The molecular mechanisms linking amyloid-β, the prion protein and tau in Alzheimer’s disease

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<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>α7nAchR</td>
<td>Alpha-7 nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>AβM/O</td>
<td>Amyloid beta monomer/oligomer</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>ADDL</td>
<td>Aβ-derived diffusible ligand</td>
</tr>
<tr>
<td>AEP</td>
<td>Asparagine endopeptidase</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid intracellular domain</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>BACE1</td>
<td>Beta-site APP cleaving enzyme 1</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>CBD</td>
<td>Corticobasal degeneration</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>Computerised tomography</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent resistant membranes</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>fAD</td>
<td>Familial Alzheimer's disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td>Fcγ receptor Iib</td>
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<tr>
<td>FSG</td>
<td>Fish skin gelatin</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontal temporal dementia</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GPI-anchor</td>
<td>Glycosylphosphatidylinositol-anchor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoropropanol-2-ol</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparin sulphate proteoglycan</td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>KO</td>
<td>Knock-Out</td>
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<tr>
<td>KPI-domain</td>
<td>Kunitz protease inhibitor-domain</td>
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<tr>
<td>Lambda-PP</td>
<td>Lambda protein phosphatase</td>
</tr>
<tr>
<td>LirB2</td>
<td>Leukocyte immunoglobulin-like receptor B2</td>
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<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>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MBS</td>
<td>Mes buffered saline</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>mGluR5</td>
<td>Metabotropic glutamate receptor 5</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSD</td>
<td>MesoScale Discovery</td>
</tr>
<tr>
<td>MBSM</td>
<td>Phosphate buffered saline containing calcium and magnesium ions</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween-20</td>
</tr>
<tr>
<td>PD</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emissions tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filament</td>
</tr>
<tr>
<td>PGRMC1</td>
<td>Progesterone receptor membrane component 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Cellular prion protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Scrapie prion protein</td>
</tr>
<tr>
<td>PSEN</td>
<td>Presenilin</td>
</tr>
<tr>
<td>PSP</td>
<td>Progressive supranuclear palsy</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute 1640 medium</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide electrophoresis</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
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<tr>
<td>SH</td>
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<td>STEP61</td>
<td>Striatal enriched tyrosine phosphatase 61</td>
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<td>STI1</td>
<td>Stress-inducible protein 1</td>
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<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
</tr>
</tbody>
</table>
The molecular mechanisms linking amyloid-β, the prion protein and tau in Alzheimer’s disease

2016

Several lines of evidence suggest that the expression of the cellular prion protein (PrP⁰) is altered with age and in sporadic Alzheimer’s disease, however, published results have been contradictory. Furthermore, a relationship between the expression of PrP⁰ and Tau has started to emerge. We have revealed a specific relationship between the expression of PrP⁰ and Tau in neuroblastoma cell lines and transgenic mouse models. In addition, we identified that the expression levels of PrP⁰ are reduced in multiple brain regions following the progression of sporadic Alzheimer’s disease. Furthermore, the reduction in PrP⁰ expression significantly correlated with the reduction in Tau expression and coincided with an increase in Tau pathology. In addition, data from neuroblastoma cell lines implicated the glycosylphosphatidylinositol (GPI)-anchor and in part the localisation of PrP⁰ to lipid rafts in mediating these alterations to Tau. We hypothesise that the reduction in PrP⁰ expression reflects a primary mechanism in Alzheimer’s disease pathogenesis and indirectly triggers the reduction in Tau expression which subsequently contributes to neuronal destabilisation and disruption to neuronal function. Soluble oligomeric forms of amyloid-beta (Aβ) are the primary pathogenic species in Alzheimer’s disease and strongly correlate with the presence and severity of cognitive decline. PrP⁰ acts as a high affinity neuronal receptor for Aβ oligomers and triggers pathogenic signaling cascades which induce synaptic impairment and further exacerbate neuronal destabilisation. We demonstrated that Flotillin-1 and the lipid raft localisation of PrP⁰ are essential for the binding of Aβ oligomers to PrP⁰. Furthermore, the metabotropic glutamate receptor, mGluR5 plays a pivotal role in the aberrant signaling of PrP⁰, and this PrP⁰/mGluR5 complex provides a mechanistic link between extracellular Aβ oligomers and intracellular Tau phosphorylation, by Fyn kinase, Pyk2 and possibly by inactivation of the protein phosphatase, PP2A. Considering there is now strong evidence that Tau is the mediator of Aβ induced toxicity, the reduction in Tau levels mediated by PrP⁰ may be a protective mechanism. Aβ oligomers interact with a multitude of neuronal receptors in addition to PrP⁰. It is likely that activation of multiple receptor complexes and signalling cascades are responsible for synaptic impairment and Tau phosphorylation induced by Aβ, however, these complexes remain to be fully determined. Investigating Aβ oligomer induced Tau phosphorylation in vitro has proven challenging, however, we suggest that a functional, mature, neuronal model is necessary to induce the complex mechanisms linking extracellular Aβ oligomers and the phosphorylation of intracellular Tau. A greater understanding of the complex relationship between Aβ, PrP⁰ and Tau will aid in our understanding of the molecular mechanisms underlying Alzheimer’s disease and in the discovery of novel therapeutic targets for this progressive neurodegenerative disease.
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Chapter 1. Introduction

1.1 Alzheimer’s disease

1.1.1 Alzheimer’s disease: an increasing social and economic burden

Alzheimer’s disease is a progressive, neurodegenerative disease and the most prevalent form of dementia. There are currently over 850,000 people living with dementia in the UK today. By 2025 this number is expected to rise over 1 million and exceed 2 million by 2050 (http://www.alzheimersresearchuk.org). Dementia costs the UK economy over £24 billion a year and globally over £818 billion each year (http://www.alzheimersresearchuk.org). Age is the single major risk factor for Alzheimer’s disease. With increasing life expectancy and currently no treatments available to halt or slow disease progression, Alzheimer’s disease is fast becoming an increasing economic and social burden to the ever-ageing population. Increasing our understanding of the complex mechanisms underlying disease pathogenesis and neurodegeneration of this devastating disease is crucial for the development of novel therapeutics.

1.1.2 Symptoms and pathological hallmarks of Alzheimer’s disease

Alzheimer’s disease is characterised by the progressive loss of memory and cognitive function as a result of substantial neuronal cell death, predominantly of basal forebrain cholinergic neurons innervating the cerebral cortex and hippocampus (Perry et al., 1978, Mufson et al., 2008). In late stages of disease progression there is a widespread decline in neurons with various neurotransmitter end-bodies, causing widespread atrophy, see Figure 1.1. Loss of innervation to the cerebral cortex and hippocampus affects planning, problem solving, and the formation and recall of memories, constituting the main symptoms underlying Alzheimer’s disease. Other symptoms of Alzheimer’s disease include elevated anxiety and depression, and problems with communication and language (http://www.alzheimers.org.uk).
**Figure 1.1 Substantial neuronal loss and brain atrophy in Alzheimer’s disease**

Coronal sections of a healthy adult brain (left) and Alzheimer’s disease brain (right). In Alzheimer’s disease there is substantial neuronal loss and global brain atrophy. Widening of the lateral ventricles and loss of the hippocampus, a region involved in memory and learning is particularly prominent. The original image was obtained from Professor Seth Love (The University of Bristol, UK) and rights have been granted to reuse the image.

Structural changes to the brain in patients with cognitive decline are identified using imaging techniques such as computerised tomography (CT) and magnetic resonance imaging (MRI) (Johnson et al., 2012). Brain atrophy, particularly a reduction in hippocampal volume is associated with increasing cognitive decline in Alzheimer’s disease (Cardenas et al., 2011). Functional MRI (fMRI) and positron emission tomography (PET) imaging techniques are also used to assess the activity and connectivity between neuronal networks underlying memory and cognitive function (Johnson et al., 2012).

In addition to substantial neuronal cell loss and loss of neuronal networks, Alzheimer’s disease is characterised by two insoluble protein aggregates; amyloid-beta (Aβ) peptide plaques which accumulate around the outside of neurons and intracellular neurofibrillary Tau tangles (NFT) (Serrano-Pozo et al., 2011). Aβ plaques and neurofibrillary tangles are the two main neuropathological hallmarks of the disease, **Figure 1.2**. Changes in Aβ and Tau in the cerebrospinal fluid (CSF) are used as biomarkers for disease pathogenesis and strongly correlate to cognitive impairment (Olsson et al., 2016). These two molecular pathologies can also be visualised in patients using specialised imaging techniques to
analyse the extent and progression of neuropathology (Scholl et al., 2016, Vlassenko et al., 2012).

![Amyloid-β plaques and Neurofibrillary Tau tangles](image)

**Figure 1.2 Neuropathological hallmarks of Alzheimer’s disease**

Histology staining of amyloid-β plaques (left) and neurofibrillary Tau tangles (NFTs) (right) in the hippocampus of an Alzheimer’s disease brain. The NFT were stained with the anti-phosphorylated Tau antibody, AT8 (pTau Ser202/Thr205). The original images were obtained from Tammaryn Lashley (University College London, UK) and rights have been granted to reuse the image.

1.1.3 The amyloid-cascade hypothesis

The amyloid-cascade hypothesis is the dominant model for Alzheimer’s disease pathogenesis. In the original publication of the hypothesis by Hardy and Higgins in 1992, the authors stated that Aβ “is the causative agent of Alzheimer’s pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition” (Hardy and Higgins, 1992). Despite over two decades of research, this original hypothesis largely still holds true although fails to capture the true complexity of the disease. Although increased production and aggregation of Aβ is key to the initiation and pathogenesis of disease, on its own Aβ is not sufficient to cause Alzheimer’s disease with multiple molecular, cellular and genetic imbalances all contributing to the neuronal loss, reduction in network connectivity and dementia in Alzheimer’s disease (Herrup, 2015, Selkoe and Hardy, 2016). Furthermore, increasing evidence suggests Tau is the mediator of Aβ toxicity in Alzheimer’s disease (Oddo et al., 2006b, Roberson et al., 2007).
1.2 The Aβ peptide

1.2.1 Proteolytic processing of APP

The Aβ peptide is generated by the sequential proteolytic cleavage of the amyloid precursor protein (APP). Three groups of proteases are involved in the proteolytic processing of APP; alpha (α)-secretase, beta (β)-secretase and gamma (γ)-secretase. Members of the membrane bound ADAM (a disintegrin and metalloprotease) family mediate α-secretase activity (De Strooper et al., 2010). Members include ADAM9, ADAM17 and ADAM10, with evidence suggesting that ADAM10 is the principal neuronal α-secretase (Kuhn et al., 2010). The β-site APP cleaving enzyme 1 (BACE1) is highly expressed in the brain and is the principal β-secretase (Vassar, 2014). In comparison, γ-secretase is a high molecular weight multi-subunit complex which requires Presenilins for secretase activity (De Strooper, 2007) (For further information on APP secretases see the following reviews (De Strooper et al., 2010, Haass et al., 2012)). However, recently the identification of novel APP secretases add to complexity of APP proteolytic processing (For recent review see (Andrew et al., 2016)).

APP is processed by two pathways; the amyloidogenic and non-amyloidogenic pathways, see Figure 1.3. Both APP processing pathways are normally active in the brain and Aβ is generated under physiological conditions (Haass et al., 1992) however, APP is predominantly processed by the non-amyloidogenic pathway and α-secretase which precludes Aβ production. Amyloidogenic processing of APP by the sequential cleavage of APP with β- and γ-secretases generates Aβ peptides which range in length from 36-43 amino acids. Peptides containing 40 amino acids (Aβ1-40) are the most abundant in the brain and account for 80-90% of Aβ in the brain, followed by Aβ1-42 which accounts for around 5% of Aβ peptides in the brain (Murphy and LeVine, 2010).
Figure 1.3 APP proteolysis and Aβ peptide generation

The transmembrane protein APP is processed by two pathways; the non-amyloidogenic and amyloidogenic pathway. In the amyloidogenic pathway Aβ peptides are generated by sequential cleavage with beta (β)-secretase and gamma (γ)-secretase. Firstly, β-secretase cleaves APP releasing a secreted N-terminal fragment, sAPPβ and a membrane bound C-terminal fragment, C99. The membrane bound fragment is subsequently cleaved by γ-secretase at various positions to generate Aβ ranging in length from 36-43 amino acids and the amyloid intracellular domain (AICD). APP can also be cleaved by alpha (α)-secretase producing a secreted N-terminal fragment (sAPPα) and a membrane bound C-terminal fragment, C83. Cleavage of APP by β-secretase occurs within the Aβ sequence and therefore precludes Aβ formation. Again the membrane bound fragment can be cleaved by γ-secretase to produce a secreted P3 fragment and AICD fragment (O’Brien and Wong, 2011, Zhang et al., 2011b).

APP is a single pass type 1 transmembrane protein with a large extracellular domain. The physiological function of APP and APP metabolites remains relatively unknown (Andrew et al., 2016). However, APP is known to play a role in neuronal survival/outgrowth and to regulate various signal transduction mechanisms (Dawkins and Small, 2014). Alternative splicing of the APP gene generates three major isoforms; APP770, APP751 and APP695, comprising of 770, 751 and 695 amino acids, respectively (Zhang et al., 2011b). The two longer APP isoforms contain an additional Kunitz protease inhibitor (KPI) domain in the extracellular domain of the protein.
The deposition of Aβ is a primary event in both sporadic and familial Alzheimer’s disease (O’Brien and Wong, 2011). Interestingly the levels of KPI-domain containing APP isoforms increase in Alzheimer’s disease and are associated with increased Aβ production and deposition in the brain (Menendez-Gonzalez et al., 2005, Ho et al., 1996). The processing of APP by secretases is altered by genetic mutations of autosomal dominant Alzheimer’s disease but may also be altered in sporadic Alzheimer’s disease contributing to increased Aβ levels (Tyler et al., 2002). Furthermore, the ratio of Aβ40/42 peptides shifts and levels of Aβ1-42 are increased. This longer form of Aβ is more hydrophobic and prone to aggregation, and constitutes the principal Aβ species deposited in the brain (Jarrett et al., 1993). Furthermore, an impairment of Aβ clearance and degradation mechanisms contributes to increased amyloid pathology in sporadic Alzheimer’s disease (Bates et al., 2009, Wildsmith et al., 2013, Ries and Sastre, 2016, Baranello et al., 2015).

1.2.2 Genetic mutations and altered Aβ production

Genetic analysis from rare autosomal dominant familial Alzheimer’s disease (fAD) provides the strongest evidence for Aβ initiating neuronal loss and for support of the amyloid-cascade hypothesis. Mutations in three genes APP, PSEN1, and PSEN2 are known to be associated with fAD. To date over 50 mutations in APP, over 200 in PSEN1 and around 15 in PSEN2 have been identified (http://www.molgen.vib-ua.be/ADMutations). Individuals with these rare mutations tend to develop Alzheimer’s disease before the age of 65 and so are described as early-onset Alzheimer’s disease. It is estimated that only around 1-5% of all Alzheimer’s disease cases, both familial and sporadic are early-onset (Zhu et al., 2015).

Most mutations in APP are clustered around the secretase cleavage sites of APP and alter Aβ production. For example, the Swedish mutation (APP K670M/N671L) increases the production of total Aβ levels (Mullan et al., 1992), the Arctic mutation (APP E693G) increases the propensity of Aβ to aggregate (Nilsberth et al., 2001) and the Indiana mutation (APP V717F) increases the ratio of Aβ42/40 peptides (Murrell et al., 1991). PSEN genes encode Presenilin proteins which are catalytic components of the γ-secretase enzyme complex. PSEN pathogenic mutations increase γ-secretase cleavage of APP and subsequently increase Aβ generation (Sisodia and St George-Hyslop, 2002, De Strooper, 2007, Wolfe, 2012).
In addition, genome wide association studies (GWAS) have identified multiple genetic risk factors in sporadic Alzheimer’s disease. The strongest genetic risk factor is the APOE-ε4 allele which increases the risk of disease 4-fold and 12-fold for heterozygous and homozygous carriers, respectively (Verghese et al., 2011). The apolipoprotein E4 (APOE4) lipoprotein influences Aβ metabolism by promoting aggregation and disrupting clearance (Musiek and Holtzman, 2015). Other genetic risk factors include: BIN1, PICALM, SORL1, CLU, TREM2 and CD33 and are roughly grouped to influence three pathways; immune system/inflammatory responses, cholesterol/lipid metabolism and endosomal vesicle recycling (Van Cauwenberghe et al., 2016, Lacour et al., 2016).

1.2.3 Aβ assemblies and aggregation

Following cleavage from APP by γ-secretase Aβ loses its helical structure and rapidly aggregates to form oligomers, protofibrils and fibrils before ultimately forming insoluble plaques rich in beta-sheet structures (Benilova et al., 2012). Increasing evidence suggests that smaller soluble, oligomeric conformations of the Aβ peptide are the pathogenic species rather than larger insoluble aggregates (for review see (Walsh and Selkoe, 2007)). Soluble Aβ oligomers induce the inhibition of hippocampal long-term potentiation (LTP) and impaired spatial memory in vivo (Lauren et al., 2009, Walsh et al., 2002, Mucke et al., 2000, Ferreira and Klein, 2011, Lambert et al., 1998) and also strongly correlate to Tau phosphorylation (Chabrier et al., 2012). Furthermore, soluble Aβ oligomers strongly correlate with the presence and severity of cognitive decline (McLean et al., 1999, Lue et al., 1999).

Various ‘toxic’ soluble oligomeric forms of Aβ have been isolated from transgenic mouse models, human Alzheimer’s disease brain tissue, cell culture medium and generated from synthetic Aβ peptides (Benilova et al., 2012). These assemblies include, low molecular weight oligomers such as dimers and trimers (Walsh et al., 2002), Aβ*56 (56 kDa dodecamer assembly) (Lesne et al., 2008), high molecular-weight oligomers (Rushworth et al., 2013) and annular Aβ (Lasagna-Reeves et al., 2011c). Many authors use the term ADDL (Aβ-derived diffusible ligand) to describe soluble toxic oligomers. However, the size of the ADDL species varies depending on the conditions for preparation (Lambert et al., 1998, Chromy et al., 2003). These differences are possibly a result of the dynamic equilibrium...
between species (Klein et al., 2001). For clarification, oligomeric forms of Aβ are defined by their size and conformation throughout this thesis.

1.3 Toxic mechanisms of Aβ

The misfolding and aggregation of proteins is a feature of many neurodegenerative diseases, classed as proteinopathies and include: Aβ/Tau and Alzheimer’s disease; α-Synuclein and Parkinson’s disease; Huntingtin and Huntington’s disease; the prion protein (PrP) and transmissible spongiform encephalopathies (TSEs) (Bayer, 2015). Despite differences in the brain regions affected, clinical symptoms and progression of disease there may be common molecular mechanisms by which misfolded and aggregated proteins induce neurotoxicity (Soto, 2003). Indeed altered membrane permeabilisation, oxidative stress, neuroinflammation, calcium dyshomeostasis and excitotoxicity have all been identified as mechanisms of Aβ toxicity but are also key features of multiple neurodegenerative disorders. Many of these mechanisms converge to induce synaptic dysfunction and neuronal death, as summarised in Figure 1.5. A greater understanding of the mechanisms of synaptic impairment and neuronal death induced by protein aggregates may aid in the discovery of novel therapeutic targets for various neurodegenerative diseases (Golde et al., 2013).

1.3.1 Membrane permeabilisation

Many amyloidogenic proteins including Aβ alter membrane permeabilisation which subsequently alters the neuronal ionic and calcium homeostasis. Calcium dysregulation has been widely reported in Alzheimer’s disease and many signalling pathways which are essential for synaptic and neuronal functions are dependent on calcium (Glabe and Kayed, 2006, Demuro et al., 2005, LaFerla, 2002, Yu et al., 2009). To alter membrane permeabilisation amyloidogenic proteins may directly associate with the neuronal membrane but also form channel- or pore-like structures (Green et al., 2004) (Figure 1.5). A specific Aβ oligomer structure termed annular Aβ alters membrane permeabilisation by forming pore-like structures in the plasma membrane (Kayed et al., 2009). These specific Aβ aggregates have been isolated in Alzheimer’s disease brain tissue and are distinct from
fibril forming oligomers (Lasagna-Reeves et al., 2011c, Lashuel et al., 2002, Lashuel and Lansbury, 2006).

1.3.2 Oxidative stress

An imbalance between the production of reactive oxygen species (ROS) and antioxidants results in oxidative stress and damage to proteins, lipids and nucleic acids. Oxidative stress has been implicated in both ageing (Finkel and Holbrook, 2000) and neurodegenerative diseases (Barnham et al., 2004). Oxidative stress disrupts multiple signalling pathways, impairing neuronal/synaptic function and induces neuronal damage/death (Butterfield et al., 2013, Zhao and Zhao, 2013). Aβ may mediate oxidative stress by inducing mitochondrial dysfunction (Lin and Beal, 2006) or by the reduction of copper (Cu) and iron (Fe) metals, both of which are redox active metals and produce the redox active species, hydrogen peroxide, following reduction (Maynard et al., 2005, Perry et al., 2002, Huang et al., 1999). Furthermore, increased levels of Cu, Fe and the redox silent metal zinc (Zn) may also contribute to amyloidogenesis and toxicity in Alzheimer’s disease (Maynard et al., 2005, Loef and Walach, 2012). Soluble oligomers are also linked to oxidative stress and ROS generation via N-methyl-D-aspartate (NMDA)-receptor activation and increased calcium influx (De Felice et al., 2007). Again aberrant calcium signalling via this mechanism affects multiple pathways and triggers synaptic impairment (Kamat et al., 2016).

1.3.3 Inflammation

A common link between many neurodegenerative diseases is inflammation (Amor et al., 2014, Heppner et al., 2015). GWAS studies have identified variants in TREM2 (Guerreiro et al., 2013, Colonna and Wang, 2016) and CD33 (Bradshaw et al., 2013) with increased risk of Alzheimer’s disease and are implicated in altering inflammatory responses but also Aβ clearance, supporting a role of neuroinflammation in Alzheimer’s disease pathophysiology. In addition, activated microglia and astrocytes are known to surround insoluble amyloid plaques (Metcalf and Figueiredo-Pereira, 2010). Soluble Aβ oligomers also interact with multiple receptors on the surface of microglia and astrocytes. The scavenger receptor SCARA-1 may be involved in the phagocytosis and clearance of soluble Aβ and thereby protecting against Aβ-induced synaptic impairment and toxicity (Frenkel et al., 2013). On
the other hand, Aβ binding to the scavenger receptor CD36 and receptor for advanced glycation end products (RAGE) induces microglial activation and the release of pro-inflammatory cytokines such as TNFα (tumour necrosis factor alpha) and IL1β (interleukin 1 beta) (Wilkinson and El Khoury, 2012, Origlia et al., 2010) subsequently inducing synaptic dysfunction and oxidative stress (Moore et al., 2002) (Figure 1.5). Furthermore, in a tauopathy mouse model, prominent microglial activation and synaptic dysfunction was observed prior to NFT formation (Yoshiyama et al., 2007). One hypothesis is that small protein aggregates, such as oligomers, induce neuroinflammation which can subsequently exacerbate protein aggregation.

### 1.3.4 Intracellular Aβ

Not long after the original reports of Aβ accumulating in extracellular plaques, intracellular Aβ was also described in the literature (Grundke-Iqbal et al., 1989). Intracellular Aβ, most of which ends at residue 42 appears to accumulate before the onset of extracellular Aβ and has been associated with proteasomal dysfunction, synapse dysfunction, loss of the α7-nicotinic acetylcholine receptor (α7nAChR) and cognitive impairment (Takahashi et al., 2002, Oddo et al., 2006a, Billings et al., 2005, Sakono and Zako, 2010, Gouras et al., 2000). Aβ can be produced intracellularly by the proteolytic cleavage of APP in the endoplasmic reticulum (ER) and trans-Golgi network (TGN). Extracellular Aβ also binds to neuronal receptors including the low density lipoprotein receptor-related protein 1 (LRP1) (Rushworth et al., 2013) and α7nAChR (Nagele et al., 2002) which mediate the internalisation of Aβ (Figure 1.5). The extent of the relationship between extracellular and intracellular Aβ remains to be determined. However, there is evidence that intracellular Aβ may act as a source for extracellular plaques (for a review on intracellular Aβ see (LaFerla et al., 2007)).

### 1.3.5 Cholesterol and lipid raft microdomains

Cholesterol has been implicated in the regulation of Aβ production, aggregation and clearance but also in mediating the toxic actions of Aβ (Ricciarelli et al., 2012). GWAS studies for Alzheimer’s disease have identified multiple susceptibility genes implicated in cholesterol metabolism (Lacour et al., 2016), including APOEε4 and CLU which encodes...
clusterin (also known as apolipoprotein J) both of which have been implicated in
cholesterol transport and Aβ clearance (Desikan et al., 2014). Furthermore, in vivo
hypercholesterolemia accelerates the deposition of Aβ in the brain (Shie et al., 2002) and in
vitro the amyloidogenic processing of APP and production of Aβ peptides is modulated by
cholesterol (Rushworth and Hooper, 2010).

Cholesterol is a key component and modulator of lipid raft microdomains. These are
distinct regions of the plasma membrane rich in cholesterol, sphingolipids, phospholipids
with saturated hydrocarbon tails and GPI-anchored proteins, Figure 1.4. Lipid raft
microdomains play a key role in signal transduction (Simons and Toomre, 2000, Simons and
Ehehalt, 2002) and are implicated in the pathogenesis of Alzheimer’s disease (Hicks et al.,
2012, Rushworth and Hooper, 2010). For example, the amyloidogenic processing of APP is
dependent on cholesterol via lipid raft microdomains (Rushworth and Hooper, 2010).
Although only a small proportion of APP is localised to lipid raft microdomains, this
localisation is crucial for β-secretase cleavage and Aβ generation. In comparison, α-
secretase cleavage of APP occurs predominantly in non-raft fractions of the plasma
membrane and is not dependent on cholesterol (Chen et al., 2006, Ehehalt et al., 2003).

**Figure 1.4 Cholesterol rich lipid raft microdomains**

Lipid raft microdomains are composed of cholesterol, sphingolipids and phospholipids with
saturated fatty acid chains. The highly saturated chains enable tight packing and an ordered
composition compared to the rest of the plasma membrane containing phospholipids with
largely unsaturated chains. Lipid raft microdomains are estimated to be 25-100 nm in
diameter and their structure and tight packing results in their resistance to solubilisation by
non-ionic detergents such as Triton X-100. Lipid rafts are enriched in GPI-anchored proteins
and acylated proteins due to interaction with saturated fatty acid chains (Allen et al., 2007,
Rushworth and Hooper, 2010).
Furthermore, lipid raft microdomains are key for the assembly, compartmentalisation and signalling of multi-protein receptor complexes. These complexes are implicated in binding Aβ oligomers and the subsequent pathogenic signalling cascades and synaptic impairment mediated by oligomeric Aβ (Jarosz-Griffiths et al., 2016) (Figure 1.5). Aβ oligomers preferentially bind to excitatory neurons containing NMDA-receptors which are localised to lipid raft microdomains (Lacor et al., 2007, Frank et al., 2004). NMDA-receptor dependent calcium influx subsequently triggers multiple signalling cascades which are essential for synaptic plasticity and function (Tsien et al., 1996, Paoletti et al., 2013, Hunt and Castillo, 2012). These data implicate lipid raft microdomains in normal synaptic function and in the pathogenesis of Alzheimer’s disease. Furthermore, many of these complexes provide a mechanistic link to extracellular Aβ oligomer induced phosphorylation of intracellular Tau. The cell surface signalling complexes involved in the binding of Aβ and the subsequent phosphorylation of Tau and synaptic impairment are explored in further detail in Section 1.4.

In Alzheimer’s disease the composition of lipid raft microdomains is altered and there is an increase in membrane order and viscosity (Fabelo et al., 2014, Martin et al., 2010), possibly favouring the amyloidogenic processing of APP and aberrant signalling mediated by Aβ.
Oligomeric conformations of Aβ mediate synaptic impairment and neuronal death by multiple mechanisms. Cell surface signalling multi-protein complex(es) mediate Aβ oligomer binding and the subsequent excitotoxicity, Tau phosphorylation/truncation and synaptic impairment but also mediate Aβ internalisation. Aβ oligomers also bind to receptors on microglial cells triggering release of pro-inflammatory cytokines triggering neuroinflammation. Aβ oligomers also interact with or form pore-like structures in the plasma membrane to alter membrane permeability and calcium dyshomeostasis further disrupting synaptic function. In addition to internalisation of cell-surface receptors, intracellular Aβ may also be produced in the endoplasmic reticulum or the trans-Golgi network contributing to toxic mechanisms such as mitochondrial dysfunction and oxidative stress.
1.4 Aβ cell surface signalling complexes

Aβ interacts with multiple receptors at the neuronal surface. These include: α7nAChR (Snyder et al., 2005, Parri et al., 2011), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA)-receptors (GluR2 subunit) (Zhao et al., 2010), EphA4 and EphB2 receptors (Cisse et al., 2011b, Vargas et al., 2014), Frizzled receptor (Magdesian et al., 2008), Insulin receptor (Zhao et al., 2008), Fcγ receptor Iib (FcγRIIb), leukocyte immunoglobulin-like receptor B2 (LilrB2) (Kim et al., 2013), Na+/K+-ATPase neuron-specific subunit (NaKα3) (Ohnishi et al., 2015), p75 neurotrophin receptor (p75NTR), Sigma-2/PGRMC1 (progesterone receptor membrane component 1) (Izzo et al., 2014) and PrPc (Lauren et al., 2009).

As briefly described multi-protein signalling complexes, some of which are dependent on the integrity of lipid raft microdomains are crucial for the binding and subsequent synaptic impairment induced by Aβ. Furthermore, some of these receptors and signalling complexes provide a possible mechanistic link to Tau. However, cell-surface signalling complexes linking Aβ to Tau are largely not well understood or characterised. Aberrant changes to Tau contribute to synaptic impairment and toxicity and reducing Tau prevents the excitotoxicity, synaptic impairment and cognitive decline induced by Aβ in vivo (Shipton et al., 2011, Roberson et al., 2007, Oddo et al., 2006b), therefore identifying the mechanisms linking Aβ and Tau may aid the identification of novel therapeutic strategies. Below three multi-protein signalling complexes based around PrPc, Eph receptors and the α7nAch receptor are proposed that may provide a mechanistic link between Aβ, Tau phosphorylation and synaptic impairment.

1.4.1 PrPc signalling complex

The best described and most widely documented cell-surface Aβ signalling complex centres around the lipid raft localised, GPI-anchored protein, PrPc. Prefibrillar, soluble Aβ oligomers of high molecular weight derived from synthetic peptide (Lauren et al., 2009) and soluble Aβ dimers derived from Alzheimer’s disease brain extracts (Larson et al., 2012) bind to PrPc with high affinity in nanomolar concentrations.

Aβ oligomer binding to PrPc induces activation of the Src kinase, Fyn (Lauren et al., 2009). Fyn subsequently phosphorylates the NR2B subunit of NMDA-receptors at tyrosine 1472 facilitating the interaction between NMDA-receptors and the scaffolding protein PSD95.
The increased association between NMDA-receptors and PSD95 results in an initial increase in surface expression of NMDA-receptors and increased excitotoxicity (Salter and Kalia, 2004, Aarts et al., 2002). This initial increase is followed by a loss of surface expression of NMDA-receptors, triggering dendritic spine loss and synaptic impairment (Um et al., 2012, Rushworth et al., 2013).

The internalisation of NMDA-receptors is mediated by the phosphotyrosine phosphatase, striatal enriched tyrosine phosphatase 61 (STEP61). Aβ activates STEP which subsequently dephosphorylates the NR2B subunit at tyrosine 1472 thus opposing the actions of Fyn (Kurup et al., 2010). Furthermore, STEP is also able to dephosphorylate and inactivate Fyn (Xu et al., 2015). It should be noted that there is no direct evidence that STEP61 is activated by Aβ oligomer binding to PrP⁶¹.

Aβ binding to PrP⁶¹ and the subsequent neurotoxicity is dependent on the integrity of lipid raft microdomains (Rushworth et al., 2013) and two transmembrane receptors, the low density lipoprotein (LDL)-receptor-related protein-1 (LRP1) and the metabotropic glutamate receptor, mGluR5, see Figure 1.6. Interestingly recruitment of both LRP1 and mGluR5 to lipid raft microdomains enhances in the presences of Aβ (Rushworth et al., 2013, Renner et al., 2010). LRP1 and mGluR5 have been separately identified to play a key role in the Aβ/PrP⁶¹ signalling complex (Rushworth et al., 2013, Um et al., 2013), however, it is yet to be determined whether they are present in the same complex or represent distinct PrP/Aβ signalling complexes.

Fyn also phosphorylates Tau at tyrosine 18 (Tyr18), thus this cell-surface complex links Aβ oligomers to Tau pathology. Tau phosphorylated at Tyr18 is present during neuronal development and is absent in adults, however, it is found in NFTs in Alzheimer’s disease (Lee et al., 2004). In addition, Fyn also phosphorylates and activates the Alzheimer’s disease risk gene product Pyk2 (PTK2B: Protein tyrosine kinase 2B) (Lambert et al., 2013). Pyk2 is enriched in the post-synaptic density and is involved in synaptic plasticity by interacting with NMDA-receptors via PSD95 (Huang et al., 2001, Seabold et al., 2003). The transmembrane receptor, mGluR5, mediates the association between PrP⁶¹ and Pyk2, however, this interaction is reduced following Aβ interaction with PrP⁶¹ possibly by Fyn activation, further contributing to synaptic dysfunction (Haas and Strittmatter, 2016).

Furthermore, LRP1 mediates the subsequent internalisation of Aβ/PrP⁶¹ via clathrin-dependent endocytosis and trafficking to the endosomes and lysosomes where it is subsequently degraded (Rushworth et al., 2013, Taylor and Hooper, 2007).
internalisation of Aβ/PrP<sup>C</sup> is thought to contribute to Aβ oligomer mediated toxicity (Chafekar et al., 2008).

![Diagram](image)

**Figure 1.6 Aβ oligomer induced PrP<sup>C</sup> signalling complex**

Aβ oligomers bind to PrP<sup>C</sup> with high affinity on the postsynaptic density of neurons and induce the formation of a transient signalling complex within lipid raft microdomains involving the transmembrane receptors LRP1 and mGluR5. This signalling complex mediates the activation of Fyn kinase which phosphorylates Tau at tyrosine 18 but also the NR2B subunit of NMDA receptors at Tyr1472. Phosphorylation of NMDA-receptors at this site facilitates interaction with the scaffolding protein PSD95, increasing surface expression and inducing excitotoxicity. STEP61 is also activated by Aβ and subsequently dephosphorylates NR2B subunits opposing Fyn action and inducing the internalisation of the NMDA receptors and synaptic impairment. Aβ oligomers bound to PrP<sup>C</sup> are subsequently internalised by LRP1.

### 1.4.2 α7nAChR signalling complex

Nicotinic acetylcholine receptors consisting entirely of alpha-7 subunits (α7nAChR) are highly expressed in basal forebrain cholinergic neurons and play a key role in calcium homeostasis. The nAChR α7 subunits are localised in lipid raft microdomains and their activity is dependent on cholesterol and lipid raft integrity (Oshikawa et al., 2003). In
Alzheimer’s disease cholinergic neurons are predominantly affected resulting in disruption of innervation to the hippocampus and the cortex and α7nAChR are strongly implicated in disease pathogenesis (Parri et al., 2011).

Aβ interacts with α7nAChR triggering altered cholinergic transmission and calcium signalling (Wang et al., 2000, Fodero et al., 2004). Interaction of Aβ to α7nAChR also activates the calcium sensitive enzyme, PP2B (also known as calcineurin). PP2B subsequently dephosphorylates and activates the STEP61 which dephosphorylates the NR2B subunit of NMDA receptor at tyrosine 1472 inducing its endocytosis by reducing its interaction with PSD95 (Snyder et al., 2005), Figure 1.7. Aβ induced Tau phosphorylation (Ser202, Thr181 and Thr231) is also mediated by α7nAChR via activation of the mitogen-activated protein kinases (MAPKs), ERK and JNK-1 kinase (Wang et al., 2003).

The studies described above have predominantly used Aβ peptides or small oligomeric assemblies to investigate Aβ induced α7nAChR deficits (Wang et al., 2000, Fodero et al., 2004, Snyder et al., 2005, Wang et al., 2003). It remains to be determined the exact binding site on the α7 subunit, and the role of different oligomeric assemblies of Aβ on α7nAChR function and Tau phosphorylation.

Interestingly, PrP C interacts with α7nAChR to increase calcium influx following interaction with the stress-inducible protein 1 (STI1) promoting neuronal differentiation via protein synthesis and mediating synaptic function (Beraldo et al., 2010, Roffe et al., 2010). STI1 binds to PrP C in the vicinity of the Aβ oligomer binding site (Ostapchenko et al., 2013). Competition between Aβ mediated toxic actions and loss of STI1 neurotrophic function may contribute to PrP C/α7nAChR mediated synaptic dysfunction (Ostapchenko et al., 2013). The role of α7nAChR in other toxic actions mediated by Aβ/PrP C including Fyn kinase activation remain to be determined.
Figure 1.7 Aβ induced α7nAChR signalling complex

Aβ interacts with α7nAChR on the post-synaptic density activating the calcium sensitive phosphatase, PP2B. PP2B subsequently dephosphorylates and activates STEP61 which desphosphorylates the NR2B subunit of NMDA receptors at Tyr1472, triggering its internalisation and contributing to synaptic impairment. Aβ also induced Tau phosphorylation mediated by α7nAChR and activation of MAPK further contributing to synaptic impairment.

1.4.3 Eph signalling complexes

Eph family receptor tyrosine kinases play a key role in the development and maintenance of neurons and other cell types. Eph receptors and their ligands, Ephrins, can be divided into two subgroups; EphA and EphB (Kullander and Klein, 2002). Both EphA4 and EphB2 are highly expressed in the cortex and hippocampus and are implicated in maintaining and stabilising synaptic structure and glutamatergic neurotransmission via NMDA and AMPA receptors (Simon et al., 2009).

EphB2 activation leads to src-kinase dependent phosphorylation of the NR2B subunit of NMDA-receptors at three tyrosine residues, including Tyr1472, the site important for surface localisation (Salter and Kalia, 2004, Antion et al., 2010, Nolt et al., 2011). EphB2 levels are reduced in Alzheimer’s disease transgenic mice and in incipient Alzheimer’s disease (Simon et al., 2009). Aβ oligomers (dimers and trimers) bind to the fibronectin repeat domain of EphB2 triggering the subsequent degradation of EphB2 in the proteasome.
inducing synaptic impairment via loss of NMDA-receptors at the cell surface (Cisse et al., 2011a), Figure 1.8. Loss of NMDA-receptors via EphB2 occurs in a distinct complex to PrP<sup>C</sup> (Miyamoto et al., 2016). Reversing EphB2 depletion reverses NMDA-dependent synaptic and memory impairments in vivo (Cisse et al., 2011a). Furthermore, increasing EphB2 levels may also prevent Aβ induced loss of surface AMPA-receptors (Miyamoto et al., 2016).

In addition, activation of Ephb2 normally induces Tau dephosphorylation via phosphatidylinositol-3-kinase (PI3K)/Akt mediated GSK3β inhibition (Jiang et al., 2015). Therefore upregulation of EphB2 may prevent Aβ oligomer induced synaptic deficits by NMDA/AMPA-receptors but also attenuate Tau phosphorylation.

Aβ oligomers also interact with and activate EphA4 inducing activation of the tyrosine kinase, c-Abl and synaptic impairment mediated by loss of surface AMPA-receptors (Vargas et al., 2014), Figure 1.8. Blocking EphA4 with small molecule inhibitors rescues synaptic impairment induced by Aβ and reverses synaptic plasticity in an Alzheimer’s disease mouse model (Fu et al., 2014). Activation of c-Abl is linked to phosphorylation of Tau at tyrosine 394 but also indirectly at serine and threonine residues via activation of CDK5 (Schlatterer et al., 2011). Aβ oligomers also interact directly with AMPA-receptors to induce downregulation of AMPA-receptors (GluR2 subunit) via PP2B to induce synaptic impairment (Zhao et al., 2010).
Figure 1.8 Aβ induced Eph signalling complexes

Both EphB2 and EphA4 are involved in Aβ oligomer mediated internalisation of NMDA- and AMPA-receptors mediating synaptic impairment. In addition both have been linked to Tau phosphorylation. Aβ oligomers bind to EphB2 triggering its degradation by the proteasome. EphB2 normally phosphorylates NMDA–receptors to facilitate their surface expression. In addition EphB2 normally inhibits GSK3β preventing Tau phosphorylation. Aβ mediated loss of EphB2 triggers internalisation of NMDA-receptors and Tau phosphorylation. Aβ also interacts with EphA4 and AMPA-receptors triggering surface depletion of AMPA-receptors via PP2B and synaptic impairment.
1.5 The cellular prion protein (PrP<sup>C</sup>)

PrP<sup>C</sup> is encoded by the PRNP gene and is synthesised in the endoplasmic reticulum (ER) before transport through the Golgi network and to the plasma membrane where the majority of PrP<sup>C</sup> is localised. During synthesis in the ER, PrP<sup>C</sup> undergoes several posttranslational modifications including cleavage of the N-terminal signal peptide (amino acids 1-22) and attachment of the GPI-anchor to the C-terminus replacing amino acids 231-253. PrP also undergoes N-linked glycosylation at two sites (N181 and N197) which are subsequently extended and modified in the golgi (Harris, 2003, Campana et al., 2005). The attachment of the GPI-anchor enables the localisation of PrP<sup>C</sup> at the cell surface and specifically to reside in cholesterol rich, lipid raft microdomains (Westergard et al., 2007), Figure 1.9.

PrP<sup>C</sup> predominantly has an alpha-helical structure with a structured C-terminus and more flexible and unstructured N-terminus (Riesner, 2003), Figure 1.9. The N-terminus contains 4-5 octapeptide binding repeats, a charge cluster region and a neurotoxic region (Qin et al., 2002). The structured C-terminal region is comprised of three alpha-helices, two beta-strands and loop domains (Altmepen et al., 2012). The two N-linked glycosylation sites are also found on the C-terminus and PrP<sup>C</sup> is normally isolated as mixture of un-, mono- and di-glycosylated forms.

The N-terminal region of PrP<sup>C</sup> acts as a molecular sensor, interacting with a range of proteins and cellular components. Interaction with the heparin sulphate proteoglycan (HSPG) Glypican-1, another GPI-anchored protein, mediates the lipid raft localisation of PrP<sup>C</sup> (Taylor et al., 2009). In addition, interaction of the N-terminal domain with the transmembrane receptor LRP1, mediates the cellular trafficking of PrP<sup>C</sup> from the cell-surface to endosomes for degradation via clathrin-coated pits (Parkyn et al., 2008, Taylor and Hooper, 2007). Furthermore, the N-terminal region of PrP<sup>C</sup> interacts with beta-sheet rich toxic signals such as Aβ oligomers and the infectious aggregated form of PrP, PrP<sup>Sc</sup> and is strongly implicated in the pathogenesis and neurodegeneration of Alzheimer’s disease and Prion disease, respectively (Beland and Roucou, 2012).

PrP<sup>C</sup> also undergoes proteolytic cleavage at two sites; the α-cleavage site on the C-terminus of the protein and β-cleavage site which resides near the octapeptide binding repeats on the N-terminus of the protein. Research has implicated the α-cleavage of PrP<sup>C</sup> as neuroprotective and β-cleavage as pro-apoptotic, however, the exact roles of these PrP<sup>C</sup>
cleavage products remain to be determined (Liang and Kong, 2012, Watt et al., 2005, McDonald and Millhauser, 2014).

![Figure 1.9: Structure and lipid raft localisation of PrP](image)

**Figure 1.9 Structure and lipid raft localisation of PrP**

PrP is anchored to lipid raft microdomains on the outer (extracellular) leaflet of the plasma membrane by a GPI anchor. PrP^C predominately has an alpha-helical structure with a structured C-terminus and a more flexible and unstructured N-terminus. The unstructured N-terminus acts as a molecular sensor interacting with a range of proteins and cellular components in addition to beta-sheet rich structures such as Aβ oligomers and PrP^Sc. Two N-linked glycosylation sites are found on the C-terminus and PrP^C is normally isolated as mixture of un-, mono- and di-glycosylated forms.

### 1.5.1 Functions of the cellular prion protein

PrP plays a key role in the pathogenesis of both Alzheimer’s disease and prion disease, however, the exact function of the cellular form of PrP, PrP^C, remains largely unknown. Original reports of two PrP knockout mouse lines; Zurich and Edinburgh, revealed normal development and behaviour, at least up to 7 months of age (Bueler et al., 1992, Manson et al., 1994). Although no overt phenotype was reported in these studies, subsequent
research has identified a wide range of abnormalities implicating an intrinsic physiological role for PrP\textsuperscript{C}.

PrP knockout mice have increased sensitivity to oxidative stress (Wong et al., 2001) and inflammation (Isaacs et al., 2006). The octapeptide binding repeats of PrP\textsuperscript{C} preferentially bind copper ions which stimulate the endocytosis of PrP\textsuperscript{C} (Pauly and Harris, 1998), thus removing extracellular copper and possibly protecting against oxidative stress. PrP knockout mice also have altered sleep regulation and circadian rhythms (Tobler et al., 1997). In addition, electrophysiological studies have revealed altered neuronal excitability and increased sensitivity to induced seizures (Walz et al., 1999) and disruption to long term potentiation (LTP) and synaptic function in PrP knockout mice (Collinge et al., 1994, Manson et al., 1995, Curtis et al., 2003). PrP\textsuperscript{C} is widely expressed throughout the brain, however, it is highly expressed in the hippocampus and synaptic terminals (Sales et al., 1998), further supporting the role for PrP\textsuperscript{C} in synaptic plasticity and function. PrP\textsuperscript{C} regulates synaptic plasticity via interaction with NMDA-receptors which are abnormally activated in PrP\textsuperscript{C} knockout mice inducing excitotoxicity and synaptic impairment (Maglio et al., 2006).

1.5.2  PrP and prion disease

The misfolding of PrP\textsuperscript{C} into the β-sheet rich aggregation prone scrapie conformation (PrP\textsuperscript{Sc}) occurs in multiple neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs) or prion diseases. Prion diseases are a groups of progressive, fatal, neurodegenerative disorders that affect both humans and animals and are grouped according to whether they are sporadic, inherited or acquired (Cobb and Surewicz, 2009). Prion diseases are infectious and transmissible within and between mammalian species, for example bovine spongiform encephalopathy (BSE) in cattle is known to induce Creutzfeldt-Jakob disease (CJD) in humans (Gough and Maddison, 2010). Different prion ‘strains’ have been reported, which have different conformational and aggregation states and differ in toxicity, incubation period and distribution pattern of pathology (Prusiner, 1998, Aguzzi et al., 2007, Solforosi et al., 2013). Patients with prion disease display clinical symptoms of progressive motor dysfunction and cognitive impairment. Histological analysis of the brain reveals spongiform degeneration (vacuolation of the neuropil), astrocytic gliosis and the characteristic accumulation of misfolded and aggregated form of PrP, PrP\textsuperscript{Sc} (Prusiner, 1998).
PrP\textsuperscript{C} is essential for the susceptibility to PrP\textsuperscript{SC} and propagation of disease (Bueler et al., 1993). In the presence of the infectious PrP\textsuperscript{SC} the N-terminal α-helical region of PrP\textsuperscript{C} converts to an insoluble β-sheet rich structure by self-templating (Eghiaian et al., 2004, Riesner, 2003). Both PrP\textsuperscript{C} and PrP\textsuperscript{SC} share identical amino acid sequences, however, they have different conformations and biochemical properties. In addition to interacting with PrP\textsuperscript{SC} the N-terminal region of PrP\textsuperscript{C} is implicated in the misfolding and aggregation of PrP (Prusiner, 1998, Groveman et al., 2015). For example, PrP\textsuperscript{C} normally contains 4-5 octapeptide metal binding repeats. However, genetic mutations which increase the number of repeats are linked to prion disease and increased aggregation of the protein (Moore et al., 2006). Interaction with Glypican-1, the GPI-anchor and the subsequent lipid raft localisation of PrP\textsuperscript{C} may also mediate the conformational conversion of the protein (Taylor et al., 2009, Priola and McNally, 2009). Furthermore, PrP\textsuperscript{SC} may induce toxicity by triggering of neurotoxic signal transduction cascades upon interaction with PrP\textsuperscript{C} within lipid raft microdomains. Accumulation of PrP\textsuperscript{SC} has also been identified in the cytosol to promote prion conversion (Ma and Lindquist, 2002) and induce ER stress and impairment of the proteasome (Soto, 2008).

1.5.3 PrP and Alzheimer’s disease

PrP is strongly implicated in the pathogenesis and progression of Alzheimer’s disease. As described previously, oligomeric Aβ binds to PrP\textsuperscript{C} with nanomolar affinity to induce synaptic impairment (Lauren et al., 2009), section 1.4.1. The charge cluster region (amino acids 95-110) in the N-terminus is crucial for Aβ binding to PrP\textsuperscript{C}, as shown by PrP\textsuperscript{C} mutants and anti-PrP antibodies which block Aβ binding to this region (Lauren et al., 2009). A second cluster of basic residues in the N-terminus (amino acids 23-27) is also crucial for Aβ binding to PrP\textsuperscript{C} (Chen et al., 2010).

PrP\textsuperscript{C} normally exerts inhibitory effects on the β-secretase, BACE1, to reduce Aβ production mediated via interaction with glycosaminoglycans (GAGs) (Parkin et al., 2007), however, this is impaired upon Aβ oligomer binding to PrP\textsuperscript{C} (Rushworth et al., 2013), thus increasing Aβ levels and providing a positive feedback loop in Alzheimer’s disease. Furthermore, several lines of evidence suggest that PrP\textsuperscript{C} expression is altered with age and in sporadic but not familial Alzheimer’s disease, implicating PrP\textsuperscript{C} further in the pathogenesis of the disease (Whitehouse et al., 2010, Whitehouse et al., 2013). A reduction in expression levels
of PrP\textsuperscript{C} in Alzheimer’s disease inversely correlated with BACE1 activity and soluble A\textbeta levels (Whitehouse et al., 2013). PrP\textsuperscript{C} normally exerts protective effects on oxidative stress and neuroinflammation. However, both are implicated in Alzheimer’s disease pathogenesis (Heppner et al., 2015, Persson et al., 2014). The loss of PrP\textsuperscript{C} expression and therefore loss of protective function may contribute to oxidative stress and inflammation. PrP\textsuperscript{C} expression has also been putatively linked with alterations to Tau expression (Vergara et al., 2015), although the exact nature and implications of this relationship are unknown.

1.5.1 PrP and synaptic function

Early synapse loss and dysfunction are features of both prion disease and Alzheimer’s disease (Mallucci, 2009, Shankar and Walsh, 2009). The common methionine/valine polymorphism of Prnp at codon 129 (Met129Val) is linked with altered synaptic plasticity and learning (Buchmann et al., 2008) and increased risk of prion disease but also early-onset Alzheimer’s disease (Mastrianni, 2010, Riemenschneider et al., 2004). The exact mechanisms underlying synaptic impairment in prion disease are not fully understood. There may be a toxic loss or toxic gain of PrP function, in addition to the toxicity induced by the aggregation and accumulation of PrP\textsuperscript{SC}.

Normally PrP\textsuperscript{C} regulates NMDA-receptors to prevent excitotoxicity (Khosravani et al., 2008). In prion disease and Alzheimer’s disease there is a loss of PrP\textsuperscript{C} function and dysregulation of NMDA-receptors. A\textbeta oligomers induce synaptic deficits via NMDA-receptor excitotoxicity and dysfunction in a PrP\textsuperscript{C}-dependent manner (Lauren et al., 2009). Furthermore, an NMDA-receptor antagonist reduces the toxic effects of both A\textbeta and PrP\textsuperscript{SC} (Resenberger et al., 2011). There may also be a toxic gain of function from aberrant PrP\textsuperscript{C} signalling. The GPI anchor of PrP\textsuperscript{C} is essential for mediating the toxic actions of A\textbeta but is also essential for the clinical representation of prion disease in mice (Chesebro et al., 2005), further supporting the role of aberrant PrP signalling. In addition to this, synaptic impairment may be induced by the aggregation and accumulation of the proteins themselves. Similar to A\textbeta aggregates, small oligomeric forms of PrP\textsuperscript{SC} have been shown to be the highly infectious and toxic form (Diaz-Espinoza and Soto, 2012).
1.6  Tau

1.6.1  Structure and function

Tau belongs to the MAP2/Tau family of microtubule-associated proteins (MAPs). In mammals this family consists of the neuronal proteins MAP2 and Tau and the non-neuronal MAP4 (Dehmelt and Halpain, 2005). MAP2 shares 67% sequence identity to Tau and has similar functions; both bind to and stabilise microtubules (Lewis et al., 1988). Both proteins contain microtubule binding repeats in the C-terminal region. These motifs (or repeats) are 18 amino acids in length, separated by 13 or 14 amino acid residues (Lewis et al., 1988) and contain KXGS motifs which regulate the affinity for microtubules (Biernat and Mandelkow, 1999). Phosphorylation of the KXGS motif decreases the affinity of MAP2/Tau for microtubules and is essential for neurite outgrowth and the development of cell processes. Both MAP2 and Tau also contain an N-terminal projection domain which contains a basic proline-rich domain and acidic N-terminal inserts of 29 amino acids in length. The exact role of the N-terminal inserts remains unclear. However, the projection domain of Tau interacts with multiple protein partners and cellular components (Wang and Mandelkow, 2015).

Alternative splicing of the MAPT gene on chromosome 17 encoding Tau generates different isoforms of Tau which are developmentally regulated. MAPT spans multiple exons with alternative splicing of exons 2, 3 and 10 generating six different isoforms of Tau, see Figure 1.10. Exon 2 and exon 3 encode the acidic N-terminal inserts, whereas exon 10 encodes the second microtubule binding repeat in the C-terminus. Normally in the brain there is an equal distribution of exon 10- and 10+ isoforms generating isoforms containing three or four microtubule binding repeats, 3R and 4R, respectively (Panda et al., 2003). Three repeat isoforms have a 3-fold lower binding affinity for microtubules and act as a 3-fold less potent inhibitor of microtubule dynamics (rate of shortening and lengthening of microtubules) than four-repeat isoforms. These properties are required to enable plasticity during neuronal development (McMillan et al., 2008).
The six Tau isoforms range from 352-441 amino acids in length and differ by the presence or absence of exons 2 and 3 (E2; E3) encoding the 29 amino acids inserts in the projection domain and exon 10 (E10) which encodes the second microtubule (MT) binding repeat towards the C-terminus (Buee et al., 2000). Exon 3 is only spliced in the presence of exon 2 generating 2-3-, 2+3- and 2+3+ isoforms containing none, one or two N-terminal inserts; 0N, 1N or 2N, respectively. In the brain Tau isoforms are expressed in the prevalence of 1N>0N>2N. Normally, there is an equal distribution of exon 10+ and 10- isoforms in the brain (Boutajangout et al., 2004). Tau 352 (0N3R) is the predominant isoform expressed in the foetal brain.

Tau is an essential protein in the neuronal cytoskeleton stabilising the neuronal network and maintaining cargo trafficking (Mietelska-Porowska et al., 2014). Interestingly, mice absent of Tau are viable and breed normally with an upregulation of other MAP proteins compensating for the absence of Tau (Harada et al., 1994). However, there are deficits in aged Tau knockout mice. By 12 months of age mice display neocortical shrinkage and brain atrophy with cognitive impairments (Lei et al., 2012). Hippocampal neuronal cultures from Tau−/− mice also have delayed maturation (Dawson et al., 2001), supporting an essential role of Tau in the brain (Ke et al., 2012).
1.6.2 Tauopathies

Tau is a soluble, natively unfolded protein however, in some neurodegenerative diseases, termed tauopathies, Tau becomes abnormally phosphorylated and polymerises into insoluble aggregates termed neurofibrillary tangles formed of paired helical filaments (PHFs). Tauopathies are a group of neurodegenerative diseases and include Alzheimer’s disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and frontotemporal dementia (FTD) (Lee and Leugers, 2012).

Mutations in MAPT are linked to a subset of autosomal dominant neurodegenerative diseases including FTD with parkinsonism linked to chromosome 17 (FTDP-17). Around 36 MAPT mutations are linked to FTD (see http://www.molgen.vib-ua.be/FTDMutations). The majority of mutations either alter exon 10 splicing, microtubule interactions or fibril formation (for reviews see (van Swieten and Spillantini, 2007, Wolfe, 2009)).

Mutations which alter exon 10 splicing and promote its inclusion induce an imbalance between 3R and 4R Tau isoforms and alone is enough to induce neurodegeneration and dementia (Liu and Gong, 2008, Adams et al., 2010). Tau isoforms containing either 3R or 4R have different binding affinity for microtubules thus an imbalance may disrupt microtubule dynamics. There may also be a saturation of binding sites on microtubules for 4R isoforms resulting in increased free 4R Tau in the cytoplasm (Liu and Gong, 2008, Wolfe, 2009). Free Tau, in particular isoforms containing 4R are more prone to phosphorylation and aggregation and therefore incorporation into NFTs than Tau bound to microtubules (Sengupta et al., 2006). The N-terminal inserts are also implicated in mediating aggregation and may explain why foetal Tau which lacks both N-terminal inserts and only has 3 binding repeats is protected from pathology (Iqbal et al., 2010). It should be noted that mutations in MAPT are not linked to autosomal dominant Alzheimer’s disease. However, genetic studies have identified MAPT variants which are associated with reduced MAPT levels and reduced risk of Alzheimer’s disease (Allen et al., 2014), supporting previous work revealing a protective role for reducing Tau in an Alzheimer’s disease mouse model (Roberson et al., 2007).
1.6.3 Tau in Alzheimer’s disease

In Alzheimer’s disease, Aβ may be the initiator of complex pathogenic cascades, however, Aβ alone is not sufficient to induce Alzheimer’s disease (Musiek and Holtzman, 2015). Aβ induces pathological changes to Tau including phosphorylation, truncation and mislocalisation (Stancu et al., 2014). Oligomeric forms of Aβ are thought to be the primary pathogenic species and induce these pathological modifications to Tau (Chabrier et al., 2012, Larson et al., 2012, De Felice et al., 2008, Zempel et al., 2010). Increasing evidence suggest that Tau is crucial for Aβ mediated toxicity and the driver of toxicity in Alzheimer’s disease (Ittner et al., 2010, Pooler et al., 2015) with a reduction in Tau ameliorating Aβ toxicity (Roberson et al., 2007, Oddo et al., 2006b). Furthermore, Tau pathology correlates more closely with the cognitive decline and disease severity, both spatially and temporally than amyloid burden (Nelson et al., 2012).

Although neurofibrillary tangles are a key pathological feature of Alzheimer’s disease, research now suggests that Tau may be involved in neuronal dysfunction before the accumulation of insoluble Tau aggregates (Santacruz et al., 2005). Pre-fibrillar, soluble forms of Tau have been implicated as the ‘toxic’ species mediating synaptic dysfunction, disruption to axonal transport and cytoskeletal destabilisation, although the exact ‘toxic’ species remains unidentified (Kopeikina et al., 2012, Lasagna-Reeves et al., 2011a, Oddo et al., 2006b). Furthermore, smaller, insoluble aggregates of Tau may be protective (Cowan and Mudher, 2013) with NFTs sequestering toxic soluble aggregates of Tau (Kopeikina et al., 2012).

1.6.4 The role of synaptic Tau in Alzheimer’s disease

Normally in the brain Tau is predominantly localised in the axons and MAP2 to the dendrites and cell bodies (somatodendritic region) (Kosik and Finch, 1987). In Alzheimer’s disease Tau is mislocalised to the somatodendritic region (Zempel and Mandelkow, 2014). The mislocalisation of Tau is dependent on phosphorylation and results in a disruption to glutamate receptor trafficking and synaptic impairment (Hoover et al., 2010). One mechanism of this synaptic toxicity may be via the targeting of Fyn kinase to the somatodendritic region which is known to affect NMDA-receptor trafficking and function (Section 1.4.1). In support off this, in transgenic mice deficient of Tau targeting of Fyn to
the synaptic density no longer occurs and they are protected against NMDA-receptor mediated excitotoxicity (Ittner and Gotz, 2011). Loss of dendritic spines occurs before the formation of NFTs, suggesting pathology starts with the mislocalisation of phosphorylated Tau to the somatodendritic compartment altering synaptic morphology (Messing et al., 2013). Furthermore, Tau toxicity has shown to be dependent on aggregation with small soluble oligomeric forms of Tau implicated in early events in Alzheimer’s disease pathogenesis and are found to induce synaptic and mitochondrial dysfunction (Ward et al., 2012, Lasagna-Reeves et al., 2011b, Mocanu et al., 2008). Further work using an inducible tauopathy mouse model which expressed human Tau pseudophosphorylated at multiple sites showed that mice expressing low levels of this pathological Tau seeded the formation of oligomers, induced synaptic dysfunction and triggered neurodegeneration (Di et al., 2016).

1.6.5 Tau progression and propagation

In Alzheimer’s disease the spatiotemporal progression of Tau pathology is predictable and can be consistently grouped into stages, termed Braak stages which are defined by the localisation of tangle bearing neurons and clinical representation. Braak stages I-II are clinically silent and defined by the appearance of tangles in the transentorhinal region. Next, NFTs accumulate in the limbic region and are defined by incipient Alzheimer’s disease (Braak stages III-IV). Finally, Braak stages V-VI define severe Alzheimer’s disease where Tau pathology has spread to neocortical structures (Braak and Braak 1995). The progression of amyloid deposition is less predictable than NFTs, however, plaques are first identified and predominantly found in the neocortex (Serrano-Pozo et al., 2011). More recent work has identified Tau pathology in the brainstem before the identification of amyloid plaques, suggesting that pathology may begin earlier than originally thought and in the brain stem rather than in the transentorhinal region (Braak et al., 2011). It was later suggested that the brain stem becomes increasingly involved in disease progression rather than regions initially affected by Tau pathology (Attems et al., 2012).

Increasing research suggests that Tau is able to spread between neurons and drive disease propagation (Pooler et al., 2013b, Mohamed et al., 2013). Previously, it was thought that only dead tangle bearing neurons (ghost tangles) could passively release Tau, however, emerging research suggests Tau is able to be actively released from neurons. Tau could be
directly transferred between neurons via a physical connection such as tunnelling nanotubules or released into the extracellular space either free or in vesicles such as exosomes and subsequently taken up by the neuron. Increasing evidence supports the release of Tau into the extracellular space and Tau is identified in the CSF and can be used as an early biomarker for Alzheimer’s disease progression (Mattsson et al., 2009, Hansson et al., 2006).

Neuronal activity and amyloid pathology induce the release of Tau from healthy neurons in a calcium dependent manner and it is subsequently taken up by pre-synaptic vesicles (Pooler et al., 2013a, Pooler et al., 2015). Synaptic connectivity rather than spatial proximity has been shown to be crucial for the transfer of Tau (Calafate et al., 2015, Ahmed et al., 2014, Wu et al., 2016), possibly explaining the predictable spread of Tau pathology through the brain. The exact Tau isoform and phosphorylated state of transferred Tau are unknown and differ between studies (Mohamed et al., 2013), however, multiple phosphorylation sites and fragments have been identified in the CSF in Alzheimer’s disease (Meredith et al., 2013). In Alzheimer’s disease, neuronal excitability is increased and in combination with amyloid pathology could greatly increase the transfer of Tau, and possibly of phosphorylated forms which trigger synaptic impairment and pathology. Interestingly, microglia are also implicated in Tau propagation by phagocytosing Tau released from neurons and subsequently secreting Tau via exosomes (Asai et al., 2015). Therefore, Tau is able to spread by trans-synaptic pathways and non-trans-synaptic pathways to propagate pathology and synaptic impairment.

1.6.1 Tau phosphorylation and kinases/phosphatases

Tau phosphorylation is normally tightly regulated by kinase and phosphatase activities with around 45 phosphorylation sites on serine, threonine and tyrosine residues. In Alzheimer’s disease a dysregulation of kinase and phosphatase activity results in the overall hyperphosphorylation of Tau (Martin et al., 2011), Figure 1.11. It is likely that multiple kinases, phosphatases and phosphorylation sites are crucial for disease pathogenesis (For a full list of Tau phosphorylation sites identified in Alzheimer’s disease and the kinases implicated see http://cnr.iop.kcl.ac.uk/hangerlab/tautable).
Figure 1.11 Sites of phosphorylation on Tau

Normally in the brain Tau is phosphorylated at multiple serine, threonine and tyrosine residues (green). In Alzheimer’s disease the aberrant phosphorylation of Tau results in Tau hyperphosphorylation (black). Residues highlighted in blue have been identified both normally in the brain but also in Alzheimer’s disease. Tau phosphorylation sites are mapped onto the full-length Tau structure (Tau 441; 2N4R) (1N, 2N; N-terminal inserts, R1-4; microtubules binding repeats) (Wang et al., 2013, Martin et al., 2011).

1.6.1.1 Serine/threonine Tau kinases

Tau kinases can be grouped into three sub-groups: proline-directed kinases, non-proline directed kinases and tyrosine kinases (Martin et al., 2013b). Proline-directed kinases predominantly target serine and threonine residues in the proline-rich region and C-terminus of Tau. Kinases include glycogen synthase kinase-3β (GSK3β), cyclin-dependent kinase-5 (CDK5) and mitogen-activated protein kinases (MAPKs) such as ERK1/2 and JNK (Martin et al., 2013b).

GSK3β is essential for cell signalling and modulates a variety of cellular processes, many of which are implicated in Alzheimer’s disease (Takashima, 2006). GSK3β activity is significantly increased in Alzheimer’s disease (Leroy et al., 2007) and strongly implicated in Tau phosphorylation and the pathogenesis of Alzheimer’s disease (for review see (Hooper et al., 2008)). The neuronal-specific kinase, CDK5 is also implicated in a variety of neuronal functions (Liu et al., 2016). Upon interaction with the p35 regulatory subunit, the CDK5/p35 complex is neuroprotective, however, in Alzheimer’s disease levels of the p25 regulatory subunit increase as a result of increased intracellular calcium levels and subsequent calpain activation which cleaves p35 (Camins et al., 2006). The CDK5/p25
complex prevents the prosurvival activity and facilitates neurodegeneration via inducing synaptic dysfunction and apoptosis in addition to Tau phosphorylation (Patrick et al., 1999).

Non-proline directed kinases include the microtubule affinity-regulating kinase (MARK) and calmodulin-dependent protein kinase II (CaMKII). Phosphorylation of Tau in the KXGS motif by MARK particularly at Ser262 significantly reduces microtubule interaction (Fischer et al., 2009). Additional phosphorylation by other kinases at Ser235, Thr231 and Thr181 also contributes to the dissociation of Tau from microtubules. Tau phosphorylated at Thr231 and Thr181 is found in the CSF of patients with Alzheimer’s disease (Hampel et al., 2004, Hansson et al., 2006). Phosphorylation at Thr231 appears to be an early event in neurodegeneration and precedes PHF formation (Vincent et al., 1998). With Tau phosphorylated at AT8 (Ser202/Thr205) and PHF1 epitopes (Ser396/404) found later in NFT pathology (Augustinack et al., 2002). It is likely that there are sequential phosphorylation events of Tau in Alzheimer’s disease (Gotz et al., 2010), with certain phosphorylation events linked to microtubule dissociation and others modifying Tau to increase its propensity to misfold and aggregate. In support of this, many GSK3β sites are primed, for example, phosphorylation at Ser235 primes Tau for phosphorylation at Thr231 by GSK3β (Cho and Johnson, 2003, Goedert et al., 1994).

### 1.6.1.2 Tau tyrosine kinases

There are only five tyrosine kinase residues in Tau (Scales et al., 2011), however, tyrosine phosphorylation plays a key role in disease progression (Bhaskar et al., 2005), with Tau aberrantly phosphorylated at Tyr18, Tyr197 and Tyr394 in Alzheimer’s disease, Figure 1.11. Tyrosine protein kinases include c-Abl and the Src family tyrosine kinases (SFKs) (Scales et al., 2011).

There are nine SFK members and include Src, Lck and Fyn. Fyn is upregulated in Alzheimer’s disease and strongly implicated in the pathogenesis of the disease (Lee et al., 1998). All SFKs share a conserved structure comprising of four SH (Src homology) domains; SH1-3, a variable N-terminus region, and the SH4 domain which contains a membrane targeting region which is always myristoylated and sometimes palmitoylated (Boggon and Eck, 2004, Parsons and Parsons, 2004). The SH1 domain is the catalytic domain and autophosphorylation at Tyr416 regulates activation (Boggon and Eck, 2004). SH3 domains are involved in protein-protein interactions and recognition of the PxxP motif (where x can
be any amino acid) on proteins (Salter and Kalia, 2004). In Alzheimer’s disease the SH3 region of Fyn interacts with the PxxP motif of Tau to induce mislocalisation of Fyn to the dendrites increasing excitotoxicity and synaptic impairment mediated via NMDA-receptors (Ittner et al., 2010, Lau et al., 2016). Furthermore, tyrosine phosphorylation of Tau at Tyr18, the residue phosphorylated by Fyn kinase is important for mediating the interaction between Tau and Fyn and subsequent trafficking (Usardi et al., 2011). Fyn may also activate GSK3β and therefore indirectly alter Tau phosphorylation (Lesort et al., 1999), further exacerbating toxicity and pathology.

1.6.1.3 Tau protein phosphatases

In addition to kinase activity, phosphatase activity is also dysregulated in Alzheimer’s disease and contributes to Tau hyperphosphorylation. Tau protein phosphatases include; PP1, PP2A, PP2B and PP5 and these dephosphorylate Tau at multiple serine and threonine residues (Liu et al., 2005a). PP2A accounts for around 70% of the total phosphatase activity in the brain (Martin et al., 2013a) and in Alzheimer’s disease there is approximately a 50% decrease in PP2A activity (Liu et al., 2005a).

PP2A interacts with variety of proteins and multiple signal transduction cascades including those involved in cell development and morphology (Janssens and Goris, 2001). In addition, PP2A indirectly alters Tau phosphorylation via altering GSK3β activity to further contribute to Tau hyperphosphorylation (Qian et al., 2010). PP2B (also known as calcineurin) is a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase that preferentially dephosphorylates Tau at Ser262, the site involved in microtubule binding and at Ser396, a residue found in PHFs (Rahman et al., 2006) and PP2B activity is also strongly implicated in Alzheimer’s disease.

1.6.2 Post-translational modifications

Tau phosphorylation is the most common and most documented modification of Tau, however, Tau is subject to a myriad of modifications including; glycosylation, glycation, truncation, acetylation and ubiquitination (Martin et al., 2011). In addition to phosphorylation, other post-translational modifications are implicated in Tau toxicity and pathology.
Acetylation of Tau at lysine residues has been linked to Tau aggregation and pathology. Acetylation of soluble Tau at lysine 174 (K174) is an early event in Alzheimer’s disease pathogenesis and is implicated in slowing the turnover rate of Tau and increasing its aggregation to induce cognitive deficits in a mouse model (Min et al., 2015). Tau acetylation at K280 also promotes aggregation and is found in insoluble Tau (Cohen et al., 2011), suggesting acetylation at this residue is a marker for later stages in pathology. Furthermore, the auto-acetylation of Tau has recently been linked to the proteolytic cleavage of Tau, however, it remains to be determined as to the exact role of auto-acetylation in Alzheimer’s disease and tauopathy (Cohen et al., 2016).

Tau can be glycosylated by both N- and O-linked glycans (Schedin-Weiss et al., 2014). In Alzheimer’s disease levels of N-glycosylation increase and are implicated in PHF formation possibly via increasing the susceptibility of Tau to phosphorylation (Sato et al., 2001). On the other hand, levels of O-GlucNAcylated Tau are decreased in Alzheimer’s disease. There is evidence of competition between phosphorylation and O-GlucNAcylation for the same sites, so reduced O-GlucNAcylation may increase phosphorylation (Deng et al., 2008). However, competition between these two post-translational modifications has recently been disputed (Morris et al., 2015).

Tau is also susceptible to truncation and the proteolytic cleavage by multiple caspases and calcium-activated proteases (calpains). Tau fragments have been identified in multiple tauopathies (Hanger and Wray, 2010) and are also linked to inducing aggregation (Binder et al., 2005). The most widely documented truncation site is at aspartate 421 (D421) in the C-terminus of Tau. Tau is cleaved at this site by multiple caspases following treatment with Aβ and may precede phosphorylation and PHF formation (Gamblin et al., 2003, Rissman et al., 2004, de Calignon et al., 2010). In addition to caspases and calpains, asparagine endopeptidase (AEP) a lysosomal cysteine protease, also cleaves Tau. AEP is activated during ageing and in Alzheimer’s and may also be responsible for Tau pathology and subsequent synaptic impairment (Zhang et al., 2014, Rosenmann, 2014).
1.7 Therapeutic interventions

The current therapies available for Alzheimer’s disease patients only target the cognitive and behavioural symptoms of the disease. Drugs to target the cognitive symptoms are based on cholinesterase inhibitors and NMDA-receptor antagonists. The reversible cholinesterase inhibitors, Rivastigmine, Donepezil and Galantamine, are often used in mild to moderate cases of Alzheimer’s disease. These drugs act to prevent the breakdown of the neurotransmitter acetylcholine at the neuronal synapse thereby helping to compensate for the loss of cholinergic neurons in Alzheimer’s disease (Colovic et al., 2013, Birks, 2006). In addition, memantine is used in more severe cases of the disease. Memantine is a non-competitive NMDA-receptor antagonist which preferentially blocks the receptor channel during prolonged activation, thus acting to prevent glutamate excitotoxicity yet sparing normal synaptic activity (Parsons et al., 2013, Johnson and Kotermanski, 2006).

There are currently no therapeutic strategies available to alter or halt disease progression in Alzheimer’s disease and no new drugs have been approved since 2003, highlighting the need for novel therapeutic approaches (for recent reviews see (Godyn et al., 2016, Folch et al., 2016)) and for understanding and modelling the complex mechanisms underlying synaptic impairment and neurodegeneration in Alzheimer’s disease, and other proteinopathies.

1.7.1 Amyloid-therapies

Novel therapeutic strategies for Alzheimer’s disease have largely focused on targeting the production, aggregation and clearance of Aβ in the brain. Clearance and degradation mechanisms are impaired in Alzheimer’s disease and contribute to an increase in Aβ levels in the brain (Tarasoff-Conway et al., 2015) and have been the target of novel strategies (Yoon and Jo, 2012, Bates et al., 2009). Several large phase 3 trials of anti-amyloid immunotherapies to clear Aβ from the brain have been published, however, the results have largely been disappointing, although they may be more efficacious at earlier stages of the disease (Sevigny et al., 2016). These include the anti-amyloid monoclonal antibodies Bapineuzumab and Solanezumab (Salloway et al., 2014, Siemers et al., 2016). A recent study using the antibody Aducanumab has shown it to reduce both soluble and insoluble Aβ (possibly via microglial mediated clearance) and slow cognitive decline in patients with
prodromal or mild Alzheimer’s disease, however larger clinical trials are required (Reiman, 2016, Scheltens et al., 2016).

Soluble oligomeric forms of Aβ which are known to be the pathogenic species of Aβ have also been specifically targeted. Targeting the conformation and aggregation of Aβ oligomers has been of great therapeutic interest (Nie et al., 2011, Doig and Derreumaux, 2015). Polyphenols such as resveratrol and epigallocatechin-3-gallate (EGCG) are able to convert Aβ oligomers to unstructured oligomers which no longer bind to neuronal receptors such PrP<sup>C</sup> and are no longer cytotoxic (Ehrnhoefe et al., 2008, Ladiwala et al., 2010, Bieschke et al., 2010). Conversion of Aβ aggregates to beta-sheet rich secondary structures such as fibrils has also shown to reduce Aβ toxicity (Ahmed et al., 2010). Small compounds which stabilise Aβ in an alpha-helical conformation may also reduce the toxicity of Aβ (Nerelius et al., 2009).

### 1.7.2 Targeting Aβ receptors

Multiple therapeutic approaches have been investigated to block Aβ oligomer toxicity at receptors (Nie et al., 2011, Kumar et al., 2015), with particular focus on the high affinity neuronal receptor, PrP<sup>C</sup>. Anti-PrP monoclonal antibodies prevent the binding of Aβ oligomers to PrP<sup>C</sup> and the subsequent behavioural deficits in an Alzheimer’s disease mouse model (Chung et al., 2010, Freir et al., 2011). In addition to antibodies, small molecules also inhibit Aβ binding to PrP<sup>C</sup> and the subsequent toxicity. Risse, et al. (2015) screened 1,200 approved drugs and identified Chicago Sky Blue 6B as a small molecule able to interact with PrP<sup>C</sup> and inhibit Aβ binding and toxicity but also to prevent against prion disease in vitro (Risse et al., 2015).

In addition to Aβ binding, therapies to modulate the aberrant receptor activity induced by Aβ have also been investigated. The glutamate receptor, mGluR5 acts as a co-receptor for Aβ oligomers with PrP<sup>C</sup> and is aberrantly activated in Alzheimer’s disease, inducing excitotoxicity. Negative allosteric modulators of mGluR5 reversed synapse loss and memory deficits in an Alzheimer’s disease mouse model (Um et al., 2013). Aβ oligomers also bind to EphB2 to induce its proteasomal degradation. Reversing EphB2 levels reversed cognitive decline in an Alzheimer’s disease mouse model (Cisse et al., 2011a), therefore small molecules which block Aβ binding to EphB2 may hold therapeutic potential against synaptic
impairment but also aid to prevent Tau phosphorylation. Indeed, recently designed small peptides inhibit Aβ binding to EphB2 and rescue the depletion of EphB2 and NMDA-receptors by Aβ and improve memory deficits in an Alzheimer’s disease mouse model (Shi et al., 2016). In addition, a small antagonist which binds to the sigma-2/PGRMC1 receptor, another Aβ oligomer receptor, restored cognitive function in an Alzheimer’s disease mouse model by preventing the binding of Aβ oligomers (Izzo et al., 2014).

1.7.3 Tau Therapies

Tau plays a crucial role in neuronal stabilisation and pathogenic Tau species are increasingly being acknowledged as the driver of toxicity and pathology in Alzheimer’s disease (Pooler et al., 2013b, Oddo et al., 2006b). Targeting post-translational modifications of Tau aggregation or promoting clearance may reveal novel therapeutic avenues for Alzheimer’s disease and other tauopathies (For a recent review on Tau based therapies see (Bakota and Brandt, 2016)).

Multiple kinase inhibitors have been developed to prevent Tau phosphorylation and subsequent pathology. However, targeting kinases such as GSK3β, which is known to play a key role in normal cellular function may have detrimental effects to normal cellular functions (Llorens-Martin et al., 2014). A small molecule inhibitor of SFKs, Saracatinib (AZD0530), prevents Aβ oligomer induced Fyn activation and reversed synaptic depletion and memory deficits in an Alzheimer’s disease mouse model (Nygaard et al., 2014, Kaufman et al., 2015) and is currently being tested in a Phase II safety/tolerability trial in humans (https://www.clinicaltrials.gov).

Anti-Tau vaccines to clear Tau have also been developed targeting phosphorylated (Theunis et al., 2013) or truncated forms of Tau (Kontsekova et al., 2014). The active vaccine, AADvac-1, which targets an N-terminal truncated fragment of Tau is currently in a Phase 2 safety trial in patients with mild to moderate Alzheimer’s disease (https://www.clinicaltrials.gov). In addition to clearance, Tau aggregation has also been targeted (Wischik et al., 2014). The Tau aggregation inhibitor, methylthionium chloride, blocks Tau aggregation by oxidising cysteine residues to maintain Tau in a monomeric state blocking its misfolding and aggregation (Akoury et al., 2013). The derivative, LMXT (TRx0237) which has improved tolerability has recently been tested in a large Phase 3 trial.
However, recent reports show the drug failed to slow cognitive or functional decline in patients with mild to moderate Alzheimer’s disease (http://www.alzforum.org).

1.8 Thesis Aims

Several lines of evidence suggest that the expression of PrP$^C$ is altered with age and in sporadic Alzheimer’s disease, implicating PrP$^C$ in the pathogenesis of the disease. However, results between groups regarding the extent of these changes has been contradictory (Whitehouse et al., 2010, Llorens et al., 2013, Vergara et al., 2015, Larson et al., 2012). Furthermore, a relationship between PrP$^C$ and Tau has also started to emerge (Chen et al., 2013, Schmitz et al., 2014), however, the exact relationship between these two proteins, the mechanisms mediating this connection and the implications for the pathogenesis of Alzheimer’s disease are yet to be fully unravelled.

Small, soluble oligomeric forms of Aβ are the primary pathogenic species in Alzheimer’s disease (Walsh and Selkoe, 2007, Haass and Selkoe, 2007) and are linked to inducing pathogenic modifications to Tau, including phosphorylation (Chabrier et al., 2012, De Felice et al., 2008, Zempel et al., 2010). Several lines of evidence suggest that multi-protein complex(es) are involved in the cell surface binding of Aβ oligomers and the subsequent pathogenic signalling cascades (Jarosz-Griffiths et al., 2016), however, the role of such complexes in Aβ oligomer induced Tau phosphorylation are not well defined. The most documented neuronal receptor for Aβ oligomers and mediator of Aβ induced toxicity is PrP$^C$ (Lauren et al., 2009, Gimbel et al., 2010). Furthermore, the multi-protein complex involved in the binding of Aβ oligomers to PrP$^C$ and the subsequent toxicity has been linked to inducing the phosphorylation of Tau at tyrosine 18 (Larson et al., 2012, Rushworth et al., 2013, Um et al., 2013). However, the role of Aβ/PrP$^C$ in the phosphorylation of other Tau residues is yet to be determined. Considering there is now strong evidence that Tau is the mediator of Aβ toxicity (Oddo et al., 2006b, Roberson et al., 2007), unravelling these signalling complexes and their role in the aberrant phosphorylation of Tau will aid in our understanding of the pathogenesis of Alzheimer’s disease and aid in the identification of novel therapeutic targets for this progressive neurodegenerative disease.
1.8.1 The relationship between PrP<sup>C</sup> and Tau expression in Alzheimer’s disease

**Hypothesis:** The cellular prion protein (PrP<sup>C</sup>) mediates alterations to Tau expression with a reduction in PrP<sup>C</sup> in Alzheimer’s disease triggering changes to Tau expression levels contributing to neurodegeneration.

**Aims:**
- To explore the relationship between PrP<sup>C</sup> and Tau in cell models and transgenic mouse lines with altered PrP<sup>C</sup> expression.
- To explore how the relationship between PrP<sup>C</sup> and Tau expression is altered following the progression of sporadic Alzheimer’s disease and to correlate the changes in full-length Tau to modifications such as truncation and phosphorylation.

1.8.2 Modelling Aβ oligomer induced Tau phosphorylation

**Hypothesis:** Amyloid-beta (Aβ)-oligomers induce the phosphorylation of Tau at multiple epitopes, and the cellular prion protein (PrP<sup>C</sup>) is a key mediator of this.

**Aims:**
- To investigate Aβ oligomer induced Tau phosphorylation at multiple epitopes and the time course of events in neuroblastoma cell lines, rodent hippocampal primary neurons and induced pluripotent stem cell (iPSC)-derived neurons.
- To investigate the role of PrP<sup>C</sup> in mediating these phosphorylation events.

1.8.3 Cell-surface signalling complexes mediated by Aβ oligomers

**Hypothesis:** A cell-surface raft-based signalling complex is crucial for the binding of amyloid-beta (Aβ)-oligomers and the cellular prion protein (PrP<sup>C</sup>) and Flotillins are key components of this complex.

**Aims:**
- To determine the role of Flotillin-1 and Flotillin-2 in the cell surface binding of Aβ oligomers to PrP<sup>C</sup> and the mechanisms by which Flotillins influence Aβ binding.
- To determine the role of Flotillins in lipid raft microdomain stabilisation and localisation of PrP\text{C}
Chapter 2. Materials and Methods

2.1 Materials and Equipment

2.1.1 Molecular biology reagents

Tau381 cDNA in the bacterial pRK172 vector was a kind gift from Michel Goedert (MRC, Cambridge, UK). Tau441 cDNA in the mammalian pcDNA3.1(-) vector was a kind gift from Diane Hanger (Kings College, London, UK). The empty pIREShyg2 vector and empty pcDNA3.1(-) vector were purchased from Clontech Laboratories and Invitrogen, respectively. AflII forward and BamHI reverse primers were purchased from Sigma-Aldrich. PCR was performed using Platinum®Pfx DNA Polymerase (Invitrogen) and a Proteus II thermal cycler purchased from Helena BioSciences. Restriction enzymes, DNA ladder, CutSmart buffer1, ligase buffer and T4 ligase enzyme were all purchased from New England Biolabs. XL-1 blue competent Escherichia coli (E.coli) cells were purchased from Agilent Technologies. DNA concentration and purity was measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). QIAquick Gel Extraction Kit, QIAprep Spin Mini-preparation and plasmid purification kits (Midi and Maxi) were all purchased from Qiagen. DNA sequencing was performed by Beckman Coulter Genomics (Bishop’s Stortford, UK).

2.1.2 Cell culture reagents

NB7, SH-SY5Y cell lines and induced pluripotent stem cell (iPSC)-derived cortical neurons were provided by various members of Nigel Hooper’s laboratory (The University of Manchester, UK). For the generation of SH-SY5Y-PrP\textsuperscript{C} and SH-SY5Y-PrP-CTM cell lines see (Walmsley et al., 2001). Postnatal (PD1) Wistar rat pups were provided by Victoria Fasolino (The University of Manchester, UK). Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI 1640), Hams’s F12, phosphate buffered saline containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions (PBSM+) and phosphate buffered saline containing no metals (PBSM-) were purchased from Lonza. Minimum Essential Media (MEM), OptiMEM, fetal bovine serum (FBS) and Hygromycin B were purchased from Thermo Fisher Scientific. G418 was purchased from Sigma-Aldrich. All siRNA and Dharmafect-1 reagent was
purchased from Dharmacon. TransIT-LT1 transfection reagent was purchased from Mirus Bio.

2.1.3 Other laboratory reagents

Synthetic biotin-LC-Aβ$_{1,42}$ peptide containing a 6-carbon long chain (LC) linker between the biotin moiety and the N-terminus of Aβ was purchased from Anaspec. EDTA-free protease inhibitor cocktail and phosSTOP phosphatase inhibitor cocktail were purchased from Roche Diagnostics. PageRuler prestained protein ladder was purchased from ThermoFisher Scientific. Lambda protein phosphatase (Lambda PP) was purchased from New England Biolabs. Phospho (Thr231)/total tau multiplex immunoassay kit was purchased from Meso Scale Discovery (MSD). See Table 2.1 for a table of primary and secondary antibodies used. All other reagents and chemicals were purchased from Sigma-Aldrich and Thermo Fisher Scientific, among others.
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2.2 Methods

2.2.1 Cloning of Tau381

Polymerase chain reaction (PCR) was used to subclone Tau381 cDNA from the bacterial pRK172 vector to enable insertion of the construct into the mammalian pIREShyg2 vector for expression into mammalian cells. NdeI and EcoRI restriction sites used to clone the cDNA into the bacterial vector were not present in the pIREShyg2 vector, therefore, PCR of the Tau construct was performed using AfIII and BamHI restriction sites. AfIII forward and BamHI reverse primers were designed with sticky ends; AfIII: 5’- CCG CGG CTT AAG GAA GGA GAT ATA CAT ATG GCT GAG C -3’ (GC % = 51.3 %; Tm = 79.2 °C). BamHI: 5’- CCG GCG CCT AGG CAT GAT CAC AAA CCC TGC -3’ (GC % = 63.3 %; Tm = 84.2°C). Bases marked in red were the additional GC bases required for amplification of the construct with these restriction sites. PCR was performed using Platinum®Pfx DNA Polymerase kit prepared according to the manufacturer’s protocol, however, three step cycling was required to perform PCR amplification of the construct. The PCR product was confirmed by 1 % (w/v) agarose gel electrophoresis before being excised under ultraviolet light and purified from the excised gel slice using QIAquick Gel Extraction Kit according to manufacturer’s instructions.

In order to ligate the Tau cDNA into the pIREShyg2 vector restriction digest of the cDNA and vector was performed using AfIII and BamHI restriction enzymes for 2 h at 37°C. To stop the reaction loading buffer (50% (v/v) glycerol, 50% (v/v) ddH2O and bromophenol blue) was added to the constructs before electrophoresis on a 1% (w/v) agarose gel. cDNA was excised from the gel slice and purified using QIAquick Gel Extraction Kit according to manufacturer’s instructions.

Ligation of the vector and Tau cDNA was performed using T4 ligase buffer and T4 ligase enzyme as described in manufacturer’s instructions. Ligation was performed using various insert:vector ratios for various incubation times and temperatures, see results for further details. Products of the ligation were then transformed into XL blue competent Escherichia coli (E.coli) cells followed by QIAprep Spin Miniprep Kit to extract the DNA. DNA was eluted in 50 µl ddH2O and DNA concentration/purity determined before storage at -20°C. Ligation was also confirmed by electrophoresis on a 1% (w/v) agarose gel.
2.2.2  Tau441 plasmid DNA purification

Plasmid DNA containing the Tau441 construct was transformed into XL blue competent *E.coli* cells following the manufacturer’s protocol before transformation onto Luria Bertani (LB) medium plates containing 100 µg/ml ampicillin and incubation at 37°C for 16 h. A colony scrape was then used to inoculate 4 ml LB-medium plus ampicillin (100 µg/ml) and grown for 6 h in a shaking incubator set to 300 RPM at 37°C. This was then used to inoculate 250 ml LB-ampicillin medium and grown in a shaking incubator set to 300 RPM at 30°C overnight. To purify the plasmid DNA, bacterial cells were harvested by centrifugation at 1,500 g for 15 min. The supernatant was discarded and the pellet containing the plasmid DNA was purified using a Maxi-preparation kit. After plasmid DNA purification the pelleted DNA was re-dissolved in ddH2O and stored at -20°C following DNA concentration/purity determination and DNA sequencing.

2.2.3  Ethanol precipitation of DNA

Ethanol precipitation was performed to purify and concentrate DNA. Two volumes of cold absolute ethanol and 1:10 volume of filter sterilised 3 M sodium acetate (pH 5.2) was added to one volume of DNA and incubated at -20 °C overnight. Following incubation DNA was centrifuged for 20 min at 13,000 g. The supernatant was discarded and 300 µl cold absolute ethanol was added to the DNA pellet before being centrifuged for a further 5 min at 13,000 g. The supernatant was discarded and the DNA pellet was dried at 37 °C for 5 min before re-suspension in ddH2O to a final concentration of 1 µg/µl.

2.2.4  Aβ oligomer preparation

Aβ oligomers were prepared based on the original protocol for soluble oligomer generation by Lambert 1998 (Lambert et al., 1998) which was later modified by the same group (Chromy et al., 2003). Briefly, synthetic biotin-Aβ1-42 peptide was dissolved in 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) for 2 h at room temperature to disaggregate any preformed material. Following aliquot into sterile Eppendorf tubes, tubes were transferred to a
SpeedVac to remove traces of HFIP and moisture leaving a peptide film which was stored at -80 °C. Peptide films of biotin-Aβ1-42 were solubilised in dimethyl sulfoxide (DMSO) to 1 mM before dilution in filter sterilised Ham’s F-12 medium to a final concentration of 100 µM (total peptide). Monomeric Aβ peptide was taken at this point and the remaining solution was incubated at room temperature for 16 h to allow the Aβ to aggregate and form oligomers. Following incubation, the preparation was centrifuged at 14,000 g for 20 min to pellet out any fibrillar material, retaining the supernatant as the oligomer preparation. Monomeric and oligomeric preparations were used within 1 h of centrifugation.

2.2.5 Cell culture of human neuroblastoma cell lines

SH-SY5Y cells were routinely cultured in DMEM, supplemented with 10 % (v/v) FBS (DMEM/FBS). NB7 human neuroblastoma cells were routinely cultured in RPMI 1640 with L-Glutamine, supplemented with 10 % (v/v) FBS (RPMI/FBS). Cells were maintained in a humidified incubator at 37°C in a 5 % CO2 atmosphere. Upon reaching confluence, cell monolayers were washed once with PBSM+, before incubation with PBSM- to detach the cells from the culture flask. Cells were subsequently pelleted by centrifugation at 500 g for 3 min and resuspended into fresh media before being split into new culture flasks.

2.2.6 Transient transfection of SH-SY5Y cells

Upon reaching 60-70 % confluency cells were transiently transfected with ethanol precipitated DNA (Tau441/pcDNA3.1(-)) using TransIT-LT1 transfection reagent for 24 h. Briefly, DNA (9.5 μg) or ddH2O control and LT1 transfection reagent were combined with serum free OptiMEM in a sterile Eppendorf tube, inverted a few times and incubated at room temperature for 20 min. Cells were washed once with PBSM+ and fresh DMEM/FBS was added to the cells. The transfection complex was then added dropwise to the cells. The cell culture media was replaced following 4 h to reduce any toxicity and risk of cell death.
2.2.7 Stable transfection of NB7 cells

Upon reaching 70 % confluency cells were washed with PBSM+, displaced from the culture flask with PBSM- and pelleted by centrifugation at 500 g. The cell pellet was then re-suspended in 700 µl serum free RPMI and transferred to an electroporation cuvette. The Tau441/pcDNA3.1 construct or empty vector (30 µg) was then added to the cell suspension and cuvettes were pulsed with electricity at 250 V. The transfected cells were subsequently transferred into a new culture flask containing RPMI/FBS. The culture media was replaced the next day and cells were grown to confluence. Cells were selected using the antibiotic neomycin (G418) (1 mg/ml).

2.2.8 siRNA mediated protein knockdown

Upon reaching 60 % confluency cells were washed once with PBSM+ before fresh media/FBS was added. To transfect the cells siRNA (25 nM or 50 nM) was delivered as a complex with DharmaFECT-1 transfection reagent in serum free media according to manufacturer’s instructions. Cell were incubated for 24, 48 or 72 h, see figure legends for specific conditions used. Cell culture media was replaced following 24 h if longer time points were used.

2.2.9 Measuring the downstream effects of Aβ-oligomer incubation

Cells were cultured to confluence and washed with serum free OptiMEM before incubation with fresh Aβ oligomers (500 nM or 1 μM total peptide) diluted in OptiMEM at 37°C. To measure tau phosphorylation, cells were incubated with the oligomers for various time points as stated. Following incubation, cells were washed twice with PBSM+ before cells were harvested and lysed as described in section 2.2.10.
2.2.10 Cell lysate preparation

For NB7 and SH-SY5Y cell lines, cells were washed once in ice cold PBSM+ before detachment using ice cold PBSM-. Detached cells were pelleted by centrifugation at 500 g for 5 min at 4°C and supernatant discarded. The cell pellet was re-suspended in ice cold lysis buffer (50 mM Tris base, 150 mM sodium chloride, 0.5 % (w/v) sodium deoxycholate, 1 % (v/v) NP-40, pH 8.0). Following incubation on ice for 1 h, lysed cells were clarified by centrifugation at 14,000 g for 10 min at 4°C and the supernatant was stored at -20°C until further use. For iPSC-derived cortical neurons and rat hippocampal primary neurons, cells were washed once in ice cold PBSM+ before harvesting directly in ice cold lysis buffer. Cells were then lysed on ice for 1 h and supernatant was collected as described previously.

2.2.11 Bicinchoninic acid (BCA) assay

To generate a standard curve bovine serum albumin (BSA) in concentrations ranging from 2-10 μg/μl were loaded onto a 96 well plate. Clarified cell lysates were then loaded onto the plate in volumes ranging between 1-10 μl. All standards and lysates were diluted in ddH₂O (10 μl final volume) and loaded in duplicate. BCA assay solution and 4 % CuSO₄ were mixed in a ratio of 50:1 and 200 μl of this mixture was added to each well and reaction was incubated for 20 min at 37°C. The absorbance was then measured at 570 nm and protein concentration measured.

2.2.12 Lambda protein phosphatase (Lambda PP) mediated lysate dephosphorylation

Clarified cell lysates (60 μg total protein) were incubated with 1000 units Lambda protein phosphatase (Lambda PP) as described in manufacturer’s instructions. Reaction was incubated for 3 h at 30°C. The reaction was stopped following addition of SDS-loading dye and heat denatured at 90°C for 5 min. Dephosphorylated cell lysates were immediately electrophoresed using SDS-PAGE, as described in section 2.2.15.
2.2.13 Lipid raft preparations

Cells were cultured to confluence plus 3 days and harvested as described previously (section 1.2.10), keeping the cell pellet on ice. The cell pellet was subsequently lysed with MES buffered saline (MBS) (25 mM MES, 0.15 M sodium chloride, pH 6.5) with 0.6 % Triton X-100. Cell lysate was then passed through a 21 gauge needle (0.5 mm thickness) five times. An equal volume of 80 % (w/v) sucrose diluted in MBS was added to the cell lysate resulting in a 40 % (v/v) sucrose solution.

In a cold room a buoyant discontinuous sucrose gradient was created by sequentially pipetting sucrose solutions with a long needle to the bottom of a soft walled centrifuge tube starting with the lightest solution; 1 ml 5 % (w/v) sucrose, 3 ml 35 % (w/v) sucrose and 1 ml 40 % (v/v) sucrose solution containing the cell lysate. The sucrose gradient was then centrifuged at 100,000 g in a Beckmann L-90K centrifuge and SW55 swing bucket rotor for 18 h at 4°C. Fractions were then collected 0.5 ml at a time from the bottom of the tube using a long needle (21 gauge, 0.5 mm thickness). The insoluble lipid raft was clearly visible as a milky layer floating at the 35-5 % sucrose interface.

2.2.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Following protein determination by BCA assay samples were diluted in lysis buffer and SDS-loading buffer (1.6 mM Tris, pH 6.8, 2.2 % (w/v) sodium dodecyl sulphate (SDS), 1.6 % (w/v) dithiothreitol (DTT), 11 % (v/v) glycerol, bromophenol blue) and heat denatured at 90°C for 5 min.

Proteins were resolved by SDS-PAGE on Tris-glycine acrylamide gels. The percentage of the acrylamide gel and the amount of protein loaded varied depending on the molecular weight of the protein target being analysed, see figure legends for more details. A pre-stained molecular weight standard was used to estimate the molecular weight of the proteins loaded. Samples were electrophoresed in Tris/glycine/SDS electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) (Biorad, UK).
Aβ monomer/oligomer preparations were resolved on 10-20 % Tris-tricine precast gels. Tris/tricine gels were electrophoresed in ice cold Tris/tricine/SDS electrophoresis buffer (100 mM Tris, 100 mM tricine, 0.1 % SDS, pH 8.3) (Bio-Rad, UK).

### 2.2.15 Chemiluminescence Western blot

Following electrophoresis samples were transferred onto polyvinylidene difluoride membranes (PVDF) (GE Healthcare) (pre-wet with methanol) at 120 V for 90 min in ice cold transfer buffer (20 mM Tris, 150 mM glycine, 20 % (v/v) methanol, pH 8.3). Following transfer membranes were blocked with either 5 % (w/v) non-fat milk powder or 3 % (w/v) BSA in PBS with 0.1 % Tween 20 (PBST) overnight at 4°C. Membranes are then washed with PBST three times for 10 min and incubated with primary antibody diluted in 5 % (w/v) non-fat dried milk powder or 3-4 % (w/v) BSA in PBST overnight at 4°C. Membranes were subsequently washed three times for 10 min with PBST before incubation with HRP-conjugated secondary antibody diluted in 5 % (w/v) non-fat dried milk powder for 1 h at room temperature. Equal volumes of Pierce enhanced chemiluminescence (ECL) Western blotting substrate reagent 1 and 2 (Thermo Fisher Scientific) were combined. Membranes were incubated with the combined ECL substrate for 1 min and the horseradish peroxidase (HRP) signal was detected using a G:BOX imager (Syngene). All blocking steps, antibody incubations and washes were performed with gentle agitation.

### 2.2.16 Dot blot analysis

Fresh Aβ oligomers or monomer peptide (20 pmol) were spotted onto dry nitrocellulose membranes and air dried before blocking overnight at 4°C in 10 % (w/v) non-fat dried milk in the corresponding antibody diluent buffer; OC (0.1 M Tris, 1.51 M NaCl, 0.1% (v/v) Triton X-100, pH 7.4) and 6E10 (PBS, 0.1 % (v/v) Tween-20). Following three 10 min washes with PBST, membranes were incubated with OC and 6E10 antibodies diluted in 3 % (w/v) BSA and corresponding antibody diluent buffer overnight at 4°C. Following three 10 min washes with PBST, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Membranes were subsequently washed three times with PBST for 10 min before incubation with the combined ECL substrate for 1 min and the HRP signal was detected using a G:BOX imager (Syngene).
detected using a G:BOX imager. All blocking steps, antibody incubations and washes were performed with gentle agitation.

2.2.17 Multiplex immunoassay (pTau Thr231/total Tau)

Levels of Tau in cell lysates and culture media were determined using the phospho (Thr231)/total tau electro-chemiluminescence multiplex immunoassay plate from Meso Scale Discovery (MSD) according to the manufacturer’s instructions. All incubations were carried out at room temperature on a plate shaker. Standards were diluted in cell lysis buffer (or OptiMEM for media samples).

Briefly, plates were incubated with blocker solution for 1 h, washed four times with the plate specific wash buffer and incubated with 25 μl of standards or sample and incubated for 1 h. Plates were washed four times with specific wash buffer before incubation with anti-total Tau antibody detection solution (25 μl /well) for 1 h. Following a further four wash steps plates were incubated with Read buffer T (150 μl/well) and Tau levels determined immediately using the MESO QUICKPLEX SQ 120. Tau levels were analysed using the MSD Workbench 4.0 software.

2.2.18 Immunofluorescence microscopy

Cells were seeded onto glass coverslips in a 24 well plate. Upon reaching 70 % confluence cells were washed twice with 1 ml warmed PBS+ before fixing with either 100 % ice-cold methanol at -20°C for 5 min or with 4 % paraformaldehyde (PFA)/PBS+ for 10 min at room temperature, as stated in figure legends. Following fixation with PFA cells were washed with 1 ml 50 mM ammonium chloride to quench excessive fixative. Cells were then washed twice with PBS+ for 5 min at room temperature with gentle agitation before incubation with 1 ml blocking buffer (5 % (v/v) fish skin gelatin (FSG) in PBS+) overnight with gentle agitation at 4°C. For the visualisation of intracellular proteins following PFA fixation of cells, cells were permeabilised with 0.2 % (v/v) Triton-X 100/PBS+ for 5 min at room temperature before incubation with blocking agent.

Primary antibodies were diluted in 5 % (v/v) FSG and coverslips were incubated with the antibody solution overnight at 4°C in a dark, moist chamber. Coverslips were transferred
back into a new 24 well plate, washed twice with PBSM+ with 0.2 % Tween-20 for 5 min at room temperature with gentle agitation, then once without Tween 20.

Coverslips were incubated with fluorescently conjugated secondary antibodies for 1 h in 5 % (v/v) FSG. Coverslips were subsequently washed once with PBSM+ with 0.2 % (v/v) Tween 20 for 10 min before incubation with DAPI in PBSM+ for 10 min. Coverslips were then washed once with PBBM+ with 0.2 % (v/v) Tween-20 and once with PBSM+ for 10 min. Coverslips were then washed with ddH2O and left to dry. Slides were then mounted face down onto glass slides using Fluoromount G mounting medium (SouthernBiotech) and left to dry overnight at room temperature.

Before incubation onto cells and coverslips all solutions were filter sterilised through a 0.2 μm filter. For analysis of Aβ oligomer binding, cells were incubated with Aβ oligomers (500 nM total peptide) diluted in serum free OptiMEM (warmed to room temperature) for 10 min at room temperature before fixing cells.

Images were captured on a Delta Vision (Applied Precision) restoration microscope using a 60x / 1.42 Plan Apo objective. The images were collected using a Coolsnap HQ camera (Photometrics) with a Z optical spacing of 0.5 μm. Raw images from the centre of the cell were deconvoluted using the Softworx software. Deconvoluted images were analysed using ImageJ 2.0 (Fiji).

2.2.19 Rat hippocampal primary neuron culture

Postnatal (PD1) Wistar rat pups were killed using a schedule 1 method (cervical dislocation followed by decapitation with large sharp scissors) and the brain was removed. The hippocampus was isolated and chopped finely before incubation with trypsin (5 mg/ml) for 15 min at 37°C.

DNAase solution (1250 kU DNAase1, 0.1 mg/ml soybean trypsin inhibitor, 3 mM MgSO₄) was added to the trypsinised cells and inverted a couple of times before centrifugation at 500 g for 5 min to gently pellet the cells. The supernatant was then removed and pellet was re-suspended with DNAase solution slowly though a p1000 Gilson tip. Further DNAase solution was added before titration though a fire polished pipette. The cell solution was
then filtered through a cell strainer before centrifugation at 500 g for 5 min to pellet the cells.

The supernatant was immediately discarded and the pellet was re-suspended in MEM media supplemented with 10 % (v/v) FBS, 26 mM D-glucose and 0.05 kU Penicillin/Streptomycin and plated out onto sterile 6 well culture plates. The next day the media was replaced with MEM, supplemented with 10 % (v/v) horse serum and 160 μM FUDR (added to inhibit non-neuronal growth). The following day the media was replaced with Neurobasal A medium supplemented with 2 % (v/v) B-27, 0.05 kU Penicillin/Streptomycin, 160 μM FUDR, 0.5 mM L-Glutamine, 25 μM L-Glutamic acid. Following 13 days in culture Glutamic acid was omitted from the Neurobasal A media to prevent overstimulation and excitation of glutamate receptors. Work was conducted according to the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986 and approved by the University of Manchester, UK.

### 2.2.20 Transgenic mice and tissue homogenisation

Mice overexpressing human APP with the Swedish (K670N/M671L) and Indiana (V717F) familial Alzheimer’s disease mutations (line J20) were obtained from The Jackson Laboratory, (Line B6.Cg-Tg(PDGFβ-APPSwelnd)20 Lms/2J, stock number 006293). J20 mice were crossed with inbred PrP<sup>C</sup> knock out mice (129Ola Prnp<sup>−/−</sup>) (Manson et al., 1994). The genetic background of these mice was mixed B6/129Ola and both male and female mice were used. Wild type (Prnp<sup>+/+</sup>), PrP<sup>C</sup> knock out (Prnp<sup>−/−</sup>) and PrP<sup>C</sup> overexpressing (Tg20) mice from 129Ola genetic background, and J20 crossed mice were all obtained from Jean Manson, The Roslin Institute, University of Edinburgh, UK.

Animals were culled by cervical dislocation and brain hemispheres were frozen at -80°C. These experiments were approved by The Roslin Institute’s Animal Welfare and Ethical Review Board and were conducted according to the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986. All tissue was obtained from The Roslin Institute, University of Edinburgh, UK and subsequently homogenised at The University of Manchester, UK.

Briefly, brain hemispheres (120 mg/ml wet weight) were homogenised using an electrical homogeniser in 2% (w/v) SDS containing protease inhibitor cocktail and PhosSTOP
phosphatase inhibitor cocktail. Homogenates were subsequently clarified by centrifugation at 100,000 g for 1 h at 4°C. The supernatant was collected and stored at -80°C until further use.

2.2.21 Human brain tissue homogenisation and fractionation

Human brain tissue from the entorhinal, frontal and occipital cortex was obtained from the Manchester Brain Bank (University of Manchester, UK) with support from Brains for Dementia Research. The study had ethical approval from the London City and East NRES committee. All diagnoses were confirmed by neuropathological analysis. See Table 2.2 for demographic data and MRC identification numbers of tissue used.

Human brain tissue (50 mg/ml) was homogenised in a MES/NaCl buffer (0.1 M MES, 1M NaCl, pH 6.5) with protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail. A high salt MES buffer was chosen to allow for a better separation of soluble and insoluble Tau in the Alzheimer’s disease brain with Tris buffers often partially solubilising more highly phosphorylated species of Tau and the higher salt content for better dissociation of soluble Tau from microtubules (Diane Hanger, King College London, personal communication, 2015) see also (Hanger et al., 2002).

The homogenate was separated into two. Half was used to isolate the ‘total protein’ fraction and the other to isolate soluble and insoluble protein fractions, see Figure 2.1.

To isolate the ‘total protein’ fraction detergents were added to the homogenate (0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate), vortexed for 30 s and incubated on ice for 10 min. Homogenates were then centrifuged at 3,500 g for 10 min at 4°C to remove cell debris.

The remaining homogenate was centrifuged at 27,000 g for 30 min at 4°C using a Beckman Optima MAX-XP ultracentrifuge (TLA-110 rotor) to remove highly aggregated tau species and cell debris. The supernatant was subsequently centrifuged at 100,000 g for 30 min at 4°C. The supernatant was collected as the soluble protein fraction. The pellet was resuspended in 1% (w/v) Sarkosyl, vortexed for 1 min and incubated on a roller at 4°C overnight. The Sarkosyl homogenates were then subjected to centrifugation at 200,000 g for 30 min. The supernatant was collected as the Sarkosyl soluble fraction and pellet.
containing Sarkosyl insoluble proteins was resuspended with homogenisation buffer through a 21 gauge needle.

All fractions were stored in small volumes in sterile Eppendorf tubes at -80°C to prevent protein loss via freeze/thaw.

Figure 2.1 Human brain tissue homogenisation protocol
### Table 2.2 Identification numbers and demographic data of human tissue

<table>
<thead>
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<th>Case No.</th>
<th>Braak Stage</th>
<th>MRC ID</th>
<th>Gender</th>
<th>Age at death</th>
<th>Pathological diagnosis 1</th>
<th>Pathological diagnosis 2</th>
<th>PMD (hrs)</th>
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<td>severe Alzheimer's Disease</td>
<td></td>
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<td>36</td>
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<td>BBN_3466</td>
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<td>82</td>
<td>severe Alzheimer's Disease</td>
<td>mild SVD</td>
<td>96</td>
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<tr>
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<td>BBN_3408</td>
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<td>mild AD pathologic</td>
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<td>Alzheimer's disease</td>
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<td>mild SVD</td>
<td>97.5</td>
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</table>
2.2.1 Statistical analysis

Following Western blot analysis densitometry of the membranes was performed using Genetools analysis software (Syngene). Following immunofluorescence microscopy, deconvoluted images were analysed using Image J (Fiji) (National Institute of Health) analysis software. All data was analysed using the statistical package GraphPad PRISM (v7).

For data analysis of experiments using large immortalised cell line populations a normal distribution was assumed and parametric tests were used for analysis. For only two data sets an unpaired, two-tailed T-test was used. For multiple comparisons, a One-Way ANOVA was used and corrected for multiple comparisons using Holm-Sidak test. For mouse brain homogenates and human brain samples, due to the low sample numbers a normal distribution of data was not assumed. Therefore, non-parametric tests were used. For the analysis of the human brain tissue, statistical significance was determined using Kruskal-Wallis test with Dunn’s multiple comparisons test. Statistical significance was set to $P < 0.05$. 
Chapter 3. The relationship between PrP\textsuperscript{C} and Tau expression in Alzheimer’s disease

3.1 Introduction

3.1.1 The prion protein and Alzheimer’s disease

PrP\textsuperscript{C} is a cell surface glycoprotein attached to the exterior leaflet of cholesterol rich lipid raft microdomains by a glycosylphosphatidylinositol (GPI)-anchor (Westergard et al., 2007). PrP\textsuperscript{C} is widely expressed throughout the brain, however, is highly expressed in the hippocampus and synaptic terminals (Sales et al., 1998) and is implicated as a key mediator of signal transduction, as well as synaptic plasticity and function (Westergard et al., 2007). In Alzheimer’s disease, Aβ oligomers bind to PrP\textsuperscript{C} with high affinity triggering synaptic impairment and ultimately neuronal loss (Lauren et al., 2009), thus impairing the normal function of PrP\textsuperscript{C}. Furthermore, binding of Aβ oligomers to PrP\textsuperscript{C} is dependent on lipid raft microdomains and the formation of a transient signalling complex which subsequently mediated the synaptic impairment in addition to the aberrant phosphorylation of Tau (Rushworth et al., 2013, Larson et al., 2012). These data strongly implicate aberrant PrP\textsuperscript{C} signalling and function in the pathogenesis of Alzheimer’s disease.

In addition to synaptic function, many other neuroprotective roles of PrP\textsuperscript{C} have been described in the literature, including anti-oxidant, anti-inflammatory and anti-apoptotic activity (Roucou et al., 2004, Vassallo and Herms, 2003) and both inflammation and oxidative stress have been implicated in the pathogenesis of Alzheimer’s disease (Heppner et al., 2015, Persson et al., 2014). In addition, PrP\textsuperscript{C} normally exerts inhibitory effects on APP processing by the β-secretase, BACE1 to reduce Aβ production (Parkin et al., 2007). However, binding of Aβ to PrP\textsuperscript{C} prevents the inhibitory effects on BACE1 generating a positive feedback loop (Rushworth et al., 2013).

These data indicate that PrP\textsuperscript{C} holds many neuroprotective functions, however, some of these are inhibited upon interaction with Aβ oligomers. Interestingly, several lines of evidence suggest that PrP\textsuperscript{C} expression is altered with age and in Alzheimer’s disease, implicating PrP\textsuperscript{C} further in the pathogenesis of the disease, although it remains to be determined the implications of this on the pathogenesis of the disease.
Our research group previously reported a significant reduction in PrP$^C$ expression in the frontal cortex and hippocampus in late stage sporadic but not familial Alzheimer’s disease, with PrP$^C$ significantly inversely correlating with the Braak stages of sporadic Alzheimer’s disease, as defined by the location of Tau tangle bearing neurons (Whitehouse et al., 2010, Whitehouse et al., 2013). A second group reported no significant alterations to PrP$^C$ in the frontal cortex at late Braak stages (Llorens et al., 2013). Interestingly, PrP$^C$ has been reported to increase in the hippocampus of individuals at early Braak stages (Vergara et al., 2015). However, again these reports have been disputed. Larson, et al. 2012, reported a slight decrease in PrP$^C$ in the temporal cortex of patients with mild cognitive impairment (MCI) and a significant increase in PrP$^C$ in Alzheimer’s disease patients (Larson et al., 2012). Contrary to all these reports, another group reported no significant alteration in PrP$^C$ in either the hippocampus or temporal and frontal cortices between age-match controls, MCI and Alzheimer’s disease patients (Saijo et al., 2011).

As briefly described, there is wide variation and contradiction between reports of PrP$^C$ expression throughout the progression of Alzheimer’s disease. Western blot analysis was the primary method used by these studies to analyse PrP$^C$ expression with different anti-PrP$^C$ antibodies (Whitehouse et al., 2010, Larson et al., 2012, Vergara et al., 2015). Differences in truncation and glycosylation of PrP$^C$ may affect its detection via Western blot analysis. Therefore, differences in antibodies used, brain region and stage of disease analysed may all be responsible for the discrepancies between these results.

Recently, a relationship between PrP$^C$ and Tau expression has also started to emerge and Aβ has been implicated in altering this relationship (Chen et al., 2013, Schmitz et al., 2014, Vergara et al., 2015). Tau is crucial for neuronal stabilisation and cargo trafficking, in addition, pathogenic Tau species are increasingly being acknowledged as the mediator of toxicity and pathology in Alzheimer’s disease (Oddo et al., 2006b, Roberson et al., 2007, Pooler et al., 2013b). It is crucial to identify the exact role of PrP$^C$ in Alzheimer’s disease, be it pathogenic or protective and how this influences Tau, which is known to play a key role in disease pathogenesis.
3.1.3 Aims

The aim of this chapter was to explore the relationship between PrP^C and Tau expression. Both PrP^C and Tau have been implicated in the pathogenesis of Alzheimer’s disease, however, little is known about their exact role and how these two key players are linked. This chapter aimed to unravel and explore some of these missing links, with the focus on alterations to their expression. Initially, multiple methods were used to analyse Tau protein expression in cell models and transgenic mouse lines with altered PrP^C expression. Next, we investigated the relationship between PrP^C and Tau expression following the progression of Alzheimer’s disease. Firstly, by analysing PrP^C expression in multiple brain regions (entorhinal, frontal and occipital cortices) at various Braak stages of the disease (Braak 0-II, III-IV and V-VI). The alterations to PrP^C were then correlated to alterations in full-length Tau expression as well as modifications to Tau, such as truncation and phosphorylation.
3.2 Results

3.2.1 There is an inverse relationship between PrP<sup>C</sup> and Tau in neuroblastoma cells

Evidence suggests that PrP<sup>C</sup> expression is altered in Alzheimer’s disease (Whitehouse et al., 2010) and may be linked to alterations in Tau expression (Vergara et al., 2015). To investigate this further, Tau expression was determined by multiple methods in SH-SY5Y cells and SH-SY5Y cells overexpressing PrP<sup>C</sup> (SH-SY5Y-PrP<sup>C</sup>). Firstly, using the pan-Tau antibody, K9JA, a significant reduction of Tau by 34.6 % was measured by Western blot analysis in SH-SY5Y cells overexpressing PrP<sup>C</sup> (Figure 3.1A-B). This was supported by a second pan-Tau antibody, TAU5, which showed a 35.5 % reduction in Tau in SH-SY5Y cells overexpressing PrP<sup>C</sup> (Figure 3.1C). To confirm the results by Western blot analysis, which is a semi-quantitative method, an electrochemiluminescence immunoassay developed by Meso Scale Discovery (MSD) was used. Using the immunoassay, a 32.5 % reduction of Tau was measured in SH-SY5Y-PrP<sup>C</sup> cells compared to SH-SY5Y cells (Figure 3.1D).

Furthermore, these data were confirmed by immunofluorescence microscopy. SH-SY5Y cells endogenously express low levels of PrP<sup>C</sup>, however, following PrP<sup>C</sup> overexpression, a punctate staining of PrP<sup>C</sup> on the cell surface was observed which is representative of lipid raft localised proteins. In addition, reduced intracellular staining for Tau was observed in cells overexpressing PrP<sup>C</sup> (Figure 3.2A). In summary, Western blot analysis, immunofluorescence microscopy and immunoassay analysis all identify an inverse relationship between PrP<sup>C</sup> and Tau in this neuroblastoma cell line.
**Figure 3.1 PrP C overexpression reduces Tau levels in SH-SYSY cells**

(A) SH-SYSY and SHSY5Y cells overexpressing PrP C (SH-SYSY-PrP C) were lysed, subjected to SDS-PAGE (25 µg total protein) and immunoblotted using antibodies targeted against pan-Tau (K9JA and TAU5), PrP C (6D11) and β-actin (AC15). Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of Tau as detected by K9JA \( (n = 3 \text{ independent experiments each of } 2 \text{ replicates, mean } \pm \text{ SD}) \) (B) and TAU5 \( (n = 1 \text{ experiment of } 2 \text{ replicates}) \) (C) were represented as a percentage of SH-SYSY cells \( (* p < 0.05) \). (D) Tau levels were also analysed in 25 µg total protein using the Mesoscale Discovery (MSD) immunoassay. The levels of Tau were represented as a percentage of SH-SYSY cells \( (n = 1 \text{ experiment of } 2 \text{ replicates}). \)
Figure 3.2  PrP^C overexpression reduces Tau levels in SH-SY5Y cells as detected by immunofluorescence microscopy

(A) SH-SY5Y and SH-SY5Y-PrP^C cells were seeded onto coverslips in 24 wells plates and grown to 60 % confluence. Cells were washed, fixed and immunostained for Tau (K9JA; red) or PrP^C (SAF32; green). Nuclei were counterstained with DAPI (blue). For the visualisation of Tau (K9JA) cells were permeabilised with 0.2 % Triton X-100 for 5 minutes before fixing. Images were acquired on a Delta Vision (Applied Precision) restoration microscope using 60 x objective. The deconvoluted image presented here is a Z-section taken from the middle of the cell. The intensity setting and scale from SH-SY5Y cells was copied across to the image acquired for SH-SY5Y-PrP^C cells (n = 1) (Scale bar; 10 µm).
3.2.2 Tau recognition by Western blot analysis

It may be noted that the banding pattern of the two pan-Tau antibodies differs in SH-SY5Y cells. TAUS recognised a distinct band migrating around 50 kDa whereas K9JA recognised a wider band migrating around 55 kDa (Figure 3.1A). Tau is a highly modified protein and undergoes multiple post-translational modifications, some of which may interfere with antibody detection, however, these pan-Tau antibodies should interact with Tau irrespective of its phosphorylation state. Dephosphorylation of the cell lysate with Lambda protein phosphatase (Lambda PP) prior to immunoblotting with K9JA, revealed a more defined band around 50 kDa, similar to that recognised by TAUS, suggesting K9JA detected phosphorylated forms of Tau (Figure 3.3A). In comparison, only a slight downward shift in molecular weight of Tau was detected with TAUS following dephosphorylation of the lysate, although the band increases following dephosphorylation, suggesting TAUS preferentially recognised dephosphorylated forms of Tau (Figure 3.3A).

An explanation for the discrepancy in detection between these two pan-Tau antibodies is antibody clonality. K9JA is a polyclonal antibody, with multiple epitopes across amino acids 243-441, a region containing multiple phosphorylation epitopes (Figure 3.3B). On the other hand, the single epitope for the monoclonal antibody TAUS lies within amino acids 210-241 (Figure 3.3B). Despite these differences in epitope recognition and influence of the phosphorylation status of Tau, both antibodies detected similar Tau expression levels between SH-SY5Y and SH-SY5Y-PrPc cells (Figure 3.1A-C). Furthermore, this data supports previous reports that the foetal (0N3R; Tau352) isoform predominantly expressed by SH-SY5Y cells is highly phosphorylated (Jovanov-Milosevic et al., 2012, Simic et al., 2009).
**Figure 3.3 Sites of pan-Tau antibody recognition**

(A) SH-SY5Y cells were incubated with 1000 units Lambda protein phosphatase (Lambda PP) for 3 h at 30°C. The reaction was stopped following the addition of SDS-loading dye and 25 µg (total protein) was subjected to SDS-PAGE and immunoblotted for Tau (K9JA and Tau5).

(B) Schematic displaying Tau phosphorylation sites identified normally in the brain (green), associated with Alzheimer’s disease (black), or both (blue). Antibody epitopes are mapped onto the full length Tau (Tau441; 2N4R) structure.

### 3.2.3 The GPI-anchor is essential for the lipid raft association of PrP<sup>C</sup> and reduction in Tau

The GPI-anchor plays a key role in the signal transduction mediated by PrP<sup>C</sup> (Paulick and Bertozzi, 2008). To determine whether the GPI-anchor of PrP<sup>C</sup> mediates the alterations to Tau expression, Tau levels were analysed in cells overexpressing a C-terminal transmembrane anchored form of PrP<sup>C</sup>, PrP-CTM (Figure 3.4A). PrP-CTM has the GPI-signal peptide replaced with the transmembrane and cytosolic domains from the type I integral membrane glycoprotein, human angiotensin converting enzyme (Walmsley et al., 2001). SH-SY5Y cells overexpressing PrP-CTM display a less punctate surface distribution of PrP<sup>C</sup>,...
suggesting that it is not completely localised to lipid raft microdomains (Taylor et al., 2005). Before analysing the changes to Tau expression levels the absence of PrP-CTM in lipid-raft microdomains was confirmed. The lipid raft microdomain was extracted using buoyant sucrose gradient centrifugation and isolated in fractions 6-8 as confirmed by the marker, Flotillin-1. Normally, GPI-anchored PrP$^C$ is also extracted with Flotillin-1 in fractions 6-8 (Taylor et al., 2005). In comparison, PrP-CTM was isolated in fractions 1-2, which are non-raft fractions and localises with the known non-raft protein, the Transferrin receptor (Figure 3.4B). Furthermore, Tau expression was not reduced in SH-SY5Y-PrP-CTM cells (Figure 3.4C), suggesting that the GPI-anchor is crucial both for the lipid raft localisation of PrP$^C$ and mediating the reduction in Tau expression in SH-SY5Y cells.

### 3.2.4 PrP$^C$ deletion alters Tau expression in multiple mouse strains

To support the cell data, Tau expression was measured in transgenic mice with altered PrP$^C$ expression. Ablation of the prion protein gene ($Prnp^{-/-}$) significantly reduced Tau levels by 45.5 %, compared to wild type mice ($Prnp^{+/+}$) (Figure 3.5A-B), suggesting a positive relationship between PrP$^C$ and Tau in these mice. In addition, a downward shift in molecular weight of the Tau bands with Prnp knock out was observed (Figure 3.5A). This may be a shift in the isoforms of Tau expressed or alterations in post-translational modification of Tau in the absence of PrP$^C$.

In addition, Tau expression was analysed in Tg20 mice which overexpress PrP$^C$. Densitometric analysis revealed a 24.4 % (ns) reduction in Tau in these mice compared to wild-type mice (Figure 3.5A-B). It is worth noting that, of the four Tg20 mice analysed, the two males expressed higher levels of Tau, showing only an 8.2 % reduction compared to wild type mice (Figure 3.5C). The male Tg20 mice also expressed higher levels of PrP$^C$, indicating a positive correlation between PrP$^C$ and Tau in these mice. It is possible that the difference between sexes between Tg20 mice may be a result of differences in age. The male Tg20 mice were five weeks older than the females (27 and 22 weeks, respectively). All wild-type and knock out mice were 40-41 weeks and no significant difference between sexes in either was observed, as reflected by the lower SEM (Figure 3.5B). It is possible that if PrP$^C$ expression is altered with age in the Tg20 mice, analysis of 40 week old mice may have shown an increase in PrP$^C$ expression, thus reflecting another positive relationship between PrP$^C$ and Tau.
Figure legend on next page
Figure 3.4 Tau levels are not reduced in SH-SY5Y-PrP-CTM cells

(A) Normally PrP<sup>C</sup> is GPI-anchored to the exterior leaflet of lipid raft microdomains. Replacement of the GPI-anchor for a transmembrane region (PrP-CTM) results in the protein no longer localising to the lipid raft microdomains.  
(B) SH-SY5Y cells overexpressing PrP-CTM were lysed in the presence of 0.6 % Triton X-100 and a buoyant discontinuous sucrose gradient was created (40 %, 35 % and 5 % w/v sucrose) to isolate lipid rafts. Following centrifugation at 100,000 g, for 18 h, fractions were collected and immunoblotted for transferrin receptor (Transferrin-R), Flotillin-1 and PrP<sup>C</sup> (6D11). The insoluble lipid rafts float at the 5-35 % interface and were collected in fraction 6-8 (n = 1).
(C) SH-SY5Y, SH-SY5Y-PrP<sup>C</sup> and SH-SY5Y-PrP-CTM cells were lysed, subjected to SDS-PAGE (25 µg total protein) and immunoblotted using antibodies targeted against pan-Tau (K9JA), PrP<sup>C</sup> (SAF32) and β-actin (AC15). The samples were all analysed on the same SDS-PAGE gel and the dotted line indicates where irrelevant lands were removed (n = 1).

PrP<sup>C</sup> is known to be a high affinity receptor for oligomeric forms of Aβ triggering downstream signalling cascades (Lauren et al., 2009, Um et al., 2012). This complex is known to induce phosphorylation of Tau at tyrosine 18 (Larson et al., 2012), however, it is possible that other alterations to Tau are mediated by Aβ binding to PrP<sup>C</sup>. To investigate this possibility, Tau expression was determined in J20 mice. J20 mice overexpress human APP carrying the Swedish (K670N/M671L) and Indiana (V171F) familial mutations linked to Alzheimer’s disease which result in increased Aβ levels (Mucke et al., 2000).

APP expression in J20 mice is directed to neurons under control of a human platelet-derived growth factor polypeptide-β (PDGF-β) promoter with highest expression levels observed in the neocortex and hippocampus (The Jackson Laboratory, Maine, USA). An age-dependent increase in Aβ levels was measured in these mice, developing amyloid plaques 5-7 months of age (Mucke et al., 2000). In addition, densitometric analysis showed an age-related increase in Tau in these J20 mice; 1.62 and 2.05 fold increase at 10 and 40 weeks, respectively compared to wild-type mice (Prnp<sup>+/+</sup>) which do not overexpression human APP (Figure 5.6A-B).

To investigate the role of PrP<sup>C</sup> in mediating this increase in Tau, J20 mice crossed with PrP<sup>C</sup> knock out mice (J20/Prnp<sup>-/-</sup>) were analysed (Griffiths et al., 2011). It should be noted that
when J20 mice (C57BL/6 background) were crossed with Prnp\(^{-/-}\) mice (129Ola background) a mixed genetic background was generated (B6/129Ola). The wild type mice (Prnp\(^{+/+}\)) used as a control in this analysis were from a 129/Ola genetic background. Therefore, for further analysis Tau levels were analysed relative to 10 week old J20/Prnp\(^{+/+}\) mice.

The J20 mice crossed with PrP\(^{C}\) knock out mice revealed a significant reduction (50.6 %) in Tau expression at 40 weeks and a trend towards a reduction at 10 weeks (23.2 %) (Figure 3.6C). Interestingly, a similar level of Tau is observed at both 10 and 40 weeks in J20/Prnp\(^{-/-}\) mice (Figure 3.6A,C). Following analysis with a second pan-Tau antibody, K9JA, a band estimated to be +200 kDa was observed in 40 week J20/Prnp\(^{+/+}\) mice (Figure 3.7A). This is possibly hyperphosphorylated aggregated Tau induced by Aβ aggregates produced by the APP mutant which subsequently interact with PrP\(^{C}\). This aggregated species of Tau was not detected with the pan-Tau antibody, TAU5 (Figure 3.6A), confirming previous data that K9JA but not TAU5 recognises phosphorylated forms of Tau (Figure 3.3).
A) Genotype:  
- Prnp^{+/+} 
- Prnp^{-/-} 
- Tg20 

<table>
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<th>F</th>
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- Tau
- PrP^C
- β-Actin

B) Genotype:  
- Prnp^{+/+} 
- Prnp^{-/-} 
- Tg20 

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- Tau
- PrP^C
- β-Actin

C)  

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Figure 3.5 PrPnull mice have altered Tau expression levels compared to wild-type mice

(A) Hemi-brains from wild type (Prnp+/+), PrPnull (Prnp−/−) and PrPoverexpressing (Tg20) mice were homogenised in 2% SDS, subjected to SDS-PAGE (30 µg total protein) and immunoblotted for Tau (TAU5), PrP (SAF32) and β-actin (AC15). (B) Semi-quantitative densitometry of membranes was performed using GeneTools (Syngene). Levels of Tau are represented as percentage of Prnp+/+ (mean ± SEM), (Prnp+/+; n = 6, Prnp−/−; n = 6, Tg20; n = 4). (C) Semi-quantitative densitometry of membranes was performed using GeneTools (Syngene). Levels of Tau in female (F) and male (M) Tg20 mice was represented as percentage of Prnp+/+. All statistical analysis was performed using One-way ANOVA (ns non-significant; * p < 0.05; ** p < 0.01), (GraphPad, PRISM).
A) Genotype: $Prnp^{+/+}$, $J20/Prnp^{+/+}$, $J20/Prnp^{-/-}$

<table>
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<th>40</th>
<th>10</th>
<th>40</th>
<th>10</th>
<th>40</th>
</tr>
</thead>
</table>

70 kDa  
55 kDa

40 kDa

Tau

β-Actin

B) Graph showing Tau (% $Prnp^{+/+}$) with Genotype: $Prnp^{+/+}$, $J20/Prnp^{+/+}$, $J20/Prnp^{-/-}$ and APP Genotype: $-$, $J20$, $J20$.

C) Graph showing Tau (% $J20/Prnp^{+/+}$ 10 wks) with Genotype: $J20$, $J20$, $J20$, $J20$.

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Figure 3.6 An Alzheimer’s disease mouse model shows an age-dependent increase in Tau which is ameliorated by PrP<sup>C</sup> knock out

(A) J20 mice overexpress human APP with two familial mutations linked to Alzheimer’s disease (APP/Swe and APP/Ind). These mice were crossed with PrP null mice (J20/Prnp<sup>-/-</sup>). Hemi-brains from wild type (Prnp<sup>+/+</sup>), J20/Prnp<sup>+/+</sup> and J20/Prnp<sup>-/-</sup> mice were homogenised in 2 % SDS, subjected to SDS-PAGE (30 µg total protein) and immunoblotted for Tau (TAU5) and β-actin (AC15). (B) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of Tau in 10 and 40 week J20 mice were represented as a percentage of 40 week Prnp<sup>+/+</sup> mice (n = 2, except Prnp<sup>-/-</sup> n = 4). (C) Levels of Tau in J20 mice were represented as percentage of 10 week, J20/Prnp<sup>+/+</sup> (all n = 2). Statistical analysis was performed using One-way ANOVA (ns p > 0.05 not significant, * p < 0.05, ** p < 0.01) (mean ± SEM), (GraphPad, PRISM).

Figure 3.7 Tau aggregation in an Alzheimer’s disease mouse model is ameliorated by PrP<sub>C</sub> knock out

(A) Homogenates from 40 week J20/Prnp<sup>+/+</sup> and J20/Prnp<sup>-/-</sup> mice were subjected to SDS-PAGE (30 µg total protein) and immunoblotted for Tau (K9JA), PrP<sub>C</sub> (SAF32) and β-actin (AC15) (n = 3).
3.2.5 Full length Tau and PrP\textsuperscript{C} levels are reduced in Alzheimer’s disease

To further extend the study, the relationship between PrP\textsuperscript{C} and Tau was analysed following the progression of sporadic Alzheimer’s disease in human brain tissue. The entorhinal cortex, frontal cortex and occipital cortex from three incipient AD (Braak III-IV), three severe Alzheimer’s disease (Braak V-VI) and three age-matched controls (Braak 0-II) were used in this pilot study. To analyse both PrP\textsuperscript{C} and Tau in the same protein fraction, the detergents sodium deoxycholate and Triton X-100 were used to isolate a total protein fraction by disrupting cell membranes and solubilising lipid rafts (see methods section 2.2.21 for details).

Tau is a highly post-translationally modified protein and as identified previously pan-Tau antibodies may be influenced by the phosphorylation status of Tau (Figure 5.3). To try to overcome any bias when analysing the changes to full-length Tau expression two pan-Tau antibodies were used, K9JA (amino acids 243-441) and TAU5 (amino acids 210-241). It should be noted that, following electrophoresis, the Tau bands migrating around 55-70 kDa which represent full-length Tau (FL-Tau) were semi-quantified by densitometry. In addition, previous work in this laboratory has identified differential detection of PrP\textsuperscript{C} following Western blot analysis (Dr Isobel Whitehouse, The University of Leeds, unpublished). This may be a result of differential glycosylation or truncation of the protein altering antibody recognition (Liu et al., 2001). Therefore, two anti-PrP\textsuperscript{C} antibodies, 6D11 (amino acids 93-109) and SAF32 (amino acids 78-91), were used to analyse PrP\textsuperscript{C}.

In all three brain regions, there was a trend towards a reduction in the level of full length Tau, as detected by the TAUS antibody, with increasing Braak stages (Figure 3.8-3.10). This reduction in full length Tau correlated with increased smearing of the bands, possibly reflecting increased degradation and/or aggregation of Tau, at lower and higher molecular weights, respectively. This diffuse smear staining is more prominent in the entorhinal cortex, a region of the brain where Tau pathology is identified at an earlier Braak stage. The diffuse smear staining of Tau was detected to a greater extend with the anti-Tau antibody K9JA, which has previously been identified to detect greater levels of phosphorylated Tau than TAUS (Figure 3.3). This may explain why a trend towards an increase in Tau was detected in the entorhinal cortex in severe Alzheimer’s disease, following analysis with the K9JA antibody (Figure 3.8 B) contradicting the TAUS antibody.
Large variations in PrP\textsuperscript{C} were observed at early and moderate Braak stages in both the entorhinal and occipital cortices. However, a clear reduction in PrP\textsuperscript{C} was observed at late Braak stages in all regions. It is worth noting that no significant difference in PrP\textsuperscript{C} detection between the two antibodies was observed (Figure 5.8-5.10). PrP\textsuperscript{C} can be variably N-glycosylated at two sites, resulting in un-, mono-, or di-glycosylated forms of the protein. The 6D11 antibody appears to have greater recognition for un-glycosylated forms of the protein, observed as a band migrating around 30 kDa. In this study densitometric analysis was performed on all PrP\textsuperscript{C} forms. To identify changes to PrP\textsuperscript{C} glycosylation in these samples further analysis would be needed. Compared to the occipital cortex, higher expression levels of PrP\textsuperscript{C} were observed in the entorhinal and frontal cortex, supporting a role for PrP\textsuperscript{C} in synaptic function and memory formation (Sales et al., 1998).

3.2.6 Reductions in protein levels of PrP\textsuperscript{C} and Tau in Alzheimer’s disease is not a result of global protein loss

To confirm the reduction in Tau and PrP\textsuperscript{C} expression were protein specific and not a result of global protein loss following neurodegeneration, the levels of APP and β-III-tubulin were analysed. APP, like PrP\textsuperscript{C}, can be found on the cell surface of neurons and processing of APP plays a crucial role in Alzheimer’s disease pathophysiology (O’Brien and Wong, 2011). Unlike PrP\textsuperscript{C}, APP expression was not altered with increasing Braak stages in the any of the brain regions analysed (Figure 3.8-5.10). β-III-tubulin was also used a protein control. Tubulin is a major microtubule component, with the β-III isoform considered neuron-specific (Memberg and Hall, 1995). Following equal protein loading, no difference in β-III-tubulin levels was observed (Figure 3.8-3.10). These data suggest that there is a specific reduction in both Tau and PrP\textsuperscript{C} with Alzheimer’s disease.
A) **Entorhinal Cortex**

Braak stage: 0-II | III-IV | V-VI

- Aggregated Tau
- FL Tau (K9JA)
- Truncated Tau

**Entorhinal Cortex A)**

- Tau (TAU5)
- PrP$^c$ (SAF32)
- PrP$^c$ (6D11)
- APP
- β-III tubulin

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Figure 3.8 Altered protein expression in the entorhinal cortex following Braak stages of Alzheimer’s disease

(A) The entorhinal cortex from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5) and proteins were extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblotted for Tau (K9JA and TAU5), PrP^c (SAF32 and 6D11), APP (22C11) and β-III-Tubulin. (B-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). For Tau, only full-length (FL) isoforms were quantified. Scatter dot plots were generated and protein levels are represented relative to Braak stages 0-II (All groups; n = 3) (mean). (E) Protein levels are represented relative to the respective Braak stage 0-II (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
A) Frontal Cortex

Braak stage: 0-II  III-IV  V-VI

<table>
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<tr>
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<tr>
<td>FL Tau (TAUS)</td>
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<td>PrP^c (6D11)</td>
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<td>β-III tubulin</td>
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</tbody>
</table>

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B) **Tau**

![Graph showing protein expression (Ratio 0-II Braak) for Tau.]

C) **PrP**

![Graph showing protein expression (Ratio 0-II Braak) for PrP.]

D) **Controls**

![Graph showing protein expression (Ratio 0-II Braak) for Controls.]

E) ![Graph showing protein expression (Ratio 0-II Braak) for various proteins.]

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Figure 3.9 Altered protein expression in the frontal cortex following the Braak stages of Alzheimer’s disease

(A) The frontal cortex from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH 6.5) and proteins were extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblotted for Tau (K9JA and TAUS), PrP (SAF32 and 6D11), APP (22C11) and β-III-Tubulin. (B-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). For Tau, only full-length (FL) isoforms were quantified. Scatter dot plots were generated and protein levels are represented relative to Braak stages 0-II (All groups; n = 3) (mean). (E) Protein levels are represented relative to respective Braak stages 0-II (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
A) **Occipital Cortex**

<table>
<thead>
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<th>Braak stage:</th>
<th>0-II</th>
<th>III-IV</th>
<th>V-VI</th>
</tr>
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</table>

- **FL Tau (K9JA)**
  - 70 kDa
  - 55 kDa
  - 40 kDa
  - 35 kDa

- **FL Tau (TAU5)**
  - 70 kDa
  - 55 kDa
  - 40 kDa
  - 35 kDa

- **PrP**<sup>C</sup> (SAF32)
  - 40 kDa
  - 35 kDa

- **PrP**<sup>C</sup> (6D11)
  - 40 kDa
  - 35 kDa

- **APP**
  - 130 kDa
  - 100 kDa

- **β-III tubulin**
  - 55 kDa

*Figure continues on next page*
B) **Tau**

![Graph showing protein expression levels for Tau across different Braak stages (0-II, III-IV, V-VI).]

C) **PrP**

![Graph showing protein expression levels for PrP across different Braak stages (0-II, III-IV, V-VI).]

D) **Controls**

![Graph showing protein expression levels for APP and βIII-Tubulin across different Braak stages (0-II, III-IV, V-VI).]

E) **Total Protein Fraction**

![Graph showing total protein fraction for different conditions and Braak stages (0-II, III-IV, V-VI).]

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Figure 3.10 Altered protein expression in the occipital cortex following Braak stages of Alzheimer’s disease

(A) The occipital cortex from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH 6.5) and proteins were extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblotted for Tau (K9JA and Tau5), PrP<sup>c</sup> (SAF32 and 6D11), APP (22C11) and β-III-Tubulin. (B-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). For Tau, only full-length (FL) isoforms were quantified. Scatter dot plots were generated and protein levels were represented relative to Braak stages 0-II (All groups; n = 3) (mean). (E) Protein levels are represented relative to respective Braak stages 0-II (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).

In addition, in the entorhinal cortex, the full length Tau detected by K9JA was at a slightly higher molecular weight in later Braak stages where diffuse smear staining was observed (Figure 3.8A). This was no longer observed following dephosphorylation of the sample with Lambda protein phosphatase (Lambda PP) (Figure 3.11A) and a partial downward shift in the molecular weight is observed. These data implicate that full length Tau becomes increasing phosphorylated following the progression of Alzheimer’s disease. On the other hand, the diffuse smear staining was still observed at the higher molecular weights. It is possible that Lambda PP is unable to dephosphorylate Tau once aggregated into higher order structures. In addition, a clear reduction in full length Tau with increasing Braak stages was observed in the frontal cortex, and a trend towards a reduction in both the entorhinal and occipital cortices (Figure 3.11), confirming the reduction in Tau levels following the progression of Alzheimer’s disease.
A) **Entorhinal Cortex**
Braak stage: 0-II III-IV V-VI

70 kDa - 55 kDa - 40 kDa -

{\text{Tau (K9JA)}}

B) **Frontal Cortex**
Braak stage: 0-II III-IV V-VI

70 kDa - 55 kDa - 40 kDa -

{\text{Tau (K9JA)}}

C) **Occipital Cortex**
Braak stage: 0-II III-IV V-VI

70 kDa - 55 kDa - 40 kDa -

{\text{Tau (K9JA)}}
D) **Entorhinal Cortex**

Braak stage: 0-II  III-IV  V-VI

E) **Frontal Cortex**

Braak stage: 0-II  III-IV  V-VI

F) **Occipital Cortex**

Braak stage: 0-II  III-IV  V-VI

Figure continues on next page
The entorhinal cortex, frontal cortex and occipital cortex from nine human patients, grouped by Braak stages were homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH 6.5) and proteins extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were incubated with lambda protein phosphatase (Lambda PP) for 3 h at 30°C. The reaction was stopped following the addition of SDS-loading dye and 30 µg (total protein) was subjected to SDS-PAGE and immunoblotted for Tau, K9JA (A-C) and TAU5 (D-F). (G-I) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Scatter dot plots were generated and protein levels represented relative to Braak 0-II (All groups; n = 3) (mean). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
3.2.7 There is a significant correlation between PrP\textsuperscript{C} and Tau in Alzheimer’s disease

Correlation plots between PrP\textsuperscript{C} and full-length Tau revealed a significant correlation between the expression of the two proteins in all three brain regions. This correlation was strongest in the frontal cortex ($r = 0.929$, $p 0.002 **$). This near perfect correlation assumes that PrP\textsuperscript{C} expression closely predicts Tau levels. On the other hand, there was no significant correlation between Tau and APP in any of the brain regions analysed (Figure 3.12A-C). This further supports the hypothesis that alterations in Tau in Alzheimer’s disease are specifically linked to PrP\textsuperscript{C}.

Furthermore, linear regression analysis revealed a strong correlation between both PrP\textsuperscript{C} and Tau and the Braak stages of the disease. This correlation was strongest in the frontal cortex and weakest in the occipital cortex (Figure 3.12D-F). Alterations to both PrP and Tau strongly and significantly correlated with increasing Braak stages in the frontal cortex this is reflected in tighter confidence bands surrounding the line of best fit (Figure 3.12E).
A) Entorhinal Cortex

B) Frontal Cortex

C) Occipital Cortex

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Figure 3.12  PrP\textsuperscript{C} but not APP correlates with changes in full-length Tau following the Braak stages of Alzheimer’s disease

Correlation plots comparing the expression levels of Tau (TAUS) and PrP\textsuperscript{C} (SAF32) or Tau (TAUS) and APP (22C11) in the entorhinal cortex (A), frontal cortex (B) and occipital cortex (C). Statistical analysis was performed using Spearmans (non-parametric), two-tailed test, generating r and P values for each correlation (ns  p > 0.05 not significant, *  p < 0.05, **  p < 0.01) (GraphPad, PRISM). (D-F) Linear regression graphs show the relationship between PrP\textsuperscript{C} (SAF32) or Tau (TAUS) and the Braak stages of Alzheimer’s disease. Dashed curved lines represent 95 % confidence intervals for the solid line of best fit (green; Tau, blue; PrP\textsuperscript{C}, (GraphPad PRISM).
3.2.8 Alterations to Tau isoforms in Alzheimer’s disease

Alternative splicing of MAPT at exon 10 results in the presence or absence of the second microtubule binding repeat, leading to the expression of three- or four-repeat (3R-, 4R-) Tau isoforms. Next it was investigated whether the loss of Tau in Alzheimer’s disease was a result of both 3R and 4R containing Tau isoforms. To test the specificity of antibodies directed against either 3R- or 4R-Tau isoforms a recombinant Tau ladder containing all 6 Tau isoforms used was used (Figure 3.13A).

There was a clear reduction in both three-repeat and four-repeat Tau isoforms in the frontal cortex (Figure 3.13B,E). In comparison densitometric analysis revealed a trend towards an increase in both repeat isoforms, but predominantly 4R isoforms in the occipital cortex with increasing Braak stages (Figure 3.13C,F). In the entorhinal cortex levels of both 3R and 4R containing Tau isoforms are reduced in moderate Braak stages (III-IV), however, densitometric analysis reveals a slight increase in late Braak stages (V-VI), although this is likely a result of increased phosphorylation and degradation of Tau interfering with the quantification of full length Tau isoforms (Figure 5.13A,D).

To identify whether the decrease in PrP<sup>C</sup> specifically correlated with either 3R- or 4R- Tau isoforms correlation plots were generated. There was a significant correlation between both PrP<sup>C</sup> and 3R- or 4R- Tau isoforms in the frontal cortex, with no difference between the Tau repeat isoforms (Figure 3.14B). In comparison, in the entorhinal and occipital cortex changes in PrP<sup>C</sup> did not significantly correlate to either 3R or 4R- Tau isoforms. However, in these two brain regions the weakest correlation was between 4R Tau and PrP<sup>C</sup>, reflecting the increase in 4R- Tau isoforms in the entorhinal and occipital cortices in severe Alzheimer’s disease (Figure 3.13 and 3.14).
A) **Entorhinal Cortex**

Braak stage: 0-II  III-IV  V-VI  rTau6

70 kDa -  55 kDa -  40 kDa -  70 kDa -  55 kDa -  40 kDa -

3R-Tau

B) **Frontal Cortex**

Braak stage: 0-II  III-IV  V-VI

70 kDa -  55 kDa -  40 kDa -  70 kDa -  55 kDa -  40 kDa -

3R-Tau

C) **Occipital Cortex**

Braak stage: 0-II  III-IV  V-VI

70 kDa -  55 kDa -  40 kDa -  70 kDa -  55 kDa -  40 kDa -

3R-Tau

Figure continues on next page
Figure 3.13 Changes in Tau isoform patterns following the Braak stages of Alzheimer’s disease

The entorhinal cortex (A), frontal cortex (B) and occipital cortex (C) from nine human patients, grouped by Braak stages were homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5) and proteins were extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblotted for three-repeat or four-repeat Tau isoforms (3R-Tau and 4R-Tau, respectively). A recombinant Tau ladder expressing all six Tau isoforms (rTau6) was used to identify the Tau isoforms expressed. (D-F) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Scatter dot plots were generated and Tau isoform levels were represented relative to Braak stages 0-II (All groups; n = 3) (mean). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
A)  **Entorhinal Cortex**

![Graph A](image)

$r = 0.435$

$P = 0.242$ (ns)

B)  **Frontal Cortex**

![Graph B](image)

$r = 0.833$

$P = 0.015$ (*)

$r = 0.810$

$P = 0.022$ (*)

C)  **Occipital Cortex**

![Graph C](image)

$r = 0.683$

$P = 0.050$ (ns)

$r = -0.067$

$P = 0.880$ (ns)

Figure legend on next page
Figure 3.14 Correlating PrP\(^C\) to Tau isoforms levels

Correlation plots comparing PrP\(^C\) (SAF32) expression with 3R or 4R Tau isoform levels in the entorhinal cortex (A), frontal cortex (B) and occipital cortex (C). Statistical analysis was performed using Spearman’s (non-parametric), two-tailed test, generating r and P values for each correlation (ns \( p > 0.05 \) not significant, * \( p < 0.05 \)), (GraphPad, PRISM).

3.2.9 Alterations to Tau phosphorylation in Alzheimer’s disease

It was hypothesised that the diffuse smear staining detected at higher molecular weights when analysing full length Tau is phosphorylated and aggregated Tau species. To investigate this further and the changes to Tau phosphorylation levels in Alzheimer’s disease, two antibodies recognising Tau phosphorylated at Thr231 and at Ser396/404 (PHF1) were used. In early Braak stages, full length Tau was highly pThr231 reactive (Figure 3.15A-C). With increasing Braak stages, this decreases and increased smearing of the bands was detected, representing different phosphorylated/aggregated states of Tau and mirroring the detection measured with the pan-Tau antibody, K9JA. This diffuse staining was seen to a greater extent in the entorhinal cortex. In addition, an upward shift in full length Tau isoform bands was observed in late-stages (Braak V-VI) in the entorhinal cortex with pThr231 (Figure 3.15A). Interestingly, the pThr231 antibody detected less full length Tau in the occipital cortex at earlier Braak stages (Figure 3.15C).

The second phosphorylated Tau antibody, PHF1, did not detect full length Tau at earlier Braak stages and was more specific to later stages of the disease. Again, smear staining was detected which was seen at earlier Braak stages in the entorhinal cortex (Figure 3.15D-F), coinciding with the loss of full length Tau and supporting previous data to suggest the presence of Tau pathology at earlier Braak stages in the entorhinal cortex (Figure 3.8).
A) **Entorhinal Cortex**

Braak stage:  

<table>
<thead>
<tr>
<th>O-II</th>
<th>III-IV</th>
<th>V-VI</th>
</tr>
</thead>
</table>

![Image of Entorhinal Cortex](image1.png)

- pTau (pThr231)

B) **Frontal Cortex**

Braak stage:  

<table>
<thead>
<tr>
<th>O-II</th>
<th>III-IV</th>
<th>V-VI</th>
</tr>
</thead>
</table>

![Image of Frontal Cortex](image2.png)

- pTau (pThr231)

C) **Occipital Cortex**

Braak stage:  

<table>
<thead>
<tr>
<th>O-II</th>
<th>III-IV</th>
<th>V-VI</th>
</tr>
</thead>
</table>

![Image of Occipital Cortex](image3.png)

- pTau (pThr231)

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Figure 3.15 Altered Tau phosphorylation following the Braak stages of Alzheimer’s disease

The entorhinal cortex, frontal cortex and occipital cortex from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH 6.5) and proteins were extracted with 0.5 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate. Samples were subjected to SDS-PAGE (25 µg total protein) and the entorhinal cortex (A-D), frontal cortex (B-E) and occipital cortex (C-F) was immunoblotted for phosphorylated Tau (pTau) at Thr231 (A-C) and Ser396/404 (PHF1) (D-F).
3.2.10 Increase in hyper-phosphorylated, insoluble Tau in Alzheimer’s disease

With the advancement of Braak stages, a decrease in full length Tau and an increase in Tau phosphorylation was observed (Figure 5.8-5.16). It was hypothesised that this would correlate with increased hyperphosphorylated insoluble Tau. Multiple methods to extract insoluble Tau have been reported in the literature (Julien et al., 2012). In this study, an adapted protocol first described by Greenberg and Davies (Greenberg and Davies, 1990) using the detergent Sarkosyl was used to extract insoluble Tau. This protocol describes the isolation of paired helical filament (PHF)-Tau which migrated around 57-68 kDa following SDS-PAGE and was insoluble in the presence of Sarkosyl.

In this study, the insoluble Tau was extracted using 1% Sarkosyl followed by high-speed centrifugation separating Sarkosyl soluble and insoluble proteins. Tau was isolated in both Sarkosyl soluble and insoluble fractions (Figure 3.16 and 3.17). Analysis with the pan-Tau antibody, K9JA, detected little or no Sarkosyl insoluble Tau in early Braak stages. Insoluble Tau was first detected in the entorhinal cortex, before detection in all fractions at late Braak stages. Three bands which migrate around 60-75 kDa were identified; these are possibly PHF-associated Tau as described by Greenberg and Davies (Figure 3.16). A fourth band, migrating around 45/50 kDa was also detected in these fractions, possibly an insoluble truncated Tau species. All bands detected were also reactive to the phosphorylated Tau antibody, PHF1, suggesting that the majority of Tau extracted in the insoluble fraction is phosphorylated. In addition to these more defined bands, smear staining across the gel was detected, reflecting multiple phosphorylated aggregated and truncated Tau species. As predicted, the presence of hyperphosphorylated insoluble Tau coincided with the decrease in full length Tau in the total protein fraction (Figure 3.8-3.10).
Sarkosyl Insoluble

A) Entorhinal Cortex
   Braak stage: 0-II  III-IV  V-VI
   70 kDa - 55 kDa - 40 kDa - PHF-Tau (K9JA)

B) Frontal Cortex
   Braak stage: 0-II  III-IV  V-VI
   70 kDa - 55 kDa - 40 kDa - PHF-Tau (K9JA)

C) Occipital Cortex
   Braak stage: 0-II  III-IV  V-VI
   70 kDa - 55 kDa - 40 kDa - PHF-Tau (K9JA)

Figure continues on next page
Figure 3.16 Levels of insoluble hyperphosphorylated Tau increase in Alzheimer’s disease

The entorhinal cortex, frontal cortex and occipital cortex from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH 6.5). Following centrifugation at 100,000 g, the pellet was resuspended in 1 % (w/v) Sarkosyl and centrifuged at 200,000 g. The pellet containing the Sarkosyl insoluble proteins was subsequently resuspended in SDS-loading buffer, subjected to SDS-PAGE and immunoblotted for pan-Tau (K9JA) (A-C) and phosphorylated Tau (pTau) at Ser396/404 (PHF1) (D-F). (PHF; paired helical filament)
Tau which is soluble to the detergent Sarkosyl was also isolated. This fraction should contain membrane bound or microtubule-associated soluble Tau which is subsequently extracted using the detergent Sarkosyl. The levels of Sarkosyl soluble Tau decreased in late Braak stages in the frontal cortex but not in the entorhinal or occipital cortex (Figure 3.17). In addition, there was a trend increase for Tau in this fraction in incipient Alzheimer’s disease (Braak III-IV) in all regions (Figure 3.17). Tau isolated in this fraction migrated around 50 kDa. This is a slightly lower molecular weight than full length isoforms detected previously. In addition, a slight increase in a band migrating at 40 kDa was observed with increasing Braak stages. This is possibly another truncated form of Tau.

Membrane bound PrP(\(^{C}\)) was also extracted in the Sarkosyl soluble fraction. In all regions a trend towards a reduction PrP(\(^{C}\)) in late Braak stages was measured, with a slight increase in levels in the entorhinal and occipital cortex in moderate Braak stages (Figure 3.17), following a similar pattern to changes in Tau expression.
Sarkosyl soluble

A) Entorhinal Cortex
   Braak stage:  0-II  III-IV  V-VI
   
   55 kDa 40 kDa
   40 kDa 35 kDa

   Tau (K9JA)
   - PrP\(^c\) (SAF32)

B) Frontal Cortex
   Braak stage:  0-II  III-IV  V-VI
   
   55 kDa 40 kDa
   40 kDa 35 kDa

   Tau (K9JA)
   - PrP\(^c\) (SAF32)

C) Occipital Cortex
   Braak stage:  0-II  III-IV  V-VI
   
   55 kDa 40 kDa
   40 kDa 35 kDa

   Tau (K9JA)
   - PrP\(^c\) (SAF32)

Figure continues on next page
Figure 3.17 Alterations to Sarkosyl soluble Tau and PrP<sup>C</sup> in Alzheimer’s disease

The entorhinal cortex (A), frontal cortex (B) and occipital cortex (C) from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5). Following centrifugation at 100,000 g, the pellet was resuspended in 1 % (w/v) Sarkosyl and centrifuged at 200,000 g. The supernatant containing Sarkosyl soluble proteins were collected, subjected to SDS-PAGE and immunoblotted for pan-Tau (K9JA) and PrP<sup>C</sup> (SAF32). (D-F) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Scatter dot plots were generated and protein levels were represented relative to Braak 0-II (All groups; n = 3) (mean). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
Alterations to soluble Tau in Alzheimer’s disease

Research suggests that Tau may be involved in neuronal dysfunction before the accumulation of insoluble Tau aggregates (Santacruz et al., 2005). Pre-fibrillar, soluble forms of Tau have been implicated as the ‘toxic’ species mediating this neurotoxicity (Kopeikina et al., 2012). Furthermore, there is increasing evidence that Tau can transfer between neurons propagating tauopathy, although the mechanisms for this are largely unknown (Wu et al., 2016), and the exact species of Tau transferred is unidentified (Takeda et al., 2015, Sanders et al., 2014).

Following homogenisation, soluble proteins were isolated by sequential ultra-centrifugation speeds (See methods section 2.2.21). Western blot analysis was used to determine changes to full length Tau in the soluble fraction in addition to modifications such as truncation or phosphorylation. In all three brain regions, there was a clear trend towards a reduction in soluble Tau with increasing Braak stages, as detected by both pan-Tau antibodies (Figure 3.18). This decrease was most pronounced in the frontal cortex, and least in the occipital cortex, mirroring the total protein fraction (Figure 3.8-3.10). No distinct truncated or phosphorylated species were identified. In addition, diffuse smear staining was not observed suggesting fewer post-translational modifications of soluble Tau.

As in the total protein fraction, the pThr231 antibody recognises full length Tau in early Braak stages phosphorylated at this epitope. However, the levels of pThr231 reactivity decreased, not increased with the advancement of Braak stages (Figure 3.19). In comparison the PHF1 epitope detected little or no Tau in earlier Braak stages and increased with the advancement of the Braak stages. In the soluble protein fraction, there was little overlap of Tau phosphorylation at these two epitopes (Figure 3.19).

Looking further at the specific alterations of soluble Tau, changes in isoform expression was also analysed. Western blot analysis revealed a clear reduction in both three- and four-repeat Tau isoforms in the entorhinal cortex with increasing Braak stage (Figure 3.20A,D). Interestingly, in all regions at later Braak stages, there was a reduction in four-repeat isoform containing one or two amino (N)-terminal inserts (1N and 2N, respectively) but a prominent increase in the 0N4R isoform (Figure 3.20).
Figure continues on next page
Figure 3.18 Soluble Tau levels are altered in Alzheimer’s disease

The entorhinal cortex (A), frontal cortex (B) and occipital cortex (C) from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5), subjected to centrifugation at 100,000 g and the supernatant was collected as the soluble protein fraction. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblotted for pan-Tau (K9JA and TAUS). (D-F) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Scatter dot plots were generated and protein levels were represented relative to Braak stages 0-II (All groups; n = 3, except frontal cortex Braak 0-II; n = 2) (mean). Protein levels were represented relative to respective Braak 0-II (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
The entorhinal cortex (A), frontal cortex (B) and occipital cortex (C) from nine human patients, grouped by Braak stages was homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5), subjected to centrifugation at 100,000 g and the supernatant was collected as the soluble protein fraction. Samples were subjected to SDS-PAGE (25 µg total protein) and immunoblated for phosphorylated Tau (pTau) at pThr231 and the PHF1 epitope (pSer396/404).
Figure continues on next page
Figure 3.20  Altered soluble Tau isoform levels following the Braak stages of Alzheimer’s disease

The entorhinal cortex (A), frontal cortex (B) and occipital cortex (C) from nine human patients were grouped by Braak stages, homogenised (50 mg/ml; 0.1 MES, 1 M NaCl, pH6.5), subjected to centrifugation at 100,000 g and the supernatant was collected as the soluble protein fraction. Samples were subjected to SDS-PAGE (25 µg total protein) and immunobotted for three-repeat or four-repeat Tau isoforms (3R-Tau and 4R-Tau, respectively). (D-F) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Scatter dot plots were generated and protein levels were represented relative to Braak 0-II (All groups; n = 3, except frontal cortex Braak 0-II, n = 2, except frontal cortex Braak 0-II; n = 2) (mean). Protein levels were represented relative to respective Braak stages 0-II (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all ns p > 0.05 not significant), (GraphPad, PRISM).
3.3 Discussion

Both PrP<sup>C</sup> and Tau are strongly implicated in the pathophysiology of Alzheimer’s disease. PrP<sup>C</sup> is a high affinity neuronal receptor for oligomeric assemblies of Aβ triggering synaptic impairment (Lauren et al., 2009) and cognitive deficits in vivo (Gimbel et al., 2010). Furthermore, Aβ binding to PrP<sup>C</sup> prevents the inhibitory effects of PrP<sup>C</sup> on β-secretase cleavage of APP, increasing Aβ production and generating a positive feedback loop (Parkin et al., 2007, Rushworth et al., 2013). In addition, increasing evidence suggests that Tau is crucial for Aβ mediated toxicity (Roberson et al., 2007, Oddo et al., 2006b). Tau is known to be abnormally modified in Alzheimer’s disease, with extensive reports of aberrant Tau phosphorylation and aggregation. Hyperphosphorylated Tau not only disrupts its normal function in regulating neuronal stability and transport but results in the accumulation of neurofibrillary tangles and the generation of soluble toxic species which are thought to play a role in disease propagation (Kopeikina et al., 2012).

Several lines of evidence suggest that PrP<sup>C</sup> expression is altered with age and in sporadic Alzheimer’s disease, and interestingly, PrP<sup>C</sup> expression may alter Tau expression (Whitehouse et al., 2010, Vergara et al., 2015). This chapter further explored the connection between PrP<sup>C</sup> and Tau expression and how this relationship is altered in sporadic Alzheimer’s disease. The potential mechanisms mediating the connection between PrP<sup>C</sup> and Tau expression and the implications of this relationship for the pathogenesis of Alzheimer’s disease are discussed below.

3.3.1 What is the relationship between PrP<sup>C</sup> and Tau in cell lines?

Firstly, Tau expression was measured in cell lines with altered PrP<sup>C</sup> expression. In neuroblastoma cell lines an inverse relationship between PrP<sup>C</sup> and Tau was observed. Overexpression of PrP<sup>C</sup> significantly reduced Tau levels in SH-SYSY cells, as shown by multiple methods of detection (Figure 3.1). These data support previous studies with PrP<sup>C</sup> overexpression in neuroblastoma (Chen et al., 2013) and HEK293 cells (Schmitz et al., 2014).

What mechanisms mediate the connection between PrP<sup>C</sup> and Tau expression?
Tau is predominantly localised in the cytoplasm and PrP^C is GPI-anchored to outer leaflet of the plasma membrane. Firstly, possible indirect mechanisms between PrP^C and Tau will be explored. Signal transduction of PrP^C is mediated its GPI-anchor and preliminary data presented in this chapter identified the GPI-anchor of PrP^C as key to altering Tau expression. Neuroblastoma cells overexpressing PrP^C with the GPI-signal peptide replaced for a transmembrane region (PrP-CTM) no longer have altered Tau expression (Figure 3.4). The GPI-anchor is also crucial for the lipid raft localisation of PrP^C and PrP-CTM no longer resided within lipid raft microdomains (Figure 3.4). Whether GPI-anchor mediated signalling and/or the lipid raft localisation of PrP^C alters Tau expression remains to be determined.

In addition to the GPI anchor interaction of the N-terminal region of PrP with the heparin sulphate proteoglycan (HSPG), Glypican-1 also mediates the lipid raft localisation of PrP^C (Taylor et al., 2009). Within lipid raft microdomains PrP^C acts as a key scaffolding protein interacting with a range of proteins and cellular components. The lipid raft localisation of PrP^C and interactions with multiple receptors, such as LRP1 and mGluR5 is essential for Aβ mediated Tau phosphorylation (Rushworth et al., 2013, Um et al., 2013). However, it remains to be determined the role of Glypican-1, transmembrane spanning receptors such as mGluR5 and other lipid raft microdomain components in PrP^C mediated alterations to Tau expression.

**Could PrP^C directly interact with Tau?**

The N-terminal region of PrP^C is highly flexible and disordered (Aguzzi and Heikenwalder, 2006). This region is thought to act as a molecular sensor, specifically to toxic signals with rich beta-sheet content, such as Aβ oligomers and PrP^SC (Beland and Roucou, 2012, Resenberger et al., 2011). Interestingly, the N-terminal domain of PrP^C is capable of penetrating the plasma membrane and directly interacting with cytoplasmic compartments (Iraci et al., 2015). PrP^C has previously been linked to altering microtubule dynamics, with the N-terminal region of PrP^C capable of interacting with Tubulin (Dong et al., 2008) and Tau *in vitro* (Han et al., 2006, Canu et al., 2011, Wang et al., 2008). Thus raising the possibility that the N-terminal region of PrP^C could directly interact with Tau to influence its metabolism and turnover to ultimately reduce its expression levels. To explore this idea further, Tau expression could be analysed in SH-SY5Y cells overexpressing PrP^C lacking the N-terminal domain. However, it should be taken into consideration that the N-terminal
region is also implicated in the lipid raft localisation of PrP\textsuperscript{C} as mentioned previously (Parkin et al., 2007, Taylor et al., 2009).

The N-terminal region of PrP\textsuperscript{C} could also directly interact with Tau following internalisation. PrP\textsuperscript{C} is predominantly transported to endosomes before lysosomal degradation, however, a small proportion of PrP\textsuperscript{C} is degraded in the cytosol by the proteasome (Canu et al., 2011). Therefore, it is possible that PrP\textsuperscript{C} could exert deleterious effects in the cytosol and interact directly with Tau, either by the N-terminal region penetrating the plasma membrane or following internalisation.

*Can Tau overexpression alter PrP\textsuperscript{C}?*

Overexpression of the mature Tau isoform, Tau 441 (2N4R) in primary cortical neurons has previously been shown to alter PrP\textsuperscript{C} trafficking and reduce its surface expression (Canu et al., 2011), again describing an inverse relationship between the two proteins. Increased intracellular accumulation of PrP\textsuperscript{C} and impairment of the proteasome pathway was also identified in these cells (Canu et al., 2011). Tau overexpression may impair axonal transport resulting in the intracellular accumulation of PrP\textsuperscript{C} but also directly interacting with PrP\textsuperscript{C} preventing its recycling to the plasma membrane.

Tau is predominantly localised in the axons, however, Tau has also been detected in the nucleus (Thurston et al., 1996) and dendrites (Frandemiche et al., 2014). In Alzheimer’s disease, the levels of dendritic Tau increase with Tau capable of interacting with a range of synaptic proteins including PSD95 and Fyn kinase to trigger synaptic dysfunction (Mondragon-Rodriguez et al., 2012, Haass and Mandelkow, 2010). In addition, overexpression of Tau may increase Tau localisation to the somatodendritic compartment (Liao et al., 2014), possibly aiding in the impairments to PrP\textsuperscript{C} as described above by increased co-localisation of the two proteins.

**3.3.2 What is the relationship between PrP\textsuperscript{C} and Tau in transgenic mouse lines?**

Next, Tau expression was analysed in transgenic mice with altered PrP\textsuperscript{C} expression. In contrast to the cell line data, a positive relationship between PrP\textsuperscript{C} and Tau was identified in transgenic mice (Figure 3.5). At 40 weeks of age, Prnp knockout mice showed a significant reduction in Tau. A downward shift in the molecular weight of the full length Tau isoforms
was also observed, possibly due to a shift in isoform expression or more likely a reduction in Tau post-translational modification in the absence of PrP<sup>c</sup> (Figure 3.5).

Tau isoforms containing three-repeat domains are expressed in foetal mice, predominantly 0N3R. In contrast, adult mice express four-repeat containing isoforms and predominantly 0N4R (Hanger et al., 2002, Liu and Gotz, 2013). Following Western blot analysis a predominant band migrating around 55 kDa was observed, likely representing 0N4R (Figure 3.5). Tau is a highly regulated protein and around 100 post-translational modifications have been identified on endogenous wild-type Tau from humans and rodents, including acetylation, ubiquitination, methylation, glycation, glycosylation and phosphorylation (Morris et al., 2015). It is possible that PrP<sup>c</sup> may influence some of these endogenous modifications, in addition to mediating abnormal Tau modifications, such as phosphorylation (Larson et al., 2012).

Is the relationship between PrP<sup>c</sup> and Tau altered in an Alzheimer’s disease mouse model?

J20 mice overexpress human APP (hAPP) carrying the Swedish (K670N/M671L) and Indiana (V171F) familial mutations linked to Alzheimer’s disease. An age-dependent increase in Aβ levels was measured in these mice and amyloid plaques are observed in these mice around 5-7 months of age (Mucke et al., 2000). Data in this chapter also describes an age-dependent increase in Tau in this mouse model. This increase was not observed when the J20 mice were crossed with Prnp null mice, with levels of Tau comparable between J20/Prnp<sup>−/−</sup> mice at 10 and 40 weeks of age (Figure 3.6).

It remains unknown whether this age-dependent increase in Tau is a result of the age-dependent increase in Aβ production or as a result of hAPP overexpression. As PrP<sup>c</sup> is known to be a high affinity receptor for Aβ (Lauren et al., 2009), it is possible that Aβ triggers signal transduction pathways to alter Tau expression. On the other hand, it is possible that Tau expression is influenced by PrP<sup>c</sup> expression regardless of Aβ levels. To control for the effects of the increase in human APP in these mice, Tau expression could be analysed in the I5 mouse strain, which overexpress wild-type hAPP but do not develop increased levels of Aβ (Mucke et al., 2000).

A highly aggregated species of Tau was also identified in the J20 mice, that was absent in the J20/Prnp<sup>−/−</sup> mice, supporting evidence that Aβ interacts with PrP<sup>c</sup> to induce aberrant modifications to Tau (Figure 3.7). It should be noted that other groups report little evidence of abnormal Tau phosphorylation or aggregation in J20 mice, however,
reduction in Tau in these mice does prevent Aβ induced deficits (Roberson et al., 2007). In comparison, increased Tau phosphorylation has been reported in another hAPP mouse models (Sturchler-Pierrat et al., 1997). Note also that Morris, et al. 2015, found nine Tau modifications, including phosphorylation in J20 mice that were absent in wild-type mice. To identify these modifications the authors used mass spectrometry, a far more sensitive method to detect Tau phosphorylation than Western blot analysis or immunohistochemistry, two widely used techniques which are reliant on antibody specificity (Petry et al., 2014). In summary, an age- and PrP^C- dependent increase in Tau levels and phosphorylation were observed in J20 mice.

To support this work, data from iPSC-derived neurons from fAD patients also show altered Tau levels. Neurons carrying the London familial APP mutation (V717I), show altered β- and γ-secretase activity and increased Aβ production (Moore et al., 2015). In addition, these neurons showed increased Tau phosphorylation but also Tau levels, which is dependent on Aβ (Muratore et al., 2014). This work with iPSC-neurons was expanded by Moore, et al. 2015, who proposed a role for Aβ, but also the C-terminal APP fragment, C99 in altering Tau metabolism (Moore et al., 2015).

### 3.3.3 What is the relationship between PrP^C and Tau following the progression of sporadic Alzheimer’s disease?

Several lines of evidence suggest PrP^C expression is altered with age and in sporadic Alzheimer’s disease, although reports have been conflicting (Whitehouse et al., 2010, Larson et al., 2012, Saijo et al., 2011). To generate a more complete picture, this chapter analysed PrP^C expression in multiple brain regions (entorhinal, frontal and occipital cortex) following the Braak stages of Alzheimer’s disease. The six Braak stages of disease progression are grouped into three subgroups which are distinguished clinically and by the location of tangle-bearing neurons (AT8 immunocytochemistry). Braak stages I-II or the transentorhinal stage are clinically silent, the limbic stages of Braak III-IV are defined by incipient Alzheimer’s disease and finally Braak stages V-VI define severe Alzheimer’s disease where pathology has spread to neocortical structures (Braak and Braak, 1995).

A reduction in PrP^C expression in all brain regions was observed in severe Alzheimer’s disease (Braak V-VI) (Figure 3.8-3.10), supporting previous findings (Whitehouse et al., 2013, Whitehouse et al., 2010). Due to the relatively small sample size in this pilot study,
wide variations in the levels of PrP\textsuperscript{C} with incipient Alzheimer’s disease (Braak III-IV) were observed, however, a trend towards a reduction was observed in the entorhinal cortex. Previous findings using larger sample sizes report a slight increase (sample size; 11) (Vergara et al., 2015) and decreases (sample size; 24) (Larson et al., 2012) in PrP\textsuperscript{C} in incipient Alzheimer’s disease. A larger sample size is needed to determine a more detailed picture of the alterations to PrP\textsuperscript{C} in these three brain regions in the incipient stages of disease progression.

**Does the reduction in PrP\textsuperscript{C} relate to Tau?**

Data presented in this chapter also show a reduction in full-length Tau with increasing Braak stages (Figure 3.8-3.10). Furthermore, the levels of PrP\textsuperscript{C} in the frontal cortex, strongly correlated to reductions in Tau and increasing Braak stages (Figure 5.12). To extend this further, MAPT mRNA levels could be investigated. If no change in mRNA is detected it is likely that a post-translational mechanism is mediating alterations to the Tau protein. To dismiss the theory that this is a result of global protein loss during neurodegeneration the membrane protein APP and neuron-specific \(\beta\)-III-tubulin were also analysed. The proteolytic processing of APP plays a predominant role in the pathogenesis of Alzheimer’s disease, however, APP expression levels were not significantly altered in Alzheimer’s disease and did not correlate with PrP\textsuperscript{C}, Tau or the Braak stages of the disease (Figure 3.10). Although no change in total APP was measured, differential levels of APP isoforms and cleavage products have previously been identified in Alzheimer’s disease (Preece et al., 2004, Matsui et al., 2007).

**Tau modifications**

Following Western blot analysis, one of the most striking features was the smearing pattern of Tau along the membrane. This was observed at late Braak stages in the frontal and occipital cortices, however, was more prominent and observed at earlier Braak stages in the entorhinal cortex (Figure 3.8A). It was hypothesised that this represents increasing amounts of aggregated and truncated forms of the protein, at higher and lower molecular weights, respectively. This smearing pattern of Tau has previously been reported in Alzheimer’s disease (Tai et al., 2012, Derisbourg et al., 2015).

Tau phosphorylation is a key feature of tauopathies and Alzheimer’s disease. Increased Tau phosphorylation correlated with the reduction in full-length Tau and was first detected in the entorhinal cortex (Figure 3.15). The decrease in full-length Tau also correlated with
increased insoluble, hyperphosphorylated Tau which was insoluble in the detergent Sarkosyl (Figure 3.16). Specific truncated and aggregates species of Tau were detected with both anti-Tau and phosphorylation-specific antibodies. For example, a band migrating around 40 kDa was observed in the entorhinal cortex in severe Alzheimer’s disease with both the anti-Tau (K9JA) and phosphorylation epitope, PHF1 (pSer396/404). Previously, it was identified that aggregated and truncated species of Tau were C-terminally truncated and highly reactive to the Tau pSer396 (Derisbourg et al., 2015). In support of this, another group identified an increase in a C-terminally truncated form of Tau which migrated around 40 kDa (Sokolow et al., 2015).

Tau isoforms

Next it was investigated whether the reduction in total Tau was a loss of Tau isoforms containing both three and four microtubules binding repeats. Previously a lack of highly specific antibodies for three- and four-repeat Tau isoforms (3R- and 4R-) has limited the understanding of the distribution of Tau isoforms in pathology. In this chapter, specific antibodies targeted against either 3R- or 4R- Tau isoforms showed reductions in both isoforms in the frontal cortex, correlating with decreased total Tau detection (Figure 3.13). Interestingly, in the entorhinal cortex a reduction of 4R containing Tau isoforms was observed earlier than for 3R- Tau isoforms (Figure 3.13A). At earlier Braak stages in the entorhinal cortex, 4R- Tau isoforms may have increased propensity for truncation and/or aggregation, thus resulting in reduced full-length levels. Unlike other tauopathies such as PSP and CBD which are defined by increased 4R-isoforms, both 3R- and 4R- Tau isoforms are identified in Alzheimer’s disease pathology (Wang and Mandelkow, 2016). Espinoza et al. 2008, speculated that 4R-Tau may be the seed of pathology in Alzheimer’s disease, possibly explaining the loss of 4R- isoforms earlier in the entorhinal cortex. Interestingly, in the occipital cortex, levels of full-length 4R-Tau isoforms increased in late Braak stages (Figure 3.13C). This upregulation possibly reflects a compensatory mechanism.

Soluble Tau

Research suggests that Tau may be involved in neuronal dysfunction before the accumulation of insoluble Tau aggregates (Santacruz et al., 2005). Pre-fibrillar soluble forms of Tau are implicated as the pathogenic species of Tau, however, the exact pathogenic species remains to be identified (Kopeikina et al., 2012, Lasagna-Reeves et al., 2011a). Data presented in this chapter identified a trend decrease in soluble Tau with
increasing Braak stages (Figure 3.18). Interestingly, the changes in soluble full-length Tau and Tau isoform expression mirrors that seen in the total protein fraction.

On the other hand, patterns of Tau phosphorylation differed between soluble and total protein fractions. In the soluble fraction, a decrease in pThr231 but increase in PHF1 immunoreactivity was observed with the advancement of Braak stages. In the entorhinal cortex and frontal cortex no overlap of pThr231 and PHF1 reactivity is observed (Figure 3.16), possibly implicating specific roles for phospho-epitopes in the pathogenesis of the disease. In addition, no specific soluble oligomeric or truncated Tau species were detected, however, levels may be too low for accurate detection by Western blot analysis. Furthermore, mislocalised Tau species may play a prominent role in Tau toxicity rather than an overall increase in specific species of ‘toxic’ Tau (Hoover et al., 2010). It is interesting to note that post mortem delay can result in the dephosphorylation of soluble Tau by phosphatases but not PHF Tau and as a result true phosphorylation levels of soluble Tau may be underestimated (Matsuo et al., 1994). The post-mortem delay of samples used in this chapter ranged from 36-176 h (Table 2.2). Indeed in the late Braak stages (V-VI) longer post-mortem delay correlated with reduced PHF1 reactivity of Tau isolated in the soluble protein fraction (graph not shown).

3.3.4 Chapter Summary

Data presented in this chapter from neuroblastoma cell lines, mouse brain homogenates and human tissue show that there is a specific relationship between the expression of PrPC and Tau. Preliminary data suggests the GPI-anchor of PrPC, and possibly in part the lipid raft localisation of PrPC is crucial for mediating this relationship, although the exact molecular mechanisms remain unknown. Following analysis of human brain tissue, the advancement of Alzheimer’s disease correlated with a decrease in the level of full-length Tau (both bound to microtubules and soluble) and increased levels of insoluble, phosphorylated, aggregated and truncated Tau. With the decrease in Tau levels correlating strongly with reductions in PrPC.

The extent of the relationship between PrPC and Tau differs between neuroblastoma cell lines and mouse models. Despite the discrepancy between the two models following manipulation of protein levels, both revealed a specific relationship between PrPC and Tau.
Furthermore, data from mouse brain homogenates supported data gathered from human brain tissue and revealed a positive relationship between the two proteins in more complex models.

The implications of the reduced PrP$^C$ and Tau levels following the advancement of Alzheimer’s disease remain unknown. Many neuroprotective roles for PrP$^C$ have been described including synaptic function, and protection against oxidative stress and inflammation, all of which are altered in Alzheimer’s disease. Thus, implicating a toxic-loss of function in Alzheimer’s disease. Data presented in this chapter displays that the loss of PrP$^C$ correlates with the loss of Tau which possibly contributes to increased neuronal instability. Aβ is also known to interact with PrP$^C$ and is known to induce the aberrant Tau phosphorylation. On the other hand, a reduction in Tau is known to be protective against Aβ-induced deficits (Oddo et al., 2006b, Roberson et al., 2007), raising the possibility that PrP$^C$ may play differential roles in disease pathogenesis.

A greater understanding of the relationship between PrP$^C$ and Tau and the molecular mechanisms involved, will aid in our understanding of Tau-mediated toxicity and the interplay between different neurodegenerative diseases such as Alzheimer’s disease, tauopathies and prion diseases.
Chapter 4. Aβ oligomer induced Tau phosphorylation

4.1 Introduction

4.1.1 Tau phosphorylation and Alzheimer’s disease

Tau is a natively soluble and unfolded protein. Primarily localised in the axon, Tau promotes microtubule polymerisation and the stabilisation of the neuronal cytoskeleton (Avila et al., 2004). Tau is tightly regulated by kinase and phosphatase activities which alter its phosphorylation state. Tau phosphorylation is essential for neuronal outgrowth, development and cellular activity (Mietelska-Porowska et al., 2014) and around 40 phosphorylation epitopes on serine, threonine and tyrosine residues are found normally in the brain (Figure 4.1).

In Alzheimer’s disease an imbalance between kinase and phosphatase activities results in Tau becoming increasingly hyperphosphorylated (Figure 4.1). Hyperphosphorylated Tau has reduced affinity for microtubules which destabilises the neuronal cytoskeleton and has increased propensity to aggregate and form insoluble neurofibrillary tangles (Martin et al., 2013b). Furthermore, Aβ aggregates are implicated in mediating Tau pathology and are able to activate multiple Tau kinases (Martin et al., 2013b, Stancu et al., 2014, Johnson and Stoothoff, 2004). Specifically, smaller, soluble oligomeric forms of Aβ are the primary pathogenic species (Walsh and Selkoe, 2007) and strongly correlate with the presence and severity of cognitive decline (McLean et al., 1999, Lue et al., 1999). Furthermore, soluble oligomeric forms of Aβ are linked to inducing aberrant Tau phosphorylation (Chabrier et al., 2012, Larson et al., 2012, De Felice et al., 2008, Zempel et al., 2010).

There is now strong evidence that Tau is the mediator of Aβ toxicity, synaptic dysfunction and disease propagation in Alzheimer’s disease (Roberson et al., 2007, Ittner and Gotz, 2011, Pooler et al., 2013b, Bloom, 2014, Stancu et al., 2014). Research suggests that Tau may even be involved in neuronal dysfunction before the accumulation of Tau pathology (Santacruz et al., 2005) and pre-fibrillar, soluble, hyperphosphorylated forms of Tau have been implicated as the pathogenic species (Kopeikina et al., 2012). However, little is known about the specific cell-surface signalling complex(es) linking extracellular Aβ oligomers to the phosphorylation of intracellular Tau in Alzheimer’s disease.
Figure 4.1 Sites of phosphorylation on Tau

Normally Tau is phosphorylated at multiple serine (S), threonine (T) and tyrosine (Y) residues in the adult brain (green). In Alzheimer’s disease Tau is aberrantly phosphorylated at multiple residues (black). The residues associated with both the healthy adult brain and Alzheimer’s disease are highlighted in blue. Phosphorylation sites are mapped onto the full length Tau structure (Tau441; 2N4R).

4.1.2 What is the role of PrP<sup>C</sup> in Aβ mediated Tau phosphorylation?

PrP<sup>C</sup> plays a crucial role in the binding and subsequent synaptic impairment induced by Aβ oligomers (Lauren et al., 2009). Interaction of Aβ oligomers with PrP<sup>C</sup> induces activation of the Src family kinase, Fyn (Um et al., 2012). Fyn subsequently phosphorylates the NR2B subunit of NMDA-receptors and ultimately triggers their internalisation and loss at the cell surface triggering dendritic spine loss (Rushworth et al., 2013, Um et al., 2012). In addition, Fyn activation induces the phosphorylation of Tau at tyrosine 18, a residue which is not normally phosphorylated in adults (Lee et al., 2004, Um et al., 2012, Larson et al., 2012). As described previously, the binding of Aβ oligomers to PrP<sup>C</sup> and subsequent toxicity is dependent on the transmembrane receptors LRP1 and mGluR5, and the integrity of the lipid raft microdomain, see Figure 4.2.

The role of this Aβ/PrP<sup>C</sup> signalling complex in the phosphorylation of Tau at other epitopes remain to be determined. In addition, EphB2 and α7nACh receptors are also linked to mediating Aβ oligomer induced Tau phosphorylation, however, full details of these signalling complexes remain to be fully determined (Wang et al., 2003, Jiang et al., 2015). Specific signalling complexes such as these may mediate the phosphorylation of Tau at certain epitopes resulting in specific pathogenic signalling cascades. On the other hand, the
overall phosphorylated state of Tau mediated by multiple converging signalling complexes may be toxic and detrimental to cells.

Figure 4.2 Aβ oligomer induced Tau phosphorylation at Tyr18 is dependent on PrP<sup>C</sup> and a cell surface, raft-based signalling complex

4.1.3 Aims

The aim of this chapter was to further investigate Aβ oligomer induced Tau phosphorylation and the role of PrP<sup>C</sup> in this. Unravelling the molecular links between Aβ and Tau will aid in the understanding of Alzheimer’s disease pathogenesis and reveal novel therapeutic targets for the prevention of Aβ/Tau mediated toxicity. Initially Aβ oligomer induced Tau phosphorylation at multiple epitopes and the time course of events was analysed in neuroblastoma cell lines overexpressing the full-length Tau construct, Tau441. Next Aβ oligomer induced Tau phosphorylation was investigated in primary neuronal cultures and iPSC-derived cortical neurons, comparing results in more physiologically relevant neuronal models. Furthermore, to investigate the role of PrP<sup>C</sup> in mediating Aβ induced Tau phosphorylation in iPSC-derived neurons, the anti-PrP<sup>C</sup> antibody, 6D11 which interacts with the Aβ oligomer binding site on PrP<sup>C</sup> was used and siRNA mediated knock down of PrP<sup>C</sup> was performed.
4.2 Results

4.2.1 Soluble, fibrillar Aβ oligomers of high molecular weight bind to PrPC with high affinity

Aβ oligomers were formed from a synthetic Aβ1-42 peptide as described previously (Rushworth et al., 2013). This protocol was based on the original protocol for soluble, toxic Aβ oligomer generation by Lambert, et al. (1998) which was later adapted by the same group (Chromy et al., 2003). This oligomer preparation has previously been shown to bind to PrPC with high affinity, inhibiting hippocampal long-term potentiation and inducing dendritic spine loss (Lauren et al., 2009, Rushworth et al., 2013).

As briefly described in Figure 4.3A, the synthetic Aβ peptide was first dissolved in HFIP, a β-sheet disrupting solvent used to disaggregate the peptide before conversion into oligomeric species. The HFIP was subsequently evaporated leaving a peptide film seen as a faint transparent smear. All oligomeric preparations were made fresh before use and peptide films were stored at -80°C until required. To generate the oligomeric preparation the peptide film was solubilised in DMSO to a final peptide concentration of 1 mM before dilution in Ham’s F12 medium (a high salt solution to allow aggregation of the peptide) to a final peptide concentration of 100 μM and left to aggregate for 16 h at room temperature. Any fibrillary material was subsequently pelleted out by centrifugation leaving the remaining oligomeric preparation.

Characterisation of Aβ oligomers by Tris-tricine SDS-PAGE and Western blot analysis revealed a broad smear (55-170 kDa) of high-molecular weight oligomers. Smaller oligomeric species were also observed in both the oligomer and monomeric peptide preparations (Figure 4.3B), these were likely to be artefacts of SDS-PAGE, with SDS able to induce the oligomerisation of the Aβ peptide (Watt et al., 2013). The conformation of the Aβ oligomer species was also characterised using antibodies recognising structural epitopes. Two widely used conformation dependent antibodies are the A11 and OC antibodies recognising a prefibrillar and fibrillar epitope respectively (Kayed et al., 2007, Glabe, 2008). Soluble, fibrillar Aβ oligomers as detected by the OC antibody are significantly elevated in multiple brain regions in Alzheimer’s disease but not prefibrillar oligomers detected by the A11 antibody (Tomic et al., 2009). Our oligomer preparation has previously been shown to be OC and not A11 reactive (Rushworth et al., 2013).
confirm the fibrillar epitope of the oligomers, OC reactivity of the preparation was analysed by dot blot. The OC antibody specifically recognised the oligomer preparation but not the monomeric peptide (Figure 4.3C). Routine characterisation of Aβ oligomer preparations by Western blot and dot blot analysis were used throughout this study to confirm the presence of soluble, high molecular weight Aβ oligomers with a fibrillar conformational epitope.

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Figure 4.3 Soluble, fibrillar, high molecular weight Aβ oligomer generation and characterisation

(A) Protocol for the generation of synthetic biotin-Aβ₁₄₂ monomer (AβM) and oligomer (AβO) preparations. (B) Freshly prepared AβM and AβO were separated by denaturing Tris/tricine SDS-PAGE (0.3 nmol or 0.5 nmol, total peptide) and probed with the anti-Aβ antibody, 6E10. (C) Freshly prepared AβM or AβO Aβ (20 pmol total peptide) was spotted onto nitrocellulose membranes, keeping the native structure and immunoblotted with anti-Aβ (6E10) and fibrillar epitope (OC) antibodies.

4.2.2 Aβ oligomers failed to induce Tau phosphorylation at Tyr18 in SH-SYSY-PrP<sup>C</sup> cells

Previously, Larson, et al. 2012, showed that affinity-purified Aβ oligomer species derived from Alzheimer’s disease brain extracts induced Fyn activation and Tau phosphorylation at tyrosine 18 (Tyr18) in a PrP<sup>C</sup>-dependent manner in primary neurons (Larson et al., 2012). Our synthetic Aβ oligomer preparation binds to PrP<sup>C</sup> with high affinity activating Fyn kinase in neuroblastoma cell lines and primary neurons (Rushworth et al., 2013). Therefore, we next investigated whether our Aβ oligomer preparation induced the phosphorylation of Tau at tyrosine 18 in a PrP<sup>C</sup> dependent manner in a neuroblastoma cell line. We also investigated whether the Aβ/PrP<sup>C</sup> complex induced the phosphorylation of Tau at other Tau epitopes aberrantly phosphorylated in Alzheimer’s disease.

Initially to detect Tau phosphorylation at Tyr18 the commercial, monoclonal 9G3 antibody was used. However, our Aβ oligomer preparation failed to induce the phosphorylation of Tau at Tyr18 in SH-SYSY cells overexpressing PrP<sup>C</sup> (Figure 4.4A). To confirm the specificity of this antibody siRNA targeted against MAPT was used to reduce Tau protein expression. Following siRNA treatment a 67.2% reduction in Tau was observed, however, recognition by the 9G3 antibody was not altered (Figure 4.4B,C). It is likely that 9G3 was cross-reacting with non-Tau species as previously reported (Lee et al., 2004) and thus contributing to the negative result. Recognition of a second antibody targeted against Tau phosphorylation at the tyrosine epitope (anti-pTyr18) was decreased by 69.9 % following MAPT siRNA in SH-SYSY cells (Figure 4.4D). Therefore, for following experiments the anti-pTyr18 antibody was used and not the commercial 9G3 antibody.
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Figure 4.4 Aβ oligomers failed to induce phosphorylation of endogenous Tau at Tyr18 in SH-SY5Y cells expressing PrP<sup>C</sup>

(A) SH-SY5Y cells overexpressing PrP<sup>C</sup> were incubated with 500 nM (total peptide) Aβ oligomers for the times indicated. Cells were lysed, subjected to SDS-PAGE and immunoblotted (25 µg total protein) for Tau (K9JA), Tau phosphorylated at Tyrosine 18 (9G3) and β-actin (AC15) (n = 1 experiment of 2 independent replicates). No quantification performed due to non-specificity of 9G3 antibody. (B) SH-SY5Y cells were incubated with 25 nM siRNA targeted against either Tau (MAPT) or non-targeting control (NTC) for 72 h. Cells were lysed, subjected to SDS-PAGE (25 µg total protein) and immunoblotted for Tau (K9JA), Tau phosphorylated at Tyrosine 18 with the commercial antibody (9G3) and the polyclonal pTyr18 antibody, and β-actin (AC15). (C-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of Tau and pTyr18 were represented as a percentage of NTC (n = 1 experiment of 2 independent replicates, mean ± SD).

4.2.3 Tau construct overexpression in SH-SY5Y cell lines

SH-SY5Y cell lines predominantly express the foetal isoform of Tau, 0N3R. The foetal isoform of Tau is endogenously highly phosphorylated during development but does not form PHFs (Jovanov-Milosevic et al., 2012). The high endogenous phosphorylation state of Tau may dampen any further increases in phosphorylation induced by Aβ. To overcome this issue a cDNA construct encoding a mature Tau isoform (Tau381; 1N3R) was sourced to overexpress in neuroblastoma cell lines (Figure 4.5). cDNA encoding Tau381 from the bacterial vector, pRK172 was successfully amplified with BamH1 and AflII restriction sites (Figure 4.5B), ready for ligation into the mammalian pIREShyg2 expression vector which was also cut at these sites (Figure 4.5C). For the PCR of the Tau construct with the restriction sites three step cycling was required. Two step cycling only produced a faint band at around 1.1 kb (data not shown). Following restriction digest of the vector and insert, various ligation conditions were tested (16°C overnight; 2 h at room temperature), however the Tau construct was not successfully ligated into the vector. Electrophoresis on a 1 % (w/v) agarose gel was used to confirm a successful ligation. If successful a band migrating around 6.9 kDa would be observed (Figure 4.5D). To confirm failure of ligation was not specific to the pIREShyg2 vector, the Tau construct was also ligated into the
mammalian pcDNA3.1(-) vector, however, again the Tau construct was unsuccessfully ligated into the vector (data not shown).

Next, the mature Tau isoform, Tau441 (2N4R) which was already expressed in the mammalian vector pcDNA3.1(-) was sourced (Figure 4.6A,B) and successfully transiently transfected into SH-SY5Y cells. Following SDS-PAGE, a band migrating just below the 70 kDa marker was observed (Figure 4.6C). For further studies to investigate Tau phosphorylation in neuroblastoma cell lines, overexpression of this mature Tau construct was used.

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Figure 4.5  Tau381 (1N3R) cDNA preparation and insertion into mammalian expression vector (pIREShyg2) using BamHI and AflII restriction sites

(A) Schematic comparing the structure of the full length mature Tau isoform Tau441 (2N4R) with Tau381 (1N3R) and the foetal isoform Tau352 (0N3R) which is predominantly expressed by SH-SY5Y cells. (B) PCR was used to subclone the cDNA of the Tau381 isoform from the bacterial vector pRK172. The Tau cDNA was amplified with BamHI (forward primer) and AflII (reverse primer) restriction sites using three-step PCR cycling. PCR was verified by 1% (w/v) agarose gel electrophoresis. (C) Following extraction, cDNA was purified and restriction digest of both the Tau construct and empty vector (pIREShyg2) was performed to verify PCR and to cut the vector so that the Tau sequence can be ligated into the empty cut vector (pIREShyg2; ~5.8 Kb, Tau381; ~1.1 Kb). (D) Products of the restriction digest were excised, purified and concentration confirmed. The vector and Tau381 were ligated at different vector to insert ratios for 16 h, 16 °C. Ligation was analysed by agarose gel electrophoresis. If successful a band at ~6.9 Kb should be visible.
Figure 4.6 Expression of Tau441/pDNA3.1(-) construct into mammalian cell lines

(A) Vector map of pDNA3.1(-) highlighting the restriction sites (BamHI and KpnI) used to express the full length mature Tau construct, Tau441 (2N4R). This construct was a kind gift from Diane Hanger (Kings College London, UK). (B) To confirm the expression of the Tau441 cDNA, restriction digest (1 µg cDNA) was performed using BamHI-HF and KpnI-HF restriction enzymes for 1 h at 37°C. The reaction was stopped following the addition of SDS-loading dye and subjected to agarose gel electrophoresis (1% (w/v) agarose). (C) To check the successful expression of the construct in mammalian cell lines, SH-SY5Y cells were transiently transfected with either the Tau441/pDNA3.1(-) construct or ddH₂O control for 24 h. Lysates were prepared, subjected to SDS-PAGE and immunoblotted for Tau (K9JA).
4.2.4 Aβ oligomers failed to induce the phosphorylation of overexpressed Tau in SH-SY5Y cell lines

To investigate the role of PrP$^C$ in Aβ oligomer induced Tau phosphorylation, SH-SY5Y cells, which endogenously express low levels of PrP$^C$ and SH-SY5Y cells overexpressing PrP$^C$ (SH-SY5Y-PrP$^C$) were transiently transfected with the Tau441 construct (Figure 4.7A). Unfortunately, our synthetic Aβ oligomer preparation failed to induce the phosphorylation of Tau441 at Tyr18 and two other epitopes (the PHF1 epitope and Thr231) in SH-SY5Y-PrP$^C$ (Figure 4.7) and SH-SY5Y cells (Figure 4.8), at least under the experimental conditions used (500 nM total peptide, 0-60 min). Different time points were used to investigate the time course of Tau phosphorylation at different epitopes. Previously phosphorylation at Tyr18 was induced following 60 min Aβ treatment (Larson et al., 2012), however, this may differ for other epitopes. Interestingly, a 27.4 % (ns) increase in PHF1 reactivity was observed following 20 min Aβ oligomer incubation in SH-SY5Y cells (Figure 4.8C). It should be noted that despite rigorous standardisation, phosphorylation was highly variable between experiments.

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Figure 4.7 Aβ oligomer induced Tau phosphorylation in SH-SY5Y cells overexpressing PrP<sup>C</sup> and Tau441

(A) SH-SY5Y and SH-SY5Y cells overexpressing PrP<sup>C</sup> were lysed and immunoblotted (25 µg total protein) for PrP<sup>C</sup> (6D11). (B) SH-SY5Y overexpressing PrP<sup>C</sup> cells were grown to 60% confluence before transient transfection with the Tau441 cDNA construct for 24 h. Cells were incubated in Opti-MEM in the presence of Aβ oligomers (500 nM total peptide) for the times indicated. Cell lysates were subjected to SDS-PAGE and immunoblotted for Tau (K9JA) and Tau phosphorylated at pTyr<sub>18</sub>, the PHF1 epitope and pThr<sub>231</sub>. (C) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and were represented as a percentage of Aβ control (n = 4 independent experiments each of two replicates, mean ± SEM). Statistical analysis was performed using One-way ANOVA (GraphPad, PRISM). All non-significant (ns p > 0.05).
A) Tau441: + + + + + + +
Time (mins): 0 0 20 20 40 40 60 60

70 kDa - Tau441
70 kDa - pTyr18
70 kDa - PHF1 (pSer396/404)
pThr231

B) pTyr18

C) PHF1

D) pThr231

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Figure 4.8  Aβ oligomer induced Tau phosphorylation in SH-SY5Y cells overexpressing Tau

(A) SH-SY5Y cells were grown to 60% confluence before transient transfection with the Tau441 cDNA construct for 24 h. Cells were incubated in Opti-MEM in the presence of Aβ oligomers (500 nM total peptide) for the times indicated. Cell lysates were subjected to SDS-PAGE and immunoblotted for Tau (K9JA) and Tau phosphorylated at pTyr18, the PHF1 epitope and pThr231. (C-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to total Tau levels and represented as a percentage of Aβ control (n = 4 independent experiments each of two replicates, mean ± SEM). Statistical analysis was performed using One-way ANOVA (GraphPad, PRISM). All non-significant (ns p > 0.05).

4.2.1  Aβ oligomers failed to induce Tau phosphorylation in NB7 cells overexpressing Tau

As discussed previously, the binding of Aβ oligomers to PrPC and the subsequent Fyn kinase activation requires the presence of the mGluR5 dimer (Um et al., 2013). Following Western blot analysis it was identified that SH-SY5Y cells do not express the dimeric, functional form of the glutamate receptor (Figure 4.9A). The absence of the functional mGluR5 dimer may explain why Aβ oligomers failed to induce Tau phosphorylation at Tyr18 in SH-SY5Y cell lines. Another neuroblastoma cell line, NB7, did express the functional mGluR5 dimer, seen as a broad smear around 260 kDa following SDS-PAGE (Figure 4.9A). NB7 cells also endogenously express PrPC, Fyn kinase and low levels of the 0N4R Tau isoform (Figure 4.9A). Following stable transfection with the Tau441 construct in NB7 cells, Tau expression levels were comparable to SH-SY5Y cells transiently expressing the same construct (Figure 4.9B). The level of Tau441 expression in NB7 cells was dependent on the continual treatment with the antibiotic, neomycin. Prolonged culture without this antibiotic resulted in a 50 % reduction in Tau441 expression levels (data not shown).

It has previously been reported that Aβ oligomers are able to induce Fyn activation in this cell line in a PrP dependent manner (Rushworth et al., 2013), however, failed to induce the phosphorylation of Tau441 at Tyr18 or two other epitopes (pThr231 and PHF1) under the experimental conditions used (500 nM total peptide; 0-60 min) (Figure 4.10).
Figure 4.9 Protein expression in NB7 and SH-SY5Y cell lines

(A) SH-SY5Y, SH-SY5Y cells overexpressing PrP^C and NB7 cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for Tau (K9JA, PrP^C (SAF32), Fyn and mGluR5. For mGluR5 non-reducing conditions were used. (B) NB7 cells stably transfected with pcDNA-Tau441 or empty vector and SH-SY5Y cells transiently transfected with pcDNA-Tau441 or ddH2O control were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted with an antibody targeted against pan-Tau (K9JA).
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Figure 4.10  Aβ oligomer induced Tau phosphorylation in NB7 cells overexpressing Tau

(A) NB7 cells expressing Tau441 were incubated in Opti-MEM in the presence of Aβ oligomers (500 nM total peptide) for the times indicated. Cell lysates were subjected to SDS-PAGE and immunoblotted for Tau (K9JA) and phospho-epitopes (pTyr18, PHF1, pThr231). (B-D) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and represented as a percentage of Aβ control (n = 4 independent experiments of two replicates, mean ± SEM). Statistical analysis was performed using One-way ANOVA (GraphPad, PRISM). All non-significant (ns p > 0.05).

4.2.2  Aβ oligomer induced Tau phosphorylation in rat hippocampal neurons

To induce complex downstream mechanisms such as Tau phosphorylation mediated by Aβ a more physiologically relevant model may be required. Indeed, the original study by Larson, et al. (2012) used mouse primary cortical cultures to show Aβ induced Tau phosphorylation at Tyr18. Therefore, we next sought to investigate Aβ oligomer induced Tau phosphorylation in primary neuronal cultures. A longer incubation of Aβ oligomers was used and Tau phosphorylation at Thr231 was investigated. In comparison to Tyr18 which is only known to be phosphorylated by the tyrosine kinase, Fyn, phosphorylation at Thr231 is targeted by multiple serine/threonine kinases (http://cnr.iop.kcl.ac.uk/hangerlab/tautable). In addition, phosphorylation at Thr231 appears to be an early event and precede the formation of PHFs (Vincent et al., 1998, Augustinack et al., 2002). Furthermore, phosphorylation at Thr231 reduces the affinity of Tau to bind to microtubules (Johnson and Stoothoff, 2004).

To generate a primary neuronal culture the hippocampus was isolated from postnatal (postnatal day (PD) 1) Wistar rats. The adult rat brain expresses all six Tau isoforms, however, neuronal cultures from rat pup express Tau isoforms containing three microtubule binding repeats until 10 days in vitro (DIV) where levels of four-repeat containing isoforms increases (Hanes et al., 2009). Therefore, we cultured the primary hippocampal neuronal cultures until 15 DIV where both three-repeat and four-repeat containing Tau isoforms would be expressed.
Soon after plating the neuronal cells small neurite outgrowths were visible and by 15 DIV, longer outgrowths and some network connections were visible, although cultures were relatively sparse (Figure 4.11A). Following Western blot analysis of the neuronal lysate, multiple bands ranging from 45-70 kDa were detected by the anti-Tau antibody, possibly representing the expression of multiple Tau isoforms. However, a predominant band migrating around 55 kDa was observed (Figure 4.11B). This band migrates at the same molecular weight as the endogenous foetal Tau isoform expressed in SH-SYSY cells (Figure 4.4). Therefore although by 15 DIV the neuronal cultures express multiple Tau isoforms, the foetal, 0N3R isoform is predominantly expressed.

Following incubation with Aβ oligomers (500 nM total peptide, 6 h), no obvious toxicity or cell death was observed by light microscope analysis. Furthermore, Western blot analysis revealed no increase in Tau phosphorylation following incubation with Aβ (Figure 4.11B-C). Analysis using the MSD immunoassay revealed a slight (ns) decrease in Tau phosphorylation levels in the neuronal lysate and a slight (ns) increase in Tau in the media following incubation with Aβ oligomers (Figure 4.11D-E). The levels of phosphorylated Tau in the media were too low to detect using the MSD immunoassay, therefore, we cannot conclude whether the increase in Tau in the media was a result of increased phosphorylation and release of Tau from microtubules and release from the neuronal cell.
Figure continues on next page.
Figure 4.11 Aβ-induced Tau phosphorylation in rat primary hippocampal neurons

(A) Rat primary neuronal cultures were cultured for 15 day in vitro (DIV). At this time point, longer neurite outgrowth and some network connections were visible. Image was acquired on a light microscope (EVOS Imaging System). (B) Day 15 rat primary hippocampal neurons were incubated in Opti-MEM in the presence of Aβ oligomers (500 nM total peptide) or DMSO control for 6 h. Cells were lysed, subjected to SDS-PAGE (15 μg total protein) and immunoblotted for pan-Tau (K9JA), phosphorylated Tau (pTau) (pThr231), β-III-tubulin and β-actin (AC15). (C) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and represented as a percentage of Aβ control (n = 3 replicates from single experiment, mean ± SEM). (D-E) Tau and pTau (pThr231) was also detected in the lysate (5 μg total protein) (D) and culture media (E) using the Mesoscale Discovery system (MSD) multiplex immunoassay. Statistical analysis was performed using unpaired two-tailed T-tests (GraphPad, PRISM). All non-significant (ns p > 0.05).
4.2.3 Aβ oligomer induced Tau phosphorylation in iPSC-derived cortical neurons

Human induced pluripotent stem cell (iPSC)-derived neurons hold great potential and promise for modelling genetic neurodegenerative diseases and testing potential therapeutics (Tiscornia et al., 2011, Sullivan and Young-Pearse, 2015). Neuronal cell lines derived from patients with sporadic or familial Alzheimer’s disease are able to recapture disease phenotype and display increased Aβ and Tau phosphorylation levels (Israel et al., 2012, Moore et al., 2015). Therefore, we hypothesised that iPSC-derived neurons may recapture the complexity of human neurons and provide a better model to investigate the complex downstream mechanisms induced by the exogenous application Aβ, such as Tau phosphorylation.

To investigate Aβ oligomer induced Tau phosphorylation, iPSC-derived neurons from control patients (non-disease affected individuals) were used as a model. The human iPSCs were differentiated into excitatory, glutamatergic cortical neurons, based on the original protocol (Shi et al., 2012). After 50 days from neural induction, long MAP2 positive dendrites were observed (Figure 4.12A) and endogenously expressed PrPC, Fyn kinase and the functional mGluR5 dimer (Figure 4.12B-C). In addition the neurons expressed high levels of Tau compared to the SH-SY5Y cell line (Figure 4.12D). Dephosphorylation of the neuronal lysate with Lambda protein phosphatase and analysis with anti-Tau antibodies revealed a single band migrating around 50 kDa and the same molecular weight as the foetal isoform, 0N3R (Figure 4.12E). The sole expression of the foetal Tau isoform implies that these neurons have a foetal neuronal phenotype.

Next, iPSC-derived neurons were incubated with oligomeric Aβ (1 μM total peptide for 6 h) and Tau phosphorylation at Thr231, Ser202, Thr181 and PHF1 epitope (Ser396/404) was measured using Western blot analysis (Figure 4.13). Western blot analysis showed no increase in Tau phosphorylation at Thr231 or Ser202 (Figure 4.13C/D). However, an increase (ns) at the PHF1 epitope and Thr181 (ns) was observed (Figure 4.13B/E). To investigate whether phosphorylation at these two epitopes was dependent on Aβ binding to PrPC, cells were pre-incubated with the anti-PrP antibody, 6D11 which binds to the Aβ binding site on PrPC (Lauren et al., 2009). Following Western blot analysis the heavy (~55 kDa) and light (~25 kDa) IgG chains of 6D11 were observed with certain antibodies, thus interfering with accurate quantification of Tau phosphorylation at these epitopes (Figure 4.13A). The IgG chains are not observed following detection with pThr181, however, pre-
incubation with the anti-PrP antibody did not alter phosphorylation induced by Aβ at this site (Figure 4.13E).

Due to the interference with the IgG chains of 6D11 following Western blot analysis with certain antibodies, siRNA targeted against PrP<sup>C</sup> was used to reduce protein expression. Following treatment with siRNA targeted against PrP<sup>C</sup> a 48.4% reduction in protein expression was observed (Figure 4.14). To deliver the siRNA Dharmafect1 transfection reagent was used. Delivering the complex with 4 μl Dharmafect 1 (per well of a 6 well plate) resulted in no toxicity, however, delivering the complex with 8 μl Dharmafect 1 resulted in significant toxicity and cell death as observed by light microscopy (data not shown), therefore careful optimisation of siRNA mediated knockdown in iPSC-derived neurons is required to prevent toxicity whilst yielding optimal protein knock down. Next, Aβ oligomer induced Tau phosphorylation was measured following siRNA mediated knockdown of PrP<sup>C</sup>. Following incubation with Aβ oligomers Tau phosphorylation at the PHF1 epitope was not observed in neurons treated with siRNA targeted against either non-targeting control or PrP<sup>C</sup> (Figure 4.15).

Aβ oligomer induced Tau phosphorylation was also investigated in a second iPSC-derived neuronal line (Figure 4.16). Incubation of our Aβ oligomer preparation induced a dose-dependent increase (ns) in Tau phosphorylation in this cell line at both the PHF1 epitope and Thr231, however, was not significantly reduced with pre-incubation with the anti-PrP antibody, 6D11 (Figure 4.16). In combination these data show that although iPSC-derived neurons endogenously express the components of the PrP<sup>C</sup> signalling complex they do not model Aβ oligomer induced Tau phosphorylation, with levels of Tau phosphorylation in controls and Aβ treated cell highly variable between culture plates from the same experiment and between experiments.
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Figure 4.12 iPSC-neuron characterisation

(A) OX1-19 iPSC-derived cortical neurons were grown onto coverslips until 50 days from neural induction. Cells were washed, fixed and immunostained for MAP2 (green) and nuclei were counterstained with DAPI (blue). Images were acquired on a Delta Vision (Applied Precision) restoration microscope using 60 x objective. The deconvoluted image presented here is a Z-section taken from the centre of the cell (Scale bar; 10 μm). Image was courtesy of Ben Allsop (The University of Manchester, UK).  

(B) Day 50 iPSC-neurons (OX1-19) were lysed, subjected to SDS-PAGE (25 μg total protein) under non-reducing conditions and immunoblotted for mGluR5.  

(C) Day 50 iPSC-neurons (OX1-19) were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for PrP<sup>C</sup> (SAF32) and Fyn kinase.  

(D) Day 50 iPSC-neurons (OX1-19) and SH-SY5Y cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for Tau (K9JA).  

(E) Day 50 iPSC-neurons (OX1-19) were incubated with 1000 units Lambda protein phosphatase (Lambda PP) for 3 h at 30° C. The reaction was stopped following addition of SDS-loading dye, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for Tau isoforms containing three-microtubule binding repeats (3R Tau). A recombinant Tau ladder expressing all six Tau isoforms (rTau6) was used to identify the Tau isoforms expressed. The samples were all analysed on the same SDS-PAGE gel and the dotted line indicates where irrelevant lanes were removed.
A)  

AβO: - - + + + + + - -  
6D11: - - - - + + + +  

Figure continues on next page
Figure 4.13 Aβ-induced Tau phosphorylation in OX1-19 iPSC-derived neurons

(A) Day 50 OX1-19 iPSC-derived neurons were pre-incubated with the anti-PrP antibody, 6D11 for 20 min prior to incubation with 1 μM Aβ oligomers (AβO) (total peptide) or DMSO control for 6 h. Cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for antibodies directed against Tau (K9JA), Tau phosphorylation epitopes (PHF1, pThr231, pSer202 and pThr181), and β-actin (AC15) (Heavy chain IgG; IgG_H and light chain; IgG_L).  

(B-E) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and represented as a percentage of DMSO control (all n = 4 except Aβ n = 6 replicates from single experiment) (mean ± SEM). Statistical analysis was performed using Kruskal-Wallis (GraphPad, PRISM), all non-significant.
Figure 4.14  siRNA mediated knockdown of PrP\textsuperscript{C} in iPSC-derived cortical neurons

A) Day 45-50 OX1-19 iPSC-derived neurons were incubated with 25 nM siRNA targeted against either non-targeting control (NTC) or PrP\textsuperscript{C} for 48 h. Cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for PrP\textsuperscript{C} (SAF32). (B) Semi-quantitative densitometry of membranes was performed using GeneTools (Syngene). Levels of PrP\textsuperscript{C} were represented as percentage of NTC siRNA (\( n = 2 \) independent experiments each of four replicates, mean ± SEM). Statistical analysis was performed using an unpaired, two-tailed T-test (*\( p < 0.05 \)), (GraphPad, PRISM).
Figure 4.15  Aβ oligomer induced Tau phosphorylation in iPSC-derived cortical neurons following siRNA mediated knockdown of PrP

A) Day 50-65 OX1-19 iPSC-derived neurons were incubated with 25 nM siRNA targeted against either non-targeting control (NTC) or PrP for 48 h. Neurons were subsequently incubated in Opti-MEM in the presence of Aβ oligomers (1µM total peptide) or DMSO control for 6h. Cells were lysed, subjected to SDS-PAGE (25 µg total protein) and immunoblotted for Tau (K9JA) and Tau phosphorylated at the PHF1 epitope.  (B) Semi-quantitative densitometry of membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and represented as a percentage of NTC siRNA (DMSO control) (n = 2 independent experiments each of four replicates, mean ± SEM). Statistical analysis was performed using Kruskal-Wallis test (all non-significant), (GraphPad, PRISM).
Figure 4.16 Aβ-induced Tau phosphorylation in NHDF-1 iPSC-derived neurons

(A) Day 50 NHDF-1 iPSC-derived neurons were pre-incubated with the anti-PrP antibody, 6D11 for 20 min prior to incubation with 500 nM or 1 μM Aβ oligomers (AβO) (total peptide) or DMSO control for 6 h. Cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted with antibodies directed against Tau (K9JA), and Tau phosphorylated at the PHF1 epitope and pThr231, and β-actin (AC15). (B-C) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of phosphorylated Tau were normalised to Tau levels and represented as a percentage of DMSO control ($n = 1$).


4.3 Discussion

Tau is a highly regulated protein and undergoes multiple post-translational modifications including phosphorylation and truncation (Martin et al., 2011, Morris et al., 2015). Phosphorylation is the most widely studied modification of Tau, with around 30 sites found to be aberrantly phosphorylated in Alzheimer’s disease, see Figure 4.1. Hyperphosphorylation reduces the ability of Tau to interact with microtubules causing neuronal destabilisation and increases the propensity of Tau to aggregate and form insoluble aggregates known as NFTs (Mietelska-Porowska et al., 2014). In Alzheimer’s disease, aberrant changes to Tau, including phosphorylation have been linked to Aβ and specifically to soluble oligomeric forms of the peptide (Chabrier et al., 2012, De Felice et al., 2008, Zempel et al., 2010). In addition, increasing evidence suggests that Tau is crucial for Aβ mediated toxicity (Roberson et al., 2007, Oddo et al., 2006b). These data implicate Aβ as the inducer and Tau as the mediator of toxicity in Alzheimer’s disease. However, the cellular mechanisms mediating Aβ induced Tau modifications and the exact mechanisms of Tau toxicity are yet to be fully characterised. PrPC is the most widely documented neuronal receptor for Aβ oligomers and has been linked to mediating Tau phosphorylation at tyrosine 18 by activation of Fyn kinase (Lauren et al., 2009, Larson et al., 2012), however, the role of this Aβ/PrPC complex in the phosphorylation of other Tau epitopes has not yet been investigated. This chapter aimed to investigate a suitable model for Aβ oligomer induced Tau phosphorylation at multiple epitopes and the role for PrPC in mediating these phosphorylation events.

4.3.1 Toxic Aβ oligomer preparations

Many different ‘toxic’ Aβ oligomer species have been described in the literature (Benilova et al., 2012). In this thesis, soluble, high molecular weight Aβ oligomers with a fibrillar conformational epitope were generated from a synthetic Aβ1-42 peptide. This synthetic oligomer preparation has previously been shown to bind to PrPc with high affinity, inhibiting hippocampal long-term potentiation and inducing dendritic spine loss (Lauren et al., 2009, Rushworth et al., 2013).

Smaller, soluble oligomeric forms of Aβ play a key role in early synaptic loss, are elevated in Alzheimer’s disease and strongly correlate with the cognitive impairment, severity and
onset of the disease (Lacor et al., 2004, Shankar et al., 2008, McLean et al., 1999, McDonald et al., 2010, Lue et al., 1999). Although it is likely that a more heterogeneous complex of Aβ oligomers exists in the brain, oligomers comprising only of the Aβ_{1-42} peptide were chosen. This Aβ peptide was chosen as Aβ_{1-42} has a higher propensity to aggregate than shorter Aβ peptides, has also been shown to be more toxic (Yan and Wang, 2006) and increased levels are strongly associated with Alzheimer’s disease.

Following SDS-PAGE a broad smear of high molecular weight oligomers was observed (Figure 4.3). In addition, smaller aggregates representing dimers and trimers were also observed, however, these are likely to be artefacts of SDS-PAGE (Hepler et al., 2006, Watt et al., 2013). This highlights the need for multiple methods of characterisation to prevent any misinterpretation of the oligomer preparation. Previously, atomic force microscopy (AFM) was used to determine the structure of our oligomer preparation. Using AFM, fibril free, globular particles and spheres of 5-6 nm in diameter were observed (Rushworth et al., 2013). A more accessible way to characterise the structure of Aβ oligomers is to use conformation-specific antibodies recognising a structural epitope. Although our Aβ oligomer preparation does not contain fibrils, the oligomers have a fibrillar epitope, as determined by OC immunoreactivity (Figure 4.3). Fibrillar oligomers share a common structural epitope with fibrils and may represent fibril seeds (Kayed et al., 2007). The fibrillar conformation of oligomeric Aβ is preferential for the binding to PrP^C. Remodelling oligomer conformation to non-fibrillar oligomers using the green tea polyphenol (-)-epigallocatechin gallate (EGCG) and the red wine extract resveratrol reduces binding to PrP^C and the cytotoxicity of the oligomer preparation (Rushworth et al., 2013).

A recent report highlighted that OC reactive oligomers, termed type 2 oligomers, represent the majority of Aβ oligomers in the brain. However, the authors state that these are not responsible for the cognitive decline due to their confinement around and sequestering into amyloid plaques (Liu et al., 2015). Instead the authors argue that the A11 reactive, or the type 1 oligomer, Aβ*56, described originally by Lesné et al. (2006) is associated with cognitive impairment (Liu et al., 2015). This work contradicts others which states that fibrillar, soluble oligomers correlate better with cognitive dysfunction in Alzheimer’s disease (Tomic et al., 2009) and correlate with Tau pathology in vivo (Chabrier et al., 2012).
4.3.2 Aβ oligomers failed to induce Tau phosphorylation in neuroblastoma cells

To investigate Tau phosphorylation induced by our synthetic Aβ oligomer preparation and the role of PrP<sup>C</sup> in this we overexpressed the mature Tau construct, Tau441, into SH-SY5Y cells and SH-SY5Y cells overexpressing PrP<sup>C</sup>. Our Aβ preparation binds with high affinity to SH-SY5Y cells overexpressing PrP<sup>C</sup> and mediates cytotoxicity in these cells (Rushworth et al., 2013). However, data presented in this chapter show that Aβ oligomers failed to induce the phosphorylation of Tau at Tyr18 and two other epitopes (PHF1 and pThr231) in SH-SY5Y-PrP<sup>C</sup> cells overexpressing Tau441 (Figure 4.7). Interestingly in the absence of PrP<sup>C</sup>, a trend towards an increase in phosphorylation was observed at some Tau epitopes in SH-SY5Y cells, including at Tyr18 and following 1 h Aβ incubation and at the PHF1 epitope following 20 min Aβ incubation (Figure 4.8). In support of this, Vergara, et al. (2015) found an increase in Tau phosphorylation at PHF1 following Aβ oligomer incubation of primary neuronal cultures from Prnp<sup>−</sup>−/− mice but not Prnp<sup>+/−</sup> mice.

The metabotropic glutamate receptor, mGluR5, physically interacts with PrP<sup>C</sup> to mediate Aβ oligomer binding to PrP<sup>C</sup>, the subsequent Fyn activation and synaptic impairment (Um et al., 2013). Aβ does not directly interact with mGluR5, however, Hamilton, et al. (2015) proposed that mGluR5 acts as a scaffold protein to the Aβ/PrP<sup>C</sup> complex. Furthermore, the Aβ/PrP<sup>C</sup> complex induces the aberrant clustering of mGluR5 and elevates intracellular calcium levels further exacerbating synaptic impairment (Um et al., 2013, Renner et al., 2010). In addition, allosteric antagonism of mGluR5 reverses cognitive decline in an Alzheimer’s disease mouse model (Hamilton et al., 2016). These data strongly implicate mGluR5 in the Aβ/PrP<sup>C</sup> signalling complex and subsequent synaptic impairment. We identified that SH-SY5Y cells do not express the functional mGluR5 dimer (Figure 4.9). The absence of the mGluR5 dimer possibly explains the inability of Aβ to induce Tau phosphorylation in SH-SY5Y cell lines (Figure 4.7/4.8). On the other hand, NB7 cells endogenously express the functional mGluR5 dimer, PrP<sup>C</sup> and Fyn kinase. Furthermore, oligomeric Aβ is able to activate Fyn kinase in these cells (Rushworth et al., 2013). Therefore, we next investigated Aβ oligomer induced Tau phosphorylation at Tyr18 and two other epitopes in this cell line. Unfortunately, our Aβ oligomer preparation failed to induce the phosphorylation of Tau at all three epitopes in this cell line (Figure 4.10).

In the original study by Larson, et al. (2012) identifying Aβ oligomer induced Tau phosphorylation at Tyr18 the authors used an oligomeric preparation purified from Alzheimer’s disease human brain extracts. The Aβ preparation used by Larson et al. (2012)
predominantly contained dimeric and trimeric oligomer species. Although both our high-
molecular weight oligomers (Rushworth et al., 2013) and their low-molecular weight
oligomers (Larson et al., 2012) interact with PrP\(_{\text{C}}\), they may have different potencies and so
require different concentrations and/or incubation times or it is possible that they mediate
different downstream signal transduction mechanisms. Furthermore, the authors used a
primary neuronal culture to model A\(\beta\) oligomer induced Tau phosphorylation. It is possible
that a more physiological neuronal model is necessary to induce the complex downstream
cascades mediated by A\(\beta\) such as Tau phosphorylation. Furthermore, both of our
neuroblastoma cell lines required the overexpression of a mature Tau isoform and/or PrP\(_{\text{C}}\),
therefore other fundamental proteins or protein connections may also be essential for Tau
phosphorylation yet absent in these cell lines.

The role of PrP\(_{\text{C}}\) in mediating the downstream toxicity induced by A\(\beta\) oligomers has been
disputed. Shortly after the initial study by Lauren, et al. (2009) identifying PrP\(_{\text{C}}\) as a high
affinity neuronal receptor for oligomeric A\(\beta\) and the mediator of synaptic impairment the
role for PrP\(_{\text{C}}\) in Alzheimer’s disease was challenged. Balducci, et al. (2010) confirmed the
binding of A\(\beta\) oligomers to PrP\(_{\text{C}}\) and hippocampal dependent memory impairment upon
injection of the oligomers, however, ablation of PrP\(_{\text{C}}\) failed to ameliorate this impairment
(Balducci et al., 2010). Furthermore, a second group identified that ablation or
overexpression of PrP\(_{\text{C}}\) had no effect on hippocampal synaptic plasticity in an Alzheimer’s
disease mouse model (Calella et al., 2010). These data were later confirmed in a second
mouse model of Alzheimer’s disease (Cisse et al., 2011b). Therefore, the role of PrP\(_{\text{C}}\) in
mediating A\(\beta\) toxicity may differ depending on the A\(\beta\) oligomer preparation and model
used. It is possible that different cell-surface signalling complex(es) are activated and
therefore, different downstream signalling cascades are activated and Tau phosphorylation
events are triggered with certain A\(\beta\) oligomer preparations and models.

4.3.3 A\(\beta\) oligomer induced Tau phosphorylation in primary neuronal cultures

To investigate A\(\beta\) oligomer mediated Tau phosphorylation in a more physiologically
relevant model, hippocampal primary neuronal cultures from postnatal Wistar rat pups
were generated. Neurons were cultured until 15 DIV where both 3R- and 4R- containing
Tau isoforms are reportedly expressed (Hanes et al., 2009). By this time point robust
binding of A\(\beta\) oligomers to PrP\(_{\text{C}}\) is also observed (Rushworth et al., 2013, Lauren et al.,
Primary neuronal cultures were incubated with Aβ oligomers (500 nM total peptide) for 6 h and Tau phosphorylation was analysed. A longer incubation of Aβ oligomers was used and Tau phosphorylation at Thr231 was investigated. Phosphorylation at Thr231 appears to be an early event in neurodegeneration and precede NFT formation (Vincent et al., 1998, Augustinack et al., 2002), however, our Aβ oligomer preparation failed to induce the phosphorylation of Tau at Thr231, as detected by both Western blot analysis and the MSD immunoassay (Figure 4.11). In addition, the MSD immunoassay detected a slight increase in Tau in the media following incubation with oligomeric Aβ, this is possibly a result of increased degradation and truncation of Tau induced by Aβ. In support of this, Reifert, et al. (2011) previously found an increase in Tau truncation but not phosphorylation following a 4 h exposure of DIV 15 hippocampal primary cultures to Aβ oligomers.

On the other hand, Aβ induced Tau phosphorylation in primary neuronal cultures has been widely reported (Busciglio et al., 1995, Ferreira et al., 1997, De Felice et al., 2008, Zempel et al., 2010, Jin et al., 2011, Vergara et al., 2015). Although it should be noted that a few of these studies use high concentrations of Aβ to induce these effects far beyond physiological levels, with up to 10 µM (Vergara et al., 2015, Liu et al., 2004) and even as high as 20 µM Aβ used (Ferreira et al., 1997, Busciglio et al., 1995). In addition to phosphorylation, Aβ oligomers also trigger Tau mislocalisation to dendritic spines in primary hippocampal neurons (Zempel et al., 2010, Miller et al., 2014), although this has been found to be dependent on Tau phosphorylation (Hoover et al., 2010).

Previously, Aβ has shown not to be toxic to primary cultures at earlier ages (Liu et al., 2004), possibly due to low levels of Tau which is known to be the mediator of Aβ toxicity. Although multiple Tau isoforms were expressed in our primary neuronal culture, the foetal isoform of Tau was predominantly expressed (Figure 4.11). Therefore, the low Tau expression levels and predominant expression of the foetal isoform may explain why Aβ failed to induce Tau phosphorylation in our primary neuronal preparation.

4.3.4 Aβ oligomer induced Tau phosphorylation in iPSC-derived neurons

Takahashi, et al. (2007). identified that adult human fibroblasts can be reprogrammed into iPSCs using four transcription factors; Oct4, Sox2, Klf4 and c-Myc enabling their differentiation to disease relevant cells including neurons and glial cells (Sullivan and
Young-Pearse, 2015). Therefore, iPSCs hold great potential as a model to investigate the cellular mechanisms underlying neurodegenerative diseases and to test novel therapeutics in a physiologically relevant, even patient-specific model (Tiscornia et al., 2011).

Previously rodent models have been widely used to model complex neurodegenerative diseases, however, differences in biochemistry and physiology have led to multiple therapies failing to translate to human clinical trials (Perel et al., 2007). Using human iPSCs overcomes species differences and have successfully remodelled the genetic phenotype of neurodegenerative diseases. For example, neurons derived from fAD patients have altered APP processing, increased Aβ generation and altered Tau phosphorylation (Israel et al., 2012, Muratore et al., 2014, Moore et al., 2015).

Using iPSC-derived cortical neurons from cognitively normal (control) patients we sought to investigate Tau phosphorylation induced by the exogenous application of oligomeric Aβ and the cellular mechanisms mediating this. Following 6 h application of Aβ a trend increase in phosphorylation was observed at the PHF1 epitope and Thr181 but not Thr231 or Ser202 (Figure 4.13). To investigate whether Aβ induced phosphorylation at these epitopes was PrP\textsuperscript{C} dependent, neurons were pre-incubated with the anti-PrP\textsuperscript{C} antibody, 6D11 which is known to interact with the Aβ binding site (Lauren et al., 2009). Pre-incubation with the anti-PrP\textsuperscript{C} antibody failed to prevent phosphorylation at Thr181 by Aβ (Figure 4.13), furthermore, due to the interference of the IgG chains of 6D11 we were unable to measure the alteration to phosphorylation at PHF1 (Figure 4.13). To extend this study further, the mislocalisation of phosphorylated Tau to the somatodendritic region could be investigated. Tau phosphorylated at Ser202 but not PHF1 has been found in the dendritic region following incubation with Aβ oligomers (Zempel et al., 2010).

To further identify the role of PrP\textsuperscript{C} siRNA mediated knockdown of the protein was investigated. Expression levels of PrP\textsuperscript{C} were successfully reduced in iPSC-derived neurons (Figure 4.14). Following incubation with Aβ oligomers Tau phosphorylation at the PHF1 epitope was not observed in neurons treated with siRNA targeted against either non-targeting control or PrP\textsuperscript{C} (Figure 4.15), highlighting the high variation in Tau phosphorylation levels between experiments. Tau phosphorylation induced by the exogenous application of Aβ has previously been investigated in iPSC-derived neurons. Nieweg, et al. (2015) reported a trend towards an increase in Tau phosphorylation at AT8 (Ser202/Thr205) and AT180 (Thr231) following an 8 day incubation with Aβ from 7PA2-conditioned media. However, it is important to note the lack of control experiments, low
experimental repeats and high variation between experiments in this report (Nieweg et al., 2015). High variation between experiments was also reported in our work with iPSC-neurons, highlighting the need for rigorous controls and high experimental repeat numbers when using iPSC derived neurons.

The iPSC-derived neurons used in this study solely express the foetal isoform of Tau (Figure 4.8) and as described previously this is highly endogenously phosphorylated. The high phosphorylation state is essential for neuronal development and does not induce aggregation of this isoform. In addition, the absence of mature Tau isoforms implies that the iPSC-neurons have a foetal phenotype and therefore may not capture the functional complexity of mature neurons. It has previously been reported that iPSC-derived neurons express much lower levels of synaptic and ion channel pathways than brain tissue indicating the iPSC-derived neurons are electrophysiologically immature (Handel et al., 2016), however, with increased maturation in culture, iPSC-neurons have increased capacity to fire action potentials (Bergstrom et al., 2016). Furthermore, considering foetal Tau is already highly phosphorylated, to what extent is the exogenous application of Aβ able to induce a further insult.

Tau splicing is developmentally regulated and interestingly the expression of 4R-containing Tau isoforms has been identified in iPSC-neurons, but only following 365 days in culture (Sposito et al., 2015). Culturing neurons for this length of time is not time or cost effective, therefore, methods of accelerating the neuronal aging processes are necessary. The use of cell stressors has been proposed a method of accelerating neuronal aging (Campos et al., 2014). Furthermore, certain MAPT mutations have shown to accelerate the maturation of iPSC-derived neurons and the expression of 4R-Tau isoforms (Iovino et al., 2015, Sposito et al., 2015). Culturing neurons on a 3D matrix culture has also shown to promote neuronal maturation and mature Tau splicing (Choi et al., 2014). Furthermore, using mixed population cultures with neurons and glial cells has shown to support functional neurons (Pasca et al., 2015).
4.4 Chapter summary

Modelling Aβ oligomer induced Tau phosphorylation in vitro has proved challenging. Our synthetic, oligomeric preparation binds to neuroblastoma cell lines expressing PrPC and primary neurons with high affinity and induces cytotoxicity (Rushworth et al., 2013), however, failed to induce the phosphorylation of Tau at multiple epitopes in both SH-SY5Y and NB7 cell lines. SH-SY5Y cells do not endogenously express PrPC or mature Tau isoforms, therefore, both were overexpressed in these cells. In addition, SH-SY5Y cells do not express the functional mGluR5. Other essential proteins or protein interactions necessary for Aβ oligomers to induce Tau phosphorylation may also be absent from this cell line. Although NB7 cell lines endogenously express proteins essential for the Aβ/PrPC signalling complex a more functionally complex neuronal model may be necessary. Therefore, Aβ oligomer induced Tau phosphorylation was investigated in primary hippocampal neuronal cultures. However, the failure of Aβ oligomers to induce Tau phosphorylation in this neuronal culture may again be a result of a foetal neuronal phenotype and low levels of endogenous Tau expression.

IPSC-derived neurons are capable of remodelling the disease phenotype of genetic neurodegenerative diseases including fAD and hold much promise for recapturing the complexity of human disease and modelling novel therapies. Our Aβ oligomer preparation failed to robustly or significantly increase Tau phosphorylation in iPSC-derived neuronal cultures with results between experiments were highly variable. Furthermore, iPSC-derived neurons do not model functionally mature neurons and also only express the foetal isoform of Tau. Therefore, further work is needed to generate a mature, functional neuronal model to investigate the complex mechanisms linking extracellular Aβ oligomers and the aberrant phosphorylation intracellular Tau.
Chapter 5. The role of Flotillins in Aβ oligomer binding to PrP<sub>C</sub>

5.1 Introduction

5.1.1 Cell surface lipid raft based signalling complex(es) are crucial for the binding of Aβ oligomers and subsequent toxicity

Lipid raft microdomains are distinct regions of the plasma membrane rich in cholesterol and sphingolipids and are characterised by their insolvency to detergents. Lipid rafts are essential for signal transduction and the assembly/compartmentalisation of multi-protein receptor complexes and receptor-activated components (Allen et al., 2007, Simons and Toomre, 2000).

Several lines of evidence suggest that multi-protein receptor complexes are involved in the cell-surface binding of Aβ oligomers and their subsequent neurotoxicity (as reviewed in Jarosz-Griffiths, et al. (2016)). The most widely documented cell-surface complex for Aβ oligomers centres around PrP<sub>C</sub> which is GPI-anchored to the outer leaflet of lipid raft microdomains. This Aβ/PrP<sub>C</sub> complex triggers synaptic impairment and cognitive deficits in vivo (Lauren et al., 2009) and requires the transmembrane receptors LRP1 and mGluR5 (Rushworth et al., 2013, Um et al., 2012), Figure 5.1. This lipid raft based multi-protein complex activates the tyrosine kinase, Fyn and ultimately triggers the surface depletion of NMDA-receptors (Um et al., 2012). Furthermore, as described in Chapter 4, this signalling complex provides a mechanistic link to Aβ oligomer induced Tau phosphorylation (Larson et al., 2012). In addition, binding of Aβ to PrP<sub>C</sub> activates mGluR5 and induces its aberrant clustering within lipid raft microdomains, increasing intracellular calcium levels and further exacerbating synaptic impairment (Renner et al., 2010, Um et al., 2013, Kumari et al., 2013). The association of LRP1 with lipid raft microdomains also increases in the presence of Aβ (Wu and Gonias, 2005). Both LRP1 and mGluR5 were separately identified as part of the Aβ/PrP<sub>C</sub> complex, however, it remains to be determined whether LRP1 and mGluR5 are present in the same signalling complex with PrP<sub>C</sub> or whether these represent distinct PrP<sub>C</sub> signalling complexes.

Interestingly, Fyn kinase has been implicated in mediating the clustering of Aβ within lipid raft microdomains (Williamson et al., 2008), possibly by aiding the aberrant clustering of Aβ receptors. Furthermore, phosphorylated Tau also accumulates within lipid raft
microdomains in the presence of Aβ oligomers and in Alzheimer’s disease (Kawarabayashi et al., 2004). Previously, Tau has been abnormally identified in the somatodendritic region in the presence of Aβ and has been implicated in the increased association of Fyn kinase with the post-synaptic density (Ittner et al., 2010, Zempel et al., 2010). These data implicate both Fyn and Tau in mediating the aberrant clustering and signalling of multi-protein signalling complexes in lipid raft microdomains.

Figure 5.1  Aβ oligomers bind to PrP^C in a cell surface, lipid raft based signalling complex

5.1.2  What are Flotillins and are they involved in PrP^C signalling?

Flotillins are highly conserved proteins and associate tightly to the inner leaflet of the plasma membrane by myristoyl and palmitoyl residues (Morrow et al., 2002, Neumann-Giesen et al., 2004). These abundant proteins are implicated in the assembly, compartmentalisation and signalling of lipid raft based signalling complexes (Otto and Nichols, 2011). Furthermore, Flotillins co-localise with PrP^C in lipid raft microdomains (Solis et al., 2010), however, the exact role of Flotillin in Aβ/PrP^C signalling complexes remains unknown. Flotillins may form functional connections between proteins in multi-protein signalling complexes in lipid raft microdomains (Liu et al., 2005b) and/or aid in the aberrant clustering of proteins within lipid rafts. In support of this, Flotillins are known to interact with Fyn kinase via their N-terminal sequence (Liu et al., 2005b) and have also been shown to interact with NMDA-receptor subunits (Swanwick et al., 2009).
5.1.3 Aims

The aim of this chapter was to determine the role of Flotillins in the Aβ oligomer multi-protein signalling complex. Firstly, the role of Flotillin-1 and Flotillin-2 in the cell-surface binding of oligomeric Aβ to PrP<sup>C</sup> was determined. Following small interfering RNA (siRNA) gene silencing of either Flot1 or Flot2 binding of Aβ oligomers to PrP<sup>C</sup> was measured by immunofluorescence microscopy using streptavidin to detect the biotin tag on the Aβ peptide. Next, the mechanism by which Flotillin influences Aβ binding to PrP<sup>C</sup> was explored and the role of Flotillins in the stabilisation of lipid raft microdomains and PrP<sup>C</sup> localisation was analysed following siRNA treatment.
5.2 Results

5.2.1 Flotillin proteins are isolated in insoluble lipid raft microdomains

Lipid raft microdomains are distinct compartments of the plasma membrane enriched in cholesterol, sphingolipids, phospholipids with saturated fatty acid tails and GPI-anchored proteins (Simons and Toomre, 2000). Flotillins associate tightly to the inner leaflet of the plasma membrane and are known to reside within lipid raft microdomains (Figure 5.2A). Lipid rafts are defined by their insolubility to non-ionic detergents such as Triton X-100 (Allen et al., 2007) and as a result, these detergent-resistant membrane fractions can be isolated by buoyant sucrose density gradient centrifugation.

To confirm the presence of both Flotillin isoforms, Flotillin-1 and Flotillin-2, in lipid raft microdomains in SH-SY5Y cells, cells were lysed in the presence of 0.6 % Triton X-100 and a buoyant discontinuous sucrose gradient was generated. Following centrifugation, the lipid raft microdomain was seen as a milky layer floating at the 35 % and 5 % sucrose interface. Both Flotillin isoforms were isolated in the lipid raft fractions (fractions 6-8) in SH-SY5Y cells, as determined by Western blot analysis (Figure 5.2B). The transmembrane glycoprotein transferrin receptor was used as a negative control as is known to not reside within lipid raft microdomains and was successfully isolated in the non-raft fractions (fractions 1-3) (Figure 5.2B).
Figure 5.2 Flotillins are isolated in insoluble lipid raft microdomains

(A) Schematic showing the localisation of Flotillin-1 and Flotillin-2 within the lipid raft microdomain of the plasma membrane. Flotillin-1 is anchored to the plasma membrane by a palmitoyl residue and Flotillin-2 by both a palmitoyl and myristoyl residue. (B) SH-SY5Y cells were lysed in the presence of 0.6 % (v/v) Triton X-100 and a buoyant sucrose gradient was created (40 %, 35 % and 5 % w/v sucrose). Following centrifugation at 100,000 g for 18 h, fractions were collected, subjected to SDS-PAGE and immunoblotted for transferrin receptor (Transferrin-R), Flotillin-1 and Flotillin-2. The insoluble lipid rafts floated at the 5-35 % sucrose interface and were collected in fractions 6-8.
5.2.2 The relationship between Flotillin-1 and Flotillin-2 expression

To identify the role of Flotillins in Aβ oligomer binding Flot1 and Flot2 genes were silenced using siRNA. To confirm successful siRNA mediated knockdown, protein expression was determined using Western blot analysis. Protein expression of Flotillin-1 was significantly reduced by 61.2 % and Flotillin-2 by 59.8 % in SH-SY5Y cells incubated by siRNA targeted against either Flot1 or Flot2, respectively (Figure 5.3). Interestingly, Flotillin-1 expression was also reduced following Flot2 gene silencing, and Flotillin-2 by Flot1 gene silencing (44.0 % and 30.2 % respectively) (Figure 5.3). These data indicate that Flotillin-1 stability is dependent on Flotillin-2 expression and vice versa.

Successful siRNA mediated knockdown of Flotillin-1 and Flotillin-2 was also confirmed using immunofluorescence microscopy of intact cells. For the detection of Flotillins by immunofluorescence microscopy cold methanol fixation of the cells produced brighter and clearing staining of the proteins compared to paraformaldehyde fixation with Triton X-100 permeabilisation (data not shown). Organic solvents such as methanol fix the cells by dehydration but also permeabilise the cell enabling the visualisation of intracellular proteins such as Flotillin which reside on the inner leaflet of the plasma membrane.

Following quantification, Flotillin-1 expression was reduced by 50.5 % and Flotillin-2 by 25.6 % following respective siRNA incubation (Figure 5.4). Staining for both Flotillins was predominantly observed along the edge of the cell, however, some non-specific staining was observed in the cytoplasm (Figure 5.4A,B). Levels of Flotillin-1 knockdown were comparable between Western blot analysis and immunofluorescence microscopy. In comparison, Flotillin-2 knockdown appeared far less effective following visualisation by immunofluorescence microscopy. It should be noted that the cell membrane staining appeared to be reduced following Flotillin-2 knockdown, however, the majority of cytoplasmic staining remained and it likely to be non-specific staining (Figure 5.4B).
Figure 5.3 A concomitant relationship between Flotillin isoforms in SH-SY5Y cells

(A) SH-SY5Y cells were incubated with 25 nM siRNA targeted against Flotillin-1 (Flot-1), Flotillin-2 (Flot-2) or non-targeting control (NTC) for 48 h. Cells were lysed, subjected to SDS-PAGE (25 μg total protein) and immunoblotted for Flotillin-1, Flotillin-2 and β-Actin (AC15). (B) Semi-quantitative densitometry of the membranes was performed using GeneTools (Syngene). Levels of Flotillin-1 and Flotillin-2 were represented as a percentage of NTC siRNA (mean ± SEM, n = 4 independent experiments each of 2 replicates). Statistical analysis was performed using One-way ANOVA with Tukey’s multiple comparisons (**** p < 0.0001; ** p < 0.01), (GraphPad, PRISM).
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Figure 5.4 Immunofluorescence microscopy analysis of Flotillin-1 and Flotillin-2 siRNA mediated protein reduction

(A-B) SH-SY5Y cells overexpressing PrP<sup>C</sup> were seeded onto coverslips in 24 well plates and grown to 50-60 % confluence. Cells were incubated with 25 nM siRNA targeted against Flotillin-1 (Flot-1), Flotillin-2 (Flot-2) or non-targeting control (NTC) for 48 h. Cells were washed, fixed with 100 % cold methanol and immunostained for Flotillin-1 (green) or Flotillin-2 (red) and nuclei were counterstained with DAPI (blue). Images were acquired on a Delta Vision (Applied Precision) restoration microscope using a 60 x objective. The deconvoluted images presented here are Z-section images taken from the middle of the cell. The intensity setting and scale from NTC was copied across to the image acquired for respective Flotillin siRNA (Scale bar; 10 µm).  

(C-D) The intensity of Flotillin staining was quantified using Image J (Fiji) and levels of Flotillin-1 and Flotillin-2 were represented as a percentage of NTC siRNA (mean ± SEM, n = ~10 cells over 4 images). Statistical analysis was performed using an unpaired, two-tailed, T-test (*** p < 0.001, ns = non-significant), (GraphPad, PRISM).

5.2.3 Flotillin-1 is crucial for Aβ oligomer binding to PrP<sup>C</sup>

Next, the role of Flotillins in Aβ oligomer binding to PrP<sup>C</sup> was analysed. Following treatment with siRNA targeted against either Flotillin isoform, SH-SY5Y cells overexpressing PrP<sup>C</sup> were incubated with Aβ oligomers for 10 min before immunostaining for PrP<sup>C</sup> and Aβ and visualised by immunofluorescence microscopy. Streptavidin was used for the detection of the biotin tag on the Aβ peptide. The biotin tag is small and relatively stable and does not interfere with the oligomerisation of our Aβ oligomer preparation (Rushworth et al., 2013). However, it does enable visualisation of our oligomers by immunofluorescence microscopy unlike total Aβ antibodies such as 6E10 which would also detect APP and APP fragments containing the Aβ sequence. Along the membrane of the cell a distinct punctate staining of PrP<sup>C</sup> was observed, a characteristic of lipid raft proteins (Figure 5.5). Aβ oligomers strongly associated with PrP<sup>C</sup> on the surface of the cells (Figure 5.5A), however, following incubation with Flotillin-1 siRNA, Aβ oligomer binding to cell surface PrP<sup>C</sup> was reduced by 83.2 % (Figure 5.5B,D). On the other hand, only a 6.9 % reduction in Aβ oligomer binding to cell surface PrP<sup>C</sup> was observed following Flotillin-2 siRNA (Figure 5.5C,D).
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Figure 5.5 Flotillin-1 plays a key role in Aβ oligomer binding to PrP<sub>C</sub>

(A-C) SH-SY5Y cells overexpressing PrP<sub>C</sub> were seeded onto coverslips in 24 well plates and grown to 50-60 % confluence. Cells were incubated with 25 nM siRNA targeted against Flotillin-1 (Flot-1), Flotillin-2 (Flot-2) or non-targeting control (NTC) for 48 h. Cells were washed and incubated with 500 nM (total peptide) Aβ oligomers for 10 min at room temperature. Cells were washed, fixed with 4% PFA (paraformaldehyde) and immunostained for PrP<sub>C</sub> (SAF32; green) and Aβ biotin (Streptavidin; red). Nuclei were counterstained with DAPI (blue). Images were acquired on a Delta Vision (Applied Precision) restoration microscope using a 60 x objective. The deconvoluted image presented here is a Z-section taken from the middle of the cell. The intensity setting and scale from NTC was copied across to the image acquired for the respective Flotillin siRNA (Scale bar, 10 µm). (D) Aβ oligomer binding to cell-surface PrP<sub>C</sub> was quantified using Image J (Fiji). Cell surface binding was represented as a percentage of NTC siRNA (mean ± SEM, n = ~15 cells for Aβ, n = ~5 cells per DMSO condition). Statistical analysis was performed using One-way ANOVA with Tukey’s multiple comparisons test (*p < 0.05, ns = non-significant), (GraphPad, PRISM).
5.2.4 Flotillins are essential for the lipid raft localisation of PrP<sub>C</sub>

The lipid raft localisation of PrP<sub>C</sub> is crucial for the binding of Aβ and signal transduction mediated by PrP<sub>C</sub> (Rushworth et al., 2013). Considering Flotillins are isolated within lipid raft microdomains, it was next investigated whether PrP<sub>C</sub> is destabilised from raft fractions upon loss of either Flotillin isoform and as a result reduces Aβ oligomer binding to PrP<sub>C</sub>. To explore this, lipid raft microdomains were isolated by buoyant sucrose gradient centrifugation and PrP<sub>C</sub> localisation was analysed following Flot1 and Flot2 mediated siRNA. Following both Flotillin-1 and Flotillin-2 gene silencing, PrP<sub>C</sub> was identified in non-raft fractions (1-3) as well as lipid raft fractions (6-7) (**Figure 5.6B,C**). In comparison, following treatment with non-targeting control siRNA, PrP<sub>C</sub> was predominantly isolated in lipid raft fractions (6-7) (**Figure 5.6A**).

Furthermore, a reduction in Flotillin-1 also resulted in the destabilisation of Flotillin-2 in lipid raft fractions and vice versa (**Figure 5.6**). Therefore, both Flotillin isoforms are key to the lipid raft localisation of PrP<sub>C</sub> and the other Flotillin isoform, possibly by maintaining the stability of the lipid raft domain. Interestingly, although both Flotillin-1 and Flotillin-2 siRNA equally redistributed PrP<sub>C</sub> to non-raft fractions (**Figure 5.6D**), only Flotillin-1 siRNA significantly altered Aβ oligomer binding to cell surface PrP<sub>C</sub>. 
Figure continues on next page
Figure 5.6 Flotillins are essential for the lipid raft localisation of PrP<sub>C</sub>

SH-SY5Y-PrP<sub>C</sub> cells were incubated with 25 nM siRNA targeted against either non-targeting control (NTC) (A), Flotillin-1 (Flot-1) (B) or Flotillin-2 (Flot-2) (C) for 48 h. Cells were lysed in the presence of 0.5 % (v/v) Triton X-100 and a buoyant sucrose gradient was created (40 %, 35 % and 5 % w/v sucrose). Following centrifugation at 100,000 g for 18 h, fractions were collected, subjected to SDS-PAGE and immunoblotted for transferrin receptor (Transferrin-R), Flotillin-1, Flotillin-2 and PrP<sub>C</sub> (6D11). The insoluble lipid rafts floated at the 5-35 % sucrose interface and were collected in fractions 6-8. (D) The distribution of PrP<sub>C</sub> in non-raft (NR) and lipid raft (R) fractions following incubation with siRNA targeted against NTC, Flot-1 and Flot-2. Levels of PrP<sub>C</sub> were represented as a percentage of total PrP<sub>C</sub> levels (n =1).
5.3 Discussion

5.3.1 The role of Flotillins in the raft localisation of, and Aβ binding to, PrP\textsuperscript{C}

The relationship between Flotillin isoforms

Flotillins are anchored to the inner leaflet of the plasma membrane and are implicated in the assembly, compartmentalisation and signalling of lipid raft based signalling complexes (Simons and Toomre, 2000). The Flotillin family consists of two isoforms, Flotillin-1 and Flotillin-2, which share only 50% amino acid sequence identity but similar secondary structures (Rivera-Milla et al., 2006), see Figure 5.2. Data presented in this chapter demonstrates that each Flotillin isoform stabilises the expression and lipid raft localisation of the other (Figure 5.3 and Figure 5.6). Following siRNA mediated knockdown of either isoform a reciprocal decrease in the other isoform was observed (Figure 5.3). This decrease was slightly more for Flotillin-1 following a loss of Flotillin-2, implicating that Flotillin-1 was more dependent on the presence of Flotillin-2. Interestingly, although Flot2 siRNA had a greater effect on Flotillin-1 expression, loss of Flotillin-1 induced a greater redistribution of Flotillin-2 out of lipid raft microdomains (Figure 5.6). A concomitant relationship between Flotillin isoforms has previously been shown in cell lines (Solis et al., 2007), drosophila (Hoehne et al., 2005) and mouse models (Ludwig et al., 2010, Berger et al., 2013), however, the mechanisms remain largely unknown.

Despite sharing similar structures the membrane association of the two Flotillin isoforms differs. The association of Flotillin-1 to the plasma membrane is mediated by a single palmitoylation and a hydrophobic region of the polypeptide chain. On the other hand, Flotillin-2 contains both a palmitoylation and myrisotylation site but no clear hydrophobic region of the polypeptide chain that could play a role in membrane association has been identified (Neumann-Giesen et al., 2004, Liu et al., 2005b, Otto and Nichols, 2011). These differences in membrane association may account for the differences in stability and mobility within lipid rafts between the isoforms. Furthermore, both isoforms physically interact with one another via the C-terminal region of the protein (Stuermer et al., 2001) and are capable of forming hetero- as well as homo-oligomers. Another possibility is that differences in the formation of hetero-oligomers may result in a greater instability of Flotillin-1 and formation of these oligomers is more dependent on the presence of Flotillin-2.
**Flatillin-1, but not Flatillin-2, is crucial for Aβ binding to PrP<sup>C</sup>**

Data presented in this chapter also revealed a crucial role for Flatillin-1 in Aβ oligomer binding to PrP<sup>C</sup>. Flot1 siRNA reduced cell-surface binding of Aβ oligomers to PrP<sup>C</sup> by 83.2 %. On the other hand Flatillin-2 siRNA had no significant effect on Aβ oligomer binding (Figure 5.5). It should be noted that Flot2 siRNA induced a 44.3 % reduction in Flatillin-1 protein levels (Figure 5.3), therefore a reduction in cell-surface binding of Aβ was also predicted with Flatillin-2 knockdown due to the additional loss of the Flatillin-1 isoform.

Quantification following immunofluorescence staining revealed a non-significant reduction in Flatillin-2 following siRNA treatment, thus the inability to prevent Aβ binding to PrP<sup>C</sup> may therefore in part be a result of inefficient siRNA knockdown, at least as measured by immunofluorescence microscopy. It should be noted that Flot2 siRNA clearly reduced Flatillin-2 immunoreactivity along the membrane of the cell, however, non-specific staining of Flatillin-2 was still observed in the cytoplasm of the cell. This may account for the disparities between Western blot analysis and immunofluorescent microscopy quantification.

**Flatillins are essential for the raft localisation of PrP<sup>C</sup>**

Lipid rafts are enriched in cholesterol which is implicated in mediating the assembly of lipid raft microdomains. Cholesterol holds a higher affinity for sphingolipids over unsaturated phospholipids which form the vast proportion of the plasma membrane (Simons and Ehehalt, 2002). Treatment of cells with a cholesterol depleting agent, methyl-β-cyclodextrin (MβCD), results in the destabilisation of lipid raft domains and the redistribution of both PrP<sup>C</sup> and Flatillin to non-raft fractions (Rushworth et al., 2013). It was hypothesised that a reduction of Flatillin by siRNA may also result in the redistribution of PrP<sup>C</sup> out of lipid raft microdomains. Indeed, siRNA targeted against either Flatillin isoform resulted in the redistribution of PrP<sup>C</sup> to non-raft fractions (Figure 5.6). Interestingly, the level of PrP<sup>C</sup> in raft and non-raft fractions was the same following siRNA of either Flatillin isoform despite differences in their role in Aβ binding to PrP<sup>C</sup> (Figure 5.6). Following treatment with MβCD, cell surface binding of Aβ oligomers to PrP<sup>C</sup> was also reduced by 80.6 % (Rushworth et al., 2013), a similar reduction in binding compared to that mediated by Flatillin-1 siRNA (83.2 %) (Figure 5.4), thus highlighting the importance of the lipid raft integrity on Aβ oligomer binding to PrP<sup>C</sup>.
5.3.2 Other proteins involved in the binding of Aβ to PrP\textsuperscript{C} and Flotillin interacting partners

Although both Flotillin isoforms influence the lipid raft localisation of PrP\textsuperscript{C}, only Flotillin-1 altered Aβ oligomer binding to PrP\textsuperscript{C} at the cell surface. Due to their localisation on the outer and inner leaflet, respectively, PrP\textsuperscript{C} and Flotillin cannot physically interact. The transmembrane receptors, LRP1 and mGluR5, are crucial for the binding of Aβ to PrP\textsuperscript{C} and are likely intermediate proteins coupling Flotillin and PrP\textsuperscript{C}. Knockdown of LRP1 reduces Aβ binding to PrP\textsuperscript{C} by 86.6% (Rushworth et al., 2013), similar the reduction induced by Flotillin-1 knockdown (Figure 5.6). One hypothesis is that Flotillin-1 may interact with and localise more strongly with PrP\textsuperscript{C} or other components of the signalling complex compared to Flotillin-2 and thus loss of Flotillin-1 has a greater impact on the binding of Aβ. Immunofluorescence microscopy and co-immunoprecipitation experiments could be used to analyse the co-localisation of the Flotillin isoforms with PrP\textsuperscript{C} and other components of the signalling complex such as mGluR5 and whether these interactions are strengthened in the presence of Aβ oligomers.

Caveolin

In this thesis the term lipid raft has been used to describe detergent resistant microdomains rich in Flotililns. Caveolae are another subset of lipid rafts. Caveolae are flask-shaped invaginations formed by the oligomerisation of Caveolin which is palmitoylated to the inner leaflet of the plasma membrane. Flotillin and Caveolin microdomains are distinct but do share functional similarities (Stuermer et al., 2001). Like Flotillins, Caveolin associates with cholesterol and sphingolipids (Hooper, 1999) and both caveolae and lipid raft microdomains are involved in membrane compartmentalisation, endocytosis and vesicle trafficking (Frick et al., 2007).

Interestingly, Larson et al. (2012) identified Caveolin-1 as an interacting protein with the Aβ/PrP\textsuperscript{C}/Fyn complex in human brain tissue (Larson et al., 2012). This supported previous cell line data from Mouillet-Richard, et al. (2000) where the authors also observed a Caveolin-1 dependent coupling of PrP\textsuperscript{C} to Fyn (Mouillet-Richard et al., 2000). Thus, these data in combination with data presented in this chapter implicate a role for both caveolae and Flotillin lipid raft microdomains in PrP\textsuperscript{C} mediated signal transduction. Considering caveolae and Flotillin lipid rafts are distinct domains, PrP\textsuperscript{C} mediated signal transduction cascades may differ depending on the localisation of PrP\textsuperscript{C} and in turn may reflect mediation by different activators.
Our SH-SY5Y cell line does not express Caveolin-1 (data not shown) and in the brain Caveolin-1 was previously thought to be largely absent from neurons. On the other hand, Caveolin-1 has been identified in glial cells and endothelial cells (Ikezu et al., 1998, Simons and Toomre, 2000, Viegas et al., 2006). Furthermore, PrP<sup>C</sup> is also expressed in endothelial cells and mediates Aβ transcytosis across the blood brain barrier (BBB) in a complex with LRP1 (Pflanzner et al., 2012, Taylor and Hooper, 2007). This supports the idea that the same protein complex, or interactions between proteins, can be identified on multiple cell types to mediate different mechanisms. Larson et al. (2012) used human brain tissue to identify Caveolin-1 and PrP<sup>C</sup> coupling, although only low levels of Caveolin-1 were pulled down with PrP<sup>C</sup>, suggesting a weak interaction between the proteins and/or low Caveolin-1 expression levels (Larson et al., 2012). It is possible that in such a heterogeneous tissue this Caveolin-1/PrP<sup>C</sup> complex may be present in glial cells or another cell type and not neurons. Furthermore the authors did not directly identify the role of Caveolin-1 in the Aβ/PrP<sup>C</sup>/Fyn complex. Instead we propose that the neuronal Aβ/PrP<sup>C</sup>/Fyn complex is present in Flotillin lipid raft microdomains, however, further work is needed to identify the exact role of Flotillins in this signalling complex. On the other hand, Caveolin-1 has previously been identified in primary neuronal cultures (Boulware et al., 2007) and coupling of PrP<sup>C</sup> to Caveolin-1 has also been reported in neuronal cell lines (Toni et al., 2006). Therefore, further work is necessary to unravel the role of both Caveolae and Flotillin lipid raft microdomains in the signalling of neuronal PrP<sup>C</sup>.

**Glutamate receptors**

As mentioned previously, the glutamate receptor, mGluR5 is implicated in mediating Aβ oligomer binding to PrP<sup>C</sup>, the subsequent activation of Fyn kinase and in mediating synaptic impairment (Um et al., 2013). The lipid raft association of mGluR5 increases upon activation and in the presence of Aβ, however, is not altered following Caveolin knockout (Kumari et al., 2013), thus, further supporting the role for Flotillin containing lipid raft microdomains in the formation of the Aβ/PrP<sup>C</sup>/mGluR5 signalling complex.

As mentioned previously, the ionotropic glutamate receptor, NMDA-receptor is a downstream target of the Aβ/PrP<sup>C</sup> complex. The NRB2 subunit does not physically interact with PrP<sup>C</sup> (Um et al., 2013), however, it is localised to lipid raft microdomains (Abulrob et al., 2005, Besshoh et al., 2007). Flotillin-1 physically interacts with NMDA-receptor subunits in primary hippocampal neuronal cultures (Swanwick et al., 2010). Flotillin-1 may play a crucial role in glutamatergic synapse function and formation mediated by
interactions with NMDA-receptors which are crucial for LTP in the hippocampus and underlies the synaptic plasticity essential for learning and memory. Further work is needed to identify the relationship between Flotillin isoforms and NMDA-receptors and how this relationship may be altered in the presence of Aβ oligomers.

5.3.3 Chapter summary

In summary, data presented in this chapter illustrates that the expression and lipid raft localisation of either Flotillin isoform was dependent on the expression of the other. In addition, expression of either Flotillin isoform was crucial for the lipid raft localisation of PrP\(^C\). Furthermore, Flotillin-1 but not Flotillin-2 was crucial for mediating Aβ oligomer binding to cell surface PrP\(^C\). There is evidence that Flotillins play a key role in the assembly of protein signalling complexes in lipid rafts, therefore, it is possible that Flotillins interact with specific proteins upon Aβ binding to PrP\(^C\) which are crucial to coupling Flotillin to this PrP\(^C\) signalling complex. The transmembrane receptors, LRP1 and mGluR5, are possible Flotillin interacting proteins and are already known to play a key role in Aβ binding to PrP\(^C\). On the other hand, Flotillin is a key part of lipid raft microdomains, therefore, it is possible that a reduction in the expression of Flotillin results in an overall destabilisation of lipid raft microdomains and as a result alters PrP\(^C\) binding and signalling which is dependent on the integrity and stability of lipid raft microdomains.
Chapter 6. Final discussion

6.1 Aβ, PrP<sup>C</sup> and Tau in Alzheimer’s disease

Smaller, soluble oligomeric forms of Aβ are the primary pathogenic species in Alzheimer’s disease (Walsh and Selkoe, 2007) and strongly correlate with the presence and severity of cognitive decline (McLean et al., 1999, Lue et al., 1999). Furthermore, soluble oligomeric forms of Aβ are linked to inducing pathogenic modifications to Tau, including phosphorylation (Chabrier et al., 2012, Larson et al., 2012, De Felice et al., 2008, Zempel et al., 2010). Interestingly, there is now strong evidence that Tau is the mediator of Aβ toxicity, synaptic dysfunction and disease propagation in Alzheimer’s disease (Roberson et al., 2007, Ittner and Gotz, 2011, Pooler et al., 2013b, Bloom, 2014, Stancu et al., 2014, Oddo et al., 2006b). Several lines of evidence suggest that multi-protein complex(es) are involved in the cell surface binding of Aβ oligomers and the subsequent synaptic impairment (as reviewed in Jarosz-Griffiths et al. 2016). However, the role of such complexes in Aβ oligomer induced Tau phosphorylation is not well defined.

The prion protein is the most documented Aβ oligomer receptor and mediator of Aβ-induced synaptic impairment (Lauren et al., 2009, Gimbel et al., 2010, Barry et al., 2011, Chung et al., 2010, Chen et al., 2010, Freir et al., 2011). The toxic actions of Aβ/PrP<sup>C</sup> require the formation of a multi-protein signalling complex (Rushworth et al., 2013, Um et al., 2012). Furthermore, this signalling complex provides a mechanistic link to Tau phosphorylation at tyrosine 18 (Larson et al., 2012). However, the role of the Aβ/PrP<sup>C</sup> complex in the phosphorylation of Tau at other residues is yet to be fully determined.

In addition, several lines of evidence suggest that PrP<sup>C</sup> expression is altered with age and in sporadic Alzheimer’s disease, further implicating PrP<sup>C</sup> in the pathogenesis of the disease. However, results between groups on the extent of these changes has been contradictory (Whitehouse et al., 2013, Whitehouse et al., 2010, Llorens et al., 2013, Vergara et al., 2015). Furthermore, a relationship between PrP<sup>C</sup> and Tau expression has also started to emerge and Aβ has been implicated in altering this relationship (Chen et al., 2013, Schmitz et al., 2014, Vergara et al., 2015). The exact relationship between PrP<sup>C</sup> and Tau, the mechanisms mediating this connection and the implications for the pathogenesis of Alzheimer’s disease are yet to be fully unravelled.
The aim of this body of work was to further explore the relationship between Aβ, PrP<sup>C</sup> and Tau in Alzheimer’s disease. Firstly by investigating the relationship between the expression of PrP<sup>C</sup> and Tau, how this relationship is altered following the progression of sporadic Alzheimer’s disease and the mechanisms by which PrP<sup>C</sup> influences the expression of Tau. Secondly, the cell surface signalling complex(es) connecting extracellular Aβ oligomers, PrP<sup>C</sup> and the aberrant phosphorylation of Tau was investigated. The potential implications for this work, the remaining unanswered questions and potential future directions of the research will be discussed in this chapter.

### 6.2 The relationship between PrP<sup>C</sup> and Tau expression

Data presented in this thesis revealed a specific relationship between the expression of PrP<sup>C</sup> and Tau in manipulated neuroblastoma cell lines, transgenic mouse models and human tissue from sporadic Alzheimer’s disease patients. Data from sporadic Alzheimer’s disease tissue showed a reduction in PrP<sup>C</sup> expression in multiple brain regions following the advancement of the Braak stages of the disease. Furthermore, the reduction in PrP<sup>C</sup> significantly correlated with the reduction in Tau expression, but also coincided with an increase in Tau pathology, as shown by an increase in phosphorylated and truncated Tau species and an increase in insoluble hyperphosphorylated Tau. Preliminary data from neuroblastoma cell lines also implicated the GPI-anchor and thus in part the lipid raft localisation of PrP<sup>C</sup> as mediating the alterations to Tau expression. Previously, the GPI-anchor and lipid raft localisation of PrP<sup>C</sup> was identified as essential for mediating the toxic actions of Aβ (Rushworth et al., 2013). Thus implicating a key role for the GPI anchor in PrP function and dysfunction.

One hypothesis is that the reduction in PrP<sup>C</sup> expression reflects a primary mechanism in Alzheimer’s disease pathogenesis and indirectly triggers the reduction in Tau expression mediated by the GPI-anchor and lipid raft localisation of PrP<sup>C</sup>. The reduction in Tau may contribute to neuronal destabilisation and disruption to neuronal function. Aβ oligomers may subsequently interact with PrP<sup>C</sup> on the cell surface triggering pathogenic signalling cascades which induce the aberrant phosphorylation of Tau, synaptic impairment and further exacerbate neuronal destabilisation.
Data presented in this thesis revealed no change in APP expression levels in sporadic Alzheimer’s disease. However, altered APP processing and fragments are involved in the pathogenesis of the disease (O’Brien and Wong, 2011) and has been linked to Tau proteostasis (Moore et al., 2015, Chen et al., 2013).

Moore, et al. 2015 identified a link between Tau levels and APP processing by β- and γ-secretase and implicated the APP C-terminal fragment, C99 in regulating these alterations, however, the mechanisms by which are unknown. Interestingly, PrP<sup>C</sup> normally inhibits β-secretase activity (Parkin et al., 2007, Whitehouse et al., 2013). However, the reduction in PrP<sup>C</sup> in Alzheimer’s disease and Aβ binding to PrP<sup>C</sup> reduces the inhibitory actions on β-secretase, thus altering APP processing and generation of the C99 fragment which may regulate Tau levels. Furthermore, PrP<sup>C</sup> normally exerts protective mechanisms against oxidative stress and neuroinflammation (Roucou et al., 2004), however, a loss of PrP<sup>C</sup> in Alzheimer’s and therefore an increase in oxidative stress and inflammation could further alter Tau proteostasis and/or induce aberrant changes to Tau such as phosphorylation and aggregation. It should also be taken into consideration that a reduction in Tau levels has previously been shown to protect against Aβ induced toxicity (Oddo et al., 2006b, Roberson et al., 2007), therefore, the reduction in Tau by PrP<sup>C</sup> in Alzheimer’s disease may be a neuroprotective compensatory mechanism.

Prion diseases are associated with the conversion and misfolding of PrP<sup>C</sup> into the β-sheet rich aggregation prone conformation, PrP<sup>Sc</sup> (Prusiner, 1998). Synaptic impairment, oxidative stress and inflammation are features of both prion disease and Alzheimer’s disease with aberrant PrP<sup>C</sup> function and signalling transduction cascades mediated by PrP<sup>C</sup> strongly implicated in the pathogenesis of both diseases. Furthermore, high levels of Tau but relatively unchanged levels of phosphorylated Tau have been measured in the CSF of patients with sporadic CJD (Hyeon et al., 2015). Interestingly, higher levels of phosphorylated Tau have been measured in the CSF of variant CJD patients (Goodall et al., 2006) and familial prion diseases are also linked to an increase in Tau phosphorylation (Reiniger et al., 2011). One hypothesis is that aberrant function/signalling cascades mediated by PrP<sup>C</sup> alter Tau expression and additional factors in Alzheimer’s disease and some prion diseases induce the aberrant phosphorylation of Tau. However, it remains to be determined the exact changes to Tau in the brain of sporadic CJD cases and how these link to PrP<sup>Sc</sup> conversion.
PrP<sup>C</sup> interacts with a range of proteins and cellular components, some of which may indirectly mediate the connection to intracellular Tau. PrP<sup>C</sup> is known to interact with the 37/67 kDa Laminin receptor (Rieger et al., 1997, Hundt et al., 2001). The Laminin receptor influences the stabilisation of PrP<sup>C</sup> at the cell surface (Sarnataro et al., 2016) and increased levels of the Laminin receptor have been identified in Scrapie infected cells which correlated with the accumulation of intracellular PrP<sup>Sc</sup> (Rieger et al., 1997). It remains to be determined whether the changes to the Laminin receptor occur in Alzheimer’s disease and whether these correlate to the reduction in PrP<sup>C</sup>. Furthermore, the Laminin receptor has previously been implicated in mediating the toxicity of Aβ by PrP<sup>C</sup> (Pinnock et al., 2015) and also interacts with γ-secretase possibly to influence its activity (Jovanovic et al., 2014). Therefore, inhibiting the Laminin receptor may provide therapeutic benefits to both prion disease and Alzheimer’s disease.

### 6.3 Aβ oligomer induced signalling complexes and Tau phosphorylation

PrP<sup>C</sup> plays a crucial role in the binding and subsequent synaptic impairment induced by Aβ oligomers (Lauren et al., 2009, Gimbel et al., 2010, Kostylev et al., 2015). Interaction of Aβ oligomers with PrP<sup>C</sup> activates the Src family kinase, Fyn (Um et al., 2012). Fyn phosphorylates the NR2B subunit of NMDA-receptors at tyrosine 1472 resulting in an initial increase in the cell surface expression of the receptor, an increase in intracellular calcium and excitotoxicity (Um et al., 2012). This transient increase in cell surface NMDA-receptors also triggers dendritic spine destabilisation by activating calcineurin (PP2B) in a calcium dependent manner which activates cofilin, the regulator of actin polymerisation and assembly (Shankar et al., 2007, Wu et al., 2010). The binding of Aβ oligomers to PrP<sup>C</sup> and the subsequent activation of Fyn is dependent on mGluR5 (Um et al., 2013) and LRP1 (Rushworth et al., 2013). Data presented in this thesis also revealed a key role for Flotillin-1 in mediating the binding of Aβ oligomers to PrP<sup>C</sup>, possibly by stabilising the lipid raft microdomain and localisation of PrP<sup>C</sup> within a lipid raft multi-protein complex. The interactions between Flotillin-1 and other components of the Aβ/PrP<sup>C</sup> signalling complex are yet to be determined.

The association of PrP<sup>C</sup> and mGluR5 is also implicated in the physiological function of mGluR5 and maintenance of synaptic function. It was recently reported that Aβ oligomers induce a pathological conformation of the PrP<sup>C</sup>/mGluR5 complex which prevents the
physiological activation and function of mGluR5 (Haas et al., 2016, Haas and Strittmatter, 2016). The physiological activation of mGluR5 triggers the dissociation and activation of Pyk2 and CamKII from PrP$_C$/mGluR5. Activation of the PrP$_C$/mGluR5 complex by Aβ oligomers also induces the dissociation and activation of Pyk2 but enhances the association of PrP$_C$/mGluR5 with CamKII (Haas and Strittmatter, 2016). CamKII is essential for LTP and spine growth/stabilisation, however, by enhancing its association within the PrP$_C$/mGluR5 complex, Aβ disrupts the normal function of CamKII (Jourdain et al., 2003, Barria and Malinow, 2005, Haas and Strittmatter, 2016). Pyk2 also plays a role in synaptic function and LTP and interestingly was identified as a genetic risk factor for Alzheimer’s disease (Rosenthal and Kamboh, 2014). The activation of Pyk2 is dependent on extracellular calcium, possibly by NMDA-receptor influx and Fyn kinase (Haas and Strittmatter, 2016). In addition, Pyk2 couples mGlur5 to GSK3β activation, a prominent serine and threonine Tau kinase (Kohler et al., 2013), thus possibly reflecting a mechanism by which the PrP$_C$/mGluR5 complex triggers Aβ oligomer induced Tau phosphorylation.

Normally, Tau is predominantly localised to the axons, however, in Alzheimer’s disease phosphorylated Tau has been identified in the somatodendritic region (Gotz et al., 1995). This mislocalisation of Tau correlates with dendritic spine loss and can be induced by Aβ oligomers and other cell stressors including oxidative stress and excitotoxicity (Zempel et al., 2010, Hoover et al., 2010). Furthermore, dendritic Tau is implicated in the increased association of Fyn with the post-synaptic density, enabling Fyn to be in close proximity to NMDA-receptors (Ittner et al., 2010). Fyn is able to phosphorylate Tau at tyrosine 18, a residue which is not normally phosphorylated in adults, but is aberrantly phosphorylated in Alzheimer’s disease (Lee et al., 2004, Um et al., 2012, Larson et al., 2012). Tyrosine phosphorylation of Tau at this residue facilitates the association between Tau and the SH2 domain of Fyn (Usardi et al., 2011) thus facilitating the aberrant mislocalisation of Fyn to the post-synaptic density and subsequent NMDA-receptor dysfunction.

The transient increase in cell surface NMDA-receptors is followed by internalisation and loss of NMDA-receptors at the cell surface, further contributing to dendritic spine loss and synaptic dysfunction (Rushworth et al., 2013, Um et al., 2012). Aβ is known to activate the phosphatase, STEP61 which opposes the actions of Fyn and dephosphorylates the NR2B subunit at tyrosine 1472. A reduction in phosphorylation at this site decreases the association between the NR2B subunit and the scaffolding protein PSD95 resulting in the subsequent destabilisation and internalisation of NMDA-receptors (Kurup et al., 2010, Snyder et al., 2005). Recently the scaffolding protein PSD95 has been implicated in
destabilising STEP61 in the post-synaptic density and inducing its proteasomal degradation (Won et al., 2016). The levels of PSD95 decrease with the progression of Alzheimer’s disease (Shao et al., 2011, Yuki et al., 2014) thus, PSD95 contributes to the destabilisation of NMDA-receptors directly at the cell surface but also indirectly via STEP61.

The role of PrP<sup>C</sup> in mediating the activation of STEP61 is yet to be determined however, STEP61 is known to be activated by calcineurin following Aβ binding to α7nACh receptors (Snyder et al., 2005). On the other hand, activation of mGluR5 by Aβ has been implicated in the internalisation of the GluA2 subunit of AMPA-receptors by STEP61 (Zhang et al., 2008, Zhang et al., 2011a, Herrold et al., 2013). Aβ oligomers do not directly bind to mGluR5, however, mGluR5 acts a co-receptor for Aβ binding to PrP<sup>C</sup> and this interaction mediates the activation of the glutamate receptor (Um et al., 2013). Despite this, it is hypothesised that Aβ oligomers interact with PrP<sup>C</sup> triggering activation of Fyn kinase which is subsequently followed by the activation of STEP61 by Aβ interacting with α7nACh receptors, all contributing to synaptic impairment and neuronal destabilisation.

Fyn and STEP61 are both promising targets for therapeutic intervention in Alzheimer’s disease and the inhibition of both Fyn and STEP61 rescues cognitive deficits in Alzheimer’s disease mouse models (Kaufman et al., 2015, Zhang et al., 2010). Interestingly, inhibition of Fyn but not STEP61 rescued Tau phosphorylation levels, further implicating Fyn in additional signalling cascades linking Aβ to the aberrant phosphorylation of Tau, possibly by the activation of Pyk2 which may mediate the activation of GSK3β.

The aberrant activation of mGluR5 has also been linked to Tau hyperphosphorylation via inactivation of the protein phosphatase, PP2A (Arif et al., 2014). PP2A accounts for approximately 70 % of the phosphatase activity in the brain (Martin et al., 2013a) and activity is reduced by around 50 % in Alzheimer’s disease (Gong et al., 1995). PP2A inhibition correlates with increased Tau phosphorylation and accumulation reflecting early neurofibrillary tangle formation in rodents (Gong et al., 2000, Kins et al., 2001). Thus implicating PP2A in the pathophysiology of Alzheimer’s disease, however, the role of Aβ oligomers and the cellular mechanisms by which the PrP<sup>C</sup>/mGluR5 complex mediate PP2A inactivation are unknown.

A recent study identified that the neurotoxin β-N-methylamino-l-alanine (BMAA) decreases PP2A activity and is implicated in the pathophysiology of parkinsonism-dementia of Guam, a neurodegenerative disease with parkinsonism and early-onset Alzheimer’s-like dementia associated with hyperphosphorylated Tau accumulated into neurofibrillary tangles (Arif et
The researchers identified that BMAA activates mGluR5 causing the catalytic subunit of PP2A to dissociate from mGluR5 where it is subsequently phosphorylated at tyrosine 307 by Src kinase in the cytoplasm and as a result is inactivated. Aβ oligomers are known to activate the mGluR5 receptor following binding to PrP\textsuperscript{C}, therefore, we hypothesised that Aβ oligomer/PrP\textsuperscript{C} mediated mGluR5 activation may result in PP2A inhibition, thus further contributing to Tau hyperphosphorylation in Alzheimer’s disease, Figure 6.1.

**Figure 6.1 Aβ oligomer induced PP2A inactivation?**

Activation of mGluR5 by glutamate reduces the association between mGluR5 and PP2A resulting in its dissociation and subsequent phosphorylation and inactivation. We propose that Aβ oligomers may interact with the PrP\textsuperscript{C}/mGluR5 complex, activating mGluR5 and trigger the subsequent dissociation and inactivation of PP2A which contributes to Tau hyperphosphorylation in Alzheimer’s disease.
6.4 Concluding remarks

Normally PrP\textsuperscript{C} protects neurons against oxidative stress, neuroinflammation, A\textbeta\ generation and is essential for synaptic function and plasticity (Roucou et al., 2004, Westergard et al., 2007). We revealed that PrP\textsuperscript{C} expression is reduced following the progression of Alzheimer’s disease, possibly contributing to increased inflammation, oxidative stress and A\textbeta\ levels, all features of Alzheimer’s disease and triggered by a toxic loss of PrP\textsuperscript{C} function (Heppner et al., 2015, Persson et al., 2014). We also show that the reduction in PrP\textsuperscript{C} strongly correlated with a reduction in Tau expression and coincided with increased Tau phosphorylation and truncation.

The reduction in PrP\textsuperscript{C} may reflect a primary mechanism of disease pathogenesis and the subsequent reduction in Tau possibly contributes to reduced neuronal stability and function. Furthermore, A\textbeta\ oligomers interact with PrP\textsuperscript{C} remaining on the cell surface hijacking PrP\textsuperscript{C} to induce aberrant signalling cascades. The glutamate receptor, mGluR5 plays a pivotal role in the aberrant signalling of PrP\textsuperscript{C} (Um et al., 2013, Haas and Strittmatter, 2016). PrP\textsuperscript{C}/mGluR5 signalling complexes are strongly implicated in neuronal destabilisation and synaptic dysfunction but may also provide a mechanistic link between extracellular A\textbeta\ oligomers and intracellular Tau phosphorylation, by Fyn kinase, Pyk2 and possibly by inactivation of PP2A, Figure 6.2.

A\textbeta\ oligomers interact with a multitude of receptors at the surface of neurons (Jarosz-Griffiths et al., 2016). It is likely that activation of multiple receptor complexes and signalling cascades is responsible for synaptic impairment and Tau phosphorylation induced by A\textbeta. Tau is known to be sequentially phosphorylated therefore, different signalling complexes may be involved in the phosphorylation of Tau at specific residues and possibly at different stages in the pathogenesis of disease. Considering there is now strong evidence that Tau is the mediator of A\textbeta toxicity, synaptic dysfunction and disease propagation in Alzheimer’s disease (Roberson et al., 2007, Ittner and Gotz, 2011, Pooler et al., 2013b, Bloom, 2014, Stancu et al., 2014, Oddo et al., 2006b) unravelling these signalling complexes will aid in our understanding of the pathogenesis of Alzheimer’s disease and the identification of much needed novel therapeutics.
Figure 6.2  Mechanisms of PrP<sup>C</sup> mediated dysfunction in Alzheimer’s disease

Normally in the brain PrP<sup>C</sup> mediates multiple neuroprotective mechanisms, including anti-inflammation, anti-oxidative stress, inhibition of β-secretase and maintenance of Tau levels. In addition, PrP<sup>C</sup> forms a complex with mGluR5 which mediates the activation of CamKII. CamKII mediates LTP and dendritic spine growth/stabilisation, contributing to normal synaptic function and neuronal stabilisation. On the other hand, the levels of PrP<sup>C</sup> are reduced in Alzheimer’s disease contributing to increased oxidative stress and inflammation and reduced levels of Tau. In addition to these toxic loss of functions, Aβ oligomers induce a pathological complex of PrP<sup>C</sup> and mGluR5 which prevents the actions of CamKII and induces aberrant signalling cascades resulting in Tau phosphorylation and further exacerbating dendritic spine loss and synaptic impairment.

Investigating Aβ oligomer induced Tau phosphorylation and the signalling complex(es) involved is limited by the methods used to model this. Although soluble oligomeric forms of Aβ are implicated as the pathogenic species, there is wide variation in the size and conformation of these oligomers used. It should be taken into consideration that slightly different oligomer species may interact with different receptor complexes, or at least to these complexes with different potencies, and trigger different signalling cascades. In the brain there is a heterogeneous mixture of Aβ and it is possible that more than one soluble oligomeric species is involved in the pathogenesis of Alzheimer’s disease, however, identifying the exact oligomorphic species in the brain has proven difficult. Furthermore, as
described in this thesis, modelling Aβ oligomer induced Tau phosphorylation *in vitro* has proved challenging. We suggest that a more functional, mature model is necessary to induce the complex mechanisms, such as Tau phosphorylation induced by Aβ oligomers. IPSC-derived neurons are capable of remodelling the disease phenotype of genetic neurodegenerative diseases including fAD and hold much promise for recapitulating the complexity of human disease and modelling novel therapies (Tiscornia et al., 2011, Sullivan and Young-Pearse, 2015). However, data presented in this thesis describe that they may not provide a suitable model to look at Aβ oligomer induced Tau phosphorylation. IPSC-derived neurons do not model functionally mature neurons and only express the already highly phosphorylated foetal isoform of Tau. In addition, we found that results of Tau phosphorylation (in controls and induced by Aβ oligomers) were highly variable between experiments. Although with increased maturation iPSC-derived neurons do become increasingly electrophysiologically active (Bergstrom et al., 2016, Handel et al., 2016), they still hold a foetal neuronal phenotype. Therefore, before we can model Aβ oligomer induced Tau phosphorylation and the cell surface signalling complexes involved there is need for a better cellular model to recapture a mature, functional neuronal population. This together with careful consideration to the Aβ oligomer preparation used is essential in order to further our understanding of the molecular and cellular mechanisms underlying Alzheimer’s disease.
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