Investigating the biochemical signature and *in vivo* seeding activity of amyloid-β from distinct Alzheimer’s Disease subtypes

*A thesis submitted to UCL in partial fulfilment of the requirements for the degree of Doctor of Philosophy*

David Mark Xavier Thomas
Author’s Declaration

I, David Mark Xavier Thomas, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Acknowledgements

I would like to take this chance to thank the people without whom none of this work would have been possible.

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Abstract

Multimeric assemblies of amyloid-Aβ (Aβ) are thought to play a key role in the progression of Alzheimer’s Disease (AD), but these aggregates display substantial structural heterogeneity in the human brain and it is unknown which conformational variants contribute most significantly to disease, or via which causal pathways they do so. One mechanism through which these aggregates could contribute to cognitive impairment is via binding to specific receptors at the surface of neuronal and glial cells, thereby activating neurodegenerative signalling cascades. The first work reported here describes a comparative analysis of several putative receptors, which revealed that prion protein (PrP), leukocyte immunoglobulin-like receptor B2 (LilrB2), and fc-gamma receptor type IIb (FcγRIIb) all bound nanomolar concentrations of aggregated Aβ in cellular binding assays, and recombinantly expressed PrP and FcγRIIb also both bound low nanomolar concentrations of Aβ aggregates in plate-based and solution-based in vitro binding assays. However, comparative binding affinities differed substantially depending on experimental system. Recent evidence has also suggested that the presence of certain Aβ assemblies in the post-mortem AD brain may correlate with variations in AD clinical phenotype, suggesting a mechanism similar to that of prion disease wherein distinct prion strains are associated with different clinical and pathological characteristics. Here, brain extracts from typical slowly-progressive AD patients (Slow-AD) were biochemically compared with brain extracts from atypical ‘rapidly-progressive’ AD patients (Rapid-AD), as well as a subset of patients with substantial amyloid pathology but no cognitive impairment at the time of death (High-Amyloid controls). Extensive variability in Aβ biochemical signature was observed between patients, but this did not correlate with disease phenotype. To
investigate the potential role of ‘strains’ of assemblies of Aβ, typical AD and Rapid-AD brain extracts were intracerebrally inoculated in NL-F mice, with separate groups of mice culled at 4, 8 and 12 months post inoculation (mpi). Inoculation with either Slow-AD or Rapid-AD brain extracts led to accelerated and spatiotemporally distinct deposition of Aβ in these mice, which preferentially targeted the cerebellum. However, inoculation with Rapid-AD brain extracts induced less aggressive amyloid deposition than equivalent Slow-AD inoculations, which may suggest that Aβ assemblies present in Rapid-AD brains possess distinct \textit{in vivo} seeding activity which may have contributed to the atypical clinical presentation observed in these patients. These results warrant further study of the \textit{in vivo} seeding activity of Aβ assemblies in Slow-AD and Rapid-AD brains, which may support the targeting of unique therapeutics for distinct subtypes of AD.
Impact Statement

Alzheimer's Disease (AD) is one of the largest social and economic challenges facing modern society, and treatments are desperately needed. Despite this, many pharmaceutical companies are beginning to withdraw funding from AD and other neurodegenerative disease, due in part to the wide gaps in our understanding of the core mechanistic processes underlying these diseases. This project aimed to improve our understanding of some of the key mechanisms underlying AD pathogenesis, ultimately helping contribute to the search for treatments. In particular, this project focused on the fact that while AD is often said to be caused by “aggregates of amyloid-β”, this single term actually describes a vast plethora of structurally disparate molecular assemblies, and thus various individual assemblies may be either potently pathogenic or completely inert. This project involved studies of the amyloid-β species present in distinct clinical subtypes of AD, thus contributing to our understanding of whether specific forms of Aβ correlate with specific clinical features. If potently pathogenic forms of Aβ could be identified, it would allow much more precisely targeted therapeutics which could potentially show significantly higher efficacy. Further, these pathogenic molecules could potentially act as biomarkers for disease progression, which could aid in both longitudinal studies and clinical trials. In addition, aggregates of amyloidogenic proteins are implicated in a wide range of other neurodegenerative diseases besides AD, and the work described here may help improve understanding in those other fields.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>[θ]r</td>
<td>Mean residue ellipticity</td>
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<tr>
<td>ε</td>
<td>Extinction coefficient</td>
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<tr>
<td>θ</td>
<td>Ellipticity</td>
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<td>Wavelength</td>
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<td>Aβ</td>
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<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>aCSF-B</td>
<td>Artificial cerebral spinal fluid base buffer</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>ADDLs</td>
<td>Amyloid-derived diffusible ligands</td>
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<td>AFFFF</td>
<td>Asymmetrical flow field-flow fractionation</td>
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<td>Apolipoprotein E human gene</td>
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<td>Amyloid-β-like protein precursor</td>
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<tr>
<td>BSE</td>
<td>Bovine Spongiform Encephalopathy</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-activated protein kinase II</td>
</tr>
<tr>
<td>CBD</td>
<td>Corticobasal degeneration</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CERAD</td>
<td>Consortium to establish a registry for Alzheimer’s Disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent kinase-5</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryogenic electron microscopy</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>CWD</td>
<td>Chronic Wasting Disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DELFIA®</td>
<td>Dissociation-enhanced Lanthanide Fluorescent Immunoassay</td>
</tr>
<tr>
<td>DI H2O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DYRK1A</td>
<td>Dual specificity tyrosine-phosphorylation-regulated kinase 1A</td>
</tr>
<tr>
<td>E18</td>
<td>Embryonic day 18</td>
</tr>
<tr>
<td>EOAD</td>
<td>Early-onset Alzheimer’s Disease</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-Linked ImmunoSpot</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>fAD</td>
<td>Familial Alzheimer’s Disease</td>
</tr>
<tr>
<td>FcyRs</td>
<td>Fcy-receptors</td>
</tr>
<tr>
<td>FcyRIIb</td>
<td>Fc-gamma receptor type IIb</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FFI</td>
<td>Fatal Familial Insomnia</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell derived neurotrophic factor</td>
</tr>
<tr>
<td>GM1</td>
<td>Monosialoganglioside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC50</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>ID</td>
<td>Immunodepletion</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>iN</td>
<td>Induced Neuron</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-d-galactopyranoside</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor Tyrosine-based Inhibitory Motif</td>
</tr>
<tr>
<td>KD</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>K/O</td>
<td>Knockout</td>
</tr>
<tr>
<td>KPI</td>
<td>Kunitz protease inhibitor</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LBD</td>
<td>Lewy Body dementia</td>
</tr>
<tr>
<td>LilrB2</td>
<td>Leukocyte immunoglobulin-like receptor B2 protein</td>
</tr>
<tr>
<td>LILRB2</td>
<td>Human leukocyte immunoglobulin-like receptor B2 gene</td>
</tr>
<tr>
<td>LOAD</td>
<td>Late-onset Alzheimer’s Disease</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPT</td>
<td>Human microtubule associated protein tau gene</td>
</tr>
<tr>
<td>MEM-NEAA</td>
<td>Minimum Essential Medium Non-essential amino acids</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>MMSE</td>
<td>Mini-mental state examination</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso Scale Discovery</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>Ngn2</td>
<td>Neurogenin 2</td>
</tr>
<tr>
<td>NIA-AA</td>
<td>National Institute of Aging-Alzheimer’s Association</td>
</tr>
<tr>
<td>NIA-RI</td>
<td>National Institute of Aging and Reagan Institute</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTE</td>
<td>N-terminally extended</td>
</tr>
<tr>
<td>o-Aβ</td>
<td>Oligomeric Aβ</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>ODP</td>
<td>Ocular Dominance Plasticity</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>Protein-A Sepharose</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS containing Tween-20</td>
</tr>
<tr>
<td>PDPKs</td>
<td>Proline-directed protein kinases</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filament</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PEN2</td>
<td>Presenilin enhancer 2</td>
</tr>
<tr>
<td>PirB</td>
<td>Paired Immunoglobulin-like receptor B</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2a</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PrPC</td>
<td>Prion protein (cellular form)</td>
</tr>
<tr>
<td>PrPSc</td>
<td>Prion protein (scrapie form)</td>
</tr>
<tr>
<td>PSEN1/2</td>
<td>Presenilin 1/2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PSP</td>
<td>Progressive supranuclear palsy</td>
</tr>
<tr>
<td>Rapid-AD</td>
<td>Rapidly Progressive Alzheimer’s Disease</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sAD</td>
<td>Sporadic Alzheimer’s Disease</td>
</tr>
<tr>
<td>sAPPα/β</td>
<td>Soluble APP fragment generated by α/β-cleavage of APP</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SEC-MALS</td>
<td>Size-exclusion chromatography - multi-angle light scattering</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-domain-containing inositol phosphatase</td>
</tr>
<tr>
<td>Slow-AD</td>
<td>Alzheimer’s Disease with typical insidious decline</td>
</tr>
<tr>
<td>S.O.C media</td>
<td>Super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>ssNMR</td>
<td>Solid state nuclear magnetic resonance</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi network</td>
</tr>
<tr>
<td>TRF</td>
<td>Time-resolved fluorescence</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular Dementia</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
</tbody>
</table>

**Statement of collaboration**

Certain sections of the work described here were carried out as part of collaborative research projects at the MRC Prion Unit at UCL. In particular, section 4 and section 5 involve large-scale mouse studies, which were carried out in collaboration with staff at the MRC Prion Unit Biological Services Facility (BSF), as well the dedicated histology department at the MRC Prion Unit.
Chapter 1 – Introduction

1.1 Alzheimer’s Disease Overview

1.1.1 Preamble

In 1907, Alois Alzheimer described a form of dementia characterised by two lesions: ‘miliary foci’ and ‘tangled fibrils’ (Alzheimer, 1907). More than 100 years later, the dementia now referred to as “Alzheimer’s Disease” (AD) affects more than 40 million patients worldwide (Prince et al., 2015), yet there is not a single disease-modifying therapeutic on the market, with only symptomatic treatments available. In the early stages, AD patients experience mild episodic memory loss. Further in the disease process, patients suffer a progressive loss of mental faculties, extending to language and executive function in the mid stages, and all thinking skills in advanced stages, associated with severe atrophy of the hippocampus and neighbouring cortical regions (Ballard et al., 2011). This ultimately leads to complete dependence on caregivers, as cognitive function and muscle mass deteriorate until the patient is bedridden. Available pharmacological treatments only provide mild and transient improvements in cognitive function and daily living (Winslow et al., 2011), thus most patients are faced with an insidious and inevitable decline. The precise mechanistic processes underlying the pathophysiology of AD remain controversial, and solving this puzzle stands as one of the most important social and economic challenges of our time.
1.1.2 Classification of Alzheimer's Disease

AD patients can be subcategorised by age of disease onset, rate of disease progression, and whether the disease stems from genetic inheritance or sporadic occurrence (Table 1). More than 95% of AD patients suffer from typical 'late-onset' disease beginning after age 65 (Reitz, Brayne and Mayeux, 2011), with the majority of these being sporadic cases with no single cause. In comparison, ‘Early-onset AD’ refers to patients displaying their first symptoms before 65, and many of these cases are caused by autosomal-dominant mutations in one of three genes: amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2). When such cases show clear genetic heritability they are defined as familial-AD (fAD), but as not all fAD mutations lead to early onset AD, both fAD and sporadic AD (sAD) can present as early onset or late onset diseases.

Table 1 - Categories of Alzheimer's Disease

<table>
<thead>
<tr>
<th>Genetic categories of Alzheimer's Disease</th>
<th>Sporadic Alzheimer’s Disease (sAD)</th>
<th>Familial Alzheimer’s Disease (fAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Typical’ variant of Alzheimer’s with no single cause. Usually onset &gt; 65</td>
<td>Alzheimer’s Disease showing consistent heritability throughout generations within one family. Often onset &lt;65. Usually caused by an autosomal dominant mutation in either APP, PSEN1, or PSEN2.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age-of-onset categories of Alzheimer's Disease</th>
<th>Late-onset Alzheimer’s Disease (LOAD)</th>
<th>Early-onset Alzheimer’s Disease (EOAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any variant of Alzheimer’s Disease with onset at age &gt; 65</td>
<td>Any variant of Alzheimer’s Disease with onset at age &lt; 65</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Progression rate categories of Alzheimer's Disease</th>
<th>Slowly-progressive Alzheimer’s Disease (Slow-AD)</th>
<th>Rapidly-progressive Alzheimer’s Disease (Rapid-AD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The ‘typical’ progression rate of Alzheimer’s disease, showing insidious cognitive decline which can begin up to 20 years before death.</td>
<td>Rare subset of Alzheimer’s Disease with rapid cognitive decline and less than 2 years between earliest symptoms and death.</td>
<td></td>
</tr>
</tbody>
</table>
1.1.3 Diagnosis of Alzheimer’s Disease

Many fAD patients can be confidently diagnosed by the presence of a disease-causing mutation, but accurate diagnosis of sporadic patients is much more challenging. The current gold standard requires the identification of two hallmark proteinaceous deposits in the post-mortem brain: neurofibrillary tangles (NFTs) and senile plaques (Figure 1).

Figure 1 – Amyloid plaques and neurofibrillary tangles in the AD brain

This photomicrograph of the cerebral cortex of an AD patient shows the overlap between amyloid-β (Aβ) (reddish brown stain) and tau (black stain) pathology in an AD brain. Amyloid plaques are deposits of aggregated Aβ peptides (green arrows), while neurofibrillary tangles are composed of hyperphosphorylated tau protein (blue arrows). Within some amyloid plaques, aggregates of tau are clearly visible, and these overlapping pathologies can be classified as “neuritic plaques”. Scale bar = 50 µm. Figure adapted from (Nelson et al., 2012)
NFTs are aggregates of hyperphosphorylated tau protein which develop within the nerve cell soma from where they may extend into dendrites (Braak and Braak, 1991). Cross-sectional studies suggest that the localisation and density of NFTs follows a rough spatiotemporal progression pattern which can be split into 6 'stages' according to criteria described by Braak and Braak (Braak and Braak, 1991; Braak et al., 2011), examined by either Gallyas silver staining or anti phospho-tau immunohistochemistry (Braak et al., 2006) (Figure 2). These stages correlate with levels of synapse loss and cognitive decline in AD patients (Nelson et al., 2012). The first stage defines no visible NFTs, followed by the early stages showing NFTs predominantly in the entorhinal cortex and related areas, gradually including more areas until stages V/VI (needed for diagnosis of AD at post mortem) in which NFTs are widely distributed throughout the neocortex (Montine et al., 2012).
Neuropathological ‘stages’ of Aβ (orange) and tau (blue) pathology in AD are based on cross-sectional studies, in which the post-mortem distribution of neuropathology in a wide range of patients is examined, in order to estimate the sequence in which various brain regions exhibit deposition of these lesions (red arrows). These studies suggest a general model in which Aβ pathology first appears in the neocortex before appearing in subcortical regions. In stark contrast, tau pathology is first observed around the entorhinal cortex and brainstem and is not detectable in the outer neocortical regions until the late stages of disease. Figure adapted from (Jucker and Walker, 2011).
Senile plaques are primarily composed of aggregates of peptide fragments cut from APP. These APP fragments are defined under the single term “Amyloid-β” (Aβ), although this actually represents a heterogeneous mix of peptide fragments (see 1.2.1.4). Deposition of Aβ pathology shows less consistent progression than tau, with a variety of morphological types of Aβ deposit each of which tend to develop in different brain regions (Dickson, 1997). Thus, while post-mortem diagnosis of AD requires identification of both NFTs and Aβ pathology, precisely which type of Aβ pathology is reported varies. One subset of Aβ deposits, found at the center of clusters of dystrophic neurites with coincident phospho-tau reactivity, are named ‘neuritic plaques’, and the Consortium to Establish a Registry for Alzheimer’s disease (CERAD) Aβ staging system uses the topographical localisation and density of these lesions (Mirra et al., 1991). Alternatively, general Aβ immunoreactivity can be staged using the Thal criteria (Thal et al., 2002). The National Institute of Aging-Alzheimer’s Association (NIA-AA) recommends using both CERAD and Thal staging in combination with Braak tau staging to describe the neuropathology of AD brains (McKhann et al., 2011). However, the relationship between AD neuropathological changes and true clinicopathological “Alzheimer’s Disease” is complicated by the fact that many people who die without any cognitive impairment also have significant AD neuropathological changes in their brains (Price et al., 2009; Sperling et al., 2011). Thus, it is clear that we do not yet fully comprehend the relationship between archetypal AD neuropathological features, and the underlying disease process (Montine et al., 2012).

Pre-mortem diagnosis of AD currently requires clinical evidence of dementia, supplemented by additional features such as insidious onset and early amnestic
symptoms (McKhann et al, 2011). However, this diagnosis is highly fallible, with 10-30% of individuals clinically diagnosed with AD ultimately not displaying AD neuropathological changes at autopsy (Nelson et al, 2011). The latest guidelines from the NIA-AA have begun to shift towards a biological definition of Alzheimer’s Disease, in which a living patient can be diagnosed due solely to the presence of abnormal cerebrospinal fluid (CSF), positron emission tomography (PET) and magnetic resonance imaging (MRI) signatures (Figure 3), methods which are now becoming established in routine clinical care (Jack, Bennett, et al., 2018).
Figure 3 - Changes to AD biomarkers during preclinical and clinical phases of disease

This graph describes a hypothetical model of the sequence of biomarker changes in AD patients. Early in disease, $\text{A}^\beta_{\text{x-42}}$ levels in CSF begin to drop (denoted by an increase in “biomarker abnormality” on the above graph), shortly followed by visible amyloid accumulation via PET scan. Elevated CSF tau and/or phospho-tau follows some years later, before evidence of atrophy by MRI, or hypoperfusion/hypometabolism by PET (Ahmed et al., 2014).  
*Figure adapted from* (Jack, 2013)
1.1.4 Genetic abnormalities and risk factors associated with sporadic AD

Like many chronic diseases, most sAD cases develop as a result of multiple factors rather than a single cause. The two primary non-modifiable risk factors for AD are old age and carrying the ε4 variant of the Apolipoprotein E gene (APOE). AD prevalence increases substantially with age, with <3% of 65-74 year olds, but ~17% of 75-84 year olds, and >30% of 85+ year olds succumbing to the disease (Hebert et al., 2013). Until the true mechanisms underlying the pathophysiology of disease are uncovered, it is difficult to distinguish which features of old age simply correlate with AD prevalence, and which may be causative and impact disease onset. However, some clues have recently been uncovered through large genome-wide association studies (GWAS), which track the relative ratios of genetic variants in control and disease populations. Sporadic AD populations show consistent association with single nucleotide polymorphisms (SNPs) around genes involved in immune response, inflammation, and lipid processing (Lambert et al, 2013; Jansen et al, 2018), which strongly implicates these pathways in AD, and may suggest that age-related decline in these systems also explains age-dependent increases in AD incidence. For example, the human immune system undergoes considerable change during ageing, which ultimately leads to decreased functional capacity (Meyer, 2013), and this immunosenescence could concurrently increase susceptibility to AD. Conversely, a number of other alterations in ageing human physiology could explain the correlation, such as age-dependent impairments in proteostasis (Bishop, Lu and Yankner, 2010), sleep quality (Bliwise, 1993), or brain perfusion (Kawecki and Ebert, 2004).
The first genetic risk factor identified for sAD, with an effect size high enough to be identified without modern GWAS technologies, was APOE (Saunders et al., 1993). Many studies have shown that APOE genotype potently influences the chance of succumbing to AD (Liu et al., 2013). Across the total human population, the ε3 allele is most common, with > 50% of individuals having at least one copy, while every extra copy of the ε4 variant increases the chance of succumbing to AD, and every copy of the ε2 allele reduces that risk. Specifically, the lifetime risk of AD for the total population is roughly 12%, while for APOE ε3/ε4 heterozygotes it is 20-30%, and for APOE ε4 homozygotes it is >50% (Genin et al., 2011). The differences between the variants are two individual amino acids at position 112 and 158, with ε2 containing cysteine residues at both 112 and 158, the ε3 variant containing one cysteine and one arginine, and the ε4 variant containing arginine at both positions (Liu et al., 2013). The mechanisms of increased risk due to APOE genotype are uncertain, but ApoE protein isoforms differentially regulate Aβ peptide clearance from the brain (Castellano et al., 2011), ApoE protein can be processed into neurotoxic fragments (Scheltens et al., 2016), and ApoE protein may directly impact tau pathology (Shi et al., 2017).

A variety of lifestyle-related factors are also thought to contribute to general dementia risk, including obesity, vascular health, mental inactivity, smoking status, low educational attainment, and mid-life hearing loss (Nyberg et al., 2014; Scheltens et al., 2016; Livingston et al., 2017). In total, these modifiable factors may account for more than 30% of all AD cases, thus the potential for primary prevention remains substantial (de Bruijn et al., 2015; Scheltens et al., 2016; Livingston et al., 2017).
1.1.5 Alzheimer's Disease impact and implications

When deaths are categorised according to the International Classification of Diseases version 10, specifically designed by the World Health Organization to determine leading causes of death within countries and populations, Alzheimer’s Disease is now the leading cause of death in England and Wales (Office for National Statistics, 2018), accounting for 12.8% of all deaths registered in 2018. Furthermore, during the long disease course the total costs to society amount to more than £30,000 per patient per year (Alzheimer’s Society, 2014). If a substantial intervention is not discovered, these problems are only set to increase as the average age of the global population continues to rise. Despite this, there are an astounding number of fundamental questions regarding AD pathophysiology which remain unanswered. Part of this stems from a willingness to accept poor molecular definitions of clinical targets, as the precise structure of many of the key molecules in the proposed pathophysiological pathways of AD remain a mystery (see 1.3.4). The greatest advances in our understanding of the disease have stemmed from studying the archetypal neuropathological lesions present in the end-stage AD brain and the processes which lead to their creation. From this, the greatest successes have been beginning to understand the roles played by two key proteins – APP and tau.
1.2 Molecular and cellular biology of amyloid precursor protein and tau

1.2.1 APP

1.2.1.1 APP expression and structure

APP is a transmembrane protein expressed in most tissues throughout the human body (Uhlen et al., 2015). Within the central nervous system (CNS), the most highly expressed variant is the 695 amino acid (AA) APP\textsubscript{695}, which outnumbers the 751 and 770 AA variants by roughly 2-fold and 20-fold respectively (Tanaka et al., 1989; Kang and Müller-Hill, 1990). At a cellular level, transcriptomics reveals that APP mRNA expression is highest in neurons, but is also significantly present in oligodendrocytes and endothelial cells, while being low in astrocytes and microglia (Zhang et al., 2014; Darmanis et al., 2015). To date, no full atomic structure has been obtained for any of the three major APP isoforms, however a large number of isolated domains have been characterised (Coburger, Hoefgen and Than, 2014). The bulk of the protein is present extracellularly, and > 70% of the extracellular AA participate in typical α-helical or β-sheet structure (Gralle et al., 2002). Within this region, two generic extracellular domains, E1 and E2, have been implicated in cellular adhesion, metal binding and dimerization, while possible protease inhibition activity is provided by a Kunitz protease inhibitor (KPI) domain exclusively present in the longer APP\textsubscript{751} and APP\textsubscript{770} isoforms (Dawkins and Small, 2014). The transmembrane domain comprises a single flexible α-helix, and contains multiple intramembrane cleavage sites, and the short C-terminal APP intracellular domain (AICD) comprises 49 AA with no stable tertiary structure, although small
regions may transiently populate reverse-turn or helix conformations (Kroenke et al., 1997; Ramelot, Gentile and Nicholson, 2000).

1.2.1.2 APP physiological function

The gene encoding APP in humans (APP) is part of a conserved gene family including Amyloid-β-like protein (APL-1) in C.elegans, amyloid-β-like protein precursor (APPL) in D.melanogaster, and APP, amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2) in mammals (Müller and Zheng, 2012). This suggests conserved function between species, with varied putative roles suggested including cellular adhesion, axon pruning, neuronal migration and other neurotrophic functions. The first suggested physiological role of APP, based on its structure, was that it may act as a receptor (Kang et al., 1987), with its large extracellular region interacting with various ligands, followed either by adaptor proteins binding to the intracellular domain, or the intracellular domain being cleaved and released into the cytosol to stimulate downstream pathways (Chakrabarti and Mukhopadhyay, 2012; Pardossi-Piquard and Checler, 2012). Alternatively, APP may form dimers (Soba et al., 2005), either as cis-dimers between APP molecules on the same cell (Kaden et al., 2008), or as trans-dimers between APP molecules present on adjacent cells (Deyts, Thinakaran and Parent, 2016). Mice with the mouse APP gene (App) knocked out (K/O) are viable, but show various deficits including reduction in brain weight, impairments to long-term potentiation (LTP) and spatial learning (Ring et al., 2007), motor dysfunction, and brain gliosis (Perez et al., 1997). This suggests APP is not necessary for development, but may still modulate neuronal development and function. However, knocking out the mouse genes for both APP and APLP2 concurrently leads to early postnatal lethality (Heber et al.,
2000), suggesting there is some necessary but redundant developmental functionality of the APP family members. Conditional K/O of App in either presynaptic motor neurons or in postsynaptic muscle of adult mouse brains (using transgenic mice expressing App with Cre-recombinase under either the neuronal rat nestin promoter, or the muscle creatine kinase promoter) lead to similar neuromuscular synapse defects as germline App deletion, suggesting loss of APP function in late life could have a direct deleterious effect (Wang et al., 2009). Although other conditional knockout models of APP have been produced (Mallm et al., 2010), they have not yet been well studied. Interestingly, overexpression of just a soluble ectodomain fragment of APP (sAPPα) alone is sufficient to rescue the anatomical, behavioural and electrophysiological abnormalities of App K/O, which suggests sAPPα is the key APP metabolite for neuronal development and function (Ring et al., 2007).

1.2.1.3 APP metabolism

Within the mammalian nervous system, APP is extensively metabolised to produce a plethora of bioactive fragments. The two most studied APP processing pathways are described as the “Amyloidogenic” and “Non-amyloidogenic” pathways, so named due to the propensity of the resulting peptide fragments to form insoluble ‘amyloid’ aggregates (see 1.2.1.4). In both pathways, APP usually first follows the typical sorting mechanisms through the endoplasmic reticulum (ER) and Golgi before delivery to the axon and undergoing fast anterograde transport through to synaptic terminals (Koo et al., 1990). Alternatively, APP may be transported from the Trans Golgi Network (TGN) directly into an endosomal compartment (O’Brien and Wong, 2011). At multiple points in the pathway, APP can be proteolytically cleaved by three distinct protease activities. These are
described under the single terms α-secretase, β-secretase and γ-secretase, but α-secretase activity can be carried out by an entire family of proteases, and γ-secretase activity is produced by a large protease complex (Haass et al, 2012).

The term ‘secretase’ describes the fact that when these proteases cleave the intramembrane APP, a substrate is released or “secreted” from the membrane. It is a slight misnomer as not all secretase activity occurs at the cell surface, so the cleaved substrates are often released intracellularly and even within organelles. In general, the amyloidogenic and non-amyloidogenic pathways follow a similar sequence, with a primary cleavage event releasing an N-terminal fragment, followed by an intra-membrane cleavage event which releases a further 2 fragments. The principal difference between the pathways is the relative length of fragments released by each step, which is determined by the different proteases and ensuing cleavage sites.

The amyloidogenic pathway begins with β-secretase cleavage at either the first residue (canonical β-cleavage site) or 11th residue (β’ cleavage site) of the Aβ peptide sequence, which amounts to the AA 671 or 682 in APP770. The first residue of the Aβ peptide sequence is an aspartic acid, and thus can be referred to as the “Asp1” site. This cleavage sheds a large soluble ectodomain named soluble amyloid precursor protein β (sAPPβ), while a 99 AA peptide named C-terminal fragment β (CTFβ) remains embedded in the membrane. β-secretase activity is carried out by the membrane-anchored aspartyl protease β-site APP cleaving enzyme 1 (BACE1) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), which is highly expressed within neurons. Subsequently, CTFβ is cleaved within the membrane-spanning domain by the γ-secretase complex. This complex first carries out ‘epsilon cleavage’ to release
the APP intracellular C-terminal domain (AICD) (Weidemann et al., 2002), before successive carboxypeptidase activity cleaves off tripeptides (Takami et al., 2009) until the final release of the Aβ peptide (Steiner, Fluhrer and Haass, 2008). The precise sequence of this Aβ peptide varies greatly dependent on the precise site of γ-secretase cleavage (see 1.2.1.4) This γ-secretase complex comprises at least one of the presenilins (PSEN1 or PSEN2), Nicastrin, anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2) (De Strooper, 2003; Edbauer et al., 2003).

The non-amyloidogenic pathway instead begins with α-secretase cleavage between Lysine 687 and Leucine 688 of APP770. Crucially, this lies within the Aβ peptide region, which precludes production of Aβ peptides via this pathway. This cleavage releases a 612 AA soluble ectodomain named sAPPα, while an 83 AA peptide named CTFα remains associated with the membrane. In cells, this α-cleavage occurs at the plasma membrane (Sisodia, 1992), and proteinase inhibitor profiling shows that it depends on zinc metalloproteinases (Roberts et al, 1994; Zhang et al, 2011). Three specific candidates proposed as α-secretases are ADAM9, ADAM10 and ADAM17, which have thus become therapeutic targets for modulation of APP processing, as increased α-secretase activity could potentially reduce the production of Aβ and other fragments of the amyloidogenic pathway (Bandyopadhyay et al., 2007). Following α-cleavage, the remaining CTFα peptide is further cleaved at an intramembrane site by the same γ-secretase complex, releasing the resulting two fragments from the membrane – p3 and AICD (Buoso et al., 2010).
Thus, overall the amyloidogenic pathway produces sAPPβ, Aβ and AICD, while the non-amyloidogenic pathway produces sAPPα, p3 and AICD (Figure 4), although the exact length of all of these peptides can vary slightly dependent on the precise α, β and γ cleavage sites. All of these peptides have been implicated in both physiological and disease-associated roles, and the field continues to be further complicated by an ever-growing list of potential APP proteases and associated cleavage sites (Andrew et al., 2016), many of which appear to be physiologically relevant (Willem et al., 2015; Baranger et al., 2016), although none of which have yet been genetically associated with human disease. Of all of these fragments, by far the most studied is the end product of the amyloidogenic pathway – Aβ.
APP Processing occurs through two mutually exclusive pathways. The amyloidogenic pathway begins with APP cleavage by β-secretase to release sAPPβ. The remaining CTFβ fragment is then further cleaved by γ-secretase to produce AICD and Aβ. The Non-amyloidogenic pathway instead begins with α-secretase cleavage, which occurs within the Aβ sequence and therefore precludes the production of Aβ by this pathway. This step produces sAPPα and CTFα, the latter of which is further cleaved by γ-secretase to produce the p3 fragment and AICD. Figure adapted from (Peers, Pearson and Boyle, 2007).
1.2.1.4 Amyloid-β

The term ‘Aβ’ describes a group of similar peptides produced during the amyloidogenic processing of APP. These peptides are prone to aggregation, and deposits of aggregated Aβ are the primary component of the senile plaques observed in the AD brain (Masters et al., 1985). The first biochemical characterisation of Aβ peptides from human brain arose from studies into the cerebrovascular amyloid present in AD and Down's syndrome patients. Here, the amyloid component of meningeal vessels in these patients brains was isolated, solubilised in 6 M guanidine hydrochloride, and two strong peaks were visualised at roughly 4,200 daltons by high performance liquid chromatography (HPLC) (Glenner and Wong, 1984a, 1984b). These two peaks showed identical N-terminal sequences up to residue 24, starting at what is now defined as the canonical N-terminus of Aβ, asparagine 1 (Asp1). Subsequent work then identified that a protein with identical N-terminal sequence and molecular mass comprised the plaque cores in AD and in aged individuals (Masters et al., 1985). While this biochemical characterisation revealed the canonical N-terminus of Aβ, the C-terminal residues were less clear, with various groups reporting that the peptide ended after 40, 42, or 43 amino acids (Kang et al., 1987; Mori et al., 1992). Studies of APP processing have since suggested that all of these peptide variants are natively produced, as the γ-secretase dependent C-terminal cleavage can occur at many points between AA 37 and 43 of the Aβ domain (Haass et al., 2012). Additionally, more severely C-terminally truncated Aβ variants appear to be produced if APP is sequentially cleaved by β-secretase followed by α-secretase (Portelius et al., 2006, 2011). In addition to this C-terminal heterogeneity, there is also substantial variability in the N-terminus of human brain-derived Aβ species, with many variants detected that are N-
terminally truncated relative to the canonical Asp1 site (Iwatsubo et al., 1996; Saido et al., 1996; Tekirian et al., 1998; Arai et al., 1999; Wiltfang et al., 2001; Miravalle et al., 2005; Güntert, Döbeli and Bohrmann, 2006; Lewis et al., 2006; Murayama et al., 2007; Portelius et al., 2010; Wildburger et al., 2017). Further, many post-translationally modified Aβ variants have been detected (Kummer and Heneka, 2014). These include pyroglutamylated variants in which N-terminal glutamate residues are dehydrated and cyclised to pyroglutamate (Figure 5), which seem to comprise a substantial portion of senile plaques (Mori et al., 1992; Härtig et al., 2010), and be enriched in the amyloid deposits of AD patients compared to non-demented patients displaying β-amyloidosis (Portelius et al., 2015).

![Figure 5 - Mechanism of pyroglutamate formation](image)

Pyroglutamate forms via a dehydration reaction in which the amino group of an N-terminal glutamate residue cyclises to form a lactam. This can result either spontaneously or via the action of a glutaminyl cyclase enzyme. (Schilling, Wasternack and Demuth, 2008)

Other post-translational modifications of Aβ include phosphorylation at serine 8 or serine 26 (Milton, 2001; Kumar et al., 2011), and nitration or glycosylation at tyrosine 10 (Halim et al., 2011; Kummer et al., 2011) (Figure 6), but the physiological significance of these post-translational modifications to disease is not yet clear.
A wide range of Aβ species have been detected in human brain, with substantial heterogeneity in N-termini, C-termini and post-translational modifications. The single term ‘Aβ’ is often used to describe any one of these peptides, but individual species can be more specifically described using subscript numbers to denote the N and C-termini. For example, Aβ beginning at the canonical N-terminus (Asp1) and ending at alanine 42 can be described as Aβ1-42. In order to only specify a single terminus, the opposite terminus can either be omitted or replaced with the subscript ‘x’. For example, the terms Aβx-40 and Aβ40 both refer to any Aβ peptide with a C-terminus at valine 40.
Aggregates of Aβ peptides in amyloid plaques are not simply amorphous clumps (although some amorphous Aβ aggregates may be present), but rather consist primarily of highly ordered amyloid fibrils. The precise structure of these fibrils remains uncertain (Xiao et al., 2015; Gremer et al., 2017; Qiang et al., 2017), but a core feature of all amyloid fibrils is ‘cross-beta’ structure wherein parallel β sheets stack perpendicular to the fibril axis (Figure 7). Substantial evidence suggests the Aβ species responsible for driving AD pathophysiology may in fact be soluble oligomeric intermediates of this aggregation reaction (see 1.3.4), however very little is known about the native structure of these oligomers in the human brain, and the mechanisms through which they contribute to disease remain imprecisely defined.

**Figure 7 - Hypothetic aggregation pathway for an amyloid fibril**

Amyloids can be biophysically defined as any protein or multimeric protein assembly containing cross-beta structure, where beta sheets stack parallel to the fibril axis. This diagram shows a simplified hypothetical aggregation pathway for the formation of a heteromeric amyloid fibril. Many proteins exist in solution with little secondary structure (a), but can form into β-sheets consisting of β-strands connected by hydrogen bonds between their peptide backbones (red and blue arrows). Many amyloid fibrils are thought to originate with the formation of small oligomers such as dimers (b), before extension in the lateral plane leads to formation of large fibrils (c). In the final fibril, β-sheets stack perpendicular to the fibril axis, forming highly stable repeating structures.
1.2.2 Tau

1.2.2.1 Tau structure, splicing and expression

The human microtubule associated protein tau (MAPT) gene comprises 16 exons located over roughly 100 kilobases of the long arm of chromosome 17 (Neve et al., 1986). While the protein has been detected in the peripheral nervous system, kidneys, and skeletal muscle tissue, RNA levels are highest in the CNS (Uhlen et al., 2015). Here, expression is by far the highest within neurons, although trace levels may be detectable within oligodendrocytes and astrocytes (Darmanis et al., 2015). Alternative splicing of exons 2, 3 and 10 leads to the production of the 6 primary protein isoforms found in the human adult brain (Wang and Mandelkow, 2016). As exons 2 and 3 are found near the N-terminus of the protein, the isoforms can be defined based on whether they have neither exon, only exon 2, or both exon 2 and 3, using the nomenclature 0N, 1N or 2N. Each of these 3 variants can then exist either with or without exon 10. Exon 10 represents the second of four carboxy-terminal microtubule binding repeat domains, thus isoforms containing exon 10 are defined as “4R” (for 4 repeat domains), and isoforms lacking exon 10 are defined as “3R”. These categories can be combined to define all 6 isoforms which vary in length from 352 AA to 441 AA (Figure 8). An additional “big tau” isoform is found in the peripheral nervous system, which is equivalent to the 2N4R variant plus an extra 242 residues from exon 4A (Mandelkow and Mandelkow, 2011). In the human foetal brain, only the shortest tau isoform (0N3R) is expressed, whereas all six isoforms are found in the adult brain (Goedert et al., 1989). Here, levels of 3R and 4R are approximately equal, while the ratio of 0N, 1N and 2N variants is roughly 4:5:1 (Goedert et al., 1989; Guo, Noble and Hanger, 2017). Mice show distinctly
different expression levels, with the adult murine CNS containing almost exclusively 4R tau variants (Takuma, Arawaka and Mori, 2003).

Figure 8 - Tau Exons and spliceforms in human brain
Alternative splicing of tau in the human brain produces six distinct isoforms through the alternative splicing of exons 2, 3 and 10. These isoforms are classified by the presence of 0, 1, or 2 N-terminal domains (0N, 1N, 2N variants), and the presence or absence of the second microtubule-binding repeat domain (3R vs 4R variants). Figure adapted from (Park, Ahn and Gallo, 2016).

The amino acid composition of tau is highly hydrophilic, with the longest 2N4R variants containing 80 serine or threonine residues (Mandelkow and Mandelkow, 2011). Due to this, tau does not adopt precise tertiary or even secondary structure, rather existing primarily in an intrinsically unstructured state (Schweers et al., 1994; Mukrasch et al., 2009). Intrinsically unstructured proteins and domains are common, and typically only fold following interaction with their binding target (Dyson and Wright, 2005). Despite lacking precise tertiary interactions, the highly acidic N-terminal region of tau preferably folds over into the vicinity of the repeat domains, forming a weak and transient “paperclip” structure rather than a complete random coil (Jeganathan et al., 2006).
1.2.2.2 Tau physiological function

The archetypal physiological role of tau is binding to microtubules, subsequently stabilising them and regulating cytoskeletal dynamics (Weingarten et al., 1975; Caceres and Kosik, 1990). Specifically, the “microtubule binding domain” encompassing the alternatively-spliced repeat domains in the C-terminal half of the protein binds directly to the interface between α-tubulin-β-tubulin heterodimers (Kadavath et al., 2015), while the N-terminal domain of tau projects away from microtubules (Guo, Noble and Hanger, 2017). In human neurons, this activity is most prominent in axons, wherein tau induces the nucleation and elongation of axonal microtubules (Kanai, Chen and Hirokawa, 1992), subsequently regulating axon outgrowth and neuronal plasticity (Arendt, Stieler and Holzer, 2016). 4R isoforms of tau more effectively carry out this microtubule stabilisation than 3R isoforms, most likely due to the additional repeat within the microtubule binding domain (Goedert and Jakes, 1990). Tau appears to be so key for neurite outgrowth that overexpressing tau in non-neuronal cell lines can lead to the formation of long neurite-like processes (Knops et al., 1991), whereas antisense oligonucleotides against tau in primary neurons inhibit neurite formation (Caceres and Kosik, 1990). Aside from acting as structural support for axons, microtubules also act as the scaffolding for axonal transport by dynein and kinesin motor proteins. Tau competes with both motors for microtubule binding, thereby regulating the axonal transport of both organelles and proteins (Stamer et al., 2002). Despite this, Tau K/O or overexpressing mice show few axonal transport abnormalities (Kawecki and Ebert, 2004), suggesting as yet unidentified redundant mechanisms may exist (Wang and Mandelkow, 2016).
Outside of axons, tau may also be present in dendrites (Ittner et al., 2010) and nuclei (Rady, Zinkowski and Binder, 1995). In dendrites, tau is purported to target the Src kinase Fyn to the post-synapse, leading to the phosphorylation and activation of N-methyl-D-aspartate (NMDA) receptors and potentially facilitating excitotoxic mechanisms (Ittner et al., 2010). Nuclear tau may protect neuronal DNA and RNA under hyperthermic conditions, but the precise mechanisms remain unclear (Violet et al., 2014).

1.2.2.3 Tau post-translational modifications

Tau is notorious for being extensively post-translationally modified in the human brain. Key examples include phosphorylation (Grundke-Iqbal, Iqbal and Tung, 1986), glycation (Ledesma et al., 1994), nitration (Reyes et al., 2008), acetylation (Cohen et al., 2011), sumoylation (Dorval and Fraser, 2006) and truncation (Mondragón-Rodríguez et al., 2009; Mietelska-Porowska et al., 2014). Many of these modifications are required for tau to carry out its purported physiological functions, but aberrant processing also likely enables the pathological tau aggregation seen in AD and other neurodegenerative diseases.

More than 40 phosphorylation sites in Tau have been experimentally observed (Wang and Mandelkow, 2016), with the majority found on serine-proline and threonine-proline motifs (Morishima-Kawashima et al., 1995). These phosphorylations are developmentally regulated, with foetal tau carrying approximately 7 phosphates per molecule compared to 2 phosphates per molecule in the healthy adult brain (Kanemaru et al., 1992). This process reverses in AD and other neurodegenerative conditions, where the phosphorylation of tau returns to or even exceeds foetal levels (Schober et al., 2014).
Most tau phosphorylation in human brain is carried out by proline-directed protein kinases (PDPKs) such as glycogen synthase kinase-3β (GSK-3β) (Hooper, Killick and Lovestone, 2008), cyclin-dependent-like kinase-5 (CDK5) (Kimura, Ishiguro and Hisanaga, 2014), and dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) (Ryoo et al., 2007), with some other phosphorylation activity carried out by calcium/calmodulin-activated protein kinase II (CaMKII) (Ghosh and Giese, 2015) and protein kinase A (PKA) (Martin et al., 2013) amongst others (Iqbal, Liu and Gong, 2016). Excessive tau phosphorylation in the diseased brain may be due to the over-activation of these kinases, or alternatively due to decreased activity of phosphatases. In the human brain, ~70% of tau dephosphorylation is carried out by protein phosphatase 2a (PP2A) (Gong et al., 2000), which has its activity reduced in the grey and white matter of the AD brain by ~20% and ~40% respectively (Gong et al., 1993; Wang and Mandelkow, 2016).

A plethora of other potential modifications to tau have been studied both individually, and in their ability to modify tau phosphorylation. Multiple tau asparagine, serine and threonine residues have been identified as sites of glycosylation, in which large oligosaccharide residues are attached to the amine radical of asparagine or the hydroxyl radical of serine or threonine (Martin, Latypova and Terro, 2011). This glycosylation may increase subsequent tau phosphorylation (Liu et al., 2002), which is supported as glycosylation is higher in human AD tau aggregates than in normal human tau (Wang, Grundke-Iqbal and Iqbal, 1996). Furthermore, deglycosylation of tau aggregates from human brain converts them from paired helical filaments (PHFs) into bundles of straight filaments and restores their physiological binding activity (Takahashi et al.,
Conversely, glycosylation of specific serine or threonine residues with N-acetyl-glucosamine (named O-GlcNAcylation) is markedly decreased in AD brain, thus correlating negatively with increasing tau phosphorylation (Liu et al., 2009). This suggests that certain tau glycosylation events may either increase or decrease subsequent tau phosphorylation, and thus be either beneficial or detrimental in AD.

1.2.2.4 Tau in Disease

Similar to APP, tau plays roles both in sporadic and autosomal-dominant forms of neurodegenerative disease. Deposition of hyperphosphorylated tau aggregates is one of the pathological hallmarks of AD, while mutations within the MAPT gene can dominantly lead to a class of neurodegenerative diseases called ‘tauopathies’, including frontotemporal dementia (FTD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). By definition, all tauopathies present with deposition of large tau aggregates, although the precise morphology and localisation of these is incredibly variable (Schneider et al., 1997; Seelaar et al., 2011). It remains uncertain whether mechanisms of tau-driven pathophysiology are similar between these sporadic and genetic cases.

Autosomal-dominant mutations typically confer pathogenicity by either loss-of-function or gain-of-function of the encoded protein. The fact that tau K/O mice show relatively mild phenotypes (Harada et al., 1994) counts as evidence against a loss-of-function mechanism for tau in human disease, but this lack of severe phenotype may be explained by upregulation of compensatory proteins such as microtubule associated protein 1a (Harada et al., 1994), and such redundant mechanisms may not be as flexible in the aged brain. No conditional MAPT K/O
mouse model has so far been described, but they will be key for identifying the potential effects of mature-onset loss-of-function of tau (Denk and Wade-Martins, 2009). Over 40 mutations in and around the MAPT locus lead to tauopathy, but the precise disease progression and neuropathology varies. At the molecular level, a general pattern can be seen in which the mutations either increase production of 4R tau (and subsequently lead to high levels of 4R tau neuropathology) (D’Souza et al., 1999; Stanford et al., 2000; Iseki et al., 2001; Kobayashi et al., 2003; Kouri et al., 2014), increase the propensity of tau to aggregate (Hutton et al., 1998; Pastor et al., 2001; Nicholl et al., 2003; Rossi et al., 2012; Tacik et al., 2015), or reduce tau’s ability to bind to microtubules and enhance microtubule assembly (Bugiani et al., 1999; Rizzini et al., 2000; Iseki et al., 2001; Neumann et al., 2001; Pastor et al., 2001; Hayashi et al., 2002; Poorkaj et al., 2002; Rosso et al., 2002; Spina et al., 2007; Kovacs et al., 2011; Deramecourt et al., 2012). There are many exceptions to these cases however, with some mutations increasing 3R tau production (Momeni et al., 2009), or increasing tau’s ability to enhance microtubule assembly in vitro (Pickering-Brown et al., 2004; Iyer et al., 2013). Thus, although expression of mutant tau is able to directly lead to neurodegenerative disease, the precise mechanisms through which this is achieved – and whether multiple mechanisms play a role – remains uncertain. In sAD, the deposition of a specific form of hyperphosphorylated tau aggregates (neurofibrillary tangles) is one of the hallmark neuropathological features required for diagnosis, but what role tau actually plays in the disease is unclear. The two main hypothesis are again that tau pathophysiology is based either on a toxic gain-of-function driven by hyperphosphorylated tau aggregates or mislocalised hyperphosphorylated monomers, or a toxic loss-of-function in which hyperphosphorylated and/or
aggregated tau fails to adequately stabilise microtubules or protect the cell from stress (Wang and Mandelkow, 2016). In either case, in AD it appears tau pathology is dependent on initiation by APP dyshomeostasis, and much work has been carried out to attempt identify the precise relationship between these two key features of disease (Hernández et al., 2010; Zempel and Mandelkow, 2012; Bloom, 2014; Nisbet et al., 2015).
1.3 The amyloid hypothesis

1.3.1 The search for the pathogenetic sequence of Alzheimer’s Disease

To truly understand a disease, you need to identify the causal sequence which leads from the earliest initiation through to the final disease state. Every step in this sequence is a potential target for therapeutic intervention, while every epiphenomenon which arises along the path is an avenue for wasted resources. Neurodegenerative diseases are far more complex than simple A→B causality, with multiple coincident pathologies and feedback pathways, but it must remain a top priority to identify the biological sequence which leads to neurodegeneration in order to successfully focus the search for therapeutics. In AD, the archetypal theory of the temporal sequence of pathophysiology is the amyloid hypothesis, which posits that β-amyloidosis is the primary event which begins a cascade of pathological changes ultimately leading to neurodegeneration.

1.3.2 Evidence for β-amyloidosis as the primary driver of AD

By definition, all AD patients show severe Aβ deposition throughout the brain, including regions which serve memory and executive function. This fact has long facilitated the simple inference that these deposits – or processes associated with them - may cause the clinical phenotype of AD (Blessed, Tomlinson and Roth, 1968). However, the theory that β-amyloidosis initiates the disease primarily gained traction after it was found that the precursor to Aβ resides on chromosome 21 (Kang et al., 1987), coupled with the knowledge that trisomy 21 patients invariably succumb to AD at an early age (Jervis, 1948; Olson and Shaw, 1969; Wisniewski, Wisniewski and Wen, 1985). Soon after this, a raft of
mutations within the APP gene itself were identified, each of which leads to an autosomal-dominant early onset form of fAD (Goate et al., 1991; Murrell et al., 1991; Hardy and Higgins, 1992; Mullan et al., 1992). These APP mutants prove one thing with certainty: solely altering APP is sufficient to drive every feature of AD, including tau pathology, gliosis, neurodegeneration and cognitive decline. The two primary questions which remain to be conclusively answered are: 1 – Through which mechanisms does APP dysmetabolism lead to disease? 2 – Does APP dysmetabolism drive the pathophysiology of sAD in the same way it does for APP mutants?

The most popular hypothesis for which facets of APP dysmetabolism actually lead to disease – a more precise characterisation of ‘the amyloid hypothesis’ - is that alterations to the absolute or ratiometric levels of Aβ are the primary cause for all the downstream features of AD (Hardy and Selkoe, 2002). This concept is well supported in genetic cases. Down’s syndrome patients suffer from β-amyloidosis decades before the onset of tau pathology, glial pathology and cognitive impairment (Wisniewski, Wisniewski and Wen, 1985; Mann and Esiri, 1989), thus in trisomy 21 patients Aβ deposition is present long before other features of disease. Longitudinal studies of fAD patients also suggest that Aβ begins accumulating decades before brain atrophy or clinical symptoms begin to appear (Gordon et al., 2018), and that Aβ begins to accumulate almost a decade before observable tau accumulation (Quiroz et al., 2018). Also in fAD, the mutations in APP which lead to AD largely cluster at or near the sites where Aβ is normally cleaved by the α, β and γ-secretases, suggesting these mutations will alter production of Aβ peptides in the human brain (Goate and Hardy, 2012). In vitro and in mouse models, most of these mutations alter Aβ production by these
secretases, leading to longer, more hydrophobic and amyloidogenic forms of Aβ, or a higher ratio between the longer and shorter Aβ variants (Goate, 1998; Hall and Roberson, 2012). Mutants within the PSEN1 and PSEN2 components of the γ-secretase complex themselves can also lead to fAD, and also lead to similar increases in amyloidogenic Aβ production in vitro (Borchelt et al., 1996). While these data strongly implicate β-amyloidosis as the primary feature driving disease in fAD and trisomy 21, it remains possible that Aβ deposition is only an epiphenomenon with limited impact on disease progression, and some other hidden facet of APP dysmetabolism is actually driving neurodegeneration. Certainly the potential roles of other APP metabolites such as sAPPβ, AICD and CTF-β in human physiology and disease remain underexplored (Morris, Clark and Vissel, 2014), but a vast array of studies in mouse models and in vitro support a pathogenic role for Aβ species of various structure and aggregation state. In mice overexpressing mutant APP, significant Aβ deposition is associated with memory impairments (Sturchler-Pierrat et al., 1997; Moechars et al., 1999; Mucke et al., 2000; Rockenstein et al., 2001; Oddo et al., 2003; Knobloch et al., 2007; Simón et al., 2009) which in certain cases can be reversed following treatment with anti-Aβ antibodies or Aβ immunisation (Bard et al., 2000; Morgan et al., 2000; Chauhan and Siegel, 2002). In vitro, synthetic and brain-derived Aβ species also cause a variety of neurotoxic phenotypes (see section 1.3.4). Both these kinds of studies have flaws, as most mouse models of AD which display cognitive phenotypes rely on incredibly high non-physiological levels of APP overexpression (whereas APP knock-in mice show very mild cognitive impairment even at old ages (Saito et al., 2014)), and in vitro studies of Aβ toxicity are mired in controversies regarding the actual structure of Aβ species and aggregates used (Benilova, Karran and De Strooper, 2012). Nonetheless,
the majority of current evidence supports that Aβ dyshomeostasis is the initiating factor in genetic forms of AD. In sAD, the precise link between Aβ and neurodegeneration is less certain, but as fAD and sAD cohorts appear to show very similar progression rates, clinical presentations, MRI features, PET features, and neuropathological features at autopsy (Duara et al., 1993; Nochlin et al., 1993; Lista et al., 2015), the current data also supports a primary role for Aβ in sAD, although in both fAD and sAD it is clear that this role could either be as primary driver throughout the course of the disease, or as an initiating ‘trigger’ which activates a pathological cascade which then becomes autonomous at later disease stages. Overall, two key questions remain to improve our understanding of the role of Aβ in AD pathogenesis: which Aβ species are responsible, and via which mechanisms do they confer their pathogenicity?
1.3.3 Pathogenic and inert species of amyloid-β

All AD brains are riddled with amyloid plaques, but these deposits can also be found at substantial levels in healthy non-demented patients (Crystal et al., 1988; Braak and Braak, 1997; Bennett et al., 2006; Price et al., 2009; Chételat et al., 2013). This has led to some controversy over whether these deposits actually play a causative role in disease or are simply epiphenomena (Castellani et al., 2009; Morris, Clark and Vissel, 2014). Much of these data can be explained by a long pre-clinical phase of AD, with sub-threshold Aβ deposition present many years before the onset of cognitive symptoms (Morris et al., 1996; Hulette et al., 1998; Price and Morris, 1999; Sperling et al., 2011), but it remains enigmatic that a subset of patients exhibit extensive Aβ deposition (equivalent to or exceeding levels typically seen in clinical AD) without any notable cognitive decline (Crystal et al., 1988; Katzman et al., 1988; Jellinger, 1995; Rentz et al., 2010). The existence of these non-demented High-Amyloid control patients (also termed ‘pathological ageing’ or ‘high pathology controls’) may be explained in part by the roles of cognitive reserve and variability in co-pathologies (see 1.4.5), but a hugely complex milieu of molecules are described under the single term ‘Aβ’ (Figure 9) and it thus seems likely that certain Aβ proteoforms or aggregated conformations may possess bioactivity that is either pathogenic or comparatively inert.
There are a wide variety of Aβ peptides and aggregates detectable in the human brain, and these can be stratified by three levels of heterogeneity. Firstly, there are several Aβ primary sequences detectable in the human brain, and each of these can be subject to a range of post-translational modifications (see 1.2.1.4). Secondly, these different monomeric peptides are able to both homo-oligomerise and hetero-oligomerise into a range of combinations, although precisely which hetero-oligomers are present in the human brain is currently unknown. Finally, even when only considering a single peptide, a variety of stable aggregate conformations are possible, and there are likely multiple stable conformations for every homo-oligomeric and hetero-oligomeric combination of Aβ peptides.
It has thus become a substantial aim in the field to identify which Aβ species are enriched in the brains of AD patients compared to High-Amyloid controls, but such comparisons have often been inconclusive, with high amounts of overlap in the Aβ species observed in AD and High-Amyloid control patients (Moore et al., 2012; Monsell et al., 2013). Nevertheless, some differences between AD and High-Amyloid control populations have been observed, such as higher levels of sodium dodecyl sulphate (SDS)-stable Aβ dimers (McDonald et al., 2010), phosphorylated Aβ aggregates (Rijal Upadhaya et al., 2014), soluble fibrillar Aβ oligomers (Tomic et al., 2009), conformation-specific Aβ oligomers (Perez-Nievas et al., 2013), N-terminally truncated/pyroglutamate modified Aβ species (Portelius et al., 2015), and a higher ratio between Aβ oligomers and plaque burden (Esparza et al., 2013). Such studies, in combination with analysis of the toxicity of various Aβ preparations in vitro, have led to relative consensus that it is most likely not monomeric Aβ species, nor large insoluble fibrils, but rather soluble oligomeric forms of Aβ that are the key players in disease (Ferreira et al., 2015; Viola and Klein, 2015; Selkoe and Hardy, 2016).

1.3.4 Oligomeric Aβ in Alzheimer’s Disease

The easiest Aβ species to characterise lie at either end of the aggregation spectrum, either as individual monomers or as large stable fibrils. It is perhaps for this reason that most of our molecular understanding of Aβ in the human brain is of these two states. Mass spectrometry is beginning to accurately describe the primary sequence and post-translational modifications of Aβ monomers (Portelius et al., 2010, 2015), while cryo-electron microscopy (Cryo-EM) and solid state nuclear magnetic resonance (ssNMR) spectroscopy provide insights into the precise architecture of fibrils (Lu et al., 2013; Gremer et al., 2017; Qiang
et al., 2017). However, the most popular theory of Aβ pathogenicity involves the soluble oligomeric species which lie either within or adjacent to the canonical aggregation pathway (Ferreira et al., 2015; Viola and Klein, 2015). Certain oligomeric Aβ species are specifically enriched in the brains of AD patients (Tomic et al., 2009; Mc Donald et al., 2010; Perez-Nievas et al., 2013; Rijal Upadhaya et al., 2014), and collectively Aβ oligomers of various structures have been shown to be potently synaptotoxic, able to inhibit LTP in rodent primary and slice cultures, and able to induce severe memory deficits when injected into rodents in vivo (Lambert et al., 1998; Walsh et al., 2002; Hsieh et al., 2006; Shankar et al., 2008; Laurén et al., 2009; Ono, Condron and Teplow, 2009; Wilcox et al., 2011). These experimental observations show enticing similarity to the early features of memory impairment and synapse loss in AD, but unfortunately it has proven notoriously challenging to identify the structure of any disease relevant Aβ oligomer (Benilova, Karran and De Strooper, 2012). A wide range of heterogeneous structures have been reported both from in vitro Aβ aggregation reactions (Chimon et al., 2007; Glabe, 2008; Nussbaum et al., 2012; Suzuki et al., 2013; Breydo et al., 2016; Serra-Batiste et al., 2016; Tran et al., 2016), and ex vivo purification of Aβ aggregates from human brain (Noguchi et al., 2009; Jin et al., 2011; Esparza et al., 2016), but there is not yet even consensus as to whether the truly relevant species are high molecular weight aggregates (Noguchi et al., 2009), or small oligomers such as dimers (Shankar et al., 2008). Despite this lack of structural precision, a vast literature has arisen characterising the biological activities of both synthetic and brain-derived oligomeric Aβ species. Several mechanisms for these effects have been put forward, but one of the most straightforward explanations is that these oligomeric species act as ligands for receptors expressed on the surface of neuronal or glial
cells, activating downstream signalling pathways to potentiate their effects. One attractive feature of this mechanism is that if true, it should be possible to create assays which quantify the levels of receptor-binding Aβ oligomers in AD brain, and use these interactions to purify disease-relevant Aβ species. A multitude of putative receptors have been identified, however no consensus has been reached on their absolute or comparative importance to the human disease (Jarosz-Griffiths et al., 2016).

1.3.5 PrP\textsuperscript{C} as a mediator of Aβ-oligomer toxicity

One of the most scrutinised putative Aβ oligomer receptors is the cellular form of the prion protein (PrP\textsuperscript{C}). PrP\textsuperscript{C} is a glycosylphosphatidylinositol (GPI)-anchored protein expressed in the majority of tissues in the human body (Uhlen et al., 2015). While dominantly inherited mutations in this protein cause a variety of “prion diseases” (see 1.4.6), its physiological role is comparatively poorly understood. The first mice homozygous for disrupted PrP\textsuperscript{C} expression showed no obvious phenotype aside from invulnerability to prion infection (Büeler et al., 1992), but since then at least 26 phenotypes have been reported in a variety of knockout mouse models (Steele, Lindquist and Aguzzi, 2007). These phenotypes include altered sleep cycle regulation (Tobler et al., 1996; Tobler, Deboer and Fischer, 1997), immune system abnormalities (Thackray and Bujdoso, 2002; de Almeida et al., 2005; Ballerini et al., 2006), altered neuronal excitability (Collinge et al., 1994; Walz et al., 1999), and defects in peripheral myelin maintenance (Bremer et al., 2010; Nuvolone et al., 2016). While this indicates that PrP\textsuperscript{C} plays a variety of roles in multiple tissues throughout the body, no robust phenotype has yet been observed to explain the native function.
of PrP<sup>C</sup> within the central nervous system (CNS) where it is most highly expressed (Uhlen <i>et al.</i>, 2015).

PrP<sup>C</sup> was first implicated as a receptor for Aβ oligomers in an unbiased screen of 225,000 complementary DNA (cDNA) clones from an adult mouse brain library expressed in COS-7 cells, in which two independent positive clones displaying nanomolar binding affinity for Aβ oligomers were isolated and found to both encode full-length mouse PrP (Laurén <i>et al.</i>, 2009). These Aβ oligomers were produced using synthetic Aβ<sub>1-42</sub> peptide conjugated to biotin, with avidin conjugates used for detection. At nanomolar monomer-equivalent concentrations, this preparation blocked hippocampal LTP in wild type (WT) mice, but LTP in PrP K/O mice remained normal. Further, treating slices with the anti-PrP antibody 6D11 acutely reduced Aβ oligomer binding, and also protected against LTP dampening. This led to a slew of studies showing that both PrP K/O and anti-PrP antibody treatment improves memory deficits and inhibit synaptotoxicity in AD model mice (Chung <i>et al.</i>, 2010; Gimbel <i>et al.</i>, 2010; Barry <i>et al.</i>, 2011; Klyubin <i>et al.</i>, 2014). Multiple in vitro studies have since confirmed that PrP<sup>C</sup> binds with nanomolar affinity to Aβ oligomers of various structure (Chen, Yadav and Surewicz, 2010; Nicoll <i>et al.</i>, 2013; Dohler <i>et al.</i>, 2014; Ganzinger <i>et al.</i>, 2014), and that disrupting this interaction can rescue toxic phenotypes induced by Aβ (Bate and Williams, 2011; Resenberger <i>et al.</i>, 2011; Um <i>et al.</i>, 2012; Nicoll <i>et al.</i>, 2013; Peters <i>et al.</i>, 2015). In one particularly thorough study, both cell-expressed and surface-bound PrP was found to have the highest affinity for Aβ oligomers of a wide panel of tested receptors. This PrP-Aβ binding is selective for aggregated Aβ over monomers (Chen, Yadav and Surewicz, 2010; Freir <i>et al.</i>, 2011), can be inhibited using antibodies targeted at
multiple sites on PrP (Freir *et al.*, 2011) (*Figure 10*), and appears irreversible (Chen, Yadav and Surewicz, 2010). The most popular mechanism described for this toxicity is metabotropic glutamate receptor 5 (mGluR5) dependent activation of the kinase Fyn (Um *et al.*, 2013; Hu *et al.*, 2014), but more work is needed to identify effects specifically induced by thoroughly characterised *ex vivo* Aβ oligomers.
Figure 10 – Model of PrP<sub>C</sub> structure and putative Aβ binding sites

PrP<sub>C</sub> contains roughly 40% α-helical structure, with most of this condensed in a single three-helix bundle (purple and orange) (Riesner, 2003). The putative Aβ-binding sites lie within the intrinsically unstructured domain (red), but Aβ binding can also be inhibited by targeting Helix 1 of the structured domain (orange) (Freir et al., 2011). *Figure adapted from* (Purro, Nicoll and Collinge, 2018).
1.3.6 FcγRIIb as a mediator of Aβ-oligomer toxicity

Fc-gamma receptor type IIb (FcγRIIb) is a low affinity transmembrane receptor for Immunoglobulin G (IgG) most studied for its role in the immune system (Daëron, 1997). While most Fc-γ receptors (FcγRs) stimulate a positive immune response, FcγRIIb instead activates inhibitory pathways via its intracellular Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) (Smith and Clatworthy, 2010). Following cross-linking by immune complexes, this ITIM is phosphorylated by the Src kinase Lyn (Muta et al., 1994)(Malbec et al., 1998), recruiting SH2-domain-containing inositol phosphatases (SHIPs) to facilitate dephosphorylation of downstream targets (Ono et al., 1996). Recent work has found that FcγRIIb and other FcγRs are also expressed in the brain and may play a role in a variety of CNS disorders (Okun, Mattson and Arumugam, 2010; Suemitsu et al., 2010). FcγRIIb is upregulated in the hippocampus of AD brains and aged J20 mice, and application of Aβ oligomers (produced from synthetic Aβ42) upregulated FcγRIIb expression in primary neurons and SH-SY5Y neuroblastoma cells (Kam et al., 2013). This was coupled with in vitro pulldown and surface plasmon resonance (SPR) assays using recombinant rat FcγRIIb ectodomain, which appeared to show direct binding to Aβ oligomers with a K_d ~ 56 nM. Moreover, Fcgr2b K/O mice were protected against Aβ oligomer induced inhibition of LTP, and FcγRIIb deficiency rescued memory impairments in J20 mice (Kam et al., 2013). In a second paper, the same group found that both FcγRIIb K/O and antagonistic FcγRIIb antibody inhibited Aβ oligomer induced tau hyperphosphorylation, suggesting that FcγRIIb facilitates memory impairments via this pathway, which requires SHIP2 expression and involves dysregulating phosphatidylinositol 3,4-bisphosphate (PI_{3,4}P_2) metabolism (Kam
et al., 2016). While the data appear promising for some role of FcγRIIb in facilitating Aβ oligomer effects, glial expression of FcγRIIb may be responsible. Specifically, microglia appear to show substantially higher expression of FcγRIIb than neurons when using unbiased proteomic or RNA-sequencing analysis (Zhang et al., 2014; Sharma et al., 2015), and this would align more closely with the known role for other FcγRs in other immune cells throughout the body.

1.3.7 LilrB2 and PirB as mediators of Aβ-oligomer toxicity

Leukocyte immunoglobulin-like receptor B2 (LilrB2) and its mouse homolog paired Ig-like receptor B (PirB) are transmembrane receptors also primarily studied for their role in the immune system (Kubagawa et al., 1999; Shiroishi et al., 2006). Similarly to FcγRIIb, PirB is expressed on a variety of immune cells, contains extracellular immunoglobulin domains, contains the same intracellular ITIM motif, and activates similar downstream inhibitory pathways following ITIM phosphorylation by Lyn kinase (Hayami et al., 1997; Kubagawa et al., 1999). PirB ITIM phosphorylation recruits SH2-containing tyrosine phosphatase 1 and 2 (SHP-1 and SHP-2), leading to dephosphorylation of a variety of stimulatory immune mediators (Timms et al., 1998; Long, 1999; Ravetch and Lanier, 2000). Some of the ligands proposed to activate PirB are the Major Histocompatibility Complex (MHC) Class I proteins, and following the discovery that MHC Class I proteins are expressed on healthy neurons in the CNS, the role of PirB in the CNS also began to be studied (Boulanger and Shatz, 2004; Boulanger, 2009). PirB was found to be expressed on a subset of neurons in the brain, associated with synapses, and there forms complexes with SHP-1 and SHP-2 (Syken et al., 2006). Furthermore, PirB expression appears to play a role in neural function, as PirB deficient mice display higher “Ocular Dominance Plasticity” (ODP), a
measure of brain plasticity in which one eye of a mouse is removed or sutured over, and the function over time in the visual cortex associated with the remaining eye is evaluated (Syken et al., 2006). The PirB deficient mouse visual cortex improves more rapidly and to a greater extent than in WT mice, suggesting PirB plays a role in restricting brain plasticity (Syken et al., 2006). Data produced a tentative link to AD when it was later found that two mouse models of AD (APPswe and PS1dE9) show deficits in ODP from a very early age (William et al., 2012). Further, when these AD model mice were then crossed with PirB K/O mice it was found that ablation of PirB rescued a wide variety of memory deficits usually observed in these mice (Taeho Kim, Hyman, 2013). The mechanism put forward was that PirB acts as a direct receptor for Aβ oligomers, supported by data in which synthetic oligomeric Aβ1-42 was added to HEK-293 cells transiently transfected to express either PirB, or its human homolog LilrB2. Both proteins bound Aβ oligomers with nanomolar affinity, and a putative binding domain was identified in the two extracellular IgG domains D1 and D2, which show high sequence similarity between PirB and LilrB2 (Taeho Kim, Hyman, 2013). Furthermore, PirB K/O mice were immune to the LTP inhibition induced by 200 nM synthetic Aβ oligomers, suggesting an overall mechanism wherein these oligomers bind to PirB expressed on synapses and stimulate inhibitory cascades which impair synaptic plasticity (Kim et al., 2013).
1.3.8 Relevance of Aβ receptors to AD in humans

There is clear evidence suggesting that Aβ oligomer-receptor interactions may be responsible for a wide range of phenotypes observed in AD model mice. What remains to be shown is the comparative importance of each putative receptor, and the relevance of these mechanisms to the human disease. As each research group tends to focus on a single receptor, mutually incompatible conclusions are often drawn such as the observation that PirB K/O, PrP K/O, and Fcgr2b K/O mice are all invulnerable to Aβ-induced LTP inhibition (Kam et al., 2013; Kim et al., 2013; Nicoll et al., 2013). While possible that all three of these proteins are required for Aβ induced LTP inhibition, a more likely explanation stems from the fact that each group utilised different synthetic Aβ oligomer preparations and carried out slightly different assays, making the results difficult to compare. One particularly thorough study did compare the binding of synthetic oligomeric Aβ species to a wide range of receptors, and found that both cell-expressed and surface-bound PrP showed the highest affinity and sufficiency (Smith et al., 2019). However, the fact that no polymorphism within any Aβ receptor has yet been associated with AD in any genome wide association study (GWAS) (Lambert et al., 2013; Jansen et al., 2018), and no single mutation or polymorphism in any putative receptor has been found to consistently associate with increased or decreased risk of AD in whole genome analyses, leaves a dearth of evidence for the importance of Aβ receptors to the human disease. Dozens of potential Aβ receptors have been identified (Jarosz-Griffiths et al., 2016), and a variety of receptor-independent models of Aβ toxicity exist (Lashuel et al., 2002; Williams and Serpell, 2011), thus we are far from consensus about which Aβ receptors – if any – play a substantial role in AD. If any individual receptor does play a role, then it would be expected that levels of Aβ which bind
to this receptor should be increased in the brains of AD patients, thus efforts should be made to quantify levels of receptor-binding Aβ species in AD brains to ascertain how well they correlate with cognitive impairment. PrP-binding Aβ oligomers are detectable in AD brain but not healthy controls (Dohler et al., 2014), and their levels correlate with cognitive decline in a variety of mouse models (Kostylev et al., 2015), thus it would be highly interesting to compare the levels of these species in AD brains compared to High-Amyloid controls, to see whether levels of PrP-binding Aβ oligomers also correlate with cognitive impairment in humans. As Aβ levels in human brain typically show substantial variability, it would be useful to combine this work with investigations into the significant heterogeneity observed within AD populations.
1.4 Heterogeneity in Alzheimer’s Disease and the role of strains of multimeric protein assemblies

1.4.1 Heterogeneity in Alzheimer’s Disease

The underlying implication of diagnosing such a huge number of patients worldwide under the single term ‘Alzheimer’s Disease’ is that they should all be expected to undergo relatively congruent pathophysiological processes. This idea is key to almost all research in the field, but may be oversimplification to a dangerous extent. Even when only considering late onset, sAD patients (as opposed to autosomal-dominant EOAD cases), extreme heterogeneity manifests in clinical signs, biomarkers, neuropathological lesions and rates of disease progression (Van Der Vlies et al., 2009; Querfurth and LaFerla, 2010; Wilkosz et al., 2010; Van der Flier, 2016; Chen et al., 2017), thus it is possible that different pathogenic mechanisms may be at play within different AD subpopulations.

1.4.2 Variability in canonical neuropathological lesions of Alzheimer’s Disease

By definition, all AD diagnoses require the presence of substantial Aβ and tau deposition at post-mortem neuropathological assessment. The classical 1997 NIA-RI consensus recommends combining these two features into a single scale from ‘Not AD’ through to ‘High probability of AD’, with the expectation being that patients with higher amyloid load usually also have higher tau deposition. However, more than 20% of patients appear outside these guidelines, displaying high total Aβ pathology and low tau pathology, or vice versa (Nelson, Kukull and Frosch, 2010). While some of this can be explained by the known variability in standards of Braak and CERAD scoring between neuropathologists (Hogervorst
et al., 2000; Halliday et al., 2002), it is also certain that AD patients do not all die with similar levels of Aβ and tau in their brains. As Braak and CERAD scoring systems are only semiquantitative, it is not possible to carry out precise quantitative analyses of most AD neuropathology datasets. However, the variation in actual levels of pathology within even a single neuropathological ‘stage’ is huge, with one study which carried out quantitative analysis on previously ‘staged’ brains finding an interquartile range of 4 – 44% phospho-tau coverage in the entorhinal cortex of “Braak stage VI” brains, and 6.75 – 17.03% Aβ coverage in the frontal cortex of “Thal stage V” brains (Walker et al., 2017). Thus, even when only considering the two hallmark pathologies of AD, the actual levels of histological lesions vary massively between patients. To compound this issue, these singular scores for Aβ and tau belie the huge morphological heterogeneity of histopathological lesions comprised of these proteins. Specifically, much of the information about the precise protein aggregation state, conformation, and details of post-translational modification are not distinguished by typical neuropathological staining procedures. Thus, huge heterogeneity exists in the levels of specific Aβ and tau species present in the post-mortem brain across AD populations.

1.4.3 Variability in clinical symptoms in Alzheimer’s Disease

In parallel to histopathological heterogeneity in the post-mortem brain, AD patients also show huge variability in pre-mortem clinical symptoms. Prototypical AD is described as an amnestic-predominant syndrome with additional temporal-parietal malfunction, manifesting as memory decline necessarily accompanied by similar worsening of other cognitive domains (McKhann et al., 2011), but many other clinical subtypes have been described (Petersen, 1998). These
include language variants typified by early non-fluent language impairments but the relative preservation of memory (Rogalski et al., 2016), posterior cortical atrophy variants with visuospatial dysfunction (Hof et al., 1997), and frontal variants associated with dysexecutive phenotypes and prefrontal cortex dysfunction (Johnson et al., 1999; Blennerhassett et al., 2014). Furthermore, this variability is not confined to rare variants, as many reports describe the substantial clinical variability present in both sAD and fAD populations (Bird et al., 1989; Bondareff et al., 1993; Larner and Doran, 2006; Alladi et al., 2007; Snowden et al., 2007; Murray et al., 2011).

### 1.4.4 Rapidly progressive Alzheimer’s Disease

A further hypervariable feature of AD is the rate at which patients decline. The average course of AD is roughly ~8 years, but some patients experience their first symptoms >15 years prior to death, while others face much shorter disease durations. Precisely quantifying this variability is challenging for several reasons. It is difficult to precisely define ‘progression rate’ (Doody, Massman and Dunn, 2001), measurements of cognitive function are notoriously prone to error (Clark et al., 1999), and cognitive ability deteriorates at different rates during different periods of the disease (Wilkosz et al., 2010). Nonetheless, it is certain that a huge range of progression rates typify AD populations (Komarova and Thalhauser, 2011). Particularly interesting are a small subset of patients who experience a highly accelerated disease course lasting only a matter of years or even months, and some evidence suggests these patients may be suffering from a distinct clinical subtype: ‘rapidly progressive Alzheimer’s Disease’ (Rapid-AD). Rapid-AD has been variously defined as survival time less than 2 years (Schmidt et al., 2011), less than 4 years (Josephs et al., 2009), or mini-mental state
examination (MMSE) decline of >3 (Carcaillon et al., 2007), >5 (Doody, Massman and Dunn, 2001) or >8 points per year (Dumont et al., 2005), with these various definitions thus becoming very broad and including anywhere up to 30% of all AD cases. Perhaps more interesting, however, are the much smaller subset of patients who experience extremely short survival times often less than 1 year, along with clinical features strongly reminiscent of prion disease (Barcikowska et al., 1992; Reinwald, Westner and Niedermaier, 2004; Mahmoudi et al., 2010). Rapid progression of AD is associated with early motor symptoms (Mangone, 2004; Portet et al., 2009), high baseline tau and low Aβ42 levels in CSF (Snider et al., 2009), and in highly aggressive cases 14-3-3 protein is detectable in the CSF (Reinwald, Westner and Niedermaier, 2004; Mahmoudi et al., 2010), likely indicative of rapid neuronal destruction. Some studies have reported that Rapid-AD populations contain very low prevalence of the APOE ε4 allele (Schmidt, Haïk, et al., 2012; Cohen et al., 2015), which would be highly surprising and strongly suggest a unique disease mechanism is in effect. However, other publications find APOE ε4 is associated with a faster rate of decline (Cosentino et al., 2008) or has no predictive capability over progression rate (Kester et al., 2009), so the role of this allele remains uncertain.
1.4.5 Explanations for the heterogeneity of Alzheimer's Disease

All diseases have variable presentations, and often this can be explained by each human body providing a unique environment for a given disease process, rather than different variants of a disease driving different outcomes. AD is particularly susceptible to this, because as an inevitable consequence of taking place in an aging human brain, a wide variety of co-pathologies often present alongside AD. Only a minority of patients appear to die with “pure” AD neuropathology, with one study finding 27% of patients have concurrent features of vascular dementia (VaD), 16% show features of Lewy body dementia (LBD), and a further 12% showed combinations of multiple non-AD pathologies (Rabinovici et al., 2017).

In addition to the substantial overlap with vascular dementia, >95% of AD patients present with some level of cerebral amyloid angiopathy (CAA) and microvascular degeneration (Kalaria and Ballard, 1999; Jellinger, 2002), suggestive of substantial and under-investigated overlap between the mechanisms of AD and vascular pathologies. The identification of co-pathologies is also often limited by what is screened for. TDP-43 pathology has only recently been identified as a common co-pathology of AD (Amador-Ortiz et al., 2007), but a slew of analyses have since suggested it may be present in 19-57% of all AD cases (Josephs et al., 2014). It thus appears that the majority of AD patients have alternative pathological processes ongoing in the brain, many of which may lead synergistically to cognitive decline (Haroutunian et al., 2000; Schneider et al., 2009; Nag et al., 2015; Robinson et al., 2018). As these co-pathologies are so prevalent in all neurodegenerative diseases (Robinson et al., 2018), more effort should be placed on characterising the impact of secondary pathologies on clinical trial outcomes, and more investigation is warranted on the potential necessity of combination therapies to tackle multiple pathologies concurrently.
Other explanations for the observed heterogeneity in AD cohorts include the theory of ‘cognitive reserve’ in which certain life experiences such as educational and occupational attainment allow patients to tolerate higher levels of neuropathology before displaying symptoms of dementia (Stern, 2012), and the inevitable impact of genetic, environmental and lifestyle factors on both susceptibility and presentation of AD (Schmidt, Wolff, et al., 2012). While all of these features certainly contribute to the heterogeneity in AD, one other interesting possibility is that conformational variants of protein aggregates may act as pathogenic ‘strains’ which each lead to distinct biochemical pathways of neurodegeneration. This mechanism is already known to play a key role in one of the most notorious protein misfolding diseases: prion disease.

1.4.6 Prion Diseases

The Prion diseases are a set of invariably fatal neurodegenerative diseases including Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and kuru in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer and elk (Prusiner, 1998). Most human prion diseases present as rapid neurodegenerative diseases - progressing from onset of symptoms to death within a matter of months after incredibly long incubation periods, but a variety of clinical presentations characterise individual disease subtypes (Kim and Geschwind, 2015). For each of these diseases, the core mechanism relies on proteinaceous infectious agents known as prions. Uniquely for an infectious agent, prions are devoid of nucleic acids (Alper et al., 1967; Griffith, 1967) and instead consist of aggregated assemblies of misfolded PrP (Prusiner, 1998), a highly conserved and widely
expressed GPI-anchored glycoprotein enriched in neuronal and immune cell populations (Uhlen et al., 2015).

There are three distinct aetiological variants of human prion disease: sporadic, genetic, and acquired. The sporadic form accounts for more than 85% of all human cases (Chen and Dong, 2016), and is thought to arise following a single spontaneous $\PrP^C$ misfolding event. Here, the typically $\alpha$-helical protein converts to primarily $\beta$-structure, with this single misfolded species then acting as a template to recruit other correctly folded $\PrP^C$ species in an aggressive cascade which spreads in a pattern akin to a biological pathogen (Prusiner, 1998). Genetic forms of the disease arise from autosomal-dominant inheritance of mutant PrP alleles, all of which are either point mutations, truncations, or octapeptide insertions, although the mechanisms through which these mutations lead to disease are uncertain (Mead, 2006). Finally, 'acquired' forms of the disease stem either from exposure to prions through consumption of contaminated tissue (Collinge, 2001), or iatrogenic transmission during the course of medical or surgical treatment (Barrenetxea, 2012), with similar pathophysiological mechanisms taking place in both. In most cases, the end stage of prion disease is characterised by CNS build-up of protease-resistant, detergent-insoluble $\beta$-sheet-rich PrP isoforms termed $\PrP^{Sc}$ (PrP ‘scrapie’) (Meyer et al., 1986; Pan et al., 1993), alongside the archetypal neuropathological hallmarks of neuronal loss, gliosis, and spongiform change (Budka, 2003). Many facets of prion disease pathophysiology remain to be elucidated, but the underlying feature of templated propagation of protein aggregates has begun to be used as a model for studying other diseases similarly characterised by deposition of protein aggregates. While this mechanistic analogy should be used cautiously, many lessons from the prion diseases may provide useful insights.
into these other conditions, including the complex kinetic and biological properties of protein seeding and polymerisation, the impact of conformational selection of protein aggregates on the clinical presentation of disease, and the mechanistic overlap between genetic, sporadic, and acquired disease aetiologies (Collinge, 2016).

1.4.7 Nucleation-dependent polymerisation of proteins

Protein polymerisation occurs in both physiological and pathological processes, and many attempts have been made to precisely quantify its kinetic and thermodynamic properties. Early attempts based on modelling the polymerisation of actin likened it to a condensation reaction – a single-step process only occurring above a critical concentration and displaying the sigmoidal curve typical of positive cooperativity (Oosawa et al., 1959; Kasai, Asakura and Oosawa, 1962). Later work placed more emphasis on the role of ‘nucleation’, where early aggregation steps are thermodynamically unfavourable until a critical “nucleus” is formed, from which point any further polymerisation is favourable (Hofrichter, Ross and Eaton, 1974). This nucleation-dependent polymerisation model can be applied to many amyloidogenic processes, where a slow unfavourable reaction governs the conversion of monomeric or correctly folded species into a ‘nucleus’, before rapid and favourable elongation into large amyloid fibrils (Jarrett and Lansbury, 1993). These amyloid fibrils are incredibly stable, existing in a deep free energy well which prevents spontaneous breakdown (Figure 11). When a nucleus is added to a reaction from an external source, the initial rate-limiting nucleus-formation step can be skipped, and aggregation can be rapidly induced. This is described as “seeding” of the
aggregation reaction, with the terms ‘nucleus’ and ‘seed’ often used interchangeably.

Figure 11 - Energy landscape of protein folding and amyloidogenesis

Proteins are able to populate a wide variety of secondary and tertiary folds, each with distinct Gibbs free energy values. During a typical folding reaction (in green above), proteins rapidly sample increasingly stable intermediate structures. The native state of a protein then exists within a deep free energy well, and is one of the most stable conformations a given primary sequence of amino acids is able to exist in. However, in addition to their native folding pathway, amyloidogenic proteins are also able to populate β-sheet rich conformations which are able to ‘stack’ on top of one another, leading to an alternative folding pathway (in blue above) culminating in highly ordered stable amyloid fibrils. The precise structure of folding intermediates is unknown for most amyloids, and many of these intermediates may exist in their own deep free energy wells and represent a significant fraction of protein species present at equilibrium. Figure adapted from (Hartl, Bracher and Hayer-Hartl, 2011).
Many amyloidogenic aggregates are able to act as a ‘seed’ *in vitro* to induce natively folded (or unfolded) monomers of that same protein to misfold and become part of their misfolded assembly (Jucker and Walker, 2011). Thus, introducing tiny quantities of aggregated recombinant PrP peptides to a large pool of PrP monomer induces rapid fibrillisation reactions until all of the monomeric substrate is consumed (Come, Fraser and Lansbury, 1993). Similar results are seen when introducing Aβ aggregates to Aβ monomer pools (Jarrett, Berger and Lansbury, 1993), α-synuclein aggregates to α-synuclein pools (Murray et al, 2003), and tau aggregates to monomeric tau pools (Friedhoff *et al.*, 1998), but kinetic specificities differ substantially between amyloidogenic peptides. In the case of Aβ, substantial differences are seen even between Aβ1-40 and Aβ1-42 peptide variants, with the critical concentration of Aβ1-42 commonly observed to be 4-5 fold lower than equivalent Aβ1-40 experiments (Soreghan, Kosmoski and Glabe, 1994). Notably, *in vitro* Aβ fibrillisation has not been reported below micromolar concentrations, yet the Aβ levels in human CSF reside in the low nanomolar range (Olsson *et al.*, 2016). Thus, Aβ fibrillogenesis in the brain must require either: areas where localised Aβ concentrations reach the micromolar range (such as the endosomal system (Hu *et al.*, 2009)), a cofactor in the human brain which lowers the critical concentration to nanomolar values (such as metal ions (Atwood *et al.*, 1998)), involvement of alternative Aβ peptides with lower critical concentrations (such a pyroglutamate-modified Aβ (Nussbaum *et al.*, 2012)), or exposure to amyloidogenic seeds. These intricacies prove that while *in vitro* studies can help us quantify kinetic and thermodynamic constants for protein polymerisation within carefully controlled systems, they are of limited use when interpreting protein polymerisation within the behemoth chemical complexity of biological organisms.
1.4.8 Protein aggregate seeding *in vivo*

One of the key tools in studies of prion disease has long been the mouse bioassay, in which mice are intracerebrally inoculated with brain homogenate from human prion disease patients (Wadsworth, Asante and Collinge, 2010). This has proven to be one of the most faithful representations of any human disease in a mouse, as these models reproduce many features of the human disease including long clinically silent incubation periods, PrP\(^{Sc}\) deposition, spongiosis and neurodegeneration. This model has since been adapted to a variety of other neurodegenerative diseases, and now a plethora of studies have shown that Aβ (Kane *et al.*, 2000), tau (Clavaguera *et al.*, 2009), amyloid protein A (Lundmark *et al.*, 2002) and α-synuclein (Luk *et al.*, 2012) aggregate deposition can all be induced in the brains of mice (usually mice genetically engineered to produce more monomeric substrate for aggregation) by intracerebral inoculation with their respective protein aggregates (*Figure 12*).
Deposition of protein aggregates can be induced in many mouse models of neurodegenerative disease by intracerebral inoculation with human brain extracts or purified synthetic aggregates of the target protein. (A) The mouse models utilised typically either overexpress the target protein or express mutant variants which are prone to aggregation, thus the mice will usually develop some level of protein deposition during their normal lifespan (blue spots in top right image). (B) However, following intracerebral inoculation with protein aggregates or brain extract, protein aggregate deposition can be significantly accelerated. In these mice, protein aggregates can typically be observed at earlier time points than uninoculated mice (bottom centre image) and much higher levels of protein aggregate deposition can be observed from the same incubation period (bottom right image).

While most of these models do not fully recapitulate the human disease phenotype in the same way as the prion bioassay, they still provide valuable information on the mechanisms via which these proteins seed in vivo. A particular focus of study has been induction of Aβ pathology, where many models have been adapted for mice (Kane et al., 2000), rats (Frautschy, Baird and Cole, 1991), and non-human primates (DeJesus-Hernandez et al., 2011). In mice, the most common experimental paradigm has been intracerebral inoculation of AD human brain homogenate into mice overexpressing mutant forms of APP (Kane et al., 2000; Walker et al., 2002; Langer et al., 2011; Fritschi, Langer, et al., 2014). Successful seeding requires the presence of human APP (Meyer-Luehmann et al., 2006), and most mouse models expressing human APP will develop Aβ deposits in their normal lifespan, thus these models may represent
acceleration of native Aβ deposition rather than *de novo* induction of pathology. However, Aβ deposition can also be induced in human APP-transgenic rodents which would not otherwise develop Aβ within their normal lifespan (Morales *et al.*, 2012; Rosen *et al.*, 2012), and in mice expressing human APP at endogenous levels without overexpression (Ruiz-Riquelme *et al.*, 2018). The seeding activity of brain extracts is dependent on aggregated forms of Aβ (Meyer-Luehmann *et al.*, 2006), and both the supernatant and pellet from ultracentrifugation of the human brain homogenate possess seeding activity, suggesting seeds of a wide variety of sizes exist (Langer *et al.*, 2011). Furthermore, Aβ-contaminated growth hormone treatments which were capable of seeding iatrogenic Aβ deposition in humans (Jaunmuktane *et al.*, 2018) are also capable of seeding Aβ deposition in transgenic mice (Purro *et al.*, 2018), confirming that such mouse models provide readouts of Aβ seeding with direct human relevance. However, AD CSF does not effectively seed Aβ deposition (even when normalised to total Aβ levels) suggesting a key role for species of Aβ specifically present in the brain but not detectable in CSF (Fritschi, Langer, *et al.*, 2014). Unlike prion disease, oral, intraventricular, intraocular and intranasal treatment appear unable to effectively seed Aβ *in vivo*, but surgical implantation of brain-extract-coated steel wires does (Eisele *et al.*, 2009), and intraperitoneal injections can also seed pathology (albeit less effectively than intracerebral injection) (Eisele *et al.*, 2010). Aβ seeds resist inactivation by formaldehyde (Fritschi, Cintron, *et al.*, 2014) and high temperatures, but are inactivated by plasma sterilisation (Eisele *et al.*, 2009), and Aβ seeding activity is increased by sonication of brain extract (Langer *et al.*, 2011). Synthetic Aβ appears capable of seeding Aβ aggregation in mice, but with a much lower specific activity than brain-derived species. However, preincubating synthetic Aβ
on WT mouse hippocampal slice cultures in the presence of brain homogenate appears to generate substantial seeding activity, suggesting a cofactor present in the hippocampal slice is able to convert synthetic Aβ into an effective seed (Frodel, 2005). In transgenic rats, intracerebral inoculation with human brain extract also effectively seeds Aβ pathology (Frautschy, Baird and Cole, 1991; Rosen et al., 2012), and effective seeding with synthetic Aβ peptides has also been reported (Roth, Tomlinson and Blessed, 1967; Kowall et al., 1991). In all papers listed, very little characterisation is shown for inoculated synthetic Aβ peptides, so it is difficult to know if differences observed between experiments are due to unique biological mechanisms, or simply differences in the structure of Aβ peptides injected. Some work has also been done using marmosets, where inoculation with AD brain extract once again induces Aβ deposition after long incubation periods (Maclean et al., 2000; Ridley et al., 2006; DeJesus-Hernandez et al., 2011).

These mechanisms of proteopathic seeding also extend to humans. In the archetypal example, prion diseases can be transmitted between humans via blood transfusion (Puopolo et al., 2011), dura mater transfer (Wang et al., 2012), treatment with cadaver-derived growth hormone (Rudge et al., 2015), or historically by consumption of human tissues during mortuary feasts in the case of the prion disease kuru in Papua New Guinea (Collinge et al., 2006). In every case, the underlying mechanism is thought to be the transfer of misfolded ‘seeds’ of PrP to the new host, which then nucleates the host’s own monomeric PrP into aggregates which propagate throughout the body as part of the devastating wider disease process. Similar mechanisms may explain observations of substantial cerebral Aβ pathology in patients that have undergone neurosurgery
(Jaunmuktane et al., 2018), received contaminated dura mater grafts (Frontzek et al., 2016; Hamaguchi et al., 2016; Kovacs et al., 2016), or received human growth hormone treatments (Jaunmuktane et al., 2015; Ritchie et al., 2017).

1.4.9 Prion-like spread

In addition to providing insight for how processes of protein aggregation can be transmitted between patients, understanding the mechanisms of seeded polymerisation may also provide crucial understanding of how neurodegenerative diseases progress within a single brain. For example, deposition of Aβ and tau aggregates in AD patients follows a roughly predictable temporospatial pattern (Braak et al., 2011; Grothe et al., 2017; Jack, Wiste, et al., 2018), but the reasons for this are unclear. One possibility is that certain brain regions produce more favourable environments for aggregate deposition, perhaps via higher expression levels of amyloidogenic proteins or cofactors, higher brain activity levels, or reduced local clearance mechanisms. Thus, aggregate deposition could begin in these regions, and the temporospatial ‘spreading’ of pathology could be explained by ‘selective vulnerability’, with regions with progressively favourable deposition environments succumbing in a sequential fashion (Walsh and Selkoe, 2016). However, an alternate theory proposes that following the early stochastic misfolding of proteins within one brain region, the resulting spread throughout the brain is driven by direct templated misfolding and the subsequent physical transport of seeds throughout the brain (Frost and Diamond, 2010). This is likely the case in sporadic prion disease, where it is thought a single PrP misfolding event leads to aggressive widespread protein aggregation and neuronal death, as the initial stochastic
nucleus-formation is incredibly rare and unlikely to occur at multiple points within a single brain in a human lifetime. However, a critical and common mistake is to fail to delineate between physical spreading of protein aggregates and spatiotemporal spreading of aggregate deposition. The amyloid deposits observed during post-mortem neuropathological analyses represent huge, insoluble deposits comprised of many thousands of Aβ monomers which are far removed from the early aggregation steps in which Aβ monomers are converted into soluble oligomers. Thus, just because certain areas tend to show Aβ deposition earliest in the disease course, does not mean this is the same location that original oligomerisation took place, as it is possible that levels of small oligomers first rise throughout the entire soluble fraction of the brain before then depositing in certain areas due to those areas being selectively vulnerable to Aβ deposition, rather than selectively favourable environments for amyloidogenic nucleation. Unless sensitive PET ligands are created which selectively bind to smaller oligomeric species, it will be difficult to longitudinally and spatiotemporally assess the aggregation state of Aβ in the brain throughout the disease. Thus, in AD it is difficult to know whether the massive widespread deposition of protein seen in the end-stage brain of an AD patient is the result of a single stochastic nucleation event which then spreads throughout the brain, or results from a plethora of stochastic nucleation events happening throughout the nervous system in sequentially vulnerable regions. Even in the case of mouse inoculation studies it is often unclear whether the pattern of aggregate deposition observed is due to ‘spreading’ from the site of injection to connected regions, or if the injection creates a pool of aggregates throughout the CSF which then leads to deposition in selectively vulnerable regions. One alternative model which provides evidence for the former scenario involves selective overexpression of
an amyloidogenic protein within a single mouse brain region. Following overexpression of tau protein within a single brain region (either using specific promoters or stereotaxic injection of lentiviral vectors for human tau), tau pathology spreads to neurons downstream in synaptic circuits, even when those recipient neurons do not overexpress tau (De Calignon et al., 2012; Liu et al., 2012; Caillierez et al., 2013). This strongly supports that tau pathology is able to spread directly to interconnected brain regions, but similar evidence is yet to be provided for Aβ.

1.4.10 Decoupling protein seeding from toxic mechanisms in neurodegenerative disease

Whatever the mechanism of protein seeding is in neurodegenerative disease, it is key not to equate the propagation of protein aggregates with the progression of the disease itself, as we still lack a great deal of understanding as to which aggregates, intermediates or by-products of these thermodynamic processes are actually necessary and responsible for the death of cells in the degenerating brain. For example, end stage prion disease is often characterised by the build-up of PrPSc, which invites the conclusion that PrPSc may be the neurotoxic species responsible for the widespread neuronal death observed in post-mortem prion disease brains. However, multiple studies have reported mice who carry incredibly high levels of PrPSc equivalent to those seen in end stage prion disease, yet live a typical life-span without apparent neurodegeneration (Hill et al., 2000; Race et al., 2001; Thackray et al., 2002; Thackray, Klein and Bujdoso, 2003). This supports a more complex model in which smaller PrP aggregates, cofactors, or off-pathway intermediates are the true agents of toxicity. Similarly, in AD, a range of evidence suggests that oligomeric Aβ aggregates are the key
agents driving disease, but it also seems likely that certain Aβ assemblies are either pathogenic or inert (see 1.3.3) Crucially, we do not even know if relevant species are ‘on-pathway’ intermediates in the same aggregation reaction which leads to the final amyloid fibrils observed in the end-stage brain, or ‘off-pathway’ variants with entirely separate reaction kinetics. This exemplifies one further complex facet of protein aggregation in disease: the existence of multiple aggregation pathways for any given protein, in which certain pathways may lead to the production of potently toxic species, while others may produce relatively inert aggregates. In each case, observed levels of end-state, insoluble histopathological lesions may be poor representatives of the soluble phase. That differences in the structure of protein aggregates may potentially lead to staggering differences in biological activity has already been proven in prion disease, where differences in PrP aggregate conformations underlie the diversity of prion strains which cause vastly different clinical phenotypes.

1.4.11 Strains in prion disease

One of the fundamental conundrums regarding prions is their ability to encode mutable information and specify many distinct disease phenotypes despite containing no genetic material. All prions are composed of aggregated isoforms of PrP, yet multiple ‘strains’ of prions exist which can produce astoundingly disparate clinical and neuropathological signatures (Collinge and Clarke, 2007), many of which can be serially propagated in animal models (Wadsworth, Asante and Collinge, 2010; Telling, 2011). Prions replicate by recruiting the host’s own endogenous PrP\textsuperscript{C}, and yet inoculation of two genetically identical mice with two different strains of prions can lead to maintenance of the original strain.
characteristics – thus the differences between strains cannot be solely explained by differences in primary sequence of PrP (Collinge et al., 1996; Telling et al., 1996). Instead, the data appears to be transferred via ‘conformational selection’, in which prion strains are each composed of distinct PrP conformations or assembly states, and each strain will induce native PrP to adopt its own specific misfolded conformation as it joins the infectious PrP assembly (Wadsworth and Collinge, 2011). This model has been well characterised in yeast prions (Kawecki and Ebert, 2004; King and Diaz-Avalos, 2004; Tanaka et al., 2004; Diaz-Avalos et al., 2005), and are supported by data that PrP aggregates present in human and other mammalian prion strains show differences in protease resistance, western blot glycosylation patterns, thermal denaturation curves, conformational-dependent immunoassays and metal ion binding (Safar et al., 1998; Kuczius and Groschup, 1999; Wadsworth et al., 1999; Safar, Cohen and Prusiner, 2000; Biacabe et al., 2004).

One would expect that for a given primary sequence of PrP, only a finite number of stable β-sheet structures can be populated, and thus that a PrP aggregate of a given conformation could only act as a seed for a second PrP molecule of a different primary structure if that substrate PrP was also able to fold into the same conformation. This is precisely what is thought to underlie the “species barrier” of prion disease – the observation that transmission of prions between different species (or between two members of the same species with different versions of the PrP allele) is much less efficient than transmission between organisms with identical PrP primary structure (Collinge, 1999; Collinge, 2001). If the primary structure of the PrP in the host animal renders it incapable of folding into the same conformation as the PrP within the prion strain, seeding will be inefficient.
or impossible. This plays a critical role in the human prion diseases, where polymorphisms within the PrP allele are known to substantially alter human susceptibility to both transmissible and sporadic forms of the disease (Palmer et al., 1991; Mead et al., 2003), and also play key roles in strain selection (Collinge & Hill, 1996; Wadsworth et al., 2004). In a final layer of complexity, prion infected tissue can contain multiple different strains of prions in varying ratios, and in cell culture minor subpopulations can be selected for in Darwinian fashion (Li et al., 2010). This supports a model in which individual prion strains are actually comprised of a cloud of diverse PrP-containing aggregates each with competing kinetics that can interact uniquely with different hosts, and even different tissues or brain regions within a single host (Collinge and Clarke, 2007; Collinge, 2010).

1.4.12 Evidence of Aβ strains in Alzheimer’s Disease

While the concept of strains is well established in prion disease, it is not yet proven whether similar mechanisms play an important role in other neurodegenerative diseases. For misfolded protein strains to play a meaningful role in disease, two criteria must be satisfied. First, the protein in question must be able to aggregate into multiple possible conformations which can be biochemically or biophysically differentiated. Second, it must be shown that these conformations (or some specific intermediate or process of their respective aggregation pathways) play a causative role in different clinical outcomes, and are not simply epiphenomena or downstream of the true causal events. In AD, it is well established that a plethora of heterogeneous Aβ aggregates populate the brain (Tycko, 2015; Esparza et al., 2016; Liu et al., 2016), but it is less certain if – or how – these different species may lead to different disease outcomes.
One set of evidence in support of a role for Aβ strains in AD are correlative studies, wherein different Aβ structures are selectively found at post-mortem in patients who had suffered different subtypes of the disease. Some reported differences between patients with different subtypes include variable Aβ plaque morphology observed by light microscopy (Bondareff et al., 1987; Maarouf et al., 2008), different spectral characteristics following binding of amyloid-targeting dyes (Nilsson et al., 2007; Condello et al., 2018), differences in Aβ aggregate protease resistance profiles and proteomic signatures (Di Fede et al., 2018), and differences in Aβ seeding activity (Qiang et al., 2017). While differences have most commonly been reported between autosomal-dominant AD and sAD cases, multiple studies have also compared subtypes of sAD patients and revealed different Aβ signatures in posterior cortical atrophy and rapidly progressive AD cases compared to ‘typical’ AD cases (Schmidt et al., 2010; Qiang et al., 2017; Rasmussen et al., 2017). These data confirm that at post-mortem, different subtypes of AD are characterised by conformationally disparate Aβ aggregates, but they cannot inform whether these structures are responsible for the observed clinical differences, or instead if differences within the brain environments of clinically distinct patients lead to production of different Aβ structures. The strongest evidence in support of the first scenario stems from in vivo Aβ seeding models.

Many groups have reported induction or acceleration of Aβ pathology in animal models following inoculation with Aβ-containing brain extracts, but the specific pathology induced depends on both agent and host (Meyer-Luehmann et al., 2006). Inoculation of APP23 mice with APP/PS1 mouse brain extract induces Aβ deposition with morphological, conformational, and Aβ42/40 ratio similar to
APP/PS1 mice, suggesting that APP/PS1 brain extracts contain Aβ seeds which can induce pathology in their own image (Heilbronner et al., 2013). Similar results can be seen after inoculating AD model mice with human brain extract from AD subtypes. APP23 mice inoculated with brain extracts from fAD patients possessing the Arctic mutation E693G (Arctic-AD) or Swedish mutation KM670/671NL (Swedish-AD) exhibit pathology which can be differentiated in terms of Aβ isoforms and the specific presence of Aβ38-containing CAA in Arctic-AD cases (Watts et al., 2014). Critically, these differences are maintained during second passage into a subsequent mouse. Similar experiments have revealed differences between fAD-inoculated and sAD-inoculated mice (Di Fede et al., 2018), as well as between ‘typical’ AD and posterior cortical atrophy variant AD inoculated mice (Rasmussen et al., 2017). The fact that the structural characteristics are maintained during passage into a new mouse host suggests individual subtypes of AD contain distinct Aβ ‘seeds’ which are able to nucleate distinct structures of Aβ aggregates, rather than the structures simply being shaped by the different environments within the host brain. However, this still does not prove that structural differences in the Aβ aggregates play a causative role in differentiating the clinical phenotype between subtypes, and more work is needed to tie together these biophysical characteristics with actual biological effects. Further, there are a variety of other AD subtypes which have not yet been investigated using in vivo seeding models. In particular, brain extracts from Rapid-AD patients contain distinct structures of amyloid-β (Cohen et al., 2015), and seed significantly more variable Aβ fibril structures than typical AD brain extracts in vitro (Qiang et al., 2017), thus these brains extracts should be investigated for their seeding activity in vivo.
1.5 Project rationale

1.5.1 Aim 1 – Investigating the seeding of brain extracts from Rapid-AD patients in human APP knock-in mice

Deposition of amyloid-β pathology can be induced or accelerated in mouse models by intracerebral inoculation with AD brain extracts (Kane et al., 2000; Walker et al., 2002; Fritschi, Cintron, et al., 2014), and the induced pathology can vary dependent on the brain extract used (Stöhr et al., 2014). Previous work has shown that in vitro, amyloid-β purified from Rapid-AD brains seeds distinct conformations of Aβ fibrils compared to similar preparations from typical AD brains (Qiang et al., 2017), suggesting that clinical differences between these patients may have been caused by distinct conformational strains of Aβ. However, no work has yet been carried out to investigate the in vivo Aβ seeding activity of Rapid-AD brain extracts. In this work, APP knock-in mice are inoculated intracerebrally with brain homogenates from Rapid-AD and typical AD patients and culled at multiple time-points. Biochemical and histopathological quantification of the Aβ present in the brains of these mice is used to identify whether Rapid-AD brains contain distinct in vivo Aβ seeding activity.

1.5.2 Aim 2 – Investigating the levels of PrP-binding Aβ species present in AD brain compared to non-demented high pathology controls

PrP binds to oligomeric assemblies of Aβ with high affinity (Chen, Yadav and Surewicz, 2010; Nicoll et al., 2013; Dohler et al., 2014; Ganzinger et al., 2014). This interaction is responsible for a variety of synaptotoxic phenotypes in AD mouse models (Chung et al., 2010; Gimbel et al., 2010; Barry et al., 2011; Klyubin et al., 2014), and PrP-binding Aβ oligomers are found in the brains of AD patients (Dohler et al., 2014). However, it is not yet known whether PrP-binding
Aβ oligomers are enriched in the brains of AD patients compared to non-demented high pathology controls. In this work, the binding between PrP<sup>C</sup>-Aβ is characterised using a cellular transient transfection model, and this interaction is compared to multiple other putative Aβ oligomer receptors. We then use a novel plate-based assay to quantify the levels of PrP-binding Aβ species present in brain homogenate from humans who died with a range of Aβ plaque loads, including a subgroup of patients who died with high levels of amyloid pathology but no cognitive impairment.

1.5.3 Aim 3 – Investigating toxicity contained in isolated fractions of AD brain extract

Many groups which study mechanisms of toxicity in AD use extracts of human brain produced via vigorous tissue homogenization techniques. These techniques likely lead to disruption and solubilisation of large Aβ aggregates which would have been sequestered in insoluble deposits in the living human brain, as well as release of a variety of lipid and carbohydrate moieties, all of which may lead to artefactual readouts of toxicity. In this work, a gentle ‘soaking’ method for brain extraction is compared to typical homogenisation methods on both typical AD and Rapid-AD brains. We show that despite containing only a fraction of the total protein content, this diffusible extract retains the majority of the neurotoxicity, suggesting this activity stems from readily soluble species and that harsh homogenisation techniques are unnecessary.
Chapter 2– Materials and Methods

2.1 Materials and Equipment

All chemicals and reagents were of analytical grade or the highest purity available. Deionised water (DI H₂O) was obtained from a Barnstead™ Nanopure™ Diamond water system (Thermo Scientific).

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<tr>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
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### 2.1.1 Antibodies

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## 2.1.3 Equipment

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## 2.1.4 Software

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2.2 Methods

2.2.1 Human tissue samples

Anonymised post-mortem human brain tissue from frontal cortex was kindly provided by the Queen Square Brain Bank for Neurological Disorders at UCL Queen Square Institute of Neurology, the Oxford Brain Bank at Oxford University Hospitals NHS Trust, the Banner Sun Health Research Institute in Arizona, and Massachusetts General Hospital in Boston. Samples were obtained and used in accordance with the requirements of each providing tissue bank.

Ethical approval for studies involving post-mortem human tissue and mice is provided by London Queen Square Research Ethics Committee REC reference: 03/N038. The storage, biochemical analysis of human tissue samples and transmission studies to mice were performed in accordance with informed consent from all patients or a person in a qualifying relationship to the deceased, or a legal representative in accordance with applicable UK legislation and Regulatory Codes of Practice.

2.2.2 Human brain tissue extraction

2.2.2.1 Production of crude human brain homogenates for mouse inoculation

These crude homogenates were produced by Jonathan Wadsworth. Tissue specimens, stored frozen in sealed pots within a level 3 containment laboratory, were transferred into a class 1 microbiological safety cabinet and partially thawed
and placed on a petri dish. A suitable quantity of tissue (grey matter) was excised using a scalpel, sealed in a disposable plastic pot, and weighed. The tissue was then prepared as a 10% (w/v) homogenate in Dulbecco’s sterile PBS lacking Ca\(^{2+}\) and Mg\(^{2+}\) ions. The amount of PBS to add in microliters was equal to 9 times wet weight of tissue in milligrams. This calculation produced a homogenate very close to a true 10% (w/v) ratio without the necessity of having to accurately measure the total volume of tissue in PBS before the homogenization process. Homogenisation of brain tissue was achieved through the use of a glass Duall tissue grinder. The 10% homogenate was stored as aliquots in 2 ml screw-top microfuge tubes at -80 °C. 1% (w/v) inocula was prepared by 10-fold dilution of 10% (w/v) homogenate in Dulbecco’s sterile PBS lacking Ca\(^{2+}\) and Mg\(^{2+}\) ions followed by thorough vortexing. Aliquots are dispensed and stored frozen in 2 ml screw-top microfuge tubes at -80 °C.

### 2.2.2.2 Diffusible brain extract preparation

Under supervision of Wei Hong (Harvard Medical School) and Dominic Walsh (Harvard Medical School), brain extracts were produced in accordance to the protocol described in (Hong et al., 2018). Briefly, approximately 5 grams of frozen frontal cortex was excised and blood vessels removed using a scalpel and tweezers. Each chunk was then sliced into ~1mm\(^3\) chunks using a scalpel. The diced tissue was gently mixed and divided in two. One half was used to prepare homogenised ‘H extract’ and the other to prepare soaked ‘S extract’. Both extracts were prepared using artificial spinal fluid base buffer (aCSF-B) (124 mM NaCl, 2.8 mM KCl, 1.25 mM NaH\(_2\)PO\(_4\), 26 mM NaHCO\(_3\), pH 7.4) supplemented with protease inhibitors (5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM
ethyleneglycoltetraacetic acid, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin, 120 μg/ml Pefabloc and 5 mM NaF). H extracts were prepared by homogenizing tissue in 5 volumes of ice-cold aCSF-B with 25 strokes of a motorized Tefon-glass Dounce homogenizer (Fisher, Ottawa, Canada). Resulting 20% (w/v) homogenates were centrifuged at 200,000 x g at 4 °C for 110 min in a SW41 Ti rotor (Beckman Coulter, Fullerton, CA). The upper 80% of the supernatant was removed and designated as 'H-extract'. S-extracts were prepared by incubating tissue in 5 volumes (w/v) of ice-cold aCSF-B at 4 °C for 20 min with gentle side-to-side mixing. Thereafter, this suspension was centrifuged at 2000 x g for 10 min at 4 °C. The upper 90% of the supernatant was removed and centrifuged at 200,000 x g at 4 °C for 110 min in a SW41 Ti rotor. The resulting supernatant was removed and designated as S extract. H2 extracts were prepared using the pellets generated when preparing S-extracts. The 2000 x g and 200,000 x g pellets were pooled and homogenized in 5 volumes (w/v) of ice cold aCSF-B, and centrifuged at 200,000 x g 4 °C for 110 min. The upper 80% of supernatant was removed and designated as H2 extract. H, S and H2 extracts were then dialyzed against fresh aCSF-B. Fifty milliliter of extract was dialyzed (using Slide-A-Lyzer™ G2 Dialysis Cassettes, 2 K MWCO, Fisher Scientific) against a 100-fold excess of fresh aCSFB at 4 °C, with buffer changed three times over a 72 h period.

2.2.2.3 Immunodepletion of human brain extracts

To investigate Aβ-specific toxicity of human brain extracts, 1 ml aliquots of brain extract were removed from -80 °C, thawed on ice, and split into two 500 µl aliquots. One aliquot was immunodepleted (ID) using three rounds of 12 h
incubations at 4 °C with the polyclonal anti-Aβ antibody S97 pre-conjugated to 20 µl Protein A Sepharose (PAS) beads. The second aliquot was ‘mock’ immunodepleted using pre-immune serum pre-conjugated to 20 µl PAS beads. For western blot analysis, immunodepleted Aβ was eluted by boiling beads in 20 µl of 2X sample buffer (50 mM Tris, 2% w/v SDS, 12% v/v glycerol with 0.01% phenol red).

2.2.2.4 Buffer exchange of human brain extracts

For use in neuronal toxicity assays, brain extracts were buffer exchanged into the same media used to grow the cells, in order to prevent the cells experiencing shock from media change which could lead to non-specific toxicity. Specifically, 0.5 ml aliquots of brain extract were thawed on ice for 30 min, then applied to a HiTrap 5 ml desalting column (GE Healthcare, Milwaukee, WI) using a 1 ml syringe at a flow rate of 1 ml min⁻¹, and then eluted into Neurobasal medium supplemented with B27/Glutamax. Eight 250 µl fractions were collected, and these were analysed by MSD immunoassay to ascertain the precise levels of Aβx-42 present within each fraction. Fractions containing the highest levels of Aβ were pooled and used in subsequent incucyte neurite retraction assays.

2.2.3 Biochemical analysis of tissue and cell extracts

2.2.3.1 MSD Analysis

Meso Scale Discovery (MSD) electro-chemiluminescence immunoassays were carried out to quantify a variety of protein species. To quantify denatured, monomeric species, samples were first treated with 6M Guanidine Hydrochloride
(GdnHCl) overnight at 4 °C, before subsequent dilution down to ≤0.5 M GdnHCl.

For these assays, standards were diluted in PBS containing equimolar GdnHCl. For quantification of native species, samples were diluted directly in PBS. Table 2 shows the assay conditions and antibody concentrations used for each specific quantification. For all MSD assays, detection was carried out by addition of 2X MSD read buffer (150µl/well) followed by signal determination in an MSD QuickPlex SQ 120. For commercial assays, LLOD and LLOQ were automatically quantified by MSD Discovery Workbench software. For custom assays, LLOQ was defined as the lowest concentration of calibration standard which both displayed a signal higher than (mean blank signal + (9 * standard deviation of blank signal)), and had a percentage recovery > 80% and < 120%.

Table 2 - Conditions for MSD-immunoassays

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**2.2.3.2 Western blots**

Multiple buffer systems were used depending on sample origin and target protein. A general protocol using Tris-Glycine buffers and Licor infrared detection follows. Samples were diluted 1:1 in Novex™ Tris-Glycine SDS Sample buffer (2X), boiled at 95°C for 10 min, and then centrifuged at 13,000 rpm for 10 min. Samples and SeeBlue® Plus 2 Pre-stained Standard were loaded onto NuPAGE® 4-12% Bis-Tris gels and run in 1x NuPAGE® MES SDS Running Buffer for 15 min at 100 V, then 1 h at 150 V. Protein was transferred onto Optitran® BA-S 85 nitrocellulose membrane for 2.5 h at 30 V (then microwaved for 2 min in PBS when aiming to detect Aβ) and then incubated with 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid to allow labelling of molecular weight markers and lanes on membrane. Ponceau S was washed off using DI H₂O and the membrane was incubated with Odyssey Blocking Buffer (contains non-mammalian blocking reagent and 0.1% NaN₃ in PBS) for 1 h at RT. Antibodies were diluted in Odyssey® Blocking Buffer diluted 1:1 in 0.05% PBS-T and primary antibody incubations were carried out overnight at 4 °C, while secondary antibody incubations were added for 1 h at room temperature (RT). Membranes were visualised using either an Odyssey® infrared scanner or Pierce ECL Plus. Intensity of bands was quantified using Licor Image Studio Lite v5.2.
2.2.3.3 BCA assay

To generate a standard curve bovine serum albumin (BSA) in concentrations ranging from 0.03125 mg/ml to 2 mg/ml were created in DI H₂O and loaded onto a clear-bottomed 96-well plate. Samples were diluted in DI H₂O and loaded in triplicate on the same 96-well plate. BCA assay solution and 4 % CuSO₄ solution were mixed in a ratio of 50:1 and 200 μl of this mixture was added to each well, then the reaction was incubated for 20 min at 37° C. The absorbance was then measured at 570 nm and protein concentration calculated using the standard curve.

2.2.4 Preparation and characterisation of monomeric and aggregated Aβ

2.2.4.1 Preparation of oligomeric Aβ

Oligomeric Aβ (o-Aβ) was prepared according to a modified version of the Klein lab’s ‘ADDLs’ protocol (Lambert et al., 1998). 1.25 mg of lyophilised human Aβ₁₋₄₂ (or lyophilised biotinylated Aβ₁₋₄₂) was dissolved in ice-cold hexafluoro-2-propanol (HFIP) to a concentration of 1 mM, sonicated for 5 min and then incubated for 1 h at RT. HFIP was then evaporated overnight (O/N) in a fume hood and the resultant Aβ₁₋₄₂ film dissolved in anhydrous DMSO to a final concentration of 5 mM by vortexing for 5-10 min. Ham’s F12 media (F12) lacking glutamine and phenol-red- was added to produce a 100 μM Aβ solution, then the solution was incubated at RT for 72 h to induce aggregation. Next, samples were centrifuged at 13,000 x g for 15 min to pellet any large insoluble aggregates, then the upper 90% of supernatant was collected in aliquots which were flash frozen in liquid nitrogen before storage at -80 °C.
2.2.4.2 Preparation of monomeric Aβ\textsubscript{1-42}

Monomeric Aβ\textsubscript{1-42} was obtained by diluting 1 mg of lyophilised Aβ\textsubscript{1-42} in 500 µl of 6 M GdnHCl, then isolating the monomeric peak using a superdex 75 10/300 column, eluting in 50 mM ammonium bicarbonate, pH 8.5. Peptide concentration was determined using the molar extinction coefficient for tyrosine (ε\textsubscript{275} = 1361 M\textsuperscript{-1} cm\textsuperscript{-1}), then diluted to 5 µM in 50 mM ammonium bicarbonate, pH 8.5, aliquoted, and snap frozen on dry ice.

2.2.4.3 Electron microscopy of oligomeric Aβ preparations

To characterise the morphological characteristics of our oligomeric Aβ preparations, ~ 100 µM stocks of o-Aβ were thawed at RT and diluted 10-fold in 50 mM ammonium bicarbonate, pH 8.5. 300 mesh carbon coated copper grids (Electron Microscopy Sciences) were glow discharged for 40 sec using an EMS 100 x glow discharge unit (Electron Microscopy Sciences, USA). Two minutes after loading the sample, grids were blotted with filter paper (Whatman, Grade 1), washed briefly in two drops of water and then stained with Nano-W methylamine tungstate negative stain for 45 sec. Excess stain was removed with filter paper and grids allowed to dry in air. Samples were examined on Talos 120C Electron Microscope (FEI, Eindhoven, NL) at an accelerating voltage of 120 kV.

2.2.4.4 SEC-MALS of oligomeric Aβ preparations

To characterise the aggregation state of our o-Aβ preparations, aliquots of o-Aβ (500 µl) were injected onto a Size Exclusion Chromatography column (Superdex 75 10/300 GL, GE Healthcare) coupled with an Eclipse DualTec (Wyatt
Technology, Santa Barbara, CA, USA) and eluted with 50 mM ammonium bicarbonate, pH 8.5. The sample was injected at 0.5 ml \( \text{min}^{-1} \). Refractive index was measured using a Wyatt Optilab T-rEX and Light scattering was performed using a Wyatt Dawn Heleos II multi-angle light scattering module to calculate the molar mass.

### 2.2.5 Preparation of plasmid DNA

#### 2.2.5.1 Transformation of competent cells

50 µl aliquots of DH5α™-T1R MAX Efficiency competent bacteria were removed from -80 °C and thawed on ice for 30 min prior to transformation. 10 ng of desired plasmid DNA was added to chilled competent cells which were then gently mixed before incubating on ice for 30 min. Cells were then subjected to heat shock at 42 °C for 30 seconds in a heat block and placed back on ice for 3 min. 500 µl pre-warmed Super Optimal broth with Catabolite repression (S.O.C media) was added to each reaction, and cells were grown at 37 °C in a shaking incubator for ~ 1 h. During this hour, LB-agar plates containing either 100 µg/ml ampicillin or 50 µg/ml kanamycin were pre-warmed in a 37 °C incubator for 15 min, then bacteria were streaked across LB-agar plates, grown overnight at 37 °C and checked for colonies the following day. Individual colonies were picked and added to Luria broth containing either 100 µg/ml ampicillin or 50 µg/ml kanamycin to be grown for maxipreps.
2.2.5.2 Maxipreps

Plasmid DNA was isolated from bacteria using QIAprep® Spin Maxiprep Kit as per the manufacturer’s instructions, with minor modifications to the protocol. 300 ml high-copy plasmid bacterial culture was used for purification vs the 100 ml recommended. 1.5 x the recommended volumes of buffer P1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0), P2 (200 mM NaOH and 1% (w/v) SDS) and P3 (3.0 M KH$_3$C$_2$O$_2$, pH 5.5) were used for bacterial resuspension, lysis, and lysis neutralisation respectively. Buffer QF (1.25 M NaCl, 50 mM Tris-Cl, 15% (v/v) isopropanol, pH 8.5) was heated to 50 °C prior to use. Following addition of buffer P2, tubes were inverted 10 times, rather than the 4-6 recommended, to ensure efficient cell lysis.

2.2.5.3 Nanodrop quantification of plasmid DNA

DNA concentration and purity were assessed using a NanoDrop™ ND-1000 spectrophotometer measuring absorbance at three wavelengths: 230 nm, 260 nm (OD$_{260}$) and 280 nm (OD$_{280}$). Concentrations were calculated using a serial dilution to produce diluted samples at 1/10 and 1/100 the initial DNA stock concentration. For each dilution, we calculated three measurements of concentration using the automated instrument software, assuming an extinction coefficient of 50 ng-cm/µl for double-stranded DNA (dsDNA), then manually calculated the mean. This mean was then multiplied to give an estimate of the pure DNA stock concentration, and the estimated mean from the two serial dilutions were averaged to give a final estimation of DNA concentration. OD$_{260/280}$ and OD$_{260/230}$ ratios were used to assess purity, with the acceptable OD$_{260/280}$ range set at 1.7 – 2.0, and the acceptable OD$_{260/230}$ range set at 1.9 – 2.2.
2.2.5.4 DNA sequencing

Plasmid DNA concentration was determined using a NanoDrop™ ND-1000 spectrophotometer, then diluted in ddH₂O to 100 ng/µl. Samples were outsourced to Eurofins Genomics for sequencing.

2.2.6 Recombinant protein expression

2.2.6.1 Coomassie blue staining

Recombinant proteins were diluted 1:1 (or as described) in 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% Bromophenol Blue, 125 mM Tris-HCl, pH 6.8) and boiled for 3 min at 95 °C. Boiled samples were loaded onto 16% Tris-Glycine gels and run in prediluted 1X Tris-Glycine SDS-PAGE buffer (25 mM Tris base, 0.192 M glycine, 1% (w/v) SDS) for 2 h at 160 V. Gels were stained using Coomassie Brilliant Blue solution (0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid) for 2 h and then washed O/N using destain solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid) to visualise protein bands.

2.2.6.2 Construction of plasmid for expression of hFcγRIIb ectodomain

DNA for the hFcγRIIb ectodomain was custom synthesised using the GeneArt Gene Synthesis service from ThermoFisher Scientific according to the sequences previously described (Jung, Kang and Georgiou, 2010) with codon optimisation for E.coli expression. The template plasmids were then digested with NdeI/HindIII endonucleases and ligated into pET-21a (EMD Chemicals,
Gibbstown, NJ) to produce hFcγRIIb-pET21a plasmids, then transformed into DH5α™-T1R MAX Efficiency E.coli for storage as glycerol stocks.

2.2.6.3 **Induction and purification of hFcγRIIb ectodomain**

BL21 (DE3) competent *E.coli* were transformed using hFcγRIIb-pET21a DNA as per manufacturer’s instructions, then cultured in a 100 ml starter culture of Luria broth containing 100 µg/ml ampicillin. Five 1 L flasks of sterile Luria broth containing 100 µg/ml ampicillin were then each inoculated with 10 ml of starter culture and incubated shaking at 37 °C for 2 h, or until OD₆₀₀ reached ~ 0.6. At this point, 1 ml of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each flask and the cultures were incubated for 5 h. Cells were centrifuged for 30 min at 13,000 rpm and resuspended in 60 ml of bacteria lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 250 U benzonase, 1 mg/ml of lysozyme), sonicated in 3 x 2 min bursts using a SONOPULS HD 2070 homogeniser fitted with an MS73 tip, then centrifuged at 13,000 rpm for 30 min. Cell pellets were again resuspended in 60 ml bacteria lysis buffer, sonicated, and spun down at 13,000 rpm for 30 min. Cell pellets were resuspended in 60 ml bacteria wash buffer (2M Urea, 10 mM K₂HPO₄, pH 8.2), sonicated and spun down at 13,000 rpm for 30 min. Cell pellets were resuspended in 60 ml solubilisation buffer (8M Urea, 10 mM K₂HPO₄, 100 mM β-mercaptoethanol, pH 8.2) and incubated O/N at 4 °C with constant mixing. The solubilised protein was 0.2 µm-filtered and loaded onto a 5 ml HisTrap™ FF column pre-equilibrated with buffer A (8 M Urea, 10 mM K₂HPO₄, pH 8.2), and washed with 5 column volumes of buffer A. A 10 column volume linear gradient of buffer A to buffer B (8 M urea, 250 mM imidazole, 10 mM K₂HPO₄, pH 8.2) was applied to elute the protein from the column, which was then stored in buffer B at 4 °C until refolding.
2.2.6.4  Circular dichroism spectroscopy

Circular dichroism (CD) was carried out using a J-715 spectropolarimeter equipped with a xenon lamp and Peltier temperature controller, with proteins analysed in quartz cuvettes of 1mm path length. For determination of secondary structure, far-UV CD spectra were collected between 260 nm and 180 nm at 1 nm intervals, using a continuous scanning speed of 100 nm/min, an integration time of 1 second, and a bandwidth of 1.0 nm. Background signal was corrected for by subtracting spectra obtained for buffer alone from the spectra obtained for protein and buffer combined. This was subsequently expressed as mean residue ellipticity ([θ]r), which was calculated using the formula:

\[ [\theta]_r = \frac{\theta}{c \times l \times n} \]

where \( \theta \), \( c \), \( l \) and \( n \) denote ellipticity (mdeg), protein concentration (M), path length (cm), and the number of residues in the protein, respectively.

2.2.7  COS-7 experiments

2.2.7.1  COS-7 cell culture

Simian COS-7 cells were received from the Strittmatter lab at the Yale School of Medicine and cultured in Sigma-Aldrich® Dulbecco’s Modified Eagle’s Medium 6429 (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (Pen/Strep). Cells were grown in a 5% CO₂-humidified atmosphere at 37 °C until they reached ~85% confluence at which point they were sub-cultured up to a maximum of 20 passages. For passages, cells were first harvested by incubating with 0.25% trypsin-EDTA for 2 min, then resuspended in fresh DMEM and centrifuged at 300 rpm for 4 min. Cells were
resuspended in fresh DMEM and concentration calculated using an INCYTO C-Chip™ disposable haematocytometer before plating at the desired density.

2.2.7.2 COS-7 transient transfections

Transient transfections were carried out using Lipofectamine LTX® with PLUS reagent as per manufacturer instructions, with minor alterations. Briefly, COS-7 cells were plated in DMEM 24-30 h before transfection at density sufficient to reach ~85% confluence on the day of transfection (see table 3). On the day of transfection, PLUS™ reagent was mixed with plasmid DNA and left to incubate for 15 min at room temperature. Lipofectamine™ LTX reagent was then added and the solution incubated for a further 30 min. Fresh DMEM was then added to cells, before DNA-Lipofectamine® solution was added directly to wells. COS-7 cells were then incubated at 37 °C for 18-24 h before assaying for transient gene expression.

Table 3 - Lipofectamine transient transfection variables

<table>
<thead>
<tr>
<th>Culture vessel</th>
<th>Cells per well</th>
<th>DNA</th>
<th>PLUS Reagent</th>
<th>Lipofectamine® LTX Reagent</th>
<th>Volume of Lipofectamine® complex solution added to each well</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well dish</td>
<td>2 x 10⁵</td>
<td>2500 ng</td>
<td>2.5 µl</td>
<td>11.25 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>24-well dish</td>
<td>4 x 10⁴</td>
<td>500 ng</td>
<td>0.5 µl</td>
<td>2.25 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>96-well dish</td>
<td>1.5 x 10⁴</td>
<td>100 ng</td>
<td>0.1 µl</td>
<td>0.45 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
2.2.7.3 NBT/BCIP COS-7 Aβ binding assay

The binding of Aβ to cells was examined primarily using a 96-well format. On day 1, cells were seeded in 96-well cellcoat® Poly-l-lysine cell culture plates at a density of $1.5 \times 10^4$ cells / well. On day 2, cells underwent lipofectamine transient transfection protocol (see above). On day 3, one aliquot of 100 µM biotinylated o-Aβ stock was removed from -80°C, thawed at RT, and then diluted 1:100 in phenol red- and glutamine-free Ham’s F12 media (F12) in 1 ml non-stick pre-lubricated Eppendorf tubes. The supernatant from multiple Eppendorfs was then pooled, and a serial dilution was carried out using F12. Cells were removed from the incubator and growth medium was removed, followed by the addition of 100 µl of biotinylated o-Aβ serial dilution. No biotinylated o-Aβ or further components of the assay were added to the outer ring of wells to prevent variability due to edge effects, so all results presented refer only to the central 60 wells of the 96-well plate. Following biotinylated o-Aβ addition, the plate was incubated for 2 h at 20 °C, washed using a gentle plate washer wash program to ensure no cells detached from wells. Immediately after washing, 100 µl of PFA solution (4% paraformaldehyde w/v, 4% sucrose w/v in 1X PBS) was added to each well on top of remaining wash buffer, and cells fixed for 30 min. Wells were subsequently blocked for 20 min at RT with 100 µl of 3% (v/v) donkey serum containing 0.1% (v/v) Triton X-100. Neutravidin-conjugated AP was diluted 1:5000 in 1.5% (v/v) donkey serum containing 0.05% (v/v) Triton X-100, and 100 µl of this solution was added to each well, before the plate was placed at 4 °C O/N. On day 4, cells were washed three times with PBS followed by one wash with AP buffer (100 mM NaCl, 100 mM Tris-Cl, 50 mM MgCl₂, 0.1% Tween, pH 9.5). AP buffer was removed from wells and 100 µl 1-Step™ NBT/BCIP Plus Suppressor solution
was added to each well and incubated for 15 min at RT. The reaction was quenched by washing the plate four times with DI H₂O, and the plate tapped against the bench repeatedly until every well was dry. The amount of biotinylated o-Aβ bound was quantified using a Bioreader® 5000-Eβ with the settings set at: Diameter 31 μm – 982 μm; Spot gradient “medium”; Sensitivity 215; Detection “Intensity”.

2.2.8 Primary neuron experiments

2.2.8.1 Primary rat hippocampal neuron culture

Two days prior to dissection, 96-well plates were coated with 0.1 mg/ml poly-L-lysine (PLL) in borate buffer (100 mM borate, pH 8.5). For dissection, embryonic day 18 (E18) pregnant Wistar rats were sacrificed using a schedule 1 method and the foetal heads harvested and stored in ice cold dissection buffer (Gibco HBSS supplemented with 1% (v/v) MgCl₂, 1 M HEPES, 100 mM Sodium pyruvate, 20 μg ml⁻¹ gentamycin sulfate). The brain was removed, and the hippocampus and cortex dissected out, taking care to remove any meninges. Dissected hippocampi and cortices were rinsed once with HBSS, resuspended in 10 ml trypsin solution (Gibco HBSS supplemented with 0.25% trypsin) before being placed in a pre-warmed water bath at 37 °C for 18 min. Cells were washed 3x in plating media (Life Technologies high glucose DMEM supplemented with 200 mM L-Glutamine, 10% (v/v) horse serum, 20 μg/ml gentamycin sulfate), dissociated by trituration with a set of increasingly narrow glass Pasteur pipettes (heated to size using a flame). After dissociation, cells were run through a cell strainer to remove any large debris, then resuspended in fresh plating media. Live cells were counted using trypan blue and a haemocytometer, then plated
on PLL-coated 96-well plates (TPP) in 200 µl plating media. Two to six hours later, media was exchanged for 200 µl CultureOne medium (Gibco neurobasal plus medium supplemented with 2% (v/v) B27 Plus, 1X Gibco GlutaMAX™, 20 µg ml⁻¹ gentamycin sulfate, 1X Gibco CultureOne) 48 hours later, 100 µl media was removed and replaced with maintenance medium (Gibco neurobasal plus medium supplemented with 2% (v/v) B27 Plus, 1X Gibco GlutaMAX™, 20 µg ml⁻¹ gentamycin sulfate). After this, every 72 hours, 50 µl media was removed and replaced with fresh maintenance medium.

2.2.8.2  Incucyte neurite retraction assay on primary rat neurons

Approximately 24 h prior to adding AD brain extracts into culture medium, DIV12 primary hippocampal rat neurons were placed in an Incucyte S3 live-cell imaging system (Essen Bioscience, Ann Arbor, MI), and images taken every 2 h to identify any wells containing artefacts such as dead cells or glia overproliferation (such wells were not used for the subsequent experiment). Following this, plates were removed from the Incucyte and 50 µl maintenance medium removed from each well and replaced by the indicated concentration of AD brain extract diluted in fresh maintenance medium. Images were collected from three fields of view per well every 2 h for at least 80 h. Phase contrast image sets were analysed using IncuCyte S3 2018B software (Essen Bioscience, Ann Arbor, MI), and the ‘NeuroTrack’ analysis used to quantify neurite processes and cell bodies. Typical settings were Segmentation Mode = Brightness; Segmentation Adjustment = 0.8; Neurite Filtering = Best; Neurite sensitivity = 0.4; Neurite Width = 1 µm. Total neurite length (in mm) was quantified and normalized to the first value collected for each individual image.
2.2.9 iPSC-derived neuron experiments

2.2.9.1 Production of induced neurons (iNs) from human induced pluripotent stem cells (iPSCs)

The iNs used in this work were produced by Wen Liu following a published protocol (Hong et al., 2018). Briefly, Neurogenin 2 (Ngn2)-induced human neurons were prepared as summarized in (Figure 13) as described previously (Jin et al., 2018). YZ1 induced pluripotent stem cells (iPSCs) were maintained in media containing DMEM/F12, Knockout Serum Replacement, penicillin/streptomycin/glutamine, MEM-NEAA, and 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA) plus 10 μg/ml bFGF (Millipore, Billerica, MA). iPSCs were plated at a density of 95,000 cells/cm² for viral infection. Lentiviruses were obtained from Alstem with “ultrapure titres” and used at the following concentrations: pTet-O-NGN2-puro: 0.1 μl/50,000 cells; Tet-O-FUW-eGFP: 0.05 μl/50,000 cells; Fudelta GW-rtTA: 0.11 μl/50,000 cells. To induce Neurogenin 2 expression doxycycline was added on “iN day 1” (Figure 13) at a concentration of 2 μg ml⁻¹. On iN day 2, puromycin was added at 10 μg/ml and maintained in the media at all times thereafter. On iN day 4, cells were plated at 5000 cells/well on Matrigel (Corning, NY) coated Greiner 96 well microclear plates and maintained in media consisting of Neurobasal medium (Gibco), Glutamax, 20% Dextrose, MEM-NEAA and B27 with BDNF, CNTF, GDNF (PeproTech, Rocky Hill, NJ) each at a concentration of 10 ng ml⁻¹. Prior studies indicated that neurite number and expression of neural markers reached near maximal levels by iN day 14 and that iNs were fully mature by iN day 21 (Jin et al., 2018). Cells were used at iN day 21.
2.2.9.2 Incucyte neurite retraction assay on induced Neurons.

This live-cell analysis follows a similar protocol to that outlined in (Hong et al., 2018). 6 h to 24 h before treatment with AD brain extracts, iN day 21 neurons were placed in an IncuCyte Zoom live-cell imaging instrument (Essen Bioscience, Ann Arbor, MI) and images collected every 2 h for a total of 6 h in order to obtain ‘baseline’ images of current neurite length. Immediately after collection of final baseline image, 100 µl of medium was removed from each well of the iNs, and 50 µl of buffer-exchanged extract or vehicle, plus 50 µl of fresh medium was added. Following this, images were collected from four fields per well every 2 h for a total of 84 h. Phase contrast image sets were analysed using Incucyte Zoom 2016A Software (Essen Bioscience, Ann Arbor, MI). The ‘NeuroTrack’ analysis package was used to automatically define neurite processes, with typical settings of: Segmentation Mode = Brightness; Segmentation Adjustment = 1.2; Cell body cluster filter = minimum 500 μm²; Neurite Filtering = Best; Neurite sensitivity = 0.4; Neurite Width = 2 μm. Total neurite length (in mm) was quantified and normalised to the average value measured during the 6 h period prior to sample addition.
2.2.10 Mouse studies

2.2.10.1 Ethical Approval

Work with mice was performed under approval and license granted by the UK Home Office (Animals (Scientific Procedures) Act 1986); Project Licence number 70/9022 which conformed to University College London institutional and ARRIVE guidelines (http://www.nc3rs.org.uk/ARRIVE/). Individual experiments were also subject to internal scientific animal committee and animal ethics committee review.

2.2.10.2 APP knock-in mice

Transgenic mice homozygous for APP containing a humanised Aβ region plus the Swedish and Iberian mutations, designated NL-F knock-in mice, were described previously (Saito et al., 2014). NL-F mice were first speed backcrossed at JAX (Jackson laboratories, USA) and then maintained on a C57BL6J background. All mice used in experiments were heterozygous.

2.2.10.3 Mouse intracerebral inoculation experiments

Inoculation experiments were carried out as described (Purro et al., 2018). Briefly, genotypes of NL-F breeder mice were confirmed by PCR of ear punch DNA before mating and all mice included in the experiment were uniquely identified by sub-cutaneous transponders. Mice (female, aged 7–8 weeks) were randomly assigned to experimental groups, anaesthetized with a mixture of halothane and O₂, and intracerebrally inoculated in the right parietal lobe with 10 μl of 1% (w/v) human brain homogenate (prepared as described in 2.2.2.1) or
vehicle (D-PBS). At 2 days, 7 days, 1 month, 2 months, 3 months, 4 months, 8 months or 12 months post-inoculation, mice were culled by a Schedule 1 method, brains removed and divided in two sagitally. The right half was snap frozen for use in biochemical assays and the left half fixed in 10% buffered formal saline for histological analysis.

2.2.10.4 Production of mouse brain homogenates for biochemical analysis

Right hemispheres of mouse brains, stored frozen in sealed tubes, were transferred into a class 1 microbiological safety cabinet, transferred to a tube containing zirconium beads and weighed. The tissue was prepared as a 10% (w/v) homogenate in DPBS containing one tablet of Pierce protease and phosphatase inhibitors (#A32961) per 10 ml. Homogenisation was achieved by ribolysation using a Precellys 24 (Stretton Scientific) at 6,500 rpm for 1 cycle of 60 sec. Following this, tubes were spun at 400 rpm for 30 sec to allow foam to settle, aliquotted in to fresh tubes, snap frozen on dry ice and stored at -80 °C until use.

2.2.10.5 Mouse immunohistochemistry

Immunohistochemistry experiments were carried out as described (Purro et al., 2018) Samples were processed and stained by Tamzin Nazari. Tissue was fixed in 10% buffered formal saline followed by incubation in 98% formic acid for 1 h. Following further washing in 10% buffered formal saline, tissue samples were processed and paraffin wax embedded. Serial sections of 5 μm nominal thickness were taken. Protein levels were visualised by incubation with antibodies using Ventana Discovery automated immunohistochemical staining machine (ROCHE Burgess Hill, UK) and proprietary solutions. Visualization was
accomplished with development of 3’3 diaminobenzidine tetrachloride as the chromogen (DAB Map Kit, Ventana Medical Systems). Haematoxylin was used as the counterstain.

### 2.2.10.6 Immunohistochemistry image capture

Histological slides were digitised on a LEICA SCN400F scanner (LEICA Milton Keynes, UK) at 40x magnification and 65% image compression setting during export. Slides were archived and managed on LEICA Slidepath (LEICA Milton Keynes, UK). For image analysis, whole slide images were exported for processing in Definiens tissue studio.

### 2.2.10.7 Immunohistochemical quantification of parenchymal Aβ

Parenchymal Aβ, present in the form of diffuse deposits or as plaques were quantified as described previously (Jaunmuktane et al., 2015). Protocols were optimised by Silvia Purro and Matthew Ellis. Digital image analysis on whole slides was performed using Definiens Developer XD 2.6 (Definiens, Munich, Germany). Initial tissue identification was performed using resolution equivalent to x10 magnification and stain detection was performed at x20 magnification. Regions of interest (ROI) were manually selected to separate cortex, hippocampus, and cerebellum; larger artefacts such as melanin inclusions were manually selected for exclusion from analysis. Tissue detection and initial segmentation was done to identify all tissue within the image, separating the sample from background and non-tissue regions for further analysis. This separation was based on identification of the highly homologous relatively bright/white region of background present at the perimeter of each image. A
composite raster image produced by selecting the lowest pixel value from the
three comprising colour layers (RGB colour model) provided a greyscale
representation of brightness. The mean brightness of this background region
was used to exclude all background regions from further analysis. Stain detection
(brown) is based on the transformation of the RGB colour model to a Hue-
saturation-density (HSD) representation (Van Der Laak et al., 2000). This
provides a raster image of the intensity of each colour of interest (brown and
blue). A number of fixed thresholds ($T_x$) are then used to identify areas of interest
($A_x$). The thresholds used are in arbitrary units (au) with a scale of 0 au to 3 au
in HSD images. The threshold $T_{\text{brown stain}}$ was allocated the value 0.15 au, with all
pixels above this threshold classed as “stain” ($A_{\text{stain}}$) and those below as
“unstained” ($A_{\text{unstained}}$). $A_{\text{stain}}$ was excluded if the intensity of blue staining was not
significantly lower than the level of brown stain (difference less than 0.1 au) to
remove generically dark areas. The remaining $A_{\text{stain}}$ were further categorised
using threshold $T_{\text{dark brown}}$ of 0.5 au to give $A_{\text{light}}$ and $A_{\text{dark}}$. Plaques were then
constructed from these $A_{\text{light}}$ and $A_{\text{dark}}$ objects. Each $A_{\text{dark}}$ was classified as a
plaque seed; these were then grown into all surrounding $A_{\text{light}}$ to give $A_{\text{potential plaque}}$
(constructed of $A_{\text{light}}$ and $A_{\text{dark}}$) and $A_{\text{non-plaque}}$ (constructed of only $A_{\text{light}}$). A number
of exclusions were then applied: Any $A_{\text{potential plaque}}$ with area less than 20 µm$^2$ or
containing less than 3 pixels previously classified as $A_{\text{dark}}$ were reclassified as
$A_{\text{non-plaque}}$. $A_{\text{potential plaque}}$ with a relatively high stain intensity (mean brown ($\bar{B}$)
intensity higher than 0.35 au) and low variation in brown stain level (standard
deveiation in brown stain ($B\delta$) below 0.1 au), were removed into $A_{\text{unstained}}$ as
artefacts; $A_{\text{potential plaque}}$ with a $\bar{B}$ higher than 0.5 au and $B\delta$ below 0.25 au were
reclassified as $A_{\text{non-plaque}}$, as they display an uncharacteristically dark and varied
stain character for plaques; $A_{\text{potential plaque}}$ with area less than 40 µm$^2$ and elliptic
character less than 0.2 (scale of 0 (random shape) to 1 (perfect circle)) were reclassified as $A_{\text{non-plaque}}$; and $A_{\text{potential plaque}}$ with area greater than 40 $\mu$m$^2$ and relative proportion of $A_{\text{dark}}$ greater than 70% were reclassified as $A_{\text{non-plaque}}$. The remaining $A_{\text{potential plaque}}$ give our final $A_{\text{plaque}}$. For calculation of the number of plaque cores all $A_{\text{dark}}$ with area greater than 50 $\mu$m$^2$ were reclassified as $A_{\text{core}}$. For plaques containing no $A_{\text{core}}$, largest $A_{\text{dark}}$ was retained as the $A_{\text{core}}$. For each ROI the total area analysed ($A_{\text{unstained}}+A_{\text{plaque}}+A_{\text{non-plaque}}$), $A_{\text{plaque}}$, $A_{\text{non-plaque}}$, #$A_{\text{plaque}}$ and #$A_{\text{core}}$ were exported for analysis.

2.2.11 Graphical and statistical analyses

Graphical and statistical analyses were carried out using GraphPad Prism 7. Comparisons between groups were carried out by students t-tests, One-way analysis of variance (ANOVAs) or Two-way ANOVAs. Bonferroni corrections were used to control for error in multiple comparisons. For data involving the quantitative staining of plaque pathology in NL-F mice, there was a clear skew in which groups with higher mean plaque load also had higher variance in plaque load and vice versa. Therefore, these data were log-transformed prior to statistical analysis to more closely align to the parametric assumption of homogeneity of variance. Whenever analyses were carried out on log-transformed data, graphs were also presented with in this format, but anti-log labelling of axes was included for ease of comprehension.
Chapter 3 - Results and Discussion

3.1 Section 1 - PrP, FcγRIIb and LilrB2 bind to oligomeric assemblies of Aβ

3.1.1 Context and aims

Several lines of evidence suggest that soluble oligomers of Aβ peptides play a key role in AD, however the precise mechanisms remain unclear (Ferreira et al., 2015; Viola and Klein, 2015). One possibility is that these oligomers bind to specific receptors expressed at the surface of neuronal and glial cells in the CNS, acting as ligands to activate toxic downstream mechanisms (Jarosz-Griffiths et al., 2016). A variety of putative receptors have been identified, however as each lab tends to focus on a single receptor, few direct comparisons have been made. In this work, a cell-based Aβ-binding assay was used to compare the binding of synthetic Aβ oligomers to a variety of putative Aβ receptors expressed at the surface of COS-7 cells. Selected receptors were then recombinantly expressed, and plate-based binding assays used to directly compare the binding of Aβ oligomers to each receptor.

3.1.2 A preparation of synthetic Aβ oligomers consists largely of small protofibrils

Soluble oligomers of Aβ are enriched in the brains of AD patients (Tomic et al., 2009; Mc Donald et al., 2010; Upadhaya et al., 2012), but precisely which structure or aggregation state of Aβ plays a causal role in disease pathogenesis remains controversial. Numerous protocols for producing synthetic Aβ aggregates have been presented in the literature, and as different aggregation
conditions can lead to strikingly different aggregate structures it is crucial to always thoroughly characterise any aggregate preparation used in downstream assays. Our lab has previously optimised an in vitro aggregation protocol for synthetic Aβ1-42 which produces Aβ aggregates of primarily protofibrillar structure which are able to potently inhibit LTP (Nicoll et al., 2013). The Aβ oligomers used in this section were produced from synthetic biotinylated Aβ1-42 by Andrew Nicoll following this protocol, but further characterisation was carried out by electron microscopy (EM), size exclusion chromatography (SEC) and dynamic light scattering (DLS) to ensure no significant changes had occurred to the Aβ oligomers during storage at -80 °C. Electron microscopy showed that the majority of visible particles in solution remained as disperse protofibrils <100 nm in length, with very few visible clumps (Figure 14). Further analyses by Size exclusion chromatography-multi angle light scattering (SEC-MALS) revealed two peaks, one representing monomer and one representing a range of aggregated species (Figure 15). UV_{275} readings indicated that roughly 78% of the total protein was present within the aggregate peak, and the average size of molecules within this peak was estimated at 1300 kDa by MALS. Herein, the term “o-Aβ” is used as shorthand for soluble “oligomeric Aβ”, with more detail given on precise primary structure and aggregation state where possible.
100 µM biotinylated Aβ oligomers were thawed on ice, diluted to 10 µM, then added to 300 mesh carbon coated copper grids (Electron Microscopy Sciences), stained with Nano-W methylamine tungstate negative stain, and imaged using a Fei Talos L120 G2 transmission electron microscope (TEM). The most common aggregates observed appeared to be small protofibrils roughly ~100nm in length.

**Figure 14 - Electron micrograph of Aβ1-42 oligomers immobilised on a carbon grid**
100 µM biotinylated Aβ oligomers were thawed on ice, and loaded onto a Superdex 75 10/300 GL size exclusion chromatography (SEC) column and eluted in 50 mM ammonium bicarbonate, pH 8.5 buffer. UV$_{275}$ absorbance recorded two peaks at ~ 18 min and ~28 min. The SEC column was coupled to a Wyatt Optilab T-rEX (Wyatt technology) and a Wyatt dawn heleos II module (Wyatt technology) which analysed eluted sample using both multi-angle light scattering (MALS) and differential refractive index (dRI). Peaks for dRI and MALS were aligned with the peaks observed via UV$_{275}$. Readings from DLS implied that the average size of particles in the 18 min peak was 1300 kDa.
3.1.3 Using a transient transfection model to assess interactions between Aβ oligomers and surface-expressed cellular receptors

PrP<sup>C</sup> was first identified as a putative Aβ receptor in an unbiased screen wherein COS-7 cells were transfected with cDNA from an adult mouse brain cDNA library, incubated with synthetic biotinylated o-Aβ, and the levels of cell-bound o-Aβ quantified using streptavidin-linked alkaline phosphatase (Laurén <i>et al.</i>, 2009). The following work uses a modified version of this protocol, comparing the Aβ binding capacity of wild type (WT) COS-7 cells to the Aβ binding capacity of COS-7 cells overexpressing at the protein level mouse PrP (moPrP), mouse FcyRIIb (mFcyRIIb), human FcyRIIb (hFcyRIIb), human LilrB2, or as a control COS-7 cells transfected with pLNCX2 ‘empty vector’ (EV) plasmid containing no insert (Figure 16). Each of these receptors has been shown previously to bind to oligomeric Aβ species, and to facilitate Aβ-induced synaptotoxicity (Laurén <i>et al.</i>, 2009; Kam <i>et al.</i>, 2013; Kim <i>et al.</i>, 2013; Nicoll <i>et al.</i>, 2013). The EV condition is designed to act as a control for the cell stress induced by the transfection procedure, without inducing overexpression of any protein.
COS-7 cells are plated at a 15 k/well in 96-well plates at 0 DIV, which allows them to reach 95% + confluence during the course of the assay. On 1 DIV, cells are either transiently transfected with a plasmid containing a receptor construct (leading to overexpression of receptor at cell surface), mock transfected with pLNCX2 ‘empty vector’, or left untransfected. On 2 DIV, separate wells of cells are incubated with various concentrations of biotinylated Aβ oligomers for 2 h, then fixed with 4% PFA, blocked with 3% Donkey Serum, and incubated O/N with neutravidin-conjugated alkaline phosphatase (AP). After 3 DIV, NBT/BCIP substrate is added, which is converted to a purple NBT-formazan precipitate in the presence of AP. The amount of purple precipitate formed should therefore scale with the amount of AP present, which in turn should depend on the amount of biotinylated Aβ oligomers captured on the cells. Levels of purple NBT-formazan precipitate are then quantified using the Bioreader 5000-Eβ which visually counts the number of purple spots present within each well. o-Aβ = oligomeric Aβ comprised of biotinylated synthetic Aβ1-42.

**Figure 16 - COS-7 Aβ oligomer binding assay overview**
Each receptor was transfected on to at least 5 different plates of COS-7 cells during 5 separate cell passages, and on each occasion were treated with 500 nM, 250 nM, 100 nM, 50 nM or 0 nM monomer equivalent of our biotinylated o-Aβ (Figure 17).

Figure 17 – COS-7 transient transfection o-Aβ binding assay

WT, mock-transfected and receptor-transfected COS-7 cells were incubated for 2 h with 0 nM, 50 nM, 100 nM, 250 nM, or 500 nM monomer equivalent of biotinylated o-Aβ and levels of bound o-Aβ detected by incubation with neutravidin-conjugated alkaline phosphatase followed by treatment with NBT/BCIP. Data were compared using two-way ANOVA and Bonferroni post-hoc comparisons carried out stratifying each o-Aβ concentration as a single family. * indicates that for the indicated o-Aβ concentration, receptor-transfected cells bound significantly more o-Aβ than EV-transfected cells at p < 0.01. Error bars = Means ± SEM.
These data were analysed using a two-way ANOVA across all receptor types and o-Aβ concentrations, using Bonferroni post-hoc correction for multiple comparisons while stratifying each Aβ concentration as a single family. There were no significant differences between WT and EV-transfected cells at any o-Aβ concentration. At 0 nM and 50 nM o-Aβ concentration, no receptor-transfected cells showed significantly increased binding, although the mean o-Aβ binding signal was > 2-fold higher for LilrB2-transfected cells than EV-transfected cells. At 100 nM Aβ, only LilrB2-transfected cells displayed significantly higher Aβ binding (p < 0.0001) than EV-transfected cells. At 250 nM, moPrP-transfected (p = 0.0045), mFcγRIIib-transfected (p < 0.0001) and LilrB2-transfected cells (p < 0.0001) all showed significantly increased binding compared to EV-transfected cells. At 500 nM Aβ, moPrP-transfected, mFcγRIIib-transfected, hFcγRIIib-transfected and LilrB2-transfected cells all displayed significantly increased binding (p < 0.0001) compared to EV-transfected cells. Collectively, these data suggest that COS-7 cells transiently transfected with moPrP, hFcγRIIib, mFcγRIIib or LilrB2 show significantly higher binding to biotinylated o-Aβ than cells transfected with empty pLNCX2 vector. Furthermore, at 100 nM Aβ, LilrB2-transfected cells showed significantly higher binding than hFcγRIIib-transfected (p < 0.0001), mFcγRIIib-transfected (p < 0.0001) or PrP-transfected (p < 0.0001) cells. This may suggest that LilrB2 has a higher binding affinity for Aβ oligomers than PrP, mFcγRIIib, or hFcγRIIib, but it is also possible that LilrB2 was simply more highly expressed than the other receptors, as it is difficult to control for absolute levels of protein expression in transient transfection models (see 3.1.5.2).
In parallel with the analysis of Aβ binding, protein expression levels in receptor-
transfected COS-7 cells were analysed to ensure proteins were being
successfully overexpressed. For PrP and hFcyRIIb, western blot (WB) and
immunofluorescence (IF) results confirmed that these proteins were being highly
overexpressed in plasmid-transfected cells (Figure 18). However, satisfactory
results could not be obtained for all other receptors tested. Of particular note,
LilrB2 overexpression could not be confirmed in LilrB2-transfected cells, which
called into question the previous positive data in the Aβ binding assay.

Figure 18 - Western blot and Immunofluorescence data from FcγRIIb and PrP
transfected COS-7 cells

COS-7 cells were transiently transfected with plasmids containing either hFcyRIIb or
moPrP and analysed by western blot (A, D) and immunofluorescence (C, F). Bands in
western blots were quantified using Licor image studio to estimate expression levels (B,
E). No expression of FcγRIIb could be detected in WT COS-7 cells, but FcγRIIb-transfected
cells showed high expression levels both by western blot (A) and Immunofluorescence
(C). Both WT and PrP-transfected COS-7 cells showed detectable levels of PrP expression
in both western blot (D) and IF (F), but quantification of western blot signal indicated
that PrP was ~ 20-fold more highly expressed in PrP-transfected cells (E). Immunofluorescence: DAPI (blue), overexpressed receptor (green).
As antibody labelling of LilrB2-transfected cells had failed, the experimental paradigm was altered to instead use GFP-tagged constructs of human LilrB2 (LilrB2-GFP) or its homologous family member LilrB1 (LilrB1-GFP) (both of which were received as generous gifts from the De Strooper laboratory at KU Leuven). LilrB1 shares 77% sequence identity with LilrB2, but has previously been reported to not bind Aβ oligomers (Kim et al., 2013), thus LilrB1-GFP was used as a negative control. It was hypothesised that these GFP tags would not affect Aβ binding to the extracellular domain of these receptors as the tags are present on the intracellular portion of the protein. COS-7 cells were transfected with either EV, LilrB2-GFP or LilrB1-GFP, and once again treated with either 500 nM, 250 nM, 100 nM, 50 nM or 0 nM biotinylated o-Aβ (Figure 19). In this assay, LilrB2-GFP transfected cells bound significantly higher levels of o-Aβ than LilrB1-GFP or EV transfected cells at every concentration (p < 0.01; Bonferroni post-hoc correction following one-way ANOVA). Furthermore, unlike the previous data obtained using untagged receptors, this binding appeared to be saturable, with an apparent $K_d$ of ~105 nM after subtracting background signal (Figure 19). Crucially, cells transfected with LilrB1-GFP were clearly observed to overexpress GFP-tagged protein using IF, these cells did not show significantly increased o-Aβ binding at any o-Aβ concentration. This was a critical result to rule out artefactual causes of increased o-Aβ binding, as every other receptor which was successfully overexpressed had led to increased o-Aβ binding.
Figure 19 – COS-7 o-Aβ binding assays and immunofluorescence using GFP-tagged LilrB1 and LilrB2

(A) COS-7 o-Aβ binding assay comparing mock-transfected COS-7 cells to COS-7 cells transiently overexpressing LilrB1-GFP or LilrB2-GFP. Cell-bound o-Aβ was labelled using neutravidin-conjugated AP, which was then detected by addition of NBT/BCIP. Signal was quantified using a Bioreader 5000-Eβ. Data were compared using two-way ANOVA and Bonferroni post-hoc comparisons carried out across all results as a single family. * indicates that for the indicated o-Aβ concentration, receptor-transfected cells bound significantly more o-Aβ than EV-transfected cells at p < 0.01. Error bars = Means ± SEM (n = 3). (B) After subtracting background binding values obtained for EV-transfected cells, a nonlinear curve was plotted and used to calculate a one site specific binding Kd ~ 105 nM for LilrB2-GFP. Error bars = Means ± SEM (n = 3). (C) COS-7 cells were transfected with LilrB1-GFP (centre), LilrB2-GFP (right) or left untransfected (left) and stained with DAPI (cyan) and mouse DM1A anti-tubulin primary antibody, followed by Alexa-fluor 633 anti-mouse secondary antibody (blue). GFP tagged receptors are shown in green.
3.1.4 Recombinant PrP and FcγRIIb bind oligomeric assemblies of Aβ with nanomolar affinity in a competitive fashion

Cellular binding assays are advantageous in that they allow investigation of binding to a protein present in its native state at the plasma membrane, correctly folded and having undergone the appropriate post-translational modifications. However, the data are highly variable, and in this transient transfection paradigm it is difficult to control for transfection efficiency between assays. Furthermore, even though the assay was converted to 96-well format, each plate required at least four days to run and thus it was difficult to investigate a wide range of conditions in this relatively low-throughput system. To investigate a wider range of Aβ concentrations and obtain good quality binding curves and estimates of binding affinity, recombinant receptors were expressed and purified in E.coli to be used in a plate-based 384-well dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) binding assay. The two proteins chosen to be expressed and compared were the 88 AA N1 fragment of human PrP and a 184 AA ectomain fragment of human FcγRIIb.

The N1 fragment of PrP comprises AA 23-111 (Figure 20) and has previously been shown to bind synthetic o-Aβ with similar affinity to full length PrP (Nicoll et al., 2013). Human PrP N1 fragment (PrP-N1) was expressed and purified by Will Taylor for use in these assays.
The canonical isoform of human PrP contains 253 residues, but both the N-terminus (AA 1-22) and C-terminus (AA 231-253) consist of signal sequences which are cleaved in the ER soon after translation. The N1 fragment of PrP spans from AA 23-111, and contains five octapeptide repeats (OPRs) within an octapeptide repeat domain and one putative Aβ binding site (AA 95-111). The N-terminus of PrP is highly flexible with no secondary structure, and thus the PrP N1 fragment is hypothesised to exist in an intrinsically disordered state. The C-terminus contains both α-helical and β sheet structure, and one disulphide bridge between Cys179 and Cys214.
The full length FcγRIIb protein contains a highly hydrophobic 23 AA transmembrane helix, which would make it incredibly difficult to express in a soluble form, likely requiring high concentrations of detergent in solution which would have a negative effect on any downstream assays. Therefore, only the extracellular “ectodomain” of human FcγRIIb (Fcγ-Ecto) was expressed, following a previously reported protocol (Jung et al. 2010). DNA corresponding to the human FcγRIIb ectodomain was custom synthesised using the GeneArt Gene Synthesis service from ThermoFisher Scientific with codon optimisation for E.coli expression. The template plasmids were then digested with NdeI/HindIII endonucleases and ligated into pET-21a (EMD Chemicals, Gibbstown, NJ) to produce hFcγRIIb-pET21a plasmids, then transformed into BL21 E.coli. IPTG-induction reliably induced overexpression of a protein at the expected molecular weight of 22 kDa, which was enriched in the inclusion body fraction and could be purified using His-trap FF sepharose columns under denaturing conditions (Figure 21).
Figure 21 - Design and expression of FcγRIIb ectodomain recombinant construct

(A) FcγRIIb is a transmembrane protein with substantial β-structure due to two Immunoglobulin-like (Ig-like) domains found at AA 48-127 and 131-213. In addition, there is a highly hydrophobic transmembrane helix present between AA 218-240. Custom DNA was ordered equivalent to AA 43-217, containing both Ig-like domains but excluding the transmembrane and intracellular domains. (B) Custom FcγRIIb DNA was cloned into a pET21-a vector and transformed into BL21 E.coli. IPTG-induction reliably induced overexpression of a protein which ran at the expected molecular weight of 22 kDa (Black arrow) when inclusions bodies from BL21 bacteria were analysed by SDS-PAGE. (C) Inclusion bodies from BL21 bacteria were solubilised in 8 M Urea and his-tagged protein was purified using a His-trap FF column (5 ml), producing a pure band at the expected molecular weight of 22 kDa when column elution was analysed by SDS-PAGE.
Following this expression, Fcγ-Ecto and PrP-N1 were each used as plate-bound ‘Capture’ species in DELFIAAs (Figure 22).

**Figure 22 - DELFIA assays using PrP N1 or FcγRIIb as capture**

DELFIA assays are plate-based binding assays in which the detection step uses proprietary Eu-chelates which exhibit time-resolved fluorescence to increase sensitivity. For analysis of o-Aβ binding to recombinant receptor fragments, PrP-N1 fragment or FcγRIIB-ectodomain were incubated on high-bind 384-well plates for one hour with shaking, then the plates were washed and blocked with SuperBlock, after which they were incubated with biotinylated o-Aβ diluted to a range of concentrations. These biotinylated species were detected using streptavidin conjugated to Eu-chelates which exhibit time-resolved fluorescence upon activation by proprietary ‘enhancement solution’.
Both PrP-N1 fragment and Fcγ-Ecto effectively captured biotinylated o-Aβ with nanomolar affinity (Figure 23). Specifically, plate-bound PrP N1 fragment captured biotinylated o-Aβ with a Kd of ~15 nM monomer equivalent Aβ, while plate-bound Fcγ-Ecto captured biotinylated o-Aβ with a Kd of ~10 nM monomer equivalent.

Figure 23 - DELFIA assays using PrP-N1 and Fcγ-Ecto as capture species

(A) Dose response curve for biotinylated o-Aβ binding to a plate coated in immobilised PrP N1 fragment. Serial dilutions of o-Aβ were added to immobilised PrP N1 fragment for 50 min, and detected using streptavidin-conjugated Eu chelates. Points were fitted to a 5-parameter curve using Graphpad Prism 5.0, which provided an estimated Kd of 15 nM monomer equivalent o-Aβ. Error bars = Means ± SD (n = 4). (B) Dose response curve for o-Aβ binding to a plate coated in immobilised FcγRIIb ectodomain. Serial dilutions of o-Aβ were added to immobilised hFcγRIIb ectodomain for 50 min. Points were fitted to a 5-parameter curve using Graphpad Prism 5.0 which provided an estimated Kd of 10 nM monomer equivalent o-Aβ. Error bars = Means ± SD (n = 4).
Interestingly, preincubating o-Aβ with either soluble FcγRIIb-ecto or soluble PrP-N1 was also able to inhibit 10 nM monomer equivalent of Aβ oligomers binding to plate-bound PrP-N1 (Figure 24), suggesting that both receptors are able to bind to Aβ oligomers in the soluble phase. FcγRIIb-Ecto carried out this inhibition with an IC50 of ~5 nM, while PrP-N1 fragment displayed an IC50 of ~1 nM.

Figure 24 – Dose-response curve of 10 nM o-Aβ binding to plate-immobilised PrP N1 fragment in the presence of a serial dilution of protein inhibitor

20 nM o-Aβ were added 1:1 to a serial dilution of Fcy-Ecto, PrP-N1, or buffer 5 min prior to loading on a plate coated in immobilised PrP N1 fragment. Points were fitted to a four-parameter curve using graphpad Prism 5.0. Preincubating o-Aβ with FcγRIIb-Ecto inhibited binding to plate-bound PrP-N1 fragment with an IC50 of ~5 nM, while preincubating o-Aβ with soluble PrP-N1 fragment inhibited o-Aβ binding with an IC50 of ~1 nM. Error bars = Means ± SD (n = 4).
While these assays allowed reliable quantification of receptor binding to synthetic Aβ preparations, the use of biotinylated Aβ species with streptavidin for detection prevented using this assay to recognise non-biotinylated Aβ in biological samples. A new binding assay was therefore optimised using 82e1 anti-Aβ antibody for detection rather than streptavidin. For this assay, we used the meso scale discovery (MSD) system which uses electrochemiluminescence for detection rather than time-resolved fluorescence. Human PrP-N1 fragment was used as capture (Figure 25).

**Figure 25 – Diagram of PrP-MSD assay**

MSD assays are plate-based binding assays in which the detection step uses “SULFO-tag” conjugates which release light when exposed to e⁻ during the final assay stage. For quantification of PrP-N1 binding Aβ species, PrP-N1 fragment was used as capture, and 82e1 used to label Aβ.
For the PrP-MSD, both monomeric and oligomeric Aβ lacking biotinylation were needed to produce standard curves. These o-Aβ were produced using the same protocol as described above for biotinylated o-Aβ, but using unbiotinylated Aβ$_{1-42}$ as starting material. Monomeric Aβ was produced by directly diluting lyophilised Aβ$_{1-42}$ in 6 M GdnHCl, incubating O/N, then isolating the monomeric fraction using a superdex 75 10/300 GL SEC column. Both monomeric Aβ and o-Aβ preparations were then run sequentially on analytical-SEC-MALS, where UV$_{275}$ and MALS readings indicated that 78% of our oligomeric preparation consisted of aggregates with an average molecular weight of 1870 kDa (Figure 26). In comparison, these data suggested that over 97% of our monomeric Aβ preparation remained as monomer, and the average molecular weight of our monomer prep was estimated as 4.5 kDa using MALS.

![Figure 26 - Analytical SEC of monomeric and oligomers unbiotinylated Aβ preparations](image)

Monomeric and oligomeric Aβ preparations were thawed on ice and injected sequentially on a superdex 75 10/300 GL SEC column. Both preparations showed two peaks, at ~ 18 minutes and ~ 28 minutes. 98% of Monomeric Aβ eluted in the early peak with an average molecular weight of 4.5 kDa estimated by MALS, whereas for the o-Aβ preparation only 23% eluted in the monomer peak, with the majority of protein present in the ~18 minute peak with an average molecular weight of 1870 kDa estimated by MALS.
Finally, dilution curves of both o-Aβ and monomeric Aβ were analysed using the PrP-MSD assay. Four-parameter curves were plotted for both sets of dilutions, which provided an EC50 of 158,691 pg/ml for monomeric Aβ and 35,391 pg/ml for o-Aβ (Figure 27). Furthermore, at most concentrations, monomeric Aβ produced signal equivalent to the signal produced by ~ 10-fold lower concentrations of o-Aβ. This was surprising, as we were expecting much lower signal from our monomeric Aβ samples, which may suggest that higher levels of o-Aβ were present in our monomeric Aβ preparation than we had predicted, or that on-plate aggregation of monomeric Aβ had occurred during the assay.

![Graph showing dilution curves of monomeric Aβ and o-Aβ](image)

**Figure 27 - PrP-MSD Monomer vs ADDL selectivity**

PrP N1 fragment was thawed, diluted to 250 nM in DPBS, and incubated O/N in standard bind MSD multi-array plates. Dilution series of monomeric and oligomeric Aβ1-42 preparations were incubated on plate for 1 h at RT, then detected using 82e1 antibody. Four-parameter curves were plotted for each series using GraphPad Prism v7.02, with a calculated EC50 of 35,391 pg/ml for o-Aβ and 158,691 pg/ml for monomeric Aβ. Error bars = Means ± SD (n = 3).
3.1.5 Section Discussion

3.1.5.1 Results summary and conclusions

This work investigated the binding of synthetic A\(\beta\)\textsubscript{1-42} monomers and aggregates to putative ‘A\(\beta\)-receptors’ either expressed at the surface of COS-7 cells or recombinantly expressed and used as plate-based ‘capture’ species. Nanomolar monomer equivalent concentrations of biotinylated A\(\beta\) aggregates bound significantly more to cells transfected with PrP, Fc\(\gamma\)RIIb or LilrB2, suggesting the presence of these receptors at the cell membrane leads to increased A\(\beta\) binding. LilrB2-transfected cells showed the highest levels of A\(\beta\) binding for every concentration of A\(\beta\) oligomers tested, but overexpression of this protein could not be confirmed by western blot or immunofluorescence with available antibodies. However, LilrB2-GFP transfected cells also showed significantly increased binding, which was saturable with a K\textsubscript{d} \(\sim\) 105 nM, while overexpression of LilrB1-GFP did not increase binding. Recombinant fragments of PrP and Fc\(\gamma\)RIIb were also shown to bind biotinylated A\(\beta\) aggregates in a plate-based DELFIA assay with a K\textsubscript{d} of 15 nM and 10 nM respectively. Further, when preincubated with A\(\beta\) aggregates prior to addition to the DELFIA plate, these two fragments were able to inhibit binding of A\(\beta\) to plate-bound PrP-N1 fragment with an IC\textsubscript{50} of 1 nM for PrP-N1 and 5 nM for Fc\(\gamma\)-Ecto. Finally, a novel plate-based assay utilising PrP-N1 fragment for capture and anti-A\(\beta\) antibody for detection using the MSD system detected unlabelled synthetic A\(\beta\) aggregates and showed \(\sim\)10-fold selectivity for aggregated A\(\beta\) over A\(\beta\) monomers.
3.1.5.2 Cellular binding assay conclusions, critique, and future work

Collectively, data from the COS-7 Aβ binding assay confirm that aggregates of synthetic Aβ1-42 bind to a variety of proteins which are typically found at the cell surface with nanomolar monomer equivalent affinity. The highest binding for every tested Aβ concentration was observed on LilrB2 or LilrB2-GFP transfected cells. While this may suggest that LilrB2 has the highest affinity for Aβ oligomers of all our receptors tested, it is also possible that different levels of each receptor were expressed at the cell surface or that expression of certain receptors caused unexpected differences in plasma membrane lipid or protein content. Several potential future experiments could help to prove that the increased binding is caused by direct Aβ-receptor interaction.

To help ensure that similar levels of expression are observed for each receptor, quantitative western blotting could be carried out wherein recombinant peptide standards at a range of known concentrations are blotted alongside transfected cell lysates to give estimates of the absolute concentrations of receptors present. This work could be carried out in combination with surface biotinylation in order to quantify levels of surface-expressed receptor rather than total level within the whole cell. One other option would be to exclusively use receptors with a small tag such as FLAG or myc, which would enable immunoblot analysis using anti-tag antibodies to allow direct quantitative comparison between receptors. However, it would always be difficult to rule out the impact of such tags on receptor-Aβ binding, especially in receptors whose peptide chain is found entirely extracellularly (such as PrP), where the tag would necessarily be present in close proximity to putative Aβ-receptor binding sites.
To assess whether overexpression of each receptor leads to any unexpected alterations in plasma membrane architecture, characterisations could be carried out on the lipids, carbohydrates and proteins present at the surface of transfected and non-transfected cells. However, a vast plethora of such modifications exist and it would be difficult to ever prove that such alterations play no role in increased binding.

Perhaps most crucially, inhibition assays could be carried out in which anti-receptor antibodies are incubated on receptor-transfected COS-7 cells prior to addition of Aβ aggregates. If preincubation with anti-receptor antibodies reduced Aβ binding, this would provide strong evidence that the increased binding observed was due to direct receptor-Aβ interaction rather than any artefactual confounding effect.

After this work was completed, a systematic comparison of Amyloid-β receptors was published, which found that overexpression of PrP\textsuperscript{C} led to more binding of amyloid-β oligomers than overexpression of LilrB2 or FcyRIIb (Smith et al., 2019). These differing results could be potentially be due to differences in cell density, transfection protocol, or method of amyloid-β oligomer preparation.

3.1.5.3 Recombinant receptor binding assays conclusions, critique and future work

The results presented using recombinantly expressed PrP and FcyRIIb fragments in plate-based assays provide convincing evidence that these proteins directly bind to synthetic Aβ aggregates. Our results differ from another recently published comparison (Smith et al., 2019), which did not observe any significant
binding of synthetic amyloid-β oligomers to recombinantly expressed FcγRIIb. These results are most likely due to differences in protocol for production of amyloid-β oligomers, or perhaps differences in the expression/folding protocol for FcγRIIb. According to our assays, synthetic o-Aβ1-42 may bind to synthetic FcγRIIb ectodomain with higher affinity than to PrP N1-fragment, as plate-bound PrP N1 fragment captured biotinylated o-Aβ1-42 with a Kd of ~15 nM monomer equivalent Aβ, while plate-bound Fcγ-Ecto captured biotinylated o-Aβ1-42 with a Kd of ~10 nM monomer equivalent.

However, it remains to be shown how relevant these assays are to the natural environment in the human brain. Firstly, both PrP and FcγRIIb fragments were expressed in E.coli and therefore lack the full spectrum of mammalian post-translational modifications. Expression in a mammalian system such as HEK293 cells could help produce peptides much more similar to those found in the human brain. Secondly, both of these fragments displayed no secondary structure when analysed by circular dichroism (data not shown). While this is unsurprising for PrP-N1 fragment (as this region of the PrP protein is expected to exist in an intrinsically unstructured state), the Fcγ-Ecto fragment expressed contains two immunoglobulin domains, and thus would be expected to display substantial β-structure. It is possible that a step-wise dialysis refolding protocol could enable isolation of a soluble FcγRIIb ectodomain with the expected secondary structure.

In addition, it is unclear how similar the synthetic Aβ oligomers used here are to oligomeric Aβ species found in the human brain, both in terms of primary sequence and aggregation state. All the Aβ aggregates used in this work were composed of synthetic Aβ1-42, whereas Aβ species in the human brain show
extensive heterogeneity in terms of sequence and post-translational modification. As a minimum, this work could be expanded to investigate receptor binding to synthetic aggregates of Aβ₁-₄₀, but a more extensive panel of Aβ peptides could better represent the human brain. Furthermore, while the protocol used to oligomerise Aβ for this work is commonly used in labs worldwide to reliably obtain small oligomers, the conditions used are entirely non-physiological and do not even attempt to use buffers, pH, or temperatures which reflect any extracellular or subcellular compartment of the human brain. More emphasis needs to be placed on using Aβ aggregation conditions which reflect the physiological environment of the human brain to improve the biological relevance of the synthetic aggregates produced, and we recommend that this should be a first step for any future groups looking to investigate the role of Aβ receptors in human disease.

Perhaps most critically, following the creation of physiologically relevant Aβ aggregates, plate-based assays such as the PrP-MSD described here should be used to quantify levels of receptor-binding Aβ species present in real human brain extracts. If AD brain extracts or CSF could consistently be shown to contain higher levels of receptor-binding o-Aβ than control brain extracts, in an assay model which attempts to recreate both physiologically relevant Aβ species and physiologically relevant buffers and binding conditions, this would provide much more convincing evidence that receptor-binding Aβ aggregates actually exist in the human AD brain and contribute directly to disease. In the case of this project, it was decided that the evidence was insufficient to support this experimental direction, and efforts were instead refocused on the potential role of Aβ aggregate seeding and strains in AD pathogenesis.
3.2 Section 2 - Levels of Aβ oligomers in human brain homogenate correlate with disease severity but do not distinguish AD from High-Amyloid controls

3.2.1 Context and aims

Accumulation of Aβ is one of the hallmark pathologies of AD, but the signal is also highly non-specific and high levels of Aβ can be found in cognitively normal patients. One potential signal thought to be specifically enriched in AD brains are soluble Aβ oligomers (Tomic et al., 2009; Mc Donald et al., 2010; Perez-Nievas et al., 2013; Rijal Upadhaya et al., 2014), but there have been limited attempts to characterise differences in the Aβ species present in AD brains and ‘High-Amyloid control’ samples – the latter being those who have high levels of amyloid pathology without associated cognitive decline.

This section presents analyses of brain homogenate from a cross-sectional set of patients with various levels of AD pathology, ranging from healthy controls with no detectable amyloid deposition (Zero-Amyloid controls) through to severe AD patients (see detail in Table 3). In addition, one set of ‘High-Amyloid control’ patients is included, who possess high levels of amyloid pathology but almost no evidence of cognitive decline. A variety of tests were used to quantify levels of Aβ species present in each brain, with the aim of identifying a signal which can differentiate between AD patients and High-Amyloid controls.
### Table 3 - Clinical and Neuropathological data of Banner brain samples

<table>
<thead>
<tr>
<th></th>
<th>Average age of death</th>
<th>Average PMI (hours)</th>
<th>Average final MMSE score</th>
<th>Average plaque score</th>
<th>Average Braak score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-Amyloid control (n = 6)</td>
<td>86.50</td>
<td>2.81</td>
<td>28.83</td>
<td>0.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Mild-AD pathology (n = 5)</td>
<td>92.20</td>
<td>3.30</td>
<td>24.80</td>
<td>5.64</td>
<td>3.80</td>
</tr>
<tr>
<td>Moderate AD pathology (n = 7)</td>
<td>85.14</td>
<td>4.53</td>
<td>17.00</td>
<td>11.25</td>
<td>4.43</td>
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<td>Severe AD pathology (n = 6)</td>
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<td>2.80</td>
<td>3.83</td>
<td>14.50</td>
<td>5.83</td>
</tr>
<tr>
<td>High-Amyloid control (n = 6)</td>
<td>88.50</td>
<td>2.56</td>
<td>27.83</td>
<td>11.29</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Data in this table provide information regarding 30 patients at the Banner Sun Health Research Institute in Arizona. Samples are notable for the extremely low PMI, made possible by the specialised facilities at the Banner institute which enable autopsies to be carried out within a very short time frame after patient death. Braak and plaque scoring were carried out by resident neuropathologists at the Banner institute.
3.2.2 High-amyloid control patients are defined by having a very low ratio of MMSE loss compared to amyloid neuropathology

All of the samples analysed in this section were obtained from the Banner Sun Health Research Institute in Arizona, where autopsies were carried out and Amyloid and Braak scoring carried out by resident neuropathologists. Cases were selected by Claire Sarell based on reported final MMSE scores and neuropathologist-rated amyloid scores. In order to visually represent the relationship between neuropathological plaque score and final MMSE score in each of these patient groups, the ratio between plaque score and final MMSE score can be plotted for each group (Figure 28). The High-Amyloid control group showed a mean ratio between amyloid score and MMSE point loss of 0.19, vs mean ratios of 1.15, 1.20 and 1.18 for the Mild-AD, Moderate-AD and Severe-AD groups, respectively.
Thirty Patients were subcategorised according to levels of AD neuropathology (as assessed by resident neuropathologists at the Banner Sun Health Institute), with a separate subgroup created for patients with moderate levels of AD pathology, but no cognitive impairment (High-Amyloid controls). Ratio between amyloid scores and MMSE loss were calculated for each patient. Error bars = Means ± SD.

Figure 28 - Ratio of MMSE loss to amyloid score in Banner AD patients
3.2.3 A custom MSD-Immunoassay quantifies levels of Aβ_{x-42} with an LLOQ of 7.8 pg/ml

While a variety of commercial kits can quantify levels of Aβ_{x-42} in biological samples, these kits can be very expensive which inhibits analysis of a large number of samples in a range of different reaction conditions. Thus, a custom MSD-Immunoassay was optimised to allow quantification of Aβ_{x-42} for a significantly lower price. For this MSD, the mid-region anti-Aβ antibody m266 was used to ‘capture’ Aβ species, and the Aβ_{x-42} specific antibody HJ7.4 (a kind gift from the David Holtzman laboratory at Washington University) was used for detection. HJ7.4 was biotinylated by Emmanuel Risse using EZ-Link Sulfo-NHS-LC-Biotin allowing the use of streptavidin-linked SULFO-tag for the final detection step in the MSD assay. This assay proved to be highly robust and able to detect monomeric Aβ_{1-42} with an LLOQ of 7.8 pg/ml (Figure 29).
Figure 29 - Custom Aβ\textsubscript{x-42} MSD-Immunoassay standard curve

A serial dilution of monomeric Aβ\textsubscript{x-42} was analysed via a custom MSD-immunoassay using m266 for capture, biotinylated HJ7.4 for sample labelling and streptavidin-conjugated SULFO-tag for detection. Graph shows individual replicates at each concentration, with a 4-parameter curve produced using mean signal at each concentration. This standard curve robustly provided an LLOQ of 7.8 pg/ml, defined as the lowest standard concentration which both provided signal higher than (Mean blank signal + (9*Standard Deviation Blank Signals)) and also had % recovery > 80% and < 120%.
3.2.4 Total $\text{A}\beta_{x-42}$ increases with disease severity but does not distinguish AD from High-Amyloid controls

Total homogenate from each Banner brain sample was produced by Claire Sarell in DPBS using ribolysation and stored at -80 °C until further use. For analysis of $\text{A}\beta$ in the ‘total’ fraction, crude 10% homogenate was thoroughly resuspended, then diluted directly in GdnHCl to a final concentration of 5 M. These samples were incubated overnight at 4 °C, then spun down at 13,000 rpm and the supernatant removed and diluted in PBS to a final concentration of 0.1 M GdnHCl. As expected, patients with a more severe disease classification (and thus higher plaque scores) also had increased $\text{A}\beta_{x-42}$ levels in the total fraction (Figure 30). However, the level of $\text{A}\beta_{x-42}$ in High-Amyloid controls was extremely variable, with one patient in this group showing the highest $\text{A}\beta_{x-42}$ levels of all samples tested, while others showed similar $\text{A}\beta_{x-42}$ levels as those seen in the Mild-AD cases. Thus, High-Amyloid control patients had significantly higher $\text{A}\beta_{x-42}$ levels than Zero-Amyloid control patients ($p < 0.01$; Bonferroni post-hoc correction following one-way ANOVA), but not significantly different levels than any other group.
Figure 30 - Custom Aβx-42 MSD of Banner brains replace zero amyloid pathology

Crude brain homogenates from AD and control patients were incubated O/N in 5 M GdnHCl, then quantified on a custom MSD-immunoassay using m266 as capture and biotinylated HJ7.4 for detection. Error bars = Means ± SEM.

Levels of total protein present in each brain homogenate were also quantified, and interestingly it was found there was a subtle trend towards reduced total protein concentration as disease severity and amyloid pathology increased, which was reversed in High-Amyloid controls (Figure 31). While these differences were not statistically significant, when the levels of total Aβx-42 were
normalised to the levels of total protein, the levels of total Aβ_{40,42} were then significantly different between the High-Amyloid control and Severe-AD groups (Figure 32) (p = 0.0068, Bonferroni post-hoc correction following one-way ANOVA), although the difference between High-amyloid controls and moderate-AD cases remained insignificant.

Figure 31 - BCA analyses of Banner brain homogenates
Crude homogenates from control and AD brain extracts were analysed via BCA to provide estimates of total protein concentration. There was a trend towards decreased total protein concentration with increasing disease severity which was reversed in High-Amyloid controls, but differences were not significant (p > 0.05, Bonferroni post-hoc correction following one-way ANOVA). Error bars = Means ± SEM.
Figure 32 - Total Aβ₄₂ normalised to total protein
Crude brain homogenates from AD and control patients were incubated O/N in 5 M GdnHCl, then quantified on a custom MSD-immunoassay using m266 as capture and biotinylated HJ7.4 for detection. Aβ₄₂ levels were then normalised to level of total protein in each homogenate calculated by BCA assay. P-values represent Bonferroni post-hoc corrections following one-way ANOVA. Error bars = Means ± SEM.
3.2.5 1c22 and PrP-binding Aβ oligomers in human brain do not distinguish AD from High-Amyloid controls.

To characterise the levels of oligomeric Aβ species present in these human brain extracts, an MSD assay was optimised using the oligomer-selective anti-Aβ antibody 1c22 as ‘capture’ and the N-terminal specific anti-Aβ antibody 82e1 for detection. As the standard curve for this assay, the same synthetic o-Aβ oligomers were used as previously characterised for the PrP-MSD (see 3.1.4). All 30 Banner brains were analysed using the 1c22-MSD, and observed results were highly variable within each group (Figure 33). Surprisingly, signal was observed above the lower limit of quantification (LLOQ) even in samples in the Zero-Amyloid control group, even though these patients had no observable amyloid neuropathology, and nearly undetectable levels of total Aβx-42 by immunoassay. Only the Moderate-AD, Severe-AD and High-Amyloid control groups had any patients with quantified levels of 1c22-binding Aβ higher than 2000 pg/ml, however as each of these groups also included some samples with 1c22-binding oligomer levels indistinguishable from the Zero-Amyloid control group, there were no significant differences between any group.
Crude brain homogenates from AD and control patients were quantified on a custom MSD-immunoassay using 1c22 as capture and biotinylated 82e1 for detection. Aβ\textsubscript{42} levels were then normalised to level of total protein in each homogenate calculated by BCA assay. P-values represent Bonferroni post-hoc corrections following one-way ANOVA. Error bars = Means ± SEM.
One possible difference between AD patients and High-Amyloid controls could be that per a given amount of total Aβ present in the brain, AD patients exhibit high levels of small Aβ oligomers, whereas High-Amyloid controls could contain primarily insoluble plaques with few small oligomers available to enact toxic cascades. Thus the levels of 1c22-binding oligomers were normalised to the levels of total Aβ\textsubscript{42} observed via immunoassay for the Moderate-AD, Severe-AD and High-Amyloid control brains (Figure 34). However, no significant differences were seen in the ratio of 1c22-binding Aβ species:total Aβ\textsubscript{42} in these samples.

![Figure 34](image)

**Figure 34 – Ratio between 1c22-binding species and total Aβ\textsubscript{42} in Banner brain homogenates**

Crude brain homogenates from AD and control patients were quantified on two custom MSD-immunoassays, one using 1c22 as capture with biotinylated 82e1 for detection and a second using m266 for capture and HJ7.4 for detection. Levels of 1c22-binding Aβ were then normalised to levels of Aβ\textsubscript{42} in each homogenate. (A) shows all sample groups, but the axes are skewed due to the very high values obtained for Zero-Amyloid control and Mild-AD patients (due to the unexpectedly high 1c22 signal in these samples), thus (B) shows only the Moderate-AD, Severe-AD and High-amyloid control groups. Error bars = Means ± SEM.
Finally, each of these brain samples was tested on a novel MSD-immunoassay using PrP N1-fragment as capture (see 3.1.4). Disappointingly, despite testing both the ‘total’ fraction’ and the supernatant following a 16,000rpm spin for 30 min, this assay appeared unable to even discriminate between Zero-Amyloid controls and severe-AD cases (Figure 35) This was highly surprising, as the standard curve for this assay maintained an LLOQ of 352 pg/ml, with blank buffer control wells consistently providing signal levels below this. This suggests that some unknown component present in all of the human brain extracts was able to provide positive signal in this assay, or lead to artefactually high signal by disrupting the assay in some way.
Figure 35 - PrP-binding Aβ in Banner brains

(A) Crude brain homogenates from AD and control patients were quantified on a custom MSD-immunoassay using recombinant PrP 23-111 (PrP-N1 fragment) as capture and biotinylated 82e1 for detection. Error bars = Means ± SEM. (B) Crude brain homogenates from AD and control patients were spun at 16,000 rpm x 30 min, then the supernatant analysed on a custom MSD-immunoassay using recombinant PrP 23-111 (PrP-N1 fragment) as capture and biotinylated 82e1 for detection. Error bars = Means ± SEM.
3.2.6 Section Discussion

3.2.6.1 Results summary

These studies included biochemical analyses of brain homogenates from humans who died with a range of severities of AD-neuropathology and cognitive decline, as well as a high-amyloid control subgroup with substantial amyloid neuropathology but no associated cognitive decline. The overall aim of this section was to identify any biochemical signal which could differentiate between high-amyloid controls and AD cases. Overall, levels of Aβ42 quantified with a novel immunoassay were increased in samples with more severe AD neuropathology, but were not significantly different between severe-AD cases and High-Amyloid controls. However, following normalisation to total protein levels by BCA assay, there was a significantly lower concentration of total Aβ42 in High-Amyloid controls compared to severe-AD cases (p = 0.0068, Bonferroni post-hoc correction following one-way ANOVA). Levels of Aβ oligomers were analysed using an oligomer-selective immunoassay, and oligomer levels higher than 2,000 pg/ml were only observed in moderate-AD, Severe-AD and High-Amyloid control groups. However, this assay also detected Aβ in zero-amyloid-control brains, and due to substantial variability within each group there were no significant differences between any group. Finally, levels of PrP-binding Aβ oligomers were quantified in all groups, however disappointingly this assay obtained high values for samples within every group, and could not distinguish between any group.

3.2.6.2 Banner brain characterisation critique and future work

The primary issue with this work is the high level of variability observed within each patient group. One key factor in this may have been the fact that each brain
homogenate was produced from a very small piece of brain tissue weighing ~250 mg. Aβ deposition is not uniform throughout the brain, thus even within a single brain there may be huge variability in the levels of Aβ present in one small chunk of tissue compared to the chunk of tissue immediately adjacent. If possible, this work should be repeated with homogenates either produced from a single large chunk of brain tissue (>= 5 grams), or preferably from 3+ separate chunks of brain dissected from different brain regions within each patient. Furthermore, more emphasis should be placed on characterising soluble protein species, and perhaps utilising brain extraction procedures without any homogenisation to provide more accurate representations of diffusible proteins present within the living human brain (such as those seen in 3.6).

The results from the PrP-MSD immunoassay were particularly disappointing, as they failed to even discriminate between zero-amyloid control and severe-AD cases. This is difficult to explain, as the standard curve used for quantification of Aβ species still provided an LLOQ of 352 pg/ml, and thus the assay could correctly distinguish between blank buffer, and buffer containing 352 pg/ml of synthetic o-Aβ, yet the Zero-amyloid control brains with very low levels of Aβ (as determined by neuropathological assessment or Aβx-42 immunoassay) still registered as containing > 40,000 pg/ml in the total fraction, and up to 15,000 pg/ml in the soluble fraction. This strongly suggests that some component of human brain extract not related to o-Aβ produces significant signal in this assay, but it is difficult to suggest what this species could be as the detection step uses 82e1, an antibody which specifically detects the N-terminus of Aβ1-40 and will not even bind to full length APP or N-terminally truncated Aβ peptide variants.
Several steps could be taken to try and identify the source of this artefactual signal.

First, a dilution series of a Zero-amyloid control brain extract could be analysed via the current PrP-MSD to identify if the artefactual signal is at least concentration-dependent - i.e. does a 2-fold dilution of a zero-amyloid control brain homogenate produce 2-fold less signal? If so, then this would suggest there is a specific component of these brain extracts which leads to increased signal in a concentration-dependent manner, and it is possible that component could be identified.

For this assay to provide meaningful data, optimisation steps should be taken until it is at least able to differentiate between Zero-amyloid controls and Severe-AD cases. Such optimisations could include changing the capture species to full length PrP, changing the detection antibody to a different anti-Aβ antibody which targets a different epitope of Aβ, or pre-treating or ultra-centrifuging brain homogenates before analysis. Such steps will be necessary, as if this assay is not able to differentiate between healthy control and severe-AD cases, then any species which it does manage to detect are unlikely to be disease-related or contribute to the progression of AD.
3.3 Section 3 - Crude homogenates of Slow-AD and Rapid-AD brains contain highly heterogeneous populations of Aβ peptides which are not differentiated by rate of disease progression

3.3.1 Context and aims

AD patients show substantial heterogeneity in clinical phenotype, including the rate of cognitive decline. Most patients suffer from an insidious decline lasting many years (herein defined as Slow-AD), but a small subset of patients decline from cognitive independence to death in a matter of months (defined as Rapid-AD). Recent work has suggested that molecular assemblies of Aβ may differ between Slow-AD and Rapid-AD patients both in terms of seeding activity and stability (Cohen, Appleby and Safar, 2016; Qiang et al., 2017). In this section, biochemical analyses are used to compare brain homogenates from a small set of Rapid-AD patients with disease duration less than 9 months to a group of more typical Slow-AD cases with disease duration lasting multiple years. This characterisation was primarily carried out to better interpret the experiments carried out in section 4 and section 5, where extracts from these brains are inoculated into AD mouse models.
3.3.2 Slow-AD vs Rapid-AD patient summary

The brain samples used in this section (and sections 4, 5 and 6) comprise cortical brain samples from the frontal lobe of patients from the autopsy tissue archives of the Medical Research Council (MRC) Prion Unit (Rapid-AD cases), the Queen Square Brain Bank for Neurological Disorders at UCL Institute of Neurology (Slow-AD cases) and from the Oxford Brain Bank (Control case). Rapidly progressive AD (Rapid-AD) was defined as a dementia syndrome with clinical duration (calculated from notes during clinical examination combined with patient history as described by patient and family) of less than two years and an autopsy diagnosis of AD (Braak and Braak stage: V or VI, and Consortium to Establish a Registry for AD (CERAD): definite AD). Mean disease duration for the ‘Slow-AD’ group was 124 months, whereas for Rapid-AD group was 5.6 months.

Table 4 - Patient information for Slow-AD and Rapid-AD brain samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age of onset</th>
<th>Age of Death</th>
<th>Disease duration (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>n/a</td>
<td>41</td>
<td>n/a</td>
</tr>
<tr>
<td>Slow-AD 1</td>
<td>64</td>
<td>77</td>
<td>156</td>
</tr>
<tr>
<td>Slow-AD 2</td>
<td>65</td>
<td>76</td>
<td>132</td>
</tr>
<tr>
<td>Slow-AD 3</td>
<td>57</td>
<td>64</td>
<td>84</td>
</tr>
<tr>
<td>Rapid-AD 1</td>
<td>79</td>
<td>79</td>
<td>3</td>
</tr>
<tr>
<td>Rapid-AD 2</td>
<td>83</td>
<td>83</td>
<td>6</td>
</tr>
<tr>
<td>Rapid-AD 3</td>
<td>73</td>
<td>73</td>
<td>8</td>
</tr>
</tbody>
</table>
3.3.3 Aβ_{x-38} and Aβ_{x-40} peptide levels are highly variable between AD brain homogenates, while Aβ_{1-42} levels are comparatively similar

To allow direct comparison of Aβ_{x-38}, Aβ_{x-40} and Aβ_{x-42} peptide levels within a single sample of AD brain homogenate on a single plate, samples were examined using the triplex V-PLEX Plus Aβ Peptide Panel Kit from MSD, using proprietary anti-Aβ_{x-38}, anti-Aβ_{x-40} and anti-Aβ_{x-42} antibodies for capture, and 6E10 anti-Aβ for detection. Samples were analysed as ‘total’ homogenates, with crude homogenates thoroughly resuspended before being diluted in 5 M GdnHCl for 30 min, then spun down at 13,000 rpm and the supernatant diluted in DPBS before addition to the assay plate. Aβ_{x-38} levels were < 10,000 pg/ml for almost all samples, except for a single Rapid-AD brain extract (Rapid-AD 2) which displayed extremely high Aβ_{x-38} concentration of ~100,000 pg/ml (Figure 36). Aβ_{x-40} levels were < 20,000 pg/ml in most samples tested, except for a single Slow-AD brain extract (Slow-AD 2) with Aβ_{x-40} concentration of ~ 140,000 pg/ml, and a single Rapid-AD brain extract with an extremely high Aβ_{x-40} concentration of ~400,000 pg/ml (Rapid-AD 2). Comparatively, Aβ_{x-42} levels were consistently within the range of 25,000 – 60,000 pg/ml for all AD brains tested. Unexpectedly, the control human brain sample was also quantified as having ~7,000 pg/ml Aβ_{x-42} in the total fraction, despite being a 41 year old male who died with no evidence of neurodegenerative disease. While it is possible that this patient was in the very early stages of amyloid deposition to lead to this positive value, this sample did not produce any positive signal for Aβ in any of our western blot experiments, thus this result may simply have been a procedural error.
Crude brain homogenates from one control patient and six AD patients were incubated for 30 min in 5 M GdnHCl, then spun down at 13,000 rpm and the supernatant quantified on a triplex V-PLEX Plus Aβ Peptide Panel MSD-immunoassay. Error bars = Means ± SD (n = 3).
Levels of soluble Aβ peptides were also analysed and showed a very similar pattern of Aβ peptides between samples, but > 200-fold lower absolute levels than in the total fraction. For these analyses, crude homogenates were spun down 16,000 rpm x 30 min and then the supernatant directly diluted in PBS and added to the assay plate without GdnHCl treatment. Once again, only the ‘Rapid-AD2’ brain showed high levels of Aβx-38 with ~2,000 pg/ml, and both the Slow-AD2 and Rapid-AD2 brains showed high levels of soluble Aβx-40 with ~1600 pg/ml and ~5,000 pg/ml respectively (Figure 37).

![Graph showing levels of Aβ peptides](image)

**Figure 37 - Triplex Aβx-38/Aβx-40/Aβx-42 MSD-immunoassay on soluble fraction of Slow-AD and Rapid-AD brain homogenates**

Crude brain homogenates from one control patient and six AD patients were spun down at 13,000 rpm for 30 min and the supernatant diluted in PBS and quantified on a triplex V-PLEX Plus Aβ Peptide Panel MSD-immunoassay. Error bars = Means ± SD (n = 3).
3.3.4 Aβ peptides in AD brain homogenate display substantial variability in both N-termini and C-termini of Aβ fragments visible by western blot

While ELISAs allow for precise quantitative analysis of protein species in biological samples, the use of two separate antibodies for ‘capture’ and ‘detection’ means that only molecules or aggregates which are capable of simultaneously binding to both antibodies concurrently will be quantified. Furthermore, the data output provides no information on the molecular weight of the species analysed, and cannot describe whether the signal observed is due to a single species, or due to a multitude of similar species. This is particularly troublesome in the case of Aβ, as a wide range of heterogeneous Aβ peptides and aggregate conformations are present in the human AD brain, and it is likely that different species may have profoundly different biophysical and biological activities. To enable more complete characterisation of the Aβ peptides present in our AD and Control brain samples, western blots were carried out using a panel of antibodies specific to a range of epitopes on Aβ peptides (Figure 38). In each case, crude 1% homogenate was thoroughly resuspended and then diluted directly in 2X sample buffer containing 10% SDS, boiled at 100 °C for 10 min, then spun down 13,000 rpm x 10 min and the supernatant directly loaded on to four Tricine gels simultaneously. Synthetic Aβ1-40 and Aβ1-42 peptides were run on each gel as controls.
Figure 38 - Binding sites for anti-Aβ antibodies

The western blots featured in section 3 use antibodies which target a range of epitopes on Aβ. Notably, 3D6, HJ7.4 and HJ2 are end-specific to either the N-terminus or C-terminus of specific Aβ peptide variants, meaning they will not bind to full length APP as they bind to the epitope revealed by β- or γ-secretase cleavage. In comparison, 6E10, m266 and 4G8 all bind to epitopes within the Aβ domain that will also be present within full length APP and other APP metabolites and thus will cross-react with these species.
The brain homogenates were first compared using HJ2 and HJ7.4 antibodies which specifically detect Aβ₄₀ and Aβ₄₂, respectively. A variety of bands were detectable using both antibodies in all AD brains, but zero signal could be detected in the control brain using either antibody (Figure 39).

As expected, HJ7.4 successfully labelled synthetic Aβ₁₋₄₂ peptide, but did not label synthetic Aβ₁₋₄₀ peptide. In the human samples, HJ7.4 detected at least two bands at ~4 kDa in every AD brain, and a large smear of signal between 6 - 12 kDa in every AD brain. The 4 kDa species most likely represents monomeric Aβₓ₋₄₂ species of various truncation or post-translational modification, while the 6-12 kDa smear likely represents SDS-stable dimers and other aggregated species. Interestingly, the Rapid-AD brains appeared to show substantially lower signal at both 4 kDa and 7 kDa, although this difference could not be quantified due to overlapping bands between lanes and the presence of bubbles during the transfer process. Nonetheless, this result was clearly different to what we had observed in the MSD immunoassay, where Aβₓ₋₄₂ signal was similar between Rapid-AD and Slow-AD brains.

In comparison, HJ2 successfully labelled synthetic Aβ₁₋₄₀ peptide, but did not label synthetic Aβ₁₋₄₂ peptide. In the human brain homogenates, Slow-AD2 and Rapid-AD2 showed vastly more signal at both the 4 kDa and 7 kDa region than any of the other AD brains. This aligned closely to the results from our MSD triplex immunoassay, in which it was also observed that Slow-AD2 and Rapid-AD2 showed vastly higher signal for Aβₓ₋₄₀.
Although difficult to quantify, it is also interesting that for Slow-AD2 and Rapid-AD2 homogenates, when labelled with HJ7.4 there are two clear bands visible at 4 kDa, with the lower band clearly darker than the higher band. In comparison, when these same samples are labelled with HJ2, there are also two bands visible in the 4 kDa region, but the higher band is clearly darker. This may suggest that Aβ_{x-40} and Aβ_{x-42} are each more prone to certain forms of truncation or post-translational modification in the human brain, producing different ratios of proteoforms which migrate at different weights.

**Figure 39 - Western blot analysis of AD brain homogenates using C-terminus specific anti-Aβ antibodies**

Crude brain homogenates from control, Slow-AD and Rapid-AD brains were thoroughly resuspended and then diluted directly in 2X sample buffer, boiled at 100 °C for 10 min, then spun down 13,000 rpm x 10 min and the supernatant directly loaded on to two hand-poured 16% tris-tricine gels simultaneously. Synthetic Aβ_{1-40} and Aβ_{1-42} peptides were run on each gel as controls. Blots are presented with high exposure to highlight both 4kDa and 7kDa bands for each sample.
Following this, the same brains were analysed using 3D6, 6E10, m266 and 4G8. 3D6 is an N-terminal Aβ antibody which requires an intact N-terminus for binding, thus it will bind to all Aβ\textsubscript{1-x} sequences, but not to Aβ\textsubscript{2-x}, Aβ\textsubscript{3-x}, or any other N-terminally truncated or extended variant. 6E10 is also targeted towards the N-terminus, but does not require an intact N-terminus and will bind to slightly truncated Aβ peptides such as Aβ\textsubscript{2-x} as well as full length APP and other APP metabolites. 4G8 and m266 both target the mid-region of Aβ, and will bind to a range of truncated or extended Aβ peptides.

When labelled using 3D6, every AD brain displayed a single band at 4 kDa, likely relating to full length Aβ peptides beginning at the canonical Aβ N-terminus (Figure 40). Slow-AD2 and Rapid-AD2 both also display a band at 7 kDa, while only faint 7 kDa bands are visible for the other AD brains. No bands are visible for the control brain at either weight.

When using 6E10, a second monomer band becomes visible at 4 kDa in every AD brain tested. Most likely, this band represents N-terminally truncated Aβ variants which could not be detected by 3D6 because 3D6 requires an intact N-terminus.

When labelled using m266, two distinct bands are once again visible at 4 kDa, but it is interesting that in this case the lower of the two 4 kDa bands is darker, whereas the higher of the two 4 kDa bands appears darker in the blot using 6E10. This suggests that 6E10 and m266 either detect distinct Aβ proteoforms, or that each of these two antibodies shows preferential binding towards different Aβ peptide variants.
Figure 40 - Western blot analysis of AD brain homogenates using N-terminus specific and mid-region anti-\(\alpha\) antibodies

Crude brain homogenates from control, Slow-AD and Rapid-AD brains were thoroughly resuspended and then diluted directly in 2X sample buffer containing 10% SDS, boiled at 100 °C for 10 min, then spun down 13,000 rpm x 10 min and the supernatant directly loaded on to four hand-poured 16% tris-tricine gels simultaneously. Synthetic \(\alpha\)\(1-40\) and \(\alpha\)\(1-42\) peptides were run on each gel as controls. Blots are presented with high exposure to highlight both 4kDa and 7kDa bands for each sample.

Finally, when these same samples were analysed using the mid-region 4G8 antibody, a strikingly different pattern is detected. For all AD brains, 4G8 detects a large smear between 5-10 kDa, which resembles the patterns observed when staining these brains using HJ2 or HJ74. In another interesting feature, despite
showing a large smear at 7 kDa in our biological samples, at this exposure 4G8 only detects an incredibly weak band at 7 kDa for synthetic Aβ peptides. This is in stark contrast to 3D6 and 6E10 which both detect strong bands at 7 kDa for synthetic Aβ peptides, but only weak bands at 7 kDa in our biological samples.

Collectively, these data strongly suggest that there are at least 2 distinct groups of Aβ species which run at 7 kDa by western blot. Firstly, there is a~ 7 kDa Aβ species which has its N-terminus exposed for binding by 3D6 and 6E10. Notably, even our synthetic monomeric Aβ peptides show a strong 7 kDa band which can be detected using 3D6 and 6E10. This suggests that this species is a dimer, most likely formed artefactually and induced by SDS, thus explaining why it is visible even in our synthetic Aβ stocks. Secondly, there is a group of Aβ species which runs between ~6 kDa and ~12 kDa which is not labelled by any N-terminal antibody, but which produces a very strong smear when labelled with the mid-region antibody 4G8 or either of the C-terminal antibodies HJ2 or HJ7.4. Because this smear is exclusively labelled by mid-region and C-terminal antibodies, the N-terminus must either be absent or occluded in this species. As such, this species most likely represents either dimeric Aβ with the dimerisation site present near to the N-terminus (thus occluding antibody binding), or an N-terminally truncated Aβ peptide variant with no N-terminus available for binding (most likely still dimeric due to migrating at ~6-12 kDa).

Despite the substantial complexity observed in Aβ peptides present within each AD brain, there were no notable differences between the Slow-AD and Rapid-AD groups, with each containing a similar mixture of Aβ peptides.
3.3.5 Section Discussion

3.3.5.1 Results summary

This section included analysis of crude brain homogenates from 3 slowly-progressive AD patients, 3 rapidly-progressive AD patients, and 1 healthy human control. In all AD brains, a heterogeneous mixture of Aβ peptides was present with a variety of N-termini and C-termini. When total Aβ\textsubscript{x-38}, Aβ\textsubscript{x-40} and Aβ\textsubscript{x-42} levels were analysed by MSD immunoassay, one single AD brain homogenate (Rapid-AD2) was found to have vastly higher Aβ\textsubscript{x-38} content than all other brains tested, and two homogenates contained strikingly high Aβ\textsubscript{x-40} levels (Slow-AD2 and Rapid-AD2), while all AD brains contained similar levels of Aβ\textsubscript{x-42}. A similar pattern was observed in the soluble fraction of these homogenates using the same assay. When analysed by western blot, the same two brains which had shown high Aβ\textsubscript{x-40} signal in the MSD assay (Slow-AD2 and Rapid-AD2) also showed strong bands at 4 kDa and 7 kDa when labelled with anti-Aβ\textsubscript{x-40} specific antibody HJ2. When these samples were labelled with anti-Aβ\textsubscript{x-42} antibody HJ7.4, all six AD brains showed strong bands at 4 kDa and 7 kDa. The three Rapid-AD brains appeared to show substantially lower signal at 4 kDa and 7 kDa than the three Slow-AD brains, but this difference was difficult to quantify. Finally, analysing the bands present when all of these brains and peptide standards were labelled with a range of N-terminal and mid-region anti-Aβ antibodies suggests that all AD brains contain both Aβ peptides beginning at the canonical N-terminus, and N-terminally truncated Aβ peptides. Further, all AD brains appear to contain two distinct sets of Aβ species which migrate between~6 kDa and 12 kDa, with the first detectable using N-terminal antibodies, while the second can only be labelled with mid-region or C-terminal antibodies. While striking
heterogeneity in Aβ peptide fragments was identified within each AD brain, there was no clear pattern or difference in the Aβ species present in the Slow-AD and Rapid-AD groups.

3.3.5.2 Crude AD brain homogenate analyses critique and future work

Overall, no stark differences were detected in the Aβ peptides present in Slow-AD vs Rapid-AD brains, but this was unsurprising due to the small group sizes and the extreme heterogeneity of Aβ peptides in the human samples. However, the data in this section was not primarily designed to identify differences between these two groups, but rather to provide as much information as possible regarding the constituent Aβ peptides present in each brain homogenate, in order to allow better interpretation of the results obtained in section 4 and section 5 using these same brains. For a real comprehensive comparison of Aβ peptides in Slow-AD and Rapid-AD brains using standard biochemical techniques, much larger sample sizes would be required. Further, as discussed in Section 3, levels of Aβ show significant regional variability within a single brain, thus a thorough comparison would require homogenate made from very large amounts of brain tissue, or preferably multiple pieces of tissue within each brain. From these samples, a wide range of biochemical analyses could be carried out including western blots, immunoassays, mass-spectrometry analyses, and analysis of the relative stability of specific Aβ aggregates using conformation-dependent immunoassays.
3.4 Section 4 - Inoculating NL-F mice with AD brain homogenates induces accelerated and distinct Aβ deposition

3.4.1 Context and aims

Many studies have shown that Aβ pathology can be induced in a variety of animal models by intracerebral or intraperitoneal inoculation with human brain extract containing Aβ. However, the majority of this research has utilised transgenic mouse models overexpressing mutant forms of APP at non-physiological levels, and many questions remain regarding the precise mechanisms of seeding. In this work, APP knock-in mice which express endogenous levels of human APP under the mouse APP promoter were intracerebrally inoculated with brain homogenates from three AD patients and one healthy human control. For each inoculum, mice were culled at multiple time-points and both biochemical and histological analyses were used to characterise Aβ deposition in inoculated mice.
3.4.2 Seeding NL-F mice with AD brain homogenate induces accelerated 
Aβ deposition

The NL-F mouse model used in this study contains an APP knock-in construct in which the Aβ region has been humanized, and two further mutations (Swedish and Iberian) increase levels of total Aβ and the Aβ<sub>42/40</sub> ratio, respectively (Figure 41). This leads to the age-dependent deposition of Aβ in amyloid plaques as the mice age.

**Figure 41 - Mutations in the APP construct of NL-F mice**

NL-F mice use a knock-in approach to express mutant APP at wild type levels under the control of the native APP promoter in mice. This should lead both to native expression levels and expression in the correct cell types at the correct time in the mouse lifespan. Three mutations are knocked in to humanise the Aβ region, with an additional two pathogenic mutations (KM > NL and I > V) increasing both total Aβ<sub>x</sub> production and Aβ<sub>x-42/Aβ<sub>x-40</sub></sub> ratio. NL-F mice show deficits in the Y-maze at 18 months of age, but no impairment in the Morris water maze (Saito et al., 2014). (Adapted from (Nilsson, Saito and Saido, 2014)).
NL-F mice were intracerebrally inoculated with crude human brain homogenates from four of the same patients analysed in detail in section 3 (Control, Slow-AD1, Slow-AD2 and Slow-AD3). This inoculation was carried out between 7 and 8 weeks of age by staff at the MRC Prion Unit Biological Services Facility (BSF), and separate groups of mice were then culled at 4 months, 8 months, or 12 months post-inoculation. In every case, mice were inoculated in the right hemisphere, the right hemisphere was frozen for biochemical analyses, and the left hemisphere was fixed in 10% buffered formal saline for immunohistochemistry. For immunohistochemical quantification of Aβ deposition, brain slices were stained using 82e1 anti-Aβ antibody, and the number and area of amyloid plaques present were quantified using the Definiens analysis platform (Figure 42).
Figure 42 - Figure describing plaque counting in mouse slices

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4, 8 or 12 months post inoculation (mpi), at which point the left hemisphere was fixed in 10% buffered formal saline then processed and paraffin wax embedded for immunohistochemical analyses. For analysis of Aβ deposition, serial sections of 5 µm were taken, and Aβ detected by incubation with biotinylated 82e1. After staining, slides were digitised on a LEICA SCN400F scanner (LEICA Milton Keynes, UK) at 40x magnification and whole slide images were exported for processing in Definiens tissue studio. (A) Plaques were detected using an automated analysis protocol based on the transformation of the RGB image to HSD representation (Van Der Laak et al., 2000). (B) Artefacts such as this native melanin expression were manually excluded. (C) Regions of interest were manually selected to separate cortex (yellow), hippocampus (blue), and cerebellum (pink) sub-regions for separate analyses. The remaining region of interest is highlighted in red, and excluded artifacts are highlighted in green.
First, the Aβ deposition visible in inoculated mouse brain slices was quantified as the percentage area of plaque coverage across the entire brain section (Figure 44). Quantification of plaques was carried out by Silvia Purro (MRC Prion Unit at UCL). Data showed substantial heteroscedasticity, in which the variance of plaque coverage within any group was positively correlated with the mean plaque coverage within that group, thus data were log-transformed for statistical comparison. The percentage coverage of Aβ deposits in Slow-AD1, Slow-AD2 and Slow-AD3 inoculated mice at 4 months post inoculation (mpi), 8 mpi and 12 mpi were significantly higher than the Aβ deposition present in Control-brain inoculated mice at equivalent time points (all p < 0.01; two-way ANOVA followed by Bonferroni post-hoc tests). There were no significant differences in levels of Aβ deposition induced by different AD brain inocula at any single time point.
Figure 43 - Immunohistochemistry of Aβ deposition in NL-F mice at 4, 8, and 12 months post inoculation with either human control brain or AD brain homogenate.

NL-F mice were intracerebrally inoculated in the right hemisphere with either human control brain homogenate (a, c, e) or human AD brain homogenate (b, d, f) between 7-8 weeks of age, then culled at 4 (a, b), 8 (c, d) or 12 (e, f) months post inoculation (mpi). Formalin fixed mouse brain slices from the left hemisphere were stained using the anti-Aβ antibody 82e1 (Brown stain).
Figure 44 - Quantification of plaque coverage in NL-F mice inoculated with human control or AD brain homogenate

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4, 8 or 12 months post inoculation (mpi). Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified using the Definiens analysis platform. Error bars = Means ± SEM. * represents a difference with p < 0.01 in Bonferroni post-hoc tests following a 2-way ANOVA. Statistics were carried out on log-transformed data.
Levels of $A\beta_{42}$ were then quantified in the brain homogenates of inoculated mice using MSD Multi-Array Human 6E10 A$\beta$42 assay and found to follow a similar pattern as the histological analyses (Figure 45). Additional MSD-immunoassays were carried out by Beth Noble (MRC Prion Unit at UCL) and Emma Quarterman (MRC Prion Unit at UCL). At 4 mpi, 8 mpi and 12 mpi, Slow-AD1, Slow-AD2, and Slow-AD3 inoculated mouse brain homogenates contained significantly higher levels of $A\beta_{42}$ in the total fraction than Control-brain inoculated mice ($p < 0.05$; two-way ANOVA followed by Bonferroni post-hoc tests). There were no significant differences between the levels of $A\beta_{42}$ present in mice inoculated by Slow-AD1 vs Slow-AD2 vs Slow-AD3 at 8mpi or 12mpi.
NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4, 8 or 12 months post inoculation (mpi). 10% brain homogenates were produced in 10% DPBS using ribolysation. For these analyses, brain homogenates were diluted for 30 min in 5 M GdnHCl, diluted to < 0.1 M GdnHCl in DPBS and analysed using MSD Multi-Array Human 6E10 Aβ42 assay. Error bars = Means ± SEM. * represents a difference with p < 0.01 in Bonferroni post-hoc tests following a 2-way ANOVA. Statistics were carried out on log-transformed data.
3.4.3 Inoculating NL-F mice with AD brain extract induces a distinct spatiotemporal pattern of Aβ deposition compared to control-inoculated NL-F mice

While it is clear that inoculating animal models of AD with AD brain extract can induce accelerated deposition of Aβ, one understudied question is whether the observed increase in Aβ deposition in inoculated animals is simply an acceleration of native Aβ deposition processes, or if alternate novel mechanisms are being catalysed. To assess this question, different brain regions were individually quantified using Definiens software and the ratio between levels of Aβ deposition present in each brain area of AD-brain inoculated and Control-brain inoculated mice were analysed. Strikingly, all AD-brain inoculated mice showed aggressive early Aβ deposition in the cerebellum at early time points, whilst only a single Control-brain inoculated mouse presented with a single small Aβ deposit in the cerebellum even at 8mpi. To ensure this was not simply due to the Control-brain inoculated mice having less total Aβ, we compared the ratio between cerebellar and total Aβ in 4 mpi and 12 mpi Control-brain inoculated mice to 4 mpi AD-brain inoculated mice. At this time point, 4 mpi AD-brain inoculated mice have substantially less total Aβ deposition than 12 mpi Control-brain inoculated mice, however they already show substantial Aβ deposition in the cerebellum. All mice were inoculated in the right brain hemisphere, and the majority histological analysis was carried out using the left brain hemisphere. However, a set of pilot experiments were carried which did not identify any notable differences in the morphology, abundance, or localisation of Aβ deposits between the left and right hemispheres of AD-brain inoculated mice (data not shown).
Figure 46 - Relative Cerebellar vs total Aβ pathology in control vs AD-inoculated NL-F mice

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4, 8 or 12 months post inoculation (mpi). Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified for both the total brain slice, and the sub-defined cerebellum region, using the Definiens analysis platform. Plotting total plaque coverage against cerebellar plaque coverage shows that almost no cerebellar plaques are visible in 4 mpi, 8 mpi or 12 mpi NL-F mice inoculated with human control brain, but substantial deposition is observed in Slow-AD1, Slow-AD 2 and Slow-AD 3 inoculated mice even at 4mpi.
Further, when plotted as the ratio between cerebellar Aβ deposits and total Aβ deposits, 4 mpi Slow-AD1, Slow-AD2 and Slow-AD3 inoculated mice all show a significantly higher ratio of cerebellar/total Aβ than either 4 mpi or 12 mpi Control-brain inoculated mice (p < 0.01; one-way ANOVA followed by Bonferroni post-hoc tests) (Figure 47), although these data do not truly fit parametric assumptions as the majority of values for cerebellar Aβ in control mice are 0. Nonetheless, these data confirm that AD brain inoculated mice show a distinct spatiotemporal progression of Aβ deposition compared to Control-brain inoculated mice.
Figure 47 - Ratio of Cerebellum plaque coverage vs Total plaque coverage in Control-inoculated and AD-inoculated NL-F mice

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4, 8 or 12 months post inoculation (mpi). Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified for both the total brain slice, and the sub-defined cerebellum region, using the Definiens analysis platform. Values were plotted as the ratio between cerebellar plaque load and total plaque load. Error bars = Means ± SEM. * represents a difference with p < 0.01 in Bonferroni post-hoc tests following a 2-way ANOVA. Statistics were carried out on log-transformed data.
3.4.4 Section Discussion

3.4.4.1 Results summary

In this section, it was shown that NL-F mice which are inoculated with AD brain extract between 7 and 8 weeks of age show accelerated and distinct accumulation of Aβ. Specifically, slices from AD-brain inoculated mice show higher coverage of Aβ plaques by immunohistochemical analysis at 4 mpi, 8 mpi and 12 mpi than Control-brain inoculated mice at equivalent time points. Further, AD brain inoculated mice had significantly higher Aβ$_{42}$ in Gdn-treated brain homogenates than Control-brain inoculated mice as measured by MSD immunoassay at 4 mpi, 8 mpi and 12 mpi. Aβ deposition also followed different spatiotemporal progression pathways in Control-brain inoculated vs AD-brain inoculated mice, with AD-brain inoculated mice displaying enhanced cerebellar Aβ deposition at earlier time points than Control-brain inoculated mice, even after normalising for total Aβ levels. Notably, no substantial differences were observed in the Aβ deposition induced by the three AD brain extracts.

3.4.4.2 Critique and future work

These data convincingly show that inoculation of NL-F mice with Aβ-containing human brain extract induces accelerated Aβ deposition which is specifically enhanced in the cerebellum. However, the precise mechanisms through which Aβ deposition is accelerated are unclear, and it is not obvious from these data why the cerebellum is preferentially targeted. Furthermore, these data focus on characterising Aβ$_{42}$, and thus the levels of alternate Aβ peptides in these mice is unknown.
In order to identify the precise mechanisms of accelerated Aβ deposition, it will be necessary to identify which molecular species within the human brain extract are necessary for inducing Aβ deposition. The fact that control human brain extract does not accelerate Aβ deposition confirms that the mechanism depends on molecular species exclusively found in AD brains, and the most likely candidates are Aβ aggregates. While other studies have shown that immunodepletion of Aβ aggregates from AD brain extract can deplete its ability to seed Aβ deposition in AD mouse models (Meyer-Luehmann et al., 2006), this should be repeated in our current model to confirm this is also true for NL-F mice. In addition, we have previously shown that Aβ-contaminated growth hormone extracts that are able to iatrogenically induce Aβ deposition in humans (Jaunmuktane et al., 2015) are also capable of inducing Aβ deposition in this NL-F model (Purro et al., 2018). Thus, it would be interesting to more thoroughly compare the pattern of Aβ deposition induced by these growth hormone extracts with that induced by AD brain extracts. Following this, inoculation of NL-F mice with various fractions of AD brain extract could be used to identify the most potent Aβ seeds. One particularly interesting experiment could be to carry out size-exclusion chromatography on AD brain extract to separate species by molecular weight, and inoculating elution fractions from this process into NL-F mice. The total Aβ concentration present in each of these fractions would be significantly diluted compared to the original extracts, but if any one fraction contained a high proportion of the seeding-competent Aβ species, it would still be expected to seed. This would provide invaluable information of the molecular weight of the most potent Aβ seeds.
To identify why the cerebellum is preferentially targeted in this model of Aβ seeding, it may be useful to compare alternative routes of administration, such as intraperitoneal injection of AD brain extract, or inoculation into different locations within the mouse brain. If alternative administration routes produce similar patterns of accelerated Aβ deposition, this may suggest that there are native features of the cerebellar microenvironment which favour Aβ deposition in this model, such as high levels of certain lipids or surface proteins which act as deposition sites. In addition to the cerebellum, severe early Aβ deposition was also commonly observed in the corpus callosum of AD-brain inoculated mice (but not in aged control-brain inoculated mice), and as early and severe cerebral amyloid angiopathy within blood vessels (data not shown). It is worth highlighting again here that while inoculation was carried out in the right hemisphere of every mouse, all histological results presented in this work are from the left hemisphere. In several pilot experiments, no substantial differences were observed in the morphology, location or abundance of Aβ deposits between the left and right hemispheres of inoculated mice (data not shown), and thus this pattern of Aβ deposition in the blood vessels, corpus callosum, and around the folds of the cerebellum in both hemispheres collectively suggest that Aβ seeds may have escaped from the site of inoculation into the CSF and plasma, from where they may then reattach to exposed membranes present in the blood vessels, corpus callosum and cerebellum. To test this hypothesis, levels of Aβ deposition could be analysed in the peripheral nervous system and peripheral blood vessels of inoculated mice.

Finally, much more work could be carried out to characterise the precise structure and peptide content of the amyloid deposits in these mice. This NL-F
model primarily expresses Aβ_{1-42} due to the presence of the Iberian mutation in APP, thus it would be expected that the primary component of amyloid plaques observed in these mice would be Aβ_{1-42}. However, previous studies have reported substantial Aβ_{pGlu3-42} present in plaques in NL-F mice, and more thorough characterisation of the Aβ peptides in our inoculated mice may elucidate whether the plaques in AD-brain inoculated mice differ in Aβ peptide content compared to the plaques present in Control-brain inoculated mice.
3.5 **Section 5 - Inoculating NL-F mice with Rapid-AD brain homogenates leads to less aggressive but spatiotemporally similar patterns of Aβ deposition compared to inoculation with Slow-AD brain homogenates**

3.5.1 **Context and aims**

AD populations show striking heterogeneity in terms of disease progression rates, with some patients suffering from an insidious decline over multiple decades, while others face a highly accelerated disease course and decline from autonomy to death in less than a year. These latter patients can be defined as ‘Rapid-AD’ patients, and recent research has suggested their rapid decline may in part be facilitated by distinct molecular assemblies of Aβ in these patients’ brains. Previous studies have noted differences in the stability of Aβ aggregates present in Rapid-AD vs Slow-AD brains (Cohen et al., 2015), and in the structures of Aβ fibrils seeded with Rapid-AD vs Slow-AD brains *in vitro* (Qiang et al., 2017), but as of yet no work has characterised the Aβ seeding activity of Rapid-AD vs Slow-AD brain extracts *in vivo*.

In this work, NL-F mice were inoculated with crude brain homogenate from the frontal cortex of three Rapid-AD patients, and separate groups of mice were culled at 4 mpi and 8 mpi. Levels and localisation of Aβ deposition in these mice were characterised and compared with similar groups of mice inoculated with Slow-AD brain extracts to investigate whether differences in the seeding activity of Aβ species in these patients brains may explain some of the observed differences in clinical presentation.
3.5.2 NL-F mice inoculated with Rapid-AD brain homogenate display increased plaque coverage across the 'total' brain slice compared to Control-brain inoculated mice, but less severe plaque coverage than Slow-AD inoculated mice

Mice were inoculated with Rapid-AD brain homogenates, then culled and brain slices fixed and stained following identical protocols as described for Slow-AD and Control-brain inoculations in Section 4, with Aβ deposition in inoculated mouse brain slices quantified as the percentage area of plaque coverage across the entire brain. At 4 mpi, all Slow-AD and Rapid-AD inoculated groups showed increased total plaque coverage compared to control-inoculated mice (p < 0.05; one-way ANOVA followed by Bonferroni post-hoc tests), but no significant differences were observed within or between groups of Slow-AD and Rapid-AD inoculated mice (Figure 48). However, even at this time point the mean plaque coverages for Rapid-AD1 (0.021%), Rapid-AD2 (0.046%) and Rapid-AD3 (0.016%) inoculated mice were all lower than any of the mean values for the Slow-AD1 (0.048%), Slow-AD2 (0.083%) or Slow-AD3 (0.155%) inoculated mice. At 8 mpi, all mice inoculated with Slow-AD brain homogenates once again displayed significantly higher total plaque coverage than Control-brain inoculated mice (p < 0.05; one-way ANOVA followed by Bonferroni post-hoc tests). Crucially however, as opposed to results at 4 mpi, no Rapid-AD inoculated mice showed significantly increased total plaque coverage compared to Control-brain inoculated mice at 8 mpi. Furthermore, all groups of 8mpi Rapid-AD inoculated mice displayed significantly lower total plaque coverage than Slow-AD2 inoculated mice (p < 0.01; one-way ANOVA followed by Bonferroni post-hoc tests).
Figure 48 - Quantification of plaque coverage in NL-F mice inoculated with human control, Slow-AD or Rapid-AD brain homogenate

NL-F mice were intracerebrally inoculated with either human control, Slow-AD or Rapid-AD brain homogenate between 7-8 weeks of age, then culled at 4 mpi or 8 mpi. Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified using the Definiens analysis platform. (A) Plaque coverage in the total brain slice of mice culled at 4 mpi. (B) Plaque coverage in the total brain slice of mice culled at 8 mpi. Error bars = Means ± SEM. * represents a difference with p < 0.01 in Bonferroni post-hoc tests following a 2-way ANOVA. Statistics were carried out on log-transformed data.
3.5.3 NL-F mice inoculated with Rapid-AD brain homogenate display increased plaque coverage across brain slice subregions compared to Control-brain inoculated mice, but less severe plaque coverage than Slow-AD inoculated mice

NL-F mice inoculated with Rapid-AD brain extract did not show significantly increased plaque coverage across the total brain slice at 8 mpi compared to equivalent Control-brain inoculated mice, which suggested these brain extracts may have failed entirely to accelerate amyloid deposition in these mice. To investigate this, images of brain slices were divided into sub-regions and plaque coverage was quantified by region. Of particular interest was the cerebellum, where we had previously noted almost no Aβ deposition in Control-brain inoculated mice, but substantial amyloid deposition in AD-brain inoculated mice even at shorter time points.

At 8 mpi, control-inoculated NL-F mice show an average cerebellar plaque coverage of 0.052%, compared to averages of 0.87% for Slow-AD1 inoculated mice, 3.17% for Slow-AD2 inoculated mice, 1.21% for Slow-AD3 inoculated mice, 0.42% for Rapid-AD1 inoculated mice, 0.51% for Rapid-AD2 inoculated mice, and 0.45% for Rapid-AD3 inoculated mice (Figure 49). For the majority of 8 mpi control-inoculated mice, the observed cerebellar plaque coverage was 0%, which meant in this case a log-transform could not be applied to control samples. Therefore, control samples were compared against Rapid-AD inoculated samples using raw data for cerebellar plaque coverage (Figure 49, A) while Rapid-AD inoculated samples were compared against Slow-AD inoculated samples using log-transformed data (Figure 49, B). At 8 mpi, Rapid-AD1, Rapid-AD2 and Rapid-AD3 inoculated mice all showed significantly higher cerebellar plaque load than control-inoculated mice (p < 0.01; one-way ANOVA followed by
Bonferroni post-hoc tests) but all Rapid-AD inoculated groups displayed significantly less cerebellar plaque load than either Slow-AD 2 inoculated or Slow-AD 3 inoculated mice (p < 0.01; one-way ANOVA followed by Bonferroni post-hoc tests). There were no significant differences between any group of 8 mpi Rapid-AD inoculated mice and 8 mpi Slow-AD 1 inoculated mice.
NL-F mice were intracerebrally inoculated with either human control, Slow-AD or Rapid-AD brain homogenate between 7-8 weeks of age, then culled at 8 mpi. Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified for the sub-defined cerebellum region using the Definiens analysis platform. (A) Direct comparison of values for raw cerebellum plaque coverage in Control-brain inoculated and Rapid-AD inoculated mice at 8mpi. All groups of Rapid-AD inoculated mice showed significantly higher cerebellar plaque coverage than Control-brain inoculated mice at this time point (* represents p < 0.01 in Bonferroni corrected post-hoc tests following one-way ANOVA on raw data). Error bars = 1 SEM. (B) Comparison of values for cerebellum plaque coverage in Slow-AD inoculated and Rapid-AD inoculated mice at 8mpi. All Rapid-AD inoculated mice showed significantly lower cerebellar plaque coverage than Slow-AD 2 inoculated or Slow-AD 3 inoculated mice, however there were no significant differences between Rapid-AD inoculated mice and Slow-AD 1 inoculated mice. Error bars = Means ± SEM. * represents a difference with p < 0.01 in Bonferroni post-hoc tests following a one-way ANOVA. Statistics were carried out on log-transformed data.
In (3.4.3), it was seen that the ratio between cerebellar Aβ coverage and total Aβ coverage was significantly different in Slow-AD brain inoculated mice compared to Control-brain-inoculated mice (Figure 47), suggesting a different spatiotemporal progression of Aβ deposition is observed in inoculated mice. In order to assess whether a different spatiotemporal pattern was occurring in Slow-AD versus Rapid-AD inoculated mice, these same analyses were carried out with 8 mpi Control-inoculated, Slow-AD-inoculated, and Rapid-AD-inoculated samples, however there were no significant differences in the ratio of cerebellar/total plaque coverage between Slow-AD and Rapid-AD inoculated mice at 8 mpi (Figure 50).
Figure 50 - Ratio between cerebellar Aβ histology and total Aβ histology in Rapid-AD vs Slow-AD mice at 8 months post inoculation

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 8 months post inoculation (mpi). Fixed slices of total mouse brain were stained using 82e1 and Aβ immunoreactivity was quantified for both the total brain slice, and the sub-defined cerebellum region, using the Definiens analysis platform. Values were plotted as the ratio between cerebellar plaque load and total plaque load. Error bars = Means ± SEM.
3.5.4 NL-F mice inoculated with Rapid-AD brain homogenate contain higher total and soluble Aβ<sub>x-42</sub> levels than Control-brain inoculated mice, but similar levels to Slow-AD brain inoculated mice.

Every mouse used in these experiments had its right hemisphere snap frozen and later homogenised in DPBS for biochemical analyses. Levels of Aβ<sub>x-42</sub> were characterised in the total crude homogenate and in the supernatant of Control-brain inoculated, Slow-AD inoculated and Rapid-AD-inoculated mice using the custom-Aβ<sub>x-42</sub> MSD immunoassay described in (3.2.3). Supernatant was obtained using a benchtop centrifuge at 13,000 rpm for 30 min. While all groups of Slow-AD and Rapid-AD inoculated mice displayed significantly elevated Aβ<sub>x-42</sub> levels compared to Control-brain inoculated mice in both the total and supernatant fractions at both 4 and 8 months post inoculation (p < 0.05; one-way ANOVA followed by Bonferroni post-hoc tests) we were surprised to find no significant differences between Rapid-AD and Slow-AD inoculated groups at either 4 mpi or 8 mpi (Figure 51). However, there was significant variability within each group observed at all time points and in all fractions.
Figure 51 - Levels of Aβ_{x42} in total and soluble (13,000 rpm) fraction of Slow-AD and Rapid-AD inoculated mice

NL-F mice were intracerebrally inoculated with either human control or AD brain homogenate between 7-8 weeks of age, then culled at 4 or 8 months post inoculation (mpi). 10% brain homogenates were produced in 10% DPBS using ribolysation. Quantification of ‘total’ fraction was carried out by direct resuspension and dilution of crude brain homogenate in 5 M Gdn HCl. Supernatant fractions were quantified by spinning crude homogenate at 13,000 rpm x 30 min and diluting the supernatant from this spin in 5 M Gdn HCl (A) Supernatant fraction from 4 mpi inoculated NL-F brain homogenates. (B) Supernatant fraction from 8 mpi inoculated NL-F brain homogenates. (C) Total fraction from 4 mpi inoculated NL-F brain homogenates. (D) Total fraction from 8mpi inoculated NL-F brain homogenates. Error bars = Means ± SD.
### 3.5.5 Section Discussion

#### 3.5.5.1 Results summary

In this section, immunohistochemical and biochemical analyses were carried out on NL-F mice which had been intracerebrally inoculated with brain homogenates from Slow-AD patients, Rapid-AD patients, and a healthy human control. When plaque coverage across total mouse brain slices were compared, the majority of Rapid-AD inoculated mice showed significantly less Aβ coverage at 8 mpi than most Slow-AD inoculated mice, and Rapid-AD inoculated mice did not have significantly increased total plaque coverage compared to control-inoculated mice at this time point. However, when only the cerebellum was quantified for plaque coverage, all 8 mpi Rapid-AD inoculated mice did show both significantly higher cerebellar plaque coverage than Control-brain inoculated mice, and lower cerebellar plaque coverage than both Slow-AD 1 inoculated and Slow-AD-2 inoculated mice. When levels of $A\beta_{x-42}$ were quantified by MSD immunoassay, Rapid-AD inoculated mice at both 4 months and 8 months post inoculation were found to contain significantly higher $A\beta_{x-42}$ in both the total and soluble fraction than Control-brain inoculated mice, but there were no significant differences observed between Rapid-AD and Slow-AD inoculated mice.

#### 3.5.5.2 Critique and future work

One of the primary issues with this work was the low numbers of true biological replicates, with only three human Slow-AD patients, three human Rapid-AD patients and one healthy human control. However, this was unavoidable for this project due both to the rarity of Rapid-AD cases and the time and expense involved in such large scale mouse experiments. Further, as the difference in
progression rate is so incredibly high between these two groups of patients (average disease length of 124 months for Slow-AD patients vs 5.6 months for Rapid-AD patients), it was hypothesised the observed effect sizes may be very high to partially counteract the low sample sizes. While some differences were indeed observed in immunohistochemical quantification of plaque burden in Slow-AD and Rapid-AD inoculated mouse groups, these results were hampered by large intra-group variability, and furthermore were not corroborated by quantification of total and soluble Aβ_{42} levels by MSD-immunoassay. Several follow-up experiments should be carried out to further characterise the Aβ deposition induced in NL-F mice by inoculation with Rapid-AD and Slow-AD brain extracts.

Firstly, it will be worthwhile to stain and quantify Aβ histology at earlier and later timepoints in both Slow-AD and Rapid-AD inoculated mice. As part of the wider project not reported here, mice were also culled at 1 month, 2 month, 3 months, 12 months and 16 months post inoculation with Control, Slow-AD and Rapid-AD brains, but the full time-course could not be analysed due to time constraints. Analysis of these samples will alleviate concerns that the observed differences between Slow-AD and Rapid-AD inoculated mice at 8mpi could be due to unexplained factors affecting only the 8 mpi time point. Further, more care should be taken to ensure that differences observed using these techniques are not due to procedural variability during immunohistochemical staining. In this work, mouse brain slices were stained by Tamsin Nazari using the Ventana discovery automated immunohistochemical staining machine (ROCHE Burgess Hill, UK), with each batch of slides containing a positive control to ensure there were not visible levels of variation in the staining observed. However, as such high
numbers of samples were stained in total, multiple ‘batches’ of staining were carried out and it is possible that there may have been procedural variability between these batches. To alleviate these concerns, a selection of slides from 8 mpi Rapid-AD and 8 mpi Slow-AD inoculated mice which appeared to show significantly different plaque loads should be re-stained concurrently within the same batch, to ensure the differences are still present.

Secondly, a range of further biochemical and immunohistochemical analyses should be carried out using alternative antibodies to quantify the levels of multiple Aβ peptides in the brains of these mice. In particular, attempts should be made to carry out immunohistochemical analyses and biochemical analyses using the same antibody, as while the custom Aβ_{x-42} MSD-immunoassay presented in this section utilises the anti-Aβ antibodies m266 (which targets the mid-region of Aβ) and HJ7.4 (which targets the C-terminus of Aβ_{x-42}), the immunohistochemical analyses used biotinylated 82e1 (which targets only the N-terminus of Aβ_{1-x}). This alone may explain why the differences in plaque load between Rapid-AD and Slow-AD inoculated mouse groups that were seen via IHC were not corroborated via biochemical analysis. Thus, it would be worthwhile to either carry out IHC using m266 or HJ7.4, or to carry out western blots of the homogenates of these mouse brains using 82e1 for detection. In addition to adding consistency and allowing better comparison of data, it would be in itself worthwhile using a range of other anti-Aβ antibodies to better characterise the true profile of Aβ peptides found in the amyloid deposits of both AD-brain inoculated and Control-brain inoculated mice.
Finally, analyses in these inoculated mouse models should be extended to include a variety of other disease-relevant molecules aside from Aβ. In several pilot analyses, there were no visible differences by immunohistochemistry in the levels of GFAP or Iba1 in AD-inoculated mice compared to control-inoculated mice (data not shown), but these analyses should be expanded and also include biochemical analyses of the levels of other proteins implicated in neuroinflammation and neurodegeneration.
3.6 A ‘soaking’ brain extraction procedure releases only a fraction of the Aβ but contains the majority of the neurotoxicity

3.6.1 Context and aims

Many in vitro models used to study AD utilise synthetic Aβ and artificially induced aggregates to mimic the effects of biological Aβ. However, this strategy has faced heavy scrutiny in recent years as it is uncertain whether these synthetic peptides and assemblies bear any resemblance to the Aβ found natively in humans. To combat this, many researchers and funding authorities advocate directly purifying Aβ from the post-mortem human brain for use in these studies, and such strategies have often found that – for example – biologically derived Aβ species may be many orders of magnitude more potent than synthetic equivalents at inhibiting LTP and causing other neurotoxic effects. However, one common issue amongst most of these studies is the use of harsh homogenisation techniques to produce these Aβ-containing extracts. Such homogenisation likely disrupts large insoluble Aβ deposits, leading to the release of a range of fragments as ‘soluble’ species, which would have been natively sequestered in insoluble plaques in the human brain.

In this section, a novel brain extraction procedure is utilised which involves no homogenisation, and instead extracts diffusible proteins from post mortem human brain using a simple soaking method. These novel extracts are then directly compared to more typically homogenised extracts, and characterised in terms of protein content and their effect on neurite outgrowth in iPSC-derived and primary rat neurons.
To precisely compare the species released by a novel ‘soaked’ brain extraction procedure to more typical homogenisation procedures, a multi-step protocol was carried out which allowed the production of multiple types of brain extract from a single chunk of brain tissue (Figure 52). Briefly, a piece of frozen brain was sliced into small chunks, then these chunks gently mixed and split into two tubes. The brain chunks in the first tube underwent thorough homogenisation in 5 volumes (w/v) of buffer, were ultracentrifuged, and the resulting supernatant was termed “H-extract”. The brain chunks in the second tube instead underwent a novel soaking procedure. Here, the 5 volumes (w/v) of buffer was added to the tube and gently incubated at 4°C for 20 min. Following this, the chunks were gently centrifuged at 2,000 x g and the supernatant removed. The supernatant was then ultracentrifuged and the resultant supernatant was termed “S-extract”. The remaining pellets of the gentle centrifugation and ultracentrifugation during the soaked extract procedure (not including the pellet produced during production of H-extract), were combined, homogenised in 5 vol buffer, ultracentrifuged and the resultant supernatant was termed “H2-extract”. Thus three brain extracts were produced from each brain: one typical homogenate (H-extract), one soaked extract (S-extract), and one final homogenate produced from the pellet after production of the S-extract (H2-extract). This complex process was critical for allowing a proper comparison of extraction procedures within a single piece of tissue. This entire protocol was carried out on four 5 gram chunks of AD brain tissue from different patients. These included the same three Rapid-AD cases described in previous section (Rapid-AD1, Rapid-AD2 and Rapid-AD3), in addition to one new ‘typical’ AD case from Massachusetts General Hospital.
(MGH-AD). The MGH-AD sample was primarily included to ensure no mistakes were made during the complex tissue extraction procedure, as a separate piece of tissue from the MGH-AH brain had previously undergone the same extraction procedure and the results could be compared. Thus in total twelve brain extracts were produced, defined as R1-H, R1-S, R1-H2, R2-H, R2-S, R2-H2, R3-H, R3-S, R3-H2, MGH-S, MGH-H, MGH-H2.
Figure 52 - Dual brain extraction protocol diagram

This extraction procedure allows the simultaneous creation of three distinct brain extracts from a single piece of frozen brain tissue, allowing direct comparison of the impact of different extraction procedures on protein recovery.
First, all twelve extracts were analysed via BCA to identify the levels of total protein present in each sample (Figure 53). For each brain, total protein levels were similar in “H” and “S” extracts, but at least 2-fold lower in “H2” extracts. The levels of total protein within each extract type were similar (+/-20%) between brains.

**Figure 53 - BCA analysis on brain extracts**

Frozen AD brain tissue was subject to a serial brain extraction protocol which led to the production of three distinct extracts from each single piece of tissue. Total protein levels in each brain extract were compared by BCA assay using BSA as standard curve. Error bars = Means ± SD.
Next, levels of sAPPα and sAPPβ in each brain were quantified by western blot using the antibody 22c11 (Figure 54). Similarly to the total protein results observed by BCA, sAPP levels were similar in H and S extracts of each brain, but much lower in H2 extracts.

Figure 54 - Levels of total protein and sAPPα in the different brain extraction methods

Frozen AD brain tissue was subject to a serial brain extraction protocol which led to the production of three distinct extracts from each single piece of tissue. (A) Brain extracts were diluted in SDS sample buffer containing BME, boiled at 100 °C for 10 min, then spun down 13,000 rpm x 10 min and the supernatant directly loaded on to Tris-Glycine gels. Signal for sAPPα and sAPPβ was detected using 22c11. (B) Signal present across both the sAPPα and sAPPβ bands were quantified using licor image studio.
Next, Aβ_{x-42} levels were quantified in all twelve extracts by MSD-immunoassay using m266 for capture, and 21F12 (anti-Aβ_{x-42}) for detection (Figure 55). All samples were pre-treated with 5 M GdnHCl overnight at 4 °C before analysis. Strikingly, levels of both Aβ peptides were at least 10-fold higher in the H and H2 extracts of each brain than in the S extracts of the same brain.

Figure 55 - Levels of Aβ_{x-42} in different brain extracts from rapid and MGH AD brain

Frozen AD brain tissue was subject to a serial brain extraction protocol which led to the production of three distinct extracts from each single piece of tissue. Brain extracts were incubated O/N in 5 M GdnHCl, then quantified on a custom MSD-immunoassay using m266 as capture and biotinylated 21F12 for detection. Error bars = Means ± SD.
3.6.3 AD brain homogenates vary in their ability to induce neurite degeneration in iPSC-derived neurons

To investigate human-relevant biological activity contained in each type of brain extract, highly differentiated iPSC-derived human neurons were prepared by Wen Liu according to the protocols described in (Jin et al., 2018), which employs a modified version of the Neurogenin 2 single-step Südhof protocol (Zhang et al., 2013). Pre-differentiated cells were grown to 21 DIV in 96-well plates, at which time point cells induced using this protocol typically display stable levels of GluA1, PSD-95, synaptophysin, synapsin 1 and tau (see (Jin et al., 2018) Supplementary Figure 5B). At this point, cells were loaded in an Incucyte Zoom live-cell microscopy system from Essen Bioscience, using the ‘Neurotrack’ neurite analysis software package to quantify neurite length and branch points for multiple fields of view within each well of the 96-well plate (Figure 56).

![Raw Image + Neurotrack labelled neurites](image)

**Figure 56** - Neurite labelling of iPSC-derived neurons using the Neurotrack software module package from Essen Bioscience

Pre-differentiated iPSC-derived neurons were matured to 21 DIV in 96-well plates and images taken using the Incucyte ZOOM live cell microscopy system. Phase-contrast images (left panel) were analysed using the IncuCyte Neurotrack software module to identify neurites (purple, right panel).
To reduce non-specific toxicity, brain extracts were dialysed against aCSF at 4 °C for 3 x 12 hours to remove all glutamate and small molecules before bioactivity experiments, and then buffer exchanged into neurobasal medium using 5 ml HiTrap desalting columns. Peak elution fractions from the desalting column were recovered and analysed by MSD-Immunoassay using m266 for capture and 21F12 for detection, in order to quantify recovery of Aβ\(_{x-42}\) within each fraction (Figure 57). After this analysis, the four elution fractions containing the highest levels of Aβ were pooled to be used for bioactivity experiments. Due to these dialysis and buffer exchange steps, it was hypothesised that a large portion of non-specific toxicity was removed from these extracts, thus cells in downstream assays were treated with significantly higher concentrations of brain extracts than typically reported in the literature.

**Figure 57 - Recovery of Aβ\(_{x-42}\) following buffer exchange of AD brain extracts**

500 µl aliquots of AD brain extract were subject to buffer exchange using 5 ml HiTrap desalting columns in order to remove glutamate and other small molecules which could interfere with bioactivity assays. Elution from the HiTrap column was collected in 250 µl aliquots and 5 µl from each aliquot was diluted in 5 M GdnHCl and incubated O/N, then quantified on a custom MSD-immunoassay using m266 as capture and 21F12 for detection. Results from four “H-extracts” are shown here, quantified as the % of total injected Aβ\(_{x-42}\) present in each elution fraction.
These buffer exchanged brain extracts were used for neurite analysis assays, in which pre-differentiated iPSC-derived neurons were plated in a 96-well plate and matured for ~21 DIV before being exposed to varying concentrations of brain extracts and imaged every 2 hours for 80+ hours in the Incucyte Zoom live-cell microscopy system. Four images were taken per well, and each treatment condition comprised three replicate wells, thus each treatment condition comprised an average of 12 images per time point. Neurite length present in each image was quantified using Neurotrack automated analysis software.

First, the total bioactivity present in typical homogenised “H-extract” from all four brains was compared. In order to allow comparison to other homogenisation techniques, treatment concentrations are referred to in relation to a hypothetical 100% brain homogenate. For example, H, S and H2 extracts are prepared in 5 volumes (w/v) of buffer, thus can be described as a 20% brain extract. In these studies, if 50 µl of H, S, or H2 extract was added to a well of cells containing 150 µl of buffer, this would be defined as a 1:4 dilution, and thus the final reported treatment concentration would be 5%.

Levels of toxicity varied substantially in extracts prepared from different AD brains. R3-H extract significantly decreased neurite length at both 5% and 2.5% concentration (p < 0.0001 and p < 0.05; one-way ANOVA followed by Bonferroni post-hoc tests), R1-H extract induced significantly decreased neurite length at 5% (p < 0.0001; one-way ANOVA followed by Bonferroni post-hoc tests) but not at 2.5% , and both R2-H and MGH-H failed to induce significantly decreased neurite length in iPSCs at any concentration tested (**Figure 58**).
iPSC-derived neurons were grown to 21 DIV in 96-well plates and then treated with either AD brain ‘H-extract’, or fresh media before being imaged every 2 h for 80 h using the Incucyte ZOOM live cell microscopy system. Neurites in every image were analysed using Neurotrack software and plotted as neurite length normalised to length at $t = 0$ (left panels), and then as the percentage reduction in neurite length after 80 hours (right panels). 

(A) R1-H extract induced significantly decreased neurite length at 5% ($p < 0.0001$, Bonferroni post-hoc comparison following one-way ANOVA) but not at 2.5%. (B) R2-H did not decrease neurite length at any concentration tested. (C) R3-H extract significantly decreased neurite length at both 5% and 2.5% concentration (both $p < 0.05$, Bonferroni post-hoc comparison following one-way ANOVA). (D) MGH-H did not induce decreased neurite length at any concentration tested. Error bars = Means ± SEM.

Figure 58 - AD brain H-extract neurite toxicity in iPSC-derived neuronal culture
Subsequently, R1-H and R3-H were investigated to assess whether their ability to induce reductions in neurite length was dependent on Aβ. To achieve this, R1-H and R3-H extracts were split into two parts, one portion was immunodepleted using the polyclonal anti-Aβ antibody s97 conjugated to protein A Sepharose beads (PAS) (leading to reductions in Aβ_{x-42} of 68% for R1-H and 93% for R3-H), while the other portion was mock-immunodepleted with PAS beads conjugated to pre-immune serum, which led to reductions in Aβ_{x-42} of 16% for R1-H and 19% for R3-H (Figure 59).
Figure 59 - Immunodepletion of Aβ from AD brain extracts

R1-H and R3-H extracts were split into two parts, one portion was immunodepleted using the polyclonal anti-Aβ antibody s97 conjugated to protein A Sepharose beads (PAS), while the other portion was mock-immunodepleted with PAS beads conjugated to pre-immune serum (MOCK). (A, B) PAS beads using for immunodepletion of R1-H (left) and R3-H (right) were recovered after each of three immunodepletion steps, boiled directly in sample buffer containing 10% SDS and electrophoresed hand-poured 16% Tris-tricine gels. Aβ was detected by double labelling with 21F12 (anti-Aβ_{42}) and 2G3 (anti-Aβ_{40}) antibodies. (C, D) Supernatants from immunodepletion of R1-H (left) and R3-H (right) were recovered after each step and 5 µl from each aliquot was diluted in 5 M GdnHCl and incubated O/N, then then quantified on a custom MSD-immunoassay using m266 as capture and 21F12 for detection. Error bars = Means ± SD.
Surprisingly, immunodepletion of Aβ_{x-42} was not able to ameliorate the ability of these brain extracts to induce neuritic degeneration in iPSC-derived neurons (Figure 60).

**Figure 60 - AD brain H-extract neurite toxicity in iPSC-derived neuronal culture following immunodepletion of Aβ**

iPSC-derived neurons were grown to 21 DIV in 96-well plates and then treated with either Aβ-immunodepleted or MOCK-immunodepleted AD brain ‘H-extract’, or fresh media before being imaged every 2 h for 100 h using the Incucyte ZOOM live cell microscopy system. Neurites in every image were analysed using Neurotrack software and plotted as neurite length normalised to length at t = 0 (left panels), and then as the percentage reduction in neurite length after 80 hours (right panels). Error bars = Means ± SD. (A) R1-H MOCK-ID and R1-H ID extract both induce similar neuritic degeneration in iPSC-derived neurons. (B) R3-H MOCK-ID and R3-H ID extract both induce similar neuritic degeneration in iPSC-derived neurons.
Finally, the ability for H and S brain extracts to impair neurite outgrowth were directly compared on a single plate of low-density primary hippocampal rat neurons. Cells were analysed using a new Incucyte S3 live cell microscopy system. This experiment utilised Rapid-AD3 brain extracts which had been shown to be potently bioactive in assays using iPSC-derived neurons. Treatment with 2.5% R3-S or R3-H extract induced a significant reduction in neurite length over 80 h (p < 0.05; one-way ANOVA followed by Bonferroni post-hoc tests), but there was no difference in amount of neurite reduction induced by R3-S vs R3-H (Figure 61).

**Figure 61 - Effect of Homogenised vs Soaked AD brain extract on neurites in primary hippocampal rat neurons**

Primary hippocampal rat neurons were plated at 1.5 k/well and grown to 14 DIV in 96-well plates, then treated with either homogenised (R3-H) or soaked (R3-S) AD brain extract. Images were captured every 2 h for 80 h using the Incucyte S3 live cell microscopy system. Neurites in every image were analysed using Neurotrack software and plotted as neurite length normalised to length at t = 0 (left panels), and then as the percentage reduction in neurite length after 80 hours (right panels). Error bars = Means ± SD.
3.6.4 Section Discussion

3.6.4.1 Results summary

In this section, 'soaked' brain extracts (S-extracts) were produced from four AD brains and compared to more typical homogenate extracts (H-extracts) and homogenates produced from the tissue remnants of the soaking procedure (H2-extracts). Soaked extraction released the majority of total soluble proteins and sAPP, but <10% of the total $\text{A}\beta_{x-42}$. In a neurite retraction assay using iPSC-derived neurons, H extracts from Rapid-AD1 (R1-H) and Rapid-AD3 (R3-H) brains were able to induce neurite loss, while H extracts from Rapid-AD2 (R2-H) and MGH-AD (MGH-H) did not. Immunodepletion of Aβ from R1-H and R3-H was not able to ameliorate this neurite loss. Finally, the ability for R3-H, R3-S and R3-H2 extracts to induce neurite loss were compared in primary rat neurons where R3-S and R3-H both significantly induced neurite reduction by a similar amount.

3.6.4.2 Critique and future work

The key results from this section involve the characterisation of a novel tissue extraction procedure wherein brain tissue is soaked in aqueous buffer for 20 minutes rather than being thoroughly homogenised. Strikingly, this releases the majority of total protein from frozen brain tissue, while only a fraction of the total $\text{A}\beta_{x-42}$ is recovered. Furthermore, in a neurite retraction assay, soaked and homogenised extracts were equipotent at inducing neurite loss in primary hippocampal rat neurons. While this extraction procedure is a promising tool for characterising truly relevant ‘soluble’ aggregates in neurodegenerative disease, much more work is needed to characterise the molecular content of these extracts and the mechanisms of their biological activity.
Firstly, it is not clear precisely which fractions of brain tissue are extracted via this soaking procedure. As such a high proportion of the total protein is released, the majority of both intracellular and extracellular proteins are likely extracted, probably due to cell lysis caused by the combination of a freeze thaw cycle (the extraction procedure is carried out on snap-frozen brain tissue), and the soaking procedure. Quantification of proteins enriched in specific subcellular compartments (such as the lysosomes or endosomes) in soaked and homogenised extracts would provide information on whether proteins from specific subcellular compartments are efficiently extracted during soaking. Fractionation using detergents could further inform whether intramembrane proteins are efficiently extracted, and nucleic acid analysis could describe the efficiency of DNA and RNA extraction using this technique. In addition, it may be interesting to carry out these analyses immediately on fresh brain tissue which has not been frozen, to assess whether the majority of protein release observed in these experiments was indeed due to a freeze-thaw cycle. All of these characterisations are particularly relevant for interpretation of bioactivity analyses such as the neurite retraction assay described here, where the brain extracts are applied directly to cells in the extracellular medium. Intramembrane proteins, lipid fragments, lysosomal proteins, DNA and RNA would rarely if ever be present extracellularly within the native human brain, so their presence in these extracts (if applied extracellularly during in vitro neurotoxicity assays) is highly likely to lead to artefactual readouts of toxicity.

Nonetheless, these soaked extracts most likely contain fewer sources of artefactual toxicity than a typical homogenised extract. Soaking only recovered a small fraction of the total Aβx-42 recovered by homogenisation, which suggests
that typical homogenisation forcibly disrupts large insoluble Aβ deposits in the frozen brain tissue, converting them into soluble species when they would have been sequestered in insoluble plaques in the living human brain. While this study only investigated levels of Aβ_{x-42} in these extracts, it would be worthwhile to investigate a wide range of other Aβ peptides, in addition to other proteins typically found in insoluble deposits in the post-mortem AD brain such as Tau.

Despite the likelihood that these soaked extracts contain fewer sources of artefactual toxicity than typical homogenised extracts, and the extensive attempts to remove non-specific toxicity from all of these extracts using dialysis and buffer exchange, an absolute necessity for effective mechanistic studies would be the creation of similar extracts from healthy human controls. This was initially planned during this study but could not be carried out due to limited tissue availability. As noted previously, even after dialysis and buffer exchange these extracts likely contain a variety of protein and lipid species which could cause artefactual non-specific toxicity in cell-based models, and having multiple extracts from healthy human controls who died without evidence of neurodegenerative disease would provide much more compelling data that the observed effect on neurites is due to AD-related processes in these brains and not non-disease-related artefactual toxicity. After producing thoroughly characterised ‘soaked’ extracts from both control and AD brain samples, it would be possible to carry out relatively high throughput mechanistic studies by using a variety of paradigms to inhibit the observed toxicity. Such studies could involve immunodepletion of putative toxic molecules from brain extracts, preincubation of extract with antibodies or small molecules before addition to neurons, or pre-treatment of neurons with antibodies or small molecules before being exposed
to brain extracts. Ultimately, these experiments could be used to investigate both which species within the brain extract are required for toxicity, and which cellular processes are involved in mediating toxicity.
3.7 Final Conclusions and Discussion

3.7.1 In a cellular transfection model, COS-7 cells overexpressing LilrB2, PrP or Fcgr2b all bound significantly higher levels of synthetic Aβ aggregates, but this binding was only saturable at nanomolar concentrations for LilrB2-transfected cells.

In this work, a variety of putative Aβ-receptors were compared in their ability to bind o-Aβ when overexpressed at the surface of COS-7 cells. While COS-7 cells overexpressing FcγRIIb, PrP, and LilrB2 all bound significantly more o-Aβ than WT COS-7 cells, this binding was only saturable for LilrB2-transfected cells. While each of these receptors has previously been shown to bind o-Aβ by separate labs (Laurén et al., 2009; Kam et al., 2013; Kim et al., 2013), at the time of data collection this was the first time they have been directly compared. A study has since been published which suggests that overexpression of PrP C has a higher affinity for o-Aβ binding than LilB2, and showed no successful binding to FcγRIIb (Smith et al., 2019). These results differ from the work described here, which suggests that LilrB2 has a higher affinity for o-Aβ than FcγRIIb or PrP, although in both sets of work confounding factors such as receptor overexpression inducing alterations to the lipid content of the plasma membrane are also possible. To further explore these deviations in results, recombinant LilrB2 or a LilrB2 fragment should be expressed in bacterial or mammalian cells and high-throughput plate-based binding assays carried out with our own preparations of o-Aβ. If successful, these plate-based assays could be used to quantify levels of LilrB2-binding Aβ species in healthy vs AD brain. If AD brains are enriched in LilrB2-binding Aβ species, this would provide compelling evidence that such species are involved in AD pathophysiology, and similar
plate-based binding assays could be used to carry out high throughput screens for molecules or treatments which inhibit the LilrB2-Aβ binding interaction.

### 3.7.2 Recombinant FcγRIIb and PrP fragments are able to bind to synthetic Aβ aggregates when surface-immobilised and in solution

Previously, separate studies using different methodologies have shown that fragments of FcγRIIb and PrP can bind to synthetic Aβ aggregates (Freir et al., 2011; Kam et al., 2013), at the time this work was carried out no direct comparisons had been made. In this work, both PrP-N1 fragment and a custom FcγRIIb-ectodomain fragment (Fcγ-Ecto) were able to capture synthetic o-Aβ1-42 with a Kd of \(~15\) nM and \(~10\) nM, respectively. Further, preincubating o-Aβ1-42 with these fragments was able to inhibit o-Aβ1-42 binding to plate-bound PrP N1 fragment with IC50s of \(~1\) nM for PrP-N1 and \(~5\) nM for Fcγ-Ecto. The fact that plate-bound PrP-N1 appears to have a lower affinity for o-Aβ than plate-bound FcγRIIb, whereas soluble PrP-N1 has a lower IC50 than soluble FcγRIIb, may suggest that PrP-N1 fragment binds o-Aβ more effectively in solution than when plate-bound, while the reverse is true for FcγRIIb. This has interesting implications for the role of PrP in AD, as it is known that the N-terminus of PrP can be subject to ectodomain shedding from the membrane (Taylor et al., 2009), releasing a soluble ectodomain which could potentially interact with soluble Aβ aggregates in the interstitial fluid. Interestingly, this cleavage can be carried out by ADAM10 (Taylor et al., 2009) also known to be a constitutive α-secretase of APP (Kuhn et al., 2010), perhaps suggesting mechanistic overlap. The role of ‘shed’ PrP in AD could potentially be either protective or deleterious. On one hand, such interactions could inhibit Aβ binding to surface receptors and other downstream effectors, or inhibit Aβ aggregation. Alternatively, soluble forms of
PrP binding to Aβ could act to modulate Aβ aggregation, seed unique aggregation pathways, or create multi-protein aggregates with unique biological and biophysical characteristics. In order to further study this phenomenon, it would be worthwhile to characterise soluble Aβ aggregates and PrP species isolated directly from human brain using mass spectrometry or IP-WB, to test whether these two proteins natively bind in the soluble fraction of the human AD brain.

3.7.3 Human AD brain extract contains a variety of N-terminally truncated Aβ species, and two distinct classes of Aβ species which run at ~ 7 kDa by Western Blot.

While a vast literature of studies have been published in which Aβ species from biological samples has been characterised using SDS-PAGE, there is substantial controversy regarding whether the species observed using this method are representative of the native state present in human tissue or are merely artefacts (Bitan et al., 2005; Watt et al., 2013). The work presented here analysed the same individual samples of AD brain homogenate with 6 different antibodies targeting different epitopes of Aβ to investigate what impact the choice of antibody has on the species observed. The results showed striking variability, with entirely different bands, ratios between bands, and ratios between samples visible, depending on which antibodies were chosen. Most striking was the large smear observed in all AD samples between ~6 kDa and ~12 kDa when stained with the anti-Aβx-42 antibody HJ7.4. In comparison, this band was weak or absent in most AD samples when staining with 3D6, 6E10 or m266. Fascinatingly, both 3D6 and 6E10 were still able to detect a very strong band at ~7kDa in lanes containing synthetic Aβ1-42 monomer, which suggests that there are at least two distinct classes of biological Aβ species which run at 6-12 kDa by western blot.
The first is detectable by 3D6 and 6E10, therefore it must have its N-terminus exposed and be comprised of full length Aβ. The second cannot be detected by 3D6 or 6E10, and must therefore either contain dimeric Aβ with the dimerisation site present near to the N-terminus (thus occluding antibody binding), or an N-terminally truncated Aβ peptide variant with no N-terminus available for binding. To confirm that this species is natively found in the human brain, it would be worthwhile carrying out SEC on AD brain extracts prior to WB, to confirm that these 6-10 kDa species are eluted in a distinct fraction from monomeric Aβ even before any SDS is introduced. Following this, further analysis using IP-WB or IP-MS could be used to identify precisely which molecules are present in these aggregates.

3.7.4 Intracerebral inoculation of NL-F mice with AD brain extract both accelerates and alters the spatiotemporal progression of amyloid deposition

Previous studies have shown that Aβ deposition can be accelerated in NL-F mice by intracerebral inoculation with Aβ-containing brain extracts (Ruiz-Riquelme et al., 2018), but the characterisation presented for this model was limited and it was not identified whether the observed Aβ deposition was simply an acceleration of native NL-F amyloid pathology, or if unique deposition processes were being catalysed.

In this work, NL-F mice were inoculated with either human control or AD brain homogenate, and groups of mice were then culled at three time points – 4 months post inoculation (mpi), 8 mpi and 12 mpi. AD-inoculated mice had increased levels of Aβ pathology at all time points, but the induced amyloid deposition also followed a distinct spatiotemporal progression compared to
typical NL-F mice. Specifically, AD-inoculated mice showed accelerated amyloid deposition in the cerebellum beginning at 4 mpi, with cerebellar Aβ consistently accounting for 5%-90% of total Aβ deposits visible in inoculated mice. In comparison, control-inoculated mice showed almost zero cerebellar amyloid pathology even at 12 mpi, despite having extensive amyloid deposition across the total brain slice. This confirms that this intracerebral inoculation paradigm does not simply accelerate the native amyloid deposition processes in the NL-F brain, but catalyses novel sites of amyloid deposition. In future work it would be interesting to identify which components of our AD inocula are required for seeding, and how different routes of Aβ administration alter the spatiotemporal progression of amyloid pathology in these mice.

3.7.5 Intracerebral inoculation of NL-F mice with Rapid-AD brain extract leads to less aggressive acceleration of amyloid deposition than inoculation with Slow-AD brain extract by immunohistochemical analysis but not biochemical analysis.

While most AD patients suffer from an insidious disease course lasting up to 20 years, a small subset of patients suffer from highly accelerated disease progression and decline from health to death within a matter of months. Previous studies have noted differences in the stability of Aβ aggregates present in Rapid-AD vs Slow-AD brains, and in the structures of Aβ fibrils seeded with Rapid-AD vs Slow-AD brains in vitro, but as of yet no work has characterised the Aβ seeding activity of Rapid-AD vs Slow-AD brain extracts in vivo.

In this work, NL-F mice were inoculated with both Slow-AD and Rapid-AD brain homogenates and separate mouse groups culled at 4 mpi and 8 mpi. While overall a similar pattern of amyloid deposition was observed in Slow-AD-
inoculated and Rapid-AD-inoculated groups, immunohistochemical quantification suggested that the overall rate of induced Aβ deposition appeared to be slower in Rapid-AD inoculated mice, despite biochemical quantification suggesting there were similar levels of total Aβ peptides in AD-brain homogenates from Slow-AD and Rapid-AD groups. If these data can be confirmed, they imply that the Aβ species present in Rapid-AD brains less aggressively induce amyloid deposition in NL-F mice than the Aβ species present in typical AD brains.

In humans, this could potentially suggest that Rapid-AD patients actually suffer from a very slow and gradual β-amyloidosis, perhaps slow enough that compensatory mechanisms in the brain are able to cope and prevent the gradual cognitive decline seen in typical AD patients, before finally the proteostatic mechanisms in the brain are overwhelmed by the huge amyloid burden and a rapid cascade of neurodegeneration occurs, which could present clinically in rapid cognitive decline. In this novel model, rate of cognitive decline (especially in the MCI stage) would be associated with rate of amyloid deposition, rather than absolute amyloid load. This hypothesis could be tested using large longitudinal datasets of AD patients by examining the correlation between rate of change of amyloid biomarkers vs rate of change of cognitive scores in these cohorts.

Alternatively, these data could suggest that there is a specific ‘strain’ of Aβ aggregates present in rapid-AD brains which is relatively poor at inducing Aβ deposition in NL-F mice. To test this, it would be interesting to carry out serial passage experiments in which brain homogenates from AD-inoculated NL-F
mice are inoculated into new NL-F mice. If similar Aβ seeding is observed on serial passage, this would provide stronger evidence that the observed differences in inoculation by Slow-AD and Rapid-AD brains are due to unique self-propagating conformers of aggregated Aβ rather than other confounding factors.

3.7.6 A 'soaking' tissue extraction procedure releases the majority of total protein and bioactivity from AD brain, but < 10% of the total Abeta_x-42

Many researchers and funding authorities advocate directly purifying Aβ from the post-mortem human brain for use in biological studies, but methods for obtaining Aβ from brain samples typically use of harsh homogenisation techniques which are likely to disrupt large insoluble Aβ deposits, leading to the release of a range of fragments as ‘soluble’ species which would have been natively sequestered in insoluble plaques in the human brain.

In this section, a ‘soaking’ brain extraction procedure was used on frozen brain tissue from four AD patients. This protocol released the majority of total soluble proteins and sAPP, but <10% of the total Aβ_x-42. Furthermore, soaked and homogenised extracts from one piece of tissue both significantly induced neurite reduction in primary rat neurons by a similar amount. These results imply that the majority of ‘soluble Aβ’ found in typical homogenised brain extracts after ultracentrifugation were actually not readily diffusible in the living human brain, but rather were disrupted and solubilised by the homogenisation procedure. This calls into question a vast array of studies which have investigated the biophysical nature and biological activity of Aβ in homogenised extracts, suggesting both that many artefactual results may have been caused by these homogenisation-
induced components, and also that the identity and biological activities of the truly potent diffusible Aβ species (which could perhaps be those present in these soaked extracts) may have been masked by the much more prevalent homogenisation-induced kind. A wide array of studies using these soaked extracts is therefore warranted, investigating the biophysical nature and biological activity of Aβ in the diffusible fraction of AD brain.
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