Investigation of secreted amyloid precursor protein-α binding partners

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Abstract

Alzheimer’s disease is characterised by progressive loss of memory and cognitive functions. The symptoms extend to severe dementia and, in extreme cases, death. The neurological damage arises from the accumulation of a neurotoxic peptide known as amyloid-β (Aβ). This peptide arises from the processing of a membrane bound protein, amyloid precursor protein (APP). Processing of APP can follow one of two mutually exclusive proteolytic pathways; the amyloidogenic pathway involves the actions of a β-secretase (BACE) and a γ-secretase and results in the generation of the neurotoxic Aβ fragment and the N-terminal secreted ectodomain, secreted amyloid precursor protein-β (sAPPβ). The non-amyloidogenic pathway requires the action of an α-secretase (ADAM9, 10 or 17) and the γ-secretase and results in an N-terminal fragment secreted amyloid precursor protein-α (sAPPα) that has 16 amino acids more than sAPPβ.

sAPPα has been shown to be neuroprotective, neurotrophic, and has been shown to regulate long term potentiation and long term depression, models of mammalian memory. Moreover, these beneficial effects are thought to be receptor mediated. Identification of the receptor(s) with which sAPPα interacts could identify a potential therapeutic target and will assist in further understanding the biology of sAPPα.

In light of these findings, the current study aimed to use a protein pull-down approach to isolate and characterise putative receptor(s) and any intracellular binding proteins. A GST-sAPPα fusion protein was used as bait and protein samples from rat hippocampus and a human neuroblastoma cell culture line used as prey. The proteins found to interact with the GST-fusion protein were purified on a glutathione column and identified by mass spectrometry to create a portfolio of binding proteins. To ensure that the proteins found were binding specifically to sAPPα, sAPPβ (the similar proteolytic fragment of APP that lacks the 16 C-terminal residues and has been found to be 100-fold less effective as a neuroprotective molecule) and GST alone were included as controls. Proteins that bound to either of these molecules as well were put aside in order to identify proteins that uniquely bound to sAPPα. Several novel sAPPα interacting proteins of interest were identified; HMGB1, prohibitin, hornerin, protein SET and several structural proteins were identified in the study. A previous interaction with albumin preproprotein was also confirmed. Prohibitin and HMGB1 provide the most likely candidates for transducing the effects of sAPPα. Functional studies on the proteins will need to be carried out to investigate the importance of these interactions.
Acknowledgments

I would like to take this opportunity to express my immense gratitude to my supervisors. Professor Warren Tate and Dr Joanna Williams have been fantastic supervisors throughout my MSc. They took a chance with taking on a student from a different city whom neither of them had met, and for this I am very grateful. Having not completed an honours course prior to beginning my MSc, I arrived in the lab as a very green student and both Warren and the entire Tate lab took me under their wing and created an amazing environment to be a student in. Warren has always been happy for me to wander into his office with any problems and questions about my work and somehow always managed to make me feel better about them offer some sort of elegant solution. While Joanna has been based in the Anatomy Department her contribution has been invaluable. Whether it be advice at the weekly lab meetings, offering suggestions over e-mail or helping me through problems over coffee, Joanna has always been happy to give me the time of day and offer advice. I thank you both from the bottom of my heart!

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<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
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<td>AICD</td>
<td>APP intracellular domain</td>
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<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid</td>
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<td>Acidic leucine-rich nuclear phosphoprotein 32</td>
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<td>Apolipoprotein E</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<td>BACE</td>
<td>Beta-site APP cleaving enzyme</td>
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<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<td>BLAST</td>
<td>Basic local alignment sequence tool</td>
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<td>Central amyloid precursor protein domain</td>
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<td>CID</td>
<td>Collision induced disassociation</td>
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<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>COS-7</td>
<td>African green monkey (<em>Cercopithecus aethiops</em>) kidney cells</td>
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<td>CRMP-2</td>
<td>Collapsin response mediator protein-2</td>
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<td>CTF</td>
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<td>Copper binding domain</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Deoxynucleoside triphosphate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DR</td>
<td>Death receptor</td>
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<td>DTT</td>
<td>1,4-dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>Early-onset Alzheimer’s disease</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERMA</td>
<td>Environmental Risk Management Authority</td>
</tr>
<tr>
<td>ESC</td>
<td>Impulse-evoked postsynaptic currents</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GFLD</td>
<td>Growth-factor-like domain</td>
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<tr>
<td>GMO</td>
<td>Genetically Modified Organism</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group binding protein-1</td>
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<tr>
<td>IBSC</td>
<td>Institutional Biological Safety Committee</td>
</tr>
<tr>
<td>INHAT</td>
<td>Inhibitor of histone acetyltransferase</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>KPI</td>
<td>Kunitz protease inhibitor</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MQ H₂O</td>
<td>Milli-Q™ Ultrapure Water</td>
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<td>Nuclear factor kappa B</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>OD</td>
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<td>Phosphate buffered saline</td>
</tr>
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<td>PC</td>
<td>Personal containment</td>
</tr>
<tr>
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<td>Polymerase chain reaction</td>
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<tr>
<td>PHB</td>
<td>Prohibitin</td>
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<td>Phosphatidylinositol 3-kinase</td>
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<td>Protein kinase G</td>
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<td>Protein phosphatase 2-A</td>
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<td>PSEN</td>
<td>Presenilin</td>
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<td>RAGE</td>
<td>Receptor for advanced glycation endproducts</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>sAPPα</td>
<td>Secreted amyloid precursor protein alpha</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>Secreted amyloid precursor protein beta</td>
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<tr>
<td>SAD</td>
<td>Sporadic Alzheimer’s disease</td>
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<tr>
<td>SAXS</td>
<td>Small angle X-ray scattering</td>
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<tr>
<td>Abbreviation</td>
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<td>SD</td>
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<td>SDS-PAGE</td>
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<td>Neuroblastoma cell line</td>
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<td>SSC</td>
<td>Spontaneous postsynaptic currents</td>
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<td>TACE</td>
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<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<td>TEMED</td>
<td>N,N,N′,N′-tetramethylethylenediamine</td>
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<td>Transforming growth factor-β</td>
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<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tween® 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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Chapter 1 – Introduction

1.1 Alzheimer’s disease

Alois Alzheimer published the first account of this disease in 1907 where he characterised the symptoms as a progressive dementia and a progressive decline in cognitive function and memory (Alzheimer et al., 1995). The patient in question was described as ‘paranoid, disorientated, helpless, “she does not understand anything and everything is strange to her”, and completely delirious with her memory being ‘seriously impaired’. Upon post-mortem examination of the patient’s brain, Alzheimer noted some physical alterations occurring in the brain, notably, darkly staining plaques (using the silver impregnation technique, reviewed in Puchtel & Waldrop 1978) that appeared extra-cellularly and an aggregated protein intra-cellularly. In the post-mortem brain Alzheimer noted that a quarter to one third of the brain’s neurons showed these lesions.

Alzheimer’s disease (AD) can either be familial, caused by mutation, or more commonly sporadic. AD typically affects the elderly with symptoms ranging over a wide scale from early/mild such as becoming withdrawn and less spontaneous, getting lost in familiar places, and a general decline in cognitive ability, progressing to severe such as losing the ability to feed and swallow, memory becomes severely deteriorated and, in all cases eventually results in death. Recently the ‘World Alzheimer Report 2010’ (http://www.alz.co.uk/research/world-report) was released by the Alzheimer's Disease International Federation (a

![Figure 1.1. The growth in numbers of people with dementia](http://www.alz.co.uk/research/world-report)
federation for AD consisting of 73 non-profit Alzheimer Associations worldwide). This document aimed to assess the current impact of AD on the worldwide community. This report estimates the current worldwide economic burden of dementia to be an astonishing US$604 billion. The report also states that approximately 35.6 million people suffer from dementia world-wide; with this forecasted to increase to 65.6 million by 2030 and to 115.4 million by 2050. With AD being the leading cause of dementia worldwide and with no current cure or effective treatment, much research has gone into the understanding of the disease and the mechanisms behind it.

Figure 1.2. Classic pathological hallmarks of AD.

Figure showing the typical signs of AD (taken with permission from Vandenberghe & Tournoy 2005). (A) shows the darkly staining amyloid plaques, stained with anti-Aβ antibody. (B) shows amyloid plaques in the presence of neurofibrillary tangles (NFTs) stained with anti-paired helical filament antibody.

Eighty years after the physical characteristics were first noted by Alzheimer the molecular basis of the brain changes were characterised. In 1985 a group headed by Colin Masters purified and sequenced the prominent peptide in extra-cellular plaques. This small protein, now known as amyloid-β (Aβ), was a 4 kDa peptide, but higher order assemblies were also present (Masters et al., 1985). The group also noted that there are superficial similarities in the solubility of the plaque core and scrapie infectivity agent but there was no sequence resemblance.
Interestingly, Aβ aggregates are also found in the brains of elderly patients with trisomy-21 (Down’s syndrome). This, as well as a study in 1981 that assessed the genetics of the relatives of 125 confirmed AD sufferers and found a high frequency of dementing illness (Heston et al., 1981), suggests that there is a strong genetic component to AD. Two years after the identification of the Aβ peptide, Müller-Hill and colleagues hypothesised that the amyloid protein was of neuronal origin and was the cleavage product of a larger parent protein. They isolated and sequenced a gene on chromosome 21 which contained an apparently full length sequence of the amyloid protein. It was part of a larger gene, predictably named the amyloid precursor protein (APP) (Kang et al., 1987). The finding that amyloid-β (Aβ) was the core protein in the amyloid plaques in AD patients led to the belief that Aβ was the causative

![Amyloid cascade hypothesis](image)

**Figure 1.3. Summary of Aβ hypothesis**

APP, amyloid precursor protein. PS1, presenilin 1. PS2, presenilin 2. Aβ42, amyloid β peptide variant of 42 residues in length. (Taken with permission from Hardy & Selkoe 2002)
agent in AD. In 1991, Hardy & Allsop suggested that overproduction of Aβ and aggregation in senile plaques is the root cause of Alzheimer’s disease (Hardy & Allsop 1991). From this paper, what is now known as the 'amyloid hypothesis' was born. Figure 1.3 shows a schematic outline of the amyloid hypothesis.

Further evidence for the involvement of Aβ in AD came in 1990 where it was found that mutations in the APP gene that cause hereditary cerebral haemorrhage with Dutch type amyloidosis (identified in Wattendorff et al., 1982) could also cause a rise in Aβ deposition (Levy et al., 1990). This gained context when several of these mutations were also found in familial Alzheimer’s patients (Goate et al., 1991; Mullan et al., 1992). However, these mutations account only for a fraction of the cases of early onset familial Alzheimer’s disease (FAD) and so more effort was applied to discover the genetic basis behind the familial cases of the disease.

1.2 Amyloid Precursor Protein (APP)

1.2.1 APP structure

APP is part of a gene family that includes APP-like proteins APLP1 and APLP2 (Sprecher et al., 1993). Members of this family are all type 1 integral membrane proteins with a single domain spanning the cell membrane, a large extracellular ectodomain and a smaller cytoplasmic C-terminal region (Kang et al., 1987; Dyrks et al., 1988). The APP gene is localized on chromosome 21 (21q21.2-3) and is expressed in many cell types throughout the body. The promoter region of the APP gene is typical of a housekeeping gene in that the promoter contains no defined TATA box and a high GC content of 72%, and transcription initiates at multiple sites (Salbaum et al., 1988). Although the APP gene contains 19 exons (Yoshikai et al., 1990), the primary transcript can be alternatively spliced to remove exons 7, 8, and 15 to produce one of three main isoforms. Exon 7 encodes a 55 residue domain with homology to Kunitz protease inhibitors, exon 8 codes for a 19 residue domain that shares homology with the MRC OX-2 antigen that is present in neuronal membranes and exon 15 codes a small 18 residue domain that lies N-terminal to the Aβ domain. The largest isoform of APP is 770 residues in length (APP770) and contains all exons, APP751 lacks exon 8, and APP695 has both exon 7 and 8 removed (Tanaka et al., 1988). In AD research, APP695 has received most of the research attention.
as this isoform has the highest neuronal expression (the APP$_{770}$: APP$_{751}$:APP$_{695}$ mRNA expression ratio is 1:10:20 in the cortex (Tanaka et al., 1989)).

Figure 1.4 shows APP$_{695}$ with the domain organization within the protein. The protein is broadly divided into the ‘E1’ region which contains the N-terminal heparin-binding domain within the growth-factor-like domain (GFLD) and also the metal ion binding region. The Kunitz-type proteinase inhibitor (KPI) domain which is only present in APP$_{770}$ and APP$_{751}$ and the Ox domain which is only present in APP$_{770}$ are indicated by the orange arrow. The ‘E2’ region contains the central APP domain (CAPPD), the second heparin binding domain and the neuroprotective region containing the RERMS motif. The E1 and E2 domains are linked by an acidic region rich in aspartic acid and glutamic acid (and for this reason this part is sometimes referred to as the DE domain). The cytoplasmic region contains the protein interaction motif YENPTY (this includes the NPXY internalization sequence which typically signals for the internalization of a receptor via clathrin mediated endocytosis). The APP protein is post-translationally modified with typical N- and O-glycosylation (indicated by the red spheres in Figure 1.4) occurring as the protein passes through the endoplasmic reticulum (ER) and the Golgi apparatus (Weidemann et al., 1989). APP undergoes specific proteolytic cleavage at sites in the ectodomain which is addressed in Section 1.4. APP also contains a 17 residue

![Figure 1.4 Schematic of APP695 domains](image-url)

Structure of APP695 containing a growth-factor-like domain (GFLD), a copper-binding domain (CuBD), an acidic linker region, the KPI/Ox domain that are present in APP770 and APP751 only, the second heparin binding domain containing the RERMS sequence (RERMS), the central APP domain (CAPPD), a linker sequence, the sequence corresponding to Aβ and the amyloid intracellular domain (AICD). Figure adapted from Reinhard, Hébert, & De Strooper, 2005.
signal sequence that is not shown in Figure 1.4. This signal sequence is cleaved from the parent protein during membrane translocation (Dyrks et al., 1988).

APP is ubiquitously expressed in mammalian cells and has been shown to be important for a large range of cellular functions which are further explored in Section 1.2.2. Moreover, the fragments resulting from the proteolytic cleavage of the APP protein also are implicated in many cellular processes.

1.2.2 APP Functions

Despite much research into the expression patterns of APP, the functional subdomains within the molecule itself, and in to the transport of APP to various parts of the cell, the precise function of the full-length APP protein is still unclear. Establishing the precise roles for APP is complicated by the presence of homologues that could be compensating for its loss. Indeed, it has been shown that APLP1 and APLP2 function in a similar manner to APP and show partial redundancy (Heber et al., 2000). Furthermore, complementary DNA (cDNA) of APLP1 or APLP2 rescued migration deficits induced by RNAi targeted to APP (Young-Pearse et al., 2007). APP deficient mice strains are viable and fertile, but show some neurological deficits such as decreased locomotor activity, forelimb grip strength and reactive gliosis (Zheng et al., 1995). It should also be mentioned that triple knock-out of APP, APLP1 and APLP2 genes shows 100% lethality (Herms et al., 2004), showing that there is a crucial role for this family of proteins in development. The identification of the role of APP is further complicated by the possible actions of bioactive fragments that result from endogenous proteolytic cleavage. In fact, expression of secreted amyloid precursor protein α (sAPPα) is sufficient to ameliorate the deficits resulting from APP knockout, suggesting that most of APP’s actions may come from its ectodomain shedding (Ring et al., 2007). This section will summarise the known roles of APP.

1.2.2.1 Protein trafficking

During transport, APP has been found to interact with the motor protein kinesin-1 and, in binding this protein, it mediates axonal transport of the β-secretase (involved in APP processing, see Section 1.4.1) and a protein in the γ-secretase complex (also involved in APP processing, see Section 1.4.2). APP acts as a receptor mediating the transport of these proteins (Kamal et al., 2000, 2001). Moreover, it has been shown that APP can regulate the cell surface delivery of a component of the γ-secretase complex but
not the β-secretase (Liu et al., 2009) further implicating APP in protein trafficking. In studies of the herpes simplex virus, APP was found as a major part of the virus particle and likely mediated the fast anterograde transport of the viral particles (Satpute-Krishnan et al., 2006; Satpute-Krishnan, DeGiorgis, & Bearer, 2003). Another example of APP mediating protein trafficking was found in a study looking at a mouse model of Down’s syndrome. The increased dosage of APP in Down’s syndrome from the trisomy 21 resulted in decreased retrograde transport of nerve growth factor (NGF), a factor important for growth, maintenance and survival of certain neurons, and this resulted in degeneration of the forebrain cholinergic neurons (Salehi et al., 2006). APP has also been found to be involved in the endocytosis of the choline high-affinity transporter and that this is mediated through binding at the C-terminal domain (Wang et al., 2007). Generally speaking, most studies investigating the endogenous function of full length APP find that APP is in some way involved in the regulation of protein movement and in trafficking.

1.2.2.2 Cell Adhesion and movement

Many studies have implicated APP in cell adhesion and cell motility (Schubert et al., 1989; Chen & Yankner 1991; Sabo et al., 2001). From the structure in Figure 1.4 it is clear that APP contains domains found to bind specific molecules, for example, heparin (Mok et al., 1997), collagen and laminin (Beher et al., 1996; Kibbey et al., 1993). Moreover, APP has been shown to co-localise with other proteins known to be involved in cell adhesion such as β-integrin and talin (Storey, Beyreuther, & Masters, 1996; Yamazaki, Koo, & Selkoe, 1997). APP also binds an adapter protein known as Fe65 (also known as amyloid β precursor protein-binding family B member 1 (APBB1)). This interaction has been shown to modulate cell motility (Sabo et al., 2001). In this study it was shown that APP co-localises with Fe65 and that APP-Fe65 complex co-localises with actin and Mena, a protein thought to regulate actin dynamics, in lamellipodia. Also, the over-expression of both APP and Fe65 increases cell movement (Sabo et al., 2001), further suggesting APP naturally has a role in cell movement.

APP has also been implicated in the movement of growing neurons. A study, of hamster brain development, has shown that APP is developmentally regulated and rapidly transported to the growing tips of nerve fibres (Moya et al., 1994). Both full-length APP and secreted forms of APP increase during synaptogenesis and then decline once mature connections are established. This study suggests APPs role may include synaptogenesis and once mature connections have been formed the level declines. However, it has also
been shown that after synaptogenesis APP is still produced and transported (Sisodia et al., 1993; Buxbaum, Thinakaran, et al., 1998) suggesting a constitutive role, even into adulthood. Indeed, in the adult brain APP is shown to be more highly expressed in regions that undergo a higher level of synaptic modification (Löffler & Huber, 1992; Ouimet et al., 1994). Furthermore, application of both soluble and membrane bound APP to PC12 cells in culture was shown to increase the neurite length and branching without altering the number of neurites suggesting a role in neurite outgrowth (Milward et al., 1992). Moreover, RNAi interference mediated knock-down of APP causes neuronal migration abnormalities in rodents, whereas APP over-expression caused accelerated migration of neuronal cells (Young-Pearse et al., 2007), interestingly this study found that not only cDNA coding full-length APP rescued these effects but also cDNA coding for APLP1 and APLP2. The involvement of APP in cell adhesion and motility and also its involvement in synaptogenesis suggest that it may have an important role in neuronal processes such as neurite outgrowth, dendritic arborisation and synaptic plasticity.

1.2.2.3 Synaptic Plasticity

The findings above suggest a key role for APP in nerve cell growth. However, it is difficult to assign causality since studies that alter the endogenous level of APP also alter the levels of its proteolytic cleavage products that can have distinct biological effects themselves (see sections 1.5 and 1.6). This is exemplified in genetic manipulation studies where the APP gene is knocked down with consequential lower levels of both of the bioactive cleavage products, and so any biological effects may in fact, be due to a decrease in one of these cleavage products rather than a decrease in full-length APP. That being said, a large body of research certainly suggests that full length APP is involved in critical neuronal processes. Indeed, APP-null mice have been shown to exhibit motor dysfunction and brain gliosis, diminished viability and retarded neurite development (Perez et al., 1997). A study in chick cultured sympathetic and chick hippocampus neurons has shown that the binding of heparin sulphate proteoglycans to APP can induce neurite outgrowth (Small et al., 1994). In animals reared in enriched conditions, a paradigm known to enhance learning and memory, APP expression is increased up to 4-fold compared to animals reared in impoverished conditions (Huber et al., 1997). This increase in APP was accompanied by a 30% increase in APP containing synapses (Huber et al., 1997). These observations were bolstered by the finding that enriched conditions resulted in an increase in APP in hippocampus, frontal cortex and cerebellum (Teather et
al., 2002). Despite the difficulties in elucidating the precise molecular function of full-length APP it is clear that it, or one of its proteolytic fragments, is involved in the induction and maintenance of synaptic plasticity.

Another study looking at APP-null mice showed that knock-out of the APP gene also caused significant deficiencies in long-term potentiation (LTP, the currently accepted model of mammalian memory formation) and showed a decrease in pre-synaptic proteins such as synaptophysin and synapsin (Dawson et al., 1999). GABA-mediated inhibitory post-synaptic currents are also inhibited in APP-null mice (Seabrook et al., 1999).

1.2.2.4 APP as a receptor

When the parent molecule of Aβ was first discovered it was noted that it possessed many features typical of a glycosylated cell surface receptor (Kang et al., 1987). Indeed, APP has been shown to be able to act as a G-protein coupled receptor (GPCR) in an in vitro system where it was seen that APP can act in a ligand-dependent manner to activate G_0 (Okamoto, Takeda, Murayama, Ogata, & Nishimoto, 1995). It has also been suggested that physiological activation of G_0 in insects involves a homologue of APP (Swanson et al., 2005). GPCRs function through the dissociation of their α and βγ subunits that are located intra-cellularly. Once dissociated, both subunits are able to activate downstream signalling pathways. Therefore it could be postulated that APP is able to function in such a way with these subunits being attached to the intracellular domain. Interestingly, mutations associated with familial Alzheimer’s disease (FAD) that substitute a valine with either an isoleucine, phenylalanine or a glycine result in constitutive, ligand-independent G_0 activation (Okamoto et al., 1996). Over-expression of these mutants caused apoptosis through DNA fragmentation mediated by a GPCR (Yamatsuji et al., 1996). How APP might transmit signals via this method is yet to be determined but it has been suggested that dimerization of APP is involved (Brouillet et al., 1999; Hashimoto et al., 2003). These results hint towards APP acting as a receptor but as yet ligands mediating these effects have not been identified. Until fairly recently, the search for the ligand(s) had been unfruitful. However, in 2003, F-spondin, a neuronally secreted glycoprotein, was found to bind to the extracellular conserved region of APP (referred to as CAPPD in Figure 1.4). The binding of F-spondin to this region was shown to inhibit the initial cleavage step of full-length APP (Ho & Südhof, 2004). One study also suggests sAPPα as the ligand for APP as discussed in Section 1.6.4.
Another line of evidence that APP may act as a receptor is its similarity to the well characterised Notch receptor. Notch-1 is a large type-1 integral membrane protein involved in complex cell-fate decisions (Artavanis-Tsakonas, 1999). Both APP and Notch undergo regulated proteolysis to release an intracellular domain that affects gene transcription. In the case of Notch, a ligand binds the extra-cellular portion of the receptor and this induces release of the C-terminal tail intra-cellularly via a proteolysis event occurring near or within the transmembrane domain (Schroeter, Kisslinger, & Kopan, 1998). The intra-cellular domain of notch is known to bind DNA binding proteins and be transported to the nucleus where it affects transcription of target genes, in much the same way that the intra-cellular domain of APP can mediate gene transcription. Notch and APP are structurally similar and are both cleaved by the same groups (both α- and γ-) of secretases. Evidence for the involvement of the presenilins in notch processing originally came from C.elegans (Baumeister et al., 1997; Levitan & Greenwald, 1995). Mice that had the presenilin genes knocked out also showed deficiencies in notch processing (Hartmann, De Strooper, & Saftig, 1999; Shen et al., 1997; Wong et al., 1997). γ-secretase inhibitors also inhibit notch processing (De Strooper et al., 1999). There are several lines of evidence that suggest full-length APP acts as a receptor, either through the classical GPCR pathway or through a pathway that mimics Notch developmental signalling. More research is needed to further investigate this possibility.

**1.3 Mutations in AD (Familial-Alzheimer’s Disease (FAD))**

Only a small percentage of AD cases are FAD\(^1\), but they represent an important sub-population that can provide vital insight into the causes of the more common age-related sporadic AD (SAD) and to what molecules are involved in the pathogenesis of AD. It was first postulated that mutations in the APP gene might be involved in FAD when missense mutations were found in the APP gene in cases with AD (St George-Hyslop et al., 1987) and in a related disorder, hereditary cerebral haemorrhage with amyloidosis of the Dutch type\(^2\) (Van Broeckhoven et al., 1990). The first pathogenic mutations were found in exons 16 and 17 of APP from patients exhibiting hereditary cerebral haemorrhage with amyloidosis, Dutch type. A mutation was found that

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\(^1\) Familial-Alzheimer’s Disease is also referred to as Early-Onset Familial Alzheimer (FAD)

\(^2\) The mutation was found in four different families all coming from the Netherlands and so was given the name ‘Dutch Type’
substituted a glutamic acid for glutamine at position 22 of the Aβ peptide (Levy et al., 1990). This mutation has been termed the Dutch mutation. Another mutation found in the APP gene on chromosome 21 of a Swedish patient has been termed the Swedish mutation. The Swedish mutation exists at residue 595 and/or residue 596 (in APP695) and results in the generation of 6-8-fold more Aβ than wild-type (Citron et al., 1992). The Flemish mutation was also found in a Dutch family but this was distinct from the original Dutch mutation. Alanine was substituted with glycine at position 692 of the APP770 protein (Hendriks et al., 1992). This is residue 617 in the APP695 isoform. With all these genetically linked mutations resulting in AD or AD-like diseases, the importance of the APP gene had become clear.

It soon became evident that the APP gene on chromosome 21 was not the only gene involved in AD. In 1995 mutations involving a separate gene (presenilin 2 (PSEN2)) located on chromosome 1 were found to be involved with familial Alzheimer’s disease (Levy-Lahad et al., 1995). At around the same time a group also identified a mutation in chromosome 14 (presenilin 1 (PSEN1)) that is associated with very aggressive AD (Sherrington et al., 1995). To date, around 160 mutations have been identified associated with AD (AD mutation database: http://www.molgen.ua.ac.be/ADMutations/). The PSEN1 and PSEN2 genes code for presenilin1 and presenilin 2 proteins respectively. Mutations in PSEN1 inhibited the normal cleavage of APP. While α- and β- secretases were unaffected by the mutations in PSEN1 the γ-secretase was unable to cleave APP in the transmembrane region (De Strooper et al., 1998). This pointed to a roll for the presenilins in the γ-secretase mediated cleavage of APP.

In 1991, another chromosome was implicated as an AD risk factor. In a group of families that displayed FAD, no linkage was found on chromosome 21 and so the group undertook a genomic search for the linkage. They isolated a link to the proximal long arm of chromosome 19. Two years later a polymorphism was found in the predicted region. This polymorphism, termed ε4, was found in the gene encoding apolipoprotein E (APOE) (Schmechel et al., 1993; Strittmatter et al., 1993). It was seen later that this mutation increased AD risk (20% - 90%) and reduced the mean age of onset from 84 to 68 years of age (Corder et al., 1993). It is now thought that the APO family contributes to APP metabolism and is also involved in the clearance of Aβ from the brain via receptor mediated clearance across the blood brain barrier (reviewed extensively in Bu, 2009).
Nevertheless, the genetics of AD still only contributes to a small percentage of total AD cases, and the precise cause(s) of SAD is still not known. It may result from a dysregulation of the otherwise normal cleavage pattern of the full length APP protein or from a disruption in functional metal homeostasis (reviewed extensively in Adlard & Bush, 2006).

1.4 Processing of full-length APP

APP is produced in the constitutive secretory pathway. APP mRNA is translated at the rough endoplasmic reticulum and translocated through the Golgi and to the plasma membrane (reviewed in Turner et al., 2003).

![Figure 1.5. Schematic of APP processing.](image_url)
APP is post-translationally modified with N- and O- linked glycosylation, phosphorylation and also tyrosine sulfation (reviewed in Strooper & Annaert 2000).

The full-length APP protein can be sequentially cleaved by one of several groups of proteolytic enzymes called secretases. First the molecule is cleaved within the extracellular sequence by either the α- or the β-secretases. Following this, a cleavage occurs at the carboxy terminus, within the transmembrane domain by the γ-secretase complex. The position of the α-secretase cut site is within the portion of APP that generates Aβ thereby precluding its production and results in APP proceeding down what is termed the ‘non-amyloidogenic pathway’. Sequential cleavage of APP by the β- and γ-secretases results in the generation of the Aβ molecule. This section will look at each secretase and its involvement in the processing of APP, as well as how dysregulation of these enzymes could result in AD.
A figure taken with permission from Turner et al., (2003) summarizing the putative locations of APP processing in neurons. APP is first synthesised in the RER, translocated through the Golgi and once at the plasma membrane APP and its c-terminal fragments can be re-internalized and either return to the trans-Golgi network or can be degraded in lysosomes. Re-internalization pathways are indicated with solid arrows. Evolution of the AICD and its possible fate is indicated with dashed arrows. α-secretase activity can be found in the secretory vesicles, the cell surface and in the trans-Golgi network and β-secretase activity is localised to the endosomes, secretory vesicles and trans-Golgi network. Γ-secretase location varies but can be found in the endosomes, trans-Golgi network and at the cell surface. Nuc.: nucleus, RER: rough endoplasmic reticulum, Cis-G: cis-Golgi, Trans-G: trans-Golgi, Secr. Ves.: secretory vesicle, Endo.: endosome, Lys: lysosome, α: α-secretase localisation, β: β-secretase localisation, γ: γ-secretase localisation.
1.4.1 β-secretase

In 1999 the identity of the β-secretase was identified. The recently annotated *Caenorhabditis elegans* genome was used to search for candidate enzymes and then for their homologues in the human genome. β-secretase was found to be a member of the aspartyl protease family (Yan et al., 1999) and is now referred to as β-amyloid cleaving enzyme or BACE1. A β-secretase homolog termed BACE2 was also found and localised, interestingly, to chromosome 21 (Saunders et al., 1999). Although the importance of BACE2 in APP metabolism is uncertain, it has been found that there is very little expression in the brain (Vassar et al., 1999).

Based on the UniPROTKB/Swiss-Prot database, BACE1 is a type-1 transmembrane protein. It contains a 21 residue N-terminal pro-domain that is responsible for efficient exit from the ER and this is subsequently removed. Following this is a 434 residue ectodomain and a 21 residue transmembrane domain. This is followed by the 24 residue C-terminal tail, which is important for the intra-cellular sorting of the enzyme. After transport through the endoplasmic reticulum to the trans-Golgi network through the classical anterograde transport pathway, BACE1 emerges as a 75 kDa mature protein and is transported to the plasma membrane in a similar fashion to APP. The enzyme was found to have homology to the pepsin family of aspartyl proteases (Vassar et al., 1999; Yan et al., 1999; Hussain et al., 1999). The enzyme contains two aspartic protease sites, DTGS (residues 93-96) and DSGT (residues 289-292) and mutation of either of these sites renders the enzyme non-functional (Hussain et al., 1999). β-secretase mRNA is highly expressed in the brain and is also found in a variety of other tissues (Vassar et al., 1999; Yan et al., 1999), this finding is consistent with Aβ being produced normally in many different cell types (Citron et al., 1992). It has also been seen that expression is increased in the brains of patients with AD (Fukumoto et al., 2002).

BACE1, like other aspartyl proteases, has a low optimum pH of around 4.5 which explains its localisation to acidic compartments such as the Golgi, endosomes and the trans-Golgi network. The orientation of the enzyme in the membrane allows the cleavage of its substrate(s) within the lumen of these compartments. Further support for the β-secretase cleaving APP in the endosome came from a study looking at Aβ generation and noted that there was a delay in Aβ secretion which supports the idea that cleavage occurs in the endosome following endocytosis of the APP holoprotein (Perez, Squazzo, & Koo,
1996). Based on the expression pattern of BACE1 it was determined that it has a normal function in the brain (Yan et al., 1999) and is suspected to have more than one substrate.

Lipids also seem to play a crucial role in determining the activity of the β-secretase. When endogenous cholesterol is reduced by up to 50% with the inhibitor Lovastatin, β-secretase cleavage of APP was significantly inhibited, but when water-soluble modified cholesterol was added to the medium, Aβ synthesis increased 4-fold (Frears et al., 1999). The reason for this appeared to be that APP was no longer being incorporated into lipid rafts, implying that microdomain structure of the membrane and its proteins are an important factor in the β-secretase pathway (Simons et al., 1998).

![Figure 1.7. Secretase Cut Sites.](image)

A schematic of the secretase hydrolysis sites within APP. The numbering is referring to the Aβ peptide.

Taken with permission from Zhou et al., 2011.

The β-secretase is responsible for initiating the amyloidogenic processing of APP. The enzyme typically cleaves between residues 596 and 597 (according to APP$_{695}$ residue numbering). This represents the classical β-secretase cut site, but β-secretase mediated cleavage can also occur at the position marked β’ in Figure 1.7 which produces a less toxic, truncated form of Aβ (Aβ$_{11-42}$), found in plaques previously (Masters et al., 1985). Cleavage at the classical β-secretase site releases the extracellular fragment termed secreted amyloid precursor protein β (sAPPβ) and leaves a 99 residue peptide (C99) still membrane bound. The C99 transmembrane fragment consists of the Aβ fragment and the AICD. It is the cleavage of the C99 peptide by the γ-secretase complex that results in the production of soluble Aβ peptides and consequently the release of the AICD.

Given how intricately β-secretase is involved in the generation of Aβ, it is easy to see how perturbations in this pathway could result in an increase in Aβ generation. Indeed, BACE1 cleaves APP containing the ‘Swedish mutation’ 10 times faster than wild-type APP since there is a mutation within classical β-secretase cut site where residues K$_{595}$M$_{596}$ are substituted to N$_{595}$L$_{596}$. This change means BACE1 has much
higher affinity for the substrate (Yan et al., 1999). This finding sheds light on why the Swedish mutation forms such an aggressive phenotype. Despite the β-secretase pathway seeming like such a good candidate for manipulation to alter the course of AD, drugs that interfere with BACE1 activity have yielded rather disappointing results (Hills & Vacca, 2007; Schmidt et al., 2006). This could point to an as yet unidentified β-secretase homologue that is over-riding the inhibition or perhaps Aβ can exert its toxic effects at very low concentrations and the inhibition of the β-secretase is not enough to abolish Aβ production.

1.4.2 γ-secretase

In contrast to β-secretase, the “γ-secretase” is a protein complex comprising several protein subunits, but the exact function of each is still not fully elucidated. Initially it was thought that the protein presenilin-1 (PS1) (and presenilin-2 (PS2)) was the elusive enzyme but it is also known that the protein nicastrin is intimately involved in the γ-secretase processing of APP, along with anterior pharynx-defective 1 (Yu et al., 2000; Esler et al., 2002; Yu et al., 1998; Capell et al., 1998; Li et al., 2000; Zhang et al., 2000; Herreman et al., 2000; Francis et al., 2002).

PS1 and PS2 were originally identified by genetic linkage studies of certain pedigrees of AD and mapped to chromosomes 14 and 1 respectively (Cruts et al., 1995; Hutton et al., 1996). Both PS1 and PS2 are 8 transmembrane domain proteins and are located primarily in the membranes of the ER, the intermediate compartment and the cis-Golgi apparatus (Annaert et al., 1999). However, a small portion of the total presenilin pool has been found on the plasma membrane (Georgakopoulos et al., 1999; Ray et al., 1999). Confirmation that the PS1 and PS2 proteins are involved in the processing of APP came from a double KO mouse model which accumulated the CTFs and showed a decrease in the production of P9 and Aβ (De Strooper et al., 1998; Herreman et al., 2000).

PS1 and PS2 are inactive as a full length protein (Thinakaran et al., 1996). Cleavage occurs between transmembrane domains 6 and 7 but the two fragments remain bound together forming a 100-150kDa complex (Capell et al., 1998). PS1/PS2 can autocatalytically cleave (Steiner et al., 1999; Wolfe et al., 1999; Campbell et al., 2002) but the mechanism is unknown. Other studies have shown independent catalytic activity (Campbell et al., 2003; Nyabi et al., 2003).
It is not certain that the presenilins are the enzymes responsible for the $\gamma$-secretase cleavage of APP and it has been suggested that they are just important co-factors that may be involved in the assembly of the appropriate microdomains and/or have a scaffold function for APP cleavage (De Strooper et al., 1998), Notch (De Strooper et al., 1999), and APLP-1 (Naruse et al., 1998).

Further investigation into the $\gamma$-secretase complex by affinity chromatography revealed that nicastrin is another member of the $\gamma$-secretase complex and is in fact required for the complex to function (Esler et al., 2002). Nicastrin is heavily glycosylated, with four conserved cysteine residues that are positioned near the extra-cellular C-terminus of the protein. Yu and colleagues further investigated the role of nicastrin and found that it bound the CTF of APP and also bound to PS1 and PS2 and that if the highly conserved region of nicastrin was mutated or deleted it could increase or decrease A$\beta$ production (Yu et al., 2000). Interestingly, PS1/PS2 double KO mice have altered nicastrin transport and maturation (Leem et al., 2002).

Although the $\gamma$-secretase may seem like another attractive therapeutic target, it is also known to cleave the notch receptor (which bears significant resemblance to the APP protein, see Section 1.2.2.4). The notch receptor pathway is involved in many cellular processes including cell fate decisions, and so any therapy targeting the $\gamma$-secretase would also affect notch processing. Interestingly, presenilin KO mice have remarkable similarity to notch KO mice, supporting the view that the $\gamma$-secretase also processes notch (Conlon, Reaume, & Rossant, 1995).

### 1.4.3 $\alpha$-secretases

As seen in Figure 1.5, processing of APP through the $\alpha$-secretase pathway precludes A$\beta$ formation which in itself as an attractive target for therapeutic intervention. However, the proteolytic cleavage product resulting from non-amyloidogenic processing of APP (sAPP$\alpha$) has also shown to elicit a variety of powerful neurotrophic and neuroprotective abilities (Demars et al., 2011; Quast et al., 2003; Furukawa & Mattson 1998; Mattson et al., 1993; Barger et al., 1995; Caillé et al., 2004; Mucke et al., 1996; Young-Pearse et al., 2008; Turner et al., 2007). For these reasons much research has gone into the enzyme(s) responsible for processing APP via this pathway. Interestingly, many of the findings in this area are mimicked in notch signalling. In fact many signalling pathways have similar profiles such as TGF$\alpha$ and TNF$\alpha$ (Werb & Yan 1998).
The first real clues as to the identity of the α-secretase(s) came from pharmacological proteinase inhibitor profiles. These experiments suggested the α-secretase(s) were in fact a zinc metalloproteinase (Roberts et al., 1994; Parvathy et al., 1998). Several candidate metalloproteinases have been identified. They all belong to the ADAM family of proteinases (A disintegrin and metalloproteinase) ADAM9, ADAM10 and ADAM17 (ADAM17 is also known as TNF-α converting enzyme, TACE). The most promising candidates are ADAM10 and TACE. TACE was first identified as the enzyme responsible for notch receptor ectodomain shedding (Black et al., 1997; Moss et al., 1997). TACE knockout mice showed deficiencies in sAPPα secretion (Buxbaum et al., 1998).

It has been hypothesised that α-secretase cleavage consists of a constitutive component and an inducible component. Indeed, it has been shown that α-secretase mediated cleavage of APP can be stimulated via activators of protein kinase C (PKC) such as phorbol esters or activation of muscarinic acetylcholine receptors or metabotropic glutamate receptors (mGluR) (Buxbaum et al., 1993; Hung et al., 1993; Buxbaum et al., 1990; a LeBlanc et al., 1998; Nitsch et al., 1992; Felsenstein et al., 1994; Jacobsen et al., 1994), protein kinase A (PKA) (Efthimiopoulos et al., 1996), parts of the mitogen-activated protein kinase (MAPK) pathway such as extra-cellular signal-regulated kinase (ERK), and activation of the phosphatidyl inositol 3 kinase dependent pathway (Solano et al., 2000). Further evidence for the involvement of muscarinic receptors comes from the increase in sAPPα generated from M1 and M3 receptor subtype agonists (Wolf et al., 1995). Interestingly, it has also been shown that activation of muscarinic receptors is able to reduce the cellular, learning and memory deficits in a mouse model of AD (Caccamo et al., 2006), the authors state that the positive effects were due to activation of TACE and a shift in the processing of APP to the non-amyloidogenic pathway. This finding also suggests that it is TACE that is responsible for the regulated aspect of α-secretase processing of APP. Furthering this observation, a more recent study also suggested this when a TACE inhibitor prevented inducible α-secretase cleavage but not constitutive (Blacker et al., 2002).

Further elucidation of the inducible/regulated aspect and the constitutive aspect of α-secretase processing has come with studies of one of the other candidate α-secretases, ADAM10. It was found that RNAi targeted against ADAM10 reduced the constitutive shedding of sAPPα whereas RNAi against ADAM9 or ADAM17 did not affect the basal
rate of α-secretase activity (Kuhn et al., 2010). Also, knockout of ADAM10 nearly abolished the endogenous α-secretase cleavage of APP (Jorissen et al., 2010). From the studies so far it appears that ADAM17 is responsible for the regulated processing of APP in the non-amyloidogenic pathway and is able to be induced through the activation of one of many signalling pathways and that ADAM10 appears to be responsible for the constitutive α-secretase processing (although it has been shown that it can have regulated activity as well (Asai et al., 2003)).

Initially it was thought that ADAM9 was also part if the regulated aspect of α-secretase processing of APP. For example, co-expression of ADAM9 with APP shows an increase in α-secretase cleavage in response to phorbol esters (Koike et al., 1999). However, a recent paper has shown that the maturation of ADAM10 from its preproprotein is performed by ADAM9, and the findings from Koike and colleagues can be explained through the increase in ADAM9 resulting in an increase in the processing of endogenous ADAM10 into its mature form - the phorbol esters are acting on the now increased level of ADAM10. More studies to fully delineate the precise role(s) of each secretase are needed to clarify this.

The α-secretase pathway is of great interest to AD researchers as not only does it preclude Aβ (the toxic molecule in AD) but also produces the sAPPα molecule which has been shown to have many beneficial effects on the brain. Modulators of this pathway that specifically target the APP processing with no spill over into the notch processing are an attractive potential therapy.

1.5 Secreted Amyloid Precursor Protein β (sAPPβ)

The proteolytic fragment that results from the cleavage of APP by β-secretase is a 596 residue peptide that is released extra-cellularly during APP proteolysis. sAPPβ is generated immediately prior to the generation of the Aβ species and is considered part of the amyloidogenic pathway. sAPPβ is a slightly truncated form of the equivalent peptide generated in the non-amyloidogenic pathway, sAPPα. The 16 residue difference between the two peptides appears to have large implications. Indeed, sAPPβ has been found to be up to 100 fold less potent than its non-amyloidogenic counterpart in its neuroprotective functions (Furukawa et al., 1996b). The Tate research group has evidence it does not have neuroprotective activity and is even found to be slightly toxic to cells (Tate et al., unpublished data; Taylor et al., 2008). This may be explained by the recent finding that a
proteolytic fragment of sAPPβ (N-APP286) binds and activates the death receptor 6, activating widespread caspase activation resulting in neuronal death (Nikolaev et al., 2009). In contrast sAPPβ is capable of increasing axon out-growth in primary neuronal cultures (Chasseigneaux et al., 2011). Additionally, it has been found that sAPPβ is capable of mimicking the rapid induction of neuronal differentiation induced by APP in human embryonic stem cells (Freude et al., 2011). While sAPPβ is not the focus of the current study it is important to consider as it highlights the role of the C-terminal 16 residue tail that it lacks when compared to sAPPα. It is also interesting to consider the implications of the death receptor pathway that it activates.

1.6 Secreted Amyloid Precursor Protein α (sAPPα)

sAPPα is a fragment of APP produced through cleavage of APP via the α-secretase (illustrated in Figure 1.5). sAPPα contains many structural domains, many of which have been shown to have physiological activity with effects on neurogenesis, neuroprotection, LTP, neurotrophic activities, and protein synthesis. Much research has tried to elucidate what sAPPα does and what its role in the normal physiology of the brain might be. This section will look at the body of research thus far on the sAPPα molecule.

1.6.1 Structure

While the crystal structure for full length sAPPα has not been solved, sub-domains of sAPPα have been resolved (Gralle et al., 2006; Rossjohn et al., 1999; Dahms et al., 2010; Wang & Ha 2004; Xue et al., 2011; Dulubova et al., 2004). The closest model for full length sAPPα is a predicted dimer structure in complex with heparin proposed by Gralle and colleagues. Figure 1.8 is an image of the proposed dimer structure derived from modelling on the SAXS data (Gralle et al., 2006), the functional importance of this interaction is not known.
Figure 1.8. Proposed structures of sAPPα in complex with heparin

The two proposed models of dimerization of sAPPα in complex with heparin with the two sAPPα molecules in grey and red, heparin in green attached to the cell membrane with yellow representing the transmembrane domain. The orientation on the left depicts a parallel dimer and the right depicts an antiparallel dimer. Figure taken with permission from Gralle et al., 2006.

1.6.2 The effects of sAPPα

It has become apparent that sAPPα is involved in many neural processes. For example, sAPPα can modulate LTP and LTD (LTD is an activity-dependent reduction in the efficacy of neuronal synapses) in hippocampal slices. The addition of sAPPα lowered the threshold for LTP induction and produced a rightward shift in the induction curve for LTD (Ishida et al., 1997). A more recent study provided further compelling evidence that sAPPα has the ability to modulate LTP. In an in vivo system it was found that antibodies against sAPPα reduced LTP by ~50% and that application of exogenous sAPPα facilitated LTP and facilitated n-methyl-D-aspartate (NMDA)-mediated currents. Pharmacological inhibition of the endogenous α-secretases reduced LTP and NMDA mediated currents and further application of exogenous sAPPα was able to rescue all the above negative effects on LTP (Mileusnic, Lancashire, & Rose, 2005; Taylor et al.,
These findings were complemented with behavioural studies that showed the same effects on spatial learning, tested in the Morris water maze (Taylor et al., 2008).

The ability for sAPPα to exert excitoprotection arises from its ability to modulate cell excitability. One of the earliest studies on these effects showed that sAPPα causes a rapid and prolonged decrease in intracellular Ca\(^{2+}\) concentrations and this protects cells against hypoglycaemic and also glutamate insults (Mattson et al., 1993). sAPPα can also suppress action potentials and hyperpolarize neurons through the activation of K\(^+\) channels (Furukawa et al., 1996). Another way sAPPα may influence the excitability of a cell and LTP is through its ability to modulate NMDA receptor currents specifically, and interestingly it does not affect 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) or kainite mediated currents (Furukawa & Mattson 1998). This effect was not mediated through K\(^+\) channels, as K\(^+\) channel blockers did not block sAPPα function.

Growth of different cell types can also be induced by sAPPα. It has been shown to simulate fibroblast growth (Ninomiya et al., 1993), neurite outgrowth (Roch et al., 1993; Jin et al., 1994; Small et al., 1994), axonal growth (Chasseigneaux et al., 2011) and proliferation of neural progenitor cells (Caillé et al., 2004). Using a preparation of synaptoneurosomes (a preparation of metabolically active, intact whole synapses) it was shown that sAPPα application can stimulate de novo protein synthesis (Claasen et al., 2009), mediated by several signalling pathways. This increase in protein synthesis could be important for sAPPα to alter events occurring at the synapse. Indeed, sAPPα induced protein synthesis was significantly decreased in aged rats suggesting protein synthesis at the synapse may contribute to sAPPαs modulatory effects on synaptic plasticity.

In the quest to elucidate the functionally active region(s) of the full length sAPPα molecule, many sub-domains of sAPPα have been investigated. One particular region that seems important is a pentamer located in the E2 domain (RERMS) (Ninomiya et al., 1993b; Ninomiya et al., 1994; Jin et al., 1994; Mileusnic et al., 2005; Corrigan et al., 2011; Furukawa, Sopher, et al., 1996; Roch et al., 1993). Interestingly, the tripeptide RER has neuroprotective activity (Mileusnic et al., 2005; Mileusnic & Rose 2011) and this group (Mileusnic) has patented an acetylated form of it as a potential therapy (UK Patent No GB2391548). The RERMS region was identified to be important in 1993 where several groups found that it was required for sAPPα to induce fibroblast growth (Ninomiya et al., 1993a) and also neurite extension (Roch et al., 1993). The next year the
same group found that sAPP bound to cells through two distinct sites, one was heparinase sensitive and one was not. The first was the heparin binding domain in the E1 region and the second was again the RERMS located in the E2 region, and they found that mutation of the RERMS but not the heparin binding domain abolished neuritotropic activity (Ninomiya et al., 1994). Application of sAPPα was able to decrease spontaneous postsynaptic currents (SSC), but also increased the amplitude of impulse-evoked postsynaptic currents (ESC). Antibodies directed against the C-terminal region (containing the RERMS in this study) abolished the effects on ESC, but the effects on SSC remained, whereas antibodies directed against the N-terminus abolished the effects on SSC but the effects on ESC remained (Morimoto et al., 1998). This suggests a bimodal action of sAPPα.

The C-terminal 16 residues are thought to be important for sAPPα’s activity. This is the only known difference between sAPPα and sAPPβ (the ectodomain released following β-secretase cleavage of APP) and the amino acids correspond to the first 16 residues in Aβ. sAPPβ is much less potent as a neuroprotective agent than sAPPα (Furukawa, et al., 1996, Turner et al., 2007). Further evidence came from the finding that antibodies raised against the C-terminal end of sAPPα blocked its ability to reduce cell excitability but KPI or N-terminal antibodies had no effect (Furukawa et al., 1996). The C-terminal 16 amino acids in sAPPα contain a number of highly charged residues making the region very hydrophilic, and a motif with consecutive histidines that could be a metal ion binding site. A 19-mer sequence from the sAPPα C-terminal region was able to mimic the full-length sAPPα in its ability to increase K+ currents to reduce cell excitability (Furukawa, et al., 1996). Mutational studies of the sAPPα C-terminus will reveal the importance of this area and offer insight into the importance of the histidines and the hydrophilic nature of the residues. Current investigations into this in the Tate research group suggest that these residues are important for sAPPα function (Tate et al., unpublished data).

1.6.3 Signalling pathways activated by sAPPα

Of particular interest are the signalling pathways through which sAPPα mediates its effects as they offer potential points of therapeutic intervention. Many signalling pathways have been implicated but one in particular seems to be important, the cyclic guanosine monophosphate (cGMP) pathway. Evidence for the involvement of this
pathway first arose in 1995 where it was found that membrane-permeable analogues of cGMP were able to mimic sAPPα effects (Barger et al., 1995). sAPPα was able to stimulate a membrane bound guanylate cyclase to increase cGMP levels (Barger & Mattson 1995). The next year, the same group found that not only did sAPPα increase cGMP levels but also it increased NFκB dependent transcription. Antisense decrease of an inhibitor of NFκB (thereby activating NFκB) mimicked sAPPα effects. cGMP analogues were able to mimic sAPPα effects on LTP and LTD (Ishida et al., 1997). Many other studies have implicated cGMP as being a key pathway in sAPPα signalling (Claasen et al., 2009; Morimoto et al., 1998; Furukawa & Mattson 1998; Furukawa et al., 1996).

The MAPK/ERK pathway has also been implicated in sAPPα signalling, sAPPα-induced axon outgrowth was abolished with an ERK inhibitor (Chasseigneaux et al., 2011). The wide range of signalling pathways involved in sAPPα signalling may provide many opportunities for therapeutic intervention relevant to AD and imply there might be multiple receptors for activation of these different pathways.

1.6.4 sAPPα acting through a receptor(s)?

It has been postulated that sAPPα effects are mediated through an as yet unidentified receptor (or by disruption of a receptor complex mentioned below). As early as 1993 it was suggested that sAPPα effects are receptor mediated (Roch et al., 1993). A year later it was found that sAPPα bound to cells in specific places on the cell surface and that this binding was saturable which is suggestive of receptor binding (Jin et al., 1994; Ninomiya et al., 1994). These findings were replicated when it was found that sAPPα bound cells in specific patches and that this binding was saturable and could be outcompeted (when excess non-labelled sAPPα was applied). The binding was dependent on the state of differentiation of the cell (Hoffmann et al., 1999). The proposal of a specific receptor was supported by the observation that sAPPα activated membrane-bound guanylate cyclase, characteristic of a membrane associated receptor (Barger & Mattson 1995). More recently, Mileusnic and colleagues have been studying RER contained within the RERMS motif of sAPPα and they have found RER achieves its neurotrophic effects through binding two plasma membrane proteins. They re-emphasise that receptor mediated events are responsible for sAPPα activity (Mileusnic et al., 2005). Furthermore, in the periphery, sAPPα has been shown to be the ligand of the class A scavenger receptor (Santiago-García et al., 2001), although the same study found that sAPPα from APP_{695} was not a good ligand for this receptor and that APP_{770} was much
more effective. Nevertheless this shows that sAPPα has the ability to act as a ligand for a receptor.

Several proteins have been identified as putative receptors for sAPPα. One of the two membrane bound proteins (Mileusnic et al., 2005), was collapsin response mediated protein two (CRMP-2) and antibodies directed against CRMP-2 prevented the RER region from rescuing chicks from amnesia (Mileusnic & Rose 2011). Another study that looked specifically for binding interactions with the RERMS region also identified CRMP-2 as a binding partner, along with serum albumin, actin and two novel proteins of 41 and 63kDa (Pawlik et al., 2007). This paper suggested that sAPPα was acting through regulation of the semaphorin signalling complex that is intimately involved in axonal guidance and growth cone structure. Given that the two studies that have identified CRMP-2 as a binding partner are looking at the RERMS region or the RER region within that, it seems that CRMP-2 may be responsible for at least some of the effects mediated from this region. This is supported by the fact that CRMP-2 is heavily involved in axon outgrowth and cytoskeletal arrangement and that many studies have implicated the RERMS region as being important for the neuritotropic effects and axon outgrowth induced by sAPPα (Jin et al., 1994; Ninomiya et al., 1993; Ninomiya et al., 1994; Roch et al., 1993). Another potential candidate for the sAPPα receptor is the APP protein itself. A study by Gralle and colleagues found that sAPPα exhibits its neuroprotection through disrupting APP dimers and that APP dimers were necessary for sAPPα to exhibit neuroprotection (Gralle, Botelho, & Wouters, 2009).

The C-terminus of sAPPα is also important for the pro-survival effects of sAPPα. Very few studies have looked at the binding partners of the C-terminus of sAPPα and preliminary evidence from the Tate lab suggests that mutations in this region can reduce and in some cases abolish sAPPα’s ability to rescue cells from hypoglycaemic insult (Tate et al., unpublished data). One study found that the extra-cellular domain of APP binds the ATP synthase subunit α and regulates its trafficking. Interestingly, Aβ also bound to it and this suggests the C-terminal region of sAPPα is important for binding this protein (Schmidt et al., 2008). The functional importance of this interaction and how it may be altered in AD is yet to be investigated, but given that ATP is so important for a cell, altering cellular concentrations may well exacerbate AD pathology.
1.7 Aims of this study

The current study aimed to further investigate the proteins that bind sAPPα to identify the elusive receptor and to perhaps give insight into the signalling mechanisms through which sAPPα achieves its effects. To achieve this, a protein pull-down approach was used to identify a portfolio of proteins that interact with full length sAPPα but not sAPPβ. Identification of the binding partners would allow study of the candidate proteins identified and the physiological relevance of their interaction with sAPPα. Identification of the receptor(s) and their signalling pathways may also shed insight into APP’s normal physiological role in the brain.

The strategies used in the current study to achieve this were:

1. To clone and express the full length human sAPPα protein in a vector so as to express the protein as a fusion protein with a glutathione-S-transferase (GST) tag attached to the N-terminus of the protein.
2. To use glutathione Sepharose beads to immobilise the fusion protein as ‘bait’ for interacting ‘prey’ proteins (samples containing potential ‘prey’ proteins present in the brain were exposed to the bait on the beads).
3. To elute any interacting proteins along with the fusion protein, fractionate the mixture by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and identify either by Western blotting (for the candidate interacting proteins already identified by other groups) or with tandem MS/MS mass spectrometry (for the unknown bands present on the gel).
Chapter 2 - Methods

In this section, all media and solutions used are referred to by their name only, the components of each solution and their sources are listed in Appendix 1 – Materials and Solutions. All solutions were made using Milli-Q™ Ultrapure Water (MQ H₂O) unless otherwise stated. Where solutions needed to be sterile they were autoclaved at 15 psi, 121°C for >20 min or filtered through a 0.22µm filter if autoclaving was not appropriate.

In this study the use of genetically modified organisms were approved by the Environmental Risk Management Authority, New Zealand (ERMA, GMO99/UO006) and work was carried out adhering to PC1 conditions. Cell culture was performed in a class II biological safety cabinet under PC2 conditions with the approval of the Institutional Biological Safety Committee (IBSC, GMO05/UO006). Animal work was carried out in PC2 conditions under the approval of the Otago University animal ethics guidelines (Animal Ethics approval ET21/07).

2.1 Molecular Biology Techniques

Centrifugation was carried out in a Heraeus BioFuge Pico (Kendro Laboratory Products, USA) bench top microcentrifuge at room temperature, unless otherwise stated.

2.1.1 Isolation of Plasmid DNA

Plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit (QIAGEN, Australia). The columns in the kit contain a silica membrane that adsorbs DNA in the presence of high salt. Briefly, cultures previously transformed (see Section 2.1.4) with the plasmid of interest were grown overnight from a single colony in 5 mL Luria-Bertani (LB) media containing 100 µg.mL⁻¹ ampicillin. Cells were then collected by centrifugation (17,700 x g, 1 min) 1 mL at a time in 1.5 mL microcentrifuge tubes. The cell pellet was resuspended in buffer P01 (from the kit) and lysed by the addition of buffer P2 (from the kit). Following this, the lysate was neutralised and adjusted to high-salt conditions with the addition of buffer N3 (from the kit). Precipitated cellular debris was pelleted by centrifugation (17,700 x g, for 1 min). The resulting supernatant was then applied to the column to bind the DNA and the flow-through collected by centrifugation (17,700 x g, 1 min). Plasmid DNA was eluted off the column with the addition of 30 µL of sterile MQ water and incubation for 10 min at room temperature. DNA concentration
was determined as per Section 2.1.2 and stored at -20°C short term (up to 3 months) or -80°C long term (3 months and over).

2.1.2 Determination of DNA concentration

DNA concentration was determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Auckland) using the ‘Nucleic Acid’ application module and DNA-50 (double-stranded DNA) settings. The NanoDrop gave a quantity of DNA per µL based on the absorbance of the solution. Measurements at different wavelengths gave valuable information about the spectral characteristics indicating purity and quality of the sample. DNA was only used if the A$_{260}$/A$_{280}$ ratio was ~2 which indicates very little contamination with protein. The A$_{260}$/A$_{230}$ ratio probed for contaminants such as phenolate ion, thiocyanates, and other organic compounds, and a ratio above 1.7 indicates a highly pure sample according to the manufacturer.

2.1.3 Polymerase Chain Reaction (PCR)

To minimize the risk of contamination, all PCRs were performed using aerosol-resistant and nuclease-free pipette tips, and stocks of autoclaved MQ H$_2$O used only for PCR. Amplification of the DNA sequences was performed using a PTC-200 Peltier Thermal Cycler (MJ Research Inc, USA). Each reaction was 50 µL and contained 0.2 mM dNTP, 1.5 mM MgCl$_2$, 4.4 pmol forward primers, 4.4 pmol reverse primers, 10 ng human amyloid precursor protein template DNA, 1 unit Platinum® Taq DNA polymerase and PCR buffer to a final 1x concentration. A negative reaction containing no DNA control was also included to check for contamination of any solutions. The reactions were then repeated through thirty cycles of the following PCR program:

1. Denaturation (94°C for 15 s)
2. Annealing (60°C for 30 s)
3. Extension (68°C for 2 min, with the final extension step continuing for 5 min).

Following the final extension step, the thermal cycler maintained a temperature of 4°C to maintain DNA stability.

In order for the desired sequence to be inserted successfully into the chosen vector, mutagenic primers were used to create restriction sites flanking the sequence of interest. The forward primer sequence used for both sAPPα and sAPPβ contained a
CCCGGG section immediately before a CTG (the first triplet after the start codon in the gene of interest) (full sequence of the primers can be found in Appendix 2 – Primer sequences). This allowed *SmaI* to digest the DNA immediately before the first triplet of the APP sequence (including the signal sequence). The reverse primer used for sAPPα was designed to mutate the TTG codon to a TGA codon to introduce a stop signal into the APP parent sequence to generate the appropriate truncation site for sAPPα, this was immediately followed by the sequence CGCCGGCG, allowing *NotI* to cut after the introduced stop codon. A similar strategy was employed for the reverse sAPPβ sites except a TAA stop codon was introduced to replace the endogenous GAT codon.

### 2.1.4 Restriction Digests

Both plasmid and PCR products were restricted in preparation for ligation. Restriction enzymes were chosen that (i) both had 100% activity in the same buffer (so were compatible with a simultaneous digest in the same reaction) and (ii) their recognition sites were present in the multi-cloning site in the plasmid but not in the full-length APP sequence.

The initial reaction mixture contained 10 U of each restriction enzyme (*SmaI* and *NotI*, Roche Molecular Biochemicals, USA), 1x buffer H (Roche Molecular Biochemicals, USA) and 300-500 ng DNA was made up to 20 µL with MQ H₂O. Restriction digests were in 600 µL microcentrifuge tubes and were incubated in a 37°C water bath for 2 h, additional restriction enzyme (1 µL) was added and the reaction was incubated for a further 2 h. The resulting products were then purified (Section 2.1.5) and analysed via agarose gel electrophoresis (Section 2.1.9). Samples were stored at -20°C.

### 2.1.5 Purification of linear DNA

DNA that had been amplified by PCR or that had been digested was purified using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer’s instructions. This kit is able to purify up to 10 µg of DNA between 100 bp to 10 kb from a PCR reaction or restriction digest. The spin columns in this kit contained a silica-membrane that selectively adsorbs DNA in the presence of high concentrations of chaotropic salts. Briefly, the buffer PB (supplied in the kit) was added to the DNA samples and the resulting mixture was then centrifuged (17,700 x g, 60 s). Any contaminants were removed with a wash step that involved adding the supplied buffer PE
and again centrifuging (17,700 x g, 60 s). The DNA was eluted by adding 30 µL of autoclaved MQ H₂O to the centre of the column and incubating at room temperature for >10 min followed by centrifugation (17,700 x g, 60 s). The resulting solution was then quantified as per Section 2.1.2.

2.1.6 Ligation

Previously digested vector (Section 2.1.4) and insert DNA, in a 1:5 molar end ratio, were added to a ligation mixture containing 1 U T4 DNA Ligase and 1X Ligase buffer (Roche). Reactions were carried out at 4°C overnight

2.1.7 Transformation and Creation of Competent Cells

Vector DNA was transformed into appropriate cells in order for the cells to express the genetic information of the plasmid. This was used to maintain plasmids and for fusion protein expression.

2.1.7.1 Creation of Competent Cells for Transformation

To increase the efficiency with which the cells would take up the plasmid, the cells were made chemically competent with CaCl₂ (Mandel & Higa, 1970). Briefly, cells with the genetic background of interest were grown overnight (37°C) and the following day diluted to 1/100 of the original culture volume and returned to 37°C growth. The cell density was measured with a spectrophotometer by measuring the absorbance at 600 nm (OD₆₀₀) and when the culture reached an OD₆₀₀ of 0.5 it was transferred to ice for 15 min. Cells were collected by centrifugation (4°C, 10 min, 4000 x g) and the pellet gently re-suspended in 10 mL of cold 100 mM CaCl₂ and left on ice again for 15 min. Cells were collected by centrifugation (4°C, 10 min, 4000 x g) and the pellet re-suspended in 2.5 mL 100 mM CaCl₂ and 1.25 mL 60% glycerol (v/v), separated into aliquots and stored at -80°C.

2.1.7.2 Transformation of Competent Cells

Competent cells stored at -80°C were thawed on ice. Plasmid (10 ng) was added to 100 µL of competent cells in a 1.5 mL microcentrifuge tube and placed on ice for 15 min. The cells were heat-shocked (42°C for 45 s) and left on ice for a further 1 min. Sterile 2x YT (900 µL) was then added to the tube and the cells were incubated at 37°C with shaking (200 rpm) for 45 min (Innova®40 Incubator Shaker, New Brunswick
Scientific, USA). This allowed expression of the β-lactamase protein encoding ampicillin resistance for selection of successful transformants. A sample (100 µL) was then plated onto an LB/Agar plate containing 100 µg.mL\(^{-1}\) ampicillin and allowed to grow overnight.

2.1.8 PCR Colony Screen

Following transformation a PCR protocol was used as a strategy to screen for potential positive clones using the Expand High Fidelity PCR system (Roche Applied Science, Germany). The Expand PCR system is capable of amplifying DNA fragments of up to 5kb in length and has proofreading activity.

The strategy involved amplifying the DNA directly from the bacterial clones themselves and so did not require any DNA purification. Upon inspection of the growth plates following transformation, candidate clones were selected and transferred from the plate with a pipette tip or sterile toothpick and re-suspended in 6 µL of LB medium. A small amount of this suspension (3 µL) was then transferred into 10 µL of 0.5% (v/v) Tween\(^{®}\)20. 1 µL of this mixture was then transferred into 9 µL of the PCR master mix containing 0.26U enzyme, 2 pmol of primers, 200 µM dNTPs and a 1x concentration of the supplied 10x Expand high Fidelity buffer (containing 15 mM MgCl\(_2\)). To confirm presence of the insert of interest the forward and reverse primers used in the original amplification were used (these can be found in Appendix 2 – Primer Sequences). A negative control reaction which contained no DNA was also included in each experiment. Following initial denaturation of the DNA for 10 min at 94°C, the reactions proceeded through 30 cycles of the following thermal cycler parameters:

1. Denaturation for 15 s at 94°C
2. Primer annealing for 30 s at 51.5°C
3. Extension for 2 min at 72°C. The final cycle included a final extension for 7 min. Upon completion of the entire program the thermal cycler remained at 4°C.

Products resulting from the PCR reaction were analysed by agarose gel electrophoresis (Section 2.1.9). Plasmid DNA was purified from positive clones as described in Section 2.1.1.
2.1.9 Agarose Gel Electrophoresis

Plasmid and DNA samples were mixed with 2x loading dye buffer and were run on a 1% agarose gel containing 1 µg.mL\(^{-1}\) ethidium bromide in 0.5x TBE buffer. Once cast, the gel was placed into a Biorad Mini Cell Sub GT (BioRad Laboratories, Inc., USA) and the tank was filled with TBE buffer containing 3 µL of 10 mg.mL\(^{-1}\) ethidium bromide at the positive terminal. The gel was run at 90 V for about 90 min with the dye front as an indicator using a Biorad PowerPac 300 (BioRad Laboratories, Inc., USA). The fragment size was determined by comparison to a ladder of markers (Lambda DNA digested with EcoRI and HindIII). Following electrophoresis, bands were visualised by UV transillumination and digital images of gels were captured using a GelDoc imager (BioRad Laboratories, Inc., USA).

2.1.10 Sequencing of DNA

To determine if the DNA inserts present in the plasmids contained mutations, the DNA was sequenced by the Genetic Analysis Service, Otago University, Dunedin. For the sequencing reaction, 150 ng of purified plasmid was mixed with 3.2 pmol of the appropriate primers and made up to 5 µL with MQ H\(_2\)O. Samples were run by the staff at the Genetic Analysis Service on an ABI 3730xl DNA Analyser with a BigDye v3.1 sequencing kit (Applied Biosystems, USA). The resulting sequence was analysed on the sequence analysis program 4Peaks (Mekentosj, The Netherlands) and was searched using the BLAST tool (http://blast.ncbi.nlm.nih.gov). The primers used in the sequencing reactions can be found in Appendix 2.

2.2 Cell Culture Techniques

To maintain sterile conditions, all glassware was baked at 240°C for 4 h and all solutions to be used were autoclaved. Any plasticware used was purchased sterile and pre-packaged and was disposed of after use. Prior to entering the cell culture hood all equipment and solution vessels were decontaminated with 70% ethanol (v/v) and all cell culture work was carried out in an ESCO Class II Type A2 Fume Hood (Labculture, USA). The cell culture hood surfaces were wiped down with 70% ethanol (v/v) before and after each use and the cell culture hood fan was allowed to purge for at least 10 min before and after each use. All solutions to be applied to cells were pre-heated for 30 min in a 37°C water bath. Cells were visually inspected regularly using a Nikon Eclipse
TS100 (Japan) microscope to examine cell morphology and detect signs of infection from bacteria, yeast or fungi.

The cell line used throughout the current study was the SHSY-5Y neuroblastoma cell line (ATCC number: CRL-2266), derived from Homo sapiens. It is a third generation neuroblastoma line derived from a bone marrow biopsy of an individual with metastatic neuroblastoma cancer (Biedler, Helson, & Spengler, 1973). The cell line is neuron-like with multiple neurites and therefore has neuron-like characteristics making it suitable as a model neuronal cell line for use in the cell viability assays.

2.2.1 Seeding cells from frozen stocks

SH-SY5Y cell stocks were stored in liquid nitrogen in cryotubes. To thaw the cells from storage, the cells were rapidly thawed in a 37°C water bath and immediately transferred to 10 mL of Dubecco’s Modified Eagle Medium (DMEM, Gibco Invitrogen Corporation, USA) containing 4.5 g·L⁻¹ glucose, 4 mM L-glutamine and 110 mg·L⁻¹ sodium pyruvate supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco Invitrogen Corporation, USA) (DMEM + 10% (v/v) FBS) in a 15 mL Falcon™ tube (BD Biosciences, USA). The cells were then centrifuged (CL-10 centrifuge with a G26 rotor, Thermo Fischer Scientific Inc, USA) in 15 mL Falcon™ tubes (BD Biosciences, USA) at 1000 rpm for 4 min forming a pellet at the bottom of the tube. The media was then removed through aspiration, and this included the freezing media, and 10 mL of fresh, warm DMEM + 10% (v/v) FBS was added and the cells were re-suspended by gently pipetting up and down and transferred to a T75 cell culture flask and stored at 37°C plus 5% v/v CO₂ (see Section 2.2.2).

2.2.2 Growth of Cell Culture Lines

Cells were grown in 250 mL (T75) cell culture flasks with gas permeable filter caps in a Model 371 Steri-cycle CO₂ incubator (Thermo Fischer Scientific, USA) at 37°C, 5% CO₂ (v/v) and 95% humidity (v/v). The cell lines were maintained in DMEM + 10% (v/v) FBS. To minimize the risk of contamination 1% (v/v) antibiotic/antimycotic solution was added to a final concentration of 100 units·mL⁻¹ of penicillin H, 100 µg/mL streptomycin sulphate and 0.25 µg·mL⁻¹ amphotericin B (Gibco Invitrogen Corporation, USA). Cell culture medium was changed every 2-3 d to maintain an ample supply of nutrients and to remove cell waste. The addition of phenol-red to the media enabled a
visual indication (based on pH) of the presence of cell waste. The DMEM media is normally a red/pink colour but changes to an orange/yellow colour when nutrients are depleted and acidic cell waste has accumulated.

### 2.2.3 Passaging of cells

To prevent over-growth, cells were passaged when they reached approximately 80% confluency as assessed visually through a microscope (Nikon Eclipse TS100, Japan). This involved aspirating the media, washing the cells twice with 10 mL 1x phosphate buffered saline (PBS) (as the FBS contains proteinases that inhibit the function of the trypsin) followed by the addition of 2 mL of 0.25% Trypsin-EDTA (Gibco Invitrogen Corporation, USA) to dislodge the cells. The cells were then incubated (4 min at 37°C) or until the cells were dislodged from the base of the flask. To inactivate the Trypsin, 8 mL of DMEM + 10% (v/v) FBS was added and the resulting suspension centrifuged (CL-10 centrifuge with a G26 rotor, Thermo Fischer Scientific Inc., USA) at 1000 rpm for 4 min. The supernatant was then aspirated and replaced with 10 mL of fresh DMEM + 10% (v/v) FBS and the pellet of cells was re-suspended by gently pipetting up and down. The cells were then diluted between 1/10 and 1/5 depending on the original concentration. Cells were discontinued and destroyed once 15 passages had been reached.

### 2.2.4 Seeding into 24 well plates for cell viability assays

For cell viability assays, when the cells had reached 80% confluency they were passaged (Section 2.2.3) but instead of being re-plated, a 20 µL sample was then transferred to a counting chamber (Weber Scientific International Ltd, UK) to determine the cell density for seeding. Cell number was counted using a Nikon Eclipse TS100 Microscope (Japan). The suspension was then diluted in DMEM + 10% (v/v) FBS to a final concentration of 5x10^5 cells.mL^{-1}. Cell suspension (495 µL) was then aliquoted into each of the 24 wells and was then placed in the incubator overnight.

### 2.2.5 3-[4,5--dimethylthiazol-2]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

The MTT assay (Mosmann, 1983) is a colorimetric assay based on the ability of the mitochondria to reduce the MTT compound (a soluble yellow tetrazolium salt) to formazan crystals (purple insoluble crystals) giving an indirect measure of cell viability/oxidation status. This is because in mitochondria, there are dehydrogenase
enzymes and if they are active, they cleave the tetrazolium ring and hence there are changes in the absorption spectra of the salt. The formazan crystals are then solubilized and measured with a spectrophotometer.

MTT solution was prepared at a concentration of 4 mg.mL\(^{-1}\) dissolved in autoclaved 1x PBS. The solution was filter sterilized with a 0.2 µM filter and stored in partially opaque centrifuge tubes at -20°C. Cells were grown ~18 h and were then treated with varying concentrations of different molecules depending on what was being tested. The cells were then incubated at 37°C for 2 h at which time the media was replaced with DMEM + 1% (v/v) FBS with no glucose and the cells were starved of glucose for a further 2 h. After 2 h, 35 µL of the 4 mg.mL\(^{-1}\) MTT solution was carefully added to each well (resulting in a final concentration of 0.28 mg.mL\(^{-1}\)) and then the cells were incubated for a further 2 h to allow the reduction of MTT to take place. Following this final incubation period, the media was carefully removed using a syringe with a 22G needle, so as not to disturb the formazan crystals. DMSO (200 µL of a 4:1 mix) and ethanol were then added to each well to solubilize the crystals. To ensure complete solubilisation, the plates were shaken (using a Platform STR8 shaking tray) at room temperature for 20 min. Solution from each well (180 µL) was transferred to a 96-well plate and the absorbance at 562 nm was measured using an ELx808r Ultra Micro Plate Reader (Bio-Tek Instruments Inc, USA). Replicates were averaged and standard deviation calculated, P values were determined with a Students two-tailed t-test.

2.3 Protein Techniques

2.3.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the protocol described by Kolbe, Costello, Wong, Lu, and Wohlrab (1984). Polyacrylamide (10% w/v) gels were prepared using the Mini-PROTEAN 3 system (Bio-Rad Laboratories Inc, USA). Glass spacer plates with 0.75 mm spacers (or 1.5 mm where sample volumes to be loaded exceeded 20 µL) were used to cast the gel for protein analysis. Once the separating gel had been cast it was overlaid with 200-500 µL of H\(_2\)O to ensure a flat gel layer. Upon setting, the H\(_2\)O was removed and the stacking gel was then layered on top of the separating gel and the appropriate comb was inserted into the layer prior to setting. All samples added to the gels were mixed with 3x cracking buffer containing 1% β-mercaptopethanol, and
denatured by boiling at 96°C for 5 min in a heating block. Broad Range SDS-PAGE Molecular Weight Standards were diluted to 0.1 µg.µL⁻¹ with cracking buffer, denatured, and 5 µL added to each gel as a size reference. The gels were then subjected to a constant voltage of 200 V for 1 h using a Model 200/2.0 Power Supply (Bio-Rad Laboratories Inc, USA). Following the electrophoresis the gels were either stained with Coomassie Brilliant Blue (R-250) stain to view the proteins or were transferred to nitrocellulose membranes for Western blotting analysis.

2.3.2 Coomassie Staining

For viewing proteins on the SDS-PAGE gels, the gels were stained with Coomassie Brilliant Blue (R-250) staining solution. This involved immersing the gels in the stain solution for 1 h, washing in destain solution overnight, and on the following day photographing the gels before drying them between cellophane sheets held in a Perspex frame.

2.3.3 Transfer to Nitrocellulose Membrane

Proteins separated by SDS-PAGE were transferred using the Mini Trans-Blot system (Bio-Rad Laboratories Inc, USA) for Western blot analysis. Prior to the transfer, the SDS-PAGE gels, Protran nitrocellulose membrane (Whatman Schleicher & Schuell, UK), 3MM filter paper (Whatman, UK) and sponges were equilibrated with transfer buffer at room temperature for 10 min. Following this, the Bio-Rad electroblotting cassette was assembled according to the manufacturer’s instructions. The cassette was then immersed in transfer buffer and placed in a tank with an ice block. The transfer of proteins was carried out at a constant voltage of 100 V for 1 h using a Model 200/2.0 Power Supply (Bio-Rad Laboratories Inc, USA). To see the transferred proteins, the nitrocellulose membrane was stained with Ponceau S red stain for 5 min. The bands corresponding to the molecular weight markers were marked with a pencil on the membrane, and the membrane was then rinsed in MQ H₂O and used for immunoblotting.

2.3.4 Immunoblotting (Western Blot Analysis)

Following SDS-PAGE gel running and transfer to the nitrocellulose membrane, non-specific binding of proteins to the membrane was blocked by incubation with a blocking solution of 1xPBS containing 5% (w/v) skim milk powder for typically >4 h. The membranes were then rinsed in MQ H₂O and were incubated with the primary
antibody in a solution of 1x PBS containing the antibody at the appropriate concentration, 5% (w/v) skim milk powder and 0.1% (v/v) Tween 20. Incubation times and concentrations varied between antibodies and so are outlined in the legend of the appropriate figures. Membranes were then washed three times for 5 min in MQ H2O with gentle agitation, and incubated with the secondary antibody at the appropriate dilution (in 1x PBS containing 0.1% (v/v) Tween 20 (PBST)). The membranes were then washed in PBST once for 5 min and rinsed in MQ H2O four times. Enhanced chemiluminescence (ECL) was used to visualize the proteins of interest. ECL Western Blotting Detection Reagents (GE Healthcare, UK) were prepared by mixing equal volumes of Detection Reagents 1 and 2 according to the manufacturer’s instructions and this mixture was pipetted evenly onto the membranes. The membrane was visualized using a Fujifilm Intelligent Dark Box LAS-3000 Image Reader (Fukufilm Corporation, Japan) according to the manufacturer’s instructions. Exposure times are indicated in the legends of the appropriate figures. All washes and incubations were carried out on a Platform STR8 shaking tray with moderate rocking.

2.3.5 Protein concentration determination

The amount of protein in samples was quantified using one of three methods depending on the buffer in which the protein was suspended. For protein samples containing detergent the DC Protein Assay (Bio-Rad, USA) was used and for other protein samples either the Bradford assay or the QuBit® Quantification Platform was used.

2.3.5.1 DC Protein Assay

This assay is a modified form of the traditional Lowry assay (Lowry et al., 1951) designed to measure the concentration of protein in samples that may contain reagents (such as detergent) not compatible with the traditional Lowry assay. The assay is a colorimetric assay based on the reaction of protein with an alkaline copper tartrate solution and the Folin reagent. Colour development is primarily due to the presence of tyrosine and tryptophan residues (and to a lesser extent cysteine and histidine (Lowry et al., 1951)). The solution develops a blue colour and absorbance is measured at 750 nm. All the reagents necessary are present in the DC Protein Assay Kit (Bio-Rad, USA). The reaction was carried out in a 96-well plate as per the manufacturer’s instructions. Briefly, 5 µL of each sample was pipetted into each well in triplicate as well as 5 µL of standards
ranging from 0.2-2 µg.mL\(^{-1}\). For every mL of reagent A that was needed for the assay 20 µL of reagent S was added to create reagent A’. Reagent A’ (25 µL) was added to each well followed by 200 µL of reagent B. The plate was mixed on a Platform STR8 shaking tray for 15 min to allow colour development. The absorbance at 750 nm was read using an ELx808r Ultra Micro Plate Reader (Bio-Tek Instruments Inc, USA). Protein concentration was determined from a standard curve.

2.3.5.2 Bradford Assay

This assay is also colorimetric and is based on the Coomassie Brilliant Blue (G-250) dye changing from a green-red colour if it is in acidic conditions to a blue colour upon binding to the protein (Bradford, 1976). Like the Lowry assay, the Bradford assay is also sensitive to amino acid composition. The binding of the dye to the protein causes a shift in maximal absorption from 365 to 595 nm that can be monitored with a spectrophotometer. Standards of bovine serum albumin (BSA, Gibco, Invitrogen, New Zealand) were prepared from 0-2 mg.mL\(^{-1}\) and 20 µL was added to each well in a 96-well plate in triplicate. The Bradford reagent was prepared by mixing 375 µL of the Bradford reagent (Bio-Rad, USA) with 1.5 mL MQ H\(_2\)O and mixed well, 80 µL of this was pipetted into each of the wells, mixed, and allowed to develop for 15 min at room temperature. The absorbance at 560 nm was read on a Microplate Manager® (Bio-Rad, USA) which also constructed the standard curve and placed the unknown samples on the standard curve to determine protein concentration. This method was used for determination of protein concentration of the synaptoneurosome preparations as these preparations were isolated in a different laboratory and these were the reagents present.

2.3.5.3 QuBit® Quantitation Platform

The QuBit® Quantitation Platform (Invitrogen, USA) is a highly sensitive fluorescence-based quantitation assay that can be used for DNA, RNA and protein. The kit comes with specific dyes depending on what is being measured and comes with its own pre-made standards. Samples to be measured were prepared as per the manufacturer’s instructions. Briefly, the reagent was prepared by adding 1 µL of the Quant-iT™ Reagent to every 200 µL of the Quant-iT™ Buffer required for the assays. Following this, 180-199 µL of the mixed reagent was added to each tube (0.5 mL thin wall, clear PCR tubes, Axygen, Inc., USA) and was made up to 200 µL with the sample to be measured depending on what dilution was required. Tubes were vortexed for 5 s each and allowed to incubate for 15 min at room temperature. Following incubation, the
standard curve was constructed by sequentially placing each of the pre-made standards into the QuBit® fluorometer and measuring the fluorescence of each. From this the QuBit® Fluorometer calculated the standard curve and for each sample measured, placed the measurements on the standard curve and gave a reading. This was to be adjusted depending on what dilution was used in the assay. This method is highly affected by the presence of detergents and so was not used when detergent was present.

### 2.4 Fusion Protein Expression and Purification

In order to maximise the yield of the fusion protein expression, several of the expression and purification parameters were optimised. The parameters outlined in this section reflect those found to give the highest yield. The optimisation of the expression and purification system can be seen in sections 3.3 and 3.4.

#### 2.4.1 Bacterial fusion protein expression

The BL21 strain of *E.coli* that had been transformed with the pGEX-6P-3 (GE Healthcare, UK) vector containing the fusion protein DNA insert was seeded from a single colony into 5 mL of LB media containing 100 µg.mL⁻¹ ampicillin and 34 µg.mL⁻¹ chloramphenicol and was grown at 37°C overnight (~16 h) with shaking at 200 rpm (Innova®40 Incubator Shaker, New Brunswick Scientific, USA). The culture was then diluted 1/100 with fresh, warm LB media (also containing 100 µg.mL⁻¹ ampicillin and 34 µg.mL⁻¹ chloramphenicol) and grown at 37°C with shaking (200 rpm (Innova®40 Incubator Shaker, New Brunswick Scientific, USA)). The optical density at 600 nm (OD₆₀₀) was continually measured throughout the growth (as a measure of cell density) until the culture reached an OD₆₀₀ value of 0.4-0.6 at which time isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce the expression of the fusion protein under the control of the lac operon. The culture was then grown for 4 h at 25°C with 200 rpm shaking (Innova®40 Incubator Shaker, New Brunswick Scientific, USA). Hourly samples were taken for gel analysis. At the completion of the 4 h induction, the bacterial culture was split into two 250 mL centrifuge bottles (Nalgene, USA) and centrifuged at 10,000 x g for 10 min at 4°C in an Avanti J-26 XP centrifuge (Beckman Coulter, USA) and the pellet re-suspended in ice cold, autoclaved 1xPBS to 1/100 of the culture volume. This was then placed in a 15 mL Falcon™ Tube (BD Biosciences, USA) on ice and sonicated (six times for 10 s on a Sonics Vibra-Cell® (Sonics & Materials, Inc., USA) at an amplitude of 20%) The
resulting cell lysate was then centrifuged at 13,000 rpm for 15 min at 4°C (Avanti J-26 XP centrifuge, Beckman Coulter, USA) to pellet cellular debris, the supernatant was carefully removed and placed in a clean 15 mL Falcon™ tube (BD Biosciences, USA) for further processing.

2.4.2 Purification of bacterial fusion protein

Two methods of purification were used depending on the scale of the purification. For small scale purifications the GST Microspin purification module (GE Healthcare, UK) was used and for large scale purifications, batch purification using Glutathione Sepharose 4B (GE Healthcare, UK).

2.4.2.1 Purification using GST Microspin Purification Module

The column(s) to be used were first equilibrated by incubation with 1x PBS (the solution in which the cell lysate was re-suspended) and the PBS removed by centrifugation at 500 x g. Up to 600 µL of cell lysate from Section 2.4.1 was added to a GST Spintrap Purification Module (GE Healthcare, UK) and the mixture was incubated on the column overnight at 4°C. The following day the column(s) were centrifuged at 500 x g to remove the flow-through and this was kept for analysis via SDS-PAGE. The column was washed three times with 1x PBS, 100 µL of glutathione elution buffer was added and the column was then incubated at room temperature for 6 h with gentle agitation. The initial elution was collected via centrifugation and a further 100 µL of elution buffer was added and incubated overnight at 4°C with gentle agitation. Following incubation, the column was spun at 500 x g to spin the elution down. The fractions were then analysed via SDS-PAGE.

2.4.2.2 Batch Purification Using Glutathione Sepharose 4B

For larger scale preparations, the GST Microspin purification module was not appropriate due to the upper limits of volume and so batch purification was used. A 2 mL bed volume of Glutathione Sepharose 4b beads was prepared according to the manufacturer’s instructions (this will bind up to 10 mg of fusion protein). The beads were equilibrated by incubation with 1x PBS. Following this, the entire lysate from a large scale induction (typically 4-5 mL) was added to the beads in a 15 mL Falcon™ tube (BD Biosciences, USA) and this was incubated at 4°C overnight with end over end rotation. The next day the beads were collected at the bottom of the tube by centrifugation at 500 x g in an Avanti J-26 XP centrifuge (Beckman Coulter, USA) with a F14BCI-14x50cy rotor
with a 50 mL conical to 15 mL conical adapter (Fiberlite, Piramoon Technologies, Inc, Thermoscientific, USA) and the supernatant removed and retained for further analysis (referred to as the flow-through). The beads were washed in 10 mL of cold 1x PBS three times, then 2 mL of glutathione elution buffer was added, and incubated at room temperature for approximately 8 h with end-over-end rotation. The beads were centrifuged at 500 x g to recover the supernatant from the elution; another 2 mL of glutathione elution buffer was then added for an overnight elution at 4°C, with end-over-end rotation. The following day, the supernatant was removed and pooled with the 8 h elution for dialysis.

**2.4.3 Dialysis**

Following the purification procedure the sAPPα and sAPPβ fusion proteins were dialysed against 0.1x PBS. This was done to ensure that compatibility with physiological pH and ionic conditions were maintained and to minimise any destabilising effects on conformation that might occur. Briefly, dialysis tubing was rinsed in MQ H₂O and clamped at one end. The samples were carefully pipetted into the dialysis tubing and a second clamp was added above the sample to seal it in the tubing. This was then dialysed into 1000x the sample volume 1 L at a time at 4°C for 4 h per 1 L (for a 4 mL sample, it was dialysed against 1 L for 4 hours and then repeated three times with 1 L of fresh dialysis buffer). Once dialysis was complete, the top clamp was removed and the liquid was pipetted into a clean tube and quantified using the QuBit® Quantification Platform. The dialysed solution typically varied from 1-5 mg.mL⁻¹. The post-dialysis product was also run on an SDS-PAGE gel to assess purity and degradation.

**2.5 Protein Sample Preparations**

**2.5.1 SHSY-5Y Neuroblastoma Cell Lysate Preparation**

SHSY-5Y neuroblastoma cells were grown to 80% confluency in T250 cell culture flasks. The media was aspirated off and the cells washed twice with 5 mL of 1x PBS. Following the wash steps 2 mL of 0.25% (w/v) Trypsin-EDTA (Gibco Invitrogen Corporation, USA) was added to the flask and the flask returned to the incubator for 4 min or until the cells were visibly dislodged. 8 mL of DMEM + 10% (v/v) FBS was then added to the flask to inactivate the trypsin. This was then centrifuged at 1000 rpm (CL-10 centrifuge with a G26 rotor, Thermo Fischer Scientific Inc, USA) in 15 mL Falcon™
(BD Biosciences, USA) for 4 min at room temperature. The supernatant was then aspirated and the pellet washed in 10 mL of 1x PBS. The cells were centrifuged as above, the PBS was aspirated and the pellet was re-suspended in 250 µL of Low Salt Lysis buffer and placed on ice for 5-10 min. The 250 µL lysate was then transferred to a 600 µL microcentrifuge tube and centrifuged at 13,000 rpm for 1 min to pellet the insoluble material. The supernatant was carefully removed and transferred to a clean tube for further work. The lysate preparations were stored at -80°C.

2.5.2 Synaptoneurosome Preparation

2.5.2.1 Preparation of the synaptoneurosome

The animals required for the synaptoneurosome preparation were housed in the Department of Psychology, University of Otago and members of the Abraham lab (Department of Psychology, University of Otago) maintained the animals and performed the anaesthetisation and decapitation outlined below. Synaptoneurosomes (an accepted preparation for the modelling of synaptic mechanisms (Hollingsworth et al., 1985)) were prepared from the cortex of young adult (8-12 weeks) male Sprague-Dawley rats. These animals were anaesthetised with halothane and decapitated (this was done using a protocol approved by the University of Otago Animal Ethics Committee under approval number 13/04). Cortex tissue was homogenized using five strokes of a glass/Teflon Potter Elvenheim homogenizer (clearance 0.1–0.15 mm, Kontes Glass Company) in ice cold HEPES buffer (50 mM HEPES pH 7.4, 124 mM NaCl, 3.2 mM KCl, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 1.06 mM KH₂PO₄, 26 mM NaHCO₃, 1M glucose, Complete protease inhibitor (Roche, Indianapolis, USA) and 0.2 mg.mL⁻¹ chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA)) or until the lysate was visibly clear of debris. Homogenized tissue was allowed to rest for 10 min and then homogenates were passed first through 100 µm nylon filters (Tedpella: four sheets in Millipore filter holder Ny1H09000) and then through 5 µm mitex filters (Millipore, LSW P025000). The resulting solution was centrifuged (1000 x g, 15 min, 4°C) to pellet the synaptoneurosomes. The synaptoneurosomes were collected by re-suspending the pellet in ice cold HEPES buffer and quantified using the Bradford assay (Section 2.3.5.2).

2.5.2.2 Preparation of soluble proteins from synaptoneurosomes

To isolate soluble proteins from the preparation the synaptoneurosomes an aliquot of synaptoneurosomes from Section 2.5.2.1 was thawed on ice and mixed with an equal
volume of extraction buffer (containing 1% Triton X-100 (v/v)). This mixture was incubated at 4°C for 15 min with constant stirring. Following this, the mixture was centrifuged in the appropriate centrifuge for 20 min at 33,000 x g to pellet the insoluble material. This produced a fraction that contained the soluble proteins and the proteins that were not soluble in the detergent were pelleted.

2.6 LTQ-Orbitrap Analysis

2.6.1 Fractionation of proteins

Prior to identifying the proteins with mass spectrometry, the fractions containing the eluted proteins were separated on an SDS-PAGE gel (see Section 2.3.1). Once run, the gel was stained with Coomassie stain (Section 2.3.2) and then was destained overnight. The following day, the gel was rinsed thoroughly in MQ H2O, the gel was immersed in MQ H2O and was sent to the Centre for Protein Research, University of Otago, Dunedin.

The following steps were all performed by the Centre for Protein Research, University of Otago, Dunedin. These methods are directly taken from those outlined on their website (http://biochem.otago.ac.nz/cpr/protocols.html).

2.6.2 Sample preparation

Excised protein spots/bands were subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG, Cologne, Germany). The protocol for automated in-gel digestion is based on the method of Shevchenko et al., (1996). Eluted peptides were dried using a centrifugal concentrator.

2.6.3 LC-MS/MS of tryptic peptides

Samples were re-solubilised in 5% (v/v) acetonitrile, 0.2% (v/v) formic acid in water and injected onto an Ultimate 3000 nano-flow uHPLC-System (Dionex Co,CA) that was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter-tip column (75 µm ID PicoTip fused silica tubing (New Objectives, Woburn, MA) packed with C-18 material on a length of 8-9 cm) by a gradient developed from 5% (v/v) acetonitrile, 0.2% (v/v) formic acid to 80% (v/v) acetonitrile, 0.2% (v/v) formic acid in water at a flow rate of 200-500 nL/min.
2.6.4 Typical Instrument Setting for the LTQ-Orbitrap

Full MS in a mass range between m/z 300-2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an AGC target of 5e5. Preview mode for FTMS master scan was enabled to generate precursor mass lists. The strongest 5 signals were selected for CID (collision induced dissociation)-MS/MS in the LTQ ion trap at a normalised collision energy of 35% using an AGC target of 2e4 and one microscan. Dynamic exclusion was enabled with 2 repeat counts during 30 s. and an exclusion period of 180 s. Exclusion mass width was set to 0.01.

2.6.5 Data Analysis

For protein identification MS/MS data were searched against the SWISS-PROT amino acid sequence database (downloaded in April 2009) using the Mascot search engine (http://www.matrixscience.com). The search was set up for full tryptic peptides with a maximum of 3 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 10 ppm and the max. Fragment mass error 0.8 Da.

2.6.6 Format of results

The analysis was sent via e-mail in the form of an excel document containing the identities of the proteins, the peptide fragments that lead to their identification, information about the quality of the results, and the certainty of the identifications.
Chapter 3 - Results

There is a body of evidence that strongly suggests sAPPα exerts a wide range of positive effects on the brain (summarised in section 1.5.2) mediated as a ligand through a receptor or multiple receptors to transmit the signal (reviewed in section 1.5.4). To date it is not known exactly how sAPPα does this and knowledge of the mechanism could provide vital insight into attractive therapeutic targets for AD. Specifically, identification of the receptor(s) is of paramount importance to elucidate the mechanism through which the functions of sAPPα are mediated. Several studies have attempted mostly to identify the receptor(s) and have produced a small list of candidate proteins. However, these studies have looked for binding to the RERMS motif within of the sAPPα molecule. Since the C-terminal region of sAPPα can mimic some of the positive effects produced by the full length molecule (Tate et al., Unpublished Data), this region alone may be an interacting motif. The current study set out to use human sAPPα as a bait to isolate potential binding partners that could include membrane bound receptors.

3.1 Strategy

To isolate sAPPα binding partners, sAPPα was attached to a glutathione-S-transferase carrier protein to allow it to be immobilized on a glutathione column (Figure 3.1, Panel A). The strategy was to first allow proteins in the cell samples to interact with the sAPPα fusion protein (Figure 3.1, Panel B) and then immobilize the sAPPα fusion protein complex on the glutathione column (Figure 3.1, Panel C). This was a pull down approach.
An outline of the experimental strategy of the current study. **Panel A** shows the cloning, expression and purification process to produce the fusion protein. **Panel B** depicts the combining of the test protein samples (prey) and the fusion protein (bait). **Panel C** depicts the mixture being added to a Glutathione Sepharose 4B column with the fusion protein plus any interacting proteins remaining on the column. **Panel D** depicts the bound proteins being eluted off the column. **Panel E** shows the analysis of unknown proteins by MS/MS.

**Figure 3.1 Experimental Strategy Diagram**
3.2 Generation of sAPPα-GST and sAPPβ-GST fusion

To create a GST-sAPPα (or GST-sAPPβ) fusion gene (Figure 3.1, Panel A), the portion of APP that encodes sAPPα (or sAPPβ) was cloned into the pGEX GST vector (a kind gift from Catherine Day, Biochemistry Department, University of Otago) (see Figure 3.2). In order to create the sAPPα sequence, a stop codon was introduced in the primer at the α-secretase site (sAPPα) or at the β-secretase site (sAPPβ) (primer sequences and positions can be found in Appendix 2 – Primer Sequences). Both the plasmid and the sAPPα/sAPPβ sequences were restricted and directionally cloned into the GST vector. Given the importance of the C-terminal region, the GST was attached to sAPPα at its N-terminal end to reduce the risk of the GST moiety causing steric hindrance.

![Figure 3.2. sAPPα orientation in GST vector (pGEX 6P-3)](image)

An illustration of the GST vector with the sAPPα human gene sequence fragment inserted immediately after GST. Plasmid expression is under the control of the lac I protein giving tight regulation. The plasmid also encodes a gene for resistance to the antibiotic ampicillin to allow for selection.
3.2.1 Plasmid Digest Analysis

The ability of the restriction enzymes to restrict the pGEX plasmid efficiently was investigated. pGEX plasmid was isolated from DH5α cells and purified using a Qiagen QIAprep Spin Miniprep Kit (Qiagen, Australia) with yields typically ranging from 100-400 ng µL⁻¹. For directional cloning, pGEX was cut with Smal and NotI as both enzyme restriction sites were present in the multi-cloning cassette of the plasmid and the recognition sequences for these enzymes were not present in the sAPPα or sAPPβ sequences. Initial tests shows that the restriction digest with each of the restriction enzymes produced a single linear fragment (Figure 3.3, Lanes 2-4) with the uncut plasmid running higher on the gel (Figure 3.3, Lane 1).

![Figure 3.3 Restriction enzyme analysis of pGEX plasmid DNA](image)

The restriction digest of of pGEX plasmid (300 ng) detected with ethidium bromide as per Section 2.1.9. M, λHE DNA Marker. Lane 1, Uncut plasmid. Lane 2, digestion with Smal. Lane 3, digestion with NotI, Lane 4, digestion with both Smal and NotI. Marker bands (Hind III/EcoRI digest of λ DNA (λHE)) are indicated on the left by arrows.

3.2.2 Amplification of sAPPα and sAPPβ fragments

The sAPPα and sAPPβ sequences within the human APP cDNA were amplified with a PCR reaction using specific primers. Mutagenic primers (Appendix 2) were
designed to introduce a stop codon at the appropriate place and introduce a Smal restriction site at the N-terminus and a NotI restriction site at the C-terminus.

Figure 3.4 Agarose gel electrophoresis of sAPPα and sAPPβ PCR fragments

Panel A. Agarose gel electrophoresis of PCR fragment (sAPPα) detected with ethidium bromide as per Section 2.1.9. M, λHE DNA Marker, Lane 1, negative control. Lane 2, sAPPα fragment at the expected size indicated by the thick arrow. Panel B. Agarose gel electrophoresis of PCR fragment (sAPPβ) detected with ethidium bromide as per Section 2.1.9. M, λHE DNA Marker. Lane 1, negative control. Lane 2, sAPPβ fragment indicated by the thick arrow. The size of the relevant marker band is indicated to provide a comparison with the PCR products.

Figure 3.4 shows that both PCR reactions produced a single band at the expected size of approximately 1800bp. The no DNA control lanes showed no product, this demonstrated that the product was dependent on the addition of template. The purified PCR products were then digested with NotI and Smal to produce sticky ends for cloning into the prepared pGEX vector. DH5α cells were transformed with ligated plasmids and recombinants were isolated by their resistance to the antibiotic.

3.2.3 PCR colony screens

Antibiotic resistance detects cells carrying the plasmid but this can be encoded by plasmid alone or the recombinant plasmids. A PCR colony screen was performed to confirm the presence of the sAPP insert. This involved simply performing a PCR reaction with each of the selected colonies using the same primers that were used in the amplification of the fragments.
Figure 3.5 sAPPα colony screen

Agarose gel electrophoresis of the sAPPα colony screen detected with ethidium bromide as per Section 2.1.9. Lanes 1-19 on the top section of the gel represented individual colonies taken from the ligation plates. M, λHE DNA Marker with the size of the bands indicated on the right in kb. Lane 20 on the bottom section of the gel shows the PCR no DNA control. Lane 21 on the bottom section of the gel shows the PCR of a positive control (PCR off plasmid containing APP sequence).

The PCR colony screen of sAPPα transformants identified eight positive colonies (Figure 3.5, lanes 2, 4, 10, 11, 12, 14, 18, and 19) whereas the other eleven were negative (shown in Figure 3.5). The no DNA control showed no PCR product and the positive control PCR of the plasmid containing the APP gene showed a successful PCR product of the same size.

The same protocol was used to test sAPPβ colonies present on the transformant plate after ligation. Figure 3.6 shows the agarose gel profile obtained from the sAPPβ PCR colony screen. Three clearly positive colonies were identified and the other two were negative.

Plasmid DNA from the positive colonies was isolated and sent for sequence analysis to confirm that the sequences were free of mutations introduced during the PCR reactions.
Figure 3.6 sAPPβ PCR colony screen

Agarose electrophoresis gel of the sAPPβ colony screen detected with ethidium bromide as per Section 2.1.9. M, λHE DNA Marker. Lane 1-5, 5 different colonies from the sAPPβ transformation plate.

3.2.4 Sequencing of the sAPPα-GST and sAPPβ-GST vectors

Several sequence primers were used for total coverage of the sequence (Appendix 2) and ensured full, high quality sequence of the entire sAPPα and sAPPβ inserts. The primer sequences used can be found in Appendix 2. The sequencing results were then compared to the reference sequence using a BLAST search which confirmed that there were no mutations introduced by the PCR. Figure 3.7 shows the sequence region where the stop codon was introduced and illustrates the quality of the sequence obtained. Full sequence data can be found in Appendix 3 on the attached CD-ROM disc.

Figure 3.7 Representative chromatogram of the sAPPα sequence trace

A sample of the sequence data obtained from the sequencing of the sAPPα clones displayed as a chromatogram. The bases outlined by the box indicate where mutagenic primers engineered a stop codon into the sequence in order to truncate at the α-secretase cleavage site.
The sequence depicted in Figure 3.7 indicated that the stop codon was successfully introduced in frame as shown by the TGA stop codon. The chromatogram also indicated the sequence was of high quality as shown by the high peaks for each base and the very low background trace. Figure 3.8 shows the analogous key area of the sAPPβ sequencing and the chromatogram again indicates that the stop codon TAA was introduced and also that the sequence was of high quality. The sequence results for the sAPPα and sAPPβ inserts confirm that both clones are mutation free and the mutagenic primers successfully introduced a stop codon in the appropriate place.

![Stop Codon](image)

**Figure 3.8 Representative chromatogram of the sAPPβ sequence trace**

A sample of the sequence data obtained from the sequencing of the sAPPβ clones displayed as a chromatogram. The bases outlined by the box indicate where mutagenic primers engineered a stop codon into the sequence in order to truncate at the β-secretase cleavage site.

### 3.2.5 Summary

The DNA sequences encoding sAPPα and sAPPβ were directionally cloned successfully into a pGEX-6P-3 GST vector. Transformants were selected and their plasmids sent for DNA sequencing. At least one clone was isolated for each of sAPPα and sAPPβ that showed sequence identical to the reference sequence (NM_201414.2).

### 3.3 Fusion protein expression

The fusion protein recombinant vectors created were transformed into the BL21(DE3)pLysS strain of *E.coli* (Novagen, USA) for the protein expression. This strain is deficient in the proteases *lon* and *ompT* which would minimise degradation of the fusion protein. This is important to maximise the yield of the GST-sAPPs.

#### 3.3.1 Initial expression of fusion protein

In order to express the sAPPα and sAPPβ fusion proteins, the expression conditions recommended by the manufacturer of the GST vector were used. Briefly, an
overnight culture was diluted 1/10 into fresh LB media and grown at 37°C to an OD$_{600}$ of ~0.6 (as a measure of cell density). IPTG was added to a final concentration of 0.1 mM to induce expression of the fusion protein and the culture was grown for 4 h with hourly samples being taken (Figure 3.9).

![Figure 3.9 SDS-PAGE gel of sAPPα-GST fusion protein production in E.coli](image)

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue showing a time-course of the expression of the fusion protein. M, SDS-Broadrange Marker with band sizes indicated on the gel (kDa). Lane 1, pre-induction (10 µL, 1/100 sample volume). Post-Induction - Lane 2, 1 h (10 µL, 1/100 sample volume); Lane 3, 2 h (10 µL, 1/100 sample volume); Lane 4, 3 h (10 µL, 1/100 sample volume); Lane 5, 4 h (10 µL, 1/100 sample volume). The arrow indicates the fusion protein at the expected size of ~110 kDa.

The pre-induction sample (Figure 3.9, Lane 1) showed no expression of the fusion protein indicating the expression of the plasmid was under tight control. The addition of IPTG induced expression and the protein continued to accumulate over 4 h as shown by the increasing density of the band indicated by the black arrow (Figure 3.9, lanes 2-5).

3.3.2 Optimisation of growth temperature and length of time

To optimise the expression of the fusion protein the best temperature for expression was determined as well as the length of time of expression. The cells were
grown as per the experiment in Section 3.3.1 except upon induction the cells were moved to a different growth temperature. Three temperatures were investigated and samples were taken hourly for the first 5 h and one 24 h sample was taken the following day. When the expression was carried out at 18°C the band representing the fusion protein took longer to appear (Figure 3.10, upper left panel). Then at 25°C, it increased in amount to 5 h and was lower at 24 h. The culture grown at 37°C showed earlier expression compared to the 18°C and 25°C, and continued increasing in amount and plateauing at 4 h. It decreased in amount after this. In all of the expression conditions the 24 h sample showed a reduced or unchanged amount of the fusion protein (the solid arrows, Figure 3.10). There was little difference in the yield from the three temperatures tested, only the time taken to reach the maximum amount of protein expression. For all subsequent experiments 25°C and 4 h was chosen as the conditions for expression as the lower temperature has been shown to reduce misfolding and increase solubility of the fusion proteins (Furukawa et al., 1996b).
Figure 3.10 SDS-PAGE gels of fusion protein expression at various temperatures

SDS-PAGE gels (10% acrylamide) stained with Coomassie blue showing the expression analysis carried out over various temperatures. For each gel the lanes were loaded in the same order. M, SDS Broadrange Marker. Lane 1, pre-induction (10 µL, 1/100 sample volume). Post-induction - Lane 2, 1 h (10 µL, 1/100 sample volume); Lane 3, 2 h (10 µL, 1/100 sample volume); Lane 4, 3 h (10 µL, 1/100 sample volume); Lane 5, 4 h (10 µL, 1/100 sample volume); Lane 6, 5 h (10 µL, 1/100 sample volume); Lane 7, 24 h (10 µL, 1/100 sample volume). The arrows on each gel indicate the band representing the fusion protein expression at the expected size of ~110 kDa.
3.3.3 Western blot of sAPPα

A Western blot was used to confirm the identity of the suspected fusion protein. A duplicate of the 25°C gel in Figure 3.10 was assayed for the presence of the GST-sAPPα fusion protein.

![Western blot image](image_url)

**Figure 3.11 Western blot detecting presence of GST-sAPPα**

A Western blot to detect the presence of sAPPα following induction of fusion protein expression. Following transfer to the nitrocellulose membrane, the membrane was blocked overnight at 4°C. The following day the membrane was incubated for 1.5 h at room temperature with the primary anti-N-terminal APP antibody (rabbit) (1/1,000), washed and then incubated with the secondary anti-rabbit horse radish peroxidase (HRP) antibody (1/20,000) for a further 1.5 h at room temperature. ECL was used to detect the blot which was exposed for 5 min. M, SDS Broadrange Marker. Lane 1, pre-induction (10 µL, 1/100 sample volume). Post-induction - Lane 2, 1 h (10 µL, 1/100 sample volume); Lane 3, 2 h (10 µL, 1/100 sample volume); Lane 4, 3 h (10 µL, 1/100 sample volume); Lane 5, 4 h (10 µL, 1/100 sample volume); Lane 6, 5 h (10 µL, 1/100 sample volume); Lane 7, 24 h (10 µL, 1/100 sample volume). The arrow indicates the band that represents the fusion protein at the expected size of ~110 kDa.

The Western blot analysis showed a sAPPα immunoreactive band at the same position as the over-expressed protein (Figure 3.11). This confirmed that the over-expressed protein is the GST-sAPPα fusion protein.
3.3.4 sAPPβ expression

A test expression of sAPPβ was carried out to assess the amount of fusion protein produced. The expression levels of the sAPPβ fusion protein are similar to those obtained with sAPPα (Figure 3.12).

Figure 3.12 SDS-PAGE gel of sAPPβ expression

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue investigating the amount of sAPPβ-GST produced using the parameters optimised for sAPPα (4 h induction time at 25°C). M, SDS Broadrange Marker. Lane 1, pre-induction (10 µL, 1/100 sample volume). Post-induction Lane 2, 1 h (10 µL, 1/100 sample volume); Lane 3, 2 h (10 µL, 1/100 sample volume); Lane 4, 3 h (10 µL, 1/100 sample volume); Lane 5, 4 h (10 µL, 1/100 sample volume). The arrow indicates the band that represents the fusion protein at the expected size of ~110 kDa.
3.3.5 Solubility of the fusion protein

To determine if the sAPPα/sAPPβ fusion proteins were soluble in *E.coli* or insoluble in inclusion bodies, cells were collected following induction of expression (4 h, 25°C) and the soluble and insoluble fractions were prepared by sonication and subsequent centrifugation. Insoluble and soluble proteins were fractionated by SDS-PAGE.

![SDS-PAGE gel](image)

**Figure 3.13 SDS-PAGE gel analysing the solubility of sAPPα and sAPPβ fusion proteins**

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue comparing the soluble and insoluble fractions in a post induction cell lysate. M, SDS Broadrange Marker. Lane 1, insoluble fraction of sAPPα preparation (10 µL, 1/100 sample volume). Lane 2, soluble fraction of sAPPα preparation (10 µL, 1/100 sample volume). Lane 3, insoluble fraction of sAPPβ fraction (10 µL, 1/100 sample volume). Lane 4, soluble fraction of sAPPβ preparation (10 µL, 1/100 sample volume). A solid arrow on the right of the figure indicates the fusion protein band at the expected size. Two of the marker bands sizes are annotated for comparison.

The insoluble fractions (Figure 3.13, lanes 1 and 3,) show a small number of distinct protein bands present when compared with the soluble fraction (Figure 3.13, lanes 2 and 4). Importantly, the band that represents the fusion protein is much more prominent in the soluble fractions for both sAPPα and sAPPβ. In order for the sAPPα/sAPPβ fusion proteins to bind to the Glutathione Sepharose column, they had to be soluble to allow an interaction.
3.3.6 Summary

The expression yielded a clear over-expression of the fusion protein with a tight control prior to induction. While the expression was high at all temperatures tested, subsequent fusion protein cultures were grown at 25°C. This was chosen as this temperature had been found to increase solubility and achieve correct folding of similar fusion proteins (Furukawa, Sopher, et al., 1996). The fusion protein appeared to remain soluble in the cells during the induction with only a small proportion of it going into an insoluble fraction.

3.4 Fusion protein purification

3.4.1 Optimisation of conditions for fusion protein purification using the Microspin Column

To investigate the purification parameters, the GST SpinTrap Purification Module (GE Healthcare, UK) was used as it provides a fast, easy, high-throughput method of purification. The module is designed for rapid purification of fusion proteins from small scale bacterial cultures; this made it ideal for screening multiple purification parameters simultaneously. Each microspin column is able to purify up to 400 µg of fusion protein and all of the centrifugation steps were able to be done with a table-top microcentrifuge.

3.4.1.1 Initial Microspin Column Purification

To initially analyse the purification procedure, the purification was carried out in the microspin columns manufactured by GE healthcare and followed the manufacturer’s instructions in the kit. The kit instructions suggest a 20 min binding time at room temperature and a 10 min elution time, also at room temperature. To ensure all the fusion protein came off the column, an additional elution step was added of the same duration and temperature. Figure 3.14 shows the fractions resulting from the purification.
An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue of the initial sAPPα purification stages using the microspin purification column method. M, SDS Broadrange markers. Lane 1, post-induction sample (10 µL, 1/100 sample volume). Lane 2, flow-through (10 µL, 1/100 sample volume). Lanes 3-5, washes 1-3 respectively (10 µL, 1/100 sample volume). Lane 6, first elution (6 µL, 1/100 sample volume). Lane 7, second elution (6 µL, 1/100 sample volume). The size of an appropriate band in the marker lane is indicated as a comparison to the eluted protein. The dashed arrow indicates the eluted protein running at the expected size.

In this experiment, the induction was typical as indicated by the over-expressed band in Figure 3.14, lane 1. The flow-through indicated there was a large proportion of the fusion protein that had not bound column (Figure 3.14, lane 2). The wash steps removed a small amount of bound protein but the fraction was free of protein by the third wash (Figure 3.14, lanes 3-5). The first elution step showed a small amount of fusion protein, and the second elution a lower amount (Figure 3.14, lanes 6 and 7). However, upon visual inspection of the gel, it appeared that the recovery of protein in the eluted samples was low with much less fusion protein than in the sample applied to the column. The large band present in the flow-through indicated a large amount of the fusion protein was not binding to the column under these conditions.
3.4.1.2 Increased binding and elution time

To increase the proportion of the fusion protein binding to column, the binding time was increased to 16 h (overnight) and carried out at 4°C. The fractions resulting from this purification can be seen in Figure 3.15. From the intensity of the band in lane 2 and lane 7 it can be seen that proportionally more bound. The elution time was also increased to a 16 h (overnight) elution, again at 4°C. The increased binding and elution times improved the yield of the protein as seen in Figure 3.15. The flow-through lane ran as a smear and so visualisation of the fusion protein band was not possible (Figure 3.15, lane 3). The washing steps removed a large amount of protein from the column, including some of the fusion protein itself but by the third wash the lane was protein-free (Figure 3.15, lanes 4-6). The elution fraction showed a stronger band this time, but with the increased binding and elution times it appears there was more non-specific binding as shown by an increase in the number of other protein bands present in the lane (Figure 3.15, lane 7).

![Figure 3.15 SDS-PAGE gel of purification of GST-sAPPα fusion protein with overnight binding and elution using microspin column](image)

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue showing various fractions for the purification of GST-sAPPα fusion protein involving an overnight binding step and an overnight elution step. M, SDS Broadrange marker. Lane 1, pre-induction sample (10 µL, 1/100 sample volume). Lane 2, post-induction sample (10 µL, 1/100 sample volume). Lane 3, flow-through (10 µL, 1/100 sample volume). Lanes 4-6, washes 1-3 respectively (10 µL, 1/100 sample volume). Lane 7, overnight elution sample (6 µL, 1/100 sample volume). The arrow indicates the protein of interest being eluted at the expected size.
### 3.4.1.3 Summary

Using the microspin column modules, an initial analysis of the purification parameters was able to be undertaken. The initial analysis demonstrated that an overnight binding and an overnight elution step appeared to increase the final yield of the purification but also increased contaminating proteins using visual examination of the gel. The next step was to upscale the purification to a batch method (section 2.4.2.2) in order to accommodate the large amount of protein from a large-scale induction.

### 3.4.2 Optimisation of conditions for fusion protein purification using the Batch purification method (Glutathione Sepharose 4B)

Rather than do many more conditions optimising the small scale, the yield and optimisation of the large scale was investigated. Large scale inductions produce mg of protein exceeding greatly the capacity of the Microspin Modules. Glutathione Sepharose 4B was used for batch purification (GE Healthcare, UK). The beads can accommodate 8 mg.mL$^{-1}$ and so depending on the estimated protein yield; the amount used could be scaled up or down.

#### 3.4.2.1 Batch purification of GST-sAPPα fusion protein with Glutathione Sepharose 4B using manufacturers recommendations

Initially for the batch purification a preliminary purification was carried out using the manufacturer’s conditions. This was with a 30 min binding time at room temperature and a 20 min elution time at room temperature.
3.4.2.2 Batch purification of GST-sAPPα fusion protein with Glutathione Sepharose 4B using an overnight binding step and an overnight elution step

A batch purification was then carried out using the same parameters as the final microspin column experiment (overnight binding at 4°C and overnight elution step at 4°C).
Figure 3.17 An SDS-PAGE gel of a batch purification of GST-sAPPα fusion protein with Glutathione Sepharose 4B using an overnight binding step and an overnight elution step.

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue with various fractions from a batch purification of GST-sAPPα fusion protein with Glutathione Sepharose 4B using an overnight binding step and an overnight elution step. M, SDS Broadrange Marker. Lane 1, pre-induction sample (10 µL, 1/100 sample volume). Lane 2, post-induction sample (10 µL, 1/100 sample volume); Lane 3, flow-through (10 µL, 1/100 sample volume); Lane 4, wash 1 (10 µL, 1/100 sample volume); Lane 5, wash 3 (10 µL, 1/100 sample volume); Lane 6, 30 min room temperature elution (10 µL, 1/100 sample volume); Lane 7, overnight elution (10 µL, 1/100 sample volume); Lane 8, samples post-dialysis into 0.1x PBS (10 µL, 1/100 sample volume). The arrows indicate the eluted protein of interest at the expected size.

Upon visual inspection of the gel, Figure 3.17 shows that a large proportion of the fusion protein is purified with the overnight elution step. Elution for 30 min was included as a comparison in lane 6, by contrast a very small proportion of the total fusion protein eluted. Given the apparent high yield of fusion protein in this experiment, these conditions were used for all large scale fusion protein purification preparations.

3.4.2.3 Can the unbound fusion protein bind to glutathione sepharose 4B when reapplied

Given that there is still a large amount of unbound fusion protein present in the flow-through it was re-applied to the Glutathione Sepharose 4B to see whether more could be recovered or whether it was a ‘different’ fraction that could be bound due to perhaps mis-folding or aggregation. Figure 3.18 shows the flow-throughs from four sequential overnight binding steps of the same samples containing purified fusion protein.
As shown from lanes 2-4 and 6-8. Further protein could be rebound but in decreasing proportional amounts in each application.

![SDS-PAGE gel showing re-binding analysis of GST-sAPPα and GST-sAPPβ fusion proteins](image)

An SDS-PAGE gel (10% acrylamide) stained with Coomassie blue showing the analysis of re-binding both sAPPα and sAPPβ fusion proteins. Purified fusion protein was incubated with Glutathione Sepharose 4B, the flow-through collected and the flow-through was then incubated with fresh resin and this was repeated for 3 overnight binding steps M, SDS Broadrange Marker. Lane 1, the purified GST-sAPPα sample prior to incubation (10 µL, 1/100 sample volume); Lanes 2-4, the flow-through from successive overnight binding for GST-sAPPα (10 µL, 1/100 sample volume); Lane 5, the purified GST-sAPPβ sample prior to incubation (10 µL, 1/100 sample volume); Lanes 6-8, the flow-through from successive overnight binding for GST-sAPPβ (10 µL, 1/100 sample volume). The arrow indicates the protein of interest at the expected mass.

Before and between each successive overnight binding, the concentration of protein was determined in each sample using the QuBit® quantification method (section 2.3.5.3).
The determination of the proportion of total fusion protein present in each fraction (Figure 3.19) indicates that two additional overnight binding steps increases the amount of bound protein but in decreasing proportions.

### 3.4.2.4 Final purification profile

The final large scale purification was carried out according to the parameters previously investigated (overnight binding at 4°C and overnight elution at 4°C). Following dialysis the protein samples were quantified and the final sample stored in aliquots at -80°C. Samples were typically quantified with both the QuBit® Quantification platform (section 2.3.5.3) and the using the DC protein assay (section 2.3.5.1). For the DC protein assay, a standard curve (ranging from 0.5 mg.mL$^{-1}$ – 2 mg.mL$^{-1}$) was made from known concentrations of bovine serum albumin (BSA) against which to compare the samples. The final yields from these purifications (from 500 mL cultures) gave concentrations ranging from 1 to 5 mg.mL$^{-1}$ of fusion protein.
3.5 Functional validation of sAPPα-GST fusion protein

The system developed here utilises a prokaryotic expression system with a different capacity to post-translationally modify proteins than a eukaryotic cell. It is known that APP is heavily glycosylated in vivo (Weidemann et al., 1989). To determine if a lack of post-translational modifications and the presence of the GST carrier at the N-terminus of the protein affected the function of the protein, GST-sAPPα was tested in an assay measuring neuroprotection. It has been shown by the Tate group that sAPPα produced in cultured eukaryotic cells can protect a neuronal cell line (SHSY-5Y neuroblastoma cells) in culture against toxic shock from hypoglycaemic insult (Turner et al., 2007). The MTT assay (Section 2.2.5) was used to compare the ability of sAPPα expressed in the prokaryotic system developed in this study with that of the sAPPα produced in stably transfected human embryonic kidney cells in culture. Figure 3.21 shows that the sAPPα produced in the prokaryotic system developed in the current study rescued cells to the same degree as the sAPPα produced in cells where correct post-translational modification is possible (Turner et al., 2007). Glucose deprivation reduced the antioxidant capacity, measured by formation of formazan crystals, by approximately 70%, typical of an oxidative status after such an insult. However, prior or simultaneous additions of either 10 nM GST-sAPPα (prokaryotic) or 10 nM sAPPα (eukaryotic) both protected against the decrease in formazan production, restricting it to approximately 30%. Moreover, the extra sequence of GST at the N-terminus of sAPPα did not affect this function. The fact that the GST-sAPPα fusion protein was able to rescue the cells to the same extent as the sAPPα produced in culture culture suggests that it is in the conformation necessary to bind the receptor.
SHSY-5Y neuroblastoma cells were deprived of glucose for a total of 6 h with 10 nM sAPPα being added for the duration of glucose deprivation. Positive control cells (+ glucose) did not have glucose withdrawn and received vehicle during the deprivation period. Negative control cells (- glucose) deprived of glucose also received vehicle instead of sAPPα. Both sAPPα and GST-sAPPα were added to a final concentration of 10 nM in 0.1x PBS as vehicle. Each condition was performed in quadruplicate with the means displayed and error bars representing standard deviation. * = P<0.05 as measured by Students two-tailed t-test.

3.6 Investigation of binding partners using the synaptoneurosome preparation

3.6.1 Synaptoneurosome preparation

Synaptoneurosome preparations were carried out as per Section 2.5.2 and the protein concentration in the final synaptoneurosome enriched sample was determined by the Bradford Assay (Section 2.3.5.2) and samples were typically ~1 mg/mL and stored in HEPES buffer.

3.6.2 Do the candidate receptors CRMP-2 and APP bind sAPPα?

APP (Gralle et al., 2009) and CRMP-2 (Mileusnic & Rose, 2011) have both been claimed as receptors for sAPPα. To determine if these candidate receptors can be detected in the current system, the lysate obtained from Section 2.5.2.2 was incubated with the
GST-sAPPα fusion protein. The protein sample and the GST-sAPPα fusion protein were combined and placed at 4°C to allow binding. Following wash steps, proteins that bound were removed from the column by the addition of 50 µL of cracking buffer (a solution containing high concentrations of SDS and urea to denature proteins). This was then run on an SDS-PAGE gel and probed via Western blot for the candidate receptors CRMP-2 and APP (refer to Appendix 1 for antibody details).

![Figure 3.22 Western blot probing for APP in synaptoneurosome fractions](image)

A Western blot was used to detect APP in the synaptoneurosome soluble protein preparation and also detecting its presence in the eluted GST-sAPPα protein fraction. The gel represents two identical experiments (referred to as experiment 1 and experiment 2 in legend) from the same synaptoneurosome preparation. Lane M, SDS Broadrange Marker. Lane 1, flow-through from experiment one (proteins that did not bind to GST-sAPPα column). Lane 2, eluted proteins from experiment one (proteins that did bind the GST-sAPPα column). Lane 3, flow-through from experiment 2. Lane 4, eluted proteins from experiment 2. 10 µL of the flow-through and 50 µL of the eluted protein fraction was loaded onto the gel and ran for 1 h at 200 V. Following transfer to nitrocellulose membrane the membrane was blocked for 3 h at room temperature and incubated overnight with the primary anti-C-terminal APP antibody (1/1000). The following day the membrane was washed and incubated with the secondary anti-goat HRP (1/5000) for 2 h at room temperature. The blot was developed with ECL detection and was exposed for 90 s. Solid arrow indicates the expected size of the APPΔC protein (~110 kDa).
The solid arrow in figure 3.22 indicates the expected size of the APP protein (~110 kDa). The antibody used here detects a C-terminal antigenic site that is not present in sAPPα. There is an APP band in the flow-through fraction of both experiments (Figure 3.22, lanes 1 and 3) but no signal from the eluted protein fractions with GST-sAPPα (Figure 3.22, lanes 2 and 4). The weak APP signal may have precluded detection of bound APP if the amount were relatively low. Additional faint bands seen in the flow-through lanes may represent cleavage products, non-specific binding or it may in fact be reacting with different isoforms of the APP protein. This indicates that in these conditions, full-length APP does not significantly bind the sAPPα fusion protein.

The same samples were probed for the other candidate protein, CRMP-2. The solid arrow in Figure 3.23 indicates the expected size of the CRMP-2 protein. Indeed, there is a clear single band in both the flow-through fractions from each experiment as with APP and no visible band present in the eluted proteins fraction. The state of the proteins on extraction from the synaptoneurosomes may have affected their ability to bind to sAPPα compared with the native protein.
Figure 3.23 Western blot probing for presence of CRMP-2 in synaptoneurosome fractions

A Western blot probing for the presence of CRMP-2 in the synaptoneurosome preparation and also detecting its presence in the eluted GST-sAPPα protein fraction. The gel represents 2 identical experiments from the same synaptoneurosome preparation. Lane M, SDS Broadrange Marker. Lane 1, flow-through from experiment one. Lane 2, eluted proteins from experiment one. Lane 3, flow-through from experiment 2. Lane 4, proteins bound to GST-sAPPα. Flow-through (10 µL) and the eluted protein fraction (50 µL) were loaded onto the gel and ran for 1 h at 200V. Following transfer to nitrocellulose membrane the membrane was blocked for 3 h at room temperature and incubated overnight with the primary anti-C-terminal CRMP-2 antibody (1/5000). The following day the membrane was washed and incubated with the secondary anti-goat HRP (1/5000) for 2 h at room temperature. The blow was developed with ECL detection and was exposed for 210 s. Solid arrow indicates the expected size of the CRMP-2 protein (~63kDa).

The Western blot analysis of the candidate receptor proteins confirmed that they are present in the preparation as indicated by the bands seen on the flow-through lanes on both blots, but the binding of these candidate receptors to the GST-sAPPα fusion protein could not be confirmed as seen by the absence of any bands located in the eluted protein fractions.
3.6.3 Investigation of binding partners in synaptoneurosomes

In an unbiased approach to identify candidate binding partners of sAPPα that might be present in the synaptoneurosomal preparation, protein bands that eluted with GST-sAPPα were cut out of the gel and analysed via mass spectrometry to identify the interacting proteins. Cell lysate was first pre-cleared of proteins that might interact with Glutathione Sepharose 4B itself independent of GST-sAPPα by pre-incubation with Glutathione Sepharose 4B alone for 4 h at room temperature

![SDS-PAGE gel analysis](image)

**Figure 3.24 SDS-PAGE gel analysis of protein fractions from binding assay with synaptoneurosomal preparation**

Cell lysate and GST-sAPPα were incubated overnight at 4°C and then incubated with a microspin column for 6 h at room temperature and washed 3 times with low-salt lysis buffer. Bound proteins were eluted in 50 µL of cracking buffer and all 50 µL loaded onto the 1.5 mm thick gel (10% acrylamide), fractionated, and stained with Coomassie stain. Lane M, SDS Broadrange marker. Lane 1, flow-through after binding. Lane 2, wash 1. Lane 3, wash 3. Lane 4, proteins that were found in the GST-sAPPα eluted fraction. The three bands labelled as A, B and C were cut out of the gel and sent for MS/MS analysis. Protein band A was identified via MS/MS mass spectrometry to contain ‘Amyloid beta A4 protein’ and also glutathione s-transferase. Protein band B was identified as actin. Protein cluster C was identified as glutathione s-transferase.
The proteins identified in band A were identified by MS/MS as containing peptides that corresponded to both APP and GST suggesting that it is the fusion protein itself. Supporting this is the fact that the apparent molecular weight of the band corresponds to the expected molecular weight of the fusion protein, as seen in previous gels. The protein identified in band B was identified as actin which is a frequent contaminant of protein preparations and was also found to bind the RERMS region of sAPPα (Pawlik et al., 2007). The group of proteins annotated in C were identified as GST but not from Schistosoma japonicum, the species of the GST protein present in the GST vector, but instead from Rattus norvegicus indicating the identified proteins were in fact the endogenous GST present in the sample obtained from the rat hippocampus that were binding the glutathione tag on the glutathione Sepharose 4B beads.

This experiment was repeated three times with similar groups of proteins being identified in each experiment and similar numbers of proteins being present in the elution lane. Given the low number of binding proteins that were pulled down and the failure of the Western blot analysis to confirm the candidate interacting proteins, another type of protein preparation was investigated for binding partners.

3.7 Investigation of binding partners using SHSY-5Y cell lysate

Given the fact that sAPPα is able to protect neuroblastoma SHSY-5Y cells from hypoglycaemic insult it is likely receptors of sAPPα are mediating the effect. In this light, a Triton X-100 extraction of the cell lysate of SHSY-5Y neuroblastoma cells was investigated for the presence of binding proteins.

3.7.1 Analysis of detergent treated cell lysate preparation

To determine the proportion of total protein that is present in each fraction (soluble and insoluble) obtained from the cell lysate preparation (outlined in section 2.5.1), the cell lysate was analysed by SDS-PAGE. To make the loading in each lane proportional to each other, from one preparation the insoluble pellet was re-suspended in 250 µL of cracking buffer to make the total volume of each fraction equal. To make sure a clear profile of each fraction was visible, two concentrations of the samples differing 10-fold in concentration were loaded, 10 µL and 1 µL of each fraction was then loaded onto an SDS-PAGE gel, fractionated, and analysed.
Figure 3.25 SDS-PAGE comparison of insoluble and soluble fractions from SHSY-5Y cell lysate preparation

SDS-PAGE gel (10% acrylamide) stained with Coomassie blue comparing the soluble fraction and the insoluble fraction resulting from the cell lysate preparation outlined in section 2.5.1. M, SDS Broadrange Marker. Lane 1, 10 µL of the soluble fraction. Lane 2, 10 µL of the insoluble fraction. Lane 3, 1 µL of the soluble fraction. Lane 4, 1 µL of the insoluble fraction.

As can be seen from the gel profile, Figure 3.25 shows that the soluble fraction (Figure 3.25, lanes 1 and 3) contains the larger fraction of protein apart from several prominent bands of lower molecular weight in the insoluble fraction (seen in lanes 2 and 4).

3.7.2 Identifying which fraction the receptor is located

A previous study has suggested that the receptor is found in detergent insoluble domains (Tikkanen et al., 2002). The location of the potential sAPPα receptor in the
detergent extract cell lysate was investigated to ensure this preparation was suitable for binding partner isolation experiments.

3.7.2.1 Investigation of the presence of CRMP-2 in SHSY-5Y cells

As mentioned in section 1.6.4, CRMP-2 is claimed as a promising candidate for a receptor for sAPPα. Western blot analysis detected CRMP-2 in the cell lysate. Figure 3.26 shows that CRMP-2 is indeed present in SHSY-5Y cells and in the soluble fraction when extracted with 1% Triton X-100. The solid arrow indicates the most prominent band is at the expected relative mass of ~63 kDa. The dashed arrow shows what is inferred to be non-specific binding to a band present in the insoluble pellet fraction that was extremely dense on the gel.
Figure 3.26 Western blot probing for CRMP-2 in SHSY-5Y neuroblastoma cell lysate

Samples (10 µL) of both the solubilised fraction and the insoluble pellet from the cell lysate preparation were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked and incubated overnight at 4°C with the primary anti-CRMP-2 antibody (1/1,000), washed and incubated for 2 h at room temperature with the secondary anti-rabbit antibody (1/20,000) and developed with ECL (30 s exposure). M, SDS Broadrange Marker. S, solubilised fraction. I, insoluble pellet.

3.7.2.2 Investigation of the presence of APP in SHSY-5Y cells

As with CRMP-2, APP has been claimed to be a potential receptor for sAPPα and so the presence of APP in the cell lysate fraction to be used in the binding experiment needed to be confirmed. Figure 3.27 shows the western blot probing for APP. Most of the antibody reactivity is present in the soluble fraction. While multiple bands are detected, one of the strongest on the gel corresponds to the expected size for APP_{695} of ~110kDa (Figure 3.27, arrow).
Figure 3.27 Western blot probing for APP in the SHSY-5Y neuroblastoma cell lysate

Samples (10 µL) of both the detergent solubilised fraction and the insoluble pellet from the cell lysate preparation were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked and incubated overnight at 4°C with the primary anti-APP antibody (1/1,000), washed and incubated for 2 h at room temperature with the secondary anti-rabbit antibody (1/20,000) and developed with ECL (30 s exposure). M, SDS Broade Range Marker. S, solubilised fraction. I, insoluble pellet. The solid arrow indicates the most prominent band which is at the expected position of ~110 kDa.

In figure 3.27, the detergent soluble protein lane on the Western blot shows several clear bands, one of which corresponds to the molecular weight of APP$_{695}$. The other bands seen could represent the different sized isoforms of APP present in neurons or may in fact be cleavage products, non-specific binding or a dimer. No bands of the expected size were detected in the insoluble lane. The APP reactivity confirms that APP is present in the cell line and also that it is present in the soluble fraction making it an appropriate preparation for binding analysis.
3.7.2.3 Investigating whether the sAPPα-receptor complex resides in the soluble or insoluble fraction

To ascertain which fraction (soluble or insoluble) the GST-sAPPα-receptor complex is found in, GST-sAPPα was added to ~90% confluent SHSY-5Y neuroblastoma cells to a final concentration of 300 nM and was incubated for 30 min at 37°C to allow the GST-sAPPα fusion protein to bind to putative receptor(s). Following this, a standard cell lysate preparation with detergent was performed (section 2.5.1) and each fraction was analysed via Western blot to locate GST-sAPPα. The steps involved in the cell lysate preparation included removal of the media and wash steps which should remove unbound sAPPα. The Western blot used anti-GST to detect the ligand (the antibody usually used to detect GST-sAPPα was not used to avoid confusion with the endogenous APP). GST-sAPPα was almost exclusively present in the soluble fraction as indicated by the solid arrow at around 100-110 kDa (Figure 3.28). The dashed arrows indicate either non-specific binding or degradation products. The dashed arrows also align with prominent bands in the cell lysate (figure 3.25) so is suggestive of non-specific binding. There may be some non-specific binding to the extremely prominent bands present at the bottom of the insoluble lane which can also be seen in the SDS-PAGE gel of the cell lysate preparation in figure 3.25.
Figure 3.28 A Western blot identifying the location of the sAPPα receptor(s) and/or binding partners from SHSY-5Y cell lysate

Insoluble fraction (10 µL) and soluble fraction (10 µL) from an SHSY-5Y cell lysate preparation (Section 2.5.1) were run on a 12.5% SDS-PAGE gel and transferred to nitrocellulose membrane and blocked overnight. The next day the membrane was rinsed and incubated with primary anti-GST antibody (1/10,000) for 1.5 h at room temperature. The membrane was then washed three times and incubated with the secondary anti-goat antibody (1/10,000) for 1.5 h at room temperature. Following three more wash steps the blot was developed using ECL and exposed for 30 s on standard sensitivity. M, SDS Broadrange Marker. I/S, insoluble fraction. S, soluble fraction. The solid arrow indicates the fusion protein at the expected height. The dashed arrows indicate possible non-specific binding or degradation products.

3.7.3 Investigation of binding partners in SHSY-5Y cell lysate

To determine which proteins from the SHSY-5Y cell lysate bound to GST-sAPPα, the lysate was combined with GST-sAPPα overnight at 4°C. Following three wash steps the proteins that remained on the column with GST-sAPPα were removed with the addition of 50 µL of cracking buffer. Various fractions were then fractionated on an SDS-PAGE gel for analysis (Figure 3.29).
Detergent extracted cell lysate was pre-cleared of proteins that might interact with glutathione Sepharose by pre-incubation with glutathione Sepharose 4B alone for 4 h at room temperature. Cleared cell lysate and GST-sAPPα were incubated overnight at 4°C and then incubated in a microspin column for 6 h at room temperature and washed 3 times with low-salt lysis buffer. Bound proteins were eluted in 50 µL of cracking buffer and all 50 µL loaded onto the 1.5 mm thick gel (10% acrylamide) and stained with Coomassie blue. M, SDS Broadrange Marker. Lane 1, cell lysate prior to pre-clearing. Lane 2, flow-through. Lanes 3-5, washes 1-3 respectively. The lane between lanes 5 and 6 was left empty to prevent any spill over into lane 6 flowing into the wash lane. Lane 6, fraction with proteins found to bind GST-sAPPα. Solid arrow indicates eluted sAPPα. The dashed arrow indicates endogenous GST.

There were a number of proteins present in lane 6 and so individual bands were not cut out for MS/MS analysis. Instead, direct injection LTQ-Orbitrap MS/MS was used. This method of mass spectrometry has the ability to analyse all of the proteins in an entire gel lane. The results of this are found in section 3.7.4.

To provide more certainty that the isolated proteins represent real interactions with GST-sAPPα and not non-specific binding, the experiment was repeated, with two important controls, sAPPβ and GST alone. sAPPβ differs from sAPPα only in the C-terminal 16 amino acids and yet is 100-fold less effective as a neuroprotective agent (Turner et al., 2007). Both samples eluted with sAPPα and sAPPβ from the gel were submitted for LTQ-Orbitrap MS/MS analysis.
Figure 3.30 SDS-PAGE gel comparing proteins from an SHSY-5Y cell lysate preparation that bound to GST-sAPPα, GST-sAPPβ and GST alone

SDS-PAGE gel (10% acrylamide) stained with Coomassie blue analysing the proteins from an SHSY-5Y cell lysate preparation that bind to sAPPα, sAPPβ and GST alone. M, SDS Broadrange Marker. FT, the flow-through from the binding experiment (60 µL, 1/10 sample volume). E, the eluted proteins interacting with GST-sAPPα, GST-sAPPβ or GST alone (50 µL, entire sample). The solid arrows indicate the likely positions of the bait proteins themselves eluted from the column. The dashed arrow is an example of a protein clearly bound in one fraction and not the others indicating binding specificity. The flow-through lanes are saturated due to the contrast being optimised for the exposure of the bands in the eluted protein lanes.

The comparison between the proteins that bound to sAPPα, sAPPβ and GST (Figure 3.30) is interesting. Firstly, while the amount of the bait protein (GST-sAPPα, GST-sAPPβ or GST alone) that was combined with the cell lysate samples was in the same range between the three samples (4 nmol sAPPα, 11 nmol sAPPβ, and 20 nmol GST, so as to load as much fusion protein as possible given the stock concentrations), the amount of GST in the eluted fraction was much larger. The most interesting observation is indicated by the dashed arrow in Figure 3.30 which shows a band that is clearly present in the sAPPα fraction but is absent in both the sAPPβ and GST fractions.
3.7.4 LTQ-Orbitrap MS/MS analysis of interacting proteins

To identify the proteins that bound the GST-sAPPα and GST-sAPPβ fusion proteins and GST alone, the samples were fractionated by SDS-PAGE and the proteins of interest were identified via tandem mass spectrometry. A shift to the LTQ-Orbitrap was made as this technology is more suited to analyse an entire lane of an SDS-PAGE gel. This involved running the SDS-PAGE gel as per Section 2.3.1 and once the gel had been stained it was placed in water and the gel was analysed at the Centre for Protein Research. The staff carried out the steps outlined in Section 2.6 and the results were e-mailed in the form an Excel spreadsheet. The spreadsheet contained a list of the proteins that were identified as putative partners, the fragments that identified them and information about the quality of the data and accession numbers. Table 2 contains the raw data obtained from the three LTQ-Orbitrap MS/MS runs.

Table 1 shows the proteins that were found to be binding partners in multiple experiments and that were not found to bind GST alone. The full list of proteins identified can be found in Table 2. Three experimental analyses were performed to identify proteins that bound sAPPα, two analyses to identify proteins that bound to sAPPβ as a comparison and one to identify the proteins that bound to GST alone. The GST alone experiment identified the proteins that bound to the GST moiety of the fusion protein and so were not considered to bind sAPPα and/or sAPPβ uniquely. Three proteins were identified in all three experiments with GST-sAPPα. The first protein APP is expected as the fusion protein itself contains a portion of full length APP. To confirm that the sequences were in fact from the fusion protein and not full length APP, the fragments that lead to the identification of APP were run in a BLAST search (http://blast.ncbi.nlm.nih.gov) and aligned against the sequence for full length APP. The fragments were mapped to the full length APP in order to see if any of the fragments aligned to an area of APP that is not present in sAPPα. This would be considered confirmation that full length APP bound to sAPPα. Upon investigation, it was found that all fragments from the MS run aligned with areas within sAPPα and so this confirmed the identification of the molecule (amyloid beta A4 protein isoform c precursor) in the MS was due to the fusion protein and not full length APP binding. This was also true for the amyloid beta A4 protein isoform c precursor identified in the sAPPβ experiments.
Table 1. List of proteins identified in multiple experiments

<table>
<thead>
<tr>
<th>sAPPα</th>
<th>sAPPβ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteins identified in all three experiments</strong></td>
<td><strong>Proteins identified in both experiments</strong></td>
</tr>
<tr>
<td>amyloid beta A4 protein isoform c precursor [Homo sapiens]</td>
<td>amyloid beta A4 protein isoform c precursor [Homo sapiens]</td>
</tr>
<tr>
<td>albumin preproprotein [Homo sapiens]</td>
<td>tubulin alpha-1A chain [Homo sapiens]</td>
</tr>
<tr>
<td>high mobility group protein B1 [Homo sapiens]</td>
<td>acidic leucine-rich nuclear phosphoprotein 32 family member B [Homo sapiens]</td>
</tr>
<tr>
<td></td>
<td>glutathione S-transferase Mu 3 [Homo sapiens]</td>
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<td><strong>Proteins identified in two experiments</strong></td>
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</tr>
<tr>
<td>keratin, type I cytoskeletal 16 [Homo sapiens]</td>
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</tr>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>protein SET isoform 2 [Homo sapiens]</td>
<td></td>
</tr>
<tr>
<td>tubulin alpha-1A chain [Homo sapiens]</td>
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</tr>
<tr>
<td>prohibitin [Homo sapiens]</td>
<td></td>
</tr>
<tr>
<td>acidic leucine-rich nuclear phosphoprotein 32 family member B [Homo sapiens]</td>
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Albumin preproprotein was found in all three of the sAPPα experiments and was found in one of the sAPPβ experiments but was not found in the GST binding experiment indicating that it uniquely binds to sAPPα and sAPPβ. High mobility group protein B1 (HMGB1) was found to bind sAPPα in all three experiments also. HMGB1 was also found with one of the sAPPβ experiments but not in the GST experiment.

From Table 1 it can be seen that several more proteins were found in two of the three sAPPα experiments. Several structural proteins were found, three isoforms of keratin and an isoform of tubulin. Several other proteins of varying functions (these are discussed in detail in Section 4.7) were isolated: hornerin, protein SET, prohibitin, and acidic leucine-rich nuclear phosphoprotein 32 family member B. Acidic leucine-rich nuclear phosphoprotein 32 family member B and tubulin alpha 1A were both also found to bind sAPPβ in both experiments. The sAPPβ experiments did not identify any proteins that bound to sAPPβ uniquely in more than one experiment. Table 2 shows a full list of the proteins identified in all the experiments.
Table 2. All proteins identified interacting with fusion proteins

<table>
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<tr>
<th>sAPPα</th>
<th>sAPPβ</th>
</tr>
</thead>
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<td><strong>Proteins identified in all three experiments</strong></td>
</tr>
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<td>amyloid beta A4 protein isoform c precursor [Homo sapiens]</td>
</tr>
<tr>
<td>albumin preproprotein [Homo sapiens]</td>
<td>tubulin alpha-1A chain [Homo sapiens]</td>
</tr>
<tr>
<td>high mobility group protein B1 [Homo sapiens]</td>
<td>glutathione S-transferase Mu 3 [Homo sapiens]</td>
</tr>
</tbody>
</table>

<table>
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<th><strong>Proteins identified in two experiments</strong></th>
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<td>tubulin alpha-1A chain [Homo sapiens]</td>
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Chapter 4 – Discussion

4.1 Context of Study

The current study aimed to generate a portfolio of proteins that bind to sAPPα and among which there could be a receptor that uses sAPPα as a ligand. To achieve this, GST-sAPP fusion proteins were expressed and purified in E.coli as ‘bait’ for the interacting proteins. Then using a protein pull-down strategy the fusion protein and interacting protein complexes were immobilized on a glutathione column before the bound proteins were eluted off the column and analysed via tandem mass spectrometry. The analysis revealed traces of many putative interacting proteins. From these, proteins that were found to interact with GST alone were removed from the list as they were assumed to be binding to the GST moiety or non-specifically, not to the sAPP protein itself. To reduce non-specific binding, the protein mixtures had been passed through column resin without glutathione to remove proteins that interacted with this medium, before being applied to the glutathione resin.

A large amount (2-8 mg) of fusion protein was produced from a 500 ml culture and the procedure has the potential to be scaled up. This GST-sAPPα fusion protein was shown to be as effective as eukaryotic sAPPα in a neuroprotective assay measuring protection from hypoglycaemic stress. This implied that the fusion protein expressed in E.coli was in a conformation similar to sAPPα derived from cultured cells making it an ideal bait protein.

4.2 Creation of sAPP vectors

For an easy one step purification procedure the GST gene fusion system (GE Healthcare, UK) was used. The pGEX-6P-3 vector was chosen as it contains an engineered cleavage site to remove the GST N-terminal extension from the fusion protein if this were necessary. The human APP sequence was amplified using PCR and a stop codon was engineered at the α-secretase cleavage site in order to truncate the full length APP protein in the correct place. Following digestion with restriction enzymes and ligation the recombinant plasmids from individual clones were sequenced to ensure the absence of mutations. Section 2.1 shows the relevant agarose gel analyses and
demonstrated that the sequence of some clones was mutation-free and that the introduction of the new stop codon introduction had been successful. Vectors containing the appropriate protein sequences (either sAPPα or sAPPβ) were then amplified, isolated, and stored for future use.

4.3 Production of sAPP fusion proteins

Given that the fusion protein production system in the current study was also being designed for large scale production for future studies a subtheme was to maximise the yield of the system. The fusion protein vectors created were transformed into BL21(DE3)pLysS strain of E.coli to minimise degradation of the fusion protein as this strain is deficient in the proteases encoded by lon and ompT.

The initial expression of the fusion protein showed a large protein band at the expected molecular weight (Figure 3.9), and the expression parameters were investigated in order to maximise the yield of the system. The temperature used for the expression of the fusion proteins was set at 25°C as this was found to give a large fusion protein band. The yield was similar between 37°C and 25°C, but the lower temperature is recommended to maintain correct folding of fusion proteins in the GST Gene Fusion System Booklet. Optimal expression time for the protein was found to be 4 h and at longer times there was a decrease in the intensity of the band indicating the fusion protein was being degraded (Figure 3.10). To confirm that the band on the gel was in fact the protein of interest, hourly samples from an induction was probed with an antibody raised against an epitope in the N-terminal region of APP. Indeed, the band of interest reacted positively with the anti-N-terminal APP antibody (Figure 3.11) confirming the identity of the fusion protein. Since all of the optimisation experiments were carried out using the GST-sAPPα fusion protein, an analysis was carried out to ensure the GST-sAPPβ fusion protein would give a similar yield using the conditions used for the GST-sAPPα protocol. A similar, if not higher yield using the same conditions was achieved (Figure 3.12). This showed that one protocol could be used for both fusion proteins.

The next step was to investigate whether the fusion proteins were soluble following sonication of the bacterial cells. After sonication and centrifugation of the resulting lysate, the soluble and insoluble fractions were separated and proportional samples each run on an SDS-PAGE gel. As expected from the solubilising properties of GST, most of the fusion protein was soluble with a small portion being present in the
insoluble fraction (Figure 3.13). The gel shows that the proportions are similar for both GST-sAPPα and GST-sAPPβ fusion proteins. The high solubility of the protein made the purification much simpler and no further steps were needed to be carried out to optimise the solubility.

4.4 Purification of sAPP fusion proteins

The GST-purification microspin kits (Section 2.4.2.1) were ideal for quick, simple analysis of purification parameters and so optimisation was carried out using these. Initially the suggested parameters from the kit manufacturer were used but there was a relatively small amount of the fusion protein in the eluted fractions when compared to the amount loaded from the cell lysate sample (Figure 3.14). The temperature and time of the interactions of a GST-protein with the glutathione resin and its elution can have a large influence on the yield. If a low final yield were seen, the kit suggested a longer incubation time at a lower temperature was most likely required. Figure 3.15 shows that with these conditions the yield improved, and the eluted fraction that was obtained contained a higher proportion of the band seen in the post-induced sample.

It can be seen from Figure 3.15 that an overnight elution step combined with an overnight binding step led to a significantly increased amount of the fusion protein band. Given the increase in yield achieved in these pilot experiments, the parameters were then used to assess the yield of the batch purification system that would be used for the large scale purification procedures. The suggested 30 min binding step and 10 min elution step both performed at room temperature were included for comparison. Figure 3.17 shows the batch purification, an elution step carried out for 8 h (Lane 7), and a further elution step carried out overnight (Lane 8). This was done to assess the amount of protein still left on the column after the initial elution. Lane 7 shows a large amount of fusion protein but there was also a significant amount derived from the second overnight elution step. The yield was determined by the QuBit® Quantitation Platform to be ~5 mg.mL⁻¹, and the parameters established were deemed to be satisfactory.

Nevertheless, in all binding experiments, there was a significant proportion of the fusion protein not binding to the column. This was determined by visually comparing the flow-through lanes to the post-induction lanes on the stained gel. To investigate whether sAPPs in the flow-through fraction would bind to the column on a second reiteration and increase the yield of pure protein or if there were a sub-population of sAPP protein that
could not bind the column or, alternatively perhaps due to mis-folding, an experiment was carried out to test these possibilities. This was done by starting with a known amount of purified fusion protein sample, applying it to the column overnight, and quantitating the flow-through to see how much of the fusion protein bound to the column. This process was repeated several times. The results are seen in Figure 3.18 and Figure 3.19, with the gel analysis and the quantitation of sAPP in each flow-through (expressed as a % of the fusion protein bound). From this experiment it could be concluded that there was a sub-population of sAPP that would not bind the column and it appeared to be in equilibrium with the proportion that would bind. A second binding reiteration of the first flow-through did increase the yield but the increase in the amount of sAPP protein obtained by adding an additional overnight binding step was not time-effective and so the protocol used in Section 2.4.2.2 was used for all further purifications.

4.5 Validation of functional activity of fusion proteins

Under physiological conditions sAPPα is produced in the mammalian brain and post-translationally modified. The sAPPα produced in the current study is produced in a prokaryotic expression system that lacks the capability to modify proteins post-translationally in the same way and so the function of the sAPPα fusion protein needed to be investigated. To do this, a cell based test system for modelling protection from toxic insults by sAPPα was used.

The MTT assay can be used to measure the oxidative status of the cell and is based on the ability of mitochondrial enzymes to form a coloured product. This only happens when the cell and mitochondria are healthy and intact. Briefly, the plated cells receive the insult, in this case removal of glucose. Following an insult period, the MTT compound is added and after a 2 h incubation the colour of the product is measured. In the assay used in the current study a concentration of sAPPα was used that had been seen to have an effect in previous studies (Furukawa, Sopher, et al., 1996; Turner et al., 2007). The glucose deprivation caused an approximately 75% reduction in the production of the coloured product, reflecting the stress on the cells. This was reduced to only a 30% reduction with the addition of eukaryotic cell-produced sAPPα (10 nM) and this effect was mimicked with the addition of 10 nM of the sAPPα fusion protein expressed in a prokaryotic expression system (Figure 3.21). This replicates an earlier report that
prokaryotic produced sAPPα can elicit similar physiological effects (Furukawa, Sopher, et al., 1996).

These findings confirm that the sAPPα produced in the current system is able to mimic sAPPα produced in a eukaryotic cell culture model as a ligand for a cascade of events leading to a global outcome for the cell. This suggests that the post-translational modifications are not necessary for the neuroprotective functions of sAPPα, and that the GST-sAPPα fusion protein has maintained a conformation that is able to bind the necessary cellular proteins to elicit neuroprotection, making it a suitable bait protein for identification of binding partners.

4.6 Investigation of suitable pools of prey protein

Prior to further binding assays to identify sAPPα binding proteins, two candidate prey protein pools were considered utilising a Triton X-100 extraction. The synaptoneurosome preparation was chosen with the aim of identifying a cell surface receptor because of its physiological relevance. It was isolated from rat cortex where sAPPα has been shown to be functioning in vivo (Taylor et al., 2008). The SHSY-5Y cell lysate preparation was also considered as a human neuronal cell line and because relatively large amounts of proteins can be obtained. It was also noted that given the ability of sAPPα to rescue this cell line from glucose deprivation, the cellular machinery necessary for sAPPα to produce these effects must be present.

4.6.1 Investigation of synaptoneurosome preparation

To assess the synaptoneurosome preparation, two approaches were taken. The first involved carrying out a binding assay followed by a Western blot of the resulting fraction binding to sAPPα to probe for candidate receptors. The second approach involved analysing the unspecified bands on the gel via tandem mass spectrometry.

4.6.1.1 Does sAPPα bind full length APP?

To investigate whether full-length APP bound the sAPPα-fusion protein in the current study, the fraction that was found to bind sAPPα bait on the column was analysed via Western blot using an anti-APP antibody. To ensure that the antibody differentiated the full-length APP from the sAPPα fusion protein itself, an antibody was used that had been raised against an epitope in the C-terminal region APP not present in sAPPα. Figure 3.22 shows that sAPPα does not bind full-length APP efficiently under these conditions.
This is shown by the presence of full-length APP in the flow-through fraction, which represents the proteins that do not interact with sAPPα. No immunopositive bands were detected in the bound fraction. Gralle and colleagues have suggested that sAPPα elicits its actions by disrupting APP dimers and thereby acting as a ligand for APP (Gralle et al., 2009). Possibly the methodology in the current study does not favour dimer formation and so did not provide the opportunity for GST-sAPPα to interact.

4.6.1.2 Does sAPPα bind CRMP-2?

Several studies have found that CRMP-2 interacts with the RERMS motif present in the E2 domain of sAPPα that has been shown to be important for many of its functions (Pawlik et al., 2007; Mileusnic & Rose 2011). To determine if full-length sAPPα binds this protein, the fractions were probed with an anti-CRMP-2 antibody. The antibody was sourced from the same company (AbCam, USA) as that used by Mileusnic & Rose as it had already been shown this antibody was of high quality (Mileusnic & Rose 2011). The flow-through fraction showed a single strong reaction at the expected position on the gel for CRMP-2 (~63 kDa) but there was no band in the bound protein fraction. This showed that in this experiment CRMP-2 did not interact with sAPPα under these conditions.

4.6.1.3 Identification of unknown proteins with tandem mass spectrometry

While APP and CRMP-2 were not detected the gels displaying the proteins in the fraction that bound sAPPα, there were some distinct proteins (Figure 3.29). The gel was analysed at the Centre for Protein Research, University of Otago to identify individual bands by Mass Spectrometry. The band labelled ‘A’ in (Figure 3.29) was suspected to be the fusion protein itself and this was confirmed by mass spectrometry, with the identity of both GST and APP detected. The band labelled ‘B’ was of great interest, but the mass spectrometry identified the band as containing GST and APP, and this led to the conclusion that it was a cleavage product of the full length fusion protein. This may be due to further cleavage from an as yet unidentified protease as has been seen with sAPPβ (Nikolaev et al., 2009). The group of bands labelled ‘C’ were found to be containing several isoforms of cellular GST.

4.6.1.4 Synaptoneurososome preparation is not suitable for the current study

The experiments analysing the binding partners present in the synaptoneurososome fraction yielded disappointing results, perhaps for reasons of sensitivity. The three repeats of the experiments yielded the same results. While this was disappointing it did raise the
intriguing possibility that there is some further regulated cleavage of the sAPPα protein as indicated by the presence of the degradation product in multiple experiments. It was decided that a different source of potential sAPP receptors was needed to develop a suitable system.

**Investigation of neuroblastoma cell lysate preparation**

The neuroblastoma preparation was chosen as it is a good neuronal cell model and relatively large amounts of material that can be obtained with relative ease, but most importantly because sAPPα is able to protect this cell line from toxic insults (Turner et al., 2007), indicating the receptor(s) and/or binding proteins required for this must be present in this cell line.

4.6.1.5 **Analysis of cell lysate preparation**

Both the triton-soluble and insoluble fractions were run on a gel to visualise the amount of protein in each fraction and Figure 3.25 shows the comparison. It can be seen that in the lanes representing the soluble fraction that the majority of the cellular protein is present in the soluble fraction. There were few proteins in the insoluble fraction; this made the fraction suitable for further analysis. Furthermore, it has been shown that proteins that bind the RERMS region are present in detergent soluble fractions (Pawlik et al., 2007). It has been suggested that the sAPPα receptor is located in detergent insoluble lipid rafts (Tikkanen et al., 2002). To determine the location of the sAPPα binding proteins, GST-sAPPα was added to cells in culture and incubated for 30 min to allow binding. Following this a normal cell lysate preparation was carried out (Section 2.5.1) and the soluble and insoluble fractions were probed with an antibody against GST (to avoid ambiguity from endogenous APP and/or sAPPs). It was clear that no fusion protein was detected in the insoluble fraction - strong immunopositive bands were seen by contrast in the soluble fraction (Figure 3.28). These experiments confirmed that the protein(s) to which sAPPα bound were found in this cell line and were extracted successfully in the protocol used in the current study.

As indicated earlier there are two main candidate receptors for sAPPα, CRMP-2 and APP, and so it had to be confirmed that the candidate receptors were present in the cells and in the fraction used to probe for sAPPα interactions. To do this, the cell lysate preparation was carried out as per Section 2.5.1. Both the soluble and insoluble fractions were run on a gel and via Western blotting were probed with antibodies against CRMP-2
and APP. Figure 3.26 showed that CRMP-2 was present in the neuroblastoma cell line and extracted in the soluble fraction. APP is also found in this cell line and appears to remain also predominantly in the soluble fraction (Figure 3.27). The APP antibody appears to have bound to several proteins non-specifically, but there is still virtually no signal in the insoluble fraction. The presence of APP and CRMP-2 in the soluble fraction allowed an investigation of the previously suggested interactions (Gralle et al., 2009; Mileusnic & Rose 2011).

4.6.1.6 SHSY-5Y cell lysate preparation is suitable

The analysis carried out on the SHSY-5Y neuroblastoma cell lysate confirmed that this preparation is suitable for the binding experiments to investigate sAPPα binding partners. The protocol used gave rise to the majority of the cellular protein being in the soluble fraction allowing access for binding analysis. The cell line was confirmed to have both the candidate receptors present in the soluble fraction. Furthermore, confirmation that the protein(s) with which sAPPα interacted was in the soluble fraction made this preparation ideal for isolation sAPPα interacting proteins.

4.7 sAPPα interacting proteins

Once the data from the mass spectrometry runs had been compiled (Section 3.7.4) the proteins that bound to GST alone were removed from the other datasets as they were considered to be non-specific binders. Following this, the remaining proteins were placed in groups based on the number of times each protein was identified in the replicate experiments. Only the proteins that were identified in at least 2/3 experiments were considered.

4.7.1 High mobility group binding protein 1 (HMGB1)

One of the proteins that was found to consistently bind sAPPα was HMGB1. The precise molecular function of this 215 residue protein is still not fully known but is thought to play a role in inflammation through interactions with toll-like receptor 4 and the receptor for advanced glycosylation end-products (RAGE) (Yang & Calakos 2010; Kokkola et al., 2005) Inflammation in the brain has been highly implicated in AD (reviewed in Holmes & Butchart, 2011; Sastre et al., 2011). More relevant to the actions of sAPPα however is HMGB1s role in neurite outgrowth. HMGB1 has been shown to induce neurite outgrowth in a cell culture model (Huttunen, Kuja-Panula, Sorci,
Agneletti, et al., 2000) and this action is mediated through the RAGE receptor (previously implicated in AD, reviewed in Yan et al., 1996). This could be related to its role in inflammation. This is interesting as sAPPα has also been seen to increase neurite outgrowth (Jin et al., 1994). HMGB1 has also been shown to be able to elicit a pro-survival effect on cells (Sajithlal et al., 2002; Huttunen et al., 2000) and that this is due to an increase in the anti-apoptotic protein BCL-2 (Sajithlal et al., 2002). Another similarity between sAPPα and HMGB1 is their ability to activate NFκB expression (Turner et al., 2007; Huttunen, Kuja-Panula, Sorci, Agneletti, et al., 2000; Huttunen et al., 1999) and both molecules are toxic at higher doses (Taylor et al., 2008; Huttunen, Kuja-Panula, Sorci, Agneletti, et al., 2000). HMGB1 has also been shown to be able to bind heparin like sAPPα (Ling et al., 2011; Rauvala et al., 2000; Wake et al., 2009). Multiple heparin binding domains in sAPPs may assist in an interaction. Furthermore, another study looking at what determines HMGB1 binding interactions found that HMGB1 binds an array of peptide sequences (Dintilhac & Bernués, 2002) and a BLAST search identified one in the N-terminal region of sAPPs. The motif found in the Dintilhac & Bernués study was DTDPPG and the sequence in sAPPs is DxDPxG. Dintilhac & Bernués also predicted some interacting proteins and validated them in the study; these validated interactors had a similar level of homology with the recognition sequences. The interaction between sAPPs and HMGB1 identified in the current study is a novel interaction and given the similar modes of interaction and signalling pathways, it is not unreasonable to hypothesise that this is a functional interaction. This could be investigated utilising tools such as RNAi knock-down or HMGB1 KO mouse models to test if sAPPα is still as effective in these experimental paradigms.

4.7.2 Protein Phosphatase Inhibitors

Two of the proteins (protein SET, acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B)) isolated are inhibitors themselves or participate in the inhibition of protein phosphatase 2A (PP2A). PP2A activity has been shown to be decreased in AD and is partially responsible for the aberrant phosphorylation of tau and neurofilaments (reviewed in Rudrabhatla & Pant 2011). Inhibition of PP2A has been shown to induce AD like symptoms (Wang et al., 2010). Protein SET has been shown to have PP2A inhibitor activity as well as ANP32B (reviewed in Santa-Coloma 2003). sAPPα may dissociate the PP2A inhibitor complex through binding these proteins and
hence relieving PP2A of the inhibition and that this could contribute to the beneficial effects seen by sAPPα.

Furthermore, protein SET and ANP32B form a complex that is an inhibitor of histone acetyltransferase (INHAT) complex that can alter transcription (Seo et al., 2001). ANP32B regulates PP2A activity during synaptogenesis implicating it in synaptic plasticity (Costanzo et al., 2006). Other members of the ANP family have been found to interact with microtubule associated proteins (including tau) specifically and regulate neuritogenesis (Opal et al., 2003). Protein SET has also been implicated in the activation of AP1 signalling (Al-Murrani, Woodgett, & Damuni, 1999). Some of the pro-survival effects elicited by sAPPα have been attributed to a decrease in AP1/c-Jun signalling (Copanaki et al., 2010), sAPPα may be binding protein SET and preventing it from activating the signalling cascade.

4.7.3 Prohibitin

Prohibitin was found to exclusively bind to sAPPα in two of the three experiments. The precise molecular function of prohibitin is still largely unknown. It was initially thought to have a role in anti-proliferative activity (McClung et al., 1995). Much more recent evidence suggests a contradictory role and implicates it in two very critical signalling pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/MAPK/ERK pathways (reviewed in Mishra et al., 2010). It is very interesting that prohibitin activates the same pathways as sAPPα has been found to activate (see Section 1.6.3) In fact prohibitin has been found to be necessary for Ras/MAPK/ERK signalling (Rajalingam et al., 2005). Another very interesting property of prohibitin is its ability to promote Ca\^{2+} efflux (McClung et al., 1995). Figure 4.1 summarises both the positive and negative effects of prohibitin on the signalling pathways and the molecules involved.
Figure 4.1 Effects of prohibitin on signalling cascades

A figure outlining the effects prohibitin (PHB) on the Akt and ERK signalling cascades. Red arrows indicate inhibition, while green arrows indicate enhancement and yellow arrows indicate crosstalk. RTK, receptor tyrosine kinase. PIP3, phosphoinositide triphosphate. IRS, insulin receptor substrate. PI3K, phosphatidylinositol 3-kinase. Shp, Src homology protein. MAPK, mitogen activated kinase. ERK, extracellular receptor kinase. OGT, O-linked N-acetylglucosamine (O-GlcNAc) transferase. TGF-β, transforming growth factor β. Figure taken with permission from Mishra et al., 2010.

While prohibitin is located mostly in the mitochondria (Ikonen et al., 1995), the proportion located in the plasma membrane may be acting to shuttle Ca\(^{2+}\) out of the cell. Hence it may be acting as a receptor for sAPPα as it too has been shown to suppress the intracellular rise in Ca\(^{2+}\) and protect cells against excitotoxicity.

It is clear that prohibitin has many interesting properties, many of which mimic the effects elicited by sAPPα. The fact that it was absent from sAPPβ binding experiments but present in sAPPα experiments may mean it contributes to the increased efficacy of sAPPα over sAPPβ. Further functional studies here are required to further elaborate the purpose of the interaction such as studies involving the knock out or reduction in the levels of this protein and assessing the effects it has on the neuroprotective ability of sAPPα.

4.7.4 Keratin and Tubulin

Four different structural proteins were also found to consistently bind sAPPs, three different isoforms of keratin and an isoform of tubulin. This finding is more difficult
to explain. The interaction could reflect sAPPs binding to a complex that is bound to these proteins and so they are included as well. Keratin has been shown to interact with other forms of amyloid previously (Hintner et al., 1988) and so the isolation of the keratin proteins could in fact be due to them interacting with the sequence that sAPPα shares with Aβ, this is supported by the absence of the keratin proteins in the sAPPβ experiments.

**4.7.5 Interacting proteins found by previous studies**

It is interesting neither CRMP-2 nor APP was recovered in the binding assays in the current study but several novel interacting proteins were found. CRMP-2 may not have been found due to the previous studies having used either isolated RERMS (Pawlik et al., 2007) or RER (Mileusnic & Rose 2011), but full-length sAPPα, as in the current study, may not have this sequence exposed and thereby not bind CRMP-2 as readily. Pawlik also isolated albumin preproprotein and this remains an interesting interaction to be further investigated. Also a previous study identified ATP synthase subunit A as a sAPPα interacting protein (Schmidt et al., 2008). The current study also found that ATP synthase subunit A bound sAPPα, but it also bound to sAPPβ and GST alone as well, suggesting the interaction is non-specific.

**4.7.6 Summary of Findings**

The current study aimed to explore a simple strategy that might identify a portfolio of proteins that interact with sAPPα. Six novel proteins were identified that interact with sAPPα and the current study has replicated a previous finding that preproalbumin binds to sAPPα. Three of these proteins (protein SET, ANP32B and prohibitin) all have functions that directly relate to AD and therefore are good candidates for further function studies using RNAi or antibody mediated knock-down of these proteins to assess the effect this has on the functions of sAPPα. The keratin (large component of skin and hair cells) identifications may indicate contamination of the samples during the MS procedure; further studies will clarify if these are genuine interactions. The identification of tubulin may reflect the presence of a complex that was attached to tubulin or may in fact represent a genuine interaction between tubulin and sAPPα, perhaps the knock-down studies mentioned above will elaborate on this by assessing the effects it would have on the properties of sAPPα.
4.8 Future Directions

The analysis carried out in the current study has identified several novel binding proteins that interact with sAPPα and may contribute to its effects. The first step would be to confirm the interactions perhaps with yeast-2-hybrid studies and repeats of the experiments here and look into the folding of the sAPPα protein by using circular dichroism. Size exclusion chromatography could also be used to further purify the final protein sample. Once the interactions are confirmed, the function of these proteins need to be investigated. To validate these interactions, functional experiments to dissect out the nature of the interaction will need to be carried out. Knock-down of the proteins of interest with RNAi or blocking with antibodies, and then assessing sAPPα function could be carried out. A more sophisticated method of finding interactive sAPPs proteins through the use of a tri-functional reagent attached to sAPP that will covalently link to the interactor is being investigated concurrently in the laboratory. Briefly, this uses a system by which UV light is used to covalently attach the bait protein to any proteins in a close vicinity to the bait and so may be a more sensitive method for identifying interactions.

The prokaryotic expression system developed here will allow large scale production of these sAPPα and sAPPβ fusion proteins and pave the way for x-ray crystallography analysis. Currently the differences in the overall structures of sAPPα and sAPPβ are not known, or how the extra 16 amino acid C-terminal extension of sAPPα is positioned in the molecule. Given the ease of manipulation of the prokaryotic expression system production of sub-domains to order will be possible which will help isolate the domain(s) responsible for the effects of sAPPα.
References


Ninomiya, H., Roch, J. M., Jin, L. W., & Saitoh, T. (1994). Secreted form of amyloid beta/A4 protein precursor (APP) binds to two distinct APP binding sites on rat B103 neuron-like cells through two different domains, but only one site is involved in neuritotropic activity. Journal of Neurochemistry, 63(2), 495-500.


Appendix 1- Materials and Solutions

All chemicals used were of analytical grade where possible. Solutions were prepared with MQ H₂O. Where sterility was required, the solutions were either sterilised by autoclaving at 121°C for 20min at 15psi or were filtered through a 0.22 µm filter. The following is a list of the components used to make the solutions and the chemical make-up of the solutions used unless they are outlined in the text.

4.9 Materials

*AbCam, USA*: anti-CRMP-2 antibody (rabbit)

*Acros Organics, USA*: Ponceau S

*Agfa, Belgium*: 18 x 24 cm X-Ray CP-BU film (blue)

*AppliChem GmbH, Germany*: agarose, glycine, SDS, tri-hydroxymethylamiomethane (Tris), HEPES, N,N,N’,N’-tetramethyl-ethylenediamine (TEMED)

*Applied Biosystems, USA*: BigDye v3.1 sequencing kit

*Biolab, Australia*: ethanol, glycerol

*Bio-Rad Laboratories Inc, USA*: 30% (w/v) Acrylamide/Bis solution 37.5:1, ammonium persulphate

*Becton Dickinson Labware, USA*: 15 ml, 50 ml sterile Falcon® tubes, 10 ml, 50 ml serological pipettes

*GE Healthcare, UK*: ECL™ Western Blotting Detection Reagents, anti-GST antibody (goat)

*Gibco®, USA (Invitrogen Corporation)*: Dulbecco’s Modified Eagle Medium with and without D-glucose, foetal bovine serum, Trypsin (0.25% w/v), Geneticin®
**Greiner Bio-One**: T25, T75 cell culture flasks

**Invitrogen Corporation, USA**: ethidium bromide, 100 bp DNA ladder

**Koch-Light Limited, UK**: bromophenol blue

**JT Baker Chemical Co, USA**: hydrochloric acid

**Merck KGaA, Germany**: bactotryptone, yeast extract

**Oxoid Limited, UK**: phosphate buffered saline tablets (Dulbecco A)

**Pierce, Thermoscientific, USA**: Anti-goat peroxidase conjugate

**Pam’s, New Zealand**: skim milk powder

**Roche Applied Science, Germany**: ampicillin, dNTPs (PCR grade dATP, dCTP, dGTP and dTTP), 1,4-dithiothreitol (DTT), λ DNA, restriction endonucleases (SmaI and NotI) and the respective buffer (Buffer H)

**Schleicher and Schuell, Germany**: Protran® nitrocellulose transfer membrane

**Sigma-Aldrich Corporation, USA**: Anti-Rabbit IgG (whole molecule) peroxidase conjugate (goat), rabbit anti-N-terminal APP antibody, bicinchoninic acid, Brilliant Blue R-250, manganese(II) chloride, MTT, dialysis tubing, β-mercaptoethanol, DMSO, reduced glutathione

**USB Corporation, USA**: Tween® 20

**Whatman Ltd, UK**: 3 MM chromatography paper, No. 1 filter paper
4.10 Solutions

Solutions for protein analysis

Cracking buffer (2x)
150 mM Tris pH 6.8
6% (w/v) SDS
8 M urea
0.3% (w/v) bromophenol blue

Ponceau S stain
0.1% (w/v) Ponceau S
0.1% (w/v) Ponceau S
5% (v/v) acetic acid

SDS polyacrylamide resolving gel, 10%
1x Kolbe resolving gel buffer
10% (v/v) acrylamide:bisacrylamide (37.5:1)
0.09% (w/v) ammonium persulphate
5 mM TEMED

SDS polyacrylamide stacking gel, 10%
1x Kolbe stacking gel buffer
10% (v/v) acrylamide:bisacrylamide (37.5:1)
0.04% (w/v) ammonium persulphate
10 mM TEMED

Kolbe lower running buffer (10x)
250 mM Tris
1.9 M glycine
pH was not adjusted

Kolbe upper running buffer (10x)
250 mM Tris
1.9 M glycine
1% (w/v) SDS
pH was not adjusted

Kolbe resolving gel buffer (4x)
3 M Tris
0.4% (w/v) SDS
Adjusted to pH 8.8 with HCl

Kolbe stacking gel buffer (4x)
500 mM Tris
0.4% (w/v) SDS
Adjusted to pH 6.8 with HCl

Glutathione Elution Buffer (1x)
50mM reduced glutathione
50mM Tris
Adjusted to pH 7.0

Transfer buffer (1x)
25 mM Tris
192 mM glycine
20% (v/v) methanol
pH was not adjusted

Low-Salt Lysis Buffer (1x)
50mM Tris
50mM NaCl
1% (v/v) Triton X-100

Extraction Buffer (1x)
32mM sucrose
12mM Tris
1% (v/v) Triton X-100
pH adjusted to 8.1 with HCl

Solutions for DNA analysis
DNA loading buffer (10x)
20% (w/v) sucrose
0.5% (w/v) bromophenol blue
1 mM NaOH

TBE (Tris-Borate-EDTA) buffer (10x)
900 mM Tris
20 mM EDTA
5.5% (w/v) boric acid
pH was not adjusted

LB (Luria-Bertani) medium
1% (w/v) bactotryptone
0.5% (w/v) yeast extract
172 mM NaCl
For agar plates, 1.5% (w/v) agar was added. After autoclaving, selective antibiotic was added once medium had cooled to 60°C

YT medium
1.6% (w/v) bactotryptone
1% (w/v) yeast extract
86 mM NaCl

Solutions for cell culture
Freezing medium
90% (v/v) FBS
10% (v/v) Dimethyl sulfoxide (DMSO)

General Solutions
PBS (phosphate buffered saline) (1x)
Dissolved 1 tablet in 100 ml MQ H2O
This results in:
80 mM di-sodium hydrogen orthophosphate anhydrous
20 mM sodium di-hydrogen orthophosphate anhydrous

PBS Tween®-20 (1x)
PBS Solution (1x)
0.1% (v/v) Tween® 20

Media for bacterial growth
## Appendix 2 – Primer sequences

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer</th>
<th>Sequence</th>
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<td>sAPPα forward</td>
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<tr>
<td></td>
<td>sAPPα reverse</td>
<td>ATAGTTTAGGGCGGCGCTCACTTTTGATGATGAACCTTC</td>
</tr>
<tr>
<td></td>
<td>sAPPβ forward</td>
<td>CGCTCCGTCCGCCTGCCCGGTTTGGC</td>
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<tr>
<td></td>
<td>sAPPβ reverse</td>
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<td>Sequencing Analysis</td>
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