Depression in Alzheimer's Disease: a role for the Locus coeruleus

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December 2022

A dissertation submitted for the higher degree of PhD

●0.1 Summary of thesis

Neuropsychiatric symptoms (NPS) are a common yet often overlooked aspect of Alzheimer's disease (AD) associated with accelerated disease progression and reduced quality of life. Amyloid pathology, both soluble oligomers and insoluble plaques, has been heavily linked to the development of NPS along with locus coeruleus (LC) noradrenergic signalling. A knock-in (KI) mouse model, *App*$^{NL\text{-}G\text{-}F}$, was used to examine this question through a battery of affective and biochemical tests to examine the link between amyloid, LC and NPS. The final experiment looked at how LC-NA signalling changes could lead to depressive symptoms seen in AD.

App$^{NL\text{-}G\text{-}F}$ mice expressing Swedish (KM670/671NL), Iberian/Beyreuther (I716F) and arctic (E694G) mutations, were tested at both a young and old time point across both sexes. Reduced anxiety was found across elevated plus maze (EPM) for females only and open field (OF) while no social or cognitive deficits were found compared to wildtype (WT) mice. *App*$^{NL\text{-}G\text{-}F}$ mice had reduced sucrose consumption with normal liking behaviour suggesting no depressive phenotype but rather an apathetic one.

Biochemical analysis revealed increased gliosis with altered neurotrophic support. Interestingly, increased gliosis was not accompanied by elevated common cytokine levels but some chemokines were increased (e.g.CCL3 & CCL4). Despite there being no changes to mature brain derived neurotrophic factor (BDNF), there were increased levels of its receptor and precursor, TrkB and proBDNF in the cortex. No LC cell loss was found at either time point. Combined with the behavioural data, this suggests amyloid causes apathy through increased gliosis and the lack of cognitive, anxious and depressive effects could be due to the lack of tauopathy and LC damage.

Disrupted noradrenergic signalling relates to depression and was examined in a DSP4, a noradrenergic specific neurotoxin, injected WT mice as well as postmortem human tissue. Only subtle trends for increased depressive behaviour were seen in the mice but no changes in tyrosine hydroxylase, the rate limiting enzyme in noradrenaline production, were seen in depressed and non-depressed AD and non-AD patients. Results remain inconclusive due to confounds but the subtle trends suggest some link between LC and depression.

Together, this work suggests amyloid causes apathy and reduced anxiety possibly through increased gliosis but the lack of cognitive, anxious and depressive effects could be down to intact LC

and normal BDNF levels. Results are discussed around whether amyloid would be a good target for NPS treatments.

●0.2 Acknowledgements

This work spanning 4 years was only made possible by the people around me who supported, trained and kept me sane throughout. With so many to thank, I will go through my journey chronologically to highlight the people and organisations who helped me tremendously throughout this entire project.

Firstly, I would not be here without the Wellcome Trust who funded this project but also the wonderful academics who interviewed me and deemed me worthy to give a PhD studentship to: Prof John Aggleton, Prof Anthony Isles, Prof Frank Sengpiel and Prof Penny Lewis. For this, I also have Dr Andrew Nelson to endlessly thank for reading through my initial application and telling me it was rubbish and rewrite the whole thing. His honesty was brutal but, without it, I would not be here writing these acknowledgements. I also want to thank Prof John Aggleton again for seeing something in me, for the encouragement, the work experience and helping fix the technical issue that allowed me to do this PhD project with my supervisors in the first place.

Speaking of supervisors, Prof Dominic Dwyer! A man who inspired me even in my first year of undergraduate to fall in love with stats. A man who has taught me so much from being a good scientist to a good writer, who read each page of this thesis countless times and spotted so many things that I would never have even thought of. Dom, I cannot thank you enough for your supervision, I count myself incredibly lucky to have had you supervise my project, help solve so many issues and have a beer (or five) with.

My other supervisor, Prof Mark Good, beyond lives up to that name! Mark, you also inspired me in my first year of university to fall in love with neuroscience, to tell stories during lectures and teach information in such a fascinating way. That continued through your supervision, you gave me more ideas than I could even follow through with. You provided me so much knowledge, so many laughs and you were a genuine joy to be around. Please, never change!

Prof Emma Kidd, my surrogate supervisor, who took me in even when you did not need to. I cannot thank you enough for all your wisdom, your time and expertise that got me through some of the most difficult parts of my PhD. I truly could not have done this work to the level I managed without you opening your lab and time to me.

To all those people who have taken their time to train me (Charlie, Jawsa, Pascal), I am grateful for your time and expertise. For all the many students who have helped me run or be my blind raters, you were all a joy to work with and I hope you are doing so well now. That includes Kirby, Leo, Hannah, Evie and Cyan who helped run my DSP4 experiments and Ralph, Hanaa and Chantal who were my blind raters to cell counts and behaviour. I also cannot thank my good friend and computer wizard, Zak, for helping me with my website to be used at conferences.

Thank you to Patricia who selflessly stepped in to ween my mice when I had COVID. Your advice, your support has been such a blessing throughout my work and I you deserve so much good in your life for all the countless aid you generously offer out. Sophie, thank you for being my office mate, my colleague and my friend. You not only gave me so many laughs but also listened to my rants and kept me sane throughout my PhD life. Also, to my other friends Matt and Peter, for the many curries and beers, for the jokes and absolute ludicrous nonsense you come out with- it never fails to amuse me. I also want to thank Ffion for supporting me, for your kind words and being a good friend. Elliot, I do not have enough words for you, my partner. You have supported me by never failing to make me laugh even on my darkest days. You really are an amazing, kind person, my best friend and partner- I am immeasurably lucky to have you.

Finally, I want to thank the human patients who donated their bodies to science and the Sudden Death Edinburgh Brain and tissue bank for generously providing the human brain samples. All 402 mice that were also used in this experiment, I thank you for your sacrifice of which I can never repay.

Thank you to you, the reader, who has just waded through a large acknowledgements section and who will now hopefully go on to read the work I have poured my heart and soul into. I hope you have a wonderful day.

●0.3 Contents

●0.4 List of abbreviations

6OHDA- 6-Hydroxydopamine

AD- Alzheimer's disease

APP- Amyloid precursor protein

AR- Adrenoreceptor

Aβ- Amyloid beta

BBB- Blood brain barrier

BDNF- Brain derived neurotrophic factor

CNS- Central nervous system

CSF- Cerebrospinal fluid

CX- Cortex

DSP4- N--N-ethyl-2-bromobenzylamine

ELISA- Enzyme-linked immunosorbent assay

EPM- Elevated plus maze

FAD- Familial Alzheimer's disease

FST- Forced swim test

HPA- Hypothalamic-pituitary adrenal axis

HPC- Hippocampus

IHC- Immunohistochemistry

KI- Knock-in

LC- Locus coeruleus

LCA- Lick cluster analysis

LPS- Lipopolysaccharide

MAO-B- Monoamine oxidase B

MCI- Mild Cognitive impairment

MDD- Major depressive disorder

MMSE- Mini-Mental state examination

mRNA- Messenger ribonucleic acid

NA- Noradrenaline

NARI- Noradrenaline reuptake inhibitor

NFT- Neurofibrillary tangle

NMDA- N-methyl-D-aspartate

NOR- Novel object recognition

NPI- Neuropsychiatric Inventory

NPS- Neuropsychiatric symptoms

NSAID- Non-steroidal anti-inflammatory drugs

OF- Open field

OiP- Object in Place

PD- Parkinson's disease

RCT- randomised control trial

ROS- Reactive oxygen species

SAD- Sporadic Alzheimer's disease

SNRI- Serotonin and Noradrenaline reuptake inhibitor

SPT- Social preference test

SSRI- Serotonin reuptake inhibitor

Tg-Transgenic

TH- Tyrosine Hydroxylase

TLR4- Toll like receptor 4

TrkB- Tropomyosin receptor kinase B

TST- Tail suspension test

WB- Western blot

WT- Wildtype

NET- Noradrenaline transporter

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●Chapter 1: General introduction●

●1.1 Overview

This introduction will outline the neuropsychiatric symptoms seen in Alzheimer's disease (AD) and why their existence exacerbates disease pathology. It will also consider the locus coeruleus (LC) dysfunction as a potential candidate to explain these symptoms. Section 1.2 will outline important aspects of AD including its neuropathies, its symptoms and both their timeline in relation to disease progression. Section 1.3 will then outline the LC-noradrenergic system that has strong links to stress, depression and anxiety as well as being a key part in many treatments for these mental health conditions (e.g. noradrenaline reuptake inhibitors, SNRIs, MAOA-B inhibitors, β-blockers). The noradrenergic system also has neuroprotective roles in maintaining the health of the entire brain including modulating inflammatory processes and providing neurotrophic support to neurons. As it is the first area to exhibit tauopathy, the early loss of neuroprotection from the LC would explain further neurodegeneration and rampant neuroinflammation which is examined in section 1.4. Section 1.5 examines how animal models have been used to look at the underpinnings of AD. Transgenic models have examined affective deficits and the LC in response to amyloid and tau pathology but they suffer from lack of consistency across models and overexpression of *APP* fragments with unknown biological consequences. Newer knock-in models have come a step toward solving this problem to create a more valid model in respect to the human condition. Work examining neuropsychiatric symptoms, neuroinflammation and the LC will be considered before outlining the three aims of this thesis and how it will go about addressing them.

●1.2 Alzheimer's disease

In 2016, Alzheimer's disease (AD) became the leading cause of death in the UK surpassing heart disease with cases increasing over two-fold since 2001 (Office of national Statistics, 2020). While prevalence in Europe was 3.31% for men and 7.13% for women (Niu et al., 2017), the estimated prevalence for preclinical AD was 22% of the population (Parnetti et al., 2019). While these increases may be partially due to improved diagnostic criteria (Donegan et al., 2017), as well as to decreases in other causes of death that occur prior to potential dementia onset, it cannot be understated how wide the impact of this disease is.

With an aging population and increased diagnosis also comes surges in emotional and financial burdens of the disease. The mental and emotional strain on caregivers of dementia patients has been shown to far exceed the carers of healthy age-matched controls due to the difficulty of everyday tasks (Ory et al., 1999). Their stress also impacts the patient in a bidirectional relationship exacerbating disease progression (Isik et al., 2019). The financial costs can include loss or reduction of income, medical treatments and, at the later stages, formal carers or institutionalisation (Castro et al., 2010). On top of that, the NHS estimate their costs of treating AD in the UK to be £2 billion a year (Morris et al., 2015) with costs for treating dementia 5.5x greater than non-dementia patients of the same age (Connections, 2014). AD is an increasing problem causing lasting distress and burden to people as well as the health care system. With few effective treatment options, it is imperative that finding ways of improving quality of life for patients and care givers is researched to limit the emotional impact of this disease.

●1.2.1 Alzheimer's disease symptoms

AD is characterised by cognitive decline and problems with learning and memory. This decline is exclusive to explicit memory (i.e., episodic and semantic; Fleischman et al. (2005)). The first recorded case of AD, Auguste D, demonstrated this with an inability to recall her last name, some objects and food items (Maurer et al., 1997). Various other studies have shown these explicit memory deficits are common among patients (Bäckman et al., 2005; Golby et al., 2005; Greene et al., 1996) while implicit memory (i.e.,, procedural memory and priming) remained intact (Eslinger & Damasio, 1986; Gabrieli et al., 1993; Rice et al., 2008). Other cognitive issues include attention deficits (Belleville et al., 2007; Bracco et al., 2014; Perry & Hodges, 1999), visuospatial dysfunction (Deng et al., 2016; Fukui & Lee, 2009; Quental et al., 2013; Salimi et al., 2018), language deficits (Ahmed & Garrard, 2012; Aramaki et al., 2016; Price et al., 1993) and executive function issues (Baddeley et al., 1986; Baddeley et al., 1991; Foley et al., 2011; Logie et al., 2004). Although a strong characterising feature of AD, cognition will not be the focus of the current thesis.

Less commonly emphasised symptoms of AD include the neuropsychiatric symptoms (NPS) that are a prevalent feature of the disease. These include depression, anxiety, agitation, apathy, aggression, disinhibition and various psychotic symptoms (e.g., hallucinations). It is estimated that above 80% of patients suffering from dementia exhibit at least one NPS (Lyketsos et al., 2002) but it is difficult to pinpoint exact prevalence due to heterogeneity of all NPS and how they are studied (Zhao et al., 2016).

While they are they not often the focus of AD research, there is clear evidence for how damaging NPSs are to the patient and others around them (Lyketsos et al., 2011). For example, they can greatly increase disease progression (Taragano et al., 2009) as well as cause an accelerated path to institutionalisation due to care giver distress (González-Salvador et al., 1999). NPS greatly hasten the progression from mild cognitive impairment (MCI) to full blown AD (Taragano et al., 2009) as well as exacerbate cognitive decline (Emanuel et al., 2011). While increases in cognitive deficits can impact regular life tasks, it is the NPS that contribute much more to the daily life distress of care givers and patients (Allegri et al., 2006). Due to great focus on the cognitive side, the affective symptoms are often under-recognised and improperly managed (Chow et al., 2002) creating an even worse daily life experience for patients and caregivers. However, the aetiology of these symptoms is complex and AD patients can present with contrasting symptoms (e.g., apathy or agitation) that makes understanding the cause very difficult (Chung & Cummings, 2000; Lyketsos et al., 2002). Together, it seems NPS are not only poorly understood but also incredibly damaging to daily life and integral to accelerating the progression of the disease. Although the mechanisms behind these symptoms are not well understood (Bruen et al., 2008), if they could be identified, then this may suggest potential treatment targets for future development that would slow disease progression and increase quality of life for patients and caregivers.

Some of the NPS will be focused on in more detail to shed some light on their impact. Apathy and depression are two of the most common and persistent NPS (Lyketsos et al., 2011) with apathy defined as a disorder of motivation and requires two of 1) reduced goal-directed behaviour, 2) reduced goal-directed cognitive activity and 3) reduced emotions for over 4 weeks (Robert et al., 2009). However, it is difficult to pinpoint since its symptoms overlap with depression (Nobis & Husain, 2018; Siafarikas et al., 2018; Zhao et al., 2016) but they are distinct (Landes et al., 2001). Patients who are diagnosed with apathy are also linked to greater caregiver distress (Chen et al., 2017; Dauphinot et al., 2015; Riedijk et al., 2006) as it leads to disengagement strategies from the carer suggesting the hardships in caring for someone with greatly reduced motivation (García-Alberca et al., 2014). Mirroring this, apathy in AD patients was also associated with decreased quality of life (Hongisto et al., 2018), greater functional decline and morbidity (You et al., 2015). In a longitudinal 10 year study, the presence of apathy was able to accurately predict time of death for patients (Spalletta et al., 2015).

Depression is also found to affect around 50% of AD patients (Lee & Lyketsos, 2003) with a 3-4 times greater prevalence than in healthy populations of the same age (Lyketsos & Olin, 2002). However, depressive symptoms seem to fluctuate over time making it difficult to get an accurate reading on prevalence and how it develops alongside AD (Olin et al., 2002). Depression is

characterised by loss of interest, changes in appetite and activity and indecisiveness (American Psychiatric Association, 2010). Like apathy, depression in patients is linked to higher caregiver distress (González-Salvador et al., 1999), worse quality of life (González-Salvador et al., 2000), increased financial costs and worsened cognitive decline (Murman et al., 2002). Research has demonstrated that AD patients with depression were more cognitively impaired and physically disabled than those without (Lyketsos et al., 1997; Rovner et al., 1989), again, highlighting the detrimental effect of NPS.

As well as presenting with cognitive deficits, Auguste D. was also described as anxious and showed bouts of screaming and paranoia (Maurer et al., 1997). Prevalence estimates from a meta-analysis suggested anxiety to be around 39% in AD patients (Zhao et al., 2016) with other research showing anxiety symptoms ranged from 8-71% while a diagnosable disorder ranged from 5-21% (Lyketsos et al., 2000; Porter et al., 2003). These vast ranges highlight the heterogeneity of anxiety. Anxiety is defined as a nervous disorder with excessive worry of uncertain outcomes. It is also a risk factor of progressing from MCI to AD independent of other NPS (Burke et al., 2018; Gallagher et al., 2011; Li & Li, 2018; Mortby et al., 2017; Petkus et al., 2016; Santabárbara et al., 2019) but is more commonly associated with early AD (Kaiser et al., 2014; Mendez, 2021).

Overall, NPS encompass a wide variety of different affective disorders that all seem to negatively impact the patient's daily life, increase caregiver distress and greatly increase the progression of AD. While they are poorly understood, researching the underlying biological mechanisms around their initiation could improve the lives of patients and better manage symptoms.

●1.2.2 Amyloid pathology in Alzheimer's disease

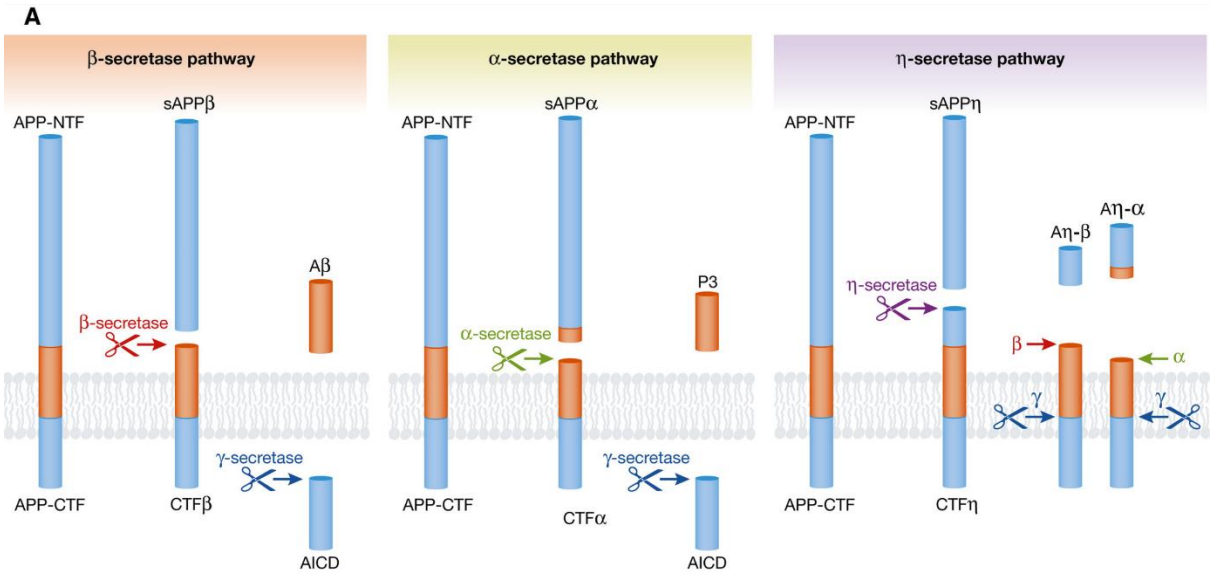

Figure 1.1 Visual representation of the possible cleavage pathways of APP (Sasaguri et al., 2017)

Cleavage of APP

One of the main hallmarks of AD that has received a great degree of attention is extracellular plaques that are visible post mortem. Plaques are comprised of aggregated amyloid beta (Aβ) fibrils cleaved from *APP* in the β-secretase pathway (fig. 1.1). *APP* is a 770 amino acid long protein that can be subject to alternative splicing creating various fragments through non-amyloidogenic (α- and η-secretase pathways; fig. 1) and amyloidogenic pathway (β-secretase pathway). The main non-amyloidogenic pathway starts with α-secretase splicing *APP* to release *sAPPα* and the remaining *C83* (*CTFα*) fragment in the membrane is spliced by γ-secretases to create *p3* and *AICD* fragment. The *C83*, *p3* and *AICD* are non-toxic and seen in healthy splicing of *APP*. The amyloidogenic pathway cleaves *APP* at the N-terminal with β-secretases, mainly BACE1, to release *sAPPβ*. The remaining membrane bound fragment, *C99* (*CTFβ*), is then cleaved by γ-secretases in the same place to release *AICD* and neurotoxic amyloid beta (Aβ), that is prone to aggregate. Two main Aβ fragments are released: $A\beta_{40}$ that is more abundant but less toxic and $A\beta_{42}$ that is less abundant, highly neurotoxic and very prone to accumulate into oligomers and then extracellular plaques (Haass et al., 2012).

The Aβ produced from the amyloidogenic pathway are highly neurotoxic and can block ion channels leading to increased oxidative stress, altered calcium signalling and overall deterioration of neuronal health (Rauk, 2008). Aβ has also been found to impair synaptic functions (Shankar & Walsh, 2009) such as disrupting long term potentiation in rodent models when given Aβ extracted from

human brain samples (Shankar et al., 2008). Soluble forms of Aβ have been shown to interfere with memory functions by disrupting dendritic spine production (Balducci et al., 2010) and are also a better correlate of AD progression than plaque number or insoluble forms (Jin et al., 2011; Kittelberger et al., 2012). However, both soluble and insoluble forms elicit microglial activation through NF-κB pathways by increasing pro-inflammatory cytokine and chemokine production (Ridolfi et al., 2013) as they attempt to clear away the toxic Aβ. In removing the amyloid, they may reduce its impact on oxidative stress and memory function, but the microglia also end up damaging surrounding cells (Dorey et al., 2014) leading to further neurodegeneration. Amyloid can also stimulate tau hyperphosphorylation through various pathways that not only reduce tau degradation but also increase hyperphosphorylation and aggregation (Sadigh-Eteghad et al., 2015). Amyloid is not the only cause of tau hyperphosphorylation as reduced glucose metabolism has also been cited (Gong & Iqbal, 2008) but it is an important one. So, increased cleavage of *APP* via β-secretase pathway leads to higher Aβ that increases oxidative stress, interferes with neuronal signalling, stimulates neuroinflammation and hyperphosphorylates tau that promotes neurodegeneration.

Amyloid cascade hypothesis

Research has shown Aβ fibrils can cause the hyperphosphorylation of tau leading to the proposal of the amyloid cascade hypothesis (Hardy & Higgins, 1992). Although it has become more detailed over the years, the basic and consistent idea is that amyloid is the initiating factor in the development of AD. Evidence in support of this comes from the observation that the highly penetrant mutations that cause familial AD all affect *APP* processing (AβPP mutations) or Aβ deposition (PSEN1 and PSEN2 mutations) that can go on to cause the tau pathology seen in AD (Hardy et al., 1998). However, these mutations merely deal with changes to the processing of *APP* by presenilin and not whether the Aβ fibrils themselves are causing other AD pathologies (Herrup, 2015). It is known that Aβ is neurotoxic (Rauk, 2008) but for it to cause AD, simply adding it to a brain would be enough to instigate the disease which is not the case. Animal models of amyloid pathology would go on to naturally develop tauopathy that does not happen without the addition of tau related transgenes (e.g. 3xTg-AD mouse model; Oddo et al. (2003)). It is possible they do not develop tauopathy simply due to not living as long as humans naturally do. Similarly, humans have been found to have plaques yet have no cognitive decline or other AD pathologies (Hyman & Gomez-Isla, 1997; Maarouf et al., 2011; Tsartsalis et al., 2018). In contrast, removing the Aβ through immunotherapies reversed cognitive deficits seen in mice models (Cramer et al., 2012; Schenk et al., 1999) but not in humans (Holmes et al., 2008). Vaccine trials against Aβ have been successful in completely abolishing plaque load from the brains of AD patients but still showed progressive tau pathologies after a 14 year follow up (Nicoll et al., 2019). Other drugs that target Aβ reduction have

shown some promise (Decourt et al., 2021) but whether the effects are clinically significant are still debated (Karran & De Strooper, 2022). The latest anti-amyloid drug gaining FDA approval is lecanemab which shows promise in reducing early biomarkers and slowing cognitive decline (Van Dyck et al., 2023). So, while it seems clear that amyloid plays an important role in AD, exactly what that entails is still uncertain.

Amyloid and neuropsychiatric symptoms

While little evidence has found direct links between cognitive decline and amyloid, there is growing support for a correlation between Aβ pathology and depression. Research has found a link between greater amyloid and tau pathology in a post mortem study of patients with and without a family history of depression (Rapp et al., 2006). However, this does not specify what the independent role of amyloid is. It could simply represent how depression can exacerbate disease progression (Taragano et al., 2009) which is what the study found in patients with an actual concurrent depression diagnosis. With the improvement of brain scanning technologies, it is now possible to examine amyloid load in live patients to monitor how it relates to depressive symptoms. It was found using positron emission tomography (PET) that patients with major depressive disorder had higher amyloid loads than healthy controls (Kumar et al., 2011; Wu et al., 2014). The idea of depression and other neuropsychiatric symptoms increasing pathology has already been established but further evidence suggests amyloid pathology could directly produce depressive symptoms along with other neuropsychiatric symptoms. Looking at samples of cognitively normal people, it was found that greater amyloid load was associated with higher depression and anxiety scores (Donovan et al., 2018; Krell-Roesch et al., 2018). Using mediation analysis, Xu et al. (2021) found that depressive symptoms were associated with higher amyloid burden in a bidirectional relationship using a cognitively normal population. However, other research has found no relationship between amyloid and depression (Babulal et al., 2020; Ehrenberg et al., 2018) or even less amyloid is linked to depression (Mackin et al., 2021). While this discrepancy could be due to sampling bias (van Dyck et al., 2021) it is more likely to highlight the complex relationship between amyloid and depression. It is likely that the link is not direct but amyloid could induce other processes or cascades that could eventually lead to depression (e.g. inflammation). It is important to include a longitudinal approach to this as amyloid fluctuations could impact processes that could then go on to the development of depression over time. A simple snapshot in time would not address this.

Looking at a longitudinal approach, it seems that amyloid burden could be a significant predictor of future depression in cognitively normal people (Almdahl et al., 2022). Mahgoub and Alexopoulos (2016) even suggest the amyloid hypothesis of late life depression acknowledging a

difference between early onset and later life diagnoses. They outline support for this with depression being a risk factor and a symptom that commonly appears during preclinical AD where amyloid accumulation is prevalent (Lyketsos & Olin, 2002). Other evidence is from patients with a lifetime history of depression have significant amyloid pathology in emotional regulation brain areas (Wu et al., 2014). Furthermore, amyloid pathology leads onto other neurobiological processes that could be associated with or impair the networks related to mood regulation (e.g. neurodegeneration, vascular damage, blood brain barrier breakdown, neuroinflammation; (Alexopoulos et al., 1997; Miller & Raison, 2016; Taylor et al., 2013). To get a better understanding of how amyloid accumulation could lead to the development of NPS, Aβ needs to be directly manipulated. While various anti-amyloid treatments are available, trials with these treatments often exclude patients with a comorbid diagnosis of depression meaning data is currently scarce in humans (Lee & Lyketsos, 2003). In animals though, giving rats injections of soluble Aβ oligomers greatly increased their depressive symptoms. This was measured with sucrose preference test and forced swim test via neuroinflammation and oxidative stress that was successfully treated with sulforaphane, a compound with potent anti-inflammatory and anti-oxidative effects (W. Wang et al., 2020). Similarly, mice given an injection of Aβ had increased immobility in the forced swim test, a measure of susceptibility to negative outlook, that was prevented with agmatine treatment which has anti-oxidant effects (de Souza et al., 2018; Kotagale et al., 2020). So perhaps the link is not a direct one but the processes that amyloid provokes could be integral to depression onset in preclinical AD.

●1.2.3 Tau pathology in Alzheimer's disease

Tau is an important soluble protein that regulates microtubule and the cytoskeletal structure of neurons in the central nervous system (CNS; Ballatore et al. (2007)). Hyperphosphorolated tau fails to bind appropriately to microtubules, and thus produces instability in the structure of a neuron's transport systems (Sengupta et al., 1998). These phosphorylated tau filaments also bind together to form neurofibrillary tangles (NFTs) that can block transport and disrupt the integrity of axons and synapses leading to cell death (Iqbal et al., 2005). This hyperphosphorylation of tau occurs in various neurodegenerative diseases such as AD, Pick's disease and Frontotemporal dementia and can explain why NFT presence correlates with cognitive decline (Grundke-Iqbal et al., 1986; Lewis et al., 2001; Nukina & Ihara, 1986).

While some theories state that the amyloid pathology causes tauopathy (Sadigh-Eteghad et al., 2015), research has shown tau issues occur first (Braak et al., 2011) with some suggesting tau is

the initiating factor in AD (Arnsten et al., 2021). While that is a difficult hypothesis to confirm, it is supported by the fact that a pre-tangle form of tau has been found prior to any amyloid pathology. It is more likely to be a bi-directional relationship with hyperphosphorylated tau impacting amyloid production and amyloid increasing hyperphosphorylated tau. Furthermore, both amyloid and tau promote inflammation. Aβ is one cause of the phosphorylation of tau by activating kinases that will cause the hyperphosphorylation e.g. Glycogen Synthase Kinase 3, cyclin-dependent kinase 5. Despite some research, the mechanisms that initially cause tauopathy are not well understood, however one theoretical suggestion is that alterations in glucose metabolism may be involved (Gong & Iqbal, 2008). While the mechanisms are uncertain, it seems that tau pathology spreads through the brain starting in the LC and entorhinal cortex before spreading to the hippocampus and neocortex (Braak et al., 2011). In AD, the spread of tau is relatively consistent so can be used to measure how progressed the disease is with Braak staging (Braak & Braak, 1991).

●1.3 Locus Coeruleus and noradrenaline system

With neuropsychiatric symptoms creating such issues with quality of life and hastening disease progression, they are likely linked to at least one of the three pathologies of AD: amyloid, tau and neuroinflammation. What exactly causes the manifestation of NPS is uncertain but getting answers could offer novel treatment options that could improve quality of life for patients and caregivers. Investigating the LC could offer useful insights into this question as it is heavily involved in the aetiology of depression, anxiety and other NPS as well as being one of the first areas to show Alzheimer's related tauopathies. This section will examine what this brain area is, what it does and how it is affected in AD.

●1.3.1 Neuroanatomy

The LC is the main hub of noradrenaline (NA) in the brain and is the sole source of NA for the hippocampus and cortex while other areas receive NA innervation from the lateral tegmental region (Loughlin, Foote, & Bloom, 1986; Loughlin, Foote, & Grzanna, 1986). It is located in the pons region of the brainstem and innervates to most of the brain, with an exception of the basal ganglia (Sara, 2009), with most of the LC's connections being ipsilateral (Mason & Fibiger, 1979; Room et al., 1981). The LC has a topographical organisation with the rostral portion of the LC innervating forebrain and the caudal to the hindbrain. Furthermore, the LC has 5 main tracts where 3 project to

the forebrain, the fourth to the cerebellum and the fifth to the spinal cord (Szot, 2012). Each LC neuron can innervate many different regions as they are highly arborized.

The LC neurons communicate primarily using the neurotransmitter NA, although some research has also suggested that they release dopamine so both neurotransmitters can work in concert on specific target regions (Sara, 2009). NA is released in two ways: through standard synaptic transmission and volume transmission (Feinstein et al., 2016). While standard synaptic transmission communicates via direct synaptic connections, volume transmission releases NA into extracellular space to stimulate any neurons with the corresponding receptors (Sara, 2009). Furthermore, NA can be transported via cerebral spinal fluid for even longer-range transmission (Taber & Hurley, 2014) showing the LC's influence far exceeds neuronal connections. NA will affect target neurons via G-protein coupled receptors, the adrenoreceptors that will be considered next.

The receptor family that is susceptible to activation with NA are the adrenoreceptors (AR) that can be split into 3 classes: α1- (A, B,C), α2- (A, B, C) and β- (1 & 2). They are widely distributed throughout the brain, and all activate different G-proteins such as β-AR couple to Gs that enhance cAMP signalling while α2-AR couple with Gi that suppress adenylyl cyclase and reduce cAMP suppressing the release of other neurotransmitters (Nasse & Travers, 2014; Yavich et al., 1997). The α1-AR couple with Gq to activate phospholipase C signalling pathway. The activation of AR in many brain regions has been found to enhance long term potentiation, working memory, memory consolidation and retrieval highlighting NA and AR importance in cognition (Sara, 2009). Adrenoreceptors are also found on microglia and astrocytes allowing NA to directly affect inflammation (Madrigal et al., 2009; Pocock & Kettenmann, 2007). For astrocytes, their main effect may not be inflammation as a key role has also been found in modulating synaptic activity through NA binding to adrenoreceptors (Wahis & Holt, 2021).

The extensive connectivity of the LC comes at the cost of having long axons that are highly susceptible to damage (Weinshenker, 2018). Consequently, the LC is often one of the first areas to exhibit pathology in neurodegenerative diseases such as AD (Braak et al., 2011). This vulnerability occurs for many reasons including the unmyelination leading to higher energy output to perform efficient action potentials which, over time, leads to high levels of oxidative stress (Theofilas et al., 2015; Weinshenker, 2018). The lack of myelin also leaves the neurons heavily exposed to neurotoxins from extracellular space and the cardiovascular system. Pamphlett (2014) demonstrated that the LC has a major exposure to blood with over 20 m of capillaries as it requires a lot of energy. Each neuron has roughly 2 capillaries wrapped around them (Mather & Harley, 2016) creating heavy exposure to environmental toxins in the blood that is exacerbated in disease conditions when the

blood brain barrier starts to diminish (Satoh & Iijima, 2019). As well as the blood, cerebral spinal fluid accumulates toxins as it acts as a filter system for the brain. The LC, being in close proximity to the fourth ventricle, is heavily exposed to these contaminants (Mravec et al., 2014). The LC neurons themselves contain neuromelanin that chelates excess heavy metals in the brain. However, when the cell dies then these heavy metals will be released further damaging surrounding cells (Zucca et al., 2014). Together, it seems that long, unmyelinated neurons with a high bioenergetic need are susceptible to oxidative stress and toxins leading to cell death. This death can lead to the loss of neuroprotection (see section 1.3.3) as well as initiating cascades that could result in damage to surrounding neurons.

●1.3.2 LC-NA involvement in stress and neuropsychiatric disorders

The LC is important in various neural processes such as sleep (Aston-Jones & Bloom, 1981) and cognition (Sara, 2009) as well as being an integral part of the stress response. As stress is important in the development of anxiety/anxiety disorders, this could explain why they are linked to the LC (Berridge & Waterhouse, 2003; Hoehn-Saric, 1982; Yamamoto et al., 2014). Furthermore, LC neurons also express receptors for a variety of substances involved in the stress response (e.g. NA, somatostatin, galanin, orexin, substance P; (Van Bockstaele & Valentino, 2013). Alongside that, NA directly stimulates corticotrophin releasing hormone from the Paraventricular nucleus of the hypothalamus via the Hypothalamic-Pituitary-Adrenal (HPA) axis (Ma & Morilak, 2005). This is an integral part of the autonomic stress response that leads to the release of cortisol into the blood (Bremner et al., 2003) that will increase heart rate, respiratory rate and decrease digestive processes to prepare the body for threats (Bruchas et al., 2009). The discrete pathway from the central nucleus of the amygdala to the LC increases its tonic activity that can induce anxiety and anxiety-like behaviours in response to stressful stimuli (Reyes et al., 2008). Research from McCall et al. (2017) showed that directly stimulating the LC-NA fibres in the basolateral amygdala alters its activity to increase anxiety-like behaviour in mice that was blocked with β-AR antagonists. Repeat firing of this circuitry could increase the excitability of the LC neurons making them much easier to fire in response to lesser stimuli (Borodovitsyna et al., 2018). In making this connection stronger, this allows for a stress response to be elicited from associated but smaller threats leading to anxiety disorders. Further evidence for this has been in using α2-AR agonists as treatment for symptoms of panic disorder (Itoi & Sugimoto, 2010) as they could reduce the stress response (i.e., panic). Hoehn-Saric (1982) also found that drugs that seem to increase NA activity will also increase anxiety while

drugs that reduce activity will also reduce anxiety showing a correlation in how easy the stress response is to elicit.

Not only does the LC elicit a stress response, stress can also increase its activity creating a feed forward system of growing stress and anxiety. Animal research found that a variety of stressors increased Tyrosine hydroxylase (TH) mRNA production in the LC (Angulo et al., 1991; Chang et al., 2000; McDevitt et al., 2009; Richard et al., 1988; Smith et al., 1991; Watanabe et al., 1995). TH is the rate limiting enzyme in the production of NA so is used to measure NA production. TH mRNA may only be an indirect measure of LC activity but other research found reductions in central NA content in response to stress (Weiss & Simson, 1989) that could highlight increased NA turnover.

As well as anxiety, the LC has links to depression. Depression is sometimes referred to as a "stress disease" because stress is one of the major causes of depression (Hammen, 2005; Van Praag, 2004). In animal models of depression, LC hyperactivity has been noted (Stone et al., 2011; Weiss & Simson, 1988) and anti-depressants can reduce LC activity (Grant & Weiss, 2001; Rovin et al., 2012; West et al., 2009). This increased LC activity could link to a highly aroused brain that is often seen in MDD (Wittekind et al., 2016) as well as being seen after repeat stress exposure. It is important to note that it is not the stress itself that causes depression but rather the individual's perception of their ability to cope with the stressor and LC activity only increases when the person feels unable to cope (Anisman & Zacharko, 1982). In rats, this inability to cope is found with chronic stress causing increased NA activity in the amygdala (Ronzoni et al., 2016) and over sensitised serotonin and NA systems in response to further stressors i.e., neurons fire more readily (Adell et al., 1988). Repeated firing of the LC-NA system can lead to increased NA turnover (Tanaka et al., 1989; Tanaka et al., 1982) that could explain why NA depletion in the brain is often found in patients suffering from major depressive disorder (Frieling et al., 2007; Klimek et al., 1997; Werner & Covenas, 2010). This could explain why some depression can be successfully treated with drugs designed to increase monoamine tissue content (e.g. SSRIs, NARIs) as well as inhibitors to enzymes that break down the monoamines (e.g. MAO-B inhibitors; (Werner & Covenas, 2010). Evidence shows that depressive patients who are successfully treated with NA reuptake inhibitors (NARIs) regain their depressive symptoms if their NA is depleted. This same process does not occur with serotonin and patients who respond to SSRIs (Berman et al., 1999; Delgado & Moreno, 2000). This discrepancy between NA and serotonin could allude to NA playing a key role in the manifestation of depressive symptoms. In support, depleting NA was enough to induce depressive symptoms but only in people with a family history of affective disorders suggesting an interplay between NA and genetic predisposition to depression (Caspi et al., 2003; Leyton et al., 2000).

However, while NA and serotonin are important in the aetiology of depression and major depressive disorder (MDD), it is important to note that not every patient responds to SSRI/NARIs (Hodes et al., 2016). Even in those that do, 50% will fall into remission (Fonseka et al., 2015) showing increases in these monoamines are insufficient to prevent depressive symptoms in some instances. Increasing these monoamines should be more seen as an add-on to depressive treatments but it is often cited that lifestyle factors and/or therapy is needed to really treat depression (Duval et al., 2022). If the cause of the disorder is not addressed then the same circuitry can keep signalling increasing the monoamine turnover and making pharmacological interventions ineffective. While not everyone responds to NARIs (Nelson, 1999), people with diminished drive, energy and interest (Joyce & Paykel, 1989; Nelson & Charney, 1981) or lethargy and fatigue (Nutt et al., 2007) were found to better respond to increases in NA. It is possible that lethargy and fatigue are due to disrupted sleep/wake cycle in that the LC-NA system plays an important role (Takahashi et al., 2010). However, the link between LC-NA disruption and depressive symptoms may not be linear as one study found when lesioning the LC in mice (Szot et al., 2016) the lowest dose of 6OHDA caused the greatest change in depressive symptoms (as measured by forced swim test and sucrose preference test). Larger doses destroyed more LC neurons but did not lead to greater changes in symptoms. This could suggest that the loss of too many LC neurons leaves too few to actually respond to stress or the damage was not specific enough to mirror the human condition. Loss of LC neurons in neurodegenerative diseases often happen in a rosto-caudal gradient (i.e., more loss in the rostral part and more sparing in the caudal LC) while a surgical lesion is non-specific (Chan-Palay & Asan, 1989; German et al., 1992). Further evidence for a causal relationship is shown by giving mice precursors to NA and serotonin, L-DOPS and L-DOPA. This treatment decreased their depressive symptoms but did not correspond to an increased NA tissue content (Szot et al., 2016). However, this could be down to the time the brain tissue was taken as it was after a particularly stressful event (FST) that reduced NA content (Koob, 1999) or changes to NA could be too small to detect but still impact behaviour.

Increased firing of the LC and reduced NA content explain some of the links between LC and depression but the LC-NA system is also heavily involved in pathways, such as BDNF, glutamate and inflammation, that are neuroprotective that have also been independently implicated in MDD aetiology. These will each be discussed now in reference to depression.

BDNF

Brain-derived neurotrophic factor (BDNF) is important in synaptic maintenance and repair that also helps increase neuron survival. It is also found to be decreased in patients with MDD (Lee &

Kim, 2010) that could be down to reduced NA levels. Evidence for this is shown through anti-depressants, such as NARIs, actually increasing BDNF (Duman, 2004). More than that, administering BDNF into central brain tissue reduced depressive symptoms in animals (Siuciak et al., 1997). Not only BDNF but also its receptor, TrkB, is downregulated in MDD (Dwivedi et al., 2003; Evans et al., 2004) showing the MDD brain is less responsive to BDNF. LC neurons produce BDNF and NA stimulates its release from other cells that could explain why reduced NA could lead to lowered BDNF levels. With BDNF directly improving depressive symptoms, it could highlight a role in loss of BDNF in the development of depressive symptoms.

Glutamate

Glutamate, a major excitatory neurotransmitter in the brain with vast innervation to the LC (Aston-Jones et al., 1986; Singewald et al., 1999), is altered in MDD (Werner & Covenas, 2010). If glutamate levels are too high then this can cause neuronal damage and excitotoxicity that can be seen in MDD (Meldrum & Garthwaite, 1990) but if they are too low then it leaves the brain in a less stimulated state. Both an increase and decrease in glutamate have been found in MDD (Hasler et al., 2007). Suffice to say, abnormal levels of glutamate could be associated with depression and could highlight the diversity in its symptoms (i.e., both insomnia and increased sleep are depressive symptoms). This is supported by the way effective depressive treatments often also normalise glutamate levels (Michael et al., 2003; Pfleiderer et al., 2003).

Inflammation

Recently, inflammation has been heavily linked to the aetiology of depression (Miller & Raison, 2016) insofar as inducing inflammation through interferon treatments has been found to increase the number of depressive symptoms in people (Miller et al., 2009; Musselman et al., 2001). This is often found with chronic, low-grade inflammation that can be modulated by NA. The β-AR on microglia and astrocytes are activated with NA and can modulate inflammatory response by suppressing pro-inflammatory states and increasing anti-inflammatory ones. This is supported by β-AR agonists reducing microglial inflammatory response (Galea & Feinstein, 1999; Minghetti et al., 1997). Furthermore, NA was able to reduce cell death of cortical neurons via reducing inflammatory processes (Madrigal et al., 2006). The LC also induces an autonomic stress response which is itself a cause of increased pro-inflammatory cytokine expression (Dieckmann et al., 2020; Marsland et al., 2017) and thus chronic inflammation (Berridge & Waterhouse, 2003). The LC is also able to release certain peptides with anti-inflammatory effects (e.g. Neuropeptide Y) that is also downregulated in MDD (Zukowska-Grojec, 1995) potentially due to reduced NA or another cause.

Moreover, effective anti-depressant treatments also seem to have anti-inflammatory effects, possibly through increases in NA and other monoamines that modulate inflammation (Dionisie et al., 2021). Further support for NA's antidepressant effects being through its impact on inflammation comes from the fact that NARIs/SSRIs often take 2-4 weeks to show behavioural change despite having a near instant impact on monoamine levels. This highlights the fact that it may not be the neurotransmitters themselves driving depression but their effects on other neural processes (e.g., inflammation; Sangkuhl et al. (2011). However, monoamine effects go beyond just inflammation which could be why direct anti-inflammatory treatments are showing only some promise (Bai et al., 2020; Köhler-Forsberg et al., 2019) as well as a lot of variance as an effective treatment (Köhler et al., 2014). Köhler et al. (2014) did a systematic review where they pooled 6 randomised control trials and found 5/6 showed improvements with anti-inflammatory medications. However, the anti-inflammatory add-on did no better than other anti-depressant medications alone suggesting no additional benefit (Köhler et al., 2014). Another systematic review found a subpopulation of MDD patients with increased pro-inflammatory cytokines in serum were unresponsive to typical monoamine treatments but responded better to anti-inflammatory medications (Arteaga-Henríquez et al., 2019). While NARI/SSRI treatments do have anti-inflammatory effects, their mechanism of use to treat depression is likely through other processes besides inflammation. Regardless, it seems inflammation is important and managing chronic inflammation levels could be key to finding better treatments for depression.

Overall, the LC-NA system is involved in many processes that are also linked to depression which could allude to the aetiology of MDD, the manifestation of symptoms or simply be correlational. It is clear that the noradrenergic system seems to be important in some stages of depression either through the stress response or NA impacting other neurological mechanisms.

●1.3.3 LC-NA system in Alzheimer's disease

In AD, there is a profound loss of LC neurons (German et al., 1992; Mann et al., 1980). Furthermore, likely due it its vulnerable nature, the LC is the first area to show AD related pathology with pre-tangles (p-tau) to the point where the Braak stages had to be updated with p-tau pathology stages a-c prior to the original 5 stages (Braak et al., 2011). From stage a, the LC neurons are positive for p-tau which continually extends further down the axons in stages b and c that can occur in early adulthood (20-30). By Braak stage 0, 8% of LC neurons are p-tau positive that will become 100% by stage IV (Ehrenberg et al., 2017). It is estimated that the LC shrinks by about 8.4% during each stage that is down to minor neuron loss as well as loss of connections (Theofilas et al., 2015; Theofilas et

al., 2017). While amyloid pathology may be important in the development of NPS, the initial build up of tau pathology is crucial in disrupting the LC-NA system so shall be examined here.

These increases in p-tau cause mitochondrial dysfunction leading to oxidative stress and reduced sensitivity of LC neurons to inputs as shown by ~50% loss of synaptophysin-immunoreactive perineuronal dots in p-tau+ LC neurons (Andrés-Benito et al., 2017). This reduced sensitivity means lower NA secretion and impaired noradrenergic transmission even before loss of neurons (Braak & Del Tredici, 2012). Loss of NA leads to reductions in its neuroprotective effects such as the release of BDNF. The retroactive transport of BDNF to LC neurons further reduces their protective aid leaving noradrenergic neurons more vulnerable (Mufson et al., 1999). Increases in aberrant tau causes stress and thus hyperactivity of the LC neurons that can then spread the tau seeds to regions the LC innervates (Chalermpalanupap et al., 2017).

Substantial cell loss occurs around Braak stage 3 (Theofilas et al., 2017) that coincides with increased Aβ deposition via reductions in inflammatory mechanisms meant to clear away toxic amyloid (Bondareff et al., 1987; Marcyniuk et al., 1986; Tomlinson et al., 1981). The reduction of LC neurons correlated with severity of dementia (Bondareff et al., 1987), increased plaques and tangles (Friedman et al., 1999) and AD duration (Mesulam, 2013; Zarow et al., 2003). However, there is data showing no loss of NA in cerebrospinal fluid (CSF; Friedman et al. (1999)): this could imply that the CSF method of NA transmission is not affected in AD while there is a loss of NA in cell terminals (Adolfsson et al., 1979). This could contribute to the loss of neuroprotective pathways that will exacerbate cell loss in the brain (Heneka, Nadrigny, et al., 2010). Support for this is found in animal work where LC lesions exacerbate AD pathology (Rommelfanger et al., 2004).

The aggregation of tau into NFT in the LC occurs roughly 10 years prior to the start of cognitive impairments (Braak et al., 2011; Grudzien et al., 2007; Theofilas et al., 2015). The loss of the LC-NA system leads to the brain attempting to compensate by creating aberrant dendritic innervation and axonal sprouting to the HPC. It also shows increases in TH mRNA to produce more NA (Szot et al., 2000; Szot et al., 2006). This is supported by work by Adolfsson et al. (1979) who found lower NA content in the brains of AD patients but not to the degree that would reflect the LC cell loss. Friedman et al. (1999) also found that despite increased sprouting there was improper NA signalling to incorrect areas that could lead to certain features of AD (Weinshenker, 2008). The reduction of LC is profound in AD that not only reduces neuroprotection, but also leads to compensatory mechanisms with unknown effects on the disease. Alongside the evidence considered in this section regarding tau pathology and dysfunction of the LC-NA system, there is also evidence for amyloid impacting this system as well (see section 1.4).

Loss of neuroprotective mechanisms

This profound loss of LC functionality throughout the progression of the disease leads to the loss of neuroprotection offered by the LC-NA system that is depicted in fig 1.2. This includes loss of control over inflammatory processes that lead to inefficient clearing of Aβ and sustained pro-inflammatory signalling (Heneka, O'Banion, et al., 2010). This state of chronic inflammation can further damage surrounding cells (Dorey et al., 2014) as well as be linked to manifestation of depression (Miller & Raison, 2016). Furthermore, there is a loss of neurotrophic factors that aid neuronal and synaptic survivability. This loss could directly reduce synapses and be a key contributor to lowered neuronal plasticity (Bramham & Messaoudi, 2005). Reduced astrocyte effectiveness also leads to excessive glutamate (Jensen et al., 2013) that elicits cell death through excitotoxicity (Malva et al., 2012). NA is also important in anti-oxidant processes so LC degeneration can lead to rampant oxidative stress causing further neurodegeneration (Troadec et al., 2001). As all of these processes can be found in patients suffering MDD, it is likely that reduction of LC neurons can manifest as depression and other neuropsychiatric symptoms seen in AD (see section 1.3.2).

Noradrenergic related treatments for AD

With disruption of LC creating rampant neuroinflammation, examining treatment options designed to boost noradrenergic signalling could stop this cycle. In mice, there has been promise of treatments with the NA precursor, L-DOPS, in improving cognitive deficits, reducing cell death, inflammation and increasing BDNF (Heneka et al., 2006; Kalinin et al., 2012). However, most clinical studies in humans focus on treating Orthostatic hypotension due to L-DOPS (Droxidopa) effects in reducing blood pressure (Kaufmann et al., 2003; Mathias et al., 2001) but little has been done to look at AD. Since it has shown promise in animals especially when administered alongside an NARI, it could offer a potential preventative measure to protect the LC in early AD. Marsh et al. (2009) found promise in treating cognitive deficits in Parkinson's disease patients with an NARI (atomoxetine). However, when 47 mild to moderate AD patients were also given atomoxetine, no changes to cognition, clinical scores or NPS were found (Mohs et al., 2009). Perhaps the mild to moderate AD patients who already show extensive neurodegeneration are not the best target for an NARI and preclinical, MCI or earlier are the best target before substantial NA-ergic signalling loss (Chalermpalanupap et al., 2013).

Serotonin noradrenaline reuptake inhibitors (SNRIs) are often used to treat depression and directly impact both monoamines. Studies giving AD patients milnacipran (SNRI) were effective at treating depressive symptoms unlike SSRIs but neither affected general Alzheimer's progression score (i.e., Mini-mental state examination; MMSE; (Mizukami et al., 2009; Mizukami et al., 2006).

This supports the idea of NA playing a role in the aetiology of NPS in AD. Nevertheless, another study looked at atomoxeinte (NARI) treatment in Parkinson's disease (PD) and MCI patients across 8 weeks but found no improvement of cognition (Ghosh et al., 2020) that initially would suggest NA is not a good treatment target. On the other hand, Levey et al. (2021) gave AD patients atomoxetine treatment and examined the biomarkers of AD. NARI significantly reduced CSF tau, p-tau and normalised protein biomarker panels linked to synaptic function (e.g. BDNF) as well as normalising brain metabolism and microglial immunity but found no effect on cognition or clinical outcomes. This could suggest atomoxetine shows promise at slowing down the progression of AD if taken for a longer length of time than eight weeks to really impact behaviour. Perhaps this sort of treatment should be measured on its ability to slow down progression opposed to reversing deficits that are already present.

A recent systematic review compiled 19 studies all looking at noradrenergic treatments on AD symptoms and found global improvements in general cognition but not it's specific subdomains (e.g. attention and visuospatial memory; David et al. (2022)). The studies that looked at NPS found no overall improvement as measured by the Neuropsychiatric inventory (NPI) or agitation but 7/8 studies that measured apathy did find significant improvements. This supports LC-NA disruption leading to apathy but lack of a significant effect in general NPS could be down to short treatment times. Furthermore, depression and anxiety were not looked at so it is hard to say what impact NARIs could have on them.

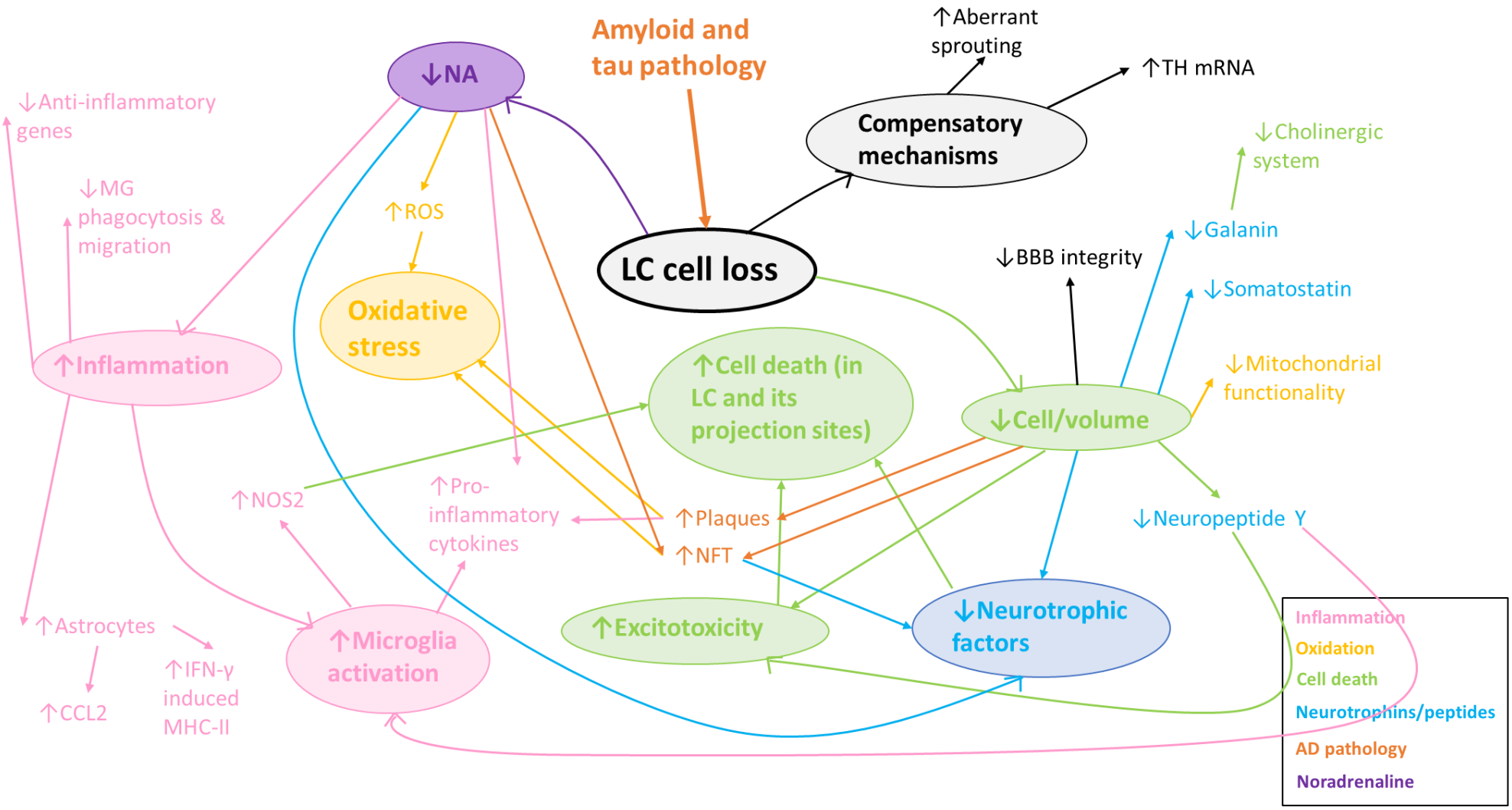

Figure 1.2 How loss of locus coeruleus in Alzheimer's disease leads to loss of neuroprotective mechanisms. *Loss of the LC leads to reductions in NA as well as cell loss which reduces control over inflammation, reduced control over oxidative stress, reduced neurotrophic support and increases in excitotoxicity. These factors have certain effects on other aspects that exacerbate Alzheimer's pathology and create compensatory mechanisms.*

●1.4 Inflammation in Alzheimer's disease

Inflammation is another hallmark of AD. While inflammation itself is incredibly beneficial to our survival, it can be damaging if not returned to a non-inflammatory state. Immune cells will activate in response to pathogens and debris in order to clear away the threat but, in doing so, can damage surrounding cells (Becher et al., 2017; Frank-Cannon et al., 2009). They can only be healed once the immune cells return to a less active state via anti-inflammatory cytokine release (Dokalis & Prinz, 2019). If the inflammation is sustained, then the detrimental effects from immune cells cannot be fixed effectively and further damage can occur. Chronic neuroinflammation has recently received a lot of attention for its part in neurodegenerative disorders such as AD as it is heavily involved in clearing the brain of threats and misfolded proteins such as Aβ and tau (Ridolfi et al., 2013).

Immune cells can traverse from the blood into the brain via the blood brain barrier (BBB). The peripheral immune system in the blood is able to communicate with their neurological counterparts to ensure the entire body is aware of threats and can respond accordingly. Nevertheless, the brain has its own resident immune cells. Microglia are neuronal macrophages that clear away debris and misfolded proteins. Like macrophages, activating microglia alter their physiology from a state of stable surveillance to amoeboid migration and phagocytic capabilities. Alongside microglia, astrocytes also maintain the homeostasis of the brain but through regulating metabolic and synaptic transmission. They also maintain neurotransmission as well as formation and maintenance of the BBB modulating what can permeate into the brain. Both microglia and astrocytes are important for maintaining neuronal health but their chronic activation has been found in a lot of diseased states (Becher et al., 2017).

In AD, there are robust reports of increases in microgliosis and astrogliosis as well as increases in pro-inflammatory cytokines (Heneka et al., 2015) that can activate other immune cells and alert them of potential threats. Microglia are commonly found surrounding plaques in the AD brain (Sastre et al., 2006) that will attempt to clear away the toxic Aβ. Increases in inflammation may initially help the clearance of the neurotoxic build-up of tau and Aβ but if immune cell activation becomes chronic then it could lead on to neurodegeneration. Furthermore, Aβ pathology stimulates the NFκB pathway by binding to microglia to induce pro-inflammatory cytokine production (Akiyama et al., 2000; Ho et al., 2005) and reactive oxygen species (Schilling & Eder, 2011). These cytokines can also stimulate β-secretase release (e.g. BACE1) that splices *APP* into toxic Aβ (Chen et al., 2012) creating a feed forward loop. On top of this, it seems microglia become less effective at clearing away the Aβ which then allows it to build up (Heneka, O'Banion, et al., 2010). One theory for why this occurs is that microglia that are repeatedly activated become primed making them more

sensitive to threats (J.-W. Li et al., 2018). A cause of primed microglia is age and various aged mouse models show exaggerated and sustained inflammatory response to LPS (Godbout et al., 2005; Godbout et al., 2008) that can be reduced if co-treated with anti-inflammatory drugs (Henry et al., 2008). Since age is the main risk factor of AD, this exaggerated and prolonged response to Aβ could partly explain the chronic inflammation seen alongside plaques.

Despite inflammatory dysregulation playing a part in the progression of neurodegenerative diseases, anti-inflammatory medications have mainly shown promise in mouse models (Varvel et al., 2009) but results have been more mixed in humans where some found improvement with NSAIDS (Côté et al., 2012; Jaturapatporn et al., 2012) but other research did not (Jaturapatporn et al., 2012). This is unsurprising as anti-inflammatory treatments are non-specific and while reducing inflammation could provide some relief against neuronal cell death, it is not addressing the cause. For more information on anti-inflammatory treatments see section 1.4.3.

●1.4.1 Neuroinflammation and LC damage feed forward model

So, with the reduction in the LC function potentially playing a role in the onset of NPS in AD, it seems neuroinflammation is integral to this. As previously discussed (see section 1.3.3), the LC develops tauopathy early on that reduces its sensitivity to inputs making the release of NA less likely. This may reduce the neuroprotection offered to regions the LC innervates to including unchecked and ineffective neuroinflammation. Alongside this, toxic amyloid fibrils are forming causing damage to cells as well as inciting the inflammatory processes. Normally, microglia and astrocytes might be able to clear away this toxic Aβ but there are some factors that could impede their efforts. The first may be the genetics of the person as large scale Genome-wide association studies (GWAS) have highlighted a genetic pathway for AD that involves inflammation as a mechanism rather than just individual risk factors (Karch & Goate, 2015). One major genetic mutation involves interfering with *TREM2*, a gene expressed on microglia and important for phagocytosis and suppressing pro-inflammatory cytokine production (Jiang et al., 2014). Other risk factors are also involved in phagocytosis (e.g. *CD33* gene) while others are important in various strands of the inflammatory processes (e.g. *CR1* is important in complement cascades). Alongside genetics, another factor is ageing that is one of the key risk factors for AD. Ageing is associated with general wear and tear of the body and this includes microglia that become primed (Heneka et al., 2015). Primed microglia are defined as cells that elicit an out of proportion inflammatory response to smaller toxic stimuli while doing a less effective job at clearing them away (J.-W. Li et al., 2018). Furthermore, there is evidence to suggest Aβ production increases as AD progresses (Selkoe, 1998; Shankar & Walsh, 2009).

Altogether, increasing production of amyloid coupled with microglia inciting a large inflammatory response while being impeded in their ability to clear the Aβ away, creates a chronic inflammatory state in the brain. This could explain why there is an early spike in neuroinflammation found even prior to amyloid deposition (i.e., during early MCI; (Fan et al., 2015; Okello et al., 2009).

During the immune response to Aβ, other toxic by-products are made in the process such as reactive oxygen and nitrogen species and pro-inflammatory cytokines (Kempuraj et al., 2016). Evidence has shown that neuroinflammation itself can be enough to produce neurodegeneration as Hauss-Wegrzyniak et al. (1998) found when injecting rats with extremely high doses of lipopolysaccharide (LPS). The LPS induced an intense and chronic inflammatory state that caused neurodegeneration and decline in spatial memory. Furthermore, amyloid pathology stimulates neuroinflammation that can also lead to a loss of cells in the rat brain (Weldon et al., 1998).

As well as damaging neural tissue, neuroinflammation has been found to exacerbate other AD pathologies. When APP/PS1 mice were given injections of LPS to mirror a bacterial infection, Zhou et al. (2019) found reduced spatial memory abilities in the Morris water maze despite no changes in $A\beta_{1\text{-}42}$. They found alterations in *TREM2* and toll-like receptor 4 (TLR4) that is expressed on microglia and will recognise pathological patterns to elicit an immune response. This was also found in a mouse expressing tauopathy, Tg4510, as LPS injections greatly accelerated the progression of tau build up (Lee et al., 2010). However, other studies did show that LPS injections helped clear away Aβ from the brains up to 2 weeks after inflammatory insult but amyloid levels returned to normal afterwards (DiCarlo et al., 2001; Herber et al., 2004).

This damage and neurodegeneration from inflammation also further exacerbates the damage to the LC. With the LC being highly vulnerable to damage with a high bioenergetic need (see section 1.3.1), it is logical that it would be impacted by inflammatory injury by stimulating increases in oxidative stress. But further support comes from mouse models given injections of LPS. Q. Wang et al. (2020) offered a detailed examination to the inflammatory impact on the LC-NA system and found the long noradrenergic neurons produced high levels of oxidative stress and mitochondrial dysfunction that was reduced with inhibition of NOX2. NOX2 is an enzyme that generates reactive oxygen species and the researchers found inhibiting it not only logically reduced oxidative stress but also spared more LC-NA neurons from neurodegeneration.

Early loss of the LC produces a feed forward model that exacerbates the inflammatory processes stimulating further neurodegeneration (fig. 1.3). While both tau and amyloid are important players in these processes, amyloid seems to have an additional role in the inflammatory issues commonly seen in AD. Alongside the loss of other neuroprotective mechanisms, a key role of

NA is sustaining an appropriate inflammatory response by promoting anti-inflammatory signalling, suppressing pro-inflammatory and ensuring microglia migrate and phagocytose effectively (Heneka, Nadrigny, et al., 2010). Loss of NA itself can create excessive neuroinflammation and accelerate AD pathologies. Lesioning the LC in various AD mouse models has shown increased microglia activation (Chalermpalanupap et al., 2018; Kalinin et al., 2007) when given an injection of DSP4, a noradrenergic specific neurotoxin. In addition, rats injected with both DSP4 and Aβ lead to an induction of key inflammatory markers, iNOS, IL-6 and IL-1β, that was attenuated if later treated with an injection of NA or isoproterenol (β-adrenergic receptor agonist; (Heneka et al., 2002)). These studies show how reduction of NA can directly impact inflammatory processes. Furthermore, loss of NA can also impede microglial ability to migrate and phagocytose toxic amyloid (Heneka, Nadrigny, et al., 2010). Microglia functionality was restored if given L-DOPS, an NA precursor (Heneka, O'Banion, et al., 2010). They also mirrored this finding *in vitro* by finding microglia could effectively clear Aβ away if given NA and a β2-AR agonist to stimulate noradrenergic function on microglia. However, a different transgenic model of amyloid pathology, APP/PS1, was given an α2-AR antagonist (fluparoxan) that should have impeded microglia functionality but found no change (Scullion et al., 2011). This discrepancy could be because the later mouse model does not have diminished NA levels so cannot model LC loss or the α2-AR receptor is not as important in NA helping microglia function. NA also helps suppress an inflammatory response by reducing NOS2. Madrigal et al. (2006) found *in vitro* that NA treatment to activated microglia suppressed secretion of NOS2 and when this media from the microglia was given to cells, this reduced cell death. Aβ itself is also able to stimulate pro-inflammatory cytokines that NA suppresses as shown by injecting rodents with Aβ to induce NOS2 expression in microglia (Heneka et al., 2002). However, if the rats were also given a NA specific neurotoxin, N-N-ethyl-2-bromobenzylamine (DSP4), then this exacerbated microglia inflammatory response in the cortex sooner, more robustly and for longer than controls. Altogether, damage to the LC-NA system leads to further rampant and ineffective inflammatory processes that go on to damage the brain creating a feed forward process.

Along with inflammatory processes, the LC-NA system is heavily involved in mechanisms that protect the brain from amyloid build up. BDNF is one example that is released after NA stimulation as well as being secreted directly from LC neurons (Rémy et al., 2001). It is integral to protecting the brain against toxic Aβ through its receptor, TrkB. Liu et al. (2015) found NA was able to protect cortical neurons from Aβ if given NA and a TrkB antagonist (K252a) but not an AR antagonist showing this NA protective mechanism is through direct BDNF:TrkB signalling. Furthermore, there are other neurotrophic factors which, when reduced, impact noradrenergic protection against Aβ. These include Neuropeptide Y (Croce et al., 2011) and Somatostatin (Saito et al., 2005).

Reduced noradrenergic signalling also leads to less control over excitotoxicity caused by excessive glutamate. Extrasynaptic NMDA receptors are stimulated by Aβ fibrils that then elicit pro-apoptotic cascades and oxidative stress signalling (Hardingham & Bading, 2010; Parsons & Raymond, 2014). Aβ can also impair the uptake and recycling of glutamate as shown by cell cultures exposed to Aβ leading to excitotoxic cell death (Arias et al., 1995; Fernández-Tomé et al., 2004; Parpura-Gill et al., 1997).

NA also plays a role in maintaining the homeostasis of oxidative stress that is another neuroprotective mechanism. Aβ itself stimulates to production of free radicals (Smith et al., 2000) and NA has anti-oxidant effects by reducing free radical production (Troadec et al., 2001). Counts and Mufson (2010) found NA was able to protect neuroteratocarcinoma cultures from $A\beta_{1-42}$ and $A\beta_{23-35}$ toxicity through preventing increases in intracellular reactive oxygen species (ROS), mitochondrial membrane depolarisation and caspase activation.

Another neuroprotective effect of the LC-NA system is through the neuromelanin in the LC neurons. Neuromelanin rich LC neurons also take up iron that is important in the formation of OH• from H_2O_2 (Zecca et al., 1996) and when iron is released back into extracellular space upon LC cell death in AD, this increases free radical production.

With the LC being so vulnerable to damage and developing tauopathy early, this reduces its functionality. Coupled with risk factors that reduce the functionality of immune cells, this creates an issue when attempting to clear away excessive amyloid leading to a chronic inflammatory state. This itself can lead to neurodegeneration and further harming the LC. Loss of noradrenergic signalling leads to widespread inflammation and loss of neuroprotection against amyloid leading to further degeneration (fig. 1.3). This is a possible feed forward model for how tau and amyloid can affect neuroinflammation and the LC. While both tau and amyloid impact the LC, understanding each neuropathies individual effect could be important to developing new treatments in the future.

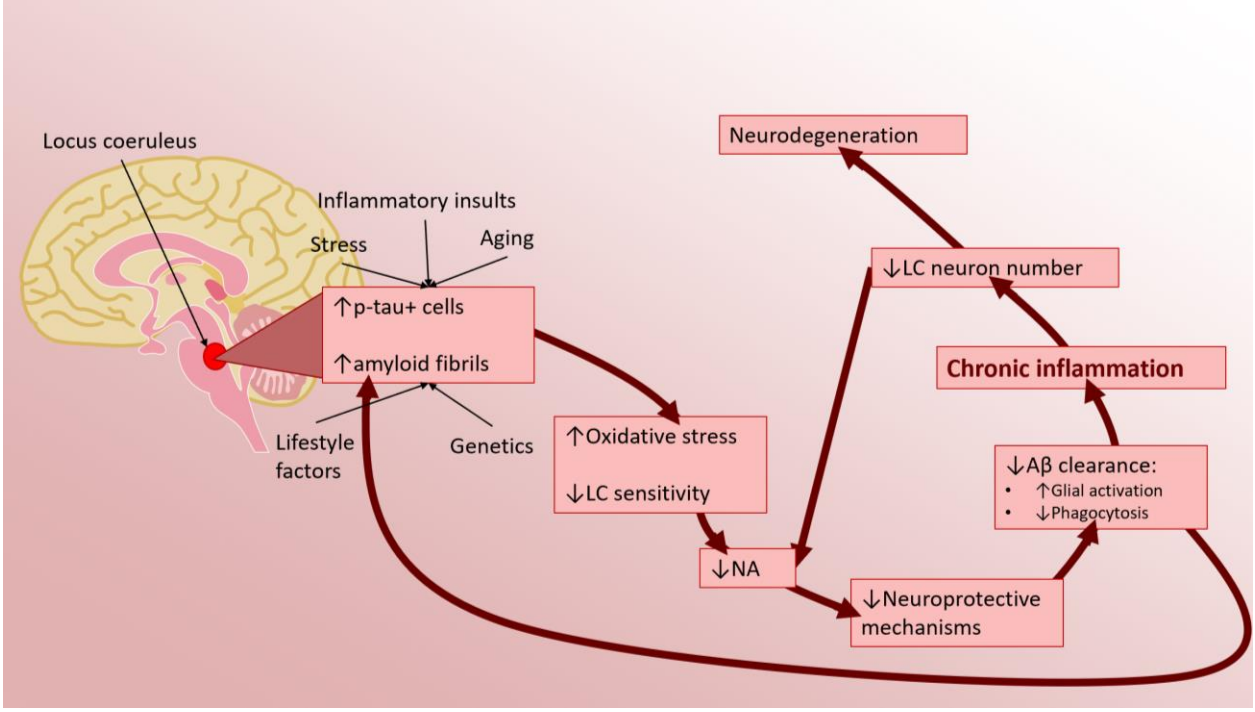

Figure 1.3 The neuroinflammation-LC disruption feed forward model. *Increases in p-tau and amyloid fibrils affect LC and reduce NA release. This reduces it's neuroprotective effects including less effective microglia which increase amyloid burden and lead to chronic inflammation. Chronic inflammation damages the LC which leads to neurodegeneration and reductions to NA in a feed forward model.*

●1.4.2 Anti-inflammatory related treatments for AD

Various anti-inflammatory treatments have been approved for drug treatments that could be used to directly reduce the detrimental effects of neuroinflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) are often used to relieve pain, reduce inflammation and treat fever (e.g. ibuprofen) and primarily work by inhibiting cyclooxygenase (COX) activity (Vane, 2014). Animal work has shown promise by giving transgenic AD mice various NSAIDs shows a dose dependent reduction in pathology. Often, NSAID treatment reduces activated microglia as well as Aβ in various transgenic mouse models ((Heneka et al., 2005; Jantzen et al., 2002; Lim et al., 2000; Van Groen & Kadish, 2005); for review see McGeer and McGeer (2007)). In humans, the results have been much less successful. A recent Cochrane review examined 14 randomised control trials (RCT) using various NSAIDs but concluded NSAIDs provide little benefit for slowing down the progression of AD (Jaturapatporn et al., 2012). However, multiple studies found that people taking NSAIDs for various reasons in life were less likely to develop AD (Breitner et al., 1995; Côté et al., 2012; Landi et al., 2003; Stewart et al., 1997). An in-depth meta-analysis noted that NSAIDs did reduce prevalence, but had no effect on cognitive decline (Rivers-Auty et al., 2020). This was suggested to stem from a healthy user bias, whereby people who take the NSAIDs are more health conscious and so less likely

to develop AD or drop out due to making healthier choices. This is indeed supported when a randomised control trial with 2528 cognitively normal people with a family history of dementia were given NSAIDs and followed up for 7 years. There was no difference between drug and placebo groups for developing AD (Group, 2013). This lack of an effect could be due to inflammatory troubles in AD are not reliant on COX related mechanisms to elicit inflammation or the effect sizes are too small to notice.

Whilst a majority of the clinical trials for anti-inflammatory treatments focus on NSAIDS, there are a variety of other treatments that reduce inflammatory impact in AD. Disease modifying anti-rheumatic drugs (DMARDs) are a group of anti-inflammatory treatments used to treat rheumatoid arthritis (RA). A recent review found RA patients treated with targeted synthetic DMARDs were at a 19% reduced risk of later developing AD (Sattui et al., 2022). In addition, Zhou et al. (2020) showed that this reduced risk of developing AD extended to other inflammatory diseases (e.g. psoriasis and inflammatory bowel diseases) including RA if treated with TNF blockers, a type of targeted synthetic DMARD. Another recent review also highlighted 3 large epidemiological studies looking at TNF blockers lead to a 60-70% reduction in odds ratios of developing AD (Torres-Acosta et al., 2020). The review also found support in smaller randomised control trials of AD patients where TNF related treatments improved their cognition. This data could suggest blocking the effects of TNFα could reduce the risk of developing AD or improve cognition in current AD patients.

Another anti-inflammatory treatment used to treat AD is resveratrol, a biomolecule produced by plants with potent anti-oxidant and anti-inflammatory effects. It has shown promise for reduced Aβ in rodent models (Chen et al., 2019) and well as cell models (Chen et al., 2019; Gomes et al., 2018). In human AD trials, resveratrol was found to improve MMSE scores, reduce Aβ and prevent decline in brain volume (Buglio et al., 2022). In addition, it was also able to slow down cell loss in MCI patients highlighting that it could be another potential preventative treatment (Buglio et al., 2022).

However, it seems quite likely that taking a single minded approach (e.g. increasing NA or reduce inflammation) is too simplistic when finding an effective treatment for AD. Multi-modal approaches may be better at tackling the disease from various deleterious approaches. Inflammation is complex and even anti-inflammatory medications can have varying effects that may not actually get to the heart of the issues in AD. While some anti-inflammatory treatments have shown promise, further trials need to be completed to ascertain best treatment practices. A lack of effect of NSAIDs in humans could simply show they are too broad and more targeted approached,

such as TNF blockers, should be considered. It is important to note that anti-inflammatory drugs will have a global effect that could interfere with the neuroprotective actions it does have.

●1.5 Animal models of Alzheimer's disease

This thesis aims to examine the impact Aβ has on the development of neuropsychiatric symptoms. Although it is debated as to whether Aβ is the main causal factor of AD (i.e., amyloid cascade hypothesis) it seems clear that it does play some crucial role in the progression of AD. Amyloid fibrils themselves are very neurotoxic (Kienlen-Campard et al., 2002; Pierrot et al., 2004; Pike et al., 1993) and stimulate inflammatory processes (Ridolfi et al., 2013) but to understand the extent of their impact, the disease needs to be modelled in non-human organisms. Various models of the human AD condition exist that allow detailed examination of biological processes as well as what would occur after direct manipulations to biological processes relevant to AD. According to Esquerda-Canals et al. (2017), a good model needs to have face validity (accurate portrayal of the human condition), have predictive validity (model can be used to make accurate predictions on the human disease) and construct validity (it should be generalisable). Non-human primates would offer the highest validity across these three conditions due to being our closest genetic relatives and some exist that even naturally develop amyloid or tau pathology (Braidy et al., 2012) but their long lifespans make them difficult to practically use in research that requires aging. On the other side, there are invertebrate models using Drosophila melanogaster or Caenorhabditis elegans that can be easily genetically modified but only exhibit limited behaviour that does not offer high face validity. Along a similar vein, there are also various cell cultures that can model the cellular mechanisms within the disease that are used for such but cannot be used to examine behavioural phenotypes (for review see Arber et al. (2017)). Rodents, being mammals, offer high amounts of face validity making them good candidates. Their genome is very well understood that makes them viable to genetic manipulation and the fact mice do not naturally develop amyloid or tau pathology (Braidy et al., 2012) offer no confounds to said genetic manipulations. Rodents also have very complex social behaviours that make them a high validity model. Advances in gene editing have allowed the creation of a vast array of different mouse and rat models of both amyloid and tau pathology with some exhibiting both. Each model has its limitations on what it can be used for so selecting the right one is important.

●1.5.1 Transgenic animal models

For over 25 years, AD has been modelled in mice through genetic modification (Hsiao et al., 1996). For amyloid models, they insert Familial AD mutations due to their high penetrant effects. These increase amyloidosis by either increasing β-secretase ability (*APP* mutations) or γ-secretase to increase $A\beta_{42}$ production (PSEN1 and PSEN2 mutations) that both promote toxic amyloid production. These mutations are inserted into the genome of a murine embryonic stem cell via micro-injection or vector and this stem cell is then injected into blastocytes that grow to term in surrogate dams. Artificial promoter genes are also inserted that modulate levels and protein expressions across different cell types. Different promoters can elicit vastly different effects from the same genetic mutations that leads to such variability across transgenic models. The earliest models (e.g. hPDAPP) were given *APP* mutations that affected β-secretase cleavage of *APP* and developed plaques in an age dependent manner (Esquerda-Canals et al., 2017). Models that utilised the PSEN1 & PSEN2 mutations did not develop plaques so were often crossed with *APP* mutations to create more aggressive amyloid pathology (double transgenic). 5xFAD mouse contains 5 separate mutations which, combined, produce intracellular $A\beta_{42}$ as early as 1.5 months old, plaques at 2 months and cognitive impairments at 4-6 months old (Oakley et al., 2006).

Transgenic models of tau pathologies also exist but suffer similar problems. Mutations that are based on either affect the alternate splicing of exon 10 to change isoform ratios or modifying tau interaction and microtubules (Buée et al., 2000). These mutations are then promoted through exogenous promoters that offer similar issues to the amyloid models (Denk & Wade-Martins, 2009). However, while tau models are able to show the effects of increased hyperphosphorylated tau they fail to capitulate the fibrillar pattern of phosphorylated tau seen in AD (Götz et al., 1995). The models are often seen as better at modelling frontotemporal dementia with Parkinsonism in chromosome 17 (Saito et al., 2019). Other models have reported to have intraneuronal excess of tau proteins leading to axonopathy or simply fail to reproduce all the features of tauopathies seen in AD (Buée et al., 2000).

Transgenic mouse model and locus Coeruleus

These various mouse models have been used to look at the effect of amyloid on neuroinflammation and LC activity. The PDAPP model (Games et al., 1995) utilises the *APP* mutation V717F to increase β-secretase activity and increase plaque deposition. German et al. (2005) found Aβ pathology did not reduce LC neuron number but did decrease the size of neurons that innervated to areas of heavily plaque load in the PDAPP mouse. This specific reduction suggests a role of Aβ in causing retrograde stress to the LC. In a double transgenic mouse, APP/PS1, researchers found that

Aβ increased inflammation as well as reduced LC neuron number more so than normal aging (Cao et al., 2021). It is possible that the inclusion of a PSEN1 mutation leads to a more aggressive effect on LC neurons hence why neuron loss was only found in the latter model. Nevertheless, it is difficult to say for certain due to different artificial promoters used between the PDAPP and APP/PS1 models. Regardless, it is clear that amyloid impacts the LC but it is uncertain what effects this has on behaviour and inflammation.

Amyloid impacts the LC and this could have drastic effects on neuroinflammation. Lesioning LC signalling with DSP4 robustly found increases in plaque load across various mouse models including APP23 (Heneka et al., 2006), APP/PS1 (Jardanhazi-Kurutz et al., 2010; Rey et al., 2012) and V717F overexpressing mice (Kalinin et al., 2007). As well as that, LC lesions can also increase neuroinflammation. DSP4 injections consistently increase microglia activation across various different transgenic models like the APP23 and V717F overexpressing mice (Heneka et al., 2006; Kalinin et al., 2006), double transgenic such as APP/PS1 (Jardanhazi-Kurutz et al., 2010) and even P301S tau model (Chalermpalanupap et al., 2018). However, Pugh et al. (2007) found no changes to amyloid, microglia or astrocyte activation in double transgenic mice, TASTPM, despite reductions in NA and LC neurons. They were using a much lower dose of DSP4 but this still did not impact activated microglia or amyloid. This is not a surprise as changes to microglial activation do not impact plaque load as injections of LPS into APP/PS1 mice only decreased Aβ fibrils but not plaques (DiCarlo et al., 2001; Herber et al., 2004). These LPS injections increased pro-inflammatory cytokines but reduced phagocytic gene expression suggesting a sustained inflammatory state was produced with reduced clearance ability (Zhou et al., 2019). Even destroying microglia did not reduce plaque load but did increase pro-inflammatory cytokines and reduce phagocytic capabilities (Unger et al., 2018). Loss of LC functionality also mirrors the effects of increased pro-inflammatory states, reduced phagocytosis and sustained plaque load to create chronic inflammation in the brain (Heneka, Nadrigny, et al., 2010).

Amyloid affecting LC's ability to control effective clearance of Aβ could also manifest as neuropsychiatric symptoms. When WT mice had their LC lesioned surgically with 6-hydroxydopamine (6OHDA), they had increases in depressive-like behaviour through increased immobility in the forced swim test and reduced affinity for sucrose in the sucrose consumption test (Szot et al., 2016). They only found this with the lowest dose of 6OHDA but, even though higher doses resulted in more LC loss, the time spent immobile reduced suggesting more neuron loss does not equate to more NPS. This could be explained as the low dose mirrors early AD where NPS are common. These depressive symptoms were even rescued in the low dose when given NA precursors, L-DOPS, suggesting early increases in NA could combat depression. However, this was not a direct

model of AD so it is unclear whether amyloid pathology can cause this and surgically lesioning the LC occurs quickly while LC degeneration in AD is a much more gradual process. Kelberman et al. (2022) gave a double transgenic amyloid rat model (TgF344-AD) LC infusions of phosphorylated tau to mirror early LC pathology. They found increased anxiety in the TgF344-AD rats regardless of tau infusion suggesting amyloid is pivotal in NPS. However, there was no change in the forced swim test suggesting a lack of depressive symptoms. That said, the validity of the FST as measure of depression has been questioned (Molendijk & de Kloet, 2015). This issue about the FST is further discussed in section 2.2.3. Braun and Feinstein (2019) gave 5xFAD mice vindeburnol, a drug that increases tyrosine hydroxylase positive cells (i.e., LC neurons) and found it normalised their anxiety and reduced plaque burden. Together, it seems amyloid has its own impact on the LC irrespective of tau pathology and this leads onto neuroinflammation and NPS.

Limitations

There are many limitations to using transgenic mouse models that make any firm conclusions from them difficult. Firstly, with so many single and double transgenic, it is difficult to compare across models due to the unknown biological variability created from different promoters (Foley et al., 2015; Webster et al., 2014). To make matters worse, it has been shown that insertion of the transgene can destroy endogenous gene loci with unknown consequences making mechanistic research all the more difficult to unpick. One such consequence could be the often reported increased lethality in these models (Sasaguri et al., 2017) where cause of death cannot be confirmed. This really highlights how little is known about what the transgenes are doing to the body and their interactions. Furthermore, a lot of these mutations and artificial promoters overproduce *APP* to unphysiologically possible levels that has been shown to be highly neurotoxic (Sasaguri et al., 2017). With that, there is also increased *APP* processing so other fragments, aside from Aβ, are produced to unnatural levels that could have unknown interactions and unanticipated effects. Together, these issues highlight how drawing mechanistic conclusions from transgenic animal models may not offer the consistency and validity needed from a model of AD pathology. For these reasons and more (for complete lists of limitations, see (Saito et al., 2014; Sasaguri et al., 2017), better models of amyloid pathology are required to detail the mechanisms that occur in the human condition.

●1.5.2 Knock-in animal models

One of the main issues is the unknown effect the artificial promoters have on the animals' genome in transgenic models but new generation models have been produced that use an

endogenous promoter alongside the FAD mutations. Reaume et al. (1996) had some success making a knock-in (KI) model with the Swedish FAD mutation and humanised amyloid sequence by altering three amino acids. The mouse expresses elevated $A\beta_{40}$ and $A\beta_{42}$ without *APP* overexpression but never formed plaques even at 22 months of age. Li et al. (2014) made KI models with the Swedish, Dutch (E693Q) and London (V717I) amyloid mutations. However, the Dutch mutation is actually found in humans with intensive cerebral amyloid angiopathy and not a cause of FAD making it a viable model but not for AD. Both these KI models did not develop plaques until crossed with transgenic mice that undermines the benefits of KI models.

Success came when Saito et al. (2014) released two viable KI models that developed plaques, inflammation and mild cognitive deficits similar to preclinical AD. Both models consisted of the Swedish double mutation (KM670/671NL) that augments the interaction of BACE1 and *APP* to increase Aβ production. The next mutation is the Beyreuther/Iberian (I716F) that promotes γ-secretase cleavage at the 42 site increasing the $A\beta_{42}$:$A\beta_{40}$ ratio. The first model that incorporates these 2 mutations is the App^{NL-F} that develops amyloid pathology and mild cognitive impairments from 12 months along with increased inflammation (Saito et al., 2014). The second KI model also includes the arctic mutation (E694G) that occurs in the central domain of the Aβ sequence making it more prone to aggregation. Due to the more aggressive amyloid pathology, plaques in the App^{NL-G-F} appear around 2-3 months of age along with extensive microgliosis and astrogliosis. Neither of these mice overproduce *APP* but there are increases in CTF-β, a fragment created after β-secretase cleavage that could have unknown interactions. It should be noted that levels of CTF-α are decreased (fragment made after cleavage with α-secretase) making the total CTF levels the same. However, the increased levels of CTF-β do not reach the unnatural heights of other transgenic models but Saito et al. also produced a KI model with just the Swedish mutation to see whether there were unwanted interactions. This App^{NL} model offers a good negative control and found no development of plaques as well as no effect on pathology or cognitive function of the mice (Masuda et al., 2016; Saito et al., 2014; Salas et al., 2018).

Limitations

Despite the benefits of this model, it still comes with a few drawbacks that should be taken into account. This model has been described by its creators as a model of early AD with mild cognitive impairment that is fine if trying to examine the NPS that appear early into the disease progression. Due to the arctic mutation in the App^{NL-G-F}, the amyloid aggregates very easily making the model unsuitable for examining Aβ degradation and anti-Aβ vaccinations as it will not break down easily. In human AD, each individual FAD mutations used in the mice are highly penetrant and

virtually guarantee early-onset of AD (Tanzi, 2012). Since this is not the case for mice, multiple mutations are used to achieve the same pathology meaning there may be unknown interactions between the mutations that cannot be accounted for. However, this is a better alternative than masses of neurotoxic *APP* fragments found in the Tg models but it should still be taken into account when interpreting results. Despite this, the model still exhibits extensive neuroinflammation and amyloid pathology seen in early AD without toxic overexpression of *APP* making it a reasonable model to examine NPS in early AD.

App^{NL-G-F} mice and NPS

The *App*$^{NL-G-F}$ model is still relatively new but some characterisation work on the model's behavioural phenotypes has already been outlined. While early research found cognitive deficits can appear between 6 months (Kaur et al., 2020; Manocha et al., 2019; Mehla et al., 2019; Saito et al., 2014) and 12 months (Latif-Hernandez et al., 2019; Masuda et al., 2016), examination of affective deficits seem to appear more robustly and sooner. Research has found reduced anxiety in the *App*$^{NL-G-F}$ in the elevated plus maze robustly appears from 6 months (Latif-Hernandez et al., 2019; Pervolaraki et al., 2019; Sakakibara et al., 2018). The anxiolytic behaviour is seemingly counterintuitive to the increased anxiety often cited in humans (Zhao et al., 2016), but this could be explained as more of a disinhibitory phenotype that is another common NPS (Lyketsos et al., 2002). These deficits could possibly appear sooner but younger cohorts have yet been tested. However, much more varied results appear when using the open field test and some found no differences between *App*$^{NL-G-F}$ and WT mice up to 10 months (Maezono et al., 2020; Whyte et al., 2018). Pervolaraki et al. (2019) even found increased anxiety between 7-9 month old KI mice compared to WT. Another study actually found a decrease in anxiety at 6 months but not at 3 or 10 months suggesting this is not an age dependent deficit. This discrepancy between the two anxiety tests highlights that both may be measuring slightly different aspects of anxious behaviour and solidifies the idea that multiple affective tests should be done (Lalonde et al., 2012). Other studies found *App*$^{NL-G-F}$ mice have reduced phobia development (Sakakibara et al., 2018), reduced fear learning and memory (Maezono et al., 2020; Mehla et al., 2019) suggesting they seem to lack a functioning internal sense of danger. This has also been found in transgenic tau mice who exhibit reduced anxiety on the elevated plus maze (EPM) but no change in the open field (OF) that authors assign to reduced anxiety with disinhibitory tendencies rather than just reduced anxiety (Cook et al., 2014; Przybyla et al., 2016). This could suggest a role of amyloid in causing disinhibition seen in early AD. In support of this, compulsive behaviour was also found in *App*$^{NL-G-F}$ mice at 8 months (Masuda et al., 2016) that could be akin to the inappropriate behaviours patients are not inhibited to perform. While it is often cited that to conclude disinhibition, you must also have a hyperactive phenotype

(Gil-Bea et al., 2007; Lalonde et al., 2012) but this seems to be more of a suggestion than a solid fact and no hyperactivity is accompanied in these anxiety tests on *App*$^{NL-G-F}$ mice (Latif-Hernandez et al., 2019; Pervolaraki et al., 2019).

With *App*$^{NL-G-F}$ exhibiting certain NPS, the question of whether this is caused by noradrenergic deficits and rampant neuroinflammation needs to be examined. Mehla et al. (2019) gave an extensive look at the LC, neuroinflammation and some behaviour in the *App*$^{NL-G-F}$. While they found plaques in the LC from 9 months, they also found reductions in the neuron number at the same age. This was accompanied by age dependent increase in astrocytosis in the cortex and hippocampus compared to WT and cognitive deficits from 9 months and spatial deficits from 6 months. The only examination of any neuropsychiatric symptoms was in showing reduced fear learning in contextual fear conditioning that could suggest a disinhibition phenotype. It is also difficult to tell what extent the changes to the LC have on the behaviour and whether a loss of neurons translates to a loss of function. Sakakibara et al. (2021) also closely examined the LC in the 12 month old *App*$^{NL-G-F}$ mouse but did not find this reduction in cell number. Instead, they found greatly impaired connectivity of noradrenergic neurons through analysing noradrenaline transporter (NET) positive cell distribution. They found reduced density in axons and aberrant connections, the latter being a compensatory mechanism where new connections are formed to replace lost ones but they innervate to the wrong brain area. The loss of NA afferents was not just limited to areas with high neuroinflammation or plaque load suggesting other mechanisms aside from retroactive stress. However, the aberrant NET connections were to places with heavy plaque load and neuroinflammation that could suggest an attempt at bring the NA to where it is most needed (i.e., where the inflammation is). Examining neurotrophic support found no changes in BDNF, somatostatin or neurotrophin-3 that either suggests no loss of neuroprotection from these or the compensatory mechanisms worked to bring neurotrophins back to base line. Despite this extensive examination of the LC, no behavioural work was done on these mice so it is still unclear whether this loss of noradrenergic function manifests as neuropsychiatric symptoms seen in AD.

Overall, mice models of Alzheimer's pathology are important in giving detailed accounts of the biological underpinnings of the disease and how this creates behavioural phenotypes. Old transgenic models created pathology at the cost of inconsistency and excess neurotoxic fragments making conclusions hard to draw from these models. The next generation of models, KI, fixed this issue and while work has shown they exhibit certain neuropsychiatric symptoms, others have shown noradrenergic deficits but few have looked at both. Examining whether increased neuroinflammation and LC loss of function can lead to affective deficits should be studied in this model of preclinical AD (e.g., when NPS appear developmentally). That is why this research will use

the *App*$^{NL-G-F}$ model to look at neuroinflammation, LC degeneration and affective deficits and relate it to LC degeneration and depression in human AD.

●1.6 Aims and outline of experiments

This literature review detailed how the LC could be integral to understanding how neuropsychiatric symptoms develop in preclinical AD. While tau pathology seems to be crucial in initial disruption to the LC, it is unclear what independent role amyloid has on this disruption and the development of NPS. The build-up of toxic Aβ leads to neuroinflammation and possible changes to noradrenergic signalling which, in turn, exacerbate inflammatory processes and NPS. This thesis will examine the question of whether changes to the LC link to the presence of neuropsychiatric symptoms.

This question will be examined in chapter 2 and 3 using a KI mouse model that offers a better model of amyloid pathology without the toxic overexpression of APP. Chapter 2 will look at the behavioural phenotypes of a large cohort of *App*$^{NL-G-F}$ and WT mice (male and female) at a young and old time point. This will examine the presence of anxiety, depression, disinhibition and social deficits at the 6 and 12 month time point and address aim one: Does amyloid pathology lead onto manifestations of affective deficits?

Chapter 3 will examine the biochemical analyses on this same young and old cohort of mice. This will assess amyloid pathology, pro and anti-inflammatory cytokines, gliosis, the LC and BDNF signalling and answer aim two: How does amyloid pathology affect the LC, neuroinflammation and neurotrophic support?

Chapter 4 will move on to examine how disruption to the LC-NA system could relate to depression that could bridge the gap between NPS in AD. Examination of TH will be done in depressed and non-depressed people both with and without a diagnosis of AD. This can show whether NA production differs between the groups and if there is a difference between the two AD groups. Furthermore, a group of mice will also have their LC-NA system experimentally disrupted with injections of DSP4 and their depressive and anxious symptoms will be examined. This will attempt to answer the question: will disruption to noradrenergic signalling lead to depressive symptoms?

Finally chapter 5 will attempt to bring together all the evidence gathered in this thesis and combine it with previous literature to answer the question: Does amyloid affect the LC and does this link to the presence of neuropsychiatric symptoms?

●Chapter 2: Behavioural assessment of the *App*$^{NL-G-F}$ mice●

●2.1 Chapter overview

In AD, neuropsychiatric symptoms are associated with worse outcomes and increased disease progression. Being able to understand the individual role amyloid has in neuropsychiatric symptom development could offer new targets for treatment options. While transgenic models have looked at this question, they are not able to selectively produce amyloid pathology without extraneous physiological effects (e.g. overexpression of APP). Knock-in (KI) models, such as the *App*$^{NL-G-F}$ mice have managed to create a better model of amyloid pathology without complications such as the overexpression of APP and other neurotoxic Aβ fragments. The *App*$^{NL-G-F}$ mouse model is examined to answer the question: Does amyloid pathology lead onto manifestations of affective deficits?

To answer this, a young (5-7 month) and an old (12-14 month) cohort of *App*$^{NL-G-F}$ mice are compared to age-matched wild-types (WT) in a battery of affective tests that include elevated plus maze, open field, social preference test and lick cluster analysis. Due to the potential heterogeneity of affective deficits, high group sizes (n=24) were used. Also, both male and female mice were used to examine the possible sex dependent deficits.

The results showed reduced anxiety, no social deficits, no anhedonia, but *App*$^{NL-G-F}$ mice did exhibit consumption changes consistent with apathy. They also showed no recognition memory deficits. These results are discussed in the context of previous findings and potential biological mechanisms.

●2.2 Chapter Introduction

The amyloid pathology seen in AD could be important in the development of neuropsychiatric symptoms. This issue can be examined in detail using animal models of amyloid pathology and behavioural testing. Transgenic models do offer some information but suffer from toxic overexpression of *APP* and other fragments. Knock-in models, such as the *App*$^{NL-G-F}$ mice (Saito

et al., 2014) address these issues by providing a model of amyloid pathology without toxic overexpression. The *App*$^{NL-G-F}$ mice were used to examine what link amyloid pathology has to the development of neuropsychiatric symptoms through the use of robust behavioural tests. Previously, it was noted that the NPS examined in mice via behavioural assays will be outlined here. Thus, anxiety is examined in section 2.2.1, social deficits in section 2.2.2, depression in 2.2.3 and cognition in 2.2.4.

●2.2.1 Anxiety

Increases in anxiety are commonly seen in Alzheimer's patients (Lyketsos et al., 2000) and the official definition, as outlined in the diagnostic statistical manual- V (DSM-V), is a negative emotional state with feelings of tension, worried thoughts, hypervigilance and physiological changes (American Psychiatric Association, 2013). Barlow (2004) defines it more simply as a future-oriented state associated with preparing for upcoming negative events. This mirrors animals' predatory imminence continuum where they prepare for a predatory attack (Fanselow & Lester, 1988). So, anxiety can be measured in mice by exposing them to potential threats and measuring avoidance and risk assessment behaviours (Pentkowski et al., 2021). Tests such as the elevated plus maze and open field both do this by having open spaces that mice naturally avoid as it leaves them exposed to predatory attack.

The elevated plus maze (EPM) consists of a cross shaped maze with two closed arms that have walls to protect against attack and two open arms that leave the mouse completely exposed. It creates a conflict between rodent's natural desire to explore novel areas and their aversion to the open arms (Pellow et al., 1985). Research found it was exposure and not novelty or height that created an anxiogenic stimulus as avoidance of the open arms still remained after repeat exposure and if the elevation was removed (Treit et al., 1993). As healthy mice will naturally venture into the open arms at least briefly, both a decrease and an increase in open arm exploration can be informative regarding anxiogenic and anxiolytic behaviour respectively. Furthermore, anti-anxiety medications increase propensity for mice to explore the open arms (Cole & Rodgers, 1993) that helps validate the test as a measure for anxiety in rodents. Similarly, medications that increase anxiety reduced time spent in the open arms (Anseloni et al., 1995; Pellow & File, 1986).

Other tests for anxiety include the open field (OF) test based on similar principles of measuring avoidance of an open area, but here it is the middle of a square arena. Time spent in the 'unsafe' inner zone and the 'safe' outer zone are recorded with more time in the inner zone

indicating less anxiety. Mice will show risk assessment behaviours toward the centre of the arena (Blanchard & Blanchard, 1971, 1989) and thigmotaxis (wall hugging) to demonstrate fear for the open space (Treit & Fundytus, 1988). Prut and Belzung (2003) offer a review of validating the OF as a test for anxiety with anxiolytic (e.g. diazepam) and anxiogenic medications (e.g. corticotrophin releasing factor receptor agonists) but warn the OF could be more of a test of general anxiety opposed to pathological anxiety. This is due to no differences in anxiety behaviour displayed after being given drugs used to treat human anxiety disorders (e.g. SSRIs).

The behavioural outputs of the OF and EPM have also been described as disinhibition. Reduced avoidance of open spaces could be explained by less inhibitions opposed to less anxiety (Gil-Bea et al., 2007) but this would likely be accompanied by hyperactivity. If their behaviour suggests anxiolytic reaction but it was simply caused by a general increase in movement then that indicates a disinhibited reaction. Other ways of measuring whether behaviour is truly down to changes in anxiety is through recording ethological behaviours such as rearing and stretching (Rodgers, 1994). These risk assessment behaviours represent an increase in anxiety and they include head dips over the open arms in the EPM or stretch-attend postures as they venture into open space (Walf & Frye, 2007). They have been validated with anxiety drug studies (Cole & Rodgers, 1994; Rodgers et al., 1994; Wall & Messier, 2000) with anxiolytics reducing risk assessment behaviour. So, to truly conclude changes to anxiety with the OF and EPM, general activity and ethological behaviours should also be measured.

Amyloid models

While anxiolytic and anxiogenic drugs affect anxiety responses to these tests, amyloid is also thought to alter anxiety in Alzheimer's disease. Evidence for this comes from animal models of amyloid pathology exhibiting changes to anxiety (Lalonde et al., 2012). Looking at the EPM, various different mouse models exhibit large differences in anxious output. They can exhibit both hypoanxiety in the Tg2576 (Gil-Bea et al., 2007; Lalonde et al., 2003) and hyperanxiety in APP/PS1 (Filali et al., 2011; Lee et al., 2006) albeit some show no difference in comparison to WT controls including J9 (Chin et al., 2005), V717F (Lee et al., 2004) and 3xTg-AD (Romano et al., 2015). Lalonde et al. (2012) suggested that changes to anxiety only occurred in animal models with plaques but some models with plaques, such as APP751SWE/LD, show no difference to controls (Blanchard et al., 2009; Le Cudennec et al., 2008). These varying results could be explained by different models using different exogenous promoters creating a vast array of biological differences across transgenic mice. However, there are even different results found within the same model (Lalonde et al., 2012) suggesting that disparities between lab handling and practices could have an impact on mouse

anxiety. For example, examining the APP/PS1 mouse, Pugh et al. (2007) found no change in anxiety compared to WT at 2, 5 or 10 months old. In contrast, Filali et al. (2011) found increased anxiety in male APP/PS1 mice at 6 months of age but no change in females and Verma et al. (2015) found increased anxiety in both sexes at 10-12 months old. Regardless, due to the vast array of biological differences through damaged genetics and unphysiological excess of *APP* fragments, transgenic models of Alzheimer's disease may not be ideal. It is near impossible to ascertain what part of amyloid pathology is causing changes to anxiety when transgenic mice vary greatly in their biology. There is also evidence to suggest large deletions of their genome occur as an effect of transgenes (Bryan et al., 2011) highlighting a need to find better amyloid models.

Anxiety has been looked at in the *App*$^{NL-G-F}$ mice and the complete timeline for research can be viewed in fig. 2.1. There is a clear discrepancy between the results from EPM and OF. From 6 months, all studies who did the EPM found an anxiolytic reaction in the *App*$^{NL-G-F}$ mice (Johnson et al., 2020; Latif-Hernandez et al., 2019; Pervolaraki et al., 2019) except for at Latif-Hernandez et al. (2019) who found no change at 8 months but that was compared to *App*^{NL} mice. However, Sakakibara et al. (2018) only found a trend in increased anxiolytic behaviour when compared to WT but it was significant against another KI model, *App*^{NL} mice. The *App*^{NL} mice are a control model for the *App*$^{NL-G-F}$ mice as they only exhibit the Swedish mutation and no plaques. The researchers found the *App*^{NL} mice to actually show anxiogenic behaviour in the EPM with decreased time spent in the open arms that continued to decrease after repeat testing. This could show support for the idea that plaques play an important role in creating reduced anxiety. The *App*$^{NL-G-F}$ mice still continued to explore the open arms after repeated trials leaving the authors to conclude anxiolytic behaviour albeit with a small sample size.

Examination of the OF test has led to a great deal more discrepancy that could highlight differences between anxiety tests. Some researchers found no difference compared to WT (Kundu et al., 2022; Maezono et al., 2020; Whyte et al., 2018). Latif-Hernandez et al. (2019) found *App*$^{NL-G-F}$ mice spent more time in the centre at 6 months of age but no difference at 3 or 10 months. This could suggest that amyloid's impact on anxiety could be dynamic and not necessarily just a progressive change with age. To further add to this point, Pervolaraki et al. (2019) and Locci et al. (2021) found *App*$^{NL-G-F}$ mice actually spent less time in the centre at 8 & 10 months respectively in stark contrast to their anxiolytic response on the EPM. It is possible that elevated plus maze and open field measure two different aspects of anxiety as previously noted. Thus multiple tests, with ethological behaviours, should be used to get a better overview of anxious behaviour.

Overall, anxiety is a common neuropsychiatric symptom seen in Alzheimer's disease that could be caused, even partially, by amyloid pathology. This is supported by animal models of amyloid exhibiting anxious behaviour during behavioural testing but biological discrepancies between models could suggest the anxious phenotype is down to confounds in the transgenic mice not found in the human condition. *App*$^{NL-G-F}$ mice also show changes to anxiety suggesting amyloid is part of the cause. While various tests have previously run the OF and EPM on this mouse model, there is a great deal of discrepancy between results. Repeating these tests with a large sample size that includes both sexes will be able to offer a more conclusive answer to the potential anxiety phenotypes displayed by these mice. This is vital for affective testing due to the heterogeneity of emotional deficits (Cummings, 2000). Furthermore, the inclusion of ethological behaviours and locomotor activity in the assessment will offer a more conclusive examination of anxious behaviour (Carobrez & Bertoglio, 2005; Rodgers et al., 1997). Using multiple anxiety tests will also add to this detailed examination.

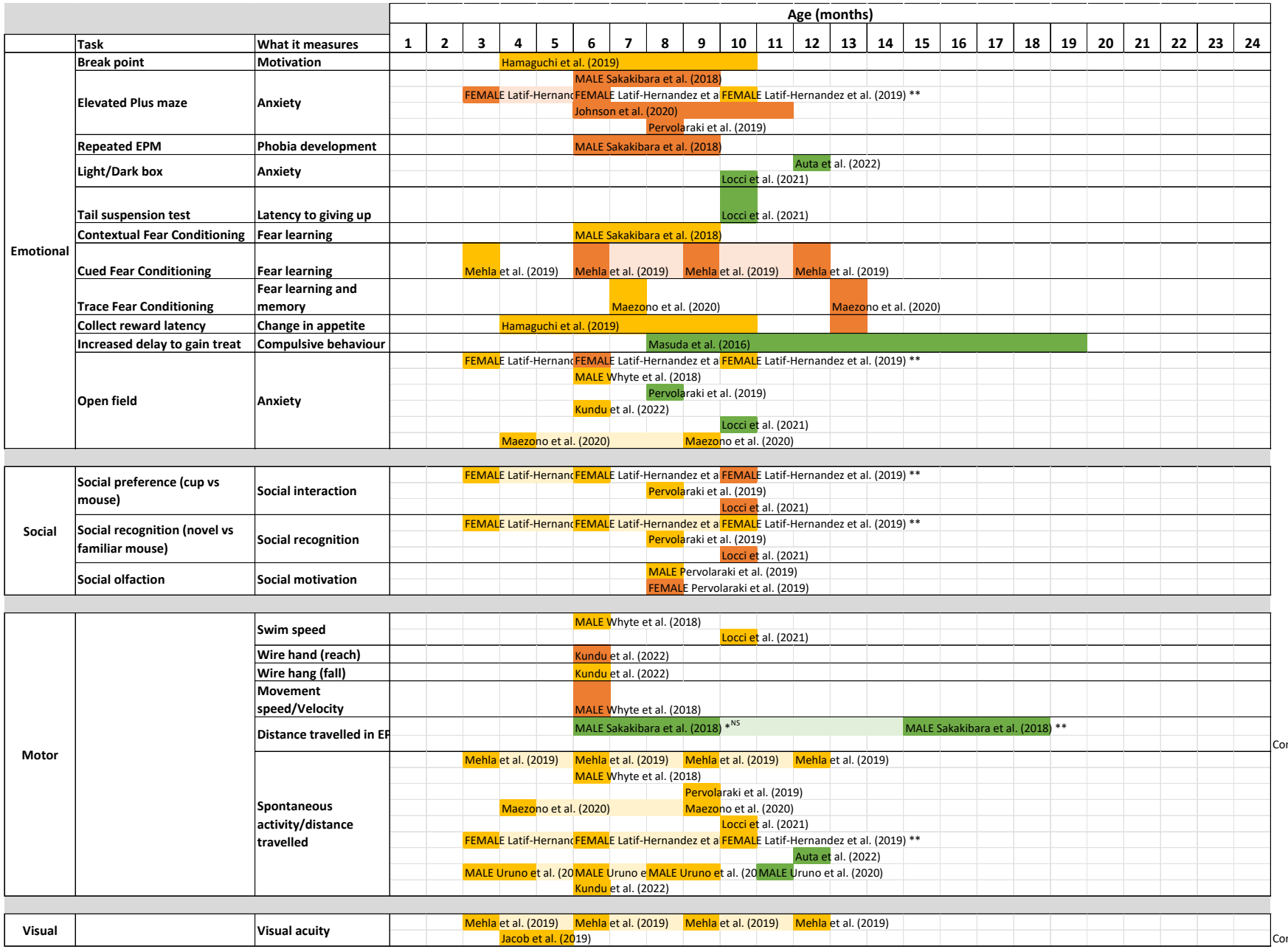

Figure 2.1 Behavioural timeline of deficits tested in the App^{NL-G-F} mouse. *Orange indicates a decrease compared to control, yellow represents no significant difference and green indicates increase compared to control. Control is C57BL/6 WT unless stated otherwise. This only represents healthy App^{NL-G-F} meaning no other manipulation was done on them e.g. early life stress. Results from experiments that include a manipulation are only showing the outcome of healthy App^{NL-G-F} compared to WT. Research used both sexes unless the sex is specifically stated. Sexes have the same outcome unless stated otherwise. The assumed blocks are areas that were not directly tested by the authors but they tested two other time points and got the same result (i.e., increase or decrease from controls). If there is any difference between time points then nothing can be assumed.*

●2.2.2 Social deficits

Alzheimer's patients can experience social deficits such as inappropriate social behaviour and a lack of desire to interact with others (Craig et al., 2005; Mega et al., 1996). Social deficits can lead to increased social isolation in AD patients that can lead to depression, loneliness, increased disease progression (Drinkwater et al., 2021) and even reduction in hallucinations (El Haj et al., 2016). As mice are social creatures, this is possible to measure by examining how they interact with conspecifics. Mice will naturally show a preference for a social interaction and even more so if that mouse is novel in some strains (Moy et al., 2007). File and Hyde (1978) originally developed the social interaction test as an ethologically relevant test for anxiety in rats. They placed two animals in an arena and measured their interaction. If interaction is increased without changing motor activity then it is defined as anxiolytic while a decrease in interaction would suggest social anxiety. It was one of the first tests that was able to examine both increases and decreases in social anxiety based on familiarity of the arena and light levels. However, the validation of this test in mice was less stable (de Angelis & File, 1979) and anxiolytic or anxiogenic drugs did not impact on interaction as would be expected (Hilakivi et al., 1989; Lister & Hilakivi, 1988). However, to reduce possibilities of fighting, Moy et al. (2007) placed the conspecific under a cup and had another empty cup to measure preference for interacting with a novel object that is social or inanimate. While the social interaction test can measure sociability, a desire to interact with conspecifics, they also wanted to measure social novelty, the ability to discriminate between novel and familiar mice. Social novelty measures recognition memory specific to conspecifics rather than objects that is assessed with novel object recognition. The ability to accurately recognise a conspecific is integral to healthy social interactions while deficits could lead to social isolation. This creates a two-phase test to initially examine sociability and then phase 2 that assesses social recognition.

Amyloid models

To test whether the build up of amyloid can induce social deficits, these tests have been run in amyloid pathology mice models. Using the double transgenic model, APP/PS1, various researchers found them less likely to interact with their conspecifics compared to WT (Filali et al., 2011; Olesen et al., 2016; Rens et al., 2019). This reduced social interaction was not fixed with an SSRI treatment (Olesen et al., 2016). This could be an anxiogenic reaction as Filali et al. (2011) found reduced sociability despite a hyperactive phenotype meaning exploratory behaviour was increased but this did not impact social activity. This did extend to phase 2, social novelty, as APP/PS1 mice sniffed the novel mouse less than WT and were unable to discriminate between their own cage mate and the

novel conspecific (Lo et al., 2013; Misrani et al., 2021). This suggests social recognition deficits along with social anxiety in the APP/PS1 as well as Tg2576 (Deacon et al., 2009).

These tests should also be confirmed in KI models (e.g. *App*$^{NL-G-F}$ mice) to see whether any deficits are down to the amyloid pathology or complications of inserting a transgene. Latif-Hernandez et al. (2019) found no social recognition deficits in female *App*$^{NL-G-F}$ mice at 3, 6 or 10 months of age but there was a trend for reduced social novelty at 10 months. However, the lack of genotype effect could be due to comparing to *App*^{NL} mice that also showed a trend for reduced social novelty meaning the result could be due to the Swedish mutation alone impacting on social behaviour. Using both sexes also produced the same result with a subtle trend for reduced social novelty (Pervolaraki et al., 2019). However, when the test involved interaction preferences with soiled versus clean bedding of conspecifics then only females showed little preference compared to males and WT (Pervolaraki et al., 2019). This could suggest reduced desire to seek out social interaction as soiled bedding indicates the presence of a conspecific. The fact it was only found in females highlights the importance of using both sexes when running sociability tests due to differences in socialising between sexes. Locci et al. (2021) did the two phase sociability and social novelty test but delayed each phase by 24hrs. They found reduced time socialising in phase 1 as measured by entries into stranger mouse chamber and less time spent interacting with them. They also found reduction in social recognition suggesting a longer delay does impact recognition memory. The discrepancy between these results could be down to a longer delay between phases or differences between labs.

Overall, using the social interaction test and social novelty test is a valid way of measuring social deficits in mice. While transgenic mice seemed to show that amyloid could cause social anxiety and social recognition deficits this did not reliably translate to *App*$^{NL-G-F}$ mice. In fact, they seemed to only show slight trends for reduced sociability and social interaction but did have a significant reduction is social motivation in females. Using a larger sample size will be able to show whether the trend for reduced sociability is reliable. Due to sexually dimorphic differences found in social interaction (Pervolaraki et al., 2019), it is important that more tests include both sexes to get a full picture of social deficits.

●2.2.3 Depression

Depression is a complex but core neuropsychiatric symptom seen in early Alzheimer's disease (Landes et al., 2001). It is difficult to test in mice due to the heterogeneity of the disorder so

most tests examine a symptom of depression e.g. anhedonia. Other methods attempt to look at a more comprehensive examination of depression such as the tail suspension test (TST) and forced swim test (FST). These put the rodent in an inescapable and uncomfortable position and measure their time immobile. A higher time immobile indicates higher feelings of hopelessness as they give up on escaping before healthy controls. While both the FST and TST have been validated with anti-depressant medications (Borsini & Meli, 1988; Stukalin et al., 2020) others have suggested they are actually a better measure of behavioural despair opposed to depression (Zhou & Liu, 2013). Furthermore, the tests themselves are stressors that could cause unneeded harm to the animals as well as impacting future testing. One alternative is sucrose preference test that measures anhedonia without excess stress caused to the animal. Anhedonia is defined as a reduction in pleasure gained from a normally pleasurable experience and the sucrose preference test measures hedonic response through amount of sucrose consumed in comparison to water. Other things being equal, reduced hedonic responses should cause a reduction in consumption of a pleasurable solution. But consumption is potentially confounded by variables such as weight of the mouse or motivation to drink fluids. Consumption has also been found to increase with sucrose concentration only to a point but after that consumption actually decreases at higher concentrations despite animals typically choosing to consume the higher concentration over the lower when both were presented concurrently (Richter & Campbell, 1940). Together, this highlights a need for a better measure of anhedonia.

Grill and Norgren (1978) measured rats taste reactivity to sucrose solutions by examining their facial features either expressing appetitive or aversive patterns. While it has high face validity across species (Berridge, 2000; Dwyer, 2012) it suffers from being very labour intensive and only encapsulates a momentary hedonic response. Often this would also require surgical intervention for optimum results in order to implant a cannula for directly infusing liquid in the rodents' mouths that is potentially stressful and may restrict other behavioural tests. Examining natural drinking itself offers an alternative method for measuring hedonic response and can be done over time without causing unneeded stress to the animal. When rodents drink, they lick at the solution in clusters and take short breaks between said clusters. Researchers inferred that higher average licks per cluster was indicative of higher liking of a solution (Davis, 1989; Davis & Levine, 1977; Davis & Smith, 1992) because average lick cluster size increased monotonically with concentration of sucrose (Spector et al., 1998). They also found the opposite when giving increasingly unpalatable quinine solutions monotonically decreased average lick cluster size (Hsiao & Fan, 1993; Spector & St. John, 1998). It has been validated as a measure of hedonic response through manipulations that would alter the enjoyment of a solution also impacting on the lick measure. For example, repeatedly pairing sucrose

with nausea through lithium chloride injection reduced the average lick cluster size (Baird et al., 2005; Dwyer, 2009). Average lick cluster size could also be increased for a bitter quinine solution if paired with sweet tastes (Pelchat et al., 1983) or with doses of benzodiazepine known to enhance hedonic response to food in humans (Higgs & Cooper, 1998). As anhedonia is a core symptom of depression, examining it in animal models of Aβ could provide better insight into whether amyloid induces a depressive-like state. Previous research has used the sucrose preference test to examine anhedonia that is confounded by various factors already discussed. Lick cluster analysis managed to address these confounds so offers a more selective approach to measuring hedonic responses.

Amyloid models

Looking at depressive-like symptoms in transgenic models has often been done with forced swim test, tail suspension and sucrose preference test. Increases in depression-like symptoms have been found across multiple models through increased immobility in the forced swim and tail suspension test in 5xFAD mice (Patel et al., 2014; Yamazaki et al., 2015). It was also found in a triple transgenic model, 3xTg-AD mice as well as reduced preference for sucrose compared to water despite no gross change in overall fluid consumption (Romano et al., 2015). However, in another model, APP23, no hedonic changes were found in the sucrose consumption test and they actually found decreases in immobility in the forced swim and tail suspension test (Pfeffer et al., 2018; Vloeberghs et al., 2007). Differences across models can be expected due to variations in physiologies but the tests also suffer from how valid they are as measures of depressive symptoms. Lick cluster analysis is not commonly chosen as a test for anhedonia due to extensive training required. However, the study that has used it with Tg2576 mice found a lack of expected increase in lick cluster to a 16% sucrose solution compared to a 4% that was found in WT indicating anhedonia (Brelsford et al., 2017). Together, this could suggest that amyloid is one cause of certain depressive symptoms seen in Alzheimer's disease but, due to the inconsistencies across models, this is difficult to conclude from transgenic animal work alone.

Very little examination of depressive-like symptoms has been done in *App*$^{NL-G-F}$ mice. Locci et al. (2021) did find increased immobility in the tail suspension test for both male and female *App*$^{NL-G-F}$ mice. Depressive symptoms have been largely overlooked so far so it is difficult to conclude whether amyloid is a cause of depressive symptoms seen in Alzheimer's disease. The current study will use the lick cluster analysis to test hedonic response to palatability in the *App*$^{NL-G-F}$ mice to see if the result found in the Tg2576 translate to an improved model of amyloid pathology.

●2.2.4 Cognition

While cognition is a complex topic, it has been heavily researched in Alzheimer's models and normal mice alike. AD patients present with a variety of memory problems with a core one being issues in recognition (Maurer et al., 1997). This can be measured in mice with a straightforward test known as novel object recognition (NOR) that relies on a rodents natural desire to explore novel stimuli and thus recall what objects have been encountered previously. Ennaceur and Delacour (1988) originally ran 3-5 minute exposure trials on two identical objects before swapping one object out for something novel after twenty-four hour delay. Exploration (defined as nose within 2cm and pointed toward the object) of the novel object was greater compared to the familiar object that increased with longer trials. Researchers inferred that increased exposure to the objects through longer trials allowed greater memory consolidation. This basic protocol has been used as the novel object recognition and variations have also been used to measure spatial and temporal memory (Dix & Aggleton, 1999). This includes the similar test, object in place (OiP) that changes one object location to see whether rodents will be able to distinguish the moved from the stationary objects. This assesses spatial memory that is also impacted in AD (Maurer et al., 1997). This OiP is often used as a test for episodic-like memory with a spatial component that we intended to use in the present study. However, due to issues outlined in section 2.5.5, the data from the young cohort was not usable so was changed for NOR during behavioural testing of the older cohort.

Cognitive testing is a staple in amyloid models due to examining the role amyloid may have of cognitive decline in Alzheimer's disease. Cognitive deficits are often found in transgenic models but it is uncertain whether these results are down to amyloid pathology (Bryan et al., 2011). *App*$^{NL-G-F}$ mice have also displayed recognition memory deficits from 9 months of age in novel object recognition (Auta et al., 2022; Locci et al., 2021; Mehla et al., 2019) but not at 6 months (Whyte et al., 2018) suggesting an age dependent decrease. However, some researchers found no cognitive deficits in the Morris water maze even up to 10 months (Latif-Hernandez et al., 2019) unlike Mehla et al. (2019) who found spatial deficits at 6 months. Sakakibara et al. (2018) also found spatial deficits in the Morris water maze at 24 months of age. Discrepancies across tests have been attributed to differences in lab practices (Whyte et al., 2018) but most suggest that any cognitive deficits found in the *App*$^{NL-G-F}$ mice are often mild. Complete display of cognitive deficits can be seen in fig. 2.2.

There are various ways to measure cognition but the NOR test provides a robust and sensitive measure for assessing recognition rather than spatial or other forms of memory. The object in place assesses spatial memory so will be run in the young cohort. To accompany this, certain

ethological behaviours will be assessed to further evaluate anxiety. Both grooming and rearing are found to increase with anxiety (Kalueff et al., 2016; Sestakova et al., 2013) while time spent stationary will be used as a check along with locomotor activity to examine whether sufficient movement occurs.

Testing cognition offers examination of level of cognitive decline that will be compared to potential affective deficits. OiP will be run in the young cohort to assess spatial memory in these mice in a way that has not been previously done with *App*$^{NL-G-F}$ mice. NOR, which offers an assessment of recognition memory, will be repeated in the *App*$^{NL-G-F}$ mice to see whether Mehla et al. (2019) findings are robust with a larger sample that includes both sexes.

●2.3 Aims and hypotheses

The present study will use a large cohort of *App*$^{NL-G-F}$ mice of both sexes to undergo a battery of affective tests at a young (5-7 months) and an old time point (12-14 months) to examine age related emotional deficits. This will include multiple tests for anxiety (EPM/Open field), social deficits (social interaction and social novelty test), anhedonia (lick cluster analysis) and recognition memory (novel object recognition). They are expected to show reduced anxiety as measured with elevated plus maze, open field and confirmed with locomotor activity and reduced risk assessment behaviours. It is unclear what changes if any will be found when examining social interaction and social novelty in the *App*$^{NL-G-F}$ mice. It is expected that there will be reductions in average lick cluster when drinking sucrose compared to age matched WT. It is also hypothesised that *App*$^{NL-G-F}$ mice will show recognition memory deficits in the novel object recognition task at the old time point but not the young.

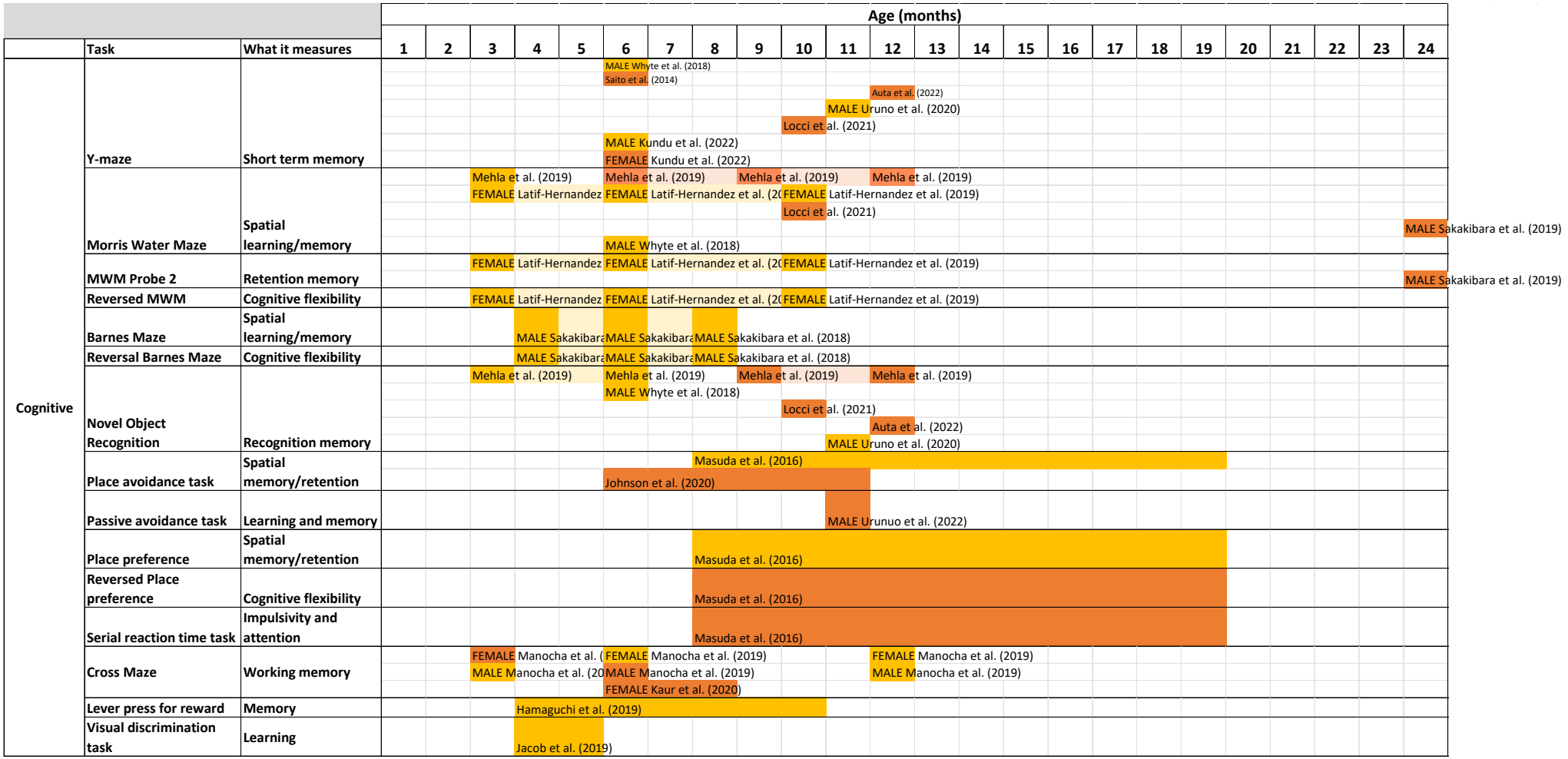

Figure 2.2 Cognitive timeline of deficits tested in the App^{NL-G-F} mouse. *Orange indicates a decrease compared to control, yellow represents no significant difference and green indicates increase compared to control. Control is C57BL/6 WT unless stated otherwise. This only represents healthy App^{NL-G-F} meaning no other manipulation was done on them e.g. early life stress. Results from experiments that include a manipulation are only showing the outcome of healthy App^{NL-G-F} compared to WT. Research used both sexes unless the sex is specifically stated. Sexes have the same outcome unless stated otherwise. The assumed blocks are areas that were not directly tested by the authors but they tested two other time points and got the same result (i.e., increase or decrease from controls). If there is any difference between time points then nothing can be assumed.*

●2.4 Methods

●2.4.1 Animals and breeding

All mice used in the breeding and experiments were of a C57BL/6J background. Four homozygous unrelated mating pairs of *App*$^{NL-G-F}$ mice were donated from in house (DRI lab, Cardiff) to create the main breeding cohort. These litters were left to age and 15 pairings were selected from different parents to create the behavioural cohort. At the same time, 15 breeding pairs of C57BL/6J wildtype mice (Charles River, UK) were paired to create the control cohort. These mice pairs were all between 10-15 weeks old at the beginning of breeding. Sires remained in the cage with the litter and each breeding pair was left to have 1-2 litters (except 2 WT pairs who had to be culled due to violence). After 21 days, litters were weened into same sex cages with their siblings and left to age before behavioural testing. Fourty-eight *App*$^{NL-G-F}$ (24 male, 24 female) and 48 WT (24 male, 24 female) mice were selected for the young cohort (aged 5 months) and the old cohort (aged 12 months) making a total of 192 mice across behavioural testing (table 2.1). Due to a sexing error in the old cohort, a cage of 4 WT females was actually 4 WT males and so group sizing reflects this.

Age	Genotype	Sex	N	Behavioural tests done
Young (5-7 months)	*App*$^{NL-G-F}$	Male	24	OF, EPM, SPT, LCA, OiP
		Female	24	
	WT	Male	24	
		Female	24	
Old (12-14 months)	*App*$^{NL-G-F}$	Male	24	OF, EPM, SPT, LCA, NOR
		Female	24	
	WT	Male	28	
		Female	22	

Table 2.1. Split of mice used in the behavioural testing. *OF=Open field, EPM=elevated plus maze, SPT=social preference test, LCA=lick cluster analysis, OiP=object in place, NOR=novel object recognition*

Housing

All mice were housed in standard cages (48 x 15 x 13 cm) with up to 5 per cage. Each cage contained a floor of wood chips, a single, clear handling tube, 1 cardboard tube and some beddernest that mice could shred to use as bedding. They had access to food and water ad lib unless

stated otherwise and were on a 12 hr light/dark cycle from 08:00-20:00 each day. Cages remained in holding rooms with stable temperature (21+-2 °C) and humidity (60+-10%) that were constantly monitored. All animals were maintained in accordance with the Animals in Scientific Procedures Act (1986) along with UK Home office licensing regulations.

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●2.4.2 Timeline of testing

The young cohort were tested at 5-7 months of age in the order shown in fig. 2.3. Approximately half the mice did the Object in place (OiP) before affective testing and the other half after affective testing to counterbalance cognitive and affective test order. The mice that did not do OiP first had their open field (OF) test recorded during arena habituation prior to social preference test to ensure OF was all mice's first encounter to the main arena. Twenty-four mice (n=6 per genotype and sex) were culled via perfusion for immunohistochemistry 3 days after the final day of behavioural testing while the other 72 mice were culled 6 days after behavioural testing and brains dissected for biochemistry (see chapter 3).

The older cohort were given a slightly revised timeline due to changing the inconclusive OiP test for novel object recognition (NOR). They were tested at 12 months and were born at the same time as the young cohort but left to age longer. Behavioural testing commenced in the order shown in fig. 2.3 with the same culling methods as the young cohort (see above).

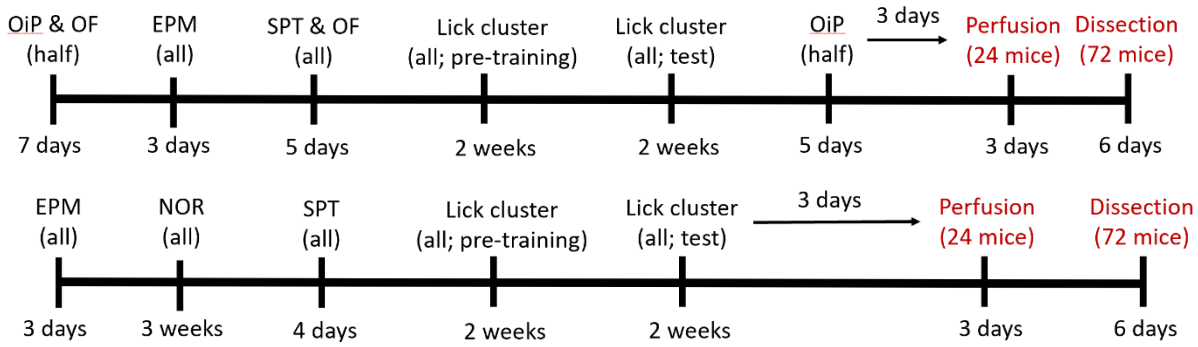

Figure 2.3. Timeline of behavioural testing for young (top line) and old (bottom line) App^{NL-G-F} and WT mice. *Top ofthe line is the test done and below is the length of time that test took to run. In total, each timeline took approximately 2 months. All mice had the same order of testing except for the young cohort where half were run through the OiP first and half at the end.*

●2.4.3 Apparatus

Main arena

All behavioural tests, aside from lick cluster analysis and elevated plus maze, were done in a square arena (60.5 cm x 60.5 cm x 40.5 cm) with clear, Perspex walls covered externally with white paper (fig. 2.4). The floor was grey and the arena was elevated 30 cm off the ground. The room was illuminated by one ceiling light and the walls of the room were covered in an assortment of black and white spatial cues placed so the mice could see them. The camera was set up directly above the arena.

Figure 2.4. The square arena used in NOR, SPT and OF.

Elevated plus maze

The elevated plus maze consisted of a cross shaped platform 75 cm above ground (fig. 2.5). Each arm was 40 cm long and 7 cm wide with opposing arms being either 'closed' with high, black Perspex walls (15 cm) or 'open' with shallow clear Perspex walls (1 cm). The maze had a grey floor and used in the same room with the same lighting and spatial cues as the main arena.

Figure 2.5. The elevated plus maze arena.

Recording equipment

The camera used throughout was a webcam (Megapixel camera module 2.0, ELP) suspended from the ceiling and plugged in to a Windows Laptop and recorded using Windows camera application. The videos were saved at 720p with 16:9 aspect ratio, 30fps and 60Hz flicker resolution onto an external hard drive and then backed up to another hard drive and the Cloud.

EthoVision XT 13 (Noldus Information Technology, Leesburg, USA) was used to track the movement of the mice post testing with appropriate behaviours, specified below, manually scored by the experimenter.

Objects

The objects used during the object in place (OiP) and novel object recognition (NOR) test were kept constant and chosen based on similar height but immovable by the mice and with limited climbing surfaces (fig. 2.6). The materials differed as some were made of plastic while others were glass or aluminium to ensure sufficient differences between them. No object produced a scent (even scent producing objects like soap were closed to ensure minimal scent) and they were placed in the arena 15 cm from the walls and 30 cm away from each other where crosses were on the floor to ensure consistent placement. Between each mouse, the objects and arena were wiped down using 70% alcohol and white roll to disrupt any scent marking.

Figure 2.6. Examples of objects used in NOR and OiP. *Objects were counterbalanced during testing meaning no object was always the novel/moved object.*

Social preference test equipment

For the social preference test, stranger mice were kept under wire cups (7.5 cm diameter) with a weight placed on top to stop the cup from moving. These were 15 cm from the walls in corners diagonal from each other with order counterbalanced.

Licking boxes

For lick cluster analysis, 16 identical drinking boxes were used (32 x 15 x 12 cm) with acrylic white walls, a wire cage lid and metal mesh floor (fig. 2.7). In the cage lid, there was slots to allow the placement of 2 stainless steel drinking spouts attached to a 50 ml plastic cylinder to hold the fluid. Spouts were placed on the left hand side for lick cluster and the other drinking holder left empty. A sensitive lickometer registered the timing of each lick to the nearest 0.01 s through MED-PC IV computer software (Med Associates Inc., St. Albans, VT, USA) and the amount of liquid consumed during the test was measured by weighing bottles before and after on a scale accurate to 2 d.p. Sucrose solutions were made with sucrose powder mixed with deionised water to create necessary weight/weight concentrations.

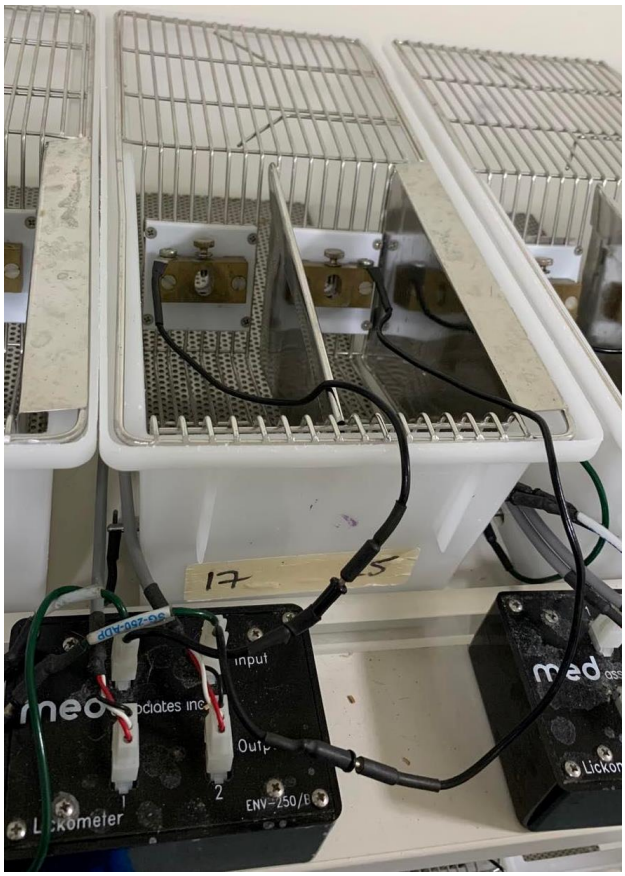

Figure 2.7. A licking box used during the lick cluster analysis. *Sixteen identical boxes were used during this test. Spout placement was consistently on the left.*

●2.4.4 Open field & habituation

Mice were individually tube handled into the centre of the main arena facing the same wall. They were allowed to freely move around the empty arena and movement was tracked for the first 5 minutes using EthoVision XT 13 to assess locomotor activity. In the program, the arena was split into an inner square zone of 50cm x 50cm (2500 cm^3) and an outer zone (fig. 2.8; 1160.25 cm^3) that constituted everything from the inner boundary to the walls (Seibenhener & Wooten, 2015). The

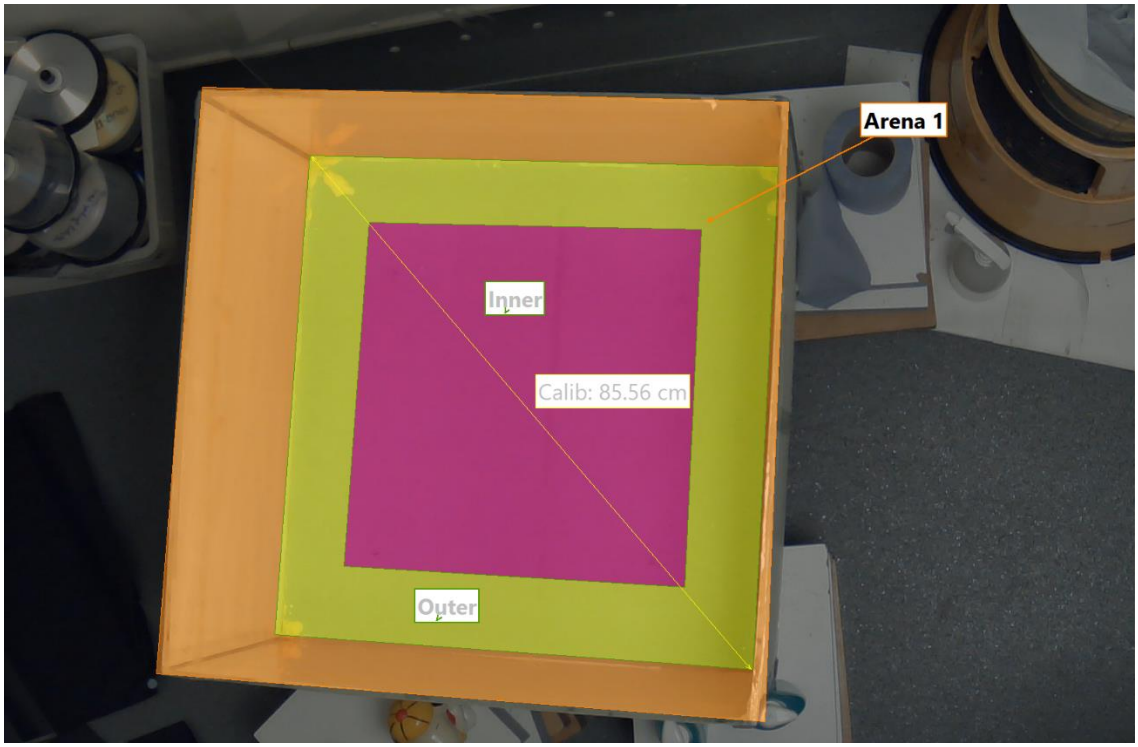

Figure 2.8. How the arena was divided in EthoVision XT 13 for the OF.

software recorded time spent by the mouse in each zone as a measure of anxiety along with distance travelled to assess locomotor activity.

This test represented the first day of habituation in preparation for either object in place/Social preference test (young cohort) or novel object recognition (old cohort) in the same arena. Due to a communication error, for the young cohort, their entire habituation consisted of one 10 minute session prior to testing but this was rectified for the older cohort. The older cohort's habituation was much more substantial and included 10 minutes a day for 4 days with day 3 having an object (not used in later testing) in the arena centre to introduce the concept of objects to the mice prior to cognitive testing (fig. 2.9). They had a 24 hr gap between habituation phases, sample phases and test making the test run across 6 consecutive days.

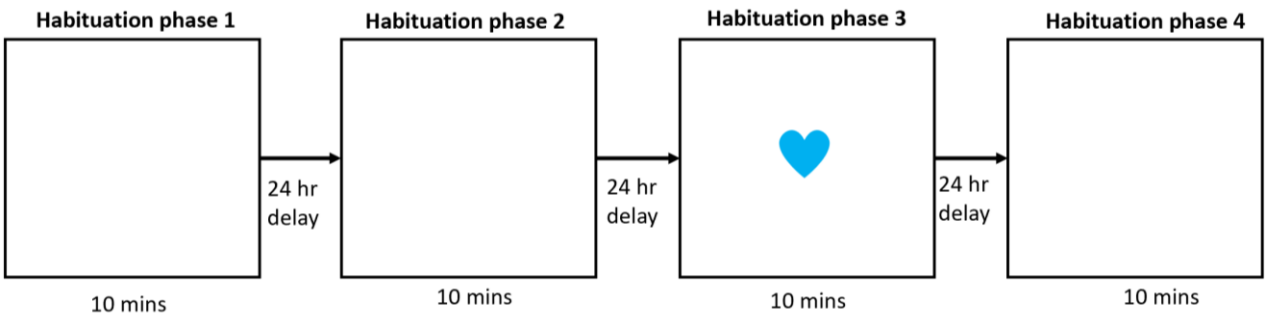

Figure 2.9. Habituation training for the NOR.

●2.4.5 Object in place

After habituation of one 10 minute session (described above), mice were tube handled into the arena with 4 different objects (fig. 2.10) for 10 minutes for their first sample phase. This exact configuration and timing was repeated twice more with 10 minute delay between each phase. After the final sample phase and 10 minute delay, 2 objects were swapped location and the mouse allowed to freely explore for 5 minutes which was recorded. Exploring the objects was taken as a way to assess interest and defined as nose =< 2 cm from the object or touching it. However, paws or body on the object with a nose pointing away was not (Ennaceur & Delacour, 1988). Exploration of the objects was manually scored by 3 independent researchers, 2 of which were blind to which objects were moved. Time spent exploring stationary objects was compared to the relocated objects.

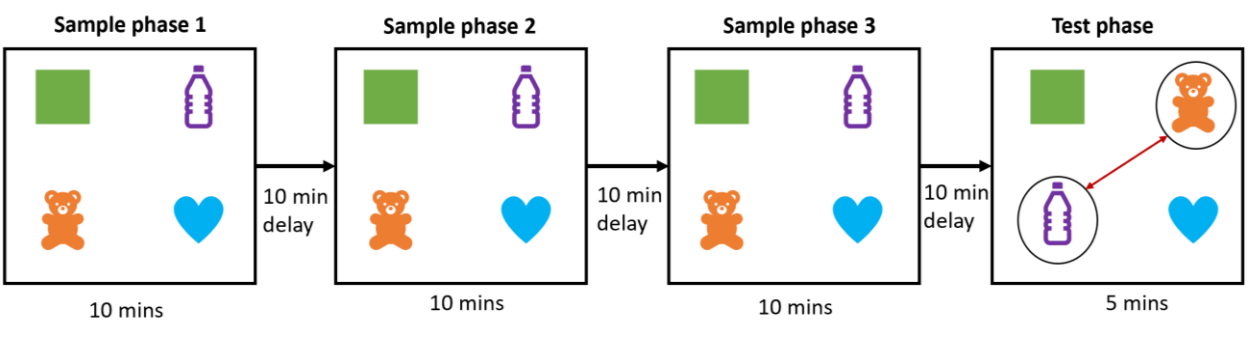

Figure 2.10. How the OiP was run. *Three identical sample phases were run after habituation and then a test phase.*

●2.4.6 Novel object recognition

Twenty-four hours after the final habituation phase described above, mice were introduced to two identical objects in diagonally opposing corners for 10 minutes. After another 24hr delay, mice were tube handled into the arena again with 1 object being swapped for a brand new one (object and place counterbalanced across groups) and their exploration lasted 5 minutes and was recorded (fig. 2.11). Interest in objects was manually recorded with the same criteria as the Object in Place (see above). Recognition memory was tested based on the premise that mice will prefer to explore what is novel to them and so time spent sniffing the novel object should be greater than the familiar object assuming memory is intact.

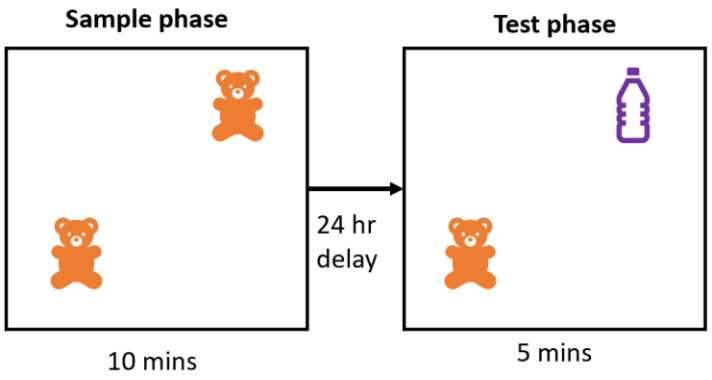

Figure 2.11. Sample phase and test phase of NOR. *This was run 24hr after the habituation phase shown in fig. 2.8*

Ethological behaviours

As well as exploration of objects, certain natural behaviours were manually recorded during the NOR. This included time spent not moving (measure of lack of exploration/anxiety; Aubele et al. (2008)), grooming (to measure displacement or avoidance of anxious stimuli; Heyser and Chemero (2012)) and rearing (measure of general environmental exploration; Aubele et al. (2008)). While time spent stationary is defined as lack of movement of limbs with feet remaining stationary, it is seen as a measure of lack of exploration (Aubele et al., 2008) and can interfere with analysis. It will be measured as a check. While rearing is defined as the lifting of forelimbs and sitting back on hind legs, it is seen as an exploratory behaviour with the aim at familiarising with the environment as well as searching for food (Amirazodi et al., 2020). Grooming is defined as using forelimbs to clean the body while sitting on haunches. Both rearing and grooming have been linked to anxiety (Kalueff et al., 2016; Sestakova et al., 2013). Furthermore, distance moved will measure general locomotor activity.

●2.4.7 Elevated plus maze

The walls and floor of the maze were completely wiped down with 70% ethanol between each mouse to remove odour scents. Mice were tube handled into the centre of the maze facing the open arm and allowed to freely explore for 5 minutes. Their movements were recorded with an overhead camera and movement tracked using EthoVision XT 13. Within the program, boxes were drawn around the open and closed arms and the mouse was automatically recorded as being in those arms when its centre point was registered within the boxes. Certain ethological behaviours were also manually recorded.

Ethological behaviours

Along with general locomotor activity, head dipping and stretch attended posture will be assessed. Head dipping is defined as lowering of head over the edge of the open arm and defined as anxious exploration (Walf & Frye, 2007; Wall & Messier, 2000). Stretch attend posture was defined as hind limbs remaining in closed/central portions and the forelimbs stretch out into the open arms. This was defined as risk assessment (Walf & Frye, 2007). Count of open arm visits was also automatically taken as a measure of reduced anxiety and is simply to complement the main discrimination ratio assessment. A count of closed arm visits was also taken.

●2.4.8 Social preference test

The experimental mice were habituated to the arena for 5 minutes before being placed back into their home cage (fig. 2.12). The first stranger mouse, same sex as the experimental mouse, was then tube handled into a wire cup and placed in one corner of the arena with a weight on top. An empty cup with a weight on top was placed in the opposing corner. Immediately, the experimental mouse was then tube handled into the centre of the area and recorded for 5 minutes with the experimenter outside the room. After the allotted time, the mouse was returned to their home cage and a second stranger mouse was tube handled into the empty cup with the first stranger mouse remaining in place to serve as a now familiar mouse for phase 2 of this test. The experimental mouse is, once again, tube handled into the centre of the cage and left for 5 minutes. Upon completion, the mouse is returned to the homecage via handling tube and the stranger mice are returned to their cages. The arena is wiped down with 70% alcohol ready for the next mouse.

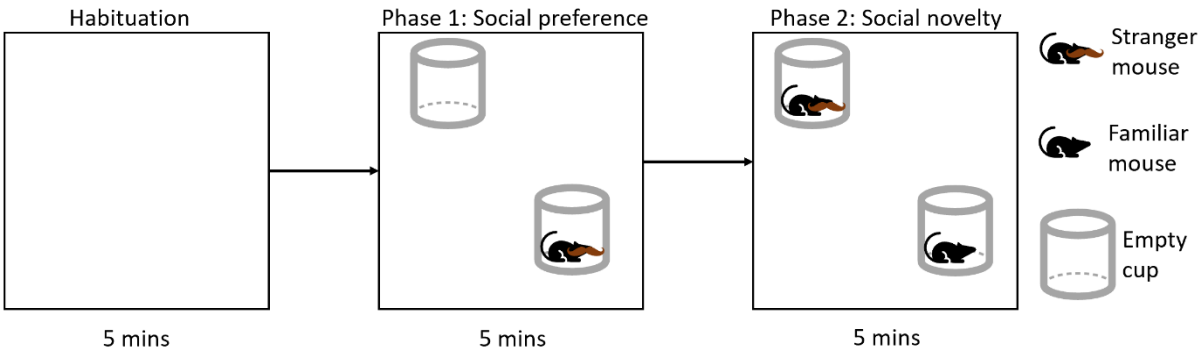

Figure 2.12. Protocol for Social preference and novelty test. *Mouse with moustache is stranger mouse but the same mouse becomes a familiar mouse by phase 2.*

Ethological behaviours

Time spent stationary and grooming were taken as measures of anxiety in the SPT (Westbrook et al., 1994). Being stationary was defined as lack of limb movement with feet remaining stationary in place and is seen as a measure of social anxiety. Grooming was defined as sitting on hind legs and using forelimbs to clean their body and is taken as a measure of displacement from a stressor (i.e., anxiety).

●2.4.9 Lick cluster analysis

Each day, mice were tube handled into the licking boxes. Pre-training lasted 10 days that consisted of all mice being given 8% sucrose solution to train them to drink. Mice were water restricted for the first 5 days of this pre-training that was then swapped over to food restriction for the remainder of the test. Food/water was taken off 4 hours before testing and returned after testing had been completed. After adequate pre-training, defined as the >90% of mice recording over 100 licks per session, they moved on to 10 days of the test. This involved giving mice either 4% or 16% sucrose solution for 5 days and swapping over for the final 5 days with order being counterbalanced. For both pre-training and test, mice were left in the licking boxes for 10 minutes with the experimenter out the room. Their licking behaviour was measured using MED equipment and consumption was recorded by change in bottle weight (2 d.p.). Mice were tube handled back to their home cages and licking boxes were only cleaned at the end of each day so cleaning fluid would not interfere with the recording.

Analysis

To measure anhedonia in mice, their licking microstructure and consumption of a 4% and 16% sucrose solution was recorded. Consumption (g) was taken by change in drinking bottle weight and lick cluster was calculated by dividing total licks in the session by total clusters (defined with a break in licking of 0.5 seconds; (Davis & Smith, 1992). A larger lick cluster size is indicative of more liking (Davis, 1989; Davis & Levine, 1977; Davis & Smith, 1992) that would be found in the 16% compared to the 4% if hedonic response is intact. For this analysis, there will be a within-subjects comparison across concentrations (i.e., 4% vs 16%) and between-subjects factors will be genotype, age and sex.

Exclusion criteria

Due to the sensitive nature of the recording equipment, data points would need to be excluded if there was a suggestion of equipment malfunction that day. For this, any day where mice had 0 licks were removed due to lack of data. The first day the mice had on a new concentration was also excluded due to the previous concentration potentially producing contrast effects. Next, volume consumed per 1000 licks was calculated (Consumption (g)*1000/total licks) as a way to assess spout issues (e.g., low amount could suggest spout blockage while unexpectedly high amount could suggest a leak). Any data point outside a pre-set range was deemed a spout malfunction and thus excluded. Furthermore, one entire day was excluded due to a global equipment malfunction that was not easily fixed until the following day. The remaining data points were deemed possible and accurate and were thus averaged across each mouse and each concentration. Any mouse without at least 2 good days at a concentration (and thus unable to give an accurate average) was also removed from the data pool.

●2.4.10 Statistical approach

NHST and Bayesian analysis methods

All null hypothesis significance testing (NHST) was done using SPSS (version 26; IBM corp., 2019) while Bayesian statistics were completed in JASP (version 0.14.0.0; (JASP team, 2022). NHST is able to demonstrate how unlikely the observed data would be assuming the null hypothesis is true but is unable to distinguish between evidence for the null and inconclusive data when a $p>.05$ is found. Conversely, Bayesian statistics calculate the strength of the evidence provided by the dataset for both alternate and null hypotheses. Bayesian statistics will be used for non-significant results in order to assess whether it is a probable null result or inconclusive data.

Bayesian statistics utilise Bayes factors that relate to the probability of finding the observed data under a model based on the null or an alternate hypothesis. When using the bayes factor, BF_{01}, the number will range from 0 to infinity where 1 would suggest inconclusive data. However, the greater the number above 1 suggests evidence in favour of the null and, in contrast, the smaller the number below 1 shows evidence in support of the alternate hypothesis. When using BF_{01} to assess the strength of evidence, Jeffreys (1998) offers guidelines to understand them. They say a BF_{01} between 1-3 offers weak or inconclusive evidence while between 3-10 suggest supporting evidence and above 10 would indicate strong evidence for the null.

Factorial Bayes ANOVA will be implemented as previously described (Rouder et al., 2012; Rouder et al., 2017). The default prior scales for fixed and random effects were used and results reported as the analysis of effects – this gives a $BF_{exclusion}$ that is equivalent to BF_{01} when considered across all models that include the factor or interaction of interest.

Ratios and measurements used for behavioural testing

This chapter outlines the results to the behavioural testing that is calculated through discrimination (DR) and preference ratios. Ethovision XT 13 automatically recorded the movement of the mouse while exploration of objects and stranger mice (i.e., as used in SPT and NOR) was measured manually by the experimenter. All ethological behaviours from EPM, SPT and NOR were also recorded manually in Ethovision by the experimenter. Samples of videos were also given to at least one other scientist, they provided scores (blind to condition) of manually recorded data points and these independent scores were correlated with those of the primary experimenter (not blind). This inter-rater reliability score is given in the corresponding behavioural tests results section.

Cumulative timings of exploration or behaviour were imported into excel and ratios calculated using variables outlined in table 2.2. These DRs were then used in ANOVA testing that offer examination of desired behaviour without the confound of general exploration differences between animals. This allows all mice to be assessed on the same scale while still providing an accurate measure of their desired behaviour (Sivakumaran et al., 2018). Raw scores have been examined and commented on if there is an important discrepancy between raw and ratio data.

For lick cluster analysis, average lick cluster was calculated by measuring total licks and total clusters based on a pre-determined pause interval of 0.5 seconds (Davis & Smith, 1992) and finding the mean number of licks per cluster (total licks/total clusters). Any single lick was counted as a '1 lick bout' and were removed from the lick total before calculation. Consumption was measured by a change in bottle weight before and after the test.

Test	Desired behaviour	Total exploration
Open field	Time spent in inner zone (s)	Total exploration time in the maze (s)
Elevated plus maze	Time spent in open arms (s)	Total exploration time in the maze (s)
Social preference test	P1&2: Time spent exploring stranger mouse (s)	P1&2: Time spent exploring both cups (s)
Novel object recognition	Time spent exploring novel object (s)	Time spent exploring all objects (s)

Lick cluster analysis	Total licks (without 1 lick bouts)	Total clusters (defined by a break in licking of 0.5s)

Table 2.2. A list of how all discrimination and preference ratios were calculated. *Desired behaviour/Total exploration was used to reach a value.*

Testing assumptions

Assumptions for ANOVA were analysed graphically in SPSS due to the statistical tests (i.e., Levines test, Bartlett's test, Shapiro-wilks) often proving inaccurate (Atkinson, 1985; Kozak, 2009; Rasch et al., 2011). Due to the randomised design, each observation was seen as independent. Normality of residuals was assessed using Q-Q plots (example in fig. 2.13) or histograms where large variations indicate a violation. However, due to the large sample sizes of mice used in behavioural testing, evidence suggests it is often robust to violations of normality (Blanca Mena et al., 2017; Glass et al., 1972). Therefore, small variations from the line will be overlooked but any large discrepancies will be considered a violation (examples of violations are shown in fig. 2.14 that are from data examined in chapter 3). For Q-Q plots, large discrepancies are defined as the dots clearly not in a straight line or majority of the points being over 0.5 point away from the line (fig. 2.14b).

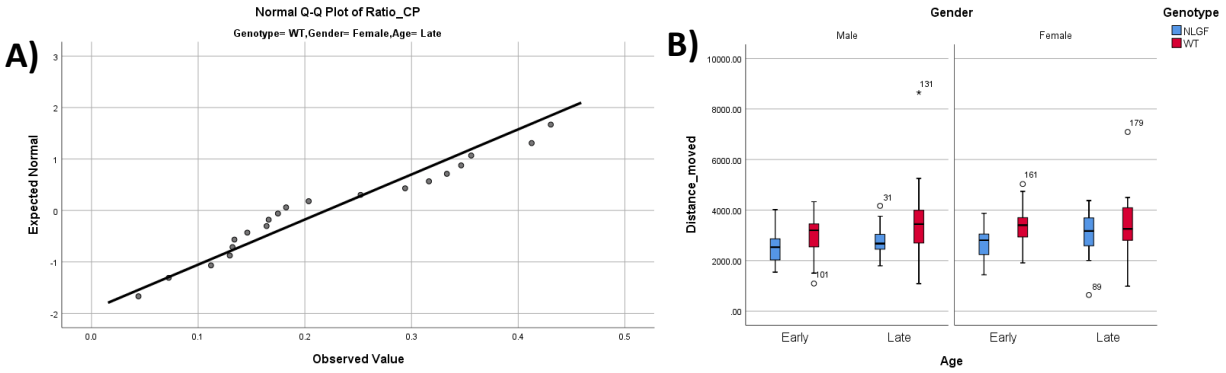

Figure 2.13. Examples of the A) Q-Q plot to assess assumption of normality and B) boxplot to assess assumption of homogeneity of variance. *Normality is not violated if the points are on the line but here there are small deviations which can be expected but as they are still quite close to the line, this is deemed okay. The boxplots are all of similar size but with small differences so also deemed okay.*

Histogram violations were defined as a visible skew. Sphericity was only examined in repeated measures ANOVA (i.e., raw timing calculations, Lick cluster analysis) but large sample sizes often mean Mauchly's test over estimates violations. Homogeneity of variance was tested with boxplots of the variances to see whether box size was equal across groups (example in fig. 2.13). However, the equal and large sample sizes suggest ANOVA to be less sensitive to issues of heterogeneity (Glass et al., 1972) so only large discrepancies (fig. 2.14c) will be considered violations. Large discrepancies are defined as boxes being over 2 times larger than another.

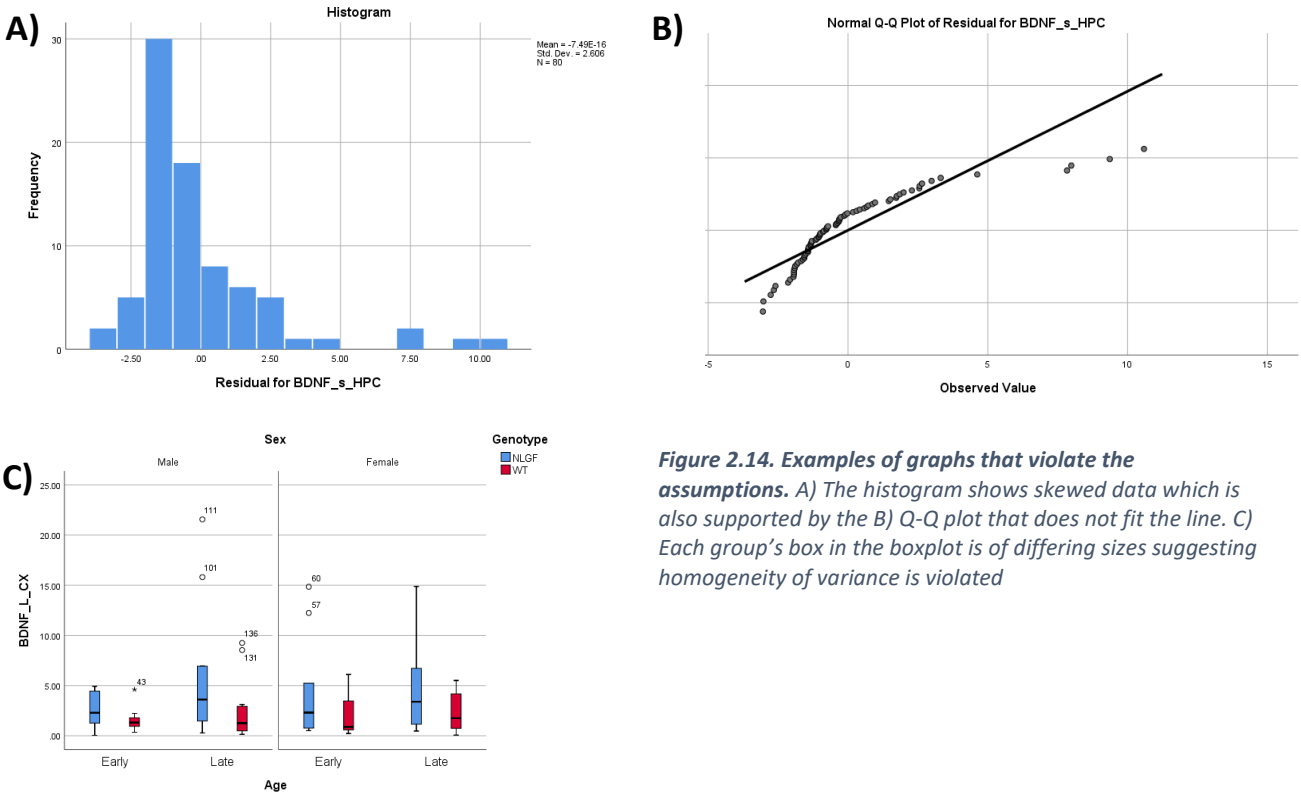

Figure 2.14. Examples of graphs that violate the assumptions. *A) The histogram shows skewed data which is also supported by the B) Q-Q plot that does not fit the line. C) Each group's box in the boxplot is of differing sizes suggesting homogeneity of variance is violated*

●2.5 Results

●2.5.1 Open Field

Anxiety and locomotor activity were assessed using the open field test with EthoVision XT 13 used to automatically track the movement of mice around the arena. Based on the idea that rodents find exposed areas unsettling so prefer to stick close to walls, the arena was split into 2 zones: an outer (safe) zone and an inner (unsafe) zone (see fig 2.8). This safety bias was confirmed by comparing time spent in the inner and outer zone for all animals collapsed across genotype, sex and age ($t(190)=28.055$, $p<.001$) showing a general preference for the outer zone. One mouse (*App*$^{NL-G-F}$, female, young) failed to record so their data was not included (Young *App*$^{NL-G-F}$ male (n=24), young WT male (n=24), young *App*$^{NL-G-F}$ female (n=23), young WT female (n=24), old *App*$^{NL-G-F}$ male (n=24), old WT male (n=28), old *App*$^{NL-G-F}$ female (n=24), old WT female (n=20)).

Movement

Locomotor activity was used to assess general exploratory behaviour as a measure of agitation or apathy. The distance moved in the arena was automatically recording during tracking. Fig 2.15b Suggests *App*$^{NL-G-F}$ mice move less than WT with a slight increase with age. Analysis using a three-way ANOVA confirmed this with main effect of genotype ($F(1, 183)=17.501$, $P<.001$, $\eta^2=.087$) and age ($F(1, 183)=5.538$, $p=.02$, $\eta^2=.029$) but no difference in sex ($F(1, 183)=2.006$, $p=.158$, $\eta^2=.011$, $BF_{exclusion}=4.659$). All the two and three way interactions were non-significant (Genotype*Age, $F(1,183)=.169$, $p=.681$, $\eta^2=.001$, $BF_{exclusion}=.2.706$; Genotype*Sex, $F(1, 183)=.032$, $p=.859$, $\eta^2<.001$, $BF_{exclusion}=6.224$; Age*Sex, $F(1, 183)=.304$, $p=.582$, $\eta^2=.002$, $BF_{exclusion}=7.227$; Genotype*Age*Sex, $F(1, 183)=1.365$, $p=.244$, $\eta^2=.007$, $BF_{exclusion}=61.623$). Overall, WT moved more than *App*$^{NL-G-F}$ mice and the older cohort move more than the younger group. There is no suggestion of interactions between any factors.

To assess speed of movement, total distance travelled was divided by total time spent moving. Both were automatically recorded by EthoVision. Fig. 2.15c suggests *App*$^{NL-G-F}$ mice are slower than WT that is confirmed with a three way ANOVA, $F(1, 183)=11.043$, $p=.001$, $\eta^2=.057$. There was also a significant effect of age ($F(1, 183)=9.873$, $p=.002$, $\eta^2=.051$) and a near significant effect of sex ($F(1, 183)=3.544$, $p=.061$, $\eta^2=.019$, $BF_{exclusion}=1.473$). The older animals seem to move faster in transit compared to the young and while there is a trend for increased speed in females, this was not significant with bayes factors suggesting inconclusive result. All interactions were non-significant (Genotype*sex, $F(1, 183)=3.211$, $p=.075$, $\eta^2=.017$, $BF_{exclusion}=.989$; Genotype*age, $F(1, 183)=1.548$, $p=.215$, $\eta^2=.008$, $BF_{exclusion}=1.168$; Sex*age, $F(1, 183)=.899$, $p=.344$, $\eta^2=.005$, $BF_{exclusion}=2.349$; Genotype*sex*age, $F(1, 183)=2.011$, $p=.158$, $\eta^2=.011$, $BF_{exclusion}=3.751$) but bayes factors suggesting more inconclusive results. This suggests that *App*$^{NL-G-F}$ mice move slower than WT in transit and the old cohort more faster than the young. This will be taken into account when analysing open field data as a possible movement deficit.

Anxiety score in the Open field

Despite *App*$^{NL-G-F}$ mice moving less and slower than WT and young less than old, this does not seem to impact the anxiety score. This is because *App*$^{NL-G-F}$ mice move slower and if this was the reason for a change in anxiety score then it would be expected for them to spend less time in the unsafe zones. This is not found and so this anxiety scoring will be deemed reasonable as a measure of anxious behaviour. To assess anxiety, discrimination ratios (time spent in inner zone(s)/total exploration time(s)) were calculated to represent preference for the unsafe zone where a higher score means more anxiety (fig. 2.15a).

A three way ANOVA compared discrimination ratios (DR) across genotype, age and sex and found a significant main effect of age ($F(1, 183)=177.409$, $p<.001$, $\eta^2=.492$) with a higher DR in young animals suggesting anxiety profiles generally increase with age (fig 3.3a). A main effect of genotype, $F(1, 183)=3.976$, $p=.048$, $\eta^2=.021$, was also found with the lower DR found in the *App*$^{NL-G-F}$ mice which suggests an anxiolytic phenotype. Sex was non-significant with an inconclusive bayes factor ($F(1, 183)=2.112$, $p=.148$, $\eta^2=.011$, $BF_{exclusion}=.489$) as was the genotype*sex interaction ($F(1, 183)=.226$, $p=.635$, $\eta^2=.001$, $BF_{exclusion}=2.75$) and the three way interaction ($F(1, 183)=.042$, $p=.839$, $\eta^2>.001$, $BF_{exclusion}=2.965$) all showing consistent bayes factors. However, there was a significant interaction between genotype*age ($F(1, 183)=4.384$, $p=.038$, $\eta^2=.023$) and sex*age ($F(1, 183)=9.061$, $p=.003$, $\eta^2=.047$) which will be examined closer below.

Simple effects testing found a significant difference of DR across genotypes but only at the young time point ($F(1, 183)=8.369$, $p=.004$, $\eta^2=.044$) and not the old ($F(1, 183)= .005$, $p=.944$, $\eta^2<.001$, $BF_{exclusion}=4.65$) confirming young but not old *App*$^{NL-G-F}$ mice have spent more time in the inner zone. There is also a significant sex effect at the young time point ($F(1, 183)=9.978$, $p=.002$, $\eta^2=.052$) that does not appear in the old cohort ($F(1, 183)=1.21$, $p=.273$, $\eta^2=.007$, $BF_{exclusion}=2.429$) with young males spending more time in the centre zone compared to young females. Altogether, this suggests that young *App*$^{NL-G-F}$ mice show reduced anxiety compared to the young WTs that disappears with age. There is also less anxiety in the young males in comparison to the young females that seems to disappear by the older time point but with a more inconclusive result. This difference disappears with age and all groups increase in anxiety score. It is possible that this loss of an effect in the older cohort is a floor effect but this seems unlikely as all animals still spent time in the inner zone.

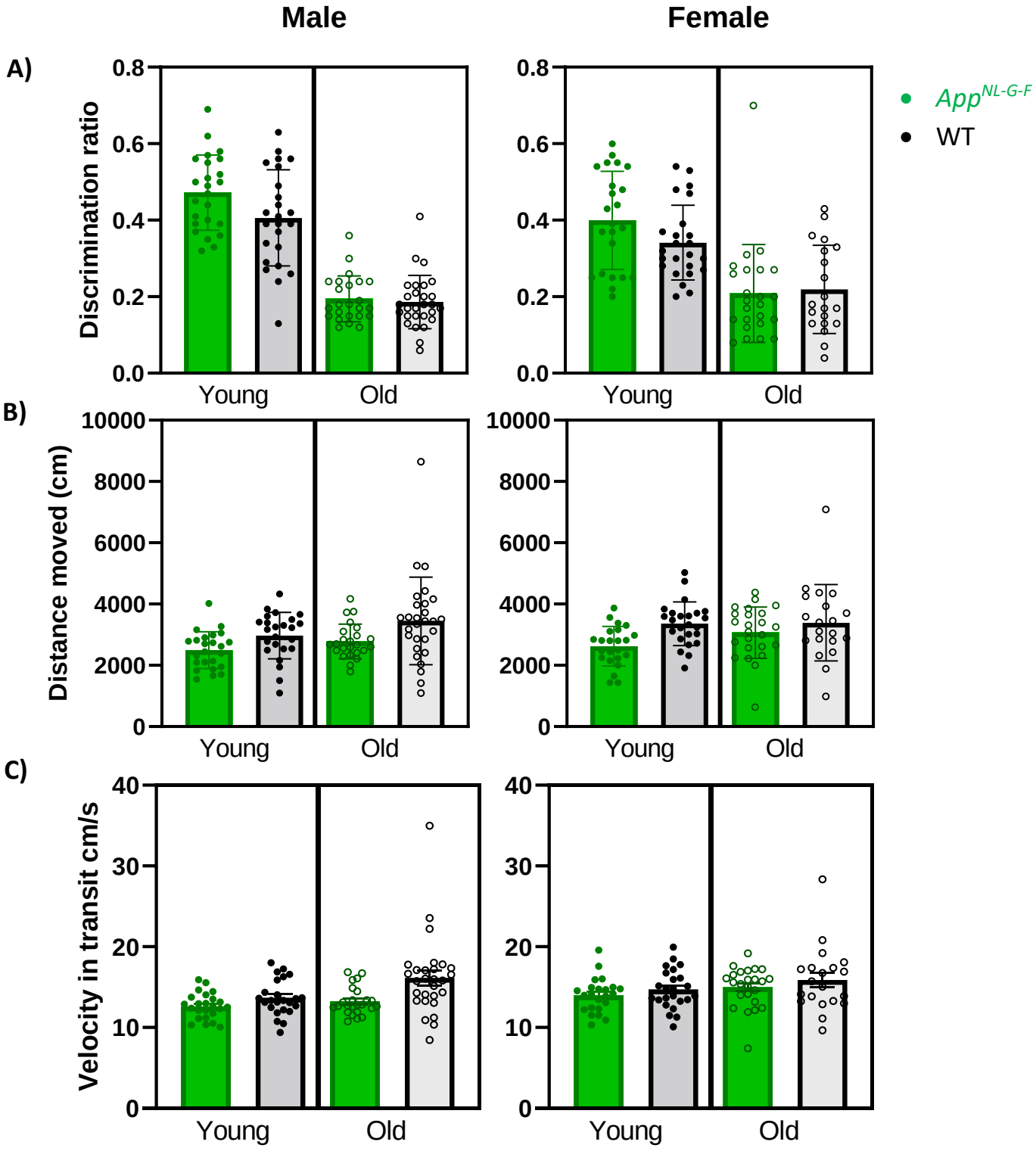

Figure 2.15. Assessment of anxiety and movement between App^{NL-G-F} and WT mice in the open field test. *A) Mean discrimination ratio=time spent in centre zone/total exploration time(s). B) Mean total distance travelled measured by EthoVision XT 13. C) Mean velocity in transit=distance moved (cm)/time spent moving (s). Error bars represent +- SE and each dot is one mouse. Young App^{NL-G-F} male (n=24), young WT male (n=24), young App^{NL-G-F} female (n=23), young WT female (n=24), old App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

●2.5.2 Elevated plus maze

Anxiety in the same cohort of *App*$^{NL-G-F}$ and WT mice was assessed through the Elevated plus maze (EPM) based on the premise that spending more time in the exposed open arms leaves the mice vulnerable to predatory attack and thus suggests a reduction in anxiety. This safety bias was confirmed by comparing time spent in the open compared to the closed arms across all animals regardless of groups ($t(189)=30.924$, $p<.001$) suggesting a general preference for the closed arms. Movement across the EPM was automatically recorded using EthoVision XT 13 where the arena was split into open arms, closed arms and the centre of the cross. Two mice were excluded from analysis (1 young WT male, 1 young *App*$^{NL-G-F}$ female) due to recording malfunction (Young *App*$^{NL-G-F}$ male (n=24), young WT male (n=23), young *App*$^{NL-G-F}$ female (n=23), young WT female (n=24), old *App*$^{NL-G-F}$ male (n=24), old WT male (n=28), old *App*$^{NL-G-F}$ female (n=24), old WT female (n=20)).

Movement

To ascertain whether there were any movement differences between groups, total distance moved was recorded by EthoVision XT 13. This will also be used in conjunction with discerning a potential reduction in anxiety with disinhibition. The three-way ANOVA found a main effect of age ($F(1, 182)=118.706$, $p<.001$, $\eta^2=.395$) with younger animals moving more than older mice as visualised in fig. 2.16a. There was also a main effect of sex ($F(1, 182)=13.417$, $p<.001$, $\eta^2=.069$) where females moved more than males. However, there was no effect of genotype, $F(1, 182)=2.319$, $p=.13$, $\eta^2=.013$, $BF_{exclusion}=2.71$, with a bayes factor suggesting an inconclusive result. All two-way interactions returned non-significant (Genotype * Age, $F(1, 182)=.001$, $p=.978$, $\eta^2<.001$, $BF_{exclusion}=4.254$; Genotype * Sex, $F(1, 182)=1.792$, $p=.182$, $\eta^2=.01$, $BF_{exclusion}=2.171$; Age * Sex, $F(1, 182)=.162$, $p=.687$, $\eta^2=.001$, $BF_{exclusion}=1.903$) with bayes factors suggesting inconclusive or null results. The three-way interaction did return significant, $F(1, 182)=4.949$, $p=.027$, $\eta^2=.026$, where simple effects testing comparing sex effects found significance in the young WT ($F(1, 182)=4.101$, $p=.044$, $\eta^2=.022$) and old *App*$^{NL-G-F}$ mice ($F(1, 182)=14.877$, $p<.001$, $\eta^2=.076$) with females moving more in both groups compared to males. There was no significant sex difference for young *App*$^{NL-G-F}$ ($F(1, 182)=1.455$, $p=.229$, $\eta^2=.008$, $BF_{exclusion}=2.145$) or old WT mice ($F(1, 182)=.063$, $p=.802$, $\eta^2<.001$, $BF_{exclusion}=3.313$). Overall, the lack of significant genotype difference in movement suggests no locomotor deficits but females and younger mice were more likely to move more compared to old and male mice.

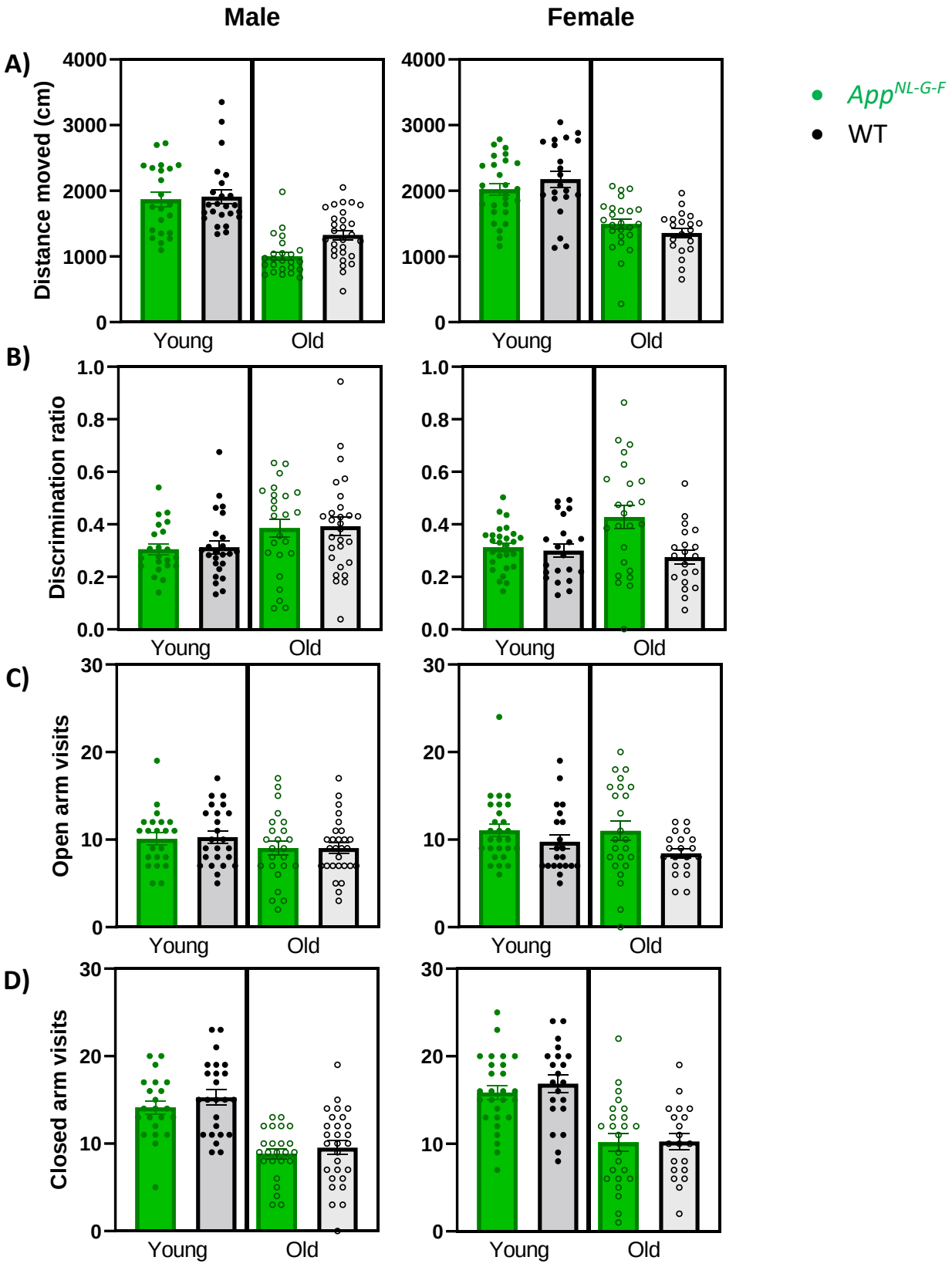

Figure 2.16. Assessment of anxiety through elevated plus maze for App^{NL-G-F} and WT. *A) Discrimination ratio= time in open arms/total exploration time (s). B) Distance moved as measured by EthoVision XT 13 over the EPM test. C) Total count of open arm visits. D) Total count of closed arm visits. Bars represent mean and error bars represent +- SE with each dot corresponding to a mouse. Young App^{NL-G-F} male (n=24), young WT male (n=23), young App^{NL-G-F} female (n=23), young WT female (n=24), App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

Anxiety score in the Elevated Plus maze

Discrimination ratios (DR; time spent in open arms (s)/total exploration time (s)) were calculated and displayed in fig. 2.16b where a higher DR represents preference for the unsafe zone. Total exploration time was time spent in open, closed and centre areas. A higher DR suggests reduced anxiety. This will be compared across genotypes, ages and sexes.

Using DR in a three-way ANOVA found no main effect of genotype ($F(1, 182)=3.069$, $p=.081$, $\eta^2=.017$, $BF_{exclusion}=2.875$) suggesting both *App*$^{NL-G-F}$ and WT have similar preferences for the unsafe zone. However, there was a significant two way interaction between genotype*sex, $F(1, 182)=4.44$, $p=.036$, $\eta^2=.024$, where simple effects testing found significant differences for *App*$^{NL-G-F}$ females ($F(1, 182)=7.197$, $p=.008$, $\eta^2=.038$) but not male mice ($F(1, 182)=.065$, $p=.798$, $\eta^2<.001$, $BF_{exclusion}=5.501$). Female *App*$^{NL-G-F}$ mice have a reduction in anxiety while male *App*$^{NL-G-F}$ mice do not which is supported by the moderate bayes factor. There was also a main effect of age ($F(1, 182)=8.698$, $p=.004$, $\eta^2=.046$) where older mice generally showed lower anxiety compared to younger mice. There was no main effect for sex, $F(1, 182)=.889$, $p=.347$, $\eta^2=.005$, $BF_{exclusion}=5.95$, and all other interactions were non-significant (Genotype * Age, $F(1, 182)=2.719$, $p=.101$, $\eta^2=.015$, $BF_{exclusion}=2.059$; Age * Sex, $F(1, 182)=.635$, $p=.427$, $\eta^2=.003$, $BF_{exclusion}=6.105$; Genotype * Age * Sex, $F(1, 182)=2.734$, $p=.1$, $\eta^2=.015$, $BF_{exclusion}=7.468$). Taken together, while there is no general reduction in anxiety for *App*$^{NL-G-F}$ mice, there is evidence to suggest reduced anxiety for female *App*$^{NL-G-F}$ mice but not males. There is also a general decrease in anxiety score as mice age.

Arm visits

To further assess anxiety, entries into the open arms and closed arms were also automatically counted throughout the recording. For the open arms, the three way ANOVA (fig. 2.16c) found no main effect of genotype ($F(1, 182)=2.828$, $p=.094$, $\eta^2=.015$, $BF_{exclusion}=3.581$), age ($F(1, 182)=2.865$, $p=.092$, $\eta^2=.015$, $BF_{exclusion}=3.92$) or sex ($F(1, 182)=.701$, $p=.403$, $\eta^2=.004$, $BF_{exclusion}=7.151$). All interactions were also non-significant (Genotype * Age, $F(1, 182)=.439$, $p=.509$, $\eta^2=.002$, $BF_{exclusion}=11.002$; Genotype * Sex, $F(1, 182)=3.461$, $p=.064$, $\eta^2=.019$, $BF_{exclusion}=5.771$; Age * Sex, $F(1, 182)=.178$, $p=.674$, $\eta^2=.001$, $BF_{exclusion}=17.868$; Genotype * Age * Sex, $F(1, 182)=.224$, $p=.637$, $\eta^2=.001$, $BF_{exclusion}=137.629$;) with high bayes factors suggesting evidence for the null hypothesis.

Examining the closed arm entries the three-way ANOVA (fig. 2.16d) found no main effect of genotype ($F(1, 182)=1.457$, $p=.229$, $\eta^2=.008$, $BF_{exclusion}=6.501$) with a high bayes factor suggesting evidence for no difference. There was a significant main effect of age, $F(1, 182)=91.54$, $p<.001$, $\eta^2=.335$, with older animals venturing into the closed arms less than younger animals. There was also

a main effect of sex, F(1, 182)=4.767, p=.03, η^2=.026, with females entering the closed arms more than males. All two and three way interactions were non-significant (Genotype * Age, F(1, 182)=.317, p=.574, η^2=.002, $BF_{exclusion}$=7.488; Genotype * Sex, F(1, 182)=.099, p=.753, η^2=.001, $BF_{exclusion}$=11.686; Age * Sex, F(1, 182)=.255, p=.614, η^2=.001, $BF_{exclusion}$=3.49; Genotype * Age * Sex, F(1, 182)=.037, p=.848, η^2<.001, $BF_{exclusion}$=142.449).

Overall, there were no differences in open arm entries between any groups but for the closed arm, younger mice and females entered the closed arms more. This mirrors the distance moved results suggesting movement could be sufficient to explain these results.

Risk assessment behaviour in the EPM

To measure risk assessment behaviour, a variety of ethological behaviours were manually recorded during the EthoVision recording. Time performing each behaviour was taken and used for the below analysis.

Head dips

Defined by the head dipping over the edge of the open arm and down, this measures anxiety (Carola et al., 2002). Time spent doing so was run in a three-way ANOVA and shown in fig. 2.17a. There was no effect of genotype (F(1, 182)=.405, p=.525, η^2=.002, $BF_{exclusion}$=8.986) or sex (F(1, 182)=.196, p=.659, η^2=.001, $BF_{exclusion}$=10.788) with bayes factors suggesting support for the null. There was a significant effect of age, F(1, 182)=8.577, p=.004, η^2=.045, where time spent head dipping increased with age. When examining the interactions, the genotype*age (F(1, 182)=2.387, p=.124, η^2=.013, $BF_{exclusion}$=5.237) and sex*age (F(1, 182)=.174, p=.677, η^2=.001, $BF_{exclusion}$=12.01) came back non-significant but the genotype*sex interaction was significant, F(1, 182)=4.279, p=.04, η^2=.023. Simple effects testing found genotype comparisons for each sex to be non-significant (Male, F(1, 182)= 1.062, p=.304, η_2=.006, $BF_{exclusion}$=1.297; Female, F(1, 182)= 3.537, p=.062, η_2=.019, $BF_{exclusion}$=2.861) with females nearing significance but bayes factors returning inconclusive. The three-way interaction was non-significant, F(1, 182)=2.599, p=.109, η^2=.014, $BF_{exclusion}$=21.822. Overall, older mice spent more time head dipping compared to younger suggesting higher risk assessment activities but no differences between genotypes.

Stretching

Stretching was defined by hind legs within the closed or centre of the cross and the body outstretched in the open arms. This is seen as a cautious assessment of the unsafe area before traversing it. As shown in fig. 2.17b, there is a main effect of genotype, F(1, 182)=51.467, p<.001,

η^2=.22, with WT spending significantly more time stretching into the open arms than *App*$^{NL-G-F}$ mice. There was also a significant effect of age, F(1, 182)=49.965, p<.001, η^2=.215, where the older mice spent more time in this elongated posture to assess the open arms than the younger mice. There was no effect of sex (F(1, 182)=2.081, p=.151, η^2=.011, $BF_{exclusion}$=1.532) with the bayes factor suggesting an inconclusive result. Upon examining the interactions, genotype*sex returned non-significant (F(1, 182)=.026, p=.873, η^2<.001, $BF_{exclusion}$=2.936) as did the three way interaction (F(1, 182)=.004, p=.949, η^2<.001, $BF_{exclusion}$=2.642). The interaction between genotype and age was significant, F(1, 182)=13.688, p<.001, η^2=.07, and simple effects testing found significant genotype differences at both the young (F(1, 182)=5.988, p=.015, η^2=.032) and old (F(1, 182)=59.597, p<.001, η^2=.247) time point where *App*$^{NL-G-F}$ mice stretch less than WT. The age*sex interaction was also significant, F(1, 182)=5.826, p=.017, η^2=.031, with simple effects revealing a significant sex difference at the old (F(1, 182)=7.495, p=.007, η^2=.04) but not the young time point (F(1, 182)=.468, p=.495, η^2=.003=, $BF_{exclusion}$=3.128). Altogether, WT spend more time stretching out than *App*$^{NL-G-F}$ mice showing a more cautious phenotype and females had higher risk assessment behaviour than males but only at the older time point.

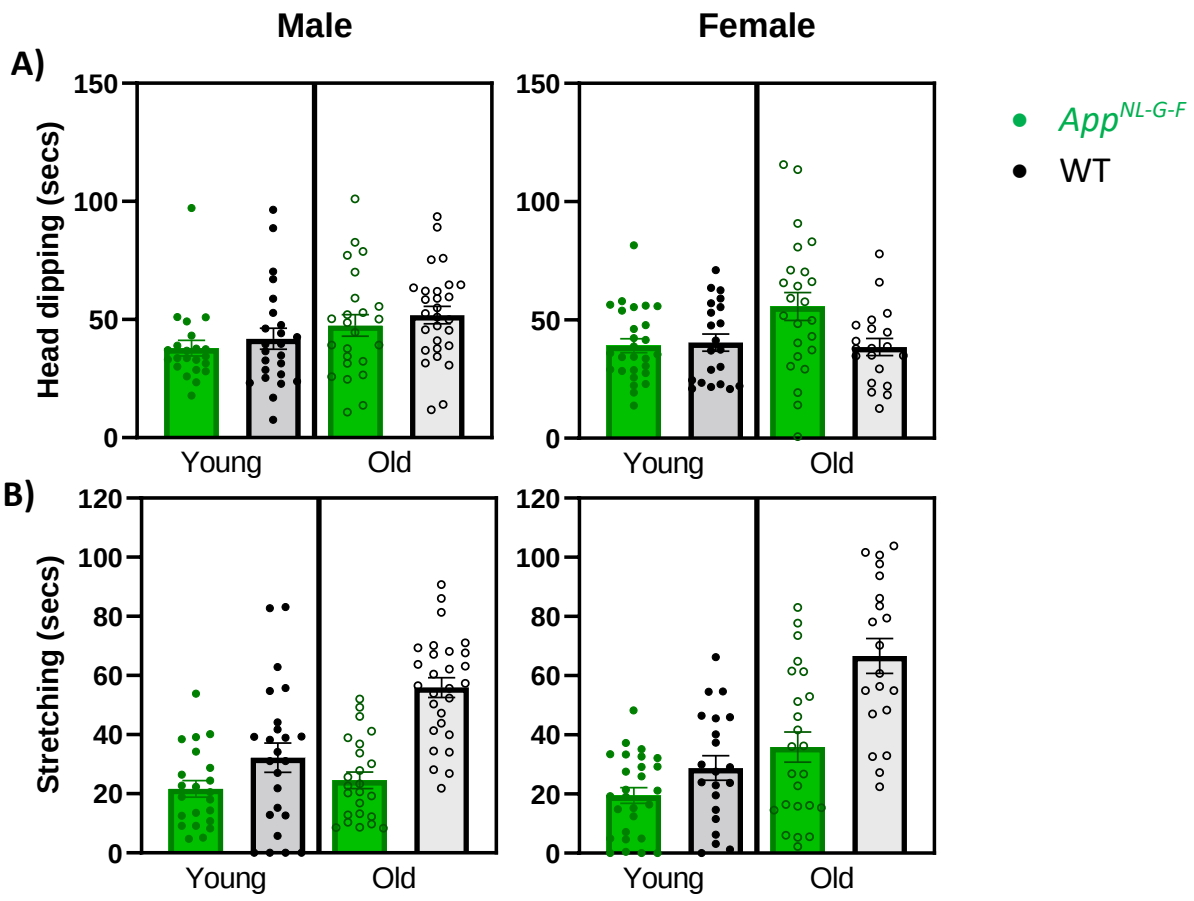

Figure 2.17. Ethological behaviours manually measured in the elevated plus maze for App^{NL-G-F} and WT. *A) Head dipping defined as head over the edge of an open arm to measure anxious exploration. B) Stretch attenuated posture into the open arms defined as hind legs remain in closed or centre sections with front paws and body stretched out into the open arm to measure risk assessment. Bars represent mean and error bars represent +- SE with each dot corresponding to a mouse. Young App^{NL-G-F} male (n=24), young WT male (n=23), young App^{NL-G-F} female (n=23), young WT female (n=24), App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

●2.5.3 Social Preference test

Social interaction and social memory were measured using the 2 phases of the Social preference test. Mouse movement was automatically recorded with EthoVision XT 13 while sniffing of each cup and ethological behaviours were measured manually. An independent researcher also scored exploration of the cups to give an inter-rater reliability score of r(7)=.956, p<.001 for phase 1 and r(7)=.758, p=.029 for phase 2. Phase 1 examined exploration of an empty cup versus a stranger conspecific. Normal mice with intact social preferences will explore the mouse more than the empty cup. Phase 2 filled that empty cup with a second novel mouse to test social memory as mice will explore the new mouse more than the familiar mouse. Due to corruption of an external hard drive, 49 recordings of the young cohort were lost. This impacts the power and thus assumptions were also

scrutinised but not violated. The final group n's are as follows: Young *App*$^{NL-G-F}$ male (n=15), young WT male (n=10), young *App*$^{NL-G-F}$ female (n=9), young WT female (n=13), old *App*$^{NL-G-F}$ male (n=24), old WT male (n=28), old *App*$^{NL-G-F}$ female (n=24), old WT female (n=20). Social preference was confirmed in both phases by a one sample t-test showing a preference for the conspecific in phase 1 ($t(142)=66.54$, $p<.001$) and a preference for the novel mouse in phase 2 ($t(143)=50.835$, $p<.001$). A preference ratio (PR; time spent sniffing novel mouse(s)/total exploration time(s)) was used for each phase independently where a higher PR shows preference for the novel mouse and thus intact social processing.

Distance moved

To measure locomotor activity, the distance moved in centimetres by the mouse was recorded automatically by EthoVision. For phase 1, the three-way ANOVA found a significant effect of genotype ($F(1, 135)=10.371$, $p=.002$, $\eta_2=.071$) where *App*$^{NL-G-F}$ mice moved further than WT (fig. 2.18c). There was no effect of age ($F(1, 135)=.083$, $p=.774$, $\eta_2=.001$, $BF_{exclusion}=1.709$) but the main effect of sex was significant ($F(1, 135)=10.416$, $p=.002$, $\eta_2=.072$) showing females moving more than males. However, there was a significant interaction between genotype*age, $F(1, 135)=6.982$, $p=.009$, $\eta_2=.049$, and simple effects testing found a significant genotype difference at the old ($F(1, 135)=26.622$, $p<.001$, $\eta_2=.165$) but not the young time point ($F(1, 135)=.123$, $p=.726$, $\eta_2=.001$, $BF_{exclusion}=3.449$). This suggests that increased locomotor activity is found in *App*$^{NL-G-F}$ mice which is exacerbated by age. There was also a significant interaction between genotype and sex, $F(1, 135)=10.656$, $p=.001$, $\eta_2=.073$, where a difference in genotype was shown for females ($F(1, 135)=19.685$, $p<.001$, $\eta_2=.127$) and not males ($F(1, 135)=.001$, $p=.974$, $\eta_2<.001$, $BF_{exclusion}=3.716$). As seen in fig. 2.18b, females move more than males and the female *App*$^{NL-G-F}$ mice move even more than male *App*$^{NL-G-F}$ mice. There was no interaction between age*sex ($F(1, 135)=.685$, $p=.409$, $\eta_2=.005$, $BF_{exclusion}=1.5$) or genotype*age*sex ($F(1, 135)=2.502$, $p=.116$, $\eta_2=.018$, $BF_{exclusion}=0.485$).

Distance moved was also recorded in phase 2 and found similar results. A main effect of genotype ($F(1, 136)=16.096$, $p<.001$, $\eta_2=.106$) and sex ($F(1, 136)=11.513$, $p=.001$, $\eta_2=.078$) was found again showing *App*$^{NL-G-F}$ mice and females moving more than WT and males respectively (fig. 2.18d). There was no effect of age ($F(1, 136)=.049$, $p=.825$, $\eta_2<.001$, $BF_{exclusion}=0.465$). The interaction between genotype*age was significant ($F(1, 136)=10.192$, $p=.002$, $\eta_2=.07$) and simple effects testing found a genotype difference only in old ($F(1, 136)=39.506$, $p<.001$, $\eta_2=.225$) but not young ($F(1, 136)=.25$, $p=.618$, $\eta_2=.002$, $BF_{exclusion}=3.389$). This suggests that *App*$^{NL-G-F}$ mice show a hyperactive phenotype but more so as they age. As in phase 1, there was also a significant interaction between genotype*sex ($F(1, 136)=13.187$, $p<.001$, $\eta_2=.088$) where there was a significant genotype difference

in females (F(1, 136)=26.874, p<.001, η_2=.165) but not males (F(1, 136)=.079, p=.779, η_2=.001, $BF_{exclusion}$=1.815). Together, this shows *App*$^{NL-G-F}$ female mice display hyperactivity compared to WT but this does not extend to *App*$^{NL-G-F}$ male mice. There was no interaction between age*sex, F(1, 136)=.329, p=.567, η_2=.002, $BF_{exclusion}$=0.651, but the three-way interaction was significant (F(1, 136)=4.387, p=.038, η_2=.031) unlike in phase 1. The simple effects revealed a significant difference between *App*$^{NL-G-F}$ and WT mice but only for the old stage females (F(1, 136)=58.67, p<.001, η_2=.301) and not the other groups (Young male, F(1, 136)=.105, p=.746, η_2=.001, $BF_{exclusion}$=1.982; young female, F(1, 136)=.95, p=.332, η_2=.007, $BF_{exclusion}$=0.985; old male, F(1, 136)=.909, p=.342, η_2=.007, $BF_{exclusion}$=0.843). This suggests that the old female *App*$^{NL-G-F}$ mice display a hyperactive phenotype compared to the old WT females. Overall, this shows that *App*$^{NL-G-F}$ mice show a hyperactive phenotype in the social preference test with females showing higher movement compared to males. These differences in genotype are more pronounced in females especially in the older time point suggesting female *App*$^{NL-G-F}$ mice become more hyperactive with age which does not seem to be as pronounced in males. This will be explored further in the discussion (see section 2.6).

Social interaction

For phase 1, a three-way ANOVA on PR scores with genotype, age and sex as factors was carried out (see fig. 2.18a). Despite a suggested trend in *App*$^{NL-G-F}$ mice reduced preference for social interaction, there was no effect of genotype (F(1, 135)=3.875, p=.051, η_2=.028, $BF_{exclusion}$=2.021), age (F(1, 135)=.504, p=.479, η_2=.004, $BF_{exclusion}$=10.004) or sex (F(1, 135)=.998, p=.319, η_2=.007, $BF_{exclusion}$=7.761). However, it should be noted that the bayes factor for genotype suggests a more inconclusive result opposed to support for the null and coupled with a p value of 0.051, supports an inconclusive result. All two and three way interactions were also non-significant (Genotype*Age, F(1, 135)=.199, p=.656, η_2=.001, $BF_{exclusion}$=15.122; Genotype*Sex, F(1, 135)=.48, p=.49, η_2=.004, $BF_{exclusion}$=9.917; Age*Sex, F(1, 135)<.001, p=.991, η_2<.001, $BF_{exclusion}$=30.085; Genotype*Age*Sex, F(1, 135)=.266, p=.607, η_2=.002, $BF_{exclusion}$=263.204) with bayes factors supporting strong evidence for the null. Overall, this suggests that social interaction does not differ between age points and sexes but the genotype result is inconclusive.

Social preference

Phase 2 changed the empty cup for a new conspecific and the three-way ANOVA revealed no effect of genotype (F(1, 136)=.334, p=.565, η_2=.002, $BF_{exclusion}$=10.437), age (F(1, 136)=2.075, p=.152, η_2=.015, $BF_{exclusion}$=6.324) or sex (F(1, 136)<.001, p=.99, η_2<.001, $BF_{exclusion}$=12.752), see Fig 2.17b. All two and three way interactions were also non-significant with matching bayes factors

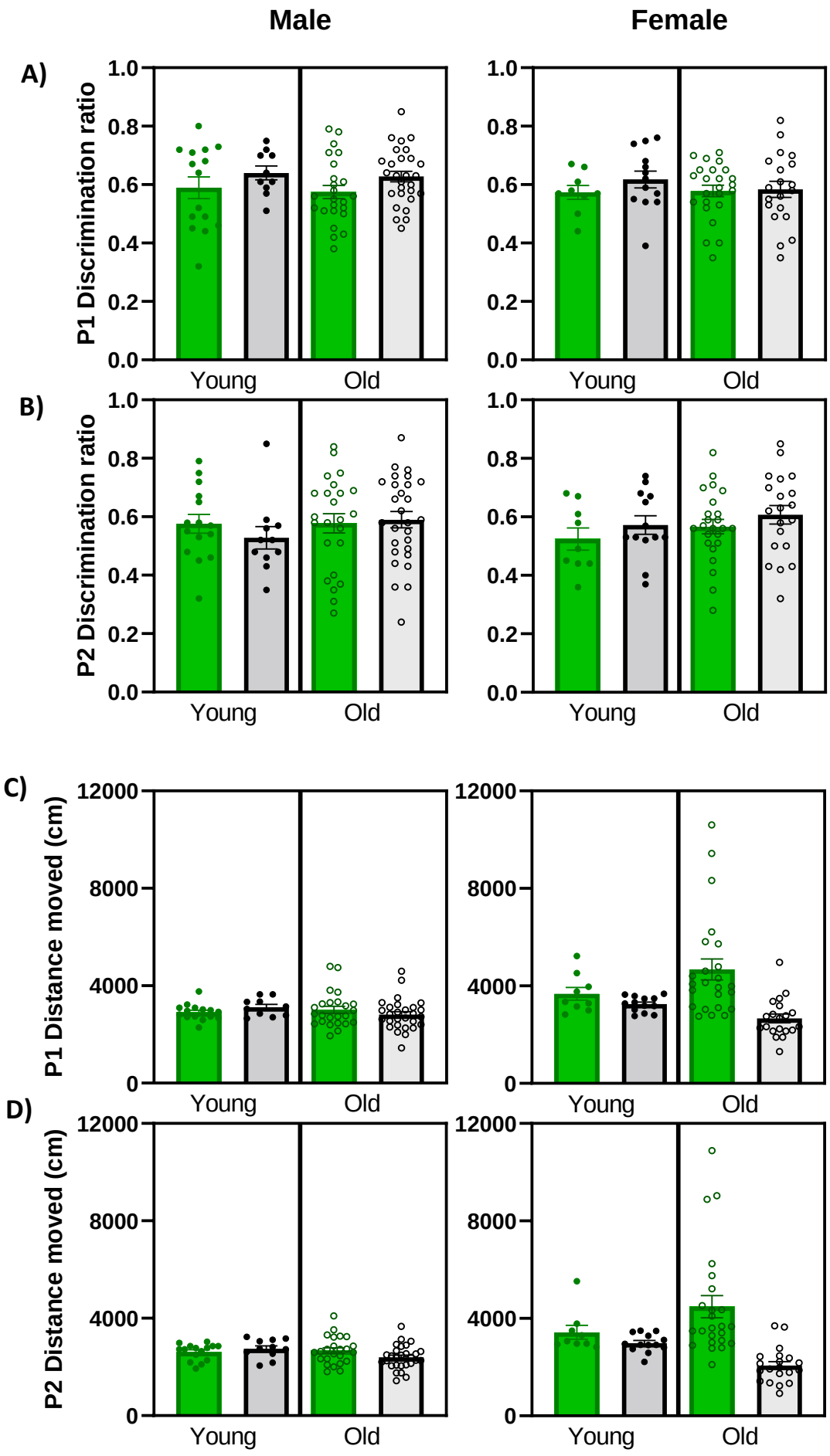

Figure 2.18. Assessment of social deficits of the App^{NL-G-F} and WT through the Social preference test (P1= Social preference, P2= Social recognition/novelty). *Discrimination ratio = exploration of stranger mouse/total exploration. A) P1 compares exploration of stranger mouse and an empty cup. B) P2 compares exploration of stranger mouse and a familiar mouse. C) Phase 1 & D) Phase 2 distance moved is automatically recorded with EthoVision XT 13. Bars represent mean, error bars represent +- SE and each dot corresponds to one mouse. Mice were recorded at a young (5-7 month old) and old (12-14 month old) App^{NL-G-F} were compared to WT. Young App^{NL-G-F} male (n=15), young WT male (n=10), young App^{NL-G-F} female (n=9), young WT female (n=13), old App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

(Genotype*Age, F(1, 136)=.299, p=.585, η_2=.002, $BF_{exclusion}$=28.788; Genotype*Sex, F(1, 136)=1.688, p=.196, η_2=.012, $BF_{exclusion}$=37.482; Age*Sex, F(1, 136)=.019, p=.892, η_2<.001, $BF_{exclusion}$=39.512; Genotype*Age*Sex, F(1, 136)=.471, p=.494, η_2=.003, $BF_{exclusion}$=582.185). Altogether, this suggests that there is no social memory issue in the *App*$^{NL-G-F}$ mice at either age point.

Ethological behaviours in SPT

Immobility

Time spent with the body remaining motionless was taken as a measure of disinterest. The three-way ANOVA is visualised in fig. 2.19a that shows a significant effect of genotype (F(1, 135)=4.439, p=.037, η_2=.032) where *App*$^{NL-G-F}$ mice show less time spent immobile. There was also a significant effect of age (F(1, 135)=5.055, p=.026, η_2=.036) as older mice spend more time motionless than younger. While there was no main effect of sex (F(1, 135)=.687, p=.409, η_2=.005, $BF_{exclusion}$=3.601), there was a significant interaction between genotype*age (F(1, 135)=5.922, p=.016, η_2=.042) where simple effects testing revealed a significant genotype difference in the old (F(1, 136)=15.969, p<.001, η_2=.106) but not the young time point (F(1, 136)=.039, p=.843, η_2<.001, $BF_{exclusion}$=1.902). All other interactions were non-significant (Genotype*Sex, F(1, 135)=2.493, p=.117, η_2=.018, $BF_{exclusion}$=1.508; Age*Sex, F(1, 135)=.674, p=.413, η_2=.005, $BF_{exclusion}$=3.501; Genotype*Age*Sex, F(1, 135)=.517, p=.474, η_2=.004, $BF_{exclusion}$=3.295). While WT may show less interest in the cup and stranger mouse, this is greatly increased with age as they spend more time motionless than *App*$^{NL-G-F}$ mice.

Immobility during phase 2 was also measured using a three way ANOVA (fig. 2.19a). Like phase 1, there was a significant main effect of genotype, F(1, 136)=8.914, p=.003, η_2=.062, with WT showing increased immobility. However, the main effect of age did not remain for phase 2 (F(1, 136)=1.866, p=.174, η_2=.014, $BF_{exclusion}$=0.221) but a significant effect of sex did (F(1, 136)=4.509, p=.036, η_2=.032) where females spent more time immobile than males. For the interactions, there was a significant genotype*age effect (F(1, 136)=8.011, p=.005, η_2=.056) with simple effects showing a genotype difference in the old (F(1, 136)=25.745, p<.001, η_2=.159) but not young time point (F(1, 136)=.009, p=.925, η_2<.001, $BF_{exclusion}$=3.483). WT, again, show more time spent immobile but only at the older stage. A significant genotype*sex interaction (F(1, 136)=11.204, p=.001, η_2=.076) lead to simple effects testing that showed the significance to be from a genotype difference in females (F(1, 135)=18.449, p<.001, η_2=.119) but not males ((1, 135)=.072, p=.789, η_2=.001, $BF_{exclusion}$=4.228). So WT have higher immobility time but only the females. The other interactions were non-significant (Age*Sex, F(1, 136)=2.931, p=.089, η_2=.021, $BF_{exclusion}$=0.219; Genotype*Age*Sex, F(1, 136)=3.647, p=.058, η_2=.026, $BF_{exclusion}$=0.084) but with bayes factors that disagree. Like phase 1, WT show

greater immobility than *App*$^{NL-G-F}$ mice that is more pronounced in the older time point and in females. However, bayes factors often suggest evidence for the alternative when NHST reports no significance.

Overall, WT spend more time immobile in both phases of the social preference test. In the first phase, older mice had increased immobility that was only found in the WT in phase 2. All of this confirms the movement findings and shows there is a genotype difference between basic locomotor activity.

Grooming

Time spent grooming was defined as licking paws and body or rubbing face and was taken as a measure of anxious locomotor activity (Carola et al., 2002). Fig. 2.19b depicts the three way ANOVA, during phase 1, that showed no genotype effect ($F(1, 135)=.42$, $p=.518$, $\eta_2=.003$, $BF_{exclusion}=9.516$) with congruent bayes factors. There was a significant age effect ($F(1, 135)=7.194$, $p=.008$, $\eta_2=.051$) showing time spent grooming was much lower in the older mice. Females also spent less time grooming as shown by a significant main effect of sex, $F(1, 135)=6.01$, $p=.016$, $\eta_2=.043$. All interactions were non-significant (Genotype*Age, $F(1, 135)=1.109$, $p=.294$, $\eta_2=.008$, $BF_{exclusion}=8.007$; Genotype*Sex, $F(1, 135)=1.092$, $p=.298$, $\eta_2=.008$, $BF_{exclusion}=10.519$; Age*Sex, $F(1, 135)=1.122$, $p=.291$, $\eta_2=.008$, $BF_{exclusion}=2.358$; Genotype*Age*Sex, $F(1, 135)=.464$, $p=.497$, $\eta_2=.003$, $BF_{exclusion}=46.997$) with bayes factors generally suggesting evidence for a null result. Together, this shows that WT and *App*$^{NL-G-F}$ mice spend just as much time grooming but this time decreases with age and is lower in females.

This was also measured in the second phase as shown in fig. 2.19b. However, during this phase, there was no main effect of genotype ($F(1, 136)=.982$, $p=.323$, $\eta_2=.007$, $BF_{exclusion}=7.309$), age($F(1, 136)=3.295$, $p=.072$, $\eta_2=.024$, $BF_{exclusion}=3.004$) or sex ($F(1, 136)=3.652$, $p=.058$, $\eta_2=.026$, $BF_{exclusion}=3.68$). All two way interactions were also non-significant (Genotype*Age, $F(1, 136)=.281$, $p=.597$, $\eta_2=.002$, $BF_{exclusion}=11.353$; Genotype*Sex, $F(1, 136)=.615$, $p=.434$, $\eta_2=.005$, $BF_{exclusion}=12.746$; Age*Sex, $F(1, 136)=2.017$, $p=.158$, $\eta_2=.015$, $BF_{exclusion}=4.78$) but the three way interaction was significant ($F(1, 136)=7.552$, $p=.007$, $\eta_2=.053$). Simple effects testing found a significant difference between genotypes only in the old females ($F(1, 136)=4.26$, $p=.041$, $\eta_2=.03$) but not the other groups (young male, $F(1, 136)=3.256$, $p=.073$, $\eta_2=.023$, $BF_{exclusion}=1.206$; young female, $F(1, 136)=1.614$, $p=.206$, $\eta_2=.012$, $BF_{exclusion}=0.863$; old male, $F(1, 136)=.082$, $p=.776$, $\eta_2=.001$, $BF_{exclusion}=3.449$). However, the bayes factors for the other groups suggest an inconclusive result and the significant difference in the older females seems to be driven by 1 mouse who groomed for 35 seconds.

Overall, there does not appear to be any differences between the groups self-grooming habits during the second phase of the social preference test.

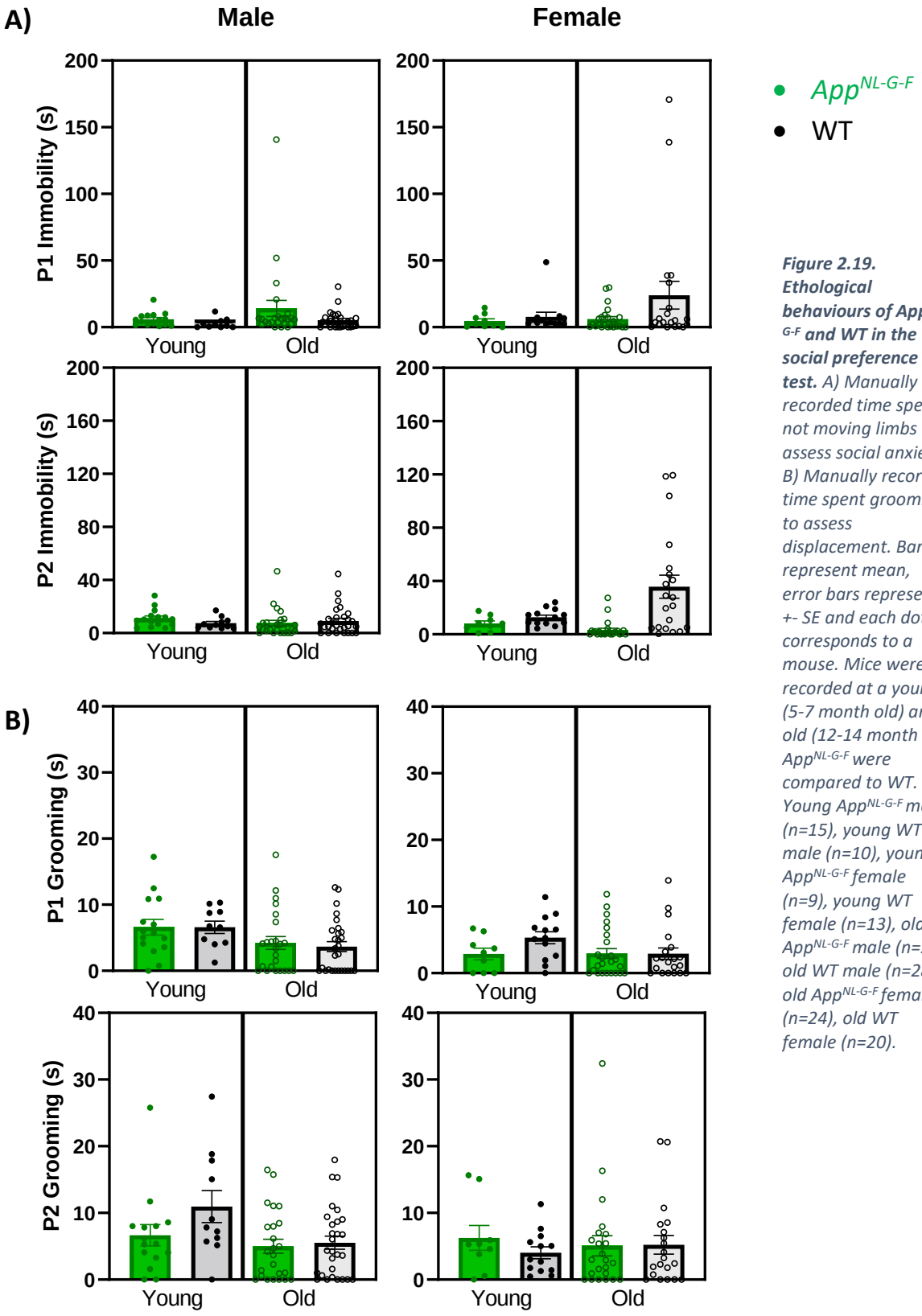

Figure 2.19. Ethological behaviours of App^{NL-G-F} and WT in the social preference test. *A) Manually recorded time spent not moving limbs to assess social anxiety. B) Manually recorded time spent grooming to assess displacement. Bars represent mean, error bars represent +- SE and each dot corresponds to a mouse. Mice were recorded at a young (5-7 month old) and old (12-14 month old) App^{NL-G-F} were compared to WT. Young App^{NL-G-F} male (n=15), young WT male (n=10), young App^{NL-G-F} female (n=9), young WT female (n=13), old App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

Overall, time spent grooming in both phases is greatly varied for all groups but there is no difference in the self-cleaning habits of *App*$^{NL-G-F}$ and WT mice. There is some suggestion that time spent grooming decreases with age and is reduced in females but this was only apparent during the first phase of the SPT.

●2.5.4 Lick cluster analysis

To check whether there was evidence of gross motor issues in licking, a three-way repeated measures ANOVA was done on their inter-lick interval which is the average time between licks in a cluster (i.e., the speed of licking). Means and standard deviations are shown in fig 2.20b. There was a main effect of age ($F(1, 164)=28.405$, $p<.001$, $\eta^2=.148$) and sex ($F(1, 164)=23.156$, $p<.001$, $\eta^2=.124$) suggesting that the older cohort have more time between each lick and females also have a higher inter-lick interval. Importantly, there was no effect of genotype, $F(1, 164)=3.679$, $p=.057$, $\eta^2=.022$, $BF_{exclusion}=.04$. However, it did approach conventional significance where *App*$^{NL-G-F}$ have a slower lick speed and the bayes factors supports the presence of an effect which will be considered in relation to bodyweight. Inter-lick interval did not change as a function of concentration either ($F(1, 163)=.872$, $p=.352$, $\eta_2=.005$, $BF_{exclusion}=3.52$) so licking behaviour was comparable across concentrations. When assessing amount consumed per 1000 licks (described in section 2.4.9) as another measure of base drinking behaviour (i.e., average amount of liquid taken in per 1000 licks) the same effects were observed. There was a main effect of age ($F(1, 164)=14.258$, $p<.001$, $\eta^2=.08$) and sex ($F(1, 164)=6.123$, $p=.014$, $\eta^2=.036$) but no effect of genotype ($F(1, 164)=3.232$, $p=.074$, $\eta^2=.019$, $BF_{exclusion}=5.469$). There was also no effect of concentration, $F(1, 163)=1.895$, $p=.171$, $\eta_2=.011$, $BF_{exclusion}=13.912$. All other additional interactions for both amount and ILI are shown in table 2.3 and are not important for this analysis. While genotype does not impact on inter-lick intervals, both sex and age do which will be taken into account when interpreting data.

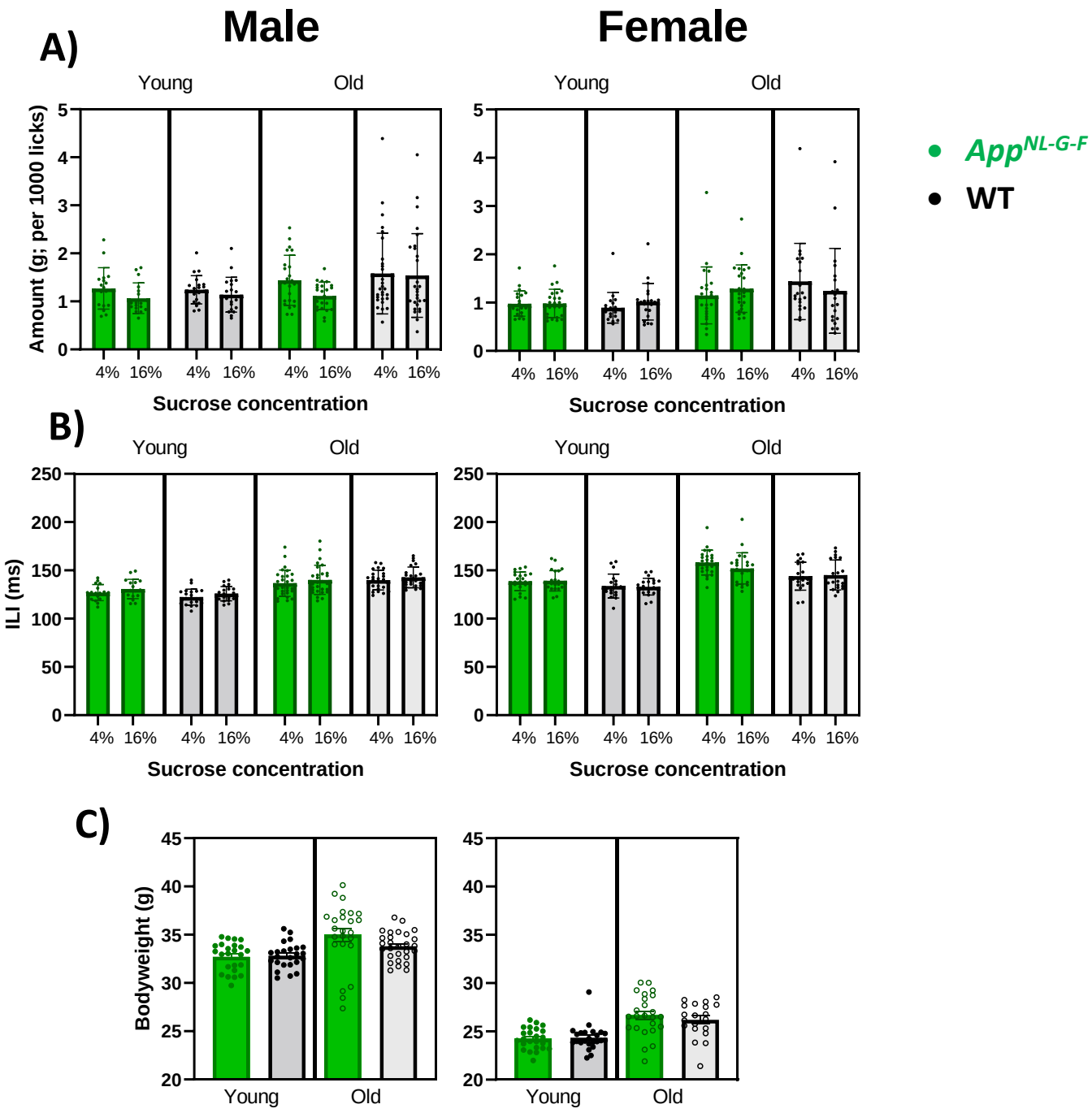

Figure 2.20 Distribution for amount per 1000 licks, interlick interval and bodyweight in the lick cluster analysis. *A) (Amount (g)*1000)/total licks to check for potential spout leakage. B) Interlick interval calculated by (total licks – 1 lick bouts)/total bouts to assess speed of licking. C) Bodyweight (g) of mice across groups. Graphs represent mean and error bars +- SEM. Mice were recorded at a young (5-7 month old) and old (12-14 month old) App^{NL-G-F} were compared to WT. Young App^{NL-G-F} male (n=17), young WT male (n=22), young App^{NL-G-F} female (n=24), young WT female (n=21), old App^{NL-G-F} male (n=23), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

Bodyweight itself has been shown to have an impact on consumption so will be considered. The average bodyweights are shown in fig. 2.20c and a three way ANOVA showed increases in bodyweight with age ($F(1, 184)=48.596$, $p<.001$, $\eta^2=.209$) and higher weights in males ($F(1,184)=927.055$, $p<.001$, $\eta^2=.834$) but no genotype differences ($F(1, 184)=1.79$, $p=.183$, $\eta^2=.01$,

$BF_{exclusion}$=2.809). All interactions for bodyweight were non-significant (Genotype*Sex, F(1, 191)=.520, p=.472, η^2=.003, $BF_{exclusion}$=3.958; Genotype*Age, F(1, 191)=2.945, p=.088, η^2=.016, $BF_{exclusion}$=1.481; Sex*Age, F(1, 191)=.888, p=.347, η^2=.005, $BF_{exclusion}$=1.288; Genotype*Sex*Age, F(1, 191)=.458, p=.499, η^2=.002, $BF_{exclusion}$=15.518). These bodyweight results could account for the differences in licking behaviour (as assessed above) between sexes and ages so will thus be considered in the licking behaviour analysis from above. Importantly, no differences in genotype were found.

	ILI					**Amount**			
	F	p	η_2	$BF_{exclusion}$		F	p	η_2	$BF_{exclusion}$
Genotype*Age	.234	.629	.001	3.158		1.935	.166	.012	4.659
Genotype*Sex	1.533	.217	.009	1.648		.686	.409	.004	7.153
Sex*Age	.031	.861	<.001	3.412		.4	.528	.002	5.396
Genotype*Sex*Age	1.216	.272	.007	12.308		.141	.708	.001	73.783
Concentration*genotype	.896	.345	.005	5.083		.249	.618	.002	27.841
Concentration*sex	7.664	.006*	.045	-		2.5	.116	.015	7.732
Concentration*age	.318	.573	.002	7.207		.218	.641	.001	17.282
Concentration*Genotype*Sex	.601	.439	.004	9.482		1.489	.224	.009	103.041
Concentration*Genotype*Age	1.404	.238	.009	47.113		.511	.476	.003	300.227
Concentration*Sex*Age	.051	.821	<.001	26.231		.048	.826	<.001	184.755
Concentration*Genotype*Sex*Age	.957	.329	.006	2117.832		2.251	.135	.014	33601.617

Table 2.3 All interactions for interlick interval (ILI) and amount (average liquid taken in per 1000 licks) for App^{NL-G-F} and WT. *This includes all analyses not alluded to in the text.*

Exclusion criteria can be read in section 2.4.9 that lead to the removal of 13 mice (7 young *App*$^{NL-G-F}$ male, 1 old *App*$^{NL-G-F}$ male, 2 young WT male and 3 young WT female). This leaves a total of 179 mice with the split shown in the caption to fig. 2.20.

Consumption

Consumption of 4% and 16% sucrose solutions were measured for each mouse and it was found that mice generally show a greater acceptance for the 16% ($F(1, 171)=66.138$, $p<.001$, $\eta_2=.279$). Looking at the between subjects measures in the repeated measures ANOVA, there was a significant effect of genotype ($F(1, 171)=5.387$, $p=.021$, $\eta_2=.031$) showing *App*$^{NL-G-F}$ mice consume less sucrose overall. There was also a significant main effect of age ($F(1, 171)=5.274$, $p=.023$, $\eta_2=.03$) where older mice drink more sucrose than the younger time point. There was no effect of sex, $F(1, 171)=.451$, $p=.503$, $\eta_2=.003$, $BF_{exclusion}=7.2$. However, when examining the within subjects factors, there was no effect of concentration*genotype ($F(1, 171)=.205$, $p=.651$, $\eta_2=.001$, $BF_{exclusion}=4.08$), concentration*age ($F(1, 171)=1.662$, $p=.199$, $\eta_2=.01$, $BF_{exclusion}=3.01$) or concentration*sex ($F(1, 171)=.898$, $p=.345$, $\eta_2=.005$, $BF_{exclusion}=8.93$). All between subjects interactions were non-significant (Genotype*Age, $F(1, 171)=.091$, $p=.763$, $\eta_2=.001$, $BF_{exclusion}=3.797$; Genotype*Sex, $F(1, 171)=.02$, $p=.889$, $\eta_2<.001$, $BF_{exclusion}=6.58$; Age*Sex, $F(1, 171)=.811$, $p=.369$, $\eta_2=.005$, $BF_{exclusion}=4.897$; Genotype*Age*Sex, $F(1, 171)=.166$, $p=.684$, $\eta_2=.001$, $BF_{exclusion}=18.76$). The majority of the interactions with concentration were also non-significant (Concentration*genotype*sex, $F(1, 171)=.157$, $p=.693$, $\eta_2=.001$, $BF_{exclusion}=110.586$; concentration*sex*age, $F(1, 171)=.294$, $p=.589$, $\eta_2=.002$, $BF_{exclusion}=63.74$; concentration*genotype*sex*age, $F(1, 171)=.013$, $p=.909$, $\eta_2<.001$, $BF_{exclusion}=2240.57$) except for a concentration*genotype*age effect ($F(1, 171)=6.885$, $p=.009$, $\eta_2=.039$) that is likely coming from the greater discrepancy in 4%/16% consumption in young WT compared to *App*$^{NL-G-F}$ mice but the reverse at the old time point. So, while there was a global change in sucrose consumption, the proportion of 4% to 16% sucrose remained similar across all groups.

Bodyweight controlled consumption

As bodyweight can impact amount of sucrose consumed, it was controlled for to see whether the main effects of genotype and age remained (consumption(g)/(bodyweight$^{0.75}$) as shown by Wright et al. (2020). The repeated measures ANOVA found the effect of concentration was significant ($F(1, 171)=66.138$, $p<.001$, $\eta_2=.279$) showing mice consumed more of the 16% solution. Looking to the main effects, the effect of genotype ($F(1, 171)=6.894$, $p=.009$, $\eta_2=.036$) and age ($F(1, 171)=7.714$, $p=.006$, $\eta_2=.04$) remained after controlling for bodyweight. This shows that *App*$^{NL-G-F}$ mice still drink less and older mice drink more when controlling for bodyweight. The difference here is the main effect of sex becomes significant ($F(1, 171)=5.315$, $p=.022$, $\eta_2=.028$) that suggests that

females actually consume more in proportion to their bodyweight than males. When taking concentration into account, the interaction with genotype ($F(1, 171)=.291$, $p=.59$, $\eta_2=.002$, $BF_{exclusion}=4.962$) and age ($F(1, 171)=2.125$, $p=.147$, $\eta_2=.011$, $BF_{exclusion}=5.258$) remain non-significant. However, the concentration*sex becomes significant ($F(1, 171)=4.006$, $p=.047$, $\eta_2=.021$, $BF_{exclusion}=2.221$) and simple effects shows there is a significant sex effect at 16% ($F(1, 171)=4.276$, $p=.04$, $\eta_2=.024$) but not 4% ($F(1, 171)=1.407$, $p=.237$, $\eta_2=.008$, $BF_{exclusion}=4.68$). For the between subjects factor, all interactions were, again, non-significant (Genotype*age, $F(1, 171)=.4$, $p=.528$, $\eta_2=.002$, $BF_{exclusion}=3.97$; genotype*sex, $F(1, 171)=.405$, $p=.525$, $\eta_2=.002$, $BF_{exclusion}=4.292$; age*sex, $F(1, 171)=1.423$, $p=.234$, $\eta_2=.008$, $BF_{exclusion}=4.491$; genotype*age*sex, $F(1, 171)=1.057$, $p=.305$, $\eta_2=.006$, $BF_{exclusion}=18.232$). All other interactions with concentration are reported but not important for this analysis (Concentration*Genotype*Sex, $F(1, 171)=.608$, $p=.436$, $\eta_2=.003$, $BF_{exclusion}=39.279$; Concentration*Genotype*Age, $F(1, 171)=6.566$, $p=.011$, $\eta_2=.034$; Concentration*Sex*Age, $F(1, 171)=.16$, $p=.69$, $\eta_2=.001$, $BF_{exclusion}=35.279$; Concentration*Genotype*Sex*Age, $F(1, 171)=.061$, $p=.805$, $\eta_2<.001$, $BF_{exclusion}=765.193$).

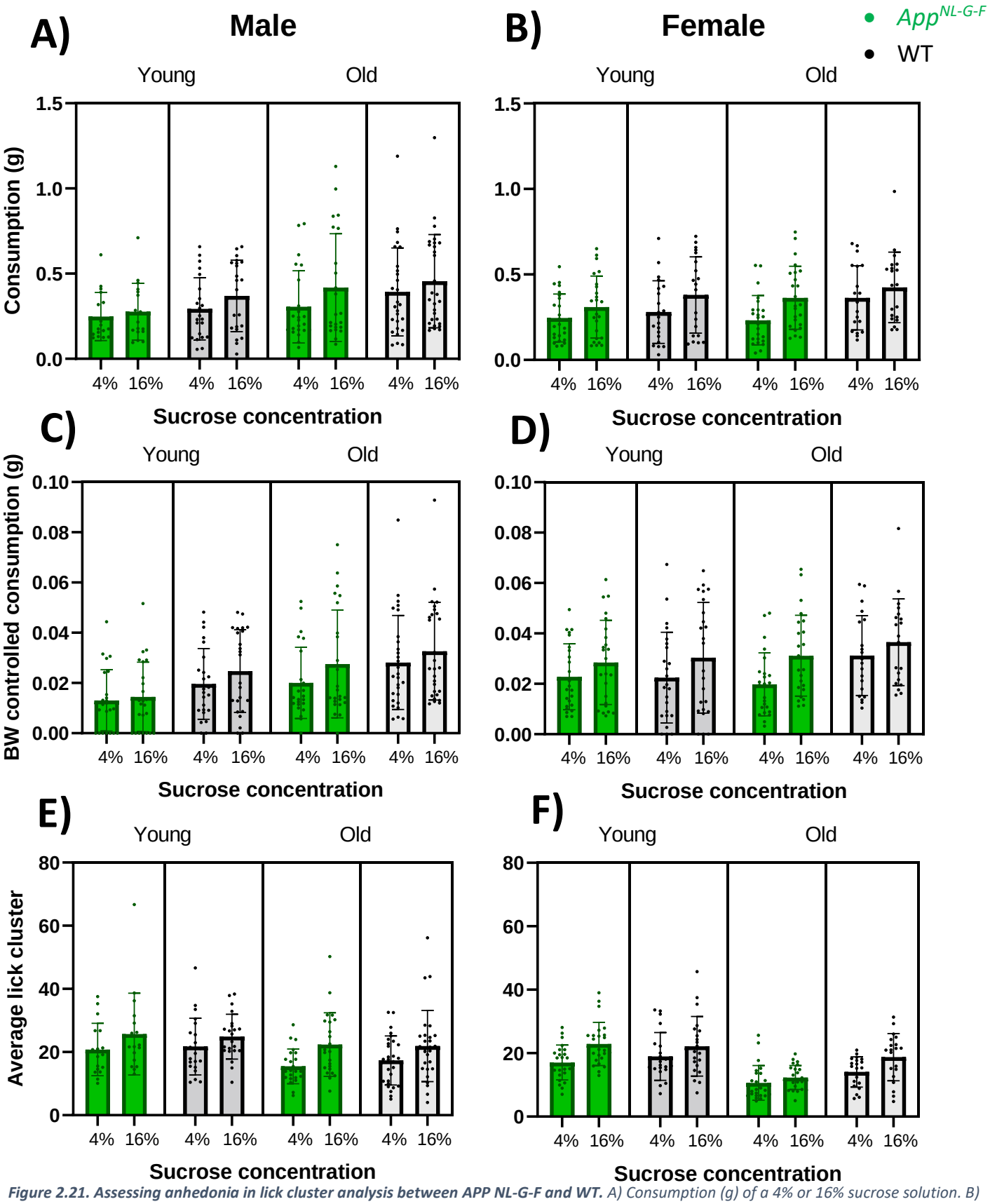

Figure 2.21. Assessing anhedonia in lick cluster analysis between APP NL-G-F and WT. *A) Consumption (g) of a 4% or 16% sucrose solution. B) Bodyweight controlled (consumption(g)/(bodyweight$^{0.75}$) consumption. C) Average lick cluster ((total licks-1 lick bouts)/total clusters) as measured with a break of at least 0.5 between licks. Bars represent mean, error bars represent +- SE and each dot is one mouse. Mice were recorded at a young (5-7 month old) and old (12-14 month old) App^{NL-G-F} were compared to WT. Young App^{NL-G-F} male (n=17), young WT male (n=22), young App^{NL-G-F} female (n=24), young WT female (n=21), old App^{NL-G-F} male (n=23), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

Lick cluster size

If the near genotype difference of slower lick speed (i.e., ILI) does impact lick cluster then it would be expected to reduced average lick cluster. This does not occur so average lick cluster genotype differences can be taken without caveats of lick speed.

A repeated measures ANOVA was done to evaluate a within subjects effect of Concentration ($F(1, 171)=49.491$, $p<.001$, $\eta_2=.224$) reflecting the typical higher palatability for higher concentrations of sucrose (fig. 2.21e-f). When looking at the main effects of between subject factors, the significant genotype effect found in consumption was not replicated in lick cluster ($F(1, 171)=2.342$, $p=.128$, $\eta_2=.014$, $BF_{exclusion}=5.014$). The bayes factor reinforces the support of a null result with no difference between genotypes. There was a significant effect of age ($F(1, 171)=25.329$, $p<.001$, $\eta_2=.129$) and sex ($F(1, 171)=16.339$, $p<.001$, $\eta_2=.087$), showing average lick cluster goes down with age and females have smaller lick clusters than males. The between subjects interactions were non-significant (Genotype*Age, $F(1, 171)=1.458$, $p=.229$, $\eta_2=.008$, $BF_{exclusion}=3.913$; Genotype*Sex, $F(1, 171)=1.352$, $p=.247$, $\eta_2=.008$, $BF_{exclusion}=4.118$; Age*Sex, $F(1, 171)=1.291$, $p=.257$, $\eta_2=.007$, $BF_{exclusion}=2.042$; Genotype*Age*Sex, $F(1, 171)=.808$, $p=.37$, $\eta_2=.005$, $BF_{exclusion}=14.596$). All within subjects interactions were also non-significant (Concentration*genotype, $F(1, 171)=.571$, $p=.451$, $\eta_2=.003$, $BF_{exclusion}=8.548$; Concentration*sex, $F(1, 171)=.692$, $p=.407$, $\eta_2=.004$, $BF_{exclusion}=4.316$; Concentration*age, $F(1, 171)=.019$, $p=.89$, $\eta_2<.001$, $BF_{exclusion}=6.007$; Concentration*Genotype*Sex, $F(1, 171)=.792$, $p=.375$, $\eta_2=.005$, $BF_{exclusion}=70.626$; Concentration*Genotype*Age, $F(1, 171)=1.065$, $p=.304$, $\eta_2=.006$, $BF_{exclusion}=71.701$; Concentration*Sex*Age, $F(1, 171)=1.503$, $p=.222$, $\eta_2=.009$, $BF_{exclusion}=14.981$; Concentration*Genotype*Sex*Age, $F(1, 171)=1.548$, $p=.215$, $\eta_2=.009$, $BF_{exclusion}=4232.51$). Overall, this suggests that while average lick cluster does not differ between genotypes, it does go down with age and females have lower lick cluster than males.

Taken together, these results suggest that there is no anhedonia in the *App*$^{NL-G-F}$ mice but they consume less suggesting a possible motivational deficit. Females have a lower lick cluster and consume more in relation to their bodyweight than males and lick slower. Older mice also have a lower lick cluster but still consume more even with a slower lick speed.

●2.5.5 Novel object recognition

To assess recognition memory, mice were presented with a novel and familiar object and time spent exploring each was manually recorded. If mice successfully recalled the familiar object

then they should spend more time exploring the novel based on that notion that mice prefer novelty (Ennaceur & Delacour, 1988). As this was recorded manually, a second independent researcher, blind to the group, also rated a sample of these runs that gave a significant inter rate reliability, r(7)=79.46, p=.028. The timing spent exploring each object was used to calculate a discrimination ratio (exploration of novel object (s)/total exploration time (s)) to represent retention of memory for the novel object. To establish if the mice were discriminating between object type, a one sample t-test against 0.5 was done, collapsing across genotype and sex, that found mice generally have a preference for the novel object, t(95)=28.686, p<.001. No mice were excluded.

Due to experimenter error, this test was only run on the older cohort so a two-way ANOVA was done on DR and visualised in fig. 2.22. Object in place was run on the younger cohort but the test was run incorrectly and so the results, being meaningless, are not reported. There was no difference between genotypes (F(1, 92)=.058, p=.81, η_2=.001, $BF_{exclusion}$=6.174) or sexes (F(1, 92)=.627, p=.43, η_2=.007, $BF_{exclusion}$=4.906) with bayes factors suggesting support for a null result. The interaction was also non-significant, F(1, 92)=.988, p=.323, η_2=.011, $BF_{exclusion}$=14.946. With Bayes agreeing, this suggests that there is no recognition memory deficit in the *App*$^{NL-G-F}$ mice at 12-14 months.

Distance moved in NOR

General locomotor activity was taken as a measure of general exploration and the two way ANOVA found no effect of genotype (F(1, 92)=1.423, p=.236, η_2=.015, $BF_{exclusion}$=3.089) or sex (F(1, 92)=1.648, p=.202, η_2=.018, $BF_{exclusion}$=2.801) and no interaction (F(1, 92)=.001, p=.978, η_2<.001, $BF_{exclusion}$=8.841). As shown in fig. 2.22b, the movement of each group is comparable with no group differences.

Raw exploratory scores in NOR

In contrast to DR data, analysis of the raw scores revealed a significant effect of genotype (F(1, 92)=8.603, p=.004, η_2=.086) showing the *App*$^{NL-G-F}$ mice have higher contact times than WT (fig. 2.20c). However, there was no effect of sex (F(1, 92)=.546, p=.462, η_2=.006, $BF_{exclusion}$=6.801) and no interaction (F(1, 92)=1.63, p=.205, η_2=.017, $BF_{exclusion}$=4.717). The interaction between object and genotype is not significant (F(1, 92)=.487, p=.487, η_2=.005, $BF_{exclusion}$=1.761) suggesting that although the *App*$^{NL-G-F}$ mice explore the objects more, they distribute exploratory activity across the familiar and novel objects in a manner consistent with WT mice.

Immobility in NOR

Time spent immobile was taken to measure anxiety and is shown in fig. 2.22d. Two way ANOVA revealed a significant main effect of genotype ($F(1, 92)=4.238$, $p=.042$, $\eta_2=.044$) as *App*$^{NL-G-F}$ mice spend less time immobile and this did not differ between sexes ($F(1, 92)=.805$, $p=.372$, $\eta_2=.009$, $BF_{exclusion}=4.225$). The interaction was non-significant, $F(1, 92)=.636$, $p=.427$, $\eta_2=.007$, $BF_{exclusion}=4.776$, altogether, showing *App*$^{NL-G-F}$ mice are less immobile during the NOR test.

Grooming in NOR

The cumulative duration of time spent grooming was taken to measure displacement behaviour as visualised in fig. 2.22e. ANOVA revealed no main effect of genotype ($F(1, 92)=1.508$, $p=.223$, $\eta_2=.016$, $BF_{exclusion}=2.873$), sex ($F(1, 92)=2.193$, $p=.142$, $\eta_2=.023$, $BF_{exclusion}=2.137$) and no interaction ($F(1, 92)=.002$, $p=.966$, $\eta_2<.001$, $BF_{exclusion}=7.478$) but bayes factors suggested the main effect results may be inconclusive rather than strong evidence for a null result. Altogether, there are no differences in time spent grooming across groups.

Rearing in NOR

Rearing was measured as an exploratory behaviour and the cumulative duration during the NOR test is shown in fig. 2.22f. There was no difference between genotypes ($F(1, 92)=3.063$, $p=.083$, $\eta_2=.032$, $BF_{exclusion}=1.619$) despite a slight trend in fig. 2.22f showing reduction in *App*$^{NL-G-F}$ mice and the bayes factor suggests this is an inconclusive result. There was also no main effect of sex ($F(1, 92)=.877$, $p=.352$, $\eta_2=.009$, $BF_{exclusion}=4.298$) and no interaction ($F(1, 92)=.674$, $p=.414$, $\eta_2=.007$, $BF_{exclusion}=5.666$). Together, this suggests that mice spend equal time rearing regardless of their sex or genotype.

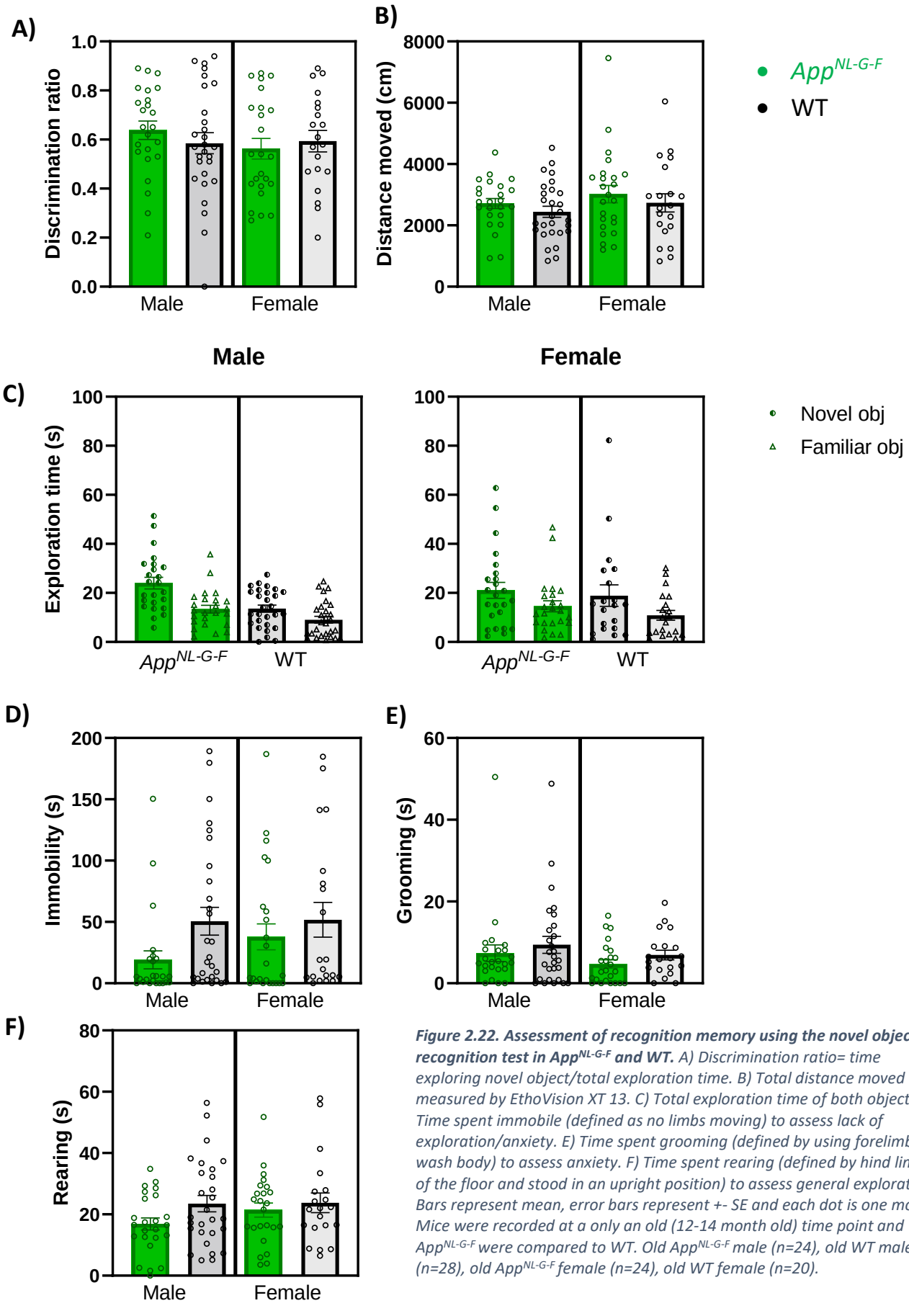

Figure 2.22. Assessment of recognition memory using the novel object recognition test in App^{NL-G-F} and WT. *A) Discrimination ratio= time exploring novel object/total exploration time. B) Total distance moved measured by EthoVision XT 13. C) Total exploration time of both objects. D) Time spent immobile (defined as no limbs moving) to assess lack of exploration/anxiety. E) Time spent grooming (defined by using forelimbs to wash body) to assess anxiety. F) Time spent rearing (defined by hind limbs of the floor and stood in an upright position) to assess general exploration. Bars represent mean, error bars represent +- SE and each dot is one mouse. Mice were recorded at a only an old (12-14 month old) time point and App^{NL-G-F} were compared to WT. Old App^{NL-G-F} male (n=24), old WT male (n=28), old App^{NL-G-F} female (n=24), old WT female (n=20).*

●2.6 Discussion

The aim of this chapter was to answer the question of whether amyloid causes the development of certain affective deficits in a novel KI mouse model, *App*$^{NL-G-F}$ mouse. Through a battery of behavioural tests, a reduction in anxiety was found in the OF and for female *App*$^{NL-G-F}$ mice in the EPM. This genotype effect vanished at the older time point in the OF. These findings somewhat confirm previous research and the discrepancy will be discussed. Despite past literature showing social interaction deficits at 12 months, this research found an inconclusive result that could show that, if there are social deficits, they are not very strong. Furthermore, recognition memory was tested in the NOR that previously showed deficits after 9 months of age but this was not found at 12-14 months. However, the novel test to examine anhedonia, lick cluster analysis, was done to show no anhedonia but did show reduced consumption despite normal liking. This, together with reduced movement across the anxiety tests is indicative of an apathetic phenotype. These findings will be discussed in relation to previous literature and the role amyloid is playing.

The *App*$^{NL-G-F}$ mice seemed to show movement deficits in the OF that could interfere with the interpretation. However, this reduced movement was only found in both anxiety tests and not when other stimuli were present in the arena (i.e., SPT and NOR) suggesting the movement deficit was not universal. Moreover, the *App*$^{NL-G-F}$ mice move less that would suggest a reduced amount of time spent in the open spaces simply by chance, but the opposite is found. This suggests that despite a movement deficit, it does not impact the interpretation of the anxiety score in OF and EPM.

While an anxiolytic phenotype was found in the OF at the young time point, this disappeared by 12 months. Latif-Hernandez et al. (2019) also found this unusual finding but Whyte et al. (2018), Kundu et al. (2022) and Maezono et al. (2020) found no difference at any age point. They also did not find any difference in distance travelled while the present study and Latif-Hernandez et al. (2019) found reduced locomotor activity. While it is likely down to differences in lab practices, it may simply highlight that findings from the OF in *App*$^{NL-G-F}$ mice are not robust. Furthermore, Pervolaraki et al. (2019) found an anxiogenic phenotype at 7-8 months. They measured this using absolute times opposed to a discrimination ratio that is subject to bias. They also split the arena into 3 layers opposed to 2: outer, middle and inner. These different parameters allow them to conclude *App*$^{NL-G-F}$ mice have higher thigmotaxis compared to control but this could be confounded by time spent in the middle zone which is not reported. Again, it seems the results of OF are more susceptible to laboratory differences.

The lack of difference between *App*$^{NL-G-F}$ and WT mice found in the OF at the older time point could possibly be a floor effect. It is possible that there would be a genotype difference if the test

was more sensitive (e.g., of longer duration). The area of the outer zone is also much larger than the area for the inner that could explain a potential floor effect. In any case, there is still an age difference showing an increase in anxiety in the OF compared to a trend to decrease in the EPM.

The EPM provides a much more robust finding that generally concurs with all the previous literature (Johnson et al., 2020; Latif-Hernandez et al., 2019; Pervolaraki et al., 2019; Sakakibara et al., 2018). From 6 months of age, there is an anxiolytic phenotype found where *App*$^{NL-G-F}$ mice spend more time in the open arms of the EPM compared to the closed arms. However, this was only confirmed in females but, due to the large body of evidence also present for males, it is likely still present but we were unable to uncover it. This also has a trend to increase with age but that is non-significant. The discrepancy between the EPM and the OF could suggest they measure different aspects of anxiety. In fact, OF was originally used as a general test of emotionality (Hall, 1934) opposed to anxiety specifically. Other suggestions are that OF is more a test of general anxiety opposed to pathological anxiety seen in disorders (Harro, 2018) due to the fact EPM creates a more anxiogenic environment (Carola et al., 2002). This makes sense as the open space in the OF is still enclosed in 4 walls while, in the EPM, it encompasses the entire room creating a far greater expanse. Therefore, it is likely that the reduction in anxiety at 5-7 months encompasses both pathological and normal anxiety but, with age, normal anxiety returns to normal while reductions in pathological anxiety increase.

Anxiolytic phenotypes can also be described as disinhibition which is also a neuropsychiatric symptom seen in AD (Chung & Cummings, 2000; Hart et al., 2003). Pervolaraki et al. (2019) found reduced glutamatergic signalling in the prefrontal cortex but not the amygdala suggesting the anxiolytic phenotypes others found were likely the result of top down processes such as disinhibition. In behaviour, the anxiolytic behaviour coupled with increased locomotor activity is often concluded to be disinhibition. However, it is more likely that reduced anxiety and disinhibition have some overlap and are not entirely distinct. The present study measured ethological behaviours to further assess anxiety and found no difference in head dipping (an anxious exploration behaviour) but did show reduced stretch attenuated posture (SAP; risk assessment behaviour; (Walf & Frye, 2007)). So, *App*$^{NL-G-F}$ mice assess risk less that is in line with disinhibitory tendencies (Lalonde et al., 2012).

Results suggest that amyloid pathology causes disinhibition and reduced anxiety. This raises the possibility that past work on transgenic Aβ mice that found increased anxiety (Lalonde et al., 2012) could be the result of complications of *APP* overexpression opposed to amyloid pathology per se. While the amygdala is often cited as important in anxiety (Moreira et al., 2007; Silveira et al.,

1993), this was found to be intact in the *App*$^{NL-G-F}$ mice unlike the PFC (Pervolaraki et al., 2019). Disruption to the frontal cortex (FC) has also been associated with disinhibition in humans with AD (Finger et al., 2017; Tanguy et al., 2022) suggesting issues in integrating various social and emotional information. Therefore, it is likely that amyloid interferes with the FC processing of integrating information that can lead to anxiolytic and disinhibitory phenotype seen in AD. It is also sensible to examine noradrenergic deficits in this development due to their role in fear and anxiety (Ma & Morilak, 2005). Using an α2-AR agonist (Clonidine), researchers found it had a dose dependent relationship on reducing fear in rodents (Davis et al., 1979). Noradrenaline is also important in the release of corticotrophin-releasing hormone (CRH) and using a CRH1 receptor antagonist promoted anxiolytic behaviour in mice (Bertagna et al., 2021). Alterations to the LC-NA system could potentially play a role in anxiolytic behaviour.

While the present study did not find any significant social deficits, previous work with the *App*$^{NL-G-F}$ mice has. Latif-Hernandez et al. (2019) found 10 month old *App*$^{NL-G-F}$ mice have a reduced affinity for the stranger mouse compared to both an empty cup and a familiar mouse. However, they did use the *App*^{NL} mice as a control opposed to WT that makes it difficult to draw comparisons. The *App*^{NL} model only exhibits the Swedish mutation but does not develop any plaques and is a good control mouse to look at the added effects of the other mutations that do cause plaques to develop (e.g. Beyreuther/Iberian and Arctic mutation). Other research has found that *App*^{NL} mice actually show an anxiogenic phenotype compared to WT while *App*$^{NL-G-F}$ mice show an anxiolytic one (Sakakibara et al., 2018) that could mean results from the present study are difficult to directly compare to this research. Further evidence for this is that, in phase 2, neither the *App*^{NL} nor the *App*$^{NL-G-F}$ mice showed a healthy preference for the stranger mouse suggesting a global deficit caused by the Swedish mutation (Sakakibara et al., 2018). Another difference is Latif-Hernandez et al. (2019) only examined proximity to the cups opposed to actual exploratory behaviour making it a far more crude measure. They also only looked at females that have been shown to have greater social deficits (Pervolaraki et al., 2019) but the present study did not reflect this. Both sexes may approach sociality differently so it is important to get data from both males and females. To further illustrate this point, Pervolaraki et al. (2019) did measure direct exploratory behaviour (e.g. sniffing of object/mouse) and found no difference at 7-8 month old mice. Although this is still quite young and may be that social deficits have not developed yet, measuring direct exploratory behaviour provides a more accurate result. Locci et al. (2021) also looked at exploratory behaviour but did find a deficit in 10 month old *App*$^{NL-G-F}$ mice. They concluded this using raw exploratory time and not a discrimination ratio that can be subject to bias by not including time spent with the cup/familiar mouse.

One big difference between the present study and past research is the configuration of the arena. While most use a 3 chamber arena, this study used opposite corners of a large square box similar to the configuration of objects in NOR. While this does not allow a measure of chamber entries, it still offers a valid measure of discrimination as there is enough distance between them to warrant distinct exploration. The use of a very familiar arena was also able to offer reduced anxiety for exploring the mice.

Examination of the ILI and amount consumed per 1000 licks revealed older mice and females had a slower lick speed. There was no difference in licking speed between genotypes. This suggests that the lower lick cluster size found in older mice could be down to the program not differentiating the slower lick speed and an actual break between clusters as well as mice with faster licking. Said differently, the program would likely break their licking into more clusters due to the fact their slow licking may go beyond the 0.5s cutoff. The same could be true for the females. However, this is not true for genotype differences so it does seem the *App*$^{NL-G-F}$ mice have similar average lick clusters but interpretations of sex and age are more complex to assess.

The lick cluster analysis found no difference in the liking of sucrose solution between genotypes suggesting a normal hedonic response. However, this was accompanied by reduced consumption that suggests a reduction in motivation or possibly an apathetic response to sucrose. This could also be reflected in *App*$^{NL-G-F}$ mice reduced locomotor activity during the anxiety tests that is also seen as a crude measure for apathy (Kosel et al., 2020). Apathy is seen as a distinct disorder from depression so will be treated as such (Landes et al., 2001). While very little direct examination of apathy has been done on transgenic and KI mouse models, a review reported apathetic phenotypes is not often found in transgenic mice. However, using break point analysis, Hamaguchi et al. (2019) found 4-10 month old *App*$^{NL-G-F}$ mice showed no difference in motivation to get a reward. Break point represents active attainment of a reward while LCA is more a passive gain which could explain the discrepancy. Research looking at locomotor activity in the *App*$^{NL-G-F}$ mice has found increased locomotor activity (Latif-Hernandez et al., 2019) or no difference (Pervolaraki et al., 2019) during anxiety tests. Whyte et al. (2018) did show reduced movement speed in the OF but no difference in path length. However, this reduced locomotor activity found in the present study was only found in the anxiety tests and not in social interaction or NOR that both include stimuli in the arena. The extra objects or conspecifics could create different motivations that an empty arena does not provide making it so locomotor activity is no different between genotypes in the Social interaction test and NOR.

While no previous work on hedonic preferences has been done in the *App*$^{NL-G-F}$ mice, transgenic mice have been examined through the sucrose preference test and lick cluster analysis. Romano et al. (2015) found 3xTg-AD mice showed reduced preference for sucrose compared to water despite total fluid consumption remaining equal over 24hrs. This dip in consumption could suggest either anhedonia or apathy since sucrose preference test can be confounded by motivation. Other mouse models of amyloid pathology undergoing the sucrose preference test show no changes in sucrose consumption compared to control (Pfeffer et al., 2018; Vloeberghs et al., 2007). The 3xTg-AD mice are some of the only triple transgenic mice who exhibit tau pathology and other transgenic models of tauopathy show reduced consumption of food pellets (Van der Jeugd et al., 2013). It is possible that reduced consumption is a result of the tau pathology and not amyloid but it is also difficult to understand whether this is a deficit of motivation or enjoyment. Lick cluster analysis is able to differentiate these and thus can be viewed as a superior test of motivation and hedonic responses. Lick cluster analysis was run on the amyloid model, Tg2576, in the same lab as the present study to find anhedonia through equal lick clusters to a 4% and 16% solution (Brelsford et al., 2017). They found an age related reduction in lick cluster size up to 16 months of age despite increased sucrose consumption that is contradictory to the present findings. Given the difference, the anhedonia in the Tg2576 is likely down to complications of the transgenic models that the KI do not experience. In any case, the present studies suggest that amyloid pathology can cause motivational deficits such as apathy.

Post mortem studies of humans rarely find a connection between amyloid build up and apathy (Lanctôt et al., 2017) but this does not offer information on amyloid pathology while patients are exhibiting apathetic behaviour. In fact, imaging studies of apathetic AD patients does show a correlation between apathy and amyloid burden in the frontal cortex and anterior cingulate (Mori et al., 2014). It has already been shown that *App*$^{NL-G-F}$ mice exhibit signalling issues in their prefrontal cortex (Pervolaraki et al., 2019) but deficits of motivation and liking are often linked to dopaminergic signalling. Hamaguchi et al. (2019) found reductions in dopamine transporter (DAT) in the caudate putamen of the *App*$^{NL-G-F}$ mice that has been linked to apathy in human AD patients (David et al., 2008). While there were no motivational impairments measured in the *App*$^{NL-G-F}$ mice up to 10 months of age, they did find apathy inversely correlated with cored amyloid plaque build-up in the dopaminergic striatum (Hamaguchi et al., 2019). However, they measured motivation opposed to apathy through break point analysis. This measures the point at which a reward that takes increasing effort to obtain becomes not worth the effort required to get it. The test differs from lick cluster analysis that does not measure perceived effort as sucrose is given freely throughout the test suggesting more of an apathetic phenotype opposed to global motivation. So, the reduction in DAT

signalling could, in part, be responsible for the apathy found in the present study. The noradrenergic system has also been heavily linked to apathy as noradrenergic treatments were found to robustly improve apathy in human AD patients in a recent systematic review (David et al., 2022). Other research, using electrophysiological recordings in monkeys, has even found NA activity to be a better correlate of motivation (Varazzani et al., 2015). Given how each system is heavily intertwined (Sara, 2009) it is likely both work in concert to provide motivation while the pathology seen in AD interferes with both systems.

Cognitive deficits using the NOR test have been found in 9-12 month old *App*$^{NL-G-F}$ mice (Mehla et al., 2019). The present study, despite being a direct replication, did not find recognition memory deficits even at 12 months of age. The main methodological difference between the two studies is in how the habituation was run that is incredibly important in cognitive tests (Antunes & Biala, 2012). While Mehla et al. (2019) habituated to the arena for 5 minutes a day for 6 days, this study did 10 minutes across 4 days. The third day also introduced the mice to the concept of objects to reduce anxiety about being shown them. It is unlikely down to too little habituation as both control groups from the present study and Mehla et al. show they can discriminate between novel and familiar objects. Perhaps the day of introducing mice to an object reduced neophobia allowing them to exhibit less anxiety during the test as increased anxiety can interfere with memory (Weinstock, 2017). It is possible that differences in anxiety are the cause for this discrepancy but an alternate explanation is this is not a robust effect as these mice are meant to model preclinical AD when cognitive deficits are mild (Saito et al., 2014). This is further supported by discrepancies found in spatial memory where some researchers find deficits at 10 months (Mehla et al., 2019) and others do not (Latif-Hernandez et al., 2019) suggesting any cognition differences may be small and inconsistent. Whyte et al. (2018) also examined differences in laboratory practices that could explain the lack of memory deficit they found in the Y-maze contradictory to Saito et al. (2014). They cited that variations in diet (Subash et al., 2016), home cage enrichment (Burman et al., 2014) and sex of the experimenter (Sorge et al., 2014) can cause increased stress that lead to the cognitive deficits seen by Saito et al. (2014) but not Whyte et al. (2018). The present study included home cage enrichment, a similar diet and handling by a female experimenter that are seen to reduce anxiety (Sorge et al., 2014). Laboratory differences could be why this study failed to see a difference in recognition memory at 12 months.

In summary, it seems the amyloid pathology exhibited by *App*$^{NL-G-F}$ mice produces some neuropsychiatric-like symptoms seen in human AD. Amyloid pathology seems to cause reduced anxiety with disinhibitory tendencies as well as apathy. Despite previous literature finding social and recognition memory deficits in aged *App*$^{NL-G-F}$ mice, the present study was unable to replicate these

findings. This could be down to differences in the anxiety caused by laboratory practices or suggest the deficit is not robust. More work should be done around possible motivational deficiencies and apathy in this mouse model to understand the extent of the deficit.

●Chapter 3: Biochemical examination of the *App*$^{NL-G-F}$ mouse●

●3.1 Chapter overview

Following behavioural assessment of the *App*$^{NL-G-F}$ mice, examination of selected biochemical processes was performed to ascertain whether there were changes to the LC, inflammation or neurotrophic support that may contribute to the behavioural changes reported in Chapter 2. *App*$^{NL-G-F}$ mice have previously shown an age-dependent increase in soluble and insoluble Aβ that was re-assessed here to confirm expression levels at the young and old time points. Reduction of LC neurons has previously shown to impact inflammation, neurotrophic support and NPS so each will be assessed for changes compared to their WT counterparts.

In overview, *App*$^{NL-G-F}$ mice exhibited higher expression of amyloid but these were not related to age except for soluble $A\beta_{40}$. It was also confirmed that *App*$^{NL-G-F}$ mice exhibited no increase in *APP* expression unlike transgenic models of amyloid pathology (Balducci et al., 2010). Inflammation also did not show an age dependent increase but did display a large increase in gliosis in the *App*$^{NL-G-F}$ mice compared to WT. The pro-inflammatory chemokines, MIP-1α and MIP-1β, were found to be increased in the *App*$^{NL-G-F}$ mice, while IL-6, IL-1β, TNFα and anti-inflammatory cytokines (IL-4, IL-10) showed no difference between genotypes. *App*$^{NL-G-F}$ did not significantly vary from WT controls for mature BDNF but there was an increase in the damaging BDNF precursor, proBDNF, in the cortex of *App*$^{NL-G-F}$ mice. BDNF receptor, TrkB, was also upregulated in the cortex of *App*$^{NL-G-F}$ mice. There was also no significant difference LC cell counts between genotypes but females did present a higher cell count than males with no change with age. Together, these results show that excess amyloid production greatly increased gliosis while only affecting some cytokine expression levels and also enhanced proBDNF without affecting BDNF:TrkB signalling. However, due to the lack of age effects, this could suggest the impact of excessive amyloid peaks prior to the early test point in these experiments.

●3.2 Chapter Introduction

●3.2.1 Amyloid pathology

APP processing is described in section 1.2.2 but will be expanded on here. Amyloid pathology is one of the key hallmarks of AD. The amyloid aggregate known as plaques were some of the first pathologies detailed by Alois Alzheimer (Maurer et al., 1997) and became a strong focus for research. However, extensive investigation found no link between plaques and cognitive decline or plaques and dementia severity (Selkoe, 1994; Terry et al., 1991) or even synapse loss (Lue et al., 1999; Masliah et al., 1993; Masliah et al., 1990). Furthermore, the removal of plaques had little to no impact on progression of AD (Holmes et al., 2008). While this calls into question whether amyloid actually impacts AD, it should be noted that plaques mainly consist of insoluble amyloid fibrils that are not the only form found in the brain. Smaller, soluble amyloid fragments also aggregate into oligomers that are now seen as a much worse threat to neural processing in AD (Kayed et al., 2003; Walsh et al., 1999). Soluble amyloid is very neurotoxic and can produce downstream effects that lead to increased Aβ production (Kittelberger et al., 2012), inflammation (White et al., 2005), glucose metabolism issues (Yang et al., 2020) and oxidative stress (Tabner et al., 2011). Increased soluble Aβ is correlated to synapse loss in AD (Dahlgren et al., 2002; Lacor et al., 2004; Lue et al., 1999; Walsh et al., 2002). These soluble oligomers also induce astrocytosis and microgliosis (Ledo et al., 2013; Narayan et al., 2014) while disrupting microglial metabolism (Tarczyluk et al., 2015). They also cause increased cytokine release from microglia that has been shown to disrupt cognition (Bomfim et al., 2012). BDNF axonal transport is also disrupted by AB oligomers more than it is disrupted by tau (Ramser et al., 2013; Takach et al., 2015). The *App*$^{NL-G-F}$ mice produce humanised amyloid in place of their endogenous murine Aβ.

Both soluble and insoluble Aβ are created through the amyloidogenic pathway in the cleavage of *APP*. This cleavage creates Aβ monomers of varying lengths with $Aβ_{40}$ and $Aβ_{42}$ being 40 and 42 amino acids long respectively making up the majority (Wildburger et al., 2017). Both of these fragments are cleaved at the N-terminal of *APP* but also include smaller fragments cleaved at the same site. The brain has various mechanisms in place to clear away these aberrant proteins that include neprilysin, insulin degrading enzyme, removal via the blood brain barrier (BBB) or phagocytosis via miroglia (Wilcock et al., 2004). These pathways can become ineffective with age or LC degeneration (Heneka et al., 2006) leading to Aβ build up and aggregation into insoluble plaques and soluble oligomers. Furthermore, Aβ itself disrupts these clearance pathways making aggregation more likely. They can reduce astrocytic ability to degrade proteins and increase BACE1 production (Roßner et al., 2005), an important enzyme in Aβ production. It has also been shown that, in AD,

macrophages may undergo apoptosis while attempting to degrade Aβ oligomers which then get released into extracellular space. This does not occur in the healthy populace (Zaghi et al., 2009). This feed forward model of Aβ inducing further Aβ production along with decreased clearance leads to aggregation into plaques and oligomers. Both the monomers and aggregates can impact other downstream processes leading to cognitive deficits and NPS (Hardy & Selkoe, 2002; Ledo et al., 2013).

App^{NL-G-F} mice exhibit extensive amyloid pathology (Saito et al., 2014) and research measuring its expression is summarised in fig. 3.1. The Swedish mutation (KM670/671NL; Citron et al. (1992)) and beyreuether/Iberian mutation (I716F; Guardia-Laguarta et al. (2010); Lichtenthaler et al. (1999)) lead to increased β-secretase and γ-secretase activity at the 42 site of *APP* respectively. This increases Aβ overall but also promotes $A\beta_{42}$ production increasing the $A\beta_{40}$: $A\beta_{42}$ ratio. The final mutation, arctic (E694G; Hashimoto et al. (2011)), increases the likelihood of aggregation into plaques and oligomers (Saito et al., 2014). Examination of amyloid pathology reveals an age dependent increase in $A\beta_{42}$ between 2-9 months, while $A\beta_{40}$ remains stable (Saito et al., 2014). Manocha et al. (2019) found that both soluble and insoluble $A\beta_{40}$ significantly increased at 6 months compared to WT. Soluble $A\beta_{42}$ significantly increased at 12 months while insoluble $A\beta_{42}$ increased from 3 months, likely due to the effects of the beyreuther/Iberian mutation. These were measured using enzyme-linked immunobsorbant assay (ELISA) that is a fast, reliable, sensitive and commonly used method of assessing amyloid pathology post mortem (Schmidt et al., 2005).

The current study will also assess amyloid levels using similar analysis to determine the extent of pathology in the 7 and 14-month old experimental animals. Using ELISAs also allows direct comparison to other research that has assessed amyloid in the *App*$^{NL-G-F}$ mice. This makes it easier to assess robustness of the above findings (Manocha et al., 2019; Saito et al., 2014) while affording an informative comparison to potential differences in the behavioural assays found across studies. Soluble and insoluble species of amyloid were assessed in the cortex as it is a primary site of amyloid pathology build up in humans (Braak & Braak, 1991) and rodents (Saito et al., 2014). Overall, soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ were examined in the cortex of *App*$^{NL-G-F}$ mice firstly to ensure the amyloid manipulation was effectively modelled and, secondly, to see whether there was an age dependent increase from the time points measured (7 and 14 months old). This was then used to see whether amyloid and age have an impact on the development of NPS through affecting LC, inflammation and neurotrophic support.

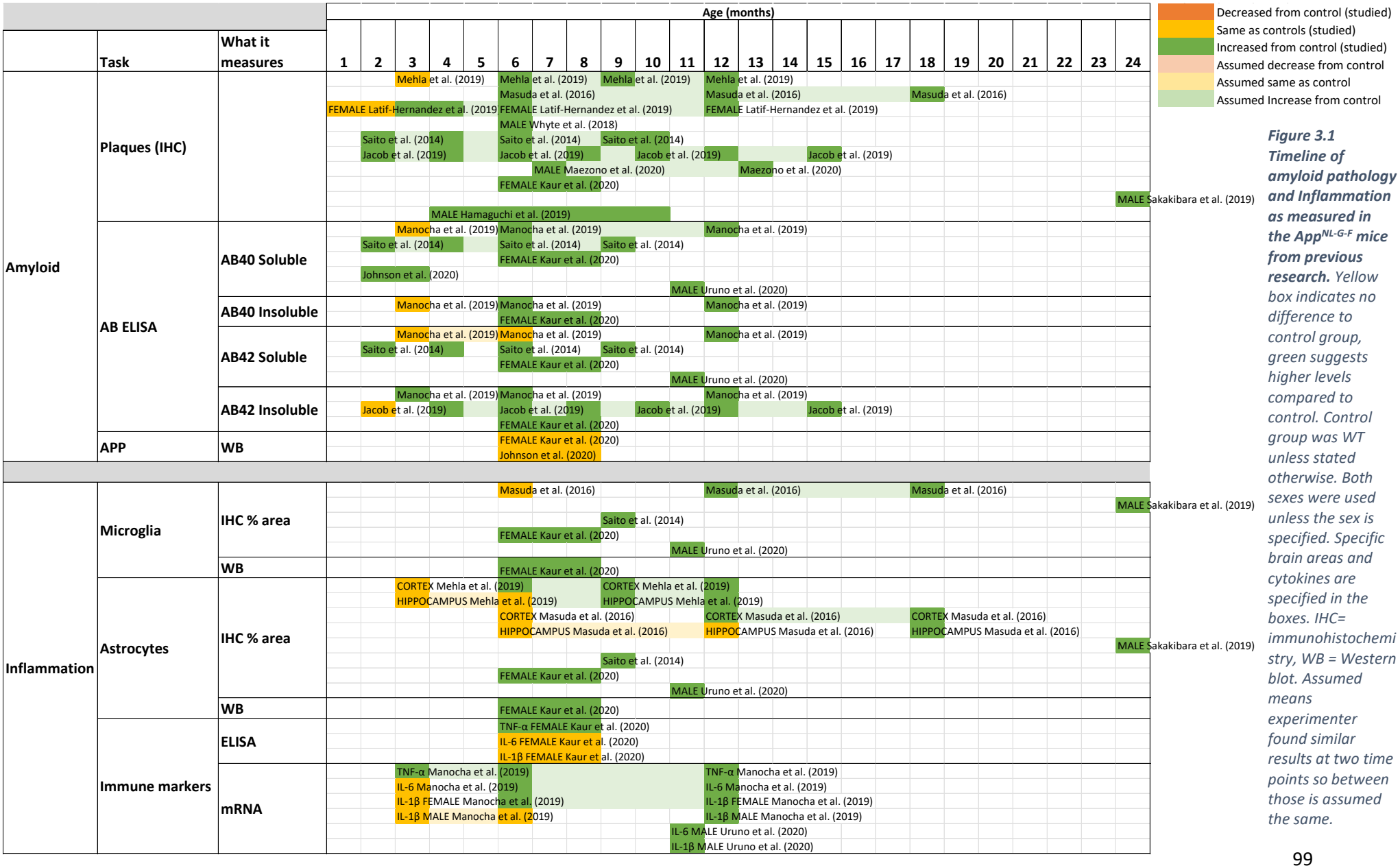

Figure 3.1 Timeline of amyloid pathology and Inflammation as measured in the App^{NL-G-F} mice from previous research. *Yellow box indicates no difference to control group, green suggests higher levels compared to control. Control group was WT unless stated otherwise. Both sexes were used unless the sex is specified. Specific brain areas and cytokines are specified in the boxes. IHC= immunohistochemistry, WB = Western blot. Assumed means experimenter found similar results at two time points so between those is assumed the same.*

●3.2.2 Locus coeruleus

Normal aging can cause a reduction in LC neurons especially in the rostral parts that innervate to the forebrain (Manaye et al., 1995). This is likely due to the vulnerability of LC neurons making them highly susceptible to damage (see section 1.3.1). Indeed, higher LC neuron count in older age is associated with reduced cognitive decline in healthy individuals (Wilson et al., 2013) suggesting LC activity can be neuroprotective to the effects of aging. In AD, normal age-related cell loss is exacerbated as shown by Kelly et al. (2017) who found 30% reduction in cell counts between MCI and healthy controls and a further 25% reduction between MCI and AD patients (Tomlinson et al., 1981). The loss of neurons results in reduction of axonal connections as well as NA (Feinstein et al., 2002) that also reduces the ability to modulate inflammation (Heneka et al., 2002; Kalinin et al., 2006), neurotrophic support (Bramham & Messaoudi, 2005) and oxidative stress (Troadec et al., 2001). Depression and other neuropsychiatric symptoms have been heavily linked to reduced NA (Wittekind et al., 2016) as a result of overactive LC neurons and higher NA turnover rather than cell loss. Increased LC activity and reduced NA is also found as a result of LC cell loss as shown by LC-lesioned mice who exhibit increased depressive-like behaviour (Szot et al., 2016). Reduction in cell count in the LC is also shown to increase Aβ deposition (Bondareff et al., 1987; Marcyniuk et al., 1986) that could be due to reduction in NA signalling or loss of neuroprotective mechanisms. Together, this suggests that LC cell loss is a good indicator of poorer cognitive and neuropsychiatric outcomes.

It is important to note that the base neuron count in the LC is sexually dimorphic in rats (Pendergast et al., 2008) that could lead to potential functional differences (Bangasser et al., 2011). It is not clear whether higher base LC cell count is neuroprotective (Wilson et al., 2013) or could make them more susceptible to the effects of stress (Bangasser et al., 2011) but what is important is the amount lost. Higher average neuronal counts have also been found in human females compared to males (Busch et al., 1997; Ohm et al., 1997). Sex differences in cell count should be an important consideration into any examination of the LC.

Reduction in cell count is not the only disruption to the LC. Even if cell body count remains comparable between mutant and WT mice, there could be changes to neural connections, reduction in NA or it's adrenergic receptors. In AD, cell loss is also accompanied by compensatory mechanisms that attempt to restore LC innervation and signalling (Szot et al., 2000; Szot et al., 2006). This includes increased NA synthesis (Szot et al., 2000) leading to normal or even elevated NA levels in the CSF but not axon terminals (Friedman et al., 1999). This compensation was also accompanied by aberrant sprouting from surviving LC neurons (Szot et al., 2006) leading to improper signalling that

was also found in LC-lesioned rats (Dyon-Laurent et al., 1993; Fritschy & Grzanna, 1992; Sara et al., 1992). The existence of these compensatory mechanisms makes examination of the LC more complex as no change in NA or axonal sprouting may simply be down to the compensation. Cell counts are not among these compensatory mechanisms and are generally used to examine the extent of the damage. So, despite there being a plethora of potential deficits in LC-NA signalling, a cell count offers a global overall picture of general LC health.

Early p-tau build up in the LC disrupts its glucose metabolism leading to reduced sensitivity and eventually cell death (Braak & Del Tredici, 2011).(Braak & Del Tredici, 2012; Grudzien et al., 2007). However, there is evidence to suggest amyloid pathology is also important in LC disruption. While plaques are not present in the LC until the later Braak stages, soluble amyloid appears much earlier prior to LC degeneration (Braak et al., 2011). Betts et al. (2019) found a link between reduced LC signal measured via MRI and increased CSF Aβ levels but no relation to tau levels. Another MRI study found tau levels did relate to reduced LC signal but only in individuals with a higher amyloid burden (Jacobs et al., 2018). In mice, loss of TH+ neurons was found in an amyloid mouse model, APP/PS1, that was reduced with antibodies that targeted amyloid (Liu et al., 2011). This anti-amyloid treatment was found to only significantly impact insoluble amyloid suggesting a role for plaques. They also only looked at TH+ neurons, which does not differentiate between dopamine or noradrenaline neurons but reducing insoluble amyloid fibrils seems to have a protective effect on mouse LC. The exact mechanism for amyloid's impact on the LC is not fully clear but it is known that oligomers could impair synaptic transmission of the LC (Lacor et al., 2004) reducing its neuroprotection. It could therefore also impair inflammatory, neurotrophic and oxidative processes leading to increased cell death.

LC integrity in *APP* mouse models

Previous animal work in transgenic mice has shown LC degeneration. Research has robustly found a reduction in LC neurons measured by TH-Immunoreactivity (IR) in the APP/PS1 model (Cao et al., 2021; Liu et al., 2013; O'Neil et al., 2007) as well as the 5xFAD (Kalinin et al., 2012). This loss was not found in the 23 month old PDAPP mouse but they did find reduced size in the rostral area of the LC which innervates the hippocampus and cortex (German et al., 2005). APP23 mice also showed no significant LC cell loss compared to WT (Szot et al., 2009). The discrepancy across models could be down to the extent of amyloidosis exhibited as both APP/PS1 and 5xFAD show extensive amyloid pathology (Hall & Roberson, 2012). Furthermore, the APP/PS1 mouse uses the promoter *MoPrP* that produces a 15-fold increase in *APP* expression and affects microglia and non-neuronal tissue, while PDAPP mutations would mostly be expressed in the brain. 5xFAD mice are known as expressing

some of the most aggressive amyloid pathology, with plaques developing as early as 1.5 months (Oakley et al., 2006). The massive increase in *APP* expression, the amyloidosis and its impact on microglia could all affect LC degeneration and these aggressive phenotypes are not present in the human condition. Regardless, this does cause the question of how much impact amyloid pathology has on the LC, either directly or through neuroinflammation (Gomez-Arboledas et al., 2018; Hong et al., 2016; Rajendran & Paolicelli, 2018) or neurotrophic support (Braun et al., 2017).

The *App*$^{NL-G-F}$ mice offer a more valid build-up of amyloid pathology but research differs on whether or not they exhibit LC neuron degeneration. Mehla et al. (2019) showed from 9 month old, *App*$^{NL-G-F}$ mice show reduced LC cell count as measured by TH-IR, that coincides with significant plaque deposition, compared to WT. However, when Sakakibara et al. (2021) re-examined this issue, no such cell loss was detected even at 24 months old. They explained this discrepancy through differences in lab practices or calculation methods such as Sakaibara et al. using more slices of the LC to produce their cell count average, so this needs to be repeated to see which finding is more robust. However, when examining the connections of the LC through NET, they found a significant reduction in axonal sprouting in 12 & 24 month old *App*$^{NL-G-F}$ mice compared to both *App*^{NL} and WT mice. They also discovered loss of connections was not limited to areas with plaques or reactive gliosis suggesting neither insoluble amyloid or inflammation caused axonal degeneration. It is possible that the cause relates to soluble amyloid but that was not directly measured. Interestingly, they also found no reduction in BDNF, NGF or NT-3 levels suggesting that reduced neurotrophic support was also not the cause of reduced axonal sprouting of the LC neurons.

Given the lack of consensus in the literature regarding LC cell counts in *App*$^{NL-G-F}$ mice, LC density will also be assessed in the current cohort of mice. As a cell count requires spatial representation, an immunohistochemical stain was used on a subset of mice from both age points, sex and genotypes. While there are other ways of assessing the functionality of the LC (e.g. NET stain, examination of NA levels) the cell count method offers a good first step as a gross measure of LC integrity, which is not confounded by potential compensatory mechanisms.

●3.2.3 Inflammation

Amyloid promotes activation of immune cells leading to an inflammatory response. While it does this directly, it also has indirect effects through amyloid's impact on the LC. The direct path means all aberrant Aβ fragments can activate microglia by binding to toll-like receptors (TLR) and activating the NF-κB pathway leading to the production of pro-inflammatory cytokines and

chemokines (Fulop et al., 2013). The increased expression of pro-inflammatory cytokines signal to other microglia to activate in order to deal with the aberrant protein (Hoozemans et al., 2006). This process is able to successfully clear away aberrant Aβ if working efficiently alongside other clearance pathways, such as insulin degrading enzyme (IDE), neprilysin or removed via the BBB (Wilcock et al., 2004). Manipulations that increase inflammation have even been linked to reducing plaque load in Tg amyloid mouse models (Ghosh et al., 2013) and improve memory deficits (Yamanaka et al., 2012). However, this increased inflammation was also accompanied by worsened tau pathology (Ghosh et al., 2013) that has been confirmed *in vitro* by inflammation triggering phosphorylation of tau (Griffin et al., 2006; Sheng et al., 2001). Furthermore, astrocytes, which are an important neural immune cell, can digest Aβ oligomers but this induces an influx of calcium ions into the cell leading to the generation of reactive oxygen species and thereby increasing oxidative stress (Narayan et al., 2014). In addition, oligomers can then impair astrocytic metabolism reducing their clearance ability (Tarczyluk et al., 2015). This impairment could then lead to less amyloid clearance and thus a build-up of the protein. Evidence in mice has also shown that soluble oligomers cause microglia to release pro-inflammatory cytokines, TNF-α and IL-1β (Ledo et al., 2013), which impairs cognition in mice (Bomfim et al., 2012).

Amyloid impairs immune cell activation as well as microglial ability to clear away Aβ. This amyloid build-up is further caused by the pro-inflammatory cytokines increasing *APP* expression in neurons and microglia (Forloni et al., 1992) as well as stimulating both γ-secretase cleavage (Liaoi et al., 2004) and β-secretase cleavage of *APP* (Sastre et al., 2008). This increased accumulation of amyloid is shown in transgenic amyloid mice (Lee et al., 2008; Sheng et al., 2003) and *App*$^{NL-G-F}$ mice (Xie et al., 2021) given an inflammatory insult (injection of LPS) that significantly increased Aβ deposition.

Amyloid also impacts LC processing which increased neuroinflammation. Transgenic amyloid mouse models with LC lesions have amplified microglial and astroglial activation in the cortex and hippocampus (Kalinin et al., 2006). This inflammatory response to amyloid was found to occur sooner, more robustly and with a longer duration than without the LC lesion (Heneka et al., 2002). Sustained inflammation also lead to increased amyloid deposition and cognitive decline (Feinstein et al., 2002). Thus, evidence of LC cell loss would lend support to the view that alterations in this circuit contribute to brain inflammatory processes elicited by amyloid production.

Increased gliosis

Both increased gliosis and cytokines themselves impact clearance of amyloid, so each will be examined independently in the current cohort of mice. Increased gliosis refers to the activation of

the main neural immune cells: microglia and astroglia. Both have primary roles in the clearance of Aβ. Some evidence suggests astrocytes are part of the early defence against amyloid accumulation as Aβ was found inside astrocytes only in areas without insoluble amyloid fibrils (Nielsen et al., 2009). Astrocytes also release neurotrophic factors and provide a protective barrier between Aβ deposits and neurons (Roßner et al., 2005). Microglia are the resident macrophage of the brain and can be found in different activation stages just like macrophages in the blood. They have a state that is amoeboid in shape allowing for easier migration and phagocytosis of threats (Lucin & Wyss-Coray, 2009). Microglia can also have a more branched shape that is associated with anti-inflammatory processes. When microglia become activated, it migrates and deals with the threat that also means releasing pro-inflammatory cytokines to alert other immune cells of the issue. Once the threat has been dealt with, the microglia return to a more surveillance based state and release anti-inflammatory cytokines to suppress inflammatory response (Lucin & Wyss-Coray, 2009). In early stages of AD, microglia are increased in the brains but, as the disease progresses, there is higher microglia activation. In other words, as AD progresses, a sustained pro-inflammatory state is found.

Macrophages, monocytes found in the blood, are also important in this process. The released pro-inflammatory cytokines attract macrophages to cross the BBB from microvessels (Fiala et al., 2005). These will aid in the clearance of Aβ fibrils and are shown to increase in AD patients brains between Braak stage III and V compared to aged matched controls (Fiala et al., 2002). However, in AD, macrophages attempting to digest Aβ may undergo apoptosis which then spills the still intact Aβ fragments into blood vessels which does not occur in healthy controls (Zaghi et al., 2009).

Research looking at reactive gliosis in the *App*$^{NL-G-F}$ mice has shown increased microglial and astrocytic activation (fig. 3.1). Saito et al. (2014) found increased microgliosis and astrocytosis at 9 month old. This was measured using IHC staining and percentage of area with activated microglia. Masuda et al. (2016) replicated the observation with increased microgliosis from 12 months and astrocytosis from 18 months in cortex and hippocampus. However, Mehla et al. (2019) found increased astrocytosis from 6 months in the cortex and 9 months in the hippocampus as measured through IHC staining and percentage area. They did not find an increase in 3 month old *App*$^{NL-G-F}$ mice. Kaur et al. (2020) measured astrocytosis and microgliosis through optical density and found both increased in *App*$^{NL-G-F}$ mice at 6 months. Although there is some disagreement about when the increase starts and in what brain area, it is clear that there is an age-dependent increase that starts between 3 and 9 months old. IHC protocols were used here and are good at showing spatial information but western blot protocols may be superior in finding a straight difference in protein expression in a tissue sample.

Astrocytosis and monocyte activation will be examined in the young and old cohort of *App*$^{NL-G-F}$ mice using western blotting. This will provide a measure of whether amyloid impacts total immune cell level as age increases. Gaining a global picture of protein level can help discern when a significant increase in inflammation occurs in the *App*$^{NL-G-F}$ mice. Examination in the cortex and hippocampus will also allow direct comparison to previous studies that use *App*$^{NL-G-F}$ mice as well as both areas being significant regions with amyloid build up (Braak & Braak, 1991). Overall, this will reveal whether there is an age dependent increase in inflammatory markers between 6 months and 12 months in both the cortex and hippocampus.

Increased expression of cytokines

Cytokines are chemical signals that communicate with various cells and can induce and cease inflammatory reactions (Rubio-Perez & Morillas-Ruiz, 2012). They are also involved in various other biological events (e.g. cell proliferation, cytotoxicity/apoptosis, cell growth and differentiation) but their role in inflammation will be the focus of this section. Levels of pro-inflammatory cytokines (PIC) are increased in the brains of AD patients (McGeer & McGeer, 2003) and they may be involved in a vicious cycle wherein Aβ initiates PIC release via microglia which in turn increases Aβ production (McGeer & McGeer, 1997). Evidence has also shown PIC levels correlate with progression of AD (Alasmari et al., 2018) as well as causing neurodegeneration (Smith et al., 2012). PIC have also been shown to disrupt Aβ transport leading to it accumulating (Evseenko et al., 2007) as well as increasing *APP* expression (Forloni et al., 1992). Overall, this evidence shows that PIC increase brain inflammation but they also have an independent role in accelerating Aβ production/deposition.

While there are a variety of PIC, the main cytokines that are increased in AD are IL-1β, IL-6 and TNF-α (McGeer & McGeer, 1997; Walker et al., 1997) all of which seem to have neurotoxic effects. In contrast, TGF-β, as an anti-inflammatory cytokine (AIC), has protective effects (Allan & Rothwell, 2001).

Interleukin 1 (IL-1) is a family of PIC, the most prominent being IL-1β. IL-1β is an important initiator of immune responses and is released from activated microglia cells in pro-forms, that is cleaved by the protease caspase- 1 to make its mature form (Wang et al., 2015). Oligomers have been found to increase this cleavage to mature form in microglia via ROS-dependent inflammasome processes leading to increased oxidative stress (Parajuli et al., 2013). Hunter et al. (2012) also found increased IL-1β expression around plaques in human AD and Tg animal models suggesting its importance in initiating inflammation. However, some research has also shown IL-1β has a beneficial role. Sustained overexpression of IL-1β reduced Aβ related pathology by modulating plaque degradation via microglia as well as promoting non-amyloidogenic cleavage of *APP* (Tachida et al.,

2008). So elevated levels of IL-1β could highlight a neurotoxic effect while excessive overexpression could be neuroprotective.

Interleukin 6 (IL-6) is a multifunctional cytokine important for host defence (Wang et al., 2015). In AD, it has been shown to be elevated and found around plaques in humans and animals (Wang et al., 2015). Research has also shown that IL-6 can induce hyperphosphorylation of tau (Spooren et al., 2011) and memory problems (Weaver et al., 2002) suggesting a negative impact from elevated levels in AD.

Tumour necrosis factor alpha (TNF-α) is important in initiating and regulating cytokine cascades during the inflammatory response. Using the 3xTg-AD triple transgenic mouse model, increased TNF-α lead to higher Aβ in the short term and enhanced inflammation, tau pathology and neuronal death in the long term suggesting a detrimental impact on AD pathology (McAlpine et al., 2009). It has also been shown to cause cell proliferation, cell migration and apoptosis via it's activation of various kinases (Montgomery et al., 2013).

AIC, such as IL-10, IL-4 and TGF-β, suppress the production and activity of PIC (Rubio-Perez & Morillas-Ruiz, 2012). All are required for efficient inflammatory response as PIC can offer host defence and AIC can then induce repair of the inflammatory damage (Plata-Salamán et al., 1998). If there is an imbalance such as high PIC and low AIC then this can lead to amplification cycle of inflammatory cellular activation as well as apoptosis via cytotoxicity (Plata-Salamán et al., 1998). This imbalance is commonly seen in AD (Plata-Salamán et al., 1998).

Interleukin 10 (IL-10) is a primary AIC important for neuronal homeostasis and cell survival (Strle et al., 2001). IL-10 is able to reduce synthesis of IL-1 and TNF-α and inhibit LPS or Aβ-mediated PIC production *in vitro* (Franciosi et al., 2005). It also gives a dose dependent decrease in IL-6 production from Aβ in microglia cells and promotes the more branched states of microglia (Arosio et al., 2010). A recent study found significantly decreased levels of IL-10 in the serum of AD patients (Culjak et al., 2020).

Transforming growth factor beta (TGF-β) is initially synthesised as an inactive precursor that requires activation before exerting its effect (Norgaard et al., 1995). TGF-β is an important regulator of cell proliferation, differentiation and formulation as well as being able to convert an active inflammatory site into one dominated by reparations (Letterio & Roberts, 1997). TGF-β is also able to actively suppress actions of T cells, B cells and TNF-α production (Norgaard et al., 1995). However, despite these neuroprotective effects, TGF-β has been found to be increased in the CSF of AD

patients suggesting either its ineffective or it actually has a neuropathological role (Chao et al., 1994).

Past research assessing cytokine expression in the *App*$^{NL-G-F}$ mice has shown that TNF-α expression is increased from 3 months, IL-1β from 6 months and IL-6 from 6 months for females and 12 months for males (Manocha et al., 2019). This was measured through mRNA expression in cortices using real time PCR (fig. 3.1). This supports the idea of amyloid causing a pro-inflammatory state with PIC adding to the inflammatory milieu. No work has yet looked at AIC so it is unclear whether they would be increased, as seen in some AD patients, or decreased.

The present research will expand on the work of Manocha et al. (2019) by looking at IL-6, IL-1β and TNF-α expression using western blotting as it will directly measure protein levels opposed to an indirect measure of mRNA. The addition of AIC will also offer insights into whether the anti-inflammatory status is affected alongside pro-inflammation. Again, both cytokine expression will be measured in both the cortex and hippocampus as both areas are subject to high amyloidosis and this will also allow direct comparison to immune cell count. In addition, a 23-cytokine multiplex assay was run to examine a plethora of PIC and AIC in conjunction with the western blots. This test had never been run on *App*$^{NL-G-F}$ mice prior to this. Being a more sensitive measure, it will be able to show whether WB are able to accurately detect cytokines as well as measuring 23 different inflammatory proteins. It is expected that increases in astrocytosis and monocyte expression would also lead to an increase in PIC and decreases in AIC. However, as TGF-β is found to be increased in AD, this is expected to also be increase in the *App*$^{NL-G-F}$ mice. It is unclear whether the multiplex and western blot measures will agree over cytokine expression. Overall, the gliosis and cytokine expression measures will provide a clear picture of the inflammation occurring in the brains of *App*$^{NL-G-F}$ mice.

●3.2.4 Neurotrophic support

Brain derived neurotrophic factor (BDNF) is one of various neurotrophins that regulate neuronal survival, differentiation and plasticity via activating TrkB and p75^{NTR} receptors (Tanila, 2017). BDNF is downregulated in AD (Ferrer et al., 1999; Phillips et al., 1991) but the results are not so clear in Tg mice (Tanila, 2017). Signalling through BDNF connecting to TrkB enhances long term potentiation (Rösch et al., 2005) and prevents $A\beta_{42}$ impairment of neuroplasticity (Zheng et al., 2010). So BDNF signalling has various beneficial effects on brain health and neural plasticity.

In MCI, lower BDNF levels were correlated with cognitive function suggesting a reduction in plasticity and synapse health (Peng et al., 2005). The receptor, TrkB, was also found to be

downregulated in post mortem AD tissue showing the brain becomes less sensitive to the beneficial effects of BDNF (Ferrer et al., 1999; Salehi et al., 1996). TrkB knockout mice demonstrate this by having severe deficits in learning (Minichiello et al., 1999). Furthermore, Murer et al. (1999) found neurons with tau tangles did not exhibit any BDNF as measured in immunoreactivity suggesting tau could play a role in its downregulation. The researchers also concluded that neurons that still had BDNF and TrkB were not immune to degeneration so their protective properties are not without fault.

While loss of BDNF and TrkB is robust in humans, in animal models there seems to be a lot of variation. Some studies report a decrease in BDNF in APP/PS1 mice (Peng et al., 2009), while others report increased levels (Burbach et al., 2004; Szapacs et al., 2004) in the same model. One paper suggested BDNF has differing biological conditions in rodents and in humans that could explain this discrepancy (Radka et al., 1996) but it is also possible the animal researchers did not differentiate between proBDNF and mature BDNF.

BDNF is cleaved from proBDNF that is roughly twice the size of BDNF (13kDa compared to 32kDa;Koshimizu et al. (2010)). While BDNF offers a lot of neuroprotective properties through TrkB signalling, proBDNF elicits apoptosis via activation of p75^{NTR} receptors (Koshimizu et al., 2010). ProBDNF also elicits synaptic depression (Niculescu et al., 2018; Wang et al., 2021) and the ratio of ProBDNF:BDNF was increased in the CSF of AD patients compared to controls (Fleitas et al., 2018). In addition, proteases responsible for cleaving proBDNF to BDNF are inhibited in AD (Zheng et al., 2010). Amyloid can directly inhibit these proteases (Zheng et al., 2010) as well as disrupt the axonal transport of BDNF (Poon et al., 2013). So, BDNF:TrkB are both downregulated in AD while proBDNF is increased due to reduction in cleavage proteases leading to synaptic depression and apoptosis.

Research looking at BDNF signalling in *App*$^{NL-G-F}$ mice is limited but one study examined it using immunohistochemistry and found no change in BDNF compared to WT (Sakakibara et al., 2021). However, while immunoreactivity can distinguish between BDNF and proBDNF (Telegina et al., 2019), it is much easier to visualise using western blot techniques as both BDNF and proBDNF respond to anti-BDNF antibodies. This will be rectified in the present study by using western blotting to distinguish between BDNF and proBDNF in a clearer way due to their difference in size wherein proBDNF is over double the size of BDNF. Due to such polarising effects on the brain, this distinction is important. Furthermore, western blotting is better at giving a global amount of protein rather than the extrapolation from a few focused brain regions used in IHC techniques. Protein levels will be examined in both the cortex and hippocampus as amyloid builds up in these regions (Braak & Braak, 1991).

BDNF levels have been shown to decrease in the cortex as Braak stage increases (Tanila, 2017) as well as the hippocampus (Phillips et al., 1991). Additionally, amyloid burden in the cortex of MCI patients has been heavily linked to both cognitive symptoms and NPS (Krell-Roesch et al., 2018) making both the cortex and hippocampus important structures to examine BDNF levels in. Measuring TrkB will also provide a more complete picture of the changes amyloid makes to BDNF signalling as it will indicate whether the brain's sensitivity to BDNF is altered as the *App*$^{NL-G-F}$ mice age. Overall, western blotting will highlight whether amyloid changes BDNF:TrkB signalling in the cortex and hippocampus of *App*$^{NL-G-F}$ mice through aging and whether proBDNF is also affected.

●3.3 Aims and hypotheses

The main aim of this chapter is to answer the question: How does amyloid pathology affect the LC, neuroinflammation and neurotrophic support? To examine this, neural tissue from the same young and old *App*$^{NL-G-F}$ and WT mice from behavioural chapter 2 will be used to assess each of these aspects. For the LC, immunohistochemical analysis will reveal a cell count to see if amyloid causes age dependent cell loss. As previous research is inconclusive, there is no prediction for what will be found. Neuroinflammation will be examined in a few ways. The first is through astrocytosis and monocyte activation via western blotting that will highlight whether amyloid does impact neuroinflammation in these mice. Previous research has shown amyloid does cause an increase in immune cell activation so it is expected to be increased in *App*$^{NL-G-F}$ mice. The second way is through pro and anti-inflammatory cytokine expression. Prior research is scarce here but, as they are increased in AD, we anticipate higher expression in the *App*$^{NL-G-F}$ mice. Finally, neurotrophic support will be assessed through BDNF, proBDNF and its receptor, TrkB, to show whether amyloid impacts any of them. BDNF and TrkB are often downregulated in AD so it is expected the same will occur in *App*$^{NL-G-F}$ mice. ProBDNF, on the other hand, is increased in AD so higher levels are anticipated in the *App*$^{NL-G-F}$ mice. Soluble and insoluble amyloid will also be examined to see whether there are age dependent increases which are anticipated.

●3.4 Methods

●3.4.1 Animals

These are the same animals used in chapter 2 but only a selection was taken for biochemical analysis (table 3.1, 3.3, 3.4) and immunohistochemistry (table 3.2). Due to the different tissue

collection methods for western blotting and immunohistochemistry, different mice were used for each. The mice used in ELISAs and multiplex assay were the same as the ones used in western blotting. For western blotting, there was a total n of 80. For immunohistochemistry, there was an n of 48. ELISA only looked at amyloid in *App*$^{NL-G-F}$ mice so had an n of 39. The multiplex assay could fit a maximum of 76 samples on the plate so the maximum was used. See section 2.4.1 for breeding and housing.

Western blotting

Age	Genotype	Sex	N
Young (5-7 months)	*App*$^{NL-G-F}$	Male	10
		Female	10
	WT	Male	10
		Female	10
Old (12-14 months)	*App*$^{NL-G-F}$	Male	10
		Female	10
	WT	Male	10
		Female	10
Total			**80**

Table 3.1 Group sizes for App^{NL-G-F} and WT mice used in western blotting

Immunohistochemistry

Age	Genotype	Sex	N
Young (5-7 months)	*App*$^{NL-G-F}$	Male	6
		Female	6
	WT	Male	6
		Female	6
Old (12-14 months)	*App*$^{NL-G-F}$	Male	6
		Female	6
	WT	Male	6
		Female	6
Total			**48**

Table 3.2 Group sizes for App^{NL-G-F} and WT mice used in immunohistochemistry. *Mice were different from western blotting, ELISA and multiplex assay*

ELISA

Age	Genotype	Sex	N
Young (5-7 months)	*App*$^{NL-G-F}$	Male	10
		Female	10
Old (12-14 months)	*App*$^{NL-G-F}$	Male	10
		Female	9
Total			**39**

Table 3.3 Group sizes for App^{NL-G-F} mice used in ELISA. *Only App^{NL-G-F} mice were used as this was a measure of human amyloid.*

Multiplex cytokine assay

Age	Genotype	Sex	N
Young (5-7 months)	*App*$^{NL-G-F}$	Male	10
		Female	9
	WT	Male	8
		Female	9
Old (12-14 months)	*App*$^{NL-G-F}$	Male	10
		Female	10
	WT	Male	10
		Female	10
Total			**76**

Table 3.4 Group sizes for App^{NL-G-F} and WT mice used in the multiplex cytokine assay. *Samples were the same as those used in western blotting and ELISA.*

●3.4.2 Murine tissue collection

Two separate tissue collection techniques were done with perfused mouse brains used for immunohistochemistry and dissected brain for biochemistry (western blot, ELISA and Multiplex assay) techniques.

Perfusion

Mice were anaesthetised using an I.P. injection of 0.05 ml of Pentobarbitol with their reflexes checked by squeezing of the paws and tail tip. Once the mouse was unresponsive, they were placed on the perfusion grid and heart exposed and posterior end of the left ventricle was severed.

Cold PBS was flushed through their system using Watson Marlow SCIQ 323 peristaltic pump until the blood ran clear that was then swapped to cold 4% PFA until the body was stiff. The brain was then carefully extracted and left in 4% PFA for 48 hrs before being transferred to 30% sucrose solution. Once the brains had sunk, they were then moved on to the microtome for slicing (see section 3.4.3).

Dissection

Mice were culled using cervical dislocation with their head quickly removed and a blood sample extracted from the body (this was not done for perfused animals). The brain was removed from the skull and dissected out into cerebellum as well as the left and right hippocampus, frontal cortex and cortex. The brain was placed with cortices facing upwards and the cerebellum was removed by parsing out the area from the cerebellar peduncles. The olfactory bulb was removed and a midsagittal cut between the hemispheres was done to remove the cortices from the subcortical regions. The hippocampus was defined as a lighter coloured long, thin area that was separated from the inner cortex using a curved probe. The PFC was defined as a 1 mm cut at the anterior of the cortex with the remaining tissue taken as cortex. This was done for both hemispheres but only the right hemisphere was used for analysis. They were immediately snap frozen in liquid nitrogen and left in -80 ^{o}C freezer before being used for biochemistry (see section 3.4.4).

●3.4.3 Immunohistochemistry

Microtome

Brains that had sunk in the 30% sucrose solution were then ready to be sliced using a microtome (Jung SM 1400). Brains were fixed to the microtome plate using OCT at -40 ^{o}C. The entire brain was cut at 30 µm thickness and split into 5 pots per brain. They were stored in cryoprotectant and left at -20 ^{o}C until ready for staining.

Tyrosine hydroxylase stain

This stain was to look for the presence of noradrenaline neurons throughout the brain. It started by washing the brains in 0.1 M TBS (Tris-buffered saline, pH 7.4) 3 times for 10 minutes before being quenched in a solution of methanol (10%), 30% hydrogen peroxide (10%) in distilled water (80%). The sections were, again, washed in 0.1 M TBS 3 times for 10 minutes before being blocked in a solution of 3% normal goat serum in Tx-TBS (TBS with 0.1% Triton X-100) for 1 hour. The primary antibody (Tyrosine Hydroxylase, Abcam, #AB152) was put into a solution of 1% normal goat serum in Tx-TBS at a dilution of 1:1000 and the brain tissue was left overnight at room temperature

on a shaker. The sections were triple washed in 0.1 M TBS again and then left for 3 hours in the secondary antibody solution with 1% normal goat serum and 0.1 M TBS before another round of triple washing with 0.1 M TBS. An ABC kit (2BScientific, #PK-6101) was made up with 1% normal goat serum and left for 2 hours before being washed in 0.1 M TBS and then fresh TNS (0.05 M Tris base, pH 7.4). The sections were stained using a DAB solution (2BScientific, #SK-4100) made up fresh on the day which was left until tissue started going light brown (approximately 1-3 minutes). The stain was finished by triple washing with TNS for 5 minutes and then double wash of 0.1 M TBS for 10 minutes before being mounted on gelatinised slides and airdried overnight. The slides were then dehydrated using a treatment of ascending series of alcohols for 5 minutes each (70%, 95%, 100%), cleared in xylene and then cover slipped with DPX.

Slides were imaged on a brightfield microscope where 3 pictures of the Locus Coeruleus were taken from consecutive slices from both hemispheres (coordinates: A/P: -5.4 mm from bregma; M/L:+-0.9 mm; D/V:-3.25 mm from skull surface; Szot et al. (2012)). The area was defined using a mouse atlas at the above coordinates as well as with the DAB stain highlighting the LC cells. Cells were manually counted using ImageJ (National Institutes of Health, USA; Bourne (2010)) and an average was taken for each mouse. These values were then compared across genotypes, sexes and time point to examine noradrenaline neuron count.

●3.4.4 Biochemistry

Homogenate extraction

Right cortex and right hippocampal tissue were suspended in a solution of 2% sodium dodecyl sulphate (SDS), Protease inhibitor cocktail III (1:100, #539134, Millipore) and Phosphatase inhibitor cocktail V (1:50, #524629, Millipore). They were homogenised using Precellys 24 Dual (Bertin Technologies, Montigny le Bretonneux, France) machine at 5500 rpm for 2 x 30 seconds with 30 second delay before being rotated at 4 °C overnight. Samples were placed in the Ultra Centrifuge (Optima LE-80K, Beckman Coulter, USA) and rotated at 28,300 rpm (100,000 g) for 1 hour at 4 °C before carefully removing the supernatant without disturbing the pellet. The supernatant was diluted 1:3 in EC sodium buffer (20 mM Na_2HPO_4, 0.2 mM EDTA, 0.4 M NaCl, 0.2% BSA) before being stored at -80 °C. These samples were used for Western blotting, soluble Aβ ELISA and multiplex assay testing.

For insoluble Aβ ELISA analysis, the insoluble pellet was resuspended in 70% formic acid (Honeywell, USA, # F0507) at 150 mg/ml of original wet tissue weight. They underwent further

centrifugation as above (100,000 g) for 1 hour at 4 °C before being diluted in neutralising buffer (1 M Tris, 0.5 M Na_2HPO_4, pH 11) at a factor of 1:20 and being stored at -20 °C.

Bicinchoninic Acid Protein assay

To examine protein concentrations in each sample, diluted samples underwent BCA analysis using bicinchonic acid protein (BCA) assay kit (#23225, ThermoScientific, UK). A series of standards were made up by diluting the BSA (2 mg/ml) in dH_2O to create known concentrations ranging from 2-0.008 mg/ml. 20 µl of each standard were pipetted, in duplicate, into a 96 well plate along with 2 dH_2O blank and a duplicate of SDS, inhibitor cocktail with EC buffer solution. 5 µl of each sample were pipetted in triplicate to the plate before 200 µl BCA working reagent (50:1 Reagent A to Reagent B) was added to each well. The plate was mixed for 15 seconds before being incubated at 37 °C for 30 minutes. Absorbance was read at 562 nm using Infinite M Plex and Tecan i-Control software (Tecan, Switzerland) and duplicates were checked for accuracy by ensuring readings did not vary more than 0.1. Once samples were cleared, a standard curve was produced from the BSA standards that was used to calculate protein concentration of each sample. This allowed equal protein amounts across samples for western blot.

Optimising antibodies

To determine the best primary and secondary antibody dilutions, various combinations were trialled on small strips of the gel for western blot. All blocking was initially done in 5% non-fat milk TBS-T before being tested in 5% BSA (FisherScientific, UK, #BP1605-100) TBS-T if milk produced no results. Primary antibody concentration was varied to start before experimenting with differing secondary antibody concentration to see what would give the clearest looking bands with as little background as possible. The results of the trialled antibodies are in table 3.5.

Target	Blocking	Primary antibody	Concentration	Secondary Concentration
APP	5% NF milk TBST	Mouse monoclonal (Merck; mab348)	1:1000	1:2000
BDNF	5% NF milk TBST	Rabbit monoclonal (Abcam, UK; Ab108319)	1:1000	1:2000
IL-1β	5% BSA TBST	Rabbit monoclonal (Abcam, UK; Ab234437)	1:1000	1:15,000
IL-6	5% BSA TBST	Rabbit monoclonal (Cell signalling technology, USA; 12912)	1:1000	1:2000

TGF-β	5% BSA TBST	Rabbit polyclonal (Cell signalling technology, USA; 3711s)	1:2000	1:10,000
GFAP	5% NF milk TBST	Rabbit monoclonal (Abcam, UK; Ab68428)	1:10,000	1:5000
TNF-α	5% BSA TBST	Rabbit monoclonal (Cell signalling technology, USA; 11948s)		
IL-10	5% BSA TBST	Rat monoclonal (Abcam, UK; Ab189392)		
Trkβ	5%NF milk TBST	Rabbit monoclonal (Abcam, UK; ab187041)	1:1000	1:10,000
Iba-1	5%NF milk TBST	Rabbit monoclonal (Abcam, UK; ab178847)	1:1000	1:2000
NGF	5% BSA TBST	Rabbit polyclonal (Abcam, UK; ab6199)	1:250	1:2000
β-actin	-	Mouse monoclonal (Merck, A3854)	1:10,000	-

Table 3.5. The final primary and secondary concentrations for each antibody used in western blotting. *TNFα, IL-10 and NGF were repeatedly trialled but no usable results were given*

Tests for TNF-α and IL-10 were postponed due to too much background and unclear bands even after multiple attempts. NGF was completed but not included due to high background and unclear bands suggesting insufficient amounts of these proteins in the samples.

Western blot

Samples were diluted 2:1 in 3x sample buffer (0.2 M TrisBase, 0.4 M SDS, 30% glycerol, 15% β-mercaptoethanol, 3% bromophenol blue) before being heated to 70 °C for 15 mins or for 5 mins if not initial use. Pre-made 10-20% gels (Fisher Scientific UK, #15426824) were filled with 30 mg of sample calculated using BCA analysis in gel tanks (Fisher Scientific UK, #10093492) filled with running buffer (25 mM Tris Base, 192 mM glycine, 0.1% SDS). Electrolysis was run for 90 mins at 130 V to separate out proteins according to size. Proteins were then transferred to 0.2 µm nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) for 45 minutes at 38 mA using semi-dry transfer buffer (42.9 mM Tris base, 38.9 mM glycine, 0.038% SDS, 20% methanol). Each membrane was briefly washed in TBS-T before staining with ponceau (0.1% Ponceau S, 5% acetic acid) for 5 minutes and de-stained in distilled water for 5 minutes to ensure a successful transfer of proteins. Membranes were then blocked for 1 hour before being incubated in their primary antibodies (table 3.5) overnight at 4 °C. Membranes were washed thrice in TBS-T and placed in their secondary antibodies (table 3.5) in blocking reagent for 1 hr at room temperature. A final wash of 3 x 15

minutes was done before imaging using Syngene GBOX Chemi-XX6 gel doc system and software (Syngene, Cambridge, UK). Blots were either imaged using ECL (Thermo Fisher scientific, UK, #32106; β-actin, GFAP) or West Dura (Thermo Fisher scientific, UK, #34075; APP, BDNF, IL-1β, IL-6, TGF-β). Multiple images were taken to ensure the best exposure was selected to reduce background while retaining strong bands.

Procedure

Each gel was run, pictures taken and then left in stripping buffer for 12 minutes before incubation in β-actin for 2 hours in whatever blocking buffer was used for that target antibody (table 3.5). A β-actin signal was developed for each gel to ensure adequate normalisation. Each target was run on a different gel except for *APP* & BDNF and Iba-1 & TrkB (due to large size difference). These were run on the same gel but the membrane was cut between the 55kDa and 70kDa bands on the ladder prior to primary antibody incubation. β-actin was then run on the lower half of the membrane (BDNF/Iba-1). Gels across the same target spanned multiple days.

Analysis and quantification

Signal was calculated using ImageJ (Bourne, 2010). Blots were inverted and straightened to ensure bands were as level as possible (fig. 3.2a). A rectangle was drawn around all bands and a signal peak graph was developed (fig. 3.2b). A straight line was drawn below each peak from lowest part of the dip to the lowest part of the next dip and the area was selected and measured. The area of each peak was taken as a percentage of total area across all peaks to be used in analysis. The exact same protocol was done for the corresponding β-actin blot for that gel (fig. 3.2c).

Protein signal was normalised to the house keeping protein (β-actin) by protein signal %/ β-actin signal % for each band to give the normalised result. As the first band on every gel was the reference mouse (same mouse across all gels), each mouse normalised result was divided by the reference mouse normalised result. The corresponding values were taken as the signal for each mouse for analysis.

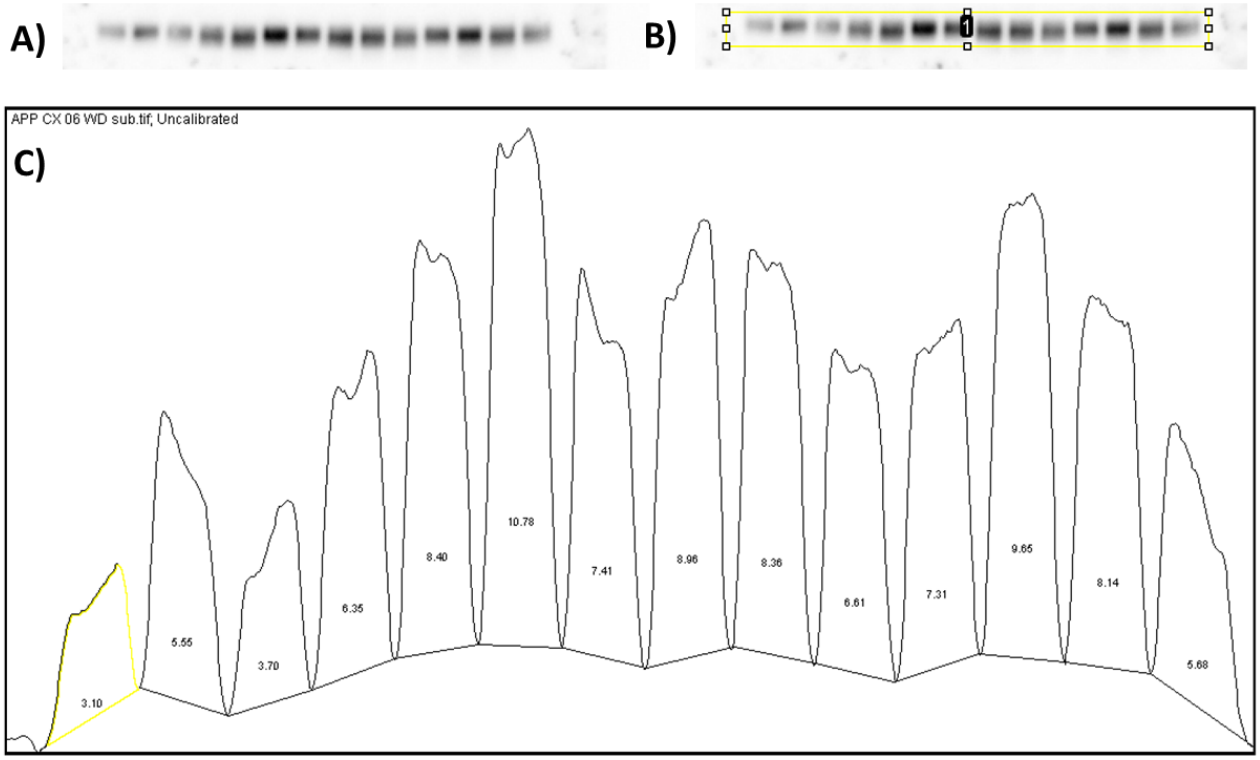

Figure 3.2. Quantification of western blots. *A) Signal image was taken, inverted and straightened. B) A rectangle was drawn around all bands. C) Intensity of colour graph was created, straight lines drawn to connect bottom of peaks, area of each peak selected (example shown on first peak) and percentage peak was calculated by imageJ.*

ELISA

To assess Aβ$_{40}$ and Aβ$_{42}$ levels in *App*$^{NL-G-F}$ mice, ELISA kits were purchased from Invitrogen (California, USA). Homogenate extraction was used for soluble Aβ and insoluble pellet for insoluble Aβ (see section 3.4.4). Protocols were carried out according to the manufacturer's instructions and correct sample dilutions were optimised prior to testing (table 3.6). Each 96-well plate required a series of sample dilutions created from the provided Aβ standard dissolved in filter sterilised reconstitution buffer (55 mM $NaHCO_3$, pH 9; all Aβ$_{40}$ kits) or distilled water (the Aβ$_{42}$ kit) and diluted in the standard diluent buffer provided. Chromogen and water blanks were also present to remove background signal prior to analysis. Human ELISA kits (Aβ$_{40}$: #KHB3481; Aβ$_{42}$: #KHB3441) assessed protein levels in *App*$^{NL-G-F}$ due to the fact their amyloid is humanised (Saito et al., 2014).

Human Aβ$_{40}$ and Aβ$_{42}$ ELISA

Samples were already diluted from homogenate extraction (soluble 1:3, insoluble 1:20) but, following optimisation, were diluted again for ELISAs. Final dilutions used are shown in table 3.6. Fifty microlitres of samples and standards were pipetted in duplicate and incubated for 3 hrs at room temperature with 50 µl detection antibody. Wells were washed four times with 1x wash buffer

and then 100 µl of 1x horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was added to each well and left for 30 minutes at room temperature. Wells were aspirated and washed another 4 times before adding 100 µl stabilised chromogen and left until the colour was developed enough (i.e., the highest standard was dark enough but still able to be read by the machine) which was between 10-20 minutes. The reaction was stopped with 100 µl of the provided stop solution turning the blue colour to yellow which was then analysed.

	Dilution from extraction	Diluted in ELISA	**Final dilution**
$A\beta_{42}$ soluble	1:3	1:3	**1:9**
$A\beta_{42}$ insoluble	1:20	1:50	**1:1000**
$A\beta_{40}$ soluble	1:3	1	**1:3**
$A\beta_{40}$ insoluble	1:20	1:20	**1:400**

Table 3.6. Final dilutions of samples used for human amyloid ELISA kits.

Analysis

The plate was placed in the Infinite M Plex and Tecan i-Control software (Tecan, Switzerland) and absorbance was read at 450 nm. A standard curve was generated from the standards minus blanks using sigmoidal, 4PL (equation: *Y=Bottom + (Top-Bottom)/(1+(IC50/X)^HillSlope*)) on graphpad prism 9.4.1. Sample absorbance, minus blank and chromogen blank, was interpolated off the standard curve to give concentrations. These were multiplied by dilution factors to give the concentration of each sample. This was analysed in SPSS.

Multiplex cytokine assay

The assay was outsourced to IndoorBio (Cardiff, UK) and run by Dr Maria Oliver. To assess multiple cytokine expression levels at once, the Bio-Plex Mouse Cytokine 23-plex assay (#M60009RDPD, Bio-Rad) was used. It utilised Luminex xMAP® system (Luminex Corporation, Austin, TX) in which 6.5 µm magnetic polystyrene beads containing unique fluorophores are used as the assay surface in a suspension array. Cytokine specific antibodies were coupled to unique bead sets allowing multiple cytokines to be measured in one assay.

A 10x stock of antibody-coupled beads were diluted 10 fold in Bio-Plex Pro assay buffer and 50 µl added to all wells of a 96-well 1.2 µm filter plate (# MSBVN1250, Sigma). Vacuum filtration removed assay buffer leaving the beads that were twice washed in 100 µl of Bio-Plex Pro wash buffer after which was removed with vacuum filtration. Washed beads were incubated for 30 mins (shaking at 850 rpm) with standards, blanks (sample diluent) and samples (diluted 1:2 in Bio-Plex

sample diluent). When incubation had 10 minutes remaining, a 1x cocktail of biotin-conjugated detector antibody to cover each cytokine target was diluted from 10x stock with assay buffer. Beads were washed as previously described and incubated for 30 minutes with 25 µl of 1x biotin-conjugated detector antibody cocktail with 850 rpm shaking. Ten minutes prior to the end of incubation, 1x streptavidin-phycoerythrin (SA-PE) was diluted from 100x stock with assay buffer. Beads were again washed following incubation and the bound detector antibody was made quantifiable by adding 50 µl of SA-PE for 10 mins and shaking at 850 rpm. Beads were washed and resuspended in 125 µl assay buffer by shaking for 30 seconds at 850 rpm.

The median fluorescence intensity of specific bead sets (50 beads per cytokine/sample in 125 µl) was measured using a fully calibrated and validated Bio-Plex 200 instrument (Bio-Rad). Median intensity of each cytokine from each sample was processed using accompanying Bio-Plex Manager software. Running multiple analyses on the same samples in this multiplex assay raises the potential problem of type 1 error rate inflation. However, it is also the case that the antibodies are cytokine specific. Thus, it would be possible to argue that a correction for multiple comparisons is appropriate (23 different analyses on the same sample), or is not appropriate (each analysis is based on cytokine-specific antibodies). Therefore, the results from each cytokine were subject to separate ANOVA analysis; but the results will also be considered in light of a Bonferroni correction (where $p < 0.00217$ - i.e., 0.05 / 23 as there were 23 cytokines included in the analysis). This helped determine effects of genotype, age and sex for each individual cytokine (table 3.7).

List of cytokines in assay			
Eotaxin (CCL11)	MIP-1α (CCL3)	IL-2	IL-10
G-CSF	MIP-1β (CCL4)	IL-3	IL-12p40
CM-CSF	RANTES (CCL5)	IL-4	IL-12p70
IFNγ	TNFα	IL-5	IL-13
KC	IL-1α	IL-6	IL-17A (mCTLA-8)
MCP-1	IL-1β	IL-9	

Table 3.7. Complete list of the 23 cytokines measured in the multiplex assay

●3.4.5 Statistical approach

Assumptions and bootstrapping

The use of NHST and Bayesian statistics is identical to what is outlined in section 2.4.10. Assumptions were, again, examined graphically as outlined in section 2.4.10. Briefly, histograms and Q-Q plots examined assumption of normality, boxplots represented equal variance for ANOVA. Samples were still independent and no within-subjects ANOVAs were done making the assumption of sphericity not relevant. However, as the sample sizes were smaller, this suggests the data is more sensitive to violations in assumptions so graphical assessment was less robust. Violations such as the skewed histogram, Q-Q plot and uneven boxplots seen in fig. 2.14, lead to the use of bootstrapping to remove the need for assumptions to be met in ANOVA (Field & Wilcox, 2017).

The bootstrapping was run with 2000 iterations using the Bias-corrected and accelerated (BCa) confidence interval type which offers a more accurate representation. The sampling method was simple with a 95% confidence interval and was run in SPSS.

The way bootstrapping works is by randomly assigning group identity to each data point and running the desired ANOVA. This is repeated by the number of iterations given (in this case 2000) to create the sampling distribution. The ANOVA will then be run against this sampling distribution rather than an assumed normal distribution. A significant result will show if the actual data gives an F statistic that is below 5% probability of occurring (i.e., the tail ends of the bootstrapped sampling distribution histogram). This comparison does not rely on any assumptions so can be run without the need of ANOVA assumptions being met. It should be noted that it can only be run on between subjects data which is what primarily occurs in this chapter.

The bootstrapping methods applied here are appropriate only for between-subjects data, although conceptually similar approaches are possible with repeated measures or mixed designs. This method is beginning to be used more in the academic community (example of bootstrapping being used; Filshtein et al. (2019)).

●3.5 Results

●3.5.1 Amyloid pathology

APP

App^{NL-G-F} mice exhibit amyloid issues in the absence of an increase in *APP* and to confirm this, levels of *APP* were taken in the cortex and hippocampus and western blot performed (fig. 3.3a-b). As expected, in the cortex, there was no difference between the genotypes ($F(1, 72)=.227$, $p=.635$, $\eta_2=.003$, $BF_{exclusion}=8.881$), sexes ($F(1, 72)=.006$, $p=.938$, $\eta_2<.001$, $BF_{exclusion}=9.804$) or across age points ($F(1, 72)=2.538$, $p=.116$, $\eta_2=.034$, $BF_{exclusion}=3.268$) with high bayes factors suggesting strength for the null. All interactions were also non-significant (Genotype*Age, $F(1, 72)=.04$, $p=.843$, $\eta_2=.001$, $BF_{exclusion}=15.232$; Genotype*Sex, $F(1, 72)=.238$, $p=.627$, $\eta_2=.003$, $BF_{exclusion}=29.098$; Age*Sex, $F(1, 72)=.003$, $p=.955$, $\eta_2<.001$, $BF_{exclusion}=16.59$; Genotype*Age*Sex, $F(1, 72)=.013$, $p=.908$, $\eta_2<.001$, $BF_{exclusion}=253.546$). Overall, this supports other evidence showing no overexpression of *APP* in the *App*$^{NL-G-F}$ model across either time point or sex.

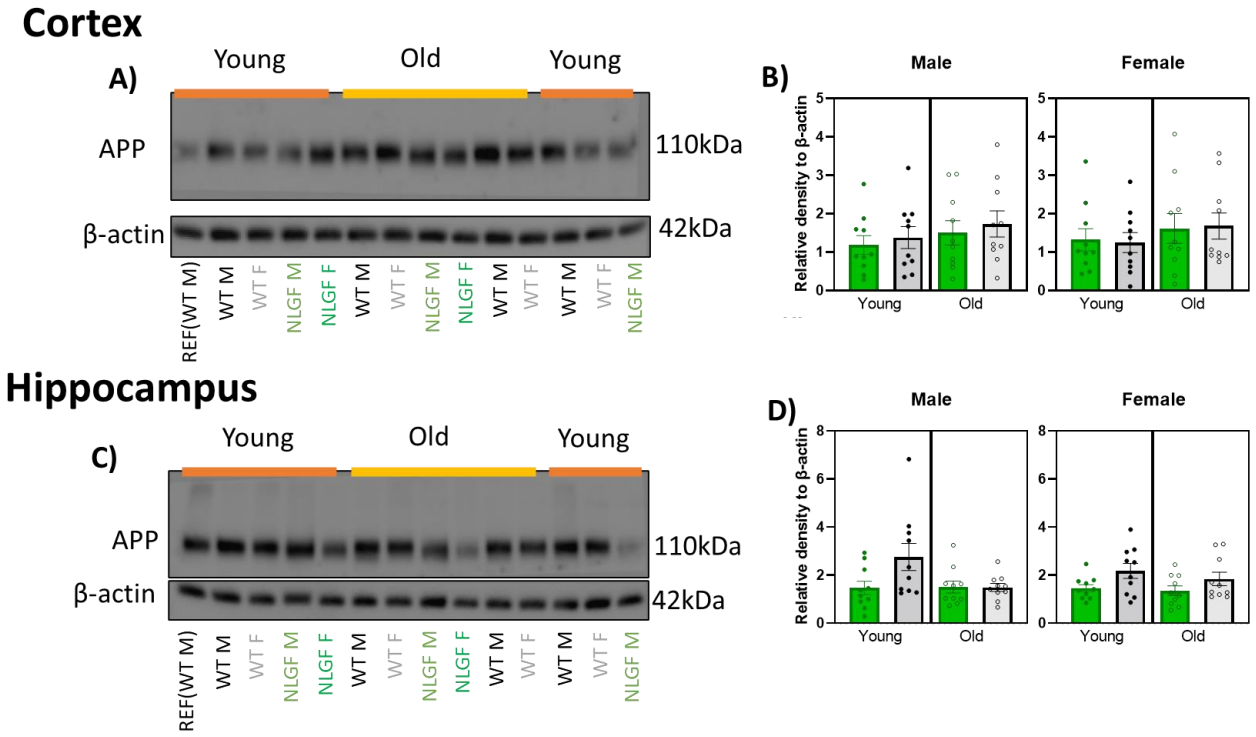

Figure 3.3 Expression levels of APP in the cortex and hippocampus of App^{NL-G-F} and WT mice with ß-actin*. Quantification of APP expression relative to ß-actin in cortex (no significance) and hippocampus (genotype effect, ANOVA, p<.01) across genotypes, sexes and ages. A) Cortex, APP western blot. B) Graph showing APP signal relative to ß-actin in the cortex. C) Hippocampus, APP western blot final image example. D) Graph showing APP signal relative to ß-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=10), old WT male (n=10), old App^{NL-G-F} female (n=10), old WT female (n=10).*

When looking at *APP* expression in the hippocampus (fig. 3.3c-d), there is a significant effect of genotype ($F(1, 72)=8.556$, $p=.005$, $\eta_2=.106$) where *App*$^{NL-G-F}$ actually have reduced signal relative to WT. However, age ($F(1, 72)=3.8$, $p=.055$, $\eta_2=.05$, $BF_{exclusion}=1.032$) and sex ($F(1, 72)=.206$, $p=.651$, $\eta_2=.003$, $BF_{exclusion}=6.087$) effects show no differences and none of the interactions were significant (Genotype*Age, $F(1, 72)=3.247$, $p=.076$, $\eta_2=.043$, $BF_{exclusion}=.722$; Genotype*Sex, $F(1, 72)=.005$, $p=.943$, $\eta_2<.001$, $BF_{exclusion}=4.309$; Age*Sex, $F(1, 72)=.858$, $p=.357$, $\eta_2=.012$, $BF_{exclusion}=4.29$; Genotype*Age*Sex, $F(1, 72)=1.606$, $p=.209$, $\eta_2=.022$, $BF_{exclusion}=1.624$). Altogether, this suggests no overexpression of *APP* in the *App*$^{NL-G-F}$ and a significant reduction in the hippocampus so this model successfully creates amyloid pathology without *APP* overexpression. The reduction of *APP* in the *App*$^{NL-G-F}$ mice will be examined in the discussion.

Amyloid ELISAs

Both soluble and insoluble fragments were extracted from the cortex and used to assess $A\beta_{40}$ and $A\beta_{42}$ levels at both time points in the *App*$^{NL-G-F}$ mice (fig. 3.4). Each type of amyloid was present at all time points suggesting the *App*$^{NL-G-F}$ mice do exhibit humanised amyloid. Two-way

ANOVA was run on each test but, due to violations of assumptions, both Aβ$_{42}$ fragments were bootstrapped for analysis of variance.

Examination of soluble Aβ$_{40}$ shows an increase in age ($F(1, 35)=7.555$, $p=.009$, $\eta_2=.178$) but no sex difference ($F(1, 35)=.208$, $p=.651$, $\eta_2=.006$, $BF_{exclusion}=3.259$) with bayes factors agreeing with the null result. The interaction between sex*age was also non-significant, $F(1, 35)=.056$, $p=.815$, $\eta_2=.002$, $BF_{exclusion}=2.774$. For soluble Aβ$_{42}$, there was no age dependent increase, $F(1, 35)=1.451$, $p=.236$, $\eta_2=.04$, $BF_{exclusion}=2.422$, but the bayes factor suggest this could be inconclusive. There was no effect of sex ($F(1, 35)=.296$, $p=.59$, $\eta_2=.008$, $BF_{exclusion}=3.758$) and the interaction returned non-significant, $F(1, 35)=.01$, $p=.919$, $\eta_2<.001$, $BF_{exclusion}=6.622$.

The insoluble Aβ$_{40}$ fragment showed no increase across time ($F(1, 35)=.761$, $p=.389$, $\eta_2=.021$, $BF_{exclusion}=2.592$) and no effect of sex ($F(1, 35)=2.33$, $p=.136$, $\eta_2=.062$, $BF_{exclusion}=1.528$) but bayes factors highlight this could be an inconclusive result. This also extends to the non-significant interaction of sex*age, $F(1, 35)=1.957$, $p=.172$, $\eta_2=.053$, $BF_{exclusion}=2.258$. For insoluble Aβ$_{42}$, there was no effect of age ($F(1, 35)=1.531$, $p=.224$, $\eta_2=.042$, $BF_{exclusion}=2.057$) or sex ($F(1, 35)=.185$, $p=.67$, $\eta_2=.005$, $BF_{exclusion}=3.36$) and no sex*age interaction ($F(1, 35)=2.386$, $p=.131$, $\eta_2=.064$, $BF_{exclusion}=2.84$).

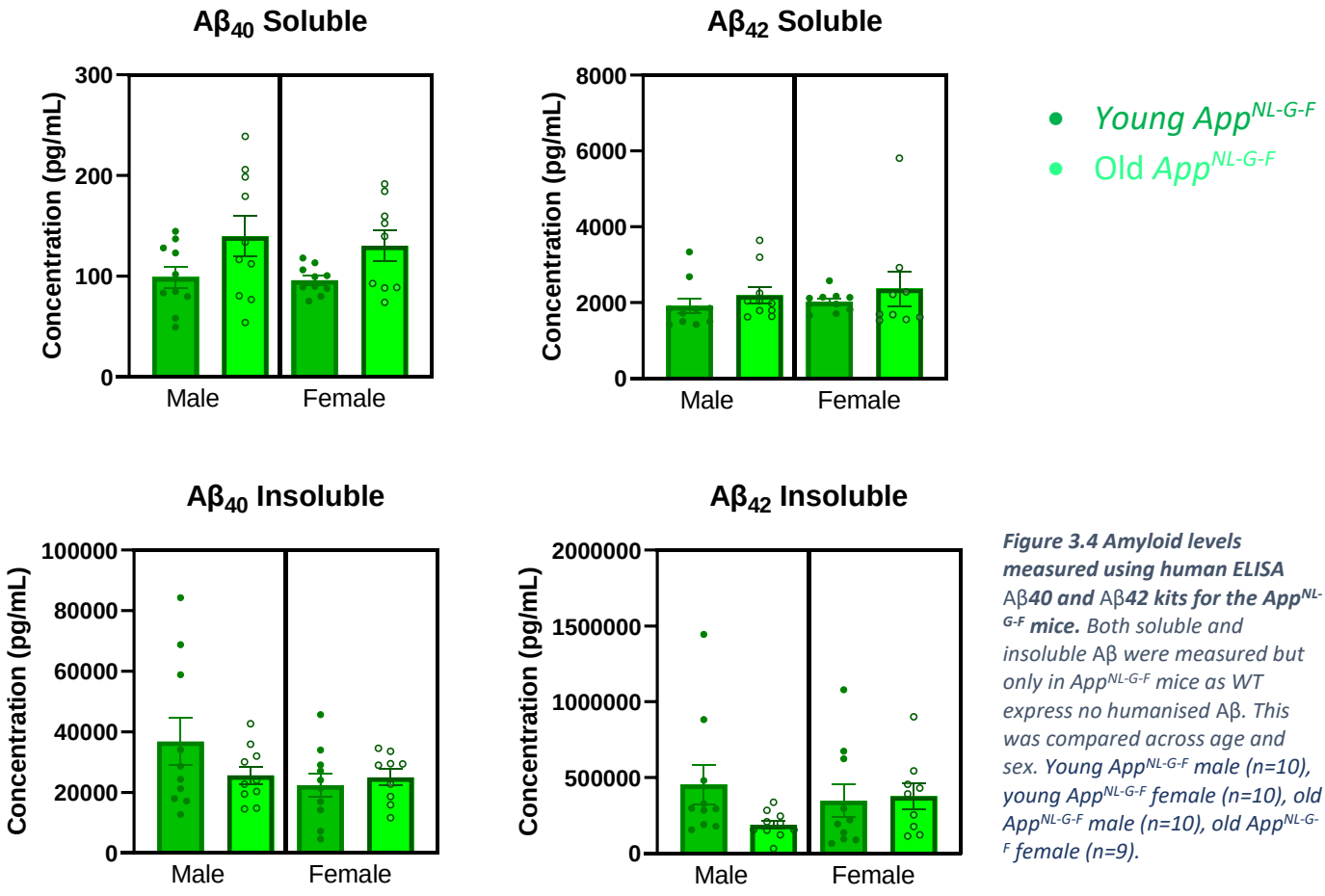

Figure 3.4 Amyloid levels measured using human ELISA Aβ***40 and*** Aβ***42 kits for the App^{NL-G-F} mice.*** *Both soluble and insoluble* Aβ *were measured but only in App^{NL-G-F} mice as WT express no humanised* Aβ*. This was compared across age and sex. Young App^{NL-G-F} male (n=10), young App^{NL-G-F} female (n=10), old App^{NL-G-F} male (n=10), old App^{NL-G-F} female (n=9).*

The Bayesian exclusion factors suggest evidence for the effect of age and the interaction being more inconclusive opposed to a strong null. Overall, there appears to be a significant age increase in soluble $A\beta_{40}$ but not in any other fragments and no differences across sex in the cortex.

●3.5.2 Locus coeruleus

Brains were sliced and stained for Tyrosine hydroxylase and cells were counted manually across 3 slices of Locus Coeruleus and an average for each mouse was used for the ANOVA.

The data is visualised in fig. 3.5 and shows no genotype difference ($F(1, 40)=2.238$, $p=.143$, $\eta_2=.053$, $BF_{exclusion}=2.444$) and no loss of cells with age ($F(1, 40)=.58$, $p=.451$, $\eta_2=.014$, $BF_{exclusion}=4.71$)

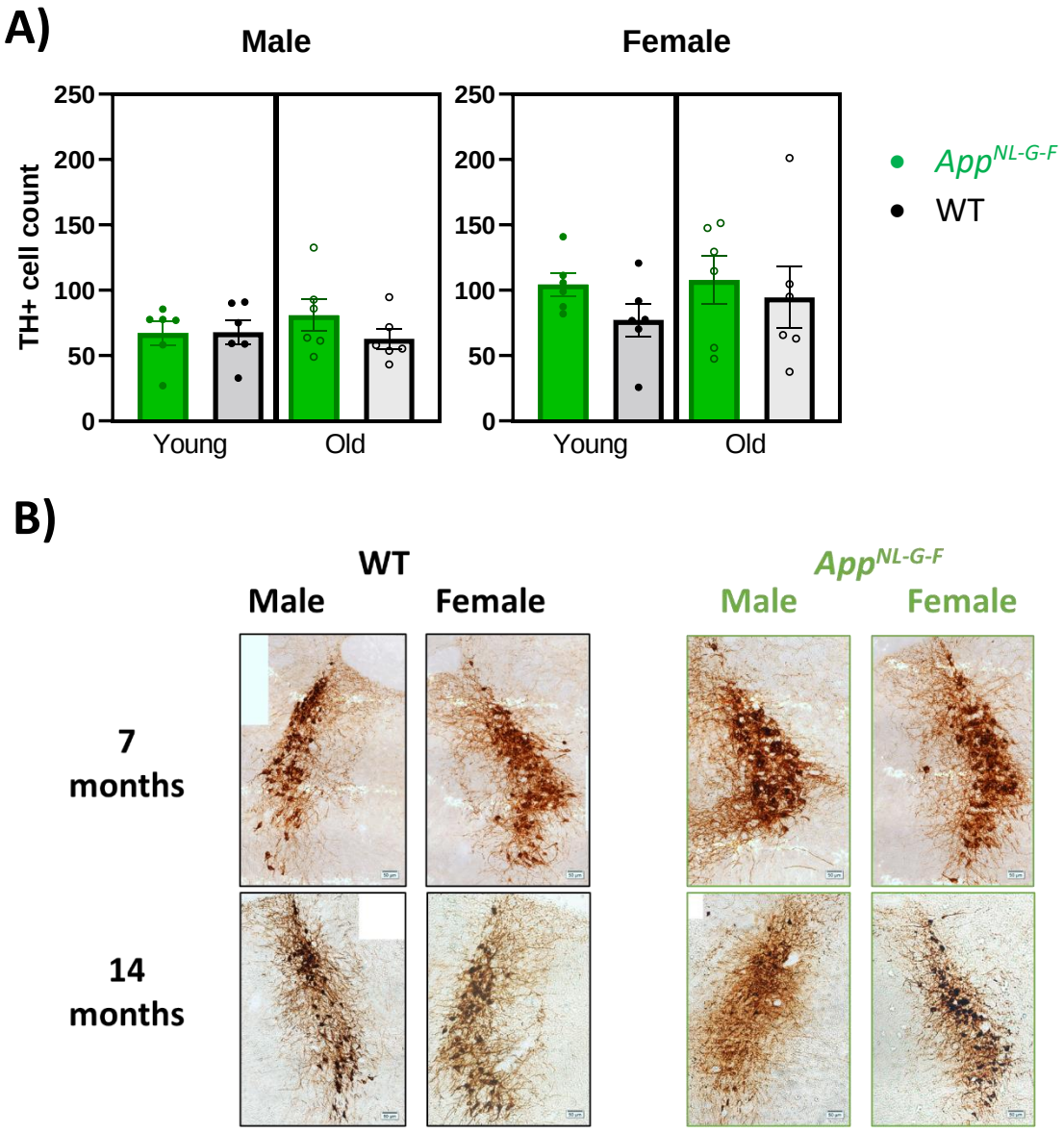

Figure 3.5 Cell count of TH+ cells in the Locus Coeruleus of App^{NL-G-F} and WT mice. *Quantification of amount of tyrosine hydroxylase+ cells in the Locus Coeruleus of male and female, App^{NL-G-F} and WT mice at young (7 month) and old (14 month) ages. ANOVA showed significant effect of sex (p=.01). A) Graph showing TH+ cell count across groups (error bars represent +- SEM). B) Example images of mouse LC. Young App^{NL-G-F} male (n=6), young WT male (n=6), young App^{NL-G-F} female (n=6), young WT female (n=6), old App^{NL-G-F} male (n=6), old WT male (n=6), old App^{NL-G-F} female (n=6), old WT female (n=6).*

with bayes factors suggesting inconclusive or weak evidence for the null respectively. However,

there was a significant sex effect (F(1, 40)=7.4, p=.01, η_2=.156) with females having a higher cell count than males. All interactions were non-significant (Genotype*Sex, F(1, 40)=.354, p=.555, η_2=.009, $BF_{exclusion}$=2.676; Genotype*Age, F(1, 40)=.016, p=.901, η_2<.001, $BF_{exclusion}$=7.067; Age*Sex, F(1, 40)=.097, p=.757, η_2=.002, $BF_{exclusion}$=4.302; Genotype*Sex*Age, F(1, 40)=.724, p=.4, η_2=.018, $BF_{exclusion}$=18.466). Overall, there appears to be no significant loss of TH+ Locus Coeruleus cells in the *App*$^{NL-G-F}$ mice and no age-related degradation but females show a higher count than male mice.

●3.5.3 Inflammation

GFAP

To assess astrogliosis in the cortex, a western blot targeting GFAP was run and signal strength relative to β-actin was put into a three-way ANOVA (fig. 3.6). It is important to note that while fig. 3.6a-b shows no signal from the WT, this is simply due to the large signal from *App*$^{NL-G-F}$ mice. Covering the *App*$^{NL-G-F}$ mouse bands did result in the WT band being visible. This is not a failing of the antibody.

As seen in fig. 3.6b, there is a profound genotype effect (F(1, 72)=64.058, p<.001, η_2=.471) where *App*$^{NL-G-F}$ have higher GFAP signal compared to WT in the cortex. Despite a trend in increased signal with age, this does not reach significance, F(1, 72)=1.092, p=.299, η_2=.015, $BF_{exclusion}$=4.674, and there is also no sex effect , F(1, 72)=.033, p=.857, η_2<.001, $BF_{exclusion}$=7.923. All interactions were non-significant (Genotype*Age, F(1, 72)=1.254, p=.267, η_2=.017, $BF_{exclusion}$=3.13; Genotype*Sex, F(1, 72)=.057, p=.812, η_2=.001, $BF_{exclusion}$=6.825; Age*Sex, F(1, 72)=.28, p=.598, η_2=.004, $BF_{exclusion}$=14.389; Genotype*Age*Sex, F(1, 72)=.222, p=.639, η_2=.003, $BF_{exclusion}$=54.981) and the high bayes factors confirmed the strength of each of these null results. Overall, this data shows a sharp increase in astrogliosis in the *App*$^{NL-G-F}$ mice that does not differ between ages and sexes.

When assessing astrocyte activity in the hippocampus, the western blot signal relative to β-actin was used in a three-way ANOVA (fig. 3.6c-d). As in the cortex, a strong genotype effect was found (F(1, 72)=68.418, p<.001, η_2=.487) where *App*$^{NL-G-F}$ show increased GFAP signal compared to WT. However, the increase with age was significant (F(1, 72)=4.895, p=.03, η_2=.064) but sex remained non-significant (F(1, 72)=.761, p=.386, η_2=.01, $BF_{exclusion}$=5.285). The genotype*age interaction was not significant (F(1, 72)=3.544, p=.064, η_2=, $BF_{exclusion}$=.504) with the bayes factor

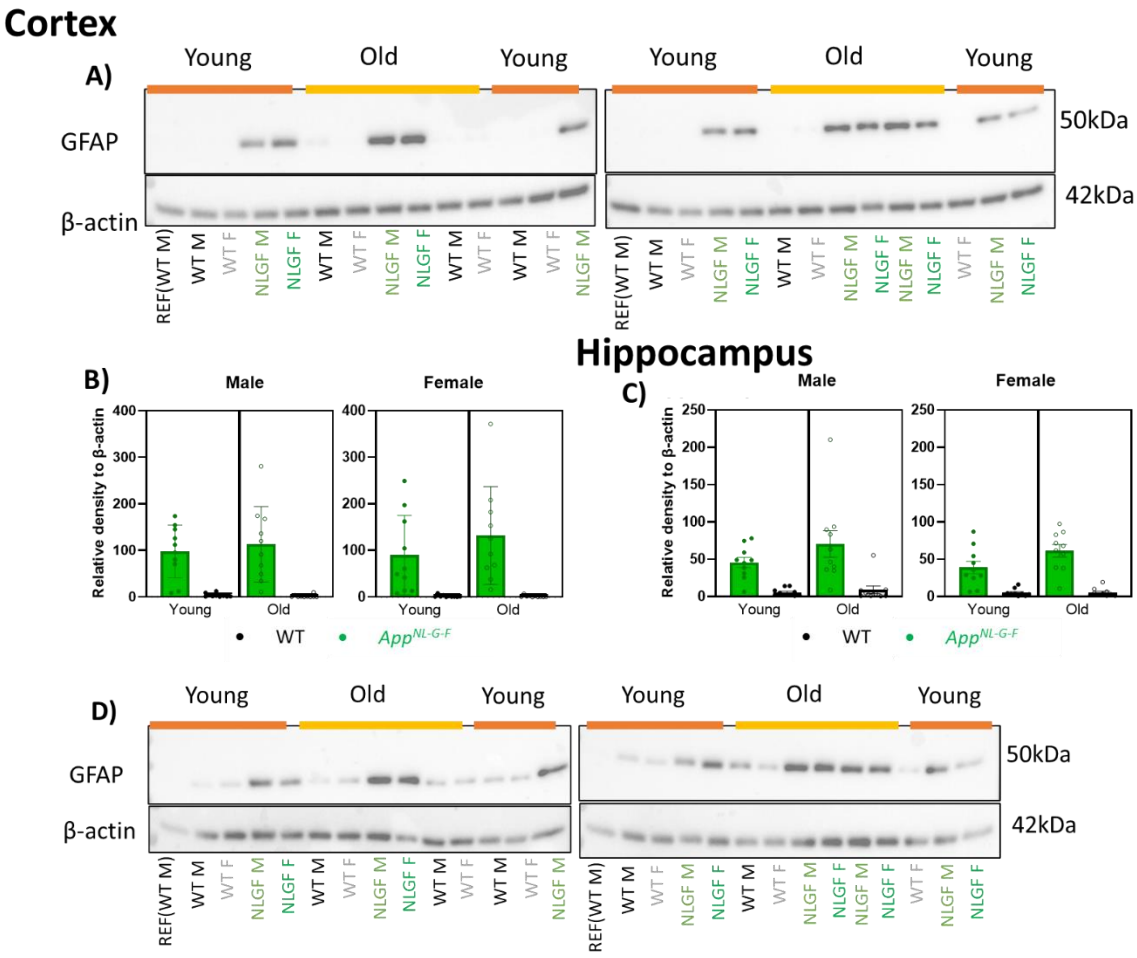

Figure 3.6 Expression levels of GFAP in the cortex and hippocampus of App^{NL-G-F} and WT mice with ß-actin. *Quantification of GFAP expression relative to ß-actin in cortex (genotype effect, ANOVA, p<.001) and hippocampus (genotype effect, ANOVA, p<.001) across genotypes, sexes and ages. A) Two examples of GFAP western blots run with their respective ß-actin blots. WT bands were present but not visible due to the high signal from App^{NL-G-F}. B) Bar graph showing GFAP signal relative to ß-actin in the cortex. C) Bar graph showing GFAP signal relative to ß-actin in the hippocampus. D) Two examples of GFAP western blots run with their respective ß-actin blots. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

suggesting inconclusive data but all other interactions were non-significant (Genotype*Sex, F(1, 72)=.261, p=.611, η_2=.004, $BF_{exclusion}$=4.605; Age*Sex, F(1, 72)=.063, p=.803, η_2=.001, $BF_{exclusion}$=5.4; Genotype*Age*Sex, F(1, 72)<.001, p=.986, η_2<.001, $BF_{exclusion}$=13.185). Together, it appears astrogliosis is greatly increased in *App*$^{NL-G-F}$ mice in both brain areas that is exacerbated with age in the hippocampus but not in the cortex. This reveals increased inflammation in the *App*$^{NL-G-F}$ mice.

Iba-1

Ionized calcium-binding molecule 1 (Iba-1) is a target found on macrophages and microglia and so will be used as a general inflammatory assessment of the brain (fig. 3.7). For both the cortex and hippocampus, data violated assumptions for ANOVA so bootstrapping was run in analysis. For the cortex, a three-way ANOVA revealed a significant effect of genotype ($F(1, 70)=9.271$, $p=.003$, $\eta^2=.117$) in the cortex and, as shown in fig. 3.7, *App*$^{NL-G-F}$ have higher signal compared to WT. There were no differences in age ($F(1, 70)=2.467$, $p=.121$, $\eta^2=.034$, $BF_{exclusion}=2.541$) or sex ($F(1, 70)=.184$, $p=.669$, $\eta^2=.003$, $BF_{exclusion}=7.363$). All interactions were non-significant (Genotype*Age, $F(1, 70)=1.528$, $p=.221$, $\eta^2=.021$, $BF_{exclusion}=2.058$; Genotype*Sex, $F(1, 70)=.114$, $p=.737$, $\eta^2=.002$, $BF_{exclusion}=6.644$; Sex*Age, $F(1, 70)=.328$, $p=.569$, $\eta^2=.005$, $BF_{exclusion}=10.011$; Genotype*Age*Sex, $F(1, 70)=.536$, $p=.467$, $\eta^2=.008$, $BF_{exclusion}=37.027$).

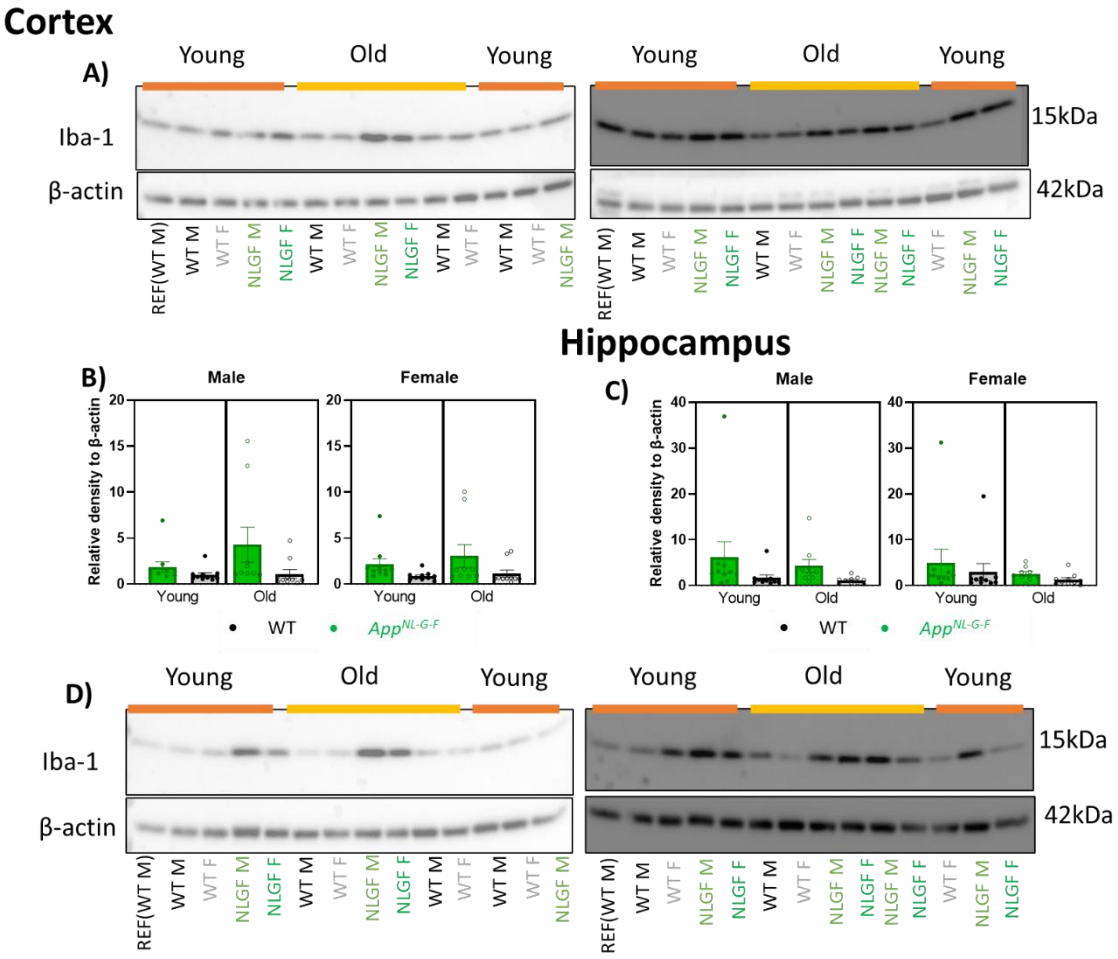

Figure 3.7 Expression levels of Iba-1 in the cortex and hippocampus of App^{NL-G-F} and WT mice with β-actin. *Quantification of Iba-1 expression relative to β-actin in cortex (genotype effect, ANOVA, p<.01) and hippocampus (genotype effect, ANOVA, p<.05) across genotypes, sexes and ages. A) Two examples of Iba-1 western blots run with their respective β-actin in the cortex. B) Bar graph showing Iba-1 signal relative to β-actin in the cortex. Error bars represent +-SEM. C) Bar graph showing Iba-1 signal relative to β-actin in the hippocampus. Error bars represent +-SEM. D) Two examples of Iba-1 western blots run with their respective β-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

For the hippocampus, ANOVA showed a significant effect of genotype, F(1, 70)=4.289, p=.042, η^2=.058, just as in the cortex. Fig. 3.7 shows *App*$^{NL-G-F}$ have a higher signal of Iba-1 compared to WT but there were no significant effects of age (F(1, 70)=1.489, p=.226, η^2=.021, $BF_{exclusion}$=4.643) or sex (F(1, 70)=.074, p=.786, η^2=.001, $BF_{exclusion}$=8.404). Furthermore, there were no significant interactions (Genotype*Age, F(1, 70)=.15, p=.7, η^2=.002, $BF_{exclusion}$=6.333; Genotype*Sex, F(1, 70)=.67, p=.416, η^2=.009, $BF_{exclusion}$=8.203; Sex*Age, F(1, 70)=.101, p=.752, η^2=.001, $BF_{exclusion}$=16.764; Genotype*Age*Sex, F(1, 70)=.01, p=.919, η^2<.001, $BF_{exclusion}$=101.393).

Overall, *App*$^{NL-G-F}$ have a higher rate of inflammation in the cortex and hippocampus compared to WT and this did not differ across sexes or age points.

Pro-inflammatory cytokines

IL-1β

The pro-inflammatory marker, IL-1β, expression was measured using western blotting (fig. 3.8) and standardised to β-actin for analysis with bootstrapping due to non-normal data. Both the precursor fragment (pro) and mature fragment (secreted/cleaved) after cleavage will be assessed as both indicate increased PIC levels and could show whether there are changes in amount cleaved. The three-way ANOVA on the secreted/cleaved fragment (fig. 3.8b) revealed no differences between the genotypes (F(1, 72)=.868, p=.355, η_2=.012, $BF_{exclusion}$=6.116), sexes (F(1, 72)=1.516, p=.222, η_2=.021, $BF_{exclusion}$=5.969) or ages (F(1, 72)=2.164, p=.146, η_2=.029, $BF_{exclusion}$=3.629) with moderate bayes factors suggesting evidence in favour of null effect. All the two and three way interactions also were non-significant (Genotype*Age, F(1, 72)=.371, p=.544, η_2=.005, $BF_{exclusion}$=10.128; Genotype*Sex, F(1, 72)=.762, p=.383, η_2=.01, $BF_{exclusion}$=13.215; Age*Sex, F(1, 72)=1.111, p=.295, η_2=.015, $BF_{exclusion}$=7.146; Genotype *Age*Sex, F(1, 72)=.748, p=.39, η_2=.01, $BF_{exclusion}$=49.282). For the cleaved fragment, there were no differences across groups in the cortex.

Western blotting also looked at secreted IL-1β expression in the hippocampus and results are shown in fig. 3.8d-f. The bootstrapped three-way ANOVA found no genotype difference (F(1, 72)=.236, p=.629, η_2=.003, $BF_{exclusion}$=8.367), sex difference (F(1, 72)=.025, p=.874, η_2<.001, $BF_{exclusion}$=9.792) or age difference (F(1, 72)=1.384, p=.243, η_2=.019, $BF_{exclusion}$=6.454). The two and three way interactions (Genotype*Age (F(1, 72)=.033, p=.857, η_2<.001, $BF_{exclusion}$=21; Genotype*Sex, F(1, 72)=.639, p=.427, η_2=.009, $BF_{exclusion}$=24.501; Age*Sex, F(1, 72)=.356, p=.552, η_2=.005, $BF_{exclusion}$=23.771; Genotype*Age*Sex, F(1, 72)=.022, p=.882, η_2=.001, $BF_{exclusion}$=300.885) were also non-significant with high bayes factors supporting this null result. In the cortex and hippocampus, there were no group differences in expression of Il-1β cleaved fragment.

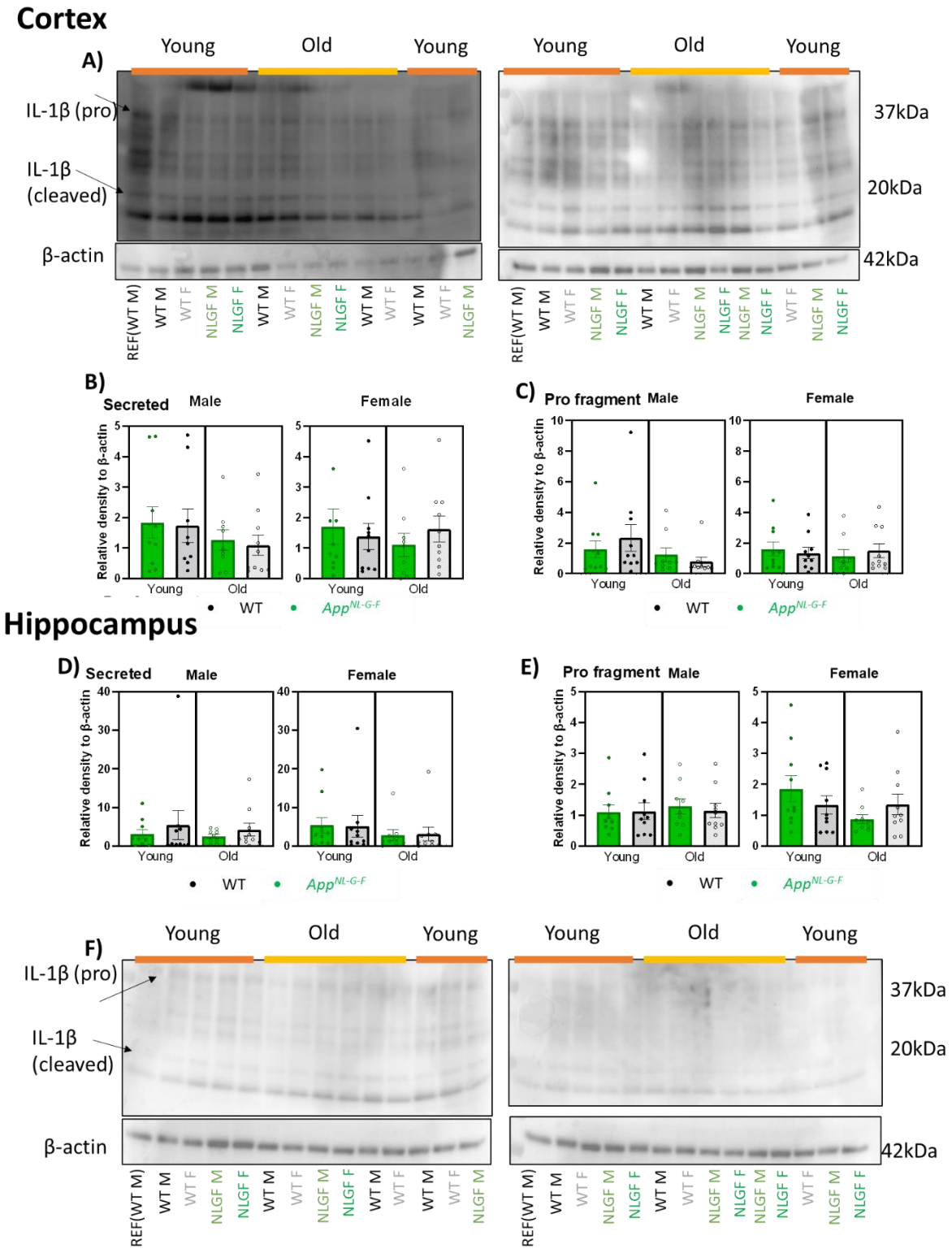

Figure 3.8 Expression levels of IL-1ß in the cortex and hippocampus of App^{NL-G-F} and WT mice with ß-actin*. Quantification of IL-1ß expression secreted fragment and precursor (pro) fragment in the cortex and hippocampus with no significant effects across genotypes, age points and sex. A) Two examples of IL-1ß western blots run with their respective ß-actin in the cortex. B & C) Bar graph showing IL-1ß signal (secreted/cleaved & pro) relative to ß-actin in the cortex. Error bars represent +-SEM. D & E) Bar graph showing IL-1ß signal (secreted/cleaved & pro) relative to ß-actin in the hippocampus. Error bars represent +-SEM. F) Two examples of IL-1ß western blots run with their respective ß-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

The precursor to secreted IL-1β was also measured with western blotting and results are shown in fig. 3.8c for the cortex. Due to violating assumption of normality, bootstrapping was done on the ANOVA. Like the cleaved fragment, there were no differences between genotypes ($F(1, 72)=.06$, $p=.808$, $\eta_2=.001$, $BF_{exclusion}=9.048$), sexes ($F(1, 72)=2.023$, $p=.159$, $\eta_2=.027$, $BF_{exclusion}=8.838$) or ages ($F(1, 72)=3.433$, $p=.068$, $\eta_2=.046$, $BF_{exclusion}=2.754$) and all interactions were non-significant (Genotype*Age, $F(1, 72)=.169$, $p=.682$, $\eta_2=.002$, $BF_{exclusion}=14.035$; Genotype*Sex, $F(1, 72)=.682$, $p=.412$, $\eta_2=.009$, $BF_{exclusion}=27.559$; Age*Sex, $F(1, 72)=.175$, $p=.677$, $\eta_2=.002$, $BF_{exclusion}=8.988$; Genotype*Age*Sex, $F(1, 72)=.002$, $p=.963$, $\eta_2<.001$, $BF_{exclusion}=96.037$). The Bayesian ANOVA shows strong evidence for the null hypothesis aside from age which may lean more toward an inconclusive result.

The precursor to Il-1β was measured in the same way and expression was analysed using a three-way ANOVA (fig. 3.8e) for the hippocampus. Like the secreted fragment, there was also no effect of genotype ($F(1, 72)=.041$, $p=.84$, $\eta_2=.001$, $BF_{exclusion}=9.327$), sex ($F(1, 72)=.853$, $p=.359$, $\eta_2=.012$, $BF_{exclusion}=6.137$) or age ($F(1, 72)=.883$, $p=.351$, $\eta_2=.012$, $BF_{exclusion}=6.411$) and the non-significant interactions meant no further testing was done (Genotype*Age, $F(1, 72)=1.037$, $p=.312$, $\eta_2=.015$, $BF_{exclusion}=15.501$; Genotype*Sex, $F(1, 72)=.01$, $p=.92$, $\eta_2<.001$, $BF_{exclusion}=21.064$; Age*Sex, $F(1, 72)=2.038$, $p=.158$, $\eta_2=.028$, $BF_{exclusion}=8.712$; Genotype*Age*Sex, $F(1, 72)=2.067$, $p=.155$, $\eta_2=.029$, $BF_{exclusion}=54.07$). The Bayes factors also suggested weak to strong evidence for the null supporting the idea that there is no difference in IL-1β expression between all groups examined in the hippocampus. Therefore, the pro-inflammatory profile does not appear to change between *App*$^{NL-G-F}$ and WT, sexes, or age.

Overall, expression of the pro-inflammatory cytokine, IL-1β, in either its secreted or precursor form does not differ across genotype groups in the cortex or hippocampus. This also suggests that the cleavage of IL-1β is not impacted in *App*$^{NL-G-F}$ mice.

IL-6

Expression of the pro-inflammatory marker, IL-6, was measured using western blot (fig. 3.9) and analysed using three-way ANOVA. There was no effect of genotype ($F(1, 72)=.036$, $p=.85$, $\eta_2=.001$, $BF_{exclusion}=10.153$), age ($F(1, 72)=.125$, $p=.724$, $\eta_2=.002$, $BF_{exclusion}=10.029$) or sex ($F(1, 72)=.007$, $p=.934$, $\eta_2<.001$, $BF_{exclusion}=10.35$) as all groups show great variance (Fig. 3.9b). All interactions were also non-significant (Genotype*Age, $F(1, 72)=.335$, $p=.564$, $\eta_2=.005$, $BF_{exclusion}=29.084$; Genotype*Sex, $F(1, 72)=.844$, $p=.361$, $\eta_2=.012$, $BF_{exclusion}=26.111$; Age*Sex, $F(1, 72)=.034$, $p=.855$, $\eta_2<.001$, $BF_{exclusion}=34.17$; Genotype*Age*Sex, $F(1, 72)=.86$, $p=.357$, $\eta_2=.012$,

$BF_{exclusion}$=328.717) with high bayes factors supporting this null result. Overall, this suggests no difference in IL-6 expression in the cortex in any of the groups.

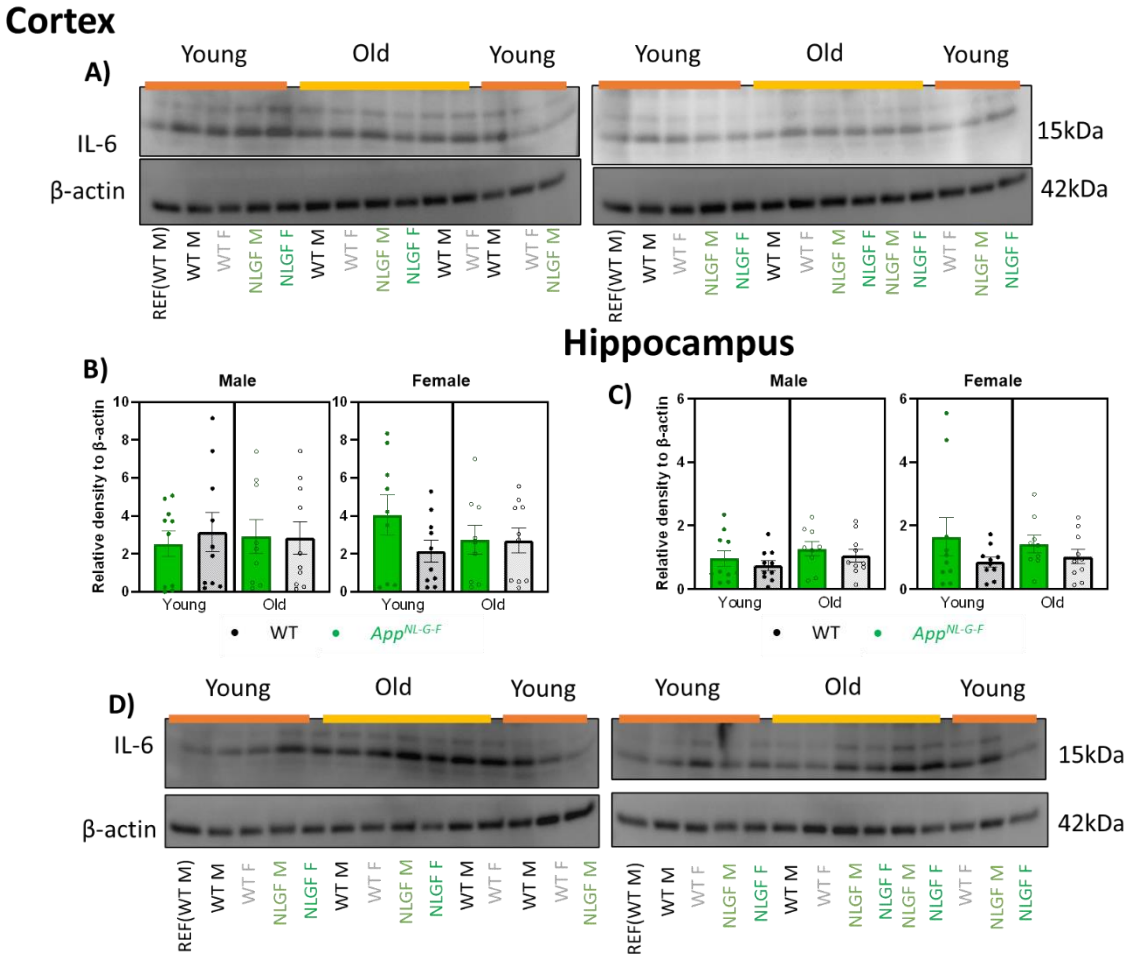

Figure 3.9 Expression levels of IL-6 in the cortex and hippocampus of App^{NL-G-F} and WT mice with ß-actin. *Quantification of IL-6 expression in the cortex and hippocampus with no significant effects across genotypes, age points and sex. A) Two examples of IL-6 western blots run with their respective ß-actin in the cortex. B) Bar graph showing IL-6 signal relative to ß-actin in the cortex. Error bars represent +-SEM. C) Bar graph showing IL-6 signal relative to ß-actin in the hippocampus. Error bars represent +-SEM. D) Two examples of IL-6 western blots run with their respective ß-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

IL-6 expression was also measured in the hippocampus that showed a trend of increased expression in *App*$^{NL-G-F}$ which approached statistical significance (Fig. 3.9c-d), F(1, 72)=3.815, p=.055, η_2=.052, $BF_{exclusion}$=1.792, but the bayes factor suggests an inconclusive result. There was no main effect of age (F(1, 72)=.452, p=.504, η_2=.006, $BF_{exclusion}$=7.167) or sex (F(1, 72)=1.189, p=.279, η_2=.017, $BF_{exclusion}$=5.117) and all interactions were non-significant (Genotype*Age, F(1, 72)=.24, p=.626, η_2=.003, $BF_{exclusion}$=8.88; Genotype*Sex, F(1, 72)=.781, p=.38, η_2=.011, $BF_{exclusion}$=5.972; Age*Sex, F(1, 72)=.63, p=.43, η_2=.009, $BF_{exclusion}$=12.979; Genotype*Age*Sex, F(1, 72)=.219, p=.641, η_2=.003, $BF_{exclusion}$=68.114). IL-6 expression appears to be numerically higher in the *App*$^{NL-G-F}$ consistent with

increased inflammation in the hippocampus but not the cortex, however, this was an inconclusive result.

Anti-inflammatory cytokines

The anti-inflammatory marker, TGF-β, was assessed using western blot in the cortex and hippocampus. Two fragments were analysed, the precursor and mature TGF-β that both offer measurements for TGF-β levels but the precursor is non-active (fig. 3.10). Three-way ANOVA with bootstrapping revealed no effect of genotype ($F(1, 72)=.126$, $p=.724$, $\eta_2=.002$, $BF_{exclusion}=9.647$), age ($F(1, 72)=.384$, $p=.537$, $\eta_2=.005$, $BF_{exclusion}=8.713$) or sex ($F(1, 72)=.266$, $p=.607$, $\eta_2=.004$, $BF_{exclusion}=9.34$) for the monomer fragment in the cortex. Furthermore, all interactions were non-significant (Genotype*Age, $F(1, 72)=.769$, $p=.383$, $\eta_2=.011$, $BF_{exclusion}=22.361$; Genotype*Sex, $F(1, 72)=.241$, $p=.625$, $\eta_2=.003$, $BF_{exclusion}=28.445$; Age*Sex, $F(1, 72)=.094$, $p=.761$, $\eta_2=.001$, $BF_{exclusion}=28.783$; Genotype*Age*Sex, $F(1, 72)=.042$, $p=.839$, $\eta_2=.001$, $BF_{exclusion}=338.859$) and with bayes factors greatly over 10, this would suggest strong evidence for this null result. This suggests no difference in this anti-inflammatory marker across all groups.

Mature TGF-β expression (monomer) was also measured in the hippocampus using western blotting and the data is shown in fig. 3.10d. The ANOVA included bootstrapping due to violating assumptions. There were no differences between genotypes ($F(1, 72)=.025$, $p=.874$, $\eta_2<.001$, $BF_{exclusion}=10.33$), sexes ($F(1, 72)=.033$, $p=.857$, $\eta_2<.001$, $BF_{exclusion}=10.179$) or ages ($F(1, 72)=.534$, $p=.467$, $\eta_2=.008$, $BF_{exclusion}=8.14$) with high bayes factors agreeing with a null result. All interactions were non-significant so no further testing was done (Genotype*Age, $F(1, 72)=.058$, $p=.811$, $\eta_2=.001$, $BF_{exclusion}=29.304$; Genotype*Sex, $F(1, 72)=.004$, $p=.952$, $\eta_2<.001$, $BF_{exclusion}=34.786$; Age*Sex, $F(1, 72)=.03$, $p=.862$, $\eta_2<.001$, $BF_{exclusion}=28.157$; Genotype*Age*Sex, $F(1, 72)=.144$, $p=.705$, $\eta_2=.002$, $BF_{exclusion}=463.193$). The anti-inflammatory profile appears to not change between *App*$^{NL-G-F}$ and WT at different ages and sexes.

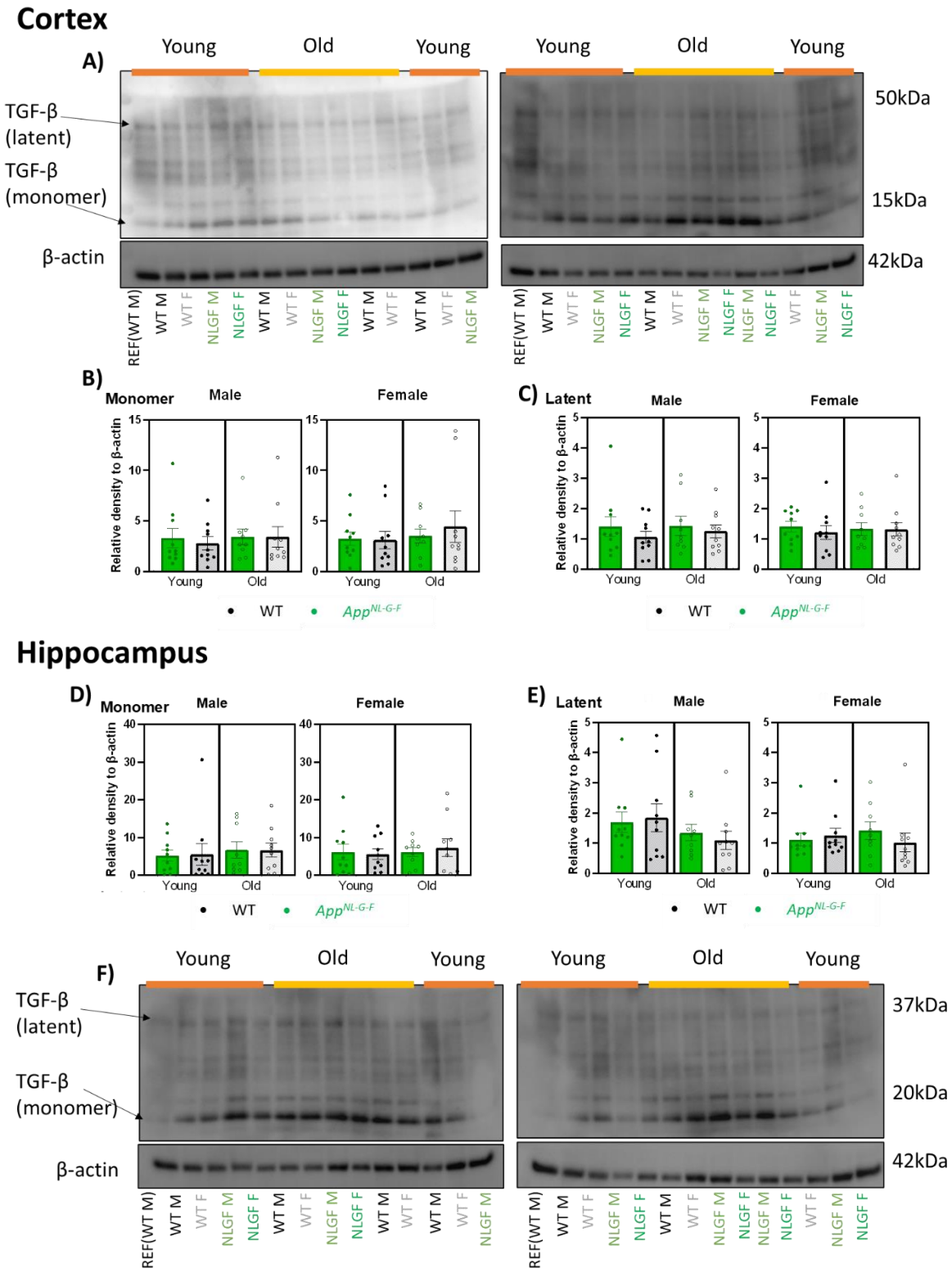

Figure 3.10 Expression levels of TGF-β in the cortex and hippocampus of App^{NL-G-F} and WT mice with β-actin. *Quantification of TGF-β expression monomer fragment and latent fragment in the cortex and hippocampus with no significant effects across genotypes, age points and sex. A) Two examples of TGF-β western blots run with their respective β-actin in the cortex. B&C) Bar graph showing TGF-β signal relative to β-actin in the cortex. Error bars represent +-SEM. D&E) Bar graph showing TGF-β signal relative to β-actin in the hippocampus. Error bars represent +-SEM. F) Two examples of TGF-β western blots run with their respective β-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

The latent or precursor fragment band was analysed on the same western blots and results of the ANOVA are visualised in fig. 3.10c but showed no main effect of genotype ($F(1, 72)=.427$, $p=.516$, $\eta_2=.006$, $BF_{exclusion}=8.605$), sex ($F(1, 72)=.03$, $p=.864$, $\eta_2<.001$, $BF_{exclusion}=10.304$) or age ($F(1, 72)=.002$, $p=.964$, $\eta_2<.001$, $BF_{exclusion}=10.372$) suggesting no expression differences of TGF-β between groupsin the cortex. The interactions were also non-significant (Genotype*Age, $F(1, 72)=.798$, $p=.375$, $\eta_2=.011$, $BF_{exclusion}=23.122$; Genotype*Sex, $F(1, 72)=.209$, $p=.649$, $\eta_2=.003$, $BF_{exclusion}=28.379$; Age*Sex, $F(1, 72)=.076$, $p=.784$, $\eta_2=.001$, $BF_{exclusion}=33.666$; Genotype*Age*Sex, $F(1, 72)<.001$, $p=.996$, $\eta_2<.001$, $BF_{exclusion}=454.72$) with corresponding bayes factors suggesting strong evidence for a null result.

The latent fragment of TGF-β was assessed in the hippocampus as above and visualised in fig. 3.10e. The three-way ANOVA with bootstrapping revealed no signal differences between genotypes ($F(1, 72)=.18$, $p=.673$, $\eta_2=.003$, $BF_{exclusion}=8.413$), sexes ($F(1, 72)=1.762$, $p=.189$, $\eta_2=.025$, $BF_{exclusion}=4.025$) or ages ($F(1, 72)=1.336$, $p=.252$, $\eta_2=.019$, $BF_{exclusion}=4.697$) with the bayes factors suggesting weak evidence for the null. The interactions were also non-significant (Genotype*Age, $F(1, 72)=1.152$, $p=.287$, $\eta_2=.016$, $BF_{exclusion}=12.279$; Genotype*Sex, $F(1, 72)=.031$, $p=.862$, $\eta_2<.001$, $BF_{exclusion}=16.096$; Age*Sex, $F(1, 72)=1.71$, $p=.195$, $\eta_2=.024$, $BF_{exclusion}=6.048$; Genotype*Age*Sex, $F(1, 72)=.02$, $p=.887$, $\eta_2<.001$, $BF_{exclusion}=78.67$) suggesting no difference in the anti-inflammatory marker TGF-β across groups.

Overall, the anti-inflammatory profile does not appear to differ between *App*$^{NL-G-F}$ or WT across ages or sexes in the cortex or hippocampus for either fragment assessed.

Multiplex cytokine assay

A multiplex cytokine assay was run on the *App*$^{NL-G-F}$ and WT to give a more sensitive analysis of various important cytokines. The primary ones of interest are: IL-6, IL-1β, TNF-α for PIC and IL-4 and IL-10 for AIC. Although these were the initial focus, other cytokines that were examined showed results of interest, some of which will also be focused on. These include markers that found a significant genotype difference which are IFNγ, MIP-1α (CCL3), MIP-1β (CCL4), IL-9 and IL-12p70. The means and standard deviations for all markers are shown in table 3.8 while all three-way or two-way ANOVA and bayes factors are shown in table 3.9. A summary of important results is also presented detailing direction of significant findings (table 3.10). Due to the potential of increased type 1 error rate, the results of individual ANOVAs are reported but also highlighted if they pass the Bonferroni correction (i.e., $p < 0.00217$). Table 3.9 will highlight significant results but those that pass the Bonferroni corrections will be highlighted in orange. All graphs collapse across sex due to the lack of significant sex differences to improve clarity.

Cytokine	Age	Genotype	Sex	Mean	Standard deviation	n
Eotaxin	Young	NLGF	F	13.47111	5.862884	9
			M	14.34	3.410448	10
		WT	F	11.34556	5.217246	9
			M	11.64625	3.966466	8
	Old	NLGF	F	12.192	2.949459	10
			M	13.311	3.272911	10
		WT	F	9.766	6.36177	10
			M	12.735	4.603357	10
G-CSF	Young	NLGF	F	15.29111	7.973795	9
			M	17.636	3.927853	10
		WT	F	11.59778	7.638257	9
			M	13.8375	10.19073	8
	Old	NLGF	F	15.919	7.209889	10
			M	19.133	9.066759	10
		WT	F	13.304	9.904103	10
			M	16.79	8.282388	10
GM-CSF	Young	NLGF	F	25.63	5.889034	9
			M	25.292	5.258876	10
		WT	F	20.88556	10.12988	9
			M	21.83875	6.064356	8
	Old	NLGF	F	26.499	6.46949	10
			M	31.708	5.608616	10
		WT	F	24.78	13.40729	10
			M	30.475	7.503546	10
IFNg	**Young**	**NLGF**	**F**	**21.67667**	**7.825629**	**9**
			M	**21.082**	**2.954803**	**10**
		WT	**F**	**17.71**	**7.599461**	**9**
			M	**18.46625**	**5.095351**	**8**
	Old	**NLGF**	**F**	**13.267**	**2.800147**	**10**
			M	**19.078**	**3.144027**	**10**
		WT	**F**	**12.531**	**7.561707**	**10**
			M	**14.012**	**5.063334**	**10**
KC	Young	NLGF	F	15.43778	3.512438	9
			M	13.751	3.73022	10
		WT	F	12.59	6.151652	9
			M	15.1275	4.536168	8
	Old	NLGF	F	16	4.872264	10
			M	18.663	4.395928	10
		WT	F	12.316	7.801524	10
			M	14.886	5.754761	10
MCP-1	Young	NLGF	F	91.09222	27.94967	9
			M	101.44	21.07239	10
		WT	F	74.19	37.15779	9

Cytokine	Age	Genotype	Sex	Mean	Standard deviation	n
			M	77.97	22.35392	8
	Old	NLGF	F	133.013	49.86016	10
			M	109.093	36.16505	10
		WT	F	105.971	62.67627	10
			M	145.485	25.85302	10
MIP-1a	**Young**	**NLGF**	**F**	**18.71778**	**1.829978**	**9**
			M	**16.267**	**2.599915**	**10**
		WT	**F**	**3.091111**	**1.259866**	**9**
			M	**3.4675**	**0.934845**	**8**
	Old	**NLGF**	**F**	**13.538**	**2.846592**	**10**
			M	**17.249**	**1.823181**	**10**
		WT	**F**	**2.619**	**1.559383**	**10**
			M	**3.043**	**0.855623**	**10**
MIP-1b	**Young**	**NLGF**	**F**	**99.24333**	**36.52185**	**9**
			M	**108.188**	**16.68633**	**10**
		WT	**F**	**70.59444**	**34.6856**	**9**
			M	**71.975**	**20.78705**	**8**
	Old	**NLGF**	**F**	**60.503**	**21.33121**	**10**
			M	**78.235**	**10.91504**	**10**
		WT	**F**	**43.271**	**33.18875**	**10**
			M	**46.034**	**28.31955**	**10**
RANTES	Young	NLGF	F	7.577778	7.496907	9
			M	6.859	6.105754	10
		WT	F	5.676667	6.826308	9
			M	5.81125	6.386992	8
	Old	NLGF	F	3.83	6.258644	10
			M	7.323	6.431762	10
		WT	F	4.649	6.142644	10
			M	6.795	7.71753	10
TNFa	**Young**	**NLGF**	**F**	**51.08333**	**13.54863**	**9**
			M	**59.666**	**13.5801**	**10**
		WT	**F**	**52.00889**	**22.58462**	**9**
			M	**49.28375**	**9.936422**	**8**
	Old	**NLGF**	**F**	**37.554**	**11.21479**	**10**
			M	**49.51**	**7.443604**	**10**
		WT	**F**	**35.406**	**22.27959**	**10**
			M	**40.2**	**12.71818**	**10**
IL-1a	Young	NLGF	F	2.644444	0.618549	9
			M	2.562	0.53262	10
		WT	F	2.275556	1.078043	9
			M	2.33125	1.355101	8
	Old	NLGF	F	2.339	0.485946	10
			M	2.934	0.680608	10

Cytokine	Age	Genotype	Sex	Mean	Standard deviation	n
		WT	F	2.309	1.319448	10
			M	2.526	0.690237	10
IL-1b	**Young**	**NLGF**	**F&M**	**4.8975**	**.14886**	**4**
		WT	**F&M**	**4.8975**	**.39567**	**4**
	Old	**NLGF**	**F&M**	**5.498**	**.67173**	**10**
		WT	**F&M**	**5.322**	**.63723**	**10**
IL-2	Young	NLGF	F	6.715556	2.123741	9
			M	6.709	1.141358	10
		WT	F	5.656667	3.076707	9
			M	5.64	1.042319	8
	Old	NLGF	F	4.848	1.992959	10
			M	6.514	1.31015	10
		WT	F	4.9	2.786523	10
			M	6.578	1.572837	10
IL-3	Young	NLGF	F	2.382222	0.72289	9
			M	2.648	0.641003	10
		WT	F	2.184444	0.935055	9
			M	2.17625	0.425069	8
	Old	NLGF	F	2.598	0.609276	10
			M	2.761	0.538959	10
		WT	F	2.354	1.274478	10
			M	2.81	0.567646	10
IL-4	**Young**	**NLGF**	**F**	**5.647778**	**1.607566**	**9**
			M	**5.254**	**0.899842**	**10**
		WT	**F**	**4.973333**	**2.164399**	**9**
			M	**5.98625**	**1.876379**	**8**
	Old	**NLGF**	**F**	**6.486**	**1.558533**	**10**
			M	**7.437**	**1.669005**	**10**
		WT	**F**	**5.461**	**3.316221**	**10**
			M	**6.024**	**1.899346**	**10**
IL-5	Young	NLGF	F	2.274444	2.820714	9
			M	3.603	2.042292	10
		WT	F	2.318889	2.308736	9
			M	2.055	2.241536	8
	Old	NLGF	F	3.334	2.59992	10
			M	5.073	1.356417	10
		WT	F	3.651	2.7051	10
			M	4.316	2.752551	10
IL-6	**Young**	**NLGF**	**F**	**9.422222**	**2.324101**	**9**
			M	**8.911**	**2.561803**	**10**
		WT	**F**	**8.74**	**3.860512**	**9**
			M	**9.3375**	**2.663873**	**8**
	Old	**NLGF**	**F**	**6.059**	**2.265124**	**10**

Cytokine	Age	Genotype	Sex	Mean	Standard deviation	n
			M	**9.334**	**2.432384**	**10**
		WT	**F**	**6.352**	**4.446701**	**10**
			M	**7.653**	**2.910376**	**10**
IL-9	**Young**	**NLGF**	**F**	**25.43333**	**11.22421**	**9**
			M	**27.533**	**6.024279**	**10**
		WT	**F**	**21.62222**	**11.14329**	**9**
			M	**19.22625**	**4.326216**	**8**
	Old	**NLGF**	**F**	**11.699**	**3.525978**	**10**
			M	**17.043**	**2.454081**	**10**
		WT	**F**	**10.606**	**8.076829**	**10**
			M	**12.326**	**5.443106**	**10**
IL-10	**Young**	**NLGF**	**F**	**30.61333**	**9.499568**	**9**
			M	**29.21**	**5.591788**	**10**
		WT	**F**	**26.24667**	**11.44568**	**9**
			M	**26.7625**	**8.900584**	**8**
	Old	**NLGF**	**F**	**26.725**	**5.921727**	**10**
			M	**32.997**	**8.591273**	**10**
		WT	**F**	**22.908**	**13.66902**	**10**
			M	**28.346**	**9.855928**	**10**
IL-12p40	Young	NLGF	F	12.02	18.0473	9
			M	10.743	17.55315	10
		WT	F	14.71556	17.59356	9
			M	16.0625	17.23818	8
	Old	NLGF	F	14.11	18.31416	10
			M	29.333	16.42034	10
		WT	F	14.583	18.89071	10
			M	14.899	19.31163	10
IL-12p70	**Young**	**NLGF**	**F**	**117.7822**	**26.70694**	**9**
			M	**120.925**	**20.89068**	**10**
		WT	**F**	**104.7756**	**41.75561**	**9**
			M	**104.7275**	**8.554512**	**8**
	Old	**NLGF**	**F**	**72.892**	**16.10935**	**10**
			M	**89.668**	**12.99623**	**10**
		WT	**F**	**67.412**	**39.75332**	**10**
			M	**71.949**	**10.15257**	**10**
IL-13	Young	NLGF	F	28.45222	19.30441	9
			M	24.075	17.81753	10
		WT	F	19.48556	19.56664	9
			M	24.4675	16.20435	8
	Old	NLGF	F	17.507	19.75929	10
			M	34.266	7.862376	10
		WT	F	23.657	17.52914	10
			M	32.549	15.51844	10

Cytokine	Age	Genotype	Sex	Mean	Standard deviation	n
IL-17A	Young	NLGF	F	4.272222	1.370117	9
			M	4.312	0.585241	10
		WT	F	4.144444	2.276379	9
			M	3.75625	0.441877	8
	Old	NLGF	F	4.713	1.541875	10
			M	5.204	0.54023	10
		WT	F	4.482	2.593093	10
			M	5.593	1.120695	10

Table 3.8. Means, standard deviations and group sizes for each cytokine examined between App^{NL-G-F} and WT ages and sexes. *Values in bold are the targets that will be discussed.*

IL-6

IL-6 is a very important PIC and the multiplex assay found no genotype or sex effect but did find a significant age effect. However, this did not survive Bonferroni corrections. This replicates the data shown in Fig. 3 where IL-6 levels were higher in the younger cohort. There were no significant interactions (see table 3.9). Bayes factors generally agree for a lack of genotype effect and interactions but suggest the sex result may be inconclusive.

IL-1β

Levels detected for multiple samples were below the detection threshold so were discounted. This left young *App*$^{NL-G-F}$ male = 1, young *App*$^{NL-G-F}$ female = 3, young WT male = 2, young WT female = 2, old *App*$^{NL-G-F}$ male = 5, old *App*$^{NL-G-F}$ female = 5, old WT male = 4, old WT female = 6. Due to this large loss of data, sex was discounted due to some low group sizes. Both a three-way ANOVA which included undetectable values coded as 0's was done and a two-way without. Removal of the 0's did not impact the significance. However, due to multiple violations of assumptions, bootstrapping was performed and results of this two-way ANOVA without 0's are shown in table 3.9.

IL-1β is another important PIC seen to increase in AD. However, the assay showed no genotype effect but did show levels increased with age (fig. 3.11e) which did not survive Bonferroni corrections. There were no significant interactions (see table 3.9). Moderate BF_{excl} suggest results are inconclusive but that could also reflect the small sample sizes not being sufficient to give a conclusive result.

TNF-α

Another prominent PIC that was not able to be detected with western blot but the multiplex assay showed no genotype or sex effect (fig. 3.11f). There was a large increase in levels in the younger cohort compared to the older but there were no significant interactions (see table 3.9). The

age effect did still remain after Bonferroni corrections. Higher bayes factors agree with results being null rather than inconclusive except the sex effect (BF_{excl} =1.812) which suggests inconclusive.

IL-4

IL-4 is a common AIC which sometimes sees changes in AD. However, this assay found no significant differences in genotype or sex. There was a near significant age effect and fig. 3.13i shows levels are higher in the older cohort. There were no significant interactions (see table 3.9). High BF_{excl} values agree that these are all null results.

IL-10

IL-10, another prominent AIC, was unable to be detected with western blot but the assay managed to measure it (fig. 3.11j). However, no significant genotype, sex or age effects were found along with no interactions (see table 3.9). This suggests there are no differences in IL-10 levels between any groups. The high bayes factors all agree with these being null rather than inconclusive results.

Cytokine	Effect	DFn	DFd	F	p	Partial eta sq	BFexcl
Eotaxin	Age	1	68	0.437	0.511	0.006	6.922
	Genotype	1	68	3.416	0.069	0.048	2.086
	Sex	1	68	1.544	0.218	0.022	4.091
	Genotype*Age	1	68	0.184	0.669	0.003	9.077
	Sex*Age	1	68	0.476	0.493	0.007	11.165
	Genotype*Sex	1	68	0.092	0.763	0.001	7.044
	Genotype*Sex*Age	1	68	0.327	0.57	0.005	84.236
G-CSF	Age	1	68	0.812	0.371	0.012	6.345
	Genotype	1	68	2.735	0.103	0.039	2.822
	Sex	1	68	2.247	0.139	0.032	3.179
	Genotype*Age	1	68	0.113	0.737	0.002	9.969
	Sex*Age	1	68	0.079	0.78	0.001	11.298
	Genotype*Sex	1	68	0.00049	0.982	0.00000722	6.889
	Genotype*Sex*Age	1	68	0.003	0.96	0.00003690	114.584
GM-CSF	**Age**	**1**	**68**	**7.181**	**0.009****	**0.096**	**0.396**
	Genotype	1	68	2.273	0.136	0.032	3.034
	Sex	1	68	2.427	0.124	0.034	1.98
	Genotype*Age	1	68	0.503	0.481	0.007	3.342
	Sex*Age	1	68	1.936	0.169	0.028	1.633
	Genotype*Sex	1	68	0.058	0.811	0.00085	5.444

Cytokine	Effect	DFn	DFd	F	p	Partial eta sq	BFexcl
	Genotype*Sex*Age	1	68	0.012	0.914	0.00017	20.187
IFNγ	**Age**	**1**	**68**	**15.236**	**0.00022*** **	**0.183**	**0.015**
	Genotype	**1**	**68**	**5.815**	**0.019***	**0.079**	**0.637**
	Sex	1	68	2.106	0.151	0.03	1.976
	Genotype*Age	1	68	0.023	0.88	0.00034	1.832
	Sex*Age	1	68	1.927	0.17	0.028	1.422
	Genotype*Sex	1	68	0.336	0.564	0.005	2.945
	Genotype*Sex*Age	1	68	1.223	0.273	0.018	6.779
KC	Age	1	68	1.036	0.312	0.015	4.579
	Genotype	1	68	3.363	0.071	0.047	1.912
	Sex	1	68	1.56	0.216	0.022	4.322
	Genotype*Age	1	68	1.512	0.223	0.022	4.401
	Sex*Age	1	68	0.81	0.371	0.012	7.96
	Genotype*Sex	1	68	0.719	0.399	0.01	5.757
	Genotype*Sex*Age	1	68	0.786	0.379	0.011	26.491
MCP-1	**Age**	**1**	**68**	**17.734**	**0.00007640*** **	**0.207**	**0.012**
	Genotype	1	68	0.77	0.383	0.011	3.998
	Sex	1	68	0.707	0.403	0.01	4.523
	Genotype*Age	1	68	1.978	0.164	0.028	2.162
	Sex*Age	1	68	0.002	0.967	0.00002530	3.701
	Genotype*Sex	1	68	2.588	0.112	0.037	3.982
	Genotype*Sex*Age	1	68	3.921	0.052	0.055	3.703
MIP-1α	**Age**	**1**	**68**	**8.761**	**0.004****	**0.114**	**0.007**
	Genotype	**1**	**68**	**968.08**	**>0.00000001*** **	**0.934**	**0.000000000000005285**
	Sex	1	68	1.433	0.235	0.021	0.031
	Genotype*Age	1	68	3.679	0.059	0.051	0.045
	Sex*Age	**1**	**68**	**13.016**	**0.00059*** **	**0.161**	**0.008**
	Genotype*Sex	1	68	0.071	0.79	0.001	0.075
	Genotype*Sex*Age	**1**	**68**	**12.62**	**0.00070*** **	**0.157**	**0.012**
MIP-1β	**Age**	**1**	**68**	**24.806**	**0.00000460*** **	**0.267**	**0.0003074**
	Genotype	**1**	**68**	**21.787**	**0.00001480*** **	**0.243**	**0.001**
	Sex	1	68	1.584	0.212	0.023	2.816
	Genotype*Age	1	68	0.397	0.531	0.006	1.24
	Sex*Age	1	68	0.172	0.679	0.003	3.188
	Genotype*Sex	1	68	0.847	0.361	0.012	2.53

Cytokine	Effect	DFn	DFd	F	p	Partial eta sq	BFexcl
	Genotype*Sex*Age	1	68	0.091	0.763	0.001	13.002
RANTES	Age	1	68	0.292	0.591	0.004	8.517
	Genotype	1	68	0.186	0.667	0.003	9.299
	Sex	1	68	0.674	0.415	0.01	6.928
	Genotype*Age	1	68	0.277	0.601	0.004	25.387
	Sex*Age	1	68	1.021	0.316	0.015	15.467
	Genotype*Sex	1	68	0.006	0.936	0.00009450	24.969
	Genotype*Sex*Age	1	68	0.128	0.722	0.002	229.837
TNFα	**Age**	**1**	**68**	**12.66**	**0.00069*** **	**0.157**	**0.037**
	Genotype	1	68	2.272	0.136	0.032	2.432
	Sex	1	68	2.655	0.108	0.038	1.812
	Genotype*Age	1	68	0.021	0.886	0.00031	3.029
	Sex*Age	1	68	0.616	0.435	0.009	2.239
	Genotype*Sex	1	68	1.772	0.188	0.025	2.827
	Genotype*Sex*Age	1	68	0.089	0.766	0.001	15.017
IL-1α	Age	1	68	0.129	0.72	0.002	8.818
	Genotype	1	68	1.6	0.21	0.023	4.875
	Sex	1	68	0.916	0.342	0.013	5.884
	Genotype*Age	1	68	0.039	0.844	0.00057	16.304
	Sex*Age	1	68	1.045	0.31	0.015	14.697
	Genotype*Sex	1	68	0.085	0.771	0.001	14.113
	Genotype*Sex*Age	1	68	0.396	0.531	0.006	155.188
IL-1β	**Age**	**1**	**24**	**4.365**	**.047**	**.154**	**0.699**
	Genotype	1	24	.129	.723	.005	2.884
	Genotype*Age	1	24	.129	.723	.005	2.743
IL-2	Age	1	68	1.035	0.313	0.015	3.732
	Genotype	1	68	1.184	0.28	0.017	5.094
	Sex	1	68	3.225	0.077	0.045	1.577
	Genotype*Age	1	68	1.472	0.229	0.021	6.639
	Sex*Age	1	68	3.315	0.073	0.046	2.222
	Genotype*Sex	1	68	0.00000104	0.999	0.00000002	7.364
	Genotype*Sex*Age	1	68	0.00014	0.99	0.00000210	22.312
IL-3	Age	1	68	2.597	0.112	0.037	3.175
	Genotype	1	68	1.514	0.223	0.022	4.853
	Sex	1	68	1.557	0.216	0.022	4.149
	Genotype*Age	1	68	0.456	0.502	0.007	8.132
	Sex*Age	1	68	0.265	0.609	0.004	7.82

Cytokine	Effect	DFn	DFd	F	p	Partial eta sq	BFexcl
	Genotype*Sex	1	68	0.00073	0.978	0.00001080	11.109
	Genotype*Sex*Age	1	68	0.651	0.422	0.009	69.932
IL-4	Age	1	68	3.761	0.057	0.052	1.528
	Genotype	1	68	1.694	0.197	0.024	3.404
	Sex	1	68	1.36	0.248	0.02	4.745
	Genotype*Age	1	68	1.862	0.177	0.027	3.053
	Sex*Age	1	68	0.239	0.626	0.004	6.086
	Genotype*Sex	1	68	0.31	0.579	0.005	8.381
	Genotype*Sex*Age	1	68	0.963	0.33	0.014	20.285
IL-5	**Age**	**1**	**68**	**7.706**	**0.007****	**0.102**	**0.383**
	Genotype	1	68	0.777	0.381	0.011	5.19
	Sex	1	68	2.473	0.12	0.035	2.24
	Genotype*Age	1	68	0.233	0.631	0.003	5.241
	Sex*Age	1	68	0.369	0.546	0.005	3.06
	Genotype*Sex	1	68	1.462	0.231	0.021	5.295
	Genotype*Sex*Age	1	68	0.055	0.815	0.00081	28.682
IL-6	**Age**	**1**	**68**	**6.318**	**0.014***	**0.085**	**0.536**
	Genotype	1	68	0.347	0.558	0.005	6.501
	Sex	1	68	2.792	0.099	0.039	1.713
	Genotype*Age	1	68	0.165	0.686	0.002	6.351
	Sex*Age	1	68	2.589	0.112	0.037	1.298
	Genotype*Sex	1	68	0.096	0.757	0.001	8.189
	Genotype*Sex*Age	1	68	1.221	0.273	0.018	15.444
IL-9	**Age**	**1**	**68**	**40.973**	**0.00000002*****	**0.376**	**0.000001593**
	Genotype	**1**	**68**	**7.416**	**0.008****	**0.098**	**0.319**
	Sex	1	68	1.057	0.308	0.015	2.841
	Genotype*Age	1	68	0.918	0.341	0.013	1.097
	Sex*Age	1	68	1.25	0.268	0.018	2.382
	Genotype*Sex	1	68	1.521	0.222	0.022	2.357
	Genotype*Sex*Age	1	68	0.018	0.895	0.00026	8.259
IL-10	Age	1	68	0.045	0.833	0.00066	7.986
	Genotype	1	68	3.052	0.085	0.043	2.483
	Sex	1	68	1.531	0.22	0.022	3.974
	Genotype*Age	1	68	0.036	0.851	0.00053	11.558
	Sex*Age	1	68	2.074	0.154	0.03	7.811
	Genotype*Sex	1	68	0.015	0.902	0.00023	7.587
	Genotype*Sex*Age	1	68	0.099	0.754	0.001	61.738

Cytokine	Effect	DFn	DFd	F	p	Partial eta sq	BFexcl
IL-12p40	Age	1	68	1.374	0.245	0.02	4.5
	Genotype	1	68	0.129	0.72	0.002	7.283
	Sex	1	68	0.891	0.349	0.013	5.767
	Genotype*Age	1	68	1.766	0.188	0.025	9.553
	Sex*Age	1	68	0.875	0.353	0.013	6.589
	Genotype*Sex	1	68	0.552	0.46	0.008	16.572
	Genotype*Sex*Age	1	68	1.124	0.293	0.016	47.034
IL-12p70	**Age**	**1**	**68**	**39.852**	**0.000000024 *****	**0.37**	**0.000002257**
	Genotype	**1**	**68**	**5.114**	**0.027***	**0.07**	**0.866**
	Sex	1	68	1.109	0.296	0.016	3.711
	Genotype*Age	1	68	0.067	0.796	0.00099	2.075
	Sex*Age	1	68	0.618	0.434	0.009	3.275
	Genotype*Sex	1	68	0.443	0.508	0.006	4.342
	Genotype*Sex*Age	1	68	0.152	0.697	0.002	19.174
IL-13	Age	1	68	0.538	0.466	0.008	5.957
	Genotype	1	68	0.07	0.793	0.001	8.882
	Sex	1	68	2.803	0.099	0.04	2.334
	Genotype*Age	1	68	0.688	0.41	0.01	16.775
	Sex*Age	1	68	2.55	0.115	0.036	4.076
	Genotype*Sex	1	68	0.009	0.924	0.00013	12.326
	Genotype*Sex*Age	1	68	1.206	0.276	0.017	44.288
IL-17A	**Age**	**1**	**68**	**6.297**	**0.014***	**0.085**	**0.625**
	Genotype	1	68	0.141	0.708	0.002	7.389
	Sex	1	68	0.805	0.373	0.012	4.272
	Genotype*Age	1	68	0.363	0.549	0.005	7.516
	Sex*Age	1	68	1.948	0.167	0.028	2.627
	Genotype*Sex	1	68	0.019	0.891	0.00028	9.51
	Genotype*Sex*Age	1	68	0.562	0.456	0.008	36.628

Table 3.9. ANOVA and bayesian output for each three-way ANOVA per cytokine. *All were three-way ANOVA comparing genotype, age and sex between App*$^{NL-G-F}$ *and WT except for IL-1ß which was a two way comparing genotype and age. Includes degrees of freedom, F statistic, P value, partial eta squared and BFexcl. * = p<.05, **=p<.01, ***=p<.001. Bold and orange shows the result survived the Bonferroni correction (i.e.,, had p< 0.00217).*

Cytokine	Genotype effect	Age effect	Interactions
Eotaxin	-	-	-
G-CSF	-	-	-
GM-CSF	-	Higher in older cohort	-
IFNγ	Higher in *App*$^{NL-G-F}$	Higher in younger cohort. **(Survived corrections)**	-
KC	-	-	-

MCP-1	-	Higher in older cohort. **(Survived corrections)**	-
MIP-1α	Large effect; higher in *App*$^{NL-G-F}$ **(Survived corrections)**	Higher in younger cohort	Sex*Age effect **(Survived corrections in females)**, Genotype*Sex*Age effect. **(Survived corrections in *App*$^{NL-G-F}$ females)**
MIP-1β	Large effect, higher in *App*$^{NL-G-F}$ **(Survived corrections)**	Large effect, higher in the younger cohort. **(Survived corrections)**	-
RANTES	-	-	-
TNF-α	-	Large effect, higher in younger cohort. **(Survived corrections)**	-
IL-1α	-	-	-
IL-1β	-	Higher in older cohort	-
IL-2	-	-	-
IL-3	-	-	-
IL-4	-	Near significant age effect; higher in older cohort	-
IL-5	-	Moderate effect, higher in older cohort	-
IL-6	-	Higher in younger cohort	-
IL-9	Moderate effect; higher in *App*$^{NL-G-F}$ **(Survived corrections)**	Large effect; higher in younger cohort	-
IL-10	-	-	-
IL-12p40	-	-	-
IL-12p70	Higher in *App*$^{NL-G-F}$ **(Survived corrections)**	Large effect, higher in younger cohort	-
IL-13	-	-	-
IL-17A	-	Higher in older cohort	-

Table 3.10. Summary of three-way ANOVA results between App^{NL-G-F} and WT mice for each cytokine. *If the effect survived Bonferroni corrections (i.e.,, $p < 0.00217$) then it is noted in orange.*

IFNγ

Interferon gamma (IFNγ) is an important activator of macrophages and other pro-inflammatory immune cells. There were significantly higher levels in the *App*$^{NL-G-F}$ compared to the WT mice with no effect of sex (fig. 3.11a). There was a significant main effect of age with IFNγ levels being elevated in the younger cohort. While the genotype effect did not remain after Bonferroni corrections, the main effect of age did. All interactions were non-significant. However, the bayes factors for sex and all two-way interactions are all below 3 suggesting inconclusive results.

MIP-1α

Macrophage inflammatory protein-1 alpha (MIP-1 α; also known as CCL3) is a chemokine important in activating inflammatory immune cells. Results showed a strong main effect of genotype with levels being higher in the *App*$^{NL-G-F}$ mice which did remain after Bonferroni corrections. There was also a significant age effect showing elevated levels in the younger cohort (fig. 3.11b) which did not survive the corrections. There was no main effect of sex but there was a significant sex*age and genotype*sex*age interaction (table 3.9), both of which survived Bonferroni corrections.

Simple effects testing was done on the sex*age interaction to find a significant age effect only in females ($F(1, 68) = 21.638$, $p<.001$, $\eta^2=.241$) but not males ($F(1, 68) = .209$, $p=.649$, $\eta^2= .003$, $BF_{excl} = 3.128$) with higher levels in the young females. The three way interaction only showed a significant age effect in the *App*$^{NL-G-F}$ females ($F(1, 68) = 36.348$, $P<.001$, $\eta^2 = .348$) but not male mice ($F(1, 68) = 1.379$, $p = .244$, $\eta^2 =.02$, $BF_{excl} = 1.801$) or WT (Females; $F(1, 68) = .302$, $p = .584$, $\eta^2 = .004$, $BF_{excl} = 2.062$, Males; $F(1, 68) =.229$, $p=.634$, $\eta^2 =.003$, $BF_{excl} = 1.716$). MIP-1α levels were lower in the *App*$^{NL-G-F}$ old female mice. However, the very low BF_{excl} values for all main effects and interactions disagree with the NHST for sex, genotype*age and genotype*sex findings. This suggests that these may potentially be an effect but further testing is required.

MIP-1β

Macrophage inflammatory protein-1 beta (MIP-1β; also known as CCL4) has a similar role to MIP-1α in pro-inflammation. There was a significant genotype effect with *App*$^{NL-G-F}$ mice have higher levels of this chemokine as well as being higher in the younger cohort similar to MIP-1α (fig. 3.11c). Both the genotype and age effects remained after applying Bonferroni corrections. Unlike MIP-1α, there were no significant interactions. There was also no significant sex effect and bayes factors generally agreed with results being null except for the genotype*age interaction which is inconclusive (table 3.9).

IL-9

Interleukin-9 is secreted by immune cells (CD4+ helper cells) and is a PIC. There was a significant genotype effect with higher levels found in the *App*$^{NL-G-F}$ mice as well as a significant age effect with elevated levels in the young cohort which survived Bonferroni corrections (fig. 3.11d). All interactions were non-significant. The BF_{excl} values generally suggest inconclusive results except for the three-way interaction which is in support of the null (table 3.9).

IL-12p70

Interleukin 12p70 is produced by macrophages and thus is pro-inflammatory. The results show a significant effect of genotype with *App*$^{NL-G-F}$ mice exhibiting elevated levels (fig. 3.11h). There

was also a significant age effect with protein levels being lower in the older cohort but there was no sex effect or no significant interactions. The effect of age survived Bonferroni corrections. Majority of the bayes factors for non-significant results agree with null findings except for genotype*age interaction which is inconclusive (table 3.9).

Overall, it seems quite a few pro inflammatory cytokines are elevated in the *App*$^{NL-G-F}$ mice (IFNγ, MIP-1α, MIP-1β, IL-9 and IL-12p70) but there were also some prominent PIC that showed no genotype difference (IL-6, TNF-α, IL-1β) agreeing with western blot results. If the Bonferroni corrections are applied, only a few retained their significant genotype effect (MIP-1α, MIP-1β). For the anti-inflammatory side, there were no effects of genotype on IL-10 or IL-4. Therefore, amyloid build up does seem to impact only some PIC but has little impact on the anti-inflammatory cytokines in *App*$^{NL-G-F}$

There were also some differences between ages but a lot of the PIC actually had higher levels in the younger cohort (IFNγ, MIP-1α, MIP-1β, IL-9 and IL-12p70, IL-6, TNFα) with only one showing an increase with age (IL-1β). As these showed no interaction with genotype, this is unlikely to be caused by amyloid build up. These findings will be discussed further in the discussion.

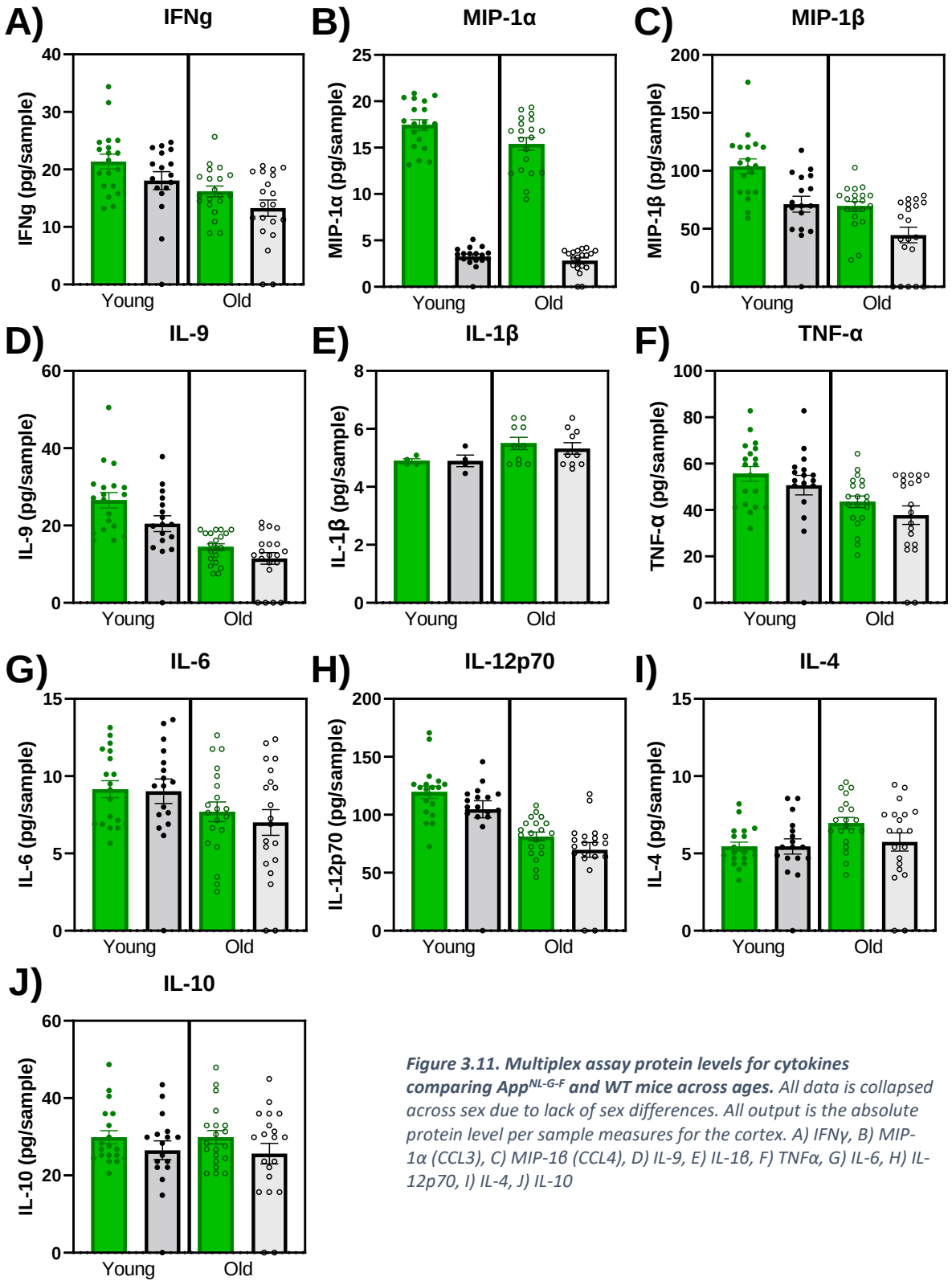

Figure 3.11. Multiplex assay protein levels for cytokines comparing App^{NL-G-F} and WT mice across ages. *All data is collapsed across sex due to lack of sex differences. All output is the absolute protein level per sample measures for the cortex. A) IFNγ, B) MIP-1α (CCL3), C) MIP-1β (CCL4), D) IL-9, E) IL-1β, F) TNFα, G) IL-6, H) IL-12p70, I) IL-4, J) IL-10*

●3.5.4 Neurotrophic signalling

BDNF

BDNF expression in the cortex and hippocampus was measured using western blot with the monomer band at ~15kDa standardised to β-actin. The results are depicted in fig. 3.12 and show no significant genotype ($F(1, 72)=2.002$, $p=.161$, $\eta_2=.027$, $BF_{exclusion}=4.142$), age ($F(1, 72)=.805$, $p=.372$, $\eta_2=.011$, $BF_{exclusion}=4.835$) or sex ($F(1, 72)=1.156$, $p=.286$, $\eta_2=.016$, $BF_{exclusion}=4.255$) difference in the cortex. There was also no genotype*age ($F(1, 72)=.172$, $p=.68$, $\eta_2=.002$, $BF_{exclusion}=10.234$), genotype*sex ($F(1, 72)=.553$, $p=.459$, $\eta_2=.008$, $BF_{exclusion}=7.974$) or genotype*sex*age ($F(1, 72)=1.005$, $p=.319$, $\eta_2=.014$, $BF_{exclusion}=22.144$) interaction. The high bayes factors offer strong evidence for the null across all these effects. However, the age*sex interaction did reach significance, $F(1, 72)=4.49$, $p=.038$, $\eta_2=.059$, and so simple effects testing was done that revealed a significant sex effect at the young time point ($F(1, 72)=5.1$, $p=.027$, $\eta_2=.066$) but not the old ($F(1, 72)=.545$, $p=.463$, $\eta_2=.008$, $BF_{exclusion}=2.386$) showing young females have higher signal than young males.

In the hippocampus, BDNF expression was measured using western blot, standardised to β-actin and analysed using three-way ANOVA with bootstrapping due to assumption violations. Analysis revealed no difference in genotype ($F(1, 72)=1.181$, $p=.281$, $\eta_2=.016$, $BF_{exclusion}=5.039$), sex ($F(1, 72)=1.27$, $p=.263$, $\eta_2=.017$, $BF_{exclusion}=5.945$) or age ($F(1, 72)=.407$, $p=.526$, $\eta_2=.006$, $BF_{exclusion}=7.306$) with bayes factors above 3 suggesting evidence for the null. All interactions were non-significant (Genotype*Age, $F(1, 72)=.099$, $p=.754$, $\eta_2=.001$, $BF_{exclusion}=16.267$; Genotype*Sex, $F(1, 72)=.061$, $p=.806$, $\eta_2=.001$, $BF_{exclusion}=14.007$; Age*Sex, $F(1, 72)=.67$, $p=.416$, $\eta_2=.009$, $BF_{exclusion}=14.174$; Genotype*Age*Sex, $F(1, 72)=2.394$, $p=.126$, $\eta_2=.032$, $BF_{exclusion}=82.135$).

For mature BDNF, it seems females show greater expression compared to males but only when young. This difference disappears with age and does not differ between *App*$^{NL-G-F}$ or WT. In the hippocampus, there was no change in mature BDNF expression in the hippocampus for any of the groups.

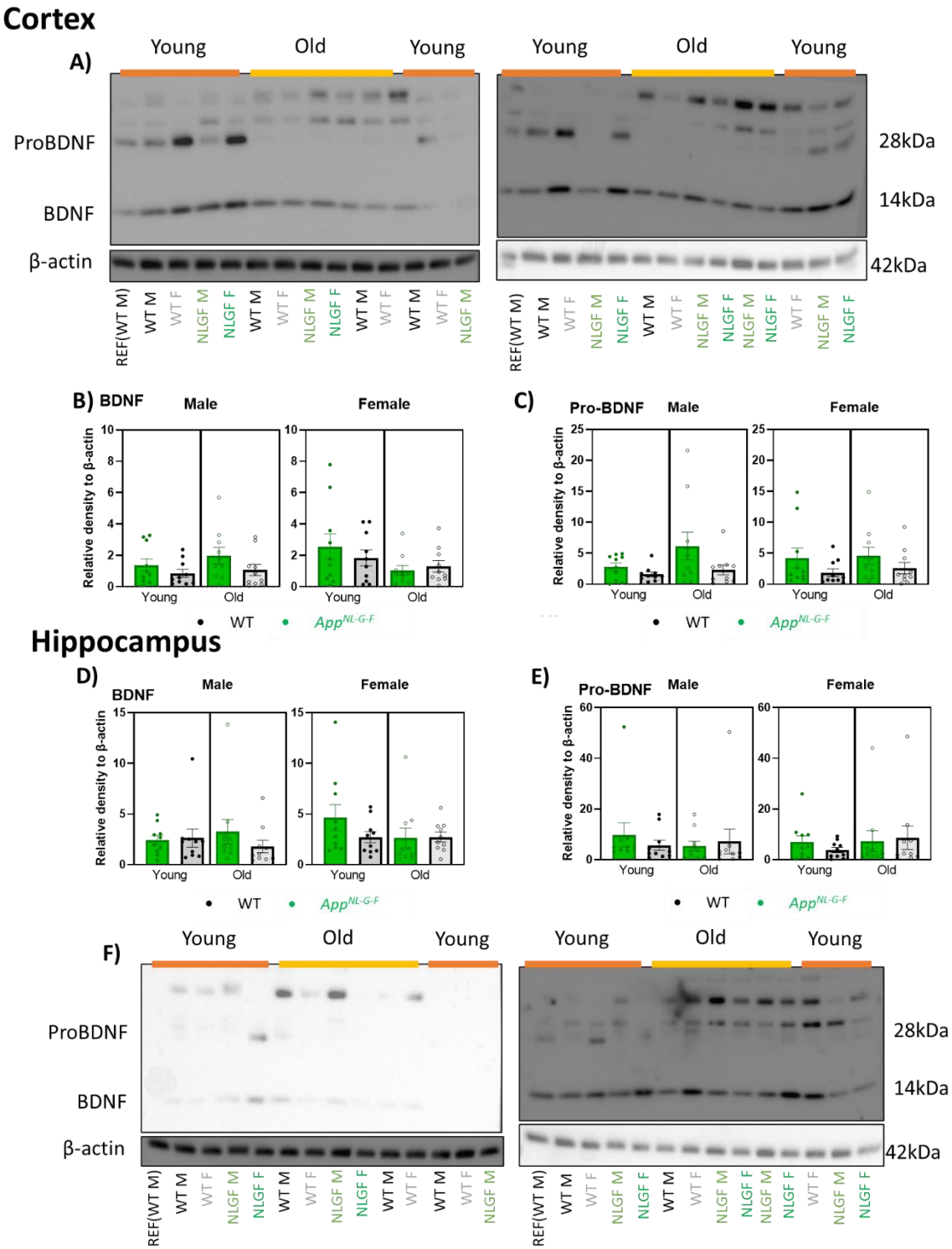

Figure 3.12 Expression levels of BDNF in the cortex and hippocampus of App^{NL-G-F} and WT mice with β-actin. *Quantification of BDNF expression mature fragment and precursor (pro) fragment in the cortex and hippocampus. Genotype effect in the precursor fragment in the cortex (ANOVA, p<.01). A) Two examples of BDNF western blots run with their respective β-actin in the cortex. B) Bar graph showing BDNF signal relative to β-actin in the cortex. Error bars represent +-SEM. C) Bar graph showing proBDNF signal relative to β-actin in the cortex. Error bars represent +-SEM. D) Bar graph showing BDNF signal relative to β-actin in the hippocampus. Error bars represent +-SEM. E) Bar graph showing proBDNF signal relative to β-actin in the hippocampus. Error bars represent +-SEM. F) Two examples of BDNF western blots run with their respective β-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=10), old WT male (n=10), old App^{NL-G-F} female (n=10), old WT female (n=10).*

Pro-BDNF

Pro-BDNF is a precursor to BDNF measured at the band at ~32kDa (fig. 3.12) and standardised to β-actin for this ANOVA with bootstrapping. It is associated with increased apoptosis in contrast to mature BDNF which offers neuroprotection. This analysis revealed a significant genotype effect ($F(1, 72)=7.114$, $p=.009$, $\eta_2=.09$) with *App*$^{NL-G-F}$ having higher signals than WT. There was no main effect of age ($F(1, 72)=2.068$, $p=.155$, $\eta_2=.028$, $BF_{exclusion}=3.119$) or sex ($F(1, 72)=.006$, $p=.937$, $\eta_2<.001$, $BF_{exclusion}=7.978$). All two way (Genotype*Age, $F(1, 72)=.423$, $p=.517$, $\eta_2=.006$, $BF_{exclusion}=3.56$; Genotype*Sex, $F(1, 72)=.001$, $p=.971$, $\eta_2<.001$, $BF_{exclusion}=7.687$; Age*Sex, $F(1, 72)=.1.14$, $p=.289$, $\eta_2=.016$, $BF_{exclusion}=10.763$) and three way interactions ($F(1, 72)=.68$, $p=.412$, $\eta_2=.009$, $BF_{exclusion}=49.902$) were non-significant with high bayes factors indicating strength for the null. This data suggests *App*$^{NL-G-F}$ have a greater expression of Pro-BDNF in their cortex compared to WT that is not affected by age or sex.

Pro-BDNF was also measured in the hippocampus. There was no difference in signal between genotypes ($F(1, 72)=.043$, $p=.837$, $\eta_2=.001$, $BF_{exclusion}=9.591$), ages ($F(1, 72)=.18$, $p=.673$, $\eta_2=.002$, $BF_{exclusion}=9.835$) or across sexes ($F(1, 72)=1.838$, $p=.179$, $\eta_2=.025$, $BF_{exclusion}=10.361$) (Fig. 3.12). Furthermore, all interactions were non-significant (Genotype*Age, $F(1, 72)=1.589$, $p=.212$, $\eta_2=.022$, $BF_{exclusion}=21.694$; Genotype*Sex, $F(1, 72)=.466$, $p=.497$, $\eta_2=.006$, $BF_{exclusion}=34.527$; Age*Sex, $F(1, 72)=.007$, $p=.935$, $\eta_2<.001$, $BF_{exclusion}=26.216$; Genotype*Age*Sex, $F(1, 72)=1.82$, $p=.181$, $\eta_2=.025$, $BF_{exclusion}=321.494$) with bayes factors near or above 10 suggesting strong evidence for the null opposed to an inconclusive result. Overall, there is no difference in pro-BDNF expression in the hippocampus of *App*$^{NL-G-F}$ mice and WT.

Altogether, there is a significant increase in Pro-BDNF in the cortex of *App*$^{NL-G-F}$ mice but not in mature BDNF or in the hippocampus. There is also a significant increase in BDNF expression in young females that vanished with age but not in Pro-BDNF or in the hippocampus. The increase in Pro-BDNF in *App*$^{NL-G-F}$ mice indicates higher neurotoxic potential.

TrkB receptor

TrkB is a receptor for BDNF in the brain and signal data for the cortex is shown in fig. 3.13. A three-way ANOVA showed a significant genotype effect ($F(1, 70)=17.148$, $p<.001$, $\eta^2=.197$) with *App*$^{NL-G-F}$ mice showing a higher TrkB signal relative to β-actin. There were no age ($F(1, 70)=1.113$, $p=.295$, $\eta^2=.016$, $BF_{exclusion}=2.408$) or sex effects ($F(1, 70)=1.139$, $p=.29$, $\eta^2=.016$, $BF_{exclusion}=5.056$). There was a significant genotype*age effect ($F(1, 70)=4.592$, $p=.036$, $\eta^2=.062$) and simple effects testing revealed a significant age effect for the *App*$^{NL-G-F}$ ($F(1, 70)=4.978$, $p=.029$, $\eta^2=.066$) but not WT

mice (F(1, 70)=.609, p=.438, η^2=.009, $BF_{exclusion}$=2.174). TrkB signal appears to increase with age but only for the *App*$^{NL-G-F}$ mice and not the WT. All other interactions were non-significant (Genotype*Sex, F(1, 70)=.044, p=.834, η^2=.001, $BF_{exclusion}$=4.758; Sex*Age, F(1, 70)=.035, p=.852, η^2=.001, $BF_{exclusion}$=8.197; Genotype*Age*Sex, F(1, 70)=1.176, p=.282, η^2=.017, $BF_{exclusion}$=10.888).

TrkB signal data for the hippocampus is shown in fig. 3.13c-d and ANOVA analysis revealed no significant genotype effect (F(1, 70)=2.25, p=.138, η^2=.031, $BF_{exclusion}$=3.507) unlike in the cortex. There was also no age (F(1, 70)=1.309, p=.256, η^2=.018, $BF_{exclusion}$=5.077) or sex (F(1, 70)=1.145, p=.288, η^2=.016, $BF_{exclusion}$=5.477) effect and all interactions were non-significant (Genotype*Age, F(1, 70)=.078, p=.781, η^2=.001, $BF_{exclusion}$=10.417; Genotype*Sex, F(1, 70)=.248, p=.62, η^2=.004, $BF_{exclusion}$=9.793; Sex*Age, F(1, 70)=.445, p=.507, η^2=.006, $BF_{exclusion}$=11.226; Genotype*Age*Sex, F(1, 70)=1.062, p=.306, η^2=.015, $BF_{exclusion}$=73.784). Together, this shows that levels of TrkB in the hippocampus did not differ across genotypes, ages or sexes.

While a significant increase in TrkB was found in the cortex for *App*$^{NL-G-F}$ which increased with age, this effect was not found in the hippocampus.

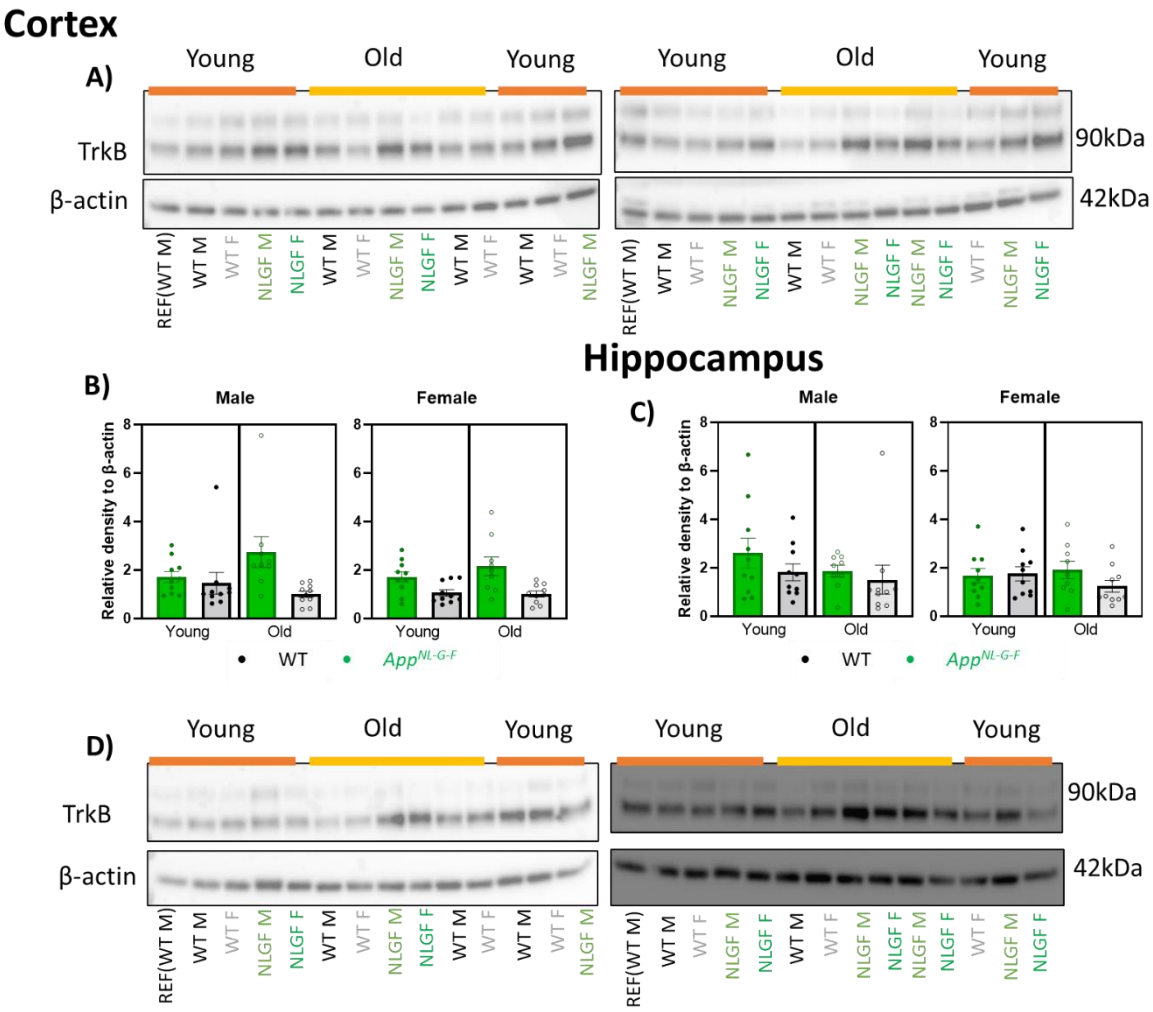

Figure 3.13 Expression levels of TrkB in the cortex and hippocampus of App^{NL-G-F} and WT mice with ß-actin*. Quantification of TrkB in the cortex (genotype effect, ANOVA, p<.001) and hippocampus (no significance). A) Two examples of TrkB western blots run with their respective ß-actin in the cortex. B) Bar graph showing TrkB signal relative to ß-actin in the cortex. Error bars represent +-SEM. C) Bar graph showing TrkB signal relative to ß-actin in the hippocampus. Error bars represent +-SEM. D) Two examples of TrkB western blots run with their respective ß-actin in the hippocampus. Young App^{NL-G-F} male (n=10), young WT male (n=10), young App^{NL-G-F} female (n=10), young WT female (n=10), old App^{NL-G-F} male (n=9), old WT male (n=10), old App^{NL-G-F} female (n=9), old WT female (n=10).*

●3.6 Discussion

In order to answer the question of how amyloid pathology affects the LC, neuroinflammation and neurotrophic support, various biochemical techniques were conducted on cortical and hippocampus tissue from age-matched *App*$^{NL-G-F}$ and WT mice. Results confirmed the presence of humanised Aβ in the absence of *APP* overexpression but only soluble Aβ$_{40}$ increased with age. This amyloid pathology did not impact LC cell count or mature BDNF expression. However, BDNF receptor, TrkB was upregulated in the cortex as well as the neurotoxic proBDNF. Examination of inflammation found a massive increase in astrogliosis in *App*$^{NL-G-F}$ mice as well as an upregulation of monocytes that did not show a corresponding increase in key PIC or decrease in TGF-β. MIP-1α

(CCL3) and MIP-1β (CCL4) were upregulated in the *App*$^{NL-G-F}$ mice but amyloid did not impact AIC expression at either age point. Each section will be examined in more detail and a summary of results is shown in table 3.11.

Topic	Measure	Result	What it means
Amyloid	**APP**	Decreased in *App*$^{NL-G-F}$ hippocampus	*App*$^{NL-G-F}$ do not overexpress *APP* but decrease will be examined
	Aβ**40 sol**	Present; increase with age	*App*$^{NL-G-F}$ exhibit humanised amyloid; but only soluble Aβ40 increases with age
	Aβ**42 sol**	Present; inconclusive result of age	
	Aβ**40 insol**	Present; inconclusive result of age	
	Aβ**42 insol**	Present; inconclusive result of age	
Locus coeruleus	**Cell count**	Higher count in females; no genotype effect	Amyloid does not appear to lead to reduction of LC cells in *App*$^{NL-G-F}$ mouse; females have higher base LC cell count than males
Inflammation	**GFAP**	Higher signal in *App*$^{NL-G-F}$ at both time points and in both CX and HPC	Amyloid in *App*$^{NL-G-F}$ causes higher astrocytosis in CX and HPC; any age progression likely occurs prior to our early time point due do no age effect
	Iba-1	Higher signal in *App*$^{NL-G-F}$ at both time points and in both CX and HPC	Amyloid in *App*$^{NL-G-F}$ causes higher microgliosis in CX and HPC; any age progression is not captured in out experiment so could occur prior to the early time point or later than out late time point
	IL-1β	WB: No change; MCA: no genotype effect but increase with age	MCA is a more sensitive measure but both agree no increase in IL-1β
	IL-6	WB: No change; MCA: no genotype effect	MCA is a more sensitive measure but both agree no increase in IL-6 caused by amyloid
	TGF-β	WB: No change	Amyloid does not appear to affect TGF-β expression
	TNF-α	WB: Unable to be detected; MCA: no genotype effect but increase with age in *App*$^{NL-G-F}$ males	Amyloid does not appear to affect TNF-α expression except for an age related increase in *App*$^{NL-G-F}$ males
	MIP-1α (CCL3)	Higher in *App*$^{NL-G-F}$ including higher in *App*$^{NL-G-F}$ females specifically	Amyloid causes higher levels of MIP-1α (CCL3)
	MIP-1β (CCL4)	Higher in *App*$^{NL-G-F}$	Amyloid causes higher levels of MIP-1β (CCL4)
	IL-4	No genotype effect	Amyloid does not appear to affect AIC

	IL-10	No genotype effect	
Neurotrophic support	**BDNF**	No genotype effect	Amyloid does not appear to affect BDNF expression in *App*$^{NL-G-F}$
	proBDNF	Higher in the CX of *App*$^{NL-G-F}$	As BDNF levels are similar, suggests the brain is producing more but amyloid is impacting it's cleavage to mature BDNF in the CX
	TrkB	Higher in the CX of *App*$^{NL-G-F}$	Together with BDNF result, suggests amyloid is causing the brain to want to increase BDNF:TrkB signalling but is also interfering with cleavage to mature BDNF; Only in the CX and not the HPC though

Table 3.11. Summary of results from this chapter. *HPC = hippocampus, CX = cortex, LC = Locus coeruleus, BDNF = Brain derived neurotrophic support, WB = Western blot, MCA = Multiplex cytokine assay*

One issue with transgenic models of amyloid pathology is the confound of *APP* overexpression which we confirmed not to occur in *App*$^{NL-G-F}$ mice. The WT mice did show an increase in *APP* expression in the hippocampus that could be due to coming from differing genetic lines to the *App*$^{NL-G-F}$ mice but my predecessor also found an increase of *APP* in WT mice that were siblings to the *App*$^{NL-F}$ mice (Freeman, 2019). Why there is an increase in unclear but, as murine *APP* expression is rarely looked at in WT mice, this may simply present a difference in expression of a humanised *APP* to endogenous mouse APP. This is just speculation but is unlikely having an impact as murine amyloid has not been found to be neurotoxic (Krohn et al., 2015).

ELISA confirmed the expression of humanised Aβ in *App*$^{NL-G-F}$ mice. However, there was no age increase in insoluble $A\beta_{40}$ or soluble and insoluble $A\beta_{42}$ suggesting levels peak at an earlier age than was assessed in the present study. The Iberian/Beyreuther mutation explains increased levels of both soluble and insoluble $A\beta_{42}$. As the arctic mutation increases amyloid aggregation, this could be responsible for the high and early levels of soluble and insoluble $A\beta_{42}$ and insoluble $A\beta_{40}$. Manocha et al. (2019) looked as early as 3 months and did show age effects from 6 months old further confirming the need to look earlier. This will be important in interpreting data as the absence of age effects could be the result of amyloid levels already peaking at the earlier time point.

One interesting finding is the trend in reduction with age in insoluble $A\beta_{40}$ and $A\beta_{42}$ but only in males. Although this did not reach significance, it does pose interesting implications for how amyloid builds up between sexes. Reasons for the sexually dimorphic decrease are unclear but Manocha et al. (2019) did also find a similar age reduction. However, they found a significant reduction of insoluble amyloid mainly in females. The reduction could highlight successful clearance attempts of neprilysin or immune cell phagocytosis but future research would need to measure these to confirm. Nevertheless, successful clearance would not explain the sexual dimorphic finding.

As the LC plays a role in effective phagocytic capability of immune cells, it is possible that sex differences in the structure create differences in clearance capabilities but this would require in depth examination of phagocytosis between sexes. This reduction in insoluble amyloid will be taken into consideration when examining Aβ impact on all other factors measured in this thesis.

There was no reduction in TH+ cells in the LC of the *App*$^{NL-G-F}$ mice at either age point. This supports Sakakibara et al. (2021) who also found no cell loss even at 24 months old but did show branching deficits using NET. Although the present study is unable to corroborate the latter observation, it does add to the idea that amyloid alone is not sufficient to cause gross cell loss in the *App*$^{NL-G-F}$ mice. Previous transgenic models with extensive amyloid pathology have shown LC cell loss (Cao et al., 2021; Kalinin et al., 2012; Liu et al., 2013; O'Neil et al., 2007) but this could be explained by unphysiological neurotoxic levels of amyloid, *APP* and its fragments. The fact Mehla et al. (2019), who also used *App*$^{NL-G-F}$ mice, did find a loss of cells in 9 month old mice could simply highlight differences in laboratory practices and cell calculating techniques. They did also have a lower power (n = 4 per group, both *App*$^{NL-G-F}$ and WT mice at 4 time points) and only used males for IHC analyses but these likely have little impact and would simply fail to examine the sexually dimorphic nature of the LC.

While we found no reduction in TH+ cells, we cannot conclude that the LC is functionally normal. There are various other ways the LC could become damaged such as aberrant branching, loss of adrenoreceptors, loss of NA or even changes to electrophysiological signalling. In AD, most of these become impacted as the disease progresses but measuring it is complicated by compensatory mechanisms that attempt to restore LC:NA signalling. Future research should delve deeply into all aspects of LC:NA signalling to corroborate Sakakibara et al. (2021) finding of reduction in LC branching. One reason for the LC's importance in AD is its neuroprotective mechanisms (see fig. 1.2) that are commonly caused by the actions of NA. NA is integral to LC signalling but difficult to measure without the correct equipment which the present study did not have access to. Measuring adrenoreceptors would also be able to highlight whether amyloid alters the brain's sensitivity to NA. Understanding the true extent of amyloid's impact on LC function requires a lot of further assessment but would be able to show whether increased inflammation caused by Aβ is partly mediated by loss of neuroprotection.

Previous research suggested that the LC is sexually dimorphic (Bangasser et al., 2011; Busch et al., 1997; Ohm et al., 1997; Pendergast et al., 2008) which the present study found with females having a higher cell count compared to male mice. Although it is uncertain what functional impact this has, it has been suggested that the higher cell count in females makes them more susceptible to

stress and affective deficits. This is supported with higher prevalence of depression and anxiety in females compared to males (Bebbington, 1998) making it an important consideration when examining NPS in AD. As AD itself is also more common in females (Schmidt et al., 2008), this could suggest that impact on the neuroprotective mechanisms of the LC is more disturbed in females. Examination of the LC in AD between sexes could highlight whether females may be more appropriate for NA related treatments.

Previous work in the *App*$^{NL-G-F}$ mice has robustly shown increases in gliosis but with discrepancies arising on when increased immune cell peaks. Some research found immune cell activation still increasing up to 18 months (Masuda et al., 2016) while others found it to be 6 months in the cortex and 9 months in the hippocampus (Mehla et al., 2019). The present study found a significant increase at 7 and 14 months in both the cortex and hippocampus with no significant age increase, suggesting gliosis peaks earlier than 7 months in *App*$^{NL-G-F}$ mice. This discrepancy is likely down to differing calculation methods as much of the previous literature utilised IHC and % area to find changes in immune cell activation while the present study used western blotting. IHC methodology would provide a better visual representation of microglia activation state and where they are in relation to plaques but are less able to give a measure of general increase in immune cells across an entire brain region. Western blotting provides an assessment of gross changes in inflammation in the brain. This data shows an increase from 7 month old *App*$^{NL-G-F}$ mice in amount of astrocytes and monocytes in the cortex and hippocampus. Together, this indicates that assessing when global inflammatory status peaks would require tests at an earlier time point than 7 months. Examining how inflammation progresses would also offer insight into how this affects other neural processes.

Monocytes also had increased protein expression which includes macrophages and microglia. While microglia would have been informative to examine independently of macrophages, a measure of monocytes provided another global measure for immune cell activity. Microglia are important in AD and the clearance of Aβ and will activate in response to aberrant proteins. Activated microglia can lead to neurotoxic damage over time. However, what is more telling would be whether amyloid and LC damage impacts their ability to phagocytose that has been shown in LC-lesioned WT mice (Heneka, Nadrigny, et al., 2010) and amyloid transgenic models (Kalinin et al., 2012). Seeing whether amyloid, independent of tau and neurotoxic *APP* fragments, is sufficient to impact microglia's ability to migrate and phagocytose could highlight potential clearance issues in the progression of AD. Whether this changes as a function of LC:NA signalling would reveal the role of the LC in Aβ clearance. For these reasons, an in-depth analysis of microglia mobility and

phagocytosis using the *App*$^{NL-G-F}$ mouse, could offer a more humanised examination of amyloid's impact on its own clearance.

To accompany the increase in gliosis and astrogliosis, cytokines were examined both in western blot and a multiplex assay that offered a more sensitive examination of protein levels. PIC, such as IL-6, TNF-α and IL-1β, were expected to increase as is seen in AD (McGeer & McGeer, 1997, 2003; Walker et al., 1997) as well as the *App*$^{NL-G-F}$ (Kaur et al., 2020; Manocha et al., 2019; Uruno et al., 2020). However, both western blot and multiplex assay agreed there was no genotype difference in the common PIC. This disagrees with previous work on *App*$^{NL-G-F}$ mice as Manocha et al. (2019) found increases in TNF-α from 3 months, IL-6 from 6 months and IL-1β from 6 months in females and 12 months in males. Uruno et al. (2020) also found increased IL-6 and IL-1β levels from 11 months. However, they indirectly examined cytokine expression by measuring mRNA levels that only indicates whether these proteins are being produced rather than total levels. ELISA's offer a direct and highly sensitive measure of protein at the time of tissue collection (Schmidt et al., 2005) which Kaur et al. (2020) did on these three PIC but found only TNF-α levels to be increased between 6-8 months compared to WT. A multiplex assay behaves similarly to an ELISA in that it is a sensitive and direct measure but, with the use of fluorophores, can measure multiple proteins at once. The discrepancy between our results and the previous findings could be down to subtle differences between ELISA and multiplex assay or lab practices. Results for IL-1β, although agreeing with Kaur et al. (2020) lack of genotype difference, were of particular trouble in the present study due to majority of samples being below the measurable threshold. Although no firm conclusions should be drawn from this result, the findings suggest relatively low levels of IL-1β in all groups measured.

With only a few PIC showing an increase in the *App*$^{NL-G-F}$ mice (IFNγ, MIP-1α, MIP-1β, IL-9, IL-12p70) and even fewer surviving Bonferroni corrections (MIP-1α, MIP-1β), this could highlight a differentiation between different inflammatory proteins. Evidence suggests that in AD, Aβ activates certain cytokines as well as astrocytes that release more chemokines (such as MIP-1α and MIP-1β). These go on to activate microglia and other monocytes, aiding them in migration which then release PIC such as IL-6, TNF-α and IL-1β. These PIC then induce a secondary activation of astrocytes that further exacerbate Aβ pathology (Liu et al., 2014). However, these results only seem to show an increase in two of the relevant chemokines and not the primary cytokines suggesting amyloid build up directly activates AG, MIP-1α, MIP-1β and monocytes but not the secondary activation. Tau pathology or LC degeneration could have more impact on the secondary activation and further exacerbation of amyloid pathology. This could highlight one possible way tau and amyloid pathology interact during the development of AD. However, it is not so clear cut as some cytokines that are released by AG were not increased in the *App*$^{NL-G-F}$ in the present study (e.g. RANTES, MCP-1,

Eotaxin). Nevertheless, each cytokine and chemokine play overlapping but differing roles and not every one could be impacted by amyloid. So, the lack of IL-6, TNF-α and IL-1β increases could show how tau and amyloid interact to produce a feed forward loop of increasing neuroinflammation.

The lack of genotype differences in AIC was surprising as their role is to suppress the pro-inflammatory markers that are elevated in AD and thus, AIC should logically decrease. Neither IL-10, IL-4 or TGF-β showed any genotype effect which could highlight the complexity of measuring inflammation through cytokine expression. However, research has also shown that increased IL-10 levels can be detrimental to the clearance of Aβ (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015; Kiyota et al., 2012) so the relationship between AIC and PIC will not be as clear cut and ratios may be more informative rather than absolute protein levels. Furthermore, although the multiplex assay is highly sensitive, minute changes to protein expression could be sufficient to alter impact on microglia but are not statistically significant. It is also possible that alterations in AIC appear much later in AD progression but as *App*$^{NL-G-F}$ are a model of amyloid pathology in preclinical AD (Saito et al., 2014), the expected reductions of AIC have not occurred yet. Overall, the results show that amyloid build up seems to impact only a select few PIC but has little to no impact on AIC in this model. This could suggest that the increase in PIC is primarily driven by increased secretion and not reduced suppression. However, it is also important for future research to consider the ratios between AIC and PIC as an imbalance is what is commonly seen in AD rather than reduction in absolute values.

Neurotrophic support is altered in the *App*$^{NL-G-F}$ mice. While reductions in BDNF and TrkB are found in human AD, this was not so clear cut in transgenic mice (Burbach et al., 2004; Peng et al., 2009). The present study found no difference in mature BDNF expression in the *App*$^{NL-G-F}$ mice but did find an increase in TrkB in the cortex. This goes against what is commonly found in humans that could suggest tau or other human-centric processes not present in a murine model are responsible for reductions in BDNF. Looking at BDNF expression in a KI tau mouse model may be able to discern whether tauopathy causes BDNF reductions. This would be supported by the work of Murer et al. (1999) who found neurons with tau tangles did not produce BDNF. The *App*$^{NL-G-F}$ mouse have also been shown to have few or weak cognitive deficits (Latif-Hernandez et al., 2019; Masuda et al., 2016; Mehla et al., 2019) that could suggest amyloid does not lead to gross loss of synapses due to normal BDNF expression. However, to confirm this would require examination of synaptic health. The increase in TrkB suggests an increased need for BDNF signalling in the brain perhaps in response to increased need for synaptic repair from amyloid damage. It is possible that reductions in BDNF are only found at a later stage of AD or are caused by tau both of which are beyond the scope of the *App*$^{NL-G-F}$ mouse.

This lack of loss of mature BDNF is also found in previous work with the *App*$^{NL-G-F}$ mouse (Sakakibara et al., 2021). They examined BDNF expression through IHC while the present study looked at a more global expression using western blot. As differing methods that measure different aspects of BDNF levels agree, it suggests this is a robust finding in the cortex and hippocampus. The previous work in transgenic mice could not agree of whether BDNF was increased, decreased or no change (Tanila, 2017) but this data suggests amyloid alone does not cause a loss of mature BDNF. For TrkB, the increase found in *App*$^{NL-G-F}$ mice would need to be replicated to corroborate this finding.

This current research also differentiated between the opposing effects of BDNF and its precursor, proBDNF and found an increase in proBDNF in the cortex. In humans, proBDNF is upregulated so this finding suggests amyloid causes the increase in AD. It is impossible to say whether tau does play a role but it seems clear that amyloid can cause proBDNF upregulation. As only the precursor is increased and not the cleaved, mature BDNF fragment, that supports the idea that Aβ impairs this cleavage (Zheng et al., 2010). As proBDNF initiates downstream apoptotic effects, cell death in the cortex could be increased in *App*$^{NL-G-F}$ mice leading to potential learning and memory deficits. However, this was not found within this thesis (chapter 2) as well as other previous research (Latif-Hernandez et al., 2019; Whyte et al., 2018). The effects of increased proBDNF could be too minimal to detect in behaviour or require a model of more advanced AD which the *App*$^{NL-G-F}$ mice are not. Examining the full detrimental effects of proBDNF could offer insight into the potential impact of amyloid on learning and memory.

While the results of all the western blots are interesting, some caveats should be considered to the process and interpretation of results. As shown through the example blots (e.g. fig 3.5), there is a high level of variability across some readings (e.g. the cytokines and BDNF). These differences could be down to gels being run on different days (i.e., temperature can greatly impact how the western blot runs; Taylor and Posch (2014)). The use of the reference mouse (i.e., a mouse that was run on every gel and results were standardised to) was an attempt to control for these effects but variance can still occur. Future research would ideally repeat the western blots to ascertain their validity across days but the present research was unable to complete this. In addition, total protein load out was controlled by measuring sample concentration via BCA analysis and standardising to a house keeping protein (β-actin). However, this practice is based on the assumption that β-actin is equally expressed in any tissue or condition but recent research has found this to be incorrect (Moritz, 2017). Better methods of measuring total protein loadout on the western blot are being developed to counteract this assumption. Another key issue with the use of house keeping proteins as a standard are their high levels of expression. Their bands are very dark suggesting saturation (e.g.

fig. 3.9) and so differences between readings may be masked by ceiling effects (Moritz, 2017). While this could be enhanced by loading less protein, that could then make it harder to detect proteins that are in very small amounts.

Another issue to consider is that some blots retained high levels of background (e.g. fig. 3.8) that included bands with unknown identities (e.g. BDNF, IL-6, IL-1β, TFG-β). Only the bands at the correct sizes were counted toward the result but their presence could highlight potentially non-specific binding of the primary antibody. The multiplex assay did agree with the findings of these cytokine western blots which could speak to their validity. It is still important for these to be replicated in the future as the results do not agree with past literature.

There were also issues in how the western blot bands were quantified. While the method described is a common practice, no steps were taken to account for differences in thickness of the bands. A thicker band would produce a larger peak regardless of signal intensity. Furthermore, during the running of the blots, some bands ended up bending or producing a smile at the right side. This was continuously a problem but no practical solution seemed to fix it. While this does not impact the signal, it did make reading the bands in imageJ a lot harder due to the need to capture all bands in a rectangle. For blots with high background (i.e., other bands of unknown identities), other bands could have made it into the rectangle selection tool when creating the peaks (fig. 3.2b). Together, the results of the western blot should be taken with caution and repeated in the future with better techniques (e.g. measuring total protein staining, ensuring straight bands, repeated multiple times) to ensure the validity of the data.

Overall, the lack of age differences in amyloid and inflammation suggests the need to examine *App*$^{NL-G-F}$ mice at an earlier time point than 7 months in order to investigate an effect of aging. While both inflammation and amyloid were increased in the *App*$^{NL-G-F}$ mice, this fails to tell us whether neural or behavioural phenotypes would be impacted as amyloid and inflammation increase. There was also no evidence of LC cell loss but the LC could still be altered in other ways not examined here. The data also show that BDNF is not impacted by amyloid while TrkB and neurotoxic proBDNF are upregulated. TrkB increase could be in response to a higher need for synaptic repair. Amyloid also seems to block the cleavage of proBDNF to BDNF causing a neurotoxic problem. So, it seems amyloid may play a role in increasing neuroinflammation, proBDNF and TrkB while not altering LC cell count and mature BDNF.

●Chapter 4: Noradrenergic signalling disruption and depression●

●4.1 Chapter overview

To examine whether the LC-NA disruption that occurs in AD is also responsible for the development of depression and anxiety in AD, both animal and human work was performed. The animal work experimentally disrupted noradrenergic signalling with injections of DSP4, an NA-specific neurotoxin. Anxiety and depression were then tested through the EPM and sucrose preference test with little or no evidence for DSP4-related changes. The discrimination ratio in the EPM was no different but DSP4 mice did have fewer entries into the open arms compared to WT. They also consumed less sucrose overall but this difference was not significant. Despite the absence of significant effects, the numerical differences allow for the possibility that DSP4 has subtle effects on NPS development. This subtlety is discussed and could also highlight a need for longer DSP4 treatments to better mirror the gradual development of depression.

The human work compared depressed, non-depressed, AD and non-AD post-mortem LC tissue and examined TH production through optical density measures of a TH-IHC stain. This provides a measure for NA production that past research suggests is increased in depressed and AD patients to reflect higher spontaneous activity in the LC. This increase in TH in the depressed or AD group was not replicated and there was a slight trend for reduction in the AD & depressed group which is the opposite of what was expected. However, optical density as a measure may require larger sample sizes due to how much noise it can produce as well as ensuring the placement in the LC is controlled for.

Overall, examining whether LC-NA disruption in AD leads to NPS still remains a very relevant question but the work here should stand as pilot studies to better research it in the future. The work may not find supporting evidence for a link between LC-NA disruption and NPS but this lack of effect is more likely down to issues in measurement rather than a definitive null conclusion.

●4.2 Introduction

Depression is one of the most common NPS in AD (Lee & Lyketsos, 2003; Lyketsos et al., 2011) and is associated with worse cognitive decline (Murman et al., 2002) and worse quality of life (González-Salvador et al., 2000). As previously discussed (section 1.3.2), the LC-NA system is heavily linked to depression due to stress creating an overactive LC and high NA turnover (Tanaka et al., 1989; Tanaka et al., 1982). Dysfunction in the LC-NA system also occurs early in AD (Braak & Del Tredici, 2011) around the time certain NPS, like depression, become prevalent (Taragano et al., 2009). This has led to theories suggesting damage to the LC-NA system can create the neurological environment that leads to depression.

Repeated exposure to stress means the LC has increased firing and can become more sensitive to stressors. An overactive LC leads to increased production of NA despite no cell loss (Zhu et al., 1999). This suggests that the LC neurodegeneration that occurs in AD is not the primary driver of this link but rather production of NA. This is supported by the fact that depression can appear in AD prior to major LC cell loss (Rosenberg et al., 2013). Tyrosine hydroxylase (TH) is the rate limiting enzyme in the production of NA so is used as an indirect measurement of NA production. If TH expression increases then it suggests NA production increases as well (fig. 4.1). For these reasons, the question of whether depression is linked to AD through LC-NA system was examined primarily through NA and TH.

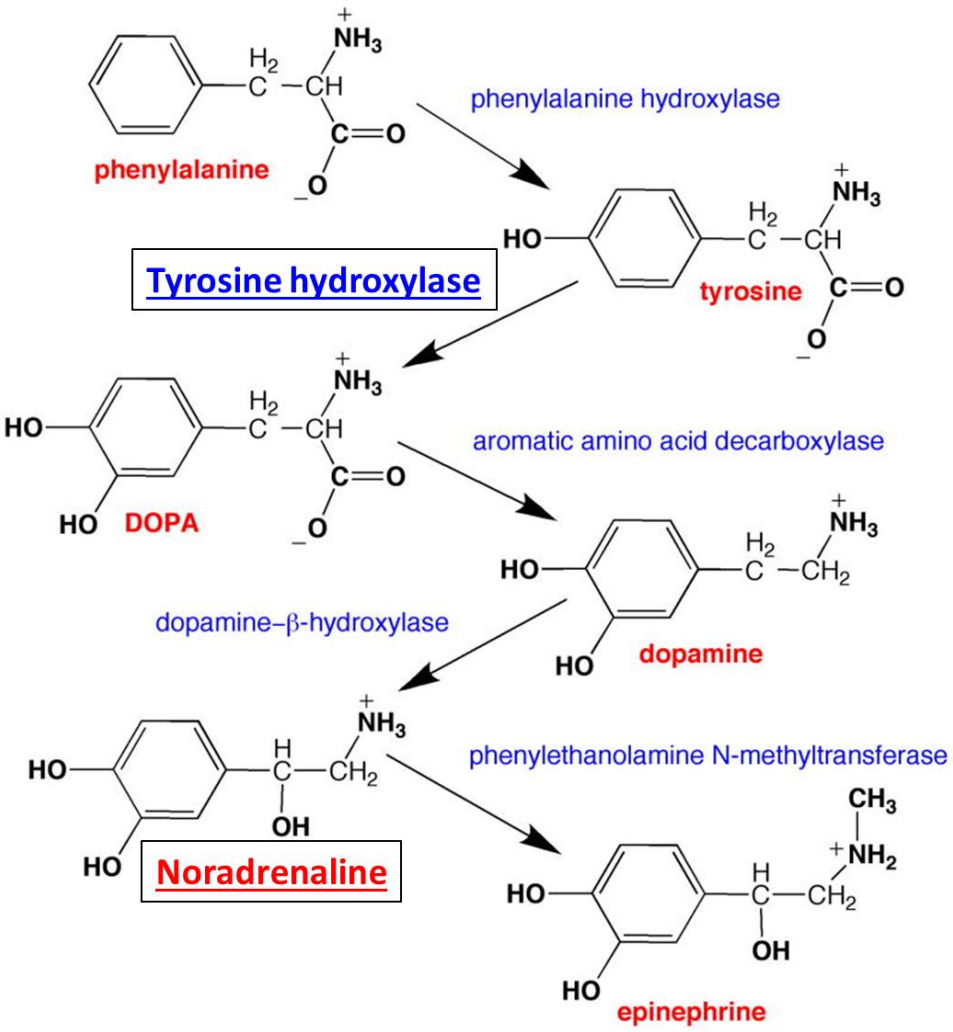

Figure 4.1 The biosynthetic pathway to produce noradrenaline. *Tyrosine hydroxylase is the rate limiting enzyme meaning if TH is reduced then the maximum NA that can be made is also reduced. Picture originally* from Daubner et al. (2011)

●4.2.1 Tyrosine hydroxylase in AD

In AD, tau reduces the sensitivity of LC neurons leading to lower NA secretion and reduced NA transmission prior to neurodegeneration (Andrés-Benito et al., 2017; Braak & Del Tredici, 2012). This reduced sensitivity also leads to stress and the subsequent hyperactivity of LC neurons (Chalermpalanupap et al., 2017) that can mirror what occurs in a depressive state.

Examining NA production in AD, Szot et al. (2000) found higher TH mRNA in AD patients compared to healthy age-matched controls. They concluded that increased NA production could lead to the higher NA levels found in CSF that could be over compensating for loss of noradrenergic transmission (Elrod et al., 1997; Raskind et al., 1984; Tohgi et al., 1992). However, there was a loss of

NA in cell terminals (Adolfsson et al., 1979) in AD patients suggesting a discrepancy between standard NA transmission and CSF transmission. It is possible that CSF transmission is favoured in AD as it allows long distance travel throughout the brain. This is just speculation though but it is clear that NA tissue content is reduced in AD that often leads to increased TH (Zhu et al., 1999). This would explain why Szot et al. (2000) found higher TH mRNA despite reduced NA. The enzymes monoamine oxidase A & B (MAO-A, MAO-B) degrade NA and are found to be upregulated in the AD brain (Kennedy et al., 2003). Together, this suggests that there is an increase in NA production through higher TH as well as increased NA degradation. This could offer an explanation as to why lower NA levels are found in AD despite higher TH expression.

Examination of the LC as a whole could also be helpful as LC neurodegeneration can lead to a reduction in NA signalling as a consequence. Research utilising neuromelanin-sensitive MRI (NM-MRI) takes advantage of the high levels of neuromelanin in LC cells to assess LC integrity. Reduced LC integrity has been heavily linked to loss of NA terminals in the brain (Sommerauer et al., 2018) as well as smaller LC volume (Keren et al., 2015) suggesting it relates to LC neurodegeneration. Evidence showed it was AD pathology driving reduction of LC integrity rather than simply an age phenomenon (Jacobs et al., 2021). Cassidy et al. (2022) used NM-MRI in live AD patients with age and gender matched healthy controls to determine LC integrity decline was progressive and correlated with Braak stage and cognitive decline. Furthermore, they found LC integrity was correlated with NPS severity but only in the AD group. This could be due to the low levels of NPS in the healthy control group but does highlight a potential role for LC signalling in the development of NPS. However, NPS were highly varied within the AD group, but this could simply highlight the heterogeneity of the blanket term “NPS” and it may be of more use to examine a select few such as depression and anxiety.

●4.2.2 Tyrosine hydroxylase in depression

A previous section established that the LC is heavily linked to depression (see section 1.3.2). Briefly, the LC is involved in aiding the stress response and repeated exposure to stressors leads to higher sensitivity of the LC and thus increased firing (Grant & Weiss, 2001; Rovin et al., 2012; West et al., 2009). Increased firing leads to high NA turnover (Tanaka et al., 1989; Tanaka et al., 1982) which could explain why NA depletion occurs and why a lot of anti-depressants work to restore NA levels (Werner & Covenas, 2010). NA depletion upregulates TH as it attempts to increase production (Zhu et al., 1999) but MAO is also increased in depression (Meyer et al., 2006) which degrades NA. This could explain why MAO inhibitors are also common anti-depressants as they work to regulate NA

levels. Although LC-NA signalling dysregulation occurs in depression, whether it is a causal factor needs to be determined.

There is evidence to suggest that LC-NA dysregulation is a causal factor for the development of depressive symptoms. Medically depleting NA has been found to induce depressive symptoms (Zubenko et al., 1990) especially in people with a family history of affective disorders (Caspi et al., 2003; Leyton et al., 2000). Treatments with NARI's have also been shown to successfully treat depression (Werner & Covenas, 2010) but only as long as treatment continues. This suggests that NA reductions are one potential neural origin to depressive symptoms but should not be considered a direct causal factor as environmental factors are often the initial cause. LC-NA dysregulation should be viewed as one potential neural basis for creating depressive symptoms. Anti-depressants have also been shown to downregulate TH in the LC of rats (Nestler et al., 1990) as well as reduce stress-induced increases in LC activity (Valentino et al., 1990). Zhu et al. (1999) found increased TH levels in patients with major depressive disorder (MDD) compared to healthy controls despite no cell loss. Together, this suggests that some anti-depressant pharmacology may work through direct effects on the LC-NA signalling system.

However, there is also evidence to contradict LC-NA dysregulation as a key factor in depressive symptom development. Not every depressed patient responds to noradrenergic-based treatments (Hodes et al., 2016; Nelson, 1999) and many patients will fall into remission despite continued treatment (Fonseka et al., 2015). However, this simply highlights the heterogeneity of MDD and how NA dysfunction is not the only neural basis. Furthermore, anti-depressants designed to increase monoamines will do so almost instantly but changes in symptoms happen over a long period of time (Brunello et al., 2003). If NA dysfunction was the neural basis then symptom changes would be expected to happen alongside increased NA. Nevertheless, it may take time for the increased NA signalling to create changes just as the development of depression is also a gradual decline. While NA signalling dysregulation does not occur in all depressed patients, the next logical question to address is: does the presence of NA signalling dysregulation always lead to depression? This is a difficult question to answer due to challenges in measuring NA directly in live patients and how depression can fluctuate from day to day. However, examining AD patients who commonly have NA dysregulation does show that, although depression is common, it does not occur in every patient (Lee & Lyketsos, 2003). I have already noted that there is high variance in LC integrity even within AD (Cassidy et al., 2022) so there could be possible subsets of AD patients with specific NA dysfunction that are more likely to show depressive symptoms.

It seems clear that NA dysregulation is not the only neural basis as there are other monoamines and not everyone responds to NARIs. Nevertheless, there is still evidence suggesting it is one possible neural basis for the development of depressive symptoms. Perhaps there are a subset of people who would respond to NARIs due to some underlying NA dysregulation. Evidence has found patients with more motivation related symptoms do respond better to NARIs (Brunello et al., 2003) meaning NA dysfunction could be the neural basis for motivation related symptoms.

●4.2.3 LC-NA signalling lesions in animal models

One way to examine whether the LC-NA system has a causative role in the development of depression and other NPS is to experimentally disable it in animals. Szot et al. (2016) partially lesioned the LC of WT mice and found increased immobility in the forced swim test (FST) and lower sucrose consumption. Both of which were attenuated with L-DOPS, an NA precursor, treatment. While this offers strong evidence for a causative role in depressive symptom development, they also found that a larger lesion did not lead to increased symptoms in FST. In fact, the smaller concentration of neurotoxin, 6OHDA, that made the lesion created the greatest increase in depressive symptoms while subsequent additions in 6OHDA reduced immobility on the FST. Nevertheless, they concluded that a larger LC lesion could mirror the cell loss that occurs in late AD and the depressive symptoms could be caused by a higher number of surviving neurons firing more erratically. This further highlights that it is not cell loss that directly causes depressive symptoms but rather cell loss can lead to further dysregulation of the LC-NA system.

One way to examine this noradrenergic signalling disruption without cell loss is with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4), a selective noradrenaline neurotoxin. While it does not cause neurodegeneration, it does affect adrenoreceptor availability, reduce NA and its transporter (NET) and increase irregular firing in the LC (Szot et al., 2010). This could mirror what occurs to the LC-NA signalling in depression to a much better degree than a 6OHDA lesion. It should be noted that it is not a specific LC lesion but will affect all noradrenergic neurons so can offer a more comprehensive look at NA signalling rather than specifically LC dysregulation. J Harro et al. (1999) injected rats with DSP4 and found the lowest dose (10 mg/kg) led to increased immobility in the FST while higher doses (30 mg/kg, 50 mg/kg) reduced immobility agreeing with the lesion work above. To accompany this, they found no cell loss but did find a reduction in NA tissue content as well as an increase in β-adrenoreceptors that greatly increase noradrenergic neuron firing.

Along with depression, DSP4 injections were also found to affect anxiety. Harro et al. (1995) found DSP4 led to increased neophobia which is in line with DSP4 creating overactive NA signalling. Other research has suggested increased anxious behaviour in monkeys who had their LC electrically stimulated to mirror increased firing seen in anxious individuals (Redmond et al., 1976). In contrast, Lapiz et al. (2001) found a medium dose of DSP4 (25 mg/kg) presented less anxiety like behaviour when assessed in the elevated plus maze. However, this was only found in group-housed rats and not when they were singly housed. They stressed the importance of environmental cues on creating anxious behaviour suggesting that LC dysregulation alone may not be sufficient to create behavioural change.

Little work solely focusing on WT mice injected with DSP4 has been done but Szot et al. (2010) examined the biochemical changes that occur in WT rats after such an injection. They found reduced NA tissue content but not solely in areas innervated by the LC suggesting DSP4 is not LC-specific but rather NA-specific. Furthermore, 2 weeks after the initial injections, there was an increase in spontaneous activity that quickly returned to normal after 3 months which mirrors the increase in LC activity in pathological depression and anxiety. It is important to note that rats could have a different sensitivity or reaction to DSP4 than mice that does occur with a similar injection compound, MPTP, which interferes with dopaminergic signalling (Sedelis et al., 2000). It is also possible to examine control (i.e., WT injected with DSP4) in research that examines depressive symptoms. One study found no difference between saline and DSP4 injected WT mice on the FST or time in the centre of the OF suggesting DSP4 has no impact on depressive or anxious behaviour (Choudhary et al., 2018). However, they did find the DSP4 group were much faster at initially entering the centre of the OF that could indicate reduced anxiety. Y. Li et al. (2018) found the DSP4 injected mice had higher immobility in the FST and TST compared to saline injected WT. There were no differences between injection groups for EPM or sucrose preference test. Other research looked at anxiety in the OF and also found no significant difference between saline or DSP4 injected WT (Jardanhazi-Kurutz et al., 2010). There are discrepancies in whether a 50 mg/kg injection of DSP4 does induce anxious or depressive behaviour which is why the present study will examine this in WT mice.

DSP4 treatments are not a good reflection of the LC damage that occurs in AD but can potentially offer a good insight into the depressive neural basis. For this reason, the present study looked at the effects of DSP4 on C57BL/6 mice in an anxiety test, EPM, and a depressive-symptom test, sucrose preference. Mice were singly housed to create an environmental stressor to see if the work from Lapiz et al. (2001) could be replicated. The sucrose preference test was run similarly to Szot et al. (2016) to see whether DSP4 created a similar reduction in consumption over time that

could indicate anhedonia or apathy. This will assess the question of whether disruption to LC signalling can lead to depressive and anxious symptoms in a mouse.

●4.2.4 Could NA dysfunction lead to depression in AD

Through both animal models and human studies, it seems clear that some part of noradrenergic signalling plays a role in the aetiology of depression and anxiety. Research seems to point to reductions in NA, or increased LC firing as key candidates rather than cell loss. Measuring TH expression in the brains of depressed, non-depressed, AD and non-AD patients would assess NA production and efficiency. TH has shown to be increased in both depression and AD independently that could suggest the effect of AD pathologies on the LC-NA system could lead to depressive symptoms. The present study will measure TH expression across depressed, non-depressed, AD and non-AD patients to see whether the increase in TH occurs and whether it is exacerbated in the depressed AD group. Higher TH would be indicative of higher firing rate and NA production supporting the LC-NA system as one neural basis for depression.

●4.3 Aims and hypotheses

To examine the role noradrenergic signalling has on the development of depressive symptoms, NA signalling was disrupted in WT mice and TH expression was examined in post-mortem human LC brain tissue. A group of C57BL/6 mice were either injected with 50 mg/kg of DSP4 or saline and tested in the elevated plus maze and a sucrose consumption test to assess anxiety and depression respectively. This would reveal whether disruption of LC signalling without cell loss is sufficient to produce depressive and anxious symptoms. The second part of this study compares TH expression across depressed, non-depressed, AD and non-AD patients post-mortem. An immunohistochemical stain for TH was done and intensity of expression measured to assess NA production. TH expression had previously been examined in depressed and AD patients separately but this represents whether there are alterations to NA production when conditions are combined. It is hypothesised that the DSP4 mice will show alterations in anxious and depressive symptoms. Furthermore, an increase in TH is predicted in the AD and depressed group but of prime interest will be how TH is expressed in the AD & depressed group. Together, this work will help to answer the question of whether disruption to noradrenergic signalling can lead to depressive symptoms.

●4.4 Methods

●4.4.1 Animals

Housing

Thirty-two male C57BL/6J WT mice (Charles River, UK) were individually housed and given ad lib access to food and water. Their cages were identical to those outlined in section 2.4.1. They had a 12hr light/dark cycle from 08:00-20:00 each day with home cages remaining in holding rooms with stable temperature (21 +-2 °C) and humidity (60 +- 10%) being constantly monitored. All animals were maintained as is outlined in the Animals Scientific Procedures Act (1986) and UK Home office licensing regulations.

DSP4 injection

Half the animals were given a DSP4 injection (n = 16) while the other half were given saline (n=16). Mice were injected with 50 mg/kg i.p DSP4 (Sigma Aldrich, #C8417) or saline on day 1 and day 7 and then once a month for the time taken to complete behavioural testing (see fig. 4.2). Behavioural testing began on day 10 giving mice 3 days to recover from their second injection.

●4.4.2 Behavioural testing

Timeline of testing

The timeline of injections and behavioural tests is shown in fig. 4.2 with length of the test shown below. Nest construction, splash test and social preference test were run but data is not shown in this thesis due to not being relevant to the aims of this chapter. The lick cluster analysis

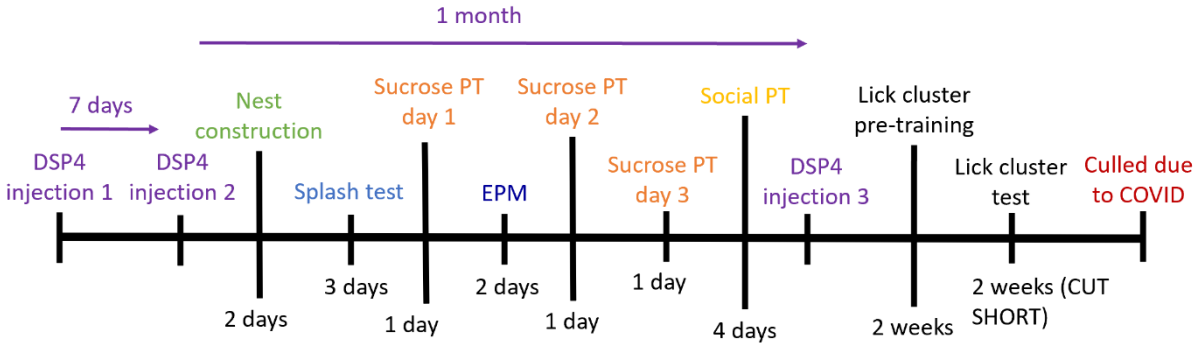

Figure 4.2 Timeline of injections and behavioural testing done on DSP4 and saline injected WT mice. *Top line is the test done and below is the length of time that test took to run. Sucrose preference test was run on the same day of the week each week so separated by 7 days.*

was run but is not included as the COVID-19 2020 lockdown prevented completion. Mice were also culled without brain tissue being extracted due to the abrupt lockdown caused by COVID-19.

Elevated plus maze

This was run identical to the EPM in chapter 2 (section 2.4.7). Briefly, mice were tube handled into the centre of the arena (section 2.4.3) and their exploration was recorded for 5 minutes. Time spent in the open and closed arms was measured using EthoVision XT 13 by tracking the centre point of the mouse. Distance travelled, velocity and entries into open and closed arms were also used to assess general anxiety behaviour.

Sucrose preference test

The equipment used to measure sucrose consumption was the same boxes and bottles used in lick cluster analysis (section 2.4.3 & 2.4.9). Two bottles were used, one contained a 1% sucrose solution and the other normal water. Placement of the sucrose in the left or right spout slot was counterbalanced across mice. Each mouse was tube handled into an individual box and left for 3hrs with free access to both solutions. This was done once a week for 3 weeks to see whether changes to consumption would occur as exposure increased (see Szot et al. (2016)). Consumption was measured by change in bottle weight (2 d.p.) over the test. While licking microstructure was recorded, the data was unusable due to equipment failure and so will not be considered.

●4.4.3 Human samples

All tissue was obtained following ethical approval and in accordance with the Human Tissue act (2004; Scotland, 2006). Paraffin embedded brain tissue sections (4µm thickness) of the pons were obtained from the Sudden Death Edinburgh Brain and Tissue Bank. Pons tissue with the LC on was requested for AD and non-AD patients with a history of at least 1 depressive episode or none at all (non-depressed groups). This formed the 4 groups used in this study: healthy controls, people with depression, AD, AD with depression. Tissue was requested from both sexes and age-matched to the best of their ability. Control cases were specified to have no history of neurodegenerative disease while AD cases had to be above Braak stage III to ensure accurate diagnosis. Table 4.1 details mean and SD for ages, post-mortem interval as well as group size. Post-mortem interval (PMI) indicates the amount of hours from death to neural tissue removal. PMI and pH of the brain have been known to affect tissue quality (Ferrer et al., 2007) so will be considered in the analysis to ensure tissue degradation does not impact results. Table 4.2 outlines the information for each

individual subject including unique identifying codes, cause of death and Braak stage. There were two people in the non-depressed, healthy control group who presented with very early AD pathology so will be removed for analysis. This is because the LC is one of the first brain areas to be affected by AD pathology so even early signs of amyloid could indicate confounding effects. One slide from each person underwent IHC staining with individual cells' optical density assessed.

Disease group	Depression	Age			PMI			Sex split	Total N
		Mean	SD	Range	Mean	SD	Range		
Non-AD	**Non-depression**	45.4	18.1365	19-71	85	21.65	40-115hr	5 female, 5 male	10
	Depression	44	19.2354	19-74	57.89	22.88	43-114hr	4 female, 5 male	9
AD	**Non-depression**	80	13.86	60-94	64	27.04	29-103hr	2 female, 3 male	5
	Depression	81	12.97	62-90hr	90	15.64	73-109hr	2 female, 2 male	4

Table 4.1 Means, standard deviations and ranges for age and PMI in human samples used in IHC*. PMI= Post-mortem interval. Also includes sex split and total number of samples per each group: Depression, non-depressed, healthy control, AD*

Code	BBN	Age	Sex	PMI (hr)	pH	Cause of death	Braak stage	Thal stage
Non-AD control, Non-depression								
SD045/17	BBN001.31503	57	F	73hr	6.08	1a) Cardiomegaly	1	1
SD030/18	BBN001.34150	63	M	115hr	6.41	1a) Ruptured atherosclerotic abdominal aortic aneurysm	0	
SD036/08	BBN_2455	20	F	40hr	6.5	1a. Suspension by a ligature	0	

SD036/17	BBN001.30916	71	M	71hr	5.97	1a) Haemopericardium 1b) Ruptured acute myocardial infarct 1c) Coronary artery atheroma and thrombosis	2	3
SD006/09	BBN_2465	25	M	81hr	6.4	1a. Multiple Injuries 1b. Road Traffic Collision (passenger)	0	
SD028/18	BBN001.34116	53	F	107hr	6.18	1a) Ascertained	0	
SD005/15	BBN_24779	46	F	76hr	6.26	1a) Complications of ischaemic heart disease and hepatic steatosis 2. Obesity	0	
SD047/15	BBN001.26976	19	M	101hr	6.24	1a) Unascertained	0	
SD054/13	BBN_19687	51	F	87hr	5.8	1a) Metastatic carcinoma	0	
SD053/15	BBN001.26797	49	M	94hr	6.2	1a) Coronary artery atheroma and cardiomegaly	0	
Non-AD control, depression								
SD011/08	BBN_2430	23	M	47hr	0	1a. Acute Methadone and Diazepam Toxicity	0	
SD023/08	BBN_2442	24	F	47hr	6.4	1a. Suspension by ligature	0	
SD001/11	BBN_2540	74	M	46hr	6.3	1a. Pulmonary thromboembolism	0	

						1b. Deep vein thrombosis		
SD032/07	BBN_2416	56	F	43hr	6.46	1a. Asphyxia 1b. Occlusion of airways by plastic bag 2. Chronic anxiety and depression Other. Acute influence of medication	0	
SD006/07	BBN_2394	40	F	57hr	6.25	1a. Fatal alcohol, diazepam and methadone poisoning	0	
SD042/06	BBN_2384	19	M	51hr	6.26	1a. Fatal Amitriptyline poisoning	0	
SD019/05	BBN_2316	51	F	114hr	6.23	1a. Combined effects of hypertensive heart disease and cirrhosis 2. Diabetes (Type 2) Other. Heart disease	0	
SD001/05	BBN_2299	49	M	72hr	0	1a. Suspension by ligature	0	
SD048/12	BBN_7626	63	M	44hr	6.08	1a) Nortriptyline toxicity	0	
Alzheimer's disease, non-depression								
SD037/18	BBN001.35096	72	M	103hr	6.18	1a) Stroke	6	5

SD027/20	BBN001.36346	90	F	60hr	6.1	1a) Aspiration pneumonia	6	5
SD005/20	BBN001.36066	94	M	29hr	5.98	1a) Aspiration pneumonia 1b) Dementia	6	5
SD021/16	BBN001.28796	60	F	54hr	5.95	1a) Alzheimer's Disease	6	5
SD038/19	BBN001.35811	83	M	72hr	6.13	1a) Bronchopneumonia 1b) Frailty 1c) Transitional cell carcinoma of the bladder 2) Right extracapsular fracture of neck of femur Mixed dementia (vascular and alcohol related brain damage) Peripheral vascular disease	6	4
Alzheimer's disease, Depression								
SD039/17	BBN001.30973	89	F	96hr	6.03	1a) Vascular dementia 1b) Hypertension 2. Atrial fibrillation Ischaemic heart disease Chronic kidney disease	6	5

SD027/16	BBN001.29135	90	M	73hr	6.44	1a) Bronchopneumonia 2. Alzheimer's Disease	6	3
SD005/16	BBN001.28410	62	F	109hr	6.04	1a) Inanation 1b) Alzheimer's disease	6	5
SD039/15	BBN001.26500	81	M	83hr	6.25	1a) Acute pyelonephritis 1b) Chronic indwelling catheter 1c) Alzheimer's Disease 2. Ischaemic heart disease 2. Hypertension	6	5

Table 4.2 Details of all people used in IHC stain for TH. *Split into depression, non-depressed, AD and non-AD groups. IHC = Immunohistochemistry, TH = Tyrosine hydroxylase, AD = Alzheimer's disease*

●4.4.4 Immunohistochemistry

Tyrosine hydroxylase DAB stain

Paraffin-embedded slices of the pons were stained with an antibody to tyrosine hydroxylase to undergo an assessment of the optical density of TH expression in the LC. First, tissue was dewaxed twice in xylene for 6 minutes each, then rehydrated in a descending series of ethanol each for 3 mins before being placed in distilled water 2 x 3 minutes. Antigen retrieval was done by heating the tissue at 95 °C in a microwave in 0.01 M citric acid (pH 6.0). Slides were cooled by running them under a tap of cold water and washed in 0.1 M PBS (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 1.5 M NaCl, pH 7.4) for 10 minutes. Endogenous peroxidases were deactivated in 20% methanol, 1.5% H_2O_2 in 0.1 M PBS before being washed thrice in PBS-T for 5 minutes (0.1 M PBS with 0.03% Triton X-100). Slides were blocked in 1% filtered Bovine serum albumin (BSA), 3% normal horse serum and 0.1% Triton X-100 in 0.1 M PBS for 30 minutes. Four drops of avidin per ml (Avidin/Biotin blocking kit, VectorLab, #SP-2001) was then added to the fresh blocking buffer and left on the tissue for another 30 minutes to

complete the block. After 3 more washes in PBS-T, sections were incubated in the same blocking buffer with primary antibody (1:500, #AB152, Abcam) and 4 drops/ml of biotin (VectorLab, #SP-2001) for 48 hrs at 4 °C. After another 3 0.1 M PBS-T washes, sections were incubated in the blocking buffer with biotinylated secondary antibody (1:100; horse anti-rabbit, #BA-1100-15, 2BScientific) for 2 hrs in a wet chamber at room temperature. Sections were then left in 1 drop/2.5 ml of reagent A, 1 drop/2.5 ml of reagent B from ABC HRP peroxidase kit (VectorLab, PK-4000) in PBS with 0.1% Triton X-100 for 45 minutes at room temperature. Sections were washed in fresh 0.05 M TNS (0.05 M Tris base, pH 7.4) before being visualised in freshly made 0.1% DAB (3,3'-Diaminobenzidine tetra- hydrochloride, Sigma Aldrich, #D5637) with 0.0036% hydrogen peroxide in 0.05 M TNS for 2-3 minutes (until the staining appeared). Sections were washed in distilled water for 5 minutes and left to airdry overnight at room temperature. They were rehydrated in distilled water (5 minutes) and then counterstained with 0.5% methyl green (Sigma Aldrich, #M8884) in 0.1 M citric acid for 60 minutes at room temperature in a wet chamber. Three more washes in distilled water followed before cells were cleared twice in xylene for 6 minutes each. Slides were cover-slipped with DPX (Sigma Aldrich, #06522) and entire slide imaged with a Zeiss Axio Scan Z1 microscope (Zeiss, Germany).

Optical density analysis

Analysis was run in Fiji (ImageJ, Schneider et al. (2012)) and depicted in fig. 4.3. Before analysis, a global calibration was set up in accordance with official ImageJ protocol (https://imagej.nih.gov/ij/docs/examples/calibration/). This used a standard Kodak optical density step tablet (fig. 4.3) where measurements were taken across multiple shades and set to Rodbard calibration to set it up for optical density. Pictures were loaded and colour deconvolution (DAB and methyl green) was run to extract the brown colouring from the image (fig. 4.4). The brown image was converted to 8-bit black and white to assess luminosity. A region of interest (fig 4.5) was drawn and used across all images which was placed on 20 different random cells/section to get multiple measures of luminosity. Three measurements were also taken from the background for each section and the average of these background readings was subtracted from the average cell optical density for that section to give the final luminosity value used in analysis. Two-way ANOVA was run to assess differences between depression status and Alzheimer's groups.

Figure 4.3 Standard kodak step tablet used to set global calibration

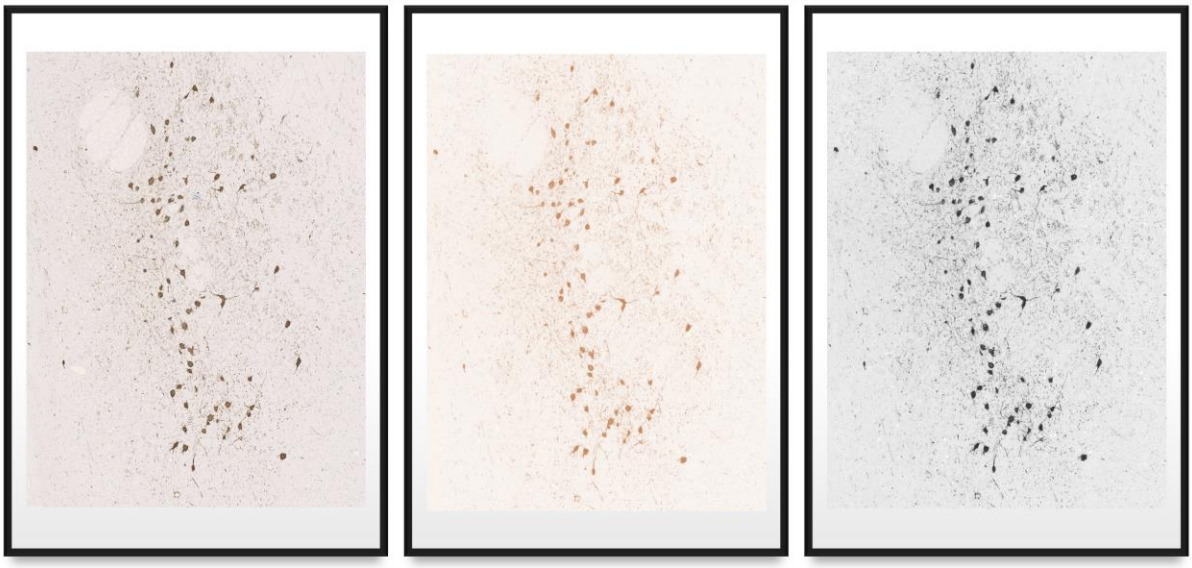

Figure 4.4. The process of going from the original image to the black and white extraction of brown colourisation used in analysis. *A) Image from the microscope B) brown colouring extracted C) Turned into a black and white 8-bit picture to simply assess luminosity*

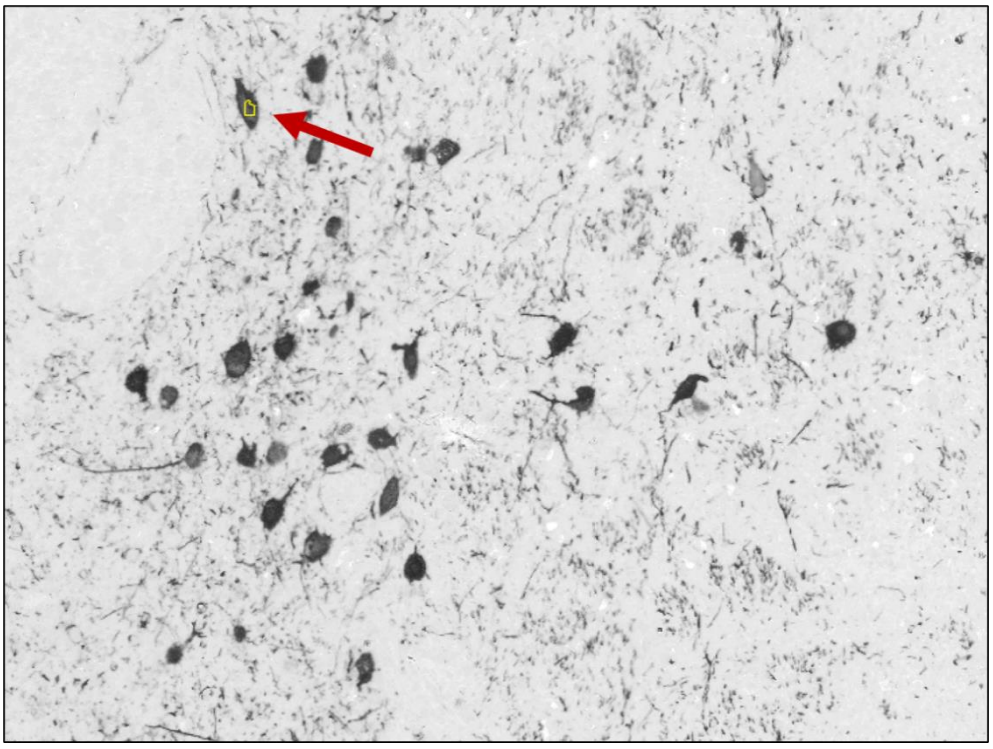

Figure 4.5. Example of the small selection shape on one cell body. *It was moved to 20 cells to take optical density measurements and then 3 of the background.*

●4.4.5 Statistical approach

All data was analysed using null hypothesis significance testing in SPSS (version 26; IBM corp', 2019) with Bayesian analysis run in JASP (version 0.14.0.0; JASP team (2022)). As outlined in

previous chapters (section 2.4.10), NHST will be the main analysis tool but Bayesian analysis will be run on all non-significant data to ascertain whether the lack of significance is evidence for the null or an inconclusive results. Briefly, as BF_{excl} between 1-3 would suggest inconclusive while above 3 would indicate evidence for the null (Jeffreys, 1998).

For behavioural testing, a discrimination ratio was used to analyse the EPM along with open arm entries as measured automatically by EthoVisionXT 13. Motor deficits are assessed by measuring total distance moved as well as velocity in transit (average speed / total time spent moving). The sucrose preference test used raw sucrose consumption across 3 days as well as a preference ratio (sucrose consumed / total liquid consumed) to assess anhedonia. For IHC with human samples, the optical density was calculated by averaging the signal and then taking away the average background signal. Two-way ANOVA analysed the differences between AD or no AD and depression or no depression.

Assumptions were tested as seen in section 2.4.10 and violations of ANOVA assumptions were addressed by using bootstrapping in the same way as described in section 3.4.5. As group sizes are not equal and sample sizes are not large, any deviations from assumptions will be taken as violations.

●4.5 Results

●4.5.1 Elevated plus maze

Anxiety was assessed in C57BL/6J mice injected with a noradrenergic specific neurotoxin, DSP4, or saline using the EPM. As previously outlined (section 2.5.2), increased time in the open or 'unsafe' arms indicates reduction in anxiety. Mice's general preference for the closed arms was confirmed with a one sample t-test, $t(31) = 14.885$, $p < .001$, regardless of injection substance. Movement across the EPM was automatically recorded using EthoVision XT 13 where the arena was split into open and closed arms (identical to *App*$^{NL-G-F}$ in section 2.5.2). For the anxiety score, a discrimination ratio was calculated by time in open arms (s) / total time exploring (s).

Movement

To check whether there were any movement differences between groups, total distance travelled across the arena was examined with a one-way ANOVA and visualised in fig. 4.6a. There was no significant effect of DSP4 on total distance moved ($F(1, 30)=.241$, $p=.627$, $\eta^2=.008$, $BF_{excl}=2.711$).

Furthermore, to assess speed of movement, average velocity was divided by total time spent moving to give velocity in transit (fig.4.6b). A one way ANOVA revealed no significant effect of DSP4, $F(1, 30)=.908$, $p=.348$, $\eta^2=.029$, $BF_{excl}=2.102$. Together, this suggests that the injection of DSP4 has no effect on movement and created no motor deficits.

Anxiety score

The assessment of anxiety used a discrimination ratio where a higher score indicates preference for the unsafe zone or less anxiety. Visualised in fig. 4.6c, the ANOVA showed no significant effect of group ($F(1, 30)=.126$, $p=.725$, $\eta^2=.004$, $BF_{excl}=2.834$) showing DSP4 does not impact proportion of time spent in the open arms.

To further assess anxiety, entries into the open arms was also automatically counted using EthoVision (fig. 4.6d). The ANOVA did show a significant effect of group, $F(1, 30)= 4.793$, $p=.036$, $\eta^2=.138$, with DSP4 having fewer entries into the open arms than the saline group.

Overall, DSP4 did not create any movement deficits but also did not impact proportion of time spent in the open arms. DSP4 did seem to reduce number of open arms entries which will be considered in the discussion.

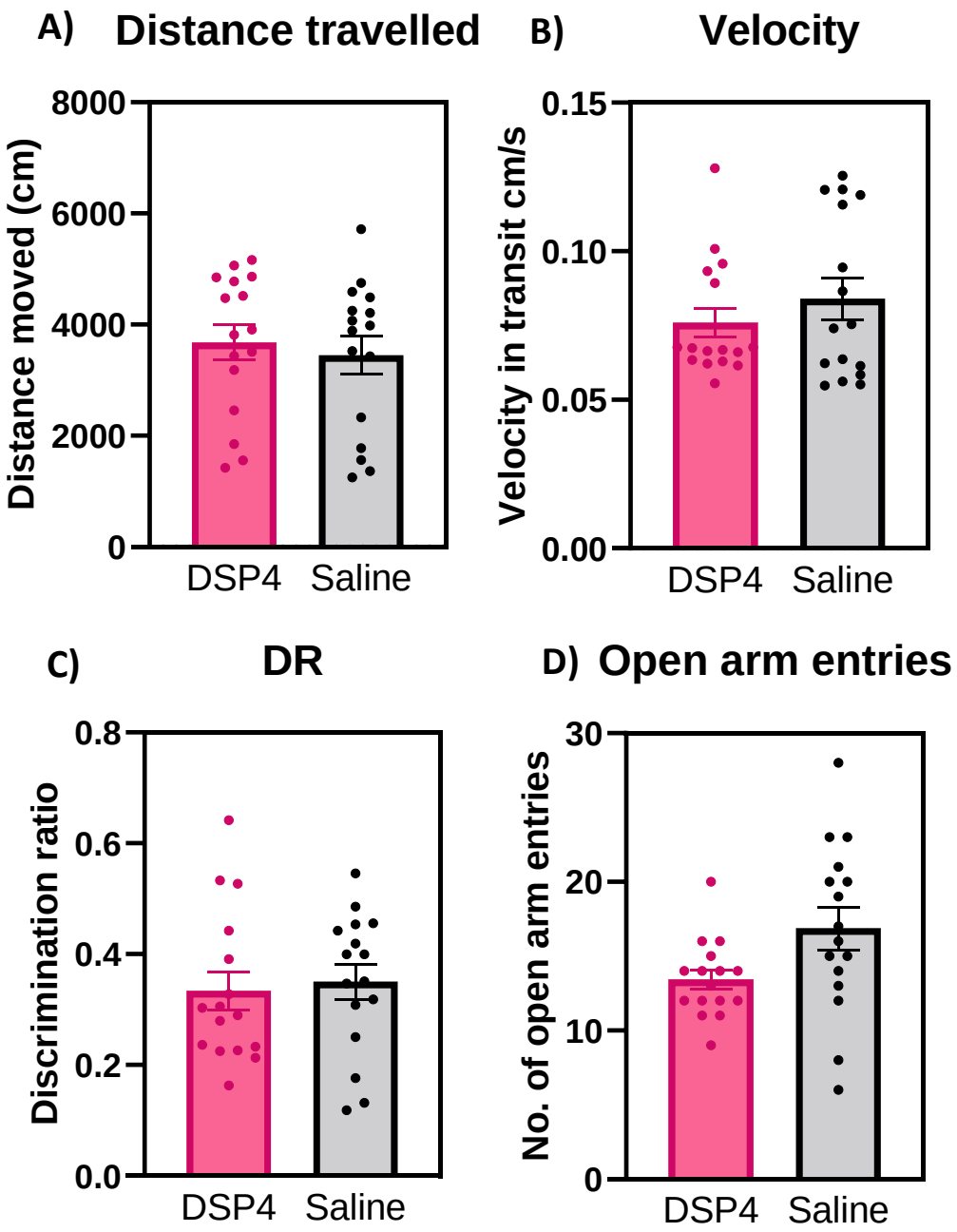

Figure 4.6 EPM results forthe DSP4 and saline injected groups. *A) distance travelled over 5 min EPM test (cm), B) Velocity in transit as measured by average speed / total time spent moving, C) Discrimination ratio in the EPM to measure anxiety- a higher*

●4.5.2 Sucrose preference test

To measure anhedonia in mice injected with DSP4, the sucrose preference test was run 1 day a week for 3 weeks. This timing component was to see whether there was a group difference in their exposure to sucrose over time. A healthy mouse is expected to consume more sucrose with repeated exposure. Both total sucrose consumed and a preference ratio will be considered to

answer this question. The preference ratio is calculated by sucrose (g) / total liquid consumptions (g) to control total fluid consumption which has been known to impact raw consumption measures.

A repeated measures ANOVA was run to assess preference ratio across each day (fig. 4.7a). It revealed no effect of group ($F(1, 30)=.398$, $p=.533$, $\eta^2=.013$, $BF_{excl}=3.25$) or day ($F(1, 30)=3.64$, $p=.066$, , $\eta^2=.108$, $BF_{excl}=1.307$) but bayes factor for the latter suggested inconclusive results. The group*day interaction was also non-significant, $F(1, 30)=1.014$, $p=.322$, , $\eta^2=.033$, $BF_{excl}=3.539$, with bayes factors agreeing with a null result. Despite the DSP4 group visually showing differences in preference ratio across the days, this was not significant and the lack of interaction suggests no simple effects testing was required.

Raw sucrose consumption was also considered (fig. 4.7b). Repeated measured ANOVA found no effect of group ($F(1, 29)=.463$, $p=.501$, $\eta^2= .016$, $BF_{excl}=3.341$), day ($F(1, 29)=2.037$, $p=.164$, $\eta^2=.066$, $BF_{excl}=4.828$) or interaction ($F(1, 29)=.266$, $p=.61$, $\eta^2=..009$, $BF_{excl}=19.749$) suggesting no differences in raw sucrose consumed across days or between groups.

Together, this indicates that injection of DSP4 does not create a significant anhedonic effect but graphically, there does appear to be trends in reduced consumption which will be considered in the discussion.

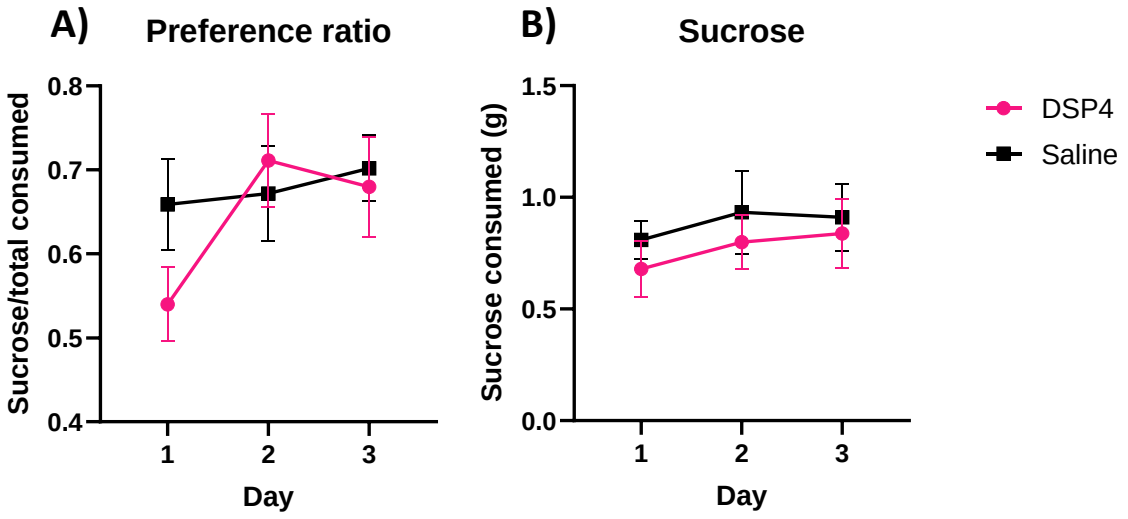

Figure 4.7 Sucrose preference test for the DSP4 and saline injected mice across days with a week delay. *A) preference ratio calculated by total sucrose/total liquid consumed- this includes the water also given during the test. B) total raw sucrose consumed in g. DSP4: n=16, saline: n=16, bars and errors bars represent mean and SEM and how they change over time.*

●4.5.3 Human TH levels

Tyrosine hydroxylase is the rate-limiting enzyme in the production of NA so changes to its levels indicate alterations to NA production. TH was measured using average optical density minus

background signal between healthy control, healthy depressed, AD and AD depressed subjects (fig. 4.9). Two healthy, non-depressed samples were excluded for having early AD pathology (final group sizes: non-AD & non-depressed = 9, non-AD & depressed = 8, AD & non-depressed = 5, AD & depressed = 4).

As previously stated (section 4.4.3), PMI, brain pH, sex and age could all impact the TH optical density measurement. These were checked with correlations and t-tests to see whether they significantly related to the TH signal which would implicate them as confounding factors. For this, BF_{01} is identical to BF_{excl} as there is only one factor to consider. Pearson's r correlations revealed no association between TH signal and age ($r(26)=.155$, $p=.451$, $BF_{01} = 3.135$), PMI ($r(26)=.121$, $p=.555$, $BF_{01} =3.48$) or brain pH ($r(26)=-.275$, $p=.174$, $BF_{01} = 1.706$). As sex is a categorical variable, an independent t-test was run to show no significant effect, $t(24)=-1.013$, $p=.321$, , $BF_{excl} = 1.888$. The Bayes factors for sex and pH do suggest inconclusive factors which means no impact on results cannot be completely ruled out.

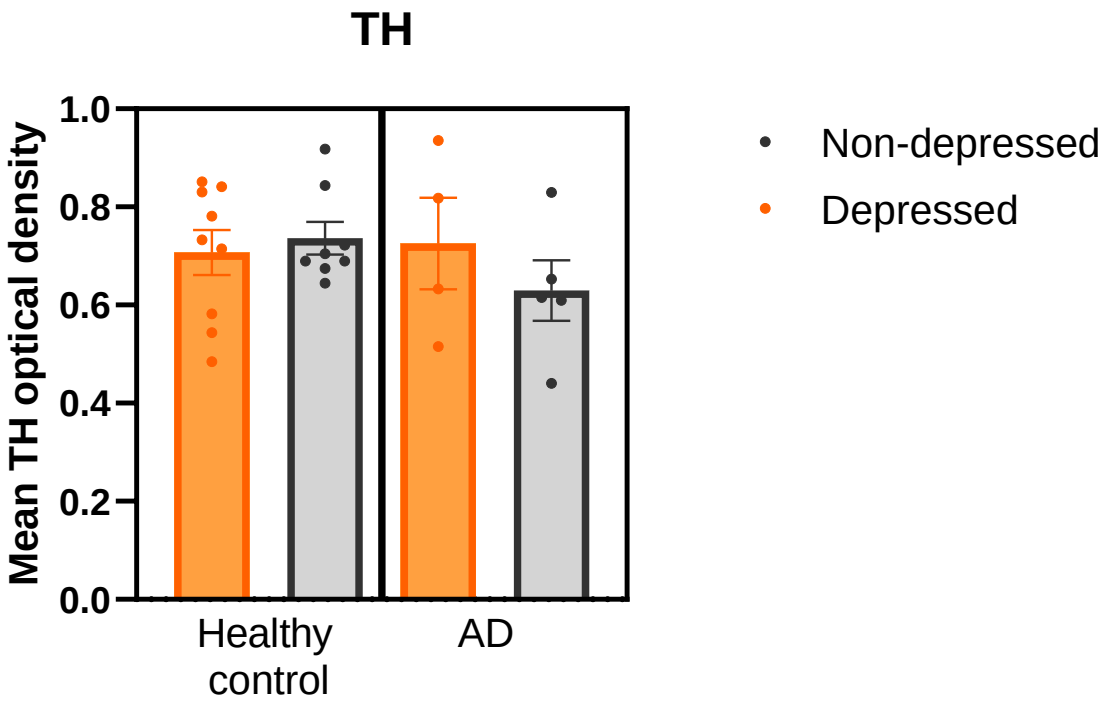

Figure 4.8 TH optical density mean measurements across depression and AD groups. *Bars indicate mean with error bars showing +- SEM. Group sizes: healthy & non-depressed = 9, healthy & depressed = 8, AD & non-depressed = 5, AD & depressed = 4*

To measure optical density of TH, a two-way ANOVA was run (fig. 4.8). It revealed no effect of AD ($F(1, 22)=.78$, $p=.387$, $\eta^2=.034$, $BF_{excl}=2.532$) or depression ($F(1, 22)=.505$, $p=.485$, $\eta^2=.022$, $BF_{excl}=3.181$). The disease*depression interaction was also non-significant, $F(1, 22)=.611$, $p=.443$, $\eta^2=.027$, $BF_{excl}=4.708$, with high bayes factors agreeing with a null result.

Visually, the graph indicates a slight trend in reduced TH expression for the AD & depression group which will be examined in the discussion. However, overall, there appears to be no significant difference in TH expression between all four groups in their production of NA. These results are also not confounded by age, sex, PMI or brain pH after death suggesting degradation in tissue has not impacted the results.

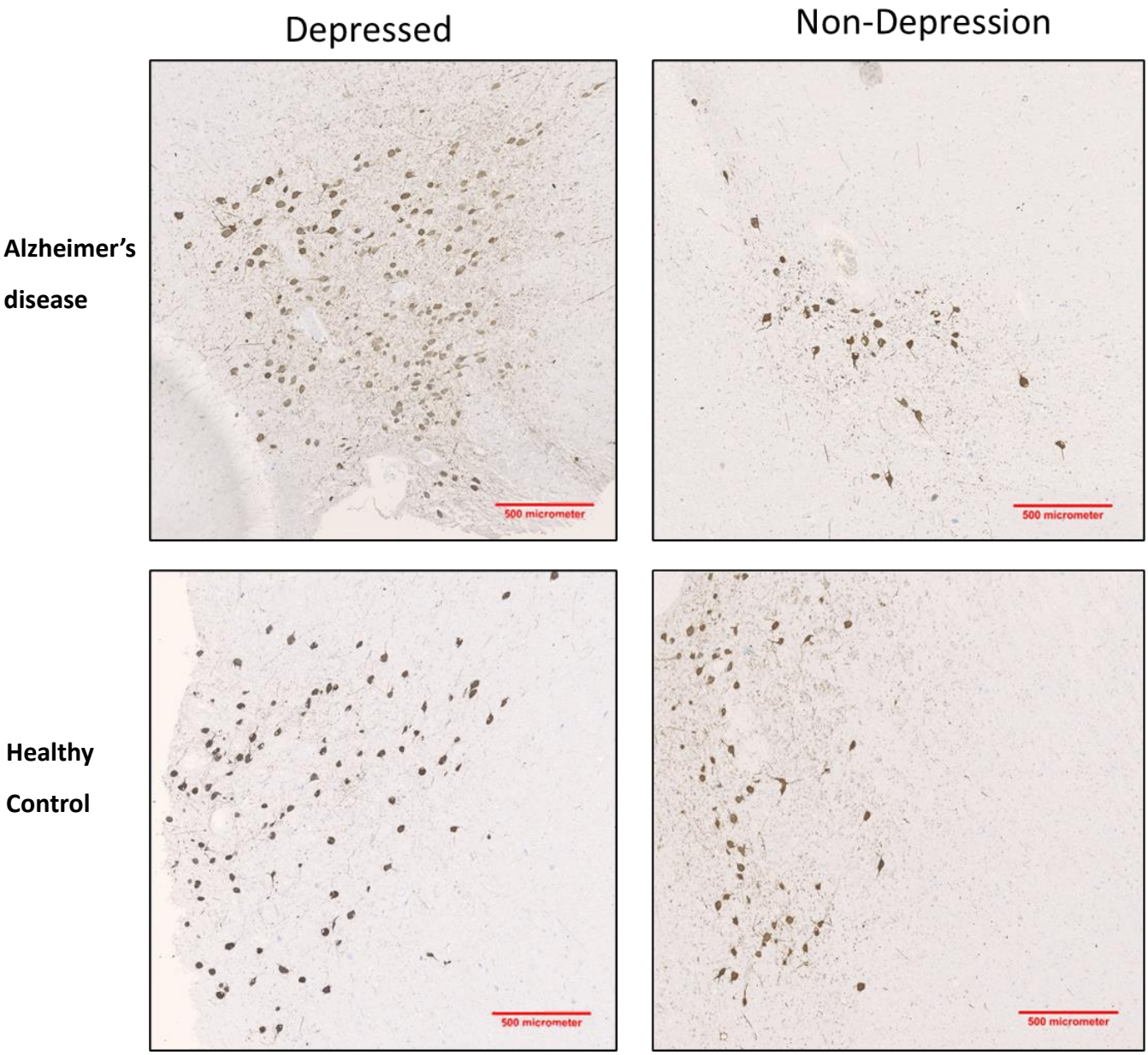

Figure 4.9. Representative images of the LC in each human group. *LC cells stained for TH and visualised with DAB with a methyl green counterstain.*

●4.6 Discussion

This work set out to answer the question: will disruption to noradrenergic signalling lead to depressive symptoms? This was done through a noradrenergic-specific neurotoxin, DSP4, being injected into WT mice and through examination of NA production across depressed, non-depressed, AD and non-AD human patients. The DSP4 work looked at anxiety and depression and found no significant differences in either anxious behaviour or hedonic responses. However, there were very subtle changes such as a slight decrease in raw sucrose consumption across days in the DSP4 group. There were also no changes between time spent in the 'unsafe', open arms as a proportion to total exploration but there was a reduction in open arm entries. These results will be discussed below. The work in humans found no significant difference in the optical density of TH between the depressed, non-depressed, AD and non-AD groups suggesting NA production is similar. However, there were a few subtle trends that will be discussed and could highlight that the impact of NA signalling on NPS is not so simple to measure.

Firstly, it was important to determine whether any anxiety-related behaviour measurements in the EPM could be influenced by movement. For this reason, total distance travelled and velocity in transit were measured and found no significant differences between DSP4 and saline injected groups. This suggests that disruption to noradrenergic signalling does not create motor deficits that could interfere with the interpretation of anxiety related measures. However, this is in contrast to Jardanhazi-Kurutz et al. (2010) who found a 20% reduction in distance travelled in DSP4-WT mice using the open field. Concentration of DSP4 and injection volumes were equal between studies that suggests that the type of arena used could impact motivation to explore or it is simply down to lab differences like how the animals are handled and thus, not a specific DSP4-impacted effect. Furthermore, reduced movement in the *App*$^{NL\text{-}G\text{-}F}$ mice was suggested to be indicative of apathy but the lack of movement differences here suggests no apathetic phenotype in DSP4 mice.

Anxiety-related behaviours were, therefore, examined using the EPM. As DSP4 did not affect discrimination ratios, this suggested that disruption to noradrenergic signalling does not lead to changes in anxiety as was hypothesised. Jardanhazi-Kurutz et al. (2010) used raw time spent in the 'unsafe', centre of the OF and found a non-significant trend toward reduced time spent in the centre. In other words, DSP4-WT mice showed higher anxiety levels compared to saline-WT but it was not statistically significant. However, when DSP4 treatments had been given monthly for 12 months, the older DSP4-WT mice showed a large reduction in time spent in the centre compared to 12-month-old saline-WT mice. This could indicate that a longer DSP4 treatment could lead to changes in how the brain develops which results in increased anxiety. The present study did not find

any such reductions to time spent in unsafe zones but the EPM was run after the 2 initial DSP4 injections and so noradrenergic signalling had been disrupted for less than a month (see fig. 4.2 for timeline). Together, this could indicate that a longer DSP4 treatment is more indicative of the repeated activation of LC neurons seen in anxiety as well as depression so should be examined in future research.

To add to this, although no changes to DR were found, the DSP4 group did enter the open arms significantly less than the saline group. In some research, open arm entries is used as the primary indicator of anxious behaviour (Pellow et al., 1985) but it does not take into account actual time spent in the unsafe zones. Lower open arm entries could suggest the mice simply move less but there were no motor deficits which points to an indication of increased anxiety. As this is not reflected in a lower DR, it could suggest this is a subtle increase in anxiety. Changes to open arm entries have been previously physiologically validated (Pellow et al., 1985) but were also accompanied with increased time spent in the open arms. The present study did not find that which could suggest the effect is not robust or is subtle. Future DSP4 research should utilise a plethora of anxiety tests with ethological behaviours to make more concrete conclusions about its effect on anxious behaviour.

To assess anhedonia, the sucrose preference test was done on the DSP4 and saline-injected mice. Both raw sucrose consumption and sucrose consumed as a fraction of total liquid consumed were used. The latter controlled for general thirst of the animals while total sucrose could indicate a general hedonic response. However, without measurement of lick microstructure or facial expression during test, it is impossible to pars out whether reduction in sucrose consumed is due to a hedonic or motivational deficit. This was done as the test was performed in the same licking boxes used for lick cluster analysis (see section 2.5.4) but the data was unusable due to equipment malfunction. The lick cluster test was also run on these animals but not completed due to COVID. This full test for anhedonia will, therefore, be left for future research as it will be helpful in uncovering possible hedonic or motivational deficits.

The present study found no significant changes to total sucrose consumed or preference ratio across multiple testing days. However, there was a subtle trend in reduced consumption across all days in the DSP4 group compared to the saline which, again, suggests a small effect. When looking at the preference ratio, the first day showed the largest difference (fig. 4.7) with lower preference ratio for sucrose in the DSP4 group compared to the saline group. Even then, this difference was not significant but could simply indicate increased water consumption especially since total sucrose consumed was only marginally reduced in the DSP4 group. Y. Li et al. (2018) also

found no difference in sucrose consumed between DSP4 and saline injected mice which agrees with the present study. Work in rats also found similar findings (Jaanus Harro et al., 1999). Ammar et al. (2001) also looked at rats and found DSP4 consistently reduced sucrose consumed over a 7 day period. There was also no difference in day as sucrose consumption remained low. However, they used an intraoral cannula to give sucrose that could have caused stress during surgery. Furthermore, Szot et al. (2016) looked at sucrose consumption after a chemical lesion to the LC and found reduced sucrose consumption. They also ran the test across multiple days to see whether repeat exposure to sucrose would lead to increased consumption as it would in healthy animals or not. They found, after lesion, a decrease in overall consumption and this worsened with repeat exposure suggesting an anhedonic effect. The present study found no such decrease over time suggesting the repeat exposure to a pleasurable experience leads to further indulgence or, worded differently, an intact hedonic response. Overall, the sucrose preference test is not able to differentiate between motivation and hedonic deficits but is able to indicate the presence of some form of related deficit. Future research should use the lick cluster analysis for reasons stated above but should also examine it on longer DSP4-treated mice to see the effects of prolonged noradrenergic disruption.

DSP4 is a noradrenergic specific neurotoxin that disrupts signalling through its effects on NET and adrenoreceptors (Szot et al., 2010). As it does not lead to actual LC cell loss, it is not a good model of AD (Szot et al., 2006). However, it does mirror depressive noradrenergic disruption (Itoi & Sugimoto, 2010) so should be a good representation of depression. Some studies suggested DSP4 only showed an effect in conjunction with other factors such as environmental stressors (Lapiz et al., 2001) or AD pathology (Chalermpalanupap et al., 2018; Jardanhazi-Kurutz et al., 2010). Prominent examples are amyloid mouse models such as APP23 (Heneka et al., 2006), APP/PS1 (Jardanhazi-Kurutz et al., 2010; Rey et al., 2012) or tau models such as P301S (Chalermpalanupap et al., 2018). In each of these examples, DSP4 exacerbated the amyloid or tau pathology present in the model or increased microglial activation (Kalinin et al., 2006). The fact only subtle effects were found in this study could simply be that impact on noradrenergic signalling requires an interaction with a biological or environmental stressor to exacerbate detrimental effects. However, J Harro et al. (1999) found increased immobility in the FST in DSP4-injected rats but only at the lower doses. This could either highlight that smaller impacts to noradrenergic signalling are what lead to depressive symptoms or rats do respond to DSP4 differently than mice. Regardless, research seems to show greater impact after prolonged DSP4 treatments (Jardanhazi-Kurutz et al., 2010) that would make sense as depression develops gradually over repeat exposure to stressors. Therefore, it would be logical to assume that depressive symptoms come about after prolonged disruption to noradrenergic signalling but future research should examine this in DSP4-injected WT mice.

While there were subtle differences between DSP4 and saline-injected groups, it cannot be confirmed the extent of the impact of DSP4. This was due to COVID stopping neural tissue extraction so it cannot be confirmed whether NA, NET or adrenoreceptors were affected but can easily be rectified in the future. This present study should be taken as a pilot study and the subtle differences found do suggest some small impact that does point to noradrenergic signalling disruption leading to depressive symptoms.

To examine NA production in humans, optical density measurements (OD) were done on TH-IHC stained LC brain tissue in depressed, non-depressed, AD and non-AD people. While previous work showed increased TH in post mortem tissue of depressed patients (Zhu et al., 1999) as well as AD patients (Szot et al., 2000), the present study did not replicate these findings. The reasoning for this could simply be that OD as a measure is subject to a lot of variation. Past research found that OD can be affected by length of tissue incubation even though the number of cells positively labelled is unaffected (Kuo et al., 1994). While the present study was aware and attempted to control for this by removing the background signal, it is possible that increased incubation in DAB would have a larger impact on the cells than the background. I also attempted to control for length of DAB stain by keeping it stable across days but, due to the variability in the samples, the visual appearance of the stain did take longer to appear in some samples than others. While in practice this was only a 30 second difference, it could affect the outcome for the OD. Furthermore, a small sample size would be much more affected by high noise suggesting a need for a much larger number of samples. It may also help to stain multiple brain slices per patient to see whether there is noise even within the same person. Examining changes to NA signalling post-mortem is subject to a lot of variation due to differences in how the tissue is stored after death. While we tested such factors and concluded no confounding effects, it could still add further noise to the OD signal. However, the same would be said for all post-mortem analyses such as the TH mRNA (Szot et al., 2000) and TH-IR measures (Zhu et al., 1999) alluded to in the introduction to this chapter. As both studies on post-mortem tissue do find increased TH in AD and depression respectively, this suggests that the issues are in the low sample number per group with high variation from tissue handling. In addition, the present study was unable to standardise where caudally in the LC was stained as the samples simply had to meet the criterion of containing the LC. Past research has shown that the LC is not uniformly affected as the caudal and middle sections show more disruption in depression (Zhu et al., 1999) and rostrocaudal areas are more degraded in AD (German et al., 1992; Theofilas et al., 2017). This could be another reason why there was so much noise and something else to standardise for future research. Future research should examine this question with larger sample sizes and control for the

placement in the LC to get a more conclusive answer to TH changes between AD, non-AD, depressed and non-depressed people.

Another way to better examine this question would look at it in live patients. The cutting-edge work using NM-MRI is able to examine the entire LC in live patients. While this does not measure actual NA or NA production (i.e., TH), it could give a general measure for LC integrity and how that differs between depressed and non-depressed people. Being able to scan the entire LC would be highly beneficial as research shows dysregulation to the LC is not uniform throughout and the middle and caudal regions (Zhu et al., 1999). This would also be able to assess the effect of time as depression is not stable and symptoms can waver across time periods. Taking multiple scans across a person's life could see whether depressive symptoms change analogous to LC integrity. Doing this in AD and non-AD patients would also show whether LC integrity is associated with depressive symptoms over time and whether it changes as AD progresses. However, the main issue is that NA signalling seems to be the primary disruption linked to depressive symptoms and LC integrity does not measure that directly, although they are related (Cassidy et al., 2022). This could also include a measure of NA in the CSF but this would become a highly invasive strategy.

Overall, seeing how NA signalling changes in AD and whether that causes depression is a good question and, if answered, could lead to better targeted treatment options. Studying it has shown to be incredibly difficult as post-mortem measures can offer up a lot of noise, imaging methodology is unable to measure NA production directly and animal work can only examine depressive-like symptoms. However, each method can offer up a difference perspective of evidence toward whether NA signalling disruption relates to depression in AD making a combination of tests the best approach. The LC-NA system is complex but there is strong evidence that suggests it plays a role in the aetiology of depression in AD. While this chapter did not find supportive evidence of that, its shortcomings suggest the evidence to be inconclusive and therefore requires future study. Together, this highlights the difficulties involved in studying the link between depression and AD but, doing so, will be able to uncover better targets for treatment.

●Chapter 5: General Discussion●

●5.1 Thesis overview

This thesis set out to investigate whether amyloid plays a role in the development of NPS seen in AD. This link was hypothesised to be mediated by damage to the Locus coeruleus, an area linked to depression and anxiety that is affected early on by AD pathology. Chapter 2 reported the use of a KI mouse model of amyloid pathology to assess what age dependent affective deficits they exhibited. Various protein markers related to inflammation, neurotrophic support, LC and amyloid were then measured in their brain tissue (Chapter 3). Chapter 4 took a closer look at the LC by inhibiting noradrenergic signalling in WT mice with DSP4 injection and examining NA production differences between depressed, non-depressed, AD and non-AD post-mortem human brain tissue.

●5.2 Summary of findings

The thesis question: Does amyloid affect the LC and does this link to the presence of NPS, was addressed through three experiments. The first was a large scale behavioural and biochemical examination of a KI mouse model, *App*$^{NL\text{-}G\text{-}F}$, to see what impact amyloid could have independent of tau. The second was an assessment of DSP4 injected mice and the third was examination of TH in post mortem human brain tissue. Findings are summarised in table 5.1.

Topic	Measure	Result	What it means
Behaviour (chapter 2)	Anxiety (EPM)	*App*$^{NL\text{-}G\text{-}F}$ females spent more time in the open arms compared to all other groups. This occurred at both time points	*App*$^{NL\text{-}G\text{-}F}$ females showed reduced anxiety compared to WT unlike males. There is no age related deficit
	General anxiety (OF)	Young *App*$^{NL\text{-}G\text{-}F}$ mice spent more time in the inner zone which disappeared by the old time point.	*App*$^{NL\text{-}G\text{-}F}$ mice showed reduced anxiety at the young time point but not the old.

	Social interaction and recognition memory (SPT)	Phase 1: *App*$^{NL-G-F}$ showed a trend for reduced preference for the stranger mouse (not significant). Phase 2: No differences between groups	Phase 1: Suggests *App*$^{NL-G-F}$ mice have reduced sociability Phase 2: Suggests intact social recognition memory
	Anhedonia & apathy (Lick cluster)	*App*$^{NL-G-F}$ mice showed similar average lick cluster but had reduced consumption	This suggests *App*$^{NL-G-F}$ mice have intact hedonic response but may present with apathy.
	Recognition memory (NOR)	All groups were able to discriminate the novel and familiar object	*App*$^{NL-G-F}$ have intact recognition memory
Biochemistry (chapter 3)	Inflammation	Western blot data revealed an increase in astrocytes and monocytes. Cytokine data revealed an increase in MIP-1α and MIP-1β after Bonferroni corrections. There was also an increase in *App*$^{NL-G-F}$ for IFNγ, IL-9, IL-12p70 which did not survive corrections.	Amyloid increases astrocytes and activated monocytes as well as MIP-1α and MIP-1β and possible IFNγ, IL-9 and IL-12p70
	Locus Coeruleus	Females had higher cell count compared to males but no differences in genotype or age	Suggests amyloid does not lead to substantial neurodegeneration of the LC at either time point
	Neurotrophic support	No changes for BDNF signal but proBDNF and TrkB were increased in *App*$^{NL-G-F}$ mouse cortex	Suggests amyloid causes increases to BDNF sensitivity through more TrkB receptors as well as blocks cleavage of proBDNF
LC-NA disruption &	DSP4	DSP4 injection lead to subtle trends for increased entries into the open arms	Potential disruption to LC signalling could lead to subtle trends for

depression (chapter 4)		(EPM) and reduced consumption of sucrose (not significant)	increased anxiety and anhedonia or apathy.
	Human TH	No significant changes between depressed-AD, non-depressed AD, depressed-HC, non-depressed-HC	Limitations may have caused this non-significant result e.g. placement in the LC of the brain slice, length of time spent incubating in DAB

Table 5.1 Summary of findings across thesis with key differences and non-differences highlighted.

●5.3 Examination of the *App*$^{NL-G-F}$ model

As stated above, the behavioural assessment concluded that *App*$^{NL-G-F}$ mouse exhibited significant reductions to anxiety and apathetic phenotypes. They did not exhibit social deficits, recognition memory issues or anhedonia. The biochemical assessment concluded the amyloid manipulation worked as intended and that it caused increased gliosis, altered neurotrophic signalling but no changes to LC cell count. Some of these findings are in opposition to what is seen in human AD so comparisons will be drawn and explanations offered for discrepancies. It is important to note that the older time point was chosen to be aged 12-14 months due to previous research suggesting this age was when amyloid pathology peaked. However, behavioural deficits could still occur after this time point.

●5.3.1 Amyloid and general behaviour

The main purpose behind examining amyloid was to confirm whether the *App*$^{NL-G-F}$ mice exhibited humanised Aβ which they did. No correlations between actual amyloid levels and other measures could be done due to needing a much larger sample size to be meaningful, however, possible trends will be examined. As amyloid is the main driver of pathology in the *App*$^{NL-G-F}$ mice, it's age-related effects will be looked at in relation to behaviour.

Insoluble $A\beta_{40}$ and $A\beta_{42}$ both decreased with age but only in males. Due to the lack of sex effects across all the behaviour and biochemical results, it could highlight how insoluble amyloid may not be as impactful on NPS as soluble amyloid. This is difficult to confirm without direct correlational analysis as other factors could impact insoluble plaque deposition. Whilst this goes

against previous research in humans that does find a strong link between plaque load and depression (Meynen et al., 2010; Rapp et al., 2006), this research only concludes a correlation rather than which effect causes which. It is perfectly possible that depression caused neural changes, such as reducing effectiveness of noradrenergic signalling, that lead to reduced clearance of insoluble Aβ. There is also evidence that suggests plaques have little impact on AD pathology as even cognitively normal people have been found to have plaques in their brain (Mormino & Papp, 2018; Sperling et al., 2011). This tells us very little about whether it affects NPS but does seem to support the idea that soluble amyloid oligomers may be more detrimental to AD pathology than insoluble plaques.

Looking at soluble $A\beta_{40}$ and $A\beta_{42}$ in the *App*$^{NL\text{-}G\text{-}F}$ mice showed only $A\beta_{40}$ increases with age. The lack of age effects in the behaviour could highlight that they are more impacted by the stable $A\beta_{42}$ levels. However, $A\beta_{42}$ does have a non-significant increase with age trend. Looking at absolute amyloid levels ignores its potential knock-on effects or how non-significant changes to amyloid could have significant changes to other processes. Furthermore, the absolute $A\beta_{42}$ levels were much higher compared to $A\beta_{40}$ due to the Beyreuther/Iberian mutation in the *App*$^{NL\text{-}G\text{-}F}$ mice so directly comparing the two oligomers may be misleading. Previous work found that injected Aβ oligomers (both $A\beta_{40}$ and $A\beta_{42}$ lengths) into WT mice increased depressive symptoms as measured by forced swim test, tail suspension test and sucrose preference test (Ledo et al., 2013). A few years later, the researchers concluded that it was the oligomers effect on microglia and other inflammatory processes that impacted serotonin levels and thus, lead to depressive symptoms (Ledo et al., 2016). As this thesis also found decreased sucrose consumption and increased inflammation, this offers a possible mechanism for how soluble oligomers in the *App*$^{NL\text{-}G\text{-}F}$ mice lead onto decreased sucrose consumption. Future work could examine this mechanism in more detail by measuring serotonin levels and whether altering them pharmacologically would impact depressive symptoms in amyloid model mice.

Overall, I cannot say for sure whether the absolute levels of amyloid impact behaviour but it can be concluded that the presence of humanised amyloid does cause various behavioural and biochemical changes in the *App*$^{NL\text{-}G\text{-}F}$ mice. These biochemical changes will now be examined as knock-on effects of amyloid pathology that could explain both the behavioural phenotypes exhibited and not present in the *App*$^{NL\text{-}G\text{-}F}$ mice.

●5.3.2 Amyloid, apathy and depression

Depression is a common symptom in human AD (Lyketsos et al., 2002) and was investigated using lick cluster analysis. LCA measured anhedonia that is a prominent symptom of depression but found no such reduction in liking a pleasurable substance. However, depression is a multifaceted and complex condition made up of many symptoms and not just anhedonia. It also includes lack of self-care and sadness, the latter of which is difficult to measure in a mouse objectively. The former could be measured by examining grooming levels and has been done in the past using the splash test or nest construction (Planchez et al., 2019). Measuring a wider range of symptoms would offer a better general picture of depressive-like behaviour in a mouse but as anhedonia is a core symptom, this does suggest amyloid pathology may not be the root cause of depression in AD.

The biochemical examination of the *App*$^{NL\text{-}G\text{-}F}$ mice also supports this as two possible mechanisms for causing depression related to neurotrophic support and the LC also showed no differences. There were no changes in mature BDNF levels in the brains of *App*$^{NL\text{-}G\text{-}F}$ mice and often lower levels of BDNF are associated with depression (Lee & Kim, 2010). There was also no neurodegeneration of the LC, however, I cannot rule out possible noradrenergic signalling changes in the LC that are linked to the development of depression (see section 1.3.2). As previously stated, future research should use electrophysiological recordings to examine LC activity as higher activity is associated with depressive phenotypes (Borodovitsyna et al., 2018). Levels of NA, it's receptors and transporters (NET) would also offer a larger picture of noradrenergic signalling health and whether increased signalling does in fact lead to depression.

However, the final biochemical marker, inflammation, has previously been shown to be related to depression through both human and murine research. Experimentally increasing inflammation has been shown to also increase depressive symptoms in humans (Miller et al., 2009; Musselman et al., 2001) and mice (Kubera et al., 2013; Vichaya et al., 2019). This thesis found massive increase in inflammation in the *App*$^{NL\text{-}G\text{-}F}$ mice that would be expected to increase depressive symptoms. However, taking a closer look at the mouse work that supports this claim may suggest inflammation only relates to motivational deficits in depression and not the whole condition. Kubera et al. (2013) interpreted decreased sucrose consumption as suggesting anhedonia but did not differentiate between motivational and hedonic deficits being the cause of the change in sucrose consumption. So, this mirrors the present findings of reduced sucrose consumption. Vichaya et al. (2019) examined a variety of aspects of depression after an injection of LPS to induce inflammation. They found inflammation led to motivational deficits as measured by reward expectancy, effort allocation and break point analysis as well as decreased consumption of sucrose. So again, the

mouse work only shows inflammation leads to deficits of motivation that are found in depression. Animal work that used amyloid pathology to induce inflammation also supports these claims. Mice injected with Aβ oligomers were found to exhibit more depressive-like symptoms through the TST, FST and sucrose preference test (Ledo et al., 2013). Whilst this work included the TST and FST that are more a measure of propensity to give up, they can be improved with anti-depressant medications (Slattery & Cryan, 2012). The researchers did also find decreased sucrose consumption as the present research did but they were, again, unable to differentiate between whether this is a motivational deficit or anhedonia. Previous work with transgenic mice was able to show the transgenic amyloid model, Tg2576 mice, did exhibit anhedonic phenotype using lick cluster analysis (Brelsford et al., 2017). To add to this, unpublished work in our lab ran lick cluster analysis on a cohort of *App*$^{NL-F}$ mice but found no change in licking or sucrose consumption. Combined with the lack of anhedonia found in the *App*$^{NL-G-F}$ mice, this suggests an artefact of the transgenic model other than amyloid pathology was the cause for anhedonia in the Tg2576 mouse. A similar story is seen in humans. Experimentally increasing inflammation led to more 'sickness behaviour' that relates to the motivational deficits of depression rather than dysphoria or suicide ideation (Miller et al., 2009; Musselman et al., 2001). Overall, evidence suggests that inflammation links to only the motivational deficits seen in depression.

The present research did find motivational deficits in the LCA which I concluded to be apathy. The above evidence suggests that increased inflammation could be the cause and recent experimental research also supports this. Ledo et al. (2016) found serotonin and microglial activation both lead onto the development of depressive symptoms in mice injected with Aβ oligomers. Their conclusion of depressive symptoms was based on reduced consumption of sucrose so would be better classified as reduced motivational deficits. They also found increased immobility in the FST but whether that measures depression was discussed in section 2.2.3. Furthermore, they also injected mutant mice with impaired toll like receptor 4 receptors (TLR4), an important receptor in microglial activation. Doing so stopped any increase in motivational deficits suggesting they are mediated by inflammation. They also found the TLR4-/- mice injected with Aβ oligomers did not show decreased serotonin unlike TLR+/+ and vehicle injected mice. Together, the research showed that soluble amyloid oligomers were able to induce inflammation that also decreased serotonin and lead to motivational deficits. This is a plausible explanation for what is occurring in the *App*$^{NL-G-F}$ mice as, although the present work did not measure serotonin levels, past work has shown 5-HT to be decreased in these KI mice (Wang et al., 2018). Furthermore, I was able to show increased microglial activation and motivational deficits in the *App*$^{NL-G-F}$ mice mimicking the above mechanism. This offers

an explanation for the presence of apathy in human AD that is caused by amyloid induced inflammation and serotonin signalling alterations.

Apathy and depression are distinct disorders (Landes et al., 2001). Apathy is defined through its motivational deficits while depression also includes dysphoria and suicide ideation. The above offers a plausible explanation for amyloid causing apathy in AD but the lack of dysphoria suggests depression is the result of other pathologies seen in AD. One example would be tauopathy and while there is evidence that shows tau pathology links to depression more so than amyloid does (Babulal et al., 2020) this correlation offers no mechanistic explanation. As very little examination on dysphoria in AD has been done, it is difficult to firmly conclude if tau is the cause or it's interaction with amyloid. One explanation could be tau's early effects on reducing LC signalling efficiency lead on to dysphoria and depression (Braak & Del Tredici, 2011). It is also possible that the dysphoria is linked to human psychology and feelings of sadness are linked to the person's perception of their own condition. However, research shows that depression appears prior to cognitive decline so there is likely a biochemical cause but uncovering it would require more mechanistic explanation of tau and/or other AD pathologies (e.g. oxidative stress, altered glucose metabolism etc.).

Altogether, it seems that amyloid can induce motivational deficits through inflammation, this is not sufficient to also include dysphoric symptoms seen in depression. Dysphoria should be given more focus in the future to examine what pathologies in AD could cause it. Treatments for apathy could possibly benefit from suppressing inflammation or early amyloid suppression but these will be focused on in more detail in section 5.5.3.

●5.3.3 Amyloid and anxiety

The present research found amyloid caused reductions in anxiety in the *App*$^{NL\text{-}G\text{-}F}$ mouse model. However, increased anxiety is what is often seen in human AD (Lyketsos et al., 2011) so the logical question is, why did this study find the opposite effect.

There is a large plethora of research that supports a link between amyloid pathology and anxiety in AD. Various PET scans have found such a link in humans (Donovan et al., 2018; Johansson et al., 2020; Pietrzak et al., 2014) but these are simply correlational and could merely represent both anxiety and increased amyloid pathology have a common denominator. If we look to the mechanistic cause, research highlights early tangle pathology in the entorhinal cortex as a risk factor for future anxious development (Ehrenberg et al., 2018). The entorhinal cortex is responsible for decreased sensorimotor gating (filtering out irrelevant stimuli) meaning lesser stimuli that would be

ignored are now focused on causing increased anxiety. Evidence for this has been shown in rats with entorhinal cortex lesions that could not inhibit their startle response to softer noises in prepulse inhibition tests (Goto et al., 2002, 2004). The entorhinal cortex is one of the earliest brain areas to exhibit AD related pathology in the form of tau tangles that could explain the lack of an anxiety phenotype found in an amyloid model. Furthermore, increased anxiety could be caused by the psychological factor of humans becoming aware of their own cognitive decline (Mendez, 2021). This is difficult if not impossible to model in mice due to requiring a perception of their own loss of function that cannot be confirmed to occur in rodents. In addition, the present study found no cognitive decline in the *App*$^{NL-G-F}$ mice, but it seems safe to conclude that modelling the psychological aspect of AD should be primarily examined in humans.

The present study did find an anxiolytic or disinhibited phenotype that could be explained by amyloid pathology. Disinhibition is itself an NPS but this is often seen in humans through social disinhibition and making inappropriate comments (Keszycki et al., 2019). In mice, disinhibition is seen as reduced anxiety phenotype accompanied by increased movement that the present study did not find but did show that *App*$^{NL-G-F}$ mice examine risk a lot less. This mirrored what previous research showed using Tg2576 who had reduced stretching and concluded the mice exhibit disinhibition (Ognibene et al., 2005). I previously concluded that reduced anxiety and disinhibition have a large overlap and attempting to discern them may not be feasible. Furthermore, little work on disinhibition in AD has been done (Keszycki et al., 2019) so understanding the mechanism that could cause it is difficult. Some research found disinhibition could be successfully treated with SSRIs in non-AD forms of dementia (Herrmann et al., 2012; Swartz et al., 1997). This could highlight amyloid impacting serotonergic signalling which was previously found in the *App*$^{NL-G-F}$ mice. Wang et al. (2018) found reductions in 5-HT levels in the CA1 region of the hippocampus of *App*$^{NL-G-F}$ mice. Although the present study did not look at 5-HT levels directly, future work could examine whether SSRI treatment would improve the disinhibition/reduced anxiety seen here. Other signalling disruption could also occur in the cholinergic systems which is heavily linked to arousal. What seems clear is that there is no concrete idea around how disinhibition appears in these rodents but it is a very underexamined NPS. It is likely that amyloid causes some disruption to cholinergic or serotonergic signalling which leads to reduced anxiety/disinhibition. Other pathologies, such as tau, may override this reduced anxiety by effects on the entorhinal cortex. Overall, tau pathology should be the key focus of future work looking at anxiety in AD. Amyloid should be focused on is respect to disinhibition.

●5.3.4 Neurotrophic support and cognition

AD is commonly characterised by cognitive decline that was examined through recognition memory deficits in the *App*$^{NL-G-F}$ mice. The present study found no such deficits in the older cohort where previous work with these animals had found memory issues (Auta et al., 2022; Locci et al., 2021; Mehla et al., 2019). I previously concluded that this finding may not be robust or simply represent a subtle cognitive decline that would be accurate to the early AD pathology *App*$^{NL-G-F}$ mice represent. To combine it with the biochemical work, examination of neurotrophic support could also offer a mechanistic explanation to a lack of cognitive deficits.

BDNF has a key role in synaptic repair that helps stop neurodegeneration and cognitive decline (Tanila, 2017). The present study found no loss of BDNF in the hippocampus or cortex suggesting damage to synapses could be repaired without issue. This could be helped by no LC neurodegeneration as LC cells are a common source of BDNF to the areas it innervates to (Mufson et al., 1999). The LC also releases PPAR-γ that works to protect neurons (Klotz et al., 2003) and has been shown to increase BDNF in amyloid injected rats (Prakash & Kumar, 2014). The retroactive transport that leads to BDNF returning to aid the LC synapses could also help explain why the LC suffered no significant cell loss (Ishida et al., 2000). So, healthy levels of BDNF could both be responsible for cortex, hippocampus and LC synapse health as well as lack of cognitive deficits.

BDNF receptor, TrkB, was found to be significantly increased in the cortex of *App*$^{NL-G-F}$ mice. Increased TrkB would enhance the cortex's sensitivity to BDNF which is the opposite of what occurs in human AD (Ferrer et al., 1999). The reason for this could be that neurodegeneration reduced the number of cells that can actually present TrkB which does not occur in *App*$^{NL-G-F}$ mice. Decreasing TrkB in 5xFAD amyloid mice greatly increased memory deficits (Devi & Ohno, 2015) and various drug trials have found success in targeting BDNF:TrkB signalling to treat cognitive decline (Rolfo et al., 2015). Together, this could help explain why the present study found no recognition memory problems in the *App*$^{NL-G-F}$ mice and future work could examine experimental reductions of BDNF or TrkB to see if they could lead to cognitive issues. The present study did only successfully examine two types of memory (recognition and social) but spatial and temporal memory are also commonly affected in AD. Cognition was not the focus of this thesis question though and is commonly examined in all mouse models of AD pathologies.

ProBDNF is the precursor to BDNF and was also found to be increased in the cortex of *App*$^{NL-G-F}$ mice. Increased proBDNF is often linked to higher apoptosis which is possible as we did not measure cell loss in the cortex. The reason for the increase could be compensatory mechanisms to improve synapse health with the idea of cleaving it to mature BDNF. However, past work has already

shown that amyloid pathology interferes with the cleavage which could explain the elevated levels. In any case, the normal BDNF:TrkB levels likely helped maintain cognition and the increase proBDNF could have some negative effects that would need to be examined in the cortex.

●5.3.5 *App*$^{NL-G-F}$ behavioural conclusions

Overall, the behavioural phenotypes found to be caused by amyloid pathology were apathy and reduced anxiety. The significant NPS not seen were anxiety, social deficits, recognition memory deficits, social memory deficits or depression. It is logical to conclude that all the symptoms found in the *App*$^{NL-G-F}$ mice are caused by direct or indirect knock-on effects of amyloid pathology. Along that same idea, the NPS not present are likely caused by other AD related pathologies such as tau and tauopathy cascades. For example, amyloid leads to increased inflammation that is the cause of motivational deficits like apathy. It seems tau causes early disruption to the entorhinal cortex and LC that could lead on to increased anxiety and depression respectively. Furthermore, evidence suggests amyloid is not the primary cause of neurodegeneration, unlike tau, that likely leads on to reduced BDNF:TrkB signalling and cognitive deficits. The *App*$^{NL-G-F}$ mouse appears to offer a good model of amyloid pathology independent of tauopathy and the research here could help better target treatments. Future work could examine all these concepts in more detail to see if they are the main causes of NPS.

●5.4 Is the *App*$^{NL-G-F}$ mouse model good?

I have just discussed how amyloid could lead to the biochemical and behavioural effects found in this thesis but this was based on the premise that the *App*$^{NL-G-F}$ mouse is a good model of amyloid pathology. This section will evaluate this claim to see if the *App*$^{NL-G-F}$ mouse is indeed a sensible model for examining amyloid related problems.

Firstly, compared to older amyloid models, the *App*$^{NL-G-F}$ KI models are a huge step forward. This has previously been outlined in detail in section 1.5.2 but, briefly, transgenic models struggle to create a clear model of mechanistic investigation due to various biological confounding factors. This included *APP* overexpression, unphysiologically high levels of amyloid, missing areas of genome and inconsistency across different models. The *App*$^{NL-G-F}$ mice do not struggle with these biological confounds making them a more accurate model to the human condition. However, *App*$^{NL-G-F}$ mice

still show some inconsistencies in findings suggesting that the model is susceptible to environmental factors.

●5.4.1 *App*$^{NL-G-F}$ mice as a representative of the human condition

To produce the amyloid pathology seen in *App*$^{NL-G-F}$ mice, 3 mutations from familial AD (FAD) were compiled to create increased amyloid production and aggregation seen in human AD. However, in humans, 1 mutation is enough to not only cause the amyloid pathology but tauopathies as well. These mice require 3 mutations to model just the amyloid pathology which highlights the interspecies issue. Furthermore, no human has ever had all 3 mutations found in the *App*$^{NL-G-F}$ mice and the interactions between them could have unknown and unphysiological consequences not seen in humans. This should always be kept in mind when drawing conclusions between the *App*$^{NL-G-F}$ and human AD.

While amyloid pathology is easily modelled in mice using FAD genetic mutations, this means the models may have limited relevance to sporadic AD (SAD). SAD is caused by a combination of environmental and genetic factors over a long period of time while FAD is genetically determined. Despite differences in pathogenesis, research shows the amyloid pathology remains similar across both familial and sporadic AD (Lantos et al., 1992; Lista et al., 2015). So, while mouse models based on FAD mutations (i.e., *App*$^{NL-G-F}$ mouse) can be generalised to SAD, care should be taken especially when examining the development of amyloid pathology. The present thesis did not do this as its primary focus was simply on what the presence of amyloid did. Nevertheless, differences in the pathogenesis of amyloid pathology could have differing knock-on effects that are currently unknown so care should be taken when generalising to all AD cases. An important consideration is FAD amyloid build up is created by increased anabolism of Aβ peptides while SAD is determined by decreased catabolism of Aβ (Trujillo-Estrada et al., 2022). Taken together, the *App*$^{NL-G-F}$ mouse is a reasonable model but care should be taken if generalising to SAD especially when examining pathogenesis mechanisms.

The *App*$^{NL-G-F}$ mouse produces amyloid pathology incredibly quickly unlike the similar model, *App*$^{NL-F}$. Practically, this is good for scientists and likely why *App*$^{NL-G-F}$ mouse is the more commonly used model of the two (Saido et al., 2022). However, the arctic mutation leads to Aβ with a higher affinity to aggregate making it near resistant to degradation. It makes the model unsuitable for research investigating metabolism, clearance or deposition of Aβ including immunotherapies (Saido et al., 2022). While the present thesis did not look at any of these factors, it is possible that it had

indirect effects on how amyloid pathology was presented. For example, resistant Aβ peptides could lead to alterations in how immune markers respond as they are unable to clear the aberrant protein away. Care should be taken when extrapolating the findings from *App*$^{NL-G-F}$ mice to humans.

AD is a progressive disorder so to successfully model it also requires progressive deficits. This progression was examined through age effects throughout this thesis but often found very little or subtle age effects. While this could suggest the *App*$^{NL-G-F}$ mice are not a good model of AD progression, it more likely highlights how fast the amyloid pathology develops. Our early time point still showed a lot of deficits indicating the need to look at the mice younger than 6 months to examine progression. Previous work looking at the earlier time points of the *App*$^{NL-G-F}$ mice does find that these pathologies do develop. For example, 3 month old *App*$^{NL-G-F}$ mice did not exhibit increased astrogliosis while they did at 6 months (Mehla et al., 2019). Therefore, the lack of age effects found in this thesis are not a mark against the *App*$^{NL-G-F}$ mice as a model but rather the time points chosen for this research.

Criteria originally set out by Willner (1986) suggest a good model must have good face, predictive and construct validity. Face validity implies a model should express a phenotype similar to human pathology which the *App*$^{NL-G-F}$ mice have managed by using humanised amyloid expressed using murine endogenous promoters. This model does not express tau pathology or neurodegeneration like in human AD but that is beyond the model's scope as it only models amyloid pathology. Predictive validity refers to how well the results from the tests can translate to human disease. It is difficult to firmly confirm whether the *App*$^{NL-G-F}$ mouse does this as it is still a relatively new model but predictions will be made later on in this thesis that can be tested. Construct validity states that the biological mechanisms should be the same in both model and humans. The *App*$^{NL-G-F}$ mouse models the genesis of FAD well but the heavy genetic component makes translating this to SAD quite difficult. So, the *App*$^{NL-G-F}$ mouse is a good model of amyloid pathology in FAD but care should be taken if generalising this to all SAD cases. Recent reviews have called for a need for better models of SAD (Trujillo-Estrada et al., 2022).

●5.5 The effects of amyloid pathology

This section will now use the evidence presented in this thesis to examine what amyloid pathology is doing in the brain. The main focus will be on the development of NPS, the LC, inflammation and whether future treatments should continue to focus on reducing amyloid pathology.

●5.5.1 Amyloid's role in the development of NPS

Previously, it had been established that the *App*$^{NL-G-F}$ mice exhibit reduced anxiety/disinhibition and apathy as well as concluding they are a good model of amyloid pathology. Furthermore, it was suggested that apathy is likely caused by increased inflammation leading to motivational deficits and reduced anxiety by changes to serotonergic and cholinergic signalling. Therefore, it is logical to conclude that the presence of amyloid pathology seen in human AD leads on to increased inflammation and interference of serotonergic and cholinergic signalling. This is not to say these effects are exclusive to amyloid pathology but they are partially caused by the build up of Aβ. Furthermore, amyloid alone does not lead onto an anxiogenic phenotype or depression so these are likely caused by other factors such as tau. Research has shown that amyloid pathology can exacerbate tau pathology (Götz et al., 2001; Oddo et al., 2004) which could still make Aβ the initiating step in anxiety and depression in AD. However, the main take away is that amyloid pathology alone is not sufficient to create these integral NPS seen in AD. The presence of environmental stressors, genetics or other biological underpinnings could also interact with amyloid pathology to exacerbate it as research has shown with LPS injections (Sheng et al., 2003), stress (Jeong et al., 2006) and DSP4 (Jardanhazi-Kurutz et al., 2010) all exacerbating Aβ deposition. The present research did not set out to look at any of these interactions as I wanted to answer the basic question of what amyloid was doing outside of other effects.

Amyloid does not lead to neurodegeneration of the LC. It has already been established that the primary cause of neurodegeneration in human AD is tauopathy (Iqbal et al., 2005) but it is still possible that amyloid leads to some amount of cell death. However, the present research found no cell loss in the LC but that was the only area of the brain examined. Other research has also concluded that amyloid does not cause substantial neuron loss (Ballatore et al., 2007) which is thought to cause a lot of the symptoms seen in AD. However, deficits begin prior to cell loss so it is possible that amyloid is impacting signalling in some other way. This is shown also by past research with the *App*$^{NL-G-F}$ mice showing disruption to signalling without cell loss to noradrenaline system (Sakakibara et al., 2021), serotonin system (Wang et al., 2018), dopamine system (Hamaguchi et al., 2019) and cholinergic system (Mehla et al., 2019).

Amyloid leads to increased inflammation. This includes increased activation of monocytes and astroglia but not with certain cytokines. The cytokines, such as IL-6, TNF-α and IL-1β, are messengers that are released from various immune and neuronal cells to further exacerbate the immune response. The present research found these were not increased by amyloid but other

research using the *App*$^{NL-G-F}$ mice disagreed (Manocha et al., 2019; Uruno et al., 2020). The discrepancy could highlight differences in measurement techniques or that it is not a robust finding unlike increased gliosis. A few PIC were elevated in *App*$^{NL-G-F}$ mice, MIP-1α and MIP-1β, that are more important in the initial activation of immune cells. Together, this suggests that amyloid does cause increased activation of immune cells but not subsequent exacerbation. The increase in inflammation is an attempt to clear away the excess amyloid which is hypothesised to be reduced in AD. I cannot say whether amyloid impacts this clearance as the arctic mutation in the *App*$^{NL-G-F}$ mouse makes catabolism of amyloid difficult. Future research should examine amyloid's impact on the clearance mechanisms including gliosis through their ability to migrate and phagocytose but in the *App*$^{NL-F}$ mouse that does not harbour the arctic mutation. Damage to the LC caused disruption in microglial ability to phagocytose and migrate (Heneka, Nadrigny, et al., 2010) that could be impacted by amyloid (Sakakibara et al., 2021), tau or both. Overall, we can conclude that amyloid caused increased inflammation but cannot say anything on its effectiveness at amyloid catabolism. This is important for understanding the process of amyloid build up which goes beyond this thesis.

●5.5.2 Amyloid, LC and depression

The effect of amyloid on the LC and subsequent depression was examined in multiple ways in this thesis. Firstly, LC cell number and anhedonia was measured in the *App*$^{NL-G-F}$ mouse. Secondly, human LC neurons were measured for TH density but this was not limited to just amyloid pathology. Finally, DSP4 was injected into WT mice without amyloid pathology to see the effects of noradrenergic signalling disruption on anxiety and motivation.

It has previously been established that amyloid does not cause LC cell loss but it's changes to noradrenergic signalling that better relate to depression. This was not found in the *App*$^{NL-G-F}$ experiment due to not seeing anhedonia and being unable to confirm any disruption to noradrenergic signalling. When NA signalling was disrupted using DSP4 injected into WT mice, they exhibited subtle trends that suggested anhedonia and increased anxiety but were ultimately non-significant. Conclusions from that work suggested the DSP4 treatment should be prolonged to better mimic the gradual progression of depression (Szot et al., 2016). The subtle trends do support the fact noradrenaline plays a role in the development of depressive symptoms. It is unfortunate the lick cluster analysis was not completed in these mice as it would be able to discern whether changes to sucrose consumption were due to a motivational deficit or anhedonia. Future work should utilise this test or include some measure of liking with sucrose consumption to be able to make this distinction in animal work.

The logical next step on from this work was to lesion the LC in the *App*$^{NL-G-F}$ mice and measure potential changes to anxiety and depressive symptoms. Originally, this was the planned final experiment to this thesis but COVID related disruption led to refocusing the experimental work. For this reason, the LC lesion work was left for future directions and so the planned experiments will be outlined here. For a surgical legion, *App*$^{NL-G-F}$ and WT mice should be aged to 6 months before undergoing surgery to partially lesion one or both LC. After their recovery, examination of their depressive and anxious symptoms should commence including tests such as EPM, OF and lick cluster analysis. Sucrose preference test, FST and TST could also be used but the stress induced by the latter two suggests they are not ideal tests of depression. As little difference between 6 and 12 months was found in the current studies, it is likely not needed to do an age comparison here as the development of NPS already peaked around 6 months old. The partial lesion is also important as previous work has shown depressive symptoms develop with smaller lesions due to needing higher amounts of surviving neurons (Szot et al., 2016). The second experiment would utilise DSP4 injections throughout the *App*$^{NL-G-F}$ mice lifetime. *App*$^{NL-G-F}$ and WT mice would be injected monthly with DSP4 until 6 months of age at least to mimic a prolonged disruption to NA signalling seen in depression. A similar battery of tests would be run to measure depressive and anxiety symptoms. Both of these experiments would better show how amyloid and LC disruption interact to develop NPS. So, while the present study can conclude amyloid pathology is not sufficient, the inclusion of LC lesion could show the role the LC plays in the development of some NPS in AD. The DSP4 treatment would mimic early tau disruption to LC signalling while the 6OHDA lesion would model the later stages after tau caused LC cell death.

The human work examined NA production differences between depressed, non-depression, AD and non-AD patients. While it was hypothesised that the depressed and AD groups would exhibit higher TH due to an overactive LC, no significant differences were found. This evidence is not sufficient to discount the idea that noradrenergic signalling is disrupted in AD and depression as the section of the LC was not controlled for. Evidence has found differences in TH in various parts of the LC making the placement of the brain slice a confounding factor (Zhu et al., 1999). Overall, this evidence is not conclusive and future work should examine this but control for where in the LC is measured.

With everything looked at in this thesis, it seems amyloid is not sufficient to have enough impact on LC signalling to cause depression in AD. However, this link has not yet been sufficiently examined as I cannot confirm what signalling disruption amyloid causes. Overall, tau is known for causing disruption to LC signalling in AD so may be a better candidate to examine as the cause for depression in AD.

●5.5.3 Amyloid: a good treatment target?

The amyloid cascade hypothesis, which has remained popular for decades, suggests that amyloid is the causing factor of AD. Despite a lot of research stating the contrary (for review see Herrup (2015)), amyloid reducing treatments have still been at the forefront of Alzheimer's research. The present investigation aimed to examine amyloid pathology's role in the development of NPS but has concluded that it leads to apathy and reduced anxiety. The question still remains of whether amyloid is a worthwhile target to prevent its direct or indirect effects such as these affective deficits.

While the logical conclusion may be to use amyloid treatments in AD patients to treat apathy or disinhibition, this is unlikely to be effective. Firstly, research found removing amyloid from a human brain did not impact the development of AD or cognitive decline (Holmes et al., 2008) that suggests Aβ build up has little impact on disease progression at later stages of AD. Said differently, the main impact of amyloid deposition is its indirect effects that should be prevented rather than cured. Recent drugs that reduce amyloid (e.g. aducanumab) have found success in clearing the brain and also some effect of slowing cognitive decline (Haddad et al., 2022). One clinical trial, EMERGE, looked at aducanumab's effects on NPS that did show an 87% decrease when given a high dose of the drug (Cummings et al., 2021). The study did not specify which NPS were improved but was also not replicated in a similar clinical trial entitled ENGAGE (Budd Haeberlein et al., 2022). To add to this, a very recent anti-amyloid drug, lecanemab, has successfully reduced clinical score of AD symptom severity but only by half a point (Biogen Inc., 2022). The results are statistically significant but that may not necessarily mean they are meaningfully significant (Mahase, 2022). This discrepancy could suggest aducanumab will affect only a subset of people but more research would need to be done investigating what that subset entails. It is more likely that reducing amyloid is not a robust and consistent treatment option for reducing NPS in current AD patients. From the present research, I have concluded that it is the knock-on effects of amyloid (e.g. inflammation) which create more disruption that direct amyloid effects. With this logic, it would be better to prevent these knock-on effects by early suppression of amyloid deposition. This idea is supported by a genetic variant in *APP* gene that lowers Aβ production by approximately 40% throughout life protects against later development of AD (Jonsson et al., 2012; Kero et al., 2013). Future research looking at amyloid treatments should focus on the effects of early and prolonged reductions of amyloid rather than in established AD and MCI cases where the knock-on effects have already occurred. Amyloid treatments should be combined with other medications that address the knock-on effects of amyloid to create a better treatment plan. For example, aducanumab could be combined with anti-

inflammatory treatments to help treat apathy. Aβ treatments alone should not be expected to have an all encompassing treatment effect on people already with full blown AD or MCI.

Altogether, the research seems to be suggesting that amyloid does have some detrimental effects such as apathy, increased inflammation and disinhibition. Amyloid is still a neurotoxic substance that does impact processes in the brain (Shankar et al., 2008; Varvel et al., 2008; Whalen et al., 2005) but it's full effect is likely not seen without tauopathy. Despite this, amyloid related treatments have not shown a lot of promise likely due to being administered after major Aβ deposition. This should not discount these treatments but should be seen as a preventative measure rather than a cure. Amyloid is not sufficient to cause some common NPS that suggests other pathologies are more detrimental contributors, mediators or accomplices. The logic derived from the amyloid cascade hypothesis states that prevention of amyloid pathology would also reduce tauopathy. Whether this hypothesis holds true is still hotly debated but, in any case, tau should be heavily focused on to see what role it plays in the development of AD and NPS. The reason for this is that a lot of hypothesised mechanisms for the development of NPS involve tauopathy (e.g. LC dysfunction). Seeing the independent role of amyloid has been vital in discerning what role it could play in AD progression but, in doing so, it seems a lot of issues in AD relate to other pathologies.

●5.6 Is Tau the way forward?

This chapter has repeatedly emphasised the importance of examining tauopathy. Whilst there are other pathologies (i.e., increased oxidative stress, inflammation; Gong and Iqbal (2008)) in AD that could be focused on, tau is a prominent one alongside amyloid. Furthermore, evidence has shown tau to be the primary cause of neurodegeneration and signalling dysruption as well as exacerbating amyloid pathology (Ballatore et al., 2007). It appears very early on in life even prior to amyloid pathology (Braak & Del Tredici, 2011) with its earliest signs appearing in the LC. I will outline the prominent reasons for why looking at tau could be very important in the development of NPS in AD and what future research should do.

●5.6.1 The tau-NPS link

Early tau pathology is known as pre-tangles (p-tau) and can disrupt the signalling of a neuron without leading to cell death. The LC develops early p-tau which was outlined in detail in section 1.3.3. Briefly, the p-tau makes the LC less sensitive to inputs (Andrés-Benito et al., 2017) that then

impaired noradrenergic signalling and loss of neuroprotection. As aberrant tau increases, this creates stress that leads to LC hyperactivity (Chalermpalanupap et al., 2017). The early signalling disruption and hyperactivity mirror LC signalling in depression that could be a potential cause for depressive symptoms early on in AD (Rosenberg et al., 2013). In addition, neuroprotective mechanisms are greatly reduced that leave the whole brain vulnerable to damage and aberrant protein deposition. While this thesis only briefly touched on the neuroprotective mechanisms, it did show that amyloid was not sufficient to reduce neurotrophic support on synapses. This work has also shown that amyloid creates increased inflammation but has not examined whether it also reduced microglial phagocytic capabilities which also occur as a result of LC damage. The other neuroprotective mechanisms (outlined in fig 1.2) also highlight excitotoxicity, oxidative stress and other neurotrophins such as neuropeptide Y, galanin and somatostatin. While amyloid oligomers from the bloodstream could impact these processes in the early stages of AD, tau may be a better bet to look at for the reasons stated above. Future research could look at the extent that tau (or even amyloid) pathology reduces the neuroprotective effects from LC-NA signalling as this could be a causal factor for neuropathologies developing in AD. This is also why noradrenergic treatments should be given early as maintaining this level of neuroprotection could prevent pathological build up.

The hyperactive LC would also lead to increased anxiety (see section 1.3.2). Alongside the LC, the entorhinal cortex is also one of the earliest brain areas to exhibit tau pathology. Both of these brain systems have been implicated in the development of anxiety which provides a solid basis for examining tauopathy in anxiogenic development in AD.

Overall, as tau is the more likely culprit of loss of NA-derived neuroprotection, damage to the different brain systems could be better attributed to tauopathy. This would include damage to cholinergic, serotonergic and noradrenergic systems that could cause a plethora of NPS. While amyloid should not be discounted, tauopathy has shown direct impact to the relevant neural networks that lead to NPS.

●5.6.2 Why was not tau the focus of this thesis

The above evidence was present in the creation of this project which creates the question of why focus on how amyloid relates to NPS was looked at rather than tau. The reasons for this were twofold: 1) research was showing amyloid was having an impact and 2) amyloid mouse model technology was far superior to models of tauopathy. For the first point, the present research still

concludes that amyloid plays a role in the development of some NPS as well as neuroinflammation. Examining amyloid's impact independent of tau would also be able to better discern what NPS were caused by what. With the results of this thesis, it can now be concluded that motivational deficits may be the result of amyloid-related inflammation but, at the same time, has also shown which NPS are not caused by amyloid (i.e., depression). In regards to the second point, tau models were still relying on transgenic technology which put into question how effective they would be at showing the effect of tau without confounding effects. This lead to wanting to characterise the possible phenotypes in these mice.

However, since the genesis of this project, a KI tau mouse model has been successfully produced called the MAPT KI (Saito et al., 2019). This mouse produced tau pathology using KI technology that reduces the confounding effects of the transgenic models (e.g. missing genomes, exogenous promoters). Future work should examine this mouse to see what impact tau has on the LC, NPS and neuroprotective mechanisms. Recent research has crossed the MAPT with the *App*$^{NL\text{-}G\text{-}F}$ mouse to produce the double KI model (Borcuk et al., 2022). This double KI can show how amyloid and tau interact. Borcuk et al. (2022) found that, when compared to each of the single KI models, tauopathy was exacerbated while amyloid pathology was not suggesting amyloid acts to exaggerate tauopathy when present but not vice versa. To the best of my knowledge, no work has looked at the impact to NPS which would be a good future direction.

Overall, tau is still a good target to examine but this does not negate the importance of examining amyloid's independent effects. New models are surfacing which seem to address key problems with past models that would make examining tauopathy much more accurate in the MAPT KI mouse. Seeing how amyloid and tauopathy interact is also a key component to the development of AD and can be done using the double KI model.

●5.7 Conclusions

The present thesis set out to examine the role amyloid plays in the development of NPS via its effects on the LC. Examination using an amyloid model, the *App*$^{NL\text{-}G\text{-}F}$ mouse, found apathy, disinhibition along with increased inflammation and altered neurotrophic signalling. Notably, recognition memory, social memory, social interaction and depressive deficits were not found to be caused by amyloid. LC cell loss seen in AD was not caused by Aβ. The link between the LC and depression was further examined with DSP4 injected WT mice who only showed subtle trends toward motivation deficits and anxiety. The human work examined changes to noradrenaline

production, but confounding effects made the results inconclusive. Altogether, this work has shown the impact of amyloid and offered possible explanations, but the important effects not found suggest a need to look at the other key pathology in AD, tau. Recent advances in tau modelling have presented a more accurate mouse model that could be used to answer the question of what role tau plays in the development of NPS via damage to the LC. Amyloid was important to examine but future work should primarily focus on tau and how it interacts with amyloid pathology to develop NPS.

●Chapter 6: References●

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