Regulation of the metabolism of the Alzheimer's amyloid precursor protein by contactin 5 and BIN1

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Alzheimer's disease (AD) is a progressive form of dementia, which currently has no cure. The metabolism of the amyloid precursor protein (APP) is an important determinant in the development of AD. APP is proteolytically processed either in the amyloidogenic pathway, generating the AD-causing amyloid-beta (Aβ) peptide, or in the non-amyloidogenic pathway which prevents the generation of Aβ. The role of four different proteins, BIN1, contactin 5, neurofascin and Thy-1, in the regulation of APP proteolytic processing was investigated. Contactin 5, neurofascin and Thy-1 have been shown to interact with APP, whereas BIN1 has been genetically related to AD by genome-wide association studies. Over-expression of each protein in cell lines showed that BIN1 and contactin 5, but not neurofascin or Thy-1, regulate the production of Aβ. Contactin 5 over-expression prevented Aβ generation by inhibiting cleavage of APP by the γ-secretase. Western blots of human brain samples showed that contactin 5 levels in the brain are not altered during aging or in AD. BIN1 over-expression decreased APP levels, and decreased Aβ, whereas BIN1 knockdown increased APP, suggesting that BIN1 redirects APP from processing in the amyloidogenic pathway to a compartment of the cell where it is degraded. BIN1 did not alter either Aβ uptake, or the endocytosis or cell surface levels of APP. BIN1 levels were shown to be reduced in the brain during aging and in AD. This has led to the hypothesis that during aging and AD, BIN1 levels decrease, so more APP is trafficked into the amyloidogenic pathway rather than being degraded, resulting in more Aβ generation so increasing the risk of developing AD. This is the first time a mechanism for the role of BIN1 in AD has been suggested.

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Abbreviations

ABC	ATP binding cassette
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
AICD	Amyloid intracellular domain
APLP	Amyloid precursor-like protein
APP	Amyloid precursor protein
Aβ	Amyloid-beta
AβM	Amyloid-beta monomer
AβO	Amyloid-beta oligomer
BACE1	Beta-site amyloid precursor cleaving enzyme 1
BCA	Bicinchoninic acid
BIN	Bridging integrator 1
BSA	Bovine serum albumin
CNS	Central nervous system
CNTN	Contactin
CNTNAP	Contactin associated protein
CTFα	C-terminal fragment alpha
CTFβ	C-terminal fragment beta
DAPI	4',6-diamidino-2-phenylindol dihydrochloride
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSG	Fish skin gelatine
GPI	Glycosyl phosphatidylinositol
GSAP	Gamma-secretase activating protein
GWAS	Genome-wide association study
HEK	Human embryonic kidney
HRP	Horse radish peroxidase
IDE	Insulin degrading enzyme
KO	Knockout
KPI	Kunitz protease inhibitor
LB	Luria-Bertani
LRP1	Low density lipoprotein receptor-related protein 1
LTP	Long term potentiation
NEP	Neprilysin
NF155/NF186	Neurofascin 155 kDa neurofascin 186 kDa
NFT	Neurofibrillary tangles
NICD	Notch intracellular domain
NMDA	N-methyl-D-aspartate
NSE	Neuron-specific enolase
PBS	Phosphate buffered saline
PFA	Para-formaldehyde
PKC	Protein kinase C
PM	Post mortem
PS	Presenilin
S.D.	Standard deviation
SAD	Sporadic alzheimer’s disease
sAPPα	Soluble amyloid precursor protein alpha

sAPPβ	Soluble amyloid precursor protein beta
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
SORLA	Sorting protein-related receptor
TGN	Trans-Golgi network

1 Introduction

1.1 Discovery of Alzheimer's disease

Alzheimer's disease (AD) is a progressive form of dementia characterised by amyloid plaques and neurofibrilary tangles in the brain (figure 1.1) (Hardy and Allsop, 1991). It is named after Dr Alois Alzheimer, who discovered the disease in 1906. In 1901 Alzheimer observed a 51 year old patient, Auguste Deter, who presented symptoms such as loss of memory, delusions, and drastic mood swings. In 1906 when Auguste Deter died Alzheimer examined her brain and identified the plaques and tangles that are characteristic of the disease, and so was the first person to associate the symptoms of the disease with the brain pathology.

1.2 Current therapies for AD

Dementia affects 33.3 % of people over the age of 65 (figures from Alzheimer's Research UK). AD is the most common form of dementia and currently there is no cure or treatment that will slow down or halt the progress of the disease: current therapeutics focus on treating just the symptoms of the disease. SSRI (selective serotonin re-uptake inhibitors) are frequently used to treat depression or anxiety in Alzheimer's patients, while other psychiatric symptoms (such as delusions) are commonly treated with the drug Risperidone.

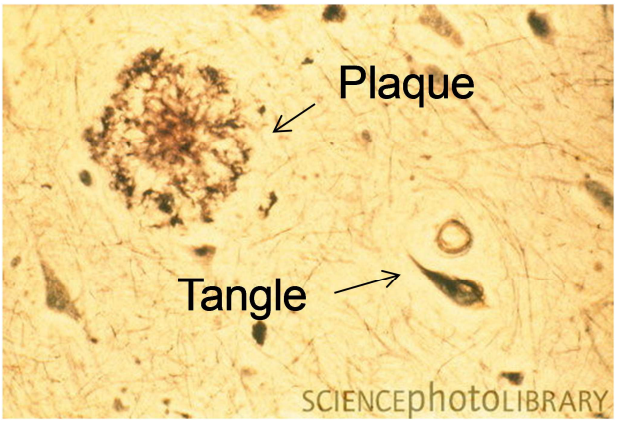

Figure 1.1 Pathological hallmarks of AD. Image showing Alzheimer's pathology in the brain, an amyloid plaque is at the top left of the image surrounded by black tau tangles. Image taken from sciencephoto.com.

Cholinesterase inhibitors (such as Donepezil and Galantamine) can be used to prevent the breakdown of the neurotransmitter acetylcholine, and improve general cognition (Citron, 2002). The NMDA (N-methyl-D-aspartate) receptor antagonist Memantine has been used in severe AD cases (over-stimulation of NMDA receptors has been linked to neuronal death in AD) to improve cognition and motor skills (Lleo et al., 2006; Mucke, 2009). Both Donepezil and Memantine have been shown to improve cognition in patients with severe AD, cholinesterase inhibitors in mild AD, and currently no drugs can benefit cognition in patients with mild cognitive impairment (O'Brien and Burns, 2011; Howard et al., 2012). Although NMDA receptor agonists and choline esterase inhibitors induce a small improvement in cognition, neither of these are disease modifying drugs – they cannot alter the pathology of AD and so do not halt or slow down progress of the disease.

1.3 Amyloid-β

The amyloid plaques that are characteristic of the disease are composed of a peptide called amyloid-beta (Aβ) (Masters et al., 1985). Aβ is secreted from brain cells throughout life; however in AD, Aβ aggregates to form toxic species which kill surrounding neurons. It is the death of neurons leading to the loss of synapses and neuronal networks which causes AD. Aβ is secreted from cells in a monomeric form, however it can aggregate into oligomers (AβOs), which range in size and structure, from dimers and trimers to much larger multimers that can be up to 200 nm in size. AβOs can aggregate further forming Aβ fibrils, which eventually deposit as the plaques of Aβ seen in AD (Shankar and Walsh, 2009).

Aβ is generated from cleavage of a larger membrane spanning protein, amyloid precursor protein (APP). APP can be proteolytically processed by one of two mutually exclusive pathways (figure 1.2). APP can be cleaved initially by α-secretase to generate a secreted N-terminal fragment – soluble APPα (sAPPα), and a C-terminal fragment which remains in the membrane – C83/CTFα. The C-terminal fragment is then cleaved by γ-secretase to generate p3 (a fragment which is secreted), and the C-terminal stub called amyloid precursor protein intracellular domain (AICD). This is the non-amyloidogenic pathway; 90 % of the APP in the cell is processed in the non-amyloidogenic pathway (Gandhi et al., 2004). Alternatively APP can be cleaved first by β-secretase, to generate the secreted fragment soluble APPβ (sAPPβ) and the β C-terminal fragment – C99/CTFβ. The C-terminal fragment is then cleaved by γ-secretase to generate Aβ (the toxic fragment implicated in AD), and the C-terminal stub AICD (Evin and Weidemann, 2002; Vardy et al., 2005). Under normal circumstances only 10 % or less of the APP in the cell is processed in the amyloidogenic pathway generating Aβ. The exact location of cleavage in C99 by γ-secretase varies generating Aβ molecules of varying lengths. The most important Aβ species for AD are $A\beta_{40}$ and $A\beta_{42}$ (40 and 42 amino acids long, respectively). Longer Aβ species are more prone to aggregation and are more toxic (Pike et al., 1995; Klein et al., 1999). The proteolytic processing of APP is discussed in further detail in section 1.6.

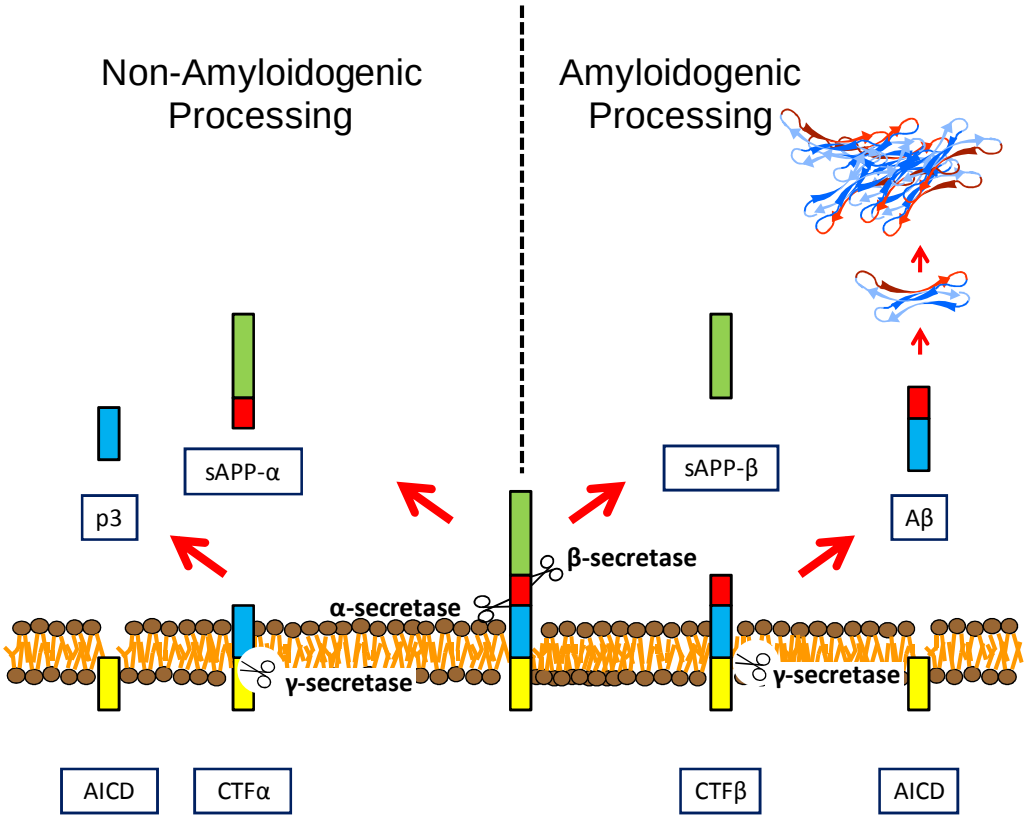

Figure 1.2 Proteolytic processing of APP. APP is processed on either the non-amyloidogenic pathway (left) or the amyloidogenic pathway (right). On the non-amyloidogenic pathway APP is cleaved initially by α-secretase, generating sAPPα and C83/CTFα. CTFα is then cleaved by γ-secretase generating p3 and AICD. On the amyloidogenic pathway APP is initially cleaved by β-secretase, generating sAPPβ and C99/CTFβ. CTFβ is then cleaved by γ-secretase generating Aβ and AICD. The Aβ peptides can build up and aggregate forming the plaques that are the hallmark of AD.

1.4 The amyloid cascade hypothesis

The amyloid cascade hypothesis proposes that the primary cause of AD is aggregation of the amyloid-β peptide, and has been the most widely accepted theory in the field since it was proposed in 1991 by John Hardy and David Allsop (Hardy and Allsop, 1991) then updated by John Hardy and Gerry Higgins (Hardy and Higgins, 1992). Since then it has been re-appraised several times by John Hardy and Dennis Selkoe (Hardy and Selkoe, 2002; Hardy, 2006). There are two forms of AD, familial/early onset AD (FAD) and sporadic/late onset AD (SAD). Both FAD and SAD are characterised by build up and deposition of amyloid in the brain, but FAD occurs earlier in life (around 50 years as opposed to 80). FAD is caused by inheritance of dominant mutations in genes which drastically increase the production or aggregation of the Aβ peptide (Murrell et al., 1991; Citron et al., 1992; Tomita et al., 1997; Su and Ni, 1998). It is this observation that forms the basis of the amyloid cascade hypothesis. Hardy, Higgins and Selkoe proposed that in FAD mutations in the APP and presenilin genes (the presenilin proteins form part of the γ-secretase complex involved in the production of Aβ) cause increased production of $A\beta_{42}$, which then accumulates and aggregates. These aggregates have subtle detrimental effects on synapses and activate microglia, which then cause synaptic injury, altering signalling pathways in neurons so altering the activities of kinases and phosphatases. This leads to, among other things, hyperphosphorylation of tau protein. Hyperphosphorylated tau aggregates and is unable to perform its normal function: stabilising microtubules. The combined effects of altered signalling pathways, hyperphosphorylated tau, and activated microglia causes neuronal death, resulting in dementia (figure 1.3) (Hardy and Selkoe, 2002; Ballatore et al., 2007).

The amyloid cascade hypothesis is also applied to SAD, though the initial event that causes increased levels or increased aggregation of Aβ is yet to be determined.

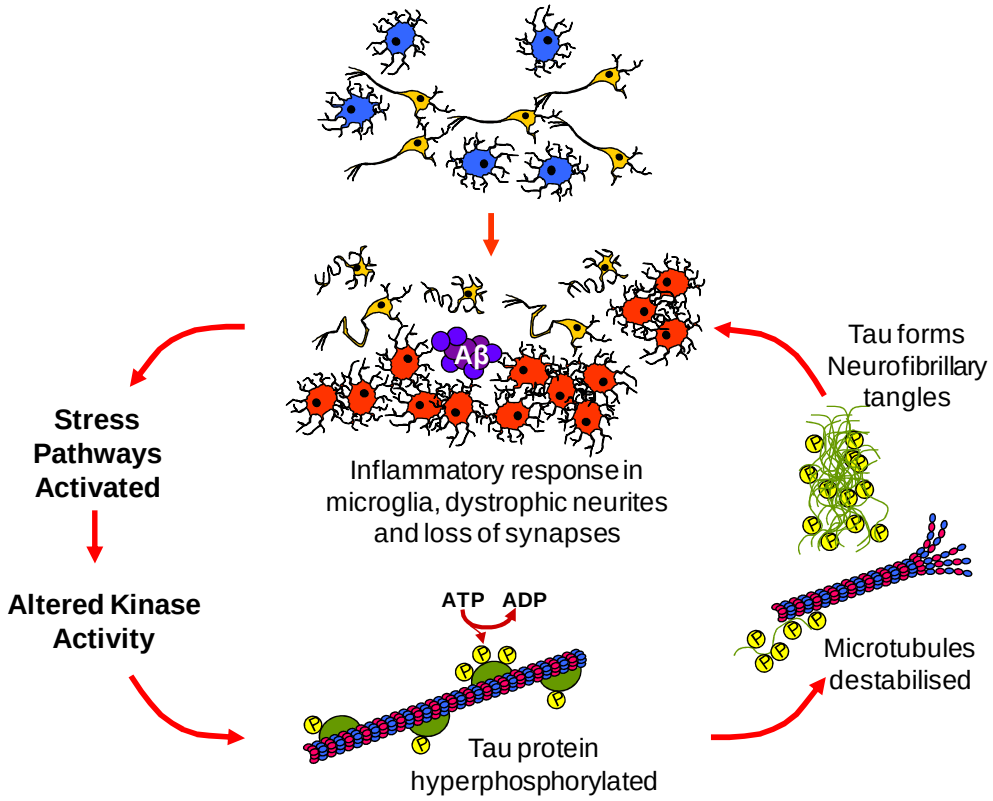

Figure 1.3 The amyloid cascade hypothesis. Mutations in APP or presenilin proteins (in FAD), or an unknown initiating event (in SAD) cause a build up and aggregation of the Aβ peptide. The Aβ aggregates are toxic to neurons (shown in yellow), causing neuronal death and dystrophy, synaptic loss, and activate microglia (the immune system cells of the brain, shown in blue, active microglia are shown in red) stimulating an inflammatory response. The activated microglia and Aβ aggregates activate stress pathways in neurons altering kinase and phosphorylase activity causing the microtubule binding protein tau to become hyperphosphorylated. Hyperphosphorylated tau no longer binds to microtubules causing them to become de-stabilised, and aggregates to form neurofibrillary tangles within the neuron. This causes further damage to the neuron and disrupts transport around the cell, causing further neuronal loss and activation of microglia.

1.4.1 Critical analysis of the hypothesis

Since its proposal there has been a lot of criticism of the amyloid cascade hypothesis. Many people argue that it is out of date and amyloid toxicity alone cannot explain the development of AD. This has lead to several attempts to update and modify the amyloid cascade hypothesis making it specific for sporadic AD, and to accommodate new findings. For example it has been suggested that amyloid deposition along with some initiating injury (such as head trauma, diabetes, life stresses or vascular events) cause a chronic inflammatory response in the brain. This inflammatory response triggers a shift in cellular physiology re-initiating cell cycle proteins (which are normally turned off) putting these neurons at an increased risk of death (Chen et al., 2010; Herrup, 2010). However it should be pointed out that the initiating injuries proposed to cause cell cylcle re-initiation can also cause chronic hypoxia in the brain. Chronic hypoxia has been linked to AD, as it causes an increase in generation of Aβ peptides which then dyrupt calcium homeostasis of neurons (Webster et al., 2006; Peers et al., 2007; Peers et al., 2009), providing and alternative explanation for the link between these “initating injuries” and development of AD.

Many people have also argued that hyperphosphorylated tau aggregates rather than Aβ are the primary cause of AD, but there is a significant volume of evidence supporting the amyloid cascade hypothesis. For example, mutations in presenilin proteins and APP that increase $A\beta_{42}$ or alter the ratio of $A\beta_{40}$ and $A\beta_{42}$ cause familial AD (Murrell et al., 1991; Citron et al., 1992; Tomita et al., 1997), and recreating these mutations in mice causes amyloid pathology (Masliah et al., 1996; Su and Ni, 1998) as well as AD-like symptoms (such as memory deficits) demonstrating that altered amyloid metabolism alone is sufficient to cause AD. Aβ is an aggregation-

prone peptide and exists in a natively unfolded conformation – it is well known that aggregation-prone molecules can be detrimental to the survival of cells, and Aβ has been shown to be toxic in tissue culture models (Lambert et al., 1998; Grace et al., 2002) suggesting it is the toxic causative species of AD. Mutations in tau do cause dementia, but do not cause any amyloid deposition. Tau tangles occur in other neurodegenerative diseases suggesting that in AD tau pathology occurs downstream from amyloid pathology (Oddo et al., 2003; Bolmont et al., 2007). Furthermore, introducing APP mutations into transgenic tau mice increases the number of neurofibrillary tangles (NFT) of tau, further confirming that amyloid pathology can induce tau hyperphosphorylation and NFT formation (Hardy and Selkoe, 2002; Hardy, 2006). Neurons derived from fibroblasts taken from AD patients have been reverted back into stem cells, then re-differentiated into neurons. In some cases these neurons secrete increased levels of Aβ compared to control cells, and have increased levels of phosphorylated tau, confirming the importance of both amyloid and tau metabolism in development of AD pathology (Israel et al., 2012). A major criticism of the amyloid cascade hypothesis is the existence of non-demented individuals who have extensive amyloid and tau pathology in their brains, yet lack any symptoms of AD. Recent evidence, though, shows that they do not have oligomeric forms of Aβ at the synapses whereas patients with AD pathology and AD symptoms do (Bjorklund et al., 2012) suggesting that both the aggregation state and location of Aβ is important for the development of the disease. Some of the most convincing evidence in support of the amyloid cascade hypothesis comes from the very recent discovery of a mutation in APP that is protective against AD. The $A_{673}T$ mutation in APP decreases β-cleavage of APP and so decreases Aβ generation (Jonsson et al., 2012). The mutation was shown to be enriched in a group of people over the age of 85 without any form of dementia compared to over-85s with AD, demonstrating that

preventing Aβ generation can protect against AD in the human brain (Jonsson et al., 2012).

However, unanswered questions remain about the amyloid cascade hypothesis. Aβ load does not correlate well with severity or progression of AD, Aβ plaques have been identified in cognitively normal patients, and although drugs designed to reduce Aβ load or Aβ aggregation have been successful in AD mouse models, they have not been successful in humans. Despite years of research and drug development no amyloid altering drugs have passed clinical trials for AD (Pimplikar, 2009), demonstrating that there is still much to learn about the development and pathogenesis of AD.

1.5 The metabolism of amyloid precursor protein

1.5.1 APP isoforms and homologues

Aβ is generated from proteolysis of the transmembrane protein APP. APP is located on chromosome 21 (21q21) and is highly conserved (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987a; Robakis et al., 1987b) suggesting it carries out vital functions in the cell. There are several isoforms of APP; the main isoforms are 695, 751 and 770 (695, 751 and 770 amino acid residues long respectively) (Ponte et al., 1988). The 751 and 770 isoforms are expressed by most cells, whereas the 695 isoform is predominantly neuronal (Weidemann et al., 1989; Kang and Muller-Hill, 1990). The 751 and 770 isoforms contains the kunitz inhibitor domain (Ponte et al., 1988). APP comes from a superfamily of proteins: there are APP homologues in other species as well as the human homologues amyloid precursor-like protein 1 and amyloid precursor-like protein 2 (APLP1 and APLP2) (Wasco et al., 1992; Sprecher et al., 1993; Wasco et al., 1993; Webster et al., 1995). APLP1

and two have a similar structure to APP, they have similar alternative splicing (Sandbrink et al., 1994), they are phosphorylated and glycosylated in a similar way to APP (Suzuki et al., 1997; Eggert et al., 2004), and they undergo similar processing events to APP. APLP1 and 2 are cleaved by the APP α-secretases, BACE1 and γ-secretase (Gu et al., 2001; Scheinfeld et al., 2002; Walsh et al., 2003; Eggert et al., 2004; Li and Sudhof, 2004; Endres et al., 2005; Cong et al., 2011; Hogl et al., 2011). γ-secretase cleavage of APLP proteins generates an intracellular transcriptionally active fragment which is translocated to the nucleus (Scheinfeld et al., 2002; Walsh et al., 2003; Li and Sudhof, 2004), like AICD generated from APP (for details about AICD see section 1.7.2). Unlike Aβ, the fragments generated from the APLPs following proteolytic processing do not aggregate and are not toxic (Eggert et al., 2004; Minogue et al., 2009). The expression of APP, APLP1 and APLP2 all overlap in the brain (Crain et al., 1996; McNamara et al., 1998) suggesting they have redundant or overlapping functions, however knockout mice show subtly different phenotypes, and there are differences in the subcellular localisation of APP and APLPs demonstrating that there are differences in their functions (Kim et al., 1995; Heber et al., 2000).

1.5.2 The physiological function of APP

The normal role of APP in the cell is still unclear, though many functions have been proposed. Much of the evidence for physiological functions of APP comes from knockout studies of APP homologues in other species. Since the phenotypes caused by knockout of APP homologues can often be rescued by expression of human APP (Luo et al., 1992) it is reasonable to assume that its functions are conserved. It has been suggested that APP may have a role in development – for example blocking APP with an anti-APP antibody can prevent induction of neuronal

differentiation of embryonic stem cells (Freude et al., 2011), and knockout of the *Caenorhabditis elegans* homologue of APP, APL-1, causes larval death due to disrupted development of the larvae (Hornsten et al., 2007). Over-expression of human APP and APLP proteins in *Drosophila melanogaster* affects the development of mechano-sensory organs (e.g. the sensory hairs on the insect's legs) (Merdes et al., 2004). APP may also have roles in stem cell differentiation and development of neurons (Hiltunen et al., 2009). Knockout and over-expression studies of the *Drosophila* APP homologue APPL have shown that APPL has a role in the development of synapses and in the transport of both organelles and proteins along axons, indicating APP may be involved in the development and the normal functioning of neurons (Torroja et al., 1999; Gunawardena and Goldstein, 2001). APP can bind several cell surface proteins, so may perform this function by acting as a receptor or through signal transduction. APP has been shown to promote synaptogenesis by functioning as an adhesion molecule; Wang et al. (2009) proposed that APP has a role in the formation of synapses at the neuromuscular junction via direct interaction between APP at the synapse and muscle (Wang et al., 2009). APP can also bind to glycosaminoglycans and collagens so may be important for cell adhesion and neural outgrowth (Evin and Weidemann, 2002).

APP has been shown to be involved in the regulation of endocytosis of the choline transporter. The choline transporter removes choline from the synaptic cleft, so APP may regulate acetylcholine metabolism (acetylcholine is a neurotransmitter with roles in learning, memory and muscle control) (Wang et al., 2007). APP can reduce Cu^{2+} to Cu^{+}, so may be an anti-oxidant (Evin and Weidemann, 2002). APP can also bind Fe^{2+} and facilitates its oxidation it to Fe^{3+}, and loss of APP expression causes iron accumulation so it has been suggested that that APP acts as a ferroxidase in neurons (Duce et al., 2010).

1.5.3 APP knockout mice

APP knockout mice have been generated (Zheng et al., 1995; Zheng et al., 1996). These were shown to be fairly normal: they had normal brain structure, and were fertile, however the brains of some mice displayed reactive gliosis (activation of microglia which suggests there was some neuronal damage) and all knockout mice had decreased body weight, decreased grip strength and decreased locomotor activity. Mice with the APP gene disrupted (by insertion of a neomycin resistance gene) also show these phenotypes, however unlike the APP knockout mice they did not have normal brains – some mice did not have a corpus callosum, and the mutant APP mice performed less well than wild type in a Morris water maze test (Muller et al., 1994). A possible explanation for the discrepancy between these studies is that the neomycin insertion mice were not complete knockouts for APP – they expressed very low levels of a truncated form of APP.

The single knockout studies suggest that either APP does not have a major physiological role or that its functions are conserved by another protein. This idea is backed up by knockout of other members of the APP protein family. Knockout of APLP2 in mice did not cause any drastic phenotypes – the mice were fertile, of average size, were not cognitively impaired and displayed normal axon outgrowth. However 80% of APP/APLP2 double knockout mice died within a week of birth, the mice that survived were decreased in weight and displayed ataxia, trouble self righting, and developed gliosis by 9 months of age (von Koch et al., 1997) suggesting that APP and APLP2 can substitute for each other functionally in the brain. Although there were no gross changes in the phenotype of APP knockout mice, later studies identified several subtle deficits such as in increase in sensitivity

to seizures (Harper et al., 1998; Steinbach et al., 1998), altered neuron morphology and decreased synaptic plasticity (Seabrook et al., 1999),

1.5.4 APP synthesis and trafficking in the secretory pathway

APP is a single span transmembrane protein with its C-terminus on the cytoplasmic side of the membrane (Dyrks et al., 1988; Yamazaki et al., 1995). It is synthesised in the cell body on endoplasmic reticulum ribosomes, then is trafficked through the secretory pathway to the cell surface of axons. Once it reaches the cell surface APP is endocytosed and either recycled or degraded (figure 1.4) (Tienari et al., 1996). APP is synthesized in an immature form, which matures when it is N-glycosylated in the ER and O-glycosylated in the Golgi (Dyrks et al., 1988; Pahlsson et al., 1992; Saito et al., 1993; Graebert et al., 1995; McFarlane et al., 1999) and sulphonated, (Schubert et al., 1989) increasing the molecular weight by 4-5 kDa (Graebert et al., 1995). Most APP then remains stored in the trans-Golgi network (TGN) (Weidemann et al., 1989; Graebert et al., 1995). N- but not O-glycosylation is required for cleavage of APP by the secretases and secretion of the soluble APP fragments (Yazaki et al., 1996; Tomita et al., 1998; McFarlane et al., 1999) (though it is not clear whether N-glycosylation of APP itself, or N-glycosylation of other proteins is required for the normal trafficking of APP (Pahlsson and Spitalnik, 1996)). APP is transported from the Golgi to the cell surface, then endocytosed in a clathrin-dependent manner (Culvenor et al., 1995; Jung et al., 1996; Marquez-Sterling et al., 1997; Cossec et al., 2010).

The majority of APP is recycled back to the cell surface, but some APP is trafficked to endosomes (the acidic compartment where β-cleavage occurs (Schrader-Fischer and Paganetti, 1996)), or to lysosomes where it is completely degraded (Caporaso et al., 1992; Lai et al., 1995; Yamazaki et al., 1996). γ-cleavage

of APP occurs after β-cleavage, and has been reported in a number of subcellular locations (Kaether et al., 2006). Very little APP is found at the cell surface at any given time – it is either very rapidly endocytosed (70 %) or cleaved by α-secretase (30%) (Koo et al., 1996). The half life of APP is relatively short (3.5 hours), APP is glycosylated (so becomes the mature form) within two hours of being synthesised, and is transported along the axon to the synapse on the fastest wave of axon transport, and is degraded within 2-4 hours of glycosylation (Lai et al., 1995; Lyckman et al., 1998).

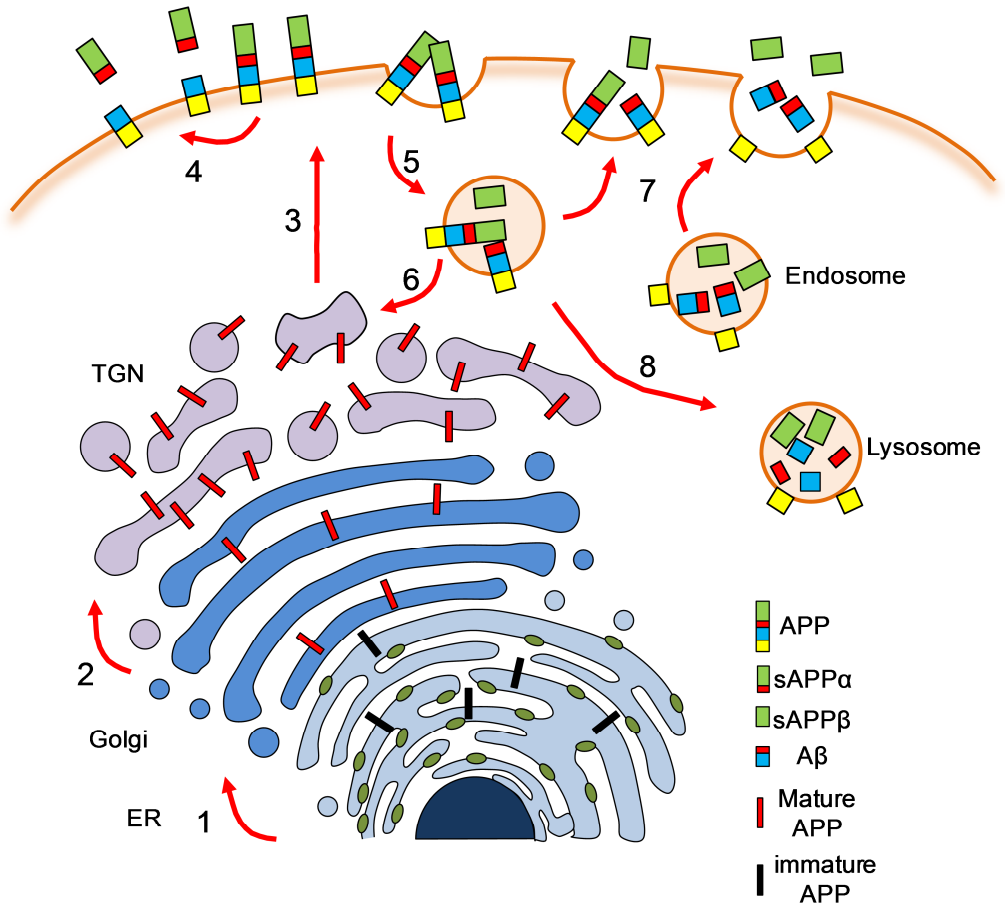

Figure 1.4 Amyloid precursor protein trafficking. APP is synthesized in the rough endoplasmic reticulum (ER) then transported to the Golgi where it is glycosylated and sulphonated to become the mature form of the peptide (1). Most of the APP in the cell resides in the trans-Golgi network (TGN) (2). APP is trafficked to the cell surface (3), when it reaches the cell surface some is cleaved by α-secretase generating sAPPα (4), but most is re-endocytosed (5). After endocytosis APP can be transported back to the TGN (6) or is cleaved by β-secretase and γ-secretase generating sAPPβ and Aβ which are both secreted (7). Some APP is completely degraded after it has been endocytosed (8).

The trafficking of APP depends on both the correct glycosylation of the ectodomain, and also on a signal sequence in the C-terminus (Tienari et al., 1996). The GYENPTY and the YSTI motifs in the C-terminus of APP are required for the internalisation and then either recycling of APP back to the cell surface or its degradation in lysosomes (Lai et al., 1995). The phosphorylation state of residues within the C-terminus of APP is important in the regulation of APP endocytosis. Phosphorylation of Y_{682} and Y_{687} in the GYENPTY motif increases endocytosis of APP, and so increases β-cleavage of APP (Rebelo et al., 2007; Barbagallo et al., 2010). The GYENPTY motif is the most important determinant for APP endocytosis (Perez et al., 1999; Cescato et al., 2000) but it, and the YTSI motif, are also important for insertion of APP into vesicles of the TGN and transport to the cell surface (Rebelo et al., 2008; Vieira et al., 2009). Phosphorylation of proteins that bind the C-terminus of APP is also important in regulating its metabolism. For example X11L binds the APP C-terminus and stimulates APP endocytosis. X11L can be phosphorylated by Src kinase, phospho-X11L stimulates APP endocytosis and transport to endosomes and lysosomes, whereas non-phosphorylated X11L stimulates APP endocytosis then recycling back to the cell surface (Chaufty et al., 2012). The endocytosis of APP and the role of endocytosis in AD are discussed in section 1.11. For a review of APP trafficking see Selkoe et al 1996 or Thinakaran and Koo 2008 (Selkoe et al., 1996; Thinakaran and Koo, 2008).

1.5.5 Lipid rafts and the role of cholesterol in APP processing

Amyloidogenic processing of APP occurs in lipid rafts (Ehehalt et al., 2003). Lipid rafts are small nanometer sized domains within the cell membrane which have a specific lipid composition – they are typically high in cholesterol and sphingolipids (Lingwood and Simons, 2010). Diffusion of proteins located in these

microdomains is limited to the individual lipid raft whereas non-raft resident proteins are much more mobile and diffuse throughout the cell surface (Giannone et al., 2010). It has been suggested that lipid rafts cluster with other lipid rafts facilitating the interaction of proteins between rafts, and may act as a way to control protein interactions at the cell surface (Simons and Gerl, 2010). This may be one way of regulating the proteolytic processing of APP, as amyloidgenic processing (β-cleavage) occurs in lipid rafts, whereas non-amyloidogenic processing (α-cleavage) occurs in non-raft regions of the membrane (Lakshmana et al., 2008).

APP metabolism is strongly influenced by cholesterol levels. Expression of the cholesterol exporting channels ABCA7 and ABCG1 in cells, both of which are highly expressed in the hippocampus, have been shown to reduce APP processing (Tansley et al., 2007; Chan et al., 2008). Knockout of the cholesterol exporting channel ABCA1 in mice followed by treatment with a cholesterol acceptor decreased the cholesterol content of cell membranes and decreased $A\beta_{40}$ secretion (Burns et al., 2006). The interaction between APP and BACE1 occurs within lipid rafts, so reducing the cholesterol levels reduces the area of lipid rafts, causing APP to shift out of lipid rafts and into non-raft areas where it will interact with α-secretase rather than BACE1, increasing α-cleavage (Kojro et al., 2001; Ehehalt et al., 2003). Increasing cholesterol levels increases APP and BACE1 co-localisation so increases β-cleavage of APP and Aβ generation (Marquer et al., 2011). APP itself binds cholesterol in a 1:1 ratio; this interaction may help partition APP into lipid rafts and so increase amyloidogenic processing of APP. Furthermore the cholesterol binding site in APP is made up from residues in the transmembrane domain which are adjacent to the α-cleavage site. It has been suggested that the binding of APP to cholesterol may prevent the α-secretase–APP interaction so promoting amyloidogenic processing of APP (Barrett et al., 2012). Cholesterol can also

influence the activity and the specificity of γ-secretase, Osenkowski et al showed that increasing the cholesterol composition of liposomes containing purified γ-secretase increased the generation of Aβ then further increases in cholesterol decreased Aβ generation. Cholesterol levels also influenced the length of the Aβ produced with higher concentrations of cholesterol increasing production of longer Aβ species relative to shorter Aβ (Osenkowski et al., 2008). Many proteins that regulate the location of APP (i.e. raft vs. non-raft regions) regulate the processing of APP. For example LRP1 (Low density lipoprotein receptor-related protein 1) binds the C-terminus of APP and re-locates it to lipid rafts, increasing Aβ and sAPPβ generation while decreasing sAPPα production (Lakshmana et al., 2008).

1.6 The proteolytic processing of APP

The different proteolytic cleavages of APP occur in distinct subcellular locations and are carried out by specific secretases (see figures 1.2 and 1.4).

1.6.1 α-cleavage

There are several candidate proteases for α-secretases, all of which are members of the ADAMs (A Disintegrin And Metalloprotease) family of proteases: ADAM9, 10 and 17. Increasing expression of each of ADAM9, 10 and 17 increases sAPPα production, whereas knocking down expression decreases sAPPα production. ADAM9, 10 and 17 are all expressed in the brain (Asai et al., 2003; Allinson et al., 2004). It appears that all of these cleave APP *in vivo*, and they may be able to functionally substitute for each other. For example, knockout of ADAM10 causes a reduction in sAPPα, though not complete inhibition (Hartmann et al., 2002; Vardy et al., 2005). Although ADAM17 is capable of cleaving APP, its inhibitor profile and kinetics do not match with physiological observations for α-secretase (Parvathy et

al., 1998), so it is most likely not the main enzyme responsible for α-cleavage of APP (Allinson et al., 2003). ADAM17 cleavage of APP is stimulated by protein kinase C activation, this occurs at the cell surface and intracellular compartments (such as the late Golgi/ TGN), so it maybe involved in stimulated rather than constitutive α-cleavage of APP (Jolly-Tornetta and Wolf, 2000).

1.6.2 β-cleavage

β-secretase has been identified as BACE1 (β-site APP cleaving enzyme). BACE1 was identified in a screen of a cDNA library from HEK (human embryonic kidney) cells, when expression of one of the cDNA clones increased generation of Aβ (Vassar et al., 1999). Purification of this clone demonstrated that BACE1 had the substrate specificity, optimum pH and inhibitor profile for β-secretase. BACE1 is an aspartyl protease, with a transmembrane domain and an active site made from two separate motifs. It is expressed at low levels by most tissues of the body, including the brain. In the brain BACE1 expression is highest in the hippocampus, the cortex and cerebellum, whereas expression in glial cells is very low/non-existent (however BACE1 expression has been detected in reactive astrocytes surrounding amyloid plaques in mouse brain (Rossner et al., 2001)). Expression of HA-tagged BACE in HEK cells showed it is localised to the Golgi, and endosomes (where it is at its optimum pH), with small amounts in the ER and lysosomes (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE1 in the endoplasmic reticulum is in its immature form, it is cleaved by a furin in the Golgi to form mature BACE1 (Benjannet et al., 2001). BACE1 has a transmembrane domain that is required for its activity (Yan et al., 2001). Most BACE1 cleavage of APP occurs in the late TGN and endosomes (an acid environment is required for activity), however there is some

in the ER (Schrader-Fischer and Paganetti, 1996; Benjannet et al., 2001; Yan et al., 2001). BACE1 as β-secretase is reviewed in (Cole and Vassar, 2007).

1.6.3 γ-cleavage

γ-secretase is a complex composed of several sub units: Aph1a, Pen-2, nicastrin and either presenilin 1 or 2 (De Strooper, 2003; Vardy et al., 2005) (but it can function in the absence of nicastrin) (Zhao et al., 2010). γ-secretase is an aspartyl protease, and the presenilin subunit (either PS1 or PS2 can be present) provides the catalytic aspartic acid residues. Pen-2 is required for an activating cleavage of the presenilin. The other subunits may only be required for stability and maturation of the complex (De Strooper, 2003). Mutations in γ-secretase account for more than half the FAD cases demonstrating its importance in the generation of Aβ in AD (Vardy et al., 2005). γ-secretase cleavage of APP has been reported in a number of subcellular locations such as the nuclear envelope, the ER, the trans-Golgi network (TGN), the cell surface and late endosomes. The majority of γ-cleavage however, occurs after APP has been transported to the cell surface, then endocytosed (Kaether et al., 2006; Hare, 2010). It has also been reported that $A\beta_{40}$ and $A\beta_{42}$ generation occurs in separate subcellular locations (Hartmann et al., 1997; Xu et al., 1997).

There is some variability in the cleavage sites of the secretases on APP. α-secretase cleaves APP at L_{17} of Aβ, but other α-cleavage sites have been identified around this area (at E_{11} and Q_{15}). These are called α', and are thought to be due to several different enzymes being responsible for α-cleavage (Simons et al., 1996). There is also an additional β-cleavage site, called β' (the β-cleavage site is D_1 of Aβ, whereas the β' site is at E_{11}). Both β and β' cleavage of APP are carried out by BACE1 (Vassar et al., 1999). The γ-secretase cleavage site is not very sequence

specific which means a range of different sized Aβ peptides are produced: it can vary from 39 to 43 amino acids long (Vardy et al., 2005). It has been hypothesised that this is because γ-secretase initially cleaves C99 at the ε-site genereating $A\beta_{48}$ or $A\beta_{49}$, then continutes "nibbling" at the C-terminus of Aβ sequentially removing two or three amino acids at a time resulting in the generation of a range of Aβ species (Takami et al., 2009).

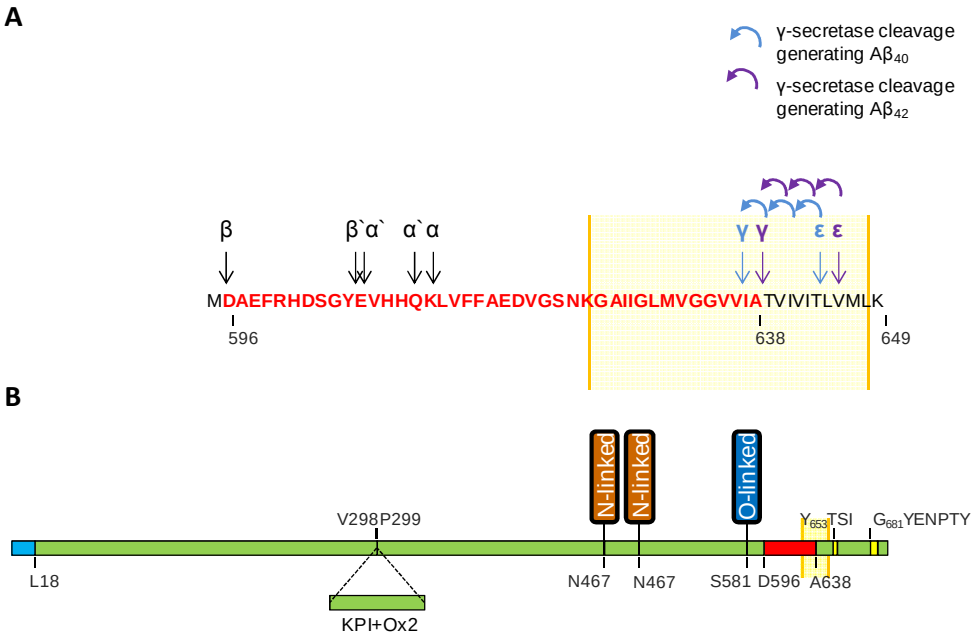

Figure 1.5 Amyloid precursor protein structure and cleavage sites. (A) The cleavage points of APP by α- β- and γ-secretase. α and α' cleavages are carried out by α-secretase, β and β' cleavages are carried out by BACE1, and γ and ε cleavages are carried out by the γ-secretase complex. Cleavage at the β- and either of the γ-sites generates Aβ (shown in red). Numbering of the residues corresponds to the 695 isoform of APP. The yellow box represents the membrane. (B) Scale diagram of APP_{695} isoform. The signal sequence is shown in blue, Aβ in red, and motifs important for endocytosis and trafficking in yellow. The pale yellow box represents the membrane. "N-linked" represents the N-linked glycosylation on APP. "O-linked" represents the complex O-linked glycosylation. The location of the KPI and Ox2 domain splice site is shown by the insert.

1.7 The functions of APP metabolites

The different proteolytic cleavages of APP result in the generation of a number of different fragments, and these have been demonstrated to have different roles within the cell. sAPPα has been shown to be neuroprotective, whereas sAPPβ is toxic. The intracellular fragment AICD can be transported to the nucleus where it is transcriptionally active and alters gene expression. It is unclear what the physiological function of Aβ is but it appears to have roles in cell survival, and may be involved in modulation of current through ion channels (Plant et al., 2006).

1.7.1 Neuroprotective functions

sAPPα is has been demonstrated to be protective against proteosomal stress, UV induced stress, excitoxicity (Mattson et al., 1993; Copanaki et al., 2010), and it decreases the effects of neuronal injury (Thornton et al., 2006). Over-expression of ADAM-10 (α-secretase) in an AD mouse model increases sAPPα, and decreases Aβ sAPPβ levels, which improves both LTP (long term potentiation, a process that is important in the formation of memories) and learning and memory deficits usually associated with these mice (Postina et al., 2004). Addition of sAPPα or sAPPβ to primary neurons increases axon growth and decreases the number and growth of dendrites (Jin et al., 1994). The axonal growth effects of sAPPα are mediated by MAP kinase (Chasseigneaux et al., 2011). sAPPα and sAPPβ have both been shown to induce neural differentiation in embryonic stem cells (Freude et al., 2011), suggesting they (and APP) may have roles in development. The C-terminal fragment produced by α-cleavage of APP may also have neuroprotective functions. CTFα can inhibit γ-secretase: it binds γ-secretase but is a poor substrate (this is modulated by residues 17-23 of CTFα), so inhibits Aβ production (Tian et al., 2010). Even Aβ in its monomeric rather than aggregated form has been shown to be neuroprotective to

developing neurons again suggesting a role for APP in development (Giuffrida et al., 2009). Aβ at low concentrations is neuroprotective and enhances LTP, it is only at higher concentrations when it becomes toxic (Puzzo et al., 2012).

1.7.2 Regulation of gene expression

The C-terminal fragment of APP can bind to the intracellular protein Fe65, and is transported from the cytoplasm to the nucleus of the cell. In the nucleus it binds the protein Tip60 and forms the AFT transcriptional complex (so called because it is a complex of AICD, Fe65 and Tip60). Nuclear AICD has been shown to regulate the expression of several genes including neprilysin, aquaporin, p53, BACE1 and many others (Belyaev et al., 2010; Ohkawara et al., 2011; Beckett et al., 2012). There is a lot of disagreement in the genes regulated by AICD identified by different groups – it has been suggested that this is due to the use of different cell lines and cell culture techniques. AICD is proposed to alter gene expression by recruiting the MED12 subunit of RNA polymerase II to promoter sites (Xu et al., 2011). Only AICD generated by βγ-cleavage (not αγ-cleavage) of the 695 isoform of APP is transported to the nucleus and alters gene expression (Belyaev et al., 2010).

1.7.3 Toxicity to cells

Aβ aggregates are toxic to cells, they cause neuronal dystrophy and death when incubated with neurons in culture (Grace et al., 2002). Aβ can exist in a number of different forms, it is initially generated from cleavage of APP as a monomer, but it has a high propensity to aggregate and can form many oligomeric species, as well as large fibrils. Initially it was thought that the large fibrils of Aβ which make up the plaques characteristic of AD were the cause of the disease, however the toxic species of Aβ is now widely considered to be the oligomeric Aβ aggregates. The oligomeric forms of Aβ correlate better with cognitive decline than

Aβ filbril load (Tomic et al., 2009). AβOs have been isolated from AD brains (demonstrating their physiological relevance). They have been shown to be toxic to neuronal cultures, to inhibit LTP and to decrease synaptic density (Walsh and Doherty, 1991; Lambert et al., 1998; Wang et al., 2002; Deshpande et al., 2006; Shankar and Walsh, 2009). There are a larger number of different oligomeric aggregates, with a range of different sizes (from dimers and trimers, to much larger protofibrils which can be up to 200 nm in diameter) (Shankar and Walsh, 2009). It has been suggested that the toxicity of Aβ oligomers may be caused by aggregates of Aβ interacting with cellular proteins disrupting their normal cellular functions (Olzscha et al., 2011).

Aβ can be modified after it has been generated, for example the formation of pyroglutamate Aβ. These modified forms of Aβ may be more toxic than un-modified Aβ, and have also been shown to cause AD-like symptoms in mice, for example increased neuronal death, and altered behaviours (Alexandru et al., 2011). Once Aβ has begun to aggregate in the brain it has been shown to act as a seed inducing further Aβ aggregation. This has been demonstrated by injecting Aβ containing extracts from transgenic AD mouse brains into the brains of younger transgenic mice which induced amyloid pathology in the brain at a much younger age (Langer et al., 2011).

Tau protein, which becomes hyperphosphorylated and forms the tangles found within neurons that are characteristic of AD, may be required for the toxicity of Aβ oligomers. Phosphorylation of tau is increased by Aβ, inhibition of GSK-3β (which phosphorylates tau) and tau knockout prevents Aβ mediated impairment in long-term potentiation (Shipton et al., 2011).

Besides Aβ, sAPPβ also exherts detrimental effects towards neurons. sAPPβ can be further cleaved into 35 and 55 kDa fragments, and once cleaved sAPPβ can bind the death receptor DR6. It has been postulated that binding of sAPPβ to the death receptor DR6 activates the receptor, stimulating pruning of axons in development and cell death following nutrient deprivation (Nikolaev et al., 2009).

It has been suggested that AICD and not Aβ is the toxic species in AD (Nakayama et al., 2008; Ohkawara et al., 2011). Evidence for this comes from studies in which AICD, rather than the whole of APP, was over-expressed in mice. Over-expression of AICD in mice caused age-dependent neuronal loss in the hippocampus and a decrease in working memory of the mice. There was no change in Aβ or Aβ plaque levels in the brains of these mice, and AICD over-expression caused an age dependent increase in tau pathology. This suggests that AD symptoms could be caused solely by increased AICD, rather than Aβ (Ghosal et al., 2009).

1.8 Proteins that regulate APP processing

There are many examples of proteins which can regulate APP processing and so alter the levels of Aβ, sAPPα, and sAPPβ secreted by cells, and therefore provide potential therapeutic targets for AD (reviewed in (Tang and Liou, 2007)). Proteins that regulate the processing of APP can be broadly split into two categories – proteins that alter the processing of APP by binding to APP or to the secretases preventing the APP-secretase interaction (direct modulators) and proteins that alter the processing of APP by altering its trafficking and its location in the cell (indirect modulators).

1.8.1 Direct modulation of APP processing

An example of a direct modulator of APP processing is the protein BRI2 (Integral membrane protein 2B). BRI2 is a membrane spanning protein that was identified in a screen for APP interacting proteins using the split ubiquitin system (Matsuda et al., 2005). It is expressed in the brain and found in the axons of neurons (as is APP). BRI2 is found at especially high levels in dystrophic neurites surrounding plaques, and mutations in BRI2 cause an AD-like dementia (Fotinopoulou et al., 2005). BRI2 co-immunoprecipitates with APP, and expression of BRI2 in cell lines causes a decrease in $A\beta_{40}$, $A\beta_{42}$, sAPPα and CTFα but increases sAPPβ. Expression of BRI2 in mouse models of AD decreases both $A\beta_{40}$ and $A\beta_{42}$ (Fotinopoulou et al., 2005). BRI2 expression also reduces expression from APP transactivator genes, suggesting it causes a decrease in the levels of AICD (Matsuda et al., 2005). BRI2 can interact with mature, but not immature APP, and can interact with CTFβ but not CTFα. A mutant form of BRI2 that is retained in the ER is not able to alter APP processing, and APP-BRI2 complexes have been identified on the surface of cells suggesting BRI2 binds to APP once it has been trafficked to the cell surface (Matsuda et al., 2011). Deletion mutants of APP were created and the interaction of these with BRI2 was determined by co-immunoprecipitation of the mutants with BRI2. This showed that the 17 amino acids of APP adjacent to the membrane spanning domain (on the N-terminal side) are essential for BRI2 binding APP. These 17 amino acids are in the N-terminal region of Aβ (Matsuda et al., 2005). These studies led to the hypothesis that APP and BRI2 are synthesised in the ER then trafficked separately through the secretory pathway where they are glycosylated to become the mature forms of the proteins. At the cell surface, or following endocytosis, BRI2 binds to APP covering the α- and γ-cleavage sites preventing access by secretases and so decreasing sAPPα, Aβ, CTFα, and AICD

production. The β-cleavage site remains exposed so APP is cleaved by β- rather than α-secretase resulting in increased sAPPβ production (Fotinopoulou et al., 2005; Matsuda et al., 2005; Matsuda et al., 2011).

1.8.2 Indirect modulation of APP processing

One of the most well characterised examples of an indirect modulator of APP processing is SORLA (sorting protein-related receptor). SORLA was predicted to be involved in transport of proteins between the Golgi and endosomes as it interacts with many adaptor proteins that act as shuttles between these compartments. SORLA expression is decreased in AD in the frontal cortex suggesting it may have a role in AD (Andersen et al., 2005). Expression of SORLA in cell lines caused a decrease in Aβ, sAPPα, and sAPPβ secretion from the cell, suggesting it has a role in APP processing. Surface plasmon resonance and analytical ultracentrifugation studies showed that SORLA binds the 695, 751 and 770 isoforms of APP in a 1:1 ratio and APP and SORLA co-localise when expressed in cell lines (Andersen et al., 2005). This interaction caused APP to accumulate in intracellular compartments reducing cell surface APP levels, which means it did not come into contact with the secretases, and so explains the reduction in APP proteolytic processing caused by SORLA expression (Andersen et al., 2005). Expression of a mutant form of SORLA that traffics to the cell surface rather than remaining in intracellular vesicles caused an accumulation of cell surface APP. To confirm these effects *in vivo* SORLA knockout mice were created, and these mice had increased sAPPα and Aβ production. The APP-SORLA interaction was confirmed *in vivo* by co-localisation of SORLA and APP in mouse brain (Andersen et al., 2005). The expression of SORLA is regulated by brain-derived neurotrophic factor (BDNF), which is known to have protective effects in AD. BDNF increases the expression of SORLA, and so

causes a decrease in Aβ production due to the effects of SORLA on APP trafficking (Rohe et al., 2009).

For further examples of proteins that interact with and regulate APP processing see table 1.1.

Table 1.1 A selection of proteins that regulate APP processing

Protein	Effect	Mechanism
ABCA7	Decreases sAPPα, sAPPβ, Aβ, CTFβ, CTFα and AICD	**Indirect** Exports cholesterol from the cell, so decreases cholesterol in the membrane, though this hasn't been proven (Chan et al., 2008).
ABCG1	Increases $A\beta_{40}$, $A\beta_{42}$, sAPPβ and sAPPα	No mechanism proposed (Tansley et al., 2007).
ApoER2	Increases Aβ, decreases C-terminal fragments of APP,	Multiple contradictory effects on APP processing are reported for ApoER2. This is because different isoforms of the receptor have different effect (Fuentealba et al., 2007; King (Thesis), 2011).
BDNF	Decreases Aβ	**Indirect** Increases expression of SORLA which prevents APP transport into early endosomes (Rohe et al., 2009).
BRI2	Decreases Aβ, sAPPα and AICD, increases sAPPβ	**Direct** Binds APP preventing access to the α- and γ-cleavage sites, but leaving the β-cleavage site exposed (Fotinopoulou et al., 2005; Matsuda et al., 2005; Matsuda et al., 2011).

Continued overleaf

Table 1.1 A selection of proteins that regulate APP processing (continued)

Protein	Effect	Mechanism
CD74	Decreases secreted Aβ (not cytoplasmic Aβ)	**Indirect** Re-locates APP to intracellular structures preventing amyloidogenic cleavage of APP (Matsuda et al., 2009).
Cellular prion protein (PrP^c)	Decreases Aβ	**Direct** PrP^c interacts with BACE1 *via* glycosaminoglycans so prevents BACE1 from interacting with and therefore cleaving APP (Parkin et al., 2007).
Flotillin-2	Increases Aβ	**Indirect** Flotillin is required for endocytosis of APP so removing flotillin decreases APP endocytosis and therefore β-processing of APP (Schneider et al., 2008).
F-spondin	Decreases β-CTF	The mechanism is unknown, but it binds to APP, and therefore may prevent the APP-BACE1 interaction (Ho and Sudhof, 2004).
Grp94/Grp 78	Decrease sAPPα and increase sAPPβ	**Indirect** Acidosis and ER stress increase expression of Grp 78 and 94 which bind the KDEL domain of APP retaining it in the ER preventing α-processing and increasing β-processing (Xiang et al., 2010).

Continued overleaf

Table 1.1 A selection of proteins that regulate APP processing (continued)

Protein	Effect	Mechanism
LRP1	Increases Aβ and sAPPβ, decreases sAPPα	**Indirect** Interacts with the C-terminal of APP targeting APP to lipid rafts promoting amyloidogenic processing, (Lakshmana et al., 2008).
LRRTM3	Increases Aβ and sAPPβ	Modulates β-cleavage of APP but not through altering BACE1 activity (Majercak et al., 2006).
NEEP21	Decreases sAPPα, sAPPβ, CTFβ, Aβ, and changes the ratio of CTFβ:CTFα. The effects of NEEP21 knockdown on APP processing are different in different cell lines.	No mechanism proposed (Norstrom et al., 2010).
Nogo receptor	Decreases Aβ, sAPPβ and sAPPα	**Direct** Binds APP and prevents the secretases interacting with APP decreasing all cleavages of APP (Park et al., 2006; Park and Strittmatter, 2007).
Reticulon family proteins	Decrease Aβ and sAPPβ	**Indirect** Re-locates BACE1 from the Golgi to the ER where it is less active resulting in decreased β-cleavage of APP (He et al., 2004; Hu et al., 2007; Shi et al., 2009).

Continued overleaf

Table 1.1 A selection of proteins that regulate APP processing (continued)

Protein	Effect	Mechanism
SNX33	Increases sAPPα	**Indirect** Prevents endocytosis so APP remains at the cell surface preventing cleavage by β-secretase and promoting cleavage by α-secretase (Schobel et al., 2008).
SORLA	Decreases Aβ, sAPPα and sAPPβ	**Indirect** Interacts with APP and retains it in the ER preventing it coming into contact with the secretases decreasing all cleavages of APP (Andersen et al., 2005).
X11 proteins	Decrease Aβ, sAPPβ, and CTF-β	**Indirect** X11 proteins bind the GYENTPY motif in the C-terminal of APP, and have been proposed to prevent its lipid rafts location, so reduce β-processing (Kondo et al., 2010).

1.8.3 The *in vivo* APP interactome

In 2008 a mouse *in vivo* brain interactome for APP was published (Bai et al., 2008). This was performed by perfusing the brains of mice with para-formaldehyde, which cross-links proteins in close proximity to each other and so cross-links any proteins that are interacting. The brain tissue was then homogenised and APP (along with any cross-linked proteins) was immunoprecipitated using antibodies to the C and N terminus. Proteins that had been co-purified with APP were identified by liquid chromatography and tandem mass spectrometry analysis. Several proteins that had already been identified as APP interactors were confirmed, and some of the novel interactions were confirmed by co-immuno-precipitation experiments, validating the methodology of this approach. The interactomes for APLP1 and 2 were also investigated and were different from the interactome of APP, which demonstrates the specificity of the approach, yet some proteins were found in all three interactomes. Many novel APP interacting proteins were identified (though the authors point out that these may not necessarily be direct interactions), and different groups of proteins were pulled out depending on the APP antibody used in the immunoprecipitation step. Antibodies directed to the C-terminus of APP mostly identified cytoplasmic proteins (possibly involved in the trafficking of APP through the secretory pathway), whereas N-terminal APP antibodies identified many extracellular proteins (Bai et al., 2008).

The *in vivo* brain interactome is an important step in identification of APP interacting proteins, as unlike *in vitro* interaction studies (such as yeast 2 hybrid) it shows proteins which do interact with APP in a physiological setting, rather than proteins which can interact with APP in an artificial environment. Three proteins identified by Bai et al., (2008), neurofascin, contactin proteins, and Thy-1, were

selected, and the role of these protein in APP metabolism was investigated (see chapter 3).

1.8.3.1 Neurofascin

Neurofascin is a type I membrane protein with six Ig domains and five fibronectin type III domains (Hortsch, 1996), and is highly glycosylated with both O- and N-linked glycosylation (Volkmer et al., 1992) (figure 1.6). There are a number of isoforms of neurofascin which are differentially expressed throughout development: isolation of neurofascin mRNA from embryonic chick brains identified 50 different neurofascin isoforms (Hassel et al., 1997). Neurofascin isoforms have been shown to interact with several proteins, and regulating the isoform of neurofascin expressed can modulate these interactions (Pruss et al., 2006). Different isoforms of neurofascin have distinct functions. For example the NF186 isoform (so called because it migrates at a molecular weight of 186 kDa on SDS-PAGE) has been shown to inhibit neuronal outgrowth, whereas the NF155 isoform promoted neurite outgrowth (Koticha et al., 2005). Neurofascin knockout mice have been generated, but died 6-7 days after birth. Analysis of the mouse brains before six days of age showed that their neurons appeared normal, however conduction velocities were decreased compared to wild type mice (Sherman et al., 2005).

Neurofascin is involved in saltatory conduction. This is a method of rapid nerve impulse conduction, which requires myelination of axons. Axons are myelinated by Schwann cells (glial cells), myelination causes formation of nodes of Ranvier on axons, these form at the points where axons and the myelinating glia interact. Neurofascin has a role in assembling and recruiting proteins to the node and paranode, and also mediates the neuron – glia interaction. NF155 on the Schwann

cell interacts with contactin on the neuron, and NF186 on the neuron interacts with gliomedin on the Schwann cell (Sherman et al., 2005). The role of the NF186 isoform in APP proteolytic processing is further investigated in chapter 3.

1.8.3.2 Contactins

The contactins are a subgroup of six proteins (contactins 1-6) all of which are glycosyl phosphatidylinositol (GPI) anchored proteins from the Ig superfamily, with four fibronectin type III domains followed by six Ig domains (Shimoda and Watanabe, 2009) (figure 1.6). The six contactins have 40-60% identity (Kamei et al., 2000), and are highly expressed in the brain and CNS (a contactin precursor was identified as one of the major glycoproteins in the brain (Huang et al., 2004)) with different expression patterns for different contactins (Reid et al., 1994; Watanabe et al., 1995; Kamei et al., 2000). The contactins have been implicated in neuronal outgrowth and cell-to-cell interactions: contactin 1 is located at synaptic sites and addition of a soluble form of contactin 1 to dorsal root ganglia cultures increased the proportion of neurons extending processes (Durbec et al., 1992; Berglund and Ranscht, 1994). Contactin knockout mice have been generated, and knockout of the different contactins cause different phenotypes. Contactin 1 knockout mice, although normal at birth, develop ataxia and do not survive for longer than 18 days. Furthermore the cerebellum of contactin 1 knockout mice is 17 % smaller than that of wild type mice (Berglund et al., 1999). Contactin 2 and contactin 5 knockout mice, on the other hand, did not have any major deleterious phenotype (Fukamauchi et al., 2001; Li et al., 2003).

There are several reasons to believe that contactins may have an important role in AD. A study aiming to identify potential biomarkers for AD found a 1.9-fold increase in isoform 1 of contactin 1 and a 2.4-fold increase in contactin 2 in the CNS

of AD patients compared to controls (Yin et al., 2009). Expression of contactin 1 was decreased in hippocampus and cerebellum of rats aged over 30 months (Shimazaki et al., 1998), and contactin 2 expression was decreased in the hippocampus of 8 and 20 month old mice compared to 8 week old mice (Tachi et al., 2010). Contactin 1 has been shown to bind the notch receptor and caused γ-secretase dependent generation and nuclear localisation of notch intracellular domain (Hu et al., 2003; Hu et al., 2004), so contactins may also have a role in γ-secretase dependent release of AICD. Contactins 2, 3, and 4 have been shown to bind APP, and contactin 5 to bind APLP1 (Ma et al., 2008; Osterfield et al., 2008). Expression of contactin 2 in cell culture caused an increase in CTFα, CTFβ and a γ-secretase dependent increase in AICD showing that contactin 2 could be involved in regulation of APP proteolysis (Ma et al., 2008). Contactin 4 has also been shown to increase both APP and CTF-α when over-expressed in cell culture (the increase in CTFα was greater than that of APP), demonstrating that contactin 4 may also have a role in regulating APP proteolysis (Osterfield et al., 2008). Contactins may also have a genetic link to AD: contactin 5 was one of 13 genes showing an association with AD in a genome wide association study (Harold et al., 2009). An association of contactin 5 with neuroimaging measures for AD (such as white matter lesion volume and entorhinal cortex thickness) has also been reported (Biffi et al., 2010). The role of contactin 5 in the proteolytic processing of APP was investigated in chapter 3.

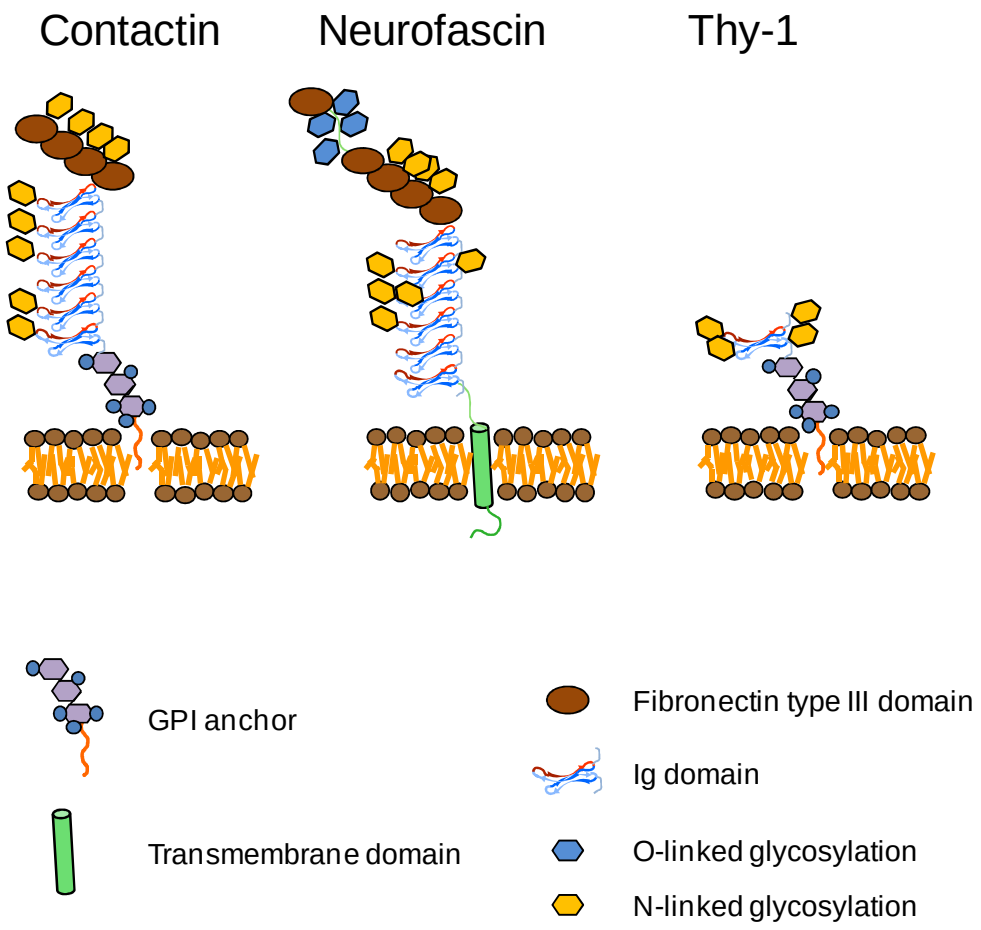

Figure 1.6 The structure of the proteins contactin 5, neurofascin 186, and Thy-1. The transmembrane anchors, the N- and O-glycosylation and the fibronectin type III domains and Ig domains of contactin 5, neurofascin, and thy-1.

1.8.3.3 Thy-1

Thy-1 (also called CD90) is another GPI anchored membrane protein and is also a member of the Ig superfamily of proteins (Walsh and Doherty, 1991) (figure 1.6). Thy-1 has been implicated in many cellular functions. It is important in cell-to-cell and cell-to-matrix interactions, it may act as a tumor suppressor in certain cancers, it may have a role in apoptosis, and has immunological functions (Rege and Hagood, 2006). Thy-1 is expressed in mature but not in developing neurons, and in continually branching axons Thy-1 expression is only found in areas of the axon where growth is complete (Xue et al., 1991). The GPI anchor of Thy-1 also means it is localised to lipid rafts, where amyloidogenic processing takes place. Thy-1 can be shed from its GPI anchor, and its GPI anchor is thought to be important for cell signalling (Haeryfar and Hoskin, 2004). Its expression and location at the cell surface has also been demonstrated in several neuroblastoma cell lines (Kemshead et al., 1982).

Thy-1 has been previously implicated in AD. Distribution of Thy-1 in neurons was found to be altered in AD brains compared to age-matched control brains. Additionally there was decreased Thy-1 staining in AD brains, though it is not clear whether this reflects loss of Thy-1 expressing neurons, or decreased Thy-1 expression in neurons (Leifer and Kowall, 1992).

1.9 Genome-wide association studies

Initial genetic research into AD was driven by identification of mutations that caused familial AD, but the vast majority of AD cases (95%) are sporadic AD, and these are not caused by dominant mutations in genes such as APP and the presenilins (Pimplikar, 2009). There is, however, a genetic component to sporadic

AD, and recent advances in technology have allowed genome-wide association studies (GWAS) with large enough sample numbers to detect single nucleotide polymorphisms (SNPs) that are related to sporadic AD. Several genes have been related to sporadic AD in this way, and different studies have identified different genes. Genes that have been consistently identified by several GWAS include *CLU* (encodes the protein clusterin), *BIN1* (bridging integrator 1), *PICALM* (phosphatidyl inositol clathrin associated lymphoid myeloid), *CD2AP* (CD2 associated protein), *EPHA1* (ephrin receptor A1), *CR1* (complement receptor 1), *ABCA7* (ATP binding cassette transporter A7), *CD33*, and the *MS4A* gene cluster (Harold et al., 2009; Bertram and Tanzi, 2010; Seshadri et al., 2010; Carrasquillo et al., 2011; Hollingworth et al., 2011; Hu et al., 2011; Wijsman et al., 2011). It has been estimated that these nine genes could explain up to 50% of sporadic AD genetics (Morgan, 2011).

Hollingworth and collegues grouped the genes that have been linked to sporadic AD into groups of related functions, and suggests that this gives us an insight into processes involved in sporadic AD (Hollingworth et al., 2011). The proteins CLU, CR1, ABCA7, CD33 and EPHA1 all have roles in immune system function, PICALM, BIN1, CD33 and CD2AP are involved in processes occurring at the cell membrane, while APOE, CLU and ABCA7 are involved in lipid processing. No function has been assigned for the MS4A4 gene cluster so these do not yet fit into any group (Harold et al., 2009; Bertram and Tanzi, 2010; Seshadri et al., 2010; Hollingworth et al., 2011).

1.10 The importance of endocytosis in the development of Alzheimer's disease

Several of the genes identified from SAD GWAS are involved in endocytosis (BIN1, PICALM, and CD2AP) suggesting alterations in endocytic pathways have a role in the development of AD. Endocytosis has an important role in AD pathology, and is involved in Aβ generation (Koo and Squazzo, 1994), Aβ toxicity to neurons (Song et al., 2011), and Aβ clearance from the brain by transport of Aβ across the blood brain barrier (Mackic et al., 1998). Other studies have also implicated endocytic proteins as contributors to AD. In 2011 using a yeast model of Aβ toxicity Treusch et al., screened a library of yeast open reading frames (approximately 90% of the yeast genes) for modulators of Aβ toxicity (Treusch et al., 2011). Twelve of the modulators identified had human homologues, three of which were endocytosis related genes, including the yeast homologue of PICALM, and the yeast homologue of synaptojanin (which interacts with BIN1) (Treusch et al., 2011). Furthermore, defects in endocytic pathways have been demonstrated in several models of AD.

1.10.1 Evidence for endocytosis disruptions in Alzheimer's disease

Slices from sporadic AD but not familial AD brains have enlarged early endosomes, suggesting the endocytic pathway is up-regulated in sporadic AD (Cataldo et al., 2000). These changes in endosome size were also observed in regions of the brain where there was not yet any detectable amyloid deposition showing that defects in the endocytic pathway occur early in AD pathogenesis. Alterations in endosome size are not seen during normal aging or in other neurodegenerative diseases, suggesting defects in endocytosis are a specific factor for AD (Cataldo et al., 2000). The clathrin mediated endocytosis proteins clathrin,

dynamin and PICALM have been shown to be up-regulated in the cortex of aged AD model mice (Thomas et al., 2011). Levels of endocytic proteins (dynamin, synaptophysin, and AP180) have been shown to be decreased in AD brains and in an AD mouse model. Levels of dynamin and synaptophysin were decreased in the brains of rats injected with $A\beta_{42}$, and this significantly reduced the performance of these rats in the Morris water maze test demonstrating that this treatment resulted in cognitive defects (Yao and Coleman, 1998; Yao et al., 1999; Cao et al., 2010). Treatment of hippocampal neurons with Aβ has been shown to decrease endocytosis of a fluorescent dye showing Aβ can disrupt endocytic pathways (Cao et al., 2010; Bate and Williams, 2011). Aβ has been shown to alter clathrin distribution and decrease endocytosis in a yeast model of AD (Treusch et al., 2011). APPswe (an FAD causing mutant of APP) expressing neurons grown in culture gradually accumulate intracellular Aβ, and endosome trafficking and synapses become altered suggesting that Aβ can cause abnormalities in the endocytic pathway in neurons (Tampellini et al., 2011). Endocytosis is important in three main areas of Aβ toxicity in AD; Aβ generation, Aβ uptake by cells, and Aβ clearance from the brain.

1.10.2 The role of endocytosis in Aβ generation

APP is transported to the cell surface, then endocytosed back into the cell, so most APP is found in intracellular vesicles (Cossec et al., 2010). β-cleavage of APP occurs in endosomes, where the β-secretase (BACE1) is located and the environment is acidic providing the optimum pH for BACE1 activity (Vassar et al., 1999). Endocytosis has been shown to be required for Aβ generation both *in vitro* using cell lines (Koo and Squazzo, 1994), and *in vivo* in mouse brains. Inhibition of endocytosis decreases Aβ generation (Koo and Squazzo, 1994; Ehehalt et al., 2003;

Cirrito et al., 2008; Schneider et al., 2008; Goodger et al., 2009), and has been shown to increase α-cleavage of APP (which precludes Aβ formation) (Schobel et al., 2008) in cultured cell lines. γ-cleavage of APP has been shown to occur in a recycling compartment of cells, rather than in the secretory pathway, therefore Aβ generation must occur after APP has reached the cell surface and been endocytosed (Kaether et al., 2006; Hare, 2010). Endocytosis is required for synaptic activity, and the concentration and deposition of Aβ in the brain is highest in areas where synaptic activity of neurons is highest (Bero et al., 2011). Synaptic activity also enhances the binding of AβOs to synapses, so endocytosis may have a role in targeting the AβOs to synapses, by causing zinc release from neurons (Deshpande et al., 2009). Endocytosis has been shown to be required for synaptic activity-dependent Aβ secretion (Cirrito et al., 2008). As well as being generated within endosomes, Aβ can aggregate into toxic AβOs inside endosomes (Takahashi et al., 2004).

1.10.3 The role of endocytosis in Aβ toxicity to cells

There is a significant volume of evidence that Aβ is endocytosed by neurons, and that this is required for its toxicity. $A\beta_{42}$ added to rat cortical neurons was taken up by, and accumulated in, the cells. This was prevented by both general endocytosis inhibitors and inhibition of clathrin-mediated endocytosis demonstrating that Aβ can be endocytosed in a clathrin-dependent way (Yu et al., 2010a; Cizas et al., 2011; Song et al., 2011). Cells with high levels of internalised $A\beta_{42}$ showed increased lysosomal and endosomal activity, and re-location of the endosomal protein cathepsin D throughout the cell, demonstrating that Aβ alters the endosomal pathway. Cortical neurons treated with $A\beta_{42}$ undergo apoptosis, however this was prevented by general endocytosis inhibitors showing that endocytosis is important

for AβO toxicity (Song et al., 2011). A high concentration of AβOs was used in this study (10 μM), however, AβOs have been shown to decrease cell viability at more physiological concentrations (500 nM) and this has been prevented by Ca^{2+} chelators and treatment with methyl-β-cyclodextrin, both of which inhibit endocytosis (Cizas et al., 2011). However, not all reports confirm that the endocytosis of AβOs is clathrin dependent. Yu et al., (2010) showed that although endocytosis of AβOs was required for toxicity in a neuroblastoma cell line, this was not prevented by inhibiting clathrin-mediated endocytosis, whereas general endocytosis inhibitors and inhibitors of clathrin-independent endocytosis were able to reduce the toxic effects of AβO treatment (Yu et al., 2010a). AβOs taken up by cells co-localised with transferrin demonstrating that they are taken up by endocytosis (again this was prevented by inhibiting endocytosis). Unlike transferrin, AβOs are transported to lysosomes and accumulate rather than being degraded which suggests a potential mechanism for their toxicity (Chafekar et al., 2008). Finally it has been suggested that amyloid oligomers may exert their toxicity by aggregating with cellular proteins, which would require their uptake into the cytosol (Olzscha et al., 2011).

1.10.4 The role of endocytosis in Aβ clearance from brains

A major mechanism of clearance of Aβ from the brain is its rapid transport across the blood brain barrier (Shibata et al., 2000). Most Aβ injected to mouse brains is removed by this mechanism, very little is degraded in the brain (it can then be degraded in blood and removed from the body by the liver) (Shibata et al., 2000; Zlokovic, 2011). Aβ was rapidly taken up by human brain microvascular endothelial cells in an *in vitro* model of the blood brain barrier, and this uptake was prevented by endocytosis inhibitors (Mackic et al., 1998) suggesting that endocytosis also has a role in transcytosis of Aβ across the blood brain barrier. Aβ clearance from the

central nervous system (CNS) has been shown to be decreased in sporadic AD (Mawuenyega et al., 2010). Aβ has been shown to bind to the protein p75NTR, a receptor on the cell surface. Binding of ligands to the p75NTR receptor can stimulate clathrin-dependent endocytosis, and the high expression of p75NTR in smooth muscle and endothelial cells of the choroid plexus suggests it may have a role in the transcytosis of Aβ from the brain to the blood, as well as in the degradation of Aβ (Zhou and Wang, 2011). Defects in endocytosis of p75NTR not only prevented the removal and degradation of Aβ, but also stimulated its aggregation acting as a seed for Aβ aggregation (Aβ aggregates are high in areas of the brain where p75NTR is expressed) (Zhou and Wang, 2011). Aβ has been shown to be internalised by human primary smooth muscle cells into endosomes, and this is inhibited by pre-treatment with trypsin suggesting receptor-mediated endocytosis is responsible for the uptake. Uptake was also inhibited by incubating cells with a lipid deficient serum, implicating the involvement of a receptor from the low-density-lipoprotein receptor family (Urmoneit et al., 1997). For example anti-LRP1 (low density lipoprotein receptor-related protein 1) antibodies decrease Aβ clearance from brain, and LRP-1 expression levels are high in brain microvessels and decrease with age (Shibata et al., 2000). Several other membrane receptors have been implicated in Aβ trancytosis across the blood brain barrier.

1.10.5 BIN1

Chapter 4 of this thesis focuses on investigation of BIN1 in APP and Aβ metabolism. BIN1 was identified as a sporadic AD linked gene by GWAS, and is involved in endocytosis, therefore is likely to have a role in either Aβ production, up-take or clearance.

1.10.5.1 The splicing and expression of BIN1

BIN1 was first identified in a yeast 2-hybrid screen which aimed to identify myc interactors. It is located at chromosome 2q14 (Negorev et al., 1996) and is localised to the nucleus. When its gene was mapped, BIN1 was found to be close to other oncogenes which, along with its ability to bind myc, suggested it has roles in cancer and regulation of the cell cycle (Negorev et al., 1996). Meanwhile, analysis of rat brain homogenates identified a protein that was highly homologous to the protein amphiphysin and had a role in clathrin-mediated endocytosis, so it was named amphiphysin II (Tsutsui et al., 1997). Several groups have shown that amphiphysin II is actually an alternatively spliced form of BIN1 (Ramjaun et al., 1997; Tsutsui et al., 1997). There are several alternative splicings of the BIN1 gene, and different isoforms are expressed by different tissues (Tsutsui et al., 1997). The widely expressed isoform of BIN1 consists of a BAR (BIN1/Amphiphysin/RVS167 related) domain, a region unique to BIN1, a nuclear localisation sequence followed by another region unique to BIN1, and its C-terminus contains regions involved in protein-protein interactions (a myc binding domain and an SH3 (src homology) domain). The brain specific isoform of BIN1 lacks the nuclear localisation signal, and contains four extra brain-specific domains (figure 1.7) (Wechsler-Reya et al., 1997). This has led to the suggestion that the widely expressed isoform of BIN1 is located in the nucleus, where it has functions relating to cell cycle regulation and cancer, whereas the brain-specific isoform is located in axons and at nodes of Ranvier (Butler et al., 1997) and has a different function.

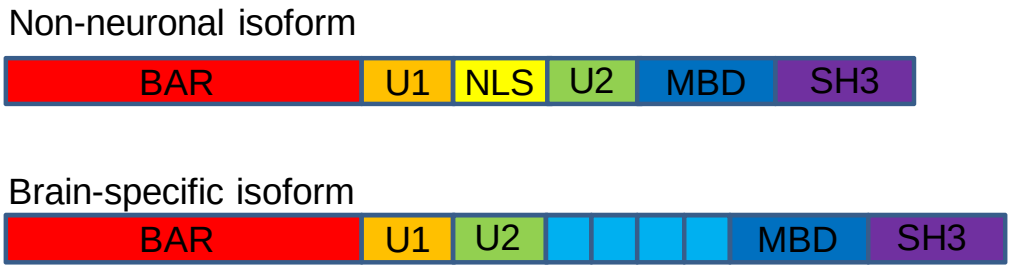

Figure 1.7 The non-neuronal and brain specific isoforms of BIN1. The different splice-forms of non-neuronal and brain specific BIN1. BAR is BIN1/Amphiphysin/RVS167 related domain, U1 and U2 are region unique to BIN1, NLS is the nuclear localisation sequence, MBD is the myc binding domain, SH3 is the src homology domain. The brain-specific isoform contains four brain-specific exons shown in blue.

1.10.5.2 The functions of BIN1

Immuno-fluorescence staining of rat brains showed a punctuate distribution of BIN1 in neurons suggesting it is located at synapses. Further evidence for this synaptic location was the co-localisation of BIN1 staining with staining for the protein synaptophysin which is localised at synaptic terminals (Ramjaun et al., 1997). The highest levels of BIN1 were found in cerebellum, hippocampus CA1 and CA3 regions, and pontine nucleus. BIN1 staining was very similar to amphiphysin I staining, suggesting it may have a very similar role in endocytosis to amphiphysin I. Immuno-electron microscopy has located BIN1 even more precisely to the outer surface of synaptic vesicles providing further support for a role in endocytosis (Wigge et al., 1997). Furthermore, generation of an amphiphysin I knockout mouse resulted in not only the absence of amphiphysin I but also BIN1. This mouse had defects in synaptic vesicle recycling suggesting that BIN1 functions with amphiphysin I during endocytosis (Di Paolo et al., 2002). BIN1, like amphiphysin I, has been shown to bind dynamin, clathrin, and the adaptor protein AP2: these molecules are required for clathrin-mediated endocytosis (Ramjaun et al., 1997). BIN1 and amphiphysin I can form a heterodimer, which can bind two dynamin molecules. As a heterodimer BIN1 and amphiphysin I are able to increase the rate of guanosine triphosphate (GTP) hydrolysis which provides the required energy for endocytosis (the monomeric forms are unable to do this) suggesting that the heterodimer may initiate dynamin oligomerisation then assists in the conformational changes required for fission of the vesicle (Wigge et al., 1997). The importance of amphiphysin for endocytosis was demonstrated by amphiphysin I knockout mice. The amphiphysin I knockout mice had decreased synaptic vesicle recycling, and only 50 % of the mice survived to 10 months of age. They also performed significantly worse in the Morris water

maze test than wild type animals. Interestingly, when amphiphysin I was knocked out levels of BIN1 were also reduced (shown by immuno-fluorescence and western blot) despite BIN1 mRNA levels being unaltered suggesting amphiphysin I is required for the stability of the BIN1 protein (Di Paolo et al., 2002). A BIN1 knockout mouse line has been created. BIN1 knockout did not alter amphiphysin I levels, so the stability of amphiphysin I is not dependent on BIN1. Levels of amphiphysin in mouse brain peak at six months of age then decreased with aging (Yang et al., 2008). BIN1 knockout also did not cause any structural alterations in the brain (Muller et al., 2003). BIN1 knockout mice die shortly after birth, so experiments were conducted on mouse embryonic fibroblasts. Surprisingly, BIN1 knockout fibroblasts showed increased uptake of fluorescently-labelled transferrin as compared to wild type cells and there was no alteration in the number of clathrin-coated synaptic vesicles (Muller et al., 2003). As previously mentioned, BIN1 has a number of different isoforms which are expressed throughout the body, so the premature death of these mice may not be due to loss of function of the brain-specific isoform of BIN1.

1.11 Alzheimer's disease therapeutics

Over the last decade much effort has been put into developing anti-amyloid therapeutics for AD. The attempted strategies can be split into two main groups – therapies that aim to decrease the production of Aβ, and therapies that aim to increase the degradation of Aβ. There have also been attempts to create drugs that prevent the aggregation of Aβ (Kawasaki and Kamijo, 2012; Powell et al., 2012; Sharma et al., 2012), though this is not a popular approach as it is mechanistically very difficult. Scyllo inositol has been shown to form a complex with $A\beta_{42}$ converting it from a structured to unstructured comformation that is not toxic to

neurons (McLaurin et al., 2000), however scyllo inositol did not cause a significant improvement in clinical trials (Salloway et al., 2009).

1.11.1 Preventing Aβ generation

1.11.1.1 Inhibiting β-secretase

The β-secretase, BACE1, is the rate limiting step for the generation of Aβ and so has been a very attractive target for AD therapeutics. Coupled with this, β-secretase activity is increased in the brains of AD patients, suggesting that decreasing the activity of BACE1 could provide an effective treatment for AD. However, there are difficulties with targeting BACE1. The active site is large which means generation of specific inhibitors is difficult – several of the initial attempts at generation of BACE1 inhibitors were not viable as they used peptide-based substrate analogues which inhibited other aspartic proteases in the brain (such as BACE2 and cathepsin D) (Citron, 2002; Nawrot, 2004). This could lead to unacceptable side effects. In 2002 the first crystal structure of an inhibitor in complex with BACE1 was solved, and this allowed structure-based design of inhibitors. This study demonstrated that inhibitors with a low K_i for BACE1 can be generated without filling the entire active site (Hong et al., 2002) which is advantageous as it allows molecules to be small enough to cross the blood brain barrier. Several BACE1 inhibitors with high specificity and low K_i have been developed (Ghosh et al., 2008; Bjorklund et al., 2010), but none of these have reached the clinic. β-secretase knock out mice have been created – initially these appeared to have a normal phenotype lending support to the idea that BACE1 inhibition is a viable therapeutic avenue for AD treatment (Benjannet et al., 2001; Luo et al., 2001; Yan et al., 2001). However, closer examination of BACE1 knockout mice several years later showed they had some abnormal cognitive and emotional functions as well as altered insulin

metabolism (Kobayashi et al., 2008; Meakin et al., 2012). Although BACE1 knockout in transgenic mouse models of AD can rescue the AD phenotype (Ohno et al., 2004; Nishitomi et al., 2006; Cole and Vassar, 2007; McConlogue et al., 2007; Kimura et al., 2010), the recent demonstration that BACE1 knockout mice present deleterious phenotypes suggests complete inhibition of BACE1 would have detrimental side effects.

1.11.1.2 Inhibiting γ-secretase

An alternative to inhibition of β-secretase is inhibition of the other enzyme required for generation of Aβ – γ-secretase. Many molecules that inhibit γ-secretase and therefore prevent generation of Aβ have been developed (Siemers et al., 2007; Fleisher et al., 2008), yet like BACE1 inhibitors none have succeeded in clinical trials. In many cases the reason for this has been unacceptable side effects (such as altering gastro-intestinal and immune cell function) caused by the drugs: knockout of γ-secretase in mice is lethal suggesting that it performs vital functions in the cell (Shen et al., 1997; Wong et al., 2004). γ-secretase is involved in the proteolytic processing of other molecules apart from APP – for example the Notch protein (De Strooper et al., 1999) which is important in development (Daudet et al., 2007; Boulton et al., 2008; Nagase et al., 2011) as well as several other proteins (e.g. ApoER2 receptor, cadherins, low density lipoprotein receptors, and β-subunits of voltage gated sodium channels) so complete inhibition of γ-secretase would prevent all of these proteolytic cleavages. Furthermore γ-cleavage of APP generates not only Aβ but also AICD which may be important for the functioning of the cell (Chang and Suh, 2010). There is also evidence that complete inhibition of γ-secretase may cause decreases in memory (Tamayev and D'Adamio, 2012). This has led to the development of γ-secretase modulators (Kukar et al., 2008). These modulate the

activity of γ-secretase rather than inhibiting it completely, for example decreasing the production of $A\beta_{40}$ and $A\beta_{42}$, but increasing $A\beta_{37}$ and $A\beta_{38}$ (less toxic species of Aβ), while not altering AICD generation (Kounnas et al., 2010), or γ-cleavage of other substrates (Fraering et al., 2005).

1.11.1.3 Activating α-secretase

An alternative to inhibiting the Aβ generating secretases is to activate the α-secretase, as cleavage of APP by α-secretase prevents the generation of Aβ. α-secretase can be activated by activating protein kinase C (PKC). PKC activators that reduce Aβ generation have been identified in cell culture though it remains unclear whether this reduction is due to increased α-cleavage or activation of Aβ degrading enzymes (Nelson et al., 2009). There are potential problems with targeting α-secretase: the α-secretase cleaves numerous other substrates, including Notch. Altering Notch cleavage has caused many problems for γ-secretase targeting AD therapeutics (Bozkulak and Weinmaster, 2009), suggesting that drugs targeting α-secretase may have similar side effects. Furthermore there have been several reports that altering α-secretase activity does not affect Aβ generation (Blacker et al., 2002; Gandhi et al., 2004; Peng et al., 2007), which casts doubt on whether an α-secretase activator would be effective *in vivo*. There are several proteins capable of performing the α-cleavage of APP, which can functionally substitute for each other (see section 1.6.1) so the protein targeted may be an important factor in whether the increased α-cleavage results in a change in Aβ generation.

1.11.2 Activating Aβ degradation

1.11.2.1 Activating Aβ degrading enzymes

Rather than preventing Aβ generation, another therapeutic strategy which has been explored is to increase the activity of Aβ degrading enzymes, thus increasing

Aβ clearance. The main Aβ degrading enzymes *in vivo* are neprilysin (NEP) and insulin degrading enzyme (IDE), (Kurochkin and Goto, 1994; Qiu et al., 1998; Iwata et al., 2000). Over-expression of IDE or NEP in mouse models of AD decreased both soluble Aβ and Aβ plaques, reduced markers of pathology in the brains (such as gliosis and dystrophic neurites), and reduced the incidence premature death of the mice (Leissring et al., 2003; El-Amouri et al., 2008). Potential for an Aβ degrading drug has been demonstrated by Jacobsen et al., (2009) who targeted plasminogen, another enzyme which can degrade Aβ. Jacobsen et al. identified a small molecule inhibitor which increases the generation of plasminogen from its inactive precursor. Transgenic mice dosed with this compound had increased Aβ degrading activity and reduced Aβ levels in their brain, as well as an improvement in LTP and memory (Jacobsen et al., 2008). Activators of IDE have been developed (Song et al., 2003; Cabrol et al., 2009) but none have reached the clinic.

1.11.2.2 Immunotherapy

One of the most successful potential therapies for AD is immunotherapy. Immunotherapy aims to use anti-amyloid antibodies to target Aβ for destruction by the immune system. There are two main forms of immunotherapy, passive and active. Passive immunotherapy involves treating patients with anti-Aβ antibodies, whereas in active immunotherapy patients are vaccinated with Aβ and the anti-Aβ antibodies are generated by the patient's own immune system. This approach was very successful in mouse models, however clinical trials had to be halted early as some patients developed brain inflammation. Analysis of the data from this halted trial showed that even though treatment with the antibody decreased Aβ plaques in the brain, there was no change in total Aβ levels and no improvement of cognitive function (Kokjohn and Roher, 2009). The anti-Aβ antibody drug bapineuzumab

which passed initial clinical trials (Salloway et al., 2009) has recently failed phase 3 clinical trials on the basis that it did not result in a change in cognition (http://www.pfizer.com/news/press_releases/pfizer_press_release.jsp?guid=2012072 2012072en&source=RSS_2011&page=3, Pfizer press release). The latest immunotherapy drug in development, CAD106, has passed initial clinical trials and did not cause severe adverse side effects, but it remains to be determined whether it has any positive effect on the symptoms or Aβ load of AD patients (Winblad et al., 2012). Other recent work has aimed at developing antibodies specifically to Aβ oligomers. The advantages of conformation specific antibodies is that they do not recognise Aβ monomers (so do not get mopped up binding just to monomer), do not bind to sAPPα or APP (so do not prevent these performing their normal function in the brain) and do not bind to Aβ fibrils (binding to fibrils could increase the risk of an inflammatory response in the brain). These have been successful in mouse models (Hillen et al., 2010).

Despite much effort being put into the development of therapies targeting Aβ, and their success in AD mouse models, none of these have passed clinical trials (e.g. (Malamas et al., 2009)). It has been suggested that this is because at the point AD symptoms become evident, too much neuronal damage has occurred for any treatments to be successful: too many neurons have died, too many synapses have been lost and amyloid deposition is too great to reverse. Treatments may need to start in the preclinical or mild cognitive impairment stages of the disease to have any symptomatic benefit to patients (Golde et al., 2011).

1.12 Aims

The metabolism of APP is an important determinant in the development and pathogenesis of AD, yet relatively little is currently known about the regulation of APP, and how this is altered in AD. Several drugs targeting APP metabolism (inhibiting Aβ production) have failed in clinical trials demonstrating the current lack of a full understanding of the role of APP in the pathogenesis of AD. Several proteins have been identified that can regulate the processing of APP, and many of these are altered in AD or during aging. Since the metabolism and proteolytic processing of APP is so tightly regulated by a large number of APP-interacting proteins, studies of these proteins represent a window into the normal regulation of APP, and the potential to identify new drug targets for AD. The first part of this study investigates three novel APP interacting proteins, neurofascin, Thy-1 and contactin 5. Due to their proximity to APP and links to Alzheimer's disease we hypothesised that these proteins have a role in the regulation of APP metabolism, and, specifically, Aβ generation.

Recent GWAS into sporadic AD have identified several novel genes associated with AD, and these have in turn highlighted the importance of certain cellular processes in the development of AD. GWAS have shown us that processes at the cell membrane, specifically endocytosis, are involved in the development of AD. Endocytosis has been shown to be dysregulated very early in AD pathology, and plays an important role in the metabolism of APP and the Aβ peptide. Endocytosis (or lack of endocytosis) of APP determines whether the toxic aggregation prone Aβ peptide or the non-toxic p3 peptide are generated from APP cleavage. Endocytosis is also required for the uptake and arguably for the toxicity of Aβ by neurons, as well as for the clearance of the Aβ peptide from the brain. In the

second part of this thesis we hypothesised that the endocytic protein BIN1 is involved in the generation of Aβ by altering the endocytosis of APP, or in the endocytosis of toxic Aβ oligomers by cells.

2 Materials and methods

2.1 Materials

Unless stated otherwise, chemicals and reagents were from Sigma-Aldrich (Pool, Dorset, UK). Absorbance 96 well plate assays were determined using an OPSYS MR plate reader from Dynex (Worthing, West Sussex, UK).

2.1.1 Antibodies

HRP-conjugated secondary antibodies were from Sigma-Aldrich Ltd (Pool, Dorset, UK), AlexaFluor® conjugated secondary antibodies and Texas-red conjugated streptavidin were from Invitrogen Life Sciences (Paisley, Scotland, UK). Primary antibodies used in western blotting are detailed in table 2.2, and primary antibodies used in immuno-fluorescence microscopy and Aβ ELISA are detailed in the appropriate methods.

2.1.2 Molecular biology general materials

Yeast extract and tryptone were from Melford (Ipswich, Suffolk, UK), agar was from Oxoid (Basingstoke, Hampshire, UK). Miniprep and maxiprep plasmid purification kits, and gel extraction kits were from Qiagen (Crawley, West Sussex UK). NcoI, KpnI, and XbaI were from New England Biolabs (Hitchin, Hertfordshire, UK) and Eco0109I was from Fermentas (Glen Burnie, Maryland, USA) *E.coli* XL1 blue competent cells were from Stratagene (Wokingham, Berkshire, UK). All polymerase chain reaction reagents were from Invitrogen Life Sciences (Paisley, Scotland, UK) except primers which were purchased from Sigma-Aldrich Ltd (Pool Dorset, UK). One kb DNA ladder was from New England Biolabs

(Hitchin, Hertfordshire, UK). Petri dishes were from Sterilin (Fischer Scientific UK Ltd, Loughborough, Leicestershire, UK).

2.1.3 General tissue culture materials

Unless stated otherwise all tissue culture reagents were from Lonza (Basel, Switzerland). Sterile pipettes were from Sterilin (Newport, UK), flasks, plates and cryovials were from Nunc A/C (Rosklide, Denmark). Fetal bovine serum was from Biosera (East Sussex, UK). OptiMEM reduced sera medium was from Invitrogen Life Sciences (Paisley, Scotland, UK). TransIT-LT1 transfection reagent was from Mirus (Madison, WI, USA). siRNA and siRNA reagents were from Dharmacon (Lafayette, CO, USA). HEK-APP_{695} cells, SH-SY5Y cells and N2a-APP_{695} cells were from Dr Katherine Kellet (University of Leeds).

2.2 Cloning methods

2.2.1 Cloning cDNA into a eukaryotic expression vector

BIN1 in pcDNA3.1(+) (NM_139343) was obtained from Source Bioscience (Nottingham, UK), Neurofascin (IMAGE 40068186), contactin (IMAGE 40146650) and Thy-1 (IMAGE 6137513) cDNAs were obtained from Source Bioscience and cloned into the eukaryotic expression vector pIRESneo (Clontech, Saint-Germain-en-Laye, France). APP_{695} in a pIREShyg vector was provided by previous members of the Hooper lab and has been described previously (Belyaev et al., 2010). pIRESneo and pIREShyg vectors contain an internal ribosome entry sequence (IRES) which allows polycistronic mRNA molecules to be transcribed. The IRES sequence in these vectors is between the gene of interest and the antibiotic resistance gene, and means the gene of interest and the antibiotic resistance gene are translated from a single mRNA transcript. This is advantageous as it means that expression of

the antibiotic resistance gene in the absence of the gene of interest does not occur, therefore all cells that survive the selection procedure express the gene of interest (Rees et al., 1996).

2.2.2 Culturing *E.coli*

2.2.2.1 Luria-Bertani media (LB media)

A sterile pipette tip was used to pick a single colony from a LB-agar plate. The pipette tip was deposited in 5 ml LB media (10 g tryptone, 5 g yeast extract and 5 g NaCl were dissolved in 1 litre of de-ionised water, then autoclaved and allowed to cool to 37 ^{o}C or below). Either ampicillin or kanomycin was added to the media to a final concentration of 100 µg ml^{-1} and the culture incubated for 8 hours at 37 ^{o}C in an orbital shaker at 250 rpm. For experiments where a culture volume larger than 5 ml was required the 5 ml starter culture was used to inoculate a conical flask containing 200 ml LB media supplemented with antibiotic. The 200 ml culture was incubated at 37 ^{o}C at 250 rpm in an orbital shaker overnight.

2.2.2.2 LB-agar plates

Agar was dissolved in LB media (15 g agar per litre of media) then autoclaved and allowed to cool to below 50 ^{o}C. Ampicillin or kanomycin was added to a final concentration of 100 µg ml^{-1} then the LB-agar was poured into petri dishes and allowed to set at room temperature. LB-agar plates were stored for up to one month at 4 ^{o}C. One hundred microlitres of *E.coli* growing in LB media were spread across the surface of pre-warmed LB-agar plates then incubated over-night at 37 ^{o}C. Colonies from LB-agar plates or *E.coli* from a glycerol stock were picked using a heat-sterilised metal loop and streaked across the surface of a pre-warmed LB-agar plate, then incubated over-night at 37 ^{o}C.

2.2.3 Glycerol stocks

Nine hundred microlitres of *E.coli* growing in exponential phase in LB media were added to 100 µl of 80 % glycerol in a cryovial then stored at -80 ^{o}C.

2.2.4 Plasmid purification

Plasmids were extracted from *E.coli* and purified using a QIAspin miniprep kit from QIAGEN according to the manufacturer's protocol. DNA was eluted with 50 µl warm ddH_2O or the elution buffer provided in the kit. The concentration of DNA was determined by absorbance at 260 nm using a nanophotometer from Implen (München, Germany), or samples were subjected to agarose gel electrophoresis, stained with ethidium bromide, and the intensity of the sample was compared to the intensity of a standard (1 kb DNA ladder, New England Biolabs). The ratio of absorbance at 260 nm to 280 nm was used to determine the purity of DNA.

2.2.5 Polymerase chain reactions (PCR)

PCR was carried out using a Proteus II thermal cycler (Helena Biosciences, Gateshead, Tyne and Wear, UK). The reaction cycle varied according to the cDNA being amplified. For specific reaction cycles see table 2.1, for primer sequences see table 2.2. Reaction volumes were made to 50 µl with sterile ddH_2O. The amplification of the correct product was confirmed by agarose gel electrophoresis. PCR products were excised from the gel using a scalpel and purified by gel extraction using a QIAgen gel extraction kit.

Table 2.1: PCR Reactions

Reaction	Reaction Mix	Cycle
Cloning neurofascin	1x amplification buffer, 0.3 mM each dNTP, 1.35 mM $MgSO_4$, 0.3 mM primer p3, 0.3 mM primer p4, 200 ng neurofascin cDNA (IMAGE 40068186), 1 U pfx polymerase, 2x enhancer solution	94 ºC for 5 minutes, **(**94 ºC for 30 seconds, 55 ºC for 30 seconds, 68 ºC for 4 minutes**) x30**, 72 ºC for 10 minutes, 4 ºC hold.
Cloning Contactin 5	2x amplification buffer, 0.3 mM each dNTP, 1.35 mM $MgSO_4$, 0.3 mM primer p1, 0.3 mM primer p2, 200 ng contactin cDNA (IMAGE 40146650), 1 U pfx polymerase, 2x enhancer solution	94 ºC for 5 minutes, **(**94 ºC for 30 seconds, 55 ºC for 30 seconds, 68 ºC for 4 minutes**) x30**, 72 ºC for 10 minutes, 4 ºC hold.
Cloning Thy-1	2x amplification buffer, 0.3 mM each dNTP, 1.35 mM $MgSO_4$, 0.3 mM primer p5, 0.3 mM primer p6, 200 ng Thy-1 cDNA (IMAGE 6137513), 1 U pfx polymerase, 1x enhancer solution	94 ºC for 5 minutes, **(**94 ºC for 30 seconds, 55 ºC for 30 seconds, 68 ºC for 1 minute**) x30**, 72 ºC for 10 minutes, 4 ºC hold.

Table 2.2 Primer sequences. All primers are shown in a 5` to 3` direction.

Name	Primer	Sequence
p1	Forward primer for cloning contactin 5	GATGCTCGTGA**GCGGCCGC**ATGGCTTCCTCTTG GAAACTAATGCT
p2	Reverse primer for cloning contactin 5	CATAG**GAATTC**TCACCAGGAAGTTGAAGGAAT CATCA
p3	Reverse primer for cloning neurofascin 186	GCTAG**GAATTC**TTAGGCCAGAGAGTAGATAGC ATTGAC
p4	Forward primer for cloning neurofascin 186	GATGCTCGTGA**GCGGCCGC**ATGGCCAGGCAGC CACCGCC
p5	Forward primer for cloning Thy-1	GATGCTCGTGA**GCGGCCGC**ATGAACCTGGCCA TCAGCATCGCT
p6	Reverse primer for cloning Thy-1	CATAG**GAATTC**TCACAGGGACATGAAATCCGTG GCC
p7	Primer for sequencing contactin 5	TCTTGGAAACTAATGCTGTTTCTG
p8	Primer for sequencing contactin 5	GAAATGGAACAGAAATAGATCTGGA
p9	Primer for sequencing contactin 5	GGTGAAAAACACAGTGACGAAT
p10	Primer for sequencing contactin 5	GGGAGCCCTCTCCGAT
p11	Primer for sequencing contactin 5	GGATCCTAAATGCTTCCAAATC
p12	Primer for sequencing contactin 5	CCAGGCCCACCTGG
p13	Primer for sequencing contactin 5	GGGGAAGGCTTCGGCTATAT

Continued overleaf

Table 2.2 Primer sequences (continued)

Name	Primer	Sequence
p14	Primer for sequencing contactin 5	TAACAGGATTAGAAGGAAATACGTTATATC
p15	Primer for sequencing neurofascin 186	CCAGGCAGCCACCGC
p16	Primer for sequencing neurofascin 186	ATGCAGCCTTCCTCCTCTG
p17	Primer for sequencing neurofascin 186	GGTGTCTAAATCTCCTCTGTGGC
p18	Primer for sequencing neurofascin 186	ATCGCCTCC
p19	Primer for sequencing neurofascin 186	GCAGGGCTGTGTACCAGTG
p20	Primer for sequencing neurofascin 186	GGGCACCACGGTGCA
p21	Primer for sequencing neurofascin 186	CAGTTCCAACCTGGGGTCT
p22	Primer for sequencing neurofascin 186	AGGCTGAAAATGACTTCGGG
p23	Primer for sequencing neurofascin 186	TACCGTGGGTGCGACG
p24	Primer for sequencing neurofascin 186	TCCCGGGATGACATACACG
p25	Primer for sequencing Thy-1	GCCAT CAGCATCGCT CTC
p26	Primer for sequencing Thy-1	CCCCACAGTGCCAAAGAG

Continued overleaf

Table 2.2 Primer sequences (continued)

Name	Primer	Sequence
p27	Primer for sequencing contactin 5	GGCATCTGAGGAATGATAAAGATT
p28	Primer for sequencing neurofascin 186	ACGACAGGGTCTAGGTTTTCCT
p29	Primer for sequencing genes inserted to pIRESneo (binds at the promoter)	TTTTGGCACCAAAATCAACG

2.2.6 Agarose gel electrophoresis

Agarose powder to make a 0.8 % w/v gel was added to TAE buffer (40 mM Tris-HCl, 10 mM sodium acetate, 2 mM EDTA pH 8.0) and heated in short bursts in a microwave until the agarose had dissolved. The agarose was then poured into a gel cast and allowed to set. Samples and 1 kb DNA ladder were diluted 1/3 with sample buffer (50 % glycerol, 0.25 % bromophenol blue) loaded onto the gel and subjected to electrophoresis at 100 V for 1 hour. Agarose gels were stained with 10 µl ethidium bromide diluted in 20 ml TAE buffer for approximately 30 minutes protected from light, and visualised under U.V. light.

2.2.7 Restriction digests

2.2.7.1 For cloning

Neurofascin, contactin and Thy-1 PCR products amplified from the vectors supplied by Source Bioscience, and empty pIRESneo, were digested with restriction enzymes. Approximately 2 µg contactin 5, neurofascin, and Thy-1, or 5 µg empty pIRESneo, were incubated with 20 U EcoRI, 20 U NotI-HF, 0.2 µg BSA in 1x NEB buffer 4 at 37 ºC for 16 hours. The restriction enzymes were then heat-inactivated by 10 minute incubation at 65 ^{o}C. To prevent the linearised pIRESneo plasmid form re-circularizing digested plasmid was incubated for 30 minutes at 37 ºC with 0.5 U Antarctic phosphatase in 1x Antarctic phosphatase buffer (New England Biolabs, Hitchin, Hertfordshire, UK).

2.2.7.2 To confirm insertion of genes into pIRESneo

Plasmids were purified from transformed colonies using a Qiagen spin-mini prep kit, then 10 µl of each mini-prep were incuated with either 20 U Eco0109I in

1x Fermantas buffer tango buffer 4, 20 U KpnI, 0.2 µg BSA in 1x NEB buffer 1 or 20 U NcoI in 1x NEB buffer 3 for 4 hours at 37 ^{o}C.

2.2.8 Ligation

Ligations were performed using a Quick Ligation kit from New England Biolabs (Hitchin, Hertfordshire, UK) according to the manufacturer's instructions. A 3 molar excess of insert was ligated into 50 ng of pIRESneo using 2000 U Quick T4 DNA ligase in 1 x Quick Ligation Reaction Buffer (New England Biolabs, Hitchin, Hertfordshire, UK). This was incubated for 5 minutes at room temperature then used immediately in transformation of XL1 blue *E.coli* cells.

2.2.9 Transformation of XL1 blue cells

β-mercaptoethanol (1.7 µl) was added to 100 µl XL1 blue competent cells which were then incubated for 10 minutes at 4 ºC, with mixing by gentle swirling every 2 minutes. The ligation reaction was added to the cell suspension, and incubated for a further 30 minutes at 4 ºC. Cells were heat shocked for 45 seconds at 42 ºC, then incubated for 2 minutes at 4 ºC. Nine hundred microlitres of S.O.C. media (2 % tryptone (w/v), 0.5 % yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 42 ºC were added to cells and incubated for 1 hour at 37ºC with shaking. Bacteria were spread onto LB-agar plates with 100 µg µl^{-1} ampicillin as a selection marker, and incubated overnight at 37 ºC.

2.2.10 Ethanol precipitation of DNA

One tenth volume of 3 M sodium acetate pH 5.2 and two volumes of absolute ethanol at -20 ^{o}C were mixed with the DNA then incubated for 1 hour at -20 ºC. DNA was pelleted by centrifugation at 11,337 *g* at 4 ºC for 20 minutes, then washed with 300 µl 80 % (v/v) ethanol at -20 ^{o}C, and centrifuged again at 11,337 *g* for 5

minutes at 4 ºC. The supernatant was discarded and the pellet was air dried at 37 ºC, then re-suspended using sterile ddH_2O.

2.2.11 Sequencing

Insertion of genes into pIRESneo was confirmed firstly by restriction digestion, and then by sequencing. Sequencing reactions were performed by Dundee Sequencing Services using forward primers designed using the OligoPerfect^{TM} primer design tool by Invitrogen (see table 2.2 for primer sequences). Sequencing results were then aligned with the expected template DNA using the freely available web tool MultAlin (http://multalin.toulouse.inra.fr/multalin/multalin.html).

2.3 Tissue culture methods

2.3.1 Tissue culture

SH-SY5Y (human neuroblastoma), HEK (human embryonic kidney) and N2a (mouse neuroblastoma) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, 1 ml per 5 cm^2 surface area) supplemented with 10 % fetal bovine serum (FBS), in a humidified incubator at 37ºC with 5 % CO_2. Once cells had reached 90 % confluency they were rinsed twice with phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} (PBS-M, diluted from 10x stock) then harvested by a 5-10 minute incubation at 37 ºC in PBS-M (1 ml per 16 cm^2 surface area). The resulting cell suspension was diluted into DMEM and transferred to a fresh flask.

2.3.2 Counting cells

Cells were rinsed twice with PBS-M, then harvested by a 5-10 minute incubation in PBS-M. Cells were pelleted by centrifugation at 1,409 *g* for 3 minutes and resuspended in an appropriate volume of DMEM. Cells were diluted 1/2 in

trypan blue, 20 µl cell suspension was pipetted onto a haemocytometer, and cells that had not taken up trypan blue stain within the grid areas at each corner of the slide were counted. The mean count for each grid was taken.

2.3.3 Stable transfection

SH-SY5Y or HEK cells were stably transfected with expression vectors by electroporation. Contactin 5 in pIRESneo vector was linearised before transfection; this was performed by digesting plasmid with XbaI (20 U XbaI, 1x NEB buffer 4, and 30 µg cDNA, incubated for 2 hours at 37 ºC). Ethanol precipitated DNA was used in transfection of SH-SY5Y and HEK cells. Cells were grown to approximately 80 % confluency in a T80 cm^2 flask in DMEM supplemented with 10 % FBS, then harvested by washing into PBS-M and pelleted by centrifugation at 1,409 *g* for 3 minutes. Cell pellets were re-suspended in 700 µl DMEM (without FBS), then mixed with either 30 µg DNA (transfection) or an equivalent volume of ddH_2O (control) in a 4 mm gap width electroporation cuvette (Geneflow, Staffordshire, UK). Cells were electroporated using an Electro Cell Manipulator 630, with a pulse at 250 V (Resistance = none, Capacitance=1650 µF). Electroporated cells were resuspended in 5 ml warm DMEM (with 10 % FBS), and transferred to a T80 cm^2 flask containing 10 ml DMEM. Cells were grown to approximately 70 % confluency, then selected for cells expressing the protein of interest using DMEM supplemented with either 1 mg ml^{-1} G418 (for pIRESneo and pcDNA3.1(+) vectors), 0.15 mg ml^{-1} hygromycin (for pIREShyg vectors). Once control cells had been killed off, transfected cells were cultured for a further 7 days in DMEM supplemented with either G418 or hygromycin.

2.3.4 Cryopreservation of cells

Cells were grown to confluence in T80 cm^2 flasks, and then harvested by a 5-10 minute incubation in PBS-M. Cells harvested from one T80 cm^2 flask were pelleted by centrifugation at 1,409 *g* for 3 minutes. The cell pellet was resuspended in 1 ml of freeze media – DMEM supplemented with 27 % (v/v) FBS and 10 % (v/v) dimethylsulphoxide (DMSO), then transferred to a cryovial and cooled to -80 °C in a Mr Frosty for 24 hours. Cells were transferred to liquid nitrogen for long term storage.

2.3.5 Transient transfection

N2a-APP_{695} cells were grown to approximately 50 % confluency in a 6-well plate then tranfected with contactin 5 cDNA using TransIT-LT1 transfection reagent according to the manufacturer's instructions (volumes of reagents were altered slightly to increase transfection efficiency). Briefly, 6 µl room temperature TransIT-LT1 transfection reagent and 2 µg ethanol precipitated DNA were diluted in 250 µl OptiMEM reduced sera media, incubated for 20 minutes at room temperature, then added drop-wise to one well of a 6-well plate. Cells were incubated for 24 hours with transfection mixture then washed twice with PBS and incubated for 48 hours with OptiMEM reduced sera media.

2.3.6 siRNA transfection

Cells were transfected with 50 nM ON-TARGET plus SMARTpool siRNA from Dharmacon. siRNA was diluted to 0.5 µM in OptiMEM reduced sera media, and Dharmafect 3 was diluted to 40 µl ml^{-1} in OptiMEM and incubated for 5 minutes at room temperature. The diluted siRNA was added to the diluted Dharmafect and incubated for 20 minutes at room temperature. The

siRNA/Dharmafect mixture was diluted with DMEM, so that the final siRNA concentration was 50 nM, then this mixture was used to replace the media on cells. Cells were incubated for 24 hours with the transfection reagents then washed twice with PBS and incubated with OptiMEM reduced sera media.

2.4 Western blotting methods

2.4.1 Preparation of cell lysates

Cells were washed twice with PBS-M then were harvested by scraping into PBS-M, and pelleted by 10 minute centrifugation at either 3,913 *g* or 15,115 *g* at 4ºC. Cells were lysed by re-suspension in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 % (w/v) Sodium deoxycholate, 1 % (v/v) NP-40, pH 8.0) with EDTA complete protease inhibitor cocktail (Roche Diagnostics, West Sussex, UK), and incubated at 4ºC for 30 minutes. Cell lysates were then centrifuged at >13,000 *g* for 10 minutes at room temperature. Supernatants were stored at -20 ºC.

2.4.2 Preparation of concentrated media samples

OptiMEM reduced sera medium was removed from cells and centrifuged for 10 minutes at either 3913 *g* or 15,115 *g* at 4 ºC to pellet any cells. Supernatant was then concentrated approximately 50 fold using Vivaspin 100,000 Da molecular weight cut off polyethersulfone membrane concentrator (Sartoris Stedim Biotech, Surrey, UK) by centrifugation at 3913 *g* at 4 ºC. Concentrated media samples were stored at -20 ºC.

2.4.3 BCA protein assay

Protein concentrations of samples were determined by bicinchoninic acid (BCA) assay. Lysates were diluted in ddH_2O on a 96 well plate (10 µl sample per

well) and 200 µl 4 % $CuSO_4$ diluted 1/50 in BCA reagent was added. The plate was incubated at 37 ^oC for 20-30 minutes, enough time to allow sufficient purple colour to develop in sample and standard wells, then the absorbance measured at 570 nm. A standard curve made up with BSA diluted in ddH_2O was used to determine the protein concentrations of lysates.

2.4.4 Sodium dodecyl sulphate gel electrophoresis

A BCA assay was performed on samples in order to standardise the concentration of protein loaded. Samples were denatured by heating to between 90 and 100 ºC for 4 minutes in 1x sample buffer (1.6 mM Tris pH 6.8, 2.2 % (w/v) SDS, 1.6 % (w/v) DTT, 11 % (v/v) glycerol) followed by centrifugation at >13,000 *g* for 1 minute at room temperature. Samples were electrophoresed on polyacrylamide gels (running buffer: 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3, from Bio-rad, Hempstead, Hertfordshire, UK), then transferred to a PVDF (polyvinylidine difluoride) membrane (transfer buffer: 150 mM glycine, 20 mM Tris, 20 % (v/v) methanol). Percentage acrylamide of gels, voltage, and transfer times were varied according to the molecular weight of protein being analysed (see table 2.3).

2.4.5 Western blotting

Membranes were blocked in PBST 5 % (w/v) milk (marvel milk powder made up in PBS 0.1% (v/v) Tween: PBST), rinsed three times briefly in PBST then incubated with primary antibody diluted in PBST 2 % (w/v) BSA for overnight at 4 ^oC (see table 2.3 for dilution). Membranes were rinsed three times briefly with PBST, then washed three times for 10 minutes in PBST, with brief rinses in between. HRP-conjugated secondary antibodies were diluted 1/4000 in PBST 2 % (w/v) BSA (see table 2.3) and incubated for a 1 hour at room temperature with

membrane. Membranes were rinsed three times briefly in PBST, then washed for 10 minutes in PBST followed by two 10 minute washes in PBS without Tween. Membranes were developed by 1 minute incubation using 4 ml luminol enhancer solution and 4 ml peroxide solution (Thermo Scientific, Waltham, MA, USA). Membranes were imaged using a LAS3000 imager (GE healthcare Buckinghamshire, UK), exposure times varied. Pre-stained molecular weight standards (Fermentas, Glen Burnie, Maryland, USA) were used to estimate molecular weight of proteins. Membranes were stripped in 1 mM glycine pH 2.0 for 30 minutes, then re-probed for actin. As a control for loading, membranes were stained with 0.1 % (w/v) amido black, 1 % (v/v) acetic acid for 2 minutes at room temperature then destained with H_2O to visualise total protein.

Table 2.3: Antibodies and conditions used in western and dot blotting

Antigen	Antibody	Dilution	Secondary	Conc. Poly-acrylamide	Transfer
Neurofascin	Ab31457 (Abcam, Cambridge, UK)	1/200	Goat anti-rabbit	7%	3 hours 300 mA
Contactin 5	AF3030 (R&D systems, Abingdon, UK)	1/500	Rabbit anti-goat	7%	80 minutes 120 V or 3 hours 300 mA
Thy-1	MAB2067 (R&D systems, Abingdon, UK)	1/2000	Rabbit anti-mouse	N.A.	N.A.
APP (full length)	22C11 (Millipore, Billerica, MA, USA)	1/2500	Rabbit anti-mouse	7%	80 minutes 120 V or 3 hours 150 mA
sAPPβ	1A9 (a gift from GlaxoSmithKline, Harlow, UK)	1/2500 incubated overnight at 4˚C	Rabbit anti-mouse	7% or 10%	80 minutes 120 V or 3 hours 150 mA
sAPPα	6E10 (Covance, New Jersey, USA)	1/4000	Rabbit anti-mouse	7% or 10%	80 minutes 120 V or 3 hours 150 mA

Continued overleaf

Table 2.3: Antibodies and conditions used in western and dot blotting (continued)

Antigen	Antibody	Dilution	Secondary	Conc. Poly-acrylamide	Transfer
APP (C-terminal)	A8717 (Sigma Aldrich, Poole, Dorset, UK)	1/750	Goat anti-rabbit	4-17%	50 minutes 380 mA
GFP	EGFP (Clontech, Saint-Germain-en-Laye, France)	1/5000	Rabbit anti-mouse	12%	80 minutes 120 V
PICALM	HPA019053 (Sigma-Aldrich)	1/1000	Goat anti-rabbit	10%	80 minutes 120 V
BIN1	Ab54764 (abcam, Cambridge, UK)	1/1000	Rabbit anti-mouse	10%	80 minutes 120 V
Actin	Ac15 (abcam, Cambridge, UK)	1/5000	Rabbit anti-mouse	N.A.	N.A.
Fibrilar Aβ epitope	OC (gift from C. Glabe, University of California, USA)	1/5000	Goat anti-rabbit	N.A	N.A.
Pre-fibrillar Aβ epitope	A11 (gift from C. Glabe, University of California, USA)	1/1250	Goat anti-rabbit	N.A.	N.A.

2.5 Dot blotting

One µg AβOs was spotted onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), then air dried for 15 minutes. Membranes were blocked for 4 hours in 10 % (w/v) milk (marvel milk powder made up in Tris A buffer (0.1 M tris, 0.85 % (w/v) NaCl, 0.1 % (v/v) Triton X-100, pH 7.5)), rinsed three times briefly in PBST then incubated with primary antibody diluted in Tris A buffer containing 3 % (w/v) BSA overnight at 4 ^{o}C (see table 2.3 for dilutions). Membranes were rinsed 3 times with PBST, then had three 10 minute washes in PBST, with brief rinses in between. HRP-conjugated secondary antibodies were diluted 1/4000 in Tris A buffer containing 2 % (w/v) BSA (see table 2.3) and incubated for 1 hour at room temperature with the membrane. Membranes were rinsed 3 times in PBST, then washed for 10 minutes in PBST followed by two 10 minute washes in PBS without Tween. Membranes were developed by a one minute incubation in 4 ml luminol enhancer solution and 4 ml peroxide solution. Membranes were imaged using LAS3000 imager (GE healthcare, Buckinghamshire, UK), exposure times were varied.

2.6 Fluorescence microscopy

2.6.1 Method A, for determining Aβ internalisation:

2.6.2 Growth of cells

Thirteen mm glass coverslips (VWR, West Sussex UK) were coated with fish-skin (FSG) gelatine by a 30 minute incubation with 200 µl 0.1 % (v/v) FSG in a 24-well plate at 37ºC. FSG was then removed and SH-SY5Y cells were plated onto the coated coverslips in order to be approximately 50-60% confluent the following day.

2.6.3 Fixing and staining cells

Following treatment with AβOs coverslips were rinsed three times with PBS (with Ca^{2+} and Mg^{2+}, PBS+M)) then fixed by a 10 minute incubation with 4 % paraformaldehyde (PFA, diluted in ddH_2O from a 16 % stock from Alfa Aesar, Ward Hill, MA, USA). The PFA was removed and quenched with 1 ml 50 mM NH_4Cl. Coverslips were washed three times for 5 minutes with PBS+M, then cells were permeabilised by a 4 minute incubation in PBS+M 0.2 % (v/v) Triton X-100. Coverslips were rinsed twice with PBS+M then blocked for 18 or more hours at 4ºC with 5 % FSG in PBS+M (sterilised using a 0.2 µm filter). Cells were then stained with primary antibody: coverslips were transferred to a moist chamber and incubated for 1 hour at room temperature with 20 µl anti-BIN1 antibody (ab54764) diluted 1/500 in 5 % (v/v) FSG. Coverslips were transferred back to the 24-well plate and washed three times for 5 minutes with PBS+M. Coverslips were transferred to the moist chamber and stained with secondary antibodies: 20 µl AlexaFluor® 488 conjugated donkey anti-mouse (secondary for BIN1) and Texas-red conjugated streptavidin (secondary for biotinylated Aβ) diluted 1/500 in 5 % FSG were incubated with coverslips for 1 hour at room temperature protected from light. Coverslips were then transferred back to 24-well plate and washed for 10 minutes with PBS+M. Nuclei were stained by 10 minute incubation with 2 µg ml^{-1} 4',6-diamidino-2-phenylindol dihydrochloride (DAPI, Invitrogen Life Sciences, Paisley, Scotland, UK) at room temperature. Coverslips were washed a further two times with PBS+M for 10 minutes each. Coverslips were then mounted face down onto glass slides using Fluoromount G (SouthernBiotech, Birmingham Alabama, USA). Slides were dried overnight at 4 ºC then visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision, Issaquah, Washington, USA).

2.6.4 Method B, for determining cell surface APP and NMDAR:

SH-SY5Y-APP$_{695}$-BIN1 and SH-SY5Y-APP$_{695}$-pcDNA3.1(+) cells were plated at 50,000 cells per well on 13 mm glass coverslips in a 24-well plate, so that they were 80 % confluent the following day. The cells were washed twice with PBS+M at 4 ^{o}C then incubated for 30 minutes with primary antibody diluted 1/200 in PBS+M (6E10 for APP, SAB2500699 (Sigma Aldrich, Poole, Dorset, UK) for the GluN2B subunit of the NMDA receptor) at 4 ^{o}C. Cells were washed twice with 4 ^{o}C PBS+M, then incubated for 10 minutes with DMEM at either 37 ^{o}C or 4 ^{o}C. Cells were washed twice with PBS+M at 4 ^{o}C then fixed, permeabilised and stained with secondary antibodies using the same procedure as Method A. Secondary antibodies used were Alexa Fluor 488 conjugated donkey anti-mouse for APP or AlexaFluor® 488 conjugated donkey anti-goat for NMDA receptor.

2.6.5 Taking and quantifying images

Cells were visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision, Issaquah, Washington, USA), and data stacks at excitation 360 nm and emission 457 nm (DAPI, (nuclei)), excitation 490 nm and emission 526 nm (FITC, for APP, BIN1, or NMDAR), and excitation 555 nm and emission 617 nm (Texas-red, for biotin tagged Aβ) were taken for 20 cells per treatment condition. Data stacks were deconvolved using SoftWoRx software (Applied Precision, Issaquah, Washington, USA). Images were analysed using Image J software. For each cell an image from the middle of the Z-stack representing a slice through the centre of the cell was selected. For Aβ measurements the total fluorescence intensity of Aβ in and around the cell and the fluorescence intensity of Aβ close to the edges of the cell were measured. The % of

Aβ fluorescence inside the cell (as opposed to bound to the edge of the cell) was determined.

2.6.6 Preparation of Aβ oligomers

Synthetic amyloid-beta oligomers (AβOs) were prepared from biotin tagged monomeric $A\beta_{42}$ peptide (biotin-LC-$A\beta_{1-42}$, from AnaSpec, Freemont, CA, USA) as described by (Chromy et al., 2003). Lyophilised $A\beta_{1-42}$ peptide was re-constituted in 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) then dried under a N_2 gas stream to form a peptide film, which was stored at -80ºC. The $A\beta_{42}$ peptide film was resuspended in dimethyl sulphoxide (DMSO) then diluted 1/10 using Ham's F12 medium (Invitrogen Life Sciences, Paisley, Scotland, UK) to a 100 µM concentration of Aβ, and this was incubated for 16 hours at room temperature to allow the Aβ to aggregate and form oligomers. The oligomer preparation was centrifuged for 20 minutes at 16,000 *g* to pellet any fibrillar material. As a vehicle control DMSO was diluted 1/10 in Ham's F12 medium, incubated for 16 hours at room temperature and centrifuged at 16,000 *g* for 20 minutes.

2.7 Flow cytometry

2.7.1 Method A, for cell surface NMDA receptor levels

Cells were plated in a 6-well plate at 500,000 cells per well and incubated for 48 hours at 37 ^{o}C. The following procedure was carried out at 4 ^{o}C. Cells were washed twice with 500 µl PBS+M, then harvested by scraping into 500 µl PBSM and pelleted by centrifugation at 800 *g* for 5 minutes. The cell pellets were resuspended in 200 µl blocking buffer (5 % (v/v) fish skin gelatine in PBS+M) and blocked for 20 minutes. Primary antibody was diluted 1/100 into the cell suspension (2 µl SAB2500699 was added to each sample), and incubated for 1 hour. Cells were

pelleted by centrifugation at 800 *g* for 5 minutes, then washed by resuspending in 500 µl PBS+M. Cells were re-pelleted, then resuspended in 200 µl blocking buffer and secondary antibody (AlexaFluor® 488 conjugated donkey anti-goat) was diluted 1/100 into samples and incubated for 1 hour. Cells were pelleted by centrifugation at 800 g for 5 minutes, then washed by re-suspending in 500 µl PBS+M. Cells were then re-pelleted and re-suspended in 500 µl PBSM 2.5 mM EDTA. One microlitre of 2 mg ml^{-1} DAPI was added to each sample less then 1 minute prior to analysis. Fluorescence from >10,000 live cells per sample was measured using a BD LSRFortessa cell analyser from BD biosciences (San Jose, CA, USA).

2.7.2 Method B, for endocytosis of APP

Cells were plated in a 12-well plate at 200,000 cells per well and incubated for 48 hours at 37 ^{o}C. The entire procedure was carried out at 4 ^{o}C unless otherwise stated. Cells were washed twice with PBS+M and incubated for 30 minutes with 300 µl primary antibody (6E10) diluted 1/200 in PBS+M. Cells were washed twice with PBS+M, then incubated for 10 minutes with DMEM at either 4 ^{o}C or 37 ^{o}C. Cells were then washed twice with PBS+M at 4 ^{o}C, then harvested by scraping into 500 µl PBS+M. Cells were pelleted by centrifugation at 800 *g* for 5 minutes, then re-suspended in 200 µl blocking buffer and blocked for 20 minutes. The addition of secondary antibody (AlexaFluor® 488 conjugated donkey anti-mouse) and analysis procedure was the same as for method A.

2.8 General assays

2.8.1 Amyloid-Beta Enzyme-linked Immunosorbent Assay (Aβ ELISA)

The Aβ ELISA method was developed by (Haugabook et al., 2001), antibodies were a kind gift from Drs Chris and Liz Eckman (Mayo Clinic, Jacksonville, FL,

USA, current address Atlantic Health System, Morristown, NJ, USA). Maxisorb ELISA plates were coated with capture antibody by 24 hour incubation at 4 °C with 50 µg ml^{-1} anti-$A\beta_{1-40}$ (33.1.1) or anti-$A\beta_{1-42}$ (2.1.3.35.86) antibody diluted in PBS-M (100 µl per well). Wells were then blocked for 4 hours at room temperature with 300 µl Block ACE (Serotec, Toronto, Ontario, USA) 0.05 % (w/v) NaN_3 in PBS-M. Wells were washed two times with 300 µl PBS-M, then samples and standards ($A\beta_{1-40}$ and $A\beta_{1-42}$ from Bachem, Bubendorf, Switzerland) were diluted in EC buffer (5.75 mM NaH_2PO_4, 15 mM Na_2HPO_4, 2 mM EDTA, 0.4 M NaCl, 0.2 % (w/v) BSA, 0.05 % (w/v) CHAPS, 0.4 % (w/v) Block ACE, 0.05 % (w/v) NaN_3, pH 7.0) in the plate and incubated at 4 °C for 24 hours. Wells were washed two times with PBS, then HRP conjugated detection antibody (anti-$A\beta_{1-40}$ 13.1.1 1/1000, anti-$A\beta_{1-42}$ 4G8 (from Covance, New Jersey, USA) 1/2000) was diluted in 3.4 mM NaH_2PO_4, 17 mM Na_2HPO_4, 2 mM EDTA 1 % (w/v) BSA pH 7.0 and incubated for 20 hours at 4 °C. Wells were washed three times with PBS containing 0.05 % (v/v) Tween then developed using a 1:1 ratio of TMB 2-component peroxidise substrate kit (Kirkegaard and Perry, Gaithersburg, Maryland, USA), the reaction was stopped with 1 M H_3PO_4 and absorbance read at 450 nm.

2.9 Human brain samples

2.9.1 Cohorts 1, 2 and 3

Brain tissue was obtained from the Medical Research Council London Neurodegenerative Diseases Brain Bank (Institute of Psychiatry, King's College London), the study had approval from the Leeds (Central) Research Ethics Committee. Brain tissue was homogenised by Dr Isobel Whitehouse as reported in (Whitehouse et al., 2010). Briefly, samples were homogenised in 9 volumes of

PBS-M, 0.5 % (v/v) NP-40, 0.5 % (w/v) sodium deoxycholate, pH 7.4 with Roche complete protease inhibitor cocktail (Roche Diagnostics, West Sussex, UK), using an electric homogeniser. Samples were centrifuged at 14,000 *g* for 10 minutes, pellets were discarded. The protein concentration of supernatants was determined by BCA assay.

2.9.2 Cohort 4

Brain tissue was obtained from South West Dementia Brain Bank, University of Bristol, UK; the study had approval from North Somerset and South Bristol Research Ethics Committee and Leeds (Central) Research Ethics Committee. The diagnosis of AD in AD cases, and the absence of AD in control cases was made according to the criteria of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Morris et al., 1989). Neuron-specific enolase (Miners et al., 2009), soluble Aβ and insoluble Aβ (van Helmond et al., 2010a, b) had been determined previously. For western blotting, samples were homogenised by Dr Isobel Whitehouse as detailed above in cohorts 1, 2, and 3. For Aβ and NSE measurements brain samples were homogenised and assays performed by Dr Scott Miners from the University of Bristol.

2.10 Data analysis

Data were analysed using GraphPad Prism software. Data were tested for normal distribution using Kolmogorov-Smirnov normality test. If the data were normal significance was determined using the Paired t-test or Pearson correlation accordingly. For non-normally distributed data non-parametric statistical analysis were used, either Mann-Whitney, or Spearman's correlation coefficient.

3 The effect of proteins from an *in vivo* APP interactome on APP processing

3.1 Introduction

The proteolytic processing of APP has been shown to be modulated by interactions with several different cellular proteins. By regulating APP metabolism these proteins regulate the production of Aβ. Several large studies have identified proteins which interact with APP (Bai et al., 2008; Kohli et al., 2012). This means there is a large number of proteins with the potential to regulate APP processing and Aβ generation, yet the effects of most of these have not been characterised fully.

Three proteins were identified from the mouse *in vivo* APP interactome by Bai et al., (2008) for further investigation into their role in the regulation of APP metabolism: neurofascin, Thy-1 and contactin 5 (CNTN5). These proteins were selected due to their sub-cellular location – they are all cell surface glycoproteins, and contactin and Thy-1 are located in lipid rafts (Kemshead et al., 1982; Shimoda and Watanabe, 2009) – which means they have a high chance of interacting with APP, and of regulating its processing. Furthermore Thy-1 and proteins from the contactin family have been previously implicated in AD. Thy-1 expression is reduced and its sub-cellular location is altered in the brain during AD (Leifer and Kowall, 1992). Proteins from the contactin family have been identified as potential CSF biomarkers for AD (Yin et al., 2009). CNTN1, 2, and 4 regulate proteolysis of APP (Hu et al., 2003; Hu et al., 2004; Ma et al., 2008; Osterfield et al., 2008), and CNTN5 has been associated with AD neuroimaging measures and identified as an AD linked gene in GWAS (Biffi et al., 2010). Although CNTN1 was identified by

Bai et al., (2008) in the *in vivo* APP interactome, studies in this chapter focus on CNTN5. There are several reasons for this: CNTN5 has been genetically linked to AD by GWAS whereas CNTN1 has not. CNTN1 is involved in notch processing (Hu et al., 2003; Hu et al., 2004). If CNTN1 was found to regulate APP processing the potential to develop it into a drug target in the future may be limited as an AD drug targeting CNTN1 is likely to have similar side effects to the γ-secretase inhibitors that have failed in clinical trials. CNTN5 knockout mice are viable, and do not display a deleterious phenotype (Li et al., 2003) unlike CNTN1 knockout mice which died prematurely (Berglund et al., 1999), suggesting an AD drug targeting CNTN5 is less likely to have undesirable side effects.

3.1.1 Aims

We hypothesied that neurofascin, Thy-1, and contactin 5 would alter APP metabolism. To investigate this, APP and each protein was expressed in HEK cells, and the effect of over expression on full length APP, sAPPα, sAPPβ and Aβ determined by western blotting (for APP, sAPPα, and sAPPβ) and ELISA (for Aβ). This showed CNTN5 expression dramatically altered Aβ metabolism, so the mechanism of this was further investigated by examining the effect of CNTN5 expression on APP C-terminal fragments. To determine how our findings in cell culture models may relate to the human brain CNTN5 levels in human brain samples, during aging, and in sporadic and familial AD were measured by western blotting.

3.2 Cloning and over-expression of neurofascin 186, Thy-1 and contactin 5

IMAGE human cDNA clones of neurofascin 186 (NF186, IMAGE 40068186), Thy-1 (IMAGE 6137513), and contactin 5 (IMAGE 40146650) were obtained from Source Bioscience. The cDNA of interest was amplified by PCR from the vector in which it was supplied using primers with a 5`NotI and a 3` EcoRI restriction site.

Forward primers were:

NF186: GATGCTCGTGAGCGGCCGCATGGCCAGGCAGCCACCGCC,

Thy-1: GATGCTCGTGAGCGGCCGCATGAACCTGGCCATCAGCATCGCT,

CNTN5:

GATGCTCGTGAGCGGCCGCATGGCTTCCTCTTGGAAACTAATGCT

Reverse primers were:

NF186: GCTAGGAATTCTTAGGCCAGAGAGTAGATAGCATTGAC,

Thy-1: CATAGGAATTCTCACAGGGACATGAAATCCGTGGCC,

CNTN5: CATAGGAATTCTCACCAGGAAGTTGAAGGAATCATCA.

The resulting product was the cDNA of interest flanked by NotI and EcoRI restriction sites. The PCR product was gel purified, then PCR product and pIRESneo vector were digested with NotI and EcoRI, and the cDNA was ligated into pIRESneo. The ligation reaction was used to transform XL1-blue competent *E.coli* cells, which were then plated and incubated overnight. Transformed colonies were picked and plasmids were purified. Correct insertion of each gene into pIRESneo was assessed by restriction digest reactions.

NF186 insertion was confirmed by NcoI digestion. Expected fragments from digestion of pIRESneo with NcoI are 3389 bp and 1865 bp, whereas expected fragments from digestion of pIRESneo with an NF186 insertion are 4733 bp, 3389 bp, and 858 bp. Digestion of plasmid purified from colony N2 showed the expected fragments (figure 3.1A) so insertion of NF186 into pIRESneo was confirmed by sequencing (see appendix). The new plasmid generated was named pIRESneo-NF186 (figure 3.1B). HEK-APP_{695} cells were transfected with pIRESneo-NF186 (empty pIRESneo was transfected in to use as a negative control) then cells were selected with the antibiotic G418 to generate a cell line stably expressing NF186 (figure 3.1C). In NF186 expressing cells the neurofascin antibody detected not only a band of 186 kDa (the expected molecular weight of the NF186 isoform) but also several bands of lower molecular weight, which were not present in vector only cell lysates. When the neurofascin gene was originally cloned in 1992 (Volkmer et al., 1992) multiple forms were detected in plasma membrane preparations for neurofascin protein, some of which were identified as proteolytic cleavage products of longer isoforms of neurofascin. This may account for the extra lower molecular weight forms seen here.

Correct insertion of Thy-1 into pIRESneo was assessed by restriction digest reactions using Eco0109I. Expected fragments from digestion of pIRESneo with Eco0109I are 4844 bp, 356 bp and 54 bp, whereas expected fragments from digestion of pIRESneo with a Thy-1 insertion are 4672 bp, 702 bp, 356 bp and 54 bp. Digestion of plasmid purified from colony T1 showed the expected fragments (figure 3.2 A) so insertion of Thy-1 into pIRESneo was confirmed by sequencing (see appendix). The new plasmid generated was named pIRESneo-Thy-1 (figure 3.2 B). HEK-APP_{695} cells were transfected with pIRESneo-Thy-1 (empty pIRESneo was transfected in to use as a negative control) then cells were selected with the

antibiotic G418 to generate a cell line stably expressing Thy-1. Expression of Thy-1 in cells was confirmed by dot blot of cell lysates. Dot blotting, as opposed to western blotting, was performed because the Thy-1 antibody MAB2067 recognises an epitope that is lost when Thy-1 is denatured (as occurs in western blotting) (Figure 3.2 C).

Correct insertion of CNTN5 into pIRESneo was assessed by restriction digest reactions using KpnI. Expected fragments from digestion of pIRESneo with KpnI are 4411 bp, and 843 bp, whereas expected fragments from digestion of pIRESneo with a CNTN5 insertion are 4411 bp, 3320 bp, and 929 bp. Digestion of plasmid purified from colony C6 showed the expected fragments (figure 3.3 A), so insertion of CNTN5 into pIRESneo was confirmed by sequencing (see appendix). The new plasmid generated was named pIRESneo-CNTN5 (figure 3.3 B). HEK-APP_{695} cells were transfected with pIRESneo-CNTN5 (empty pIRESneo was transfected in to use as a negative control) then transfected cells were selected with the antibiotic G418 to generate a cell line stably expressing CNTN5. Expression of CNTN5 in cells was confirmed by western blot of cell lysates (Figure 3.3 C).

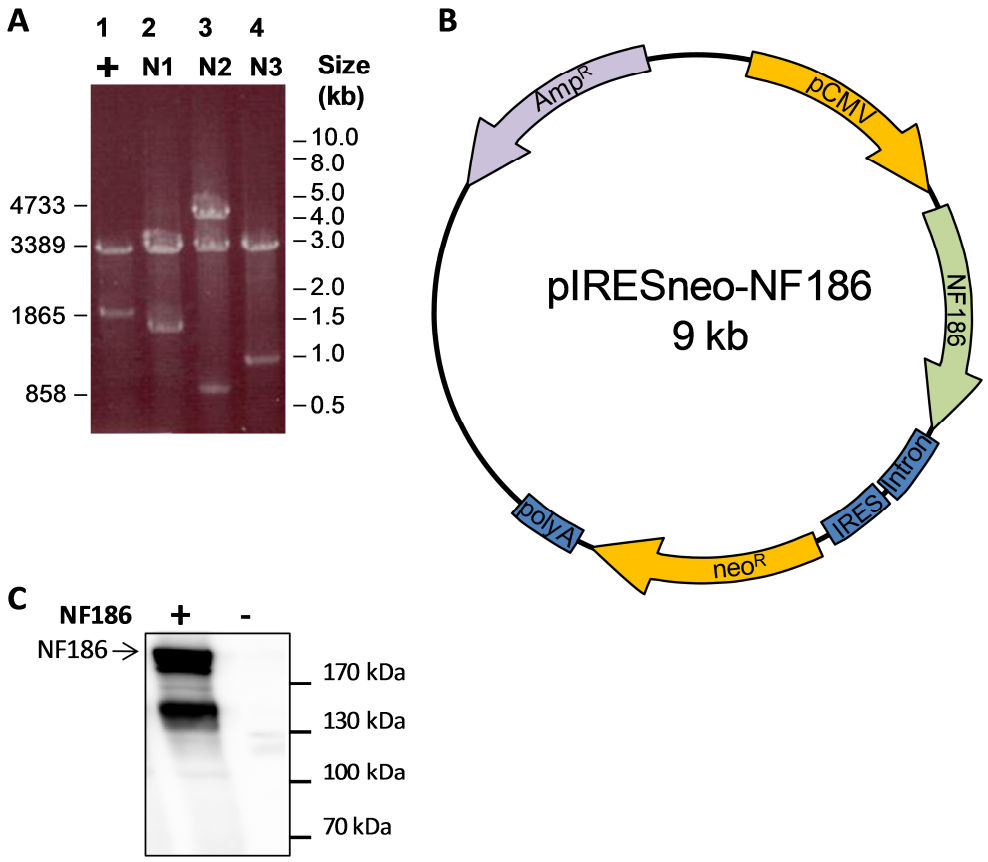

Figure 3.1 Expression of NF186 in HEK-APP$_{695}$. (A) NcoI digests of: lane 1, empty pIRESneo; lanes 2-4, plasmid purified from colonies transformed with NF186 pIRESneo ligation reaction. Colony N2 contained the expected fragments for insertion of NF186 into pIRESneo at the correct sites (4733 bp, 3389 bp, and 858 bp). (B) Map of the plasmid pIRESneo-NF186. pCMV is the promoter from human cytomegalovirus, Intron is a synthetic intron which enhances the stability of the mRNA, IRES is the ribosome entry site from encephalomyocarditis virus, neo^R is the neomycin phosphotransferase gene which confers neomycin resistance, polyA is the polyadenylation signal. Amp^R is the ampicillin resistance gene. All elements but the gene of interest (NF186) come from the pIRESneo plasmid. (C) Western blot for neurofascin (with ab31457) of HEK-APP$_{695}$ cell lysates (60 µg) transfected with pIRESneo-NF186 (+) or pIRESneo (-).

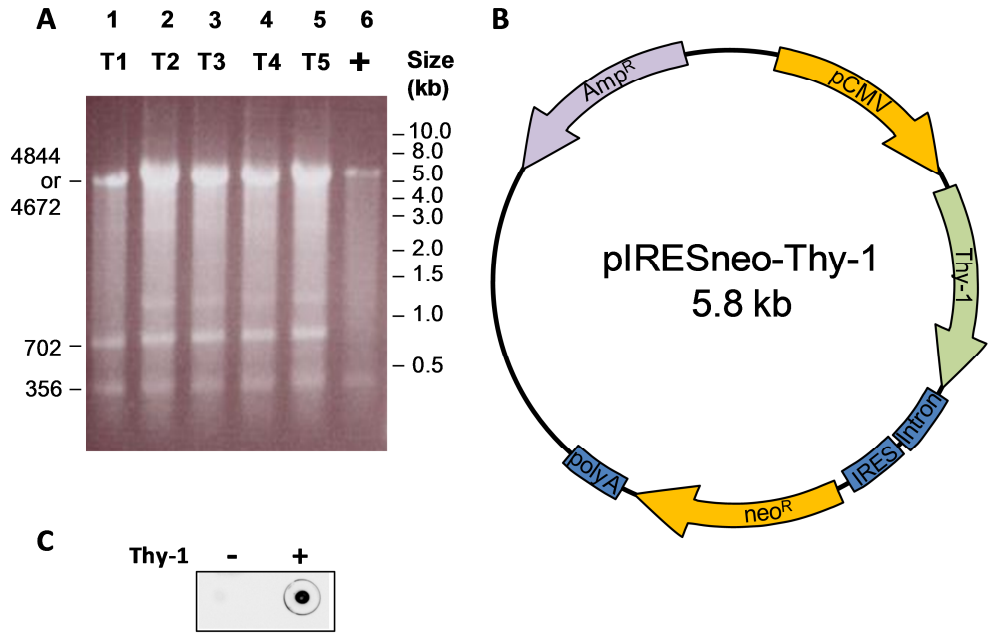

Figure 3.2 Expression of Thy-1 in HEK-APP$_{695}$. (A) Eco01095I digests of: lanes 1-5, plasmid purified from colonies transformed with Thy-1 pIRESneo ligation reaction; lane 6, empty pIRESneo. Colony T1 contained the expected fragments for insertion of Thy-1 into pIRESneo at the correct sites (4672 bp, 702 bp, 356 bp and 54 bp). (B) Map of the plasmid pIRESneo-Thy-1.For details of plasmid components see figure 3.1. (C) Dot blot for Thy-1 (with MAB2076) on HEK-APP$_{695}$ cell lysates (40 µg) transfected with pIRESneo-Thy-1 (+) or pIRESneo (-).

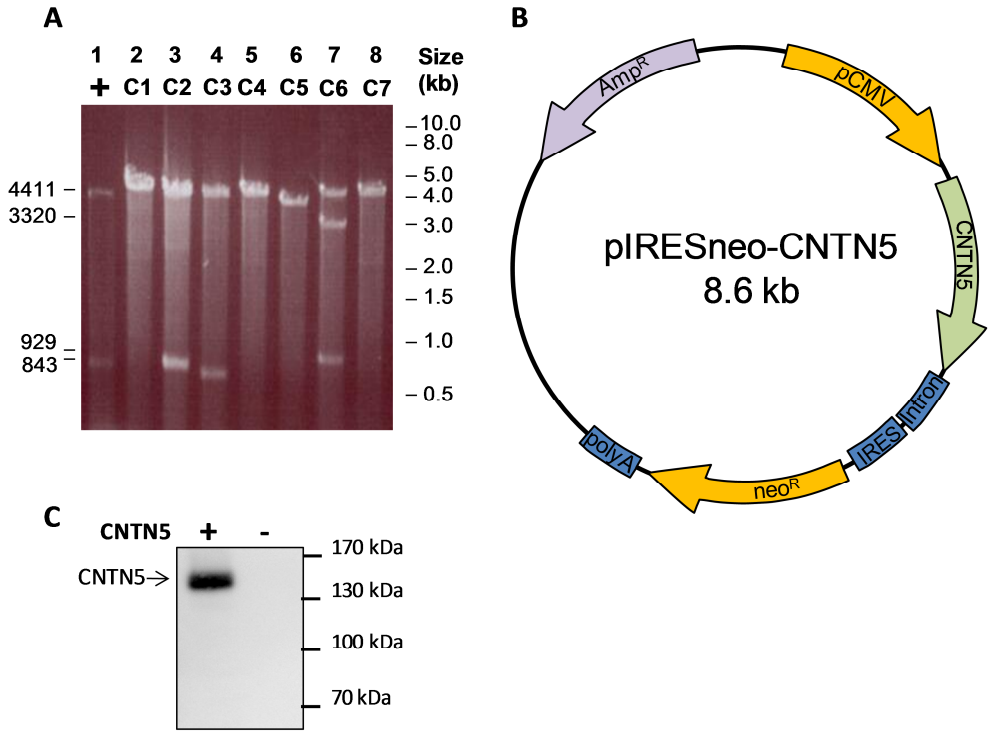

Figure 3.3 Expression of CNTN5 in HEK-APP$_{695}$. (A) KpnI digests of: lane 1 empty pIRESneo; lanes 2-8, plasmid purified from colonies transformed with CNTN5 pIRESneo ligation reaction. Colony C6 contained the expected fragments for insertion of CNTN5 into pIRESneo at the correct sites (4411 bp, 3320 bp, and 929 bp). (B) Map of the plasmid pIRESneo-CNTN5. For details of plasmid components see figure 3.1. (C) Western blot for CNTN5 (with AF3030) on HEK-APP$_{695}$ cell lysates (30 µg) transfected with pIRESneo-CNTN5 (+) or pIRESneo (-).

3.3 Cell line selection and experimental conditions

HEK cells are a Human Embryonic Kidney cell line. Although they are a kidney cell line they expresses many neuronal proteins (Shaw et al., 2002) and so were selected for use due to their comparative ease of transfection compared to neuronal cell lines. Although HEK cells express endogenous APP, in order to detect sAPPβ in conditioned media by western blotting APP_{695} was over-expressed in the cells (HEK-APP_{695} cells). To determine the effect of each gene on APP processing, each protein was over-expressed in HEK-APP_{695} cells then APP, sAPPα, sAPPβ, and Aβ were measured. Before these experiments were undertaken the appropriate length of incubation for conditioning media (to measure sAPPα and sAPPβ) was determined. HEK-APP_{695} cells were incubated for either 10, 12, 14, 24, 36 or 48 hours with OptiMEM reduced sera media. Conditioned medium was harvested and cells were harvested and lysed.

The APP levels in cell lysates and sAPPα and sAPPβ levels in conditioned media were measured by western blotting. APP was detected in cell lysates at all time points, and APP expression did not change over time (figure 3.4 A). Western blots for APP showed 4 bands, these correspond to the different isoforms of mature and immature APP. The bands corresponding to the 695 isoform are more intense than the 751/770 isoforms as the cells are over-expressing APP_{695}. The resolution of the gel is not sufficient to distinguish the 751 and 770 isoforms so these appear together as one band. sAPPα was detected at all time points, and more sAPPα accumulated in the media the longer the incubation (figure 3.4 B). Two bands corresponding to sAPPα were detected at later time points (36 and 48 hours). The lower molecular weight band is sAPPα generated from the 695 isoform of APP ($sAPP\alpha_{695}$), and the upper band is sAPPα generated from the 751/770 isoforms of

APP ($sAPP\alpha_{751/770}$). As with APP, there is less $sAPP\alpha_{751/770}$ than $sAPP\alpha_{695}$ because the cells over-express the 695 isoform of APP. Again, the resolution of the gel is not sufficient to distinguish the 751 and 770 isoforms so these appear as a single band. sAPPβ was only detected in conditioned media after a 36 hour incubation (figure 3.4 C). Under normal circumstances the majority of APP is processed *via* the non-amyoidogenic α-cleavage pathway, so there is much less sAPPβ than full length APP or sAPPα. No band corresponding to $sAPP\beta_{751/770}$ was detected; this is because it is not over-expressed so very little is produced. Although sAPPβ was detected after a 36 hour incubation significantly more was detected at 48 hours. In order to detect sufficient sAPPβ to measure both increases and decreases in sAPPβ levels a 48 hour incubation was used for subsequent experiments.

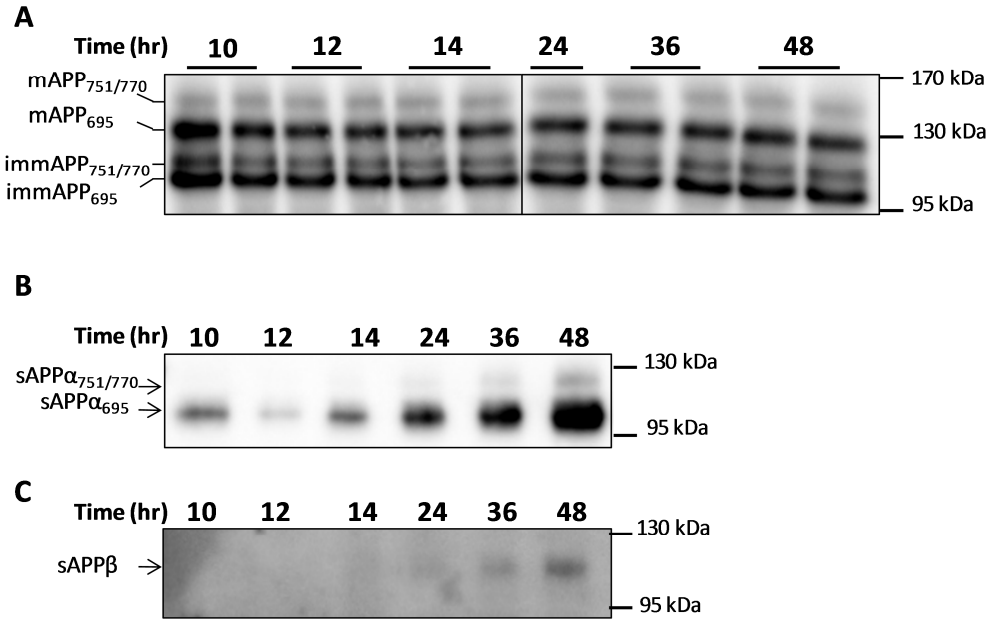

Figure 3.4 Timecourse of APP, sAPPα and sAPPβ levels over 48 hours in HEK-APP$_{695}$. (A) Western blot for APP (with 22C11) on cell lysates (40 µg). (B) western blot for sAPPα (with 6E10) on conditioned media (30 µg). (C) Western blot for sAPPβ (with 1A9) on conditioned media (30 µg). The vertical line in blot A indicates two separate gels. Although the time points shown in this experiment have been run on separate gels, in repeat experiment all samples were run on a single gel.

3.4 The effect of neurofascin 186 on APP processing

HEK-APP_{695} cells expressing NF186 or empty vector were incubated for 48 hours, then medium was harvested and cells were harvested and lysed. Cell lysates were western blotted for NF186 and APP, and conditioned media was western blotted for sAPPα and sAPPβ (figure 3.5 A). Aβ levels in conditioned media were determined by ELISA (figure 3.5 E). NF186 over-expression did not alter APP_{695} levels in cell lysates, but it did cause a significant 15 % reduction in $APP_{751/770}$ ($p=0.03$) (figure 3.5 B). NF186 over-expression did not significantly alter sAPPα (figure 3.5 C), sAPPβ (figure 3.5 D) or Aβ (figure 3.5 E).

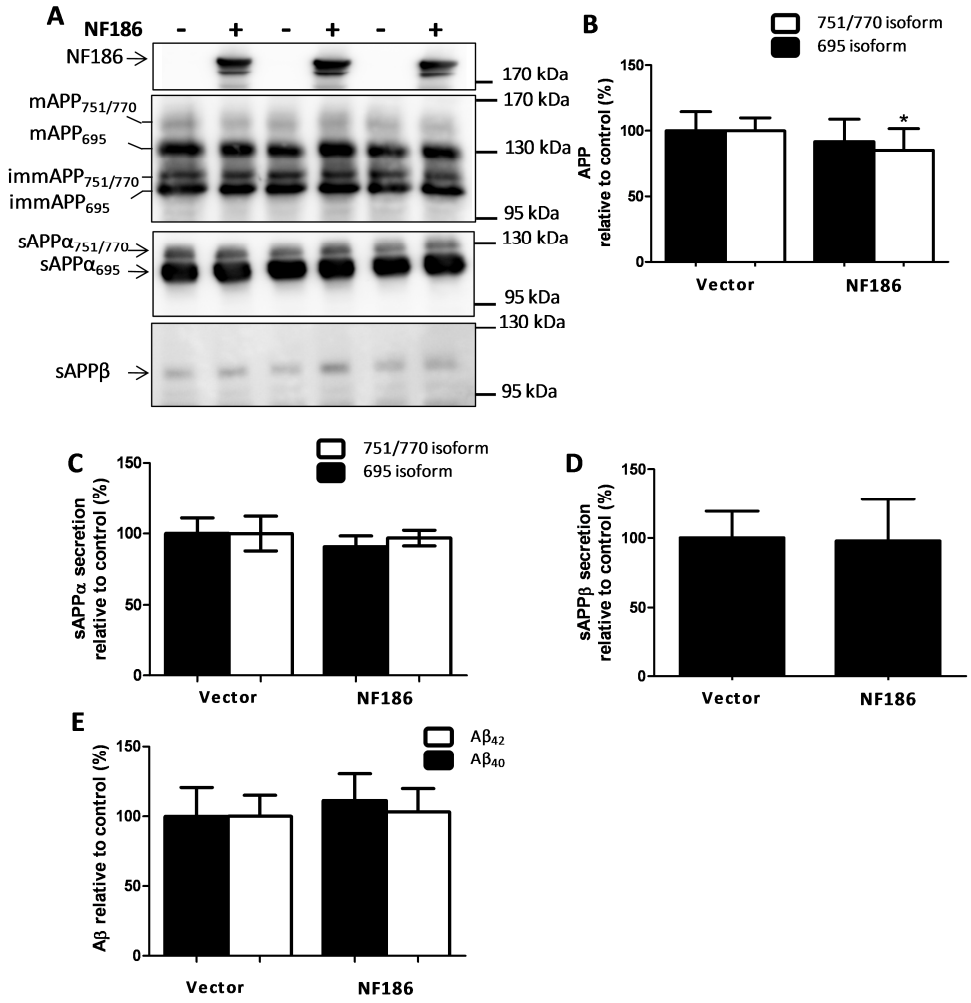

Figure 3.5 The effect of NF186 over-expression on APP proteolysis. (A) Representative western blots on HEK-APP$_{695}$ cell lysates (40 µg) expressing NF186 (+) or empty vector (-) for neurofascin, with ab31457 (top panel), APP with 22C11 (second panel) and conditioned media (30 µg) for sAPPα with 6E10 (third panel) and sAPPβ with 1A9 (fourth panel). (B) Quantification of western blots for APP. (C) Quantification of western blots for sAPPα. (D) Quantification of western blots for sAPPβ. (E) ELISA to determine Aβ in conditioned media. Data are mean ± S.D. n=6 (*$p<0.05$).

3.5 The effect of Thy-1 on APP processing

HEK-APP_{695} cells expressing Thy-1 or empty vector were incubated for 48 hours, then media was harvested and cells were harvested and lysed. Cell lysates were dot blotted for Thy-1 (figure 3.6 A) and western blotted for APP. Conditioned media was western blotted for sAPPα and sAPPβ (figure 3.6 B). Aβ levels in conditioned media were determined by ELISA (figure 3.6 F). Thy-1 over-expression caused a significant 18 % reduction in APP_{695} ($p=0.02$), and a reduction in $APP_{751/770}$ although this was not significant (figure 3.6 C). Thy-1 over-expression also caused a significant 22 % decrease in sAPPβ ($p=0.004$) (figure 3.6 E). Thy-1 over-expression did not significantly alter sAPPα (figure 3.6 D) or Aβ (figure 3.5 E).

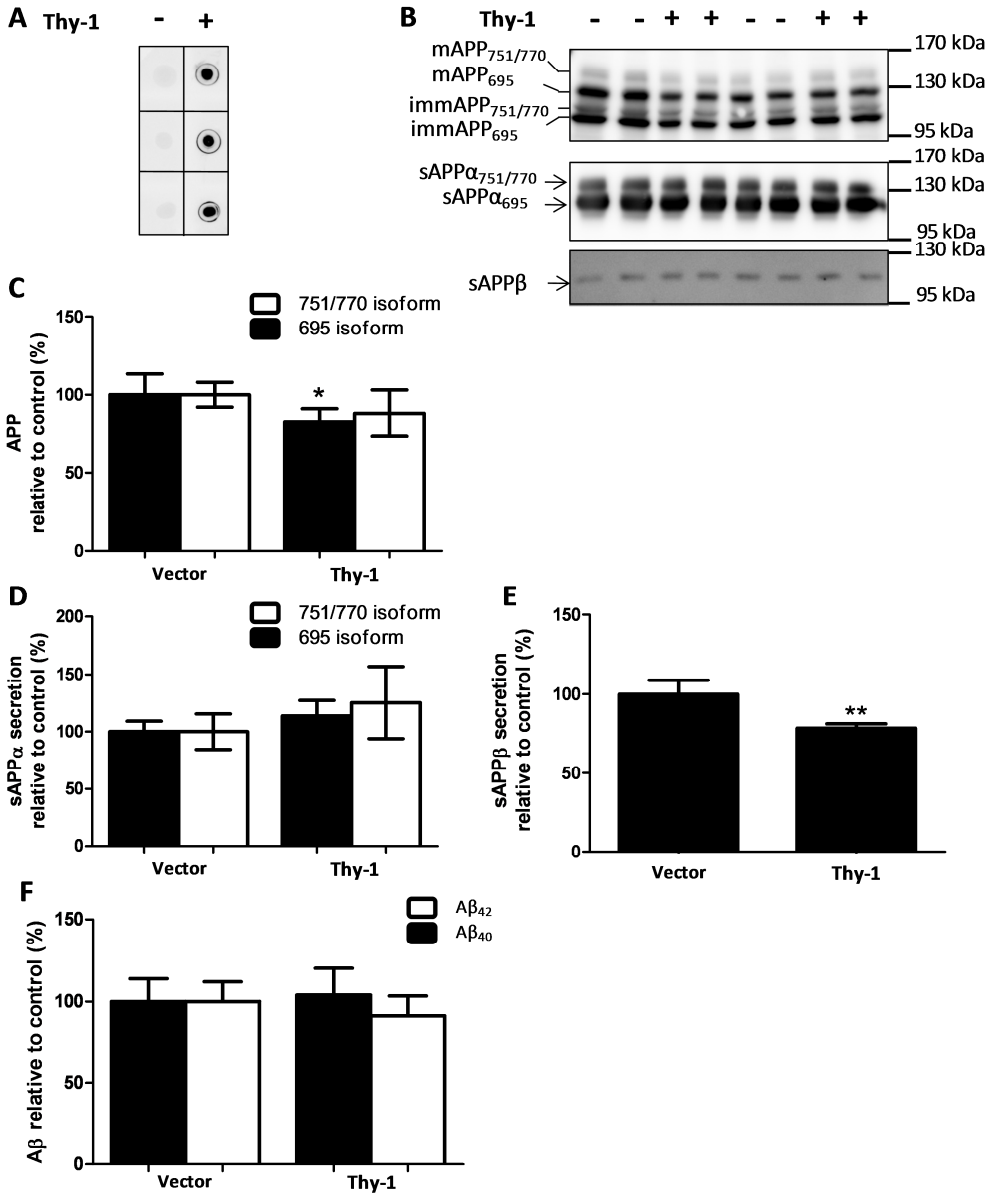

Figure 3.6 The effect of Thy-1 over-expression on APP proteolysis (legend overleaf)

Figure 3.6 The effect of Thy-1 over-expression on APP proteolysis. (A) Dot blot on HEK-APP_{695} cell lysates (40 µg) expressing Thy-1 (+) or empty vector (-) for Thy-1 using MAB2076. (B) Representative western blots on cell lysates expressing Thy-1 (+) or empty vector (-) (60 µg) for APP with 22C11 (top panel) and conditioned media (30 µg) for sAPPα with 6E10 (second panel) and sAPPβ with 1A9 (third panel). (C) Quantification of western blots for APP. (D) Quantification of western blots for sAPPα. (E) Quantification of western blots for sAPPβ. (F) ELISA to determine Aβ in conditioned media. Data are mean ± S.D. n=4 (*$p<0.05$), (**$p<0.01$).

3.6 The effect of contactin 5 on APP processing

HEK-APP_{695} cells expressing CNTN5 or empty vector were incubated for 48 hours, then medium was harvested and cells were harvested and lysed. Cell lysates were western blotted for CNTN5 and APP, and conditioned media was western blotted for sAPPα and sAPPβ (figure 3.7 A). Aβ levels in conditioned media were determined by ELISA (figure 3.7 E). CNTN5 over-expression caused a significant 40 % increase in APP_{695} ($p=0.02$), and a significant 22 % increase in $APP_{751/770}$ ($p=0.02$) (figure 3.7 B). CNTN5 over-expression also caused a significant 11 % increase in $sAPP\alpha_{695}$ ($p=0.001$), but there was no change in $sAPP\alpha_{751/770}$ (figure 3.7 C). CNTN5 over-expression did not have a significant effect on sAPPβ (figure 3.7 D). CNTN5 over-expression caused a 95 % decrease in $A\beta_{40}$ ($p=0.003$). $A\beta_{42}$ was decreased to below the limit of detection of the assay (figure 3.7 E). To confirm these data in a neuronal cell line CNTN5 was transiently expressed in N2a-APP_{695} (a mouse neuroblastoma cell line), cells were incubated for 48 hours then Aβ in conditioned media was determined by ELISA. CNTN5 expression in N2a cells caused a significant 79 % decrease in $A\beta_{40}$ ($p=0.0019$) and a significant 82 % decrease in $A\beta_{42}$ ($p=0.0003$).

NF186, Thy-1 and CNTN5 all regulated the metabolism of APP, but only CNTN5 caused a change in Aβ in conditioned media. Therefore the mechanism by which CNTN5 decreased Aβ was investigated further.

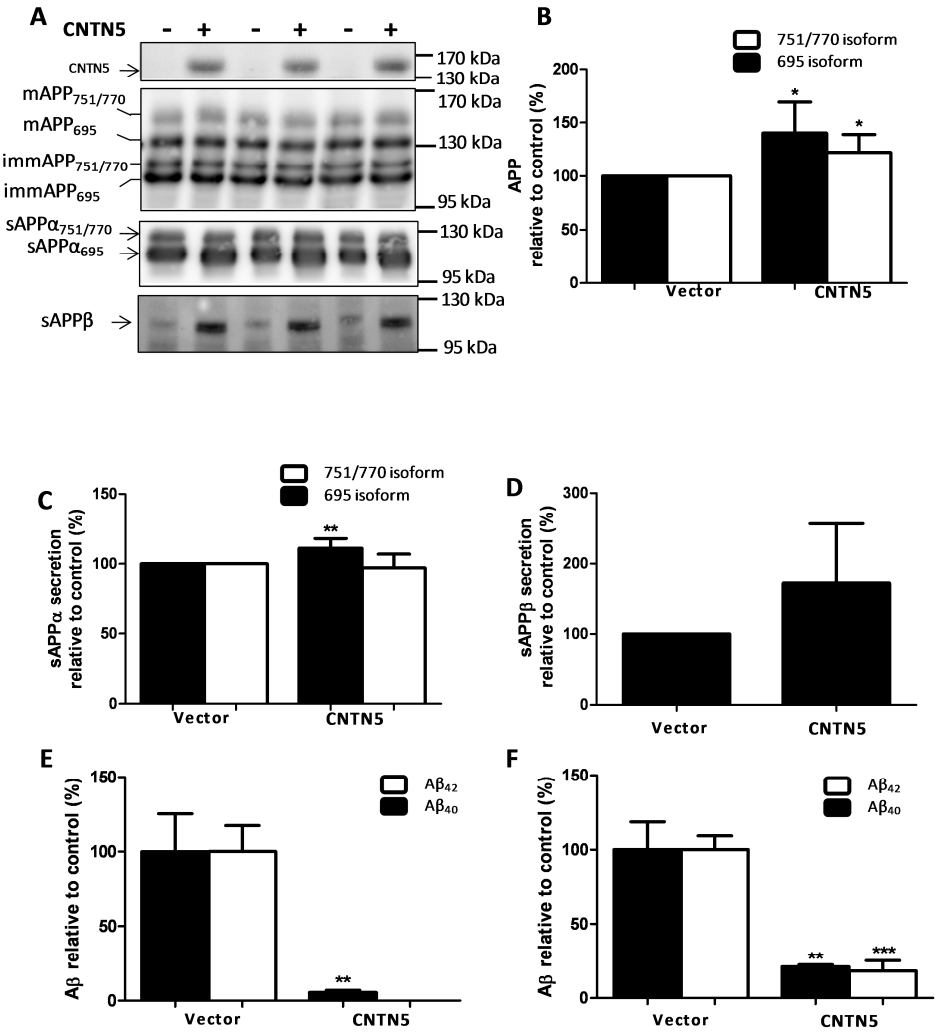

Figure 3.7 The effect of CNTN5 over-expression on APP proteolysis. (A) Representative western blots on HEK-APP$_{695}$ cell lysates (40 µg) expressing CNTN5 (+) or empty vector (-) for CNTN5 with AF3030 (top panel), APP with 22C11 (second panel) and conditioned media (35 µg) for sAPPα with 6E10 (third panel) and sAPPβ with 1A9 (fourth panel). (B) Quantification of western blots for APP. (C) Quantification of western blots for sAPPα. (D) Quantification of western blots for sAPPβ. (E) ELISA to determine Aβ in conditioned media. (F) ELISA to determine Aβ in conditioned media from N2a-APP$_{695}$ cells transiently transfected with CNTN5 cDNA or empty vector. Data are mean ± S.D. n=6 (*p<0.05), (**p<0.01), (***p<0.001).

3.7 The effect of contactin 5 on γ-secretase

To determine whether CNTN5 was decreasing Aβ in the media by decreasing Aβ secretion (so causing a build up of Aβ inside the cells) intracellular Aβ was measured in the presence and absence of CNTN5 expression. HEK-APP_{695} cells expressing CNTN5 or empty vector were incubated for 48 hours, and then cells were harvested and lysed. Aβ in cell lysates was measured by ELISA. CNTN5 over-expression did not alter intracellular Aβ (figure 3.8 A), suggesting CNTN5 does not inhibit Aβ secretion.

CNTN5 over-expression decreased Aβ but not sAPPβ, suggesting CNTN5 inhibits γ-secretase cleavage of APP. To investigate this HEK-APP_{695} cells expressing either CNTN5 or empty vector were incubated for 48 hours, then cells were harvested and lysed, and C-terminal fragments of APP (C83, C99 and AICD) measured by western blotting (figure 3.8 B). CNTN5 over-expression caused an increase in both C83 and C99, confirming the hypothesis that CNTN5 inhibits γ-secretase cleavage of APP. Due to the instability of AICD it was not robustly detected.

To further investigate the action of CNTN5 on γ-cleavage of APP we sought to knock down expression of endogenous CNTN5, and determine the effect on Aβ levels in conditioned media. Cell lysates from a number of cell lines were western blotted for CNTN5 to identify a cell line in which CNTN5 was endogenously expressed. Cell lines examined were Sw13 (a human adrenal cell line), HepG2 (a human hepatocellular carcinoma cell line), CHO (a Chinese hamster ovary cell line), HuVEC (human umbilical vein endothelial cells,), pHFF (human foreskin fibroblast cells), A549 (a human alveolar basal epithelial cell line), A431 (a human epidermoid carcinoma cell line), MCF (a human breast adenocarcinoma cell line), HeLa (a

human cervical adenocarcinoma cell line), N2a (a mouse neuroblastoma cell line), NB7 (a human neuroblastoma cell line), SH-SY5Y (a human neuroblastoma cell line), HEK (a human embryonic kidney cell line), H226 (a human lung squamous cell carcinoma cell line), T84 (a human colonic carcinoma cell line), HuH7 (a human hepatocarcinoma cell line), H1395 (a human lung adenocarcinoma cell line), and LS1034 (a human caecal carcinoma cell line). As a positive control for CNTN5 expression a lysate from SH-SY5Y cells over-expressing CNTN5 was loaded onto gels. None of the tested cell lines had detectable levels of CNTN5 (figure 3.9). It is worth noting that the anti-CNTN5 antibody is directed against human CNTN5, whereas N2a and CHO cells are not of human origin.

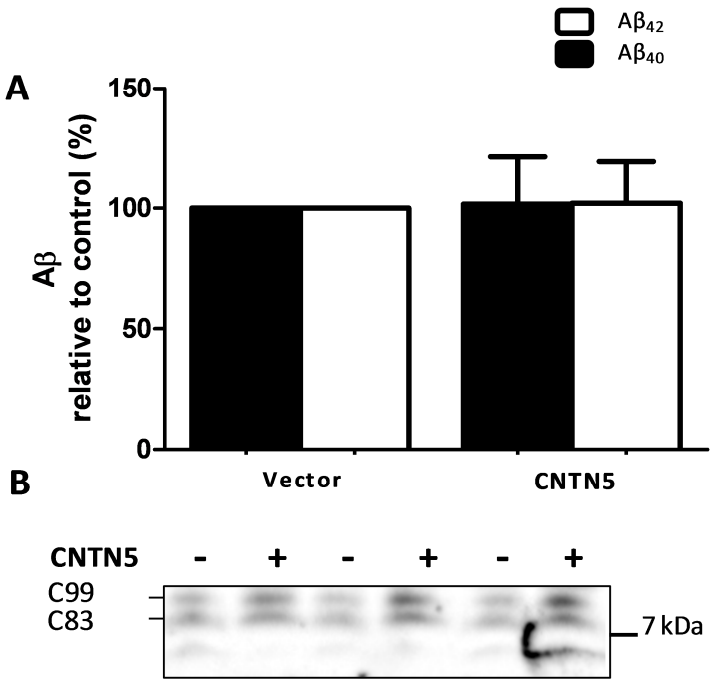

Figure 3.8 The effect of CNTN5 on γ-secretase cleavage of APP. (A) ELISA on cell lysates to determine intracellular Aβ. (B) Representative western blot on cell lysates (50 µg) for C-terminal fragments of APP with A8717. Aβ ELISA data are mean ± S.D. n=6.

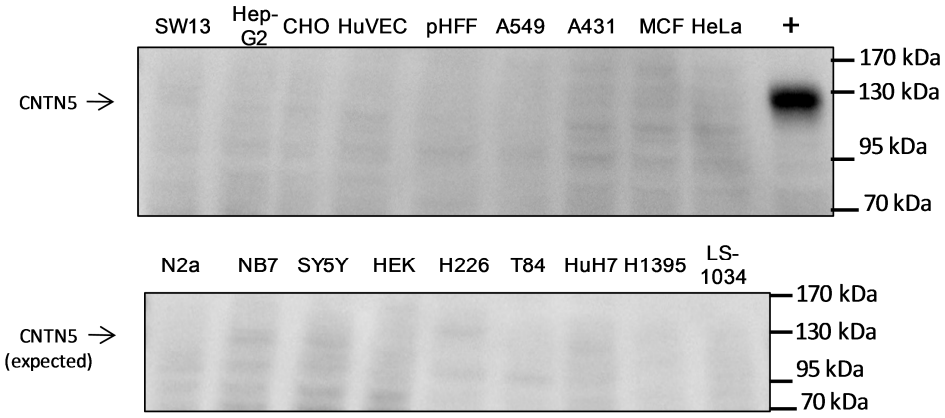

Figure 3.9 Endogenous CNTN5 in cell lines. Western blots on cell lysates (30 µg) for endogenous CNTN5 using AF3030. Positive control (+) is SH-SY5Y cells over-expressing CNTN5.

3.8 Contactin 5 in the human brain

The level of CNTN5 in the human brain during aging and Alzheimer's disease, and the correlation of CNTN5 with Aβ in the human brain, was investigated by western blotting human brain samples for CNTN5. Four different cohorts of brain samples were investigated. Aging cohort 1 (table 3.1) are human hippocampal and temporal lobe samples from individuals who had died between the ages of 20 and 88.

Sporadic AD cohort 2 (table 3.2) are hippocampal and temporal lobe samples from patients with sporadic AD, and from age matched controls. There was no significant difference in the mean age (79 years) between sporadic AD and age matched control hippocampal samples (p=0.97). For temporal lobe samples the mean age was 79 for sporadic AD and 78 for age-matched control, the age was not significantly different (p=0.90). The post mortem delay was not significantly longer for age-matched control samples than for sporadic AD samples (mean 36 hours *versus* 19 hours, p=0.09 for hippocampus, p=0.10 for temporal lobe).

Familial AD cohort 3 (table 3.3) are hippocampal samples from familial AD patients and from age-matched control patients. The mean age for familial AD samples was 68, and the mean age for age matched control samples was 63. Ages were not significantly different (p=0.86). The post mortem delay was significantly longer for age-matched control samples (mean 41 hours *versus* 13 hours, p=0.01).

Sporadic AD cohort 4 (table 3.4) are temporal lobe samples from sporadic AD patients and from age-matched controls. The mean age for sporadic AD samples was 83, the mean age for age matched control samples was 76, the ages were not significantly different (p=0.20). There was no significant difference in post mortem delay between control and sporadic AD samples in cohort 4 (51 hours *versus* 39

hours, p=0.10). Soluble and insoluble Aβ, as well as neuron-specific enolase (NSE) measurements have been carried out on these samples previously by Dr Scott Miners (Miners et al., 2009; van Helmond et al., 2010a, b). There was no significant difference in mean NSE or mean soluble Aβ between sporadic AD and age-matched control samples (figure 3.9 A and B respectively). However, there was a significant increase in mean insoluble Aβ in sporadic AD samples compared to the age matched control samples (a mean value of 139 nM Aβ in sporadic AD compared to 20 nM Aβ in age-matched control samples, $p<0.0001$).

Table 3.1 Details of brains from aging cohort 1: Human hippocampal and temporal lobe samples from individuals who had died between ages 20 and 88. PM = post mortem.

Gender	Age (y)	Cause of death	PM delay (h)
Aging - Hippocampus			
F	26	Carcinoma of the lung	44
M	25	Ruptured aortic arch aneurysm	18
F	20	Sudden accident	38
F	33	Pulmonary embolus	96
M	37	Acute necrotic pancreatitis	27
M	49	Pulmonary oedema	44
M	49	Coronary artery occlusion	32
M	58	Myocardial infarction	23
M	55	Syringomyelia	24
M	57	Left ventricular failure	45
F	62	Haemothorax	81
M	65	Coronary artery occlusion	29
F	76	Congestive heart failure	28
M	79	Septicaemia secondary to faecal peritonitis	24
F	80	Left ventricular failure/bronchopneumonia	31
F	88	Carcinomatosis	43
Aging - Temporal lobe			
F	26	Carcinoma of the lung	44
M	25	Ruptured aortic arch aneurysm	18
F	20	Sudden accident	38
M	49	Pulmonary oedema	44
M	49	Coronary artery occlusion	32
F	44	Hepatic necrosis	10
M	58	Myocardial infarction	23
M	55	Syringomyelia	24
M	57	Left ventricular failure	45
F	62	Haemothorax	81
F	65	Perforated peptic ulcer	30
M	67	Myocardial infarction	41
M	76	Bilateral bronchopneumonia	41
F	79	Chronic obstructive airway disease	38
M	82	Bronchopneumonia	48
M	85	Left ventricular failure	48
F	88	Carcinomatosis	43

Table 3.2 Details of brains from Sporadic AD cohort 2: Sporadic AD and age matched control human hippocampal and temporal lobe samples. PM = post mortem.

Gender	Age (y)	Cause of death	PM delay (h)
Sporadic AD - Hippocampus			
F	82	Dementia	12
F	88	Subdural haematoma	19
F	69	Unknown	16
F	82	Myocardial infarction	5
M	65	Unknown	23
M	87	Unknown	18
M	80	Unknown	41
Control - Hippocampus			
M	79	Septicaemia secondary to faecal peritonitis	24
F	79	Chronic obstructive airway disease	38
M	85	Left ventricular failure	48
F	87	Carcinoma of the breast	22
M	80	Carcinoma of prostate	11
F	62	Haemothorax	81
F	80	Left ventricular failure/bronchopneumonia	31
Sporadic AD - Temporal lobe			
F	82	Dementia	12
F	88	Subdural haematoma	19
F	69	Unknown	16
F	82	Myocardial infarction	5
M	65	Unknown	23
M	87	Unknown	18
M	80	Unknown	41
Control - Temporal lobe			
M	79	Septicaemia secondary to faecal peritonitis	24
F	79	Chronic obstructive airway disease	38
M	85	Left ventricular failure	48
F	87	Carcinoma of the breast	22
M	80	Carcinoma of prostate	11
F	62	Haemothorax	81
F	77	Pulmonary embolus	29

Table 3.3 Details of brains from Familial AD cohort 3: Familial AD and age matched control hippocampal and temporal lobe samples. PM = post mortem.

Gender	Age (y)	Cause of death	PM delay (h)	Mutation
Familial AD - Hippocampus				
M	61	Bronchopneumonia	15	APP717 (VAL-GLY)
F	69	Bronchopneumonia	10	APP717 (VAL-ILE)
F	62	Bronchopneumonia	23	APP717 (VAL-ILE)
M	42	Bronchopneumonia	6	PS1 DELTA4
F	72	Unknown	4	APP717 (VAL-ILE)
F	65	Pneumonia	22	PS1 (E280G)
Control - Hippocampus				
F	62	Haemothorax	81	-
M	57	Left ventricular failure	45	-
F	79	Chronic obstructive airway disease	38	-
M	37	Acute necrotic pancreatitis	27	-
M	79	Septicaemia secondary to faecal peritonitis	24	-
M	65	Coronary artery occlusion	29	-

Table 3.4 Details of brains from Sporadic AD cohort 4: Sporadic AD and age matched control temporal lobe samples. PM = post mortem.

Gender	Age (y)	PM delay (h)
Sporadic AD		
M	69	48
F	70	25
M	74	50
F	74	53
F	77	43
F	78	77
F	78	9
F	78	35
M	79	28
M	80	31
F	81	42
F	81	66
F	83	43
M	85	58
M	85	66
F	87	72
F	87	67
F	88	79
F	89	71
F	89	82
F	90	21
F	91	37
F	91	70
F	96	53
Control		
F	43	12
F	48	79
M	53	7
M	62	4
M	64	23
F	72	24
M	73	36
M	77	10
M	78	12
M	79	24
M	80	106
F	80	92
F	81	103
M	82	30
M	82	3
F	82	37
M	82	56
M	84	48
F	84	17
F	88	62
F	88	28
M	90	45
M	90	48
F	93	18

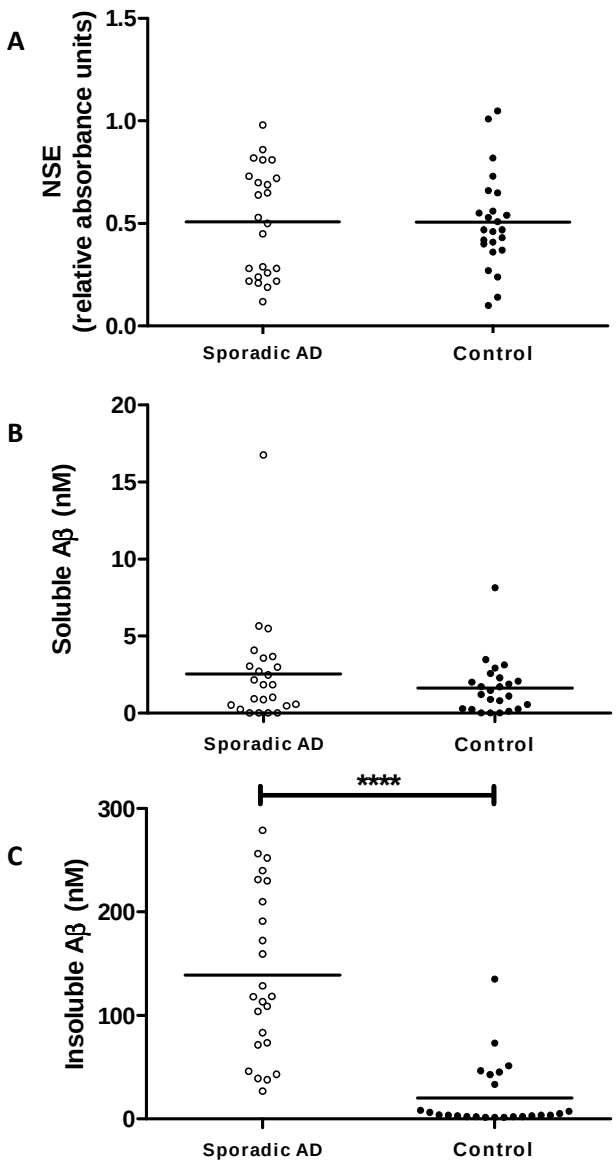

Figure 3.10 Aβ and neuron-specific enolase in Sporadic AD cohort 4. (A) Neuron-specific enolase (NSE) levels in sporadic AD and age-matched control human temporal lobe homogenates determined by ELISA. (B) Soluble Aβ extracted from sporadic AD and age-matched control human temporal lobe samples determined by ELISA. (C) Insoluble Aβ extracted from sporadic AD and age-matched control human temporal lobe samples determined by ELISA. n=24, (****$p<0.0001$). Data were obtained by Dr Scott Miners from the University of Bristol.

Quantification of western blots for CNTN5 on aging cohort 1 hippocampal (figure 3.10 A) and temporal lobe (figure 3.10 B) homogenates showed CNTN5 did not correlate with age in the hippocampus (figure 3.10 C) or the temporal lobe (figure 3.9 D). Quantification of western blots for CNTN5 on Sporadic AD cohort 2 hippocampal (figure 3.11 A) and temporal lobe (figure 3.11 B) homogenates, and on familial AD cohort 3 hippocampal homogenates (figure 3.11 C) showed no difference in CNTN5 in sporadic AD brains (figure 3.11 D) or familial AD brains (figure 3.11 E) compared to age-matched control brains. Quantification of western blots for CNTN5 on sporadic AD cohort 4 temporal lobe homogenates (figure 3.12 A) showed no difference in CNTN5 in sporadic AD brains compared to age-matched control (figure 3.12 B). There was no correlation of CNTN5 with either soluble (figure 3.12 C) or insoluble Aβ (figure 3.12 D).

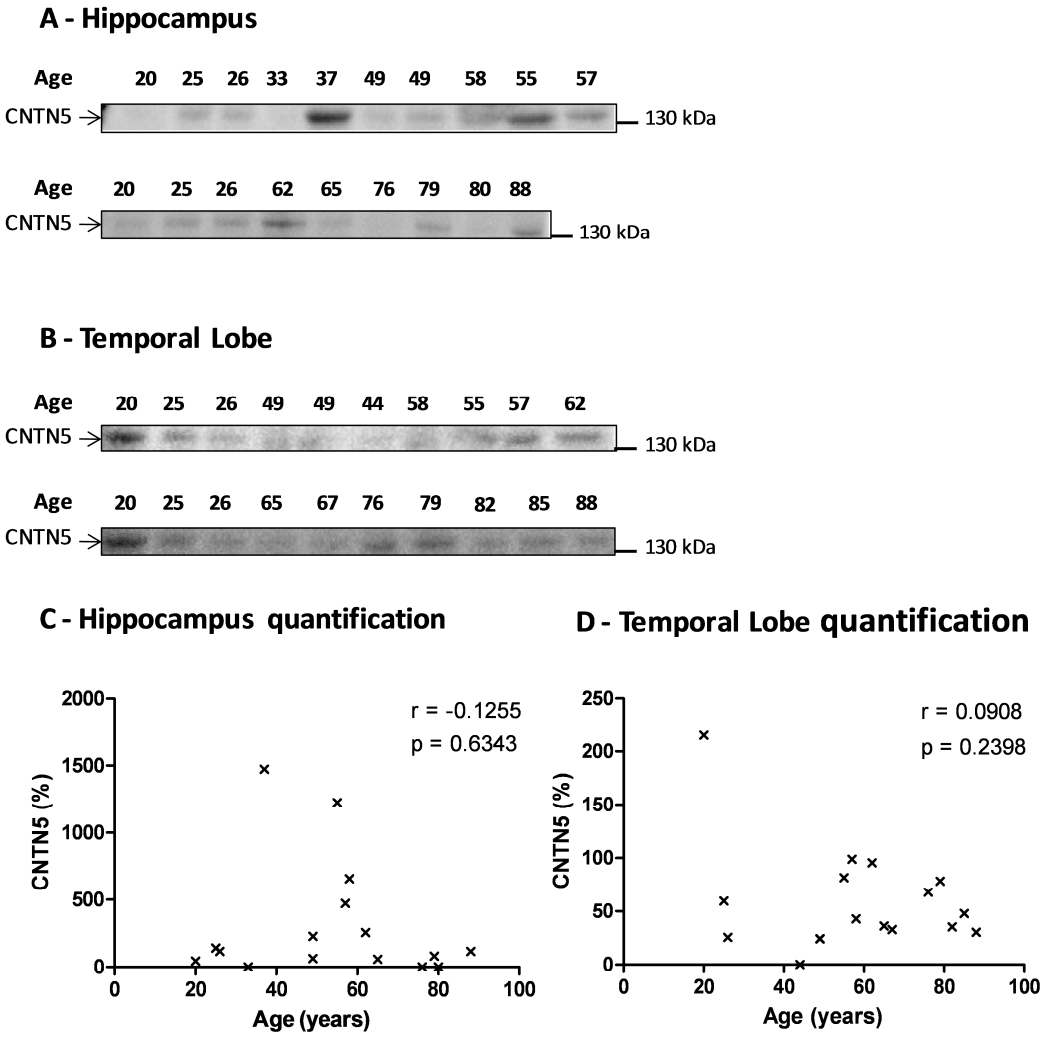

Figure 3.11 CNTN5 in the aging brain, aging cohort 1

Figure 3.11 CNTN5 in the aging brain, aging cohort 1. (A) Western blot for CNTN5 on human hippocampal homogenates (50 µg) from aging cohort 1 using AF3030. (B) Western blot for CNTN5 on human temporal lobe homogenates (50 µg) from aging cohort 1 using AF3030. (C) Quantification of western blots on hippocampal homogenates for CNTN5. Samples 20, 25 and 26 were loaded on both gels so that CNTN5 levels could be normalised according to these samples allowing comparison between gels. The y-axis % CNTN5 refers to CNTN5 as a percentage the average of CNTN5 in samples 20, 25 and 26. Pearson correlation showed no significant correlation between CNTN5 and age in human hippocampal samples. (D) Quantification of western blots on temporal lobe homogenates for CNTN5. Pearson correlation showed no significant correlation between CNTN5 and age in human temporal lobe samples.

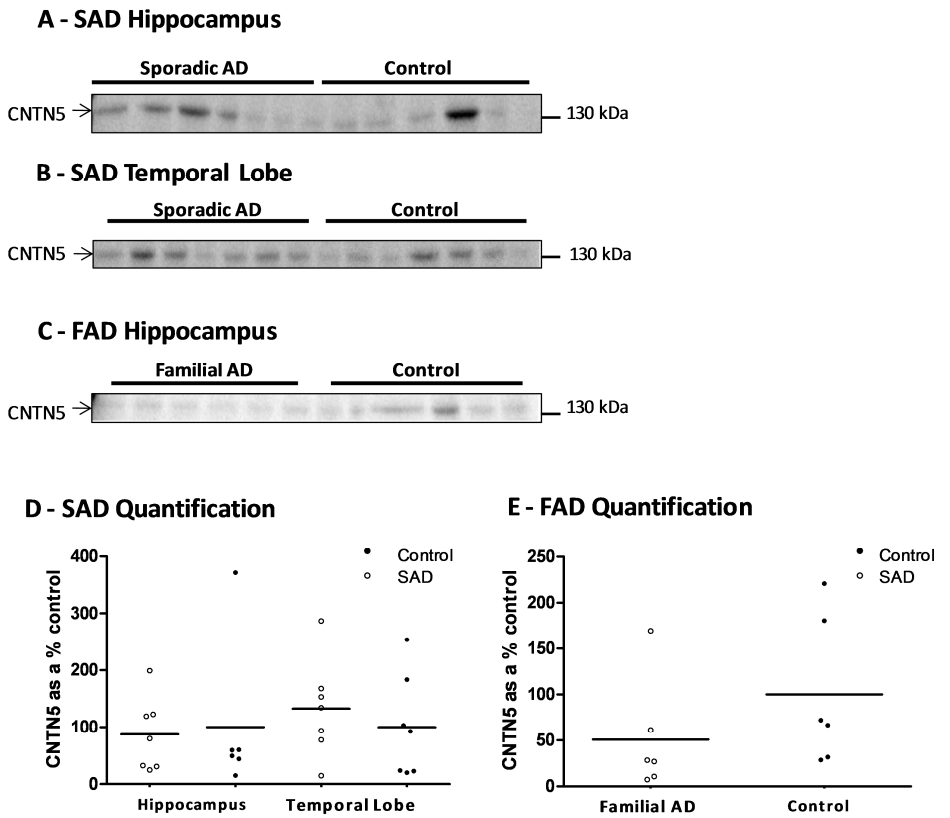

Figure 3.12 CNTN5 in the human brain in sporadic AD cohort 2 and familial AD cohort 3. (A) Western blot for CNTN5 on sporadic AD cohort 2 SAD and age-matched control human hippocampal homogenates (50 µg) using AF3030. (B) Western blot for CNTN5 on sporadic AD cohort 2 SAD and age-matched control human temporal lobe homogenates (50 µg) using AF3030. (C) Western blot for CNTN5 on familial AD cohort 3 FAD and age-matched control hippocampal homogenates (50 µg) using AF3030. (D) Quantification of western blots for CNTN5 on sporadic AD cohort 2 SAD and age matched control human hippocampal and temporal lobe homogenates (n=7). (E) Quantification of western blots for CNTN5 on familial AD cohort 3 FAD and age-matched control human hippocampal homogenates (n=6).

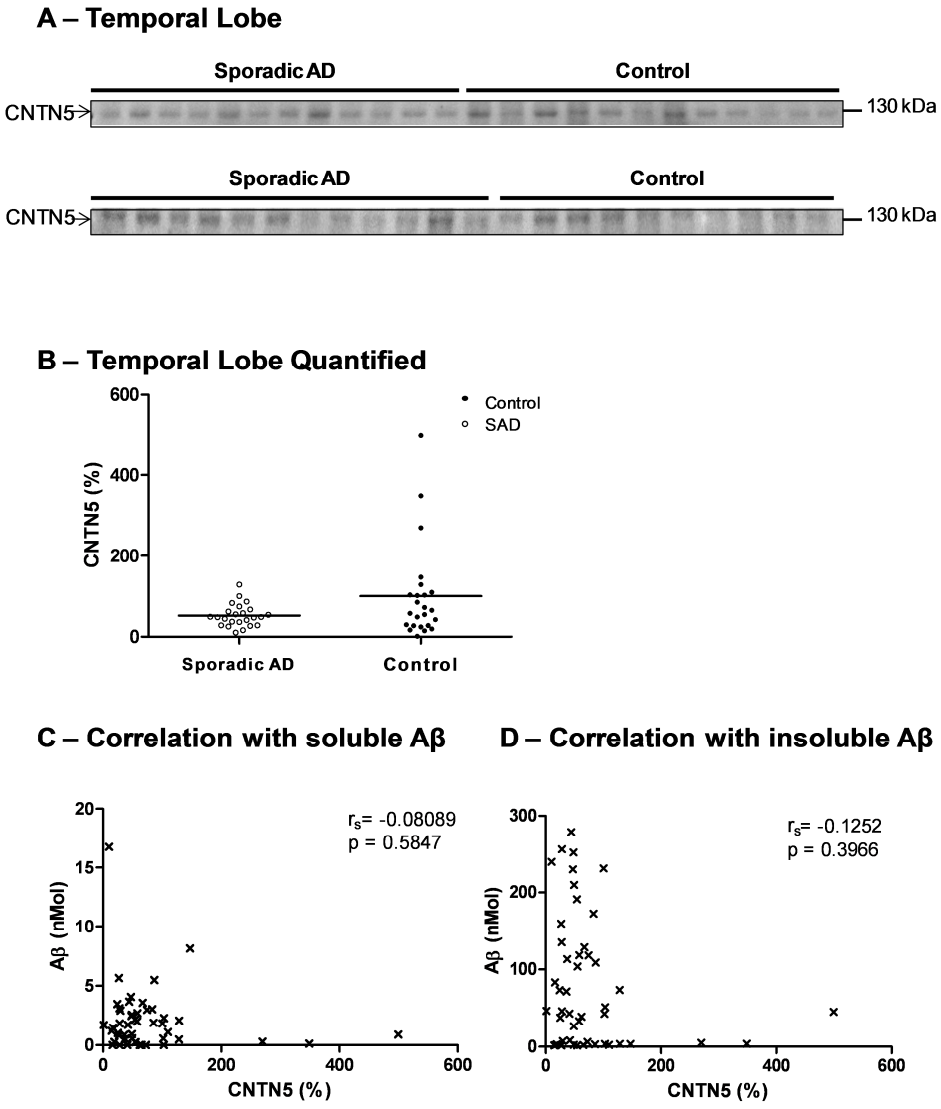

Figure 3.13 CNTN5 and Aβ levels in sporadic AD cohort 4 and age-matched control temporal lobe. (A) Western blot for CNTN5 on sporadic AD cohort 4 SAD and age-matched control human temporal lobe homogenates (40 µg, cohort 4) using AF3030. (B) Quantification of western blots for CNTN5 on sporadic AD cohort 4 SAD and age-matched control human temporal lobe homogenates (n=24). (C) Spearman's correlation analysis showed no correlation between CNTN5 and soluble Aβ in human temporal lobe samples. (D) Spearman's correlation analysis showed no correlation between CNTN5 and insoluble Aβ in human temporal lobe samples.

3.9 Discussion

Expression of neurofascin 186 did not significantly alter β- or α-cleavage of APP. There was only a small (approximately 15 %) reduction in $APP_{751/770}$ levels in NF186 expressing cells, with no change in the 695 isoform of APP. As neurofascin modulates $APP_{751/770}$ but not APP_{695} this suggests if it does interact with APP, it binds to the KPI domain which is absent in the 695 isoform. Neurofascin has been shown to be required for clustering ion channels and molecules that regulate formation of action potentials, as well as synaptic stability and levels of gamma-aminobutyric acid (GABA) recptor levels at the synapse (Kriebel et al., 2011; Zonta et al., 2011). Modulation of GABA receptors has been shown to alter APP metabolism increasing sAPPα production (Marcade et al., 2008), though no changes in sAPPα were demonstrated here.

Thy-1 appears to be a promising candidate for involvement in AD, as although expression of Thy-1 did not alter sAPPα secretion, it caused a significant 22 % decrease in sAPPβ in the media, and a decrease in APP_{695} in cell lysates. The decrease in sAPPβ is likely due to the decreased APP levels, since the decrease in sAPPβ is approximately equivalent to the decrease in APP. However, the decreased sAPPβ did not extend to a decrease in Aβ production. This is surprising, as β-secretase cleavage of APP is the rate limiting step in the generation of Aβ (Zhang et al., 2011), therefore it would be expected that a decrease in sAPPβ would cause a decrease in Aβ. These data suggests that there are further mechanisms in place regulating the generation of Aβ.

Both neurofascin and Thy-1 caused a reduction in full length APP. There have been contradicting reports, but it appears that APP mRNA levels are increased in the brains of patients with AD (Palmert et al., 1988; Schmechel et al., 1988; Vitek,

1989; Matsui et al., 2007), suggesting that dyregulation of neurofascin and Thy-1 expression (Thy-1 protein levels have been demonstrated to be reduced in AD brains (Leifer and Kowall, 1992)) could contribute to the alterations in APP seen in AD. Neither neurofascin or Thy-1 over-expression caused a change in the levels of $A\beta_{40}$ or $A\beta_{42}$, so investigations into these proteins were not continued.

CNTN5 caused multiple changes to APP metabolism: an increase in APP, an increase in sAPPα and a decrease in Aβ. Since CNTN5 over-expression caused a dramatic decrease in both $A\beta_{40}$ and $A\beta_{42}$ secretion this protein became the focus of further investigations. CNTN5 expression did not alter intracellular Aβ compared to cells expressing the vector alone, therefore is not preventing secretion of Aβ so causing it to accumulate within the cell, suggesting it is inhibiting the production of extracellular Aβ. It could also be possible that CNTN5 reduces Aβ by increasing Aβ degradation for example by activating NEP or IDE or other Aβ degrading proteases. This possibility requires further investigation.

Cleavage of APP by both β- and γ-secretase is required for Aβ generation (Evin and Weidemann, 2002). The consistent decrease in Aβ, even in the absence of a decrease in sAPPβ levels, suggests that CNTN5 may be modulating Aβ levels *via* γ- rather than β-secretase. In order to confirm that the action of CNTN5 on Aβ secretion was via γ-secretase the levels of C-terminal fragments of APP were determined. Western blotting showed that CNTN5 expression caused an increase in the levels of C83 (CTFα) and C99 (CTFβ). These proteins are substrates for γ-secretase so a build up of both C-terminal fragments suggests that CNTN5 had inhibited γ-secretase. AICD was not consistently detected by western blotting of HEK-APP_{695} cell lysates, so the effect of CNTN5 over-expression on AICD generation could not be accurately determined. Furthermore as AICD is not

transcriptionally active in non-neuronal cell lines (Belyaev et al., 2010) measurement of AICD in HEK cells would not indicate whether AICD is functional.

In order to further elucidate the role of CNTN5 in APP processing we considered knocking down endogenous CNTN5 in a cell line and measuring the levels of full length and secreted APP, and Aβ. However, we were unable to identify a cell line expressing endogenous CNTN5 either by western blotting for CNTN5 protein or using RT-PCR to amplify CNTN5 mRNA (data not shown). Work by Eckerich *et al*., (2006) demonstrated that although high levels of CNTN expression were found in primary glial cells, no CNTN was detected in cell lines derived from these cells (Eckerich et al., 2006). This suggests that CNTN5 expression is not required for *in vitro* growth and that its expression is quickly lost.

Our data suggested that CNTN5 regulates the production of Aβ, so to determine whether CNTN5 might be related to AD we measured the levels of CNTN5 in sporadic AD, familial AD and age matched control brains and in a series of aging brain samples. This showed that in the hippocampus and the temporal lobe there was no difference in CNTN5 levels in either sporadic or familial AD. Also there was no change in the levels of CNTN5 in either the hippocampus or the temporal lobe during aging. If CNTN5 has a role in the development of AD it appears not to be related to CNTN5 expression levels. CNTN5 may, however, contribute to the development of AD without any alteration in its expression level.

The subcellular location of CNTN5 may be altered in AD which may prevent its inhibition of γ-secretase. The subcellular location of CNTN1 is regulated by CNTNAP1 (contactin associated protein 1). CNTN1 binds CNTNAP1 (Peles et al., 1997), and when bound to CNTNAP1 glycosylation of CNTN1 is prevented and it is targeted to the cell surface at paranodes of the axon. However, when CNTN1 is not

bound to CNTNAP1 it is glycosylated and targeted to nodes of Ranvier rather than paranodes (Rios et al., 2000; Gollan et al., 2003). Much less is known about the interaction between CNTN5 and CNTNAP1 or CNTNAP5 (contactin associated protein 5), but it is reasonable to assume that like CNTN1, the subcellular trafficking of CNTN5 is regulated by CNTNAP1 or CNTNAP5. Altering the subcellular/cell surface location of CNTN5 may alter the proteins with which it can interact. Over-expression of CNTN5 in our cell culture system may have saturated the binding to CNTNAP leaving CNTN5 free to interact with γ-secretase components and inhibit Aβ generation. CNTN5 has been shown to interact with APLP1 at the synaptic membrane (Shimoda and Watanabe, 2009; Shimoda et al., 2012). APLP1 can influence the proteolytic processing of APP, therefore regulating the CNTN5-APLP1 interaction may indirectly regulate the processing of APP. In one model for the endocytosis of APP the adaptor protein Fe65 links APP with LRP1 via interactions with the GYENPTY motif and Y_{682} in the cytoplasmic domain of APP, and this complex formation is required for endocytosis of APP (and therefore for β-secretase cleavage of APP and ultimately Aβ generation). APLP1 can also form a complex with Fe65, competitively preventing APP from interacting with LRP1 so inhibiting APP endocytosis (Neumann et al., 2006), therefore preventing β-secretase cleavage and Aβ generation. The interaction of CNTN5 with APLP1 may act to prevent the APLP1-Fe65-LRP1 interaction so allowing more APP-Fe65-LRP1 complex to form therefore allowing more endocytosis of APP. However, this would be expected to increase the generation of Aβ whereas over-expression of CNTN5 inhibited Aβ production suggesting this model cannot explain the effects of CNTN5 in our system. Further investigation, particularly into the sub-cellular location of CNTN5 in primary neurons is required to fully understand the role of CNTN5 in APP processing.

4 The effect of BIN1 on amyloid-β metabolism

4.1 Introduction

Over the last few years several genome-wide association studies (GWAS) on sporadic Alzheimer's disease (SAD) have been performed, and these have identified a number of genes that are related to SAD (Harold et al., 2009; Bertram and Tanzi, 2010; Seshadri et al., 2010; Carrasquillo et al., 2011; Hollingworth et al., 2011; Hu et al., 2011; Wijsman et al., 2011). These genes have implicated several molecular and cellular processes in the development of AD, such as immune system functions, lipid metabolism and processes at the cell membrane such as endocytosis (Hollingworth et al., 2011). Endocytosis is very important in Aβ metabolism, as it is involved in the production and uptake (therefore potentially the toxicity) and clearance of Aβ by brain cells. One of the genes involved in endocytosis that has been implicated in sporadic AD by GWAS is BIN1. Here the role of BIN1 in the development of AD was investigated, focusing on whether BIN1 regulates the uptake or generation of Aβ.

4.1.1 Aims

Due to its role in endocytosis it was hypothesised that BIN1 is involved in either the uptake or generation of Aβ by cells and altering the levels of BIN1 would alter the amount of Aβ generated (by altering APP endocytosis) or internalised by a cell. This was investigated by knockdown and over-expression of BIN1 in SH-SY5Y cells, a human neuroblastoma cell line. To investigate the role of BIN1 in Aβ uptake, a synthetic preparation of AβOs, ADDLs (amyloid-β derived diffusible ligands), was used. ADDLs are high molecular weight oligomer with a fibrillar

conformation, they correlate with cognitive decline in AD, and have been shown to bind to and be toxic to cells (Lambert et al., 1998; Wang et al., 2002; Deshpande et al., 2006). Cells were treated with AβOs, then Aβ uptake was assessed by fluorescence microscopy. To investigate BIN1 in APP metabolism and Aβ generation, BIN1 was either over-expressed or knocked down in SH-SY5Y cells, and then full length APP, sAPPα, sAPPβ, and Aβ were measured by western blotting (APP, sAPPα and sAPPβ) and ELISA (Aβ). Endocytosis is the key factor influencing whether APP is processed in the amyloidogenic or the non-amyloidogenic pathway (Weidemann et al., 1989). The effect of BIN1 on APP endocytosis was determined using flow cytometry to measure cell surface APP following an incubation at either 4 ^{o}C (to prevent endocytosis) or 37 ^{o}C (allowing endocytosis). Finally, BIN1 was shown to regulate APP protein levels. APP has been shown to influence the levels of cell surface N-methyl-D-aspartate (NMDA) receptors, so we hypothesised that BIN1 expression would regulate cell surface NMDA receptor levels. NMDA receptors are involved in long term potentiation and are required for learning and memory (Morris et al., 1986), over-stimulation of NMDA receptors causes neuronal death by excitotoxicity (Manev et al., 1989), and NMDA receptor trafficking and cell surface levels has been shown to be disrupted in AD (Snyder et al., 2005). The effect of BIN1 on cell surface NMDA receptor levels was determined using flow cytometry. Finally, to investigate BIN1 in the human brain during aging, and in familial and sporadic AD, the level of BIN1 in human brain samples was determined by western blotting.

4.2 Optimisation of BIN1 knockdown in SH-SY5Y cells

To investigate the role of BIN1 in the development of sporadic AD, BIN1 was knocked down in the human neuroblastoma cell line SH-SY5Y. SH-SY5Y cells were transfected with siRNA directed against BIN1, incubated for 48 hours, then cells were harvested and lysed. Cell lysates were western blotted for BIN1 (figure 4.1 A, C and E). The optimal conditions for BIN1 knockdown were 50 nM siRNA (figure 4.1 B) and 6 µl Dharmafect 3 (per well of a 6-well plate) (figure 4.1 D). BIN1 knockdown was greater than 75 % after both a 48 and a 72 hour incubation (figure 4.1 F) so both of these incubation times were used, depending on the experimental set-up.

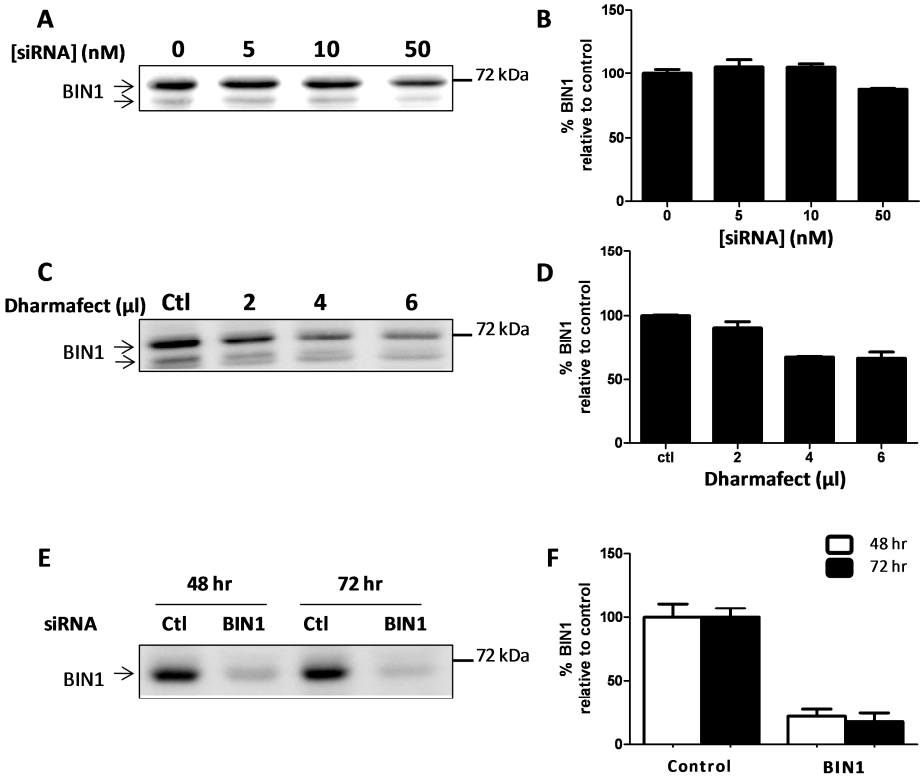

Figure 4.1 Optimising knockdown of BIN1 in SH-SY5Y cells. (A) Western blot for BIN1 on cell lysates (40 µg) transfected with increasing concentrations of BIN1 siRNA (8 µl Dharmafect 3, 72 hours post-transfection), using ab54764. (B) Quantification of western blot in A. (C) Western blot for BIN1 on cell lysates (40 µg) transfected with increasing volumes of Dharmafect 3 (50 nM BIN1 siRNA or RNAse free water (control), 72 hours post-transfection), using ab54764. (D) Quantification of western blot for BIN1 in C. (E) Western blot for BIN on cell lysates (40 µg) either 48 or 72 hours post-transfection (50 nM BIN1 siRNA or RNAse free water (control), 6 µl Dharmafect 3), using ab54764. (F) Quantification of western blot in E.

4.3 The effect of BIN1 on Aβ uptake

AβOs (the toxic species in AD) are taken up into neurons by endocytosis, and this may be an important event for their toxicity (Cizas et al., 2011; Song et al., 2011). To investigate the role of BIN1 in AβO endocytosis, a synthetic preparation of soluble AβOs was used. An SDS-polyacrylamine gel and western blot for Aβ confirmed the presence of high molecular weight oligomers in the AβO preparation (figure 4.2 A). The fibrillar epitope of the AβOs was confirmed by dot blot with the fibrillar conformation specific OC antibody (figure 4.2 B). Absence of a pre-fibrillar epitope was confirmed by dot blot with the conformation specific pre-fibrillar A11 antibody (figure 4.2 B). SH-SY5Y cells were transfected with 50 nM BIN1 or non-targeting control siRNA and incubated for 48 hours. Cells were then incubated for 30 minutes at 37 ^{o}C with 400 nM (total peptide) AβOs then fixed, permeabilised and stained for Aβ and BIN1. Aβ was detected inside cells transfected with BIN1 and with non-targeting control siRNA. There was very little co-localisation of BIN1 with Aβ (figure 4.2 C). There was no significant difference in the proportion of Aβ inside cells transfected with BIN1 compared to cells transfected with non-targeting control siRNA (figure 4.2 D).

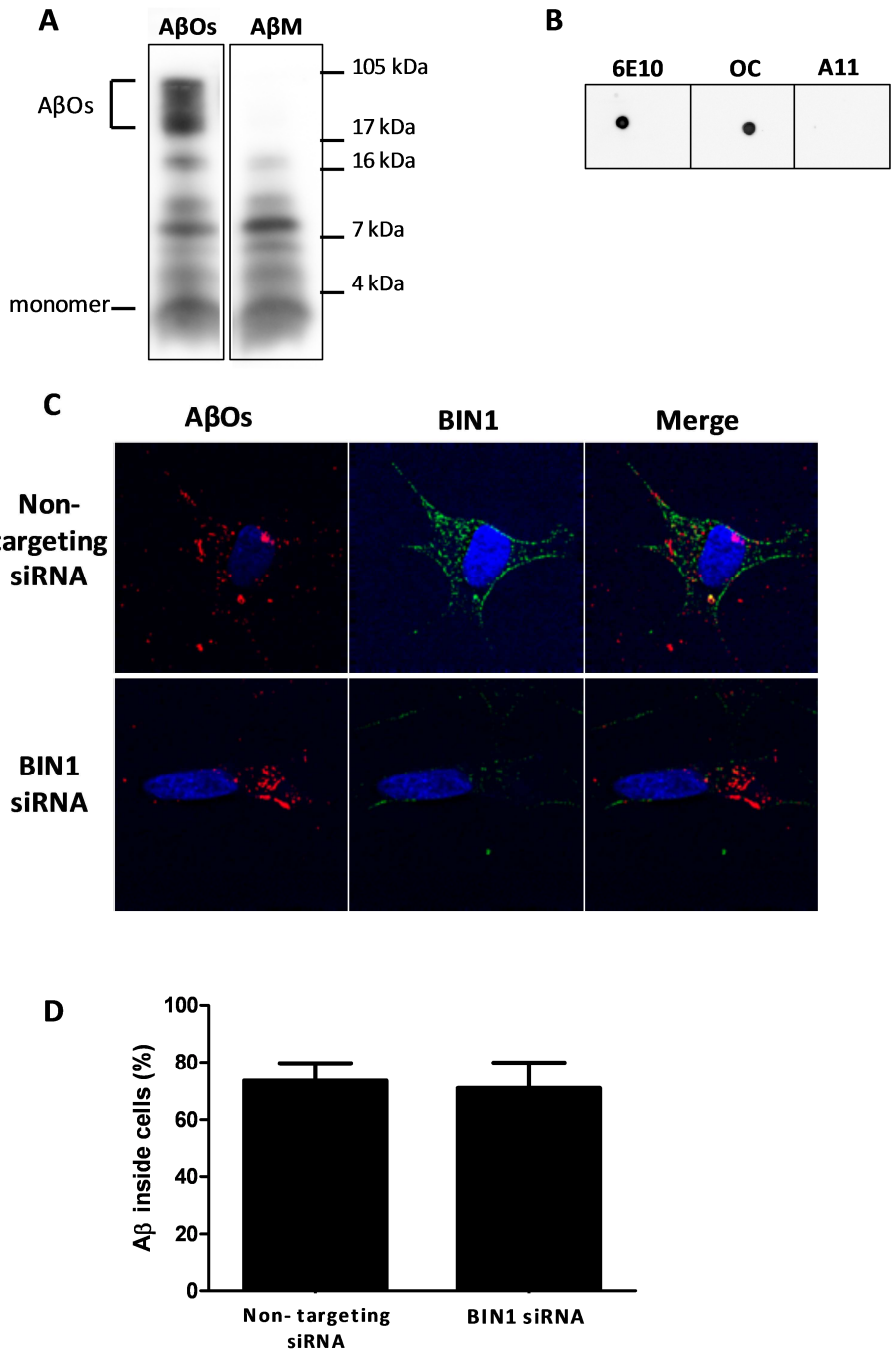

Figure 4.2 The effect of BIN1 knockdown on Aβ uptake by SH-SY5Y cells
(legend overleaf)

Figure 4.2 The effect of BIN1 knockdown on Aβ uptake by SH-SY5Y cells. (A) Western blot of Aβ oligomers (AβOs, 4 µg) and Aβ monomers (AβM, 4µg), blotted for Aβ using 6E10. (B) Dot blots of AβOs (1 µg) for total Aβ using 6E10 (first panel), for fibrillar Aβ using OC (middle panel) and non fibrillar Aβ using A11 (last panel). (C) SH-SY5Y incubated with 400 nM (total peptide) biotin-tagged AβOs fixed, permeabilised and stained for AβOs using Texas-red conjugated streptavidin (red), and BIN1 using ab54764 (green); nuclei were stained with 2 µg ml^{-1} DAPI (blue). (D) Quantification of Aβ staining inside cells in fluorescence images expressed as a percentage of total Aβ staining (total Aβ = Aβ inside +Aβ on the edge of cells). Aβ staining was measured using Image software. Aβ inside cells was mearured by drawing round the cell and determining the fluorescence intensity inside that area. Aβ on the edge of cells was measured by drawing a line around the cell and measuring the fluorescence intensity on the line. 19 cells from two separate experiments were measured for quantification. Data are mean ± S.D.

4.4 The effect of BIN1 on APP processing

To determine the effect of BIN1 on APP processing, BIN1 was knocked down in SH-SY5Y cells over-expressing APP_{695} (SH-SY5Y-APP_{695}). SH-SY5Y-APP_{695} cells were transfected with either siRNA targeted against BIN1, or a non-targeting control sequence, and incubated for 24 hours. Cells were washed and incubated for 48 hours with serum free medium, then medium was harvested and cells were harvested and lysed. Cell lysates were western blotted for BIN1 and APP, conditioned media was western blotted for sAPPα and sAPPβ (figure 4.3. A). Aβ levels in conditioned media were determined by ELISA (figure 4.3 E). BIN1 was knocked down by 65 % ($p<0.001$) in cells transfected with BIN siRNA (figure 4.3 B). BIN1 knockdown caused a significant 7 % increase in APP_{695} ($p=0.04$) but did not alter $APP_{751/770}$ in cell lysates (figure 4.3 C). BIN1 knockdown did not significantly alter sAPPα (figure 4.3 D), sAPPβ (figure 4.3 E) or Aβ (figure 4.3 F).

To confirm the results of the knockdown of BIN1 on APP processing the converse experiment was performed and BIN1 was over-expressed in SH-SY5Y-APP_{695} cells. The neuronal isoform of BIN1 in the vector pcDNA3.1(+) was obtained from Source Bioscience. SH-SY5Y-APP_{695} cells were transfected with BIN1 in pcDNA3.1(+) (empty vector was transfected in as a control), then transfected cells were selected with the antibiotic G418 to generate a cell line stably expressing BIN1: SH-SY5Y-APP_{695}-BIN1. SH-SY5Y-APP_{695}-BIN1 and SH-SY5Y-APP_{695}-vector cells were incubated for 48 hours, then medium was harvested and cells were harvested and lysed. Cell lysates were western blotted for BIN1 and APP, conditioned medium was western blotted for sAPPα and sAPPβ (figure 4.4 A). Aβ levels in conditioned media were determined by ELISA (figure 4.4 E). The neuronal isoform of BIN1 appeared as a doublet band on western blots. There is no evidence

in the literature for post-translational modification of BIN1 (e.g. by glycosylation or phosphorylation), so the lower molecular weight band may result from cleavage of the full length BIN1 by an un-identified protease. BIN1 over-expression caused a significant 19 % decrease in APP_{695} in cell lysates ($p=0.03$) but did not significantly alter $APP_{751/770}$ (figure 4.4 B). BIN1 over-expression did not significantly alter sAPPα (figure 4.4 C), or sAPPβ (figure 3.5 D). BIN1 over-expression caused a significant 10 % decrease in $A\beta_{40}$ ($p=0.005$) but no change in $A\beta_{42}$ (figure 4.4 E). Together these data show that BIN1 expression regulates APP and Aβ levels.

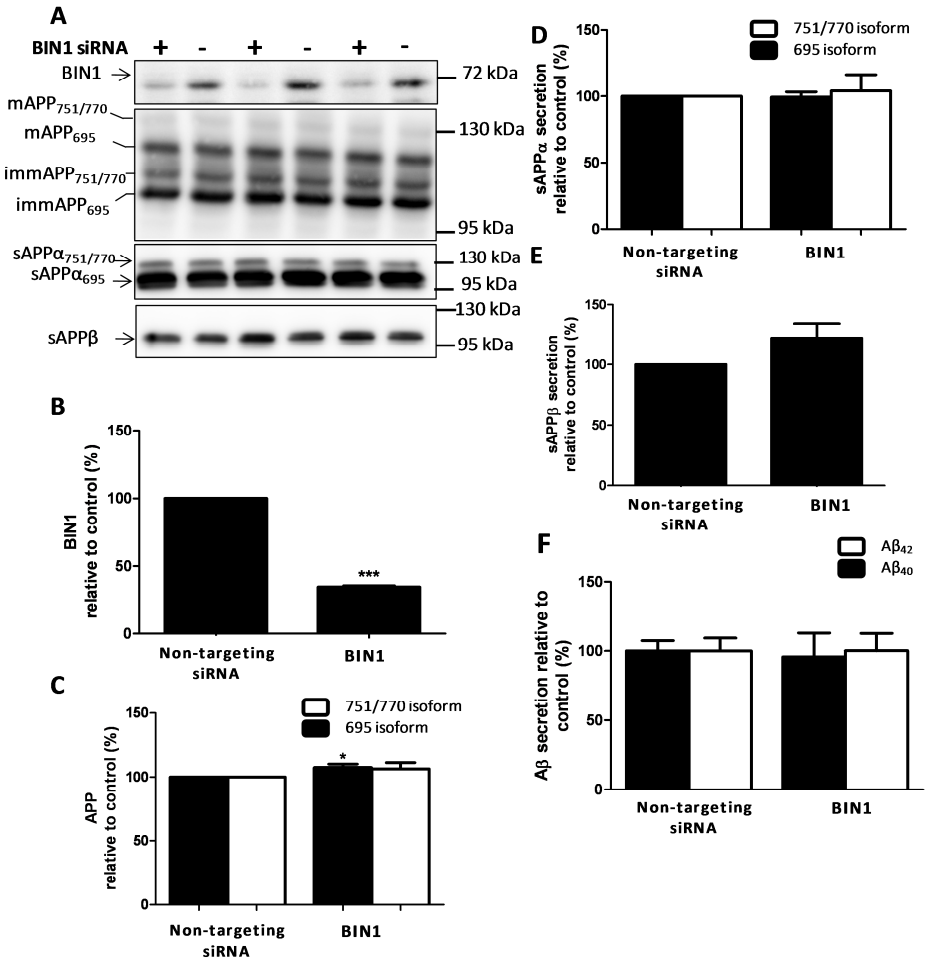

Figure 4.3 The effect of BIN1 knockdown on APP proteolysis. (A) Representative western blots on cell lysates (30 µg) transfected with siRNA for BIN1 (+) or non-targeting control (-) for: BIN1 with ab54764 (top panel), APP with 22C11 (second panel) and conditioned media (40 µg) for sAPPα with 6E10 (third panel) and sAPPβ with 1A9 (fourth panel). (B) Quantification of western blots for BIN1. (C) Quantification of western blots for APP. (D) Quantification of western blots for sAPPα. (E) Quantification of western blots for sAPPβ. (F) ELISA to determine Aβ in conditioned media. Data are mean ± S.D. n=3 (*$p<0.05$).

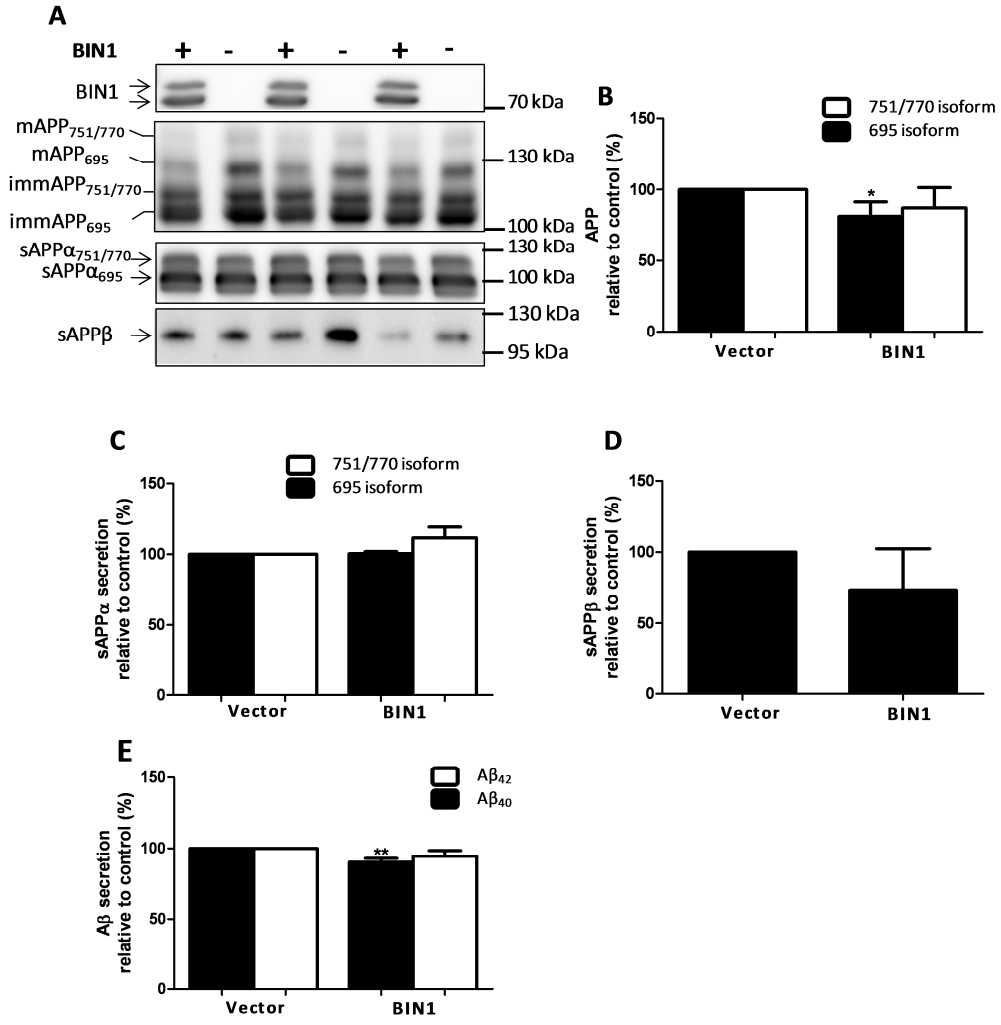

Figure 4.4 The effect of BIN1 over-expression on APP proteolysis. (A) Representative western blots on SH-SY5Y-APP$_{695}$ cell lysates (30 µg) transfected with BIN1 cDNA (+) or empty vector (-) for: BIN1 with ab54764 (top panel), APP with 22C11 (second panel) and conditioned media (30 µg) for sAPPα with 6E10 (third panel) and sAPPβ with 1A9 (fourth panel). (B) Quantification of western blots for APP. (C) Quantification of western blots for sAPPα. (D) Quantification of western blots for sAPPβ. (E) ELISA to determine Aβ in conditioned media. Data are mean ± S.D. n=3 (*$p<0.05$)(**$p<0.01$).

4.5 The effect of BIN1 on APP endocytosis

Altering the expression of BIN1 alters the levels of APP in the cells, and Aβ secreted, suggesting that it affects the amount of APP processed in the amyloidogenic pathway. Processing in the amyloidogenic pathway requires endocytosis of APP, therefore BIN1 may play a role in regulating the endocytosis of APP. Cell surface APP in SH-SY5Y-APP_{695} cells expressing BIN1 or empty vector was labelled, cells were incubated for 10 minutes at either 4 ^{o}C or 37 ^{o}C, then cells were either fixed, permeabilised, and stained for APP (figure 4.5 A), or cell surface APP was determined by flow cytometry (figure 4.5 B). Cells that had been incubated at 4 ^{o}C showed APP fluorescence at the cell surface with very little staining inside the cells. Cells that had been incubated at 37 ^{o}C showed APP fluorescence in intracellular vesicles with considerably less APP at the cell surface (figure 4.5 A). Flow cytometry showed a significant 60 % decrease in cell surface APP in vector transfected cells ($p=0.04$), and a significant 70 % decrease in cell surface APP in BIN1 over-expressing cells ($p<0.0001$) at 37 ^{o}C compared to 4 ^{o}C (figure 4.5 C). However, over-expression of BIN1 did not significantly alter the amount of APP endocytosed (figure 4.5 C, compare vector and BIN1 at 37 ^{o}C). BIN1 over-expression did not alter cell surface APP levels (figure 4.5 D). These data suggest that BIN1 does not regulate APP endocytosis.

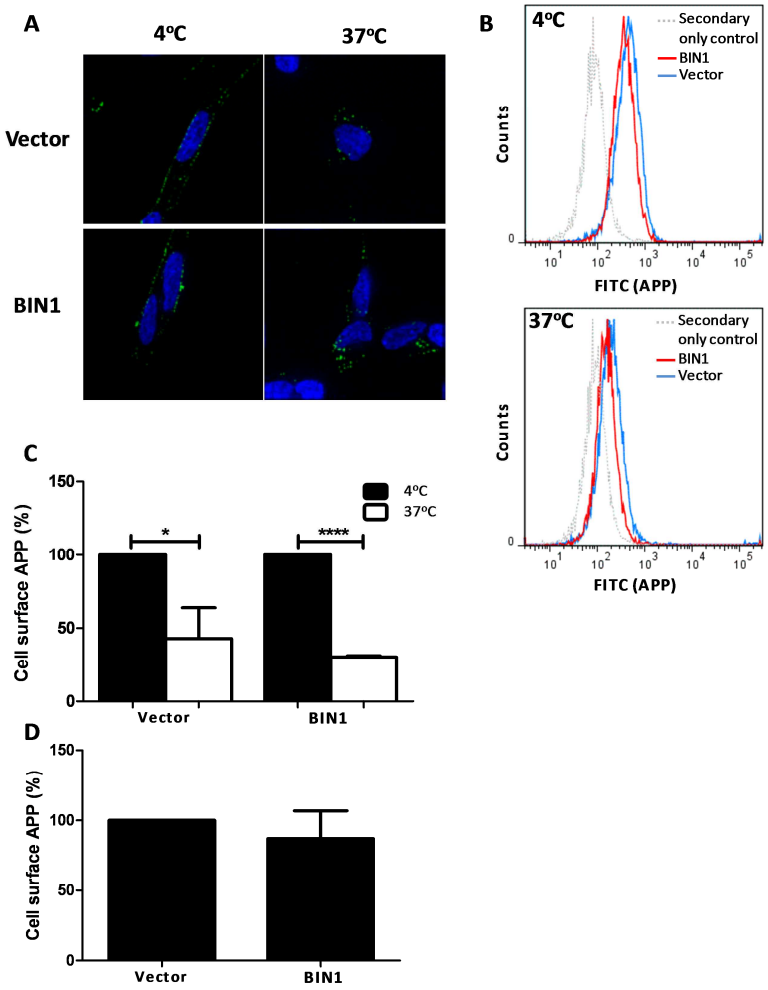

Figure 4.5 The effect of BIN1 over-expression on APP endocytosis. (A) SH-SY5Y-APP_{695} cells transfected with either BIN1 cDNA or empty vector stained with anti-APP antibody 6E10 were incubated for 10 minutes at either 4 ^{o}C or 37 ^{o}C fixed, permeabilised and stained for APP (green), nuclei were stained with 2 $\mu g ml^{-1}$ DAPI (blue). (B) Representative histograms showing cell surface APP (FITC fluorescence) after a 10 minute incubation at either 4 ^{o}C (top) or 37 ^{o}C (bottom). (C) Geometric mean of cell surface APP expressed as a percentage cell surface APP at 4 ^{o}C. (D) Geometric mean of cell surface APP in samples incubated at 4 ^{o}C, expressed as a percentage cell surface APP on cells expressing the empty vector. Data are mean ± S.D., n=3. (*$p<0.05$)(****$p<0.0001$).

4.6 The effect of BIN1 on cell surface N-methyl–D-aspartate receptors

Cell surface NMDA receptors have been shown to be regulated by APP expression (Cousins et al., 2009; Innocent et al., 2012). The data presented here demonstrate that BIN1 regulates APP levels and therefore may regulate cell surface NMDA receptors *via* APP. Cell surface NMDA receptors in SH-SY5Y-APP_{695} cells expressing BIN1 or empty vector were labelled using an anti-GluN2B antibody, then cells were either fixed, permeabilised and stained for NMDA receptors (figure 4.6 A) or cell surface NMDAR was determined by flow cytometry (figure 4.6 B). The GluN2B subunit of NMDAR is not trafficked to the cell surface unless it has formed the whole NMDAR complex, therefore GluN2B at the cell surface accurately reflects cell surface levels of NMDAR containing the GluN2B subunit. (McIlhinney et al., 1998). BIN1 over-expression did not cause a significant change in cell surface NMDAR (figure 4.6 C).

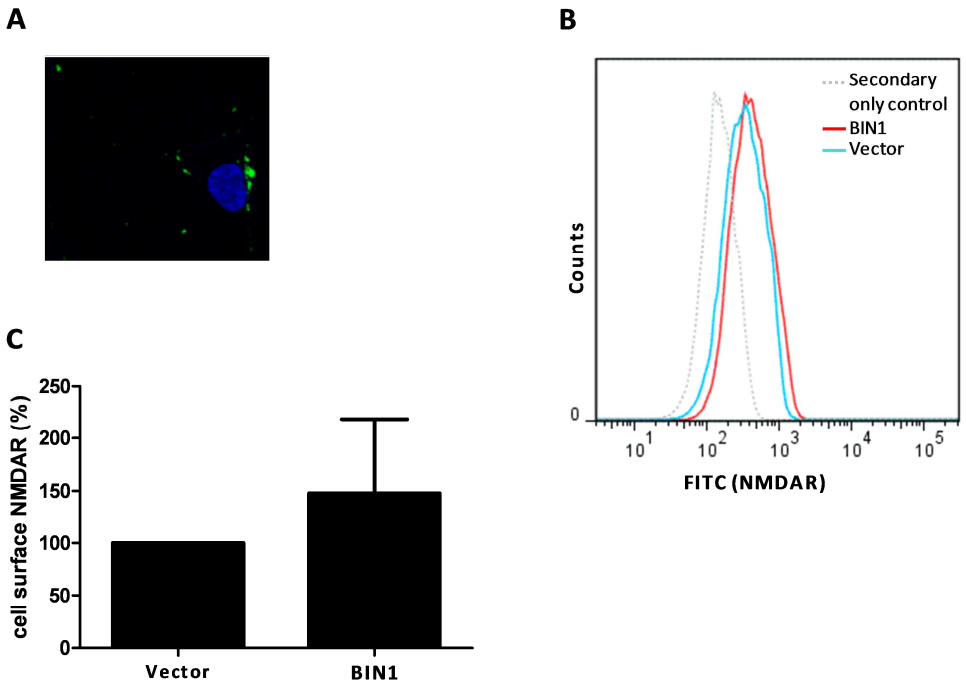

Figure 4.6 The effect of BIN1 over-expression on cell surface NMDA receptors. (A) SH-SY5Y-APP$_{695}$ cells were fixed, permeabilised and NMDA receptors stained with SAB2500699 (green), nuclei were stained with 2 µg ml^{-1} DAPI (blue). (B) Representative histograms showing cell surface NMDA receptors (FITC fluorescence) on SH-SY5Y-APP$_{695}$ transfected with BIN1 cDNA or empty vector. (C) Geometric mean of cell surface NMDA receptors on SH-SY5Y-APP$_{695}$ transfected with either BIN1 cDNA or empty vector. Data are mean ± S.D. n=3.

4.7 BIN1 in the human brain

BIN1 in the human brain during aging and Alzheimer's disease, and the correlation of BIN1 with Aβ in the human brain was investigated. Four different cohorts of brain samples were investigated: aging cohort 1 (table 3.1), sporadic AD cohort 2 (table 3.2), familial AD cohort 3 (table 3.3) and sporadic AD cohort 4 (table 3.4). For details on the brain samples in each cohort see chapter 3.

A preliminary western blot for BIN1 on a selection of temporal lobe homogenates from sporadic AD cohort 4 showed prominent bands at approximately 90 kDa and 60 kDa (figure 4.7 A). The predicted molecular weight of the neuronal isoform (and the longest isoform) of BIN1 is approximately 70 kDa, however BIN1 migrates at an aberrantly high molecular weight on SDS-PAGE (Ramjaun et al., 1997). The neuronal isoform of BIN1 expressed in SH-SY5Y cells migrates as a doublet at approximately 90 kDa (figure 4.7 B) suggesting the 90 kDa band in human brain homogenates is the neuronal isoform of BIN1. The lower molecular weight 60 kDa band is also detected in SH-SY5Y cell lysates, and its intensity is decreased by BIN1 siRNA (figure 4.4 A), demonstrating that this band is another isoform of BIN1. Both the 90 and 60 kDa isoforms of BIN1 were quantified.

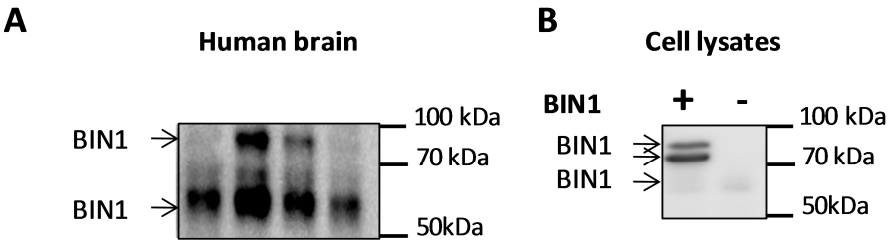

Figure 4.7 BIN1 detected in human brain homogenates and SH-SY5Y cell lysates. (A) Western blot of a selection of human temporal lobe homogenates (40 µg) from cohort 4 for BIN1 using ab54764. (B) Western blot SH-SY5Y-APP$_{695}$ cell lysates (30 µg) expressing the neuronal isoform of BIN1 for BIN1 using ab54764.

Quantification of western blots for BIN1 on aging cohort 1 hippocampal (figure 4.8 A) and temporal lobe (figure 4.8 B) homogenates showed a significant negative correlation between both 60 kDa BIN1 (figure 4.8 C, p=0.03) and 90 kDa BIN1 (figure 4.8 E, p=0.01) and age in the hippocampus, suggesting BIN1 decreases in the hippocampus with age. There was no significant correlation between 60 kDa BIN1 (figure 4.8 D) or 90 kDa BIN1 (figure 4.8 F) and age in the temporal lobe.

Quantification of western blots for BIN1 on Sporadic AD cohort 2 hippocampal (figure 4.9 A) and temporal lobe (figure 4.9 B) homogenates, and on familial AD cohort 3 hippocampal homogenates (figure 4.9 C) showed no significant difference in 60 kDa BIN1 in sporadic AD brains compared to age-matched control samples (figure 4.9 D). The 90 kDa neuronal isoform of BIN1 was not detected in sporadic AD cohort 2 brains. There was no significant difference in either 60 kDa (figure 4.9 E) or 90 kDa BIN1 (figure 4.9 F) in familial AD brains compared to age-matched controls.

Quantification of western blots for BIN1 on sporadic AD cohort 4 temporal lobe homogenates (figure 4.10 A) showed a significant decrease of both 60 kDa BIN1 (figure 4.10 B, p=0.03) and 90 kDa BIN1 (figure 4.10 C, p=0.01) in sporadic AD brains compared to age-matched controls. There was no correlation of either 60 kDa (figure 4.10 D) or 90 kDa BIN1 (figure 4.10 F) with soluble Aβ. There was no correlation of 60 kDa BIN1 with insoluble Aβ (figure 4.10 E), but there was a significant negative correlation of 90 kDa BIN1 with insoluble Aβ (figure 4.10 G, p=0.046). These results support the data from cell culture models showing that increased BIN1 results in decreased Aβ levels.

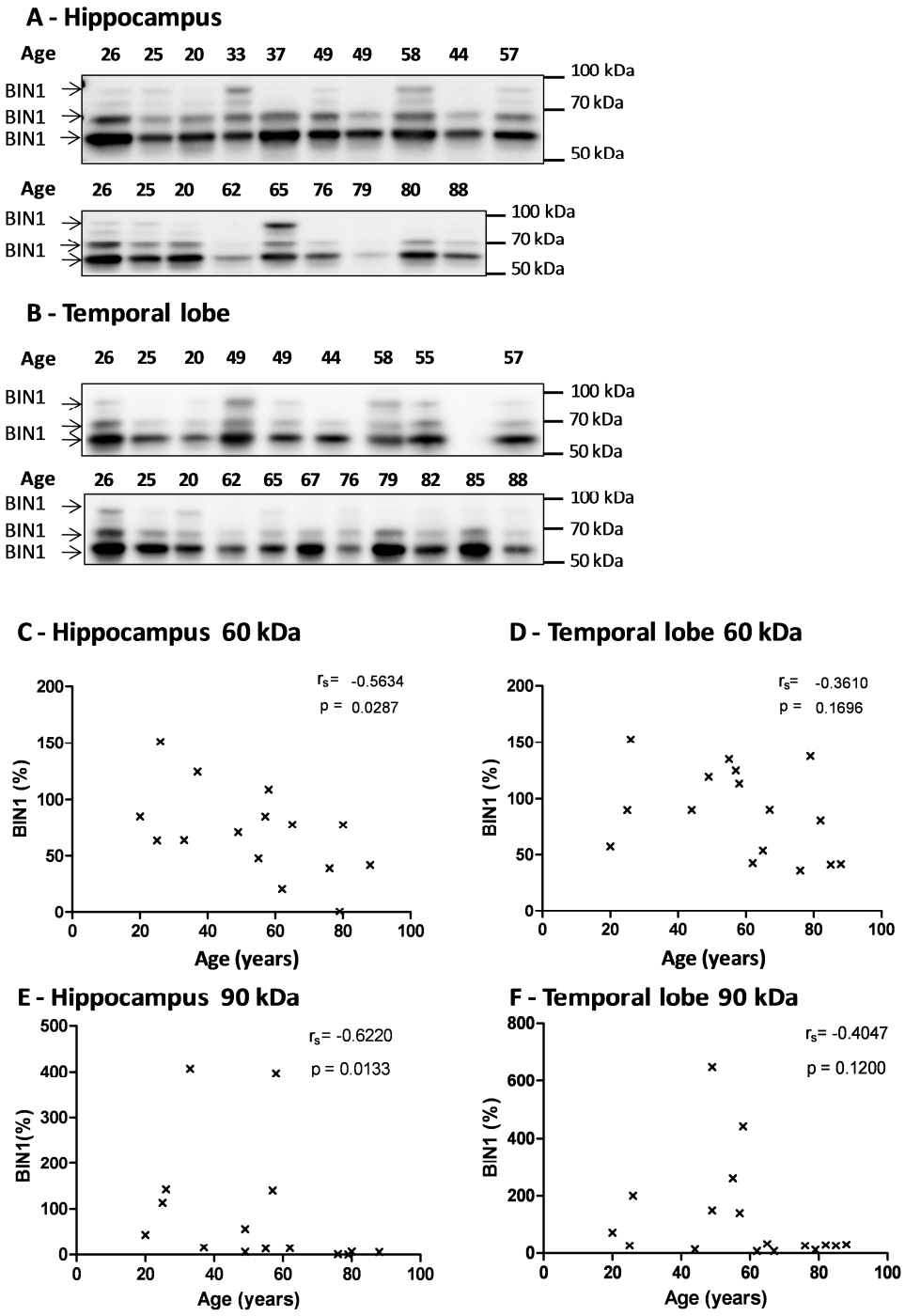

Figure 4.8 BIN1 in the aging brain, aging cohort 1 (legend overleaf)

Figure 4.8 BIN1 in the aging brain, aging cohort 1. (A) Western blot for BIN1 on human hippocampal homogenates (50 µg) from aging cohort 1 using ab54764. (B) Western blot for BIN1 on human temporal lobe homogenates (50 µg) from aging cohort 1 using ab54764. (C) Quantification of 60 kDa BIN1 from western blots on hippocampal homogenates for BIN1 (both bands indicated by arrows were quantified together). Spearman's correlation showed a significant ($p<0.05$) correlation between 60 kDa BIN1 and age in human hippocampal samples. (D) Quantification of 60 kDa BIN1 from western blots on temporal lobe homogenates for BIN1. Spearman's correlation showed no significant correlation between 60 kDa BIN1 and age in human temporal lobe samples. (E) Quantification of 90 kDa BIN1 from western blots on hippocampal homogenates for BIN1. Spearman's correlation showed a significant ($p<0.05$) correlation between 90 kDa BIN1 and age in human hippocampal samples. (F) Quantification of 90 kDa BIN1 from western blots on temporal lobe homogenates for BIN1. Spearman's correlation showed no significant correlation between 90 kDa BIN1 and age in human temporal lobe samples.

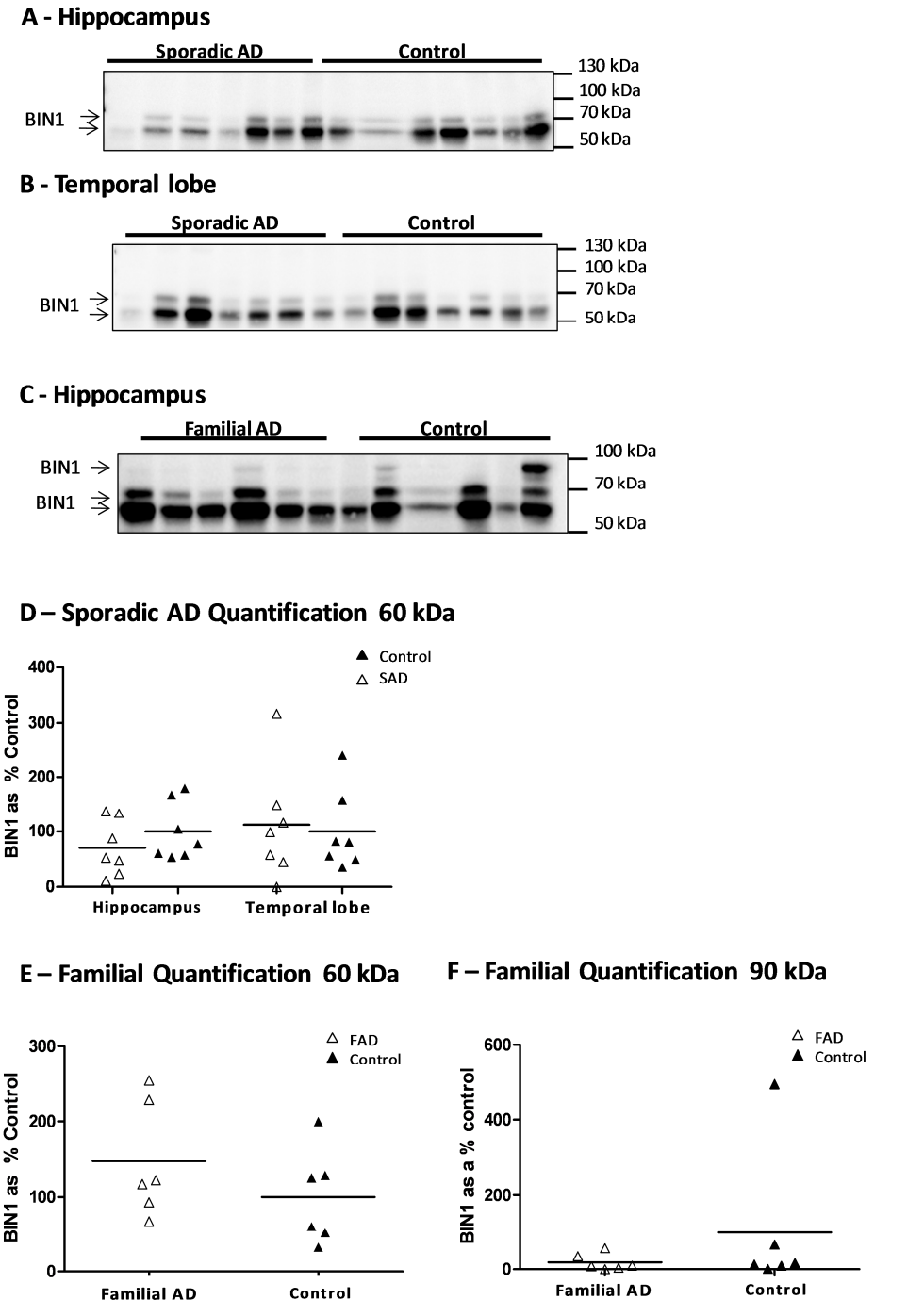

Figure 4.9 BIN1 in the human brain in sporadic AD cohort 2 and familial AD cohort 3 (legend overleaf)

Figure 4.9 BIN1 in the human brain in Sporadic AD cohort 2 and Familial AD cohort 3. (A) Western blot for BIN1 on Sporadic AD cohort 2 SAD and age-matched control human hippocampal homogenates (50 µg) using ab54764. (B) Western blot for BIN1 on Sporadic AD cohort 2 SAD and age-matched control human temporal lobe homogenates (50 µg) using ab54764. (C) Western blot for BIN1 on Familial AD cohort 3 FAD and age-matched control hippocampal homogenates (50 µg) using ab54764. (D) Quantification of 60 kDa BIN1 from western blots for BIN1 on sporadic AD cohort 2 SAD and age matched control human hippocampal and temporal lobe homogenates (n=7). (E) Quantification of 60 kDa BIN1 from western blots for BIN1 on Familial AD cohort 3 FAD and age-matched control human hippocampal homogenates (n=6). (F) Quantification of 90 kDa BIN1 from western blots for BIN1 on Familial AD cohort 3 FAD and age-matched control human hippocampal homogenates (n=6).

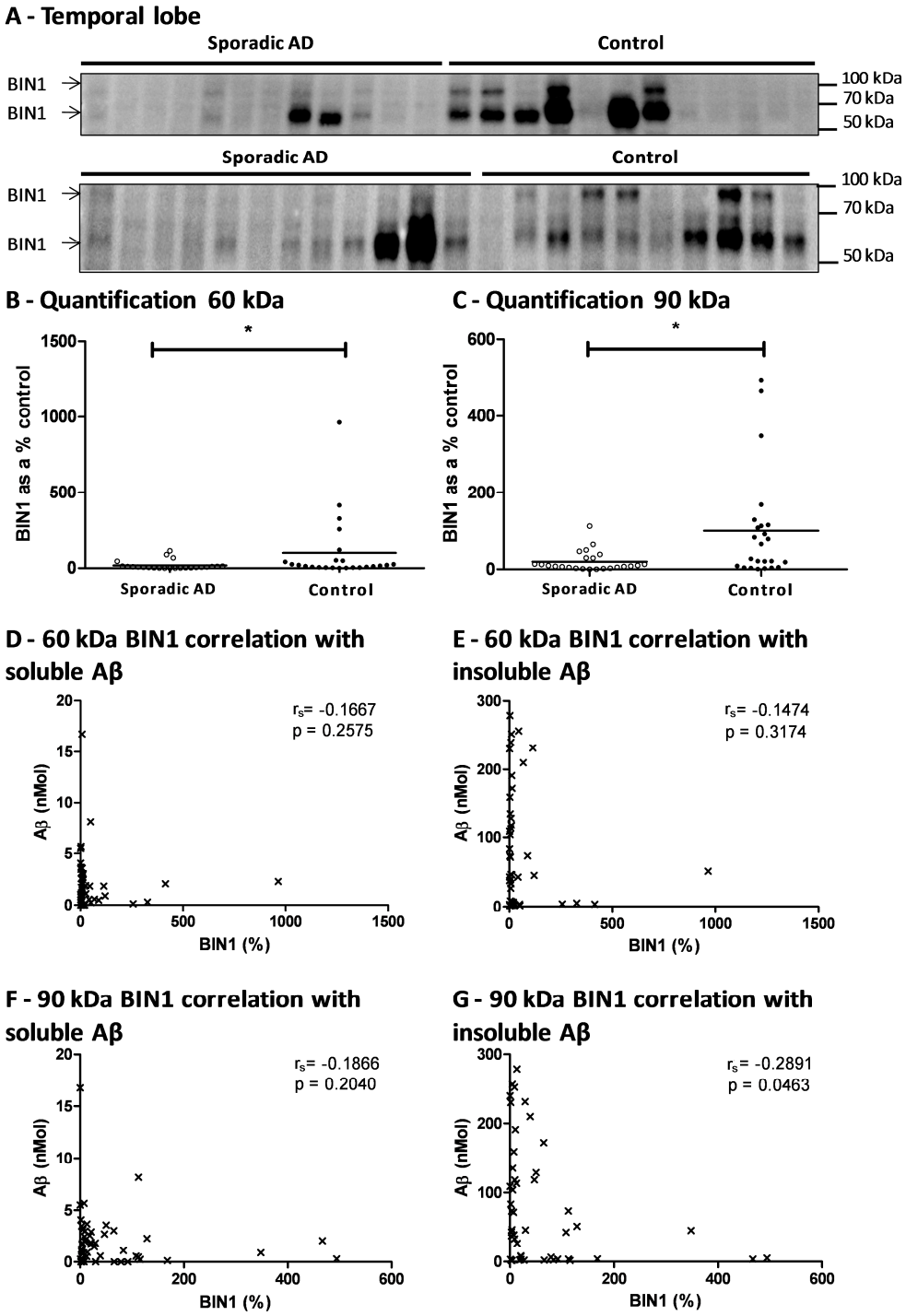

Figure 4.10 BIN1 and Aβ levels in sporadic AD cohort 4 and age-matched control temporal lobe (legend overleaf)

Figure 4.10 BIN1 and Aβ levels in Sporadic AD cohort 4 and age-matched control temporal lobe. (A) Western blot for BIN1 on Sporadic AD cohort 4 SAD and age-matched control human temporal lobe homogenates (40 µg) using ab54764. (B) Quantification of 60 kDa BIN1 from western blots for BIN1 on Sporadic AD cohort 4 SAD and age-matched control human temporal lobe homogenates (n=24). (C) Quantification of 90 kDa BIN1 from western blots for BIN1 on Sporadic AD cohort 4 SAD and age-matched control human temporal lobe homogenates (n=24). (D) Spearman's correlation analysis showed no correlation between 60 kDa BIN1 and soluble Aβ in human temporal lobe samples. (E) Spearman's correlation analysis showed no correlation between 60 kDa BIN1 and insoluble Aβ in human temporal lobe samples. (F) Spearman's correlation analysis showed no correlation between 90 kDa BIN1 and soluble Aβ in human temporal lobe samples. (G) Spearman's correlation analysis showed a significant correlation ($p<0.05$) between 90 kDa BIN1 and insoluble Aβ in human temporal lobe samples.

4.8 Discussion

Endocytosis is critical in the generation and the uptake of Aβ. BIN1 is involved in endocytosis in neurons (Ramjaun et al., 1997; Wigge et al., 1997) so the role of BIN1 in both of these processes was investigated.

BIN1 knockdown in SH-SY5Y cells did not affect the uptake of Aβ and BIN1 did not co-localise with Aβ, which suggests that BIN1 does not regulate the uptake of Aβ by neurons. Aβ is generated within cells, and then secreted to the extracellular space, where it can then be re-internalised by neurons. Several groups have shown that Aβ is taken up by endocytosis, and the uptake of Aβ may be required for Aβ toxicity (Cizas et al., 2011; Song et al., 2011). Studies have shown that AβO binds to specific receptors on neurons (for example the cellular prion protein and RAGE, receptor for advanced glycation end products) (Sturchler et al., 2008; Lauren et al., 2009; Kudo et al., 2012), so it is possible that the SH-SY5Y cells used do not express the required Aβ-receptor proteins (SH-SY5Y cells for example do not express the prion protein). Other groups have shown that Aβ is internalised in an endocytosis-independent manor (Kandimalla et al., 2009), in which case expression of BIN1 would not be expected to alter Aβ uptake. It is also not clear whether Aβ needs to be internalised in order to mediate toxic effects on the cell, or whether Aβ can initiate intracellular signalling cascades upon binding to cell surface receptors which result in cell death. It is possible that BIN1 may be involved in the regulation of these signalling cascades or the cell surface expression of these receptors. It is also important to note that during endocytosis BIN1 forms a heterodimer with amphiphysin 1, a BIN1 homologue. Amphiphysin 1 is capable of forming homodimers in the absence of BIN1 (Wigge et al., 1997), so BIN1 knockdown may

not have a significant effect on endocytosis in the absence of also reducing amphiphysin I.

BIN1 did not regulate Aβ uptake, so the effect of BIN1 on Aβ generation and APP proteolytic processing was determined. BIN1 knockdown significantly increased APP levels, and caused a trend towards increased sAPPβ. BIN1 over-expression caused a significant decrease in both APP and Aβ, as well as a trend towards decreased sAPPβ. BIN1 did not affect sAPPα levels. These data suggest that BIN1 regulates Aβ generation, and leads to the hypothesis that high levels of BIN1 decrease Aβ generation so are protective against development of AD.

Increasing BIN1 expression decreased APP levels, and decreased Aβ but did not alter sAPPα, which suggests that BIN1 regulates APP that is processed in the amyloidogenic rather than the non-amyloidogenic pathway. Endocytosis of APP from the cell surface is the key event influencing whether APP is processed by α-or β-secretase, and so dictating through which pathway APP is processed. To determine whether BIN1 can regulate the endocytosis or the cell surface levels of APP, cell surface APP was measured by flow cytometry after a 10 minute incubation at either 4 or 37 ^{o}C. At 4 ^{o}C, which reflects steady state cell surface APP levels, there was less APP at the cell surface in cells expressing BIN1, however, this difference was not significant. Both in cells expressing BIN1 or empty vector there was a significant decrease in cell surface APP after a 10 minute incubation at 37 ^{o}C indicating that endocytosis of APP occurred in both cases. This suggests that BIN1 is not regulating the total amount of APP endocytosed; therefore it is not regulating β-cleavage of APP by regulating the gross endocytosis of APP. Since the decrease in Aβ is accompanied by a decrease in full length APP this suggests that rather than

simply retaining APP intracellularly BIN1 is re-directing APP that is normally processed in the amyloidogenic pathway to be degraded in another pathway.

Altering the expression of BIN1 regulates APP levels; this may have several implications for the functioning of neuronal cells and AD. APP has been shown to regulate both cell surface NMDAR levels (Cousins et al., 2009; Innocent et al., 2012) and cell surface choline transporter levels (Wang et al., 2007).

Innocent et al., (2012) have shown that increasing APP expression increases cell surface NMDAR levels (Innocent et al., 2012). Activation of NMDAR has been shown to be important for synaptic plasticity and long term potentiation, which is involved in learning and memory (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995). Several different proteins have been implicated in the trafficking and metabolism of NMDAR, and Lau and Zukin have proposed that dysregulation of this trafficking may have a role in the development of AD (Lau and Zukin, 2007). Furthermore the drug Memantine, an NMDAR antagonist, has had some success in treating severe AD (Winblad and Poritis, 1999; Forstl et al., 2011). To determine the functional consequences of altering BIN1 expression levels on cell surface NMDAR levels, BIN1 was over-expressed in SH-SY5Y-APP_{695} cells, and cell surface NMDAR levels were measured by flow cytometry. The functional NMDAR is a tetramer composed of four subunits, normally subtypes of GluN1 and GluN2 subunits (Paoletti and Neyton, 2007). GluN1 or GluN2 subunits are not transported to the cell surface unless in a full NMDAR complex (McIlhinney et al., 1998), so using an anti-GluN2B antibody directed against a part of the NMDA receptor that is extracellular allows measurement of full/functional NMDAR at the cell surface. This showed that increasing BIN1 expression (so decreasing APP expression) caused a trend towards increased (rather than decreased as was reported previously) cell surface NMDAR containing GluN2B, suggesting that if BIN1 regulates cell

surface NMDAR it is by a different mechanism than altering APP expression. NMDAR function has been shown to decrease with age (Gonzales et al., 1991; Wenk et al., 1991; Magnusson et al., 2002), and it has been hypothesised that chronic decreases in NMDAR function can lead to neurodegenerative changes in the brain, by causing excessive release of acetylcholine and glutamate (Giovannini et al., 1994; Moghaddam et al., 1997) which causes hyperstimulation and excitotoxicity to the post synaptic neuron (Newcomer and Krystal, 2001). High levels of BIN1 may be neuroprotective by keeping NMDAR levels high so counteracting the age related decrease in NMDAR.

To investigate BIN1 in the human brain during AD and aging BIN1 in four different cohorts of brain samples was determined by western blotting. Two isoforms of BIN1, one at 60 kDa and one at 90 kDa were detected in human brain samples. It is clear that both these bands are BIN1: the 60 kDa band is detected in SH-SY5Y cells lysates, and is reduced by treatment with BIN1 siRNA, and the 90 kDa band corresponds to the neuronal isoform of BIN1 over-expressed in SH-SY5Y. Both isoforms of BIN1 decreased with age in human hippocampal samples, but not temporal lobe samples. There was no significant difference in the amount of BIN1 in sporadic AD or familial AD brain samples compared to age matched control samples in cohorts 2 and 3, but there was significantly less BIN1 in sporadic AD temporal lobe samples compared to age matched control samples in cohort 4. These data show that BIN1 is reduced in the brain in aging and in AD, and therefore agree with the hypothesis that high levels of BIN1 expression are protective against development of AD. Reduced levels of the 90 kDa isoform of BIN1 were associated with increased levels of insoluble Aβ in cohort 4. There was no significant association between the 60 kDa isoform of BIN1 and Aβ suggesting that only the neuronal isoform has a role in regulating Aβ metabolism. The non-neuronal

isoforms of BIN1 are located in the nucleus and do not have a role in endocytosis, so it is unlikely that they would influence Aβ generation (Butler et al., 1997; Elliott et al., 2000). The 90 kDa isoform of BIN1 did not correlate with soluble Aβ levels in the temporal lobe. However, there was no difference in the levels of soluble Aβ in sporadic AD temporal lobe samples compared to age-matched controls, suggesting that the form of soluble Aβ detected by the ELISA is not related to sporadic AD.

It is possible that BIN1 may have a non-endocytic related role in the development of AD. The nuclear isoform of BIN1 (the isoform expressed by most somatic cells rather than just neuronal cells) has no effect on endocytosis, but is involved in caspase-independent programmed cell death (Elliott et al., 2000). Programmed cell death contributes to neuronal death in AD, and cells undergoing caspase-independent programmed cell death have been reported in the AD brain (Yu et al., 2010b). However, a number of endocytosis-related proteins have been implicated in the development of AD by GWAS (e.g. PICALM, BIN1, CD2AP (Tebar et al., 1999; Meyerholz et al., 2005; Harel et al., 2008)) and there is a lot of evidence that endocytosis is important for AD pathogenesis. Furthermore the neuronal isoform of BIN1, but not the non-neuronal isoforms of BIN1, has a role in endocytosis (Elliott et al., 2000). Together with the data presented here showing that BIN1 alters the processing of APP, this makes it highly likely that BIN1 is involved in the development of AD due to its endocytic rather than its cell death promoting functions.

Together these data suggest that increased levels of BIN1 reduce the development of sporadic AD by decreasing the amount of APP, and decreasing the amount of Aβ produced by neurons. Furthermore, BIN1 levels decrease in the brain with age, which may contribute to the increased risk of developing AD with

increasing age. BIN1 has been related to SAD by two single nucleotide polymorphisms (SNPs), rs744373 and rs7561528 both of which lie approximately 30 kb upstream of the start of the BIN1 gene. The consequence of this is that the primary or the three-dimensional structure of BIN1 is not altered by either of these SNPs; however, they may cause alterations in the expression of BIN1 such as altering timing of expression, altering expression levels or altering the isoform expressed. In light of the evidence shown here it would be interesting to determine whether either of these SNPs are in regions of the DNA involved in regulation of BIN1 expression.

5 Discussion

5.1 Contactin 5

The Proteins studied in chapter 3, neurofascin, Thy-1 and CNTN5, were all identified from an *in vivo* APP interactome study (Bai et al., 2008). All three of these proteins had some effect on the regulation of APP metabolism, altering either levels of full length APP, sAPPα, sAPPβ or Aβ. Table 1.1 (section 1.8) shows several proteins, all with roles in the regulation of APP. This demonstrates the complexity of the regulation of APP metabolism, and in turn the complexity of Alzheimer's disease: dysregulation of one or more of these regulatory proteins may contribute to the development of AD. Although all the proteins investigated in chapter 3 appeared to be involved in the regulation of APP metabolism, the most dramatic results were obtained with CNTN5.

5.1.1 Contactin proteins and γ-secretase

Over-expression of CNTN5 in HEK-APP_{695} cells caused a decrease in Aβ secretion, and an increase in the C-terminal fragments C83 and C99 in the cells. This decrease in the γ-secretase cleavage product (Aβ) and increase in the γ-secretase substrates (the C-terminal fragments) suggests that CNTN5 inhibits γ-secretase. Other members of the contactin family have been shown to be regulators of γ-secretase: CNTN1 is capable of inducing γ-secretase dependent release of the Notch intracellular domain (NICD) (Hu et al., 2003), and CNTN2 has been shown to cause γ-secretase dependent release of the AICD (Ma et al., 2008). γ-secretase is required for generation of Aβ, and for this reason inhibition of γ-secretase is being investigated as an AD therapeutic strategy. Treating mice with a potent γ-secretase

inhibitor caused weight loss, a decrease in the number of B and T cells, altered morphology in the gastrointestinal tract, and 40 % of the mice on a higher dose of the inhibitor had died by day 15 of the trial (Wong et al., 2004). Furthermore PS1 knockout mice die within minutes of birth (Shen et al., 1997). Regardless of the adverse effects observed in mice, γ-secretase inhibitors have been developed as potential AD therapeutics, but none of them have passed clinical trials. The most recent example is Semagacestat (LY450139), a γ-secretase inhibitor developed by Eli Lilly. This drug showed promise in early trials, it did not cause adverse side effects and it decreased plasma Aβ levels (Siemers et al., 2007; Fleisher et al., 2008). However, in phase three trials this drug performed worse than the placebo, and increased the risk of skin cancer, so trials were discontinued (http://newsroom.lilly.com/releasedetail.cfm?releaseid=499794, Eli Lilly press release). This suggests that complete inhibition of γ-secretase may not be a suitable therapeutic strategy for treatment of AD.

5.1.2 Contactin 5 as a γ-secretase modulator

More recently, due to the problems with γ-secretase inhibitors, research has moved towards the development of γ-secretase modulators. γ-secretase modulators can differentially affect the activity of γ-secretase towards Aβ and AICD generation, without affecting γ-secretase cleavage of other substrates (such as Notch). Some examples of γ-secretase modulators are γ-secretase activating protein (GSAP) and the drug Gleevec (Eisele et al., 2007; He et al., 2010). These modulators have the potential to inhibit the generation of Aβ without the unpleasant side effects caused by complete inhibition of γ-secretase. The drug Gleevec (Imatinib) decreases Aβ secretion and increases AICD generation in cell culture, by modulating γ-secretase activity via GSAP. The model proposed for this is that GSAP binds the γ-secretase-

APP ternary complex, altering the structure of the complex and so promoting cleavage of APP at the γ-site and thus increasing the generation of Aβ (figure 5.1). Gleevec prevents GSAP from binding to the γ-secretase-APP ternary complex, so γ-secretase cleaves predominantly at the ε-site of APP reducing Aβ and increasing AICD generation. Increased generation of AICD could then further decrease Aβ levels by causing an AICD induced increase in neprilysin (NEP) expression, leading to increased Aβ degradation (Eisele et al., 2007; He et al., 2010). If CNTN5 is a γ-secretase modulator, rather than an outright γ-secretase inhibitor, it could have potential as a target for AD therapeutics, so further work should focus on this. To determine whether CNTN5 could be a γ-secretase modulator the effect of CNTN5 on generation of AICD from APP, as well as the effect of CNTN5 on the cleavage of other γ-secretase substrates, needs to be determined. Measurement of AICD by western blotting in HEK-APP_{695} cells expressing CNTN5 was attempted but not achieved. An alternative way to measure AICD generation is to measure expression of genes whose expression is regulated by AICD, such as NEP. Increased generation of AICD results in increased expression of NEP, and this can be measured by quantitative reverse transcription PCR for NEP mRNA (Pardossi-Piquard et al., 2005).

It has previously been demonstrated that transcriptionally active AICD is only generated in neuronal cell lines, rather than in non-neuronal cell lines such as HEK cells (Belyaev et al., 2010). The effect of CNTN5 on γ-secretase has been demonstrated in both HEK cells and the neuronal cell line N2a cells, so NEP and AICD levels could be determined in N2a cells over-expressing CNTN5. Both HEK and N2a cells are immortalised cell lines so further studies into the effect of CNTN5 on Aβ generation could be carried out in primary neurons. Determining the effect of knockdown of CNTN5 expression on APP metabolism was not carried out as no cell

lines endogenously expressing CNTN5 were identified. Primary glial cells express CNTN, but this expression is quickly lost when the cells are grown *in vitro* (Eckerich et al., 2006), suggesting that CNTN5 may be expressed in primary neuronal cells. If that is the case CNTN5 expression can be knocked down in primary neuronal cells, then the effect of this on Aβ and NEP can be determined by ELISA and quantitative reverse transcription PCR, respectively.

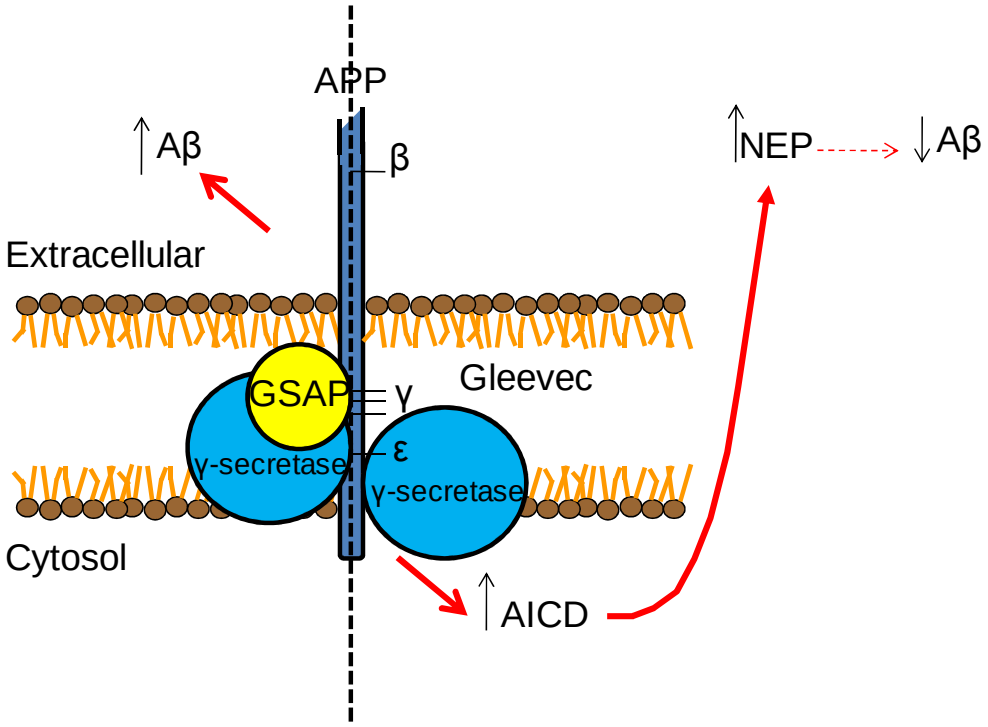

Figure 5.1 GSAP and Gleevec as γ-secretase modulators. On the left γ-secretase activating protein (GSAP) binds the γ-secretase-APP ternary complex and causes γ-secretase to cleave APP at the γ-site generating Aβ. On the right in the presence of Gleevec, GSAP does not bind the γ-secretase-APP ternary complex. In the absence of GSAP γ-secretase cleaves APP predominantly at the ε-site generating AICD. The AICD upregulates expression of neprilysin, which increases Aβ degradation, further reducing Aβ levels.

5.1.3 Contactin 5 and Notch processing

The second important factor that could determine whether CNTN5 is a viable therapeutic target for AD is whether CNTN5 regulates the cleavage of Notch by γ-secretase. The involvement of γ-secretase in Notch proteolysis and generation of the NICD has previously been a problem for γ-secretase inhibiting AD therapeutics. Notch has also been shown to have a role in cancer and both oncogenic and tumour suppressive roles have been demonstrated (Klinakis et al., 2011). Mutations that inactivate Notch have been found in patients with leukaemia, and inducing these mutations in mouse haematopoietic stem cells caused a leukaemia-like disease (Klinakis et al., 2011), demonstrating further potential complications for therapeutics that alter the activity of γ-secretase. This demonstrates the importance of Notch cleavage in cells, so it is vital that γ-secretase targeting drugs do not interfere with Notch cleavage and NICD generation. The effect of CNTN5 on NICD generation can be determined by expression of CNTN5 and NotchΔE in cell lines, then examining the effect of CNTN5 expression on levels of NICD. NotchΔE is a truncated form of Notch. Cleavage of Notch by γ-secretase occurs after an initial cleavage event, which is activated by receptor binding, generating NotchΔE. Therefore NotchΔE, rather than the full-length Notch protein is the substrate for γ-secretase (Fortini, 2002).

5.2 BIN1

5.2.1 BIN1 expression and Aβ

BIN1 has been genetically linked to sporadic AD by GWAS (Harold et al., 2009; Bertram and Tanzi, 2010; Seshadri et al., 2010; Carrasquillo et al., 2011; Hollingworth et al., 2011; Hu et al., 2011; Wijsman et al., 2011) so investigations

into the effect of BIN1 on APP metabolism can not only identify potential therapeutic targets for AD, but also shed some light onto the mechanisms involved in the of development of sporadic AD, as BIN1 levels were found to be decreased both during aging and in sporadic AD brain samples. Examination of BIN1 levels in human brain samples suggest that high levels of BIN1 protein in the brain are protective against the development of sporadic AD. Data from over-expression and knockdown studies of BIN1 in SH-SY5Y cells provide a potential mechanism for the protection afforded by BIN1. Higher levels of BIN1 expression reduced total APP levels, leading to reduced processing of APP in the amyloidogenic pathway, ultimately resulting in reduced Aβ generation. This is the first time a potential role for BIN1 in the development of sporadic AD has been identified.

The data in chapter 4 suggests that modulating expression of BIN1 could be a potential therapeutic intervention for AD. Variation at two SNPs 30 kb upstream of the start codon of BIN1 appears to influence the development of sporadic AD (Harold et al., 2009; Seshadri et al., 2010). In light of the data presented here it would be interesting to determine whether either of the AD-causing SNPs cause a decrease in expression of the neuronal isoform of BIN1 in the brain. If these SNPs result in reduced BIN1 expression, an increase in Aβ generation would be expected, which may lead to an increased chance of developing AD. A recent study by Jonsson et al., (2012) shows that a mutation in APP which decreases Aβ generation decreases the risk of developing AD. This shows that Aβ levels throughout life affect the development of AD. It also suggests that if treatment with an Aβ lowering drug begins early in the disease process then lowering Aβ could be an effective therapeutic approach for treating AD. Identification of carriers of the AD-related BIN1 SNPs could identify individuals at risk of developing AD, and early

intervention with Aβ lowering therapies has the potential to prevent AD development.

5.2.2 BIN1 expression and APP

Another avenue for further investigation is the effects of the BIN1 induced changes in APP levels. Increasing BIN1 expression decreased APP levels, whereas decreasing BIN1 expression increased APP levels. APP expression has been shown to alter synaptic strength and the number of functional synpases (Priller et al., 2006). Wang et al., (2007) showed that an increase in APP expression increases endocytosis of the high affinity choline transporter from pre-synaptic terminals, so decreasing the uptake of choline from the synaptic cleft. Re-uptake of choline is important for the re-synthesis of the neurotransmitter acetylcholine. Acetylcholine has important functions in learning and memory, and many of the current treatments for AD focus on increasing levels of acetylcholine in the synapse. This means increases in APP expression may contribute to symptoms such as reduced memory and reduced synaptic activity by reducing the amount of acetylcholine synthesis (Wang et al., 2007). Acetylcholine synthesis and choline uptake are decreased in AD, causing decreases in cholinergic function (Francis et al., 1999; Sharp et al., 2009) which correlates with cognitive decline (Garcia-Alloza et al., 2005).

The decreased levels of acetylcholine in AD has been therapeutically targeted using acetylcholine esterase inhibitors, such as Donepezil and Galantamine, to inhibit the breakdown and so increase levels of acetylcholine. These drugs have been shown to be neuroprotective in cells in culture, and can causes a temporary stabilisation or less than predicted decline in cognitive function in AD patients (Wilkinson et al., 2004; Francis et al., 2005) showing that increasing acetylcholine can have a therapeutic benefit. APP expression has also been shown to modulate

LTP – in mouse models both increases and decreases in expression of APP decrease LTP (Matsuyama et al., 2007). This means that the increase in APP caused by a loss of BIN1 could cause a decrease in LTP so decrease learning and memory formation. Innocent et al., (2012) showed that APP expression regulates cell surface NMDAR expression and so potentially can regulate the strength and activity of synapses (Innocent et al., 2012).

Data in chapter four showed that BIN1 expression in SH-SY5Y cells, however, did not cause sufficient APP alterations to affect cell surface levels NDMA receptor containing the GluN2B sunbunit. NMDA receptors are composed of a GluN1 subunit, and one of more GluN2 subunits. There are four different GluN2 subunit, GluN2(A-D) (Hynd et al., 2004). The work in chapter four only investigated NMDA receptors containing the GluN2B subunit, therefore further work could investugate whether the BIN1 induced alterations in APP can affect cell surface levels of NMDA receptors containing the GluN2A, C or D sunbunits. It remains to be determined whether the BIN1 induced increases in APP expression can modulate any other processes, but if so then therapeutics targeting this increase in APP have potential to prevent AD symptoms caused by decreased acetylcholine, decreased LTP and alterations in cell surface NMDA receptors.

5.3 Concluding remarks

Alzheimer's disease is a complex disease: many proteins regulate the processing of APP and the generation of Aβ, many proteins regulate the binding and toxicity of Aβ to neurons and many proteins and cell types are involved in the clearance of Aβ from the brain. Dysregulation of these proteins has been shown to have the potential to influence the development of AD, yet it is not clear which of these contribute to the development of AD *in vivo*. It is likely that dysregulation of

several pathways is involved in the development of the disease. Over the last decade numerous large studies have been carried out, such as GWAS and APP interactome studies, which have identified proteins with an involvement in AD. However there has been a severe lacking of research into how these newly identified genes and proteins influence the development of AD. For example the AlzGene database (www.alzgene.org) currently contains details of 1395 studies and 695 genes that have been shown to be related to AD, yet with the exception of APOE no mechanism for the involvement of any of these genes in the development of AD has been published. In order for this newly generated information to advance the field of AD detailed studies into these proteins need to be carried out. Recent work by Bali et al., (2012) has begun this, by knocking down expression of 25 SAD related genes, and determining the effect this has on sAPPβ and $A\beta_{40}$, and $A\beta_{42}$. This research showed that knockdown of 17 out of 24 SAD related genes caused an increase in Aβ production (Bali et al., 2012). The results generated by Bali et al., support the data presented here showing that BIN1 knockdown causes an increase in amyloidogenic cleavage of APP. The work presented in this thesis furthers the investigations by Bali and co-workers, showing that BIN1 over-expression has the opposite effect to BIN1 knockdown on APP processing as well as showing that BIN1 is not involved in the up-take of AβOs.

The work presented here is a detailed analysis of the involment of two novel proteins, BIN1 and CNTN5, in the regulation of APP processing and Aβ generation. CNTN5 and BIN1 regulate APP processing at different points – BIN1 regulates the levels of APP and the amount of APP directed down the amyloidogenic pathway, whereas CNTN5 regulates the γ-secretase cleavage of APP (figure 5.2). Both of these reduce Aβ generation, and represent potential targets for future AD therapeutics.

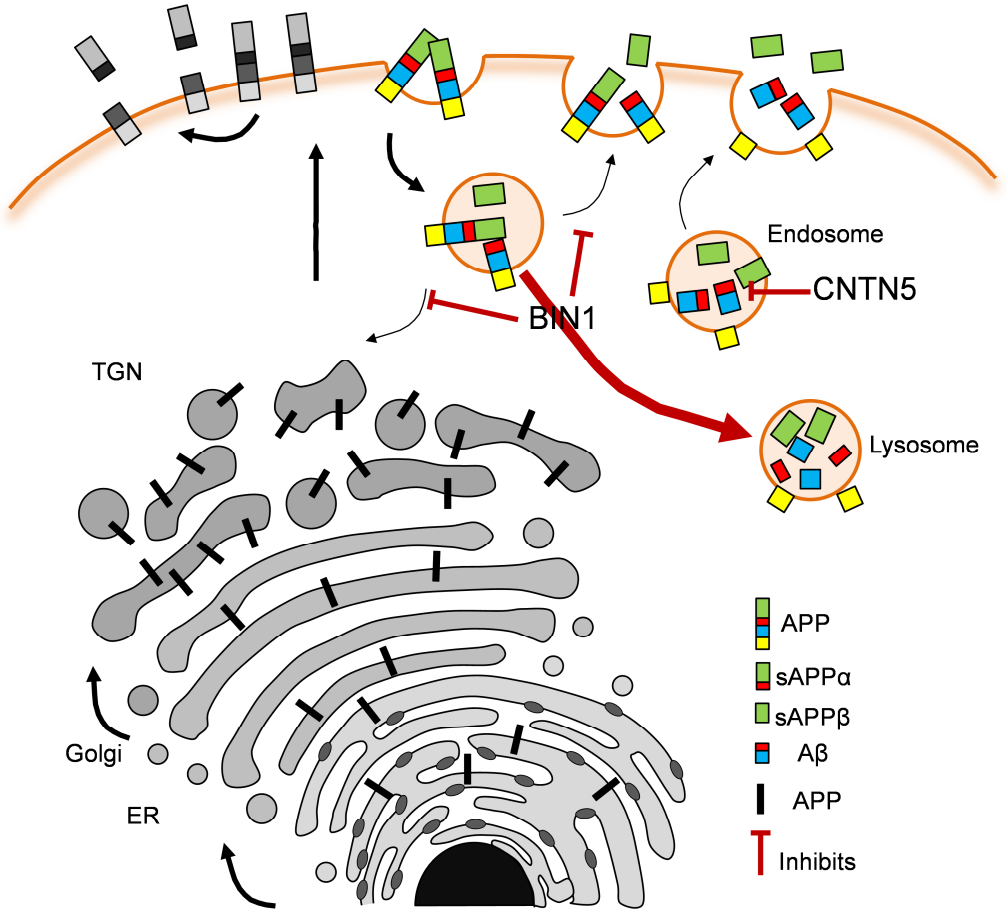

Figure 5.2 The role of BIN1 and CNTN5 in the regulation of APP processing. BIN1 and CNTN5 regulate the processing of APP after it has been synthesized, transported to the cell surface and endocytosed. BIN1 is involved in the trafficking of APP re-directing it from processing on the amyloidogenic pathway to compartments of the cell where it is degraded, so reducing the amount of APP that is cleaved on the amyloidogenic pathway. CNTN5 inhibits γ-secretase cleavage of APP reducing Aβ generation.

6 References

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7 Appendix

7.1 DNA sequences of constructs used in over-expression studies

7.1.1 Empty pcDNA3.1(+) sequence

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7.1.2 BIN1 insert sequence

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7.1.3 pcDNA3.1(+)-BIN1 (pcDNA3.1(+) with a BIN1 insert)

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4351 GCCGAACTGT TCGCCAGGCT CAAGGCGCGC ATGCCCGACG GCGAGGATCT
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4451 GCCGCTTTTC TGGATTCATC GACTGTGGCC GGCTGGGTGT GGCGGACCGC
4501 TATCAGGACA TAGCGTTGGC TACCCGTGAT ATTGCTGAAG AGCTTGGCGG
4551 CGAATGGGCT GACCGCTTCC TCGTGCTTTA CGGTATCGCC GCTCCCGATT
4601 CGCAGCGCAT CGCCTTCTAT CGCCTTCTTG ACGAGTTCTT CTGAGCGGGA
4651 CTCTGGGGTT CGAAATGACC GACCAAGCGA CGCCCAACCT GCCATCACGA
4701 GATTTCGATT CCACCGCCGC CTTCTATGAA AGGTTGGGCT TCGGAATCGT
4751 TTTCCGGGAC GCCGGCTGGA TGATCCTCCA GCGCGGGGAT CTCATGCTGG
4801 AGTTCTTCGC CCACCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA
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5151 CAGCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT
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5251 GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC
5301 ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA
5351 AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC
5401 TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG
5451 CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC
5501 CCTCGTGCGC TCTCCTGTTC CGACCCTGCC GCTTACCGGA TACCTGTCCG
5551 CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCATAGCTC ACGCTGTAGG
5601 TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA
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5701 AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT
5751 AACAGGATTA GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA
5801 GTGGTGGCCT AACTACGGCT ACACTAGAAG AACAGTATTT GGTATCTGCG
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6001 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG
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6101 TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC
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6201 TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG
6251 CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC
6301 CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC
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6851 ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT
6901 CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA
6951 AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT
7001 ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT
7051 GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA
7101 GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TC
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7.1.4 Empty pIRESneo sequence

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  51 TGCTCTGATG CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT
 101 GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA ACAAGGCAAG
 151 GCTTGACCGA CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG
 201 CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT GATTATTGAC
 251 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
 301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG
 351 CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT
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 451 AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCCC
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 601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA
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 951 TAGTAACGGC CGCCAGTGTG CTGGAATTAA TTCGCTGTCT GCGAGGGCCA
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1101 GGTGATGCCT TTGAGGGTGG CCGCGTCCAT CTGGTCAGAA AAGACAATCT
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1651 GTGCCACGTT GTGAGTTGGA TAGTTGTGGA AAGAGTCAAA TGGCTCTCCT
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2951 GGGATGCGGT GGGCTCTATG GCTTCTGAGG CGGAAAGAAC CAGCTGGGGC
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4851 GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG
4901 GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT
4951 ATCAGGGTTA TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA
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5051 CGTC
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7.1.5 Contactin 5 insert sequence

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 101 TTGTTAAGAA TTAAGAAGAG TTCATCTTCA TCTCTCTTTG GTTCCAAAAC
 151 CAGACCACGA TACAGCAGCC CTTCATTAGG AACACTGAGT GCTTCTTCAC
 201 CCAGCTGGCT AGGGGCAGCT CAGAATTATT ATTCCCCCAT CAATCTTTAT
 251 CATTCCTCAG ATGCCTTCAA ACAAGATGAA AGTGTGGACT ATGGGCCAGT
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 451 CAGTTTGATA GATGGCACCT TCATTATAAG CAATCCAAGT GAAGCAAAGG
 501 ATTCTGGTCA TTATCAGTGT TTAGCAACCA ACACTGTGGG GAGTATTCTT
 551 AGTAGAGAAG CTACACTGCA GTTTGCCTAT CTGGGAAATT TTAGTGGCCG
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3101 CCACTCCCAG ATGCTGGAGT CTATATTATT GAAGTTCGAG CATATAGTGA
3151 AGGAGGAGAT GGAACAGCTA GTTCTCAAAT TAGGGTACCA TCATATTCAG
3201 GTGGAAAAAT CACAAGTGCA CAGTCGACCC TTCACACTCT CTCCACATCT
3251 TCGTCATCAG TCACCTTGCT CTTGGCATTG ATGATTCCTT CAACTTCCTG
3301 GTGA
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7.1.6 pIRESneo-CNTN5 (pIRESneo with a CNTN5 insert)

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  51 TGCTCTGATG CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT
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 251 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
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7.1.7 Neurofascin insert sequence

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7.1.8 pIRESneo-NF186 (pIRESneo with a neurofascin 186 insert)

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6701 TGTCTGCGAG GGCCAGCTGT TGGGGTGAGT ACTCCCTCTC AAAAGCGGGC
4751 ATGACTTCTG CGCTAAGATT GTCAGTTTCC AAAAACGAGG AGGATTTGAT
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4901 ATCTGGCCAT ACACTTGAGT GACAATGACA TCCACTTTGC CTTTCTCTCC
4951 ACAGGTGTCC ACTCCCAGGT CCAACTGCAG GTCGAGCATG CATCTAGGGC
5001 GGCCAATTCC GCCCCTCTCC CTCCCCCCCC CCTAACGTTA CTGGCCGAAG
5051 CCGCTTGGAA TAAGGCCGGT GTGCGTTTGT CTATATGTGA TTTTCCACCA
5101 TATTGCCGTC TTTTGGCAAT GTGAGGGCCC GGAAACCTGG CCCTGTCTTC
5151 TTGACGAGCA TTCCTAGGGG TCTTTCCCCT CTCGCCAAAG GAATGCAAGG
5201 TCTGTTGAAT GTCGTGAAGG AAGCAGTTCC TCTGGAAGCT TCTTGAAGAC

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5251 AAACAACGTC TGTAGCGACC CTTTGCAGGC AGCGGAACCC CCCACCTGGC
5301 GACAGGTGCC TCTGCGGCCA AAAGCCACGT GTATAAGATA CACCTGCAAA
5351 GGCGGCACAA CCCCAGTGCC ACGTTGTGAG TTGGATAGTT GTGGAAAGAG
5401 TCAAATGGCT CTCCTCAAGC GTATTCAACA AGGGGCTGAA GGATGCCCAG
5451 AAGGTACCCC ATTGTATGGG ATCTGATCTG GGGCCTCGGT GCACATGCTT
5501 TACATGTGTT TAGTCGAGGT TAAAAAAACG TCTAGGCCCC CCGAACCACG
5551 GGGACGTGGT TTTCCTTTGA AAAACACGAT GATAAGCTTG CCACAACCCG
5601 GGATAATTCC TGCAGCCAAT ATGGGATCGG CCATTGAACA AGATGGATTG
5651 CACGCAGGTT CTCCGGCCGC TTGGGTGGAG AGGCTATTCG GCTATGACTG
5701 GGCACAACAG ACAATCGGCT GCTCTGATGC CGCCGTGTTC CGGCTGTCAG
5751 CGCAGGGGCG CCCGGTTCTT TTTGTCAAGA CCGACCTGTC CGGTGCCCTG
5801 AATGAACTGC AGGACGAGGC AGCGCGGCTA TCGTGGCTGG CCACGACGGG
5851 CGTTCCTTGC GCAGCTGTGC TCGACGTTGT CACTGAAGCG GGAAGGGACT
5901 GGCTGCTATT GGGCGAAGTG CCGGGGCAGG ATCTCCTGTC ATCTCACCTT
5951 GCTCCTGCCG AGAAAGTATC CATCATGGCT GATGCAATGC GGCGGCTGCA
6001 TACGCTTGAT CCGGCTACCT GCCCATTCGA CCACCAAGCG AAACATCGCA
6051 TCGAGCGAGC ACGTACTCGG ATGGAAGCCG GTCTTGTCGA TCAGGATGAT
6101 CTGGACGAAG AGCATCAGGG GCTCGCGCCA GCCGAACTGT TCGCCAGGCT
6151 CAAGGCGCGC ATGCCCGACG GCGATGATCT CGTCGTGACC CATGGCGATG
6201 CCTGCTTGCC GAATATCATG GTGGAAAATG GCCGCTTTTC TGGATTCATC
6251 GACTGTGGCC GGCTGGGTGT GGCGGACCGC TATCAGGACA TAGCGTTGGC
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6501 CCCCCGTGCC TTCCTTGACC CTGGAAGGTG CCACTCCCAC TGTCCTTTCC
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6651 ATAGCAGGCA TGCTGGGGAT GCGGTGGGCT CTATGGCTTC TGAGGCGGAA
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6851 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG
6901 CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT
6951 TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG
7001 CGCGGGGAGA GGCGGTTTGC GTATTGGGCG CTCTTCCGCT TCCTCGCTCA
7051 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC
7101 TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
7151 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
7201 CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
7251 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
7301 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
7351 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
7401 CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
7451 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
7501 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
7551 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT
7601 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
7651 GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA
7701 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
7751 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
7801 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
7851 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC
7901 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
7951 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
8001 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
8051 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG
8101 CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
8151 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC
8201 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT
8251 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG
8301 GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
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8401 CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
8451 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT
8501 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
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8601 ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
8651 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
8701 TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC
8751 AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG
8801 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
8851 ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
8901 GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
8951 AAAAGTGCCA CCTGACGTC
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7.1.9 Thy-1 insert sequence

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   1 ATGAACCTGG CCATCAGCAT CGCTCTCCTG CTAACAGTCT TGCAGGTCTC
  51 CCGAGGGCAG AAGGTGACCA GCCTAACGGC CTGCCTAGTG GACCAGAGCC
 101 TTCGTCTGGA CTGCCGCCAT GAGAATACCA GCAGTTCACC CATCCAGTAC
 151 GAGTTCAGCC TGACCCGTGA GACAAAGAAG CACGTGCTCT TTGGCACTGT
 201 GGGGGGTGCCT GAGCACACAT ACCGCTCCCG AACCAACTTC ACCAGCAAAT
 251 ACAACATGAA GGTCCTCTAC TTATCCGCCT TCACTAGCAA GGACGAGGGC
 301 ACCTACACGT GTGCACTCCA CCACTCTGGC CATTCCCCAC CCATCTCCTC
 351 CCAGAACGTC ACAGTGCTCA GAGACAAACT GGTCAAGTGT GAGGGCATCA
 401 GCCTGCTGGC TCAGAACACC TCGTGGCTGC TGCTGCTCCT GCTCTCCCTC
 451 TCCCTCCTCC AGGCCACGGA TTTCATGTCC CTGTGA
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7.1.10 pIRESneo-Thy-1 (pIRESneo with a Thy-1 insert)

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   1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC
  51 TGCTCTGATG CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT
 101 GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA ACAAGGCAAG
 151 GCTTGACCGA CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG
 201 CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT GATTATTGAC
 251 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
 301 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG
 351 CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT
 401 AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC TATTTACGGT
 451 AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCCC
 501 CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA
 551 CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
 601 TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA
 651 TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA
 701 TGGGAGTTTG TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA
 751 ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG
 801 GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA CTGCTTACTG
 851 GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTTGGTACC
 901 GAGCTCGGAT CGATATCTGC GGCCGCATGA ACCTGGCCAT CAGCATCGCT
 951 CTCCTGCTAA CAGTCTTGCA GGTCTCCCGA GGGCAGAAGG TGACCAGCCT
1001 AACGGCCTGC CTAGTGGACC AGAGCCTTCG TCTGGACTGC CGCCATGAGA
1051 ATACCAGCAG TTCACCCATC CAGTACGAGT TCAGCCTGAC CCGTGAGACA
1101 AAGAAGCACG TGCTCTTTGG CACTGTGGGG GTGCCTGAGC ACACATACCG
1151 CTCCCGAACC AACTTCACCA GCAAATACAA CATGAAGGTC CTCTACTTAT
1201 CCGCCTTCAC TAGCAAGGAC GAGGGCACCT ACACGTGTGC ACTCCACCAC
1251 TCTGGCCATT CCCCACCCAT CTCCTCCCAG AACGTCACAG TGCTCAGAGA
1301 CAAACTGGTC AAGTGTGAGG GCATCAGCCT GCTGGCTCAG AACACCTCGT
1351 GGCTGCTGCT GCTCCTGCTC TCCCTCTCCC TCCTCCAGGC CACGGATTTC
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1451 GGAATTAATT CGCTGTCTGC GAGGGCCAGC TGTTGGGGTG AGTACTCCCT
1501 CTCAAAAGCG GGCATGACTT CTGCGCTAAG ATTGTCAGTT TCCAAAAACG
1551 AGGAGGATTT GATATTCACC TGGCCCGCGG TGATGCCTTT GAGGGTGGCC
1601 GCGTCCATCT GGTCAGAAAA GACAATCTTT TTGTTGTCAA GCTTGAGGTG
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1701 TGCCTTTCTC TCCACAGGTG TCCACTCCCA GGTCCAACTG CAGGTCGAGC
1751 ATGCATCTAG GGCGGCCAAT TCCGCCCCTC TCCCTCCCCC CCCCCTAACG
1801 TTACTGGCCG AAGCCGCTTG GAATAAGGCC GGTGTGCGTT TGTCTATATG
1851 TGATTTTCCA CCATATTGCC GTCTTTTGGC AATGTGAGGG CCCGGAAACC
1901 TGGCCCTGTC TTCTTGACGA GCATTCCTAG GGGTCTTTCC CCTCTCGCCA
1951 AAGGAATGCA AGGTCTGTTG AATGTCGTGA AGGAAGCAGT TCCTCTGGAA
2001 GCTTCTTGAA GACAAACAAC GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA
2051 CCCCCCACCT GGCGACAGGT GCCTCTGCGG CCAAAAGCCA CGTGTATAAG
2101 ATACACCTGC AAAGGCGGCA CAACCCCAGT GCCACGTTGT GAGTTGGATA
2151 GTTGTGGAAA GAGTCAAATG GCTCTCCTCA AGCGTATTCA ACAAGGGGCT
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2251 GGTGCACATG CTTTACATGT GTTTAGTCGA GGTTAAAAAA ACGTCTAGGC
2301 CCCCCGAACC ACGGGGACGT GGTTTTCCTT TGAAAAACAC GATGATAAGC
2351 TTGCCACAAC CCGGGATAAT TCCTGCAGCC AATATGGGAT CGGCCATTGA
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2501 TTCCGGCTGT CAGCGCAGGG GCGCCCGGTT CTTTTTGTCA AGACCGACCT
2551 GTCCGGTGCC CTGAATGAAC TGCAGGACGA GGCAGCGCGG CTATCGTGGC
2601 TGGCCACGAC GGGCGTTCCT TGCGCAGCTG TGCTCGACGT TGTCACTGAA
2651 GCGGGAAGGG ACTGGCTGCT ATTGGGCGAA GTGCCGGGGC AGGATCTCCT
2701 GTCATCTCAC CTTGCTCCTG CCGAGAAAGT ATCCATCATG GCTGATGCAA
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2801 GCGAAACATC GCATCGAGCG AGCACGTACT CGGATGGAAG CCGGTCTTGT
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2951 ACCCATGGCG ATGCCTGCTT GCCGAATATC ATGGTGGAAA ATGGCCGCTT
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4701 CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA
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4951 GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG
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