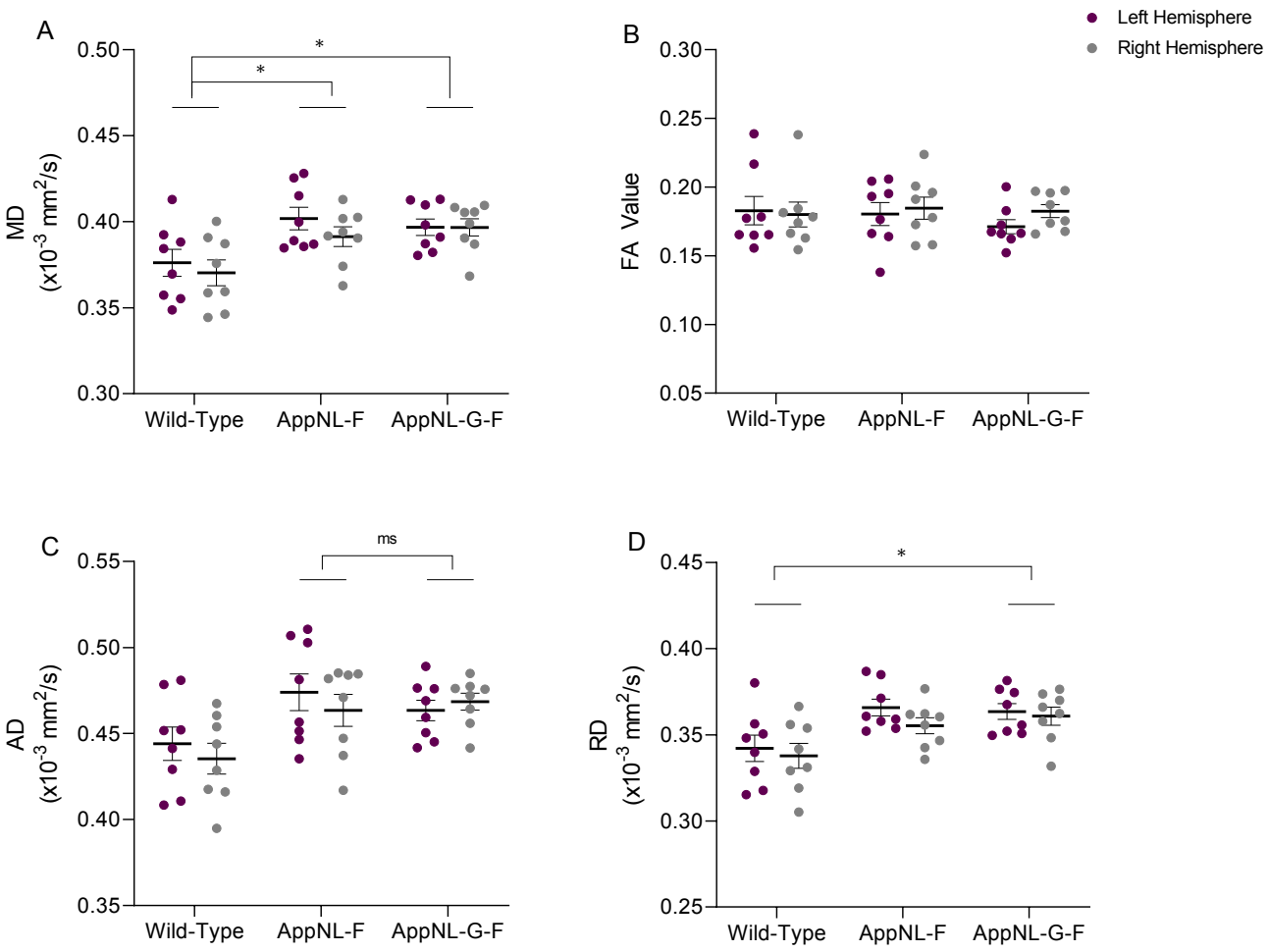


Figure 3.10. **Tissue diffusion properties within the orbitofrontal cortex at 17-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.2.2 Anterior Cingulate Cortex (17 months)

Results are illustrated in Figure 3.11A-D. There were no effects of hemisphere or interactions between hemisphere and genotype. There was an effect of genotype on FA ($F_{(2,21)}$ = 5.09, p = 0.016). FA was significantly lower in the App^{NL-G-F} mice compared with the wild-type (p = 0.021) and App^{NL-F} mice (p = 0.046); there was no difference on FA between the wild-type and App^{NL-F} mice (p = 0.931). There were no differences between genotypes in either MD ($F_{(2,21)}$ = 1.63, p = 0.219) or AxD ($F_{(2,12.95)}$ = 0.530, p = 0.601). For RD, although there was an effect of genotype ($F_{(2,11.23)}$ = 3.95, p = 0.050), the pairwise comparisons did not reach statistical significance.

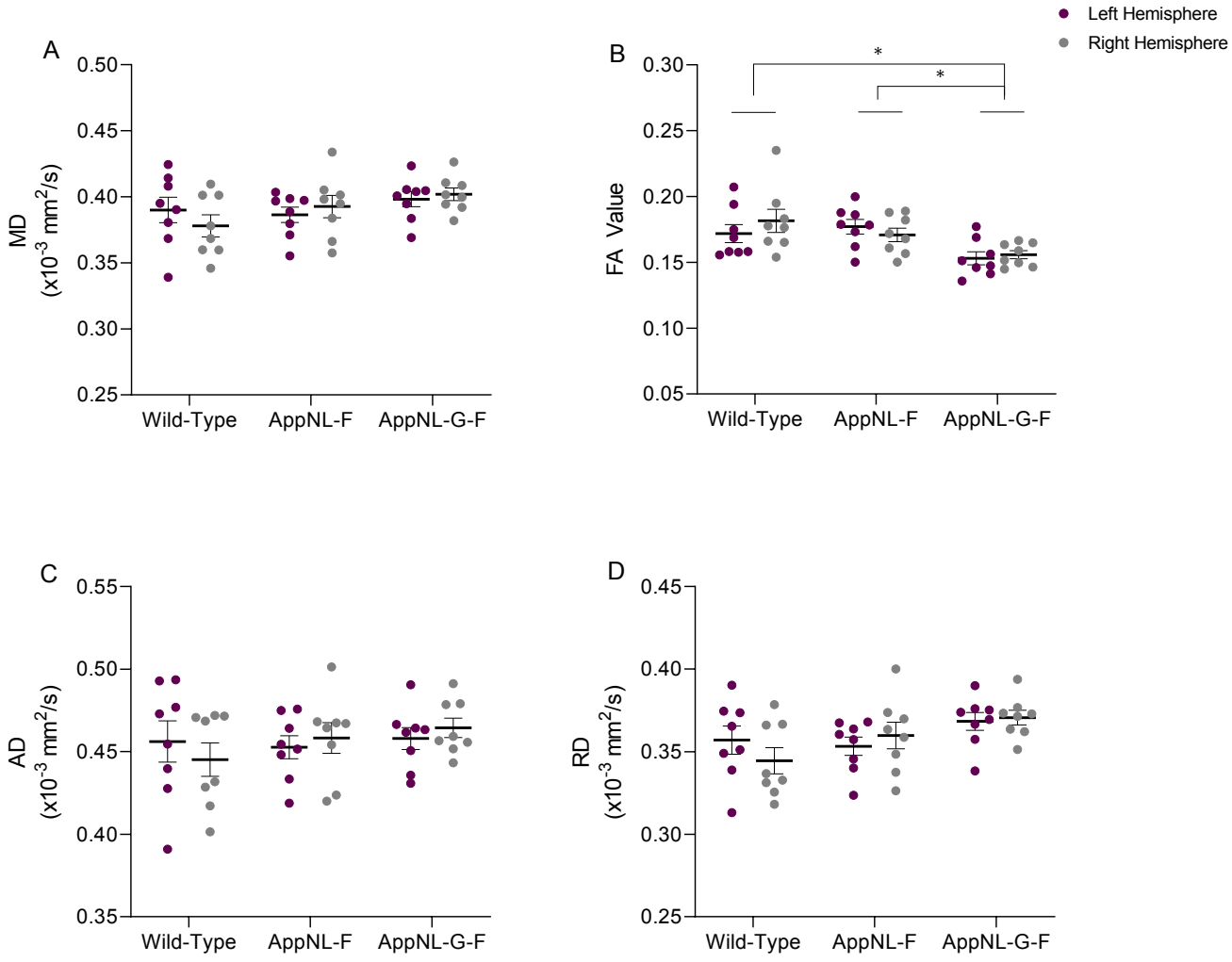


Figure 3.11. **Tissue diffusion properties within the anterior cingulate cortex at 17-months.**
(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.2.3 Amygdala (17 months)

Results are illustrated in Figure 3.12A-D. There were no effects of hemisphere or interactions between hemisphere and genotype on the tissue diffusion properties measured within the amygdala. Furthermore, there were no significant changes between genotypes when assessing the MD of the amygdala tissue ($F_{(2,21)}$ = 0.66, p = 0.524), the AxD ($F_{(2,21)}$ = 0.02, p = 0.985) or the RD ($F_{(2,21)}$ = 1.36, p = 0.280). FA was marginally significant ($F_{(2,21)}$ = 3.37, p = 0.054), which was driven by the lower FA in the App^{NL-G-F} mice relative to the App^{NL-F} mice (p = 0.045).

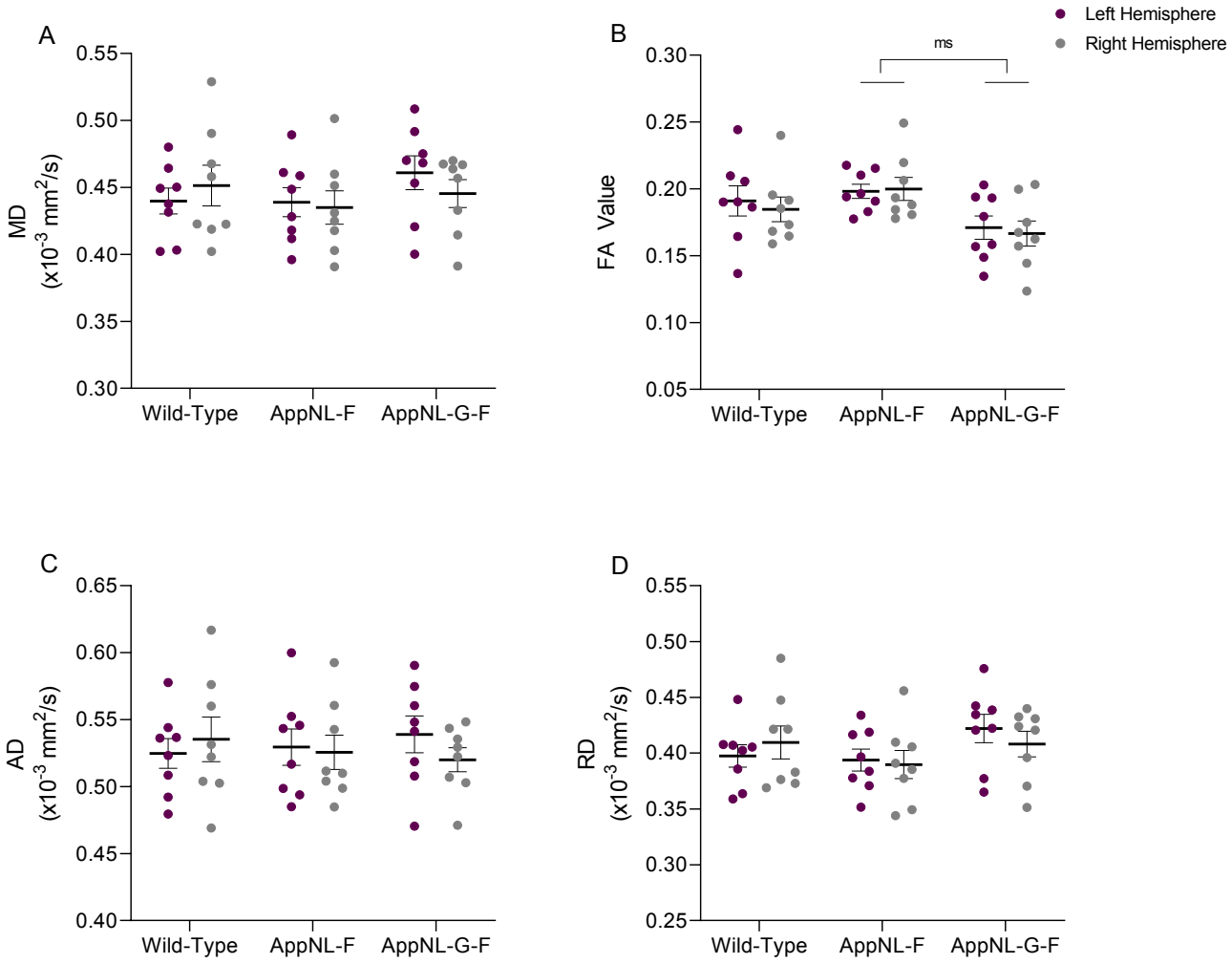


Figure 3.12. **Tissue diffusion properties within the amygdala at 17-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.2.4 Dorsal Hippocampus (17 months)

Results are illustrated in Figure 3.13A-D. There was a marginal effect of hemisphere when assessing RD, $F_{(2,21)} = 4.07$, $p = 0.056$ (L > R), otherwise, there were no effects of hemisphere or interactions between hemisphere and genotype. The FA of the tissue within the dorsal hippocampus was similar across genotypes ($F_{(2,21)} = 2.02$, $p = 0.158$). However, there were differences in MD ($F_{(2,21)} = 5.95$, $p = 0.009$), AxD ($F_{(2,21)} = 4.64$, $p = 0.021$) and RD ($F_{(2,21)} = 6.21$, $p = 0.008$). The App^{NL-G-F} mice had a significantly higher MD ($p = 0.020$) and RD ($p = 0.012$) in comparison to the wild-type mice, and a significantly higher MD ($p = 0.017$), RD ($p = 0.022$) and AxD ($p = 0.023$) in comparison to the App^{NL-F} mice. There were no significant changes in tissue diffusion properties between the App^{NL-F} mice and the wild-type mice.

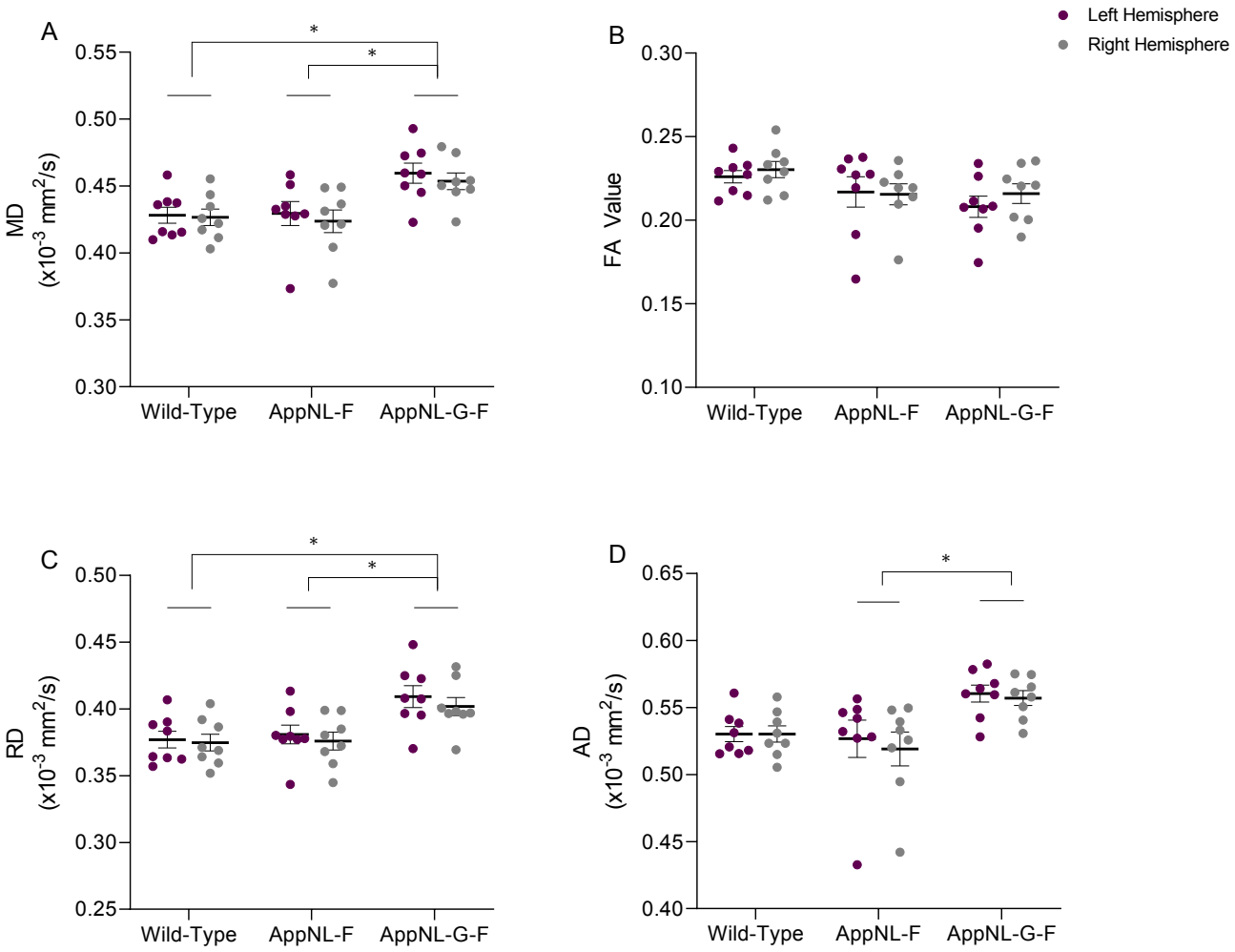


Figure 3.13. **Tissue diffusion properties within the dorsal hippocampus at 17-months.**

(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.3.2.5 Ventral Hippocampus (17 months)

Results are illustrated in Figure 3.14A-D. MD and AxD were significantly higher in the left hemisphere compared to the right hemisphere. Hemisphere and genotype interacted on RD ($F_{(2,21)}$ = 4.96, p = 0.017) due to a significant difference within the App^{NL-F} mice ($t_{(7)}$ = 3.68, p = 0.008; L > R), and a significantly higher RD within the left hemisphere of the App^{NL-G-F} mice relative to the wild-type mice ($F_{(2,11.69)}$ = 6.67, p = 0.012; p = 0.006). There was no effect of genotype on either FA ($F_{(2,21)}$ = 2.22, p = 0.134) or RD ($F_{(2,10.58)}$ = 3.74, p = 0.059). There were main effects of genotype on MD ($F_{(2,10.39)}$ = 4.68, p = 0.036) and AxD ($F_{(2,12.56)}$ = 5.83, p = 0.016). The App^{NL-G-F} mice had a significantly higher MD compared with the wild-type mice (p = 0.049) and App^{NL-F} mice (p = 0.048). For AxD, there was a significant increase within the App^{NL-G-F} mice compared with the App^{NL-F} (p = 0.017) but not the wild-type mice (p = 0.064).

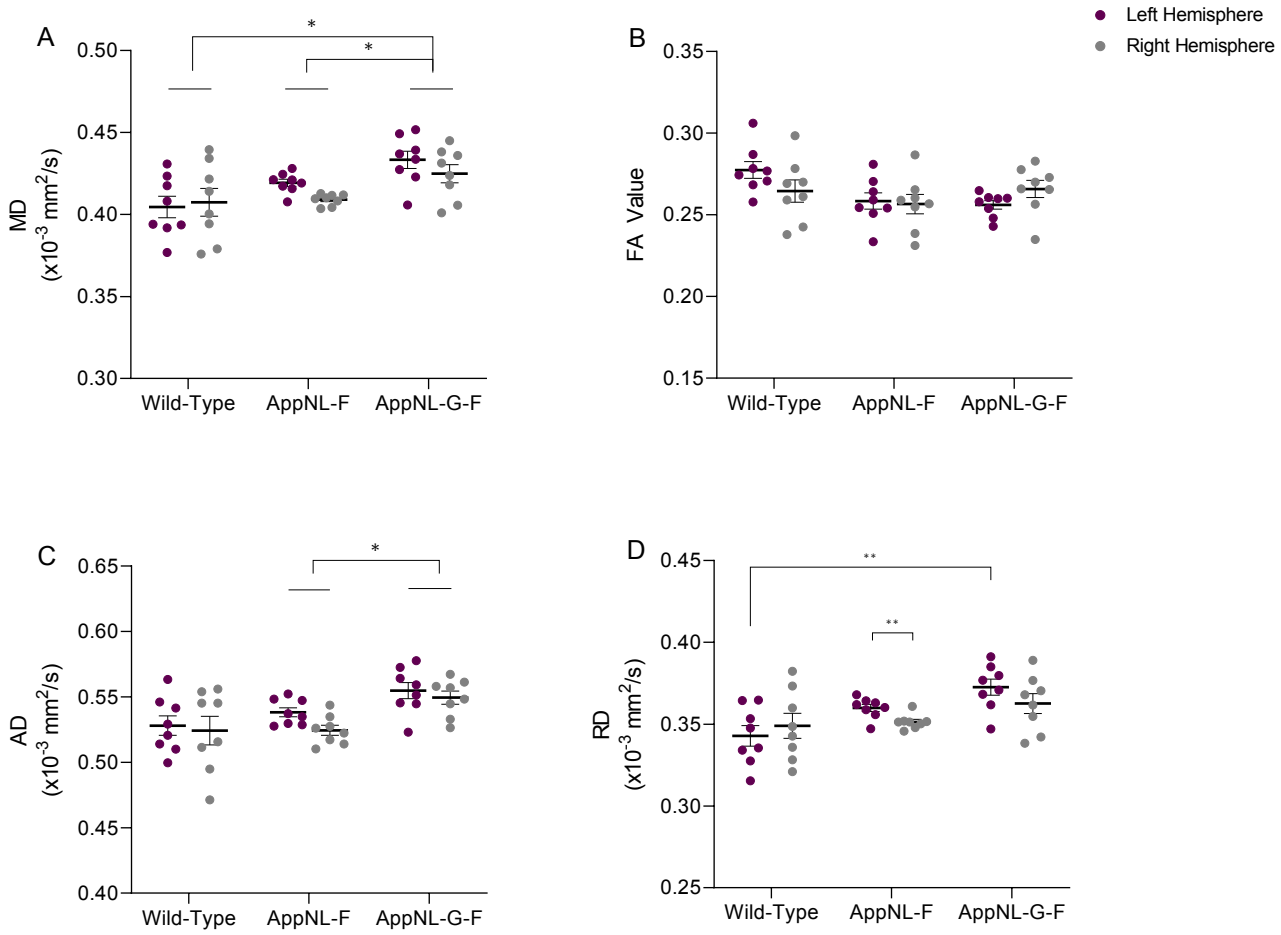


Figure 3.14. **Tissue diffusion properties within the ventral hippocampus at 17-months.**
(A) Mean Diffusivity **(B)** Fractional Anisotropy **(C)** Axial Diffusivity **(D)** Radial Diffusivity

3.4 Materials, Methods & Results (Immunohistochemistry)

3.4.1 Animals

Following *ex vivo* MR imaging, 10 brains were selected from the younger set of mice for immunohistochemical analysis: wild type (*n*=2; 1 male), App^{NL-F} (*n*=4; 2 male) and App^{NL-G-F} (*n*=4; 2 male). The tissue from the older cohort was preserved for future research initiatives, and was thus unavailable to the author for immunostaining.

3.4.2 Tissue sampling

The brains were rinsed of Fomblin Y-1800 using repeated PBS washes aided by agitation from a laboratory rocker, then cryoprotected by immersion in a solution of 30% sucrose / 0.1M PBS and stored at 4 °C for a minimum of 72 h. Immediately prior to cryosectioning, the olfactory bulb and cerebellum were removed with a perpendicular cut to the rostral isocortex and inferior colliculus using a single edge razor blade. The brain was then hemisected along the longitudinal fissure to separate the left and right hemispheres. With the aid of a brain matrix, three slices were made to the left hemibrain at 3 mm, 5 mm and 7 mm from the rostral-most boundary. The sections were placed on individual blocks of frozen optimal cutting temperature compound (OCT), covered with liquid OCT, and then placed in the cryostat-microtome to freeze at -19 °C for 30 minutes. The OCT embedded tissue was then sliced into 30 μm coronal sections with the cryostat-microtome set to -19 °C. Tissue samples corresponding to the same Bregma coordinates reported in the DTI analysis were taken and placed into multi-well culture plates filled with PBS.

3.4.3 Immunohistochemical procedure

Immunohistochemical detection of extracellular Aβ deposits was performed on free-floating 30 μm coronal tissue slices following the protocol outlined in Ly, Cai and Song (2011). At each stage of the process the culture plate was gently shaken on a laboratory rocker for the time specified. All washes were done using a solution of 0.3% v/v Triton-X 100 in PBS (Tx-PBS), and all steps were carried out at room temperature unless noted otherwise. The tissue was first placed in a solution of 88% formic acid / 12% distilled H_2O for 15 minutes to perform antigen retrieval (*i.e.*, to unmask the antigenic epitope due to the cross-link effect of aldehyde fixatives). The tissue was then washed 3x for 5 minutes. The tissue was next incubated with 0.5% H_2O_2 in Tx-PBS for 30 minutes to

eliminate endogenous peroxidase activity and prevent non-specific background staining during chromogenic detection. The tissue was next incubated with 2% bovine serum albumin (BSA) (Acros Organics, UK) in Tx-PBS for 1 h to prevent the non-specific binding of antibodies. The tissue was next incubated overnight at 4 °C with monoclonal 6E10 primary antibody (BioLegend; San Diego, USA) in a concentration of 1:500 [6E10 to 2% BSA / Tx-PBS]. Anti-Aβ 6E10 reacts to amino acid residues 1-16 of Aβ, and binds to abnormally processed isoforms and its precursor form. The tissue was then washed 3x for 10 minutes. The tissue was next incubated for 2 h with biotinylated goat anti-mouse immunoglobulin (IgG) secondary antibody (Vector Laboratories, UK) in a concentration of 1:500 [IgG to 2% BSA/ Tx-PBS]. The tissue was then washed 3x for 10 minutes. The tissue was next incubated for 30 minutes with Avidin-Biotin Complex (VECTASTAIN ELITE® ABC Kit; Vector Laboratories, UK) to amplify the target antigen signal [1 drop Reagent A to 1 drop Reagent B to 2.5 ml of 2% BSA / Tx-PBS]. The tissue was then washed 3x for 10 minutes. The tissue was next incubated for 5 minutes (no shaking) with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, UK) in order to visualize the Aβ immunostained profiles [1 drop DAB to 1 drop peroxidase to 2 drops buffer to 2.5 ml of distilled H_2O]. The tissue was then washed 3x for 10 minutes and mounted onto gelatin-coated slides. The slides were dehydrated in a drying oven for a minimum of 1 h, and then cleared twice for 5 minutes with Xylene (Fisher Chemical, UK) and cover slipped with DPX Mountant medium (Sigma-Aldrich, UK). Lastly, digital photomicrographs were acquired with a Leica DM2000 light microscope at 40x magnification using the Leica application suite (LAS) v4.12.0.

3.4.4 Immunohistochemistry Results

Immunohistochemistry was performed using the anti-Aβ 6E10 to visualize the extent of Aβ plaques within orbitofrontal and anterior cingulate cortices, anterior amygdala and ventral and dorsal hippocampus of the wild-type, App^{NL-F} and App^{NL-G-F} mice. Aβ deposition was qualitatively assessed and compared between genotypes. As shown in figure 3.15, the App^{NL-G-F} mice accumulated vast amounts of Aβ plaques in all ROI by 9 months of age, whereas the App^{NL-F} mice accumulated very few plaques at this age point. There were no Aβ plaques observed in the wild-type mice.

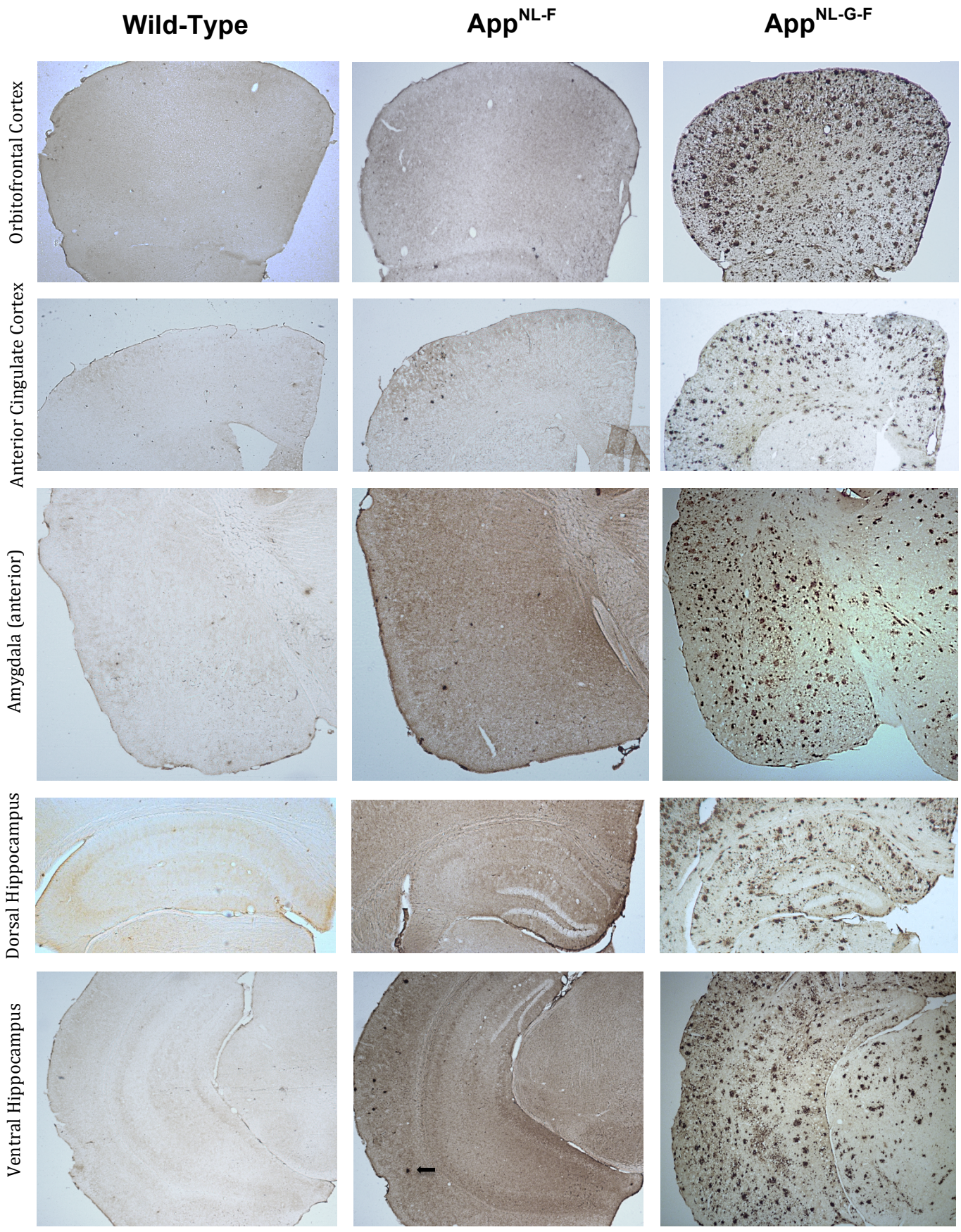


***Figure 3.15.* Aβ plaques in the APP knock-in mice.** Representative photomicrographs illustrating the extent of Aβ deposition in the wild-type, App^{NL-F} and App^{NL-G-F} mice (~9 months old), visualized with 6E10. Aβ plaques were easily observed throughout the App^{NL-G-F} brain, and to a far lesser extent in the cortex of the App^{NL-F} brain (black arrow indicates an Aβ plaque). Aβ plaques were not observed in the wild-type mice. *The author performed all tissue preparation, immunohistochemistry and imaging.*

3.5 Discussion

We performed *ex vivo* DTI on the brains of male and female App^{NL-F} and App^{NL-G-F} mice at ages 9 and 17-months in order to capture aspects of neuropathology that occur in regions traditionally associated with the early phases of AD, and the social and anxiety-like behaviours investigated in this thesis. These neural regions were the orbitofrontal and anterior cingulate cortices, the anterior amygdala and the ventral and dorsal hippocampus. As this is the first study to apply DTI to these novel and much-improved mouse models of AD, the principal aim in exploring these neural regions for potential microstructural change was to help facilitate future research endeavours employing these model mice. The more immediate aim was to identify a putative signature of neuropathology associated with the behavioural changes described in chapter 2. This is a topical initiative in AD research (Cuthbert, 2019; Lanctôt et al., 2017; Canevelli et al., 2016) and more generally psychiatric illness (Kas et al., 2019; Cuthbert & Insel, 2013), and is a necessary first step in identifying neural regions that warrant further investigating for underlying altered mechanisms.

A key question is how the DTI-derived scalar indices relate to biological change, and how the differences and changes in biology influence each measure of diffusivity individually and as an accumulative pattern. There are currently no clear answers to these questions. As Le Bihan and Lima (2015) point out, exploiting the full potential of the DTI technique to obtain information on tissue microstructure will require more research on the mechanisms that govern diffusion of water in neural tissue. However, on the simplest level, it is understood that tissue hinders the diffusion of water. In white matter, where tissue is homogeneous and assumes a shape, the FA, MD, AxD and RD can reflect the degree of myelination, cell death and edema, axonal injury and demyelination, and loss of oligodendrocytes and reactive astrocytosis, respectively (Weiner, 2017). However, in grey matter, where net diffusion is not expected to conform to any one direction, the primary measure to probe tissue microstructure is MD (Weston, 2015; Alexander, 2007). As cellular microstructure breaks down and there are fewer obstacles to diffusion, MD is expected to increase. Conversley, cellular swelling or increased cellular density is expected to reduce MD. Previous studies on grey matter MD in AD report increases of hippocampal MD and whole brain grey

matter MD in participants with MCI as compared to matched controls (Fellgeibel et al., 2004). Furthermore, increases in grey matter MD distinguished between MCI patients who later went on to develop AD and MCI patients that remained stable (Douaud et al., 2013; Scola et al., 2010). Thus, in line with most all other studies that apply DTI to the AD brain, we focus primarily on MD. However, FA, AxD and RD have been reported for completeness.

The microstructural integrity of the five neural regions investigated was intact in the 9-month old App^{NL-F} mice relative to the age-matched wild-type control mice. At the 17-month age point, the MD was significantly increased in the orbitofrontal cortex of the App^{NL-F} mice compared with the age-matched wild-type control mice but not the App^{NL-G-F} mice. This was the only DTI-derived microstructural alteration found in the App^{NL-F} mice. This finding is not trivial and will be discussed in further detail. DTI-derived microstructural alterations can precede overt behavioural change (Alexander et al., 2017; Ryan et al., 2013) and occur early in AD pathogenesis (Weiner et al., 2015). Thus, it was indeed possible that we would have observed greater change in the microstructural integrity of the neural tissue of the App^{NL-F} mice. Shah et al. (2018), using resting-state functional MRI (rsfMRI), detected hypersynchronized functional connectivity in App^{NL-F} mice as young as 3 months. BOLD functional connectivity was significantly increased in the hippocampal and frontal/cingulate networks, and there was hyposynchrony of BOLD functional connectivity at 7-months. Although our mice were at least 2 months older in age, this may suggest that measures of structural change derived from DTI data are not related to changes in BOLD functional connectivity. Nevertheless, given the minor AD-related pathology in these mice combined with the minor behavioural changes in preference for social novelty at the 8-month age point and social olfaction at the 15-month age point, the limited DTI-derived neuropathology is unsurprising. In contrast, there were DTI-derived abnormalities observed in the 9 and 17-month old App^{NL-G-F} mice.

First, in regards to the anterior amygdala, we found no DTI-derived neuropathology in the 9 or 17-month old App^{NL-G-F} mice; however, FA was marginally decreased in the 17-month old App^{NL-G-F} mice relative to the App^{NL-F} mice. Next, with respect to the DTI-

derived neuropathology in the hippocampus of the 9-month old App^{NL-G-F} mice, the AxD was significantly increased in the dorsal region relative to the wild-type mice, and the MD and RD was significantly increased in the ventral region relative to the wild-type mice and the App^{NL-F} mice. The 9-month old App^{NL-G-F} mice also exhibited a significantly increased MD, AxD and RD in the orbitofrontal cortex, and a marginally increased MD and significantly increased AxD in the anterior cingulate cortex, compared with the wild-type mice and the App^{NL-F} mice; the FA in the orbitofrontal cortex was significantly increased relative to the App^{NL-F} mice only. With respect to the DTI-derived neuropathology in the 17-month old App^{NL-G-F} mice, in the dorsal hippocampus, the MD, AxD and RD was significantly increased relative to the wild-type mice and the App^{NL-F} mice. In the ventral hippocampus, the MD was significantly increased relative to the wild-type and the App^{NL-F} mice, whereas AxD was significantly increased relative to the App^{NL-F} mice only. Additionally, the 17-month old App^{NL-G-F} mice had a significantly higher MD and RD in the orbitofrontal cortex compared with the wild-type mice, and a significantly lower FA in the anterior cingulate cortex relative to the wild-type and App^{NL-F} mice.

Changes in the rate of tissue water diffusion were far more common in the App^{NL-G-F} mice than the App^{NL-F} mice, and were always based on increased diffusivity. There was one change in FA, and that was a lower FA value in the anterior cingulate cortex of the 17-month old App^{NL-G-F} mice relative to the wild-type and App^{NL-F} mice; there was a marginally lower FA value in the amygdala of the 17-month old App^{NL-G-F} mice relative to the App^{NL-F} mice. Nearly all of the same changes that occurred in the App^{NL-G-F} mice relative to the wild-type mice also occurred relative to the App^{NL-F} mice; the two exceptions were that there were no differences between the APP knock-in strains for the dorsal hippocampus at 9-months or the orbitofrontal cortex at 17-months. A key finding is that App^{NL-G-F} mice display DTI-derived neuropathology at 9-months and 17-months, and these alterations occur in the same manner relative to the wild-type mice as they do the App^{NL-F} mice. In contrast, the App^{NL-F} mice do not exhibit DTI-derived pathology at 9-months, but do exhibit an increase in MD in the orbitofrontal cortex at 17-months.

The most salient behavioural finding from chapter 2 was the increased open arm activity observed in the 8 and 15-month old App^{NL-G-F} mice that we suggest may represent a disinhibtion-like phenotype, in line with findings from Latif-Hernandez et al. (2017) and Ognibene et al. (2005). Disinhibition is a well-known, and equally distressing behavioural feature of AD (Zhao et al., 2016; Steinberg et al., 2008; Lesser & Hughes, 2006; Chung & Cummings, 2000; Mega et al., 1996). It could be manifested within the current study as a failure to inhibit the choice to enter the open arms. Based on a review by Bannerman et al. (2004), the hippocampus may function in part to compare different response alternatives and select optimal responses. In the case of an unconditioned test of anxiety like the elevated plus-maze, this involves selecting between conflicting approach and avoid responses. The plus-maze juxtaposes mice natural propensity to explore novel environments, such as the open arms of the maze, with mice propensity to avoid the open spaces in favour of the protected closed arms. Evidence from both rodents and humans suggests that the ventral hippocampus (or anterior in humans) plays a role in mediating these sorts of approach-avoidance conflict processing. Both McHugh et al. (2004) and Bannerman et al. (2002) demonstrated that lesions to the rat ventral hippocampus resulted in an anxiolytic-like elevated plus-maze profile, and Bannerman et al. (2004) draws associations between ventral hippocampus lesions and behavioural disinhibition and reduced anxiety. Intriguingly, we found DTI-derived neuropathology in the ventral hippocampus of the 9 and 17-month old App^{NL-G-F} mice as described by significant increases in MD. It is possible that the DTI-derived neuropathology in the ventral hippocampus is linked to the ostensibly anxiolytic plus-maze profile observed in these mice. This behavioural result would also predict DTI-derived changes in the orbitofrontal cortex and anterior cingulate cortex.

The anterior cingulate cortex is a brain region that has been well established with mediating anxiety/fear (Etkin, Egner & Kalisch, 2011; Davidson, 2002). Additionally, the anterior cingulate cortex and orbitofrontal cortex have a well-established link to behavioural decision making, especially for affective stimuli (Dias et al., 1996). Furthermore, Yu and Frank (2015) highlight how hippocampal-prefrontal cortex interactions in the rat could provide a neural substrate for deliberative decision-

making. With that in mind, the 9-month old App^{NL-G-F} mice had a significantly increased MD in the orbitofrontal cortex combined with a marginally increased MD in the anterior cingulate cortex. The 17-month old App^{NL-G-F} mice had a significantly increased MD in the orbitofrontal cortex combined with a significantly lower FA in the anterior cingulate. Interestingly, the 17-month old App^{NL-F} mice also displayed an increased MD in the orbitofrontal cortex, suggesting that this microstructural alteration emerges early in AD pathogenesis. Resting-stage functional MR imaging (rsfMRI) has shown that connectivity of the medial prefrontal cortex to other regions is abnormal in the 3-month old App^{NL-G-F} mice (but not 7-month or 11 month) with the anterior cingulate cortex being the most altered region (Latif-Hernandez et al., 2017). Furthermore, these areas are susceptible to degeneration in early AD pathology (Huang et al., 2002; Scheff and Price, 2001). Together, these results suggest that the App^{NL-G-F} mouse may enable modeling of the neurobiological links between emergent disinhibition-type behaviour and AD.

A few observations are reported with regard to the immunohistochemical analysis. Aβ deposition does not appear to be sufficient to cause DTI-derived neuropathology, otherwise the author is unsure how to reconcile the fact that the amygdala remained unaffected in the 9 and 17-month old App^{NL-G-F} mice despite similar amounts of Aβ deposition compared to regions that did display marked changes in DTI-derived neuropathology (at least by our assessment; and Hamaguchi et al., 2019; Saito et al., 2014). However, given that we do see marked neural microstructural change in the 9-month old App^{NL-G-F} mice, but not the 9-month old App^{NL-F} mice, we can infer some degree of association between Aβ deposition and DTI-derived neuropathology or, perhaps more accurately, the accumulative effects (e.g., inflammation, synapse loss etc.) of a more advanced stage of AD-pathology that is marked by the build of Aβ plaques. Thus, the soluble Aβ that is present in large quantities in the App^{NL-F} mice at 9-months relative the age-matched wild-type control mice (Saito et al., 2014) does not appear sufficient to cause changes to the microstructural integrity of the neural regions we investigated. Understanding more precisely the reasons for the DTI-derived neuropathology in the 9-month old App^{NL-G-F} mice, contrasted with the lack of change in the 9-month old App^{NL-F} mice, is an opportunity for future investigation.

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