The role of amylin in Alzheimer’s disease

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CHAPTER 1: INTRODUCTION ................................................. 14

1.1 Protein misfolding disorders ............................................ 14
  1.1.1 Amyloid aggregation and oligomerisation ....................... 14
  1.1.2 Relationship between PMDs and ageing ......................... 16

1.2 Type II diabetes mellitus .................................................. 18
  1.2.1 Pathological hallmarks of T2D ..................................... 19
  1.2.2 Physiological role of amylin ....................................... 23
  1.2.3 Amylin aggregation is a central event in T2D ................... 24
  1.2.4 Proposed toxic species of amylin ................................. 25

1.3 Alzheimer’s disease ........................................................... 26
  1.3.1 Symptoms and pathological hallmarks of AD .................... 28
  1.3.2 Generation of Aβ .................................................... 29
  1.3.3 Amyloid cascade hypothesis ....................................... 31
  1.3.4 Cell surface receptors of Aβ oligomers ........................... 31
  1.3.5 Tau .................................................................. 34
  1.3.6 Other pathological features of AD ................................. 35

1.4 Connections between T2D and AD ..................................... 36
  1.4.1 Etiological links between T2D and AD ............................ 36
  1.4.2 “Type 3 diabetes” .................................................... 36
  1.4.3 Toxic oligomer species .............................................. 37
  1.4.4 Pathology in apparently non-diseased individuals .......... 38
  1.4.5 Non-canonical amyloid deposition in AD and T2D .......... 38
  1.4.6 Amylin deposition in the brain .................................... 39
  1.4.7 Repurposing antidiabetic drugs for AD ......................... 40

1.5 Thesis aims ..................................................................... 42
  1.5.1 Investigating the role of amylin in the brain in AD and T2D... 43
CHAPTER 2: MATERIALS AND METHODS .............................................. 44

2.1 Cell Culture ................................................................................. 44
  2.1.1 Cell Lines .............................................................................. 44
  2.1.2 iPSC differentiation protocol .................................................. 44

2.2 Animal tissue ........................................................................... 45

2.3 Human Brain samples ............................................................... 46
  2.3.1 Ethics and permissions ............................................................ 46
  2.3.2 Homogenisation protocol ....................................................... 48

2.4 Sample Preparation ................................................................. 49
  2.4.1 Preparation of cell lysates and media ........................................ 49
  2.4.2 Lysis buffer and loading sample preparation ......................... 49
  2.4.3 Bicinchoninic acid (BCA) assay ............................................. 50

2.5 Oligomer preparation ................................................................. 50
  2.5.1 Peptide film preparation ........................................................ 50
  2.5.2 Amyloid-β preparation .......................................................... 50
  2.5.3 Amylin preparation ................................................................ 51
  2.5.4 Small molecule inhibitors ...................................................... 51

2.6 Western Blotting and Dot blotting ............................................. 51
  2.6.1 Western Blotting .................................................................. 51
  2.6.2 Dot blotting .......................................................................... 52

2.7 Glutaraldehyde cross-linking assay .......................................... 54

2.8 Atomic Force Microscopy .......................................................... 54
  2.8.1 Protocol ................................................................................ 54
  2.8.2 Data acquisition .................................................................... 54
  2.8.3 Data analysis .......................................................................... 54

2.9 Thioflavin-T Assay ..................................................................... 55

2.10 Immunofluorescence microscopy ............................................. 55
  2.10.1 Protocol ............................................................................... 55
  2.10.2 Antibodies ........................................................................... 56
  2.10.3 Microscopes .......................................................................... 56
  2.10.4 Data Analysis ........................................................................ 56

2.11 Immunohistochemistry ............................................................. 57
  2.11.1 Fixing and slicing .................................................................. 57
  2.11.2 DAB immunohistochemistry ............................................... 57
  2.11.3 Slide scanner and quantification ........................................... 58
  2.11.4 Congo red staining .............................................................. 58
2.12 Multiplex immunoassays and ELISAs ................................................................. 58
  2.12.1 Multiplex electro-chemiluminescence immunoassays .................................... 59
  2.12.2 Sandwich ELISA .......................................................................................... 59
2.13 PCR .................................................................................................................... 59
  2.13.1 RNA isolation and generation of cDNA ........................................................ 59
  2.13.2 RT-PCR ......................................................................................................... 60
  2.13.3 Resolving PCR product by agarose gel ......................................................... 60
  2.13.4 RT-PCR data analysis ................................................................................... 61
2.14 Mass Spectrometry ............................................................................................. 61
  2.14.1 Ion-mobility mass spectrometry ................................................................... 61
  2.14.2 Tandem mass spectrometry ......................................................................... 63
2.15 Statistical Analysis ............................................................................................. 66
  2.16 Nomenclature ................................................................................................ 66

CHAPTER 3: INVESTIGATING THE ROLE OF AMYLIN IN THE BRAIN IN AD AND T2D 67
3.1 Introduction .......................................................................................................... 67
  3.1.1 Similarities between AD and T2D ................................................................. 67
  3.1.2 Complications associated with T2D correlate with amylin deposition in peripheral organs ......................................................................................... 68
  3.1.3 Amylin deposition in the brain ..................................................................... 69
  3.1.4 Chapter Aims ................................................................................................ 70
3.2 Results .................................................................................................................. 71
  3.2.1 Amylin is deposited in the brain ................................................................ 71
  3.2.2 Amylin deposits co-localise with Aβ CAA and form heterocomplexes ......... 81
  3.2.3 Quantification of amylin in human brain fractions by multiple methods ....... 87
  3.2.4 The IAPP gene is expressed in the occipital lobe and up-regulated in AD .......
  ............................................................................................................................. 100
  3.2.5 Amylin upregulates Aβ secretion in OX1-19 neurons .............................. 101
3.3 Discussion ............................................................................................................ 103
  3.3.1 Amylin is deposited in the human brain ..................................................... 103
  3.3.2 Effect of amylin on pericyte viability .......................................................... 107
  3.3.3 Expression of IAPP mRNA in the brain and up regulation in AD ............. 108
  3.3.4 Mechanism of amylin mRNA in increase of Aβ secretion ..................... 110
3.4 Chapter Summary ............................................................................................... 112

CHAPTER 4: INVESTIGATING WHETHER AMYLIN AND AΒ SHARE DOWNSTREAM SIGNALLING .................................................................................................. 114
4.1 Introduction ......................................................................................................... 114
CHAPTER 5: THERAPEUTIC POTENTIAL OF QUERCETIN IN ALZHEIMER'S DISEASE

5.1 Introduction ...........................................................................148
5.1.1 Therapeutic targets in AD ..................................................148
5.1.2 Comparing therapeutic strategies in AD ...............................148
5.1.3 Small molecule inhibitors of Aβ aggregation .......................149
5.1.4 Aims ..................................................................................152

5.2 Results ..................................................................................153
5.2.1 Quercetin prevents the oligomerisation of Aβ .......................153
5.2.2 Quercetin does not alter the structure of preformed oligomers ....160
5.2.3 Quercetin prevents Aβ oligomer binding to SH-SYSY-PrPc cells ..165
5.2.4 Quercetin reduces Aβ1-40 and Aβ1-42 production ..................167

5.3 Discussion ..............................................................................168
5.3.1 A critical appraisal of quercetin as an aggregation inhibitor .......168
5.3.2 How does quercetin affect preformed Aβ oligomers? ...............171
5.3.3 How may quercetin mediate a reduction of APP processing? ........172

5.4 Chapter summary .................................................................174

CHAPTER 6: FINAL DISCUSSION ....................................................176

6.1 Amylin deposition: a link between AD and T2D or indicative of other problems? 176
6.2 Does amylin contribute to AD pathology? ..................................177
6.3 Further discussion of IAPP expression in the brain .......................180
List of figures

Figure 1.1 Schematic of amyloid aggregation .........................................................15
Figure 1.2 Pancreas anatomy and the islets of Langerhans. .................................20
Figure 1.3 Islet amyloidosis in diabetic human pancreas .....................................22
Figure 1.4 Demonstration of the brain atrophy in AD ...........................................28
Figure 1.5 Pathological hallmarks of AD ...............................................................29
Figure 1.6 Pathways of APP processing ...............................................................30
Figure 1.7 Sequence analysis of amylin and Aβ ....................................................40
Figure 2.1 Age at death comparison between human samples ...............................46
Figure 2.2 Human brain tissue homogenisation protocol .....................................48
Figure 3.1 Amylin antibody testing in hIAPP+/− mouse pancreas .......................72
Figure 3.2 Immunohistochemistry of Aβ in human samples ...............................73
Figure 3.3 Immunohistochemistry of amylin in human occipital lobe .................75
Figure 3.4 Immunohistochemistry of amylin in human temporal lobe ...............78
Figure 3.5 Serial staining of Aβ and amylin shows co-deposition in vasculature ......81
Figure 3.6 Quantification of pericyte viability .......................................................83
Figure 3.7 Ion mobility mass spectrometry demonstrates that amylin and Aβ interact......84
Figure 3.8 Amylin and Aβ1-42 form heterocomplexes ........................................86
Figure 3.9 Soluble and Insoluble Aβ levels from fractionated human brain tissue ....88
Figure 3.10 Amylin in human brain fractions .......................................................90
Figure 3.11 Selective reaction monitoring method for Aβ .....................................92
Figure 3.12 Selective reaction monitoring method for amylin ..............................93
Figure 3.13 Separation of amylin and Aβ standards by HPLC ..............................94
Figure 3.14 Retention time and chromatogram of Aβ in human brain fractions ......95
Figure 3.15 Retention time and chromatogram of amylin in human brain fractions ....96
Figure 3.16 IAPP is expressed in the brain and is up-regulated in AD .................98
Figure 3.17 Intra-neuronal amylin staining ........................................................99
Figure 3.18 Amylin increases secreted Aβ isoforms in OX1-19 neurons .............101
Figure 3.19 Amylin impairs autophagy to increase Aβ ......................................102
Figure 3.20 Potential role of amylin in AD .........................................................113
Figure 4.1 Characterisation of Aβ oligomers and aggregation ..............................120
Figure 4.2 Characterisation of Aβ oligomers by atomic force microscopy (AFM) ......121
Figure 4.3 Characterisation of amylin aggregation .................................................123
Figure 4.4 Characterisation of amylin oligomers by AFM ........................................124
Figure 4.5 Amylin monomer and oligomer species phosphorylate Fyn ..........................125
Figure 4.6 Effect of Phospholipase-C pretreatment on amylin induced Fyn phosphorylation .............................................................................................................................127
Figure 4.7 Effect of 6D11 pretreatment on amylin induced Fyn phosphorylation ........129
Figure 4.8 PrPc expression in iPSC derived cortical neurons ..................................132
Figure 4.9 Aβ0 induces Fyn phosphorylation in OX1-19 neurons ..............................133
Figure 4.10 Amylin induces Fyn phosphorylation in OX1-19 neurons ........................134
Figure 4.11 Tau phosphorylation in OX1-19 neurons ................................................137
Figure 5.1 Structures of quercetin and rutin ..............................................................151
Figure 5.2 Characterisation of Aβ oligomers and aggregation .....................................151
Figure 5.3 Rutin prevents Aβ fibril but not oligomer formation ..................................154
Figure 5.4 Quercetin prevents Aβ aggregation ..........................................................155
Figure 5.5 AFM demonstrates quercetin prevents Aβ aggregation and produces smaller oligomers..................................................................................................................156
Figure 5.6 Modelling AFM data shows QOT preparation significantly smaller than Aβ0 ...157
Figure 5.7 Rutin binds Aβ1-42 ....................................................................................159
Figure 5.8 Biochemistry characterisation of the effect of quercetin on preformed Aβ oligomers.........................................................................................................................162
Figure 5.9 AFM characterisation of the effect of quercetin on preformed Aβ oligomers ..163
Figure 5.10 Characterisation of QOT preparation by AFM ........................................164
Figure 5.11 Quercetin prevents Aβ binding to cells ....................................................166
Figure 5.12 Quercetin reduces production of Aβ isoforms ..........................................167
Figure 5.13 Potential therapeutic actions of quercetin in AD .....................................175
Figure 6.1 Possible contributions of amylin to AD ....................................................187

List of tables

Table 1.1 Native proteins associated with PMDs .........................................................16
Table 1.2 Table of reported Aβ and amylin receptors ..................................................33
Table 2.1 Media used in neuronal induction and differentiation of iPSCs .....................45
Table 2.2 Identification and demographic data of human samples ...............................47
Table 2.3 Primary antibodies used in western blotting and dot blotting ......................53
Table 2.4 Table of antibodies used in immunofluorescence microscopy .....................56
Table 2.5 Table of antibodies used in immunohistochemistry .....................................58
Table 2.6 Table of primer designs used in RT-PCR experiments .................................61
Table 2.7 IMMS instrument parameters ....................................................................62
Table 2.8 HPLC gradient. .................................................................63
Table 2.9 LC-MS/MS Quatpump gradient. .........................................................64
Table 2.10 Triple-Quad source conditions. .......................................................64
Table 2.11 SRM methods for amylin and Aβ. .....................................................65

List of appendices

Appendix 1 Intra-neuronal amylin staining (T-4150) ........................................224
Appendix 2 Intra-neuronal amylin staining (H-017-03) .....................................225
Appendix 3 Ion-mobility mass spectrometry instrument parameters .................226
Appendix 4 Evidence of Aβ aggregation in tip during IMMS ..............................229

Word count: 52,907
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ADAM10</td>
<td>A Disintegrin and metalloproteinase domain-containing protein 10</td>
</tr>
<tr>
<td>ADDL</td>
<td>Aβ-derived diffusible ligand</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid intracellular domain</td>
</tr>
<tr>
<td>AMPL</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AMPK</td>
<td>5'-AMP activated Kinase</td>
</tr>
<tr>
<td>AMY1/2/3</td>
<td>Calcitonin receptor + RAMP isoform 1, 2 or 3</td>
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<tr>
<td>AMYm</td>
<td>Amylin monomer preparation</td>
</tr>
<tr>
<td>AMYo</td>
<td>Amylin oligomer preparation</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein-E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>Aβm</td>
<td>Aβ monomer preparation</td>
</tr>
<tr>
<td>AβO</td>
<td>Aβ oligomer preparation</td>
</tr>
<tr>
<td>BACE1</td>
<td>Beta-site APP cleaving enzyme 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca2+/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<td>DAPI</td>
<td>Diamidino-2-phenylindole</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EphA4</td>
<td>Ephrin type A receptor 4</td>
</tr>
<tr>
<td>EphB2</td>
<td>Ephrin type B receptor 2</td>
</tr>
<tr>
<td>fAD</td>
<td>Familial AD</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fetal growth factor-2</td>
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<tr>
<td>FOXG1</td>
<td>forkhead box protein G1</td>
</tr>
<tr>
<td>FTD</td>
<td>frontotemporal lobar dementia</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>GSK3</td>
<td>Glycogen synthase kinase-3</td>
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<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoropropanol-2-ol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IL-1β / -6</td>
<td>Interleukin-1β /-6</td>
</tr>
<tr>
<td>IMMS</td>
<td>Ion-mobility mass spectrometry</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1A/1B-light chain 3</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography linked tandem mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LRP1</td>
<td>Low density lipoprotein (LDL) receptor-related protein 1</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule associated protein-2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPT</td>
<td>Microtubule associated protein tau</td>
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<tr>
<td>mGlur5</td>
<td>Metabotropic glutamate receptor 5</td>
</tr>
<tr>
<td>NLPR3</td>
<td>Nod-like receptor protease-3</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OC</td>
<td>Structural antibody designed against fibrillar oligomers</td>
</tr>
<tr>
<td>OG</td>
<td>Occipital lobe grey matter</td>
</tr>
<tr>
<td>OW</td>
<td>Occipital lobe white matter</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired box protein-6</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween-20</td>
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<tr>
<td>PDGFRβ</td>
<td>Platelet derived growth factor receptor-β</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PMD</td>
<td>Protein misfolding disorder</td>
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<td>Cellular prion protein</td>
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<td>Pyk2</td>
<td>Protein tyrosine kinase 2</td>
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<tr>
<td>RAMP1/2/3</td>
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<td>Reactive oxygen species</td>
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<td>RPMI</td>
<td>Roswell park memorial institute 1640 medium</td>
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<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<td>Sex determining region-Y-box-2</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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<tr>
<td>T2D</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>TBR1</td>
<td>T-box brain 1</td>
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<td>TDP-43</td>
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<td>Toll-like receptor 2</td>
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<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
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<td>TW</td>
<td>Temporal lobe white matter</td>
</tr>
<tr>
<td>α-syn</td>
<td>α-synuclein</td>
</tr>
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Type II diabetes mellitus (T2D) and Alzheimer’s disease (AD) share aetiology and have a high incidence of co-morbidity. Evidence suggests that both diseases are caused by the pathogenic aggregation of an intrinsically disordered native amyloid peptide. Furthermore, T2D and AD share risk factors such as age, obesity and vascular health. Recent studies demonstrate that amylin, an amyloidogenic pancreatic hormone deposited in the pancreas in T2D, is also deposited in the brain in AD. We hypothesised that amylin directly contributes to AD through deposition in the brain and activation of pathogenic signalling cascades. We provide evidence to validate that amylin is deposited in the brain parenchyma and vasculature. Furthermore, we present data demonstrating amylin \((IAPP)\) expression in the brain is significantly elevated in AD; and that amylin treatment increases amyloid-\(\beta\) (A\(\beta\)) secretion in neuronal culture. Soluble oligomeric species of A\(\beta\) cause AD by initiation of downstream signalling cascades that dysregulate kinase activity, promote tau phosphorylation and result in neuronal death. One such pathway involves A\(\beta\) oligomer activation of the Src-family kinase Fyn, through binding to the cellular prion protein (PrP\(^C\)) receptor complex. We provide evidence that amylin activates Fyn in neuroblastoma and stem cell derived neurons, this activation is possibly mediated through PrP\(^C\). Together the data presented in this thesis demonstrate multiple modes of action whereby amylin may directly propagate or indirectly exacerbate AD-associated processes. Amylin aggregation, deposition, up-regulation and signalling should be considered one of several links between T2D and AD. The pathogenic actions of A\(\beta\) and amylin are mediated by oligomer species. Therefore therapeutics which prevent oligomerisation or oligomer action may be valuable in AD and T2D. One such class of therapeutic are flavonoids. Our collaborators have recently demonstrated the flavonoids rutin and quercetin reduce amylin aggregation and extend lifespan in diabetic animal models. As a result of this we investigated the anti-amyloidogenic and anti-oligomeric properties of the flavonoid quercetin against A\(\beta\). Quercetin treatment prevented A\(\beta\) oligomerisation, cell binding of pre-formed A\(\beta\) oligomers and also reduced APP processing in cell models. These data suggest quercetin is a multimodal therapeutic with potential utility in AD and T2D and should be explored for further drug development.
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Chapter 1: Introduction

1.1 Protein misfolding disorders

Protein misfolding disorders (PMDs) are defined by the presence of aggregated or misfolded protein as a key pathological marker or pathogenic cause of disease. A key feature of all such diseases is the adoption of conformational changes in secondary or tertiary structure that promote self-aggregation of native protein. A subclass of PMDs is amyloidosis disease. Amyloidosis diseases occur following the spontaneous adoption of irregular conformational changes in secondary or tertiary structure, enabled by the local environment, by a native amyloid which cause self-association and aggregation (Figure 1.1), without alteration in the primary sequence of the amyloidogenic protein that constitutes a pathogenic cause or pathological hallmark of disease. Aggregation in amyloidosis diseases can be facilitated by chaperones or promoted by post-translational modification. Proteins known to aggregate in disease are listed in Table 1.1.

1.1.1 Amyloid aggregation and oligomerisation

An amyloid is an intrinsically disordered or globular soluble protein prone to self-aggregation in response to local environmental or genetic cues. These cues can include changes in local concentration, environmental pH, availability of chaperones, post-translational modifications and changes in localisation (further discussed in Knowles et al. (2014)). The ability of a protein to misfold is dictated by its primary sequence, which defines the protein’s propensity to engage in hydrogen bonding, electrostatic interactions or interactions with solvent molecules (Dobson et al. 1998).

Changes in the local environment can lead to misfolding, wherein the monomeric amyloids adopt an array of α-helical and β-sheet motifs and self-associate, generating a diverse range of small oligomeric intermediates of varying stability (Bernstein et al. 2009; Smith et al. 2010). The general pathway of aggregation is described in Figure 1.1. The majority of oligomeric intermediates, particularly α-helix containing species, will be off-target oligomers - but species rich in β-sheet structures will form highly ordered on-pathway oligomers. Cross β-sheet structures form between on-pathway oligomers to create protofibrils, which are bound by hydrogen bonds. Protofibrils are then extended by nucleation, which is the addition of on pathway oligomers or complementary monomers to the free ends, creating an insoluble fibril. In certain instances, the structure of these on-pathway oligomers and
Protofibrils can act as seeding species, promoting further amyloid aggregation. Fibrils rich in β-sheet motif appear to be common to most amyloids and PMDs (Rambaran and Serpell 2008), with subtle differences in the overall structure determined by the primary sequence and points of self-association.

Fibrils associate with one-another, and sequester other cell debris, to form large insoluble plaques. In some diseases, particularly systemic amyloidosis, these plaque deposits correlate well with disease progression, but in other cases such as neurodegenerative dementias the quantity of plaque-like deposits rarely correlates with disease severity (Chiti and Dobson 2006; Haass and Selkoe 2007). Large plaque like or fibrillar species of many different amyloids have consistently demonstrated low cytotoxicity (Caughey and Lansbury 2003), leading researchers to focus on on-pathway and off-pathway oligomers as mediators of toxicity (Bucciontini et al. 2002; Caughey and Lansbury 2003; Campioni et al. 2010).

Figure 1.1 Schematic of amyloid aggregation
Native amyloidogenic proteins are usually soluble intrinsically disordered monomers. Changes in local environment encourage protein misfolding through adoption of α-helix or β-sheet secondary structures. These structures promote self-association and oligomer formation. Oligomers fall into two categories, on-pathway oligomers and off-pathway oligomers. On-pathway oligomers are generally β-sheet rich, and will self-associate to form protofibrils. Protofibrils are elongated by nucleation until they become insoluble fibrils. Fibrils associate with one another to create large insoluble plaques; these structures are dynamic and can cede smaller oligomers to the local environment.
Table 1.1 Native proteins associated with PMDs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-synuclein</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td></td>
<td>Dementia with Lewy bodies</td>
</tr>
<tr>
<td>β-2 Microglobulin</td>
<td>Haemodialysis-related amyloidosis</td>
</tr>
<tr>
<td>Amyloid-β (Aβ)</td>
<td>Alzheimer's disease (AD)</td>
</tr>
<tr>
<td>Amylin</td>
<td>Type II diabetes mellitus (T2D)</td>
</tr>
<tr>
<td></td>
<td>Rejection of pancreatic islet transplantation</td>
</tr>
<tr>
<td>Huntingtin (CAG expansion)</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>Immunoglobulin light chain</td>
<td>AL amyloidosis</td>
</tr>
<tr>
<td>Insulin</td>
<td>Injection-localised amyloidosis</td>
</tr>
<tr>
<td>Medin</td>
<td>Aortic medial amyloidosis</td>
</tr>
<tr>
<td>Prion protein (PrPC)</td>
<td>Kuru</td>
</tr>
<tr>
<td></td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td></td>
<td>Fatal familial insomnia</td>
</tr>
<tr>
<td></td>
<td>Gerstman-Straussler-Scheinker syndrome</td>
</tr>
<tr>
<td></td>
<td>Mad cow disease</td>
</tr>
<tr>
<td>Serum amyloid A protein</td>
<td>AA amyloidosis</td>
</tr>
<tr>
<td>Superoxide dismutase 1 (SOD1)</td>
<td>Amyotrophic lateral sclerosis (aka: motor neurone disease, Lou Gehrig's disease)</td>
</tr>
<tr>
<td>Tau</td>
<td>Alzheimer's disease (AD)</td>
</tr>
<tr>
<td></td>
<td>Frontotemporal lobar dementia (FTD)</td>
</tr>
<tr>
<td></td>
<td>Chronic traumatic encephalopathy</td>
</tr>
<tr>
<td>TDP-43</td>
<td>Amyotrophic lateral sclerosis (aka: motor neurone disease, Lou Gehrig's disease)</td>
</tr>
<tr>
<td></td>
<td>Chronic traumatic encephalopathy</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>Familial amyloid polyneuropathy</td>
</tr>
<tr>
<td></td>
<td>Senile systemic amyloidosis</td>
</tr>
</tbody>
</table>

Table uses selected information from Chiti and Dobson (2006).

1.1.2 Relationship between PMDs and ageing

Increases in food production, vaccination and hygiene regimens have steadily increased life expectancy across the globe. Ageing is the greatest non-genetic risk factor for developing cardiovascular disorders, cancers and PMDs (Niccoli and Partridge 2012). As a result of increased longevity there has been a rapid increase in prevalence of PMDs, such as Alzheimer's disease (AD) and type II diabetes mellitus (T2D) (Chiti and Dobson 2006). The exact mechanism behind age-induced susceptibility to PMDs and other disorders is unclear, but it would appear to be deeply rooted in natural selection pressures promoting phenotypes that provide advantages for the young, but may cause long term damage or
promote cancer formation during ageing. These concepts are reviewed in Kirkwood (2008) and Blagosklonny (2009).

There may be other common failures of pathways that influence longevity that contribute to the promotion of PMDs, in particular neurodegenerative dementias, in ageing - these are reviewed in Niccoli and Partridge (2012). Examples of such pathways include the nutrient-sensing pathway, cellular autophagy and the systemic inflammatory response. Nutrient sensing pathways are mediated through the insulin receptor and internal levels of adenosine triphosphate (ATP) to regulate AMPK activity. This promotes activity of the longevity promoting transcription factor FOXO and inhibition of mTOR, both of which up-regulate cellular autophagy. Pharmacological activation of this pathway with rapamycin has been demonstrated to extend life in several animal species (Bjedov et al. 2010; Glick et al. 2010).

Cellular autophagy, which literally translates to ‘self-eating’, is the self-degradation of cellular components. Autophagy is basally active in a house-keeping role and up regulated in periods of starvation. As a house-keeping mechanism autophagy is employed to target damaged organelles, misfolded proteins and aggregated proteins to the lysosome (Glick et al. 2010). Autophagy becomes impaired with age, and impairment of autophagy is a major feature of many PMDs (Rubinsztein et al. 2011). Interestingly, the longevity enhancing effects of rapamycin are prevented if autophagy is pharmacologically impaired, highlighting it’s overall contribution to healthy lifespan (Glick et al. 2010).

In addition to cellular autophagy, misfolded protein may also be cleared by the ubiquitin-proteasome system. This major intracellular protein degradation mechanism involves the conjugation of ubiquitin to misfolded protein by ubiquitin ligases, and further ubiquitination creates polyubiquitin adducts which are recognised by the proteasome causing substrate degradation. Protein aggregation impairs the ubiquitin-proteasome system, and as such it is implicated in many PMDs (Bence et al. 2001). The role of the ubiquitin-proteasome system in AD is discussed by Gong et al. (2016), but is not further addressed in this thesis.

Accumulation of cell debris, aggregated amyloids, invading pathogens, free radical oxygen species and certain metabolites gradually contributes to a pervasive elevation in the basal activity of the innate immune response during aging. This process is loosely referred to as ‘inflammaging’ and results in increased basal inflammatory markers such as interleukin (IL)-1β, IL-6 and TNFα due to activation of innate immune sensors such as the nod-like receptor protease-3 (NLRP3) inflammasome; this is reviewed in Franceschi and Campisi (2014). These inflammatory markers contribute to the heightened sensitivity of phagocytes around the body (Shaw et al. 2010), and also downregulate pro-longevity signalling and hormone secretion (Bendtzen et al. 1986; Southern et al. 1990). Heightened inflammation, and inflammation-mediated cell loss, is a contributing factor in many PMDs, including AD and T2D (Calle and Fernandez 2012; Heneka et al. 2015).
1.2 Type II diabetes mellitus

Diabetes mellitus was first described 3000 years ago by the ancient Egyptians, before being named ‘diabetes’ by the Greek physician Apollonius of Memphis in the third century BC (Ahmed 2002; Zajac et al. 2010). Diabetes literally translates to ‘siphon’, in reference to the symptomatic excessive urination typical of the disease. The term ‘mellitus’ originates from the Latin for ‘sweet’ and was added to the disease title in 1675 after Thomas Willis, a British physician, described the sweetness of patient blood and urine (Ahmed 2002; Zajac et al. 2010). Now, the term diabetes mellitus describes a group of complex endocrine and metabolic disorders which result in a systemic increase in glucose, termed hyperglycaemia.

Diabetes mellitus is separated into two distinct diseases (Himsworth 1936), type I diabetes mellitus and type II diabetes mellitus, although it is now generally accepted that nuanced exceptions to these categories exist (Alberti et al. 1998). Type I diabetes mellitus, also referred to as insulin-dependent diabetes mellitus, is an autoimmune and metabolic disorder in which T-cells destroy the secretory β-cells of the islets of Langerhans in the pancreas (Burn 2010). This results in progressive impairment and eventual abolition of glucose stimulated pancreatic insulin secretion, causing chronic hyperglycaemia at a young age (Burn 2010). Due to one of the greatest medical discoveries of all time (Banting and Best 1922), insulin isolation and supplementation now enables management of type I diabetes, but is by no means a cure and patients may still suffer from complications of diabetes and reduced lifespan. Type II diabetes mellitus (T2D), also referred to as non-insulin dependent diabetes mellitus, is a chronic metabolic disorder characterised by global insulin resistance, resulting in impaired glucose uptake from the blood, pervasive gluconeogenesis and hyperglycaemia. Resistance to insulin in T2D causes hyperglycaemia, which in turn up-regulates insulin secretion by pancreatic β-cells, eventually resulting in β-cell loss and a relative reduction in circulating endocrine hormones. T2D treatment strategies revolve around diet and lifestyle changes, insulin secretagogues (sulphonylureas), insulin sensitising agents such as metformin, or glucose removal strategies (SGLT2 inhibitors), and in some cases, insulin supplementation is required late in disease after severe pancreatic damage (Olokoba et al. 2012).

Diabetes is extremely prevalent and incidence has been steadily increasing globally, particularly in low income countries (Whiting et al. 2011; Ogurtsova et al. 2017). The international diabetes federation recently estimated that as of 2015, 415 million people had diabetes mellitus and that it caused 5 million deaths worldwide (Ogurtsova et al. 2017). Ogurtsova et al. (2017) also estimate global annual spending on diabetes care was $673 billion (USD) in 2015. T2D represents the overwhelming majority of these cases, with several studies estimating T2D represents 87-91% of the total diabetic population (Boyle 2017).
et al. 1999; Bruno et al. 2005; Holman et al. 2015). Lack of distinction between the type of diabetes and estimation that up to 50% of diabetes is undiagnosed complicates therapeutic intervention and the interpretation of the epidemiological studies linking diabetes with other disorders (Whiting et al. 2011; Ogurtsova et al. 2017).

Despite the disease management options available for diabetes mellitus, chronic hyperglycaemia still causes many complications and up to a 30% reduction in lifespan (Ogurtsova et al. 2017). Diabetics are at risk of cardiovascular disease, diabetic heart failure, diabetic retinopathy, diabetic nephropathy, renal failure, peripheral neuropathy and diabetic cognitive dysfunction (Alberti et al. 1998; Kodl and Seaquist 2008; Saedi et al. 2016; Luchsinger et al. 2007; Roberts et al. 2014a; Roberts et al. 2014b).

1.2.1 Pathological hallmarks of T2D
Insulin resistance induced hyperglycaemia is the defining feature of T2D (Kahn 2003). In its physiological role insulin is a glucose homeostasis regulating hormone, produced and secreted from the β-cells of the islets of Langerhans in the pancreas (pancreas anatomy and islet function are described in Figure 1.2). The pancreas is a major exocrine and endocrine organ located below the duodenum. The major exocrine components of the pancreas are the acini and central ducts and its endocrine elements are contained in the islets of Langerhans. Insulin is a major endocrine hormone expressed in and secreted from the β-cells of the islets of Langerhans. Insulin secretion is regulated by a number of factors including circulating metabolites, hormones and neural factors, but most importantly insulin secretion is regulated by glucose following ingestion of food (Cluck et al. 2005). Glucose is transported into the β-cell where it is metabolised. Oxidation of the glucose metabolites results in a change in the ATP/ADP ratio, increasing the amount of ADP in the cell, which in turn inhibits ATP-dependent K+ channels causing β-cell depolarisation (Cook and Hales 1984; Ashcroft et al. 1984). Depolarisation induces Ca2+ influx through voltage-gated Ca2+ channels to trigger the exocytosis of the secretory granules in which the exocrine hormones, insulin and amylin, are stored (Keahey et al. 1989; Bhattacharjee et al. 1997; Lukinius et al. 1989).
Figure 1.2 Pancreas anatomy and the islets of Langerhans.

The pancreas is a major exocrine and endocrine organ located in the peritoneum below the duodenum. The pancreas is divided into the curled head region, body region and tail region, these regions are largely homogenous, although a greater intensity of islets of Langerhans are found in the tail region. The pancreatic bile duct enters at the head of the pancreas and branches throughout the organ. The pancreas is made up of many lobules which are formed by accumulated acinar units around the duct branches; these lobules contain the islets of Langerhans. There are millions of islets in the pancreas, but they only constitute between 1-2% of pancreas mass. The islets are composed of 3 endocrine cell types: α-cells, which are generally located on the peripheral edge of the islet, secrete glucagon - a catabolic hormone that promotes gluconeogenesis and glycogenolysis; β-cells, which are the most numerous cell type, secrete insulin and amylin from shared secretory granules; δ-cells, which are the least abundant cell type, secrete somatostatin, which acts to inhibit secretion of growth hormone as well as α- and β-cell hormone secretion. Histological stain of islet of Langerhans taken from Histology for Pathologists (2007) published by Lippincott Williams & Wilkins.
Insulin is secreted into the blood, where its central action is to promote glucose uptake in all tissues. This function is best studied in skeletal muscle, which is the principle site of insulin stimulated glucose uptake (DeFronzo et al. 1985). At the cell surface insulin binds the insulin receptor, a receptor tyrosine kinase. This promotes phosphorylation of the insulin-receptor substrate (IRS) isoforms IRS-1 and IRS-2. IRS isoforms promote the activation of phosphatidylinositol 3-kinase (PI3K) through recruitment of regulatory subunits (Shepherd et al. 1997). PI3K activation mediates multiple actions of insulin (Burgering and Cofer 1995; Zierath et al. 2000). Firstly, PI3K enacts downstream signalling via Akt to promote the translocation of glucose transporter isoform 4 (GLUT4) from internal stores to the cell surface resulting in increased glucose uptake from the blood (Guma et al. 1995). Insulin signalling through PI3K or mitogen activated protein kinase (MAPK) family kinase cascades results in inhibition of glycogen synthase kinase-3 (GSK3) (Cross et al. 1995). GSK3 is a constitutively active serine/threonine kinase that inhibits glycogen synthase by phosphorylation; insulin signalling via PI3K or MAPK causes phosphorylation of GSK3, resulting in GSK3 inhibition and promotion of glycogen synthase activity (Sutherland et al. 1993). Glycogen synthase is a glycosyltransferase which acts to store cytosolic glucose as a polymeric chain of glucose molecules, termed glycogen (Zierath et al. 2000). Insulin signalling through PI3K, MAPK, Akt, protein kinase-C, protein kinase-A, cyclin-dependent kinase 5, GSK3, phosphodiesterase-ɛ-B and other signalling enzymes act to inhibit gluconeogenesis, prevent lipolysis, reduce apoptotic signalling and promote genes regulating cell survival and growth factor expression. Detailed accounts of insulin signalling pathways can be found in Zierath et al. (2000) and Cohen (2006).

The summation of these actions is that the post-prandial secretion of insulin from the pancreatic-β cells causes removal of circulating glucose and promotion of glucose storage as glycogen in muscle and liver tissue or triglycerides in adipose tissue. Post-prandial insulin stimulated glucose storage and utilisation is greatly impaired in T2D (DeFronzo et al. 1985; Shulman et al. 1990). This is tightly entwined with the hyperglycaemia characteristic of diabetes mellitus. It is unclear at what stage of the complex actions of insulin signalling that resistance occurs, and it not fully understood to what degree it precedes and is exacerbated by β-cell dysfunction. Whatever the mechanism it would appear that insulin resistance is malleable, as weight loss, insulin sensitising agents or insulin therapy have all been demonstrated to restore normal glycaemia in T2D patients (Zierath et al. 2000). Despite this, longitudinal studies demonstrate that an underlying progressive decline in islet β-cell function will lead to T2D (Holman 1998; Weyer et al. 1999).
Another major feature of T2D is β-cell loss. It has long been known that depositions of insoluble aggregated material occurs in the pancreas in T2D (Opie 1901b). This study noted the hyalinisation of the pancreatic islets of Langerhans (Figure 1.3), infiltration of amyloid to surrounding pancreas tissue and loss of pancreatic islets (Opie 1901b). 70 years later quantitative studies of amyloid deposition in the pancreas demonstrated that over 95% of diabetic patients present with amyloid deposition in the islets (Westermark 1972). The insoluble amyloid species proved positive for Congo red staining which enabled correlation of degree of amyloidosis with the number of islets positive for amyloidosis (Westermark 1972). Interestingly, this correlation showed as the number of islets affected increased, the amount of amyloid in the previously affected islets increases also, suggestive of a positive feedback loop or seeding mechanism.

These amyloid deposits were discovered to be comprised of the amyloidogenic pancreatic hormone amylin (aka islet amyloid polypeptide), which is produced and stored in β-cells with insulin (Westermark 1972; Westermark et al. 1987b; Cooper et al. 1988a). Amylin amyloidosis has subsequently been demonstrated to have a major role in islet β-cell dysfunction and T2D pathogenesis (this is further discussed in 1.2.3).
1.2.2 Physiological role of amylin

Amylin is a 37 amino acid pancreatic hormone encoded by the *IAPP* gene on chromosome 12 and is part of the calcitonin gene peptide superfamily (Wimalawansa 1997). It is produced in high levels in pancreatic β-cells in the islets of Langerhans. It is stored in dense core secretory granules, often with insulin, and the content of amylin in the granules is 1-2% that of insulin (Lukinius et al. 1989;Clark et al. 1989;Buchanan et al. 2007). Like insulin, *IAPP* expression generates a precursor protein, the 89-amino acid (aa) residue – preproamylin. This precursor has a large N-terminal surplus sequence and a small surplus sequence at the C-terminal. Prohormone convertase 2 (PC2) has been reported to cleave the majority of this large N-terminal surplus sequence to give proamylin. This undergoes subsequent cleavage by prohormone convertase 1/3 (PC1/3), carboxypeptidase E and the full length peptide is amidated by peptidyl amidating mono-oxygenase complex (PAM) (Badman et al. 1996;Higham et al. 2000;Yonemoto et al. 2008) to generate the mature peptide – Amylin.

Amylin acts as a satiety hormone to control blood glucose, reduce food intake and regulate energy balance, these functions are reviewed in Mietlicki-Baase and Hayes (2014). Administration of amylin or amylin receptor agonists will reduce food intake and blood glucose in rodents, primates and humans (Lutz et al. 1994;Bello et al. 2008;Chapman et al. 2005). In skeletal muscle and peripheral tissues amylin acts on native receptors to counteract certain signalling pathways of insulin, resulting in decreased glycogen synthesis (Cooper et al. 1988b) and promotion of glycogenolysis (Young et al. 1991). This may be mediated by activation of GSK3β (Abaffy and Cooper 2004). The native receptors for amylin are heterodimers consisting of a class B G protein-coupled receptor (GPCR) - the calcitonin receptor - and a receptor activity modifying protein (RAMP). There are 3 subtypes of RAMP proteins; these proteins are intrinsic membrane proteins that share a common topology but have less than a 30% sequence identity (Poyner et al. 2002;Sexton et al. 2009). The large extracellular N-terminal consists of ~100 amino acids attached to a single transmembrane domain and is followed by a small intracellular component (10 aa) (Muff et al. 2001;Sexton et al. 2001). These receptors respond to calcitonin, CGRP and amylin with varying affinities, but CTR/RAMP₁ (AMY₁) and CTR/RAMP₃ (AMY₃) appear to have the highest affinity for amylin. Due to preferential coupling to the Gₛ protein (but occasional coupling to G<sub>q</sub>), activation of AMY receptors will cause increased cAMP via adenylate cyclase and subsequent increase in cytosolic Ca<sup>2+</sup> (Fu et al. 2012).

Amylin is a neuropeptide and readily crosses the blood-brain barrier (BBB) (Banks and Kastin 1998). Studies using autoradiographic localisation have been used to map amylin binding sites in the brains of murine and primate models (Sexton et al. 1994;Paxinos et al. 2004). These studies found amylin binding in multiple areas associated with feeding behaviour such
as the area postrema, and recent work also demonstrates amylin regulation of reward in the nucleus accumbens via manipulation of ventral tegmental area dopaminergic signalling (Mietlicki-Baase et al. 2015b; Mietlicki-Baase et al. 2017). AMY\textsubscript{3} is widely expressed in the CNS and has been demonstrated to be expressed in brain regions related to memory and cognitive function, which is of potential relevance to diabetic cognitive impairment and dementia (Jhamandas et al. 2011; Fu and Jhamandas 2013; Roberts et al. 2014a). Amylin signalling has been reported to act on a number of downstream effectors including: PKA, AKt, AMPK, MAPK, CDK5, GSK3 and other intermediaries. The resultant effect of this signalling is interpreted differently throughout the literature inferring that amylin signalling may have neuroprotective effects (Qiu and Zhu 2014), neurotoxic effects (Konarkowska et al. 2006; Fu et al. 2013) and crosstalk with other peripheral axis hormones such as leptin (Moon et al. 2011).

1.2.3 Amylin aggregation is a central event in T2D
While amylin aggregation is not widely considered a pathogenic cause of T2D, a growing body of evidence is accumulating to suggest this is the case. Quantitative studies demonstrate 95% of T2D patients have aggregated amylin (Westermark 1972). Amylin also contributes to metabolic dysfunction; repeated injection of amylin causes hyperglycaemia and insulin resistance in murine models (Molina et al. 1990); and rats heterozygous for human amylin (hIAPP\textsuperscript{+/-}) develop amyloid deposition, insulin resistance and hyperglycaemia (Janson et al. 1996; Soeller et al. 1998; Wong et al. 2008). Smooth muscle cells in vitro and isolated rat muscle will also become resistant to insulin following amylin pre-treatment (Leighton and Cooper 1988a; Lee and Cooper 2002). The mechanism of amylin induced insulin resistance is unknown, but may be due to upregulation of lipolysis and competition for insulin signalling pathways (Ye et al. 2001). An extremely interesting observation is that spontaneous T2D also occurs in animals with amyloidogenic amylin such as cats (Ma et al. 1998) and many primates (de Koning et al. 1993; Guardado-Mendoza et al. 2009; Davis et al. 1994) but animals with non-aggregating amylin, such as mice and rats, do not generate spontaneous T2D (Ciobotaru 2013). Large insoluble pancreatic amyloid deposits, comprised of amylin and associated with \(\beta\)-cell loss, are also a major feature in these non-human models (Davis et al. 1994; Ma et al. 1998; de Koning et al. 1993; Guardado-Mendoza et al. 2009; Hubbard et al. 2002). Several changes in the 20-29 amino acids of amylin, that are distinct between animals that develop spontaneous diabetes and those that do not, seem to dictate this propensity towards aggregation behaviour (Betsholtz et al. 1989a).

In support of the central role of amylin aggregation in T2D, human genetics studies have discovered a missense mutation in the \textit{IAPP} gene resulting in S20G change in the amylin sequence. The S20G mutation strongly promotes non-insulin dependent diabetes mellitus.
(T2D) (Sakagashira et al. 1996;Lee et al. 2001). This mutation does nothing to alter IAPP expression, but multiple in vitro studies demonstrate that the S20G substitution increases the aggregation propensity of amylin (Sakagashira et al. 2000;Ma et al. 2001;Duan et al. 2012). Furthermore, mutations in the amylin promoter region have been associated with increased incidence and predisposition to T2D in the Maori people of New Zealand (Poa et al. 2003). This further supports the central role of amylin aggregation in the development of T2D.

Whether β-cell dysfunction precedes the development of hyperglycaemia and insulin resistance is a topic of continued debate. Longitudinal human population studies have compared glucose response and insulin sensitivity in non-hyperglycaemic individuals (Weyer et al. 1999). Follow up with the participants demonstrated that of the individuals who originally presented with impaired glucose response, 78% had continued or worsened insulin secretion in response to glucose, whereas insulin sensitivity remained un-impaired in most cases (Weyer et al. 1999). This indicates that β-cell dysfunction precedes insulin resistance and supports data from the UK prospective diabetes study which reached the same conclusion (Holman 1998;King et al. 1999;Kahn 2003). Islet β-cell toxicity in early T2D may be mediated by amylin aggregates, which have been repeatedly demonstrated to promote β-cell toxicity through mechanisms including cell membrane disruption, autophagy impairment, endoplasmic reticulum stress, promotion of mitochondrial dysfunction, dysregulation of Ca\(^{2+}\), activation of apoptotic signalling and promotion of localised inflammation (Bai et al. 1999;Casas et al. 2007;Engel 2009;Gurlo et al. 2010;Lim et al. 2010;Brender et al. 2012;Park et al. 2012;Shigihara et al. 2014;Kim et al. 2014b;Bram et al. 2014;Westwell-Roper et al. 2016;Gurlo et al. 2016).

These data implicate amylin aggregate species in β-cell dysfunction in early T2D. Amylin induced β-cell dysfunction primes the subsequent insulin resistance and hyperglycaemia characteristic of T2D. Furthermore, these studies strongly indicate amylin aggregate species mediate the β-cell loss observed in late T2D pathology. Amylin up regulation, aggregation and deposition should be seen as major pathogenic mechanisms underlying T2D in humans and other animals.

### 1.2.4 Proposed toxic species of amylin

As with other amyloidosis diseases, the notion that toxicity is due to oligomeric species of amylin rather than large fibrils – the toxic oligomer hypothesis – has attracted increasing interest in recent years (Haataja et al. 2008). It is clearly established that addition of mature fibrils does not cause toxicity in islet β-cells, whereas freshly prepared aqueous solutions of
human amylin cause β-cell apoptosis (Janson et al. 1999; Konarkowska et al. 2006). Non-aggregating amylin analogues are not toxic; therefore the potential to aggregate is a major determinant of amylin toxicity. In support of oligomers as the medium of toxicity, rifampicin, which prevents amyloid fibril formation, but does not affect oligomerisation, demonstrates that toxicity persists when fibrilisation is prevented but oligomerisation persists (Haataja et al. 2008). The same group demonstrated using 2 separate transgenic mouse models that such oligomers form inside islet β-cells (Lin et al. 2007) and hypothesise that toxicity is due to increased ER stress and impairment of the unfolded protein response and autophagy (Marchetti et al. 2007; Haataja et al. 2008). Amylin oligomers are yet to be fully isolated from T2D patient pancreas, but have been noted in human insulinoma tissue (O'Brien et al. 1994; Gurlo et al. 2010) and have been observed in T2D patient islet β-cells using structural antibodies and immunogold labelling (Gurlo et al. 2010). Despite these observations, the contribution of oligomeric species of amylin to β-cell dysfunction and T2D pathogenesis is not fully understood.

Amylin has been demonstrated to initiate signalling that results in islet β-cell apoptosis. Exposure of isolated mouse islet β-cells to human amylin causes up regulated expression of the cell death signalling proteins Fas and FADD (Zhang et al. 2008). Externally applied amylin co-localises with Fas at the cell surface. Amylin treatment caused JNK-1 phosphorylation and caspase 8 cleavage, followed by islet β-cell apoptosis. These effects were prevented by anti-Fas antibody pre-incubation. These effects were not caused by the non-amyloidogenic rat amylin, suggesting aggregation is central to amylin induced apoptosis. There is also evidence that Aβ oligomer species bind Fas and share this signalling pathway (Su et al. 2003). In addition to apoptotic signalling, amylin activates inflammatory pathways via activation of toll-like receptor 2 (TLR2) (Westwell-Roper et al. 2016). TLR2 activation is dependent on oligomer species, as non-aggregating amylin analogues and amylin fibril species were incapable of inducing TLR2 stimulated proIL-1β production. Interestingly, this work suggests that amylin aggregate species generated early in the aggregation process act as activators of TLR2 mediated proIL-1β expression and oligomer species generated later in the oligomerisation process act as NLRP3 activating species, causing IL-1β maturation and secretion (Masters et al. 2010; Westwell-Roper et al. 2016). As is the case with the Fas receptor, there is evidence that Aβ also binds the TLR2 receptor (Jana et al. 2008). A full list of amylin receptors is included in Table 1.2.

1.3 Alzheimer’s disease

Alzheimer’s disease (AD) is a neurodegenerative disorder that causes severe and progressive global dystrophy of the brain (Figure 1.4). This results in a profound retrograde amnesia as well as increasingly impaired comprehension, judgement, speech
and motivation as the disease progresses. AD is, for the most part, a sporadic disorder of ageing. The pathological dementia, first described 110 years ago (Alzheimer 1907), is the result of many years of cumulative pathological changes prior to the emergence of clinical symptoms (Jack et al. 2010). As with other PMDs, age is the greatest risk factor for AD. Age of onset is often a useful indicator of genetic contribution to disease and AD onset before the age of 65 (termed familial AD (fAD) or early onset AD) is often the result of pathological AD mutations or allelic predisposition. fAD only constitutes 5% of total cases, therefore the vast majority of AD cases occur after 65 and are of unknown origins, this is termed sporadic AD.

AD is a dementia. Dementias are a group of neurodegenerative diseases characterised by a progressive retrograde amnesia. There are 47 million people living with dementia worldwide, and the majority of these cases are caused by AD. Alzheimer’s Disease International estimate the annual global cost of dementia at $808 billion (USD), the majority of this burden is placed on carers (Prince et al. 2016). AD and dementia are now the leading cause of death in the UK (Patel 2016). Death is often due to an opportunistic infection, such as pneumonia, that is brought about and exacerbated by the disabilities caused by AD. These co-morbidities mean people living with AD and dementia require complex care needs, and may go some way to explain why dementia patients are estimated to occupy 25% of hospital beds in England (Lakey 2009). The incidence of dementia is increasing (Prince et al. 2014;Prince et al. 2015;Prince et al. 2016). Global population growth, and an increasingly ageing population makes the incidence of dementia likely to increase further, and the global burden on health care likely to worsen (Prince et al. 2015). These factors could well make the treatment and management of dementia, and other diseases of ageing, one of the defining scientific challenges of our time.
**Figure 1.4 Demonstration of the brain atrophy in AD**

Coronal sections of formalin fixed healthy brain (left) and a brain with advanced AD (right). Note the global volume loss, enlargement of the lateral ventricle (A), loss of the hippocampus (B), widening of the Sylvian fissure (C) and widening of the brain sulci (D). Original image was obtained by Prof. Seth Love (University of Bristol) and is used with permission.

1.3.1 **Symptoms and pathological hallmarks of AD**

The pathological characterisation of AD has remained the same since the original report by Alzheimer (1907). AD is characterised by extracellular deposits of Aβ and neurofibrillary tangles and dystrophic threads of tau in dystrophic neurites in the brain (Figure 1.5). Aβ plaques were also separately associated with senile dementia in 1907 (Fischer 1907). On a gross pathology scale, AD patient brains show widespread atrophy (Figure 1.4), but in particular there is a large degree of loss of cholinergic neurons of the septal-hippocampal and basal forebrain-neocortical pathways (Whitehouse et al. 1982). The profound loss of neurons in the hippocampus causes the characteristic retrograde amnesia symptomatic of the disease, but it is damage to the neocortex and limbic system that result in gross impairment of cognitive function, often accompanied by behavioural disturbances and wandering behaviour.
Figure 1.5 Pathological hallmarks of AD
Histological staining of Aβ containing plaques (left) and hyperphosphorylated tau in dystrophic neurites and neurofibrillary tangles (right) in AD patient neocortex. Aβ image (left) is a representative histological stain using 4G8 antibody in AD temporal lobe. Tau image copyright is owned by Dr. Mark Cohen and FrontalCortex, Inc.

While sporadic AD does not have a uniform timeframe or symptomatic presentation, pathology tends to spread through the brain in a predictable pattern used for pathological staging (Braak and Braak 1991; Thal et al. 2002b). Pathology appears to start in the entorhinal cortex or locus coeruleus and then spread to other areas, such as the hippocampus and basal forebrain. The pathology then spreads to become nearly global, with some areas such as the cerebellum and brainstem being spared. The mechanisms of this spreading are not pinned down and are highly interesting, soluble oligomeric species of Aβ, neuron-to-neuron spreading of misfolded tau and prion like seeding of Aβ and tau have all been suggested (Haass and Selkoe 2007; Mohamed et al. 2013; Brundin et al. 2010).

1.3.2 Generation of Aβ
Genetic evidence implicates the generation of excess Aβ as the causative factor of fAD (Hardy and Selkoe 2002). Aβ is a cleavage product generated by the sequential cleavage of a transmembrane protein called amyloid precursor protein (APP). APP is an integral membrane protein that exists in the trans-Golgi network, endosomes and lipid rafts of the plasma membrane. There are eight splice variants of the APP gene; the predominant isoform in the CNS is the 695 amino acid isoform (APP695). APP undergoes cleavage in an amyloidogenic or non-amyloidogenic manner described in Figure 1.6.
Figure 1.6 Pathways of APP processing

In amyloidogenic proteolysis, APP cleavage at the β-site by BACE1 results in a C99 fragment and a soluble APP-β fragment (sAPPβ). The C99 fragment undergoes further cleavage by the γ-secretase complex (presenilins-1 or -2, nicastrin, anterior pharynx defective-1 and presenilin enhancer-2 (De Strooper 2003)) to yield monomeric Aβ and the amyloid precursor protein intracellular domain (AICD) fragment. Non-amyloidogenic proteolysis by ADAM10 at the α-cleavage site generates a soluble APP-α fragment (sAPPα) and C89 fragment. C89 also undergoes further processing by the γ-secretase complex but does not generate amyloidogenic protein. Recent evidence suggests APP is also cleaved by η and δ secretases; the evidence for these cleavage events is discussed in Andrew et al. (2016).

Aβ is produced both centrally and peripherally in most cell types throughout life. Although the endogenous role of Aβ is currently unknown there is an increasing body of evidence that demonstrates Aβ, in particular soluble oligomeric species, act as endogenous antimicrobial defences (Kumar et al. 2016;Spitzer et al. 2016). Interestingly, antimicrobial activity has also been reported for amylin, and this action is fundamentally dependent on its aggregation properties. In addition to this, invading viral, fungal and bacterial pathogens have all been found associated with plaques of Aβ and amylin in humans and animals (Kumar et al. 2016;van der Kolk et al. 2011;Miklossy and McGeer 2016). This interesting idea places the amyloidogenic properties of Aβ and amylin centrally in an endogenous role, but does not provide evidence of a causal link between infection and AD or T2D. Studies investigating infectious burden with AD are beginning to demonstrate a positive correlation between burden and AD (Bu et al. 2015), but do not explain conservation of these peptides in animals with non-aggregating Aβ or amylin such as rodents.
1.3.3 Amyloid cascade hypothesis

The amyloid cascade hypothesis was first fully described in Hardy and Higgins (1992) and is widely accepted in the AD research community. The hypothesis posits that Aβ generation and deposition as plaques is the causative agent of AD, and the neurofibrillary tau pathology, vascular damage, inflammation and dementia follow as direct result of this deposition (Hardy and Higgins 1992; Hardy and Selkoe 2002). Aside from Aβ plaques being a major pathological feature of AD, this hypothesis is based on genetic evidence that demonstrates mutations in APP or the γ-secretase complex that produce more Aβ cause fAD (Crawford et al. 1991; Chartier-Harlin et al. 1991; Goate et al. 1991; Mullan et al. 1992). Furthermore, patients with Down’s syndrome, which is caused by trisomy of chromosome 21 - where the APP gene is located (St George-Hyslop et al. 1987), develop large amounts of plaques and neurofibrillary tangles in the brain by age 40 (Mann et al. 1989). The hypothesis was further supported by the discovery that the greatest genetic risk factor for sporadic AD - APOE4 status - relates to APOE mediated amyloid clearance, as well as discovery that mutations in APP that reduce Aβ production protect against AD (Jonsson et al. 2012). The hypothesis has adapted to include new evidence demonstrating that soluble oligomeric species of Aβ are significantly more bioactive and neurotoxic than Aβ fibrils (Haass and Selkoe 2007).

The amyloid cascade hypothesis has pushed therapeutic targeting almost exclusively towards Aβ, with little success, although data from ongoing trials of Aβ targeting therapies are beginning to show positive results (Sevigny et al. 2016). Despite this, the amyloid cascade hypothesis is still subject to much criticism, summarised in Herrup (2015), and fails to answer why significant amyloid deposition can occur in some individuals who do not develop dementia.

1.3.4 Cell surface receptors of Aβ oligomers

Lambert et al. (1998) pre-treated neurons with trypsin and demonstrated that soluble Aβ oligomer species were no longer able to induce toxicity. This experiment demonstrated that cell surface proteins mediated the neurotoxic effects of Aβ oligomers. Through binding cell surface proteins, Aβ species hijack native signalling cascades to cause kinase dysregulation (De Felice et al. 2008). This disrupts many cell processes, but crucially it causes increased phosphorylation of the microtubule associated protein tau. This causes tau dissociation from the microtubule and results in neurofibrillary tau tangles (De Felice et al. 2008). A list of cell surface receptors and whether they cause tau phosphorylation is found in Table 1.2. Cell surface receptors of Aβ are further discussed in Chapter 4.
The cellular prion protein (PrP\textsuperscript{C}) has been demonstrated to be a high affinity A\textsubscript{β} oligomer receptor localised to lipid raft domains at the post-synaptic density (Lauré et al. 2009; Um et al. 2012; Kostylev et al. 2015b; Haas and Strittmatter 2016; Rushworth et al. 2013; Alier et al. 2011). A\textsubscript{β} oligomer species that bind to PrP\textsuperscript{C} correlate with cognitive decline in animal models and humans (Um et al. 2012; Kostylev et al. 2015b). A\textsubscript{β} oligomer binding to PrP\textsuperscript{C} stabilises a receptor complex consisting of PrP\textsuperscript{C}, dimerised metabotropic glutamate receptor-5 (mGlur5), the low density lipoprotein related receptor protein 1 (LRP1) and Fyn kinase. This results in the activation of Fyn and subsequent LTP impairment and tau phosphorylation (Lee et al. 2004; Um et al. 2012; Haas and Strittmatter 2016). This is further discussed in Chapter 4.

A\textsubscript{β} has been reported to share signalling with amylin. Both amyloids are reported to cause cell toxicity and LTP impairment in isolated mouse hippocampal neurons by inducing Ca\textsuperscript{2+} dysregulation following binding to the AMY\textsubscript{3} receptor (Jhamandas et al. 2011; Fu et al. 2012; Kimura et al. 2012). In this model A\textsubscript{β} application increased expression of AMY\textsubscript{3} components and resultant toxicity of A\textsubscript{β} application was prevented by the use of an AMY\textsubscript{3} antagonist (Fu et al. 2012). This mechanism is yet to be validated and the ability of A\textsubscript{β} species to bind to AMY\textsubscript{3} has been disputed (Gingell et al. 2014). The receptors for amylin are listed in Table 1.2.
Table 1.2 Table of reported Aβ and amylin receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Binding?</th>
<th>Downstream action</th>
<th>pTau</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>a7-nicotinic acetylcholine receptor</td>
<td>✓</td>
<td>ERK / MAPK signalling</td>
<td>✓</td>
<td>(Parri et al. 2011)</td>
</tr>
<tr>
<td>a-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)</td>
<td>✓</td>
<td>AMPAR internalisation</td>
<td>-</td>
<td>(Zhao et al. 2010)</td>
</tr>
<tr>
<td>Amylin receptor 3</td>
<td>?</td>
<td>PKA, Akt, MAPK signalling</td>
<td>-</td>
<td>(Fu et al. 2012; Gingell et al. 2014)</td>
</tr>
<tr>
<td>apoE</td>
<td>✓</td>
<td>Peptide targeting for degradation. Transportation across the BBB</td>
<td>X</td>
<td>(Liu et al. 2013)</td>
</tr>
<tr>
<td>CD36</td>
<td>✓</td>
<td>Inflammasome activation</td>
<td>-</td>
<td>(Stewart et al. 2010)</td>
</tr>
<tr>
<td>EphA4</td>
<td>✓</td>
<td>c-Abl mediated synapse loss</td>
<td>-</td>
<td>(Vargas et al. 2014)</td>
</tr>
<tr>
<td>EphB2</td>
<td>✓</td>
<td>EphB2 targeting for degradation and NMDAR internalisation</td>
<td>-</td>
<td>(Cisse et al. 2011)</td>
</tr>
<tr>
<td>Fas</td>
<td>?</td>
<td>JNK1/3 activation. Apoptosis</td>
<td>-</td>
<td>(Su et al. 2003; Zhang et al. 2008)</td>
</tr>
<tr>
<td>Heparan sulphate proteoglycan</td>
<td>✓</td>
<td>Endocytosis mediating mechanism</td>
<td>-</td>
<td>(Kanekiyo et al. 2011; Oskarsson et al. 2015b)</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>✓</td>
<td>GSK3β, CaMKII signalling</td>
<td>✓</td>
<td>(Zhao et al. 2008b; Christopoulos et al. 1999; Hernandez et al. 2013)</td>
</tr>
<tr>
<td>Na+ / K+ -ATPase neuron-specific α3 subunit</td>
<td>✓</td>
<td>Ca2+ dyshomeostasis</td>
<td>✓</td>
<td>(Ohnishi et al. 2015)</td>
</tr>
<tr>
<td>N-methyl-D-aspartate receptor (NMDAR)</td>
<td>✓</td>
<td>NMDAR internalisation</td>
<td>-</td>
<td>(Lacor et al. 2007)</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>✓</td>
<td>Fyn, Pyk2, CaMKII activation. NMDAR internalisation</td>
<td>✓</td>
<td>(Laurén et al. 2009; Haas and Strittmatter 2016)</td>
</tr>
<tr>
<td>Receptor for advanced glycation end products (RAGE)</td>
<td>✓</td>
<td>MAPK, JNK signalling</td>
<td>-</td>
<td>(Origlia et al. 2010)</td>
</tr>
<tr>
<td>Toll-like receptor 2</td>
<td>✓</td>
<td>Pro-inflammatory signalling</td>
<td>X</td>
<td>(Jana et al. 2008; Westwell-Roper et al. 2016)</td>
</tr>
</tbody>
</table>

✓ = Demonstrated; - = not demonstrated; X = demonstrated not to; ? = conflicting data

pTau = downstream signalling induces tau phosphorylation in response to Aβ

Table partially based on information available in Jarosz-Griffiths et al. (2016).
1.3.5 Tau

The neurofibrillary tangles which are a diagnostic hallmark of AD are made of aggregated hyperphosphorylated tau and tau fragments. Although there are no genetic mutations in MAPT which cause AD, tau is an essential part of AD pathology. This is because tau aggregation and spread is extremely neurotoxic (Ward et al. 2012). It has been demonstrated multiple times that neurofibrillary tangles are much better correlate of cognitive decline than Aβ deposition (Braak and Braak 1991). And tau has been demonstrated to be essential to Aβ associated neuronal death (Leroy et al. 2012). This implicates tau as the toxic mediator of Aβ signalling in AD (Bloom 2014). Tau toxicity is evident from the fact that individuals with Aβ plaques but no tau pathology do not suffer cognitive impairment, and that tau aggregation and dysfunction is a fundamental cause of other dementias (Poorkaj et al. 1998; McKee et al. 2009).

In its normal role, tau is a neuron specific microtubule stabilising protein mainly localised to axons. Alternative splicing of MAPT results in 6 isoforms of tau in human adult neurons, but in foetal development only 1 isoform is expressed. Tau is heavily post-translationally modified to regulate its function, the exact purpose of this modification is unclear but recent thinking suggests a role in synaptic plasticity. There are approximately 45 phosphorylation sites on tau which are predominantly located in the proline rich domain and microtubule-binding domain which are common to all isoforms (Noble et al. 2013). These sites have been demonstrated to be regulated by a multitude of kinases. A list of tau phosphorylation sites and kinases that phosphorylate them has been compiled by Diane Hanger’s lab (2017) (http://cnr.iop.kcl.ac.uk/hangerlab/tautable) and are reviewed in Martin et al. (2013). However, in many cases kinase phosphorylation of tau is evidenced by in vitro co-incubation, the presence of a consensus sequence or a loss of phosphorylation following use of a non-specific inhibitor; this further complicates understanding of the role of different kinases in AD pathology. The major tau kinases are GSK3β, CK1/2 and MAPK (Cavallini et al. 2013; Martin et al. 2013).

Synthetic and AD brain derived preparations of Aβ have been demonstrated to hyperphosphorylate tau. This causes dissociation from the microtubule and promotes the missorting of tau into somatodendritic spines (Hoover et al. 2010). This hyperphosphorylated and mislocalised tau is crucial in mediating Aβ induced AMPAR and NMDAR dysregulation (Ittner et al. 2010; Mairet-Coello et al. 2013). Furthermore, mislocalised soluble tau species may also be ejected from the synapse during increased neuronal activity (Wu et al. 2016; Furman et al. 2017). Oligomeric species of tau can spread from neuron to neuron in this way, forcing endogenous tau to take on pathological qualities (Lasagna-Reeves et al. 2012), potentially explaining the staging spreading of tau pathology.
from the entorhinal cortex to hippocampus and limbic system to the frontal lobe, as characterised in *Braak and Braak (1991)*.

1.3.6 **Other pathological features of AD**

1.3.6.1 **Vascular pathology**

In addition to Aβ plaques and neurofibrillary tangles of tau, *Alzheimer* (1907) also described ‘arteriosclerotic change’ in the vascular tissues in his original account of AD. The vasculature pathologies common in AD include amyloid inclusions into the vessel walls - termed cerebral amyloid angiopathy (CAA), vascular stenosis, small vessel disease and lacunar infarcts.

CAA is the deposition of Aβ aggregates that occurs in the leptomeningeal and cortical vessels. This sort of deposition is not uncommon in cognitively normal elderly people, and is not necessarily present in all cases of AD. However, the immunohistochemical pattern of deposition can distinguish between CAA types; only CAA-Type 1 correlates with APOE4 status and AD diagnosis (Thal et al. 2002a). CAA-Type 1 occurs in leptomeningeal and cortical arteries, veins, arterioles, venuoles and the cortical capillaries; whereas CAA-Type 2 occurs in leptomeningeal and cortical vessels with the exception of capillaries (Thal et al. 2002a). CAA is the primary cause of non-hypertensive intracerebral haemorrhage in the elderly (Viswanathan and Greenberg 2011) and has also been shown to cause vascular stenosis in patients with a genetic predisposition to CAA (Natte et al. 2001).

In addition to CAA, AD patients often present with large lacunar infarcts or microinfarcts. These infarcts can also damage the neurovascular unit, which is the point where capillaries, glia and neurons coincide to form the blood brain barrier (BBB). The Rotterdam and Nun imaging studies demonstrate the emergence of lacunar infarcts and neurovascular uncoupling prior to symptom emergence (Snowdon et al. 1997; Ott et al. 1998a). This has been built on by recent work demonstrating the psychosis induced by AD positively correlates with cortical microinfarcts and arteriosclerosis (Ting et al. 2016). This should not be surprising given that vascular perturbations alone are sufficient to cause vascular dementia (Roman et al. 1993). The vasculature damage observed in AD is studied in greater detail in Chapter 3.

1.3.6.2 **Neuroinflammation**

Neuroinflammation actively contributes to AD pathogenesis, as reviewed in *Heneka et al.* (2015). This is evidenced by GWAS data associating single nucleotide polymorphisms in the inflammatory genes *TREM2* and *CD33* with increased AD risk (Guerreiro et al. 2013; Griciuc et al. 2013). It is unclear how these genes promote pathology, but growing evidence implicates impaired function of TREM2 and CD33 in altered microglial response to Aβ (Xiang
et al. 2016; Villegas-Llerena et al. 2016). Microglia are the phagocytes of the central nervous system and engage in control of invading pathogens, neural circuit maintenance and neuronal remodelling (Heneka et al. 2015). In response to pathological triggers such as the amyloid species, tau tangles and vascular lesions seen in AD, microglia migrate to the source of the trigger and activate an innate immune response, characterised by the secretion of IL-1β (Lee and Landreth 2010). This is achieved by activation of the NLRP3 inflammasome.

Interestingly, it has recently been suggested that activation of the inflammasome by Aβ can act as a precursor to amyloid plaque formation. This hypothesis suggests inflammasome complex, which is rich in aggregated ASC protein, is ejected from dying microglia where it then acts as a nucleus for Aβ plaque formation (Heneka 2016). Multiple studies have found that extended use of non-steroidal anti-inflammatory drugs (NSAIDs) can reduce AD risk (summarised in (Heneka et al. 2015)). The mechanism of this effect is unknown and data linking NSAID use with AD has proved variable. This may be because only certain classes of NSAID inhibit the NLRP3 inflammasome (Daniels et al. 2016), and that this is the prerequisite requirement for inflammatory intervention influencing AD risk (Yin et al. 2017).

1.4 Connections between T2D and AD

1.4.1 Etiological links between T2D and AD

A strong etiological link between diabetes and AD has been consistently demonstrated in large population and imaging studies (Leibson et al. 1997b; Ott et al. 1999; Arvanitakis et al. 2004; Xu et al. 2009). The mechanisms underlying this link are unknown, and there are several common elements to AD and T2D pathology which may explain this aetiology. Population studies have attempted to dissect which elements of the diabetic phenotype best correlate with AD incidence. Hyperinsulinaemia, insulin resistance and high fasting glucose concentration have all been independently associated with AD incidence or severity in population studies (Luchsinger et al. 2004; Schrijvers et al. 2010; Ohara et al. 2011). In addition to this, large increases in sugar metabolites have been observed in multiple regions of the AD brain and advanced glycation end products are observed in the brain of AD and T2D patients (Smith et al. 1994; Xu et al. 2016a; Ahmed 2005).

1.4.2 “Type 3 diabetes”

There is clear evidence that insulin resistance and impaired insulin signalling deficiency contribute to AD and neurodegeneration (for a review see de la Monte and Wands (2008)). This has led to a group of researchers calling AD-like neurodegeneration ‘type 3 diabetes’
AD is associated with regional defects in glucose metabolism very early in disease pathogenesis (Minoshima et al. 1997). These defects strongly correlate with insulin resistance and early T2D (Luchsinger et al. 2004; Baker et al. 2011). Rats intra-cerebrally injected with streptozotocin, a glucose transport inhibitor used to model T2D, develop cognitive deficits, neurodegeneration and increased hallmarks of AD, including immunohistochemical staining for Aβ, phosphorylated tau and activated GSK3β (Lester-Coll et al. 2006). Insulin resistance induced by a high sugar diet will cause memory impairment in rat models and is a frequent observation following prolonged T2D in humans (Cao et al. 2007; Luchsinger et al. 2007).

It has previously been demonstrated that non-diabetic AD patients have many markers of brain insulin resistance which correlated with cognitive decline (Talbot et al. 2012). This has been expanded upon by a recent study where comparison of cognitively normal patients and AD patients revealed significant associations between markers of insulin resistance and islet β-cell dysfunction with defects in episodic memory, executive function, global cognition and increases in pathological CSF markers such as total tau and phosphorylated tau (Laws et al. 2017). The greatest density of insulin receptor expression in the brain is in the entorhinal cortex and hippocampus, where insulin signalling has neuroprotective properties and modulates synaptic plasticity (Hill et al. 1986; Wan et al. 1997; Chiu et al. 2008). Impairment of insulin signalling by Aβ species will promote GSK3β activation and tau phosphorylation (Zhao et al. 2008a; Hernandez et al. 2013). These data are evidence for a supportive role of insulin in hippocampal function and highlight failure of insulin signalling in AD pathogenesis.

1.4.3 Toxic oligomer species

Soluble oligomeric species of Aβ and amylin have been suggested as the pathology and toxicity inducing agent in AD and T2D, reviewed by Haass and Selkoe (2007) and Haataja et al. (2008). In both instances, this follows the gradual acceptance that large fibrillar species of the amyloids are neither particularly cytotoxic nor bioactive. These hypotheses arose because of the great similarity between amylin oligomers and Aβ oligomers. This principle is best demonstrated by studies using conformational antibodies. Conformational antibodies generated against amylin or Aβ oligomers will cross react with oligomeric species of the other amyloid (Kayed et al. 2007). Furthermore, Aβ oligomers and amylin oligomers can both be used to generate antibodies which will effectively reduce insoluble Aβ species following vaccination in mouse models (Rasool et al. 2012). In addition to this, molecular imaging probes designed against Aβ plaques in the brain demonstrate reactivity with amylin deposits in the pancreas (Yoshimura et al. 2014). This conformational similarity means that receptors associated with Aβ oligomer binding may also bind amylin aggregate species; Aβ
receptors and their amylin binding status is listed in Table 1.2. These data highlight the similarity in the oligomeric species of Aβ and amylin, while the data discussed in 1.2.3 and 1.3.2 demonstrates the central role of amyloid aggregation in AD and T2D, highlighting the potential benefits of aggregation inhibitors in both diseases. The similarities between Aβ and amylin oligomer species is further discussed in Chapter 4.

1.4.4 Pathology in apparently non-diseased individuals

A perennial criticism of the amyloid cascade hypothesis is the fact that many people develop amyloid plaques, in some cases quite extensively, without cognitive impairment or other pathological features of AD (Haroutunian et al. 1998; Price and Morris 1999; Villemagne et al. 2011). Interestingly, amylin amyloidosis of the pancreatic islets, without an apparent clinical diagnosis of T2D, can also occur (Bell 1959; Westermark 1972). Quite what this commonality means for the relationship of AD and T2D, or the common properties of amyloid-induced cell loss, remains unclear. It should also be noted that other pathological features key to AD, such as widened sulci and narrowed gyri (Figure 1.3D) also frequently occur in elderly patients without apparent dementia (Terry et al. 1981; Castellani et al. 2010). Resilience to symptomatic disease, in both AD and T2D, may be controlled by a complex matrix of lifestyle and genetic factors that are yet to be fully understood.

1.4.5 Non-canonical amyloid deposition in AD and T2D

While essential for diagnosis and research, categorisation of dementias into diseases separated by immunohistochemical markers is in many ways limiting the understanding of the heterogeneous nature of disease. Symptoms are not universal, and many amyloids diagnostic of one dementia can be found in patients diagnosed with a separate disease. Indeed, amyloid lesions are often found in patients with no cognitive impairments (Haroutunian et al. 1998; Villemagne et al. 2011). For example, TDP-43 has been demonstrated to aggregate in a proportion of amyotrophic lateral sclerosis patient tissues, a proportion of chronic traumatic encephalopathy patient tissue and in some otherwise healthy brains with hippocampal sclerosis (Neumann et al. 2006; Arai et al. 2006). It has subsequently been demonstrated that aggregated TDP-43 is also present in a proportion of patients with AD and dementia with Lewy bodies (Arai et al. 2009; Amador-Ortiz et al. 2007). Potentially even having a pathological staging similar to tau or Aβ (Josephs et al. 2014; Braak and Braak 1991; Thal et al. 2002b).

In addition to this, some reports suggest up to 50% of AD patients present with aggregated α-synuclein in the brain (Raghavan et al. 1993; Clinton et al. 2010). Lewy bodies of misfolded α-synuclein are more often associated with dementia with Lewy bodies or Parkinson’s...
disease (Chiti and Dobson 2006). Likewise, Aβ deposition has also been recorded in cases of dementia with Lewy bodies (Ballard et al. 2006). This further serves to demonstrate the heterogeneous presentation of pathological lesions in neurodegenerative diseases.

Non-canonical amyloid deposition also occurs in the pancreas in T2D. Aβ deposition and phosphorylated tau deposits have been reported in the pancreatic islets of Langerhans positive for amylin aggregation in humans (Miklossy et al. 2010), as well as in the triple transgenic-AD mouse model (Vandal et al. 2015) and a hIAPP-APP genetic cross mouse model, which is representative of AD with co-morbid T2D (Wijesekara et al. 2017). These data demonstrate that non-canonical amyloid deposition is a feature in T2D and also serve to highlight the association between Aβ and amylin in the diabetic pancreas.

Recent evidence from animal models demonstrates that injection of Aβ or amylin aggregate species will promote misfolding of amylin and Aβ in both the brain and pancreas of murine models (Oskarsson et al. 2015a; Moreno-Gonzalez et al. 2017; Wijesekara et al. 2017). These data raise the interesting possibility of cross-seeding and heterocomplex formation between amyloids, providing in vivo evidence of heterocomplex formation as has been observed in vitro (Andreetto et al. 2010; Rezaei-Ghaleh et al. 2011; Seeliger et al. 2012; Young et al. 2014; Young et al. 2015a; Baram et al. 2016).

1.4.6 Amylin deposition in the brain

In addition to the non-canonical deposits described above, amylin has been reported to be deposited in the brain of T2D patients with comorbid diabetes, and AD patients, with no apparent diabetic phenotype (Jackson et al. 2013). This paper reports amylin plaques in the brain parenchyma, amylin positive inclusions in the brain vasculature and mixed plaques of Aβ and amylin in human AD brain tissue that is absent from controls (Jackson et al. 2013). Amylin accumulation in the brain was also replicated in human amylin transgenic rats were it was associated with increased neuroinflammation and behavioural deficits (Jackson et al. 2013; Srodulski et al. 2014). The mixed Aβ-amylin plaques observed by Jackson et al. (2013) are indicative of the heterocomplexes predicted from sequence analyses (Figure 1.7) and produced in in vitro experiments (Andreetto et al. 2010; Seeliger et al. 2012; Young et al. 2014). Like Aβ, soluble amylin species detectable in the plasma will decrease in AD patients (Adler et al. 2014). The mechanism behind this relationship is unclear, it is possible this is as a result of increased deposition in the brain or elsewhere in the body, or alternatively, it may be an indication of undiagnosed diabetes causing reduced amylin secretion (Ludvik et al. 1991).

A further similarity between amylin and Aβ is that levels of both peptides are regulated by the same degradation mechanisms. Intracellularly, amylin and Aβ are both targeted to the
lysosome for degradation by cellular autophagy (Bharadwaj et al. 2012; Nixon 2007; Rivera et al. 2014). Extracellular amylin and Aβ monomer species both undergo cleavage by the metalloproteases neprilysin and insulin degrading enzyme (IDE) (Guan et al. 2012; Bennett et al. 2003; Vekrellis et al. 2000; Takaki et al. 2000). Shared degradation by neprilysin and IDE means that peripheral increases in amylin secretion during T2D are likely to increase central Aβ levels. This may provide another mechanism whereby increased amylin secretion in T2D promotes AD pathology.

Amylin deposition in the brain of AD patients and amylin-Aβ heterocomplexes are further discussed in Chapter 3.

Figure 1.7 Sequence analysis of amylin and Aβ

Amylin

KCN TATCATQR LANFLVHS S N FGAL S S T NVGS NTY - NH₂

Aβ

DAEFRHDGSYG EVHQQKLVF F AEDVGSNKG A IGLMVGGVVIA(-)

1.4.7 Repurposing antidiabetic drugs for AD

Impairments of insulin signalling and localised hyperglycaemia in the brain during AD (see 1.4.3) has created an interest in repurposing antidiabetic and insulin sensitising agents utilised in T2D in AD.

Metformin is very widely prescribed to T2D patients across the globe, in the UK over 80% of T2D patients receive metformin and over 90% of newly diagnosed T2D received the drug (Sharma et al. 2016). Metformin has been demonstrated to cross the BBB and re-sensitise neurons to insulin in AD models (Gupta et al. 2011). A longitudinal population study
evaluating the risk of AD diagnosis following treatment with different antidiabetic medications demonstrated that rather than ameliorate AD risk, chronic metformin treatment mildly increased AD risk (Imfeld et al. 2012). In addition to this, insulin supplementation, sulphonylurea use and thioglitzone use had no impact of AD risk (Imfeld et al. 2012). This study demonstrates that despite the significant contribution of insulin signalling and insulin resistance to AD pathology, commonly prescribed insulin sensitising agents do not reduce AD risk.

An alternative to these approaches may lie in an emergent strategy for the treatment of T2D - the use of small molecule inhibitors of amylin aggregation. A class of bioactive small molecule employed in this regard are flavonoids. Quercetin and the quercetin glycoside rutin are flavonoids that have recently been demonstrated to prevent amylin oligomerisation and fibril formation (Aitken et al. 2017b). Dietary supplementation of these flavonoids restores normoglycaemia, reduces insulin resistance and significantly extends lifespan in IAPP+/− mice (Aitken et al. 2017a; Aitken et al. 2017b). Quercetin has also been reported to act as an insulin secretagogue (Bardy et al. 2013). These actions and the well characterised nature of such flavonoids in humans make these peptides attractive therapeutics in T2D (Booth et al. 1956; Boyle et al. 2000; Murota et al. 2010; Aziz et al. 1998; Boomgaard et al. 2010). The anti-amyloidogenic properties demonstrated by these flavonoids towards amylin suggest they may have therapeutic potential targeting Aβ in AD.
1.5 Thesis aims

T2D and AD share a deep etiological link, which are not mechanistically understood (Leibson et al. 1997b; Ott et al. 1999; Biessels and Kappelle 2005; Steen et al. 2005; de la Monte and Wands 2008; Mittal et al. 2016; Laws et al. 2017). These diseases are both amyloidoses, both share vascular and inflammatory phenotypes and risk of both diseases increases with age (Gotz et al. 2013; Mukherjee et al. 2015). The amyloids central to both diseases form highly similar oligomer structures, cause similar impairments in cell function and there is a growing body of evidence that these amyloids can interact to cross-seed one another and cause co-deposits (Jackson et al. 2013; Young et al. 2015a; Oskarsson et al. 2015a; Wijesekara et al. 2017). This co-deposition, and independent deposition of amylin, has been observed in the brain parenchyma and vasculature of AD patients and in diabetic patients with cognitive dysfunction (Jackson et al. 2013). However, Jackson et al. (2013) is an observational study reliant on immunohistochemistry, and deposition is yet to be confirmed to be statistically elevated in AD, nor has the contribution of T2D been elucidated. Amylin deposition in the brain of AD patients provides an interesting connection between AD and T2D, and may underlie the etiological link between these diseases.

The highly comparable aggregates have demonstrated sufficient tertiary structure homology to cross react with the same conformational epitope antibodies (Kayed et al. 2007) and imaging probes (Yoshimura et al. 2014). This homology makes the sharing of cell surface receptors likely, but thus far no receptors or signalling cascades associated with AD pathology have been demonstrated to be shared between Aβ and amylin. The propensity of a localised amyloid to aggregate is a central pathogenic feature of both AD and T2D. Therefore, manipulation of amyloid aggregation is an attractive therapeutic option, particularly if it can be sufficiently broad as to target both amylin and Aβ aggregation. The ability of the flavonoids rutin and quercetin to ameliorate amylin aggregation has already been demonstrated, but investigation into the actions of these flavonoids on Aβ oligomer formation and function is yet to be established.

Understanding the link between AD and T2D will highlight key elements of both pathologies for further therapeutic intervention and help underpin mechanisms that can help promote healthy ageing.

Aims:
1.5.1 Investigating the role of amylin in the brain in AD and T2D

**Hypotheses:** Amylin is deposited as insoluble aggregates in the brain of AD and T2D patients and amylin deposition will be exacerbated by co-morbid T2D-AD. Amylin and Aβ will form heterocomplexes and cross-seed one-another. Due to increased amylin secretion, T2D will reduce markers of vascular health.

**Objectives:**

- Investigate amylin deposition by multiple techniques in control, T2D, AD and T2D-AD human brain samples.
- Investigate amylin-Aβ hetero-complex formation
- Investigate effect of T2D on brain vasculature
- Investigate effect of amylin on Aβ production and clearance

1.5.2 Investigating whether amylin and Aβ share downstream signalling

**Hypothesis:** Amylin oligomers will activate downstream signalling pathways associated with Aβ oligomers and AD pathology by binding to cell surface receptors.

**Objectives:**

- Investigate amylin monomer and oligomer activation of Fyn kinase
- Investigate amylin binding to PrP<sup>C</sup>
- Model PrP<sup>C</sup> signalling and tau phosphorylation induced by Aβ<sub>2</sub> and amylin in neurons derived from control patient induced pluripotent stem cells.

1.5.3 The therapeutic potential of quercetin in AD

**Hypotheses:** The flavonoids rutin and quercetin will prevent the oligomerisation of Aβ by binding to Aβ monomers and will also promote the disaggregation of preformed oligomers.

**Objectives:**

- Incubate rutin and quercetin with monomeric Aβ to determine the effect of flavonoids on oligomerisation and fibril formation
- Investigate the effect of flavonoid incubation on preformed Aβ oligomer stability
- Investigate the effect of flavonoid incubation on Aβ oligomer binding
- Investigate the effect of flavonoids on Aβ production
Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Cell Lines
SH-SY5Y human neuroblastoma cells were cultured in DMEM (Lonza) supplemented with 10% (v/v) foetal bovine serum. These conditions were also used for SH-SY5Y cells stably expressing the human cellular prion protein (SH-SY5Y-PrP<sup>C</sup>) or stably expressing the 695aa isoform of amyloid precursor protein (SH-SY5Y-APP<sub>695</sub>). NB7 cells were cultured in RPMI media (Sigma) supplemented with 10% foetal bovine serum. All cells were stored in incubators at 37°C in 10% CO<sub>2</sub> environment. All handling of cells was performed in a sterile environment and cells were regularly checked for mycoplasma infection.

2.1.2 iPSC differentiation protocol
Neuronal differentiation from induced pluripotent stem cells (iPSCs) and neuron maintenance was performed by Alys Jones, Kate Fisher, Kate Kellett, Nicola Corbett, Helen Rowland, Heled Jarosz-Griffiths and I. The protocol employed in this study is based on Shi et al. (2012). Media refered to in this description is listed in Table 2.1. The control patient derived OX1-19 clone iPSCs, provided by Sally Cowley (Oxford, UK), were cultured in mTESR media (STEMCELL Technologies) supplemented with penicillin and streptomycin. Once the iPSC culture reached 100% confluency neuronal induction was initiated by addition of neuronal induction media, this was termed ‘day 0’. By day 10-11 cells formed a dense neuroepithelial sheet and were passaged by dispase (STEMCELL Technologies) from a 12 well plate to a 6 well plate pre-coated in laminin. Cells were then maintained in neural maintenance media (NMM) that was supplemented with 20µg/ml FGF2 (Sigma) when rosettes began to form. Following 4 days of FGF2 treatment cells were passaged with dispase and if necessary rosettes were isolated using STEMdiff Rosette Selection Reagent (STEMCELL Technologies) according company guidelines at a 1:1 ratio. On day 25 cells were passaged into single cell suspension by accutase (Sigma) mediated passage. Cells were further cultured in NMM until day 35-40 when they were then passaged by accutase for a second time. Cells were then maintained using NMM until the desired maturation day (day 50, 65 and 80 neurons were used in the present study). Markers of iPSC pluripotency (Oct-4, Nanog, Sox2), non-proliferation (PAX6, FOXG1, MAP2), neuronal maturation (Tbr1, SATB2, βIII-tubulin, PSD95 and vGlut1) and the emergence of glia (GFAP and S100) were regularly checked by immunofluorescence and RT-PCR.
Table 2.1 Media used in neuronal induction and differentiation of iPSCs

Table produced by Helen Rowland (Unpublished) and is reproduced here with permission.

<table>
<thead>
<tr>
<th>N2 Media</th>
<th>B27 Media</th>
<th>Neural Maintenance Media (NMM)</th>
<th>Induction Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM F12 Media (Life Tech)</td>
<td>Neurobasal Media (Life Tech)</td>
<td>N2 Media</td>
<td>SB431542 (10mM) (R&amp;D Systems)</td>
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<tr>
<td>Penicillin (50µg/ml) (Life Tech)</td>
<td>Penicillin (50µg/ml) (Life Tech)</td>
<td>B27 Media</td>
<td></td>
</tr>
<tr>
<td>Streptomycin (50mg/ml) (Life Tech)</td>
<td>Streptomycin (50mg/ml) (Life Tech)</td>
<td>1:1 ratio</td>
<td>Noggin (500ng/ml) (R&amp;D Systems)</td>
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<td>Non-essential amino acids (55mM) (PAA)</td>
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<td></td>
<td>Sterile filtered (0.22µm pore filter)</td>
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<tr>
<td>2- Mercaptoethanol (10uM) (Life Tech)</td>
<td>1X B27 (Life Tech)</td>
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<td></td>
</tr>
<tr>
<td>Insulin (5µg/ml) (Sigma-Aldrich)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L-glutamine (1mM) (Life Tech)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1X N2 (Life Tech)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2 Animal tissue

Human amylin transgenic mouse tissue was kindly provided by Prof. Garth Cooper via the University of Auckland. Human amylin transgenic mice were developed in a FVB/n genetic background through pronuclear micro-injection of RT-PCR generated human amylin cDNA under regulatory control of the insulin-II promoter (Wong et al. 2008). Tissue was harvested immediately after sacrifice and halved. One half was snap frozen for further biochemical analyses and the other half was mounted in OCT (Tissue-Tek) for immunohistochemical analyses.
2.3 Human Brain samples

2.3.1 Ethics and permissions
Tissue was supplied by the Manchester Brain Bank at Salford Royal Foundation NHS trust following approval by the Newcastle and North Tyneside 1 research ethics committee (REC reference: 09/H0906/52+5). Frozen sections of occipital grey and white matter were supplied separately and temporal white matter alone was also supplied. Fixed sections of mixed grey and white matter from the temporal and occipital lobes were also supplied under this application. Diagnoses of neurological pathologies were confirmed by neuropathological analyses. Diabetic status was inferred by self-reporting and medication history. Details of human samples and demographic data are detailed in Table 2.2. There were no significant differences in age of death between control, diabetes, Alzheimer’s disease or Diabetes and Alzheimer’s disease groups determined by two-way ANOVA (Figure 2.1).

Figure 2.1 Age at death comparison between human samples
Comparison of age of death demonstrates no significant differences between patient groups and no significant effects of AD or T2D. Data represent mean ± SEM. Data were analysed with a two-way ANOVA.
<table>
<thead>
<tr>
<th>Case No.</th>
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<th>MRC ID</th>
<th>Sex</th>
<th>Age at death</th>
<th>Diabetes status</th>
<th>Diabetes medication</th>
<th>Clinical diagnosis</th>
<th>Pathological diagnosis 1</th>
<th>APOE</th>
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<tbody>
<tr>
<td>DPM09/37</td>
<td>0-I</td>
<td>BBN_3399</td>
<td>M</td>
<td>90</td>
<td>Type II</td>
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<td>Vascular dementia</td>
<td>Age changes only</td>
<td>33</td>
</tr>
<tr>
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<td>Moderate SVD</td>
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<td>BBN_5770</td>
<td>M</td>
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<td>N/A</td>
<td>Vascular dementia</td>
<td>Mild Alzheimer type changes, mild TDP-43 in hippocampus</td>
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<td>Severe Alzheimer's disease</td>
<td>44</td>
</tr>
<tr>
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<td>BBN_3470</td>
<td>M</td>
<td>72</td>
<td>Type II</td>
<td>Metformin</td>
<td>Alzheimer's disease</td>
<td>Alzheimer's disease</td>
<td>34</td>
</tr>
<tr>
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<td>M</td>
<td>73</td>
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<td>N/A</td>
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<td>Alzheimer's disease; Mod SVD</td>
<td>44</td>
</tr>
<tr>
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<td>BBN_3476</td>
<td>F</td>
<td>87</td>
<td>Type II</td>
<td>No information available</td>
<td>Cognitively normal</td>
<td>Mild AD pathology in temporal lobe</td>
<td>33</td>
</tr>
<tr>
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<td>V-VI</td>
<td>BBN_11027</td>
<td>F</td>
<td>85</td>
<td>Type II</td>
<td>Metformin and insulin</td>
<td>Alzheimer's disease</td>
<td>Alzheimer's disease; Moderate to severe SVD. Very mild DLB</td>
<td>34</td>
</tr>
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<td>76</td>
<td>Type II</td>
<td>No information available</td>
<td>Cognitively normal</td>
<td>Mild AD changes in temporal lobe; Very mild CAA, Moderate SVD in BG</td>
<td>33</td>
</tr>
<tr>
<td>DPM13/39</td>
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<td>BBN_17991</td>
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<td>Vascular dementia</td>
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<td>23</td>
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<td>78</td>
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<td>Metformin</td>
<td>Alzheimer's disease</td>
<td>Alzheimer's disease; Secondary TDP-43 proteinopathy</td>
<td>44</td>
</tr>
</tbody>
</table>

DLB = dementia with Lewy bodies; SVD = small vessel disease

Table 2.2: Identification and demographic data of human samples.
2.3.2 Homogenisation protocol

Workflow is outlined in Figure 2.2. Between 0.6-0.95g of human brain was suspended in ice cold 50mM TEAB/ 2% SDS buffer supplemented with protease inhibitor cocktail 4% (v/v) (Roche) and PhosSTOP phosphatase inhibitor cocktail 10% (v/v) (Roche) to a final volume of 120mg/ml. The samples were then homogenised on ice using an electrical homogeniser in the fume hood, using probe setting intensity ‘1’ in 20s intervals with 30s
cooling between each interval until a smooth consistency was achieved. Samples were then centrifuged at 3500xg for 15min at 4°C. This generated a pellet, supernatant and floating fatty layer. The supernatant was removed by needle and syringe and the pellet and fatty layer were discarded. 2ml of the supernatant was pipetted into a thick-walled Bruker ultracentrifuge tube and any remaining supernatant was set aside and frozen as the total protein fraction. The 2ml supernatant was then centrifuged at 112,000xg at 4°C for 1h in an Optima MAX TL ultracentrifuge (Beckman). The supernatant was removed at set aside as the TEAB fraction. The pellet was then resuspended in 300μl 70% formic acid by gentle pipetting and allowed to stand for 30min on ice. The samples were then centrifuged at 112,000xg at 10°C for 1h. The supernatant was eluted using a needle and syringe, this step was repeated until no visible pellet remained. All samples were then stored at -80°C until required.

2.4 Sample Preparation

2.4.1 Preparation of cell lysates and media
Following the end of an experiment media was removed and either discarded or supplemented with protease and phosphatase inhibitors at 1:100 and 1:250 inhibitor to media ratios respectively. Media was then centrifuged at 3,000xg at 4°C for 10min. The resulting supernatant was transferred to a new Eppendorf and stored at -20°C. Cells were then washed once in ice cold PBS before being lysed by cell scraper in a volume of lysis buffer (see below) supplemented with protease and phosphatase inhibitor cocktail as in 2.4.2. Cell samples then underwent centrifugation at 14,000xg for 10min at 4°C. The resultant supernatant was then removed and assayed for protein concentration as in 2.4.3. The samples were then stored at -20°C. Plates (6 well) were lysed in 100μl lysis buffer per well; T-80 flasks were lysed in 800μl per flask.

2.4.2 Lysis buffer and loading sample preparation
Lysis Buffer: 50mM Tris, 150mM NaCl, 0.5% (w/v) Sodium deoxycholate, 1% (v/v) NP-40, 0.1% SDS. Make up to final volume (200ml) in dH2O and pH 8.0. Stored at 4°C.

All lysates were then supplemented with protease inhibitor cocktail 4% (v/v) (Roche; Cat. No. 05 892 791 001) and PhosSTOP phosphatase inhibitor cocktail 10% (v/v) (Roche; 05 892 791 001). Samples destined for SDS-PAGE were made up to an optimal equal protein concentration supplemented with dH2O up to 80μl. This was then added to 20μl 5x sample buffer (100mM Tris/HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, pH 6.8) supplemented with dithithreitol (DTT) to a final concentration of 100mM. All
samples were boiled at 95°C for 5 minutes then chilled prior to storage at 20°C or gel loading.

2.4.3 Bicinchoninic acid (BCA) assay
Protein concentration were interpolated against a standard curve ranging between 2 and 10μg bovine serum albumin, with each well to a final volume of 10μl and each point performed in duplicate. Sample wells contained a final well volume of 10μl comprising of 1-3μl sample plus 7-9μl dH2O, performed in duplicate on the same 96well plate as the standard curve. Finally a working reagent of BCA solution (Thermo-Fisher Scientific) mixed with 4% (w/v) CuSO4.5H2O in a ratio of 50:1 was added to the wells and incubated at 37°C for 20min. Measurements were then made using a ELx800 plate reader (Bio-tek) and data were extracted and concentrations determined against the standard curve using Gen5 plate-reader software (Bio-tek). Concentration values were further analysed using Excel software (Microsoft).

2.5 Oligomer preparation

2.5.1 Peptide film preparation
Aβ1-42 (Anaspec; AS-24224), Aβ1-42-LC-biotin (Anaspec; AS-24640) and human amylin1-37 (Bachem; H-7905) were purchased as a lyophilised powder from the supplier and immediately stored at -20°C. The peptide was then made up to 1mg/ml in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), briefly vortexed and left to stand for 3h to disrupt any aggregates. This was then divided into 25μl aliquots and the final weight of aliquoted peptide was determined by starting volume / total final volume as some volume is lost due to evaporation. Samples were then dried using a SPD131DDA vacuum concentrator (Savant). A vacuum was applied at slowest ramp speed (ramp 1) for 1h 30m whilst samples were under centrifugation. The resultant peptide films were then stored at -80°C.

2.5.2 Amyloid-β preparation
Peptide films were solubilised in DMSO up to a final concentration of 1mM. Oligomeric preparations were made up to 100μM in Ham’s F-12 (Sigma; N6658) and incubated at room temperature for between 16-20h. Oligomer preparations were then centrifuged at 14,000xg for 20 min to pellet any large aggregate species; the resultant supernatant was then removed and transferred to a fresh tube. This was then immediately used. For monomeric control preparations 1μl of the 1mM preparation was aliquoted, stored at 4°C for between 16 - 20h and diluted to 100μM Ham’s F-12 (Sigma) immediately prior to use.
2.5.3 Amylin preparation

Peptide films were solubilised in 20mM ammonium acetate (pH 7.4) up to a final concentration of 1mM. Oligomer preparations were made up to 100μM in Ham’s F-12 and incubated at room temperature for 24h, 6h or 3h. For monomeric control preparations 1μl of the 1mM preparation was aliquoted and snap-frozen in liquid nitrogen, this was then stored at -20°C and made up to 100μM in Ham’s F-12 immediately prior to use.

2.5.4 Small molecule inhibitors

Peptide films due to be incubated with small molecule inhibitors were solubilised to 1mM in DMSO. The peptide films were then diluted in Ham’s F-12 containing the relevant concentration of inhibitor to a final concentration of 100μM Aβ before incubation for 24h. This preparation was then centrifuged at 14,000g for 20 min and the supernatant aliquoted to a new Eppendorf before being immediately used in experiments. In some experiments oligomers of Aβ were generated as in 2.5.2 then incubated with small molecule inhibitors. Following the final centrifugation step, oligomer preparations were diluted 1:2 in Ham’s F-12 medium containing small molecule inhibitors to give the desired concentration. This was incubated at room temperature for 30min before being used in experiments. Rutin and quercetin were solubilised in 10% NH₄OH /Ham’s F12 (v/v) and diluted to the desired concentration in F-12. In all experiments final concentrations of NH₄OH were below 0.1%.

2.6 Western Blotting and Dot blotting

2.6.1 Western Blotting

Proteins were resolved by gel electrophoresis using 1.5mm thick 12% acrylamide Tris-glycine gels. SDS-PAGE was carried out in Tris/glycine/SDS electrophoresis buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 0.1% (w/v) SDS) at 45mA per gel for 1h. Amyloidogenic proteins were resolved on 1.5mm thick 10-20% Acrylamide gradient Tris-tricine precast gels (Biorad) at 45mA per gel for 1h 45min. All proteins were then transferred to PVDF membrane (equilibrated in 100% methanol for 1 min) via a wet transfer system at 120V for 72min in transfer buffer (20% (v/v) Methanol, 20.5mM Tris, 150mM glycine). Membranes were blocked in 5% fat free powdered milk (w/v) / PBS(+Ca²⁺ and Mg²⁺) supplemented with 0.2% (v/v) Tween (PBST) for 1h at room temperature before being incubated in primary antibody (Table 2.3) overnight. The following day the membrane was washed three times in PBST for 5min each before being incubated in secondary antibody (1:5000; 3% BSA/PBST) for 1h at room temperature. This was followed by two 5min washes in PBST and a final wash in PBS. Antibody binding was imaged using chemiluminescence by the ECL.
method (Sigma) as per manufacturer’s instructions and captured using the G-Box imaging system (Syngene).

2.6.2 Dot blotting

Oligomer and monomer preparations of amyloids were made as described in 2.5.2-2.5.4 and diluted to 100μM, 20μM and 10μM concentrations. 1μl of each concentration was spotted onto nitrocellulose membrane and allowed to dry for 10 min. The membrane was then blocked in 5% Milk / PBS(+Ca²⁺ and Mg²⁺) supplemented with 0.2% Tween (PBST) for 1h at room temperature before being incubated in OC primary antibody (Table 2.3) overnight. The following day the membrane was washed three times in PBST for 5min each before being incubated in secondary antibody (1:5000; 3% BSA/PBST) for 1h at room temperature. This was followed by two 5min washes in PBST and a final wash in PBS. Antibody binding was imaged using chemiluminescence by the ECL method (Sigma) as detailed in 2.4.1. The imaged membrane was then stripped using 100mM glycine buffer pH 2 for two hours before being washed three times in PBST and then reprobed with 6e10 antibody.
<table>
<thead>
<tr>
<th>Target</th>
<th>Dilution</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>6E10 - Aβ1-17</td>
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<td></td>
</tr>
<tr>
<td>4G8 - Aβ17-24</td>
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<td>A11</td>
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<tr>
<td>Amylin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4150 (amylin1-37)</td>
<td>1:1,000</td>
<td>Peninsula Labs</td>
</tr>
<tr>
<td>H-017-03</td>
<td></td>
<td>Phoenix Pharmaceuticals</td>
</tr>
<tr>
<td>(N-terminal amylin)</td>
<td>1:1,000</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>1:2,000</td>
<td>Sigma-Aldrich (#A5441)</td>
</tr>
<tr>
<td>Fyn</td>
<td>1:1,000</td>
<td>Abcam (#ab125016)</td>
</tr>
<tr>
<td>pFyn (pY416)</td>
<td>1:1,000 / 1:500</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pFyn (pY416)</td>
<td></td>
<td>dependent on batch</td>
</tr>
<tr>
<td>K9JA</td>
<td>1:2,000</td>
<td>DAKO (#A0024)</td>
</tr>
<tr>
<td>pTau</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHF1 (pS396/404)</td>
<td>1:1,000</td>
<td>A kind gift from Peter Davies, Albert</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Einstein College of Medicine, USA</td>
</tr>
<tr>
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<td>1:1,000</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>University, USA</td>
</tr>
<tr>
<td>pT181</td>
<td>1:1,000</td>
<td>Cell Signalling (#12885)</td>
</tr>
<tr>
<td>pS202</td>
<td>1:1,000</td>
<td>Cell Signalling (#11834)</td>
</tr>
<tr>
<td>Saf32 - PrPc</td>
<td>1:3,000</td>
<td>Bioquote Ltd (#189720)</td>
</tr>
</tbody>
</table>
2.7 Glutaraldehyde cross-linking assay

Peptide films were re-suspended up to 1mM in 10mM Tris-HCl pH 8.0. Peptide (2μl) was added to 2μl of TRIS-HCl pH 8.0 or 2μl of co-incubating peptide. 2μl TRIS-HCl pH 8.0 and 0.05% glutaraldehyde was then added and the mixture incubated at room temperature for 15min. The glutaraldehyde was quenched by the addition of 0.05M sodium borohydride for 15min at room temperature. Sample buffer (5x) (2.4.2) was then added to the mixture and the samples were resolved by electrophoresis on 12% acrylamide TRIS-Glycine SDS gels.

2.8 Atomic Force Microscopy

2.8.1 Protocol
Oligomeric and monomeric samples of Aβ and amylin were prepared as described previously in 2.5.2 and 2.5.3. Samples were diluted in Ham’s F-12 buffer to 10μM and a 20μl drop was placed onto freshly cleaved mica film. Protein was then allowed to adhere to the surface of the mica for 90s (Aβ) or 120s (Amylin). Following this the sample was washed three times in dH2O before being immediately dried under a stream of N2 gas. Samples were then covered and analysed within 24h. During acquisition a 5μm x 5μm section sample was measured in tapping mode with a data scale of 40nm over 516 lines with an aspect ratio of 1.00. Data were captured at a scan rate ranging from 0.3 to 0.996Hz. In tapping mode, the integral gain was 0.03, the proportional gain was 0.045 and the amplitude setpoint was 250mV in all experiments.

2.8.2 Data acquisition
Atomic force microscopy (AFM) was performed on a multimode picoforce AFM instrument (Bruker UK Ltd, Coventry). Tapping mode AFM was performed on samples under ambient conditions. Bruker MPP-11100-10 silicon cantilevers with an aluminium reflex coating (Bruker UK Ltd, Coventry) were used in all experiments. Tip accuracy was regularly checked using a NGR-21010 3D reference grid (Veeco, Phoenix, AZ).

2.8.3 Data analysis
Data extraction was performed using Gwyddion v2.42 opensource software and data analysis was performed in Microsoft excel. The drying process forces oligomeric structures to become ellipsoid in shape on a mica surface, where they would normally be spherical in solution. During data analysis this is accounted for by calculating the volume of each ellipsoid oligomer using the following equation: 

\[ V_{\text{ellipsoid}} = \frac{4}{3\pi} \times r^2 \times \text{height}, \]

then interpolating the diameter of a spherical oligomer of a corresponding volume by rearranging
the equation: $V_{sphere} = \frac{4}{3\pi} r^3$. These interpolated data were then used in figures and statistical analyses. The distribution analyses of height data acquired by AFM presented in Figures 5.6 and 5.10 were performed by Dr. Jack Rivers-Auty, University of Manchester. Generalized linear modelling was used to evaluate the effect of oligomer size and treatment on frequency (MASS package) (Venables and Ripley 2002). Size and treatment were modelled as fixed effects and experiment (n=3) as a random effect. Within-experiment design with random intercepts was used for all models. The significance of inclusion of a dependent variable or interaction terms were evaluated using log-likelihood ratio. Homoscedasticity and normality were evaluated graphically using predicted vs residual and Q-Q plots, respectively. All analyses were performed using R (version 3.3.3).

2.9 Thioflavin-T Assay

Peptide films were solubilised and made up to 1mM before being diluted to 250μM in buffer (Ham’s F-12, 20mM ammonium acetate pH 7.4) immediately before addition to 1mM Thioflavin-T solution in the same buffer. This mixture (100μl) was then immediately loaded onto a black 96 well plate (Nunclon; 137101) and fluorescence was measured in arbitrary units of fluorescence values every 5 minutes for 48 hours at a controlled room temperature of 22°C using the following excitation and emission wavelengths (excitation wavelength 450; emission wavelength 510). Each sample was loaded in triplicate with appropriate negative controls to account for the background fluorescence of the buffer.

Data was extracted as arbitrary units of fluorescence and corrected for background at each time point before being plotted against time.

2.10 Immunofluorescence microscopy

2.10.1 Protocol

Cells were seeded to an appropriate confluence on glass coverslips and underwent treatments as described in 2.4.1. Cells were washed three times in PBS (Lonza) before treatment and then washed a further three times in PBS prior to fixation in 3% paraformadehyde (PFA)/PBS for 10min at room temperature. This was removed and the excess PFA quenched using 50mM Ammonium chloride (NH4Cl) prior to 2 further washes in PBS. Cells were then blocked in 5% fish skin gelatin/PBS for 3h at room temperature or overnight at 4°C. Coverslips were then incubated in 1° antibody in a moist chamber for 1h
at room temperature or overnight at 4°C according to manufacturer’s recommendations. Following incubation, coverslips were washed three times in PBS before being incubated in 2° antibody overnight. The next day coverslips were washed once for 10min in PBST (0.2%Tween) before being bathed in 4’,6-Diamidino-2-Phenylindole (DAPI) (Sigma) (1:2000) for 2 min at room temperature. Coverslips were then washed for 10 min in PBST and two times in PBS before being dried overnight and mounted using Fluromount-G mounting media.

2.10.2 Antibodies
All antibodies were made up in filter sterilised 5% fish skin gelatin/PBS. All secondary antibodies were used at a 1:500 dilution.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Source</th>
<th>Secondary Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ1-42- LC-Biotin</td>
<td>-</td>
<td>-</td>
<td>Strepavadin-conjugate</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>PrPC</td>
<td>Saf32</td>
<td>1:3,000</td>
<td>Alexa-flour 488</td>
<td>Thermofisher</td>
</tr>
</tbody>
</table>

2.10.3 Microscopes
Data was either captured on snapshot widefield microscopes (Olympus Life Sciences, UK) or Delta-Vision high end widefield microscopes (GE Healthcare Life Sciences, UK). Image stacks captured on the Delta-Vision high end widefield microscopes were subjected to deconvolution prior to quantitative analysis. All images subject to quantitative analysis were captured at 60x magnification 1x1 binning, fluorescence channel exposure times were optimised per biological n against a positive control.

2.10.4 Data Analysis
Quantification of cell surface Aβ binding was analysed in Image-J. In each experiment the optimal exposure time per channel was determined using a positive control and then maintained throughout the experiment. Binning and image size was kept constant across all experiments. For cell surface quantification the optimal brightness and contrast of each channel was set in each experiment and those conditions were maintained throughout the experiment. Binding at the cell surface was quantified by tracing the outline of whole cells using the freehand line tool in Image-J and expressed as a mean arbitrary fluorescence over the cell surface. Line width = 6 pixels. At least 20 cells were measured per experiment.
2.11 Immunohistochemistry

2.11.1 Fixing and slicing

2.11.1.1 Mouse pancreas samples
Mouse pancreas samples were snap frozen and embedded in optimal cutting temperature (OCT) medium before storage at -80°C. Upon thawing samples were sliced in 8μm thick sections from the splenic end of the pancreas using a CryoStat microtome at -20°C and placed on slides before storage at -20°C prior to Congo-Red staining or DAB (3,3'-diaminobenzidine) immunohistochemistry.

2.11.1.2 Human brain samples
Human brain samples were sourced from the Manchester Brain Bank. Paraffin embedded wax sections (6μm thick) of mixed grey and white matter from the occipital and temporal lobes were provided from each case outlined in Table 2.1.

2.11.2 DAB immunohistochemistry

2.11.2.1 DAB staining protocol
Immunohistochemistry staining was performed by Yvonne Davidson, University of Salford. Stained slides were provided and then imaged, quantified and analysed. Slides underwent antigen retrieval as detailed in Table 2.5, in cases where sequential antigen retrieval steps were employed slides were washed 3 times in distilled water in 5min intervals before the second retrieval step. Following antigen retrieval the Vectastain kit - Elite PK-6102 Mouse IgG (Vector labs) was used. Slides were washed 3 times in distilled water as above before incubation in 0.3% hydrogen peroxide (H₂O₂) in methanol at room temperature for 30min to quench endogenous peroxidase activity. Slides were then washed 3 times in distilled water as above before a hydrophobic barrier is applied around the tissue section using ImmEdge Pen (Vector labs). Slides were then washed in PBS for 5 min before blocking in normal serum (Vectastain kit) for either 30min at room temperature or overnight at 4°C. Excess serum was then removed and the sections were then incubated with primary antibodies made up in blocking buffer for 1h at room temperature. Sections were then washed 3 times in PBS in 5 min intervals before incubation with biotinylated secondary antibody (Vectastain kit) for 30min at room temperature. Slides were then washed 3 times in PBS as above before addition of avidin-biotin complex (ABC) reagent for 30mn at room temperature followed by a further 3 washes in PBS. Slides were then treated with DAB solution (Sigma) for 5 min at room temperature before a 5min wash under running water. Slides were then counterstained with the nuclear stain haematoxylin for 3s per slide. Slides
were washed once in distilled water before undergoing a dehydration protocol of 5min washes in the following buffers: 70% industrial methylated spirits (IMS), 90% IMS, 100% IMS, Xylene, Xylene and were then immediately mounted in DPX mountant (Sigma; 06522) and dried.

Table 2.5 Table of antibodies used in immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody (antigen)</th>
<th>Antigen retrieval steps</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G8 (Aβ17-24)</td>
<td>Citric acid</td>
<td>1:3,000</td>
<td>Biolegend (#SIG-39220)</td>
</tr>
<tr>
<td>T-4150 (Amylin)</td>
<td>Citric acid and formic acid</td>
<td>1:1,500</td>
<td>Peninsula Laboratories</td>
</tr>
<tr>
<td>H-017-03 (Amylin)</td>
<td>Citric acid</td>
<td>1:1,000</td>
<td>Phoenix Pharmaceuticals</td>
</tr>
</tbody>
</table>

2.11.3 Slide scanner and quantification

The whole tissue sections were imaged using the Panoramic 250 Flash Slide Scanner (3D Histech) at a 20x magnification. Images were viewed and acquired in the Caseviewer v2.1 software (3D Histech). Quantification was performed blind using the Histoquant plugin (3D Histech). An example of what was considered to be positive staining was chosen to create a threshold scenario and then this threshold scenario was saved and applied to all samples stained with that antibody. In cases where a subtype of staining was specified, such as “vasculature amyloid” then incidences of that subtype were manually counted after application of the threshold mask. Data was then un-blinded and further analysed in Excel (Microsoft) and Prism (Graphpad).

2.11.4 Congo red staining

Congo red staining images were kindly provided by Dr. Jaqueline Aitken, Univeristy of Auckland. Congo red staining was performed on mouse pancreas extracted and fixed in OCT as described in 2.2. Fixed pancreas tissue was cryosectioned into 8μm slices and stained with congo red solution (1% (w/v) congo red) for 15-20min followed by saturated lithium carbonate (30s). Islet amyloid species were then visualised by polarisation microscopy. Images were taken at 60x magnification using a light microscope.

2.12 Multiplex immunoassays and ELISAs
2.12.1 Multiplex electro-chemiluminescence immunoassays
96-well multiplex electro-chemiluminescence plates were purchased from MSD Technologies and prepared as per the assay dependent instructions provided. Generally, each plate was blocked in a provided blocking reagent for 1h with shaking prior to 3 x washing steps in a 1 x Tris-based washing solution. For experiments using cell media the samples and standards were diluted in HEPES to a final concentration of 50mM before being incubated on the plate for 1h with shaking. This was followed by three washing steps as above. The plate was then incubated with an assay dependent antibody kit for 1h with shaking prior to a final three times washing step and incubation with read buffer for 10min at room temperature. The plate was then measured immediately using the MesoQUICKPLEX SQ 120 (MSD Technologies). Data were retrieved using the MSD workbench 4.0 software, before further analysis in Microsoft office and Prism. Assay is referred to as Mesoscale immunoassay throughout the text.

2.12.2 Sandwich ELISA
Amylin sandwich ELISA was purchased from Millipore (EZH-52K) and performed as per the manufacturer’s instructions. Briefly, solid black plates pre-coated with a monoclonal antibody raised against the N-terminal region of amylin were washed in supplied wash buffer. Supplied assay buffer was added to each well and a standard curve was generated using dilutions of a supplied standard. The standard curve and assay accuracy was tested against two supplied quality controls. 25μg of sample diluted into 50μl water was incubated on the plate for 1h with shaking. The plate was washed 3 times with the supplied wash buffer before incubation in a detection conjugate for 2h with shaking. The plate was washed as above before addition of the substrate solution. The plate was incubated in the dark and after 20 min was measured and if the supplied quality control samples were at the expected concentration then stop solution was added for 15 min in the dark. The plate was then measured at excitation 355nm/emission 460nm and data extracted in relative fluorescence units. Concentration was then interpolated against a standard curve and this was used in further analyses.

2.13 PCR

2.13.1 RNA isolation and generation of cDNA
RNA isolation was performed using the Trizol method. No more than 100mg of tissue was homogenised in 1ml of Trizol using a disposable Pestle Pellet homogeniser tip (Sigma) on ice. Homogenates were then centrifuged at 12,000xg for 10 min at 4°C. The resultant fat layer was removed and supernatant pipetted to a new tube, the pellet was discarded.
Chloroform (0.2ml) was then added to the supernatants and the mixture was shaken by hand for 15s before standing at room temperature for 10mins. The samples were then centrifuged at 12,000xg for 10min at 4°C. This separated the sample into 3 layers, a bottom layer containing protein, an interface containing DNA and the top aqueous layer containing RNA. This top aqueous phase was carefully removed and transferred to a new Eppendorf tube. Isopropanol (0.5ml) was then added and the mixture was incubated at room temperature for 10 min before being centrifuged at 12,000xg for 15 min at 4°C. The supernatant was removed and discarded and the pellet was re-suspended in 70% ethanol / RNAase-free water by repeat pipetting. The samples were then vortexed, centrifuged again at 12,000xg for 5 min at 4°C. The resultant supernatant was discarded and the pellet was dried under the fume hood for 7 min 30s before being suspended in 30μl RNAase-free water. RNA purity was analysed by Nanodrop (Thermofisher). Only samples with 260/280 ratios above 1.7 were used in further experiments. Complementary DNA (cDNA) was generated using the random hexamers, dNTP mix, RNAase OUT and Superscript III (Thermofisher) as per the manufacturer’s instructions. Up to 5μg of RNA was used per reaction.

2.13.2 RT-PCR
cDNA (50ng) was added to 10pM forward and reverse primers (Table 2.6) and 2xSybr green master mix (Biorad) made up to a final volume of 20μl in milli-Q water in an 96-well reaction plate (Thermofisher; N8010560). The samples were briefly centrifuged to ensure uniformity across the plate and before loading. For each PCR cycle samples were held at 50°C before being raised to 95°C for 25 min. The temperature was then dropped to the 60°C annealing temperature for primer binding for 1s, raised to 95°C for 15s and then held at 60°C for primer annealing for 60s, the reaction was stopped by heating to 95°C for 15s. This protocol cycle was performed 40 times per experiment.

2.13.3 Resolving PCR product by agarose gel
A 2% agarose in TAE solution was heated in the microwave until dissolved and then cooled under a stream of water. Ethidium bromide was added to the solution at a final ratio of 1:10,000 and the gel was cast. The gel was submerged in 1xTAE and the samples and 100bp ladder was added. Samples (50ng) were removed from the RT-PCR reaction and briefly centrifuged to collect the reaction product. The 20μl product was then added to 4μl PCR-dye. The 100bp ladder and samples were then loaded onto the gel and resolved at 100V for 2h. Fluorescence was immediately detected using the G-Box. Endo-βH1 cells were used as a positive control for IAPP expression (Ravassard et al. 2011).
Table 2.6 Table of primer designs used in RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ → 3’)</th>
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</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FWD: CCTGTTCGACAGTCAGCGC</td>
</tr>
<tr>
<td></td>
<td>REV: CGACCAATCCGTTGACTCC</td>
</tr>
<tr>
<td>IAPP</td>
<td>FWD: TGAAAGTCATCAGGTTGAAAAGC</td>
</tr>
<tr>
<td></td>
<td>REV: AGGCGCTGCGTTGCA</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>FWD: GACACGAGGAGGTCATCGT</td>
</tr>
<tr>
<td></td>
<td>REV: GCTGAGATCACCACCACCTTTAAA</td>
</tr>
<tr>
<td>PPIA</td>
<td>FWD: TGCTGGACCCAACAAATG</td>
</tr>
<tr>
<td></td>
<td>REV: TGCCATCCAACCACTCAGTCT</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>FWD: TGCACATTGTGAAGAGAAAAACA</td>
</tr>
<tr>
<td></td>
<td>REV: CTTGGCATTCTTGCAGCTCA</td>
</tr>
</tbody>
</table>

2.13.4 RT-PCR data analysis
The baseline and threshold were manually adjusted for each gene of interest. Data were interpolated against a standard curve generated on the same plate. Samples were then corrected to GAPDH. Data were assessed for normality and then an appropriate log transformation was performed. In experiments where a ratio was performed, data were interpolated and corrected to GAPDH as above, then a ratio was performed before an appropriate log transformation.

2.14 Mass Spectrometry

2.14.1 Ion-mobility mass spectrometry
Ion mobility mass spectrometry (IMMS) was performed using the Synapt-G2S and Synapt-G2Si ion mobility mass spectrometers (Waters UK, Elstree). Ions were produced by nano-electrospray ionisation in both positive and negative mode dependent on experimental design. Instrument parameters are outlined in Table 2.7 (for full machine conditions see Appendix 3). Peptide films were solubilised and diluted in 20mM ammonium acetate pH7.4 (pH was modified using hydrochloric acid). Rutin was suspended in the same buffer containing 0.3% NH₄OH and was added to the peptide immediately prior to ionisation. Source conditions were as consistent as possible between samples to ensure proper comparability. Source conditions were approximately equal to the following values: source voltage ~1.6kV, cone voltage 30V and nitrogen nebulizing gas pressure of 0.15psi and source temperature of 80°C. Mass spectra were analysed and extracted using MassLynx v4.1 software (Waters) and data relating to ion drift time through the gas cell was analysed.
and extracted using Driftscope v2.7 software (Waters). Figure 5.6 was generated in collaboration between Dr. Ashley Phillips and I, we both contributed equally to this work.

### Table 2.7 IMMS instrument parameters

<table>
<thead>
<tr>
<th>Instrument Parameter</th>
<th>Negative mode</th>
<th>Positive Mode</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G2S</td>
<td>G2Si</td>
<td>G2S</td>
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<tr>
<td>Capillary (kV)</td>
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<td>~1.6</td>
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</tr>
<tr>
<td>Cone (V)</td>
<td>30</td>
<td>15 - 30</td>
<td></td>
</tr>
<tr>
<td>Extractor / Offset (V)</td>
<td>60</td>
<td>45 - 80</td>
<td></td>
</tr>
<tr>
<td>Source Temp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM Resolution (V)</td>
<td>Tuned according to experiment</td>
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<td></td>
</tr>
<tr>
<td>HM Resolution (V)</td>
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<td>0</td>
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<tr>
<td>Pre-filter (V)</td>
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<td>2</td>
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<td>1</td>
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<tr>
<td>Trap Collision Energy (V)</td>
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<td>0 - 6</td>
<td></td>
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<tr>
<td>Transfer Collision Energy (V)</td>
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</tr>
<tr>
<td>Source gas flow ml/min</td>
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<td>0</td>
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<tr>
<td>Trap Gas flow (ml/min)</td>
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</tr>
<tr>
<td>Trap wave Velocity (m/s)</td>
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</tr>
<tr>
<td>Trap wave Height (V)</td>
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<tr>
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<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Step Wave 1 Out Velocity (m/s)</td>
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<td>300</td>
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<tr>
<td>Step Wave 2 Height (V)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
2.14.2 Tandem mass spectrometry

2.14.2.1 Sample preparation

Human samples were homogenised and fractionated as in 2.3.2. Formic acid fractions from human samples were dried down using the SPD131DDA vacuum concentrator (Savant) (Ramp 1) at 40°C until dry to remove the formic acid. The protein films were then resuspended in 100mM triethylammonium bicarbonate (TEAB) buffer to a final volume of 100μl. Samples were then sonicated (Microson SL200) for 30s on ice at 22.5Hz using probe setting intensity ‘3’ until in the film was in suspension prior to vortexing. The protein concentration of the samples was assayed using the BCA method and 100μg of protein was aliquoted to be used for reduction, alkylation and trypsinisation. Samples were reduced by incubation with 5mM DTT for 45min at 65°C with shaking. Samples were then alkylated with 15mM iodoacetamine for 20min at room temperature. Samples were incubated with 5μg porcine trypsin (Promega) at 37°C overnight with shaking. The reaction was stopped by putting the samples on ice before drying in a centrifugal concentrator (Eppendorf). Samples were stored at -20°C until required.

2.14.2.2 High performance liquid chromatography

Dried trypsinised samples were suspended in 95% H₂O / 5% Acetonitrile + 0.1% NH₄OH before being placed in the sample tray for injection. Injected samples were subjected to the HPLC gradient laid out in Table 2.8. A Zorbax 300-extend-C18 3.5 micron diameter column (Agilent) and Agilent 1200 series HPLC with UV detector and fraction collector were used in all experiments. Fractions were collected into a 2ml deep 96well plate. Collected fractions were then dried in a centrifugal concentrator (Eppendorf) without heating overnight or 6h before storage at -20°C. Variable wavelength detection was at 214nm to detect peptide bonds.

Table 2.8 HPLC gradient.

<table>
<thead>
<tr>
<th>Time</th>
<th>A%</th>
<th>B%</th>
<th>Flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>00:00</td>
<td>97</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>05:00</td>
<td>97</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>30:00</td>
<td>73</td>
<td>27</td>
<td>0.75</td>
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<td>35:00</td>
<td>50</td>
<td>50</td>
<td>0.75</td>
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<td>100</td>
<td>0.75</td>
</tr>
<tr>
<td>42:00</td>
<td>97</td>
<td>3</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Solvent A = H₂O + 0.1% NH₄OH. Solvent B = Acetonitrile + 0.1% NH₄OH
2.14.2.3 LC-MS/MS protocol

Samples and dried fractions collected from the HPLC were resuspended in 95% H₂O / 5% Acetonitrile containing 0.1% formic acid at a final concentration of 2μg/μl. These samples were then subject to liquid-chromatography tandem mass spectrometry (LC-MS/MS). Sample (5μl) was injected into the 1260 Infinity model liquid chromatography system (Agilent) and separated over a C18 3μm diameter column at 25°C using the gradient specified in Table 2.9. Ions were created by positive mode electrospray ionisation under source conditions outlined in Table 2.10 in a G6460 Triple-Quad mass spectrometer (Agilent). Source conditions and experiment parameters were identical between all reported runs.

Table 2.9 LC-MS/MS Quatpump gradient.

<table>
<thead>
<tr>
<th>Time</th>
<th>A%</th>
<th>B%</th>
<th>Flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>90</td>
<td>10</td>
<td>0.15</td>
</tr>
<tr>
<td>5.00</td>
<td>90</td>
<td>10</td>
<td>0.15</td>
</tr>
<tr>
<td>35.00</td>
<td>15</td>
<td>85</td>
<td>0.15</td>
</tr>
<tr>
<td>40.00</td>
<td>15</td>
<td>85</td>
<td>0.15</td>
</tr>
<tr>
<td>41.00</td>
<td>90</td>
<td>10</td>
<td>0.15</td>
</tr>
<tr>
<td>47.00</td>
<td>90</td>
<td>10</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Solvent A = H₂O + 0.1% FA. Solvent B = Acetonitrile + 0.1% formic acid.

Table 2.10 Triple-Quad source conditions.

<table>
<thead>
<tr>
<th>Triple-Quad source conditions</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Gas Temp (°C)</td>
<td>300</td>
</tr>
<tr>
<td>Gas Flow (l/min)</td>
<td>10</td>
</tr>
<tr>
<td>Nebulizer (psi)</td>
<td>45</td>
</tr>
<tr>
<td>Sheath Gas Temp (°C)</td>
<td>250</td>
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<tr>
<td>Sheath Gas Flow (l/min)</td>
<td>11</td>
</tr>
<tr>
<td>Capillary Voltage (kV)</td>
<td>4</td>
</tr>
<tr>
<td>Chamber Current (μA)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2.14.2.4 Selected reaction monitoring (SRM) method

SRM method generation was performed under the supervision of Dr. Isabel Riba-Garcia. The SRM method was generated using reduced and alkylated tryptic digests of synthetic human amylin and Aβ₁-₄₂ generated as described in 2.14.2.1. For both peptides the most abundant tryptic fragment was used as the precursor ion. The precursor ion was then isolated and fragmented and the resultant product ions were characterised. Detectable product ions resulting from fragmentation and predicted from the peptide sequence were
then used to make the SRM method outlined in **Table 2.11**. All product ions were subject to a dwell time of 200ms, fragmentor value of 135V and cell accelerator voltage of 7V. The collision energy used was optimised per product ion and is laid out in **Table 2.11**.

**Table 2.11 SRM methods for amylin and Aβ.**

<table>
<thead>
<tr>
<th>Product Ion (m/z)</th>
<th>CE</th>
<th>Residues</th>
<th>Ion</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylin T3 precursor ion (3⁺ = 909.5 m/z)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>909.5</td>
<td>12-37</td>
<td></td>
<td>(R)LANFLVHSSNFGAILSSTNVGSNTY (3⁺)</td>
<td></td>
</tr>
<tr>
<td>793.4</td>
<td>28</td>
<td></td>
<td>b’12</td>
<td>LANFLVHSSNFGAI (2⁺)</td>
</tr>
<tr>
<td>849.9</td>
<td>15</td>
<td></td>
<td>b’13</td>
<td>LANFLVHSSNFGAIL (2⁺)</td>
</tr>
<tr>
<td>903.7</td>
<td>8</td>
<td></td>
<td>Precur sor ion minus H2O</td>
<td>(R)LANFLVHSSNFGAILSSTNVGSNTY (3⁺) - H2O</td>
</tr>
<tr>
<td>1122.6</td>
<td>12</td>
<td></td>
<td>b’22</td>
<td>LANFLVHSSNFGAILSSTNVG (2⁺)</td>
</tr>
<tr>
<td>1264.6</td>
<td>22</td>
<td></td>
<td>b’25</td>
<td>LANFLVHSSNFGAILSSTNVGSNT (2⁺)</td>
</tr>
<tr>
<td>Aβ T3 precursor ion (2⁺ = 663.2 m/z)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>663.2</td>
<td>17 - 28</td>
<td></td>
<td>(K) LVFFAEDVGSNK (G) (2⁺)</td>
<td></td>
</tr>
<tr>
<td>405.2</td>
<td>10</td>
<td></td>
<td>y'4</td>
<td>GSNK</td>
</tr>
<tr>
<td>557.0</td>
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<td></td>
<td>y'10</td>
<td>FFAEDVGSNK (2⁺)</td>
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<td>y'7</td>
<td>EDVGSNK</td>
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<td>22</td>
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<td>AEDVGSNK</td>
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<td>966.5</td>
<td>20</td>
<td></td>
<td>y'9</td>
<td>FAEDVGSNK</td>
</tr>
<tr>
<td>1113.5</td>
<td>20</td>
<td></td>
<td>y'10</td>
<td>FFAEDVGSNK</td>
</tr>
</tbody>
</table>

CE = Collision energy

### 2.14.2.5 Data Analysis

Data acquisition and method generation was performed using the Data acquisition plug in of MassHunter software package (Agilent). Analysis of retention time, chromatograms and mass spectra was performed using Quantitative analysis plugin of the MassHunter software package vB.05 (Agilent). Analysis of the 214nm wavelength detection from the HPLC derived UV spectra was extracted using the ChemStation software package (Agilent).
2.15 Statistical Analysis

Data were analysed by one-way analysis of variance (ANOVA), two-way ANOVA or a two tailed student’s t-test for pairwise comparisons. In data subsets where experimental repeats were normalised to an arbitrary control value (such as 100) a one-sample t test was performed to compare the control value and column means, a further one way ANOVA with ad-hoc analysis would then be performed to compare column means with the arbitrary control value excluded. For multiple comparisons where a normal distribution was rejected a Kruskal-Wallis test was used. In several experiments where multiple comparisons were made an appropriate ad hoc statistical analysis was applied (specified in figure legends). All data are presented as the mean ± standard error of the mean unless stated otherwise and all statistical tests were performed in Prism (Version 7.0, Graphpad).

2.16 Nomenclature

Technical replicate - Multiple loadings of a single sample in a quantitative assay to demonstrate consistent loading and accurate measurement. Data from technical replicates were presented as a mean, then used for further analysis.

Replicate - Repeat(s) of an experimental condition within one biological n number. Experimental models considered to be parametric were generally performed with two replicates per condition per experiment. Experimental models considered non-parametric were performed with upwards of three replicates per condition per experiment. Data from replicates are presented as the mean and then used for further analysis.

n number - Biological replicate. For parametric models a biological replicate must be performed on a different day with different aliquoted treatments from any previous biological replicate, for cell culture this must also come from a separate cell passage to previous replicates. For non-parametric models a biological replicate may be performed on the same day with the same materials, for instance two animals may have been treated on the same day.
Chapter 3: Investigating the role of amylin in the brain in AD and T2D

3.1 Introduction

3.1.1 Similarities between AD and T2D
Etiological studies consistently link diabetes mellitus with an increased risk of Alzheimer’s disease (AD) and other dementias (Leibson et al. 1997a; Ott et al. 1999; Xu et al. 2009). Etiological studies linking diabetic status with AD often do not distinguish between type I diabetes mellitus (insulin dependent diabetes mellitus) and type II diabetes mellitus (T2D, non-insulin dependent diabetes mellitus). T2D represents the overwhelming majority of incidences in older adults (Whiting et al. 2011) and it is widely inferred as the major contributing factor causing the increased incidence of AD. T2D status (Leibson et al. 1997b; Ott et al. 1999), prediabetes, impaired glucose tolerance (Ohara et al. 2011), hyperinsulinemia (Luchsinger et al. 2004) are all associated with increased AD risk. Interestingly, T2D has an independent cognitive phenotype, termed diabetes associated cognitive deficit (Arvanitakis et al. 2004; Luchsinger et al. 2007; Roberts et al. 2014a; Roberts et al. 2014b). This deficit is characterised by white matter hyper-intensities and vascular damage (Last et al. 2007; Roberts et al. 2014b). As there is an established link between such vascular damage and AD risk (Breteler 2000; de Groot et al. 2000; Snowdon et al. 1997), the vascular damage associated with diabetes likely contributes to the etiological link between AD and T2D.

As well as being etiologically linked, AD and T2D are pathologically similar in that they are both amyloidosis diseases. AD is characterised by plaques of Aβ and intra-neuronal tangles of tau associated with neurodegeneration (Braak and Braak 1991). Likewise, a major feature of T2D is amylin deposition in the pancreatic islets of Langerhans, which leads to β-cell loss (Ludwig and Heitner 1967). Over 95% of T2D cases present with amylin aggregate pathology in the pancreas (Westermark 1972; Betsholtz et al. 1989b; Johnson et al. 1989; Jurgens et al. 2011). Amyloid pathology was discovered in the pancreas of type II diabetics (Opie 1901a) 6 years prior to the discovery of Aβ plaque deposition in the brain of AD patients in 1907 (Alzheimer 1907; Stelzmann et al. 1995), but has only recently come to the forefront of the debate of T2D pathogenesis (Mukherjee et al. 2015).
3.1.2 Complications associated with T2D correlate with amylin deposition in peripheral organs

In its endocrine role amylin infiltrates numerous tissues to promote satiety, decrease glucose uptake and influence renal function (Cooper et al. 1995; Wookey et al. 2003). Amylin infiltrates the renal cortex, binds G-coupled receptors and regulates water reabsorption (Wookey et al. 1996; Cooper and Wookey 1997). Up-regulation of amylin causes hypertension through amylin activation of kidney AMY receptors in rat and primate models (Haynes et al. 1997; Wookey et al. 1998). Further investigation of this diabetes associated hypertension revealed that fibrillar deposits of amylin are present in the kidneys of baboons with spontaneous diabetes (Hubbard et al. 2002). Hubbard et al. (2002) also report fibrillar amylin deposition in the blood vessels of the kidney and several other peripheral organs, including the heart. Amylin deposition in the islets of Langerhans is widespread in T2D and correlates with β-cell dysfunction and islet atrophy (Jurgens et al. 2011), a similar association has been observed in the kidney. Amylin deposition also occurs in the kidney renal cortex parenchyma and blood vessels in humans with diabetic nephropathy (Gong et al. 2007); the authors also demonstrate this deposition was highly correlated with hypertension. Gong et al. (2007) observed positive amylin deposition in 72/149 patients with biopsy confirmed diabetic nephropathy, while only 4/95 control patients were positive for amylin staining. This amylin deposition was characterised by immunohistochemistry and immunogold electron microscopy.

The influence of amylin deposition on complications associated with T2D was further explored by a separate group, who investigated amylin deposition in the heart and its relationship with diabetic heart failure (Despa et al. 2012). They compared tissue from patients with diabetic heart failure (n=25) and tissue from non-failing hearts in obese patients (n=8) with failing and non-failing heart tissue from otherwise healthy lean controls (n=7 & n=5 respectively) (Despa et al. 2012). Despa et al. (2012) used immunohistochemistry to demonstrate the presence of amylin aggregates in the ventricles and atria of failing diabetic hearts, these deposits were not present in control patients regardless of heart failure status. The authors also report an enrichment of soluble amylin oligomeric species in diabetic heart failure compared to non-failing control hearts by western blot analysis. Despa et al. (2012) further demonstrated this deposition as being present in hIAPP+/- rats, but not present in rats overexpressing rat amylin (Despa et al. 2012). These data demonstrate amylin can be deposited in the peripheral organs and implicate amylin deposition in common diabetic complications such as diabetic nephropathy and hypertension.
3.1.3 Amylin deposition in the brain

As mentioned above, T2D has a distinct cognitive phenotype and a strong etiological connection to AD (Biessels and Kappelle 2005). Because of these factors, a group headed by Florin Despa (author of Despa et al. (2012)), investigated the possibility of amylin deposition in the temporal lobe of T2D patients with vascular dementia, AD-nondiabetic patients and healthy age matched controls (Jackson et al. 2013). The temporal lobe was selected in this study as degeneration of the temporal lobe, particularly the hippocampus, is considered the major pathological hallmark of AD associated memory loss (Schuff et al. 2009). Jackson et al. (2013) found evidence of amylin deposition in the blood vessels and brain parenchyma of T2D cases but not controls. Of great interest, the authors also provide evidence of amylin deposition in the blood vessels and brain parenchyma of AD patients with no apparent comorbid T2D (Jackson et al. 2013). In addition to this pattern of staining, Jackson et al. (2013) also reported intra-neuronal amylin staining in T2D and AD cases that was absent in controls. Amylin deposition in the brain and its relationship to AD has also been explored in animal models. A hIAPP+/− rat model presents with amylin deposition in the brain associated with neuroinflammation and cognitive deficits (Jackson et al. 2013; Srodulski et al. 2014). A separate group analysed the propensity of endogenous rodent amylin to complex with Aβ in the Tg2576 AD model mouse (Fawver et al. 2014), this study demonstrated that basal levels of rodent amylin interacted with certain Aβ species to form aggregates and co-deposits (Fawver et al. 2014). Furthermore, hIAPP+/− rats present vascular and parenchymal deposition of amylin in the brain with associated impairments in behavioural tests (Srodulski et al. 2014). This model also noted amylin induced neuroinflammatory response, with increased brain IL-1β. This is interesting as a pathway responsible for IL-1β production, the NLRP3 inflammasome, is elevated in AD (Heneka et al. 2013) and has been demonstrated to be activated by amylin (Masters et al. 2010; Westwell-Roper et al. 2013). This potentially provides a role for amylin deposition in contributing to the neuroinflammation seen in AD.

Interestingly, Jackson et al. (2013) observed amylin-Aβ co-deposition in human brain parenchyma and blood vessels. Plaques immunoreactive for both amylin and Aβ were identified by co-staining immunohistochemistry and evidence of amylin-Aβ co-deposition in the blood vessels was demonstrated using serial staining immunohistochemistry for Aβ and amylin, consistent with pancreatic amylin deposition (Gellerstedt 1938; Westermark 1975). Supplementary material from Jackson et al. (2013) demonstrated that co-deposition did not always occur, and that amylin positive vessels were Congo red staining reactive. Intra-peritoneal injection of preformed amylin fibrils and amylin monomer causes amylin deposition in the brain vasculature, which was Congo red positive, as well as amylin-Aβ co-deposits in the brain parenchyma (Oskarsson et al. 2015a). Interestingly, this co-deposition appears to work both ways, as injection of Aβ seeds to a hIAPP+/− rat model increased
amylin deposition in the pancreas and caused instances of amylin-Aβ co-deposits (Oskarsson et al. 2015a). This cross seeding behaviour is highly interesting, and suggests the presence of key conformational species that can force a templating structure on both amyloids in a prion like manner. A recent study demonstrated templating amylin species may exist, showing that inoculation of homogenised hIAPP+/− pancreas or human pancreas caused hyperglycaemia, impaired glucose tolerance and formation of islet amyloid - but this did not occur if the homogenised pancreas preparations were IAPP depleted before inoculation (Mukherjee et al. 2017).

A group led by Claudio Soto has recently developed a genetic model of the amylin-AD connection by breeding mice expressing human APP with mice either heterozygous or homozygous for human amylin (IAPP) (Moreno-Gonzalez et al. 2017). APP-hIAPP+/− mice had significantly more Aβ deposition than APP overexpressing mice or their APP-hIAPP+/− counterparts. There was also evidence of parenchymal co-deposition of amylin-Aβ plaques determined by double-immunostaining immunofluorescence microscopy. Interestingly these cross bred mice had increased soluble and insoluble amounts of Aβ and a significantly worse cognitive phenotype (Moreno-Gonzalez et al. 2017). The amylin-Aβ co-deposits reported by Jackson et al. (2013) in human brain, builds on previous in vitro observations of seeding behaviour and interactions between the two amyloids (Seeliger et al. 2012; Andreetto et al. 2010). Amylin deposition and co-localisation with Aβ in the parenchyma, as well as evidence of co-deposition in blood vessels have been verified in human brain by a separate group using a proximity ligation assay (Oskarsson et al. 2015a).

These data provide evidence of amylin infiltration from the periphery to become deposited in the vasculature and parenchyma of the brain. Interestingly, spontaneous T2D studies in animals demonstrated peripheral deposition of amylin, whilst transgenic animal studies and immunohistochemistry in humans suggest that hetero-complexes, co-deposition and seeding behaviour between amyloids can occur. This raises the possibility that up-regulation of amylin in T2D promotes Aβ burden and increases AD risk.

3.1.4 Chapter Aims

The aim of this chapter was to validate observations of amylin deposition in the brain and that this deposition is increased in AD. We also hypothesised that amylin deposition will synergistically increase with co-morbid T2D. We aimed to use multiple methods to quantify any detectable soluble or insoluble amylin species following brain fractionation. We also investigated the effect of amylin treatment of a neuronal cell model, where we hypothesised amylin would upregulate Aβ in the media by competition for Aβ degrading enzymes.
3.2 Results

3.2.1 Amylin is deposited in the brain

We employed immunohistochemistry to replicate the data demonstrated by Jackson et al. (2013) and investigate the relationship between Aβ deposition and amylin deposition in human brain. Based on the evidence presented in Jackson et al. (2013), we hypothesised that AD patients would have more amylin deposition than controls and this would be increased by co-morbid T2D. Therefore, we compared brain tissue from non-diabetic controls, cognitively normal patients with T2D, AD patients and comorbid AD and T2D patient groups. We also hypothesised that due to the infiltration of amylin from the periphery and the observation of co-deposits, areas with high cerebral amyloid angiopathy (CAA) would have increased amylin deposition compared to areas associated with diffuse plaque deposition. We used two polyclonal amylin antibodies for our immunohistochemistry studies: the T-4150 amylin antibody which uses synthetic amylin1-37 peptide as an antigen (Peninsula Labs) and was used in Jackson et al. (2013); and the H-017-03 antibody (Phoenix Pharmaceuticals) which is derived against the N-terminal region of amylin. Antibodies were verified and optimised in pancreas tissue of the hIAPP+/− mouse model, which has been previously characterised (Wong et al. 2008). These mice develop human amylin deposits in the pancreas, which are positive for the amyloid stain Congo red (Figure 3.1A). Both antibodies bound with high affinity to the pancreatic islets (Figure 3.1B), with some cross reactivity in the WT IAPP mice. This is unsurprising as rodent amylin and human amylin only differ in 6 amino acids (Figure 1.7). Both antibodies demonstrated stronger staining and lower background in the hIAPP+/− mouse pancreas.

We then performed immunohistochemistry on formalin fixed human occipital lobe and temporal lobe sections for Aβ using the 4G8 antibody (Figure 3.2). This was to confirm increased Aβ deposition in AD and create an unbiased quantification protocol that would then be used for amylin (described in 2.11.3). The whole section of occipital or temporal lobe was scanned at 200x magnification. Then, an example of positive staining was chosen when samples were blinded. This exemplar was then used as a universal threshold which was applied to all samples and then the whole tissue section was quantified. Data were then presented as the area of positive staining divided by the total area. This is not the only way to analyse these data and alternative methods of analysis are discussed in 3.3.1. As expected, immunohistochemistry staining of Aβ using the 4G8 antibody, demonstrated that our AD samples had a large increase in Aβ deposition compared to controls in the occipital lobe (Figure 3.2A & B) and temporal lobe (Figure 3.2C & D). We observed no independent effect of T2D on Aβ deposition.
Figure 3.1 Amylin antibody testing in hIAPP +/- mouse pancreas

(A) Wild type and human IAPP heterozygous (hIAPP +/-) mouse pancreas sections imaged by birefringence light microscopy of Congo red staining under polarised light. n=3 in each group

(B) Immunohistochemistry of wild type and hIAPP +/- mouse pancreas using the T-4150 and H-017-03 amylin antibodies. Antibodies positively stain the amylin containing islets, n=3 in each group. Arrows indicate islets of Langerhans. Scale bar = 500μm.
Figure 3.2 Immunohistochemistry of Aβ in human samples
(continued on next page)
Figure 3.2 Immunohistochemistry of Aβ in human samples (continued)

(A) Representative images of immunohistochemistry staining of Aβ in human post-mortem occipital lobe slices with the 4G8 antibody. Scale bar = 1mm, scale bar of inset images = 200μm. (B) Quantification of occipital lobe T-4150 staining. (C) Representative images of immunohistochemistry staining of Aβ in human post-mortem temporal lobe slices with the 4G8 antibody. Scale bar = 1mm. (D) Quantification of temporal lobe H-017-03 staining. Data represent mean ± SEM. Data were analysed by two-way ANOVA with Holm-Sidak post hoc correction for multiple comparisons.*, p < 0.05. n=3 in each group.
Figure 3.3 Immunohistochemistry of amylin in human occipital lobe
(continued on next page)

(A) Representative images of immunohistochemistry staining of amylin in human post-mortem occipital lobe slices with the T-4150 antibody. Scale bar = 1mm, scale bar of inset images = 200μm. (B) Quantification of occipital lobe T-4150 staining.
Figure 3.3 Immunochemistry of amylin in human occipital lobe (continued)
Continued. (C) Representative images of immunohistochemistry staining of amylin in human post-mortem occipital lobe slices with the H-017-03 amylin antibody. Arrow indicates plaque like amylin deposition not in inset image. Scale bar = 1mm, scale bar of inset images = 200μm. (D) Quantification of occipital lobe H-017-03 staining. Data represent mean ± SEM. Data were analysed by two-way ANOVA with Holm-Sidak post hoc correction for multiple comparisons.* p < 0.05 by two-way anova; #, p < 0.05 compared to control:Non-diabetic. n=3 in each group.
We then performed immunohistochemistry for amylin using the T-4150 antibody (Figure 3.3A & B) and H-017-03 antibody (Figure 3.3C & D) in occipital lobe sections. These stains were broadly similar, with one key difference - the T-4150 antibody seemed to be better at detecting amylin deposits in the vasculature wall. Vasculature deposition was originally observed by Jackson et al. (2013) and has been subsequently replicated by the same group (Jackson et al. 2013; Srodulski et al. 2014; Verma et al. 2016). An independent study of amylin in vasculature, using a different in-house antibody, supported the case for vasculature deposition (Schultz et al. 2017) but distinguished the pericyte deposits they observed from the vessel wall stains observed in Jackson et al. (2013) and the present study. The vascular deposition of amylin is studied in greater detail in 3.2.2. As a result of this difference in staining, quantification from immunohistochemistry with the T-4150 antibody was performed by taking a blindly selected exemplar positive control and creating a threshold which was then applied to all samples. T-4150 stained samples were then analysed blind with a manual count of amylin deposits and then data were expressed as counts over area (Figure 3.3B). This method demonstrated no significant increase in amylin deposition in AD or T2D-AD, as previously reported (Jackson et al. 2013). We did however observe a significant increase between T2D and control samples, and a significant independent effect of T2D diagnosis on amylin deposit count over the whole control and AD groups. We report large plaque like deposits of amylin in occipital lobe by both antibodies (Figure 3.3A & 3.3C). These deposits were almost exclusively in the grey matter - but, as the zoomed out figure panels demonstrate, there were very few such deposits. With a maximum of 4 large deposits, as depicted in the inset panels of Figure 3.3A & 3.3C, observed in any one patient sample. This is far fewer than the dozens observable in data reported by Jackson et al. (2013).

The staining of H-017-03 largely supports that of the T-4150 antibody. Quantification by this method showed a non-significant trend towards increased area of amylin deposition in T2D and AD patient samples, however, the magnitude of this increase is not the same as that observed with T-4150 (Figure 3.3B & D). As was the case for the T-4150 antibody, there was no additive effect of comorbid diabetes, with the T2D-AD samples having very similar levels of deposition to controls. Again, as with the T-4150 antibody, the H-017-03 antibody observed large plaque like inclusions of amylin in the grey matter (Figure 3.3C). The results of Figure 3.3 are not directly comparable with the data in Jackson et al. (2013) as they performed immunohistochemistry in the temporal lobe.
Figure 3.4 Immunohistochemistry of amylin in human temporal lobe
(continued on next page)

(A) Representative images of immunohistochemistry staining of amylin human post-mortem temporal lobe slices with the T-4150 antibody. Scale bar = 1mm, scale bar of inset images = 200μm. (B) Quantification of temporal lobe T-4150 staining.
Figure 3.4 Immunohistochemistry of amylin in human temporal lobe (continued)
Continued. (C) Representative images of immunohistochemistry staining of amylin in human post-mortem temporal lobe slices with the H-017-03 amylin antibody. Arrows indicate amylin plaque like deposits. Scale bar = 1mm, scale bar of inset images = 200μm. (D) Quantification of temporal lobe staining. Data represent mean ± SEM. Data were analysed by two-way ANOVA with Holm-Sidak post hoc correction for multiple comparisons. p < 0.05, n=3 in each group.
Our immunohistochemistry staining of the temporal lobe with T-4150 (Figure 3.4A) and H-017-03 (Figure 3.4C) also did not demonstrate significant increases in amylin deposition as observed by Jackson et al. (2013). Staining with T-4150 found roughly equal number of amylin deposits across all disease categories. This is likely due to vascular deposition of amylin in larger blood vessels, and a relative absence from smaller vessels. Although only the hippocampus is presented in all figures focusing on the temporal lobe (Figure 3.2C & Figure 3.4), a large section of temporal cortex was also included in the quantification that is not presented in the images. Quantification of H-017-03 antibody staining was similar to observations from the occipital lobe; trends towards an increase in amylin deposition were seen in the T2D samples and AD samples, with AD samples producing the greatest increase (Figure 3.4D). As in the occipital lobe, there was no additive effect of comorbid diabetes, with T2D-AD samples recording similar amylin deposition to controls by this method. Quantification from the temporal lobe is directly comparable to previous reports (Jackson et al. 2013). Jackson et al. (2013) performed quantification by manual counting of amylin plaques from parenchyma from a set of subjectively captured images. As a result, the quantification in Jackson et al. (2013) predominantly includes vascular amylin deposits, and has a large potential for bias. Our results using the T-4150 antibody similarly predominantly count vascular amylin deposits, but with far less potential bias (Figure 3.4A & B). This found essentially no difference between the disease groups. Our quantification of amylin by the H-017-03 method includes vascular, plaque-like and intraneuronal amylin staining. This method found no significant differences between disease groups in this small study but showed a clear trend to increase in the T2D and AD samples. Together our immunohistochemistry data clearly show examples of amylin deposition in the occipital and temporal lobes, with significant increases in T2D and non-significant trends to increase in AD patients in certain areas. Our data do not support our hypothesis that co-morbid T2D would act synergistically with AD to increase amylin deposition. The differences in quantification between the T-4150 and H-017-03 antibodies highlight the potential impact of amylin epitope, or method of quantification, in generated results following measuring amylin deposition. This is further discussed in 3.3.1.
3.2.2  Amylin deposits co-localise with Aβ CAA and form heterocomplexes

Our immunohistochemistry, supported by several publications of amylin deposition, provide strong evidence for amylin deposits in the vasculature (Jackson et al. 2013; Srodulski et al. 2014; Oskarsson et al. 2015a; Schultz et al. 2017; Ly et al. 2017). This chapter section aims to better characterise these deposits and their consequences. Several of the above reports demonstrated amylin-Aβ co-localisation in vasculature amyloid deposits (Jackson et al. 2013; Oskarsson et al. 2015a; Ly et al. 2017). We attempted to investigate these claims by using serially sliced brain sections and comparing vessels positive for Aβ staining with the same vessel in the next serially sliced section stained for amylin with the T-4150 antibody (Figure 3.5).

![Figure 3.5 Serial staining of Aβ and amylin shows co-deposition in vasculature](image)

**Figure 3.5 Serial staining of Aβ and amylin shows co-deposition in vasculature**

Representative light microscopy of serial sections from human control, T2D, AD and T2D-AD occipital lobe sections histologically stained for Aβ (4G8) and amylin (T-4150). Arrows indicate vessels positive for amyloid deposition. All images were captured at 12x magnification.
Figure 3.5 presents exemplar cases of vessels followed through the occipital lobe serial sections. Many vessels that stained positive for Aβ CAA were also positive for amylin inclusions as detected by T-4150, denoted by arrows of vessels positive for both amylin and Aβ (Figure 3.5). However, this was not always the case. Notably, small blood vessels in white matter deeper in the brain were less prone to amylin deposition, regardless of Aβ deposition, than the larger cerebrovascular vessels and particularly the leptomeningeal arteries. Our data suggest there is extensive amylin deposition in blood vessels and the perivascular space, often co-localising with Aβ in these larger vessels. However, we did not observe much amylin deposition in the pericapillary space or small blood vessel cell walls, even in AD cases with extensive capillary CAA. This implies that amylin and Aβ can form aggregates in the same vessel, this is likely due to increased local concentration at drainage pathways in the larger vessels that lead to perivascular accumulation, as has been previously suggested for Aβ (Weller et al. 1998; Preston et al. 2003).

Our study utilised antigen retrieval to partially unfold the highly disordered amyloids and generate the best possible staining. For both the 4G8 and T-4150 staining, the sections were briefly boiled in citric acid, which can often destroy the pericytes and neuro-vascular unit connections between blood vessels and the parenchyma (Vinod et al. 2016). This left our immunohistochemistry unable to investigate reports of amylin deposition in pericytes (Schultz et al. 2017). As a result, we investigated pericyte viability by quantitative real time-PCR (Figure 3.6). To do this we quantified the pericyte marker PDGFRβ and performed a ratio to the endothelial marker VE-Cadherin to correct for the amount of vasculature in the tissue sample. Each marker was corrected to the housekeeping gene GAPDH and made relative to a preselected internal control, one of the control:non-diabetic samples, prior to the ratio calculation.

Analysis of VE-Cadherin (corrected to GAPDH) (Figure 3.6B) and PDGFRβ (corrected to GAPDH) (Figure 3.6C) demonstrated increased amounts of both measured in the AD groups. This is likely because equal weights of tissue were homogenised, with the severe brain shrinkage in AD due to neuronal loss meaning that harvesting the same weight of tissue included more vasculature in weight harvested from the AD samples. The ratio analysis in occipital lobe grey matter resulted in a significant decrease in PDGFRβ relative to VE-Cadherin in AD; with no independent effect of T2D (Figure 3.6A). These data imply a loss of pericytes in AD.
Figure 3.6 Quantification of pericyte viability

Quantitative PCR analysis of PDGFRβ, VE-Cadherin and GAPDH mRNA in human occipital grey matter samples. (A) PDGFRβ :VE-Cadherin ratio is taken as a measure of pericyte viability. PDGFRβ, VE-Cadherin were corrected to GAPDH prior to ratio being performed. Normality was graphed and analysed and then a log(y+1) transformation was performed. Results demonstrate AD causes pericyte loss. Relative VE-Cadherin (B) and PDGFRβ (C) corrected to GAPDH demonstrate increase in both measured in AD samples, this is expected due to more vasculature per g of tissue in AD compared to control. Data represent the mean ± SEM, n=3 in all cases except for AD-T2D where n=2. Data were analysed by a two-way ANOVA with Holm-Sidak post hoc analysis. *, p<0.05.
Figure 3.7 Ion mobility mass spectrometry demonstrates that amylin and Aβ interact

Negative mode ion mobility mass spectrometry (IMMS) of 20μM Aβ\textsubscript{1-42} (A & C), 20μM amylin (E & G) or a mixture of the two peptides (B, D, F & H). Data represent the arrival time distributions (ATD) of the isolated peptide charge state. Co-incubation with amylin causes a loss of observable dimer species in the [Aβ-4H]\textsuperscript{+} charge state (A-B) as well as changes in the ATD of the [Aβ-3H]\textsuperscript{+} charge state (C-D). Co-incubation with Aβ causes loss of observable dimer, trimer and tetramer species in the [Amylin-4H]\textsuperscript{+} charge state (E-F) but causes no change in the [Amylin-3H]\textsuperscript{+} charge state. Conditions used in IMMS experiments are outlined in Table 2.7 and fully listed in Appendix 3.
We have provided evidence for amylin and Aβ aggregates being deposited in the same blood vessels (Figure 3.5) and this is supported by published data demonstrating co-localisation of amylin and Aβ in human vasculature and parenchyma (Jackson et al. 2013; Oskarsson et al. 2015a). This has been experimentally replicated in animal models (Oskarsson et al. 2015a; Moreno-Gonzalez et al. 2017). Co-deposition in vasculature and parenchyma is hypothesised to be due to direct interaction of the amyloids. This hypothesis suggests different amyloids can cross seed to promote aggregation of one another, and form heterocomplexes comprised of both amyloids. There is a growing body of in vitro (Seeliger et al. 2012; Young et al. 2015a) evidence to suggest such amyloid cross seeding can occur, but limited examples of such complexes in vivo (Oskarsson et al. 2015a). We hypothesised that the deposition of amylin and Aβ observed in Figure 3.5 is due in part to heterocomplexes of amylin-Aβ, and sought to demonstrate such complexes exist.

Amylin and Aβ were originally hypothesised to interact and promote the seeding of one another following the observation of increased amylin plaques in the pancreas of AD patients and increased diffuse plaque count in AD patients with diabetes (Janson et al. 2004). Subsequently the sequences of amylin and Aβ were compared to demonstrate they have high homology, and multiple simulation studies found areas where they could interact (Andreetto et al. 2010; Andreetto et al. 2011; Berhanu et al. 2013; Zhang et al. 2015b). Fibrils containing both amylin and Aβ were then characterised by Thioflavin-T (ThT) assay, transmission electron microscopy (Yan et al. 2007) and AFM (Seeliger et al. 2012). Whilst these experiments demonstrate amylin and Aβ can clearly interact they do not describe the nature of these early oligomeric heterocomplexes. We attempted to isolate any heterocomplexes by ion-mobility mass spectrometry using synthetic preparations of Aβ1-42 and amylin (Figure 3.7).

We failed to isolate a distinct hetero-complex; instead we focused on the effect of co-incubation of amylin and Aβ on small oligomer species and changes in drift time indicative of one amyloid influencing another (Figure 3.7). We performed negative mode ionisation as Aβ has a native charge of -3 (Bernstein et al. 2005). We isolated the Aβ3+ and Aβ4+ charge states at 1503.3m/z (Figure 3.7C) and 1127.2m/z (Figure 3.7A) respectively. Isolation of the Aβ3+ + amylin (Figure 3.7D) and Aβ4+ + amylin (Figure 3.7B) was also performed. An observable dimer species occurring prior to the two conformations of Aβ4- monomer is lost in the presence of amylin. Furthermore there is a reduction in the Aβ4+ isoform drift time, indicating compaction of both monomer conformations in the presence of amylin (Figures 3.7A & B). A similar compaction occurred to both conformations of the Aβ3- charge state (Figures 3.7C & D), and there is an additional highly compacted species present. Analysis of the charge state distribution (data not shown) provides no evidence this is a stabilised dimer species.
Isolation of the amylin$^+$ and amylin$^+$ charge states were also performed in the presence and absence of Aβ at 974.1m/z ratio and 1299.9m/z ratio, respectively. Single peaks for amylin monomer, dimer, trimer and tetramer were observable in the arrival time distribution of the amylin$^+$ charge state (Figure 3.7E). These were absent upon co-incubation with Aβ (Figure 3.7F), where a slightly longer drift time observable in the amylin$^+$ monomer species. There is no major differences observable in the amylin$^+$ charge state other than compaction of the monomeric peptide (Figure 3.7G & H). Together these data demonstrate that amylin and Aβ influence one another to promote monomer compaction, but does not provide evidence for cross seeding or promotion of aggregation. A comparable study using Aβ$_{1-40}$ in positive mode ionisation managed to isolate amylin-Aβ heterocomplex species (Young et al. 2015a), with a 1:1 incubation of amylin:Aβ$_{1-40}$ causing the formation of heterocomplex dimer and trimer species.

![Figure 3.8](image)

**Figure 3.8** Amylin and Aβ$_{1-42}$ form heterocomplexes

12% Acrylamide SDS-PAGE of Aβ$_{1-42}$, amylin and Aβ$_{1-42}$ + amylin preparations cross-linked by glutaraldehyde. Samples were split into two and electrophoresed. The membranes were then blotted for Aβ with the 6E10 antibody (A) or amylin with the H-017-03 antibody (B). n=1.
We did, however, observe heterocomplex formation between synthetic Aβ1-42 and amylin by glutaraldehyde cross linking (Figure 3.8). Following crosslinking and quenching glutaraldehyde preparations were split in two and resolved by reducing SDS-PAGE. One half of the preparation was then incubated with 6E10 and the other with H-017-03. Co-incubation with amylin causes a differential aggregation pattern detectable by 6E10 (Figure 3.8A). Analysis of the same preparation by the H-017-03 antibody revealed many oligomeric species detectable with the amylin antibody (Figure 3.8B). Several, but not all of these species are at the same molecular weight as those visible by 6E10, indicating this is a mixture of amylin oligomers and amylin-Aβ heterocomplexes. The amylin monomer is not visible by H-017-03 (Figure 3.8B); this is not uncommon for amylin antibody detection of synthetic peptides. In our experience, H-017-03 and T-4150 antibodies are poor at detecting synthetic amylin oligomeric and monomeric preparations by reducing SDS-PAGE or by dot blot where the peptide is in a native conformation (data not shown). This experiment provides evidence for amylin-Aβ heterocomplexes as well as evidence that co-incubation of the peptides can promote oligomerisation.

3.2.3 Quantification of amylin in human brain fractions by multiple methods

Our immunohistochemistry data clearly demonstrate the presence of aggregated amylin deposits in human brains, with some evidence to suggest elevation in AD and T2D (Figures 3.2 - 3.5). We attempted to quantify the amount of monomeric and aggregated amylin in human brain tissue by fractionation then quantification by ELISA and validation by mass spectrometry. Our fractionation protocol was adapted from previous published reports (Rostagno and Ghiso 2009; Jullig et al. 2011) and is outlined in Figure 2.2. This method isolates monomeric and small oligomeric amyloid species, which are soluble in water and SDS, in the triethylammonium bicarbonate (TEAB) buffer supernatant after ultracentrifugation. This centrifugation step pellets insoluble large amyloid aggregates and fibrils, which can then be solubilised in 70% formic acid (FA). These are termed the soluble fraction (monomers and small oligomer species) and insoluble fraction (large insoluble aggregates).
Figure 3.9 Soluble and Insoluble Aβ levels from fractionated human brain tissue (continued on next page)
Figure 3.9 Soluble and Insoluble Aβ levels from fractionated human brain tissue (continued)
Quantification of Aβ_{1-40} (A) and Aβ_{1-42} (B) in soluble TEAB fractions and insoluble FA fractions derived from human occipital lobe grey matter (OG), occipital lobe white matter (OW) and Temporal lobe white matter (TW) by Mesoscale multiplex immunoassay. n=3 in all cases except Insoluble-TW-AD-Non-diabetic where n=2. In several instances the insoluble fraction AD samples were above the upper limit of detection (ULOD) for the assay, in these instances the data point was set to the ULOD value interpolated from the standard curve and the data were not subject to comparative analysis. The effect of AD status and T2D status on Aβ levels were analysed by two-way ANOVA with Holm-Sidak post hoc analysis. *, p<0.05; **, p<0.01.
Figure 3.10 Amylin in human brain fractions

Quantification of amylin in TEAB and FA fraction in the occipital lobe grey matter (OG), occipital lobe white matter (OW) and temporal lobe white matter (TW) by amylin ELISA. Data below the lower limit of detection (LLOD) interpolated from the standard curve were entered as 0. n=3 in all cases except Insoluble-TW-AD-Non-diabetic where n=2. The effect of AD status and T2D status on amylin concentration were analysed by two-way ANOVA with Holm-Sidak post hoc analysis. *, p<0.05.
Our fractionation protocol was validated by quantification of Aβ from human brain tissue fractions by Mesoscale multiplex immunoassay (Figure 3.9A & B). This demonstrated an enrichment of Aβ in the insoluble fraction, particularly in AD cases. In fact, there was such enrichment that after two attempts of quantification following dilution, the AD cases were still often above the upper limit of detection (ULOD) for the assay. In such cases values were entered as the highest value of the standard curve. As a result of this, no statistical comparisons could be performed on the data from the insoluble fraction - but as expected there is a clear increase in AD for all the brain regions measured. Our original hypothesis supposed that there would be increased amylin deposition in areas more prone to CAA, therefore we chose to use occipital lobe grey and white matter, which are prone to CAA to detect amylin deposition. We also chose to use temporal lobe white matter, which is relatively spared from CAA as a negative control.

Amylin was quantified in the soluble and insoluble fractions by ELISA (Figure 3.10). The ELISA utilises a monoclonal antibody raised against the C-terminal disulphide bridge of amylin. Quantification of amylin in the soluble fraction revealed a general trend across all brain regions for decreased soluble amylin monomeric species in T2D (Figure 3.10). This is consistent with hypoamylinaemia that occurs after pancreatic islet loss in T2D (Butler et al. 2003a; Butler et al. 2003b; Butler et al. 2004). There was also a trend towards increased soluble amylin in AD in the occipital lobe grey matter (OG), occipital lobe white matter (OW) and temporal lobe white matter (TW) soluble fractions. This is an interesting observation, potentially explained by co-morbid hyperglycaemia often seen with AD (Barbagallo and Dominguez 2014). That said, however, many values detected in the soluble fraction were near the lower limit of detection (LLOD) for the assay, limiting the reliability of the quantification. Quantification of amylin in the insoluble fraction generated much higher concentrations of amylin, particularly in the OG and TW. This indicates enrichment of aggregated amylin species consistent with the immunohistochemistry results (Figure 3.3). In areas measured there appeared to be a non-significant increase of insoluble amylin in AD with no discernible effect of T2D. This supports the original hypothesis of Jackson et al. (2013) suggesting there is increased amylin deposition in AD. However, we provide no evidence to suggest T2D has an independent or synergistic role on increased amylin deposition in AD.

We then sought to validate our immunohistochemistry and ELISA results with mass spectrometry. This is an important validation as antibodies against amyloids and intrinsically disordered peptides are susceptible to non-specific reactivity. While our evidence showed no cross-reactivity between amylin and Aβ antibodies (Figure 3.8), the structures of amyloids are sufficiently similar that molecular probes for Aβ aggregates have been shown to bind amylin aggregates in the pancreas (Yoshimura et al. 2014).
Figure 3.11 Selective reaction monitoring method for Aβ
(A) Sequence coverage of the tryptic fragment used as the precursor ion and map of b’ and y’ ions incorporated in the method. (B) MS/MS spectra of fragmented Aβ standard ionised in positive mode demonstrating abundances of detected y’ ions. (C) Retention time of Aβ standard total ion current (TIC) and chromatograms of monitored transitions (inset). Refer to Table 2.11 for method details.
Figure 3.12 Selective reaction monitoring method for amylin

(A) Sequence coverage of the tryptic fragment used as the precursor ion and map of b’ and y’ ions incorporated in the method. (B) MS/MS spectra of fragmented amylin standard ionised in positive mode demonstrating abundances of detected b’ ions. (C) Retention time of amylin standard total ion current (TIC) and chromatograms of monitored transitions (inset). Refer to Table 2.11 for method details.
Figure 3.13 Separation of amylin and Aβ standards by HPLC

(A) 214nm wavelength UV spectra from co-addition of synthetic amylin and Aβ standards subjected to high pH liquid chromatography gradient (see Table 2.8). Collect fractions were analysed by QQQ-MS/MS to determine where amylin and Aβ eluted. The Amylin (B) tryptic fragment used as the precursor ion eluted in the flow through prior to application of the solvent gradient, and was then retained in the column at low levels throughout the remaining fractions. The Aβ (C) tryptic fragment used as the precursor ion eluted at ~25min.
Figure 3.14 Retention time and chromatogram of Aβ in human brain fractions
(A) Representative retention time and chromatograms and inset MS/MS spectra of the fragmented Aβ precursor ion from the soluble TEAB fraction from occipital lobe grey matter for control and T2D-AD human tissue samples. (B) Representative retention time and chromatograms and inset MS/MS spectra of the fragmented Aβ precursor ion from the insoluble formic acid fraction from occipital lobe grey matter for control and T2D-AD human tissue samples. Chromatograms and MS/MS spectra were scaled to the FA soluble T2D-AD sample presented.
Figure 3.15 Retention time and chromatogram of amylin in human brain fractions

(A) Representative retention time and chromatograms and MS/MS spectra of the fragmented amylin precursor ion from the soluble TEAB fraction from occipital lobe grey matter for control and T2D-AD human tissue samples. (B) Representative retention time and chromatograms and inset MS/MS spectra of the fragmented amylin precursor ion from the insoluble formic acid fraction from occipital lobe grey matter for control and T2D-AD human tissue samples. Chromatograms and MS/MS spectra were scaled to the FA solubule T2D-AD sample presented in Figure 3.14.
We utilised synthetic peptides to create a selective reaction monitoring (SRM) method for liquid chromatography linked tandem mass spectrometry (LC-MS/MS) to evaluate relative amounts of Aβ (Figure 3.11) and amylin (Figure 3.12). These methods isolated the most abundant tryptic fragment of the peptide, then incrementally increased collision energy in the collision cell to break apart this fragment into constituent b’ and y’ ions. The Aβ and amylin methods both contain at least 4 b’ or y’ ions and 5 transitions (Figure 3.11 & Figure 3.12). Although they are separated into two figures, both the Aβ and amylin transitions are part of one larger selective reaction monitoring (SRM) method designed to measure both peptides from one sample run. We then used this method to attempt to optimise a high pH high performance liquid chromatography (HPLC) method to concentrate amylin and Aβ in our samples and remove other sample constituents (Figure 3.13).

The entire flow through of the HPLC gradient application (Table 2.8) was collected into 5min fractions. Peptide bonds were detected at 214nm (Figure 3.13A). Analysis using the SRM method demonstrated amylin was eluting before the application of the organic solvent gradient in fraction 1 (Figure 3.13B), whereas Aβ eluted after the gradient application in fraction 6. The elution of amylin before the organic solvent gradient prevented HPLC mediated concentration of the peptide from tissue samples. Due to this result HPLC concentration of the soluble and insoluble brain fractions was abandoned and LC-MS/MS was performed on un-concentrated brain fractions (Figure 3.14 & Figure 3.15).

LC-MS/MS of soluble occipital lobe grey matter using the Aβ SRM method (Figure 3.14A) revealed no detectable Aβ in the soluble brain fractions in control or T2D-AD samples. The insoluble formic acid fractions from the same control and T2D-AD samples demonstrated no observable Aβ in the controls but detectable Aβ in the T2D-AD group (Figure 3.14B). Analysis of amylin in the samples revealed no detectable amylin in the soluble or insoluble groups (Figure 3.15). Chromatograms and MS/MS spectra are scaled to the insoluble Aβ T2D-AD fraction (Figure 3.14B). This is likely due to the relatively low abundance of amylin in the brains compared to Aβ and competition for ionisation caused by not having been able to remove contaminants by HPLC. These limitations prevented our ability to provide mass spectrometry based evidence to support our immunohistochemistry and ELISA-based results.
Figure 3.16 IAPP is expressed in the brain and is up-regulated in AD

(A) Relative IAPP expression in human occipital lobe grey matter tissue samples corrected to GAPDH as quantified by qPCR. Data were graphed and assessed for normality then subjected to log(y+1) transformation. Data represent mean ± SEM, n=3 in each group. Data were analysed using a two-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. **, p < 0.01. #, p< 0.05 vs Control-Non-diabetic. (B) End product of the qPCR assay electrophoresed on a 2% agarose ethidium bromide gel. Expected product based on primer design = 60bp.
**Figure 3.17 Intra-neuronal amylin staining**

Representative immunohistochemistry of human control and T2D-AD occipital lobe (A) and temporal lobe (B) sections histologically stained for amylin with the T-4150 and H-017-03 antibodies. Arrows indicate neurons positive for amylin staining. All images were captured at 27x magnification. Full panel of images for control, T2D, AD and T2D for T-4150 and H-017-03 can be found in **Appendix 1** and **Appendix 2** respectively.
The *IAPP* gene is expressed in the occipital lobe and up-regulated in AD. Amylin is a pancreatic hormone that enters the brain to regulate satiety and feeding behaviour (Mietlicki-Baase et al. 2015a). Microarray and RNA-seq studies in humans demonstrate that the amylin gene (*IAPP*) expression most prominently occurs in the β-cells of the pancreatic islets, with some basal expression in skin, testis and very limited expression in the brain (Genecards, GCID:GC12P021354). Proteomic data would suggest there is little to no protein detectable outside of the pancreas (ProteomicsDB; ID:P10997). As a result of these observations and the vascular deposition of amylin, we hypothesised amylin was entering from the periphery and crossing the blood brain barrier where it begins to be deposited. To investigate whether amylin was being produced in the brain we performed quantitative real time PCR for *IAPP* in our occipital lobe grey matter tissue. For this experiment we generated a standard curve using the EndoC-βH1 pancreatic cell line. This cell line has been demonstrated to have insulin and amylin expression and is considered an experimental model of islet β-cells (Ravassard et al. 2011).

To our surprise *IAPP* gene expression was detectable in 11/12 tested samples, with one control being below the threshold set on the qRT-PCR (*Figure 3.16A*). In addition to this, there appeared to be a trend towards increased expression in T2D, a significant increase in AD and a potential synergistic effect on expression in comorbid T2D-AD (*Figure 3.16A*). Analysis of the melt curve data demonstrated no primer-dimerization or off target products during the PCR reaction (data not shown) and this was confirmed by electrophoresis of the PCR product on a 2% agarose gel (*Figure 3.16B*). This generated only one band at the expected product size of 60bp, and we also observe a negative in the same sample that was negative in the RT-PCR (*Figure 3.16B*, lane 5). Use of the NCBI Primer-BLAST tool demonstrated that only the *IAPP* gene had 100% homology with our primer design.

This surprising result is supported by our immunohistochemistry and in part by the observations of published reports (Jackson et al. 2013; Verma et al. 2016). We observed positive staining inside neurons with both the T-4150 and H-017-03 antibodies (*Figure 3.17*). This staining appeared to be increased in AD compared to control, although this was not specifically quantified by our method (*Figure 3.17*). This was also observed in the Jackson et al. (2013) and a subsequent publication by the same group - Verma et al. 2016, where they attributed this observation to intra-neuronal deposition of infiltrating amylin from the periphery. Our results suggest that this is not the case, and if this neuronal staining is specific to amylin and not a result of non-specific staining, then it is likely due to increased *IAPP* expression in the neurons in T2D and AD (*Figure 3.16*).
3.2.5 Amylin upregulates Aβ secretion in OX1-19 neurons

Recent evidence demonstrates that amylin treated rats have up-regulated Aβ levels in the blood (Mohamed et al. 2017) and hIAPP+/− mice have increased Aβ burden and soluble Aβ species in the brain (Moreno-Gonzalez et al. 2017). As amylin and Aβ are both degraded by neprilysin and insulin-degrading enzyme (IDE) (Vekrellis et al. 2000; Takaki et al. 2000; Bennett et al. 2003; Guan et al. 2012), we hypothesised that amylin increases Aβ by competition for Aβ degrading enzymes or clearance mechanisms.

**Figure 3.18 Amylin increases secreted Aβ isoforms in OX1-19 neurons**

Day 65 iPSC-derived cortical neurons were treated with vehicle or amylin for 24h in OptiMEM. Following treatment media was harvested assayed for Aβ1-40 and Aβ1-42 (A) and sAPPα and sAPPβ (B) using the mesoscale multiplex assay. Data were corrected to protein concentration in the media determined by BCA assay and then made relative to vehicle control. Data represent the mean ± SEM, n=3. Paired data were analysed by two way ANOVA. **, p<0.01 vs. control.

Mesoscale multiplex assay of media following treatment of day 65 OX1-19 neurons demonstrated that both 500nM and 1μM amylin treatment causes a significant increase in Aβ isoforms compared to control (**Figure 3.18A**). Subsequent analysis of the sAPPα and sAPPβ levels in the media by Mesoscale showed no increase in either soluble fragment, this result implied that amylin treatment did not influence ADAM10 or BACE1 mediated cleavage of APP, nor was amylin treatment likely to be up-regulating APP expression (**Figure 3.18B**).
Figure 3.19 Amylin impairs autophagy to increase Aβ

Day 65 iPSC-derived cortical neurons were treated with vehicle, 1μM amylin, 5μM chloroquine (CQ) or both the amylin and CQ treatments for 24h in OptiMEM. Following treatment media was harvested assayed for Aβ \(_{1-40}\) and Aβ \(_{1-42}\) (A) and sAPP\(\alpha\) and sAPP\(\beta\) (B) using the mesoscale multiplex assay. Data were corrected to protein concentration in the media determined by BCA assay and then made relative to vehicle control. Data represent the mean ± SEM, n=3. Paired data were analysed by two way ANOVA. *, p<0.01 vs. control.

We then performed treatments with the neprilysin inhibitor - phosphoramidon (n=2, data not shown). The response of the cortical neurons was highly variable in magnitude, but in both instances amylin and phosphoramidon had an additive effect when co-incubated on the cells, suggesting they are both increasing Aβ in the media by different mechanisms. Preliminary results using an in vitro Aβ degradation assay demonstrated amylin co-incubation had no effect on Aβ degradation by recombinant neprilysin (data not shown). As a result of these experiments, we investigated whether amylin mediated impairment of autophagy could be increasing Aβ in this model. Chloroquine (CQ) prevents autophagy by impairment of lysosomal acidification (Mizushima et al. 2010) and acted as a positive control for impairment of autophagy in these experiments. Co-incubation of amylin with CQ demonstrated no additive effect of amylin treatment (Figure 3.19A) with a similar trend towards increased Aβ isoform secretion in CQ groups. There were no changes in sAPP fragment secretion (Figure 3.19B). This suggests that both CQ and amylin treatment result in decreased autophagy and ultimately lysosomal degradation of Aβ through an unknown competitive mechanism.
3.3 Discussion

3.3.1 Amylin is deposited in the human brain
T2D associated insulin resistance causes responsive hyperinsulinemia, due to regulation of the insulin and amylin promoter by hyperglycaemia (Mulder et al. 1996; Cluck et al. 2005), and due to packaging of both insulin and amylin in the same secretory granules (Clark et al. 1989; Lukinius et al. 1989; Buchanan et al. 2007), hyperinsulinemia will also cause hyperamylinemia. Increased circulating amylin is potentially connected to observed amylin deposition in the heart (Despa et al. 2012) and kidney (Gong et al. 2007). This appears to have a pathogenic role in diabetic nephropathy and diabetic heart failure.

Our immunohistochemistry data (Figures 3.3-3.5) is supported by other studies demonstrating amylin deposition in the brain parenchyma and blood vessels (Jackson et al. 2013; Fawver et al. 2014; Srodlowski et al. 2014; Oskarsson et al. 2015a; Verma et al. 2016). Our method demonstrate differential results dependent on antibody used, and while we provide some evidence for increased amylin aggregates in AD by the H-017-03 antibody and ELISA method, our study is too small to conclusively prove the suggestions by Jackson et al. (2013) that amylin deposition is increased in AD.

A potential limitation of our immunohistochemistry studies is in the quantification procedure employed. For the H-017-03 antibody and Aβ 4G8 antibody a positively stained plaque was used to create a threshold which was then applied to the entirety of each section provided during quantification. As demonstrated in Figure 3.17 there is an increase in amylin positive staining inside neurons in our AD and T2D-AD cases, this will also be included in any quantification. Furthermore, the antigen retrieval method can cause occasional tears or folds in the tissue slice and vessel walls - which may contribute to false positives - although this was minimised during quantification there will be a background contribution in all slices, but particularly slices with marked neurodegeneration due to tissue volume loss. Together these considerations demonstrate that not all of the detected amylin will be large aggregates of amylin as observed in Figures 3.3 - 3.4. Alternative analyses, such as taking random images of the tissue and comparing positive staining would not have been suitable given the sparse nature of amylin plaque-like deposits. Future analyses of these data could take individual images of the whole tissue slice, then analyse positive staining above a certain size (to measure number of plaque-like deposits) or below a certain size (to measure intraneuronal amylin) to provide a more accurate quantification of specific subtypes of amylin deposition. This approach was not taken in our study due to time constraints.
The vascular deposition we observed (Figure 3.5) is supported by reported co-localisation of amylin and Aβ in the vascular wall in AD patient tissue by co-staining immunohistochemistry (Jackson et al. 2013) and proximity ligation assay in cortical brain sections (Oskarsson et al. 2015a). As mentioned, the antigen retrieval steps employed in our immunohistochemistry limit our ability to use this data to verify perivascular deposition of amylin as reported in human tissue (Schultz et al. 2017) and an animal model of intravenous injection of amylin (Oskarsson et al. 2015a). Our experimental design also limits our ability to verify claims of parenchymal co-deposition of amylin and Aβ, previously demonstrated in humans (Fawver et al. 2014; Oskarsson et al. 2015a) and animal models (Moreno-Gonzalez et al. 2017), and the serial staining of Aβ and T-4150 / H-017-03 provides no evidence to support co-localisation in the parenchyma (Figures 3.2 - 3.5). T2D has an element of brain atrophy independent of AD, with marked vasculature damage (Last et al. 2007). Vascular deposition of amylin, which we observed being up regulated in T2D (Figure 3.2B) may mediate the increased vascular damage and reduced blood flow observed in T2D brains (Novak et al. 2006; Last et al. 2007).

Our data supports observations of Aβ-amylin co-deposition in the same blood vessels. This is hypothesised to occur by cross-seeding of amyloids and the formation of heterocomplexes. We performed ion mobility mass spectrometry (IMMS) on synthetic peptide mixtures to isolate and characterise early heterocomplexes (Figure 3.7). We chose to analyse early aggregation events, as in vivo data from animal models suggests amylin cannot interact with preformed plaques and aggregates (Gaspar et al. 2010). Our IMMS experiment was unable to detect heterocomplex formation between the two amyloids, but demonstrates co-incubation of amylin and Aβ will influence aggregation propensity and conformation, demonstrating they interact. We provide evidence of heterocomplexes using a glutaraldehyde cross linking assay where incubation with Aβ caused H-017-03 antibody reactivity of several oligomeric species (Figure 3.8). These are likely to be either heterocomplexes of amylin and Aβ, an altered structure of amylin in the oligomeric species improving its reactivity with the H-017-03 antibody (which detects the N-terminal of the amylin peptide), or a mixture of these two outcomes. Given the molecular weights of the observable bands, we are likely detecting a mixture of amylin oligomers with altered structure, potentially improving the availability of the N-terminal epitope, and heterocomplexes of amylin-Aβ. This conclusion has some support in the literature. A glutaraldehyde cross linking assay between Aβ40 and amylin previously demonstrated formation of heterocomplexes and improved immunoreactivity of amylin oligomers (Yan et al. 2007).
Our observation of heterocomplexes is further supported in the literature by multiple biophysical methods such as AFM, TEM and ThT (Hu et al. 2015; Seeliger et al. 2012). But these data can be problematic to analyse as it is difficult to distinguish between heterocomplexes and altered conformation of one or both amyloids. Recent work by Young et al. utilising synthetic Aβ1-40 and amylin demonstrated heterocomplex formation in positive mode ion mobility mass spectrometry (Young et al. 2015a). This sort of hetero-complex formation is also reported between amylin and α-synuclein (Horvath and Wittung-Stafshede 2016), further suggesting amylin is a highly promiscuous amyloid with cross seeding propensity. Interestingly, there is also evidence that Aβ interacts with α-synuclein (Bachhuber et al. 2015), suggesting that common secondary structures between amyloids allow for cross-association, changes in aggregation propensity and potential therapeutic opportunities. The experimental results from co-incubation studies are particularly difficult to interpret; techniques that allow for real time monitoring of the mass of the product analysed are essential to prevent false positives. Unfortunately, such techniques often require buffers that are considerably different to the situation in vivo. That said, evidence of co-deposition of amylin and Aβ in AD brain (Jackson et al. 2013; Oskarsson et al. 2015a), transgenic animal models (Moreno-Gonzalez et al. 2017) and inducible by injection of preformed amylin aggregates (Oskarsson et al. 2015a) confirms cross-association as a real phenomenon that may have an interesting role in pathogenesis.

We utilised a fractionation protocol designed to separate the monomeric and early oligomeric amyloid proteins of interest in an SDS containing buffer from the large aggregate species which are often water insoluble. This insolubility of larger aggregates is due to the role of hydrophobic regions in self aggregation, but these species are soluble in formic acid. This fractionation protocol has been previously validated multiple times (Selkoe et al. 1986; Roher et al. 1986; Roher and Kuo 1999; Gorevic et al. 1986; Rostagno and Ghiso 2009). We analysed these soluble and insoluble human brain fractions by multiple methods to quantify soluble and insoluble amylin and Aβ. We provide strong evidence for this method succeeding in this separation from Mesoscale multiplex immunoassay and LC-MS/MS data demonstrating an enrichment of Aβ in the insoluble formic acid fraction (Figures 3.9 & 3.14). This is the first such attempt to separate the plaque-like and soluble species of amylin from the brain, although other analyses of brain homogenates by western blot have revealed amylin oligomeric species (Jackson et al. 2013). However, we did not observe these oligomeric species following SDS-PAGE of the soluble and insoluble fraction by western blot (data not shown).

The ELISA utilised in Figure 3.10 uses a monoclonal antibody raised against the C-terminal of amylin. Quantification from the soluble fraction by this method revealed a general trend for a reduction of soluble amylin species in T2D, which was significant in the occipital white
matter. It should be noted that often the soluble amylin level was near or below the lower limit of detection (LLOD) for the assay. However, there appeared to be an enrichment of amylin in the insoluble fraction (Figure 3.10B), with large, but non-significant increases seen in all brain regions tested. The ELISA data from insoluble fractions suggested there was no independent contribution of T2D to amylin deposition within insoluble aggregates, but that there is an increase in insoluble aggregate amylin in AD.

As discussed previously, amylin and Aβ share secondary and tertiary structures and are both intrinsically disordered in the monomeric form. This can make antibody based detection subject to cross reactivity, which has previously been reported (Yoshimura et al. 2014). As a result of this, we sought to validate our immunohistochemistry by LC-MS/MS. Aβ acted as a positive control for method detection following the fractionation process.

We designed selective reaction monitoring methods for Aβ and amylin tryptic fragments derived from synthetic peptides (Figure 3.11 & 3.12). This proved particularly difficult for amylin and the most abundant transition we observed was the stripping of water from inside the sequence of the amylin tryptic fragment. We then attempted to concentrate amylin from the samples using reverse phase high pH (pH 10) HPLC with acetonitrile as the organic solvent. In all the conditions attempted amylin was predominantly retained in the column or partially eluted during the flow through prior to application of the solvent gradient (Figure 3.13). As a result of this, we had to use un-concentrated brain fractions in the LC-MS/MS but were unable to detect amylin in the insoluble fraction (Figure 3.15). We could observe Aβ in the AD insoluble fractions but the method was not sensitive enough detect Aβ in control brain insoluble fractions. In addition to this, the LC-MS/MS method was unable to detect amylin from soluble or insoluble fractions of transgenic mouse pancreas expressing human amylin (data not shown) which we have demonstrated have large inclusions of amylin and a prevalence of amylin containing islet-β cells (Figure 3.1).

Unfortunately, the failure to separate and concentrate amylin by HPLC (Figure 3.10), and competition for ionisation by other proteins that arises from using fractions not separated by HPLC, severely limited the ability of the LC-MS/MS study to detect amylin in our samples. This leads us to conclude that if amylin is present that is below the concentration detectible under these experimental parameters, or alternatively, amylin is not present in these samples.

Large proteomic studies of AD samples have not detected amylin (Musunuri et al. 2014; Drummond et al. 2015; Hondius et al. 2016). This may be because these broad approaches have a relatively poor ability to detect non-abundant species but it may also be linked to amylin’s physical properties. The largest transition we observed from the most abundantly ionised amylin tryptic fragment is a water molecule stripping from the fragment sequence (Figure 3.12). MASCOT and other software often ignore neutral losses such as
water stripping and therefore are unlikely to detect amylin’s most abundant transition (Sun et al. 2008). Recently, a comparable study succeeded in isolating amylin from T2D-AD temporal lobe and validating it using HPLC linked LC-MS/MS (Verma et al. 2016). This study also used a C18 column with acetonitrile as an organic solvent as in the present study, but Verma et al. (2016) performed HPLC using low pH solvents with 0.1% trifluoric acid, whereas we used a high pH solvent mix. This may underlie the ability to elute amylin on the organic gradient in Verma et al. (2016) but not in the present study (Figure 3.13).

Following HPLC mediated enrichment of amylin, the sequence was detected in several T2D-AD patients and validated by collision induced dissociation (Verma et al. 2016). However, the authors did not perform a relative comparison against controls nor did they perform the low pH HPLC LC-MS/MS protocol on AD brains with no apparent diabetes. This helps validate previous antibody data but does not prove previous conclusions of increased amylin deposition in AD (Jackson et al. 2013).

3.3.2 Effect of amylin on pericyte viability

Pericytes are a key component of the neurovascular unit, situated on the outer basement membrane of the endothelial cells, they act to maintain the integrity of brain microvasculature (Balabanov and Dore-Duffy 1998). Pericytes are also key in mediating signalling associated with angiogenesis and regulation of blood flow by endothelial cells. One such example of this signalling is activation of platelet-derived growth factor receptor-β (PDGFRβ) downstream signalling, which regulates pericyte proliferation, migration and recruitment to the vascular wall in response to platelet-derived growth factor B secretion by endothelial cells (Daneman et al. 2010). Endothelial cells form a circular layer sealed with tight junctions, which comprise the blood-brain barrier (BBB) (Zlokovic 2005). One such tight junction protein, and marker of endothelial cells, is VE-Cadherin. In our study, VE-Cadherin is used as an endothelial marker to correct for the amount of vascular tissue input into the qPCR assay (Figure 3.6). Therefore, the ratio is a specific measure of the loss of the pericyte marker PDGFRβ, which can be taken as a broad measure of pericyte viability. It should be acknowledged that PDGFRβ is not a perfect marker for pericytes, as there is some low level expression in smooth muscle cells, but PDGFRβ knockdown causes an age dependent loss of pericytes (Sagare et al. 2013). It is also possible that VE-Cadherin is up regulated in AD in a response to small vessel disease or vasculature damage. If this were true it would skew the ratio and make it appear that PDGFRβ was being decreased, but to the authors knowledge no such mechanism of VE-Cadherin elevation in AD or associated pathologies has been described. These data demonstrate that despite the fact that PDGFRβ is an imperfect pericyte marker, it is a clear correlate with pericyte loss and is therefore suitable for our study.
Vascular damage has long been understood to play a major role in AD, evidenced by prevalence of vascular damage in the Nun study and Rotterdam study (Snowdon et al. 1997; Ott et al. 1998b). And further demonstrated by a positive correlation between vasculature damage and AD (Ting et al. 2016). Our qPCR data suggested a loss of pericyte viability in AD, with no independent effect of T2D (Figure 3.6A). This is supported by a recent study utilising ELISA-based quantification of PDGFRβ, which demonstrated large decreases in PDGFRβ in AD that correlated with Braak stage and degree of amyloid deposition (Miners et al. 2017). Our data (Figure 3.6) and the data of Miners et al. (2017) demonstrate PDGFRβ is decreased in AD. Furthermore, this pericyte loss by PDGFRβ depletion causes 2-3 orders of magnitude higher brain Aβ deposition and broadly accelerates AD pathology in APPsw/0-PDGFRβ+/− mice (Sagare et al. 2013). This is supported by a second animal model, where antagonism of PDGFRβ also resulted in elevated Aβ deposition (Lai et al. 2015). This implies damage to pericytes may reduce Aβ clearance mechanisms and promote deposition.

Vascular damage as a result of amylin deposition in pericytes could not be directly investigated in this study, but comparison of our qPCR data (Figure 3.6) and immunohistochemistry data (Figure 3.3 & 3.5) demonstrated that conditions where we observed increased amylin deposition had no decrease in the pericyte marker PDGFRβ. These data suggested that increased vascular deposition of amylin observed in T2D does not correlate with pericyte loss as has previously be suggested (Schultz et al. 2017; Ly et al. 2017).

3.3.3 Expression of IAPP mRNA in the brain and up regulation in AD

The observation of IAPP expression in the brain (Figure 3.16) was surprising, as previous microarray and proteome data would suggest there is little IAPP gene transcription or protein expression in the brain (Genecards, GCID:GC12P021354; ProteomicsDB, ID:P10997). As previously discussed, these techniques suffer from a poor ability to detect low abundance or expression. Our observations are partially supported by the MayoRNAseq study (Allen et al. 2016), who also report IAPP gene expression in the brain, although the authors do not observe significantly increased expression between control and AD in the cerebellum. Interestingly, amylin has previously been detected in brain extracts, particularly enriched in the hypothalamus (Chance et al. 1991). Interestingly, it has previously been demonstrated that rat IAPP expression in the brain, which is generally next to nil, undergoes a 25 fold increase in selected brains area in lactating rat dams (Dobolyi 2009; Szabo et al. 2012). Understanding what peripheral signal causes this change may help explain if the advanced glycation products (proteins or lipids with covalently bound sugars) upregulated in T2D and AD (Vitek et al. 1994; Sasaki et al. 1998; Ulrich and Cerami 2001; Vlassara and...
Uribarri 2004; Coker and Wagenknecht 2011) promote the increase in IAPP expression we observe in these conditions (Figure 3.16). These studies cement the idea that IAPP gene transcription occurs at a very low level in the healthy brain, and leave open the possibility of changes in disease.

In its role as a pancreatic satiety hormone, amylin expression is promoted by glucose (Mulder et al. 1996; Gasa et al. 1997; Novials et al. 1993; Hou et al. 1999) and increased amylin peptide secretion is observed in hyperglycaemic and obese individuals (Stridsberg et al. 1993; Larsson and Ahren 1995; Leckstrom et al. 1999). Studies of T2D reported increases in circulating amylin and up regulation of amylin mRNA in the pancreas of humans (Enoki et al. 1992; Hanabusa et al. 1992; Kautzky-Willer et al. 1994). This was replicated in hyperglycaemic rat models (Huang et al. 1992), which demonstrate that this increase is most likely mediated by increased circulating glucose.

This provides a potential mechanism whereby IAPP expression in the brain could be up regulated in both T2D and AD. Hyperglycaemia is the major characteristic of T2D and has been demonstrated to negatively correlate with cognitive performance test score in diabetic patients (Cox et al. 2005). Hyperglycaemia and increased glycation products are also major features of AD (Xu et al. 2016a; Xu et al. 2016b). This relationship is potentially related to the observation that glucose metabolism in the brain steadily decreases with age (Goyal et al. 2017). This observation is particularly interesting given that metabolomics studies have recently demonstrated up regulation of glucose and glycation products across a broad spectrum of brain regions in AD (Xu et al. 2016a; Xu et al. 2016b) and that glycolytic enzymes have been demonstrated to be increased by proteomics (Schonberger et al. 2001). These data demonstrate that glucose and its metabolites are generally up-regulated in aging and that this is exacerbated in T2D and AD, and also provide mechanisms by which this could result in increased IAPP expression as observed in our study (Figure 3.16).

However, translation of IAPP will result in pre-proamylin, which will need to undergo further cleavage by prohormone convertase 2 (PC2) (Badman et al. 1996; Higham et al. 2000). PC2 is relatively abundantly expressed in human brain (Genecard, GCID: GC20P017226), therefore the expressed IAPP gene product has the potential to result in amylin1-37. Our immunohistochemistry data (Figure 3.17) and the data of multiple other studies (Jackson et al. 2013; Srodulski et al. 2014; Verma et al. 2016) demonstrate increased amylin antibody reactivity inside neurons in AD and T2D-AD but an absence in controls. The immunohistochemistry we have performed utilised an antibody raised against the entire amylin sequence (T-4150) or recognising the N-terminal of amylin (H-017-03), so this positive staining strongly indicates presence of amylin in the neurons. In the above literature (Jackson et al. 2013; Srodulski et al. 2014; Verma et al. 2016), staining of the neurons is attributed to intraneuronal deposition of infiltrating amylin. However, the scope of the
staining we observe makes this unlikely in the author’s opinion. Our qPCR data would suggest this may in fact be due to local amylin production, probably induced by perturbations in glucose or glycation products.

Due to the satiety causing role of amylin in response to food ingestion, increased amylin production in the brain in response to hyperglycaemia may well contribute to appetite loss that is a common feature of AD pathology (Ismail et al. 2008). The connection between hyperglycaemia, IAPP expression and AD deserves follow up research.

3.3.4 Mechanism of amylin induced increase of Aβ secretion

We observed a large and reliable increase in Aβ isoforms in the media of iPSC-derived cortical neurons following amylin treatment (Figure 3.18). This is a very interesting observation, potentially providing a link between increased amylin secretion during T2D and increased incidence of AD in T2D patients (Biessels and Kappelle 2005), increased diffuse Aβ plaques observed with comorbid T2D-AD (Janson et al. 2004) and multiple reports of Aβ deposition in the pancreas in T2D (Janson et al. 2004; Miklossy et al. 2010; Oskarsson et al. 2015a). Our data demonstrates an increase of Aβ isoforms in media following amylin treatment (Figures 3.18 & 3.19). This observation is supported by a recent conference presentation, which provided evidence suggesting that amylin treatment of a neuroblastoma cell line causes up-regulation of Aβ in the lysate (Bharadwaj et al. 2017). This observation is also supported by animal model data; mice expressing human APP and hIAPP act as a genetic model of the link between T2D and AD and have been demonstrated to have considerably more soluble and insoluble Aβ species in the brain (Moreno-Gonzalez et al. 2017). Furthermore, i.p injection of amylin caused an increase of Aβ isoforms in the blood of Tg2756 mice (Zhu et al. 2015; Mohamed et al. 2017). Together these data support our observation that increasing extracellular amylin causes increases of Aβ in the media.

We originally hypothesised amylin would increase Aβ via competition for degrading enzymes due to common mechanisms of degradation through neprilysin and insulin degrading enzyme. Our preliminary data demonstrated this is possibly not the case, and further experiments also demonstrated the effect was not being mediated through changes in APP processing by α- and β-secretases as there was no alteration in sAPPα or sAPPβ (Figure 3.18B). Amylin up-regulation has been demonstrated to impair autophagy in pancreatic islet β-cells (Rivera et al. 2011; Rivera et al. 2014; Shigihara et al. 2014) and amylin aggregation in the islets will increase in autophagy deficient mice, resulting in a diabetic phenotype (Kim et al. 2014b). Due to this fact, we then explored the possibility that amylin’s known autophagy inhibiting properties were preventing Aβ clearance.
Autophagy is not considered a major Aβ clearance mechanism. However, there is a basal level of autophagy involved in protein quality control (Choi et al. 2013), and inhibition of this basal level will cause neurodegeneration in mouse models (Hara et al. 2006). Other studies have demonstrated that externally applied Aβ enters the autophagy pathway to be targeted for degradation in glia (Cho et al. 2014). It has also been demonstrated that enhancing autophagy will decrease intracellular Aβ levels and Aβ burden in cellular and animal models (Nixon 2007;Bharadwaj et al. 2012). This appears to be a common mechanism of amyloid clearance, as enhancement of autophagy also clears α-synuclein in cellular and animal models (Steele et al. 2013) and reduces amylin levels in pancreatic islets (Rivera et al. 2014). These data suggest that autophagy is an under-appreciated mechanism of targeting amyloid to lysosomes for degradation.

Chloroquine (CQ) is a diprotic base which inhibits autophagy by entering endosomes and lysosomes and neutralising the local pH, thereby inhibiting lysosomal protease activity (Klionsky et al. 2012;Browning 2014). There is no additional effect of amylin induced increase of Aβ following the co-addition of CQ (Figure 3.19). This implies a mechanism of action whereby both treatments result in reduced lysosomal degradation of Aβ. This is potentially because application of amylin saturates the autophagy pathways targeted to the lysosome. It has been previously demonstrated that externally applied amylin co-localises with LC3, a ubiquitin like protein which is the major determinant of substrate selection for autophagy (Schultz et al. 2017;Choi et al. 2013). Alternatively, amylin may also impair lysosomal degradation by preventing acidification of the lysosomes. Amylin has been demonstrated to up-regulate GSK3β activity (Abaffy and Cooper 2004), which in turn will cause impaired autophagy by GSK3β mediated prevention of acidification of the lysosome (Avrahami et al. 2013). These studies provide two potential mechanisms by which amylin can impair autophagy in a manner that explains our observations (Figure 3.19).

Autophagy impairment is common to the brain in AD (Wolfe et al. 2013) and the pancreatic islets in T2D (Barlow and Thomas 2015). The data above demonstrate amylin can inhibit autophagy and that impaired autophagy upregulates Aβ and that autophagy is impaired in AD and T2D. If autophagy can be considered a broader clearance mechanism for amyloids, this impairment may explain other non-traditional amyloid deposition in the AD brain (amylin, TDP-43 and α-syn (Jackson et al. 2013;Higashi et al. 2007;Raghavan et al. 1993)) and the T2D pancreas (Aβ and tau (Miklossy et al. 2010)).
3.4 Chapter Summary

The data generated from the experiments discussed in this chapter provides evidence that amylin is deposited in brain vasculature and parenchyma in human occipital and temporal lobe as previously reported (Jackson et al. 2013; Oskarsson et al. 2015a; Ly et al. 2017). We provide serial staining evidence for amylin and Aβ co-deposition in cerebral blood vessels and in vitro evidence that amylin and Aβ interact with one another and form heterocomplexes. Our data provide evidence demonstrating a trend towards increased amylin aggregates detectable by immunohistochemistry and increased insoluble amylin deposition in AD with no independent effect of T2D. In addition to this, we observed genetic evidence of pericyte loss in AD, with no independent effect of T2D, which is supported by recent ELISA quantification of PDGFRβ protein loss (Miners et al. 2017). We also provide evidence of IAPP gene up-regulation in AD with a possible independent involvement of T2D. This novel finding is supported by increased amylin positive neurons in AD detected by immunohistochemistry, which is replicated in several reports (Jackson et al. 2013; Verma et al. 2016). This result offers an alternative interpretation of previously published immunohistochemical staining data which also observes increased amylin staining in neurons in AD and T2D (Jackson et al. 2013; Verma et al. 2016). We also demonstrate that amylin addition to neuronal culture increased Aβ levels in the media, likely by impairment of autophagy-mediated targeting of Aβ to lysosomes for degradation. Together these data imply amylin is deeply involved in the pathology of T2D and AD, providing several molecular links between the two diseases. In Figure 3.20, we provide a model summarising amylin’s contribution to AD.
Figure 3.20 Potential role of amylin in AD
Schematic demonstrating the potential contributions of amylin to AD inferred from data in the present study and literature. IAPP gene expression is increased in T2D and in AD causing increased amylin in the brain and periphery. Increased amylin secretion from the pancreas or local production results in amylin deposition, which may act to seed $\beta\beta$ plaques and contribute directly to AD. In addition to this, increased amylin results in impairment of autophagy mediated $\beta\beta$ clearance in neurons, causing increased $\beta\beta$ which may go on to form aggregates and cause AD pathology. Solid line = demonstrated connection, dashed line = potential connection.
Chapter 4: Investigating whether amylin and Aβ share downstream signalling

4.1 Introduction

There is a huge body of evidence suggesting Aβ has a central role in AD. Mutations in amyloid precursor protein (APP) or PSEN1 that promote Aβ production cause familial Alzheimer's disease (fAD) (Crawford et al. 1991; Chartier-Harlin et al. 1991; Goate et al. 1991; Mullan et al. 1992; Bentahir et al. 2006), and mutations in APP that reduce Aβ production are associated with reduced risk of developing AD (Jonsson et al. 2012). In addition to this the greatest genetic risk factor for sporadic AD is apolipoprotein-E (APOE) isoform status. The main roles of APOE are closely tied to Aβ clearance and degradation (Castellano et al. 2011), Aβ aggregation (Cerf et al. 2011) and regulation of APP expression (Huang et al. 2017). These evidences form the foundation of the amyloid cascade hypothesis, which implicates Aβ aggregation and deposition as the primary initiating factor in AD (Hardy and Allsop 1991; Hardy and Selkoe 2002).

It is now widely accepted that soluble oligomers of Aβ are the predominant bioactive species (Walsh et al. 2002; Haass and Selkoe 2007; Selkoe and Hardy 2016). This is further supported by the emergent beneficial effects of Aβ oligomer targeting methods (Doody et al. 2013; Sevigny et al. 2016). However, what represents a truly pathology relevant preparation of oligomer species remains highly contentious, with a plethora of oligomers and protocols being used and characterised in the literature. This requires careful analysis of oligomerisation protocol when interpreting results and a greater effort in the research community to identify bona fide pathology relevant responses to Aβ oligomers.

4.1.1 Complex world of Aβ oligomers

The present study, and the majority of previous studies, focuses on oligomeric species of Aβ1-42. This is due to a number of factors, including an increased ratio of Aβ1-42 to Aβ1-40 seen in disease (Naslund et al. 1994), the fact the Aβ1-42 is the primary component of diffuse plaques which can be considered reservoirs of oligomers (Haass and Selkoe 2007) and increased Aβ1-42 generation with PSEN1 mutations (Scheuner et al. 1996). However it should be noted that many other Aβ species exist in a complex matrix. APP cleavage by BACE1 then subsequent C-terminal cleavage by γ-secretase yields several major Aβ species - Aβ1-40, Aβ1-42, Aβ1-43, Aβ1-37 - as well as N-terminally truncated Aβ species and pyroglutamate modified Aβ species; APP cleavage events are reviewed in Andrew et al. (2016). In addition
to amyloidogenic cleavage, APP undergoes non-amyloidogenic processing by ADAM10 and recent evidence demonstrates APP also undergoes η-cleavage (Willem et al. 2015b) and δ-cleavage (Zhang et al. 2015c). These factors complicate the strong genetic evidence linking APP and Aβ generation, in particular Aβ₁₋₄₂ generation, with AD.

Amyloid oligomers were first implicated in pathology after discovery that naturally secreted oligomers of Aβ₁₋₄₂, but not monomer species, impaired hippocampal LTP in a rat model (Walsh et al. 2002). Walsh et al. (2002) originally associated low molecular weight Aβ assemblies that appear as dimers by SDS-PAGE and size exclusion chromatography with LTP impairment, but later clarified that the neurotoxicity of these species is dependent on aggregation and stabilisation of high molecular weight fibrillar oligomers (O’Nuallain et al. 2010). Subsequently many studies have demonstrated that naturally occurring oligomers of Aβ impair cognitive function (Cleary et al. 2005; Shankar et al. 2007; Shankar et al. 2008; Li et al. 2011) (reviewed in Haass and Selkoe (2007) and Benilova et al. (2012)). Many groups have extracted soluble oligomer species from AD brain tissue (McLean et al. 1999; Lesne et al. 2006; Shankar et al. 2008; Um et al. 2012; Lesne et al. 2013). Isolation of brain amyloid has demonstrated there is a large heterogeneous population of Aβ species, and subsequent work highlights that oligomers exist in a dynamic equilibrium of oligomeric species and therefore isolation of one sub-species will quickly lead to re-establishment of the equilibrium (Hepler et al. 2006). Interestingly, recent evidence suggests this dynamic nature may be integral to Aβ induced toxicity (Yang et al. 2017). Interpretation of these data is further complicated by the potential contribution of detergent-mediated stabilisation of certain oligomer complexes during the isolation process in some protocols, potentially leading to artefacts and false positives (Watt et al. 2013).

Effort has been made to create a common nomenclature for Aβ oligomers determined by the tertiary structure they possess. This is identified by conformational antibodies designed against specific tertiary structural epitopes (Kayed et al. 2003; Kayed and Glabe 2006; Kayed et al. 2007; Kayed et al. 2010; Hatami et al. 2014). The two most commonly employed conformational antibodies are the A11 and OC antibodies. The A11 antibody is designed against prefibrillar oligomeric species (type 1 oligomers), and the OC antibody is designed against fibrillar oligomeric species (type 2 oligomers) (Lesne et al. 2006; Kayed et al. 2007). Both types of oligomer occur in humans and both are increased in AD.

4.1.2 Aβ oligomers bind cell surface receptors
The amyloid-β-derived diffusible ligand (ADDL) oligomeric preparation is a widely adopted synthetic Aβ₁₋₄₂ oligomer preparation demonstrated to bind hippocampal neurons and elicit toxicity at a low nanomolar range (Lambert et al. 1998; Laurén et al. 2009). In a landmark
study, it was demonstrated that these effects were prevented by pretreatment of the neurons with trypsin (Lambert et al. 1998), demonstrating that the ADDLs mediate toxic effects through cell surface proteins. This neurotoxicity also appeared to be mediated through the protein tyrosine kinase Fyn, after germline knockout prevented ADDL induced neuronal dysfunction (Lambert et al. 1998). Lambert et al. (1998) demonstrated oligomeric species of Aβ act as ligands for cell surface receptors, which subsequently mediate neurotoxic signalling through activation of downstream kinases.

Since then, the receptor described by Lambert et al. (1998) has been demonstrated to be a cellular prion protein (PrP<sup>C</sup>) stabilised receptor complex (Laurén et al. 2009; Um et al. 2012; Um et al. 2013; Rushworth et al. 2013). This and many other cell surface receptors have since been discovered, some are listed in Table 1.2 and are reviewed in Jarosz-Griffiths et al. (2016). Ultimately, dysregulation of cell signalling through kinases and other cell processes as a result of aberrant receptor binding causes neuronal death. Current understanding of the amyloid cascade hypothesis places soluble oligomers of Aβ as the main causative entity of AD (Walsh and Selkoe 2007; Karran and De Strooper 2016). The process postulated is as follows: Aβ soluble oligomers bind to cell surface receptors to dysregulate kinase cascades, cause tau phosphorylation and eventually lead to neuronal loss, inflammation and cognitive impairment.

4.1.3 Tau phosphorylation in AD

Tau hyper-phosphorylation, dissociation from microtubules and aggregation into oligomeric species and neurofibrillary tangles is a major element of AD pathology. Tau is markedly more neurotoxic than Aβ (Bloom 2014), and is clearly sufficient to cause degeneration and cognitive decline as evidenced by its role in multiple dementias, such as frontotemporal dementia (FTD) (Poorkaj et al. 1998) and chronic traumatic encephalopathy (McKee et al. 2009). However, the MAPT mutations that promote tau aggregation in FTD have no impact on Aβ aggregation, whilst overwhelming evidence shows familial AD mutations promoting Aβ generation also result in tau pathology, reviewed in Stancu et al. (2014). Therefore, while there is a growing body of evidence suggesting tau mediates cell toxicity, tau pathology is still very likely downstream of Aβ mediated events.

Isolated primary neurons have been used to demonstrate tau alterations induced by synthetic and AD brain derived Aβ oligomers (Busciglio et al. 1995; Ferreira et al. 1997; Takashima et al. 1998; De Felice et al. 2008; Um et al. 2012; Rushworth et al. 2013). These studies have often struggled to elucidate the cascades that result in tau phosphorylation and, as the neurons are isolated from mouse pups, they have intrinsic
problems of translatability to humans. Likewise, neuroblastoma immortalised cell models have also proved poor replicates of Aβ induced tau pathology. While animal models strongly demonstrate Aβ induced tau modification, reproduction of tau pathology is beholden to overexpression of human tau in these models (Lewis et al. 2001; Oddo et al. 2003; Oddo et al. 2004). In addition to this, there are wide variations between murine models, potentially due to genetic background. The connections between Aβ and tau are reviewed in Stancu et al. (2014). The conclusion from the above data is that while it is clear Aβ oligomeric species induce changes in tau phosphorylation that result in tau pathology, current models limit the ability to test this in a neuronal model relevant to the human brain.

Ten years ago manipulation of a small subset of genes allowed the generation of induced pluripotent stem cells (iPSCs) from adult human fibroblasts (Takahashi et al. 2007). These have since been differentiated into multiple cell types, and have been differentiated into cortical neurons (Shi et al. 2012; Israel et al. 2012). iPSC lines isolated from patients with fAD mutations faithfully recapitulate mutation induced phenotypes (Israel et al. 2012). Expression of fAD mutation will result in kinase dysregulation and increased tau phosphorylation (Israel et al. 2012; Muratore et al. 2014; Wray 2017), but attempts to generate sporadic models in neurons differentiated from control patient derived iPSC lines have proved difficult (Nieweg et al. 2015). That said, iPSCs represent an exciting possibility to create a more accurate model of acute Aβ oligomer induced neuronal effects.

4.1.4 Similarities between Aβ and amylin

As mentioned previously amylin undergoes an aggregation process that is remarkably similar to Aβ, transitioning from an intrinsically disordered monomeric peptide to small oligomeric species containing α-helices, to prefibrillar and fibrillar β-sheet rich oligomer species and eventually stable fibrils which become insoluble plaques (Nanga et al. 2009; Cao et al. 2013b; Palmieri et al. 2013). It is clear that amylin oligomers and Aβ oligomers share tertiary structure and sensitivity to the similar environmental factors (Cao et al. 2013b). Indeed, oligomers of both amyloids are so similar that the A11 and I11 structural antibodies generated against Aβ oligomers and amylin oligomers respectively will cross react with oligomers of both amyloids (Kayed and Glabe 2006; Kayed et al. 2007); furthermore, probes designed to detect insoluble Aβ deposits have been demonstrated detect amylin deposits (Yoshimura et al. 2014). Local inflammation in the pancreas and brain has been demonstrated to be mediated through a common mechanism in T2D and AD. Amylin and Aβ oligomer species both bind and activate the pro-inflammatory cell surface receptor - toll-like receptor 2 (TLR2) (Jana et al. 2008; Westwell-Roper et al. 2016). This is the only shared cell surface receptor discovered between amylin and Aβ so far.
The common tertiary structure of amylin and Aβ oligomers makes it highly likely that amylin and Aβ share other cell surface receptors. In this chapter we investigated the propensity of amylin oligomers to bind the high affinity Aβ oligomer receptor - PrPC.

4.1.5 Aims
The evidence discussed above demonstrates the high degree of homology between amylin and Aβ oligomers. This shared tertiary structure enables the amyloids to share some cell surface receptors, and makes the discovery of other shared receptors and signalling pathways quite likely. In this chapter we aimed to create an amylin oligomerisation protocol and investigate the potential of amylin to activate kinase signalling associated with the high affinity Aβ oligomer receptor - PrPC. This may highlight common mechanisms of cellular toxicity in AD and T2D.

Tau phosphorylation induced by Aβ oligomer has proved difficult to recapitulate in cell models relevant to the human brain, in this chapter we used control patient cell line (OX1-19) induced pluripotent stem cell (iPSC)-derived cortical neurons to attempt to model Aβ oligomer induced signalling and tau phosphorylation.
4.2 Results

4.2.1 Characterisation of Aβ and amylin oligomers

The Aβ oligomer preparation used throughout this and the next chapter is based on the widely used ADDL preparation (Lambert et al. 1998; Chromy et al. 2003). These oligomers are OC reactive and have been demonstrated as a high affinity ligand to PrP<sub>C</sub> (Laurén et al. 2009). Multiple other alternative oligomer preparation protocols exist; this fact is the source of much of the complexity in the literature surrounding Aβ oligomers and their ligands. We chose a derivative of the ADDL protocol due to reports from human brain studies demonstrating OC reactive oligomer species best correlate with AD onset and severity (Nicoll et al. 2013).

We utilised a synthetic Aβ<sub>1-42-LC-biotin</sub> preparation to recreate the ADDL preparation generated using synthetic Aβ<sub>1-42</sub> described in Chromy et al. (2003). The addition of a biotin tag allows specific detection of our oligomer preparation and the LC link prevents this biotin tag from interfering with oligomerisation. In Figure 4.1 our oligomer preparation derived from synthetic Aβ<sub>1-42-LC-biotin</sub> (hereafter referred to as Aβ<sub>O</sub>) is compared to oligomers of Aβ<sub>1-42</sub> generated using the ADDL protocol. When resolved by SDS-PAGE the Aβ<sub>O</sub> preparation forms a spread of oligomeric species identical to the Aβ<sub>1-42</sub> oligomeric preparation (Figure 4.1A). Monomer, dimer, trimer and tetramer species are observed in both the monomeric controls (the Aβ<sub>1-42-LC-biotin</sub> monomer preparation is hereafter referred to as Aβ<sub>m</sub>) and oligomer preparations; while the spread of oligomer species between 56kDa and 170kDa is consistent between the Aβ<sub>O</sub> and Aβ<sub>1-42</sub> oligomer preparation. Likewise, the addition of the biotin tag does not perturb oligomer structure, as the Aβ<sub>O</sub> preparation retains affinity for the OC in-register β-sheet epitope antibody (Figure 4.1B) in the dot blot assay. This assay assesses native conformation of the oligomer preparations, while reactivity with the 6E10 antibody demonstrates equal loading. We then investigated the timeframe of Aβ<sub>1-42-LC-biotin</sub> aggregation using the thioflavin-t (ThT) assay (Figure 4.1C). This assay is dependent on flavonoid association with fibril structure altering the fluorescence properties of ThT, and allows for real time measurement of fibril, not oligomer, formation (Nilsson 2004). Our data demonstrate the Aβ<sub>1-42-LC-biotin</sub> preparation forms ThT reactive fibrils after about 19-20h. Our Aβ<sub>O</sub> preparation is isolated by centrifugation after 16h aggregation and is therefore not a fibrillar ThT reactive fibril species.
Figure 4.1 Characterisation of Aβ oligomers and aggregation

(A) 5-20% Acrylamide tris-tricine SDS-PAGE of monomeric and oligomeric preparations of synthetic Aβ_{1-42}-LC-biotin (denoted Aβ in all following figures) and synthetic Aβ_{1-42} preparations, detected with the 6E10 antibody. (B) Dot blot assay of 100ng synthetic Aβ and synthetic Aβ_{1-42} monomer and oligomer preparations Membranes were immunoblotted for OC structural antibody then stripped and re-probed with 6E10 antibody. (C) Thioflavin-T fluorescence following incubation 25μM synthetic Aβ with 10μM Thioflavin-T overnight at 20°C. Blank is vehicle loading control. Data represent mean ± SEM. n=3/point.
Figure 4.2 Characterisation of Aβ oligomers by atomic force microscopy (AFM)
Representative image of height data from 10μM Aβ16 (A) and Aβ25 (B) preparations spotted onto mica collected in tapping mode AFM. Height data scale -2.5nm to 15nm, image scale 1μm. n=3, ≥50 oligomers were measured per n. (C) Interpolated height data from all Aβ25 preparation experiments and population information. Graph depicts individual interpolated oligomer sphere height as well as the mean ± SD. (D) Data shown in (B) represented as a % of total observations by size bracket.
Atomic force microscopy (AFM) was used to measure oligomer height, which was then interpolated to sphere diameter. Our Aβₘₐₜ preparation acted as a negative control and demonstrates only a few small detectable oligomers (Figure 4.2A), whereas the Aβₒ preparation clearly has many detectable spherical oligomers across a range of sizes (Figure 4.2B). The distribution of interpolated oligomer diameter is presented in Figure 4.2C, the data demonstrates that mean oligomer height is 6nm but clearly shows a range of oligomer sizes as one would expect given the spread of observable oligomers by SDS-PAGE (Figure 4.1A). This interpolated mean is consistent with previously published reports (Chromy et al. 2003; Rushworth et al. 2013). Analysis of the population distribution demonstrates the majority of oligomers have an interpolated sphere diameter of between 5-6nm.

Amylin has been demonstrated to be a highly amyloidogenic peptide, with a much greater propensity to aggregate when compared to Aβ or α-synuclein (Cao et al. 2013a; Cao et al. 2013b). In its monomeric form amylin exists as a random coil, when it acquires α-helical or β-sheet secondary structure dependent on the local environment prior to beginning to form oligomers (Nanga et al. 2011; Cao et al. 2013b). β-sheet rich oligomers then go onto form protofibrils, which are extended by nucleation at each end of the protofibril by addition of β-sheet containing oligomers or monomers (Green et al. 2004a). The resultant fibrils then bind thioflavin-t (ThT). Analysis of amylin aggregation in our buffer conditions by ThT assay demonstrates amylin began formed ThT reactive fibril species between 4-5h (Figure 4.3).

Characterisation of the timeframe of amylin oligomerisation by AFM reveals oligomer size increases prior to fibril formation and then decreases afterwards, the fibrils being removed by centrifugation prior to assay (Figure 4.4A-E). This implies the observable small oligomer species in both preparations, and possibly the 2-4nm oligomeric component of the 4h timepoint, oligomer population are off pathway or small oligomeric species constantly being turned over (Figure 4.4B & D). Our data are comparable to previous examinations of amylin oligomerisation by AFM, which demonstrated oligomers are extended by bi-directional addition of smaller oligomer species (Green et al. 2004a; Green et al. 2004b). After reaching a critical height point (>7nm) these oligomers act as protofibrils and begin to associate with one-another to promote fibril formation (Green et al. 2004a; Green et al. 2004b). Our oligomeric preparation has a mean height of 7.32nm (Figure 4.4E). This indicates we are isolating on-pathway oligomeric species, some of which are forming seeding species in our oligomer preparation - consistent with the timeframe of the ThT assay (Figure 4.3). It has been further demonstrated that such oligomer species emerge before ThT reactivity using NMR (Suzuki et al. 2012). As a result of these data, we used oligomeric species isolated at 4h aggregation in our cell treatments throughout this chapter.
Figure 4.3 Characterisation of amylin aggregation
Thioflavin-T fluorescence following incubation of 25μM synthetic amylin in 2mM ammonium acetate / F-12 buffer with 10μM Thioflavin-T overnight at 20°C. Blank is vehicle loading control. Data represent mean ± SEM. n=3/point.
Figure 4.4 Characterisation of amylin oligomers by AFM

Representative image of detectable oligomers spotted onto mica following 0h (A), 2h (B), 4h (C), and 24h (D) incubation. Height data collected in tapping mode AFM, height data scale -2.5nm to 15nm, image scale represents 1μm. (E) Interpolated sphere diameter of amylin oligomers quantified from above. Data represent mean ± SD, individual data points from 3 experiments are presented (50 random oligomers were measured per n, in instances where there were less than 50 oligomers then all observable oligomers were measured). n=2 (B & D) or n=3 (A & C). (F) Data shown in (E) represented as a % of total observations by size bracket.
Figure 4.5 Amylin monomer and oligomer species phosphorylate Fyn

(A) NB7 cells were treated with vehicle, 500nM amylin monomer or oligomer preparations for 20min at 37°C. Cell lysates (50μg total protein) were resolved by 12% acrylamide SDS-PAGE and immunoblotted for Fyn or pY416 Src antibody. Membranes were then stripped and re-probed for β-actin (AC15).

(B) Semi-quantitative densitometry of chemiluminescence following immunoblotting. Data are expressed as the ratio of pY416 Src to total Fyn normalised to vehicle control represented as a percentages (n = 4 biological replicates with at least 2 experimental replicates in each). Mean ± SEM. *, p=<0.05, statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%. Differences between treatment groups were analysed by unpaired student’s t-test. #, p<0.05.
4.2.2 Amylin causes activation of Fyn kinase

Fibrillar OC reactive and high molecular weight ADDL preparations have been demonstrated to elicit toxic downstream effects through cell surface receptors. A high affinity cell surface receptor, and major determinant of ADDL induced toxicity, is PrP<sup>C</sup> (Laurén et al. 2009; Um et al. 2013; Rushworth et al. 2013; Kostylev et al. 2015b). The studies listed above have demonstrated Aβ<sub>0</sub> bind to PrP<sup>C</sup> in lipid raft domains to stabilise a receptor complex comprising of mGluR5 dimer, LRP1 and Fyn (Laurén et al. 2009; Um et al. 2013; Rushworth et al. 2013). Binding to PrP<sup>C</sup> on the outer leaflet of the membrane causes this complex to initiate Fyn activation, which is associated with NMDAR internalisation and neuronal toxicity (Um et al. 2012).

Our lab has previously demonstrated that the Aβ<sub>0</sub> preparation binds to a PrP<sup>C</sup>-mGluR5-LRP1 receptor complex and results in Fyn phosphorylation at tyrosine position 416 (pY416) in NB7 neuroblastoma cells and rat primary hippocampal neurons (Rushworth et al. 2013). We hypothesised that due to the similar tertiary structure of amylin oligomers, that amylin will also bind to PrP<sup>C</sup> and phosphorylate Fyn. This hypothesis was tested using the NB7 neuroblastoma cell line, which our lab has demonstrated to express PrP<sup>C</sup> in lipid rafts, as well as the mGluR5 dimer and LRP1 (Rushworth et al. 2013).

Treatment of NB7 cells with 500nM amylin monomer (AMYm) or oligomer (AMYo) preparation caused a 60% increase in Fyn phosphorylation in the NB7 model compared to vehicle control (Figure 4.5). The densitometry from the pY416 Fyn immunoblotting was corrected to the densitometry for the total Fyn antibody then made relative to the vehicle control in this and all future figures. Equal protein (50μg) was loaded following assessment of protein concentration by BCA (2.4.3), demonstrated by equal actin bands (Figure 4.5A). The activation of Fyn by 500nM amylin treatment is twice that previously reported for 500nM Aβ<sub>0</sub> treatment in this model (Um et al. 2012; Rushworth et al. 2013). Our results show that AMYm is capable of activating Fyn to the same degree as the AMYo preparation, suggesting that species common to both preparations are activating Fyn. We then investigated whether amylin mediated this effect through PrP<sup>C</sup>.

In addition to these experiments we performed preliminary experiments with a non-aggregating amylin analogue - pramlintide - which did not cause Fyn activation compared to the AMYm preparation (Data not shown, n=1). This suggests that aggregation capacity and secondary structure is important to amylin mediated Fyn activation and that some of the smaller oligomeric species either mediate this effect directly or aggregate during the treatment procedure to create PrP<sup>C</sup> interacting species.

126
Figure 4.6 Effect of Phospholipase-C pretreatment on amylin induced Fyn phosphorylation

Continued on next page
Figure 4.6 Effect of Phospholipase-C pretreatment on amylin induced Fyn phosphorylation (continued)

NB7 cells were pretreated with 0.2U Phospholipase-C or Vehicle for 2h at 37°C prior to 500nM amylin monomer treatment for 20min. Cell lysates (50μg) were resolved by reducing glycine SDS-PAGE and immunoblotted for Fyn or pY416 Src antibody (A), or blotted for PrP^C (6D11 antibody) (B). Membranes were then stripped and re-probed for β-actin. (C) Semi-quantitative densitometry following immunoblotting. Data are expressed as the ratio of pY416 Src to total Fyn normalised to buffer control represented as a percentage (n = 4). Mean ± SEM. *, p<0.05, statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%. Differences between treatment groups were analysed by RM one-way ANOVA with Holm-Sidak post hoc analysis. #, p<0.05, ##, p<0.01. (D) Semi-quantitative densitometry of PrP^C following immunoblotting. Data were normalised to no-PLC controls and presented as a percentage (n =3). Data represent mean ± SEM. *, p<0.05 by Student’s t-test with Welch’s correction.
Figure 4.7 Effect of 6D11 pretreatment on amylin induced Fyn phosphorylation.

(A) NB7 cells were pre-treated with 6D11 or Vehicle for 20 min prior to 500nM amylin monomer or oligomer preparations for 20min at 37°C. Cell lysates (50μg total protein) were resolved by reducing glycine SDS-PAGE and immunoblotted for Fyn or pY416 Src antibody. Membranes were then stripped and re-probed for β-actin (AC15). (B) Semi-quantitative densitometry of chemi-luminescence following immunoblotting. Data are expressed as the ratio of pY416 Src to total Fyn normalised to buffer control represented as a percentages (n = 4 biological replicates). Mean ± SEM. *, p<0.05, statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%. Differences between treatment groups were analysed by one-way ANOVA with Holm-Sidak post hoc analysis.
PrPc is attached to the outer leaflet of the membrane by a glycosylphosphatidylinositol (GPI)-anchor (Vey et al. 1996; Madore et al. 1999). This GPI-anchor clusters PrPc into cholesterol and sphingolipid enriched domains called lipid rafts (Taylor and Hooper 2007). Treatment with exogenous phosphatidylinositol-specific phospholipase-C (PLC) cleaves the GPI anchor and sheds PrPc into the media (Caughey and Raymond 1991). We performed a 2h incubation of NB7 cells with PLC prior to treatment with AMYm, performed as in Figure 4.5. PLC treatment significantly reduced AMYm induced Fyn activation (Figure 4.6).

As expected 500nM AMYm treatment caused a significant increase in Fyn phosphorylation at pY416, this was significantly reduced following PLC treatment, with no independent effect of PLC treatment on basal Fyn phosphorylation (Figure 4.6A & C). PLC treatment caused a 40% reduction in lysate PrPc (Figure 4.6B & D), but it is unclear what proportion of this is at the cell surface as opposed to internalised stores. This figure demonstrates that an intervention to promote the shedding of PrPc reduces amylin induced Fyn phosphorylation, suggesting amylin interacts with the cell in a PrPc dependent manner to induce Fyn activation. We further investigated this using the 6D11 antibody. This antibody targets the 93-113 amino acid sequence of PrPc and has been demonstrated in multiple studies to interfere with AβO binding and prevent AβO induced Fyn activation in neuronal (Um et al. 2012; Um and Strittmatter 2013; Rushworth et al. 2013) and neuroblastoma cell models (Rushworth et al. 2013).

Pre-treatment with 6D11 for 20min prior to treatment with AMYm or AMYo did not cause any significant reduction in Fyn activation (Figure 4.7). We observed an interesting but non-significant reduction in AMYm propensity to activate Fyn following 6D11 blockade that was not observed for the AMYo preparation. This indicates that these two preparations may be interacting with PrPc at different regions to illicit Fyn activation. However, it should be noted that in these experiments we did not observe a significant increase in Fyn phosphorylation in the AMYm treatment. This is likely due to inter-lot variability of the pY416 Fyn antibody and highlights the need for further validation of this mechanism.

Together these data provide preliminary evidence of a novel downstream signalling pathway for amylin and shared cell surface receptor for Aβ and amylin oligomer species.

4.2.3 Fyn kinase activation in iPSC derived neurons

We wished to further characterise amylin mediated activation of Fyn by utilising induced pluripotent stem cell (iPSC)-derived cortical neurons. iPSC-derived neurons offer huge potential to recreate the underlying cellular conditions in which the neurodegenerative cascades described above occur. Neurons derived from patients with familial AD mutations have demonstrated an ability to faithfully recapitulate pathology (Israel et al. 2012; Koch et al. 2012; Woodruff et al. 2013; Muratore et al. 2014). However, there has been considerably
less success modelling pathological changes in neurons derived from patients with sporadic AD, which represents the vast majority of pathological cases, discussed in Wray (2017). In addition to this, we wanted to establish evidence that Aβ0 can induce Fyn phosphorylation in the iPSC derived neuron model. We used a control patient derived iPSC line (OX1-19) to generate cortical neurons. Using a control patient line minimises potential genetic predisposition to AD pathology and better enables us to place our data in the context of sporadic AD. These neurons were generated as described in 2.1.2 and based on a published protocol (Shi et al. 2012).

Our group has previously demonstrated that the OX1-19 iPSC-derived cortical neurons express PrPc, the mGluR5 dimer and LRP1 (Noble 2016). Equally important to the expression of these receptor complex components is the localisation of PrPc to lipid rafts and enrichment in synaptic densities (Jarosz-Griﬃths et al. 2016). When investigated in a SH-SY5Y cell model stably over-expressing PrPc by immunohistochemistry, PrPc had a punctate proﬁle along the cell surface membrane; this is demonstrated in Figure 4.8A. We then investigated this in day 50 post induction iPSC derived neurons (Figure 4.8B). Immunochemistry using the Saf32 anti-PrPc antibody generated a similar punctate reactivity proﬁle in the soma and axonal projections of iPSC derived neurons (Figure 4.8C & D). There also appeared to be an enrichment of PrPc in the dendritic spines and synaptic densities. This punctate pattern of immunoreactivity in Figure 4.8 suggests that PrPc is enriched in lipid rafts in iPSC-derived neurons as is the case in SH-PrPc neuroblastoma cells and rat primary hippocampal neurons (Rushworth et al. 2013). This possibility warrants further investigation using lipid raft isolation techniques (Macdonald and Pike 2005).

Treatment of day 50 iPSC-derived cortical neurons with Aβ0 caused a small but signiﬁcant increase in Fyn phosphorylation when corrected for total Fyn (Figure 4.9). In this model 6D11 pretreatment did not prevent Aβ0 induced Fyn phosphorylation. In some cases, we observed 6D11 cross linking with PrPc to independently activate Fyn (data not shown), as has previously been reported (Solforosi et al. 2004). We observed no such activation following treatment with the Aβm preparation (Figure 4.9). These data demonstrate Aβ0 activation of Fyn in iPSC derived human neurons, and that amylin treatment of the neurons caused a significant and large increase in Fyn phosphorylation, particularly following AMYm treatment (Figure 4.10). There was slightly more variability in the AMYo treatment group, which prevented the treatment from being signiﬁcantly increased. The AMYo treatment was not signiﬁcantly different from the AMYm treatment group by a student’s t-test. Interestingly, in the cortical neuron model amylin treatment once again caused greater Fyn activation than Aβ0 treatment.
Figure 4.8 PrP<sup>C</sup> expression in iPSC derived cortical neurons

(A) Representative image of SH-SY5Y cells stably overexpressing PrP<sup>C</sup> demonstrating the punctate staining pattern of PrP<sup>C</sup>. Image captured by highend wide-field immunofluorescence microscopy. Blue = DAPI nuclear stain, Green = Saf32 (PrP<sup>C</sup>). (B) Light microscopy of day 50 iPSC derived cortical neurons. Image captured at 10x magnification. Representative images of day 50 iPSC derived cortical neurons demonstrating the same punctate expression pattern of PrP<sup>C</sup> in the soma and dendrites (C) and axonal projections (D). Blue = DAPI, Green = Saf32 (PrP<sup>C</sup>), Red = MAP2 (somatodendritic marker). Scale bars = 10µm.
Figure 4.9 Aβ0 induces Fyn phosphorylation in OX1-19 neurons.

(A) Day 50 OX1-19 neurons were incubated with 1μM AβM or AβO preparations for 20min at 37°C. Cell lysates (50μg total protein) were resolved by 12% acrylamide reducing SDS-PAGE and immunoblotted for Fyn or pY416 Src antibody. Membranes were then stripped and re-probed for β-actin (AC15). (B) Semi-quantitative densitometry of chemiluminescence following immunoblotting. Data are expressed as the ratio of pY416 Src to total Fyn normalised to buffer control represented as a percentages (n = 4 biological replicates with at least 2 experimental replicates in each). Mean ± SEM. **, p<0.01, statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%. Differences between treatment groups were analysed by RM one-way ANOVA with Holm-Sidak post hoc analysis. ##, p<0.01; ns = not significant.
Figure 4.10 Amylin induces Fyn phosphorylation in OX1-19 neurons.

(A) Day 50 OX1-19 neurons were incubated with 500nM amylin monomer or amylin oligomer preparations for 20min at 37°C. Cell lysates (50μg total protein) were resolved by reducing glycine SDS-PAGE and immunoblotted for Fyn or pY416 Src antibody. Membranes were then stripped and re-probed for β-actin (AC15). (B) Semi-quantitative densitometry of chemi-luminescence following immunoblotting. Data are expressed as the ratio of pY416 Src to total Fyn normalised to buffer control represented as a percentages (n = 3 biological replicates with at least 2 experimental replicates in each). Mean ± SEM. *, p<0.05, statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%.
4.2.4 Tau phosphorylation in iPSC-derived neurons

Soluble oligomeric forms of Aβ illicit toxicity by initiating a cascade of downstream signalling events that result in tau hyper-phosphorylation, removal from microtubules and eventual aggregation (Hardy and Allsop 1991). There is a growing school of thought that suggests Aβo initiates AD by creating soluble hyper-phosphorylated tau aggregates that are extremely neurotoxic and have the propensity to spread between neurons (Bloom 2014). This mechanism is very interesting and is supported by the spread of tau misfolded species between neurons in a manner that infers vulnerability and correlates with Braak staging (Kaufman et al. 2016; Wu et al. 2016; Furman et al. 2017).

Neuroblastoma models and hippocampal neurons isolated from rats are poor models of complex neurodegenerative disease. In particular these models poorly represent the complex signalling cascades downstream of Aβo resulting in tau phosphorylation (Stancu et al. 2014). As such we decided to employ human iPSC-derived cortical neurons to investigate Aβo induced tau phosphorylation. Thus far studies utilising iPSC neurons derived from sporadic AD patients have proved variable and poorly replicated AD markers (Israel et al. 2012; Kondo et al. 2013). As such we designed our experiments to utilise neurons from control non-demented individuals and tested the ability of Aβo to induce a key AD pathology - phosphorylation of tau. We performed a number of treatments attempting to induce tau phosphorylation in iPSC derived cortical neurons from the cognitively normal patient line OX1-19.

Treatment of neurons with Aβo caused a non-significant dose dependent increase in tau phosphorylation following Aβo treatment in d76-80 neuronal culture. We investigated Y18, T181, S202 and PHF1 phosphorylation and expressed our data as a ratio to total tau detectable by K9JA C-terminal tau antibody (Figure 4.11). These are but a few of many sites abnormally phosphorylated in AD; sites of tau phosphorylation and known kinases are listed by Diane Hanger’s lab (2017) (http://cnr.iop.kcl.ac.uk/hangerlab/tautable). Only in the case of Y18 phosphorylation was the Aβo induced increase in phosphorylation significantly different from Aβm treatment (Figure 4.11B). We observed a consistent but non-significant decrease in tau phosphorylation following pre-incubation with the 6D11 antibody. In all cases blots were stripped and re-probed for actin to demonstrate equal protein loading; only the actin band from the K9JA blot is presented (Figure 4.11A). In addition to the presented results, we also investigated phosphorylation status of several kinases implicated in downstream signalling and tau phosphorylation - Pyk2, CamKII and GSK3β - in the lysates. We found expression and phosphorylation status of these kinases to be highly variable between neuronal inductions, and even between wells of a plate (n=3 data not shown). In addition to this we explored acute Aβo treatments and elongated treatments in less mature day 50 neurons where we saw no increase in tau phosphorylation.
following 6h or 24h treatment (n=2 in both cases, data not shown). These data provide a platform for creating a model of the downstream kinase cascades involved in Aβ induced tau phosphorylation in iPSC-derived neurons. Our observations of more promising resulting following neuronal maturation also provide support to previous reports suggesting increasing neuronal maturation creates a better model for reproducing pathology (Bergstrom et al. 2016).
Figure 4.11 Tau phosphorylation in OX1-19 neurons.
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Figure 4.11  Tau phosphorylation in OX1-19 neurons (continued).

(A) Day 76-80 OX1-19 neurons were incubated with 1μM Aβ42 or Aβ40 preparations for 24h at 37°C. Cell lysates (20μg total protein) were resolved by reducing glycine SDS-PAGE and immunoblotted for K9JA or phospho-tau (pY18, pT181, pS202, PHF1). Membranes were then stripped and re-probed for β-actin (AC15). (B-E) Semi-quantitative densitometry of chemi-luminescence following immunoblotting. Data are expressed as the ratio of phospho-tau to total tau normalised to buffer control represented as a percentages (n= 3 biological replicates with at least 2 experimental replicates in each). Data represent the mean ± SEM. Statistical difference from control was determined by one-sample t-test versus hypothetical value of 100%. Differences between treatment groups were analysed by RM one-way ANOVA with Holm-Sidak post hoc analysis. #, p<0.05.
4.3 Discussion

4.3.1 Characterising oligomers

All methods commonly used to characterise oligomeric species have caveats (Bitan et al. 2005; Hepler et al. 2006; Benilova et al. 2012; Watt et al. 2013). Therefore a robust oligomer preparation should be characterised by multiple methods prior to treatment before data from cell treatments can be properly interpreted. We employed SDS-PAGE, dot blot assay, ThT assay and AFM to characterise our Aβ0 preparation (Figures 4.1 & 4.2). Our synthetic Aβ1-42-LC-biotin oligomer preparation generates a spread of oligomers, reactive with the OC antibody with an average height of 6nm as expected from previous publications on this and similar preparations (Lambert et al. 1998; Chromy et al. 2003; Laurén et al. 2009; Rushworth et al. 2013). Previous studies have established a common nomenclature to better describe the wide variety of oligomer species generated in multiple buffers (Lesne et al. 2006). This nomenclature describes two types of oligomer centred on reactivity with the A11 (type 1) and OC (type 2) structural antibodies, which recognise out-of-register anti-parallel and in-register parallel β-sheets respectively (Hatami et al. 2014). By this nomenclature, our protocol generates type 2 stable soluble oligomers.

A comparable incubation protocol to ours, using TBS in place of Ham’s F-12, characterised Aβ1-42 oligomers by SDS-PAGE, AFM and dot blot to demonstrate oligomers formed in TBS form a spread of oligomeric species, which are of a comparable size by AFM but are reactive with the A11 antibody rather than the OC antibody (Cerf et al. 2009). A11 reactive oligomers are less able to bind PrPc and activate Fyn; highlighting the importance of buffer conditions and characterisation.

SDS-PAGE separation of synthetic Aβ oligomer preparations is liable to artefactual stabilisation of small oligomer formation (dimer, trimer and tetramer) (Bitan et al. 2005; Hepler et al. 2006; Watt et al. 2013). As a result of this the dimer, trimer and tetramer bands observable in the monomer and oligomer preparations following SDS-PAGE (Figure 4.1) cannot be reliably quantified. Artefactual dimer formation in the presence of SDS will also occur in thioflavin-S only containing preparations (Hatami et al. 2014) and implies a common mechanism of SDS-micelle stabilisation that may cast doubt over previous small amylin oligomers observed by SDS-PAGE (Jackson et al. 2013).

We characterised amylin aggregation by ThT and AFM (Figures 4.3 & 4.4). These data demonstrate a timeframe for the isolation of oligomer species (3-4h) and fibrils (4h+) in our chosen buffer conditions (2mM ammonium acetate / Ham’s F-12). Small oligomer species averaging between 2-4nm were prevalent both before oligomer formation and after fibril formation by AFM (Figure 4.4E & F). This implies that these species are either constantly forming from the monomer pool and are not incorporated into the oligomeric
and fibril species of amylin in this buffer or alternatively, these species may represent small nucleation oligomers, which can elongate existing fibrils (Berhanu and Masunov 2014). Interestingly, a similar background level of small oligomer species before oligomer formation and after fibril formation was observed in our Aβ aggregation timeframe experiments by AFM (data not shown). In addition to the ThT and AFM data presented, we also attempted characterisation of amylin oligomer species by SDS-PAGE immunoblotting with multiple amylin antibodies (T-4150, H-017-03 and Biorad amylin antibody). We found no or little reactivity of synthetic amylin with these antibodies, only detecting monomeric amylin species (data not shown). This is common to analyses of amylin oligomers by western blot (Trikha and Jeremic 2011) and oligomer species are often only detected following SDS-PAGE using the A11 conformational antibody. Our amylin oligomer preparation demonstrated no reactivity with the A11 or OC conformational antibodies following western blot or dot blot assays (data not shown).

Differences in the nature of our amylin aggregate species may be a result of multiple factors associated with the aggregation buffer or protocol. For example, many other amylin studies use PBS for aggregation, where they observed a shorter lag phase and A11 positive oligomers (Kayed and Glabe 2006; Kayed et al. 2007). The use of PBS means high salt concentration in the buffer, which will reduce lag phase and promote aggregation, as has been experimentally demonstrated (Young et al. 2014). Furthermore, low pH conditions commonly used (Young et al. 2014; Young et al. 2015a; Young et al. 2015b), extended lag phase and lower overall fibril formation detectable by ThT fluorescence (Jha et al. 2014). This is consistent with the acidic environment of the secretory granule, which prevents amylin aggregation (Jha et al. 2014; Brender et al. 2011). These observations serve to demonstrate that the conditions used to control amylin aggregation can have large effects on aggregation, making comparisons between research groups problematic.

We have succeeded in isolating a timepoint rich in amylin oligomer species detectable by AFM (Figure 4.4C & E). Our data also suggest that these oligomers are ‘on pathway’ oligomeric species that form insoluble aggregates that can later be removed by centrifugation, demonstrated by the absence of such species following 24h aggregation (Figure 4.4D & E). Our characterisation is limited as we do not have information of secondary structure or oligomer stability in solution. Previous studies utilising Aβ suggest oligomer species exist in a highly dynamic equilibrium, and isolation of a particular oligomer species by size exclusion chromatography will result in re-establishment of this equilibrium of numerous oligomer species (Hepler et al. 2006; Benilova et al. 2012). The principle of dynamic equilibrium is likely to also be true for amylin oligomerisation, and therefore understanding the fate of the oligomer preparation following addition to cells is extremely difficult.
4.3.2 Amylin activates Fyn kinase, potentially through PrP<sup>C</sup>

Aβ oligomer binding to PrP<sup>C</sup> stabilised receptor complexes in lipid rafts and has been demonstrated to relate to aberrant tau phosphorylation, neuronal excitability and LTP impairment via NMDAR dysregulation and correlated with cognitive decline in certain murine models and human AD patients (Laurén et al. 2009; Larson et al. 2012; Um et al. 2012; Kostylev et al. 2015a). These factors make Aβ<sub>O</sub> binding to PrP<sup>C</sup> and downstream signalling a key component of AD pathogenesis.

PrP<sup>C</sup> is localised to cholesterol-rich lipid raft domains. Following Aβ<sub>O</sub> application it acts as a scaffolding protein to stabilise a receptor complex with mGluR5, LRP1 and Fyn (Um et al. 2012; Um et al. 2013; Um and Strittmatter 2013; Rushworth et al. 2013). Fyn is an Src family kinase enriched in the lipid raft membrane regions of post synaptic densities (Williamson et al. 2008; Suzuki and Okumura-Noji 1995), where it plays a crucial role in regulating LTP via phosphorylation of the NMDAR subunits NR2A and NR2B (Suzuki and Okumura-Noji 1995; Nakazawa et al. 2001; Abe et al. 2005; Chen and Roche 2007). Increased phosphorylation at these sites promotes NMDAR internalisation (Roche et al. 2001). Furthermore, Fyn has been demonstrated to be a key mediator of AD pathology. Over expression of Fyn in AD transgenic animals models results in augmented pathology, which was rescued by genetic knockdown or pharmacological inhibition of Fyn (Chin et al. 2005; Kaufman et al. 2015). Fyn activation following Aβ<sub>O</sub> binding has also been demonstrated to promote tau phosphorylation at Y18 (Lee et al. 1998; Lee et al. 2004), this phospho-epitope was observed to be up regulated in AD post-mortem brain tissue (Lee et al. 2004; Larson et al. 2012). This is coupled with observation of PrP<sup>C</sup>-Fyn interaction in human AD brain tissue (Larson et al. 2012). Fyn inhibition reverses synaptic deficits, memory deficits and hyperphosphorylated tau in murine models (Kaufman et al. 2015) and is currently being explored as a therapeutic approach in AD (AZD0530 (Saracatinib) ClinicalTrials.gov ID: NCT01864655). These data implicate Fyn as a key mediator of neuronal impairment in AD.

Several groups have demonstrated that Aβ<sub>O</sub> bind to PrP<sup>C</sup> to cause Fyn phosphorylation (Um et al. 2012; Um et al. 2013; Rushworth et al. 2013; Kaufman et al. 2015). Work in our lab demonstrated the Aβ<sub>O</sub> preparation used in the present study (Figure 4.1 & 4.2) activates Fyn in NB7 cells (Rushworth et al. 2013). We provide evidence that amylin monomer and oligomer species activate Fyn in the NB7 neuroblastoma model (Figure 4.5) and in iPSC derived cortical neurons (Figure 4.10). Amylin induced Fyn activation was ameliorated by externally applied PLC to induce the shedding of PrP<sup>C</sup> (Figure 4.6). Therefore, peripheral or localised amylin up-regulation in AD or T2D (Figure 3.16) may contribute to the pathogenic downstream signalling through PrP<sup>C</sup>. A prerequisite for this pathogenic mechanism is the localisation and association of amylin and PrP<sup>C</sup> in lipid rafts.
Cholesterol-rich lipid raft domains have been demonstrated to be critical in amylin association with the cell surface lipid bilayer (Cho et al. 2008; Cho et al. 2009; Wakabayashi and Matsuzaki 2009; Trikha and Jeremic 2011; Seeliger et al. 2012). Amylin associates with cholesterol-rich domains, where it becomes enriched and promotes aggregation, in particular the formation of large fibrillar oligomers (Cho et al. 2009; Brender et al. 2012; Singh et al. 2015). Externally applied amylin monomeric and oligomeric species have been demonstrated to co-localise with lipid raft domains in PC12 cells (Trikha and Jeremic 2011; Trikha and Jeremic 2013). A punctate pattern of binding, reminiscent of the staining pattern for PrP<sup>C</sup> demonstrated in Figure 4.8, was also observed in this model. Amylin localisation at lipid rafts as demonstrated in PC12 cells is abolished by addition of the cholesterol depleting agent β-cyclodextrin and lovastatin, a cholesterol biosynthesis inhibitor (Trikha and Jeremic 2011). Interestingly, this did not abolish amylin binding to the membrane, rather, the removal of lipid raft rich domains impaired amylin internalisation and increased surface amylin, promoting cell death (Trikha and Jeremic 2011). Soluble amylin monomers and oligomers localised to lipid rafts were internalised by either receptor-mediated endocytosis or pinocytosis (Trikha and Jeremic 2013). Amylin endocytosis was not completely blocked by AC187 or AC253, which are inhibitors of native amylin receptors, implying the presence of other neuronal surface receptors to mediate amylin internalisation (Jhamandas and Mactavish 2012). This is interesting, given the demonstrated role of PrP<sup>C</sup> in Aβ internalisation (Pflanzner et al. 2012). Together these data provide strong evidence that amylin monomeric and oligomeric species cluster at lipid raft domains, where they are subsequently internalised and targeted for proteasomal and/or lysosomal degradation.

These data demonstrate that both PrP<sup>C</sup> and amylin localise to lipid raft domains (Walmsley et al. 2003; Trikha and Jeremic 2011). We attempted to confirm this co-localisation by immunofluorescence microscopy for amylin and PrP<sup>C</sup> in SH-SY5Y-PrP<sup>C</sup> and iPSC derived neuronal models (data not shown). This was unsuccessful due to an intolerably high background from the amylin treatment. However, there is some circumstantial evidence to suggest amylin-PrP<sup>C</sup> co-incidental localisation results in aberrant downstream signalling. Treatment of WT mouse cholinergic forebrain neuronal cultures with Aβ oligomers results in reduction in whole cell outward currents (Alier et al. 2011). This was rescued by the addition of an anti-PrP<sup>C</sup> antibody, attenuated in Prnp<sup>+/−</sup> animal neuronal cultures and absent from Prnp<sup>+/−</sup> cultures, demonstrating disruption of neuronal activity was dependent on the PrP<sup>C</sup> interaction (Alier et al. 2011). Of great interest, Alier et al. (2011) built on previous work demonstrating that amylin addition can also impair whole cell currents (Jhamandas et al. 2001; Jhamandas et al. 2003) by comparing amylin-induced impairment of neuronal function in WT and Prnp<sup>+/−</sup> neuronal cultures. Amylin treatment caused a marked decrease in neuronal whole cell currents in the WT neuronal population and no change in the Prnp<sup>−/−</sup> cultures (Alier et al. 2011). Alier et al. (2011) demonstrated amylin-induced impairment
of neuronal function is dependent on PrP\textsubscript{C} expression. In light of these data and our evidence demonstrating amylin activation of Fyn, preventable through removal of GPI anchored surface proteins, there is evidence to suggest that PrP\textsubscript{C} plays a central role in mediating amylin induced toxic downstream signalling.

The nature of this role is currently unclear, but possible mechanisms of action can be inferred from previous studies of Aβ-PrP\textsubscript{C} binding and signalling. There appears to be two potential mechanisms, which are not mutually exclusive, whereby PrP\textsubscript{C} may mediate amylin downstream toxic signalling - direct binding to PrP\textsubscript{C} or binding to PrP\textsubscript{C} stabilised complexes. As described above, Aβ oligomer application causes lipid raft domain localised PrP\textsubscript{C} to stabilise distinct receptor complexes resulting in Fyn activation and NMDAR dysregulation (Um et al. 2012; Larson et al. 2012; Um et al. 2013; Rushworth et al. 2013). Aβ binding appears to occur in charged clusters and hydrophobic regions within the PrP\textsubscript{C} N-terminal sequence (Laurén et al. 2009; Resenberger et al. 2011). This is due to hydrogen bonding between the hydrophobic chains of the β-sheet rich oligomer species and the N-terminal sequence of PrP\textsubscript{C}. Interestingly, experiments demonstrating this principle have shown that only a β-sheet rich conformation is required for binding to PrP\textsubscript{C} and causing downstream signalling, therefore this mode of binding has the potential to be common to other β-sheet rich peptides (Resenberger et al. 2011). This finding complements separate studies demonstrating that β-sheet rich OC reactive oligomers best correlate with PrP\textsubscript{C} binding species (Nicoll et al. 2013; Rushworth et al. 2013). Multiple models of amylin aggregation demonstrate the propensity of amylin monomers and oligomers to form β-sheet rich species, and OC positive species (Kajava et al. 2005; Engel 2009; Pillay and Govender 2013; Cao et al. 2013b; Hatami et al. 2014). Our experiments with 6D11 (Figure 4.7) would suggest that amylin activation of Fyn does not occur through binding to the same N-terminal region of PrP\textsubscript{C} as it does with Aβ oligomer preparations (Laurén et al. 2009). Interestingly, these data suggest that 6D11 can block amylin monomer induced Fyn activation, but not preformed oligomer induced Fyn activation (Figure 4.7). This may be due to binding to PrP\textsubscript{C} at another site or through PrP\textsubscript{C}-independent activation.

Together the discussed data highlight the potential for amylin to localise to lipid rafts and rapidly generate the β-sheet conformation or OC complimentary motif necessary for PrP\textsubscript{C} binding and Fyn activation (Figures 4.5 & 4.10). This demonstrates the potential for amylin to contribute to pathogenic downstream signalling in AD and warrants the investigation of the involvement of PrP\textsubscript{C} and Fyn kinase in the progressive loss of β-cells in the islets of Langerhans in T2D.
4.3.3 Downstream signalling in iPSC derived neuronal cultures

Our lab has previously demonstrated our Aβ̄₀ preparation causes Fyn activation in NB7 cells in a manner dependent on PrP\(^\text{C}\) and preventable with pre-incubation with the 6D11 antibody (Rushworth et al. 2013). We wished to further investigate this mechanism in iPSC-derived cortical neurons. We provide evidence of Fyn activation by the Aβ̄₀ preparation, but not the Aβ̄ₘ preparation, in day 50 cortical neurons (Figure 4.9). The day 50 time point was used because this is the earliest time point where the pluripotency markers NANOG and OCT4 are absent, and the cortical layer III markers SATB2, β-III tubulin and MAP2 are present (based on Shi et al. (2012), these markers were regularly verified in house (data not shown)). This activation was not blocked by the 6D11 antibody in this model, potentially due to batch dependent 6D11 crosslinking of PrP\(^\text{C}\) activating downstream signalling - as has previously been reported (Solforosi et al. 2004; Klohn et al. 2012). Pyk2 and CamKII have been demonstrated to be downstream of PrP\(^\text{C}\)-mGluR5 receptor complex signalling (Haas and Strittmatter 2016). Investigation of Aβ̄₀ induced activation of these kinases in this model by western blot found kinase expression and phosphorylation to be highly variable even in untreated replicates despite equal protein loading (data not shown). This was also true for GSK3\(\beta\) phosphorylation at Serine 9 (data not shown). This is likely due to the complex regulation of kinases, kinase involvement in synaptogenesis and cell morphology regulation and the high state of flux of the neurons at this time point (Ivankovic-Dikic et al. 2000; Sutherland 2011; Gambrill and Barria 2011; Bergstrom et al. 2016).

This variability in individual kinases limited our ability to study specific kinase cascades; as a result we chose to focus on the end point of kinase dysregulation by Aβ̄₀ - tau phosphorylation. Investigation of Aβ̄₀ induced tau phosphorylation at multiple epitopes (Y18, T181, S202 and S396/S404) in day 50 iPSC-derived cortical neurons found a high level of variability in cell responses at several incubation timepoints (data not shown). This supports previous reports of variability in tau phosphorylation status at T181 and S202 in iPSC-derived neurons of a comparable maturation (Nieweg et al. 2015). We concluded this was a result of increased aberrant kinase activity in the immature neurons, and decided to repeat these experiments in more mature neurons.

iPSC derived cortical neurons generated using a comparable protocol to that used in the present study were demonstrated to undergo extensive neurite outgrowth and remodelling between days 49-59 (Bergstrom et al. 2016). As a result of this we chose a timepoint after this expansion and around the emergence of electrophysiological activity (Shi et al. 2012; Bergstrom et al. 2016). Treatment of day 80 cortical neurons with Aβ̄₀ caused increases in tau phosphorylation that were not significantly increased from control across all phosphorylation epitopes (Figure 4.11). In each instance Aβ̄₀ treatment increased phosphorylation in a dose-dependent manner and more so than Aβ̄ₘ treatment.
Furthermore, 6D11 pre-treatment of the neurons appeared to have blocked tau phosphorylation at most epitopes studied. The nature of the induced effect was continuous across experimental repeats, but the magnitude of Aβ1-42 induced tau phosphorylation was very variable, with responses varying from a 30%-200% increase in PHF1 phosphorylation between inductions - accounting for the lack of significance (Figure 4.11). This result is likely down to the elevated basal tau phosphorylation, due to the foetal phenotype of the neurons even at this stage of maturation (Iovino et al. 2010) and continued kinase involvement in consolidation of neural networks until post day 100 (Bergstrom et al. 2016).

Alternative splicing of the MAPT gene and basal phosphorylation levels of tau are developmentally regulated. Early embryonic and foetal tau has an abnormally high level of basal phosphorylation compared to adult neurons and only the smallest 3 repeat variant is expressed (Hanger et al. 2009). Whereas adult neurons express 6 splice variants of tau with considerably reduced basal phosphorylation (Goedert et al. 1989). Neurons derived from iPSCs have a foetal phenotype, only expressing only the 3R isoform of tau with a high level of basal phosphorylation, rather than the 6 isoforms common to mature human neurons (Sposito et al. 2015). Cortical neurons derived from iPSC lines of patients with FAD mutations, reliably recapitulate pathology relevant to that mutation (Israel et al. 2012; Kondo et al. 2013; Muratore et al. 2014), but offer little insight to sporadic AD which represents the vast majority of cases.

Ideally, this experiment would be repeated at a later developmental stage, when expression of longer repeat tau isoforms begin to emerge (Iovino et al. 2010; Sposito et al. 2015) and basal phosphorylation levels of tau are more comparable to adult human neurons (Sposito et al. 2015). However, this is an expensive and time consuming ask, especially given the movement towards experiment replication over >4 inductions in >3 patient derived cell lines. These requirements highlight the potential benefits of cell senescence protocols which enable quicker turnover of neurons, and reduce environmental variables, such as seasonal temperatures, between induction treatments. Such protocols are beginning to emerge, but are yet to characterise the effect of accelerated maturation on the emergence of tau isoform expression or basal phosphorylation (Qi et al. 2017).
Work in iPSC-derived neurons allows for the study of specific disease mechanisms and processes, but suffers from variability between inductions which is intrinsic to aging models for such considerable periods of time. These problems will be overcome either by generation of cell senescence protocols, automation of neuronal induction and maintenance or expansion of treatment protocols and experimental powering to better reflect the variability inherent to aging separate cell populations for extended periods of time.

4.4 Chapter summary

In this chapter we provide robust characterisation of oligomers generated from synthetic Aβ and amylin. These oligomer preparations were used to test the hypothesis that Aβ and amylin oligomers share cell surface receptors and downstream signalling in cell models. Our data demonstrates that amylin, like Aβ, is a potent activator of Fyn in neuroblastoma cells and cortical neurons. We provide evidence to suggest this activation is mediated through PrP<sub>C</sub>; potentially with distinct modes of binding between our AMYm and AMYo preparations. Amylin activation of Fyn appears to be dependent on the ability to form oligomeric species, but is not exclusive to oligomers. These data provide a novel mechanism by which amylin up regulation in T2D or AD can contribute to a pathway of pathogenic signalling in AD. We also demonstrate that Aβ oligomers are capable of inducing Fyn activation in cortical neurons derived from control patient iPSCs, as has previously been demonstrated in human neuroblastoma cells and rat primary hippocampal neurons. Aβ<sub>O</sub> induced Fyn activation was not prevented by 6D11 antibody pre-incubation under our experimental conditions. Further experiments with alternative PrP<sub>C</sub> antibodies, lipid raft compromising agents such as methyl β-cyclodextrin, PrP<sub>C</sub> shedding agents such as PLC treatment or genetic knock down of Prnp may be employed to better understand the role of the Aβ<sub>O</sub>-PrP<sub>C</sub> interaction in control patient sourced iPSC-derived cortical neuronal models.

In addition to the above, we report the results of a preliminary characterisation of Aβ oligomer induced tau phosphorylation in control patient iPSC-derived cortical neurons. These experiments examine acute Aβ<sub>O</sub> treatment of neurons at different time points at various stages of maturity. We report that Aβ<sub>O</sub> treatment causes non-significant increases in tau phosphorylation, but that the degree of this phosphorylation was highly variable between neurons from different inductions. These increases were not present following pre-incubation with the 6D11 antibody. Investigation of several tau kinases revealed heterogeneous expression and activation between wells of the same plate. In addition to this, we also report that increased neuronal maturity provided more promising data, with day 80 neurons presenting with considerably more tau phosphorylation than day 50.
neurons. These data suggest mature iPSC-derived cortical neurons may be a useful model for Aβ0 induced tau phosphorylation, but that these experiments will require a higher power to account for the inherent variability of the model. Further maturation of the neurons to points of adult tau isoform expression, and/or the use of a chronic rather than acute Aβ0 treatment may further improve this model of AD pathology.

The previous chapter demonstrated the expression and deposition of amylin in AD (Figure 3.16), and the data in the present chapter demonstrates this amylin has the potential to contribute to the dysregulation of Fyn kinase, likely through a mechanism dependent on PrPc (Figures 4.5 & 4.10) which is a major pathogenic signalling pathway in AD models and key pathologic feature in AD post-mortem brain.
Chapter 5: Therapeutic potential of quercetin in Alzheimer’s disease

5.1 Introduction

5.1.1 Therapeutic targets in AD
The most widely accepted hypothesis of AD pathogenesis is the amyloid cascade hypothesis (Hardy and Selkoe 2002). This hypothesis posits that the amyloid-β (Aβ) initiates a cascade of events leading to tau phosphorylation, neuroinflammation and neurodegeneration. Assuming the amyloid cascade hypothesis is correct then the key therapeutic targets for the prevention of AD are: reducing amyloid production by decreasing BACE or γ-secretase activity, increasing non-amyloidogenic APP processing through α-secretase activation, preventing Aβ aggregation, and preventing Aβ oligomer binding to cell surface receptors. As the hypothesis supposes Aβ aggregation, deposition and actions of oligomeric species precede all inflammatory activation and tau pathology, this chapter will not address the growing therapeutic opportunities being discovered targeting neuroinflammation (Daniels et al. 2016; Brendel et al. 2017; Daria et al. 2017; Matos et al. 2017) or phosphorylated tau species (Novak et al. 2017). However, in practice a combination therapy that addresses each of these aspects will likely be required.

5.1.2 Comparing therapeutic strategies in AD
Genetic evidence demonstrating mutations that promote amyloidogenic processing of APP to cause early onset AD (Levy et al. 1990; Chartier-Harlin et al. 1991; Murrell et al. 1991; Mullan et al. 1992), or mutations that reduce such processing and have a protective effect (Jonsson et al. 2012) led to investment in APP targeting therapeutics for AD. On the whole these have been disappointing due to off-target effects, poor study design and limited bioavailability.

Therapies targeting BACE1 to reduce Aβ production have proved unproductive. A leading compound from Merck - verubecestat - recently had a phase II/III trial in mild-moderate AD cases halted after an early safety analysis found “virtually no chance of success” (Mullard 2017). Several other BACE1 inhibitors are currently in mid-late trials, but in general current BACE1 targeting therapies are dogged by pessimism. Therapies reducing Aβ production by modulating the γ-secretase complex have also proved very disappointing, mostly due to inhibition of other γ-secretase substrate cleavage events such as Notch cleavage. Clinical trials of γ-secretase inhibitors demonstrated reduced Aβ in the patient CSF, but this strategy
has ground to a halt following a recent phase III trial of Semagacestat being ended early due to significant cognitive decline and poor outcomes compared to controls (Doody et al. 2013).

Activators of α-secretase have less potential for toxicity than BACE1 and γ-secretase inhibitors, but have proved limited and non-specific. The most promising candidate is acitretin, a retinoid commonly used to treat psoriasis, which has been demonstrated to upregulate ADAM10 expression and activity, as well as reduce Aβ levels in APP/PS1 mice (Tippmann et al. 2009; Holthoewer et al. 2012). A pilot study in patients with mild AD demonstrated that acitretin treatment increased sAPPα levels in the CSF (Endres et al. 2014). These are promising early results, but the mechanism of action remains non-specific.

Multiple pharmaceutical companies have designed monoclonal antibodies to target Aβ species. These have demonstrated poor tolerance and limited therapeutic benefit (Sevigny et al. 2016). But do still represent the largest offset in disease progression so far achieved. This approach will most likely be useful early in the disease process, however it is unclear how often intervention may be required, and there are many concerns about potential adverse reactions to the treatment.

5.1.3 Small molecule inhibitors of Aβ aggregation

An alternative opportunity for targeting Aβ lies in preventing the formation of the oligomeric and aggregate species commonly hypothesised to initiate the cellular cascades that initiate and propagate the disease (Walsh et al. 2002; Cleary et al. 2005). Multiple strategies have been developed to achieve this, the most notable being synthetic glycosaminoglycans, metal chelating compounds, flavonoids and short peptide inhibitors.

Tramiprosate (3-amino-1-propanesulfonic acid) is a synthetic glucosaminoglycan. Glucosaminoglycans have been shown to bind Aβ and promote its aggregation into nontoxic aggregate species (Leveugle et al. 1994). It is to date the only aggregation inhibitor compound to go to clinical trials (Aisen et al. 2011) - producing disappointing results from a phase III trial that led to its discontinuation. Short peptide inhibitors are made by multiple strategies designed to interact with the sequence of an amyloid in such a way as to make individual monomers unavailable for incorporation into oligomers. Due to their peptidic nature these sequences require additional mechanisms of delivery to cross the BBB. One such method of generating peptide inhibitors is the use of retro-inverted sequences of the peptide sequence, as has been done for Aβ with the generation of RI-OR2 (Austen et al. 2008; Gregori et al. 2017). Peripheral treatment of RI-OR2 attached to a liposome delivery mechanism caused reduced Aβ deposition and observable oligomer species in the
APPswe/PS1ΔE9 mouse model of AD (Gregori et al. 2017). None of these peptide-based aggregation inhibitors are yet to make human clinical trials.

Resveratrol, commonly quoted as being enriched in red wine, was hailed as a promising flavonoid compound for AD treatment. Dietary resveratrol treatment causes reduced plaque burden in Tg19959 transgenic AD model mice (Marambaud et al. 2005) and reduced Aβ oligomer binding to cell models (Rushworth et al. 2013). However, clinical trials in humans proved a failure with significantly more Aβ in CSF and increased brain shrinkage following treatment (Turner et al. 2015). Epigallocatechin gallate (EGCG) represents a more promising flavonoid compound, prevalent in green tea. It has been demonstrated to reduce amyloidogenic APP processing, reduce Aβ deposition and promote ADAM10 mediated non-amyloidogenic APP processing in the APP-swedish mouse model of AD (Rezai-Zadeh et al. 2005). In addition to this, it has also been shown to force amyloids such as amylin, Aβ and tau into off-pathway non-toxic oligomer species (Ehrnhoefer et al. 2008).

As a result of these reports EGCG has been used as the lead compound for an 18 month phase II/III clinical trial in early AD patients (NCT00951834). While the data from this trial is eagerly awaited, a pilot study in patients with Down’s syndrome demonstrates flavonoid supplementation holds much promise. A 3 month treatment of 7-9mg/kg EGCG was well tolerated and resulted in lower cholesterol, LDL cholesterol and oxidised-LDL (de la Torre and Dierssen 2012). Additionally, individuals who received the EGCG intervention have improved performance in immediate and working memory tests. All of these measures returned to basal levels 3 months after discontinuation of treatment. These measures demonstrate high tolerance and compliance with the supplementation regimen which exemplifies flavonoids as a class of compounds for long term supplementation strategies.

Quercetin (Figure 5.1) is the most ubiquitous flavonol, with quercetin glycosides being present in most foods. Notable dietary sources of quercetin and quercetin glycosides are apple peel and onions (Lee and Mitchell 2012) but they are also present in alcoholic drinks made from fermented fruits such as red wine and cider (Pace-Asciak et al. 1995;Hayek et al. 1997;DuPont et al. 2002). Quercetin and its glycosides, particularly rutin (Figure 5.1), have been demonstrated to have anti-amyloidogenic properties against multiple amyloids. And as a result of compound screening for interaction with amylin performed by Prof. Garth Cooper, we have chosen to further investigate the therapeutic potential of quercetin and its glycoside rutin (quercetin-3-rutinoside).
Figure 5.1 Structures of quercetin and rutin
Molecular structures of the flavonoids quercetin (A), with labelled residues, and rutin (quercetin-3-O-rutinose) (B). Rutin is a quercetin glycoside, where quercetin undergoes the addition of the disaccharide rutinose group to the C3 position.

Amyloid deposition occurs 15-20 years before symptoms become apparent (Jack et al. 2010). Therefore, a preventative therapy targeting Aβ aggregation will likely be suggested to individuals in midlife with some existing amyloid aggregates and deposits. As such, experiments in the present study were designed to investigate the ability to prevent amyloid aggregation (Figure 5.2B) and investigate the effect of flavonoid treatment on preformed oligomers (Figure 5.2C).

Figure 5.2 Characterisation of Aβ oligomers and aggregation
(A) To make the control oligomer preparation (AβO), Aβ monomers were diluted in Ham’s F-12 and aggregated at room temperature for 16h – 20h before isolation of oligomeric species by centrifugation. (Figure legend continued on next page).
Figure 5.2 Characterisation of Aβ oligomers and aggregation (continued)

(B) To study the addition of quercetin at time 0 (Q_{OT}) Aβ monomers were diluted in Ham’s F-12 containing quercetin then aggregated and isolated as in (A). (C) To study the addition of quercetin to preformed oligomers (Q_{OP}) Aβ_{O} were prepared as in (A), the oligomer preparation was then diluted in Ham’s F-12 containing quercetin to the ratio described. In these experiments the control Aβ_{O} preparation was diluted to the same concentration in Ham’s F-12. The above description also applies to rutin addition at time-0 (R_{OT}) or to preformed oligomers (R_{OP}).

5.1.4 Aims

The aim of this chapter was to assess the therapeutic potential of the flavonoids rutin and quercetin in the context of AD. The effect of flavonoid treatment on Aβ oligomerisation and fibril formation was investigated by multiple methods. Due to the need to understand the effect of a treatment on existing aggregate species, the effect of flavonoids on preformed Aβ oligomers was also investigated. In addition to these outputs, the effects of flavonoid intervention to soluble oligomer induced cell responses, as well as downstream effects of flavonoid application to cells were investigated by multiple methods.
5.2 Results

5.2.1 Quercetin prevents the oligomerisation of Aβ

Prof. Garth Cooper conducted a screening of thousands of small molecules which yielded several lead compounds, including rutin and quercetin, as potential inhibitors of amylin aggregation (personal communication). Subsequently, further characterisation has demonstrated that rutin and quercetin can inhibit amylin aggregation in vitro and in vivo (Aitken et al. 2017b). We have expanded on this to characterise the propensity of rutin and quercetin to influence Aβ aggregation and Aβ oligomer stability. Due to the purported central role of oligomeric Aβ species in AD (Walsh et al. 2002; Hardy and Selkoe 2002; El-Agnaf et al. 2003; Cleary et al. 2005; Hardy et al. 2014; Spires-Jones and Hyman 2014), the effect of flavonoids on oligomer formation was focused upon.

Incubation with a range of rutin concentrations did not alter the oligomers formed by Aβ when added at time 0 (Rot) (Figure 5.3). Oligomer preparations resolved by SDS-PAGE and characterised by reactivity with the conformation dependent OC antibody using the dot blot assay demonstrated the Rot preparation had no alteration in the size or distribution of the oligomer population (Figure 5.3A) or conformation of the oligomer preparation (Figure 5.3B). While the above data suggest rutin cannot prevent Aβ1-42 oligomer formation, ThT assay data suggested that rutin can prevent fibril formation (Figure 5.3C). This conclusion is supported by AFM data demonstrating rutin can prevent fibril formation (Wang et al. 2012b). Potential changes in dimer, trimer or tetramers of Aβ observable on SDS-PAGE were not quantified as some of these smaller species can be stabilised by SDS during the electrophoresis (Bitan et al. 2005; Hepler et al. 2006; Hatami et al. 2014). Larger oligomer species were also not quantified by densitometry as previous work has demonstrated that the spread of oligomers observed between 65-80kDa by SDS-PAGE actually contains species of 150 - 1000kDa when detected by multi-angle light scattering (Hepler et al. 2006; Benilova et al. 2012) - due to this lack in reliability of what is being quantified the absolute presence or absence of the distinct spread of oligomers seen in the control oligomer preparation (Figures 4.1, 5.3 and 5.4) was the only measure of inhibitor efficacy used in the present study. Together the data from several techniques show that rutin cannot prevent oligomer formation but may be able to prevent stable fibril formation observable by ThT or AFM.
Figure 5.3 Rutin prevents Aβ fibril but not oligomer formation

(A) Preparations of monomeric Aβ and Aβ oligomerised in the presence of rutin were resolved by 5-20% Acrylamide tris-tricine SDS-PAGE and detected with the 6E10 antibody. n>3 (B) Dot blot assay of 100ng Aβ oligomer ± rutin (1:50 Aβ:rutin ratio). Membranes were immunoblotted with OC antibody then stripped and re-probed with 6E10 antibody. n>3. (C) Thioflavin-T fluorescence following incubation of 20μM Aβ with 2mM rutin and 10μM Thioflavin-T overnight at 20°C. Blank is vehicle loading control. Data represent mean ± SEM. n=3/point.
Figure 5.4 Quercetin prevents Aβ aggregation

(A) Preparations of monomeric Aβ and Aβ oligomerised in the presence of quercetin were resolved by 5-20% Acrylamide tris-tricine SDS-PAGE and detected with the 6E10 antibody. n>3. (B) A lower exposure image of the gel depicted in (A). (C) Dot blot assay of 100ng Aβ oligomer ± quercetin (1:50 Aβ:quercetin ratio). Membranes were immunoblotted for OC antibody then stripped and re-probed with 6E10 antibody. n>3. (C) Thioflavin-T fluorescence following incubation of 20μM Aβ with 1mM Quercetin and 10μM Thioflavin-T overnight at 20°C. Blank is vehicle loading control. Data represent mean. n=3/point.
Figure 5.5 AFM demonstrates quercetin prevents Aβ aggregation and produces smaller oligomers

Representative image of height data from 10μM Aβ_{m} (A), Aβ_{o} (B) and Q_{OT} (C) preparations spotted onto mica collected in tapping mode AFM. Height data scale -2.5nm to 15nm, image scale 1μM. n=3, ≥50 oligomers were measured per n, in conditions where less than 50 oligomers were observed then all detectable oligomers were measured. (D) Interpolated sphere diameter from all Aβ_{o} and Q_{OT} preparation experiments. Graph depicts individual interpolated oligomer sphere height as well as the mean ± SD, n=3. Comparison performed by two-tailed unpaired Student’s t-test with Welch’s correction. **** p < 0.0001.
Comparison of height data captured by AFM from AβO and QOT oligomer populations represented as a % of observations and organised by size bracket. n=3, ≥50 oligomers were measured per n, in conditions where less than 50 oligomers were observed then all detectable oligomers were measured. Separate analysis of data presented in Figure 5.4. The effect of oligomer size and treatment on size bracket frequency were analysed by linear modelling. Size and treatment were modelled as fixed effects and experiment (n=3) as a random effect. ****, p<0.0001.
We then investigated the propensity of the aglycone quercetin to inhibit Aβ oligomerisation, when added to Aβm (QOT). Quercetin prevented the formation of oligomers detectable with 6E10 antibody following SDS-PAGE and immunoblotting (Figure 5.4A). This effect occurred at upwards of a 50 times molar excess of quercetin to Aβ. A lower exposure image of the 6E10 immunoblot chemiluminescence revealed a small doublet species in the lanes corresponding to the quercetin concentrations that prevent Aβ oligomerisation, but not in the monomer, oligomer or lower quercetin concentration conditions (Figure 5.4B). This band is at a lower molecular weight than observable Aβ dimer species, and most likely represents Aβ-bound to quercetin. The Aβ 1:50 quercetin molar ratio was then used in a dot blot assay to demonstrate that Aβ aggregating in the presence of quercetin does not form OC reactive species (Figure 5.4C). The antibody 6E10 was used to demonstrate equal loading. This result indicates that in the QOT preparation no in register β-sheet containing oligomers were formed. Using the same aggregating conditions we also demonstrated that quercetin prevents the formation of ThT reactive species (Figure 5.4D). This demonstrates that no fibril species are being formed in the presence of quercetin, as recently reported (Matos et al. 2017). The antibody-dependent data are supported by height data recorded by AFM (Figure 5.5). Aggregation in the presence of quercetin created far fewer observable oligomers compared to the control untreated oligomer preparation (Figure 5.5B-C), this was not quantifiable but clear from data acquisition, and the observable oligomers present are significantly smaller than those in the oligomer preparation with a near 50% reduction in mean oligomer size from 5.95nm to 3.32nm (Figure 5.5D). Not only is the mean size of the oligomers formed in the presence of quercetin smaller, but linear modelling of the effect of oligomer size and treatment on size bracket demonstrates that a significantly greater proportion of the oligomer population is distributed amongst smaller oligomer sizes (p<0.0001) (Figure 5.6).
Figure 5.7 Rutin binds Aβ₁-₄₂

Negative mode ion mobility mass spectrometry of 20μM Aβ₁-₄₂ shows the presence of the [Aβ-4H]⁺ and [Aβ-3H]⁻ charge states (A). Co-incubation with 100μM rutin produces the [Aβ-4H]⁺ and [Aβ-3H]⁻ charge states and rutin bound to the [Aβ-4H]⁺ species (B). The bound complex is isolated in (C) at m/z range 1280. Arrival time distribution (ATD) analysis shows compaction of Aβ in both charge states in presence of rutin (D-G). Data acquired using the Synapt-G2Si system (Waters). IMMS conditions are summarised in Table 2.11 and are fully listed in Appendix 3.
We then employed ion mobility mass spectrometry (IMMS) to investigate whether flavonoids can bind Aβ\textsubscript{1-42} and their effects on small oligomer species. Our IMMS data demonstrated that rutin directly binds and complexes with the Aβ\textsubscript{1-42}\textsuperscript{+} charge state (Figure 5.7). Negative mode ionisation was employed in this study because Aβ\textsubscript{1-42} has a native charge of Aβ\textsubscript{1-42}\textsuperscript{3-} in the brain (Bernstein et al. 2005; Bernstein et al. 2009). Attempts were made to use quercetin in these experiments, but co-incubation prevented stable spray and ionisation of Aβ in negative ionisation mode. Co-incubation of Aβ and rutin caused shifts in Aβ\textsubscript{1-42}\textsuperscript{3-} and Aβ\textsubscript{1-42}\textsuperscript{4+} charge states indicating an interaction between both charge states with rutin (Figure 5.6D-G). These figures also show the presence of dimer species in the Aβ\textsubscript{1-42}\textsuperscript{3-} and Aβ\textsubscript{1-42}\textsuperscript{4+} charge states. While rutin does not prevent amyloid oligomerisation under comparable conditions (Figure 5.2), this result would appear to suggest that the presence of the flavonoid promotes small oligomer formation. However, in published reports (Bernstein et al. 2005) and in our experience WT Aβ\textsubscript{1-42} small oligomer species such as dimers are generally in very low abundance and take a long time to be detectable by IMMS. Detection of these dimers is difficult because Aβ\textsubscript{1-42} peptide will rapidly form aggregates in the glass tip under nano-electrospray ionisation (Appendix 4), this blocks the tip orifice and prevents ion production within several minutes. This was not the case in the rutin treated sample where data acquisition was possible over much longer time frames. The prevention of large aggregate formation and extended ionisation time are likely the reasons for the observation of dimer species rather than the stabilisation of small oligomer species. It is worth noting that we could not isolate higher molecular weight oligomeric species of Aβ - dodecamers and above - previously characterised by the same IMMS system and method (Bernstein et al. 2009).

5.2.2 Quercetin does not alter the structure of preformed oligomers

Realistically anti-amyloidogenic therapeutics will be recommended to patients in mid-life or with mild cognitive impairment. Such a cohort is likely to already have amyloid deposition (Jack et al. 2010) and therefore consideration should be given to the action of small molecules inhibitors with preformed aggregate species (Q\textsubscript{OP}).

Several publications have demonstrated quercetin has the ability to disaggregate preformed amyloid fibrils (Aitken et al. 2017b; Jimenez-Aliaga et al. 2011). As such we investigated whether quercetin could disaggregate preformed Aβ oligomers. Our study found that 30min incubation with quercetin had no effect on the preformed Aβ oligomers by multiple methods. Incubation for quercetin for 30min post oligomer isolation by centrifugation did not alter the profile produced by oligomers when resolved by SDS-PAGE (Figure 5.8A). Nor did this incubation alter oligomer reactivity with the OC structural antibody (Figure 5.8B), indicating the oligomers have retained an in-register parallel β-sheet structure. This is
supported by a similar study by Ladiwala et al. in which the authors investigate the propensity of many small molecules to remodel preformed soluble Aβ oligomers by dot blot, SDS-PAGE and AFM (Ladiwala et al. 2011b). The authors employed several oligomer preparations, one considered 'toxic' that is reactive with the A11 antibody - which is reactive with early oligomeric species with an out-of-register β sheet secondary structure (Kayed et al. 2010; Liu et al. 2012) and another oligomer preparation considered non-toxic and more analogous to the oligomer preparation used in our study. Incubation of toxic oligomer species with a 10-fold molar excess of quercetin or rutin caused loss of A11 reactivity and a considerable change in AFM profile - without subsequent quantitative analysis (Ladiwala et al. 2011b). However, the large amorphous structures observed with quercetin and other polyphenol treatments are likely the result of the drying process employed in this particular instance. Our data derived by SDS-PAGE (Figure 5.8) and AFM (Figures 5.9 & 5.10) demonstrated that QOP treatment did not cause observable changes in detectable oligomers. Ladiwala et al. (2011b) also characterised their non-toxic oligomer preparation. The non-toxic oligomers were not analysed by dot blot as they are unreactive with A11 - as is the preparation used in the present study - but were characterised by AFM. Ladiwala et al. (2011b) reported no observable changes to the oligomer population following quercetin treatment, which supports our data (Ladiwala et al. 2011b).

Analysis by AFM revealed that quercetin treatment of preformed oligomers did not significantly alter mean sphere diameter or population distribution as determined by linear modelling of treatment effect on size bracket (Figures 5.9 & 5.10). Although there was no significant difference between populations by the statistical modelling, the student’s t-test performed on the oligomer population (Figure 5.9D) has a significant F test value (p>0.0001) indicating the variance in the populations is different. Rutin and quercetin have previously been reported to disaggregate preformed Aβ fibrils (Jimenez-Aliaga et al. 2011; Ladiwala et al. 2011b) and amylin fibrils (Aitken et al. 2017b). However, these results relied on ThT assay derived data, which may be subject to false negatives when used with flavonoids such as quercetin (Suzuki et al. 2012) (further discussed in 5.3.2). Our evidence demonstrates the importance of Aβ preparation conditions of the pathway of flavonoid remodelling. Our data clearly shows that preformed Aβ oligomers are not disaggregated by quercetin treatment.
Figure 5.8 Biochemistry characterisation of the effect of quercetin on preformed Aβ oligomers

(A) Preparations of monomeric Aβ and preformed Aβ oligomers incubated with quercetin for 30min resolved on a 5-20% Acrylamide tris-tricine SDS-PAGE and detected with the 6E10 antibody. n=3. (B) Dot blot assay of 100ng Aβ oligomer ± quercetin (1:50 Aβ oligomer:quercetin ratio). Membranes were immunoblotted for OC antibody then stripped and re-probed with 6E10 antibody. n=3.
Figure 5.9 AFM characterisation of the effect of quercetin on preformed Aβ oligomers

Representative image of height data from 10μM Aβₘ (A), Aβ₀ (B) and Q₂₀ (C) preparations spotted onto mica collected in tapping mode. Height data scale 2.5nm to 15nm, image scale 1μM. n=3, ≥50 oligomers were measured per n, in conditions where less than 50 oligomers were observed then all detectable oligomers were measured. (D) Interpolated sphere diameter from all Aβ₀ and Q₂₀ preparations experiments. Graph depicts individual interpolated oligomer sphere height as well as the mean ± SD, n=3. Comparison performed by two-tailed unpaired Student’s t-test with Welch’s correction. ns = not significant
Figure 5.10 Characterisation of $Q_{OP}$ preparation by AFM

Comparison of height data from $A\beta_0$ and $Q_{OP}$ oligomer populations represented as a % of observations and organised by size bracket. $n=3$, ≥50 oligomers were measured per $n$, in conditions where less than 50 oligomers were observed then all detectable oligomers were measured. Separate analysis of data presented in Figure 5.8. The effect of oligomer size and treatment on size bracket frequency were analysed by linear modelling. Size and treatment were modelled as fixed effects and experiment ($n=3$) as a random effect. NS, non-significant difference ($p=0.0837$).
5.2.3 Quercetin prevents Aβ oligomer binding to SH-SY5Y-PrP<sub>C</sub> cells

The pathogenic properties of soluble Aβ oligomers are dependent on binding cell surface receptors (Jarosz-Griffiths et al. 2016) and initiating downstream kinase cascades that ultimately hyper-phosphorylate tau (Spires-Jones and Hyman 2014). Recently, work by the Strittmatter lab and our group have revealed PrP<sub>C</sub> to be a major Aβ binding partner (Laurén et al. 2009; Um et al. 2012; Rushworth et al. 2013). Therapeutic agents that can prevent oligomer species binding or target the aggregate species that initiate downstream signalling would be highly beneficial in preventing tau phosphorylation and subsequent cell death. Given the importance of PrP<sub>C</sub> as a pathogenic Aβ oligomer receptor in animal models and humans (Um et al. 2012; Nicoll et al. 2013; Rushworth et al. 2013; Kostylev et al. 2015b; Kaufman et al. 2015; Jarosz-Griffiths et al. 2016), we investigated the ability of quercetin to reduce Aβ oligomer binding in the SH-SY5Y-PrP<sub>C</sub> cell model.

Initial experiments using a 50 molar excess of quercetin as seen in Figure 5.4 demonstrated a complete abolition of cell binding (data not shown, n=1), and as a result a dose response was performed. This revealed that equimolar concentrations of Q<sub>OT</sub> (Figure 5.11B) or Q<sub>OP</sub> preparations (Figure 5.11C) caused significant reductions in Aβ oligomer binding compared to the control Aβ oligomer preparation (Figure 5.11A & D). This was a surprising and novel finding, especially considering that previous experiments revealed this concentration of quercetin had no detectable effect on oligomer production (Figure 5.4A) or on the size and population of preformed oligomers (Figure 5.8 - 5.10). Previous work by our group has demonstrated that EGCG and resveratrol treatment can significantly reduce preformed Aβ oligomer binding to the same neuroblastoma cell model in 4:1 and 10:1 molar excesses, respectively (Rushworth et al. 2013). In these cases the effects were dependent on the ability to disaggregate oligomeric species as demonstrated by loss of OC reactivity by dot blot (Rushworth et al. 2013).
Figure 5.11 Quercetin prevents Aβ binding to cells

SH-SY5Y cells stably overexpressing PrP<sup>C</sup> were treated with Aβ oligomers (A) Q<sub>OT</sub> at an equimolar ratio of Aβ:Quercetin (B) or Q<sub>op</sub> at an equimolar ratio of Aβ oligomers:quercetin (C) for 20min at room temperature before fixation. Blue = DAPI nuclear stain, Green = Sf32 (PrP<sup>C</sup>) and Red = Streptavidin conjugated to Aβ biotin tag. (D) Data points represent individual surface fluorescence from cell membranes, lines represent mean ± SD. At least 30 cells per biological replicate were measured, n=3. Data means were compared to Aβo by Kruskal-wallis test with Dunnett’s post hoc analysis. ****p<0.0001.
5.2.4 Quercetin reduces Aβ₁₋₄₀ and Aβ₁₋₄₂ production

**Figure 5.12 Quercetin reduces production of Aβ isoforms**

SH-SY5Y cells stably expressing the APP₆₉₅ isoform were treated with vehicle, 20μM rutin or 20μM quercetin for 24h in OptiMEM. Following treatment media was harvested and assayed for Aβ isoforms (A) and sAPP fractions (B) using the Mesoscale multiplex assay. Data were corrected to protein concentration determined by BCA assay then made relative to vehicle. Data represent mean ± SEM, n=3/point. Data analysed by two-way ANOVA with multiple comparisons followed by the Holm-Sidak post hoc analysis. *, p<0.05. **, p<0.01.

Preliminary experiments incubating SH-SY5Y cells stably expressing the APP₆₉₅ isoform with 20μM quercetin to measure cell toxicity revealed that 20μM quercetin, but not rutin, treatment caused a reduction in Aβ₁₋₄₀ and Aβ₁₋₄₂ in cell media (Figure 5.12A). The observed effect may be mediated by one or a combination of several mechanisms: reduction in APP processing by BACE1 or γ-secretase, up regulation of ADAM10 activity or by up regulation in Aβ clearance mechanisms. Mesoscale quantification of sAPPα and sAPPβ in cell media demonstrated that quercetin, but not rutin, treatment significantly reduced both fragments (Figure 5.12B). This means that quercetin treatment may be reducing APP processing by both ADAM10 and BACE1. Our data is supported by previous reports that flavonoids such as EGCG (Zhang et al. 2017) and quercetin (Sabogal-Guaqueta et al. 2015) reduced Aβ production. The mechanism by which Aβ production and APP cleavage by both ADAM10 and BACE1 is reduced is yet to be elucidated.
5.3 Discussion

5.3.1 A critical appraisal of quercetin as an aggregation inhibitor
As discussed in the introduction, several flavonoid compounds have been described as aggregation inhibitors previously, with resveratrol and EGCG attracting the most attention. The claims made of flavonoids in general are wide, with anti-cancer, anti-inflammatory, ROS scavenging and anti-hyperglycaemia properties listed as just a few (Sharma et al. 2013). Due to their high bioactivity and multiple effects, these claims are rarely supported by specific mechanisms of action. As such, effects of such a highly bioactive group of natural compounds can often be seen as non-specific and the threshold for evidence required to attribute therapeutic benefits to a specific compound is great.

These thresholds are also true for the anti-aggregation properties of flavonoids. Rutin and quercetin have demonstrated anti-aggregation behaviour in models of several amyloidosis. For example rutin prevents amylin aggregation (Aitken et al. 2017b) and both quercetin and rutin prevent α-synuclein aggregation (Zhu et al. 2013). Furthermore, it has recently been demonstrated that the anti-aggregation properties of quercetin may underlie its anti-inflammatory action (Domiciano et al. 2017). *Domiciano et al.* demonstrated that quercetin bound ASC protein to prevent the formation of the NLRP3 inflammasome complex and subsequent release of mature IL-1β. These effects likely underlie the beneficial effects of flavonoids in animal models of amyloidosis diseases such as AD and T2D. Orally administered rutin rescued behavioural and pathological phenotypes in the APPswe/PS1ΔE9 transgenic mouse model of AD (Xu et al. 2014). Rutin treatment reduced densitometry of A11 and WD conformational antibodies in mouse brain lysates, implying a reduction in the level of soluble oligomers. A study using a separate mouse model of AD demonstrated quercetin caused reduced amyloid burden in 3xTg-AD mice (Sabogal-Guaqueta et al. 2015). These studies both demonstrate that peripheral administration of quercetin and its glucosides is sufficient to reduce amyloid aggregation in the brain *in vivo*. Additionally, an earlier study also demonstrated that rutin pretreatment reduced inflammatory markers and microglial infiltration in a streptozotocin-induced Alzheimer phenotype rat model (Javed et al. 2012).

As a result of these experiments and a bioactivity screening performed by Prof. Garth Cooper (data not shown) we investigated whether rutin and quercetin could prevent the formation of soluble oligomers of Aβ. Our data demonstrate that quercetin but not rutin is capable of preventing Aβ oligomerisation (*Figures 5.3 - 5.6*). This was confirmed by SDS-PAGE, dot blotting and AFM. A recent publication replicates the observations of our AFM results demonstrating reduced oligomer number and size (Matos et al. 2017) and a separate study confirms this conclusion by transmission electron microscopy (Marsh et al. 2017).
Other studies have utilised ThT assays (Matos et al. 2017), AFM (Matos et al. 2017) and surface plasmon resonance (Hirohata et al. 2007) to demonstrate rutin and quercetin will prevent Aβ fibril formation - without focussing on the oligomeric species as we have done. Interestingly, the surface plasmon resonance study suggested quercetin caps the ends of protofibril species (Hirohata et al. 2007). This mode of action towards OC reactive protofibril species implies distinct binding to that which occurs at A11 positive oligomers (Ladiwala et al. 2011b). This distinction may go some way to explain why quercetin disaggregates A11 positive, but not OC positive, oligomeric preparations.

Sato et al. (2013) investigated the binding and aggregation inhibitory properties of catechol flavonols, such as quercetin and taxifolin (2,3-dihydroquercetin), with Aβ1-42 using transmission electron microscopy, circular dichroism, ThT and LC-MS (Sato et al. 2013). The structure of quercetin and taxifolin are extremely similar, differing only in the fact that quercetin has a double bond between C2-C3 of the C-ring. Despite this similarity, data acquired using the ThT assay revealed quercetin to have an IC50 of 15.3μM on Aβ1-42 aggregation, twice as potent as that reported for taxifolin (33μM). Other studies specifically attempting to understand the role of the C2-C3 double bond support its critical role in inhibiting Aβ aggregation, using AFM to demonstrate that quercetin is a much better inhibitor of aggregation than taxifolin (Matos et al. 2017). As previous work with EGCG revealed that flavonoids can covalently bind Aβ (Ishii et al. 2008) Sato et al. (2013) investigated taxifolin-Aβ interactions by LC-MS. Two commonly hypothesised types of reactions to mediate interactions between flavonoids and amyloid are Schiff base reactions and Michael addition reaction to basic residues. LC-MS experiments performed by Sato et al. (2013) discovered evidence of Michael reaction following Aβ1-42 incubation with taxifolin. This reaction could occur at the basic resides Arg5, Lys16 and Lys28 of Aβ1-42. Substitutions of these residues, particularly Lys16, prevented the inhibitory action of taxifolin. This is important as the functional groups involved in this reaction are common to both quercetin and taxifolin, making Michael addition to basic residues a likely mediator of the Aβ1-42-quercetin interaction. While Sato et al. (2013) did not find evidence of a Schiff base interaction between flavonoids and amyloids, other LC-MS experiments have demonstrated Schiff base reaction mediated binding between baicalein, another quercetin anologue, and amylin (Velander et al. 2016). Both these reactions occur at basic residues integral to the turn regions in the peptide sequence as observed by solid state NMR (Ahmed et al. 2010). Together these experiments support our isolation of a rutin-Aβ1-42 conjugate species (Figure 5.7) and a likely quercetin-Aβ1-42 conjugate species (Figure 5.4B), as well as providing evidence for a mechanism of binding to residues critical to the aggregation properties of amyloids. This binding likely underpins the anti-aggregation activity of quercetin and other flavonoids.
The data reported in this chapter and in published literature demonstrates quercetin is a potent inhibitor of Aβ aggregation. But the therapeutic potential of quercetin is dependent on its availability in the brain in concentrations analogous to \textit{in vitro} studies. The availability of dietary quercetin has been studied in \textit{in vitro} models, animal models and in humans by various methods for over 60 years. Despite this it is still unclear exactly what proportion of dietary quercetin is available in the brain, or indeed what modifications occur to quercetin post ingestion.

Ultra-high pressure liquid chromatography combined with quadrupole based time-of-flight mass spectrometry has been employed to investigate the time frame of quercetin metabolism and bioavailability in rapidly perfused tissues, such as the brain (Boonpawa et al. 2015). This study found that when given as an aglycone quercetin had a low bioavailability of 0.1% of the administered dose (between 0.4 - 12mg/kg). This is because 95-99% of ingested quercetin undergoes glucuronidation primarily in the liver (Boonpawa et al. 2015). This supports observations from rat models (Mullen et al. 2002;Mullen et al. 2008;Boonpawa et al. 2014) and pig models (de Boer et al. 2005). As a result of this the majority of the ingested quercetin had been cleared within 6h. Previous studies administering rutin by oral supplement demonstrated quercetin is detectable in the plasma for far longer than following oral quercetin administration, with levels still being elevated at 24h post administration (Boyle et al. 2000). This is because rutin does not undergo glucorindation and first pass metabolism in the liver as readily as quercetin, therefore extending its lifespan (Wu et al. 2011). Interestingly, analysis of fasted blood plasma following 6 week supplementation with rutin showed significantly increased basal quercetin concentration compared to controls (Boyle et al. 2000). This observation should be further tested in longer supplementation studies to attempt to reach basal concentrations capable of the therapeutic effects available. Supplementation of rats with quercetin glycosides demonstrated the presence of quercetin and its metabolites in the brain (Paulke et al. 2008;Ishisaka et al. 2011). A single 100mg/kg supplement of isoquercitrin caused detectable quercetin levels of 35ng/g in the brain 4h post oral gavage (Paulke et al. 2006;Paulke et al. 2008). The latter study extended this to 8 day supplementation, where the authors found accumulation of quercetin in the brain over time, reaching a maximal concentration of 367ng/g after 5 days repeated treatment (Paulke et al. 2008). It is worth noting, however, that the dietary source of quercetin glycosides is important for its adsorption, with previous studies demonstrating quercetin glycosides from onions being more bioavailable than those from apples (Lee et al. 2012;Lee and Mitchell 2012).

Together these studies support the use of quercetin glucosides such as rutin as a medium for quercetin supplementation and suggest that long term supplementation will increase
basal levels of quercetin available in the brain. Importantly, they also demonstrate a clear maximal accumulation of quercetin in the brain, presumably due to a transport gradient, meaning that supplementation will not cause accumulation of quercetin in toxic concentrations. Immunohistochemistry provides evidence that a major metabolite of quercetin, quercetin-3-O-glucuronide, is present in the striatum and hippocampus post oral administration in rodent models (Ishisaka et al. 2011) in concentrations sufficient to enact its anti-aggregation activity (Xu et al. 2014). These studies demonstrate that flavonoids can enter areas of the brain most desirable for therapeutic intervention in dementias. Further immunohistochemistry performed on human post mortem tissue revealed the likely mechanism of translocation to the brain is via the blood-cerebrospinal fluid barrier rather than the blood-brain barrier (Ishisaka et al. 2014). This was determined from evidence of quercetin-3-O-glucuronide staining in the endothelial cells of the choroid plexus, but an absence of such staining from smaller capillary vessels (Ishisaka et al. 2014).

Together the discussed evidence demonstrates quercetin to be a flavonoid that can potently bind Aβ and prevent its oligomerisation but with relatively low bioavailability. This makes it an excellent starting point for medicinal chemistry to improve translocation and bioavailability into the brain.

### 5.3.2 How does quercetin affect preformed Aβ oligomers?

Previous studies demonstrated that quercetin and similar flavonoids are capable of remodelling and disaggregating Aβ fibrils (Jimenez-Aliaga et al. 2011), but few have focused on oligomer species. Previous studies reported that EGCG and resveratrol both prevented preformed oligomer binding to SH-SY5Y-PrP<sup>C</sup> cells (Rushworth et al. 2013). This effect was dependent on the loss of OC antibody reactivity - suggestive of oligomer disaggregation. Our results demonstrate that oligomer conformation can be maintained, whilst reducing binding affinity following quercetin co-incubation (Figures 5.8 - 5.11). Both of these results fit with a previously published framework for Aβ oligomer remodelling pathways (Ladiwala et al. 2011a). This study characterised three classes of remodelling molecules: Class I can stabilise non-toxic conformations of oligomer, Class II molecules convert soluble Aβ oligomers into fibrils - but is inactive against fibrils and monomers; and Class III molecules disassemble soluble oligomers and fibrils into low molecular weight species. By these classifications, the data from this work and evidence discussed suggest quercetin acts as a Class I remodelling molecule when interacting with Aβ oligomers.

There is some evidence to suggest rutin and quercetin can act as Class III remodelling compounds. Quercetin and rutin have been demonstrated to disaggregate preformed fibrils of α-synuclein (Zhu et al. 2013), Aβ (Jimenez-Aliaga et al. 2011) and amylin (Aitken et al.
The studies by Jimenez-Aliaga et al. (2011) and Aitken et al. (2017b) draw this conclusion exclusively from ThT derived data. As ThT, rutin and quercetin are all flavonoids, there is a reasonable possibility that ThT and other flavonoids bind fibrils in the same moiety, potentially generating false negatives. This is reasonably likely, given that EGCG and ThT have been demonstrated to bind to the same sites in amylin fibrils (Suzuki et al. 2012). Even if this is not direct competition for the same binding site, ThT fluorescence is particularly dependent on surface charge of the amyloid fibril (Patel et al. 2014) and as such some conformations of fibrils may be visible by TEM but produce false negatives by ThT, as is the case for pufferfish amylin (Wong et al. 2016). Characterisation of rifampicin addition to preformed amylin fibrils by ThT assay demonstrated complete loss of fluorescence by ThT, but no change in profile by TEM at the same timepoint (Meng et al. 2008). These results demonstrate that in some cases small molecule inhibitors may not be deconstructing amyloid aggregates as suggested, but still sufficiently alter amyloid structure or surface properties to reduce cell toxicity (Wang et al. 2012b). The experiments performed by Ladiwala et al. demonstrate that the nature of the oligomeric preparation determines the ability of quercetin and other flavonoids to remodel preformed aggregates. Despite this quercetin has been demonstrated to protects cells from Aβ oligomer application (Godoy et al. 2016).

5.3.3 How may quercetin mediate a reduction of APP processing?

Treatment of N2a cells stably overexpressing APP695 with 20μM EGCG caused lower levels of secreted Aβ1-40 and Aβ1-42 (Zhang et al. 2017). This publication also reported decreased C99 levels in the cell lysates; this is explained by decreased expression of BACE1 mRNA and protein levels. Our experiments in SH-SY5Y-APP695 cells demonstrate quercetin but not rutin treatment causes a significant reduction in Aβ1-40 and Aβ1-42, as well as both sAPPα and sAPPβ (Figure 5.12).

Our results are supported by in vivo studies. Quercetin treatment of 3xTg-AD mice caused reduced plaque deposition, reduction in Aβ1-40 and Aβ1-42 and an improvement in behavioural tests (Sabogal-Guaqueta et al. 2015). The authors attributed these effects in part to BACE1 inhibition, but also observed decreased C89 and C99 protein levels by western blot. This suggested that APP processing by both ADAM10 and BACE1 was decreased (Sabogal-Guaqueta et al. 2015), supporting our observations of decreased sAPPα and sAPPβ (Figure 5.12B). Importantly this paper also demonstrates that concentrations of quercetin achievable by oral administration are capable of lowering Aβ production and BACE1 activity in the brain in vivo. The mechanism by which quercetin reduces BACE1 and ADAM10 catalytic processing of APP is unclear. Experiments utilising a non-APP fluorescent BACE1 substrate demonstrated that both rutin and quercetin could lower BACE1 activity (Jimenez-
This demonstrates that the reduction in activity is unlikely to be mediated by a direct binding to APP resulting in impaired cleavage, and opens up the possibility of direct inhibition of APP processing enzymes.

There is evidence that quercetin can directly inhibit both BACE1 and ADAM10. Autoxidation properties of flavonoids allow them to bind to cysteiny1 thiol groups, which are often key components of enzyme regulatory sites (Ishii et al. 2008). Quercetin may also have a direct interaction with the active site of BACE1 (Shimmyo et al. 2008). Shimmyo et al. (2008) demonstrated that 20μM quercetin treatment caused a reduction in Aβ1-40 and Aβ1-42 in primary neuronal cell cultures, which has since been replicated in Tg2576 cortico-hippocampal primary neuronal cultures (Ho et al. 2013). In addition to this, Shimmyo et al. (2008) employ in silico docking simulation to investigate where quercetin binds to the BACE1 active site. They concluded that hydrogen bonding to Asp32, Gln78 and Trp198 residues in the active site is sufficient for quercetin to prevent BACE1 activity. A criticism of this literature and my study is the failure to address whether other BACE1 and ADAM10 substrates in cell models are affected - this would reveal whether quercetin is directly inhibiting catalytic activity. Alternatively, this effect may be mediated by alteration of APP expression or sub-cellular localisation, or indeed altering the expression of ADAM10 or BACE1. Experiments previously performed with EGCG demonstrated that treatment had no effect on APP expression, but did reduce BACE1 mRNA and protein expression by activation of PPARγ (Zhang et al. 2017). Recent work has also demonstrated ADAM10 to be regulated by PPARα, but not PPARγ (Corbett et al. 2015). It has been previously demonstrated that quercetin treatment can regulate PPARα (Kim et al. 2014a), providing a possible mechanism for such regulation. Ultimately, the mechanism by which quercetin appears to be reducing APP processing remains unknown and it would be interesting to investigate other APP cleavage events such as ε-secretase mediated cleavage (Willem et al. 2015a).

An alternative mechanism by which Aβ may be being reduced is by quercetin-mediated up regulation of macroautophagy to cause increased lysosomal-mediated Aβ degradation. A recent study in a transgenic C. elegans model demonstrated that quercetin treatment reduced Aβ aggregation and concentration by up regulation of the macroautophagy pathway (Regitz et al. 2014), resulting in rescue of Aβ induced paralysis in the model.

Our results were serendipitous observations that raise interesting questions, for example: Does quercetin treatment alter ADAM10 or BACE1 expression? Does quercetin directly inhibit BACE1 and/or ADAM10? And does quercetin affect γ-secretase activity? These experiments will help elucidate whether our observations are mediated by regulation of protein expression or pharmacological inhibition of catalytic activity.
5.4 Chapter summary

The data discussed in this chapter evidences that quercetin has high potential for targeting multiple elements of AD pathology. These effects are briefly outlined in Figure 5.13.

First, our data extends evidence demonstrating quercetin can prevent fibril formation to include evidence that it is also able to prevent the formation of the soluble oligomer species that bind to the cell surface PrPrC receptor. In addition to this, quercetin appears to act as a class I molecule when remodelling preformed oligomers in such a way as to promote a conformation unable to bind the cell surface. This is most likely due to a direct addition of flavonoids to the Aβ molecule, as evidenced by our IMMS and immunoblotting data. Furthermore, quercetin appears to be able to reduce Aβ production, which has been the major goal of other therapeutic strategies, although the mechanism of this remains unclear.

We have also discussed evidence that demonstrates quercetin can inhibit key anti-inflammatory pathways in AD (Domiciano et al. 2017; Ruiz-Miyazawa et al. 2017) and enter the human brain from the periphery, but at a relatively low bioavailability. This can be improved by glycoside addition, as demonstrated with rutin supplementation, making the quercetin flavanol backbone an excellent framework for further drug design. Aside from the comparable anti-aggregation and anti-inflammatory actions of quercetin in diabetes, supplementation with quercetin can also reduce circulating cholesterol and hyperglycaemia, associated risk factors for diabetes and poor vascular health which are in turn risk factors for several dementias. The most important point to highlight is that unlike a multitude of other compounds that have demonstrated comparable properties in vitro, high levels of quercetin and quercetin glycoside supplementation have already been demonstrated to be very well tolerated and maintain high compliance in study participants. These are essential qualities for a preventative intervention targeting Aβ production and aggregation, as efficacy on these targets will require early and long-term treatment compliance to offset Aβ induced downstream effects. In conclusion, quercetin or quercetin derived compounds compare favourably to other treatment strategies in their ability to tackle multiple elements of AD disease pathology in a non-invasive and non-toxic manner.
Figure 5.13 Potential therapeutic actions of quercetin in AD
Figure describing the multiple therapeutic benefits of quercetin in the context of AD. Quercetin prevents amyloid aggregation (Figures 5.3 - 5.6), NLRP3 mediated inflammation (Domiciano et al. 2017), Aβ₀ binding (Figure 5.11) and reduces Aβ production (Figure 5.12). Quercetin also has anti-diabetic properties, as it has been shown to ameliorate insulin resistance (Arias et al. 2014), and pro-autophagy effects (Regitz et al. 2014).
Chapter 6: Final discussion

The potential role of amylin in AD must be viewed within the broader context of the T2D-AD relationship. Decreased insulin sensitivity, hyperglycaemia and altered glucose utilisation as well as a deep rooted link with obesity are all elements of T2D that contribute to AD pathogenesis or pathology (de la Monte and Wands 2008; Barbagallo and Dominguez 2014; Goyal et al. 2017). Amylin is unique amongst these factors in that a single nucleotide polymorphism in IAPP (rs73069071) is linked to cognitive decline and Aβ-burden (Roostaei et al. 2017). The data in this thesis suggests IAPP expression and insoluble amylin species may contribute to AD pathology.

6.1 Amylin deposition: a link between AD and T2D or indicative of other problems?

Data presented in Chapter 3 provides evidence of amylin deposition in the brain and supports the growing literature demonstrating insoluble aggregate species are present in the brains of AD and T2D patients (Jackson et al. 2013; Fawver et al. 2014; Oskarsson et al. 2015a; Verma et al. 2016; Schultz et al. 2017; Ly et al. 2017). While there is evidence of Aβ-amylin co-deposition in human brain (Jackson et al. 2013; Oskarsson et al. 2015a), as well as in vitro and in vivo evidence of cross-seeding between the two amyloids (Mukherjee et al. 2017; Wijesekara et al. 2017; Oskarsson et al. 2015a; Hu et al. 2015; Zhang et al. 2015a), none of our data suggests that in occipital lobe or temporal lobe co-morbid T2D exacerbates amylin deposition in AD (Chapter 3).

These data do not support the hypothesis that amylin aggregate species, up regulated in T2D, are preferentially deposited in AD and initiate Aβ aggregation. Instead, the view could be taken that amylin deposition should be considered another non-canonical amyloid deposition that occurs in AD, comparable to TDP-43 or α-synuclein deposition (Amador-Ortiz et al. 2007; Arai et al. 2009; Raghavan et al. 1993; Clinton et al. 2010). In addition to these well characterised non-canonical amyloids there is also evidence of cystatin, β-2 microglobulin, serum amyloid A, insulin and transthyretin aggregates deposited in AD patient brains (this is reviewed in Luo et al. (2016)). Deposition of such a variety of amyloids may be indicative of a failure of a key house keeping mechanism, such as autophagy, in AD (Nixon and Yang 2011).

There is a large body of evidence that autophagy is impaired early in AD pathogenesis (reviewed in Nixon and Yang (2011)) and autophagic vesicles are deposited in dystrophic
neurites in the AD brain (Terry et al. 1964; Nixon 2007). Autophagy is a constitutively active house-keeping mechanism that targets misfolded and aggregating peptides to lysosomes (Mizushima and Hara 2006). Impairment of this mechanism causes protein aggregation and neurodegeneration in animal models (Hara et al. 2006; Komatsu et al. 2006) and has also been demonstrated to promote neuroinflammation (Cho et al. 2014). Interestingly, the autophagic vesicles that build up in neurites express the machinery of APP processing, and accumulation of these vesicles upregulates Aβ production (Yu et al. 2005). Therefore autophagy impairment provides two pathways towards increased Aβ accumulation: decreased basal Aβ degradation and autophagosome mediated up regulation in APP processing. Amylin treatment impairs autophagy in multiple cell types (Schultz et al. 2017; Shigihara et al. 2014; Kim et al. 2014b; Wu et al. 2017) and our data suggests amylin induced increases in Aβ is mediated through autophagy impairment in neurons (Figure 3.20). Whether this impairment is mediated through a beclin-1 or LC3 dependent mechanism remains to be elucidated. In our model it is likely the upregulation of Aβ is solely due to decreased basal degradation as we observe no increase in sAPPβ as would be the case following increased APP processing by the mechanism suggest by Yu et al. (2005).

Impairment of autophagy by amylin, or other non-canonical protein aggregates deposited in AD (Winslow et al. 2010; Bose et al. 2011), may limit neuronal degradation of Aβ oligomers, tau aggregate species and provide a potential explanation for the heterogeneity of non-canonical amyloid deposition.

6.2 Does amylin contribute to AD pathology?

Like many proteins, amylin has multimodal activity dependent on environmental context. This has led to argument over whether amylin’s role in T2D is an overall positive or negative influence on disease pathogenesis, for reviews see Gebre-Medhin et al. (2000) and Mukherjee et al. (2015). Likewise, argument exists over whether amylin’s overall contribution to AD has a positive or negative influence on pathology, this is discussed in reviews by Despa and Decarli (2013), Qiu and Zhu (2014) and Lutz and Meyer (2015).

The arguments that support a detrimental effect of amylin in AD are compelling. The toxicity of amylin aggregation in islet-β cells is well documented (Leighton and Cooper 1990; MacGibbon et al. 1997; Konarkowska et al. 2006; Zhang et al. 2008; Zhang et al. 2014b), and a similar toxicity has been demonstrated in neurons (Lim et al. 2008; Lim et al. 2010; Alier et al. 2011). Amylin treatment also impairs neuronal excitability and LTP (Lim et al. 2008; Kimura et al. 2012). Expression of IAPP in AD transgenic models worsens AD pathology and decreases lifespan (Moreno-Gonzalez et al. 2017; Wijesekara et al. 2017),
and injection of amylin aggregate species isolated from pancreas will worsen AD pathology and seed Aβ deposition (Oskarsson et al. 2015a; Moreno-Gonzalez et al. 2017). This cross-seeding is reciprocal as amylin and Aβ have both been demonstrated to promote one-another's aggregation and deposition following external application in the brain and pancreas (Moreno-Gonzalez et al. 2017; Wijesekara et al. 2017). In addition to this, there is a large body of evidence that amylin is deposited as fibrillar aggregates in the brain (Jackson et al. 2013; Oskarsson et al. 2015a; Fawver et al. 2014; Verma et al. 2016; Schultz et al. 2017). In the periphery, amylin deposition correlates with organ failure (Gong et al. 2007; Despa et al. 2012; Westermark 1972) and these data demonstrate this may also be true in the CNS.

However, there is also a large body of work demonstrating beneficial effects of amylin on AD pathology. These effects are principally mediated through the amylin receptors expressed in the entorhinal cortex and hippocampal neurons as well as vasculature endothelia (Paxinos et al. 2004; Fu et al. 2013). It has been previously demonstrated that addition of amylin or non-aggregating analogues of amylin will cause the translocation of Aβ from the brain to the blood in mouse models and in vitro (Zhu et al. 2014; Mohamed et al. 2017). Translocation in this manner is preventable using amylin receptor antagonists (Mohamed et al. 2017). Furthermore, amylin-Aβ hetero-complexes have been reported to be less toxic than Aβ oligomers or amylin alone (Yan et al. 2014; Qiu and Zhu 2014). This is supported by the fact that non-aggregating analogues of amylin will also interact with Aβ to prevent its aggregation and reduce toxicity (Andreetto et al. 2011; Rezaei-Ghaleh et al. 2011; Yan et al. 2013). One such analogue, pramlintide, will prevent Aβ induced LTP impairment and rescue memory deficits in AD mouse models (Adler et al. 2014). However, pramlintide (aka symlin) is widely used in the United State of America, and to the author's knowledge no studies to date have demonstrated an association of pramlintide treatment with reduced dementia risk.

Both arguments fit existing data, for example, decreased plasma amylin recorded in AD patients (Adler et al. 2014) may be indicative of deposition in the brain or other peripheral organs, similar to decreased Aβ levels in the CSF of AD patients due to sequestration to plaques (Hansson et al. 2006). Alternatively, β-cell loss and impaired insulin / amylin secretion associated with late stage T2D may explain the decreased plasma amylin observed by Adler et al. (2014). This loss of amylin, and insulin, secretion also represents a loss of the beneficial effects of both hormones. Lutz and Meyer (2015) propose a biphasic action for amylin in AD: in early- to mid-T2D hyperamylinaeemia promotes deposition in the peripheral organs and the brain; there amylin causes impairments in neuronal and vascular function, whilst increasing Aβ levels and deposition. Whereas in late-T2D, β-cell dysfunction or loss causes hypoamylinaeemia, resulting in impaired translocation of Aβ from the brain.
This is an interesting model, but is somewhat complicated by our observations of increased \textit{IAPP} expression in the brain of AD patients (Figure 3.16), and a trend towards increased soluble amylin in non-diabetic AD patients - but is consistent with the depressed levels of soluble amylin observed in T2D brain regions (Figure 3.10). These data suggest that amylin supplementation in AD may be beneficial in some and potentially damaging in others, dependent on \( \beta \)-cell functionality. However, as the beneficial effects of amylin appear to be mediated through the amylin receptors (Zhu et al. 2015; Mohamed et al. 2017), non-aggregating amylin analogues that activate amylin receptors may provide a mechanism of isolating the beneficial effects of amylin whilst minimising potential deleterious effects (Qiu 2017; Zhu et al. 2017b). Such a treatment strategy has so far failed to consider the effect on the periphery, where amylin signalling is associated with hypertension and insulin resistance (Leighton and Cooper 1988a; Wookey and Cooper 2000; Lee and Cooper 2002), but initial results using pramlintide do look promising from a safety perspective (Zhu et al. 2017a).

Our immunohistochemistry data and ELISA data suggests there is elevated soluble and insoluble species of amylin in the brain in AD compared to controls (Chapter 3). However, this conclusion is limited by the limited number of patient sample available. Insoluble amylin species may contribute to AD pathogenesis through promotion of inflammation, cross seeding or impairment of A\( \beta \) degradation pathways. Our data also suggests amylin species can activate Fyn kinase (Chapter 4). Fyn signalling cascades are implicated in tau phosphorylation, LTP impairment and neuronal loss (Um and Strittmatter 2013). A\( \beta \) oligomer activation of Fyn occurs through PrP\( ^C \) stabilised receptor complex localised to lipid rafts (Um et al. 2012; Rushworth et al. 2013; Haas and Strittmatter 2016). It is currently unclear whether amylin acts through this same pathway. Future experiments disrupting lipid rafts, knocking down PrP\( ^C \) or interfering with known PrP\( ^C \) co-receptors such as mGluR5 or LRP1 may all be explored to investigate if amylin shares this receptor complex. Such an observation would be important, PrP\( ^C \) bound A\( \beta \) species correlate tightly with cognitive decline (Kostylev et al. 2015b), most likely through the resultant Fyn mediated NMDAR internalisation (Suzuki and Okumura-Noji 1995; Li et al. 2011; Um et al. 2012). Amylin treatment is already known to impair neuronal firing and LTP, and this has been demonstrated to have a degree of PrP\( ^C \) dependency (Alier et al. 2011). Further elucidation of amylin induced Fyn signalling is required to determine whether amylin may contribute to the amnesic phenotype of AD. Fyn activation may also contribute to diabetic cognitive decline, and investigation of Fyn phosphorylation status in T2D brain tissue should be performed.

Our results demonstrating decreased pericyte marker in AD patient occipital grey matter support previous observations of microvascular damage in AD. Our data is supported by
recently published ELISA data showing decreased PDGFRβ in AD brains (Miners et al. 2017). Serial staining immunohistochemistry data demonstrates that amylin and Aβ are deposited in the same vessels (Figure 3.5), but these experiments are limited in that they are not quantitative and cannot definitively demonstrate these are mixed deposits as previously claimed (Jackson et al. 2013; Oskarsson et al. 2015a). This could be further investigated by use of a proximity ligation assay or laser dissection of plaques followed by mass spectrometry. We did not observe an independent effect of T2D on the pericyte marker expression. Therefore these data do not support recent claims that amylin mediates pericyte loss (Schultz et al. 2017), as we observed no decrease in the pericyte marker PDGFRβ in T2D or synergistic effect of T2D co-morbidity with AD. This hypothesis is also stymied by the timeframe of hyperamylinemia in relation to the timeframe of diabetes associated cognitive decline. There are currently no secondary or downstream processes associated with amylin deposition in the vasculature which would explain the large time gap between the age when hyperamylinemia occurs (midlife) and when diabetes associated cognitive impairment arises (later life) - i.e if amylin deposition were the primary cause with no secondary mechanism, one would expect the timeframe of onset to be closer to the period of hyperamylinemia. Our experiments were primarily observational in nature, further pathological staining similar to the categorisation of Aβ CAA (Thal et al. 2002a) should be employed to see if amylin deposition in the vasculature correlates with disease severity.

6.3 Further discussion of IAPP expression in the brain

Our original hypothesis posited that amylin deposition in the brain would be composed from infiltrating amylin species as a result of increased amylin secretion from pancreatic β-cells in early T2D. This was based on a number of factors, not least that the overwhelming majority of amylin production occurs in islet-β cells, but also because amylin deposition is frequently observed in the vasculature, indicating BBB translocation (Jackson et al. 2013; Oskarsson et al. 2015a; Ly et al. 2017). Whilst we observed vasculature deposition of amylin, our qPCR data suggests that IAPP expression occurs in AD patient occipital grey matter, potentially with an independent input of T2D, but remains minimal in controls (Figure 3.16). IAPP expression is induced by local hyperglycaemia, which is a characteristic feature of the pancreas in T2D and the brain during neurodegeneration (Ramasamy et al. 2005) and is discussed in 3.3.3. This section aims to further discuss the expression of IAPP we observed in the brain in the context of the few previous examples of IAPP expression in the brain.
Basal *IAPP* expression and mature amylin peptide have previously been detected in the dorsal root ganglia in rat models (Mulder et al. 1996). This expression was shown to be responsive to glucose concentration. Amylin expressed in neurons is likely to be stored in vesicles as it is in the pancreatic β-cells, but as the low pH of vesicles in the pancreas is required to prevent amylin aggregation, local vesicle environment may determine whether the released amylin is misfolded (Jha et al. 2014). *IAPP* expression is also induced in the brain near the end of pregnancy in rat models (Dobolyi 2009; Szabo et al. 2012). *IAPP* expression in the preoptic nucleus in these rats appears to regulate maternal behaviour and is not inhibited by preventing lactation (Szabo et al. 2012).

The timing of this *IAPP* expression observed by Dobolyi (2009) and Szabo et al. (2012) is extremely interesting, because third trimester pregnancy is associated with increased insulin resistance, impaired β-cell function and hyperglycaemia in humans and animal models (Butte 2000; Di Cianni et al. 2003; Lain and Catalano 2007). In some instances, this is sufficient to induce a T2D like metabolic syndrome called gestational diabetes mellitus, reviewed in Angueira et al. (2015). The up-regulation of *IAPP* expression observed in the preoptic area of the brain and dorsal root ganglia may be caused by insulin resistance, as occurs in the pancreas. *IAPP* expression demonstrated by Dobolyi (2009) and Szabo et al. (2012) occurs at a time of demonstrated insulin resistance; and furthermore, the dorsal root ganglia regions demonstrated to basally express *IAPP* and mature amylin (Mulder et al. 1996) have been demonstrated to lack insulin sensitivity (Patel et al. 1994). These data suggest that brain hyperglycaemia and decreased neuronal insulin sensitivity in T2D and AD may underlie the up regulation of IAPP we observed (Figure 3.16).

### 6.4 Amylin induced Fyn activation: Pathological or physiological signalling?

The data presented in Chapter 4 clearly evidence amylin monomer and oligomer preparations as potent activators of Fyn kinase. Increased amylin infiltration and *IAPP* expression in T2D and AD provide amylin the opportunity to activate neuronal Fyn and potentially contribute to the cellular pathology of AD, as is discussed in 4.3. However, if amylin monomer species can activate Fyn in neurons, what does this mean in the context of the pancreatic islet β-cells?

In neurons, Fyn activation causes the phosphorylation of the NR2B subunit of NMDARs. This post-translational modification promotes NMDAR internalisation, interrupts long term potentiation and impairs neuronal function (Suzuki and Okumura-Noji 1995; Nakazawa et al. 2001; Um et al. 2012). In islet-β cells NMDARs are not a major regulator of depolarisation as they are in neurons, but instead they modulate cell physiology and β-cell survival (Otter...
and Lammert 2016). Recent evidence suggests many signalling molecules associated with neurotransmission, such as glutamate and γ-aminobutyric acid (aka GABA) regulate depolarisation of the α-, β- and δ-cells of the islets of Langerhans. Intracellular glutamate transport to the β-cell secretory granules promotes glucose stimulated insulin secretion; whilst extracellular glutamate inhibits glucose stimulated insulin secretion via the inhibitory action of NMDARs on K_{ATP} channels (Marquard et al. 2015; Isaacson and Murphy 2001).

Recent work demonstrates that NMDAR inhibition causes longer β-cell depolarisation, enhanced insulin secretion and β-cell survival (Marquard et al. 2015). This has been demonstrated in isolated mouse and human β-cells, in mouse models and in a small clinical trial in humans (Marquard et al. 2015). Normal amylin secretion into the extracellular space following glucose stimulated release from the secretory granule may enable amylin-induced Fyn phosphorylation in the β-cell. This, in turn, may regulate NMDAR levels at the cell surface, as occurs in neurons. To my knowledge, no investigation of Fyn phosphorylation status in the pancreas has been performed. Neither has the effect of amylin on NMDAR expression at the cell surface been studied. As a result of the data presented in this thesis and reviewed in Otter and Lammert (2016) a model of amylin regulation of NMDAR could be proposed. This model would propose that under physiological circumstances, amylin induced Fyn activation would mediate NMDAR internalisation, to promote neuronal survival and improve glucose stimulated insulin secretion (Otter and Lammert 2016). However, perturbations in amylin level, as occur in T2D, would dysregulate this balance. With the hyperamylinemia that occurs in early T2D promoting hyperinsulinemia; and the hypoamylinemia that occurs in mid- late-T2D promoting NMDAR mediated β-cell toxicity. This is a potentially interesting unexplored regulatory mechanism of β-cell physiology and survival. Investigation of Fyn phosphorylation status and NMDAR localisation in response to amylin treatment in β-cell models should be investigated.

### 6.5 Therapeutic viability of quercetin in AD and T2D

There is a clear need for a treatment of AD that prevents pathology advancement before symptomatic pathology. This is because neuronal loss occurs until the brain is incapable of compensating for the damage and symptoms emerge. Therefore it may be fair to say that once symptoms emerge strategies targeting factors such as Aβ may be being used too late. A preventative treatment for AD would need to be multimodal to target several elements known to occur early in AD pathogenesis. This includes prevention of Aβ aggregation and reduction of neuroinflammation. The ability to minimise the influence of AD risk factors, such as cardiac health and T2D, would also add great value to such a therapeutic. Pre-symptomatic treatment of healthy or at risk individuals would require a cheap, non-aversive
strategy. There is a large body of evidence to demonstrate quercetin can meet all of these requirements.

Data presented in this thesis (Chapter 5) demonstrates that quercetin prevents Aβ oligomerisation and fibril formation, potently inhibits Aβ oligomer binding to cell surface receptors and reduces Aβ production. These are promising in vitro results, demonstrating multiple modes of anti-amyloid activity. Our in vitro data is complemented by existing data of quercetin treatment in AD animal models. Quercetin treatment will rescue amnesic behaviour and markers of AD, such as amyloid burden, in transgenic murine models (Ho et al. 2013; Wang et al. 2014; Sabogal-Guaqueta et al. 2015). These beneficial effects are accompanied by a reduction in Aβ oligomeric species and reductions in APP cleavage fragments (Wang et al. 2014; Sabogal-Guaqueta et al. 2015; Zhang et al. 2016). This supports our conclusions that quercetin treatment reduces APP processing as sAPPα, sAPPβ (Figure 5.11), C89 and C99 (Sabogal-Guaqueta et al. 2015) levels are all reduced following quercetin treatment. Furthermore, quercetin rescued Aβ induced neuronal impairment in C. elegans and Drosophila models through up regulation of autophagy and degradation machinery, potentially decreasing the impact of non-canonical amyloids in AD (Regitz et al. 2014; Kong et al. 2016). As discussed in 5.3.1 the greatest weakness of quercetins’ therapeutic potential in AD is bioavailability. Only 0.1% of ingested quercetin will make it into the brain and even then this can be subject to secondary modifications (such as glucoronidation) which may affect its therapeutic efficacy (Boonpawa et al. 2015). Despite this quercetin represents an excellent structure for further medicinal chemistry to develop a small molecule intervention to help prevent AD pathogenesis.

A potential small molecule therapeutic for T2D would also need to address multiple factors associated with T2D pathology, such as: amylin aggregation, insulin insensitivity, β-cell dysfunction and hyperglycaemia. Hyperglycaemia is the least pressing of these qualities, as existing therapeutics such as metformin and sulphonylureas can ameliorate this pathologic trait reasonably effectively. Quercetin and quercetin-glycosides have also proved therapeutically promising in these regards.

Rutin and quercetin have both been demonstrated to prevent amylin aggregation in vitro (Yu et al. 2015; Aitken et al. 2017b). In vivo dietary rutin supplementation will dramatically extend lifespan in a hIAPP+/- model of T2D (Aitken et al. 2017a; Aitken et al. 2017b). Furthermore, rutin supplementation following STZ induced diabetes will restore normoglycaemia and a normal lipid profile in murine models (Kamalakkannan and Prince 2006; Fernandes et al. 2010). Rat models of insulin insensitivity with ageing have demonstrated dietary supplementation with rutin will prevent insensitivity occurring and rescue impaired insulin resistance (Li et al. 2016). Quercetin treatment of rats fed a high-fat diet also prevented hyperglycaemia, hyperinsulinemia and reduced insulin resistance in
a glucose tolerance test, with no changes in body weight or adiposity (Arias et al. 2014). In addition to these functions, quercetin acts as an insulin secretagogue in rat isolated pancreatic islets by pharmacological activation of Ca\(^{2+}\) channels (Bardy et al. 2013). These data demonstrate quercetin and quercetin-glycosides act as anti-diabetic agents, preventing amylin aggregation, reducing insulin resistance, promoting insulin secretion and reducing hyperglycaemia.

Quercetin has also been demonstrated to inhibit the NLRP3 inflammasome, a major part of the innate immune response integral to both AD and T2D pathology (Masters et al. 2010; Heneka et al. 2013; Westwell-Roper et al. 2013; Baldwin et al. 2016; Westwell-Roper et al. 2016; Domiciano et al. 2017). Quercetin treatment of animal models of both AD (Yin et al. 2017) and T2D (Wang et al. 2012a; Zhang et al. 2014a) will rescue NLRP3 and IL-1\(\beta\) mediated deficits. The anti-amyloid activity quecertin has demonstrated towards A\(\beta\) and amylin is central to this anti-inflammatory activity, quercetin prevention of ASC aggregation during inflammasome activation prevents caspase-1 mediate IL-1\(\beta\) maturation (Domiciano et al. 2017).

6.6 Concluding remarks

Amylin was first identified as the main component of the islet-\(\beta\) plaques in T2D 30 years ago (Westermark et al. 1987a; Cooper et al. 1988b). A growing body of evidence implicates amylin aggregation in \(\beta\)-cell dysfunction (MacGibbon et al. 1997; Bai et al. 1999; Konarkowska et al. 2006; Zhang et al. 2008; Pillay and Govender 2013), generation of insulin resistance (Leighton and Cooper 1988b; Lee and Cooper 2002; Zhang et al. 2014b) and subsequent hyperglycaemia. Amylin deposition in the periphery is beginning to be connected to common complications of diabetes, such as nephropathy (Gong et al. 2007) and heart failure (Despa et al. 2012). Now, amylin has an emergent role in AD.

The hormonal role of amylin imbues it with the ability to cross the BBB (Banks and Kastin 1998), where it acts on multiple sites throughout the limbic system and entorhinal cortex to regulate reward and feeding behaviour (Mietlicki-Baase et al. 2015b). Data presented in this thesis (Chapter 3) and other studies demonstrates that amylin can aggregate and become deposited in the brain parenchyma. Immunohistochemistry data reported in the present study, and the literature, were limited by the methods employed. Further validation of enriched amylin plaques by mass spectrometry is required to prove they are primarily composed of amylin. Our immunohistochemistry data also demonstrates deposition of amylin in the brain vasculature, as well as local expression of \(IAPP\) in AD occipital lobe grey matter. These data make it unclear whether amylin deposition is as a result of infiltrating
species from the periphery or locally produced peptide. Due to availability of human brain tissue, we are unable to support the conclusions of Jackson et al. (2013) and Verma et al. (2016) that amylin is preferentially deposited in AD. Furthermore, our data does not support the hypothesis of Schultz et al. (2017), which suggest that amylin deposition in pericytes underlies the relationship between AD and T2D.

Data resulting from amylin treatment of iPSC-derived cortical neurons suggests amylin may directly contribute to AD pathogenesis by increasing Aβ secretion from neuronal culture, this effect is likely due to impairment of basal degradation of Aβ through autophagy mediated lysosomal targeting. The role of autophagy impairment in AD pathogenesis via promotion of Aβ by non-canonical amyloid deposition warrants further investigation. Amylin aggregate species that infiltrate or aggregate in the brain may also illicit signalling cascades demonstrated to result in the cellular pathology of AD (Chapter 4). It is unclear whether the observed signalling is mediated through the same cell surface receptors as Aβ oligomer species, but the literature suggests some cell surface receptors may be responsive to structure rather than sequence and as such may play a role in multiple amyloidoses.

Early T2D and associated hyperamylinemia often occur in mid-life, at the same time that amyloid deposition is beginning and long before AD symptoms emerge (Jack et al. 2010). While etiological studies link this state with later AD diagnosis, more mechanistic studies are needed to understand how hyperamylinemia contributes to AD pathogenesis in humans. The possible contributions of amylin to AD pathogenesis are summarised in Figure 6.1.

Modelling Aβ oligomer induced tau phosphorylation and the signalling cascades involved in this process were limited by the methods employed in this study. Neurons derived from iPSCs were treated for various timecourses at 50 and 80 days maturation. Aβ induced tau phosphorylation was variable in magnitude in all conditions tested, and investigation of kinase expression and phosphorylation status also revealed high levels of variability between experimental replicates from the same induction. These results indicate that while some major features of neuronal physiology may be common across inductions at a given maturation timepoint, other features such as kinase regulation are still in a state of flux as the neurons are yet to fully mature. This suggests future work should either endeavour to identify reasons for this regulatory divergence, which may include person-to-person handling, time of day of treatment, subtle differences in lysate harvesting protocols or initial seeding density, so as to better create uniformity across and within neuronal inductions. Alternatively, experimental design could be altered to increase the power of any such experiments to account for the potential divergence these factors may cause. Despite the limitations of the present study, the iPSC derived neuronal model represents great potential for understanding human neuronal signalling and mechanistic studies of amyloid induced cell effects.
Aβ and amylin toxicity have been demonstrated to be dependent upon their propensity to form soluble oligomer species (Haass and Selkoe 2007; Haataja et al. 2008). Therefore therapeutic strategies that could prevent amyloid oligomerisation and the pathogenic actions of oligomers would be therapeutically useful in AD and T2D. Quercetin has demonstrated such properties for Aβ and amylin. Quercetin treatment has established utility in multiple animal models of AD and T2D (Wang et al. 2012a; Ho et al. 2013; Wang et al. 2014; Zhang et al. 2016; Aitken et al. 2017b). Furthermore, quercetin has been shown to counteract key cellular processes associated with AD and T2D, these include pro-autophagy actions (Regitz et al. 2014) and anti-inflammatory properties (Domiciano et al. 2017). Flavonoid supplementation represents a cheap multimodal preventative intervention that has demonstrated tolerability in humans. Further chemistry based on the structure of quercetin or alternative delivery methods should be employed to better refine this structure into a potential therapeutic.

In summary, the potential contributions of amylin to AD are numerous. In early T2D amylin up regulation may promote impairment of neuronal autophagy, increasing brain Aβ and promoting amylin deposition in the brain parenchyma. But pathological studies utilising far greater numbers of patient tissue are required to conclude that amylin deposition is a feature of AD rather than non-canonical deposition. Amylin translocation across the BBB appears to result in amylin deposition in the vasculature that sometimes co-localises with CAA, but the nature of this vascular deposition and relationship to cognitive decline is unclear. We also present data suggesting IAPP is up regulated in AD occipital grey matter, and there may well be an independent contribution of T2D to this up regulation. The mechanism of IAPP up regulation is unclear but may be related to hyperglycaemia and insulin resistance. The local or infiltrating amylin may also contribute to pathogenic signalling in neurons, but the receptors responsible for mediating this effect are yet to be elucidated. The data presented in this thesis provides support to the hypothesis that amylin represents one of several mechanistic links between the aetiology of T2D and AD.
Figure 6.1 Possible contributions of amylin to AD

Amylin secretion from the pancreas is up regulated in T2D. This promotes insulin resistance which causes β-cell dysfunction (not shown). IAPP expression in the brain is up regulated, potentially due to associated insulin resistance or hyperglycaemia. Local or peripheral up regulation of amylin leads to impaired basal autophagy in neurons, resulting in an up regulation in Aβ and Aβ aggregation and promoting non-canonical amyloid deposition. Increased soluble Aβ and amylin species promote pathogenic signalling cascades and kinase dysregulation. Kinase dysregulation causes tau phosphorylation and NFTs associated with AD. Excluded from this figure are the potential contributions to inflammation and possible loss of amylin induced Aβ translocation from the brain as a result of reduced amylin secretion in late T2D.
References


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Inflammopharmacology.


Sevigny, J., Chiao, P., Bussiere, T., Weinreb, P. H., Williams, L., Maier, M., Dunstan, R., Salloway, S., Chen, T., Ling, Y., O’Gorman, J., Qian, F., Arastu, M., Li, M., Chollate,


Supplementary Figure 1: Intra-neuronal amylin staining (T-4150)

Representative immunohistochemistry of human control, non-diabetic, T2D and T2D-AD occipital lobe (A) and temporal lobe (B) sections histologically stained for amylin with the T-4150 antibody. Arrows indicate neurons positive for amylin staining. All images were captured at 27x magnification.
**Appendix 2 Intra-neuronal amylin staining (H-017-03)**

![Representative immunohistochemistry of human control, non-diabetic, T2D and T2D-AD occipital lobe (A) and temporal lobe (B) sections histologically stained for amylin with the H-017-03 antibody. Arrows indicate neurons positive for amylin staining. All images were captured at 27x magnification.](image)

**Supplementary Figure 2: Intra-neuronal amylin staining (H-017-03)**

Representative immunohistochemistry of human control, non-diabetic, T2D and T2D-AD occipital lobe (A) and temporal lobe (B) sections histologically stained for amylin with the H-017-03 antibody. Arrows indicate neurons positive for amylin staining. All images were captured at 27x magnification.
### Appendix 3 Ion-mobility mass spectrometry instrument parameters

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Voltages are tuned to maximise ion intensity

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Appendix 4 Evidence of Aβ aggregation in tip during IMMS

Supplementary Figure 3: Aβ_{1-42} blockade of glass tip during ionisation

Typical demonstration of how Aβ_{1-42} rapidly blockades in-house generated glass tips following 5 mins ionisation in negative mode ion mobility mass spectrometry. Arrow indicates blockade of tip caused by aggregated Aβ_{1-42} species.
A Rutin-tootin’ thesis

By Ben