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Role of Phosphoinositides in the Biology of the Amyloid Precursor Protein

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BSc (Hons)**

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Abstract

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. In countries with aging populations, such as Australia, the prevalence of AD is projected to increase substantially. AD is characterised by two distinctive pathological lesions in the brain, amyloid plaques and neurofibrillary tangles. The major component of amyloid plaques is an aggregating protein termed the beta-amyloid protein ($A\beta$). $A\beta$ is formed normally from a larger precursor protein, known as the beta-amyloid precursor protein (APP). Although APP is centrally involved in the pathogenesis of Alzheimer's disease and the production of $A\beta$, relatively little is known about its normal function. Deciphering the function of APP in the brain may be essential for the development of effective AD therapeutics.

APP is a type I transmembrane glycoprotein that can be proteolytically processed by α , β - and γ -secretases to produce a number of secreted ectodomain fragments termed sAPP β , sAPP α , $A\beta$ and p3. Many studies have suggested that sAPP α may act in the maintenance and development of the central nervous system, by acting as a paracrine factor. In vitro, sAPP α has been reported to modulate the proliferation and differentiation of a variety of cell types. However, the mechanistic basis for these effects is unclear. In part, this uncertainty has arisen because the cell-surface receptor molecules that interact with sAPP α are not known.

Previous studies have reported that sAPP α may interact with a novel lipid-raft type membrane domain in the cell. Furthermore, sAPP α has been reported to bind to the lipid GM1-ganglioside. On the basis of these reports, the work in this thesis explored the hypothesis that an interaction of APP with cell surface lipids could facilitate binding and/or signalling by sAPP α .

To determine if sAPP α is able to interact with a sub-group of lipids. The relative ability of sAPP α to bind to 27 physiological lipids was examined using a protein-lipid overlay assay. This assay identified that sAPP α could bind selectively to phosphoinositide lipids (PIPs). Further, a recombinant fragment of APP corresponding to the E1 N-terminal domain (APP-E1) also bound selectively to PIPs, suggesting there is a PIP-binding region within the E1 domain of APP.

To investigate whether APP and PIP could interact on the cell surface, it was first necessary to demonstrate that PIPs are present on the cell surface. A live cell immunolabelling method was used to examine the location of cell surface PIPs. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) immunoreactivity was found to be present on the surface of cells in primary murine hippocampal cultures in discrete puncta <1 μ m in size. This observation was also confirmed using a recombinant PI(4,5)P₂ biosensor protein.

To examine whether APP could interact with cell-surface PIP, studies were performed to examine the degree of colocalisation of exogenous APP-E1 and cell-surface PI(4,5)P₂. APP-E1 that was added to primary hippocampal cultures bound to the surface of neurons in discrete puncta <1 μ m in size. The cell-bound APP-E1 and the cell-surface PI(4,5)P₂ were highly co-localised on the surface of neurons. However, cell-surface PI(4,5)P₂ was also present on glial cells in culture where APP-E1 did not bind. Furthermore the binding of APP-E1 to cells could not be inhibited using a water soluble analogue of PI(4,5)P₂. Therefore, these data suggested that APP-E1 interacts with cell-surface PI(4,5)P₂, but the interaction was not sufficient to explain why APP-E1 binds to the cell surface.

As the APP E1 domain contains a heparin-binding site, the role of this region was investigated in the binding of APP-E1 to PIP and also the binding of APP-E1 to cells. Heparin did not block the binding of APP-E1 to PIP in vitro, suggesting the heparin-binding region and the PIP-binding region in the APP E1 domain are

distinct. However, heparin did inhibit the binding of APP-E1 to cells, suggesting that the heparin-binding region of APP is required for binding to cells. Furthermore, heparitinase treatment of cells significantly reduced cell surface heparan sulfate immunoreactivity, but did not affect the binding of APP-E1 to cells. These results suggest that APP may interact with PIP on the cell surface along with another cell surface component that binds to the heparin-binding site, which is not heparan sulfate.

As PIPs are involved in many aspects of cellular physiology, it was hypothesized that APP may signal through modulation of levels of PIPs. To address this hypothesis, levels of PIPs were measured in primary cortical cultures by two methods. Firstly, a mass-spectroscopy based method was developed to measure total levels of cellular PIP. No change in total PIP levels upon sAPP α treatment could be detected using this method. Secondly, levels of cell-surface PIPs were determined using an array of anti-PIP biosensors and antibodies. Under resting conditions, only PI(4,5)P₂ was present on the surface of cells. However, in the presence of APP-E1, there was an increase in the level of cell surface PI(3,4,5)P₃ and an increase in the level of PI(4,5)P₂, indicating that APP binding to cells may result in an increase level of cell surface PIPs.

The data presented in this thesis demonstrate that APP has a novel N-terminal PIP-binding domain. This domain may play a role in the normal function of APP, by facilitating PIP-dependent signalling.

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List of abbreviations

6xHis	Hexahis affinity tag
$\alpha 7$ nAChR	$\alpha 7$ nicotinic acetylcholine receptor
ΔG	Free energy of binding
A/ENTH	Epsin amino terminal homology domain
ABCA7	ATP-binding cassette, sub-family A, Member 7
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ADDL	Amyloid beta-derived diffusible ligand
AICD	β -amyloid precursor protein intracellular domain
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA-R	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
AP180	Clathrin coat assembly protein
AP2	Clathrin adaptor protein 2
Aph-1	Anterior pharynx-defective phenotype
APLP	Amyloid precursor protein-like protein
ApoE	Apolipoprotein E
ApoJ	Apolipoprotein J/ clusterin
APP	β -Amyloid precursor protein
APP-770/751	APP isoforms containing KPI domains (collectively)
APP-E1	Recombinant protein corresponding to APP amino acids 18-286
APP _{SW}	APP carrying the Swedish mutation (K670N/M671L)
Arb. Unit	Arbitrary unit
A β	Beta-amyloid
BACE	Beta-site APP cleaving enzyme 1
BAR	Bin/amphiphysin/rvs domain
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CAPS	Calcium dependent activator protein for secretion
CCV	Clathrin coated vesicle
CD2AP	CD2-associated protein
CD33	Myeloid cell surface antigen CD33
Cdk	Cyclin-dependent kinase
CER	Ceramide
Ch	Cholesterol
CL	Cardiolipin
CLU	Clusterin
CM	Cell medium
CNS	Central nervous system
CR1	Complement receptor 1
CSF	Cerebrospinal fluid
CTF	β -amyloid precursor protein C-terminal fragment

Dab	Disabled
DAG	Diacylglycerol
DAPI	4' 6 diamidino-2-phenylindole
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor-receptor
EOAD	Early onset Alzheimer's disease
FAD	Familial Alzheimer's disease
FBS	Foetal bovine serum
FERM	Band 4.1, ezrin, radixin and moesin domain
FGF2	Basic fibroblast growth factor
FYVE	Fab1, YOTB, Vac1 and EEA1 domain
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GD3	Disialoganglioside
GFAP	Glial fibrillary acidic protein
GM1	Monosialoganglioside
Grb2	Growth factor receptor-bound protein 2
GRP1	General receptor for phosphoinositides
GST	Glutathione-s-transferase
GTP	Guanosine 5'-triphosphate
GWAS	Genome-wide association studies
HBSS	Hank's balanced salt solution
HCHWA-D	Familial hereditary cerebral hemorrhage with amyloidosis (Dutch type)
HDL	High-density lipoproteins
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HS	Heparan sulfate
I.R.	Immunoreactivity
ICC	Immunocytochemistry
IGF-1	Insulin-like growth factor 1
IP ₃	D-myo-inositol 1,4,5-trisphosphate
JNK	c-Jun NH ₂ -terminal kinase
K _d	Dissociation constant
Kir	Inwardly rectifying potassium channels
KO	Knock out
KPI	Kunitz protease inhibitor domain
LDCV	Large dense core vesicle
LOAD	Late onset Alzheimer's disease
LPA	Lysophosphatidic acid
LPC	Lysophosphocholine
LRP1	Low-density lipoprotein receptor-related protein 1

LTD	Long term depression
LTP	Long term potentiation
MAP2	Microtubule associated protein 2
MCI	Mild cognitive impairment
mGluR	Metabotropic glutamate receptor
Mint	Munc-18-interacting proteins
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS4A4E	Membrane-spanning 4-domains, subfamily A, member 4E
MS4A6A	Membrane-spanning 4-domains, subfamily A, member 6A
NFDM	Non-fat dry milk powder
NFT	Neurofibrillary tangles
NGF	Neuronal growth factor
NGS	Normal goat serum
NICD	Notch intracellular domain
NMDA	<i>N</i> -Methyl-D-aspartic acid
NMDA-R	<i>N</i> -Methyl-D-aspartic acid receptor
n.s.	Not significant
NSPCs	Neural stem and progenitor cells
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDB ID	Protein data bank ID
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
Pen-2	Presenilin enhancer 2
PG	Phosphatidylglycerol
PH	Pleckstrin homology domain
PHF	Paired helical filaments
PhyS	Phytosphingosine
PI	Phosphatidylinositol
PI(3,4,5)P ₃	Phosphatidylinositol 3,4,5-trisphosphate
PI(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PI(3,5)P ₂	Phosphatidylinositol 3,5-bisphosphate
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PI(5)P	Phosphatidylinositol 5-phosphate
PI3K	Phosphoinositide 3-kinase
PI4K	Phosphoinositide 4-kinase
PICALM	Phosphatidylinositol binding clathrin assembly protein
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PIP2	Phosphatidylinositol phosphate kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLO	Protein-lipid overlay assay

PM	Plasma membrane
PS	Phosphatidylserine
PS1	Presenilin 1
PS2	Presenilin 2
Psy	Psychosine
PTB	Phosphotyrosine binding domain
PTEN	Phosphatase and tensin homolog
PX	Phox homology domains
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
S1P	Sphingosine-1-phosphate
sAD	Sporadic Alzheimer's disease
sAPP	Secreted APP
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SH3	SRC homology 3 domain
Shc	SHC-transforming protein
SHIP	SH2- containing inositol phosphatase
SIM	Selective ion monitoring
siRNA	Small interfering RNA
SM	Sphingomyelin
SNX	Sorting nexin
SPC	Sphingosylphosphorylcholine
Sps	Sphingosine
ST	3-sulfogalactosylceramide (sulfatide)
Syt	Synaptogamin
TBS	Tris buffered saline
TG	Triglyceride
Tip60	Tat-interactive protein 60
TNF	Tumour necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
TrkA	High affinity nerve growth factor receptor
TRP	Transient-receptor potential
UPLC	Ultra-performance liquid chromatography
WB	Western blot
WT	Wild-type

Chapter 1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative condition and the leading cause of dementia in the elderly. Alois Alzheimer first described the symptoms and pathology of AD in a 51-year-old female dementia patient to a meeting of psychiatrists in Tübingen, Germany (Alzheimer, 1907). Using the newly developed Bielschowsky's silver-staining technique, Alzheimer was able to examine the post-mortem brain of the patient to identify characteristic pathological lesions. Alzheimer's colleague and mentor, Emil Kraepelin later named the condition Alzheimer's disease (Kraepelin, 1910). It was not until many decades later that it became accepted that pre-senile AD and late-onset dementia were indistinguishable and the definition of AD was expanded to include senile dementia (Blessed et al., 1968). Today, there are considered to be two forms of AD, early onset AD (EOAD), also known as familial AD (FAD), which has a clear genetic heritability and late-onset AD (LOAD), which is often referred to as sporadic AD (sAD). Both EOAD and LOAD have a similar clinical manifestation, but differ in the age of onset (Reitz et al., 2011).

1.1.1 Clinical features of AD

AD is the leading cause of dementia (Reitz et al., 2011). Dementia can be defined as an acquired syndrome of impaired cognitive function (Tarawneh and Holtzman, 2012). Dementia can present clinically as a combination of memory impairment, language disturbance, impaired motor activity, agnosia (impaired recognition) and disturbed executive function (Tarawneh and Holtzman, 2012). Currently, AD can only be confirmed as the cause of dementia by post-mortem pathological analysis, so the diagnosis of *probable* AD is used based on clinical criteria (Tarawneh and Holtzman, 2012).

The early stages of dementia are challenging to distinguish from the decline in cognitive function associated with normal ageing (Tarawneh and Holtzman, 2012). Mild cognitive impairment (MCI) can often be detected in AD patients 5 years before clinical diagnosis of probable AD (Linn et al., 1995). However, MCI cannot be used as an accurate predictor of AD or dementia, as MCI can often be caused by a short-term fluctuation in cognitive performance. The annual conversion rate of individuals with MCI to dementia is approximately 10% (Bruscoli and Lovestone, 2004; Ward et. al., 2013; Mitchell and Shiri-Feshki, 2009).

Clinical diagnosis of dementia usually occurs in the mild to moderate stages of dementia progression. In the mild stage, patients display a significant impairment of learning and memory, especially recent declarative memory. This memory impairment affects the ability to perform complex tasks, although patients with mild dementia are able to live independently (Forstl and Kurz, 1999). As the disease progresses into moderate dementia, the symptoms increase in severity, impacting many cognitive functions. Reasoning, organisation, planning, literacy, communication and facial recognition deteriorate to the extent that the patient requires close supervision (Forstl and Kurz, 1999). Patients with severe dementia are reliant on caregivers and usually require institutionalisation. Death usually occurs over a period of 5 – 8 years after diagnosis (Forstl and Kurz, 1999). Mortality in dementia patients is most often caused by bronchopneumonia or myocardial infarction (Brunnstrom and Englund, 2009; Burns et al., 1990).

1.1.2 Impact and prevalence of AD

Alzheimer's disease presents a major challenge to global healthcare systems. In Australia, the number of people with dementia in 2012 was estimated to be over 300,000 (Australian Institute of Health and Welfare, 2012). Globally in 2010, dementia was estimated to affect 35.6 million people (Prince et al., 2013). Age is the biggest risk factor for LOAD (Reitz et al., 2011). In developed countries with an aging population, the prevalence of AD is projected to increase significantly, resulting in an increased social and economic demand for care provision (Alzheimer's Australia, 2010, Prince et al., 2013). If current dementia care health policy is not altered, there will be a significant shortfall of care provision to meet the increase in people with dementia (Alzheimer's Australia, 2010). Therefore, dementia represents an emerging health care priority for the health care system in Australia (Alzheimer's Australia, 2010).

1.2 Pathological features of AD

Pathologically, AD is characterised by a number of lesions. The original description of AD described the presence of neuritic plaques, neurofibrillary tangles, and adipose inclusions in glia (Alzheimer, 1907; Alzheimer et al., 1995). In AD, there is gross brain atrophy in the late stages of the disease, degradation of synapses, and deposition of amyloid in brain blood vessels (cerebral amyloid angiopathy; Braak and Braak, 1991; Thal et al., 2008; Terry et al., 1991). Much of the basis of our understanding for the processes that underlie Alzheimer's disease has come from careful investigation of these pathological lesions.

1.2.1 Amyloid plaques

Amyloid plaques are focal pathological lesions that are characteristic of AD (Braak and Braak, 1991). Amyloid plaques are generally found in the six layers of the isocortex, and are most abundant in layers II-V (Braak and Braak, 1991). At the centre of the amyloid plaque, there is a dense core of amyloid, which is an insoluble fibrous protein aggregate that can be identified by its ability to retain amyloid stains such thioflavin or Congo red (Sipe et al., 2010). The main proteinaceous component of the amyloid core was initially identified in cerebrovascular amyloid in both Down syndrome and AD patients (Glenner and Wong, 1984b). Shortly afterward, Glenner and Wong (1984a) identified the same protein as the amyloid forming protein in AD plaques. This observation was quickly replicated by a number of studies and the protein is now known as β -amyloid ($A\beta$; Selkoe et al., 1986; Masters et al., 1985). $A\beta$ is now known to be produced from a larger precursor protein, the β -amyloid precursor protein (discussed in Section 1.3.1).

Studies of AD brain using monoclonal antibodies directed against $A\beta$ revealed that there are dense-core thioflavin positive plaques present in AD, but also immunoreactive accumulations of $A\beta$ that are not stained by thioflavin. These are termed diffuse plaques (Joachim et al., 1989; Tagliavini et al., 1989; Yamaguchi et al., 1988). Diffuse plaques may represent an early stage of neuritic plaque development (Gowing et al., 1994; Iwatsubo et al., 1994; Iwatsubo et al., 1995; Lemere et al., 1996). However, diffuse plaques have also been detected in non-demented elderly individuals and in young individuals after brain injury (Roberts et al., 1994; Armstrong et al., 1996; Tagliavini et al., 1989; Yamaguchi et al.,

1998). As the presence of diffuse plaques is not restricted to patients with AD, the conversion of diffuse plaques to dense-core plaques may be a distinguishing factor in the development of AD. Aged subjects with diffuse plaques may therefore have a pre-clinical AD phenotype (Morris et al., 1996; Villemagne et al., 2008).

The spatial deposition of amyloid plaques in AD appears to follow a temporal progression. Studies by Braak and Braak (1991) were influential in characterising this process, which were grouped into three stages. In stage A, amyloid deposits are found in the isocortex, particularly in the basal portions of the frontal, temporal and occipital lobes. In stage B, a medium density of A β deposits is present in all isocortical association areas except the primary sensory areas and the primary motor areas. In stage C, all isocortical areas have dense deposits of amyloid, including the hippocampus. Deposits are also present in subcortical structures, the striatum, thalamus, hypothalamus and sub-thalamic and red nucleus in this stage (Braak and Braak, 1991). More recently, a progression of plaque deposition with five stages has been proposed, based on immunohistochemical detection of plaques, as well as silver staining (Thal et al., 2002). It is important to note however, that although amyloid deposition does progress in a staged manner throughout particular regions of the brain, this does not correlate with the severity of symptoms of AD nor the duration of illness (Terry et al., 1991; Hyman et al., 1993).

Although A β is the main component of amyloid plaques, a number of other plaque components have also been identified in amyloid plaques by biochemical and histological techniques. These include α 1-antichymotrypsin, cystatin C,

proteoglycans, apolipoprotein E, collagen XXV, clusterin and complement inhibitor (Abraham et al., 1988; Atwood et al., 2002; Hashimoto et al., 2002; Levy et al., 2001; May et al., 1990; Namba et al., 1991; Snow et al., 1988; DeWitt et al., 1994; Vinters et al., 1990; Young et al., 1989). A more recent proteomic study using laser-capture microdissection to isolate plaques identified 480 different proteins that were present in amyloid plaques (Liao et al., 2004). However, a study with a similar approach, but with a formic acid extraction step only identified A β , suggesting that formic acid extraction may obscure the detection of some plaque proteins (Soderberg et al., 2006). Both studies showed that A β was the major component of plaques.

1.2.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are intracellular accumulations of protein that result in dystrophic, swollen tangle-bearing neurons, often found surrounding plaques. Importantly, NFTs are a feature of many neurodegenerative diseases, not only AD (Morris et al., 2011). In AD, the distribution of NFTs is less variable than that of amyloid plaques, and follows a temporal progression that has been characterised into six stages (Braak and Braak, 1991). Progression of NFT pathology is a better correlate of clinical symptoms of dementia than A β deposition (Bierer et al., 1995). In stages I-II, most of the NFTs are confined to a single layer of the transentorhinal region (Braak and Braak, 1991). Stages I-II represent nonclinical pathology and can be present in individuals as young as 20 (Braak et al., 2011). In stage III-IV (the limbic stages), the transentorhinal layer and entorhinal layer become severely affected with NFT pathology. In addition, there is modest pathology in regions of the hippocampus (Braak and Braak,

1991). Stages III-IV are thought to represent incipient AD, and are present in 10% of adults aged 50, and in 50% of adults aged 80 (Braak et al., 2011). In stages V-VI (the isocortical stage), severe NFT pathology is found in the hippocampus and isocortex and destruction of the isocortex is apparent (Braak and Braak, 1991). Stages V-VI represent full-blown AD and are present in 10% of adults aged 80 and 20% of adults aged 90 (Braak and Braak, 1995).

1.2.2.1 Biochemical characterisation of NFTs.

Electron microscopy studies of NFTs reveal that dystrophic neurons contain accumulations of 10 nm diameter fibres that form helical pairs (Kidd, 1963; Wisniewski et al., 1976). These structures are termed paired helical filaments (PHFs). Immunohistochemical and biochemical studies show that a component of these PHFs is the microtubule-associated tau protein (Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986; Kosik et al., 1986; Nukina and Ihara, 1986; Wischik et al., 1988; Wood et al., 1986). In PHFs, tau is abnormally phosphorylated (Biernat et al., 1992). In vitro phosphorylation of recombinant tau can induce assembly of tau into PHFs (Biernat et al., 1992; Hanger et al., 1992). The tau in PHFs is unable to bind to microtubules and is biologically inert (Iqbal et al., 1994). However, hyperphosphorylated tau that is not accumulated into PHFs has been suggested to actively inhibit microtubule polymerization by binding to normal tau and other microtubule-binding proteins (Alonso et al., 1994; Alonso et al., 1997). Understanding the biology of tau protein in disease remains a subject of intense investigation and is too extensive to be discussed here in further detail, however this topic has been reviewed recently elsewhere (Iqbal et al., 2009; Morris et al., 2011).

1.2.3 Cerebral amyloid angiopathy

Approximately 80% of patients with AD have amyloid accumulation in the cerebral and leptomeningeal vessels (Ellis et al., 1996). This type of deposit is termed cerebral amyloid angiopathy (CAA; Glenner and Wong, 1984; Ellis et al., 1996). CAA is characterised in arteries and veins by deposits of A β in the outer basement membrane and between smooth muscle cells (Thal et al., 2008). CAA is also present in capillaries of some individuals, where A β accumulates on the capillary basement membrane (Thal et al., 2008).

CAA is present in normal aged individuals and there is a strong increase in prevalence of CAA with age (Attems et al., 2008; Greenberg and Vonsattel, 1997; Thal et al., 2008; Sillus et al., 1993). However, CAA is distributed more widely in the brain in patients with AD, and is also more severe (Thal et al., 2003). CAA is the cause of a number of vascular complications, such as cerebral haemorrhage and ‘microbleeds’ that may contribute to the cognitive decline seen in AD (Kalyan-Raman and Kalyan-Raman, 1984; Mandybur, 1986; Benedictus et al., 2013; Thal et al., 2008). CAA pathology has been associated with the severity of dementia in AD, although further research is needed to establish whether this represents a causal or a concurrent relationship (Attems et al., 2007; Thal et al., 2003).

1.3 A β hypothesis of AD

The predominant hypothesis to explain the molecular pathogenesis of AD is the amyloid hypothesis (reviewed by Hardy and Higgins, 1992). The amyloid

hypothesis places A β as the primary effector of the biochemical and cellular changes that underlie AD. This hypothesis is based on a number of lines of evidence that will be discussed in this section. Firstly, A β is the primary component of amyloid plaques, the pathological lesion that characterises AD. Secondly, mutations that cause familial forms of Alzheimer's disease have been mapped to the β -amyloid precursor protein and also to enzymes that produce A β from the β -amyloid precursor protein (Section 1.5.1). Thirdly, transgenic animal models carrying these mutations have phenotypes that recapitulate some of the neurological and pathological lesions of AD (Section 1.5.2). Finally, A β in various aggregation states has a capacity to act in a neurotoxic manner (Section 1.3.2). This section will discuss the cellular and biochemical changes that underlie the production of A β , and also discuss evidence for a central role of A β in AD pathogenesis.

1.3.1 A β production

The biochemical process that results in the production of A β has been well studied. A major breakthrough came from the observation that A β is derived from a larger precursor protein (Kang et al., 1987). The sequence of A β was used to identify a precursor gene on chromosome 21, which was termed the β -amyloid precursor protein (APP; Kang et al., 1987; Tanzi et al., 1987; Robakis et al., 1987; Goldgaber et al., 1987). APP is a 100 – 130 kilodalton (kDa) type 1 transmembrane glycoprotein (Kang et al., 1987; Goldgaber et al., 1987). Alternative mRNA splicing produces three major isoforms of APP, which consist of 695, 751 and 770 amino-acid residues (discussed in section 1.4). The 751 and 770 isoforms are widely expressed throughout the body, but neurons express higher levels of the 695 isoform (Goedert, 1987; Goldgaber et al., 1987; Tanzi et

al., 1987). A β is produced by the action of proteolytic enzymes known as secretases on APP, which can produce A β isoforms ranging from 37 – 48 amino acids in length (Qi-Takahara et al., 2005). There are three major proteolytic activities identified in the processing of APP that form two distinct pathways: the amyloidogenic pathway and the non-amyloidogenic pathway (Fig.1.1). Under normal conditions, the majority of APP is processed via the non-amyloidogenic pathway (Sisodia et al., 1990).

In the non-amyloidogenic pathway, α -secretase cleavage occurs at lysine 687 of APP (amino-acid numbering relative to the 770 amino-acid APP isoform). This cleavage produces two fragments, an 83-residue C-terminal fragment of APP (C83), which remains in the membrane, and a large soluble ectodomain fragment (sAPP α).

Alternatively, in the amyloidogenic pathway, APP undergoes cleavage by β -secretase, which cleaves APP at methionine 671. This cleavage produces a 99-residue C-terminal fragment of APP, which also remains associated with membrane (C99), and a smaller ectodomain fragment (sAPP β) that is secreted.

Both C99 and C83, produced by α -secretase and β -secretase cleavage respectively, can then be sequentially cleaved by a third protease, γ -secretase. Cleavage of APP by γ -secretase occurs in the centre of the trans-membrane domain, and either leads to the production of A β from the C99 fragment, or a fragment termed p3 from the C83 fragment. Differential γ -secretase cleavage can occur in the processing of APP resulting in production of different isoforms of A β ranging from 37 - 48 residues in length (discussed in more detail in Section 1.4.1.3). However, the major isoforms of A β in amyloid plaques are A β 40 and A β 42 (Iwatsubo et al., 1994). The predominant A β isoform that accumulates in CAA is A β 40 (Roher et al., 1993).

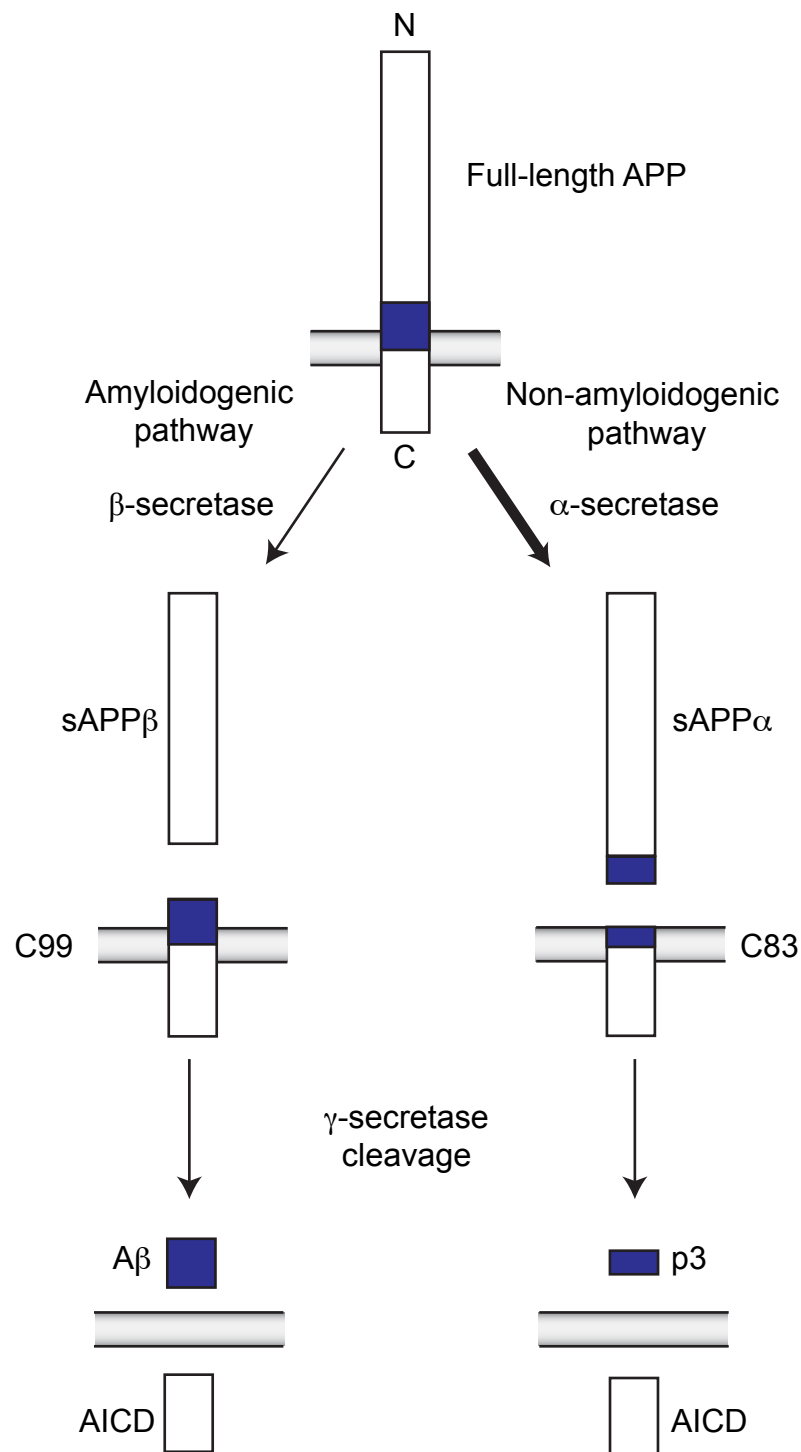


Figure 1.1. Diagrammatic representation of the amyloidogenic and non-amyloidogenic pathways of APP processing. In the amyloidogenic pathway, β -secretase cleaves APP to generate a secreted ectodomain (sAPP β) and a C-terminal fragment (CTF) that remains in the membrane, termed C99. C99 can be further cleaved by γ -secretase to produce secreted A β and an intracellular domain (AICD). The major pathway of APP processing is the non-amyloidogenic pathway (depicted by the thicker arrow). In this pathway, APP is cleaved by α -secretase to produce a secreted ectodomain (sAPP α) and a CTF fragment (C83). C83 is further cleaved to generate a secreted fragment p3 and an intracellular fragment (AICD).

1.3.1 A β aggregation

A β has a high propensity to form tightly packed cross β -sheet fibrils known as amyloid (Eisenberg and Jucker, 2012; Toyama and Weissman, 2011). Amyloid fibres are typically 5 – 15 nanometres in diameter and can be micrometres in length (Toyama and Weissman, 2011). Amyloid formation is a feature of various neurodegenerative conditions and a number of amyloid-forming proteins have been implicated in disease. Other examples of amyloid forming proteins that have been associated with disease include islet amyloid polypeptide (type 2 diabetes) and prion protein (Creutzfeldt-Jacob disease; Eisenberg and Jucker, 2012). Therefore, there is great interest in understanding the formation and biological activity of amyloids. The exact structural processes that result in the formation of A β amyloid are unresolved and current theories have been discussed in detail by a number of recent reviews (Eisenberg and Jucker, 2012; Masters and Selkoe, 2012; Toyama and Weissman, 2011).

The aggregation state of A β is strongly influenced by a number of factors. A β is partially hydrophobic and probably forms an α -helix in hydrophobic environments such as the cell membrane or organic solvents (Burdick et al., 1992). In aqueous environments, A β transitions to β -sheet structures and aggregates (Burdick et al., 1992; Kirschner et al., 1987). A low pH also promotes insolubility of A β (Burdick et al., 1992). The longer chain isoforms of A β (e.g. A β 42, A β 43) are more prone to aggregation due to additional hydrophobic residues (Jarrett et al., 1993; Burdick et al., 1992). Evidence also exists that A β 42 can “seed” the aggregation of A β 40 (Jarrett et al., 1993; Jan et al., 2008).

Consequently, the ratio of A β 40/A β 42 is thought to be a critical factor in plaque formation that may be perturbed in AD (Scheuner et al., 1996).

It is thought that a number of intermediate size aggregated A β species exist that may represent transition states between soluble A β and fibrillar A β . These include globular oligomeric species, which are water-soluble, and protofibrillar species, which are fibrillar, but shorter than amyloid fibrils. Oligomeric species have been reported to exist in various natural and synthetic preparations of A β , appearing as dimers, trimers, 12-mers and other forms (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006; Shankar et al., 2008; Hepler et al., 2006). Protofibrillar A β may represent a transition state between oligomeric A β and fibrillar A β (Walsh et al., 1997; Harper et al., 1997). Atomic force microscopy (AFM) studies of protofibrils suggest that the fibres are composed of a repeating structure of 100 nm in length, which is consistent with the idea that short protofibrils can combine to form longer fibres (Arimon et al., 2005). The significance placed on the different aggregation states of A β is largely due to observed differences in the biological interactions between various forms. The next section will discuss the relationship between the aggregation of A β and its ability to cause neurotoxicity.

1.3.2 A β toxicity

A β is thought to be a neurotoxic protein. This capacity has been postulated to be a primary cause of AD (Hardy and Higgins, 1992). However, current understanding of the toxicity of A β is complicated by its propensity to form a variety of aggregation states. Therefore, a consensus mechanism explaining the reported toxic effects of A β has not been reached, but there have been a number of

suggestions. The effects of the different reported aggregation states of A β will not be discussed here in complete detail, as this topic has been discussed comprehensively in several recent reviews (Shankar and Walsh, 2009; Benilova et al., 2012; Kaye and Lasagna-Reeves, 2013; Masters and Selkoe, 2012).

Early studies of A β toxicity found that an APP fragment containing the A β domain was toxic to primary hippocampal neurons (Yankner et al., 1989). Further experiments demonstrated that synthetic A β is neurotoxic for differentiated primary hippocampal neurons, but not undifferentiated cells (Yankner et al., 1990). This toxic effect was attributed to a region within A β between residues 25 and 35 (Yankner et al., 1990). Interestingly, at lower concentrations the A β 25 – 35 fragment was reported to have trophic effects (Yankner et al., 1990).

Subsequent studies have indicated that the aggregation state of A β is a key factor in determining its toxicity to cells. In vitro aging of A β , which increases the proportion of aggregated species, has been demonstrated to increase the toxicity of A β when compared to monomeric A β (Pike et al., 1991). However, it has also emerged that fibrillar A β is probably not the most toxic species of A β (Dahlgren et al., 2002). In recent years, lower molecular weight oligomeric forms of A β have been suggested to be the most toxic A β species (Walsh et al., 2002; Barghorn et al., 2005; Lambert et al., 1998).

A number of in vitro studies report that oligomeric A β is toxic to neurons. Lambert et al. (1998) found that a preparation of synthetic A β termed ADDLs (Abeta-derived diffusible ligands) was toxic to neurons at nanomolar concentrations. Further, they reported that oligomeric A β binds neurons close to

synaptic sites on dendrites, which has led many investigators to question if A β can affect synaptic function (Lacor et al., 2004; Barghorn et al., 2005). A number of studies have now reported effects of oligomeric A β preparations on synaptic function (Mucke and Selkoe, 2012; Shankar and Walsh, 2009). Oligomeric A β has been suggested to alter the behaviour of a number of ion channels, such as metabotropic glutamate receptors (mGluR) and the *N*-methyl-D-aspartate receptor (NMDA-R; Lacor et al., 2007; Renner et al., 2010; Shankar et al., 2007). This alteration of ion channel function has been suggested to produce effects on long-term potentiation (LTP; Lacor et al., 2007; Renner et al., 2010; Shankar et al., 2007; Shankar et al., 2008; Barghorn et al., 2005; Walsh et al., 2002). Changes in the density and morphology of excitatory dendritic spines are apparent after oligomeric A β treatment, consistent with a disruption of synapses (Lacor et al., 2007; Shrestha et al., 2006). As the levels of some soluble oligomeric A β species are reported to correlate with cognitive decline in AD better than fibrillar A β deposition, A β -induced disruption of synaptic function has been suggested to underlie cognitive decline in AD (McLean et al., 1999; Villemagne et al., 2010).

Protofibrillar species of A β are also reported to act in a toxic manner. Protofibrillar A β can induce electrophysiological changes in neurons, causing aberrant activity and inhibition of LTP (Arispe et al., 1993; Hartley et al., 2008). This disruption of normal electrophysiological function may lead to neurotoxicity (Hartley et al., 1999; Walsh et al., 1999).

A β toxicity has been postulated to be mediated by a variety of potential molecular mechanisms, which have been reviewed thoroughly elsewhere (Cappai and Barnham, 2008; Ng et al., 2007; Small, 2009; Mucke and Selkoe, 2012). One

possible mechanism of A β toxicity may be dysregulation of calcium homeostasis. A β increases the resting level of calcium in neurons, rendering them more sensitive to excitotoxicity (Mattson et al., 1992). This increased calcium influx may be caused by an effect of A β on endogenous membrane channels, a direct effect of A β on the cell membrane, or A β forming a pore in the membrane (Arispe et al., 1993; Ho et al., 2001; Kayed et al., 2004).

Importantly, it is not known which mechanism or combinations of mechanisms of A β toxicity are central to AD progression. In particular, it is not clear which molecules A β interacts with in vivo. The knowledge of these molecules will help decipher the primary mechanisms of A β -induced neurotoxicity (Mucke and Selkoe, 2012). A simple model whereby A β causes neuronal death probably cannot account for the neurodegeneration and pathology seen in AD (Small et al., 2001). Therefore, a loss of synapses is more likely to produce the cognitive decline in AD rather than cell death (Terry et al., 1991; Terry, 2000). Synapse loss is a much better correlate of cognitive decline in AD than A β plaque deposition or loss of neurons (Terry et al., 1991; Terry, 2000; Braak and Braak, 1991). Therefore, recent data that A β can interfere with synaptic function may provide a mechanistic basis for cognitive deficits in AD.

1.4 APP expression, structure and processing

APP is of great interest for AD research because of its role as the precursor of A β (Kang et al., 1987). APP is a member of an evolutionarily conserved family of proteins (Fig. 1.2). In mammals, there are three family members: APP, the amyloid precursor-like protein-1 (APLP1) and the amyloid precursor like protein-2 (APLP2; Kang et al., 1987; Slunt et al., 1994; Wasco et al., 1992; Wasco et al., 1993). Non-mammalian APP homologues also exist; *apl-1* in *C.elegans*, *APPL* in *D. melanogaster* and *appa/appb* in *D. rerio* (Daigle and Li, 1993; Luo et al., 1990; Musa et al., 2001; Rosen et al., 1989). APP family members have a relatively conserved structure.

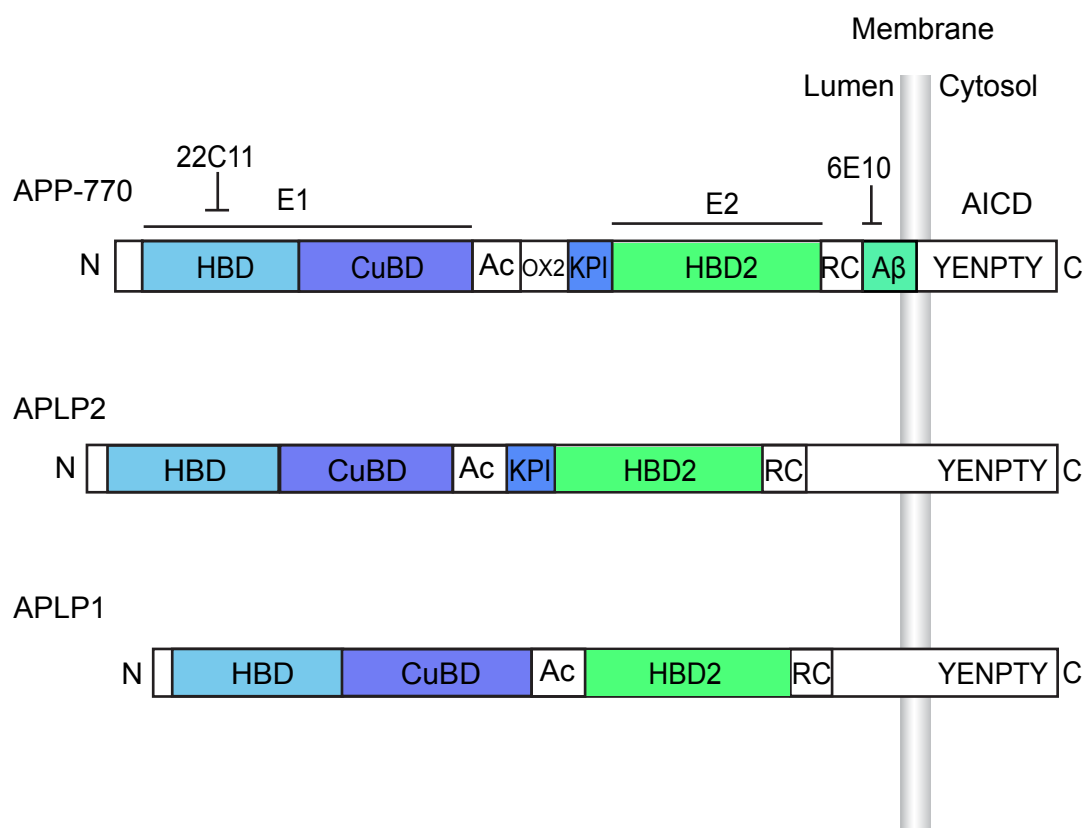


Figure 1.2. Domain structure of mammalian APP family members. HBD: Heparin binding domain. CuBD: Metal binding domain. Ac: acidic region, OX2: OX2 antigen domain. KPI: Kunitz-type protease inhibitor domain. HBD2: heparin binding domain 2. RC: random coil. YENPTY: NPxY interaction site. The epitopes of the antibodies 6E10 and 22C11 used later in the study are also depicted.

The extracellular region of APP and other family members have two structured domains, termed the E1 domain and the E2 domain, for which crystal structures are available (Dahms et al., 2010; Rossjohn et al., 1999; Wang and Ha, 2004). The E1 domain is split into a heparin-binding region and a metal-binding region (Small et al., 1994; Multhaup et al., 1996). This is followed by a short acidic region. APP-770, APP-751 and APLP2 contain a KPI domain (Tanzi et al., 1988), the physiological relevance of which is discussed in Section 1.6.4. APP-770 also contains an OX-2 antigen domain (Weidemann et al., 1989). The E2 domain is reported to contain a second heparin-binding region (Mok et al., 1997; Multhaup, 1994; Ninomiya et al., 1994). The A β region is unique to APP and is partially embedded in the cell membrane (Kang et al., 1987; Slunt et al., 1994; Wasco et al., 1992; Wasco et al., 1993). The C-terminal intracellular domain sequence is conserved throughout the APP family and features a YENPTY motif involved in interactions with other proteins and also APP trafficking (discussed in more detail in Section 1.6.5).

1.4.1 APP trafficking and post-translational modification

After expression, APP can undergo a number of post-translational modifications including glycosylation, sulfation, phosphorylation and palmitoylation (Hung and Selkoe, 1994; Selkoe, 2001; Bhattacharyya et al., 2013). APP is cotranslationally translocated into the endoplasmic reticulum, and is post-translationally modified in the Golgi. In the Golgi, an immature N-glycosylated APP species is detectable with a half-life of around 30-60 minutes that is processed to produce a mature form that is N- and O-glycosylated (Weidemann et al., 1989; Oltersdorf et al., 1990). Cells expressing an APP mutant that is not O-glycosylated demonstrate

reduced secretase processing of APP, suggesting that glycosylation occurs upstream of secretase activity. After modification in the Golgi, mature APP is then trafficked to the cell surface (Koo et al., 1996). Once at the cell surface, APP can then be internalised by clathrin-mediated endocytosis via the endosomal-lysosomal pathway (Yamazaki et al., 1996). The majority of APP is trafficked from the endosome to the lysosome, however a portion is then returned to the cell surface (Yamazaki et al., 1996). A pool of APP in the lysosome is degraded (Haass et al., 1992).

Secretase processing of APP can occur during the secretory pathway, at the cell surface, or after endocytosis (Koo et al., 1996). Ectodomain shedding (e.g. by α - or β secretase) is likely to be a pre-requisite for the action of γ -secretase, based on studies of γ -secretase function (Hemming et al., 2008; Struhl and Adachi, 2000). Therefore, the majority of β -secretase cleavage and A β production is thought to occur in acidic endosomal/lysosomal compartments, where β -secretase is most active (Vassar et al., 1999; Hussain et al., 1999; Sinha et al., 1999). This is consistent with the observation that endocytosis is required for the production of A β (Koo and Squazzo, 1994). In contrast, most α -secretase cleavage and production of sAPP α occurs at the cell surface, although some may occur during the secretory pathway (Sisodia, 1992; Tomita et al., 1998; Parvathy et al., 1999). The main cellular site of γ -secretase activity remains unresolved, however, this is likely to be in the plasma membrane (PM) and the endosomal/lysosomal system (Dries and Yu, 2008; Kaether et al., 2002; Tarassishin et al., 2004; Pasternak et al., 2004).

1.4.1.1 α -Secretase

α -Secretase cleavage of APP can be undertaken by a number of enzymes, all of which are members of the A disintegrin and metalloprotease (ADAM) family. The first APP-cleaving ADAM to be identified was ADAM 17, also known as tumour necrosis factor- α converting enzyme (TACE; Buxbaum et al., 1998). Shortly afterward, ADAM 10 and ADAM 9 were also reported to cleave APP at the α -secretase position (Koike et al., 1999; Lammich et al., 1999).

Although ADAM 10, 9 and 17 all have α -secretase activity, most recent evidence suggests that α -cleavage of APP can occur distinctly both in a constitutive manner and a regulated (i.e. inducible) manner. It is not yet resolved which ADAM is responsible for the constitutive α -secretase activity, as ADAM 10, 17 and 9 can all act in an inducible fashion, in response to phorbol ester treatment (Buxbaum et al., 1998; Lammich et al., 1999; Tomita et al., 1998). However, recent studies suggest the majority of constitutive α -secretase activity may be due to ADAM 10, at least in primary neurons (Kuhn et al., 2010; Vingtdeux and Marambaud, 2012).

Aside from the processing of APP, ADAMs have physiological roles in a variety of cellular processes. ADAMs act as sheddases, enzymes that catalyse the release of soluble ectodomains from proteins (Huovila et al., 2005). Ectodomain shedding by ADAMs is essential for the release of many cytokines and growth factor ligands. ADAM 10 is the major sheddase for epidermal growth factor (EGF) and β -cellulin (Sahin et al., 2004). ADAM 17 is the major sheddase for epiregulin, transforming growth factor α (TGF α), amphiregulin, tumour necrosis factor (TNF) and heparin-binding EGF-like growth factor (HB-EGF; Black et al., 1997; Moss et al., 1997; Sahin et al., 2004). Additionally, ADAMs also

participate in ectodomain shedding of growth factor receptors, such as human epidermal growth factor receptor 2 (HER2; Liu et al., 2006) and notch (Bozkulak and Weinmaster, 2009). Therefore, sheddase activity by ADAMs can facilitate cellular signalling in a variety of modes; either by release of secreted growth factors, or by ligand-dependent activation of cellular receptors, or a combination of both. This subject is covered in more detail by a number of reviews (Blobel, 2005; Huovila et al., 2005; Vingtdoux and Marambaud, 2012). Importantly, the fact that the APP ectodomain is released by α -secretase suggests that APP may be involved in a signalling interaction similar to other α -secretase substrates.

1.4.1.2 β -Secretase

The β -secretase cleavage of APP was shown by several groups to be undertaken by an enzyme termed β -site APP-cleaving enzyme 1 (BACE1; Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE1 is a type 1 transmembrane aspartyl protease that is expressed in the majority of tissues (Lin et al., 2000). There is also a BACE1 homologue that can cleave APP at the β -site, termed BACE2. BACE2 is predominantly expressed in peripheral tissues, with a lower expression level in the brain than BACE1 (Lin et al., 2000). BACE1 has been demonstrated to be the enzyme responsible for the majority of A β production by neurons, as neuronal A β production is abolished in BACE1 KO mice (Luo et al., 2001; Roberds et al., 2001; Dominguez et al., 2005). As a result, BACE1 has become an attractive target for the treatment of AD (Klaver et al., 2010). However, not all A β production may be BACE1-dependent as glia may produce A β in a BACE2-dependent manner (Dominguez et al., 2005). Additionally, when BACE1 is overexpressed, a second cleavage site at position

11 of A β has also been reported, although the significance of this minor cleavage site is unclear (Liu et al., 2002; Willem et al., 2004).

Studies from BACE1 KO mice indicate an important physiological function for BACE1. BACE1 KO mice have increased mortality, retinal defects and display a hyperactive phenotype compared to wild-type mice (Dominguez et al., 2005; Harrison et al., 2003; Cai et al., 2012). BACE1/BACE2 double KO mice have a further increased lethality phenotype compared to BACE1 KO mice, indicating an important role of BACE1/2 in normal physiology (Dominguez et al., 2005). Subsequent characterisation of BACE1 KO mice and over-expressing mice has revealed a role of BACE1 in myelination (Hu et al., 2006; Willem et al., 2006). This function of BACE1 is attributable to its action in cleaving neuregulin-1 and neuregulin-3, which are involved in controlling myelination (Hu et al., 2006; Willem et al., 2006; Hu et al., 2008). The combined sheddase action of BACE1 and ADAM17 on neuregulin releases an ectodomain fragment, which acts in a paracrine manner to stimulate myelination (Fleck et al., 2013). Functionally, this interaction is important for the development of muscle spindles, sensory bodies that detect muscle elongation (Cheret et al., 2013).

Other BACE1 substrates that have been identified include the sodium channel Na_v1.1 β 2 subunit, β -sialyltransferase-1, P-selectin glycoprotein ligand-1, Low-density lipoprotein receptor-related protein 1, Interleukin-1 receptor II, APLP1/2, and VEGFR1 (Klaver et al., 2010; Cai et al., 2012). Although the biological significance of BACE 1 cleavage of these substrates is not yet defined, they may have implications for the use of BACE1 inhibitors as a strategy for the treatment of AD (Klaver et al., 2010).

1.4.1.3 γ -Secretase

γ -Secretase is a transmembrane protease consisting of at least four protein subunits, presenilin (PS) 1 or 2, nicastrin, anterior pharynx-defective phenotype (Aph-1), and presenilin enhancer 2 (Pen-2; De Strooper et al., 1998; Francis et al., 2002; Scheuner et al., 1996; Yu et al., 2000). Presenilin has been shown to be the catalytic subunit, and has been demonstrated to have intrinsic catalytic γ -secretase activity (Ahn et al., 2010). However, all four subunits of γ -secretase are required for full activity (Edbauer et al., 2003). Mutations in nicastrin modulate APP processing (Yu et al., 2000), and knock down of Pen-2 or Aph-1 reduces γ -secretase processing of APP (Francis et al., 2002).

γ -Secretase has a number of important substrates other than APP, most notably Notch (De Strooper et al., 1999; Struhl and Greenwald, 1999). Mechanistic dissection of γ -secretase function in a number of these substrates has revealed a number of interesting features of γ -secretase cleavage, which has been collectively termed “regulated intramembrane proteolysis” (RIP; Ebinu and Yankner, 2002; Lichtenthaler et al., 2011). RIP cleavage occurs within the transmembrane domain of γ -secretase substrates (Lichtenthaler et al., 2011). This process requires the substrate protein to have a short ectodomain and a favourable transmembrane region (Hemming et al., 2008; Struhl and Greenwald, 1999). No primary amino acid sequence motif is shared by all γ -secretase substrates, so selectivity for substrate may be conferred by structural requirements (Hemming et al., 2008).

RIP cleavage is not thought to occur at a single peptide bond, but as a series of cleavages, starting from the C terminal end of the substrate and moving towards the N terminal region of the transmembrane domain (Fig.1.3).

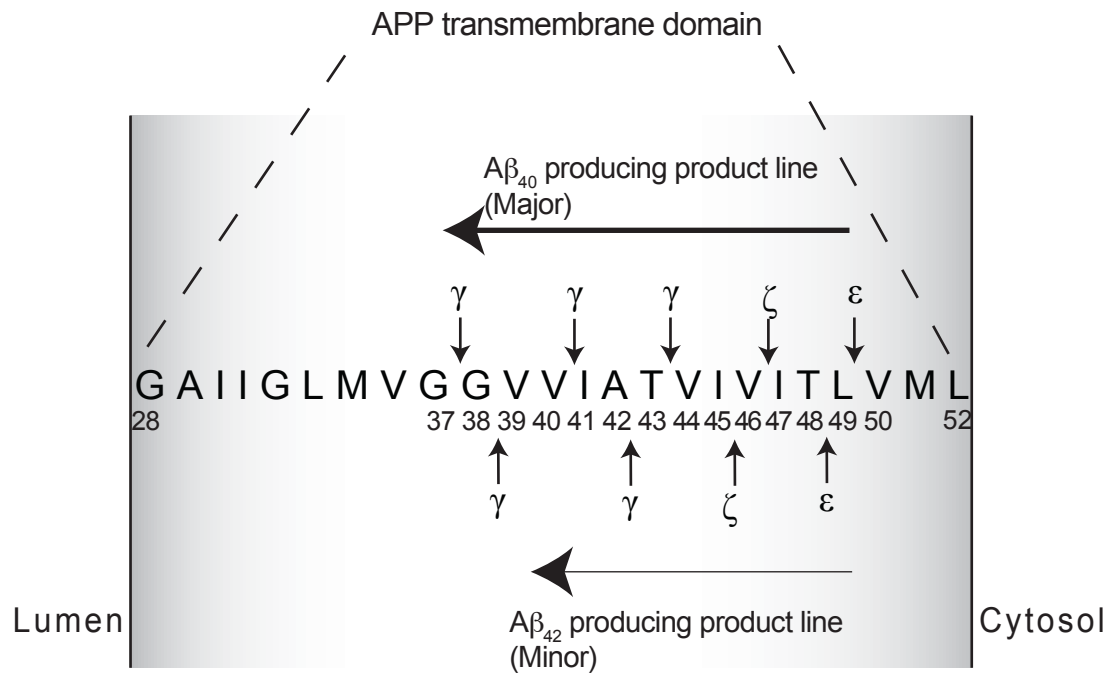


Figure 1.3. Stepwise RIP γ -secretase processing of APP. γ -Secretase cleavage of APP occurs at multiple sites, starting from the C-terminus and following the order ϵ -cleavage, ζ -cleavage and γ -cleavage. γ -Secretase cleavage has been proposed to occur in two distinct product lines, the A β_{40} producing (major) product line depicted by a thick arrow and the A β_{42} producing (minor) product line depicted by a thin arrow. Both of these production lines follow the same order and 3-dimensional interval between the ϵ -cleavage, ζ -cleavage and γ -cleavage sites, however the starting position is shifted. Amino acid numbers refer to the A β sequence. Figure based on Qi-Takahara, et al. (2005), Lichtenthaler, et al. (2011) and Haass et al. (2012).

In APP, these cleavage sites have been named as the γ -, ϵ - and ζ - sites (Gu et al., 2001; Qi-Takahara et al., 2005; Sastre et al., 2001; Weidemann et al., 2002; Yu et al., 2001; Zhao et al., 2004; Zhao et al., 2005). These cleavages occur in the order ϵ -, ζ - and γ -, however the exact residues can shift (Qi-Takahara et al., 2005). This shift may be determined by the three-dimensional orientation of the substrate in the γ -secretase active site (Qi-Takahara et al., 2005). As a result, it has been suggested that there are two production lines for the γ -secretase processing of APP; the $A\beta_{40}$ producing (major) line and the $A\beta_{42}$ producing (minor) line (Fig. 1.3; Qi-Takahara et al., 2005). Increasing membrane thickness has been demonstrated to result in the formation of less $A\beta_{42/43}$, therefore the position of the initial γ -secretase cleavage may be regulated by the lipid environment of the γ -secretase-substrate complex (Winkler et al., 2012).

1.5 Genetics of AD

1.5.1 Genetics of FAD

A small number of families worldwide carry rare heritable mutations that cause AD. Studying these mutations has given insight into the molecular etiology of AD. There are now over two hundred mutations that have been linked to dominantly inherited AD (FAD; Cruts et al., 2012). Importantly, mutations associated with AD occur in APP and presenilin, so there are key mechanistic links of FAD genes to APP processing and $A\beta$ production, which will be discussed in the following sections.

1.5.1.1 Mutations in APP

Thirty-three FAD APP mutations have been characterised in 90 families (Cruts et al., 2012). These mutations include point mutations, as well as a number of APP locus duplications (Cruts et al., 2012; Rovelet-Lecrux et al., 2006). The first pathogenic APP mutation to be discovered was associated with hereditary cerebral haemorrhage, a condition in which amyloid is deposited in cerebral blood vessels (Levy et al., 1990). Shortly afterward, the V717I mutation, which is a point substitution near the A β encoding region close to the γ -secretase cleavage site, was linked to FAD (Goate et al., 1991). This mutation was named “London” after the location of the family group that carried the gene; a convention that has been used for a number of other APP mutations.

In general, the APP point mutations are thought to accelerate AD progression by altering either the deposition of A β , the aggregation of A β or the ratio of A β ₄₀/A β ₄₂ (Selkoe, 2001; Tanzi and Bertram, 2005). The majority of APP mutations cluster in the region of the A β sequence, with many mutations found close to secretase cleavage sites (Fig. 1.4). For example, the “Swedish” mutation results in a substitution of two residues immediately N-terminal to the β -secretase cleavage site (K670N/M671L). This double mutation produces a 6 - 8 fold increase in the amount of A β that is produced (Citron et al., 1992).

More recently, an APP mutation (A673T) has been reported to be protective against late-onset AD and normal age-related cognitive decline (Jonsson et al., 2012). The A673T mutation is immediately adjacent to the β -secretase cleavage site, and results in a 40% reduction in β -cleavage of APP in vitro. This mutation provides support for a link between LOAD and A β production from APP.

APP-770 - Aβ region

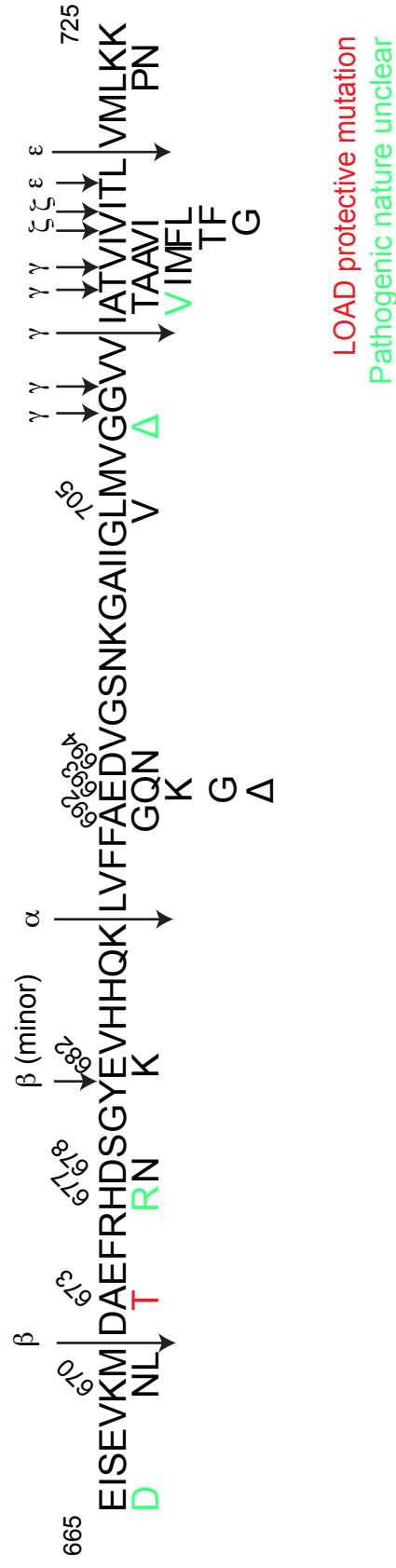


Figure 1.4. APP mutations associated with FAD. FAD-associated mutations are shown in black under the APP-770 sequence. APP mutations that have not been reported to be pathogenic are shown in green. A mutation that is protective against LOAD is shown in red. (Δ) represents a deletion mutation. Sequence numbers refer to APP-770 numbering. Major secretase cleavage sites are depicted by long arrows, and minor sites by short arrows. Figure adapted from Lichtenthaler et al., (2011); Selkoe (2001) and the Alzheimer disease and frontotemporal dementia mutation database (Cruts et al., 2012).

1.5.1.2 Presenilin mutations

The most common causes of FAD are mutations in the presenilins (Selkoe, 2001). The presenilins were initially discovered in a screen searching for mutations leading to FAD, and were named accordingly (Sherrington et al., 1995; Levy-Lahad et al., 1995). There are now 185 mutations in PS1 associated with FAD and 13 mutations in PS2 associated with FAD (Cruts et al., 2012). Interestingly, the first person to be diagnosed with Alzheimer's disease carried a PS1 mutation (Muller et al., 2013). Presenilins (PS) are multi-pass transmembrane proteins, which form catalytic subunits of the γ -secretase complex (see Section 1.4.1.3). Mutations in PS1 and other γ -secretase components have also been implicated in familial scarring acne, cardiomyopathy and heart failure (Li et al., 2006; Wang et al., 2010; Pink et al., 2013).

PS mutations associated with FAD cause an increase in $A\beta_{42}$, which may result in the earlier onset of AD symptoms (Duff et al., 1996; Scheuner et al., 1996; Potter et al., 2013). However a recent in vitro study reported that although PS mutations shift the $A\beta_{40:42}$ ratio, many PS mutations confer an overall reduction in γ -secretase activity (Cacquevel et al., 2012). Therefore, further research may reveal other detrimental effects of PS mutations, besides alteration of $A\beta$ production.

1.5.2 Transgenic animal models of AD

Transgenic animal models have provided strong evidence for $A\beta$ being the causal factor in AD. There are many transgenic animal models available of AD, which are catalogued on the AlzForum web database (<http://www.alzforum.org/res/com/tra/default.asp>). The first transgenic mouse

model of AD reported was the PDAPP mouse, which over-expresses APP carrying a mutation around the γ -secretase site (V717F). These mice develop a neuropathology similar to AD, including A β deposits, neuritic plaques, synaptic loss, astrogliosis and microgliosis (Games et al., 1995). Mice expressing human APP with the Swedish mutation (APP_{SW}), produce a 14-fold increase in the amount of A β ₄₂, develop A β plaques after 11 months and display cognitive deficits after 9 months (Hsiao et al., 1996). Mice carrying FAD mutations in PS genes produce an elevated amount of A β ₄₂ due to altered APP processing (Duff et al., 1996). When multiple FAD mutations are combined, transgenic mice carrying more than one AD mutation develop A β deposits and cognitive deficits more rapidly than single mutations. Double-transgenic mice harbouring the APP_{SW} mutation and a PS1 mutation (A264E) go on to deposit amyloid more rapidly than mice with the APP_{SW} mutation alone (Borchelt et al., 1997).

However, reproducing the complete spectrum of pathology associated with AD has not been achieved in mice carrying a single mutation. None of the APP or presenilin mice developed to date display tangle pathology with paired helical fragments (German, 2007). NFTs have been produced in Tau_{P301L} mice, which express tau carrying a mutation associated with frontotemporal dementia with parkinsonism (Gotz et al., 2001; Lewis et al., 2000). Transgenic mice that express multiple human mutations in tau, APP and PS have been reported that reproduce a wider range of AD pathology. Triple transgenic mice (3xTg-AD) carrying the Tau_{P301L}, APP_{SW} and PS_{M146V} mutations develop both plaque and tangle pathology, with intraneuronal accumulation of A β preceding both tangle formation and plaque development (Oddo et al., 2003).

1.5.3 Genetics of LOAD

Whilst the genetic risk factors for FAD have a clear influence on producing AD symptoms, the known genetic risk factors for LOAD have less clear effects and their roles in enhancing progression of AD are still poorly understood. Until recently, only a few genetic risk factors for LOAD had been identified. However, the advent of genome-wide association studies (GWAS) has revealed many more genetic variants that contribute to LOAD risk. The top ten risk genes for LOAD ranked by the Alzgene database meta-analysis are listed in Table 1-1 (Bertram et al., 2007). Apolipoprotein E (apoE), clusterin, and phosphatidylinositol-binding clathrin assembly protein (PICALM) are the most well studied genetic risk factors for LOAD and are discussed in the sections below. Mutations in BIN1, CR1, ABCA7, TREM, CD33 and MS4A6A will not be discussed here, as there are no studies that have elucidated how these proteins are mechanistically involved in AD progression. A recent systems biology study of these LOAD risk factors found no effect of siRNA knock down of any of these proteins on A β production (Bali et al., 2012). Therefore, it is likely that these LOAD risk alleles enhance AD progression through other mechanisms unrelated to an increase in A β production.

Table 1-1 Genetic risk factors for LOAD

Gene	Odds Ratio	Protein	Potential Functions
ApoE 2/3/4	3.685	Apolipoprotein E	Lipid transport
BIN1	1.116	Bridging-integrator 1	Synaptic vesicle endocytosis. Tumour suppressor
CLU	0.879	Clusterin (apolipoprotein J)	Lipid transport
ABCA7	1.229	ATP-binding cassette, sub-family A, Member 7	Lipid homeostasis
CR1	1.174	Complement receptor 1	Immune response
PICALM	0.879	Phosphatidylinositol-binding clathrin assembly protein	Endocytosis, clathrin assembly
MS4A6A	0.904	Membrane-spanning 4-domains, subfamily A, member 6A	Unknown
CD33	0.893	Myeloid cell surface antigen CD33	Unknown
MS4A4E	1.079	Membrane-spanning 4-domains, subfamily A, member 4E	Unknown
CD2AP	1.117	CD2-associated protein	Regulation of actin cytoskeleton
TREM2*	2.92	Triggering receptor expressed on myeloid cells 2	Inflammation/ phagocytosis

Table shows risk genes for LOAD, ranked by the Alzgene database meta analysis; <http://www.alzgene.org>, accessed 18 April 2013 (Bertram et al., 2007). * The TREM2 association with LOAD has been recently discovered, and has not yet been ranked by the Alzgene database meta analysis (Guerreiro et al., 2013; Jonsson et al., 2013).

1.5.3.1 ApoE

The most well established genetic risk factor for LOAD is the $\epsilon 4$ allele of apolipoprotein E gene (Strittmatter et al., 1993). The $\epsilon 4$ allele is associated with an increased risk of AD, and the $\epsilon 2$ allele is associated with a reduced risk (Corder et al., 1993; Corder et al., 1994; Strittmatter et al., 1993). Physiologically, the main role of apoE is to transport lipids, cholesterol and lipid soluble vitamins throughout the body (Mahley, 1988). For example, apoE has a key role in transporting lipids for degradation by hepatocytes in the liver, and mutations in apoE cause hyperlipoproteinemia type III (Havel and Kane, 1973; Utermann et al., 1975). ApoE is a component of chylomicrons, very low-density lipoproteins (vLDL), and high-density lipoproteins (HDL; Shore and Shore, 1973; Weisgraber and Mahley, 1978). ApoE is a ligand for the low-density lipoprotein receptor (LDL receptor), apoE receptor 2, very low-density lipoprotein receptor and the low-density lipoprotein receptor-related protein (LRP; Innerarity et al., 1978; Kim et al., 1996; Takahashi et al., 1996; Beisiegel et al., 1989).

In the CNS, apoE is mainly expressed by astrocytes (Boyles et al., 1985; Grehan et al., 2001) and astrocytic apoE has been shown to be involved in synaptogenesis and neurite outgrowth as a consequence of its role in lipid transport (Mauch et al., 2001; Nathan et al., 1994). Although apoE knock-out (KO) mice have no major cognitive deficit (Anderson et al., 1998; Piedrahita et al., 1992; Anderson et al., 1998), recent studies have demonstrated that human apoE $\epsilon 4$ carriers have subtle changes in brain structure during development (Knickmeyer et al., 2013).

1.5.3.2 Role of apoE in AD

It is not yet resolved how the apoE $\epsilon 4$ allele increases the risk of AD, however the majority of mechanistic suggestions are based on the suggestion that apoE binds A β and increases A β load in the brain (Hass et al., 1998). The apoE isotypes $\epsilon 4$, $\epsilon 3$, and $\epsilon 2$ have been suggested to bind A β with differing affinities, with $\epsilon 4$ having the weakest affinity for A β (Strittmatter et al., 1993; Yang et al., 1997; Tokuda et al., 2000). Cells have been reported to internalise apoE-bound A β via an LRP1-dependent mechanism, which has been suggested to be a possible mechanism of A β clearance (Yang et al., 1999; Kang et al., 2000). Consequently, it is suggested that the apoE $\epsilon 4$ allele, which binds A β less strongly, results in a perturbed degradation of the A β protein, predisposing individuals with the $\epsilon 4$ allele to LOAD (Kim et al., 2009; Deane et al., 2008). However, It should be noted that there is some controversy over whether apoE actually binds A β . A recent study found that apoE does not specifically bind A β , and concluded that the observed effects of apoE on A β clearance were actually due to competition for the same clearance receptor (Verghese et al., 2013). Further, genetic mouse studies do not support a role of apoE in A β clearance. PDAPP crossed with apoE knock out mice display decreased A β deposition compared to those expressing apoE (Bales et al., 1997). However, when clusterin and apoE double knockout mice are crossed with PDAPP mice, A β deposition is greatly increased compared to the PDAPP mouse model (DeMattos et al., 2004). This may suggest an overlapping effect of apoE and clusterin on A β metabolism.

An alternative suggestion for the role of apoE in AD is that apoE may modulate the aggregation of A β . Several studies have reported that in vitro apoE can

increase the fibrillisation of A β (Ma et al., 1994; Castano et al., 1995; Wisniewski et al., 1994). However, there are also reports that apoE inhibits the fibrillisation of A β (Evans et al., 1995; Wood et al., 1996). The conflicting results may be due to different lipidation states of apoE used in the studies, or isoforms of A β (Yang et al., 1999; Kim et al., 2009). More recently, apoE ϵ 4 has been shown to increase the formation of oligomeric species of A β (Cerf et al., 2011; Hashimoto et al., 2012).

Importantly, apoE has an important physiological function, which could confer increased risk of AD without being involved directly in the central disease process. The apoE ϵ 4 allele has been linked to developmental abnormalities, as ϵ 4 carriers have altered brain structure prior to birth (Knickmeyer et al., 2013). Therefore, further studies are required to elucidate the biochemical relationship between apoE function and AD progression.

1.5.3.3 Clusterin/ApoJ

Clusterin (also known as apolipoprotein J) has been identified as a risk factor for LOAD by several large GWAS studies (Harold et al., 2009; Lambert et al., 2009; Seshadri et al., 2010). Clusterin has been reported to be upregulated in AD patients, prior to its establishment as a genetic risk factor for LOAD (May et al., 1990). Like apoE, clusterin forms HDL lipoprotein complexes and has a role in lipid transport throughout the circulation and the CNS (de Silva et al., 1990; Borghini et al., 1995). Also similar to apoE, clusterin has been identified to interact with A β , and may be involved in A β clearance and aggregation (Nuutinen et al., 2007; Oda et al., 1995; Nuutinen et al., 2009). Studies in transgenic mice reported that double KO of apoE and clusterin could increase the deposition of

A β , therefore these studies provide evidence that clusterin could be involved in the metabolism of A β (DeMattos et al., 2004).

1.5.3.4 PICALM

Alleles of the phosphatidylinositol-binding clathrin assembly protein gene (PICALM) have been associated with increased risk of LOAD by a number of large GWAS studies (Seshadri et al., 2010; Harold et al., 2009). PICALM and its homologue AP180 play roles in endocytosis, by recruiting AP2 and clathrin to the membrane (Ford et al., 2001). This process is involved in the formation of clathrin-coated vesicles (Ford et al., 2001). In the brain, PICALM and AP180 have been demonstrated to play a role in the formation of synaptic vesicles (Zhang et al., 1998; Nonet et al., 1999; Xiao et al., 2012).

PICALM is predominately expressed in endothelial cells in the brain (Baig et al., 2010). One study reported slightly increased levels of PICALM expression in the frontal cortex of AD brain, however, the effects observed were not observed in other brain regions, and may need further confirmation (Baig et al., 2010). Additionally, there are reports of reduced AP180 expression in AD brain (Yao et al., 1999). Therefore, levels of PICALM and AP180 may be affected in AD.

The mechanistic link between AD pathology and PICALM is still being elucidated. There are reports that both AP180 and PICALM are able to alter the proteolytic processing of APP. Prior to the establishment of PICALM alleles as risk factors for AD, AP180 knockdown was reported to affect the production of A β (Wu et al., 2009). However, the same study was not able to find an effect of PICALM knockdown on A β production (Wu et al., 2009). A more recent study demonstrated that PICALM and APP colocalise at the cell surface after

internalization of APP (Xiao et al., 2012). Knockdown of PICALM decreased the amount of internalised APP, and PICALM overexpression increased the internalisation of APP, which provides strong evidence that APP endocytosis can be regulated by PICALM. Additionally, the same study found an increase in amyloidogenic processing of APP in PICALM over-expressing cells and a decrease in A β production when PICALM expression was knocked down. These early studies suggest that PICALM (and AP180) may affect AD pathogenesis through their endocytic function (Xiao et al., 2012).

1.5.4 Summary - Genetics of AD

The studies reviewed in this section demonstrate central involvement of a number of proteins in the pathogenesis of AD. Many mutations in APP and PS are associated with FAD. APP and PS are essential for the production of A β , which deposits in AD to form amyloid plaques. Many FAD-associated APP and PS mutations are thought to modify the production of A β . Genetic mouse models carrying human APP and PS mutations mimic some of the pathology seen in AD. Therefore, these studies provide strong evidence implicating APP, A β and PS in the pathogenesis of AD. The mechanisms by which LOAD-associated mutations increase the risk of AD are not as well understood as for FAD-associated mutations. However many studies suggest that alleles that confer risk for LOAD may also affect the biology of APP and/or A β . Therefore, further investigation of the function of these genes in AD pathogenesis is required to provide insight into the biochemical basis of LOAD.

1.6 APP function

Despite intensive research focus on the links between APP and AD pathogenesis, the normal function of APP is not resolved. Understanding the normal role of APP may be important for the development of new therapeutic strategies for the treatment of AD and also to determine the effects of current treatments that target APP processing as a strategy to reduce A β production (Citron, 2010). This section aims to summarise the main themes of research relating to APP's function in order to highlight current knowledge but also to emphasise directions for further research. This topic has also been examined by a number of other reviews (Muller and Zheng, 2012; Aydin et al., 2012; Mattson, 1997; Zheng and Koo, 2006).

1.6.1 Lessons from APP family knock-out and knock-in mice

Genetic knockout models are one of the most powerful ways of investigating the functional roles of genes. APP-KO mice are viable and fertile, indicating that APP does not play an essential role in development (Li et al., 1996; Muller et al., 1994; Zheng et al., 1995). However, APP-KO mice do have a number of subtle phenotypic abnormalities. APP-KO mice are slightly smaller, with a reduced weight of 15 - 20%, and reduced brain weight (Magara et al., 1999; Zheng et al., 1995). APP-KO mice display a number of apparent neurological deficiencies, such as a deficit in grip strength and locomotor activity (Ring et al., 2007; Zheng et al., 1995). Additionally, aged APP-KO mice have reactive gliosis in the entorhinal cortex, hippocampus and parietal cortex (Zheng et al., 1995). APP-KO mice have also been reported to have a number of phenotypes that are associated with altered neuronal function, such as hypersensitivity to kainate-induced

seizures, alterations in dendritic spine density, and reduced performance in tests of spatial memory (Dawson et al., 1999; Phinney et al., 1999; Steinbach et al., 1998). These observations provide strong evidence that APP is involved in the formation or maintenance of the nervous system.

However, the fact that the phenotype of APP-KO mice is non-lethal does not mean that APP has no developmental function. An important insight into the nature of APP function has come from the combined KO of APP and its family members APLP1 and APLP2. Like APP, APLP1-KO mice and APLP2-KO mice are both viable and fertile (Heber et al., 2000; von Koch et al., 1997). Double knock out of APLP1 and APP also does not produce a lethal phenotype (Heber et al., 2000). However, APP/APLP2 double KO mice and APLP1/APLP2 double KO mice both have a post-natal lethal phenotype (Heber et al., 2000; von Koch et al., 1997). APP/APLP2 double KO mice have impaired neuromuscular junction formation, evident in reduced numbers of synaptic vesicles, excessive nerve terminal sprouting, incorrect apposition of pre-and post- synaptic proteins, and impaired synaptic transmission (Wang et al., 2005). These synaptic deficits may be responsible for the lethality phenotype in APP/APLP2 double KO mice (Wang et al., 2005). Together, APP family KO mice suggest that APP and APLP2 have a redundant essential function throughout development.

Further insights into the biological functions of APP have come from conditional knockout studies. Knock in of sAPP α into APP/APLP2 KO mice (APPs α -DM mice) rescues the lethal phenotype (Weyer et al., 2011). Similarly, APL-1 knock out in *C. elegans* is larval-lethal, and this phenotype can be rescued by knock in of the APL-1 extracellular domain (Hornsten et al., 2007). These studies demonstrate

that the extracellular domain of APP and/or APLP2 is essential for viability. Knock in of APP carrying a non-functional YENPTY (Y682G mutation) motif into APP-KO mice (APP^{YG/YG} mice) results in altered endocytosis of APP (Ring et al., 2007). Therefore, the C-terminal Y⁶⁸²ENPTY motif of APP probably plays an important role in APP trafficking in the cell, and consequently in APP processing (Ring et al., 2007; discussed in more detail in Section 1.6.5). Importantly, crossing APP^{YG/YG} mice with APLP2-KO mice produces a lethal phenotype, presumably because the YENPTY motif in the C-terminus of APP is required for APP to function correctly (Barbagallo et al., 2011). These studies indicate that the APP family has at least two functional components involved in essential developmental functions, the extracellular secreted N-terminus, and the YENPTY-containing intracellular domain. However, rescued lethality does not exclude the possibility that other regions of APP hold non-essential developmental functions (i.e. functions which are not required for survival). For example, APP α -DM mice still display deficits in synaptic maturation and hippocampal function (Weyer et al., 2011). In a similar experimental paradigm to APP α -DM mice, sAPP β has been knocked into APP/APLP2 double KO mice. However, there was no rescue of the lethal phenotype in these mice, which indicates that the α - and β - pathways of APP processing are likely to have distinct functions (Li et al., 2010). Perhaps importantly, the studies from Li et al. (2010) and Weyer et al. (2011) raise the possibility that the first 16 amino acids of the A β sequence are somehow crucial for avoiding the lethal phenotype in the APP/APLP2 double KO mice.

1.6.2 Trophic functions of APP

APP may be involved in the growth and survival of neurons. There are a variety of reports of neurotrophic functions for APP and some of its metabolites, most of which implicate the extracellular region of APP as an important positive effector of cell growth. Studies in which recombinant full-length APP has been added into cell culture have reported that APP is able to increase neurite outgrowth and neuronal survival (Araki et al., 1991; Ohsawa et al., 1995). Similar effects on neuronal survival and neurite elongation have been reported with APP purified from human and mouse brain, that were attributable to the heparin-binding domain in the E1 region of APP (Small et al., 1994). Correspondingly, in vitro knockdown of APP expression is reported to reduce neurite outgrowth (Allinquant et al., 1995). Mice that overexpress human APP have increased numbers of synapses, suggesting a synaptotrophic role for APP (Mucke et al., 1994).

sAPP α may act in a trophic manner in a number of cell types and has been reported to increase the proliferation and/or survival of fibroblasts, keratinocytes, B109 cells, FRTL-5 cells, PC12 cells, and cortical neurons (Saitoh et al., 1989; Hoffmann et al., 2000; Ninomiya et al., 1994; Pietrzik et al., 1998; Milward et al., 1992; Jin et al., 1994; Araki et al., 1991; Young-Pearse et al., 2008). A number of these studies also found increases in neurite outgrowth in neuronal and pseudo-neuronal cells (Araki et al., 1991; Jin et al., 1994; Milward et al., 1992; Ninomiya et al., 1994). Additionally, there are some reports that infusion of sAPP α into animal models of traumatic brain injury can improve neuronal survival and

recovery (Copanaki et al., 2010; Thornton et al., 2006). Collectively, these studies provide strong evidence that sAPP α can act in a trophic manner.

1.6.2.1 APP in stem cell proliferation and differentiation

Throughout development and adulthood, pluripotent stem cell populations differentiate into distinct lineages of progenitor cells, which ultimately form the cells of the central nervous system (Gage, 2000). A number of studies have noted that APP is expressed in neuroblasts and neurons as they undergo differentiation and growth (Clarris et al., 1995; Fukuchi et al., 1992; Salbaum and Ruddle, 1994; Small et al., 1992; Masliah et al., 1992). This has led many investigators to question whether APP has a role in development in the control of stem-cell proliferation or differentiation. In addition, APP is processed in a manner that is very similar to the key developmental protein Notch, leading to the suggestion that APP could have a similar developmental function (De Strooper et al., 1999; Struhl and Greenwald, 1999; Zhang et al., 2000). This section will discuss the current evidence for a role of APP in stem cell proliferation and integration.

There is ample evidence that APP is able to positively modulate the proliferation of neural stem and progenitor cells (NSPCs) in vitro. Studies have reported that sAPP α and sAPP β can promote the proliferation of NSPCs (Ohsawa et al., 1999; Hayashi et al., 1994; Baratchi et al., 2012). Hayashi et al. (1994) also examined the effect of secreted APP-770 on NSPC proliferation and found secreted APP-770 to have a stronger effect on NSPC proliferation than secreted APP-695. A more recent study has also reported that inhibition of α -secretase can reduce NSPC proliferation and sAPP α is able to rescue this effect (Demars et al., 2011). Therefore, sAPP α appears to be able to modulate NSPC proliferation in vitro. In

vivo, sAPP α infused into the ventricles of mice binds selectively to epidermal growth factor receptor (EGFR) expressing and PSA-NCAM-expressing stem cells in the subventricular zone (Caille et al., 2004). Both the secretion of EGF and the proliferation of these EGFR-expressing cells were increased by sAPP α infusion (Caille et al., 2004). Therefore, sAPP α may regulate proliferation of NSPCs in vivo.

Aside from the reported ability of sAPP α to modify NSPC proliferation, recent evidence has demonstrated an effect of APP gene expression on stem cell proliferation. We recently reported that the expression of APP can positively modulate the proliferation of NSPCs and this effect was reduced in APP-KO NSPCs (Hu et al., 2013). The APP-driven increase in NSPC proliferation was not due to the secretion of sAPP α , but rather an effect of APP on the expression and secretion of cystatin C (Hu et al., 2013). Therefore, collectively these studies indicate that APP may act on NSPC proliferation in two modes. Firstly, through the production of sAPP α and secondly, through the modulation of other genes and proteins involved in NSPC regulation, such as cystatin C.

Further indications that APP is involved in the control of NSPC proliferation have come from observations made in AD transgene models. Although these models are complicated by the presence of disease-associated processes, they have nevertheless provided some interesting observations linking APP to NSPC function. PDGF-APP(sw, ind) mice which overexpress human APP with the Swedish and Indiana FAD mutations, have a 2-fold increase in the number of proliferating stem cells in the dentate gyrus and subventricular zone at an age of 3 months (Jin et al., 2004; Lopez-Toledano and Shelanski, 2007). However, as A β

starts to accumulate, this effect decreases (Lopez-Toledano and Shelanski, 2007). Other studies have also found increased NSPC proliferation in various AD mouse models, but suggested the effect was due to A β deposition (Kolecki et al., 2008; Sotthibundhu et al., 2009; Verret et al., 2007). Conversely, a number of studies have reported decreased NSPC proliferation in various AD mouse models (Naumann et al., 2010; Dong et al., 2004; Haughey et al., 2002; Donovan et al., 2006). Therefore, together these studies do not clarify whether it is APP expression, APP mutations, A β deposition, transgene effects, genetic background or age that is able to affect NSPC proliferation in AD mouse models *in vivo*. However, the clear effects of AD mutations and/or APP expression on NSPC proliferation in AD mouse models warrant further investigation.

APP may also play a role in regulating the differentiation of NSPCs. A recent study using human embryonic stem cells found that APP overexpression, or addition of sAPP α enhanced neuronal differentiation (Freude et al., 2011). We also found that the over-expression of APP in Tg2576 derived NSPCs increased neural differentiation, and APP knockout decreased neural differentiation (Hu et al., 2013). However, a conflicting report presents evidence that sAPP α/β may cause an increase in differentiation to a glial lineage (Baratchi et al., 2012). There were marked differences in the time scales used between these studies, so further investigation will be required to fully establish a role of APP in NSPC differentiation. It should also be noted that the APP family of proteins is not required for neural differentiation, as embryonic stem cells derived from APP triple KO mice still form neuronal precursors (Bergmans et al., 2010).

1.6.2.2 Mechanism of the trophic effects of APP

It is not clear whether APP is able to directly elicit trophic effects, e.g. by activating a growth factor receptor. Some receptors for APP that have been suggested are β 1-integrin, lipoprotein receptor related protein-1, death receptor 6, p75 neurotrophin receptor and APP itself (Young-Pearse et al., 2008; Kounnas et al., 1995; Nikolaev et al., 2009; Gralle et al., 2009). However, APP may interact with many other extracellular proteins (Bai et al., 2008). Therefore, it is unclear which of these interactions may be important.

Importantly, many indirect mechanisms of action have also been reported that make mechanistic interpretation of APP's trophic effects complex. A number of studies have demonstrated that APP is able to modify the trophic effects of other growth factors. For example, secreted APP is able to potentiate nerve growth factor (NGF) signalling, possibly by increasing the affinity of the NGF receptor for NGF (Wallace et al., 1997; Milward et al., 1992; Akar and Wallace, 1998). More recently, APP has been suggested to regulate NGF/TrkA signalling, through an intracellular interaction involving the C-terminal YENPTY phosphorylation site (Matrone et al., 2011). Curiously, NGF, EGF, basic fibroblast growth factor (FGF2) and brain-derived neurotrophic factor have all been reported to increase the expression of APP (Cosgaya et al., 1996; Lahiri and Nall, 1995; Mobley et al., 1988; Ohyagi and Tabira, 1993; Ruiz-Leon and Pascual, 2001; Villa et al., 2001; Clarris et al., 1994). Also, NGF, EGF, insulin and insulin-like growth factor 1 have been reported to increase the secretion of sAPP α (Caille et al., 2004; Slack et al., 1995; Ruiz-Leon and Pascual, 2001; Solano et al., 2000; Jacobsen et al., 2010). We have recently shown that expression of APP can affect the proliferation of neural stem cells in an indirect manner, by modulating the expression and

secretion of cystatin C (Hu et al., 2013). Therefore, these studies above highlight that the trophic actions of APP are complex, and strongly implicate APP as a modulator of cellular growth pathways. Importantly, as APP has some functional interplay with other effectors of cell growth, careful consideration of experimental conditions is needed to delineate the mechanisms underlying the trophic actions of APP.

1.6.3 Roles of APP in cell adhesion and synaptogenesis

Some studies have suggested APP could have a function in cell-adhesion. The extracellular region of APP can bind laminin, collagen type I, heparin and glypican-1 (Beher et al., 1996; Clarris et al., 1997; Kibbey et al., 1993; Williamson et al., 1996; Mok et al., 1997). Physical adhesion to these extracellular components could explain the effects of APP on neurite outgrowth (Kibbey et al., 1993; Small et al., 1994).

APP has also been proposed to have other roles in cell adhesion beside adherence to extracellular matrix components. One suggestion is that APP and its homologues APLP1/2 could form physical contacts between cells (Soba et al., 2005). In the presence of heparin, APP can form trans-dimers that might act as cell-to-cell contacts (Dahms et al., 2010; Gralle et al., 2006). Trans-dimerisation of APP has also been proposed as a mechanism for the stabilisation of synapses by APP (Wang et al., 2009). Another suggestion for a role of APP in cell adhesion is that APP may modulate other proteins involved in cell adhesion. APP has been suggested to interact with integrin, fasciclin II, contactin 4, neuroglia cell adhesion molecule, and transient axonal glycoprotein-1, which are all proteins implicated in cell-adhesion (Ashley et al., 2005; Ma et al., 2008; Osterfield et al.,

2008; Yamazaki et al., 1997; Young-Pearse et al., 2008). These studies provide a number of avenues by which APP may perform functions in cell-adhesion. However, it is hard to determine whether effects of APP on neurite outgrowth are due to increased adhesion or increased growth, and therefore further work will be needed to delineate the precise mechanisms of action.

1.6.4 Non-neuronal functions of KPI domain-containing APP isoforms (Protease nexin II)

The majority of research into the biology of APP has considered the roles of APP-695, as this is the APP isoform that is predominantly expressed in neurons. However, the secreted KPI-domain containing 770 and 751 isoforms of APP (collectively termed here APP-770/751, but also known as protease nexin II (Van Nostrand et al., 1989) have well-described roles in non-neuronal contexts that may be able to inform our understanding of APP function in the brain.

In the blood, APP is predominantly expressed in platelets (Bush et al., 1990; Gardella et al., 1990; Van Nostrand et al., 1991a; Smith and Broze, 1992). In platelets, APP, sAPP and A β accumulate in α -granules, intracellular vesicles that are used to store a variety of clotting factors (Van Nostrand et al., 1991b; Blair and Flaumenhaft, 2009). Upon platelet stimulation APP, secreted APP and A β are released, along with a number of another components of the coagulation cascade (Bush et al., 1990; Gardella et al., 1990; Smith, 1997; Smith et al., 1990; Smith and Broze, 1992; Van Nostrand et al., 1990).

APP-770/751 is a potent inhibitor of the coagulation factors XIa, IXa and Xa (Scandura et al., 1997; Smith et al., 1990; Mahdi et al., 1995; Schmaier et al.,

1993). This inhibition of coagulation factors is due to the KPI domain, which inhibits factor Xa with a K_i of 400 pM (Scandura et al., 1997; Smith et al., 1990). However, other regions of APP also participate. Inhibition of factor XIa by APP is enhanced by heparin, suggesting an involvement of one of the heparin-binding regions of APP (Smith et al., 1990). The E1 N-terminal heparin-binding domain of APP can inhibit the activation of factor XII and also inhibit platelet activation, independently of the KPI domain (Henry et al., 1998; Niwano et al., 1995). These studies demonstrate that APP has a role as a suppressor of multiple stages of the coagulation cascade, which is not only due to the KPI domain.

Consistent with this role of APP as an inhibitor of multiple stages of the coagulation cascade, APP-770/751 stops blood from clotting in vitro (Schmaier et al., 1993; Annich et al., 1999). Genetic overexpression of APP in mice decreases cerebral thrombosis and also increases the severity of haemorrhage in animal models, and knockout of APP has the opposite effect (Xu et al., 2005; Xu et al., 2007). These functions of APP are conserved among APP family members (Xu et al., 2009). There is also evidence that this function of APP action may play a role in human disease. Familial hereditary cerebral haemorrhage, Dutch type (HCHWA-D) leads to haemorrhage, but also accumulation of APP and A β in the brain vasculature (Rozemuller et al., 1993). Furthermore, mutations in APP have been linked to this condition (Levy et al., 1990; Bakker et al., 1991; Fernandez-Madrid et al., 1991; Van Broeckhoven et al., 1990). Therefore, considering APP-770/751 can inhibit blood coagulation, it has been suggested that the over-accumulation of APP in blood vessels in HCHWA-D may be a cause of haemorrhage (Xu et al., 2005). However, this suggestion has not yet been experimentally tested. The role of APP as an anti-coagulant has also led to

suggestions it could be used for the treatment of conditions such as stroke or as a biomarker for coronary syndrome (Kitazume et al., 2012; Wu et al., 2012).

1.6.5 AICD interactions and functions

The intracellular domain of APP is highly conserved among APP family members (Muller and Zheng, 2012). This region of APP has a number of phosphorylation sites and interacts with a number of cytosolic adaptor proteins (Fig. 1.5, Section 1.6.5.2). Interactions between the intracellular domain of APP and cytosolic adaptor proteins can potentially occur with full-length APP, C99, C83, and also with the γ -secretase cleavage product AICD (Schettini et al., 2010). The roles of cytosolic adaptor protein-interactions in the trafficking and function of APP will be discussed in this section.

1.6.5.1 Functional motifs and phosphorylation sites in AICD

The APP C-terminus contains a YENPTY motif (residues 682 – 687 of the APP695 isoform). This motif is conserved in many tyrosine receptor kinases and non-receptor tyrosine kinases, which are type-1 transmembrane proteins that orchestrate cellular signalling processes (Lemmon and Schlessinger, 2010). The YENPTY motif has been demonstrated to be important in the trafficking of APP (Lai et al., 1995; Marquez-Sterling et al., 1997; Perez et al., 1999).

There are three potential tyrosine phosphorylation sites in the C-terminus of APP that have been identified in vitro, tyr⁶⁵³, tyr⁶⁸², and tyr⁶⁸⁷ (APP695 numbering system; Schettini et al., 2010). Tyr⁶⁸² and tyr⁶⁸⁷ are part of the “YENPTY” motif in the C-terminus of APP. Proteins containing SH3 domains or phosphotyrosine binding (PTB) domains selectively bind tyr⁶⁸² and tyr⁶⁸⁷, respectively (Lim and Pawson, 2010; Borg et al., 1996; Tarr et al., 2002b). Tyr⁶⁵³ may be involved in the sorting of APP to basolateral membranes, however this phosphorylation site is not as well characterised as tyr⁶⁸² and tyr⁶⁸⁷ (Haass et al., 1995; Schettini et al., 2010).

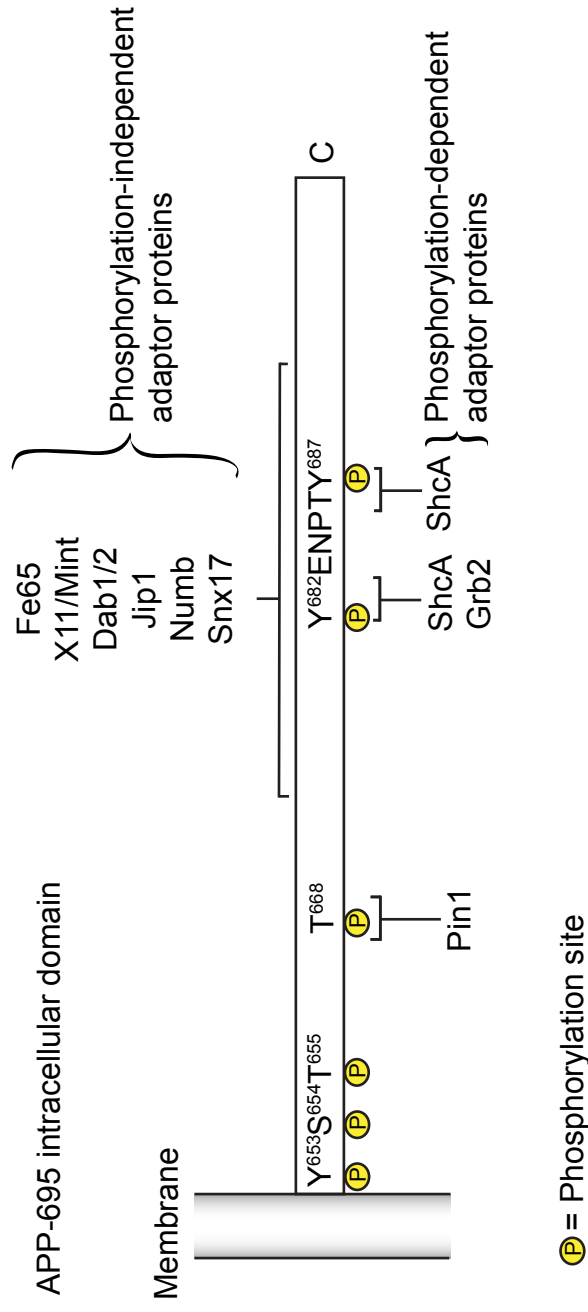


Figure 1.5. Phosphorylation sites and interaction domains in the amyloid precursor intracellular domain. Adaptor proteins that bind to APP in a phosphorylation-independent manner are shown above APP, and adaptor proteins that bind to APP in a phosphorylation-dependent manner are shown below APP. Amino acid numbering refers to APP⁶⁹⁵ isoform. Figure adapted from Schettini et al. (2010) Abbreviations: Dab; disabled, Mint; Munc-18-interacting proteins, Grb2 Growth factor receptor-bound protein 2, Jip1; Jun NH2-terminal kinase interacting protein, Pin1; Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, Snx17; sorting nexin 17.

Aside from the tyrosine phosphorylation sites, there are three serine and threonine phosphorylation sites: Ser⁶⁵⁵, thr⁶⁵⁴ and thr⁶⁶⁸. The significance of these phosphorylation sites is unclear. Some studies have reported contradictory effects of thr⁶⁶⁸ phosphorylation on APP processing (Feyt et al., 2007; Lee et al., 2003). Thr⁶⁶⁸ is phosphorylated in a cell-cycle dependent manner in the G2/M phase, suggesting that the metabolism of APP may be affected by cell-cycle state (Suzuki et al., 1994).

The kinases that have been implicated in phosphorylation of AICD are summarised in Table 1-2. Many of these kinases have been identified using in vitro phosphorylation studies of AICD fragments, but have not yet been confirmed in vivo.

1.6.5.2 Binding of adaptor proteins to AICD

The AICD has a number of reported cytosolic interacting proteins. Many of the adaptor proteins discussed below bind to the YENPTY motif in the C terminus of APP. This is a conserved sorting motif that is found in many proteins, and accordingly, many of the adaptor proteins that interact with this site in APP are reported to affect its trafficking (Bonifacino and Traub, 2003).

Table 1-2 Kinases implicated in the phosphorylation of AICD in vitro

AICD phosphorylation site	Implicated kinase	Reference
Tyr⁶⁸²	Tyrosine receptor kinase A (TrkA) Abl Src kinase Fyn kinase	Matrone et al., 2011; Tarr et al., 2002a Zambrano et al., 2001 Zhou et al., 2004 Hoe et al., 2008
Tyr⁶⁸⁷	Unknown	Rebelo et al., 2007
Ser⁶⁵⁵	Protein kinase C Ca ²⁺ /calmodulin-dependent protein kinase II	Gandy et al., 1988; Suzuki et al., 1992 Gandy et al., 1988
Thr⁶⁵⁴	Protein kinase C Cyclin dependent kinase 5 (Cdk5)	Gandy et al., 1988 Iijima et al., 2000
Thr⁶⁶⁸	Cyclin-dependent kinase 1 (Cdk1/cdc2k) c-Jun NH ₂ -terminal kinase 3 (JNK3) c-Jun NH ₂ -terminal kinase 1/2 (JNK1-2)	Suzuki et al., 1994 Inomata et al., 2003 Scheinfeld et al., 2003

Table shows potential phosphorylation sites in the C-terminal intracellular domain of APP, and kinases that have been implicated in the phosphorylation of each residue in vitro.

1.6.5.2.1 Fe65

The first protein to be identified to bind to the AICD was Fe65 (Fiore et al., 1995; Borg et al., 1996). Fe65 binds to the YENTPTY motif of AICD in a tyrosine phosphorylation-independent manner through its PTB domain (Borg et al., 1996). Phosphorylation of thr⁶⁶⁸ can inhibit Fe65 binding to AICD in vitro (Ando et al., 2001). The Fe65-AICD interaction is reported to have a number of biological consequences. Firstly, Fe65-AICD binding has been reported to affect the trafficking and proteolytic processing of APP, including the secretion of A β (Ando et al., 2001; Guenette et al., 1999; Sabo et al., 1999). As Fe65 also binds to the C-terminus of LRP1, LRP1 has been suggested to be necessary for effects of Fe65 on APP trafficking (Pietrzik et al., 2004).

A more controversial suggestion for the biological basis of the Fe65-AICD interaction is in the direct regulation of gene transcription. This idea has been formulated based on the similarities of the AICD domain to the Notch intracellular-domain (NICD). NICD is released from the plasma membrane after γ -secretase cleavage, and can translocate to the nucleus to regulate gene transcription (Andersson et al., 2011; De Strooper et al., 1999). γ -Secretase-cleaved AICD has been reported to translocate to the nucleus in a similar manner to the NICD (Cupers et al., 2001; Gao and Pimplikar, 2001). Normally AICD is prone to degradation, however Fe65 may stabilise AICD allowing it to translocate to the nucleus (Cupers et al., 2001; Kimberly et al., 2001). In the nucleus, the Fe65-APP complex has been reported to form a transcriptionally active complex in combination with tat-interactive protein 60 (tip60), a histone acetyltransferase (Cao and Sudhof, 2001; Gao and Pimplikar, 2001). A number of target genes have been reported for this interaction, KAI1 (Baek et al., 2002; von Rotz et al., 2004;

Zhang et al., 2007), APP (von Rotz et al., 2004), BACE1 (von Rotz et al., 2004), Tip60 (von Rotz et al., 2004), GSK3 β (Kim et al., 2003; Ryan and Pimplikar, 2005; von Rotz et al., 2004), EGFR (Zhang et al., 2007), p53 (Checler et al., 2007) and LRP1 (Liu et al., 2007). However, controversy surrounding the transcriptional role of AICD/Fe65 has arisen from studies that question many aspects of the AICD nuclear signalling model. γ -Secretase-induced AICD release is not necessary for Tip60 activation (Hass and Yankner, 2005). Fe65 has also been reported to signal gene transcription independently of APP (Yang et al., 2006). Additionally, many of the downstream gene targets of the proposed AICD/Fe65 complex have been questioned (Aydin et al., 2011; Chen and Selkoe, 2007; Repetto et al., 2007; Waldron et al., 2008). Therefore, it is still unclear whether the Fe65-AICD gene interaction is involved in the regulation of gene transcription.

1.6.5.2.2 ShcA/C and Grb2

Of the proteins that interact with the YENPTY motif of APP, only ShcA/C and growth factor receptor-bound protein 2 (Grb2) require tyr⁶⁸² to be phosphorylated (Tarr et al., 2002b; Zhou et al., 2004). ShcA contains an SH2 domain and a PTB domain that facilitate binding to phospho-tyr⁶⁸², and this interaction is also possibly modulated by thr⁶⁶⁸ (Tarr et al., 2002b).

Grb2 also binds to tyr⁶⁸² in a phosphorylation dependent manner (Zhou et al., 2004). This interaction may be involved in the trafficking of APP, as Grb2 alters the localisation of APP in cells (Raychaudhuri and Mukhopadhyay, 2010). Both ShcA and Grb2 are implicated in activating the mitogen-activated protein kinase 3 pathway (MAPK3, also known as extracellular signal-regulated kinase 1 ERK1),

which may represent the downstream signalling mechanism for these processes (Nizzari et al., 2007; Schettini et al., 2010).

1.6.5.2.3 X11/mint

X11/mint (Munc-18-interacting proteins) proteins also bind the APP YENPTY motif through their PTB domains (Borg et al., 1996; Tanahashi and Tabira, 1999). The X11/mint proteins are scaffolding components that play roles in polarized trafficking of proteins. Studies in *Drosophila* suggest that X11/mint proteins target APP and a number of other proteins to axons (Gross et al., 2013). A number of studies have illustrated that the X11/Mint binding to APP reduces A β production and APP processing in vitro and in vivo (Borg et al., 1998; Lee et al., 2004; Sastre et al., 1998). This may be due to the altered trafficking and metabolism of APP, as the X11/mint interaction prolongs the half-life of full length APP (Borg et al., 1998).

1.6.5.2.4 Dab

Disabled 1 (Dab1) and Disabled 2 (Dab2) are another family of proteins that bind to the YENPTY motif of APP through a PTB domain (Homayouni et al., 1999; Howell et al., 1997a; Lee et al., 2008). Dab1 is a key mediator of reelin signalling, a process that is crucial during neuronal development (Howell et al., 1997a; Howell et al., 1997b). Dab1 binds to the NPxY motifs of apoE receptor 2 (apoER2) and also vLDL receptor through its PTB domain to initiate reelin signalling (Bock et al., 2004). Phosphoinositide binding by Dab1 is also required for activity (Huang et al., 2005; Stolt et al., 2003; Stolt et al., 2004; Stolt et al., 2005). Interestingly, modulation of Dab1-YENPTY interactions alters the proteolytic processing of APP and decreases the production of A β (Hoe et al.,

2006). Dab2 has also been demonstrated to affect the trafficking of APP (Lee et al., 2008).

1.6.5.2.5 Jip1

Members of the APP family also bind the Jun NH₂-terminal kinase interacting protein (Jip1) via the YENPTY motif (Scheinfeld et al., 2002; Taru et al., 2002). This interaction enhances the phosphorylation of APP at Thr⁶⁶⁸ by JNK1 (Inomata et al., 2003; Scheinfeld et al., 2003). Inhibition of JNK phosphorylation of Thr⁶⁶⁸ is reported to prevent the ectodomain cleavage of APP and A β production and also induce degradation of APP (Colombo et al., 2009). Therefore, Jip/JNK interactions with the APP C-terminus may play a role in targeting APP for degradation.

1.6.5.2.6 Pin1

Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), is a prolylisomerase that has been found to bind to Thr⁶⁶⁸ of APP. Pin1 knockout increased the secretion of A β , while conversely Pin1 overexpression decreased the secretion of A β , suggesting that Pin1 promotes non-amyloidogenic processing of APP (Pastorino et al., 2006). Pin1 binding has been suggested to convert Thr⁶⁶⁸ from a cis-conformation to a trans-conformation to mediate this effect (Pastorino et al., 2006). However, more recently, Pin1 has also been suggested to affect the turnover of APP by inhibiting GSK3 β mediated phosphorylation at Thr⁶⁶⁸ (Ma et al., 2012).

1.6.5.2.7 Numb

Numb is a protein that binds to the intracellular domain of APP and also to Notch through its PTB domain (Roncarati et al., 2002). Numb has been implicated in a number of endocytic pathways, and directly binds to clathrin adaptor protein AP2 (Ntelios et al., 2012). Numb-APP interactions modulate the trafficking of APP, in a Numb-isoform dependent manner (Kyriazis et al., 2008). Levels of different Numb isoforms are perturbed in AD brain and also AD mouse models; suggesting a role of the Numb/APP interaction in AD (Chigurupati et al., 2011).

1.6.5.2.8 Sorting nexins

Sorting nexins (SNX) are endocytic adaptor proteins that are components of the endocytic machinery (see Section 1.7.5.2). SNX17 and SNX33 have been reported to bind to the YENPTY motif and affect the trafficking of APP (Lee et al., 2008; Schobel et al., 2008). As the α -secretase and β -secretase cleavage of APP occurs in different cellular compartments, modulation of SNX-APP interactions has also been reported to affect APP processing (Lee et al., 2008; Schobel et al., 2008). Interestingly, mutations in the Sortilin-related receptor, a protein that also interacts with sorting nexins and can affect APP trafficking, have been linked to Alzheimer's disease (Rogaeva et al., 2007).

1.6.5.3 Summary: Functional roles of the APP intracellular domain

In summary, although the C-terminal interactions of APP are complex, there are clear and overlapping links to a role for this region in controlling the trafficking and consequently the proteolytic processing of APP. This idea is supported by evidence from APP-conditional knock in mice discussed in Section 1.6.1 that have a non-functional YENPTY motif, which have altered trafficking of APP.

Importantly, many adaptor proteins that interact with the C-terminus of APP also interact with other type-1 transmembrane proteins, allowing for a broader reflection on possible roles of these adaptor proteins in the normal biology of APP.

1.6.6 Summary: Function of APP.

The reported functions of APP are diverse, although there are some broad connecting themes between current studies. There are many reports of trophic effects of APP on the proliferation, growth and differentiation of cells. However, whether these effects are due to secreted APP, full length APP, or a combination of both has not yet been conclusively determined *in vivo*. It is clear that the extracellular region of APP is involved in aspects of APP function. This is demonstrated by studies with sAPP α that show effects on cell growth *in vitro* and also conditional knock-in studies which show that the extracellular region of APP can rescue the lethal phenotype of APP/APLP2 double knockout mice. The intracellular region of APP is clearly involved in the regulation of APP trafficking through interaction with cytosolic adaptor proteins. However, it is also possible that AICD may facilitate some APP functions, for example by regulating the expression of a number of genes in combination with Fe65.

There are a number of aspects of APP biology that complicate interpretation of functional data. Firstly, there may be tissue- and cell-specific roles for APP. For example, the role of APP in the clotting cascade is apparently distinct from its function in the differentiation and growth of neural stem cells. However, the modes of action that elicit APP functions in different contexts may be similar.

Secondly, the APP homologues APLP1/2 may share functions with APP to some extent, which makes experimental determination of APP's function more difficult. This compensation is best illustrated by APP/APLP2 double KO mice, which have a lethal phenotype, whereas APP-KO and APLP2-KO mice are viable. Thirdly, the secretase processing of APP means that full length APP and its derivative proteolytic fragments may all have separate cellular actions. Secreted APP fragments may have a paracrine function, as illustrated by the reported effects of sAPP α on cell proliferation. However, it is not clear at present if the reported effects of sAPP α on cell proliferation are direct or indirect. APP ectodomain shedding to produce sAPP α can be stimulated by a number of growth factors; therefore, the physiological roles of sAPP α may be closely related to the actions of those growth factors. To fully understand the biological roles of APP, further research is required, with careful consideration of the complexities outlined above.

1.7 Phosphatidylinositol phosphate lipids: Minor lipids with major roles in cellular function

Lipids are a class of molecule that hold many roles beyond the formation of cellular membranes. In the last three decades, studies of lipid metabolism have revealed sophisticated lipid signalling interactions, which are involved in almost all aspects of cellular physiology. To explain some of the findings presented later in this thesis, this section will give a brief introduction to lipid signalling. Although the field of lipid signalling is too large to discuss here in complete detail, the reader is directed to a number of excellent reviews for further

information (Hannun and Obeid, 2008; Wymann and Schneider, 2008; Di Paolo and De Camilli, 2006; McLaughlin and Murray, 2005; Lemmon, 2008; Stace and Ktistakis, 2006; Cullen et al., 2001). This next section will discuss a particularly interesting class of lipid; the phosphorylated derivatives of phosphatidylinositol, known as phosphoinositides or PIPs.

1.7.1 Structure and nomenclature of phosphatidylinositol phosphates.

Phosphatidylinositol (PI) is a glycerophospholipid, consisting of an inositol head group, linked to two fatty-acid chains via a glycerol backbone and phosphate group (Fig. 1.6). Each of the hydroxyl groups of PI can be substituted with a phosphate group, resulting in mono, bis- and tris- phosphate PI species. These phosphorylated derivatives of phosphatidylinositol are termed phosphatidylinositol phosphates, phosphoinositides, PtdInsP or PIPs for short. There are seven different species of PIP. The mono, bis- and tris- phosphate species are therefore called PIP, PIP₂ and PIP₃ respectively. As the phosphate groups can be added at the 3, 4 or 5 position, both PIP and PIP₂ species can exist as different regioisomers, for example, monophosphate species PI(3)P, PI(4)P or PI(5)P, and the bisphosphate species PI(4,5)P₂, PI(3,5)P₂ and PI(3,4)P₂. Confusingly, PIP₂ in the older literature is often used to refer specifically to PI(4,5)P₂, however the other PIP₂ regioisomers such as PI(3,4)P₂ also hold important biological roles (McLaughlin et al., 2002; Posor et al., 2013).

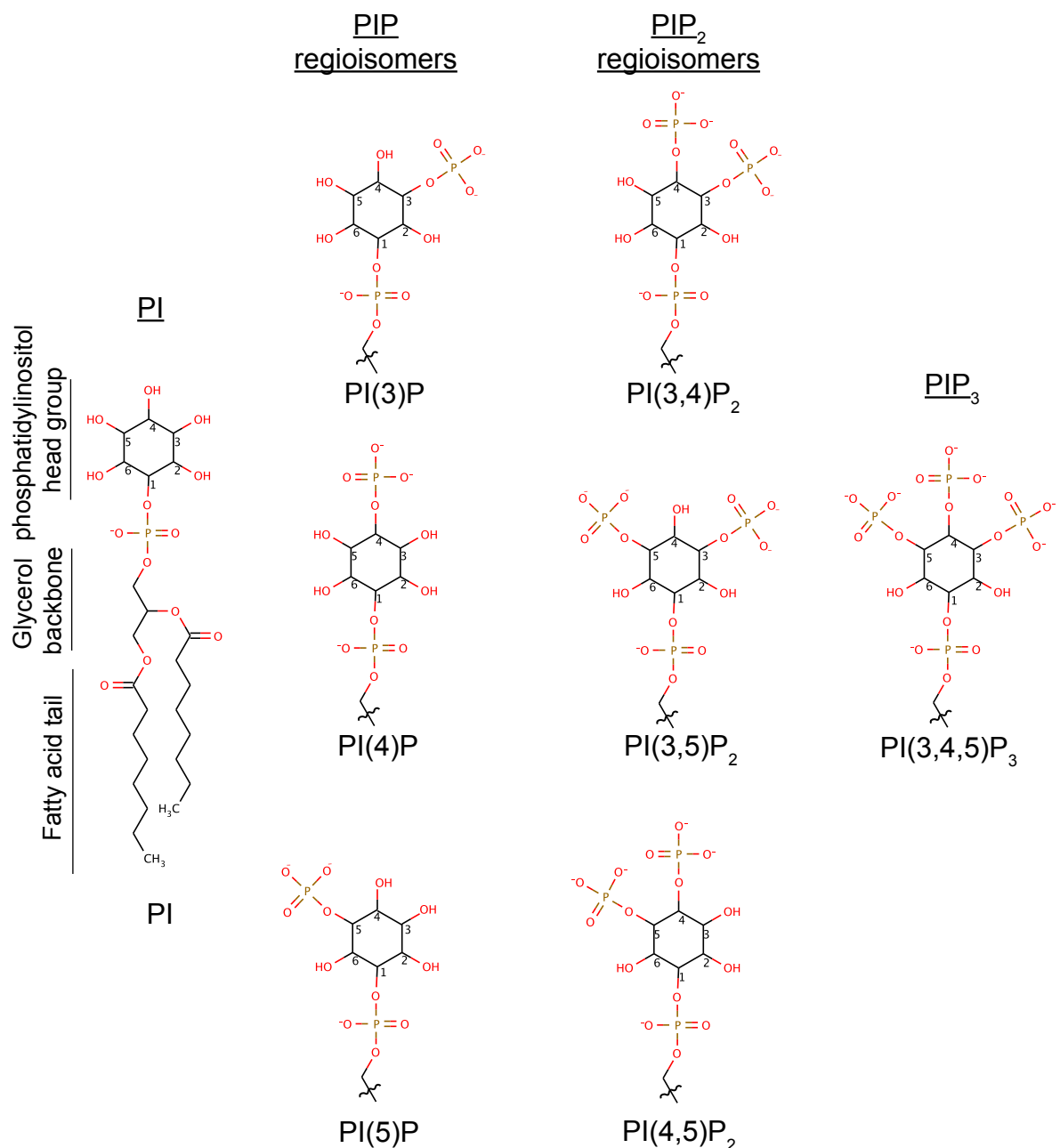


Figure 1.6. Figure shows diagrammatic representation of all seven phosphoinositides. The structure of phosphatidylinositol is shown on the left. The position of phosphate groups on the head groups of each phosphoinositide species is shown on the right. Abbreviations: PI – Phosphatidylinositol, PI(3)P - Phosphatidylinositol (3) phosphate, PI(4)P - Phosphatidylinositol (4) phosphate, PI(5)P - Phosphatidylinositol (5) phosphate, PI(3,4)P₂ - Phosphatidylinositol (3,4) bisphosphate, PI(3,5)P₂ - Phosphatidylinositol (3,5) bisphosphate, PI(4,5)P₂ - Phosphatidylinositol (4,5) bisphosphate, PI(3,4,5)P₃ - Phosphatidylinositol (3,4,5) trisphosphate.

1.7.2 Introduction to modes of PIP signalling

PIPs account for less than 1% of the total lipid content in mammalian cells (McLaughlin and Murray, 2005; van Meer et al., 2008; Lemmon, 2008). However, PIP lipids are intimately involved in many cellular processes. Levels of PIPs are tightly controlled by an elaborate network of kinases and phosphatases that can rapidly convert PIP isoforms (discussed in Section 1.7.3). This means the cell has exquisite control of the PIP composition of membranes and can alter this upon stimulation. Many proteins contain evolutionarily conserved modular PIP-binding domains, which can selectively bind to specific PIP regioisomers (Section 1.7.4). The combination of these two features of PIP biology means that proteins containing a PIP-binding domain can be targeted to a particular membrane within the cell in a reversible fashion. As different cellular compartments have different prevalent PIP compositions, PIP-protein interactions ensure that proteins are in the correct cellular location (Di Paolo and De Camilli, 2006; Varnai and Balla, 1998; Balla and Varnai, 2002). For example, endosomal membranes predominantly contain PI(3)P and endosomal proteins such as EEA1 associate with endosomes through a domain that specifically binds to PI(3)P (Gaullier et al., 1998; Simonsen et al., 1998). Further, PIPs carry a strong negative charge, and there is some indication that this is involved in the lateral clustering of proteins into discrete membrane domains to enable function (Khuong et al., 2013; van den Bogaart et al., 2011; Huang et al., 2004). Therefore, PIPs can ensure proteins are in the right location at the right time, to facilitate a variety of functions within the cell.

1.7.3 PIP metabolism

PIP levels in the cell are tightly controlled by a number of kinases and phosphatases outlined in (Fig. 1.7). A summary of the main phosphoinositide kinases and phosphatases is presented below. The structure, function and regulation of PIP kinases is discussed in more detail in a recent volume (Balla, Wymann and York, 2012a).

Figure 1.7. Metabolism of phosphoinositides. The phosphorylation of PIPs in the cell is controlled by the action of phosphoinositide kinases (shown in blue) and phosphoinositide phosphatases (shown in red, numbers correspond to enzymes in list). Phospholipase C-mediated production of IP₃ and DAG is shown in green. Figure based on Wymann (2012), Di Paolo and Di Camilli (2006) and Rusten and Stenmark (2006).

1.7.3.1 Phosphoinositide kinases

PI and PIP kinases are grouped into three main families based on their substrate specificity (Table 1-3). These are the phosphatidylinositol 3-kinases (PI3K), phosphatidylinositol 4-kinases (PI4K), and phosphatidylinositol phosphate kinases (PIPK).

PI3Ks are enzymes that phosphorylate PI and PIP at the three position of the inositol head group. There are three classes of PI3Ks, type I, II and III based on structural homology (Table 1-3A). PI3Ks generally consist of a regulatory subunit and catalytic subunit, with a conserved catalytic core (Wymann, 2012; Wymann and Schneider, 2008). PI3Ks have been intensively studied as these kinases have central roles in the regulation of cell proliferation, which means that they are strongly implicated in many human cancers (Wymann, 2012; Wymann and Schneider, 2008).

Phosphatidylinositol 4-kinases (PI4K) and phosphatidylinositol phosphate kinases (PIPks or PIP4Ks) phosphorylate PI and PIP respectively in the 4 position of the inositol head group. There are two classes of PI4Ks based on structural homology with two genes (α , β) in each class (Table 1-3B). Confusingly, PI4Ks only use PI as a substrate, whereas PI3K use both PI and PIP as substrates. PIPks (or PIP4Ks) are the enzymes that phosphorylate PIPs in the 4 positions. The PIPks are also divided into three main classes (Table 1-3C), with three genes (α, β, γ) in each class.

Table 1-3 Classes of phosphoinositide kinases

Class	Genes		Substrate	Product	References
A. Phosphoinositide 3-Kinases (PI3K)					
	Catalytic Subunits	Regulatory Subunits			
PI3K 1A	p110α, p110β, or p110δ	p85α, p55α, p50α, p85β, p55γ	PI(4,5)P ₂	PI(3,4,5)P ₃	Vanhaesebroeck et al., 2010
PI3K 1B	p110γ	p101, p84			
PI3K II	PI3K-C2α, PI3K-C2β PI3K-C2γ		PI, PI(4)P	PI(3)P, PI(3,4)P ₂	Vanhaesebroeck et al., 2010
PI3K III	Vps34	Vps15	PI	PI(3)P	Vanhaesebroeck et al., 2010
B. Phosphoinositide 4-Kinases (PI4K)					
PI4K II	α, β		PI	PI(4)P	Endemann et al., 1987; Whitman et al., 1987; Minogue and Waugh, 2012
PI4K III	α, β		PI	PI(4)P	Endemann et al., 1987; Whitman et al., 1987; Minogue and Waugh, 2012
C. Phosphatidylinositol phosphate kinases (PIPK)					
PIPK I (also referred to as PI(4)P 5-Kinase)	α, β, γ		PI(4)P	PI(4,5)P ₂	Ishihara et al., 1996; Jenkins et al., 1991; Schramp et al., 2012
PIPK II (also referred to as PI(5)P 4-Kinase)	α, β, γ		PI(5)P	PI(4,5)P ₂	Boronenkov and Anderson, 1995; Rameh et al., 1997; Schramp et al., 2012
PIPK III (also referred to as PI(3)P 5-Kinase)	PIKfyve (human)		PI(3)P	PI(3,5)P ₂	Cabezas et al., 2006; Schramp et al., 2012

Table shows classes of phosphoinositide kinases and substrates. Individual genes in each class are indicated. Functional PI3K 1 and PI3K III proteins are expressed by two genes, a regulatory subunit and a catalytic subunit.

1.7.3.2 *Phosphoinositide phosphatases*

There are currently over 35 known mammalian phosphoinositide phosphatases that remove phosphate groups from PIP (Dyson et al., 2012). These can be separated into 3-phosphatases, 4-phosphatases and 5-phosphatases, although there are some phosphatases (e.g. SAC1) that have mixed specificity (Table 1-4). By counteracting the role of many PI kinases, phosphoinositide phosphatases control many important developmental and disease related processes. A well known example is phosphatase and tensin homolog (PTEN), a phosphatase that dephosphorylates PI(3,4,5)P₃. Mutations in PTEN are frequently associated with cancer due to its critical role in deactivation of PI(3,4,5)P₃-dependent signalling (Li and Sun, 1998).

1.7.4 PIP-binding domains

Lipid-binding domains are a feature of many proteins that interact with cellular membranes. The scientific literature surrounding the discovery and characterisation of lipid-binding domains is too extensive to review comprehensively here. However, lipid-binding domains have been discussed by a number of excellent reviews (Balla, 2005; Lemmon, 2008; McLaughlin et al., 2002; McLaughlin and Murray, 2005; Carlton and Cullen, 2005).

In general, lipid-binding domains interact with acidic phospholipids, such as phosphatidylserine, phosphatidic acid, PI and PIPs (Lemmon, 2008). There is great structural diversity amongst different lipid-binding domain families, ranging from relatively ordered domains with high specificity for a particular lipid, to loosely structured regions that interact with the membrane in a broad electrostatic manner (McLaughlin and Murray, 2005).

Table 1-4. Mammalian phosphoinositide phosphatases

Name	Locus	Substrate(s)	Product(s)	Reference
3-phosphatases				
Phosphatase and tensin homolog deleted on chromosome ten	PTEN	PI(3,4,5)P ₃ PI(3,4)P ₂	PI(4,5)P ₂ PI(4)P	Li et al., 1997; Leslie et al., 2012
Myotubularins and myotubularin-related phosphatases	MTM MTMR (16 family members)	PI(3)P PI(3,5)P ₂	PI PI(5)P	Leslie et al., 2012
4-phosphatases				
Inositol polyphosphate 4-phosphatase type I	INPP4A	PI(3,4)P ₂	PI(3)P	Norris and Majerus, 1994
Inositol polyphosphate 4-phosphatase type II	INPP4B	PI(3,4)P ₂	PI(3)P	Norris et al., 1997
PI(4,5)P ₂ 4-phosphatase type I	TMEM55A	PI(4,5)P ₂	PI(5)P	Ungewickell et al., 2005
PI(4,5)P ₂ 4-phosphatase type I	TMEM55B	PI(4,5)P ₂	PI(5)P	Ungewickell et al., 2005
5-phosphatases				
Inositol-polyphosphate 5-phosphatase type II	INPP5B	PI(4,5)P ₂ PI(3,4,5)P ₃	PI(4)P PI(3,4)P ₂	Jefferson and Majerus, 1995
SH2- containing inositol phosphatase SHIP-1	INPP5D	PI(3,4,5)P ₃	PI(4,5)P ₂	Damen et al., 1996
SHIP2	INPPL1	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(3,4)P ₃	Hejna et al., 1995; Pesesse et al., 1997
Pharbin Type IV 5-phosphatase	INPP5E	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(3,4)P ₃	Asano et al., 1999; Kisseleva et al., 2000; Kong et al., 2000
Lowe oculocerebrorenal syndrome protein OCRL	OCRL INPP5F	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(4,5)P ₂	Zhang et al., 1995

Table 1-4. Mammalian phosphoinositide phosphatases

Name	Locus	Substrate(s)	Product(s)	Reference
Synaptojanin-1	INPP5G SYNJ1	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(4,5)P ₂	McPherson et al., 1996
Synaptojanin-2	INPP5H	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(4,5)P ₂	Nemoto et al., 1997
PIPP Proline rich inositol polyphosphate 5- phosphatase	INPP5J	PI(3,5)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	PI(3)P PI(4)P PI(4,5)P ₂	Mochizuki and Takenawa, 1999
SKIP Skeletal muscle and kidney inositol phosphatase	INPP5K	PI(3,4,5)P ₃ PI(4,5)P ₂	PI(3,4)P ₂ PI(4)P	Ooms et al., 2006
PLIP Phospholipid-inositol phosphatase	PLIP	PI(5)P	PI	Pagliarini et al., 2004
Sac Phosphatases				
SAC1	SACM1L	PI(4,5)P ₂ PI(3,4,5)P ₃	PI(4)P PI(3,4)P ₂	Nemoto et al., 2000
SAC2	INPP5F	PI(3)P PI(4)P	PI	Minagawa et al., 2001
SAC3	FIG4	PI(4,5)P ₂ PI(3,4,5)P ₃ PI(3,5)P ₂	PI(4)P PI(3,4)P ₂ PI(3)P	Sbrissa et al., 2007

Table shows the classes of known phosphoinositide phosphatases, genetic loci, substrates and products. Note, these phosphatases have been characterised in vitro, and are differentially expressed throughout the body.

Although PIPs are a relatively minor cellular lipid, a disproportionate number of classes of PIP-binding domains have been identified (Table 1-5). The first PIP-binding domain to be identified was in a region of pleckstrin that specifically binds to PI(4,5)P₂ (Harlan et al., 1994). Later, a homologous region of phospholipase C also was identified to bind to PI(4,5)P₂, which was then named the pleckstrin-homology (PH) domain (Lemmon et al., 1995; Garcia et al., 1995). About 250 PH domain-containing proteins are present in the human genome, making PH domains the eleventh most common protein domain (Yu et al., 2004). However, only about 10% of PH domains bind phosphoinositides with high affinity, based on studies in *S. cerevisiae* (Yu et al., 2004).

Since the identification of PH domains, other PIP-binding domain families have been discovered (Table 1-5). These include the epsin amino terminal homology (A/ENTH) domain; Fab1, YOTB, Vac1 and EEA1 (FYVE) domains; band 4.1, ezrin, radixin and moesin (FERM) domain and phox homology domains (PX). Additionally, domains that have other functions in the cell also bind to PIPs. Phosphotyrosine binding domains (PTB) are well known to bind to phosphotyrosine NPxY sorting motifs, however a number of these domains also bind PIP and this may be required for function (Huang et al., 2005; Mishra et al., 2002; Stolt et al., 2004; Stolt et al., 2005).

Many lipid-binding domains share little primary amino-acid sequence similarity, however some computational methods exist for identifying lipid-binding regions based on structural and electrostatic properties (Honing et al., 2005). There are also some examples of lipid-binding domains that may be very difficult to identify from computational methods alone. There are reports of 'split' PH domains,

Table 1-5: PIP-binding domains, binding specificity and examples of domain containing proteins

PIP-binding domain	Binding specificity	Examples of proteins containing domain
A/ENTH domain (epsin amino terminal homology)	PI(4)P PI(3,5)P ₂ PI(4,5)P ₂	EpsinR Ent3p, Ent5p AP180, PICALM, Epsin
C2 domain	PI(4,5)P ₂	Synaptogamin
FERM domain (band 4.1, ezrin, radixin and moesin)	PI(4,5)P ₂	Ezrin, Moesin, radixin, talin
FYVE domain (Fab1, YOTB, Vac1 and EEA1)	PI(3)P	EEA1, Hrs, SARA, PIKfyve
GRAM domain (glucosyl transferase, Rab-like GTPase activator and myotubularins)	PI(3,5)P ₂	Myotubularin
PDZ domain (PSD-95, Dig1, zo-1)	PI(4,5)P ₂	Syntenin
PH domain (pleckstrin homology domain)	PI(4)P PI(3,4)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	FAPP1/2, OSBP AKT, TAPP1,2 PLCδ1, dynamin BTK, AKT, ARNO, GRP1
PHD finger (plant homeo domain)	PI(5)P	ING2
PTB domain (phosphotyrosine binding domain)	PI(4,5)P ₂ PI(3,4,5)P ₃	Dab, ARH, SHC SHC
PX domain (Phox homology domains)	PI(3)P PI(5)P PI(3,4)P ₂ PI(4,5)P ₂ PI(3,4,5)P ₃	SNX2,3,7,13 SNX13 P47 ^{phox} Class II PI(3) Kinase CISK

Table shows examples of PIP-binding domains, binding specificity and examples of proteins that contain each domain. Table based on Di Paolo and De Camilli, 2006.

domains that are split into two halves that are found within separate regions of proteins, or even between two distinct proteins (Teo et al., 2006; van Rossum et al., 2005). Split PH domains can form functional lipid-binding sites when the two halves of the domain come together in their cellular context (Teo et al., 2006; van Rossum et al., 2005). Therefore, it is quite likely that the number of proteins known to contain PIP-binding domains will continue to expand.

In general, the affinity of lipid domains for their specific ligands is lower than would be expected to be biologically significant for a receptor binding to a soluble ligand. For example, the PH domain of PLC binds PI(4,5)P₂ with a K_d of 2 μM (Hirose et al., 1999; Lemmon et al., 1995). However, the restriction of lipids and lipid-binding proteins to a membrane interface (i.e. resulting in reduced dimensionality) can greatly increase the effective concentration of lipid that is encountered by the domain (McLaughlin et al., 2002). In addition, lipid-binding domains commonly act in co-operation with other binding domains, in a phenomenon known as co-incidence detection (Carlton and Cullen, 2005; Di Paolo and De Camilli, 2006; Lemmon, 2008). In co-incidence detection, multiple low affinity binding interactions can add to produce a stronger interaction. There are a number of examples of different modes of this phenomenon, illustrated in Fig. 1.8.

In summary, a rapidly expanding number of proteins have been identified to contain lipid-binding and PIP-binding domains. These can act in a variety of modes, and in combination with other binding domains, giving a diverse range of functional interactions. The next sections will discuss examples of how PIP-binding domains are used in a number of important neuronal functions.

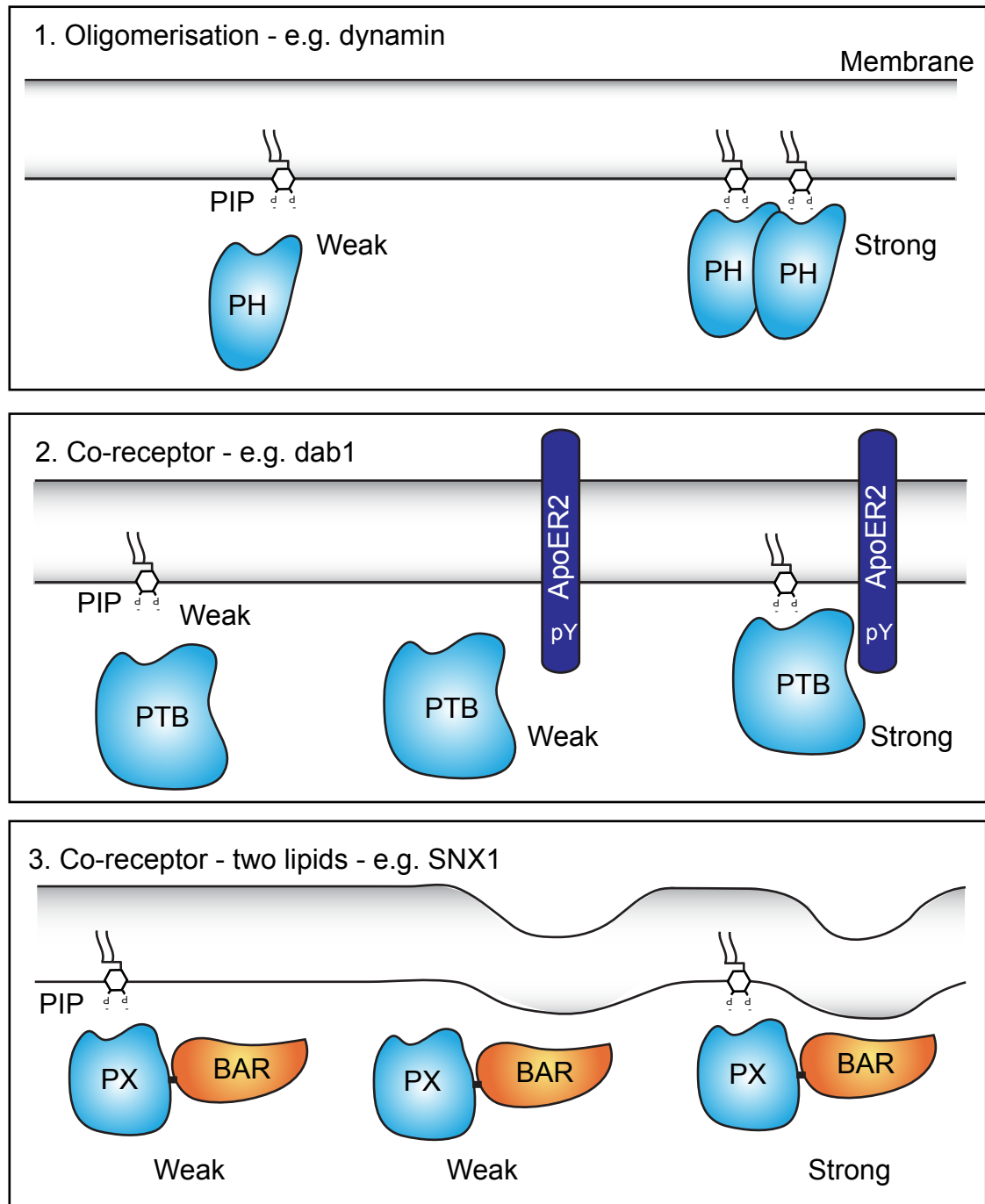


Figure 1.8. Coincidence detection by PIP-binding domains. The combination of multiple low-affinity domains can produce high specificity for recognising target membranes. Example 1: Dynamin has low affinity for membranes until it oligomerises, which results in a higher affinity interaction. Example 2: the combination of a lipid-binding domain and a tyrosine binding motif, as found in the PTB domain of dab1, can produce a high affinity interaction to a particular receptor, such as apoER2. Example 3: the combination of multiple low affinity lipid-binding domains can produce high affinity interactions. SNX1 is depicted, which contains a PI(3)P-binding PX domain and a BAR (Bin/Amphiphysin/Rvs) domain that binds to curved membranes and targets SNX1 to endosomes. Figure adapted from Lemmon (2008).

1.7.5 Neuronal roles of PIPs

PIPs are ideally suited to the control of many cellular processes, due to the combination of high turnover, regulated location and specific recognition by PIP-binding proteins (Di Paolo and De Camilli, 2006). Comprehensively reviewing the current state of knowledge regarding biological roles of PIPs will not be done here as this topic has been extensively reviewed in a recent volume (Balla, Wymann and York, 2012b). The important cellular functions involving PIPs that are not discussed here include the regulation of cytoskeleton, roles in the cell nucleus, Golgi complex function, signal transduction, and chemotaxis. Instead, this section will illustrate some ways that PIP interactions are important in neuronal function with a focus on processes that are intimately involved in synaptic transmission.

1.7.5.1 Roles of PIPs in synaptic vesicle exocytosis

Studies of large dense core vesicle (LDCV) secretion in PC12 and chromaffin cells gave early insight into the role of PIPs in exocytosis (Eberhard et al., 1990; Hay et al., 1995). PI(4,5)P₂ is required for exocytosis and PIPK1 has been identified as a factor required for LDCV vesicle priming (Eberhard et al., 1990; Hay et al., 1995). A number of components of the secretory system in LDCV exocytosis have now been identified as PIP-binding proteins (Osborne et al., 2006; Wen et al., 2012). These PIP-binding components of the secretory system include synaptogamin (Syt; Schiavo et al., 1996), calcium-dependent activator protein for secretion (CAPS; Loyet et al., 1998) and SNARE proteins (Lam et al., 2008). CAPS, SNARE and Syt all have roles in bringing secretory vesicles into

proximity with the plasma membrane (“priming”) and facilitating fusion with the plasma membrane (Frere et al., 2012).

Although LDCV exocytosis and synaptic vesicle exocytosis may operate by slightly different mechanisms, there are strong indications of the importance of PIP in synaptic vesicle exocytosis. For example, deficiency in PIPK1 γ , the main PIP kinase in neuronal synapses results in impaired neurotransmitter release (Di Paolo et al., 2004). Additionally, inhibition of PI(4)P synthesis with phenylarsine oxide decreases the release of glutamate from synaptosomes (Wiedemann et al., 1998). More recently, it has been shown that PI(4,5)P₂ and PI(3,4,5)P₃ are required for the clustering of syntaxin 1A (a SNARE protein) at neurotransmitter release sites to allow synaptic vesicle release (Aoyagi et al., 2005; Khuong et al., 2013; van den Bogaart et al., 2011). Therefore, PIPs are involved in many aspects of the regulation of correct release of synaptic vesicles.

1.7.5.2 Roles of PIPs in synaptic vesicle recycling and endocytosis

To maintain sustained signalling at the synapse, neurons must be able to recover membrane from PM-fused synaptic vesicles, to enable production of new synaptic vesicles. This process is known as synaptic vesicle recycling (Rohrbough and Broadie, 2005; Sudhof, 2004). A number of modes of endocytosis are involved in this event (Rohrbough and Broadie, 2005; Sudhof, 2004). The best-characterised PIP-dependent mechanism is clathrin-mediated endocytosis (Doherty and McMahon, 2009; Royle and Lagnado, 2003; Frere et al., 2012). The early evidence for the involvement of PIPs in synaptic vesicle recycling came from the observation that a number of proteins intimately involved in endocytosis also bind to phosphoinositides. Examples include Dynamin, AP180/PICALM, and AP2,

which are components of clathrin assemblies and all contain PIP-binding domains (Ford et al., 2001; Jost et al., 1998; Salim et al., 1996; Klein et al., 1998).

A compelling model involving PIPs for the co-ordination of clathrin-coated vesicle (CCV) formation has emerged (Frere et al., 2012; Posor et al., 2013). At sites of endocytosis, an accumulation of PI(4,5)P₂ recruits PIP-binding adaptor proteins such as epsins, AP2 or AP180 to the membrane (Jost et al., 1998). Adaptor protein binding to the membrane is essential for assembly of the clathrin coat (Ford et al., 2001; Jost et al., 1998). Recent evidence suggests the maturation of the endocytic vesicle may then be co-ordinated by spatiotemporal regulation of PIP composition in the forming vesicle bud. The phosphoinositide phosphatase synaptojanin associates with the membrane and hydrolyses PI(4,5)P₂ to PI(4)P (Posor et al., 2013; Cremona et al., 1999). PI(4)P is then converted to PI(3,4)P₂ by the phosphoinositide kinase PI3K C2α (Posor et al., 2013). PI(3,4)P₂ production allows for the association of sorting nexin 9 (SNX9) with the forming vesicle. SNX9 is a BAR-domain (Bin/Amphiphysin/Rvs) containing protein that binds to the PI(3,4)P₂ produced by PI3K C2α (Posor et al., 2013). BAR domains are large, banana-shaped domains with a charged surface that bind anionic lipids such as PIP and induce membrane curvature (Takei et al., 1999; Peter et al., 2004). Therefore, SNX9 may help the formation of the budding vesicles (Posor et al., 2013).

Fission of the endocytic vesicle from the plasma membrane is achieved by the GTPase dynamin, which requires PI(4,5)P₂ binding for function (Salim et al., 1996; Klein et al., 1998). Once the endocytic vesicle has separated from the plasma membrane (PM), disassembly of the endocytic machinery has been

suggested to occur through the action of a number of phosphoinositide phosphatases such as synaptojanin, however the exact mechanism has not yet been elucidated (Cremona et al., 1999).

Perturbation of PIP dependent processes in endocytosis leads to a number of neuronal phenotypes. Knockout or mutation of synaptojanin, the synaptic PIP phosphatase, results in failure of synaptic vesicle endocytosis and accumulation of clathrin coated vesicles within the synapse, consistent with its proposed role in clathrin coated vesicle maturation (Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003). Loss of PIPK1 γ function in the synapse decreases the rate of synaptic vesicle endocytosis, resulting in the accumulation of bulk endosomes (Di Paolo et al., 2004). These examples illustrate that PIPs have many roles in the regulation of endocytosis.

1.7.5.3 Modulation of ion channel function by PIPs

An interesting emerging role of PIPs is as modulators and facilitators of ion channel function. PI(4,5)P₂ is known to directly regulate inwardly rectifying potassium channels (Kir channels; Rohacs et al., 2003; Du et al., 2004), voltage gated calcium channels (Wu et al., 2002) and transient-receptor potential (TRP) channels (Wu et al., 2002). Kir channels and TRP channels interact with PIPs through lipid-binding domains in the cytoplasmic regions of the proteins (Lopes et al., 2002; Nilius et al., 2008). Most of these channels are activated in the presence of PI(4,5)P₂, which has led to the suggestion that PI(4,5)P₂ binding by ion channels prevents their activity while they are trafficked to the PM (Hilgemann et al., 2001). Some TRP channels are also inhibited by PIPs, which has been

suggested to be a physiological mechanism of negative TRP channel regulation to allow proper function (Gamper and Rohacs, 2012; Cao et al., 2013).

A number of ion channels may also be regulated by PIPs in an indirect manner. NMDA-R activity can be modulated by PI(4,5)P₂ (Michailidis et al., 2007). 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor (AMPA-R) activity can also be modulated by PIPs, which are involved in the clustering of AMPA-R and control of cell surface levels of AMPA-R (Jin et al., 2006; Frere et al., 2012). As neuronal function is highly dependent on the action of ion channels, PIP-ion channel interactions therefore provide further evidence for the wide utilization of PIP in cellular function.

1.7.5.4 PI(4,5)P₂ as the metabolic precursor of IP₃ and DAG

PIPs are probably most well known for their role in Ca²⁺ signalling. The Ca²⁺ signalling pathway has been largely elucidated (Berridge et al., 2000). Activation of G-protein coupled receptors or receptor tyrosine kinases can activate phospholipase C (Berridge et al., 2000). Phospholipase C at the plasma membrane catalyses the hydrolysis of PI(4,5)P₂ into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG; Lapetina and Michell, 1973). Interestingly, phospholipase C has a higher affinity for IP₃ than for PI(4,5)P₂, which probably represents an in-built negative feedback mechanism (Garcia et al., 1995). IP₃ is water-soluble so it can diffuse into the cell and act as a second messenger. IP₃ binds to the IP₃ receptor on the endoplasmic reticulum, and stimulates the release of Ca²⁺ from the intracellular calcium stores (Streb et al., 1983; Ferris et al., 1989). The activation of the IP₃ receptor results in a rapid local increase in cytosolic calcium, that can have a number of effects in controlling cellular processes such as gene

transcription, apoptosis, proliferation and activation of ion channels (Berridge et al., 2000).

DAG also acts as a second messenger once produced from PI(4,5)P₂. Where IP₃ produces rapid cellular responses, DAG produces a longer lasting signalling response through protein kinase C (PKC; Nishizuka, 1995; Rosse et al., 2010). DAG activates PKC in combination with Ca²⁺ and other phospholipids (Rosse et al., 2010). PKC is inactive in its resting state, however the binding of its C1 and C2 domains to DAG and/or Ca²⁺ promotes a conformational change to an active form (Oancea and Meyer, 1998). Once active, PKC can phosphorylate a number of downstream signalling proteins in processes such as cell-growth and control of the cytoskeleton (Nishizuka, 1995; Rosse et al., 2010).

1.7.6 Summary

The studies reviewed in this section demonstrate that PIP-lipid signalling represents a highly sophisticated, evolutionarily conserved system that is involved in many cellular processes. The cell has a high degree of control over the location and level of PIPs, so PIP-binding by proteins can facilitate the targeting of proteins to particular cellular locations. As a result, PIP lipids appear to be involved in almost all aspects of cellular physiology (Balla, 2005; Di Paolo and De Camilli, 2006).

1.7.7 General links between PIP and AD

There are some reports of perturbed PIP metabolism in patients with AD and in experimental animals models of AD. Studies report that there is a reduction in

PI3K and PI4K activity in the AD brain, when compared to healthy controls (Bothmer et al., 1994a; Jolles et al., 1992; Zubenko et al., 1999; Wallace, 1994). A reason for this loss of PI3K and PI4K activity may be that the AD brain has reduced synapses, where there is a significant pool of PI3K and PI4K (Bothmer et al., 1994b). However, synaptic PIP kinase activity was found to be unaffected in some of these studies, potentially supporting the possibility of a selective down-regulation of PI4K in AD (Bothmer et al., 1994b; Jolles et al., 1992; Wallace, 1994).

A number of in-vitro studies suggest that the reported decrease in PI3K and PI4K activity in AD may be related to some of the pathology in AD. A β has been reported to directly inhibit PI4K in a competitive manner (Wallace, 1994; Wu et al., 2004). Levels of PI(4)P and PI(4,5)P₂ are also lowered in cells by A β treatment, and this has been suggested to be a mechanism of A β toxicity (Berman et al., 2008). Interestingly, overexpression of the synaptic PIP phosphatase synaptojanin1 reportedly reduces the toxic effects of A β in cell culture and also in mouse models of AD (Berman et al., 2008; McIntire et al., 2012). These studies suggest A β could modify PIP levels.

Some studies also suggest that PIPs may have a role in the metabolism of APP. Inhibition of PI3K with wortmannin or LY294002 inhibits A β and sAPP α secretion in cell-based systems, suggesting that PIPs can regulate APP processing (Haugabook et al., 2001; Petanceska and Gandy, 1999). Wortmannin has also been reported to significantly reduce A β deposition in Tg2576 mice, which suggests that PI3K inhibition could form a therapeutic strategy for the treatment of AD (Haugabook et al., 2001). Recently, the trafficking of APP has been shown

to be dependent on PI(3)P (Morel et al., 2013). This could explain why inhibition of PI3K is able to reduce the secretion of sAPP α and A β .

There is also some evidence to suggest that PIPs may regulate γ -secretase to some extent. PI and PI(4,5)P₂ have been reported to potently inhibit γ -secretase activity, suggesting that PIP may be an endogenous inhibitor of γ -secretase (Osenkowski et al., 2008; Osawa et al., 2008). Interestingly, cells expressing presenilin mutations associated with Alzheimer's disease have a consistently lower level of PI(4,5)P₂ (Landman et al., 2006). These reports suggest that γ -secretase activity could be affected by PIP and also affect PIP metabolism in a feedback loop. However, more research would be needed to establish whether this is the case.

In summary, there are some suggestions that PIP metabolism is perturbed in AD and that PIPs can affect the processing of APP to form A β . However, it is not known whether the alterations of PIP metabolism that have been reported are involved in AD progression or a result of the disease state.

1.8 Hypothesis and aims of the study.

The studies reviewed in this chapter demonstrate that APP is intimately involved in the pathogenesis of AD. However, despite intensive research, the normal function of APP is unclear. Further understanding of the biology of this protein may provide insight not only into the biochemical processes that are disrupted in AD, but also into the normal function of the brain.

Several functions of APP have been suggested to be mediated through the production of a secreted factor, sAPP α . sAPP α may act in a trophic manner and elicit effects on cellular proliferation, differentiation and neurite outgrowth (Saitoh et al., 1989; Hoffmann et al., 2000; Ninomiya et al., 1994; Pietrzik et al., 1998; Milward et al., 1992; Jin et al., 1994; Araki et al., 1991; Young-Pearse et al., 2008; Caille et al., 2004; Baratchi et al., 2012). This trophic capacity of sAPP α suggests that it may interact with a cell surface receptor or receptors on the signal-receiving cell. However, the cell-surface interactions of sAPP α have been incompletely characterised. Understanding the interactions of sAPP α with the surface of cells will give insight into the mechanisms of binding and possibly also mechanisms of signalling that are directly activated by APP.

Some cell-surface receptors for sAPP α have been proposed. sAPP α is known to bind to cell-surface or extracellular-matrix components such as laminin, glypican-1 and other heparan sulfate containing matrix components (Narindrasorasak et al., 1992; Williamson et al., 1996; Ninomiya et al., 1994; Small et al., 1994). However, binding interactions with glycosaminoglycans may not be the sole determinant of binding to the cell surface, as proteoglycans often act in

combination with other receptors (Spivak-Kroizman et al., 1994). Several studies have reported both heparitinase-sensitive and heparitinase-insensitive cell-surface sAPP α binding sites on cells, supporting this idea (Hoffmann et al., 1999; Ninomiya et al., 1994; Kounnas et al., 1995). In addition to its interaction with heparan sulfate proteoglycans, sAPP α may also interact with β 1-integrin, lipoprotein receptor related protein-1, death receptor 6, p75 neurotrophin receptor and APP itself (Young-Pearse et al., 2008; Kounnas et al., 1995; Nikolaev et al., 2009; Gralle et al., 2009). However, APP may interact with many other extracellular proteins (Bai et al., 2008). Therefore, it still remains to be established which of these interactions are most important in vivo.

In addition to its potential interaction with proteins and carbohydrates, there are some reports that APP may also interact with lipids. sAPP α has also been reported to localise to a novel 'lipid raft' fraction on cells (Tikkanen et al., 2002) and more recently, a direct interaction of sAPP with gangliosides has been proposed (Zhang et al., 2009). However, the interactions of APP with lipids have not been studied in detail. The studies reviewed in Section 1.7 demonstrate that lipids play active roles in many signalling processes, by targeting proteins to membranes. Therefore, the aim of the experiments presented in this thesis was to further investigate the role of APP-lipid interactions in its binding to cells.

1.8.1 Hypothesis

The central hypothesis behind the studies presented in this thesis is:

Secreted forms of APP function through direct interactions with lipids on the cell surface

1.8.2 Aims

To address the central hypothesis, the experimental aims in this thesis were to:

1. Examine the ability of secreted APP to bind to lipids in vitro;
2. Investigate the role of lipid binding in the cell-surface interactions of secreted APP;
3. Investigate which regions of APP are involved in binding to cells;
4. Examine the ability of secreted APP to affect levels of lipids in cells;
5. Investigate if APP can affect lipid-signalling pathways.

Chapter 2 Materials and methods

2.1 Materials

All materials and suppliers of materials used in this study are listed in Appendix I.

2.1.1 Buffers, solutions and cell culture media

All buffers and solutions used in this study are listed in Appendix II.

2.1.2 Antibodies and dilutions

All primary antibodies, suppliers and antibody dilutions used in this study are listed in Appendix III. All secondary antibodies and antibody dilutions used in this study are listed in Appendix IV.

2.2 Methods

2.2.1 Protein-lipid overlay assay

Commercially available hydrophobic membranes (PIP StripsTM, SphingoStripsTM, Membrane Lipid StripsTM) spotted with 27 different lipids were used to examine the binding of sAPP α to lipids in vitro. For experiments investigating the effect of diC8PI(4,5)P₂ and mucosal heparin on PI(4,5)P₂ binding, PI(4,5)P₂ was spotted on hydrophobic membranes according to the method of Dowler et al. (2002). Briefly, a 1 mM stock solution of 37:4 PI(4,5)P₂ in CH₃Cl:CH₃OH was prepared and stored at -80°C. For spotting, the PI(4,5)P₂ stock was diluted to 100 μ M concentration in 1:2:0.8 CHCl₃:CH₃OH:H₂O and 1 μ L of the PI(4,5)P₂ solution

containing 100 pmol of PI(4,5)P₂ was spotted on to Hybond C Extra nitrocellulose at 4°C. Membranes were dried for one hour before use.

Membranes were incubated with recombinant sAPP α or APP-E1 (described in Appendix I) at the concentrations indicated in figure legends overnight at 4°C in 2.5 mL of 2 mg mL⁻¹ fatty-acid free bovine serum albumin (BSA) in tris-buffered saline with tween-20 (TBS-T). Alternatively, membranes were incubated with conditioned cell medium for one hour. Membranes were then washed five times over 30 min with TBS-T. Lipid-bound sAPP α or APP-E1 was detected using an anti-6xHis antibody, 22C11 or 6E10. The concentrations of primary antibodies that were used are listed in Appendix III. Primary antibodies were incubated for 1 h at room temperature in 2.5 mL 2 mg mL⁻¹ BSA/TBS-T. Membranes were washed five times in TBS-T over 30 min, followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibody in 2.5 mL BSA/TBS-T for 1 h. Membranes were then washed in TBS-T five times over 30 min, and bound secondary antibody was detected using Immobilon chemiluminescent detection substrate which was incubated with membranes for 5 min before detection. Chemiluminescence was monitored using a Chemi-smart 5000 and images were collected using Chemicapt 5001 software (Vilber-Lourmat GmbH, Eberhardzell, Germany). For quantification of immunoreactivity, 16-bit 2x2 binned images were collected and the integrated density of luminescence for each lipid spot was measured using Image J (National Institutes of Health, Maryland, USA).

2.2.2 Neural stem and progenitor cell culture and conditioned medium collection

Neural stem and progenitor cells (NSPCs) were cultured from the cerebral cortices of newborn Tg2576 or wild-type (WT) littermate mice (P0). Brain cortices were cleared of meninges and hippocampus, and then incubated in 1×TrypLE Express for 10 min at 37°C. Tissue was disrupted mechanically with 1000 µL fine tip and then the tissue was passed through a 40 µm cell strainer (BD Biosciences, North Ryde, Australia) to remove undissociated cells. NSPCs were grown as neurospheres by culturing cells in suspension in T75 cell culture flasks at a density of 20,000 cells mL⁻¹ in proliferation medium. Neurosphere cultures were incubated in a humidified incubator at 37°C with 5% CO₂. After 7 days in culture, cells were centrifuged at 500 x g for 5 min. At this stage, the supernatant conditioned medium was collected and stored at -80°C. Neurospheres in the cell pellet were dissociated mechanically with 200 µL fine tips, cells were counted in a Bright-LineTM haemocytometer (Sigma-Aldrich Pty. Ltd. Castle Hill, Australia) and then reseeded as suspension cultures in a T75 cell culture flask.

2.2.3 Primary murine hippocampal and cortical culture

All animal use was approved by the University of Tasmania Animal Ethics Committee (Permit No. A12555). Mice were housed in the animal facility at the University of Tasmania. For immunocytochemistry, primary neuronal cultures were prepared from the hippocampi of neonatal C57BL/6xSJL mice. For western blotting and lipid extraction, primary neuronal cultures were prepared from the whole cortices of neonatal C57BL/6xSJL mice.

Dissected tissue was transferred to 0.25% (w/v) papain and 0.06% (w/v) deoxyribonuclease-1 in Hank's balanced salt solution and incubated for 15 min at 37°C. Tissue was then washed in Neurobasal medium three times and dissociated by trituration. Single cell suspensions were centrifuged at 500 x g for 5 min and re-suspended in Neurobasal plating medium. The number of viable cells was determined using the Trypan blue exclusion method, and a Bright-Line™ Haemocytometer (Sigma-Aldrich). For immunocytochemistry, hippocampal cells were plated on nitric acid-washed poly-L-lysine-coated 13 mm glass coverslips in 24-well tissue culture plates at a density of 1.25×10^5 viable cells per well. For western blotting and lipid extraction, cortical cells were plated in 12-well tissue culture plates, at a density of 1×10^6 viable cells per well. Cultures were maintained at 37° C in 5% CO₂. After 24 h, the medium was replaced with Neurobasal maintenance medium. Half of the cell culture media was replaced every 4 days and cultures were used for experiments after 10 days in vitro (DIV).

2.2.4 Immunocytochemistry

Antibody labelling was performed on live, unfixed cells except where explicitly stated. Cells were washed twice in 500 µL Neurobasal maintenance medium warmed to 37°C. After washing, all antibody incubations and washes were performed at 4°C to prevent endocytosis of the antibodies. Cells were labelled by incubation with primary antibodies in 100 µL of imaging buffer (Appendix II). Incubations with primary antibodies were performed for 30 min, and the cells were washed three times with 500 µL of imaging buffer. Alexa-fluor-conjugated secondary antibodies were incubated for 30 min in 100 µL of imaging buffer,

before washing three times with 500 μ L of imaging buffer. Cells were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min, washed twice in PBS and stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) in PBS for five min. Coverslips were then washed two times in PBS and once in dH₂O. Coverslips were mounted in DAKO mounting medium and allowed to dry overnight before imaging.

2.2.5 Microscopy and image analysis

Confocal images were acquired using a Zeiss 510 confocal microscope, using a 63x/1.4 oil immersion objective and Zen software (Carl Zeiss, Pty. Ltd. Sydney, Australia). Confocal slices were collected with one Airy unit pinhole size. Epifluorescence images were acquired using a Nikon Ti-E microscope (Nikon corporation, Tokyo, Japan) with 20x/0.5, 40x/0.95, 60x/1.27 objectives. Representative images are shown of at least 3 independent experiments.

To quantify the level of immunoreactivity, 10 random fields from 3 coverslips were collected for each treatment group. Multichannel images were acquired using a microscope with an automated stage, automated filters and Nikon Perfect Focus system. Twelve-bit, 2x2 binned images were collected using a 40x/0.95 objective. The average background intensity for each channel was defined as the sum pixel intensity measured from appropriate negative controls. For each field, the sum of all pixel intensities was measured and background was subtracted from these values. Statistical analysis was performed using Graph-Pad Prism v.6 (Graphpad software Inc. La Jolla, CA, USA). Statistical significance was assumed at a 95% confidence level was determined using an unpaired Student's t-test or

one-way analysis of variance (ANOVA) with Tukey's or Dunnett's post-hoc tests. Data with a p value < 0.05 were considered significantly different.

2.2.6 Western blotting

Cells were grown for 7 DIV before lysis and analysis of proteins. All extraction steps were performed on ice to reduce degradation of the extracted proteins. Cells were washed twice in warmed Neurobasal medium, prior to the addition of 150 μ L of lysis buffer containing protease inhibitor cocktail (Roche cOmplete ultra protease inhibitor tablets; 1 tablet 10 mL⁻¹). For the experiments investigating the phosphorylation state of Akt, phosphatase inhibitor cocktail was included in the lysis buffer (Roche PhosStop phosphatase inhibitor tablets; 1 tablet 10 mL⁻¹). Cells were scraped, triturated 15 times and transferred to an Eppendorf tube. Cell membranes were removed by centrifugation (13,000 rpm, 10 min, 4°C). The supernatant fraction was flash frozen in liquid nitrogen and stored at -80°C prior to analysis.

The amount of protein in each sample was measured using the Bio-Rad DC protein assay, using bovine serum albumin as the standard. Absorbance was measured on a Fluostar Optima microplate reader (BMG Labtech GmbH, Ortenberg, Germany). The protein concentration in each sample was normalised by the addition of an appropriate volume of MilliQ H₂O. Laemmli Sample buffer (Appendix II) was added to samples, which were then heated at 95°C for 10 min. Samples were briefly centrifuged and 15 – 25 μ g of protein was loaded on an 8% tris-glycine polyacrylamide gel. To determine the ratio of pAkt to total Akt, individual samples were run twice on two separate gels in parallel. Samples were

separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were blocked for 20 min in TBS-T containing 5% (w/v) non-fat dry milk powder (NFDM). Specific proteins were detected using primary antibodies at concentrations outlined in Appendix III. An anti-GAPDH antibody was used to verify equal loading of samples. Primary antibodies were incubated with blots in TBS-T/NFDM for 1 h at room temperature or at 4°C overnight. Blots were washed three times in TBS-T, prior to incubation with a HRP-conjugated secondary antibody for 1 hour at room temperature in TBS-T/NFDM. Blots were washed three times, and signal was detected using Immobilon chemiluminescent detection substrate according to the manufacturer's instructions. Chemiluminescence was monitored using a Chemi-smart 5000 image acquisition system and images were collected using Chemicapt 5001 software (Vilber-Lourmat GmbH, Eberhardzell, Germany). For quantification of immunoreactivity, 12-bit 2x2 binned images of chemiluminescence were collected and the integrated density of luminescence for each band was measured using Image J. Data analysis was performed using Graph Pad Prism V.6. Statistical significance was determined using one-way ANOVA with Tukey's or Dunnett's post-hoc tests, or Student's t-tests. Data with a *p* value < 0.05 were considered significantly different.

2.2.7 Computational modelling of IP₃ binding sites on APP

Modelling of potential binding sites in APP was performed using Protein Data Bank (PDB) crystal structures for the heparin-induced APP E1 dimer (PDB ID: 3KTM; chains B + D; Dahms et al., 2010) and the pleckstrin homology domain of

β -spectrin (PDB ID: 1BTN; Hyvonen et al., 1995). The binding of D-myo-inositol 1,4,5-triphosphate (Zinc database accession No. 6973597) and inositol (Zinc database accession No. 1530357) was computationally modelled using the SwissDock server (Grosdidier et al., 2011b; Grosdidier et al., 2011a). Molecular graphics and analyses were performed with the University of California San Francisco Chimera package (Pettersen et al., 2004). Docked ligands were viewed using the ViewDock function of Chimera. The predicted free energy of binding (ΔG) values were calculated using SwissDock, which uses SwissParam (Zoete et al., 2011) and CHARMM (Brooks et al., 2009) to predict ΔG . To generate figures, predicted binding sites were sorted according to predicted ΔG values using ViewDock, and binding clusters with a calculated ΔG more than was calculated for inositol were displayed. Figures were generated using Persistence of Vision RaytracerTM V3.6 (Persistence of Vision Pty. Ltd., Williamstown, Australia). Electrostatic surface calculations were performed using PDB2PQR (Dolinsky et al., 2007), APBS (Baker et al., 2001) with default settings and visualised using Chimera.

2.2.8 Lipid extraction

To measure changes in total levels of PIP, a reverse phase UPLC-MS method was developed, based on methods previously reported (Ivanova et al., 2007; Ogiso and Taguchi, 2008; Ogiso et al., 2010; Pettitt, 2010). Cells were washed in warmed Neurobasal medium, prior to scraping in PBS containing PhosStop phosphatase inhibitor cocktail (1 tablet 10 mL⁻¹) at 4°C. Cell suspensions were centrifuged (13,000 rpm, 10 min, 4°C in a microcentrifuge) and the supernatant fraction was removed. Extraction buffer (200 μ L of 1:1 CHCl₃:MeOH with 0.25% (v/v) 12N

HCL) containing 50 pmol of each non-natural internal PIP standard (37:4 PI, 37:4 PI(3)P, 37:4 PI(3,4)P₂, 37:4 PI(3,4,5)P₃) was added to the cell pellets. Cell pellets were then thoroughly mixed for 30s using a vortex mixer. The phases were split by addition of 40 µL 1M HCL and 5.7 µL 2M NaCl, and separated by a brief pulse of centrifugation. The lower organic phase (80 µL) was transferred to a new Eppendorf tube, and the solvent was removed by evaporation using a centrifugal evaporator (Centrivap: Labconco Kansas City, USA). The dried lipid was resuspended in 50 µl 1:2:0.8 CHCl₃:MeOH:H₂O, thoroughly mixed using a vortex mixer and then centrifuged in a microcentrifuge (13,000 rpm, 20 min, 4°C) to remove any cellular debris. The supernatant fraction (45 µL) was then transferred to an autosampler vial with a small volume insert (Waters Australia Pty. Ltd, Rydalmere, Australia). Samples were snap frozen in liquid nitrogen and stored at -80°C prior to analysis. Samples were analysed by UPLC-MS within 24 hours after lipid extraction.

2.2.9 UPLC-MS lipid analysis

Cell lipid extracts were analysed by UPLC-MS in a random order. Cell lipid extracts (10 µL) were injected into a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer (Waters Australia). A Waters Acquity UPLC C18 column (2.1 x 100mm x 1.7 micron particles) was used for reverse phase liquid chromatography, with 0.13% ethylamine in water as solvent A and 0.13% ethylamine in acetonitrile as solvent B at a flow rate of 0.35 mL min⁻¹. The column was eluted with an initial isocratic mobile phase mixture of 90% A: 10% B for 1 min followed by a linear gradient to 0% A: 100% B over 9 min. The column was then washed with 0% A: 100% B for a further 3 min before

re-equilibration to initial conditions over 3 min. The column temperature was 40°C and the samples maintained at 6°C prior to injection. The mass spectrometer was operated in negative ion electrospray mode, and selected ion monitoring (SIM) was used to detect the specific phospholipids. The SIM masses for each ion and cone voltage are shown below in Table 2-1. The ion source temperature was 150°C, the desolvation gas was nitrogen (950 L hr⁻¹), and the desolvation temperature was 450°C. It was noticed that signals improved after priming the column, so before each batch of samples a 37:4 PIP₂ standard solution was injected 4 times. A PIP₂ standard solution was run after every 12 samples to ensure system performance was maintained.

Table 2-1 SIM ions, dwell times and cone voltages used for UPLC-MS

Lipid (acyl chain)	Ion m/z [M-H]-	Dwell time (ms)	Cone voltage (V)
PI 37:4 standard	871.4	18	55
PI 36:4	857.4	18	55
PI 38:5	883.4	18	55
PI 38:4	885.4	18	55
PI 38:3	887.4	18	55
PI(3)P 37:4 standard	951.4	18	65
PIP 36:4	937.4	18	65
PIP 38:5	963.4	18	65
PIP 38:4	965.4	18	65
PIP 38:3	967.4	18	65
PI(3,4)P ₂ 37:4 standard	1031.4	18	70
PIP ₂ 36:4	1017.4	18	65
PIP ₂ 38:5	1043.4	18	65
PIP ₂ 38:4	1045.4	18	65
PIP ₂ 38:3	1047.4	18	70
PI(3,4,5)P ₃ 37:4 standard	1111.4	18	70
PIP ₃ 36:4	1097.4	18	70
PIP ₃ 38:5	1123.4	18	70
PIP ₃ 38:4	1125.4	18	70
PIP ₃ 38:3	1127.4	18	70

Table shows the m/z ions that were monitored using selective ion monitoring (SIM), as well as the dwell times and cone voltages used for detection of each ion.

2.2.10 Mass spectroscopy data analysis

The total SIM intensity for each lipid elution peak was calculated by integrating the elution peak area. Elution peaks were selected for measurement according to the following criteria. Firstly, peaks had to be unambiguously identified using expected m/z and elution time relative to standards and/or other acyl chain variants. Secondly, the signal-to-noise ratio had to exceed 1:10, to allow for proper measurement. To account for variations in extraction efficiency between samples, PIP levels were normalized to the applicable internal standard. For each individual sample, the pmol of lipid per 1 million cells were calculated according to the formula (Raw elution intensity / 10 pmol internal standard elution intensity) x (total lipid extract volume / injection volume). Mean values for each treatment group were derived from 5 – 12 replicate wells (1×10^6 cells each) per treatment group. Experiments shown are representative of three independent experiments. Data analysis was performed using Graph Pad Prism V.6. Statistical significance was determined using one-way ANOVA with Tukey's or Dunnett's post-hoc tests, or Student's t-tests. Data with a p value < 0.05 were considered significantly different.

Chapter 3 Results

3.1 Studies on the binding of APP to lipids

sAPP α has been reported to elicit trophic effects in a number of cell types (Hoffmann et al., 2000; Pietrzik et al., 1998; Saitoh et al., 1989; Chasseigneaux et al., 2011; Gakhar-Koppole et al., 2008; Milward et al., 1992; Small et al., 1994; Jin et al., 1994; Ninomiya et al., 1994; Wallace et al., 1997; Caille et al., 2004; Demars et al., 2011; Freude et al., 2011; Ohsawa et al., 1999). sAPP α also binds to the surface of cells (Hoffmann et al., 1999), however the interactions of sAPP α with molecules on the surface of cells have been incompletely characterised.

There have been some reports that suggest sAPP α could interact directly with lipids on the cell surface. sAPP α has been reported to localise to a lipid raft type domain (Tikkanen et al., 2002). Recently sAPP α has been reported to directly interact with GM1-ganglioside (Zhang et al., 2009). However, the ability of sAPP α to bind to different types of lipids has not been explored in detail. Therefore, the first aim of the experiments presented in this thesis was to identify the types of lipid that sAPP α is able to bind.

3.1.1 Examination of the ability of sAPP α to bind to lipids

To investigate whether secreted forms of APP are able to bind to lipids, the binding of a recombinant sAPP α with an N-terminal 6xHis affinity tag (Appendix I) to lipids was examined using a protein-lipid overlay assay. This assay allows for the comparison of a protein's ability to bind to different types of lipid (Dowler et al., 2002). Recombinant sAPP α was incubated with hydrophobic membranes onto which 27 different types of lipid were immobilised. Membranes were

washed to remove any unbound sAPP α and the lipid-bound sAPP α was detected using an anti-6xHis antibody.

No significant sAPP α immunoreactivity was associated with major lipid species such as phosphatidylcholine, phosphatidylethanolamine and cholesterol (Fig. 3.1). However, sAPP α immunoreactivity was associated with several phosphatidylinositol phosphates (PIPs) and 3-sulfogalactosylceramide (Fig. 3.1). The most sAPP α immunoreactivity was associated with the PIP species PI(3)P, PI(4)P and PI(5)P, followed by PI(3,5)P₂ (Fig. 3.1B,C). Interestingly, there was minimal sAPP α immunoreactivity associated with phosphatidylinositol, which is structurally closely related to PIPs, but lacks the phosphate groups. Furthermore, there was no significant sAPP α immunoreactivity associated with GM1 (Fig. 3.1A), which has previously been reported to be capable of binding to sAPP α (Zhang et al., 2009).

To exclude the possibility that the binding of the recombinant sAPP α was due to an interaction of PIPs with the 6xHis tag, the binding of native sAPP α produced by mammalian cells was also examined. sAPP α -containing conditioned medium (Tg2576-CM) was collected from neural stem and progenitor cell (NSPC) cultures that were derived from Tg2576 mice. These cells over-express human APP carrying the K670N/M671L Swedish mutation and secrete human sAPP α into the cell medium (Hu et al., 2013). The ability of APP in the conditioned medium to bind to lipids was then examined using the protein-lipid overlay assay. As a control, wild-type conditioned medium (WT-CM) was also collected from NSPC cultures that were derived from littermate wild-type mice, which do not express human APP.

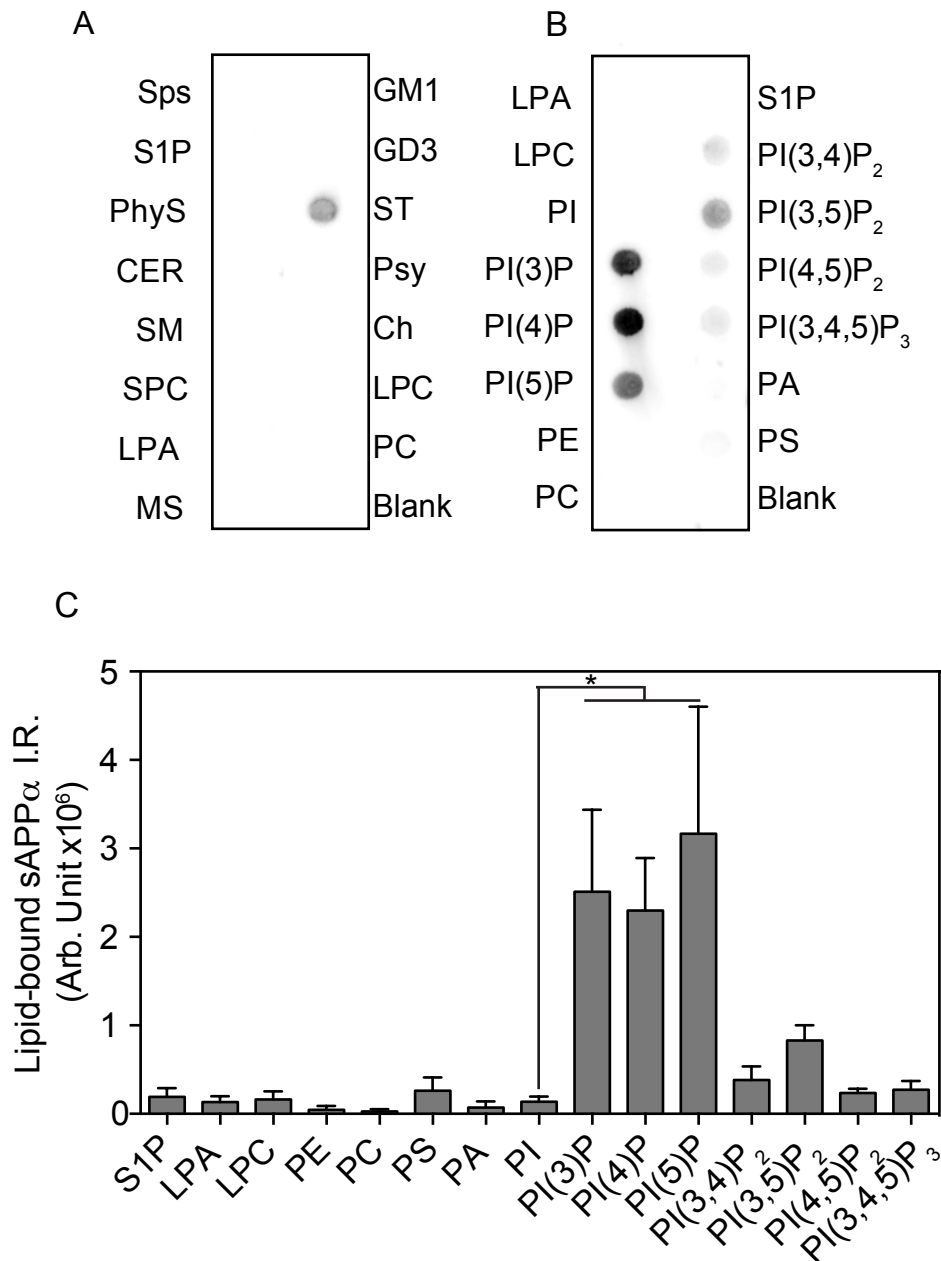


Figure 3.1. Recombinant 6xHis-tagged sAPP α binds selectively to PIP lipids and sulfatide. Panels A-C: Lipid binding preference of sAPP α for 50 pmol of each lipid was compared using a protein-lipid overlay assay. Membranes were incubated with 1 nM sAPP α overnight. Membranes were washed and lipid-bound protein was detected using a 6xHis antibody. Panel C: 6xHis immunoreactivity (I.R.) of 3 experiments as shown in panel B was quantified by densitometry. Bars show means \pm SEM of sAPP α I.R. Statistical significance was determined by 1 way ANOVA with Tukey's post-hoc test (* = $p < 0.05$ vs. PI). Abbreviations: Sps – Sphingosine, S1P - Sphingosine-1-phosphate, PhyS – Phytosphingosine, CER – Ceramide, SM – Sphingomyelin, SPC - Sphingosylphosphorylcholine, LPA - Lysophosphatidic acid, MS - Myriosine, GM1- Monosialoganglioside, GD3 - Disialoganglioside, ST - 3-sulfogalactosylceramide (sulfatide), Psy – Psychosine, Ch – Cholesterol, LPC - Lysophosphocholine, PC – Phosphatidylcholine, PI – Phosphatidylinositol, PI(3)P - Phosphatidylinositol (3) phosphate, PI(4)P - Phosphatidylinositol (4) phosphate, PI(5)P - Phosphatidylinositol (5) phosphate, PE – phosphatidylethanolamine, PI(3,4)P₂ - Phosphatidylinositol (3,4) biphosphate, PI(3,5)P₂ - Phosphatidylinositol (3,5) biphosphate, PI(4,5)P₂ - Phosphatidylinositol (4,5) biphosphate, PI(3,4,5)P₃ - Phosphatidylinositol (3,4,5) trisphosphate, PA - Phosphatidic acid, PS – Phosphatidylserine.

The conditioned medium was incubated with hydrophobic membranes onto which different lipids were immobilised. Membranes were washed to remove any unbound APP, and lipid-bound APP was then detected using the monoclonal antibody 6E10, which detects amino acid residues 672 to 688 of human APP. No lipid-associated sAPP α immunoreactivity was present using the conditioned medium collected from WT neurosphere cultures (Fig. 3.2A). When membranes were incubated with Tg2576-CM, no lipid-associated APP immunoreactivity was present for major lipid species such as phosphatidylcholine, phosphatidylethanolamine or cholesterol (Fig. 3.2B). However, sAPP α immunoreactivity was associated with PIPs, phosphatidic acid and 3-sulfogalactosylceramide (Fig. 3.2B). As the epitope of 6E10 antibody used to detect sAPP α is residues 1 - 16 of the A β region of APP, this antibody can detect both sAPP α and A β in the cell medium. Therefore, to determine if sAPP α bound to PIPs, the anti-APP N-terminus antibody 22C11 was also used to confirm that sAPP α in the Tg2576-CM binds to PIPs. When membranes were incubated with Tg2576-CM, 22C11 immunoreactivity was also associated with PIPs, demonstrating that sAPP α in the Tg2576-CM binds to PIPs (Fig. 3.2C).

3.1.2 Identification of a PIP-binding region in the E1 domain of APP

As the E1 domain of APP has a highly ordered structure, with some positively charged surfaces (Rossjohn et al., 1999), it was hypothesised that this region of APP could be responsible for the binding of sAPP α to the anionic PIPs observed in the experiments described in Section 3.1.1. Therefore, the possibility that there may be a PIP-binding domain in the E1 region of APP was examined using the protein-lipid overlay assay.

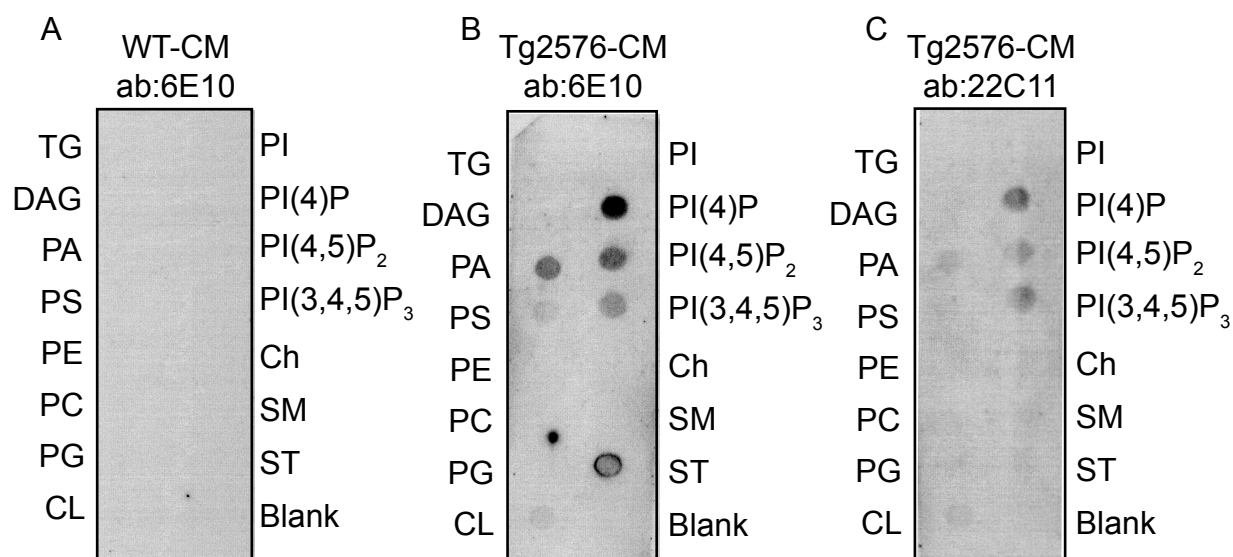


Figure 3.2. Human sAPP α secreted by human APP-overexpressing NSPC cultures binds to PIP lipids. Membrane strips were incubated with conditioned cell medium collected from WT-derived neurosphere cultures (WT-CM; panel A) or Tg2576-derived neurosphere cultures (Tg2576-CM; panels B and C) for 1h. Membranes were washed and lipid-bound APP was detected using the monoclonal 6E10 antibody (panels A and B) or the monoclonal APP N-terminal antibody 22C11 (panel C). Abbreviations not used previously: TG - triglyceride, DAG - diacylglycerol, PG- Phosphatidylglycerol.

A recombinant protein corresponding to the E1 domain of APP with a C-terminal 6xHis affinity tag (APP-E1) was incubated with hydrophobic membranes onto which lipids were immobilised. Membranes were washed, and lipid-associated APP-E1 was detected using an antibody against the 6xHis tag. Like sAPP α , the APP-E1 immunoreactivity was not associated with most lipids (Fig. 3.3). APP-E1 immunoreactivity was associated with PIPs and 3-sulfogalactosylceramide (Fig. 3.3). The APP-E1 bound more uniformly to all PIPs than was observed for sAPP α and gave a stronger chemiluminescent signal under similar incubation conditions (Fig. 3.1C vs. Fig. 3.3C). Therefore, these results suggested that APP has a PIP-binding region in the N-terminal E1 domain.

3.1.3 Competition of APP-E1 binding to PI(4,5)P₂ with a water soluble PIP analogue and mucosal heparin.

To determine that the binding of APP-E1 recombinant protein to PI(4,5)P₂ in the protein-lipid overlay assay was due to a specific interaction and could be reduced by competition, a water soluble short acyl chain PI(4,5)P₂ analog (diC8PI(4,5)P₂) was used to compete for binding. The effect of a range of concentrations of a water-soluble PIP analogue diC8PI(4,5)P₂ on APP-E1 binding to PI(4,5)P₂ was determined in the protein-lipid overlay assay. These results demonstrated that at least 5 μ M concentration of diC8PI(4,5)P₂ was required to significantly block the binding of 0.5 nM APP-E1 to 100 pmol PI(4,5)P₂ in vitro (Fig. 3.4A).

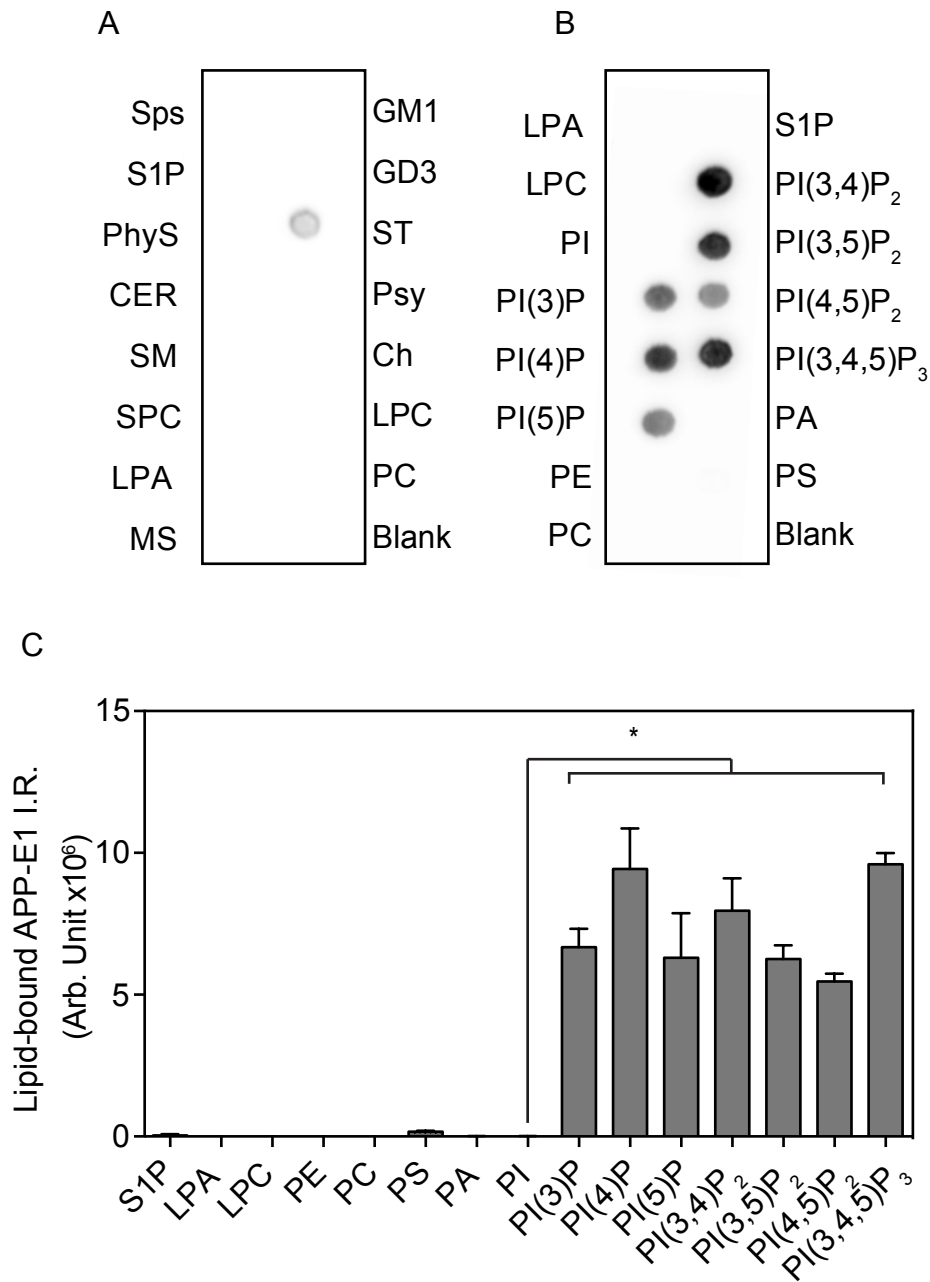
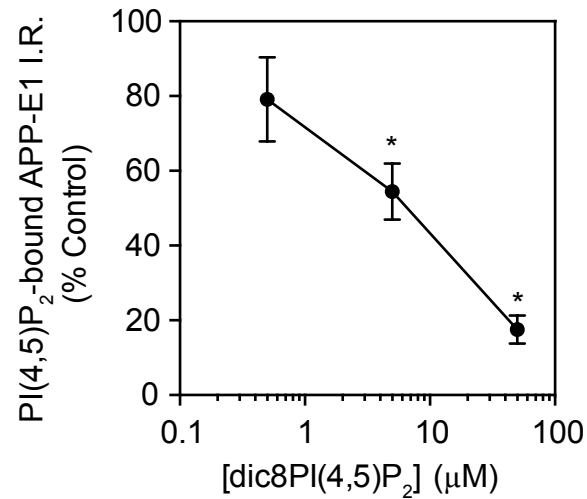


Figure 3.3. APP-E1 binds selectively to PIP lipids. Panels A -C: The lipid binding of APP-E1 was examined using the protein-lipid assay. Membranes were incubated with 1 nM APP-E1 overnight and lipid-bound protein was detected using a 6xHis antibody. Panel C: Immunoreactivity (I.R.) of lipid-bound APP-E1 from 3 experiments as shown in panel B was quantified by densitometry. Bars show means \pm SEM APP-E1 immunoreactivity. Statistical significance determined by 1 way ANOVA with Tukey's post-hoc test (* = $p < 0.05$ vs. PI).

A



B

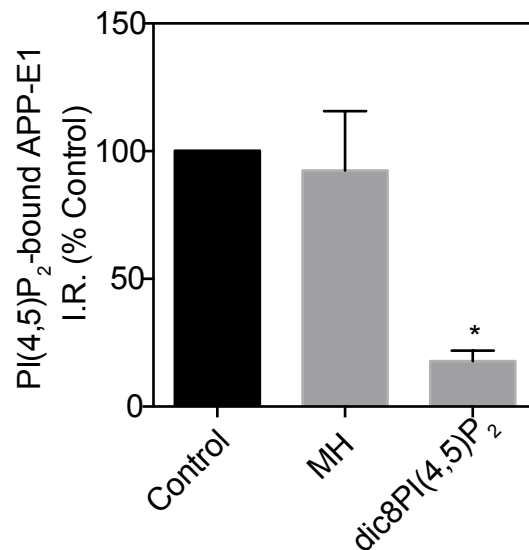


Figure 3.4. Dic8PI(4,5)P₂ but not mucosal heparin (MH) competes for APP-E1 binding to PI(4,5)P₂. Panel A: The effect of increasing diC8PI(4,5)P₂ concentration on APP-E1 (0.5 nM) binding to PI(4,5)P₂ (100 pmol) was examined in the protein-lipid overlay assay. Panel B: APP-E1 (0.5 nM) was incubated with MH (100 μg mL⁻¹, approx. 5.8 μM), dic8PI(4,5)P₂ (50 μM, 58.4 μg mL⁻¹) or PBS (control). PI(4,5)P₂-bound APP-E1 immunoreactivity (I.R.) was then quantified using densitometry. Bars show mean ± SEM of PI(4,5)P₂-bound APP-E1 immunoreactivity expressed as a percentage of PBS control from 9 replicates per treatment. Statistical significance was determined using 1-way ANOVA and Dunnett's post-hoc test (**p* < 0.05 vs control).

The amount of APP-E1 immunoreactivity associated with PI(4,5)P₂ in the presence of 0.5 μ M, 5 μ M and 50 μ M diC8PI(4,5)P₂ was observed to be 80%, 50% and 20% of control respectively (Fig 3.4A). However, only the 5 μ M and 50 μ M concentrations were significantly different from the PBS control (Fig. 3.4A). These data demonstrated that diC8PI(4,5)P₂ can compete for APP-E1 binding to PI(4,5)P₂, and also that the protein-lipid overlay assay was suitable for measuring the effect of a competitive inhibitor.

The E1 domain of APP contains a heparin-binding region (residues 95 – 110) and a metal-binding region (Small et al., 1994; Multhaup et al., 1996). As the heparin-binding region carries a positively charged surface (Rossjohn et al., 1999), it was hypothesised that this region of APP could be responsible for binding to PIPs. To investigate whether the heparin-binding domain could be responsible for the binding of APP to PIP, the ability of heparin to inhibit the binding of APP-E1 to PI(4,5)P₂ was assessed using the protein-lipid overlay assay. The concentration of heparin that was used (100 μ g mL⁻¹) was a \approx 40-fold molar excess compared to the amount of PI(4,5)P₂. This concentration of heparin has previously been demonstrated to block the binding of APP to cells (Ninomiya et. al., 1994). Heparin was not able to block the binding of APP-E1 to PI(4,5)P₂ in the protein-lipid overlay assay (Fig. 3.4B). In contrast, half the amount by weight (58.4 μ g mL⁻¹) of diC8PI(4,5)P₂ was able to block the binding of APP-E1 to PI(4,5)P₂ in the protein-lipid overlay assay (Fig. 3.4B). This suggests that the binding of APP-E1 to PI(4,5)P₂ is not via the heparin-binding domain in APP-E1 between residues 95 – 110, but through another region (Small et al., 1994; Clarris et al., 1997).

3.1.3.1 Computational modelling of the PIP-binding domain in the E1 domain of APP

The E1 domain of APP is a highly structured region, for which crystal structures are available (Dahms et al., 2010; Rossjohn et al., 1999). To identify possible binding sites for PIP in the APP E1 domain, a computational modelling approach was used. D-myo-inositol 1,4,5-trisphosphate (IP₃) is homologous in structure to the head group of PI(4,5)P₂. As a result, PI(4,5)P₂ binding proteins often bind to IP₃ with similar or higher affinity than to PI(4,5)P₂ (Lemmon et al., 1995; Garcia et al., 1995; Hyvonen et al., 1995). Therefore, the binding of IP₃ to the APP E1 domain was computationally modelled using the SwissDock protein-ligand docking tool (Grosdidier et al., 2011b; Grosdidier et al., 2011a). This tool models the interactions of small molecule ligands with proteins, to allow for visualisation of clusters of potential binding sites. The SwissDock server also estimates the free energy of binding (ΔG) based on electrostatic interactions calculated using the CHARMM fields for the protein (Brooks et al., 2009) and SwissParam field for the ligand (Zoete et al., 2011). These estimated ΔG values were used to indicate how strongly IP₃ could potentially interact with a particular binding site on the APP E1 domain.

The computational modelling of the interactions between the APP E1 domain and IP₃ suggested that there were two potential IP₃ binding pockets present on the E1 domain of APP (Fig. 3.5). The highest ranked binding pocket was at the heparin-binding loop, with energetically favorable predicted free energy of binding values (ΔG) in the range of -12.5 to -6.6 kcal mol⁻¹ (Fig 3.5).

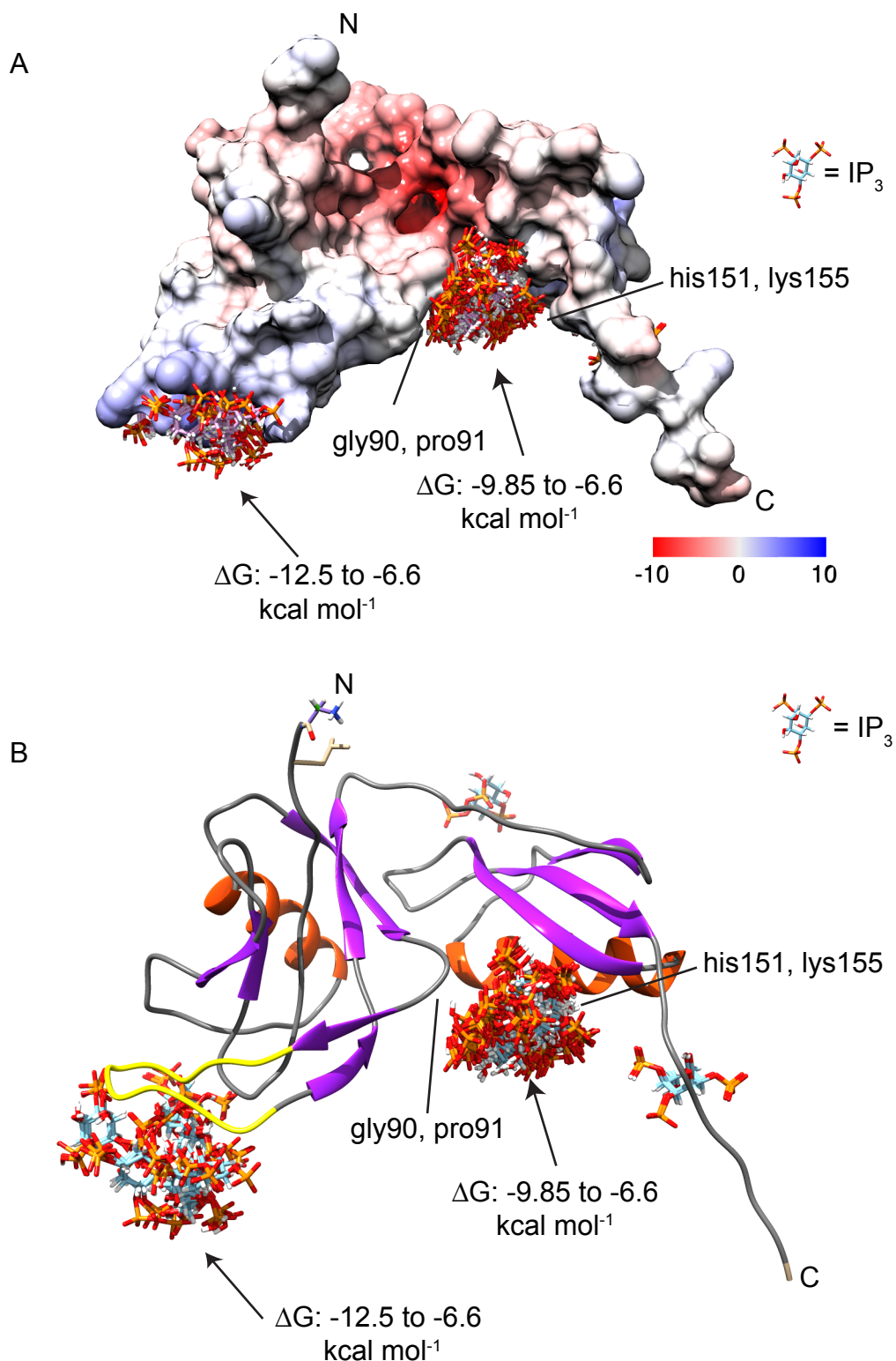


Figure 3.5. Possible binding sites of D-myo-inositol 1,4,5-trisphosphate (IP_3) on the APP E1 domain. The binding of IP_3 to the crystal structure of the APP E1 domain (PDB ID: 3KTM, Chain B) was modelled using the SwissDock protein docking server. Panel A: Electrostatic surface charge representation of the APP E1 domain. Panel B: Secondary structure cartoon representation of the APP E1 domain. Alpha-helices are coloured in orange and beta-sheets are coloured in purple. The heparin-binding domain (residues 95 - 110) has been highlighted in yellow. Clusters of predicted binding sites for IP_3 with a predicted free energy of binding (ΔG) more negative than $-6.52 \text{ kcal mol}^{-1}$ are shown. Labels indicate the range of predicted ΔG for each binding cluster. The positions of histidine 151, lysine 155, glycine 90 and proline 91 are also indicated.

Electrostatic surface modelling demonstrates this region of the APP E1 domain has a positively charged surface, which could theoretically interact with anionic PIP (Fig 3.5A). A second potential IP₃ binding pocket was present closer to the N-terminus in a pocket formed by the amino acid residues histidine 151, lysine 155, glycine 90 and proline 91. This potential binding site had a slightly less negative range of predicted ΔG values, in the range of -9.85 to -6.6 kcal mol⁻¹ (Fig 3.5).

As the E1 domain of APP may also form a dimer (Dahms et al., 2010), the binding of IP₃ was also modelled to the APP E1 domain dimer (PDB ID: 3KTM). The computational modelling suggested a number of potential binding sites for IP₃ on the APP E1 dimer (Fig. 3.6). A number of binding pockets were predicted around the heparin-binding region (residues 95 - 110) and interestingly, these had more negative predicted ΔG values (in the range of -17 to -9 kcal mol⁻¹) than was predicted for the APP E1 domain monomer (Fig. 3.6). The positively charged surface around the heparin-binding site is larger in the APP E1 domain dimer, which could explain the more negative predicted ΔG (Fig. 3.6A). Additionally, there were other potential binding pockets closer to the N-terminus of the APP E1 domain, with less negative predicted ΔG values in the range of -9 to -7 kcal mol⁻¹.

To provide context to these modelled interactions of APP with IP₃, positive and negative control models were produced. As a negative control, the binding of the APP E1 domain to inositol was computationally modelled. Inositol was used as a control as inositol is structurally similar to IP₃, but does not have the negatively charged phosphate groups.

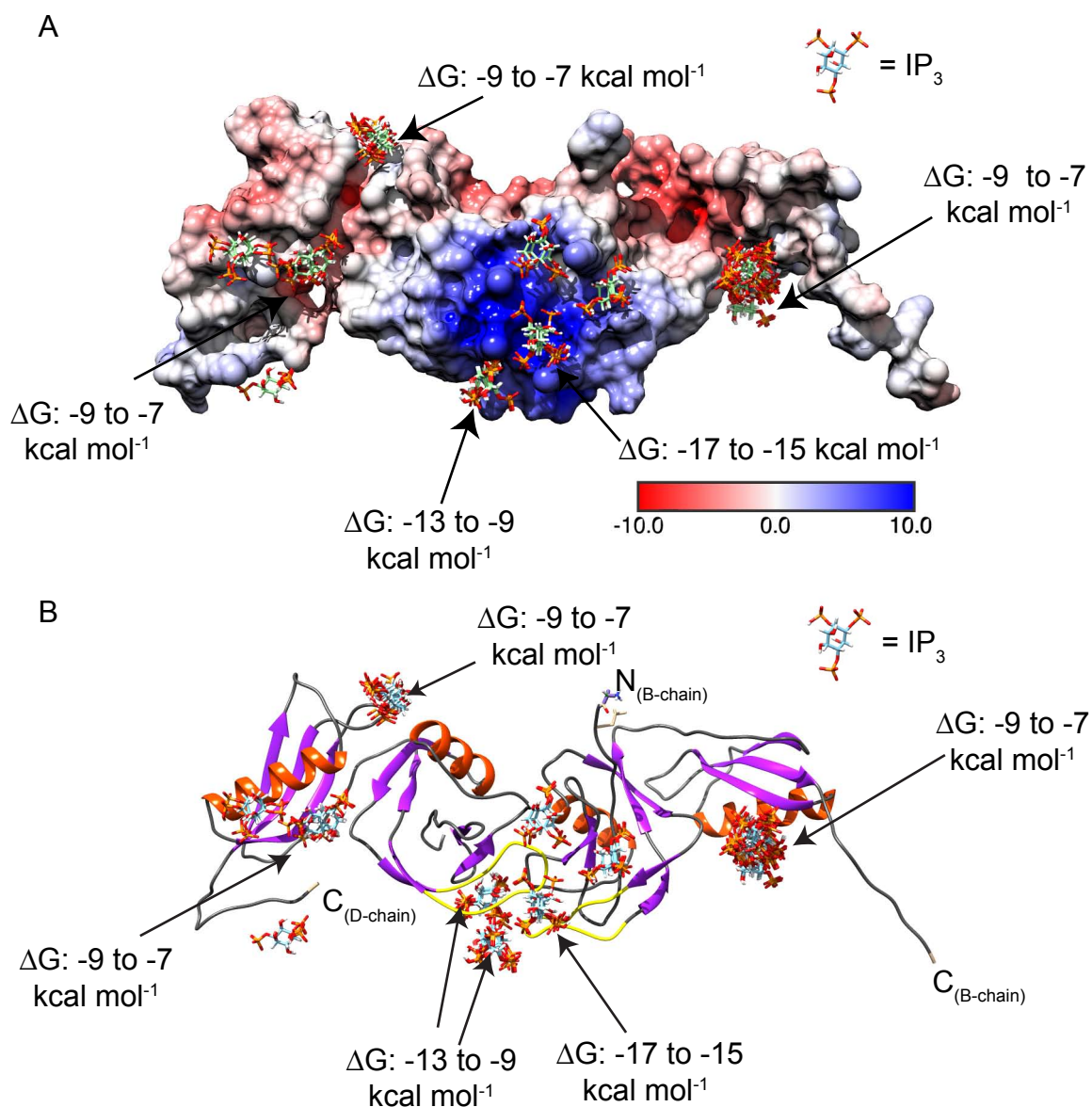


Figure 3.6. Possible binding sites for D-myo-inositol 1,4,5-trisphosphate (IP_3) on the heparin-induced APP dimer. The binding of IP_3 to the crystal structure of the heparin-induced APP E1 dimer (PDB ID: 3KTM, Chains B+D) was modelled using the Swiss-Dock protein docking server. Panel A: Electrostatic surface charge representation of the APP E1 dimer. Panel B: Secondary structure cartoon representation of the APP E1 dimer. Alpha-helices are coloured in orange and beta-sheets are coloured in purple. The heparin-binding domain (residues 95 - 110) has been highlighted in yellow. Clusters of predicted binding sites for IP_3 with a predicted ΔG more negative than $-6.7 \text{ kcal mol}^{-1}$ are shown. Labels indicate the range of predicted ΔG for each binding cluster.

The computational modelling of the binding of inositol to the APP E1 domain monomer and the APP E1 domain dimer produced a number of potential binding sites on the APP E1 domain (Fig. 3.7 A, B). The potential binding sites for inositol were distinct from those predicted for IP₃, and all had less energetically favourable predicted ΔG values (in the range of -6.7 to -4.2 kcal mol⁻¹) than was predicted for APP binding to IP₃. Therefore, these results suggested that interactions of IP₃ with the APP E1 domain were more energetically favorable than the interaction of inositol with the APP E1 domain. This was consistent with the results of the protein-lipid overlay assay, which found that APP-E1 bound to PIP but not to PI.

As a positive control model, the binding of IP₃ to the pleckstrin homology domain of spectrin (spectrin-PH) was modelled using the SwissDock server. The pleckstrin homology domain of spectrin (spectrin-PH) binds selectively to PI(4,5)P₂ with a dissociation constant (K_d) of around 40 μ M (Hyvonen et al., 1995). Further, the IP₃ binding site for spectrin-PH has been identified using X-ray crystallography (Hyvonen et al., 1995). Modelling the interactions between spectrin-PH and IP₃ produced a number of potential binding sites for IP₃ that matched the previously reported binding site (Fig. 3.7C; Hyvonen et al., 1995). The range of predicted ΔG values for these interactions were -15.8 to -7.87 kcal mol⁻¹ (Fig 3.7C). Therefore, this modelling suggested that some of the potential IP₃ binding sites identified for the APP E1 domain (predicted ΔG range -17.0 to -6.6 kcal mol⁻¹) were as energetically favorable as were predicted for the spectrin PH domain (predicted ΔG range -15.8 to -7.87 kcal mol⁻¹). Therefore, these theoretical data supported the presence of a PIP-binding region in the E1 domain of APP.

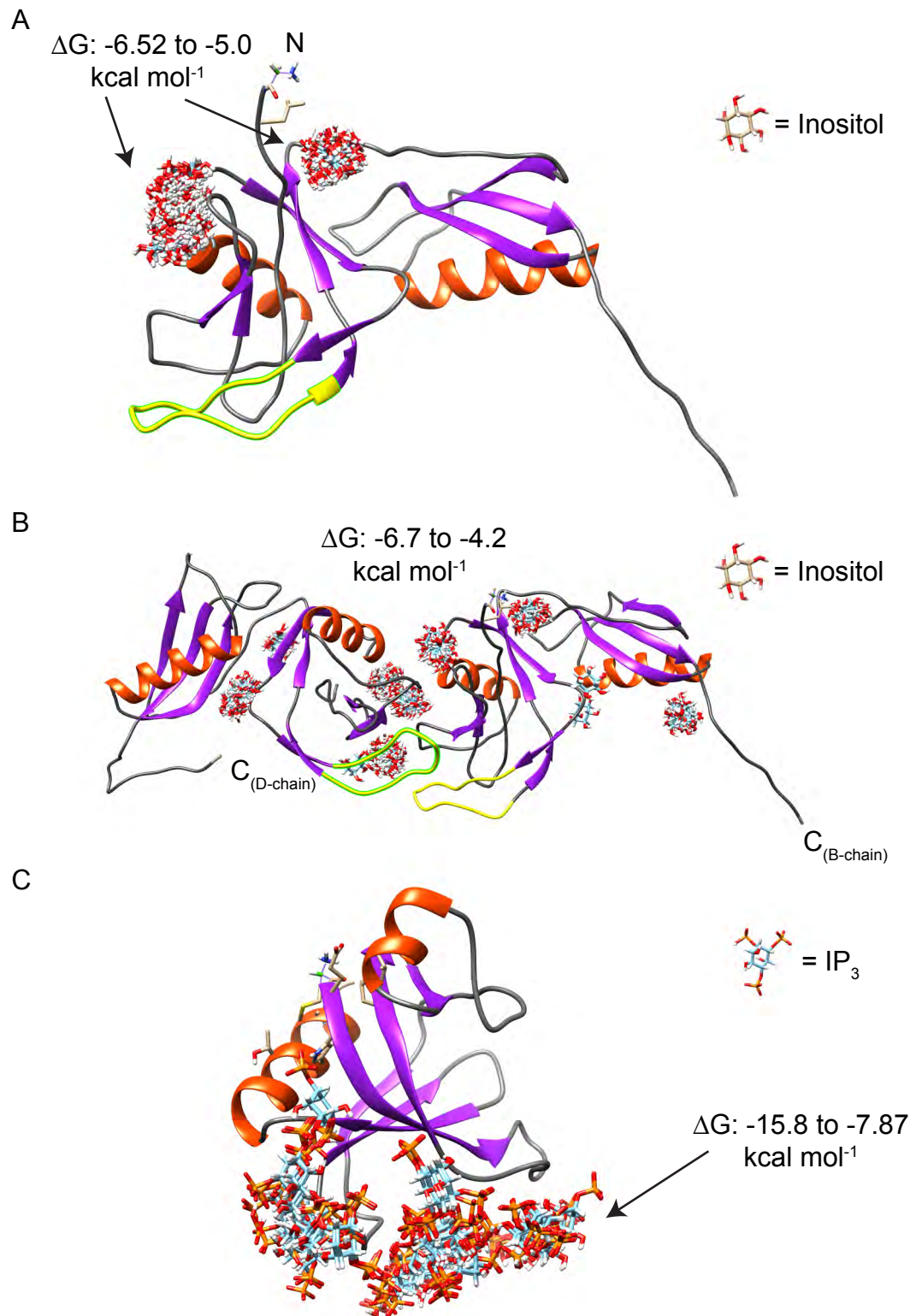


Figure 3.7. Positive and negative tests of the computational docking model. Panel A shows the predicted binding clusters and the range of predicted free energy of binding (ΔG) for inositol binding to the APP E1 domain (PDB ID: 3KTM, chain B), modelled using the SwissDock protein docking server. Panel B shows the predicted binding clusters and the range of predicted ΔG for inositol binding to the heparin-induced APP E1 dimer (PDB ID: 3KTM, chain B and D). The heparin-binding domain (residues 95 - 110) has been highlighted in yellow. Panel C shows predicted binding sites and predicted ΔG for D-myo-inositol 1,4,5-trisphosphate (IP_3) binding to the pleckstrin homology domain of spectrin (PDB ID: 1BTN). Alpha-helices are coloured in orange and beta-sheets are coloured in purple.

3.2 Studies on the binding of APP to hippocampal neurons

The central hypothesis of this thesis was that APP directly interacts with lipids on the cell surface. The results reported in Section 3.1 showed that sAPP α and APP-E1 are able to bind to PIPs in an in vitro assay. Therefore, to test the hypothesis that APP can interact with PIPs on the cell surface, a cell culture model was developed. Previous studies have reported that sAPP α binds to the surface of a number of different cell types (Hoffmann et al., 1999; Tikkanen et al., 2002). In these studies, purified sAPP α was added to cell cultures and cell-bound sAPP α was detected using immunocytochemistry (Hoffmann et al., 1999; Tikkanen et al., 2002). Therefore, the same experimental approach was chosen for the following experiments.

Primary murine hippocampal cultures were prepared at a low density of 1×10^5 cells per coverslip to enable visualisation of individual cells. Cultures were initially plated in 10% serum, which produced a glial feeder layer, with neuronal cells growing on top (Fig. 3.8A). To examine the binding of APP to cells, the primary murine hippocampal cultures were incubated with recombinant 6xHis tagged sAPP α and recombinant 6xHis tagged APP-E1, after which unbound protein was removed by washing with culture medium. Cell-bound sAPP α or cell-bound APP-E1 was then detected using an antibody directed against the 6xHis tag on the recombinant proteins.

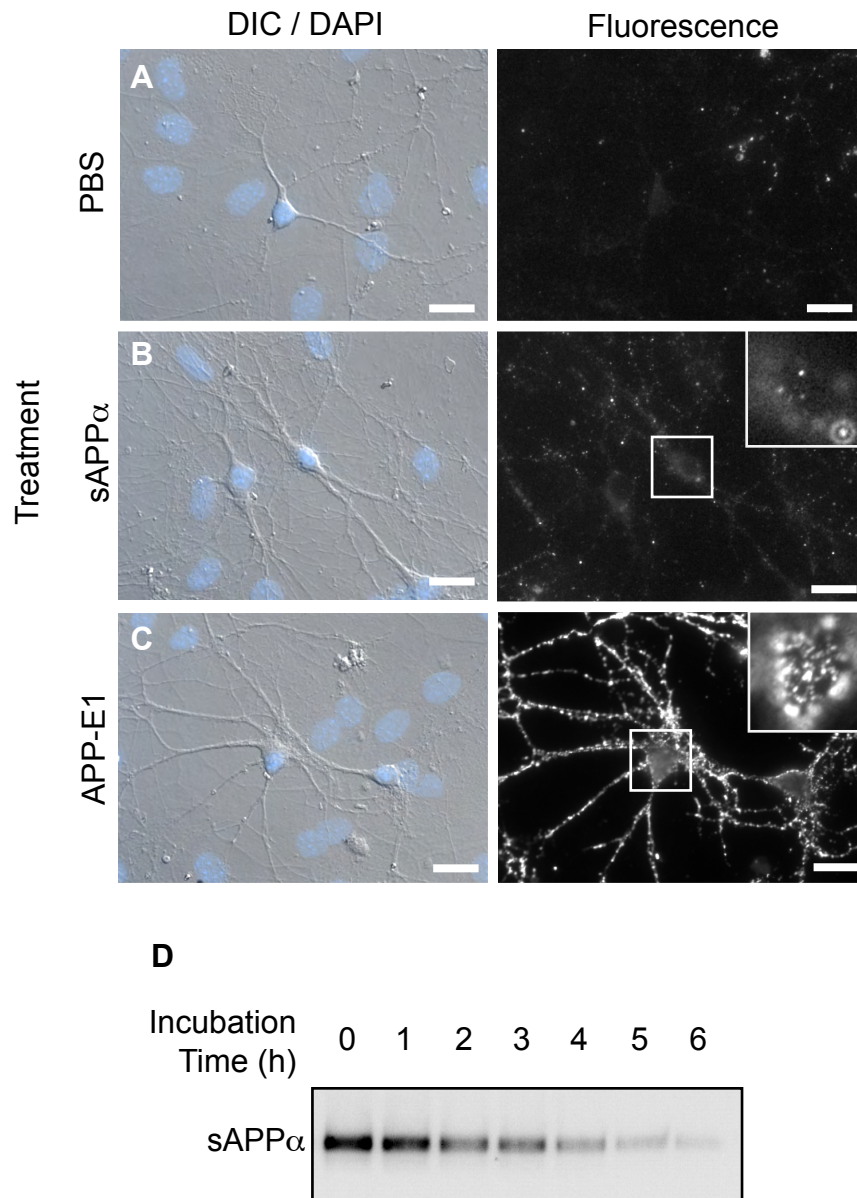


Figure 3.8. APP-E1 and sAPP α bind to neuronal cells. Primary hippocampal cultures were incubated with PBS control (panel A), 50 nM sAPP α (panel B) or 50 nM APP-E1 (panel C) for 2 h. Cells were washed, and cell-surface bound APP was detected by immunocytochemistry using an anti-6xHis antibody. The nuclei of glial cells in the feeder layer are visible in the DAPI channel on the left. Note: the contrast in panel C is lower than panels A and B. Insets show enlargement of cell soma region shown in white boxes, focussed on the cell surface. Scale bars = 20 μ m. Panel D shows a western blot of recombinant sAPP α immunoreactivity over time, showing evidence for low stability of the recombinant sAPP α . Recombinant sAPP α (350 ng) was incubated in 105 μ L tris buffered saline at 22°C. At each time point 15 μ L (50 ng equivalent) was removed, added to SDS-PAGE sample buffer and heated to 95°C for 10 minutes. The samples were then analysed by SDS-PAGE, blotted onto PVDF membranes, and immunolabelled using the monoclonal antibody 6E10.

Some sAPP α bound to cells with a punctate pattern, however, this immunoreactivity was only visible when the contrast of the images was enhanced, and the signal was difficult to distinguish from background fluorescence (Fig. 3.8B, A). However, much more immunoreactivity associated with cells was observed using the APP-E1 recombinant protein than was observed using sAPP α recombinant protein (Fig. 3.8). APP-E1 bound selectively to neurons, with a distinctive punctate pattern along the dendrites and over the surface of the cell soma (Fig. 3.8C). There was no binding of APP-E1 to the glial cells in the feeder layer (Fig. 3.8C), indicating that the cell binding of exogenously added APP-E1 is to a specific site present on neuronal cells.

Subsequent investigation suggested that the recombinant sAPP α was unstable, as the 6E10 immunoreactivity of the recombinant sAPP α spontaneously declined over time with a half-life of approximately two hours (Fig. 3.8C). This may explain why the cell-associated APP immunoreactivity was weak. Therefore, as the recombinant sAPP α was unstable and the sAPP α immunoreactivity associated with cells was weak, the APP-E1 recombinant protein was used for the majority of experiments in this study.

3.2.1 Identification of PIPs on the extracellular surface of the cell membrane

As APP-E1 was able to bind to PIP *in vitro* and was also able to bind to the surface of neurons, the possibility that APP-E1 could bind to PIPs on the surface of neurons was investigated. Although there have been some reports of the presence of PIPs on the surface of cells (Gascard et al., 1991; Fogler et al., 1987; Kale et al., 2010), most studies of PIPs have focussed on the role of PIPs on the cytoplasmic side of the plasma membrane (PM). Therefore, to investigate whether APP could interact with PIPs on the cell surface, it was first necessary to determine whether PIPs were present on the surface of cells.

3.2.1.1 Immunocytochemical detection of cell-surface PIPs

A number of studies have used monoclonal antibodies to investigate the localisation of PIPs inside the cell (Hammond et al., 2012; Hammond et al., 2009). To determine whether PIPs were present on the surface of cells, primary hippocampal cultures were incubated with anti-PIP monoclonal antibodies. Antibody labelling was performed on live cells at 4°C to minimise the possibility of disruption of the cell membrane and the consequent detection of intracellular PIPs (Rusten and Stenmark, 2006; Hammond et al., 2009).

PI(4)P, PI(3)P, PI(3,5)P₂ and PI(3,4,5)P₃ immunoreactivity was not detected on the surface of primary hippocampal cultures (Fig. 3.9A, B, D, E). However, PI(4,5)P₂ immunoreactivity was detectable on the surface of cells in primary hippocampal cultures (Fig. 3.9C). The anti-PI(4,5)P₂ antibody labelled the surface of both neurons and glia in the feeder layer (Fig. 3.9C, 3.10A). PI(4,5)P₂ immunoreactivity exhibited a punctate distribution with puncta < 1 µm present along dendrites and on the surface of the neuron soma (Fig. 3.10A). The specificity of the anti-PI(4,5)P₂ monoclonal antibody for binding to cellular PI(4,5)P₂ was demonstrated by pre-adsorbing the antibody with a 1000x molar excess of a water-soluble analog of PI(4,5)P₂ (diC8 PI(4,5)P₂). Pre-absorption of the PI(4,5)P₂ antibody abolished antibody binding to cells (Fig. 3.10B).

To confirm that the PI(4,5)P₂ immunoreactivity that was observed on the cells was extracellular and not intracellular, the localisation of the PI(4,5)P₂ immunoreactivity was compared with that of the intracellular proteins MAP2 and GFAP using both live unfixed cells and cells that had been fixed and permeabilised with 4% paraformaldehyde and 0.3% triton X-100. The principle of the experiment was that the antibodies directed against intracellular antigens would not be immunoreactive with cells when the cell membrane is intact, but extracellular antigens would be detected.

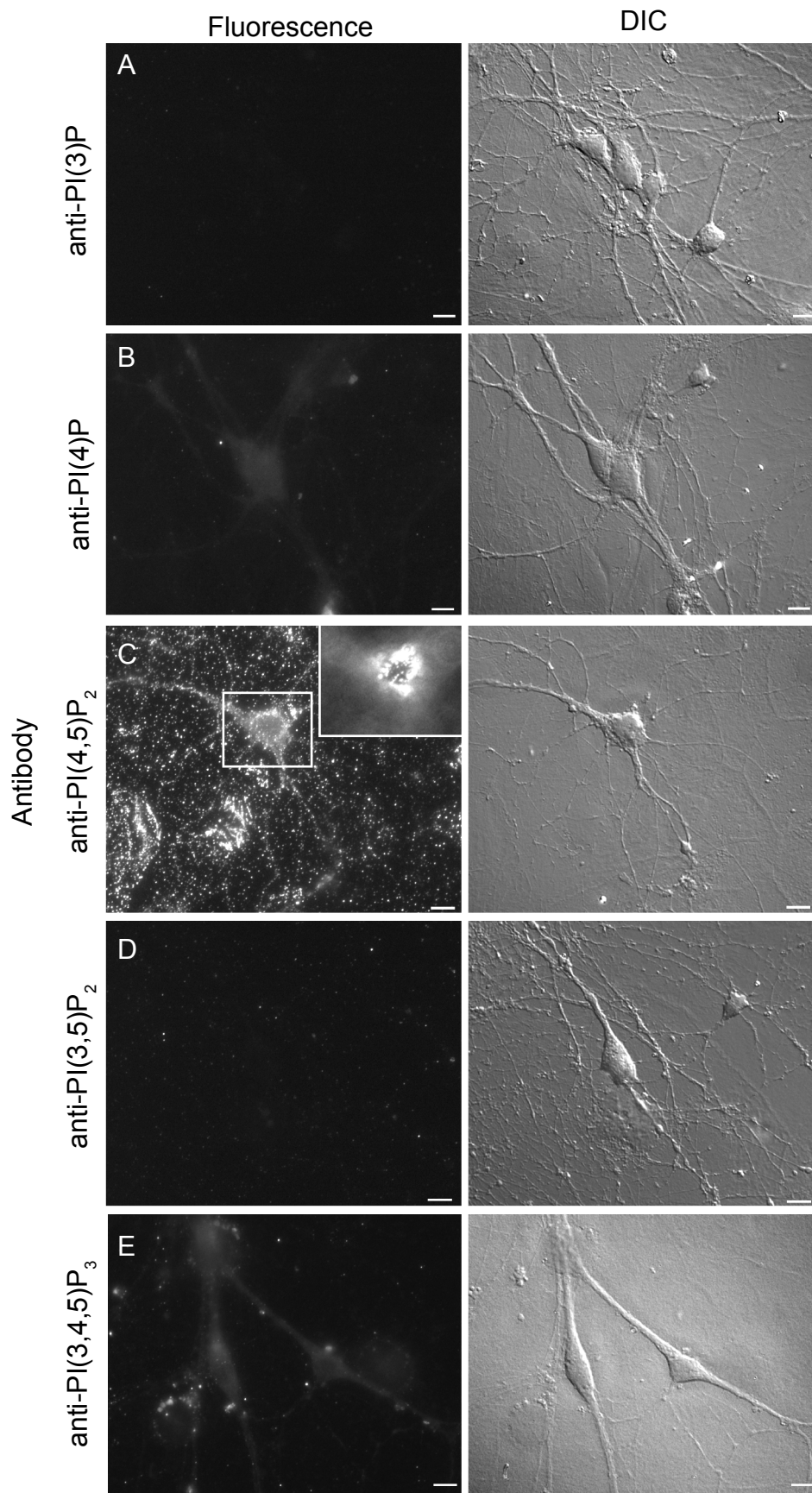


Figure 3.9. Evidence for the presence of cell-surface PI(3)P, PI(4)P, PI(4,5)P₂, PI(3,5)P₂ and PI(3,4,5)P₃ immunoreactivity on cells in hippocampal culture. Live hippocampal cultures were labelled at 4 °C with monoclonal PIP antibodies. Inset shows enlargement of region in white box focused on surface of the neuron soma. Scale bars = 10 μ m.

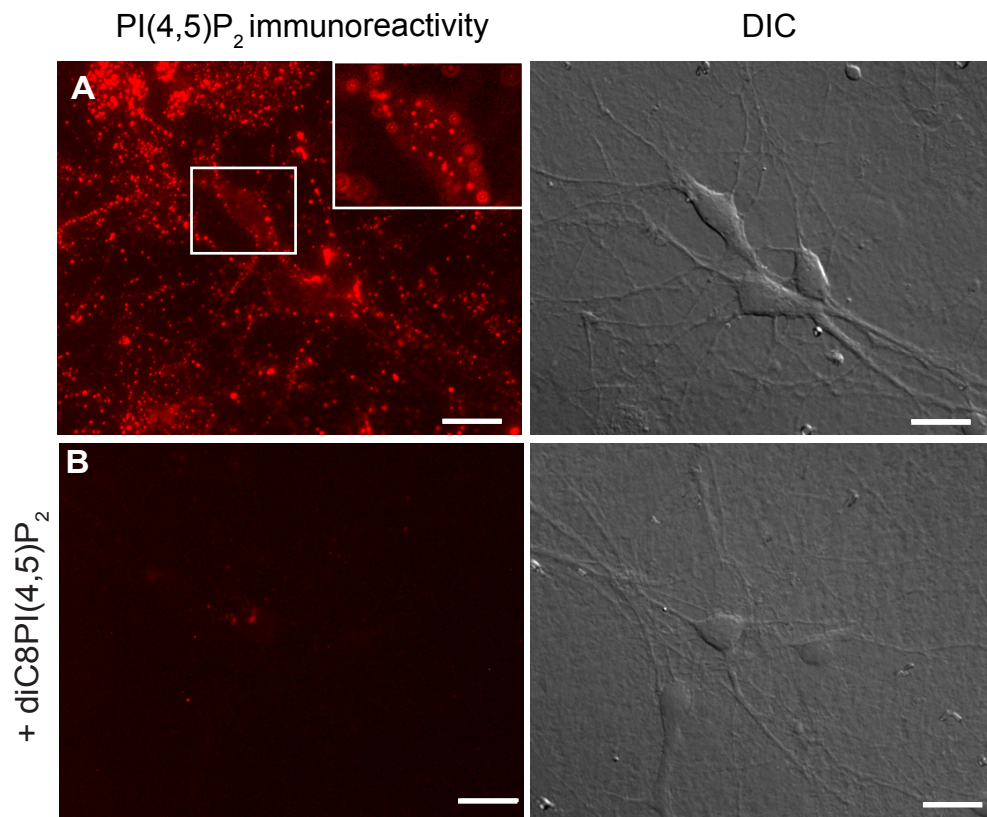


Figure 3.10. Effect of preabsorption of anti-PI(4,5)P₂ antibody with diC8PI(4,5)P₂ on cell-surface PI(4,5)P₂ staining. Primary hippocampal cultures were labelled at 4°C with a PI(4,5)P₂ antibody (Panel A), or a PI(4,5)P₂ antibody that had been preabsorbed with 20 μM diC8PI(4,5)P₂ (approx. 1000x molar excess; Panel B). Insets show enlargement of region indicated in white box, but focussed on the surface of the neuron soma. Scale bars = 20 μm.

Anti-MAP2, anti-GFAP and anti-PI(4,5)P₂ antibodies were incubated with live cells and with cells that had been first fixed and permeabilised prior to immunolabelling. MAP2 immunoreactivity was not detectable in the live-cell labelled cells (Fig. 3.11A), but punctate PI(4,5)P₂ immunoreactivity was detectable along the dendrites and over the surface of cells (Fig. 3.11C). Cells that were fixed and permeabilised prior to antibody labelling were immunoreactive for both antibodies (Fig. 3.11B, D). In the fixed/permeabilised cells, the MAP2 antibody labelled the dendrites and soma of neurons, and intracellular PI(4,5)P₂ immunoreactivity was present in neurons and glia (Fig. 3.11B, D).

Similarly, GFAP immunoreactivity was not detectable in the live-cell labelled cells (Fig. 3.11E), but punctate PI(4,5)P₂ immunoreactivity was present on the surface of glia (Fig. 3.11G). When cells were fixed and permeabilised prior to antibody labelling, GFAP immunoreactivity was present in glia and PI(4,5)P₂ immunoreactivity was present in neurons and glia (Fig. 3.11F, H). Therefore, these results demonstrated that PI(4,5)P₂ is present on the surface of both neuronal and glial cells in the hippocampal cultures.

3.2.1.2 Biosensor detection of cell-surface PIP

To confirm the specificity of the staining of PIPs on the cell surface, commercially available PIP biosensors were also used. PIP biosensors are recombinant glutathione-s-transferase (GST) -tagged PIP-binding domains that have been well characterised for their ability to bind selectively to individual PIP species. Cells were incubated with a PI(3)P biosensor (recombinant GST-tagged PX domain of p40phox; Stahelin et al., 2003), a PI(4)P biosensor (recombinant GST-tagged SidC_3C domain; Weber et al., 2006), a PI(4,5)P₂ biosensor

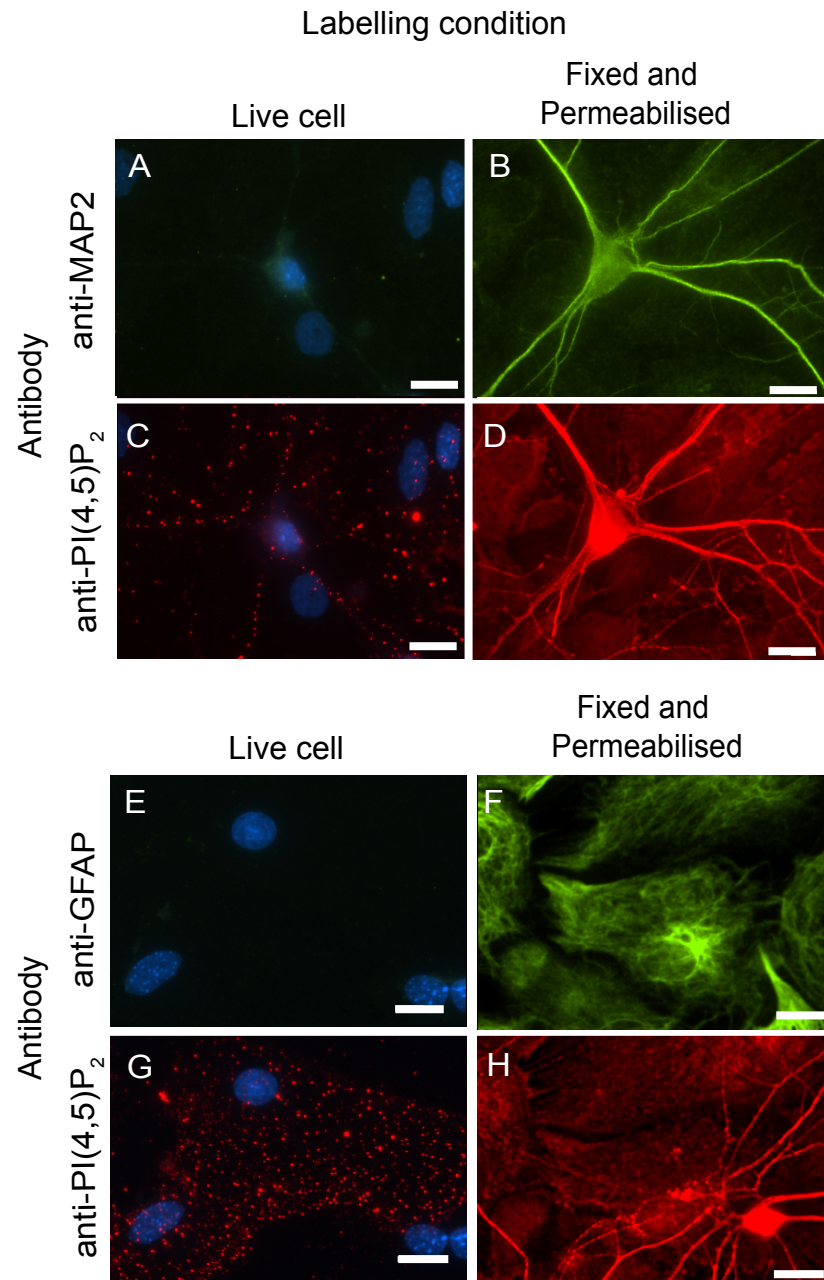


Figure 3.11. PI(4,5)P₂ immunoreactivity is present on the surface of cells in hippocampal cultures. Live or fixed/permeabilised primary hippocampal neurons were labelled with anti-MAP2, anti-GFAP and anti-PI(4,5)P₂ antibodies. DAPI staining is shown in panels A,C,E and G. Panels A, C: Extracellular MAP2 and PI(4,5)P₂ immunoreactivity of live cells labelled at 4°C. Panels B and D show MAP2 and PI(4,5)P₂ immunoreactivity, with labelling performed in parallel at 4°C on cells that have been fixed (4% PFA, 20 minutes), permeabilised (5 min, 0.3% Triton X-100 in PBS) and washed immediately prior to labelling. Note, the contrast between panels A + B, C + D, and B + D is not equivalent, as intracellular PI(4,5)P₂ immunoreactivity is much higher than extracellular PI(4,5)P₂ immunoreactivity. Contrast between panels A + C is equivalent. Panels E - H show equivalent experiment using an anti-GFAP antibody. Panels E + G show extracellular GFAP and PI(4,5)P₂ immunoreactivity of live cells labelled at 4°C. Panels F + H show GFAP and PI(4,5)P₂ immunoreactivity with labelling performed in parallel at 4°C on cells that have been fixed, permeabilised and washed immediately prior to labelling. Note: contrast between panels E + F, G + H, F + H is not equivalent. Contrast between panels E + G is equivalent. Scale bars = 20 µm.

(recombinant GST tagged pleckstrin homology domain of phospholipase C- δ 1; Kavran et al., 1998) or a PI(3,4,5)P₃ biosensor (recombinant GST-tagged pleckstrin homology domain of general receptor for phosphoinositides-1; Kavran et al., 1998). Cells were washed and then cell-bound biosensors were detected using an antibody directed against the GST affinity tags attached to the biosensors.

The PI(4,5)P₂ biosensor was the only biosensor that was observed to bind to the surface of primary neuronal cultures (Fig. 3.12D). The PI(4,5)P₂ biosensor binding pattern was similar to that observed with the anti-PI(4,5)P₂ antibody, as it stained cells with a punctate distribution on the surface of both neurons and glia. PI(4)P, PI(3)P and PI(3,4,5)P₃ biosensors stained the surface of cells poorly, or not at all (Fig. 3.12B, C, E). A low-level of biosensor binding was observed using the PI(3)P biosensor (Fig. 3.12B). The PI(3,4,5)P₃ biosensor had a high propensity to bind to extracellular debris, visible as regions of high intensity staining (Fig. 3.12E). These data supported the conclusion that PI(4,5)P₂ is present extracellularly on neuronal and glial cells. Some of the other PIPs may also be present on the surface of cells, although the detectable levels of these lipids are much lower than PI(4,5)P₂.

3.2.2 Co-localisation of exogenous APP-E1 and cell-surface PI(4,5)P₂

The data presented in Section 3.1 demonstrate that APP-E1 contains a PIP-binding domain, while the data presented in Section 3.2.1 demonstrate that PI(4,5)P₂ is present on the surface of hippocampal cells in culture. Therefore, to assess whether APP-E1 could bind to PI(4,5)P₂ on the neuronal cell surface, the distribution of PI(4,5)P₂ immunoreactivity, as well as the distribution of the

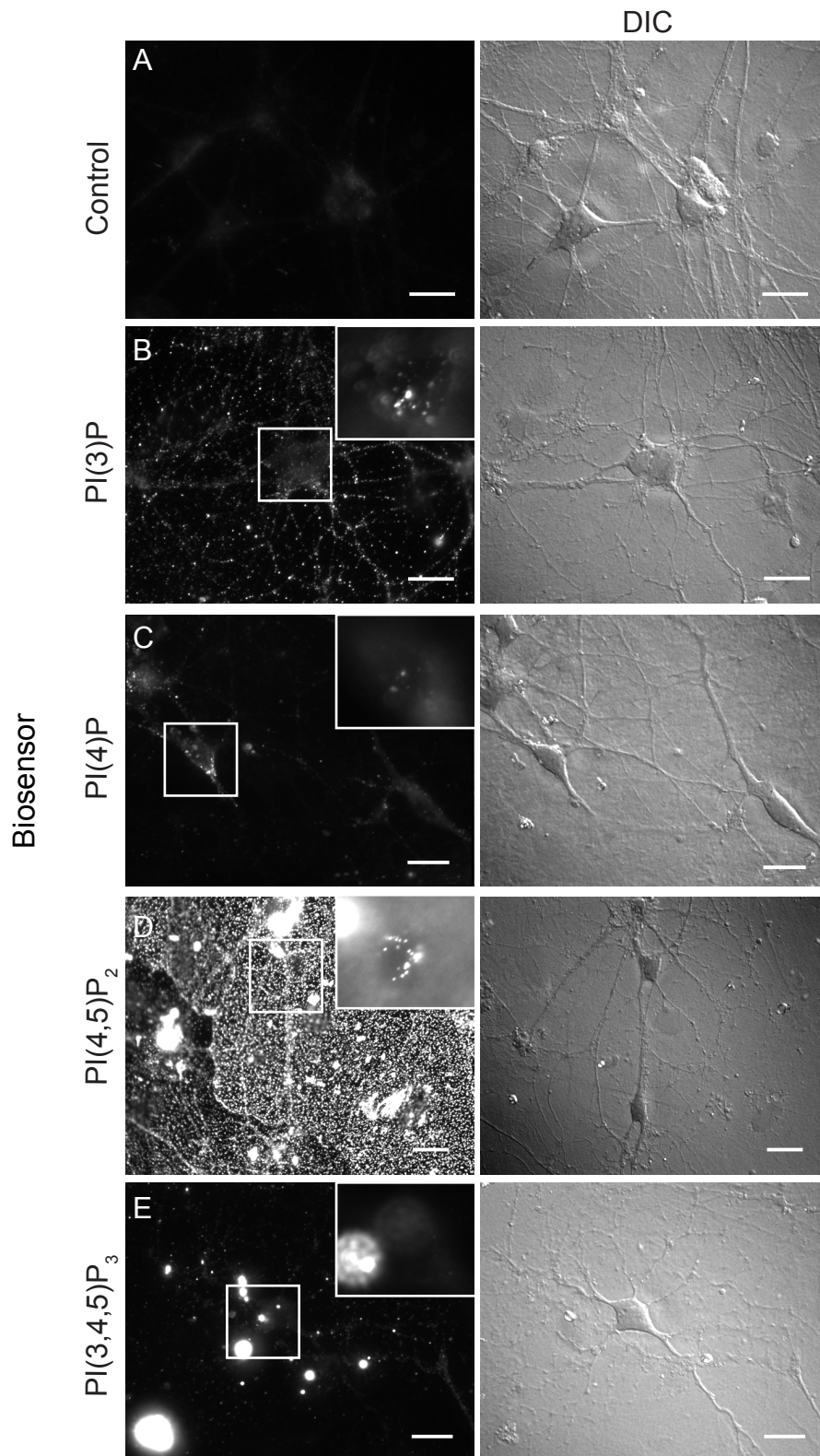


Figure 3.12. Detection of cell-surface PIPs using PIP biosensor proteins. Primary hippocampal cultures were incubated with 50 nM of each biosensor for 2h at 37°C. Cells were washed, and cell-bound biosensors were detected by fluorescence immunocytochemistry using an anti-GST antibody performed on live cells at 4°C. Insets show enlargement of the neuronal soma shown in white boxes, focused on the cell surface. Scale bars = 20 μ m

PI(4,5)P₂ biosensor and APP-E1 was examined. First, primary hippocampal cultures were incubated with APP-E1, or APP-E1 and the PI(4,5)P₂ biosensor. Cells were washed and cell-bound APP-E1 was detected using antibodies directed against the 6xHis tag and cell-bound PI(4,5)P₂ biosensor was detected using an antibody directed against the GST tag. Alternatively, the cell-surface PI(4,5)P₂ was labelled using the PI(4,5)P₂ antibody.

A high degree of co-localisation of APP-E1 and PI(4,5)P₂ was observed on neuronal cells using both the PI(4,5)P₂ biosensor and the PI(4,5)P₂ antibody (Fig. 3.13C, F). This co-localisation was confirmed using confocal microscopy (Fig. 3.14A). APP-E1 bound to neurons with a punctate pattern and PI(4,5)P₂ was present in small puncta that were highly colocalised with APP-E1 (Fig. 3.13, 3.14). However, APP-E1 was found to bind only to neuronal cells, whereas PI(4,5)P₂ immunoreactivity was present both on neurons and on the surface of glia in the feeder layer (Fig. 3.13). Taken together the data suggest that APP-E1 binds to the neuronal cell surface and localises to PI(4,5)P₂ micro-domains. The data also show that while APP-E1 colocalises with PI(4,5)P₂ on neurons, PI(4,5)P₂ cannot be the sole determinant of APP binding to the cell surface, as PI(4,5)P₂ was detected on glia where no APP-E1 was bound.

3.2.3 Competition of APP-E1 binding to cells with diC8PI(4,5)P₂

The data in Section 3.2.2 indicated that PI(4,5)P₂ and APP-E1 was co-localised on the cell surface of neurons. However, these data also suggested that the binding of APP-E1 to PI(4,5)P₂ was not sufficient for binding to neurons, as PI(4,5)P₂ was also detected on glial cells where APP-E1 did not bind.

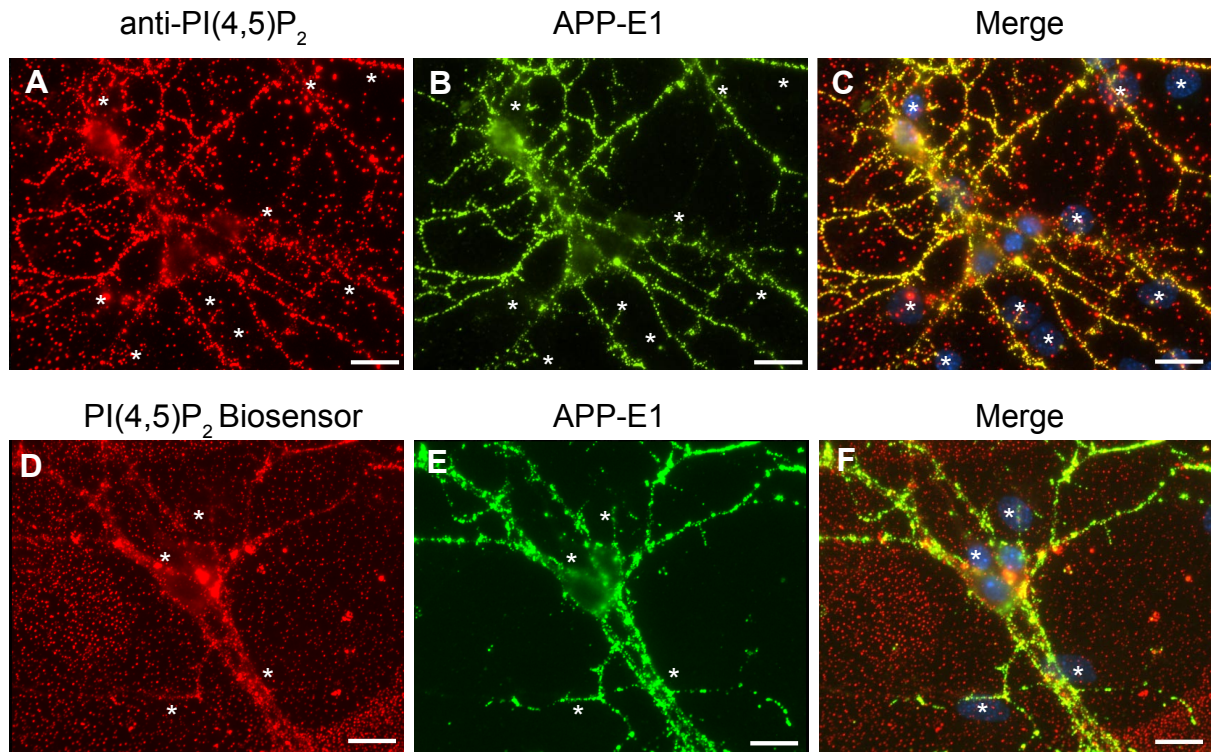


Figure 3.13. Co-localisation of PI(4,5)P₂ and APP-E1 on the neuronal cell surface. Panels A, B, C show APP-E1 immunoreactivity and PI(4,5)P₂ immunoreactivity after incubation of primary hippocampal cultures with 50 nM APP-E1 for 2h. Panels D, E, F show APP-E1 immunoreactivity and PI(4,5)P₂ biosensor immunoreactivity after incubation of cultures with 25 nM APP-E1 and 25 nM PI(4,5)P₂ biosensor for 2h at 37°C prior to detection of cell-bound APP-E1 and cell-bound PI(4,5)P₂ biosensor by immunocytochemistry at 4°C. DAPI staining of nuclei is shown in panels C and F in blue. The nuclei of glia in the feeder layer are marked with an asterisk. Scale bars = 20 μm.

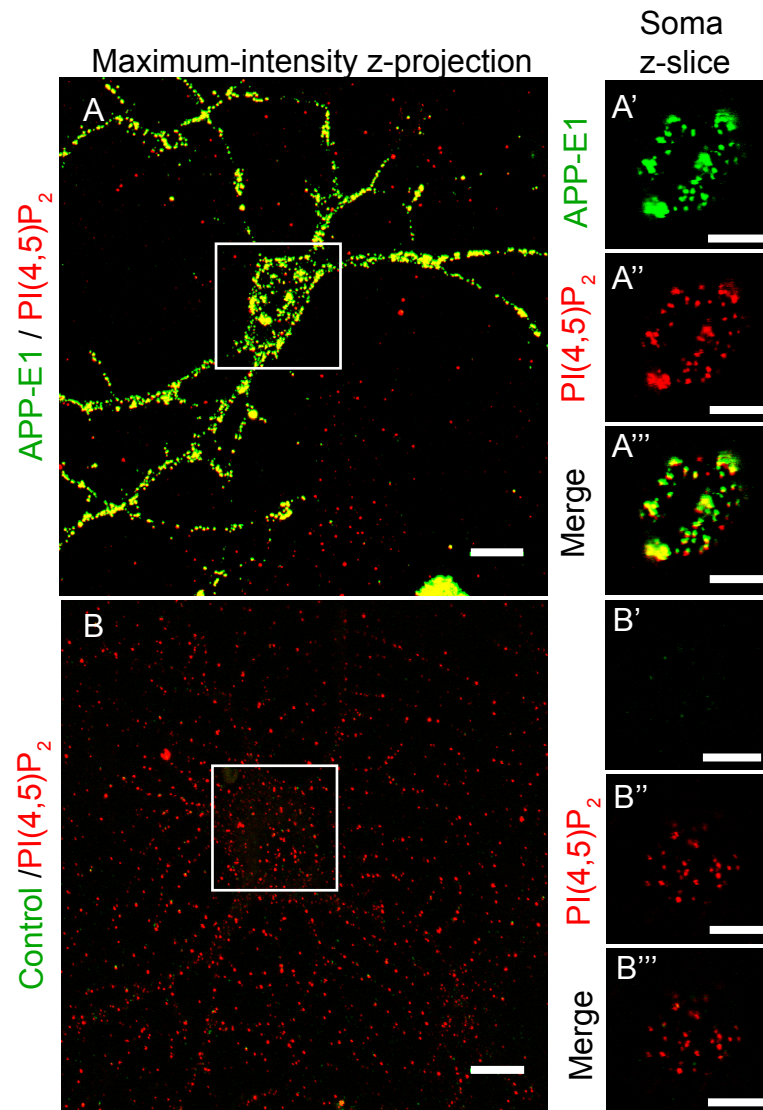


Figure 3.14. APP-E1 co-localises with cell-surface PI(4,5)P₂ microdomains. Cells were incubated with 50 nM of APP-E1 (panel A) or PBS (control; panel B) for 2.5 hours at 37°C. Cells were washed and cell-bound APP-E1 and cell-surface PI(4,5)P₂ were detected by immunocytochemistry performed at 4°C. Exogenous APP-E1 is shown in green and cell-surface PI(4,5)P₂ is shown in red. Panels A and B show maximum intensity projection of a confocal z-stack of primary hippocampal neurons. Insets (A' - B'') show images of a single 1 airy unit confocal slice of the cell soma region indicated in the white box, focused on the cell surface. Scale bars = 10 μm or 5 μm in insets.

To further examine the possibility that APP-E1 only binds to PI(4,5)P₂ on the cell surface, the ability of a water-soluble PI(4,5)P₂ analogue (diC8PI(4,5)P₂) to compete for APP-E1 binding to the cell surface was investigated. APP-E1 (10 nM) was pre-incubated with 500 μM diC8PI(4,5)P₂ for two hours, prior to incubation with primary hippocampal cultures. This concentration of diC8PI(4,5)P₂ was chosen based on the protein-lipid overlay assay data in Fig. 3.4 and was determined to be sufficient to inhibit the binding of APP to PI(4,5)P₂. After incubation, cells were washed, and cell-bound APP-E1 was detected using an antibody directed against the 6xHis tag.

The water soluble PI(4,5)P₂ analogue did not significantly reduce the binding of 10 nM APP-E1 to primary hippocampal cultures (Fig. 3.15A, B). Therefore, the results did not provide clear evidence that PIPs were the major binding site for APP-E1 on the cell surface, as diC8PI(4,5)P₂ could not compete for binding of APP-E1 to the cell surface.

3.2.4 Investigation into the role of the heparin-binding domain in the binding of APP-E1 to the cell surface

The APP-E1 recombinant protein also contains an N-terminal heparin-binding domain (residues 95 - 110). This heparin-binding domain has been reported to be important in the stimulation of neurite outgrowth (Small et al., 1994). Furthermore, APP has been demonstrated to bind to heparan sulfate proteoglycans such as glypican 1 (Williamson et al., 1996). Therefore, the cell surface receptor for APP could be a heparan sulfate proteoglycan. As the results in Sections 3.2.2 and 3.2.3 indicated that PIPs might not be the main binding site for APP-E1 on the cell surface, the role of the heparin-binding region in cell-surface binding by APP-E1 was investigated.

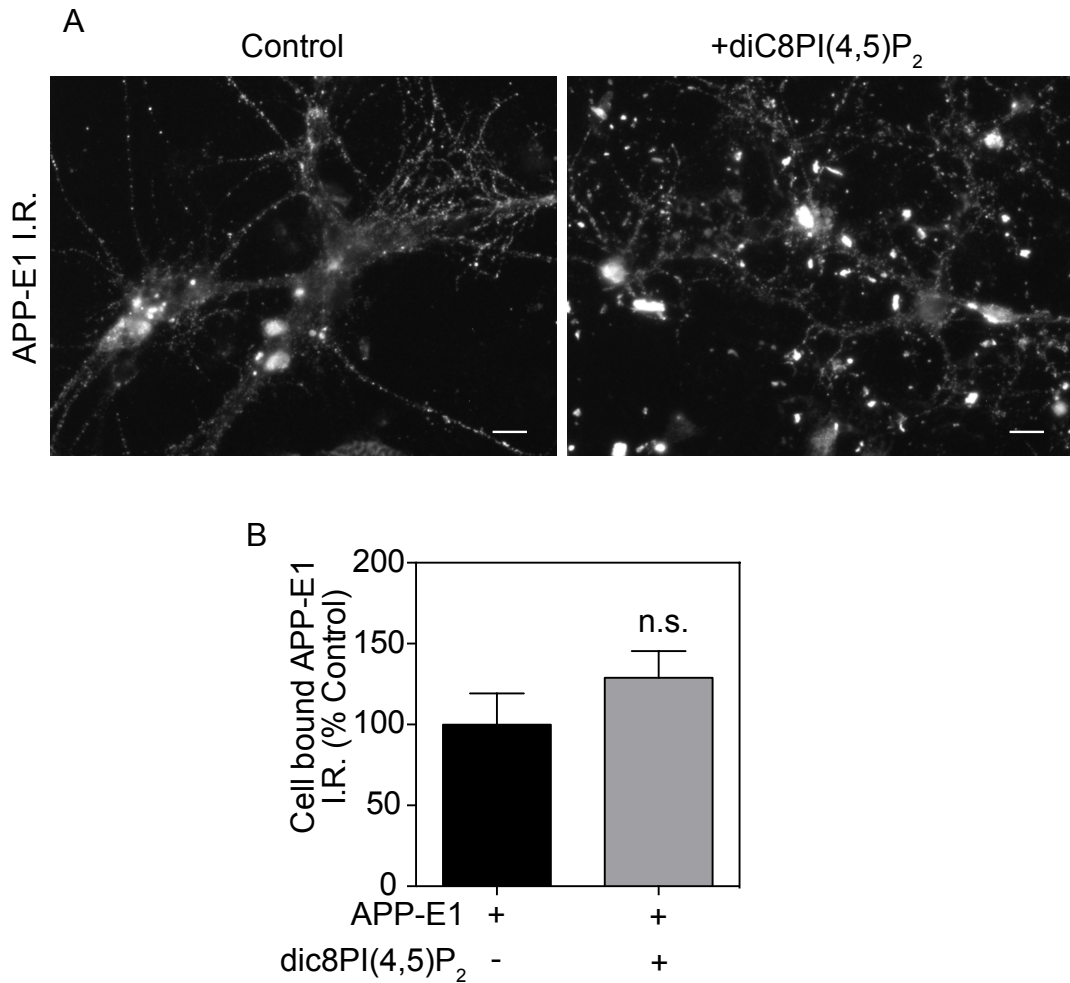


Figure 3.15. Incubation of APP-E1 with diC8PI(4,5)P₂ has no effect on APP-E1 binding to hippocampal neurons. Primary hippocampal cultures were incubated with APP-E1 (10 nM) for 2 hours at 37°C in the presence of 500 μM diC8PI(4,5)P₂ or PBS (control) prior to detection of cell-bound APP-E1 by immunocytochemistry performed at 4°C. Panel A shows representative images of APP-E1 immunoreactivity (I.R.). Scale bars = 20 μm. Panel B shows quantification of cell-bound APP-E1 immunoreactivity. Bars show mean ± SEM cell-bound APP-E1 immunoreactivity expressed as a percentage of control determined from 30 fields per treatment group. n.s. - Data not significantly different between treatment groups determined by Students t-test ($p > 0.05$)

To investigate whether the heparin-binding domain in APP-E1 could facilitate the binding of APP-E1 to cells, primary hippocampal cultures were incubated with APP-E1 in the presence or absence of a large excess of mucosal heparin. Cells were washed and cell-bound APP-E1 was detected by immunocytochemistry. Mucosal heparin ($100 \mu\text{g mL}^{-1}$) significantly reduced the amount of APP-E1 immunoreactivity associated with cells (Fig. 3.16). The concentration of mucosal heparin that blocked binding to cells was not sufficient to block binding to PI(4,5)P₂ in the protein-lipid overlay assay (Fig. 3.4). This indicated that the heparin-binding region and the PIP-binding region on APP-E1 may be distinct, and that the heparin-binding region is required for binding to the cell surface.

To investigate whether APP-E1 can bind to endogenous cell-surface heparan sulfate proteoglycans, the effect of removal of endogenous heparan sulfate on the ability of APP-E1 to bind to the cells was investigated. Primary hippocampal cultures were incubated with heparitinase, prior to incubation with APP-E1. Cells were washed and APP-E1 was detected using an antibody against the 6xHis tag. Heparitinase treatment significantly reduced the amount of cell-surface heparan sulfate immunoreactivity (Fig. 3.17A, C). However, removal of endogenous heparan sulfate with heparitinase did not affect the amount of APP-E1 that bound to cells (Fig. 3.17B, D). As endogenous heparan sulfate was present on cells in the glial feeder layer where APP-E1 did not bind, this also suggested that heparan sulfate proteoglycans do not represent the major binding site for APP-E1 (Fig 3.17A). Together the results suggested that the heparin-binding domain in APP-E1 is involved in the binding of APP-E1 to the cell surface, however the heparin-binding domain of APP-E1 may not bind to a heparan sulfate proteoglycan on the cell surface. This data suggests that there may be another type of receptor on the surface of neurons that is responsible for binding to APP.

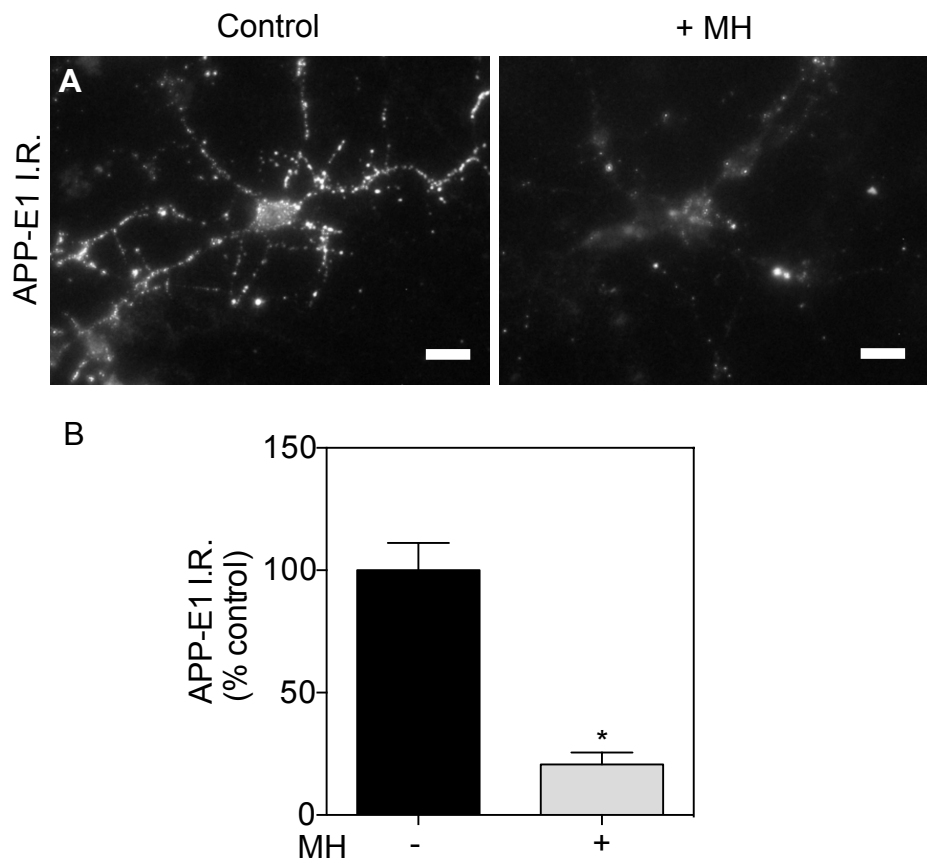


Figure 3.16. Incubation of APP-E1 with mucosal heparin (MH) blocks the binding of APP-E1 to cells. APP-E1 (25 nM) was incubated with $100\ \mu\text{g mL}^{-1}$ MH or PBS control for 1h in cell culture medium, prior to incubation with primary hippocampal neurons for 2h at 37°C . Cells were then washed and cell-bound APP-E1 was detected by immunocytochemistry at 4°C . Panel A shows representative images of cell-bound APP-E1 immunoreactivity. Scale bars = $20\ \mu\text{m}$. Panel B shows quantification of cell-bound APP-E1 immunoreactivity measured from 60 fields. Bars show mean \pm SEM APP-E1 immunoreactivity per field, expressed as a percentage of control. *Statistically significant as determined by Student's t-test ($p < 0.05$).

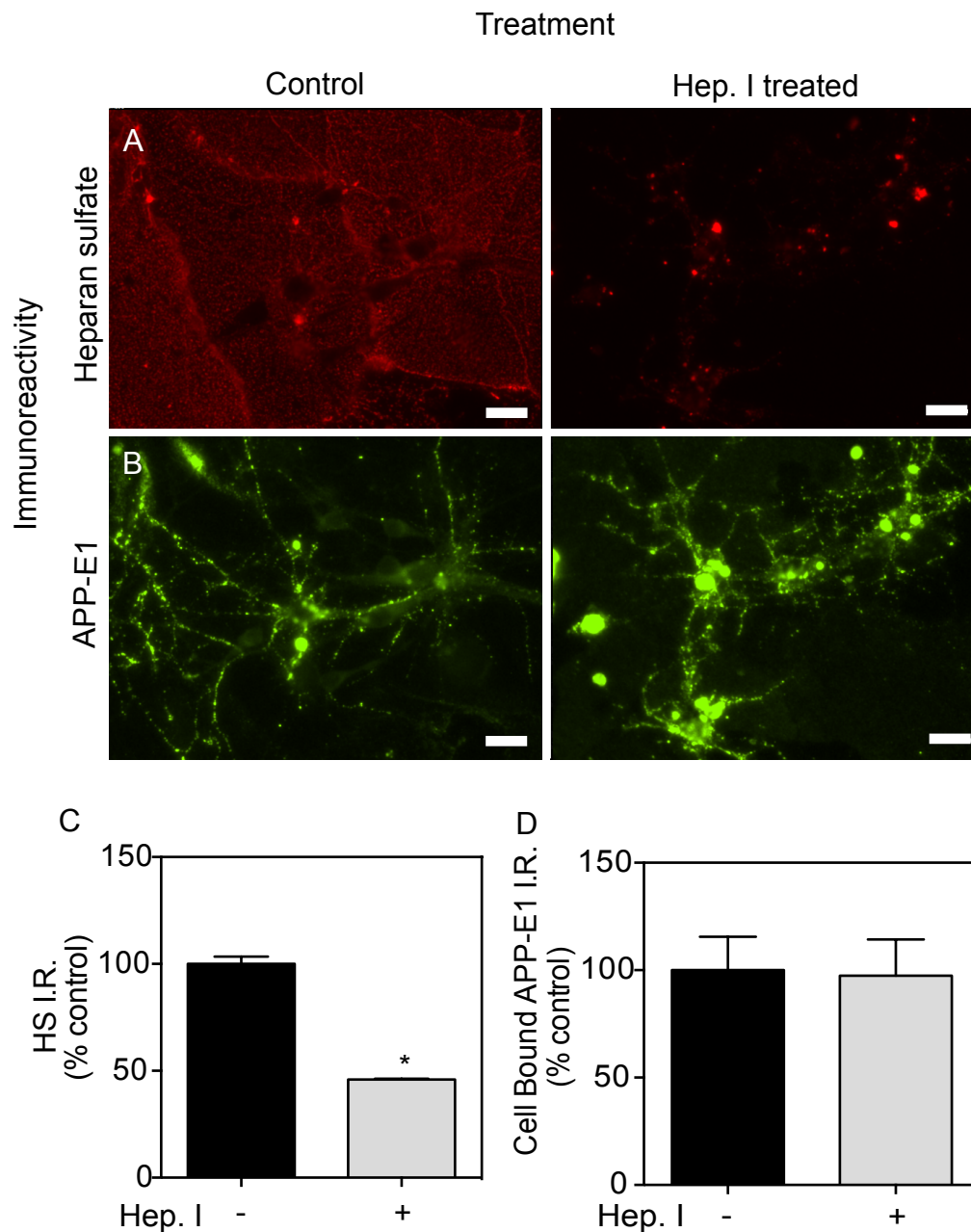


Figure 3.17. Heparitinase treatment does not affect APP-E1 binding to cells. Cells were treated with or without 0.8 mU ML^{-1} heparitinase I (Hep. I) for 24 hours, prior to incubation with 25 nM APP-E1 for 2 hours at 37°C . Cells were washed and cell-surface heparan sulfate (HS) and cell-bound APP-E1 was detected using immunocytochemistry performed at 4°C . Panels A and B show representative images of the effect of heparitinase treatment on cell-surface heparan sulfate immunoreactivity (I.R.) and corresponding effect of heparitinase treatment on cell-bound APP-E1 I.R. Scale bars = $20 \mu\text{m}$. Panels E + F show quantification of the effect of heparitinase treatment on HS I.R. and APP-E1 I.R. measured from 60 fields. Bars show mean \pm SEM I.R. expressed as a percentage of untreated controls. *Statistically significant as determined by Student's t-test ($p < 0.05$).

3.3 Studies on the biological effects of APP-PIP interactions

3.3.1 Effect of sAPP α on PIP levels in primary cortical cultures

Many important cellular signalling mechanisms are reliant on changes in the levels of PIPs, for example calcium signalling (Lapetina and Michell, 1973) and PI3K/Akt signalling (Watton and Downward, 1999; Andjelkovic et al., 1997). Therefore, changes to levels of PIPs could potentially result in effects on cellular signalling pathways. As APP was found to bind to PI(4,5)P₂, it was hypothesised that APP might be able to affect levels of cellular PIPs. Therefore, the effect of sAPP α treatment on total cellular levels of PIPs was assessed.

In order to examine the effect of APP on PIPs, a mass spectrometry (MS) method was used. The principle of this technique is that PIP lipids can be extracted from cells using an acidified-chloroform extraction (Ivanova et al., 2007; Ogiso and Taguchi, 2008; Pettitt, 2010). PIPs can then be separated by reverse-phase ultra performance liquid chromatography (UPLC), which allows for the rapid chromatographic separation of PIPs. PIPs can be selectively identified from a complex cellular extract using electrospray MS with selective ion monitoring (SIM; Ogiso and Taguchi, 2008). In selective ion mode, the MS detector only measures ions at selected m/z, which results in significantly greater sensitivity than full scan-based MS. Quantification of the relative levels of PIPs can then be inferred by integration of the chromatographic elution peaks. Theoretically, this approach allows for the detection of different acyl chain species of PIP, and also the different phosphorylated isoforms, e.g. PIP vs. PIP₂ vs. PIP₃. However, this method does not allow for the quantitative determination of individual PIP regioisomers, as these have identical mass (Pettitt, 2010).

To achieve chromatographic separation of PIPs by reversed phase UPLC on a C18 column, a mixture containing non-natural acyl chain 37:4 PI(3)P, 37:4 PI(3,4)P₂ and 37:4 PI(3,4,5)P₃ standards was first analyzed. Lipids in the eluate were detected by SIM. The solvent system gave good chromatographic separation of each one of the standards in the mixture. Elution peaks for each of the 37:4 PI(3)P, 37:4 PI(3,4)P₂ and 37:4 PI(3,4,5)P₃ standards were detected at the appropriate m/z and eluted at 6.52 min, 5.90 min and 5.63 min respectively (Fig. 3.18). There was a small elution peak detected at 5.63 min in the 37:4 PI(3,4)P₂ and 37:4 PI(3)P channels, which was attributed to an in-source fragmentation product of the 37:4 PI(3,4,5)P₃ standard (Fig. 3.18B).

To determine if cellular PIPs could be detected using UPLC-MS with SIM, lipids were extracted from cultures using an acidified-chloroform extraction. As large numbers of cells were needed, cultures from the whole cortex were used instead of hippocampal cultures. Synthetic non-naturally occurring 37:4 PI, 37:4 PI(3)P, 37:4 PI(3,4)P₂ and 37:4 PI(3,4,5)P₃ were added as internal standards to the cell extracts to correct for differences in the efficiency of the extraction procedure. PI, PIP and PIP₂ species were all detectable in the primary cortical neuron extracts (Fig. 3.19, Fig. 3.20, Fig. 3.21). The retention times for different acyl chain species followed a clear pattern consistent with the number of carbons and saturation, for example different PIP acyl chain species eluted from the column in the order 38:5, 38:4, 38:3, corresponding to the degree of saturation (Fig. 3.20 C, B, A). The amount of PIP that was detectable was approximately inversely correlated to the degree of phosphorylation. For example, the SIM intensity was an approximately an order of magnitude lower for PIP₂ (in the 10⁴ range) than for PIP (in the 10⁵ range; e.g. Fig. 3.20C vs. 3.21C). Natural PIP₃ species were not detectable using this method (data not shown), in agreement with previous studies (Ogiso and Taguchi, 2008; Ogiso et al., 2010; Pettitt, 2010).

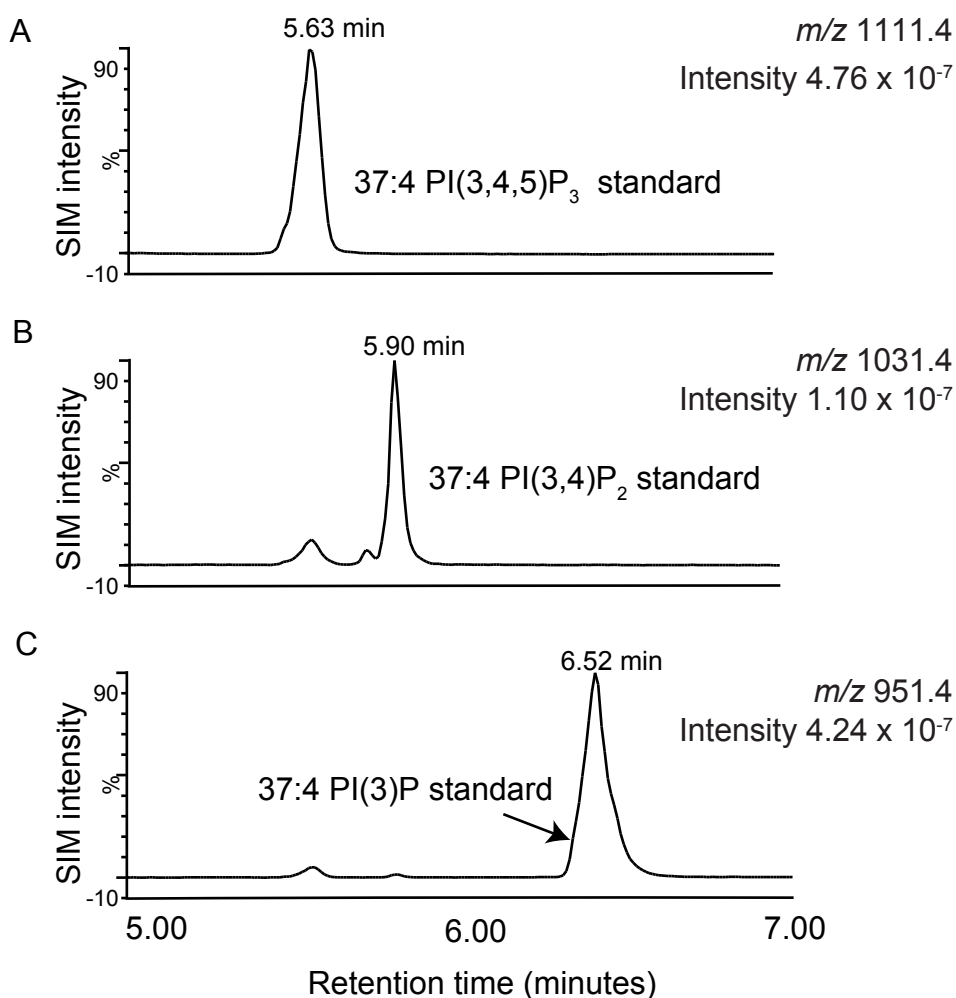


Figure 3-18. Separation of a mixture of non-natural 37:4 PIP standards by reverse phase UPLC and detection by MS with SIM. A solution containing $5 \mu\text{g mL}^{-1}$ of each PIP standard was subjected to reverse phase UPLC on a C18 column ($2.1 \times 100 \text{ mm} \times 1.7 \mu\text{m}$ particles). The column was eluted with an initial isocratic mobile phase mixture of 90% A: 10% B for 1 minute followed by a linear gradient to 0% A: 100% B over 9 minutes. PIPs were detected by SIM of the mass to charge ratios (m/z) indicated on the right. Chromatograms A, B and C show elution peaks for 42 pmol 37:4 PI(3,4,5)P₃, 46 pmol 37:4 PI(3,4)P₂ and 50 pmol 37:4 PI(3)P standards respectively, separated from a single $10 \mu\text{L}$ injection. The y axis shows SIM current intensity, expressed as a % of the maximum SIM current intensity for each m/z ion, which is indicated on the right.

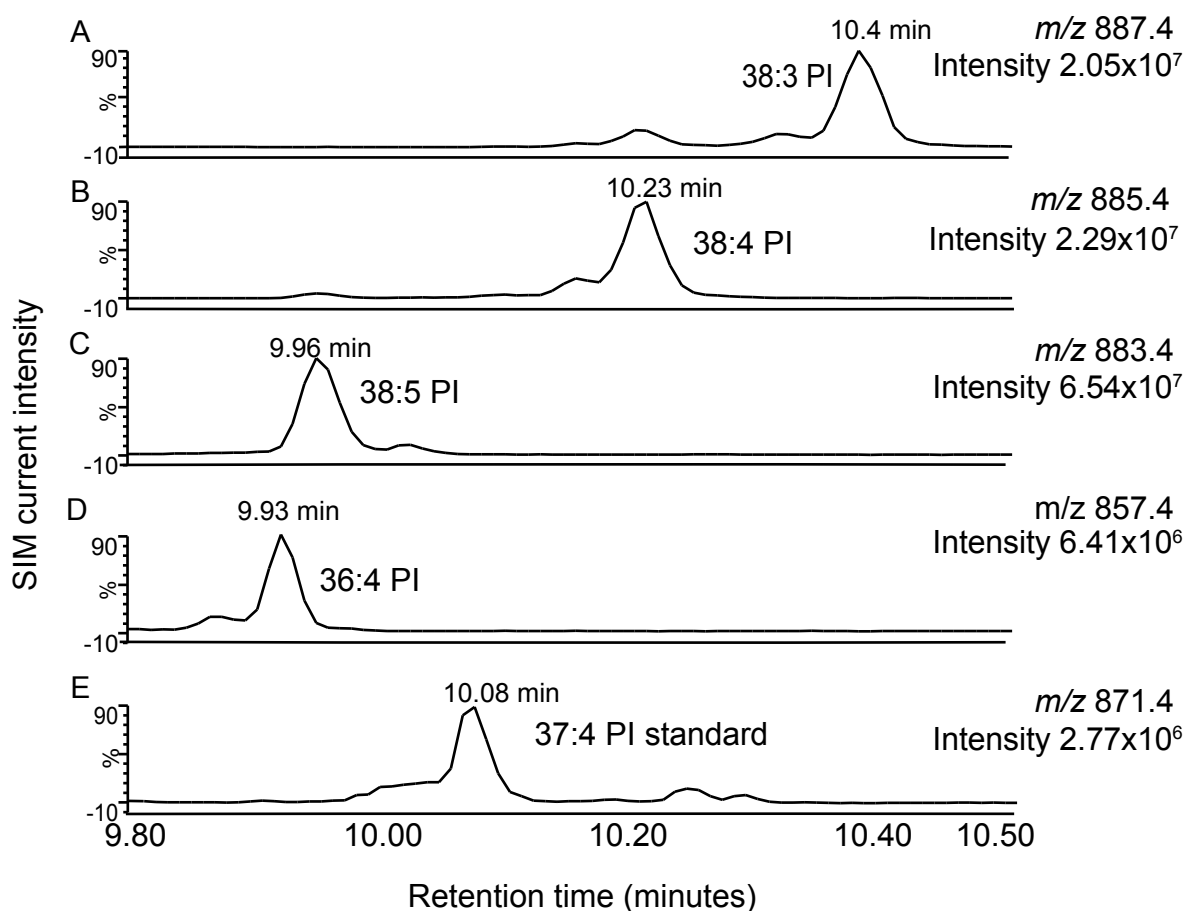


Figure 3.19. Chromatographic separation and SIM detection of PI species extracted from primary cortical cultures. Lipid extracts were subjected to reverse phase UPLC on a C18 column (2.1 x 100mm x 1.7 micron particles). The column was eluted with an initial isocratic mobile phase mixture of 90% A: 10% B for 1 minute followed by a linear gradient to 0% A: 100% B over 9 minutes. PIPs were detected by SIM of the mass to charge ratios (m/z) indicated on the right. Chromatograms A - E show the results obtained from analysis of a 10 μ L injection of lipids extracted from 1×10^6 cells. Each chromatogram shows SIM monitoring of an individual PI acyl chain species. Chromatogram E is the 37:4 PI internal standard which represents 10 pmol of standard assuming perfect extraction. The y axis shows SIM current intensity, as a % of the maximum SIM current intensity for each m/z ion, which is indicated on the right.

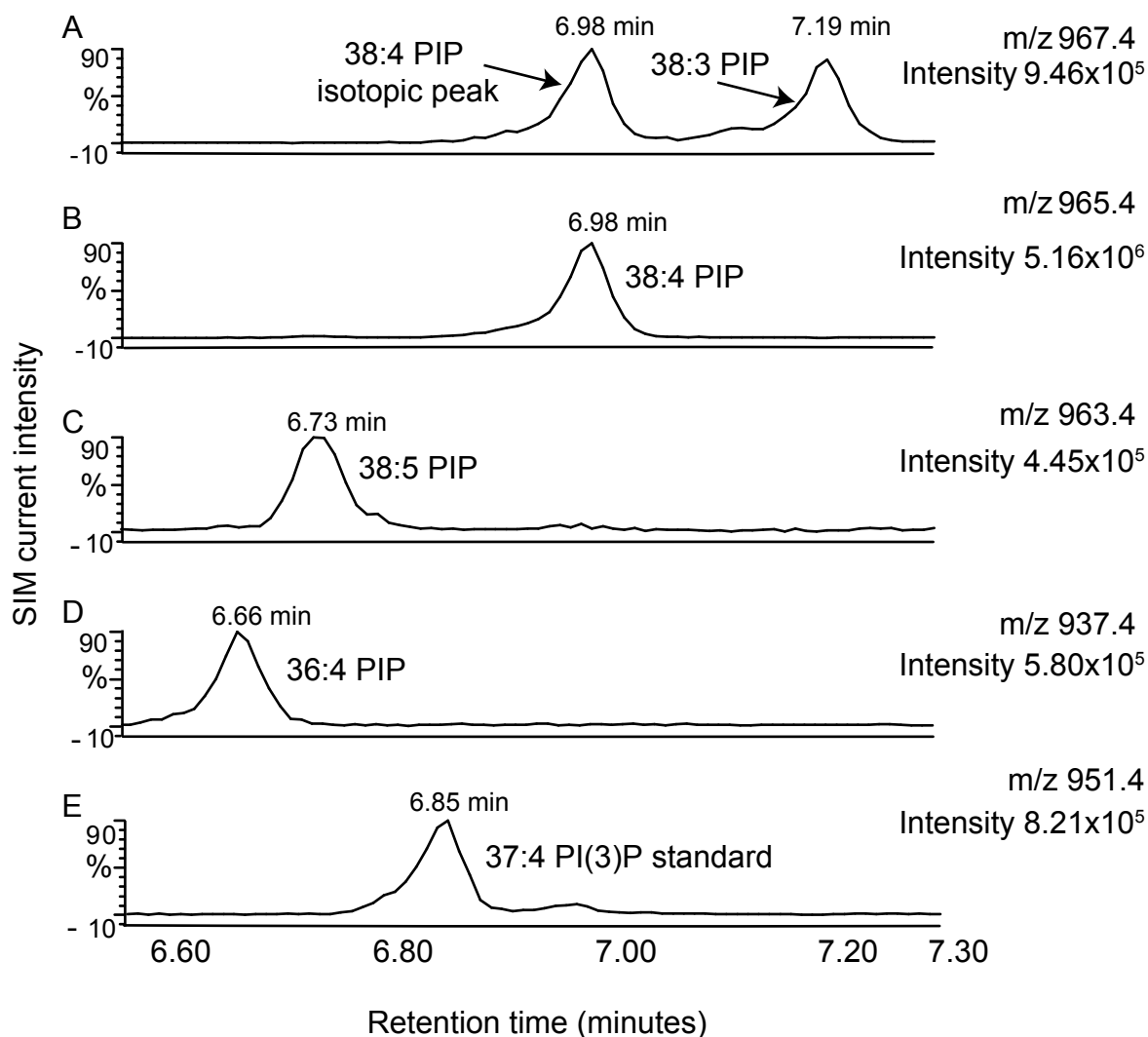


Figure 3.20. Chromatographic separation and detection by SIM of PIP species extracted from primary cortical cultures. Lipid extracts were subjected to reverse phase UPLC on a C18 column (2.1 x 100 mm x 1.7 μ m particles). The column was eluted with an initial isocratic mobile phase mixture of 90% A: 10% B for 1 minute followed by a linear gradient to 0% A: 100% B over 9 minutes. PIPs were detected by SIM of the mass to charge ratios (m/z) indicated on the right. Chromatograms A - E show the results obtained from analysis of a 10 μ L injection of lipids extracted from 1×10^6 cells. Each chromatogram shows SIM monitoring of an individual PIP acyl chain species. Chromatogram E is the 37:4 PI(3)P internal standard which represents 10 pmol of standard assuming perfect extraction. The y axis shows SIM current intensity, expressed as a % of the maximum SIM current intensity for each m/z ion, which is indicated on the right.

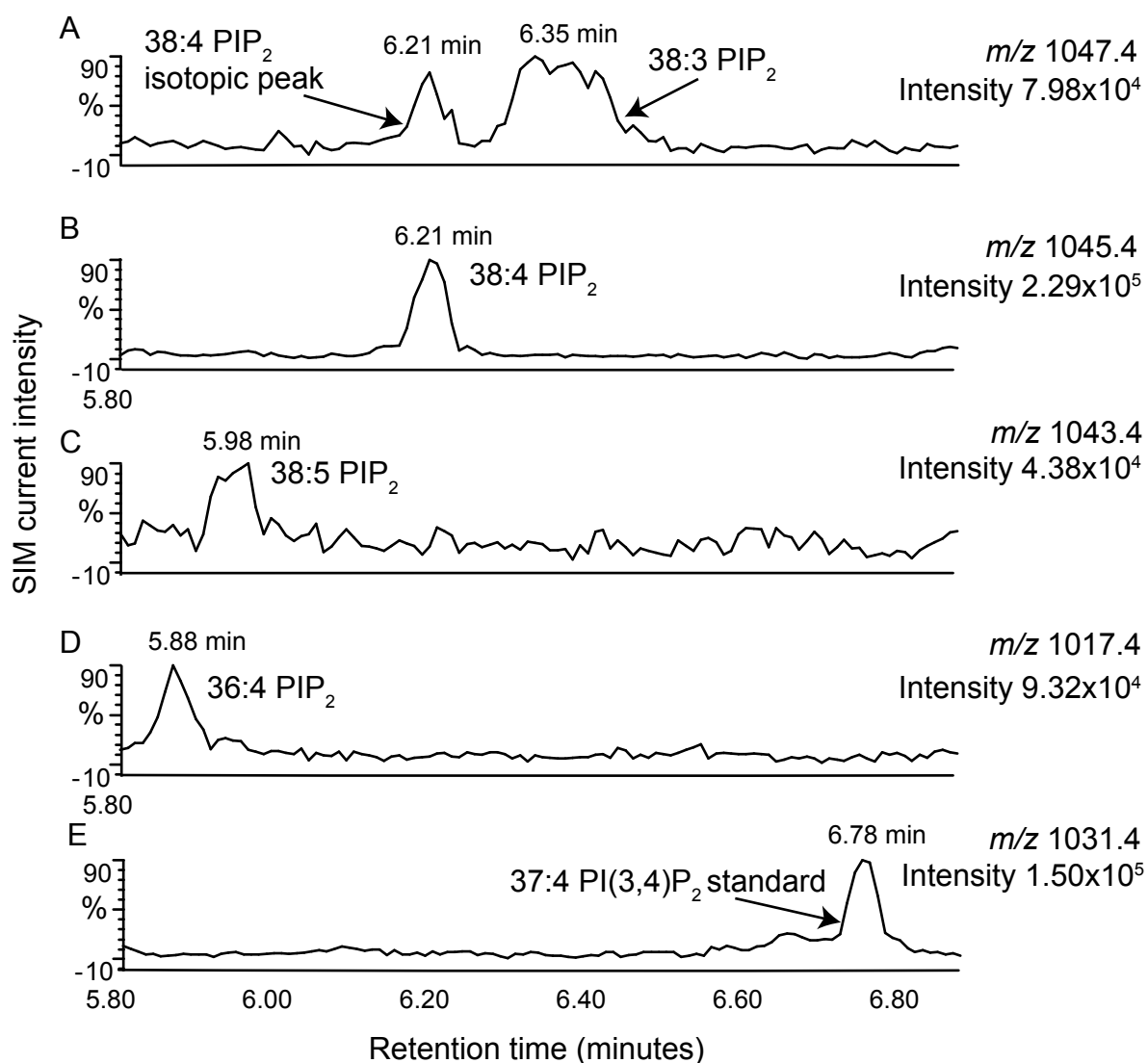


Figure 3.21. Chromatographic separation and SIM detection of PIP_2 species extracted from primary cortical cultures. Lipid extracts were subjected to reverse phase UPLC on a C18 column (2.1 x 100mm x 1.7 μm particles). The column was eluted with an initial isocratic mobile phase mixture of 90% A: 10% B for 1 minute followed by a linear gradient to 0% A: 100% B over 9 minutes. PIP_2 species were detected by SIM of the mass to charge ratios (m/z) indicated on the right. Chromatograms A - E show the results obtained from analysis of a 10 μL injection of lipids extracted from 1×10^6 cells. Each chromatogram shows SIM monitoring of an individual PIP_2 acyl chain species. Chromatogram E is the 37:4 $\text{PI}(3,4)\text{P}_2$ internal standard which represents 10 pmol of standard assuming perfect extraction. The y axis shows SIM current intensity, as a % of the maximum SIM current intensity for each m/z ion, which is indicated on the right.

The area for each elution peak was then measured to calculate the levels of individual PIPs in the cell extracts (Table 3-1). The PI, PIP, PIP₂ internal standards were added to lipid extracts at the same molar amounts (50 pmol), but were not recovered at the same levels (Table 3-1). This suggested that the PIP and PIP₂ species may undergo degradation during the lipid extraction process. Therefore, levels of PIPs in the cell extracts were corrected to the level of the internal standard, to calculate the amount of PIPs in pmol per million cells (Ogiso and Taguchi, 2008). The 38:4 and 38:3 PIPs were the most abundant acyl chain species of PIPs in the primary cortical cultures (Table 3-1). The amount of each acyl chain species was summed to estimate the total levels of PIPs in the cells, which was 143.7 ± 14.1 pmol PIP 10^6 cells⁻¹ and 127.1 ± 25.5 pmol PIP₂ 10^6 cells⁻¹ (Table 3-1). The level of PIP and PIP₂ species was approximately an order of magnitude below that of PI, which was present at 1010.81 ± 55 pmol PI 10^6 cells⁻¹ (Table 3-1).

To confirm that the assay could detect changes in levels of cellular PIPs, the effect of wortmannin on PIP levels was assessed. Wortmannin is a potent inhibitor of PI3K and at higher concentrations can inhibit PI4K (Arcaro and Wymann, 1993; Yano et al., 1993). Both PI3K and PI4K are essential for the production of PIP. Therefore, wortmannin treatment would be expected to cause a reduction in the levels of 3-phosphorylated and 4-phosphorylated PIPs. Primary cortical cells were treated with a high concentration (10 μ M) of wortmannin for 30 min prior to extraction of lipids and analysis by UPLC-MS. The primary cultures treated with wortmannin had lower levels of PIP and PIP₂ relative to vehicle-treated control cultures (Fig. 3.22). In contrast, the level of PI was unaffected by wortmannin treatment as expected (Fig. 3.22). Therefore, it was concluded that the assay method was appropriate for measuring changes in levels of cellular PIPs extracted from primary cortical cultures.

Table 3-1 Levels of PIPs in mouse primary cortical cultures

	PI		PIP		PIP ₂	
Acyl chain isoform	Raw elution intensity Arb. Unit. (±SD)	Corrected mean pmol lipid/10 ⁶ cells (±SD)	Raw elution intensity Arb. Unit. (±SD)	Corrected mean pmol lipid/10 ⁶ cells (±SD)	Raw elution intensity Arb. Unit. (±SD)	Corrected mean pmol lipid/10 ⁶ cells (±SD)
37:4 Standard	21.04 (3.9)	50.00	8.22 (1.6)	50.00	1.01 (0.2)	50.00
36:4	60.76 (9.8)	144.92 (6.4)	3.08 (0.6)	18.86 (2.1)	n.d.	n.d.
38:5	47.98 (7.0)	114.65 (7.3)	2.60 (0.5)	15.96 (1.8)	n.d.	n.d.
38:4	235.40 (36.4)	562.12 (36.2)	15.40 (2.4)	94.44 (9.1)	2.01 (0.4)	99.58 (17.3)
38:3	80.06 (20.1)	189.13 (21.6)	2.39 (0.7)	14.47 (2.3)	0.57 (0.2)	27.61 (17.8)
Total	424.20 (67.8)	1010.81(55.4)	23.47 (4.1)	143.74 (14.1)	2.59 (0.7)	127.19 (25.5)

PIPs were measured in primary cortical culture lipid extracts by UPLC-MS. Each value represents mean (± SD) derived from 12 replicates. Mean raw elution intensities, calculated from the mass chromatogram elution peak area for each lipid are shown, including the PIP internal standards. There were significant differences in the recovery of PI, PIP and PIP₂ internal standards suggesting some degradation of PIP and PIP₂ throughout lipid extraction and measurement (shown in **bold**, $p > 0.05$, 1-way ANOVA). Levels of PIPs were corrected to the amount of internal standards according to the formula: (raw elution intensity / internal standard elution intensity) x (total lipid extract volume / injection volume) to calculate the amount of lipid in pmol per million cells. n.d. = lipid was not detectable at measurable levels.

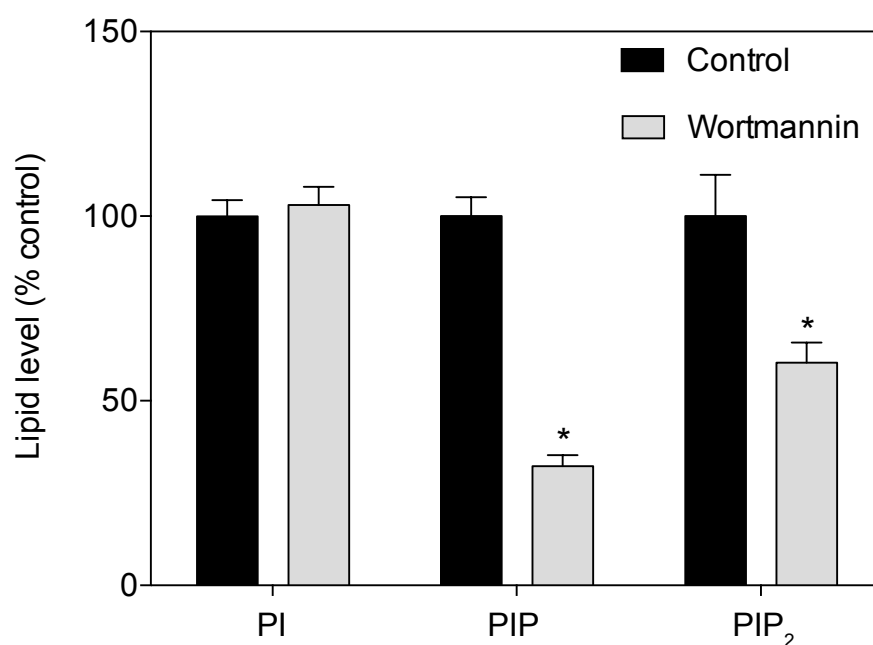


Figure 3.22. Wortmannin reduced the levels of PIP and PIP₂ in primary cortical cultures. Primary cortical neurons were treated with 10 μ M wortmannin or vehicle (DMSO) control for 30 minutes prior to lipid extraction and analysis by UPLC-MS. PIP 37:4 internal standards were used to correct for differences in extraction efficiency between samples. Graph shows the total amounts of PI, PIP and PIP₂ species, expressed as a percentage of the control. Bars show mean lipid level \pm SEM determined from 6 replicate wells containing 1×10^6 cells per treatment group. *Statistically significant as determined using individual Student's t-tests ($p < 0.05$).

As the UPLC-MS method was determined to be suitable for measuring levels of total PIPs in the primary cortical cultures, the effect of sAPP α treatment was then investigated. Cells were treated with sAPP α for 72 hours prior to the extraction of lipids and measurement of PIPs. However, no significant effect of sAPP α was found on levels of PI or on the levels of PIP or PIP₂ in the cultures (Fig. 3.23). Therefore, the results suggested that total levels of PIP and PIP₂ in primary cortical cultures were unaffected by sAPP α treatment.

3.3.2 Effect of APP-E1 on levels of cell-surface PIP

Many functions of PIPs in the cell are highly localised to specific membrane compartments and possibly also to distinct lateral domains within the membrane (Di Paolo and De Camilli, 2006; Posor et al., 2013; Khuong et al., 2013). Therefore, APP binding to PIPs could theoretically produce small, rapid changes in the levels of PIPs that would not be detectable by measuring the total cellular levels of PIPs. The experiments described in Section 3.2.2 reported evidence for discrete PI(4,5)P₂ domains on the cell surface, and APP-E1 was found to localise to these domains. Therefore, the possibility that APP-E1 could affect the amount of PIPs on the cell surface was also examined.

3.3.2.1 *Effect of APP-E1 on cell-surface PI(4,5)P₂*

The data in Section 3.2.1 showed that PI(4,5)P₂ was on the surface of primary hippocampal cultures. To examine whether APP-E1 can alter the level of cell-surface PI(4,5)P₂, cells were incubated with APP-E1 and the level of PI(4,5)P₂ immunoreactivity was measured. In the presence of APP-E1, there was a significantly higher level of PI(4,5)P₂ immunoreactivity observed on the surface of neurons, but not on glia (Fig. 3.24A, C). As APP-E1 binds selectively to the neurons in the hippocampal cultures (Section 3.2), this suggests that the effect was due to the binding of APP-E1 to the cell surface.

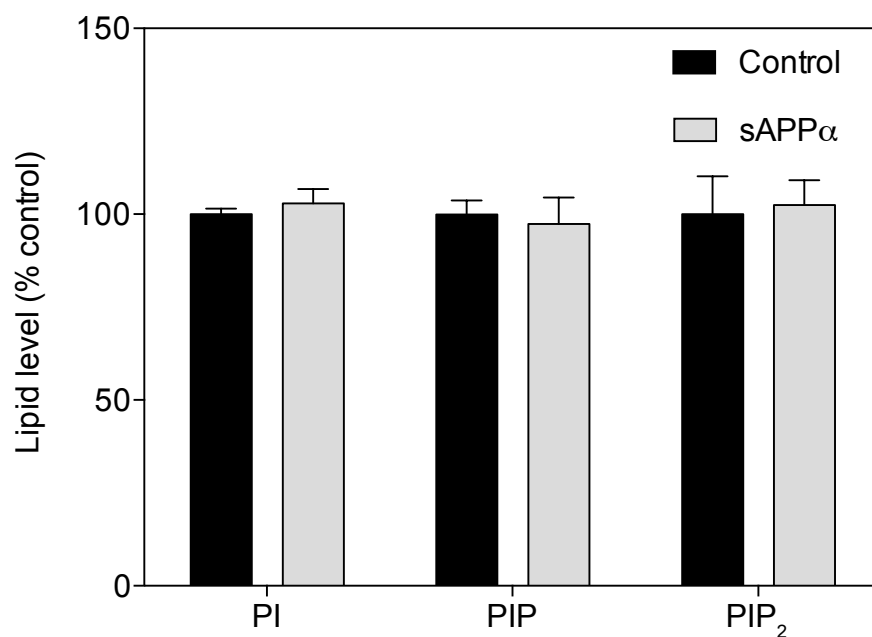


Figure 3.23. sAPP α does not affect total PIP levels in primary cortical cultures. Primary cortical cultures were treated with 1 nM sAPP α or PBS (control) for 72h prior to lipid extraction and analysis by UPLC-MS. Internal standards were used to correct for differences in extraction efficiency between samples. Graphs show the effect of sAPP α treatment on levels of PI, PIP and PIP₂ respectively, expressed as percentage of control. Bars show mean \pm SEM levels of PIPs derived from 11 replicate wells containing 1×10^6 cells per treatment group. Data not significantly different between treatment groups as determined by individual Student's t-tests ($p > 0.05$).

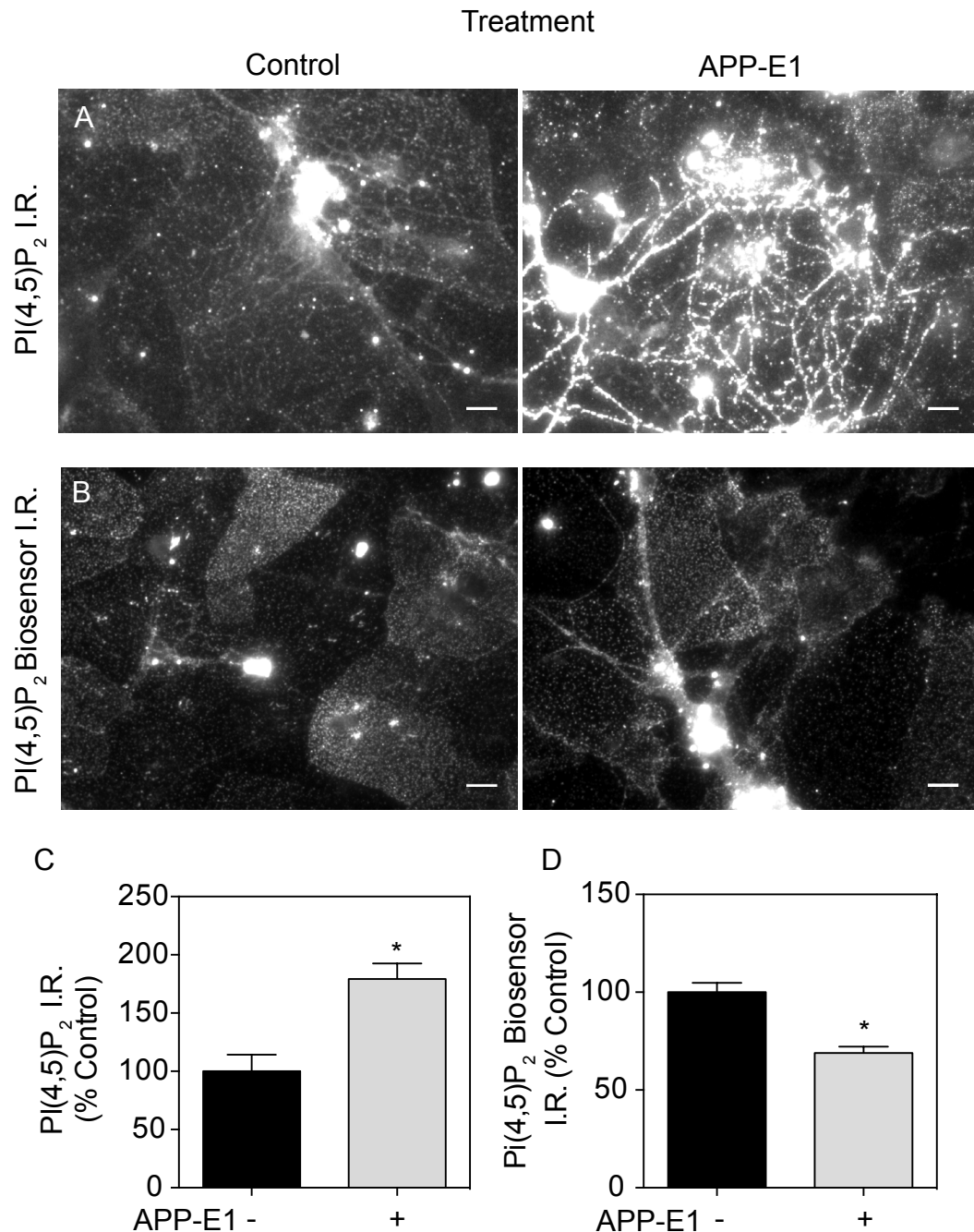


Figure 3.24. APP-E1 increases cell-surface PI(4,5)P₂ immunoreactivity, but decreases PI(4,5)P₂ biosensor binding. Panel A and C: Primary hippocampal cultures were incubated with 50 nM APP-E1 or PBS control for 2 hours at 37°C prior to labelling with the PI(4,5)P₂ antibody at 4°C. Panel A shows representative image of PI(4,5)P₂ immunoreactivity. Panel C shows quantification of PI(4,5)P₂ immunoreactivity (I.R.) from 60 fields. Panels B and D: Primary hippocampal cultures were incubated with 25 nM APP-E1 or PBS control and 25 nM PI(4,5)P₂ biosensor for 2 hours at 37°C prior to labelling of cell-bound biosensor by immunocytochemistry at 4°C. Panel B shows representative image of PI(4,5)P₂ biosensor immunoreactivity. Panel D shows quantification of cell bound PI(4,5)P₂ biosensor immunoreactivity from 60 fields. Bars show mean ± SEM I.R. Statistical significance determined using Student's t-tests. * = Significantly different from PBS control $p < 0.05$. Scale bars = 10 μ m.

Despite the previous result, it was not possible to reproduce the effect of APP-E1 on PI(4,5)P₂ when a biosensor was used to measure the PIP levels (Fig. 3.24B, D). In contrast to the result with the PI(4,5)P₂ antibody, the amount of PI(4,5)P₂ biosensor that bound to cells decreased in the presence of APP-E1 (Fig. 3.24D). As the PI(4,5)P₂ antibody was incubated with cells after APP-E1 treatment, but the PI(4,5)P₂ biosensor was incubated at an equal concentration as APP-E1 and at the same time, it was hypothesised that the PI(4,5)P₂ biosensor could compete with APP-E1. To investigate this possibility, the binding of APP-E1 to cells was assessed in the presence of a 10-fold molar excess of the PI(4,5)P₂ biosensor. Under these conditions, it was observed that the amount of APP-E1 that bound to cells was significantly reduced by the excess of the PI(4,5)P₂ biosensor (Fig. 3.25). Therefore, these results supported the conclusion that the PI(4,5)P₂ biosensor and APP-E1 can compete for PI(4,5)P₂ binding to the cell surface. This result explained why an increase in cell-surface PI(4,5)P₂ after incubation with APP-E1 was only observed using the antibody and not the PI(4,5)P₂ biosensor. Presumably, the PI(4,5)P₂ biosensor present in an excess can obstruct the binding of APP-E1 to the cell surface.

3.3.2.2 *Effect of APP-E1 on cell-surface PI(3,4,5)P₃*

As there was an increase in the levels of PI(4,5)P₂ immunoreactivity in the presence of APP-E1 (Section 3.3.2.1), the possibility that APP-E1 might affect the levels of other PIPs was investigated. Although the experiments described in Section 3.2.1 indicated that PI(3)P, PI(4)P, PI(3,4)P₂ and PI(3,4,5)P₂ were not present at detectable levels on the cell surface under resting conditions, it seemed possible that APP-E1 might increase levels of PIPs on the cell surface.

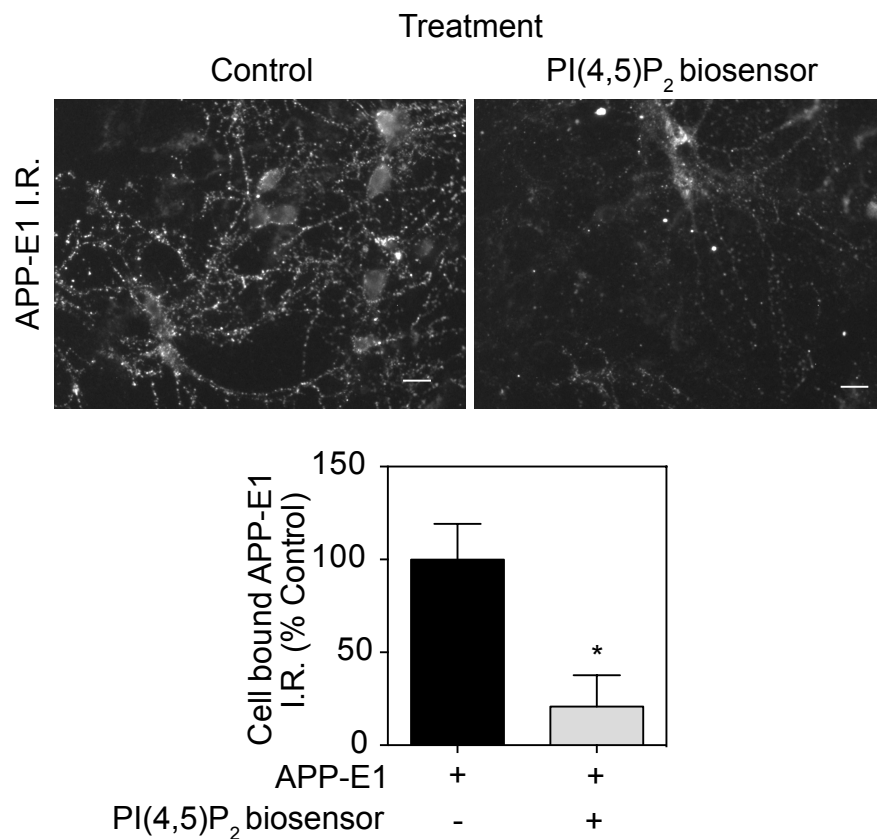


Figure 3.25. A molar excess of the PI(4,5)P₂ biosensor decreases APP-E1 binding to cells. Primary hippocampal cultures were incubated with 100 nM PI(4,5)P₂ biosensor and 10 nM APP-E1 for 2 hours at 37°C, and cell-bound APP-E1 was detected using immunocytochemistry at 4°C. Scale bars = 10 μm. Graphs show quantification of cell bound APP-E1 immunoreactivity (I.R.), derived from 30 fields per treatment. Bars show mean ± SEM APP-E1 I.R. Statistical significance determined using Student's t-test (*p < 0.05).

To examine whether APP binding to the cell surface could alter the levels of other cell-surface PIPs, the binding of the PI(3)P biosensor, the PI(4)P biosensor and the PI(3,4,5)P₃ biosensor was examined in the presence or the absence of APP-E1. Neither the PI(4)P biosensor nor the PI(3)P biosensors bound to cells in the presence or absence of APP-E1 (Fig. 3.26). However, an increase in the binding of the PI(3,4,5)P₃ biosensor to neurons was observed in the presence of APP-E1 (Fig. 3.27). The cell-bound PI(3,4,5)P₃ biosensor was highly co-localised with APP-E1 on the surface of neurons in puncta <1 µm (Fig. 3.27). This effect was not due to non-specific cross-reaction of the anti-6xHis antibody with the anti-GST antibody as the APP-E1-only control showed staining in the APP-E1 channel but no other channels (Fig. 3.27B, E). Neither was the effect due to spectral overlap or bleedthrough, because the increase in neuronal PI(3,4,5)P₃ biosensor immunoreactivity was also observed when APP-E1 was not detected using antibodies (Fig. 3.28). However, quantification of pixel intensities was not possible, because the PI(3,4,5)P₃ biosensor had a propensity to bind to extracellular debris (Fig. 3.28A, 3.12E). The high intensity staining of the extracellular debris obscured measurement of the increase in PI(3,4,5)P₃ biosensor observed on neurons in the presence of APP-E1. Despite repeated attempts to optimise the experimental conditions to reduce the amount of extracellular debris, this issue could not be resolved.

Instead, the same experimental paradigm was performed using an antibody to detect the cell surface PI(3,4,5)P₃. The PI(3,4,5)P₃ antibody did not label cells in the absence of APP-E1 and there was minimal non-specific binding to extracellular debris (Fig. 3.28B). When APP-E1 was added, cell surface PI(3,4,5)P₃ immunoreactivity was observed on neurons (Fig. 3.28B).

Treatment

APP-E1

PI(3)P Biosensor

PI(4)P Biosensor

+ - +

- - +

+ + -

- + -

+ - -

- - -

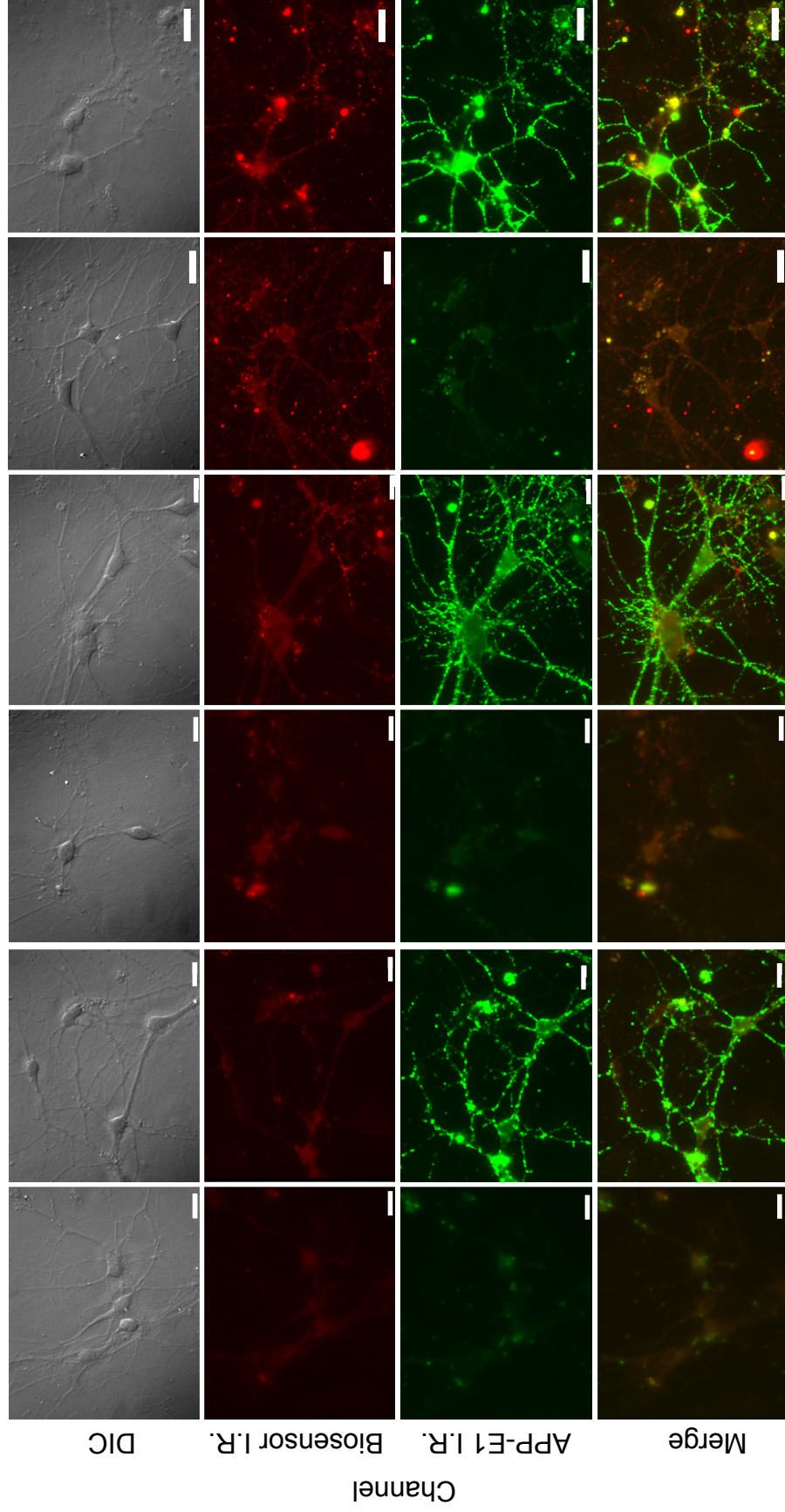


Figure 3.26. APP-E1 does not effect PI(4)P and PI(3)P biosensor binding to primary hippocampal cultures. Cells were treated with 25 nM APP-E1, 25 nM PI(3)P biosensor or 25 nM PI(4)P biosensor for 2 hours at 37°C prior to washing. Cell-bound APP-E1, PI(3)P or PI(4)P biosensor was then detected at 4°C using immunocytochemistry. Scale bars = 10 μ m.

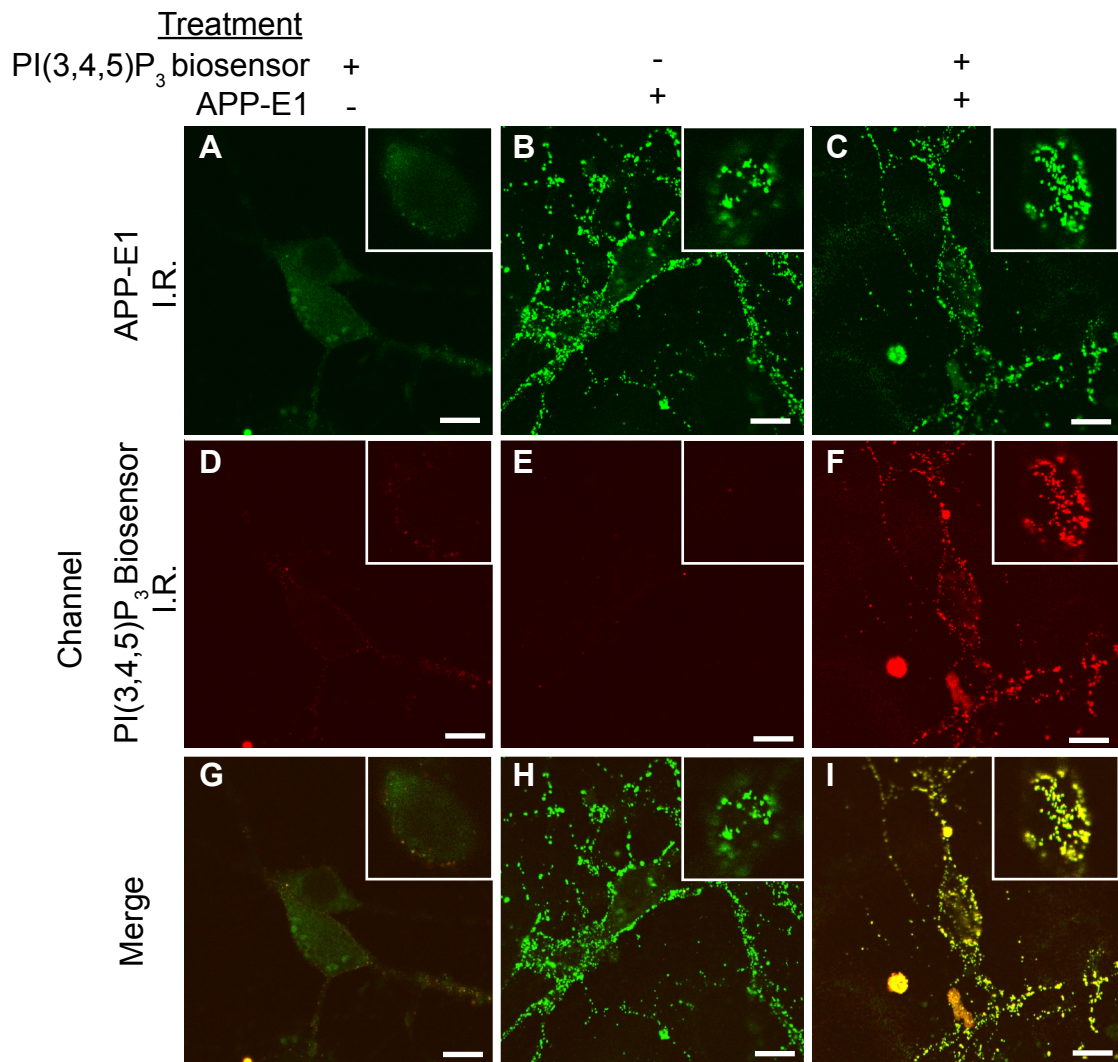


Figure 3.27. Exogenous APP-E1 induces an increase in cell-surface PI(3,4,5)P₃. Panels A - I show confocal slice of primary hippocampal cultures treated with APP-E1 (50 nM) or PBS control and/or PI(3,4,5)P₃ biosensor (50 nM) for 2 h at 37°C. Cells were washed and cell-bound APP-E1 and cell-bound PI(3,4,5)P₃ biosensor were detected by immunocytochemistry performed at 4°C. Insets show enlargement of cell-soma region focused on the cell surface. Scale bars = 10 µm.

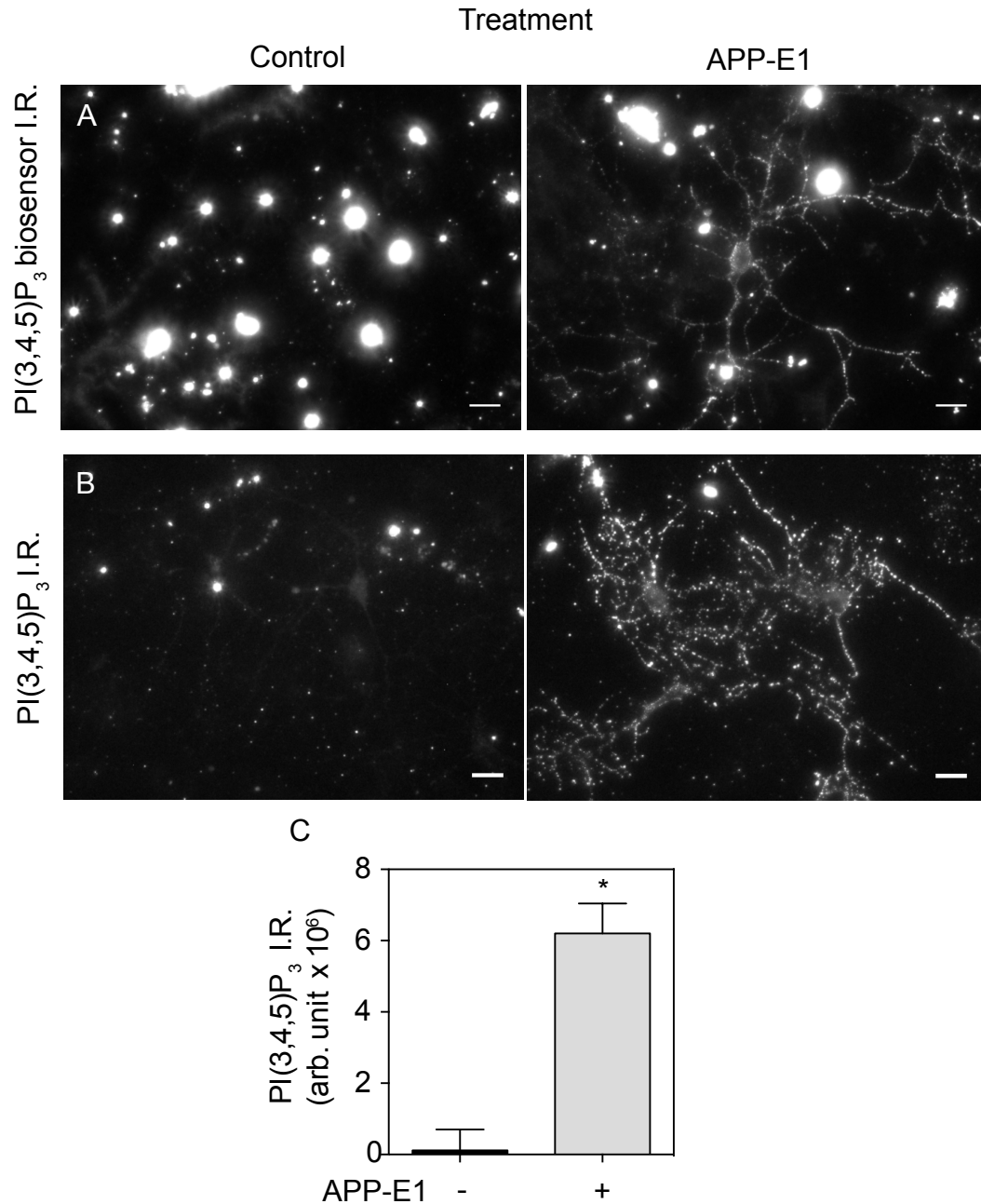


Figure 3.28. APP-E1 increases PI(3,4,5)P₃ biosensor and PI(3,4,5)P₃ antibody binding to primary hippocampal cells. Panel A: Primary hippocampal cultures were incubated with 50 nM APP-E1 or PBS control and 50 nM PI(3,4,5)P₃ biosensor for 2 hours at 37°C prior to labelling of cell-bound PI(3,4,5)P₃ biosensor at 4°C by immunocytochemistry. Panels B, C: Primary hippocampal cultures were treated with 25 nM APP-E1 or PBS control for 2 hours at 37°C prior to labelling with a PI(3,4,5)P₂ antibody at 4°C. Panel C shows quantification of PI(3,4,5)P₂ immunoreactivity from 30 fields. Bars show mean \pm SEM PI(4,5)P₂ immunoreactivity. Statistical significance determined using a Student's t-test. *Significantly different from control, $p < 0.05$. Scale bars = 20 μ m.

Quantification of pixel intensities confirmed that the presence of APP-E1 significantly increased the amount of PI(3,4,5)P₃ immunoreactivity (Fig. 3.28C). Therefore, these data suggest that the binding of APP-E1 to the cell surface induces an increase in the level of cell-surface PI(3,4,5)P₃.

3.3.3 Effect of APP-E1 on Akt phosphorylation

Many cellular signalling pathways are dependent on PIPs (Di Paolo and De Camilli, 2006). One such pathway is the PI3K/Akt pathway, which is activated by growth factors such as PDGF, NGF and IGF-1, all of which bind to receptors on the cell surface (Franke et al., 1995; Park et al., 1996; Kulik et al., 1997). Receptor activation by these growth factors results in activation of PI3K, which produces PI(3,4,5)P₃ (Franke et al., 1995; Park et al., 1996; Kulik et al., 1997). The production of PI(3,4,5)P₃ results in the downstream phosphorylation of Akt, which can be measured using phospho-specific antibodies (Andjelkovic et al., 1997). APP N-terminally truncated species similar to the APP-E1 recombinant protein used in this study have also been reported to exist in the brain (Vella and Cappai, 2012). Therefore, it was hypothesised that APP-E1 might induce an increase in cell-surface PI(3,4,5)P₃, which might result in a change in Akt phosphorylation.

To examine the effect of APP-E1 on Akt phosphorylation, primary cortical neurons were treated with APP-E1, proteins were extracted and the phosphorylation state of Akt was investigated by western blotting. Two anti-Akt antibodies were used, a phospho-specific antibody that only detects Akt that is phosphorylated at the serine 473 position, and a pan Akt antibody that detects total Akt. The ratio of the pAkt473/total Akt was used to indicate changes in the

phosphorylation state of Akt. The PI3K inhibitor wortmannin was used as a negative control (Arcaro and Wymann, 1993), and insulin was used as a positive control because insulin receptor activation results in the phosphorylation of Akt (Kulik et al., 1997). As the B27 supplement routinely used to culture these cells contains insulin, cultures were grown in the absence of B27 supplement for two hours prior to treatment.

There was a significant increase in the pAkt:total Akt ratio for the insulin-treated positive control cells, and a significant decrease in the pAkt:total Akt ratio in wortmannin treated negative control cells, which demonstrated that the assay was suitable for assessing Akt phosphorylation in primary cortical neurons (Fig. 3.29). However, there was no effect of APP-E1 on the pAkt:total Akt ratio after two hours APP-E1 treatment (Fig. 3.29). Therefore, the results did not support the hypothesis that the up-regulation of PI(3,4,5)P₃ observed on the cell surface after two hours of APP-E1 treatment would coincide with an increase in the phosphorylation state of Akt.

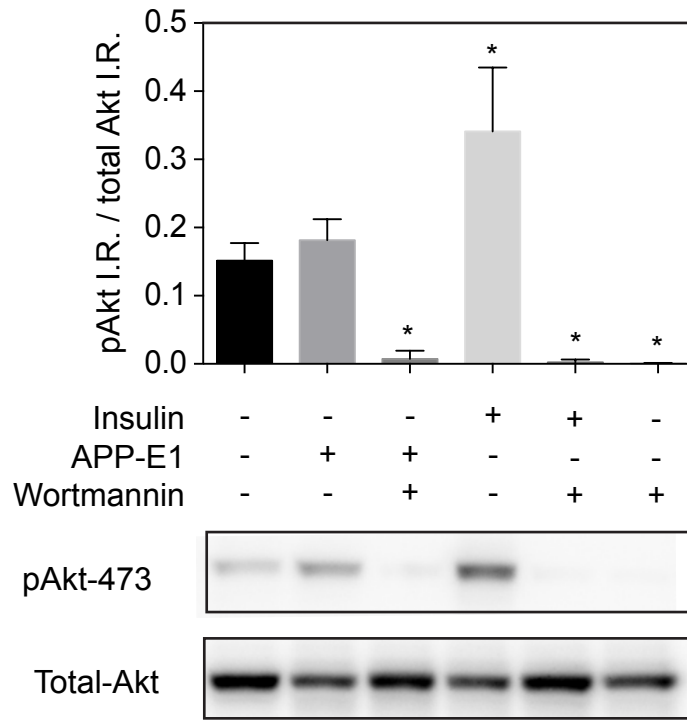


Figure 3.29. APP-E1 treatment does not affect the phosphorylation of Akt at residue 473. Primary cortical cultures were deprived of B27 supplement for 2 hours prior to incubation with a combination of 25 nM APP-E1, 25 nM insulin or 1 μ M wortmannin for 2 hours. Cells were lysed and Akt phosphorylation was analysed by western blotting. Bars show mean \pm SEM ratio of phospho (p) Akt-473 immunoreactivity / total Akt immunoreactivity, derived from 4 replicate wells containing 1×10^6 cells each. Statistical significance was determined by one-way ANOVA with Dunnett's post-hoc test. * Statistically different from untreated control ($p < 0.05$).

Chapter 4 Discussion and conclusions

4.1 Summary of results

The experiments presented in this thesis provide a number of novel conclusions relating to the biological interactions of APP and the role of PIPs in cell signalling. APP was found to contain a PIP-binding domain. This domain was located in the N-terminal E1 region of APP. The presence of the E1 PIP-binding domain was demonstrated by the experiments that showed that sAPP α and APP-E1 selectively bound to PIPs in the protein-lipid overlay assay (Fig 3.2, 3.1). Both sAPP α and APP-E1 did not bind to any major lipid species such as phosphatidylinositol, phosphatidylglycerol or phosphatidylethanolamine. Furthermore, the binding of APP-E1 to PI(4,5)P₂ could be reduced by competition with a water soluble analogue of PI(4,5)P₂ (Fig. 3.4A). This demonstrated specificity of the interaction of the APP E1 domain with PI(4,5)P₂. Computational modelling using the crystal structure of the E1 domain suggested that there could be at least two energetically favourable possible binding sites for IP₃ on the APP E1 domain (Fig. 3.5). Therefore, these data suggest that the E1 domain of APP is a bona-fide PIP-binding domain.

The data also suggested that the E1 domain of APP is sufficient for targeting secreted forms of APP to the cell surface. The APP-E1 recombinant protein bound selectively to neurons and not to glia (Fig 3.8). APP-E1 bound to the surface of neurons with a punctate distribution, in puncta less than 1 μ m in size (Fig 3.8). Therefore, the lack of APP-E1 binding to glial cells suggested that the E1 domain of APP interacts with a particular binding site that is only found on neurons (Fig. 3.8).

The results also demonstrate that mouse hippocampal cells possess discrete domains on the cell surface that are rich in PIP lipids, which were detected using specific monoclonal antibodies and biosensor proteins (Section 3.2.1). These PIP domains had a punctate distribution over the surface of neurons and glia, and were less than 1 μm in size. APP-E1 bound to neurons and was highly co-localised with the cell-surface PI(4,5)P₂ domains (Fig. 3.14 and 3.13). These data suggested that APP interacts with PI(4,5)P₂ on the surface of neurons. However, the results also suggested that the binding of APP to PI(4,5)P₂ was not sufficient for binding to cells. This insufficiency was evident as PI(4,5)P₂ was also present on glial cells to which APP-E1 did not bind (Fig. 3.13). Furthermore, the binding of APP to the surface of cells could not be reduced using competition with a water soluble PI(4,5)P₂ analogue (Fig 3.15). Therefore, the interaction of APP-E1 with cell-surface PI(4,5)P₂ was not sufficient to explain why APP-E1 binds to the cell surface.

Instead, the results suggest that APP may interact not only with PIP on the cell surface, but also another cell-surface component, which binds to the heparin-binding site. Heparin could block the binding of APP-E1 to the cell surface (Fig. 3.16), but not the binding of APP-E1 to PI(4,5)P₂ in the protein-lipid overlay assay (Fig. 3.4). This suggested that the heparin-binding domain was sufficient for binding to cells and was distinct from the PIP-binding domain. However, heparitinase treatment of cells, which significantly reduced cell surface heparan sulfate immunoreactivity, did not affect the binding of APP-E1 to cells (Fig. 3.17). This evidence, along with the high specificity of APP-E1 binding to particular cells, suggested that APP-E1 might bind to a specific cell-surface receptor that is not heparan sulfate.

As the data suggest that the PIP-binding domain in APP-E1 does not determine the binding of APP-E1 to cells, it is possible that the PIP-binding site may be involved in eliciting another biological function. Many cellular signalling pathways are PIP-dependent; therefore, it was hypothesised that changes in the levels of PIPs may reflect a biological action of APP. To determine if APP could affect levels of PIPs, the effect of sAPP α on levels of total PIPs was examined and the effect of APP-E1 on levels of cell-surface PIPs was also examined. No effect of sAPP α was found on total cellular levels of PIP. However, the binding of APP-E1 to the cell surface increased the levels of cell-surface PI(4,5)P₂ (Fig. 3.24) and cell-surface PI(3,4,5)P₃ (Fig. 3.27). The increase in cell-surface PIP was highly localised to where APP-E1 bound to neurons (Fig. 3.27). This suggests that the effect was due to the binding of APP-E1 to the cell surface. Therefore, APP-E1 may bind to the neuronal cell surface and alter the levels of PIPs.

To investigate whether APP-E1 could affect a known PI(3,4,5)P₃-dependent signalling pathway, the effect of APP-E1 on Akt phosphorylation was examined. In these experiments, APP-E1 was not found to effect the phosphorylation of Akt (Fig 3.29) after two hours APP-E1 treatment. This data suggested that two hours APP-E1 treatment does not activate the PI3K/Akt signalling pathway. Therefore, binding of APP to cell surface PIPs may alter other cellular signalling pathways.

These results support the existence of a PIP-binding domain in the E1 domain of APP. The data also suggest the presence of a specific receptor for APP, that is not heparan sulfate, which binds to APP via the heparin-binding site. The PIP-binding site may facilitate the binding of APP to the cell-surface receptor, or hold a functional role by modulating cell-surface PIP levels.

4.2 Discussion

APP has been a subject of intense investigation since it was first cloned in 1987 (Kang et al., 1987). APP is intimately involved in the pathogenesis of Alzheimer's disease as it is the precursor of A β , the protein that is deposited in the AD brain to form amyloid plaques (Glennner and Wong, 1984; Masters et al., 1985; Selkoe et al., 1986). Despite much research into the biosynthesis, structure and metabolism of APP, the normal function of APP is unresolved. Importantly, understanding the biological function of APP will give insight into systems that are perturbed in AD, and will also be important for assessing possible off-target effects for AD treatments that target APP processing.

Numerous studies suggest that APP is likely to have an important physiological function. Many studies suggest that the major secreted form of APP (sAPP α) may mediate some of these functions. For example, sAPP α is able to act in a neurotrophic and a neuritotrophic manner (Chasseigneaux et al., 2011; Gakhar-Koppole et al., 2008; Milward et al., 1992; Jin et al., 1994; Ninomiya et al., 1994; Wallace et al., 1997; Hoffmann et al., 2000; Pietrzik et al., 1998; Saitoh et al., 1989; Demars et al., 2011; Freude et al., 2011; Ohsawa et al., 1999). The fact that sAPP α can specifically bind to particular types of cells suggests the presence of a receptor for APP that could mediate trophic effects (Hoffmann et al., 2000; Reinhard et al., 2013). A number of carbohydrates and proteins have been reported to interact with APP on the cell surface (Young-Pearse et al., 2008; Kounnas et al., 1995; Nikolaev et al., 2009; Gralle et al., 2009; Narindrasorasak et al., 1992; Williamson et al., 1996; Ninomiya et al., 1994; Small et al., 1994). However, the role of lipids in the binding of APP to cells has not been

investigated in detail. Therefore, this study explored the interactions of APP with lipids to evaluate if APP-lipid interactions could contribute to APP binding to the cell surface.

The experiments presented in this thesis showed that both APP-E1 and sAPP α were highly selective for binding to PIPs and did not bind to other major types of lipid. This selectivity is illustrated by the fact that sAPP α and APP-E1 did not bind to PI, but did bind to PIPs, which only differ by the presence of one phosphate group. Interestingly, APP was not found to bind to GM1-ganglioside in this study, in contrast to a previous report (Zhang et al., 2009). Although the experiments did not find that APP bound to GM1 in the protein-lipid overlay assay, the data did suggest that the E1 region of APP was responsible for binding to PIPs, which was the same region suggested to be a lipid-binding region by Zhang et al. (2009).

PIPs are quantitatively minor lipids in the cell, but are of great biological interest as PIPs are signalling lipids that are involved in many aspects of cellular function (Di Paolo and De Camilli, 2006; Section 1.6). For example, PIPs are involved in the regulation of endocytosis (Posor et al., 2013), exocytosis (Khuong et al., 2013), Ca²⁺ signalling (Lapetina and Michell, 1973), ion channel function (Gamper and Rohacs, 2012) and cytoskeletal function (Golub and Caroni, 2005). PIP signalling represents a sophisticated and evolutionarily conserved system in eukaryotes for controlling the interactions of proteins with membranes (discussed in Section 1.7). The levels of PIPs can be rapidly and precisely controlled in the cell by the action of phosphoinositide kinases and phosphatases (Di Paolo and De Camilli, 2006). Furthermore, PIP-binding domains are highly common in biology

and are present in many proteins (Lemmon, 2008; Michell, 2008). This means that PIPs target proteins to particular cellular locations, through interactions with specific lipid-binding domains (Lemmon, 2008). As PIPs are involved in many cellular functions, it is highly likely that the PIP-binding domain in APP identified in this study is involved in the biological function of APP.

4.2.1 PIP-binding regions in APP

The E1 domain of APP has been suggested to be an important functional region of APP as it has a growth factor-like structure and contains a heparin-binding region (D1) and a metal-binding region (D2; Small et al., 1994; Multhaup et al., 1996). Furthermore, previous studies have suggested that this region of APP could bind to gangliosides (Zhang et al., 2009). For these reasons, the ability of an APP-E1 recombinant protein to bind to PIPs was examined in the protein-lipid overlay assay. These experiments found that APP-E1 bound to PIPs, suggesting that a PIP-binding domain in APP resides within the E1 domain.

Based on the crystal structures of the APP E1 domain that are available, there are some candidate regions that could be involved in PIP binding by APP-E1. (Rossjohn et al., 1999; Dahms et al., 2010). The E1 heparin-binding region (residues 95 - 110) has a large positively charged surface that could interact with PIPs (Small et al., 1994). A positively charged surface of this type is typical of many PIP-binding domains, which in general bind to PIPs through electrostatic interactions (McLaughlin et al., 2002; McLaughlin and Murray, 2005; Lumb and Sansom, 2012; Lemmon, 2008). The computational modelling of APP-E1 IP₃ interactions predicted that APP could interact with PIP at the heparin-binding site in an energetically favorable manner, consistent with the suggestion that the

positively charged heparin-binding region could bind to PIP. However, in the lipid overlay assay, heparin did not block the binding of APP to PIP, which suggested that the PIP-binding region of APP-E1 might be distinct from the heparin-binding region. The computational modelling of the E1 domain also predicted other binding pockets that could contribute to PIP binding by APP, with the largest around the amino acids residues his151, lys155, gly90 and pro91. The presence of a separate PIP-binding pocket may explain why heparin did not block the binding of APP to PIP in the protein-lipid overlay assay.

Although the computational docking models are theoretical, and are not yet supported by in vitro binding data, these models can give some indication of the affinity of PIPs for APP. The predicted ΔG values for the APP-E1 protein binding to IP₃ were comparable to that obtained with the PH domain of spectrin binding to IP₃. The spectrin PH domain binds to IP₃ with a K_d of 40 μM , which suggests that the affinity of APP-E1 for IP₃ may be in a similar range (Hyvonen et al., 1995). Importantly, these theoretical data suggest that the affinity of APP for PIP may be in a physiologically relevant range.

To determine the direct mechanism of the interaction of APP-E1 with PIP, the computationally predicted binding model will need to be experimentally verified. The binding affinities of APP for IP₃ could be determined by a direct method such as isothermal titration calorimetry (Lemmon et al., 1995). Precise binding sites for PIP on APP-E1 can be determined by a number of approaches. Some studies have experimentally identified PIP binding sites by X-Ray crystallography (Hyvonen et al., 1995). Alternatively, a mutagenesis study could be used to identify the PIP-binding site, by producing recombinant APPs with the residues that are predicted

to be involved in binding to PIPs mutated (Kale et al., 2010). An advantage of this approach would be that once a non-PIP binding mutant APP is produced, the effect of removing interactions of APP with PIPs could be characterised in cells. For example, the effect of non-PIP binding APP expression on APP trafficking could be examined.

Most PIP-binding domains do not bind to a particular PIP with absolute specificity, but will bind to a number of PIPs (Kavran et al., 1998). From the data in Fig. 3.1 and Fig. 3.3, it is not clear whether APP binds selectively to different PIP regioisomers. In the protein-lipid overlay assay, most sAPP α immunoreactivity was associated with PI(3)P, PI(4)P and PI(5)P, whereas APP-E1 immunoreactivity was associated more uniformly with all PIPs (Fig 3.1 and 3.3). Importantly, the proteins used in this study were not directly comparable, as the recombinant sAPP α had a N-terminal 6xHis affinity tag, whereas the recombinant APP-E1 had a C-terminal 6xHis affinity tag. Therefore, although it is possible that regions of sAPP α outside the E1 domain could modify the binding of the APP E1 domain to PIP, this possibility would have to be investigated using recombinant proteins expressed in the same expression system with identical affinity tags.

4.2.2 Extracellular PIP is present on the surface of neurons and glia

Most studies of the function of PIPs have considered roles for PIPs in the cytoplasmic leaflets of membranes. This has lead to the commonly held assumption that PIPs are only present intracellularly. The data presented in this study shows for the first time evidence for the presence of PIP on the outer

surface of cells of the central nervous system. Also, this study demonstrates that a number of PIP-binding proteins (the PI(4,5)P₂ biosensor, PI(3,4,5)P₃ biosensor and APP-E1) can bind to PIPs on the surface of hippocampal cells in culture. These results suggest that PIPs may target proteins to the cell surface, similar to the known roles of PIPs in the cytoplasmic leaflet of the plasma membrane (PM; Lemmon, 2008). Importantly, these data raise the possibility that many extracellular proteins could bind to PIP as a physiological mechanism of targeting to the cell surface. If this is the case, it is possible that many extracellular proteins may contain PIP-binding domains.

The presence of PIPs on the surface of cells is perhaps not surprising, as phospholipids are known to “flip” from the outer leaflet to the cytoplasmic leaflet, and “flop” from the cytoplasmic leaflet to the outer leaflet (van Meer et al., 2008). In the PM, the concentration of particular lipids such as phosphatidylserine and phosphatidylethanolamine is enriched in the cytosolic leaflet (van Meer et al., 2008; van Meer, 2011). The asymmetric distribution of lipids in the PM is maintained in an ATP-dependent manner by enzymes known as flippases (Daleke, 2007). The asymmetrical distribution of lipids is a highly dynamic process and is known to be altered in processes such as apoptosis and platelet activation (van Meer, 2011; Fadok et al., 1992; Bevers et al., 1982).

Although the majority of studies investigating the function of PIPs have only considered intracellular PIPs, a few studies have previously noted the existence of PIPs on the surface of cells. Studies of membrane asymmetry in erythrocytes have demonstrated that 20% of PI(4,5)P₂ is present in the outer leaflet of the PM, but no PIP is present (Gascard et al., 1991). The first study to generate antibodies

against PIPs noted that anti-PIP antibodies were immunoreactive with the surface of live adherent macrophages (Fogler et al., 1987). Recently, some studies have demonstrated that some eukaryotic plant pathogens can use extracellular PI(3)P as a binding site to gain entry to plant and mammalian cells (Kale et al., 2010; Lu et al., 2013). Collectively these studies and the results in this thesis demonstrate that PIPs are present on the surface of cells.

The data in this study suggested that PI(3,4,5)P₃ is present on the surface of cells, however this was only detected in the presence of APP-E1. Therefore it is possible that the levels of cell surface PIPs can be altered. It is also not clear at present if PIPs can flip/flop from one leaflet to another. If PIPs could flip from one leaflet of the membrane to the other, it is possible that PIP lipid signalling could occur in a trans-membrane fashion. However, this prospect has not been investigated.

Interestingly, the PI(4,5)P₂ immunoreactivity and the PI(4,5)P₂ biosensor data (Section 3.2.1) suggested that cell surface PIPs were present in punctate microdomains that were smaller than 1 µm in size. This is consistent with other studies that report that PIPs segregate into punctate PIP microdomains that are less than 1 µm in size in intracellular membranes (van den Bogaart et al., 2011; Wang and Richards, 2012). There has been some controversy surrounding whether PIP can form microdomains (McLaughlin et al., 2002; McLaughlin and Murray, 2005; van Rheenen et al., 2005). It is also not clear whether PIP microdomains are distinct from other type of membrane domains, such as lipid rafts (Lingwood and Simons, 2010). However, recent evidence suggests that on the cytoplasmic leaflet of cell membranes at least, PI(4,5)P₂ is not uniformly

distributed and may be organised into discrete lateral domains (Golebiewska et al., 2008; Golebiewska et al., 2011). A potential mechanism to explain the formation of PIP domains is that anionic PIPs may cluster together with cationic proteins (van den Bogaart et al., 2011). Further, the clustering of PIPs with proteins has been suggested to be important for spatial regulation of processes such as synaptic vesicle release and endocytosis (Aoyagi et al., 2005; Khuong et al., 2013; Posor et al., 2013). Therefore, the observation in this study that cell-surface PI(4,5)P₂ was present in discrete puncta provides support to the theory that PIP can form microdomains, and these domains can spatially segregate proteins.

The APP-E1 recombinant protein that bound to cells was highly localised to the PI(4,5)P₂ microdomains (Fig 3.14). Previous studies exploring the ability of sAPP α to bind to raft fractions of cells have reported that sAPP α binds to cells and localises to detergent-resistant membrane fractions (Tikkanen et al., 2002). Although the sAPP α cofractionated with markers of caveolae in the detergent-resistant membrane fractions, immunocytochemical detection of caveolar markers showed that sAPP α localised to a novel, non-caveolar type of domain (Tikkanen et al., 2002). The data presented in this thesis suggests a possible explanation for the observations by Tikkanen et al., (2002), which is that sAPP α localises to PI(4,5)P₂ rich domains on the cell surface.

4.2.3 Mechanism of APP-E1 binding to the cell surface

The data in this thesis show that APP-E1 can bind to PIP *in vitro*, and that APP-E1 localises to PI(4,5)P₂ domains on the surface of cells. However, the results did not support the hypothesis that PI(4,5)P₂ is the only binding site for APP-E1, as PI(4,5)P₂ was detected on glial cells which APP-E1 did not bind to. Also, the binding of APP to the surface of cells could not be reduced by competition with a water soluble PI(4,5)P₂ analogue. This suggested that there might be another binding site for APP on the surface of cells, which has a higher affinity than the PIP-binding site. As the binding of PIPs to a protein is commonly a low-affinity interaction (Lemmon, 2008), PIPs rarely act alone in membrane targeting interactions, but rather act as co-receptors (Di Paolo and De Camilli, 2006; Lemmon, 2008; Carlton and Cullen, 2005). Typically, the combination of a PIP-binding domain and another interaction domain in a protein results in a stronger affinity for a particular binding site (Carlton and Cullen, 2005). The data in this study suggest that the PIP-binding site could act in combination with another component, such as a glycosaminoglycan and/or a receptor.

Heparin did not block the binding of APP-E1 to PI(4,5)P₂ *in vitro*, which suggested that the PIP-binding site is distinct from the heparin-binding site. However, heparin was able to block the binding of APP-E1 to cells, which provided evidence that the heparin-binding site is involved in binding of APP-E1 to the cell surface. Importantly, the data did not support the possibility that a heparan sulfate proteoglycan was a cell-surface receptor for APP, as heparitinase treatment did not affect the binding of APP to cells. This result is consistent with a number of other studies, which report that sAPP α binding to cells is

heparitinase-insensitive (Hoffmann et al., 1999), or only partially sensitive to heparitinase (Ninomiya et al., 1994), or occurs independently of glycosaminoglycan synthesis (Reinhard et al., 2013).

A model to explain the results in this study is presented in Fig. 4.1. In the model, the APP E1 domain binds through the heparin-binding site to a high affinity receptor that is expressed on neurons. This receptor is present in cell surface PI(4,5)P₂ microdomains, and APP-E1 interacts with this cell-surface PI(4,5)P₂ through its PIP-binding domain. The PIP-binding site may therefore facilitate binding of APP to the receptor by tethering APP to the membrane, or it may play another role (discussed in Section 4.2.6).

Recently, a study was published which supports the conclusion that the APP E1 domain may bind to a receptor (Reinhard et al., 2013). Reinhard et al. (2013) explored the binding of sAPP α , the APP E1 domain and the APP-E2 domain to cells. They reported that the E1 domain of APP binds to a high affinity cell-surface receptor, but this receptor was not a heparan sulfate proteoglycan. Interestingly, the E1 domain of APP was necessary for binding to the cell surface, and the E2 domain positively modulated this interaction (Reinhard et al., 2013).

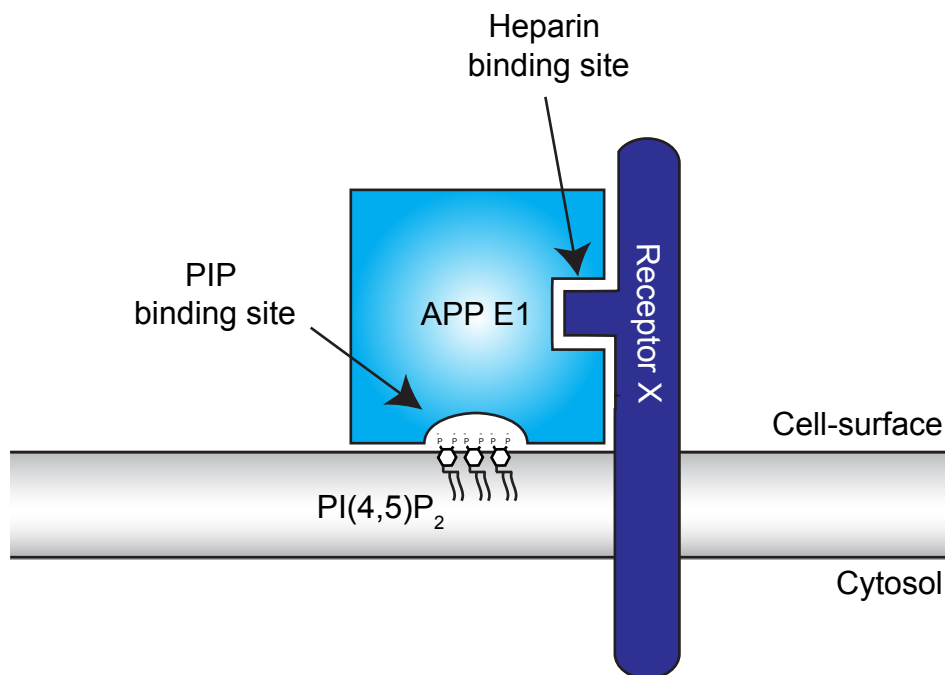


Figure 4.1. Hypothetical model of the binding of the APP E1 domain to the cell surface to explain the data in the study. In this model it is proposed that the heparin-binding site in the E1 domain interacts with a receptor (X) that is not a heparan sulfate proteoglycan. This receptor is expressed on neurons and is present in PI(4,5)P₂ microdomains on the cell surface. APP also interacts with PI(4,5)P₂ on the cell surface through the PIP binding site, which is distinct from the heparin-binding site.

Further, they reported two binding sites for the APP E1 domain on cells, a high-affinity binding site ($K_d = 10 - 80 \text{ nM}$) and a lower affinity-binding site ($K_d > 5 \text{ }\mu\text{M}$), which they suggested could be a lipid (Reinhard et al., 2013). Therefore, the low-affinity binding interaction of the APP E1 domain with cells reported by Reinhard et al. (2013) could be a result of the PIP-binding domain identified in this thesis.

The data in this study demonstrated that the APP E1 domain is able to bind selectively to neuronal cells. Therefore, these data support the presence of a specific receptor for APP that is expressed on neurons. Importantly, the presence of such a receptor suggests that APP is involved in a cell-surface signalling interaction. Many growth factors are known to signal through specific receptors (Lemmon and Schlessinger, 2010). Notably, the structure of the APP extracellular domain has been likened to a growth factor (Rossjohn et al., 1999; Reinhard et al., 2013). Many studies have also suggested that APP acts in a trophic capacity (Saitoh et al., 1989; Chasseigneaux et al., 2011; Pietrzik et al., 1998; Gakhar-Koppole et al., 2008; Milward et al., 1992; Jin et al., 1994; Ninomiya et al., 1994; Wallace et al., 1997; Caille et al., 2004; Demars et al., 2011; Freude et al., 2011; Ohsawa et al., 1999). Therefore, it is likely that the interaction of APP E1 domain with the receptor could be involved in the regulation of cellular growth.

However, the receptor for the APP E1 domain that is expressed on neurons remains to be identified. Previous studies have suggested a number of potential receptors for APP including $\beta 1$ -integrin, lipoprotein receptor related protein-1 (LRP1), death receptor 6, p75 neurotrophin receptor and APP itself (Young-Pearse et al., 2008; Kounnas et al., 1995; Nikolaev et al., 2009; Gralle et al.,

2009). Of these suggested receptors, LRP1 was reported to bind preferentially to KPI-containing isoforms of APP, therefore it is unlikely that LRP1 represents the cellular receptor for APP-E1 observed in the experiments this thesis (Kounnas et al., 1995). The APP-E1 recombinant protein that was used in this thesis was also used by the study that reported that DR6 and p75 neurotrophin receptor were receptors for APP. Therefore it is possible that DR6 and p75 neurotrophin receptor could be candidate receptors for the binding of APP-E1 to cells observed in this study (Nikolaev et al., 2009). Further, APP and β -1 integrin are expressed on hippocampal neurons, so these too are possible candidate receptors (Shi and Ethell, 2006). The method of detecting cell-bound APP-E1 used in this study could easily be applied to compare the distribution of candidate receptors to investigate whether any of these receptors are responsible for APP-E1 binding to hippocampal neurons. Importantly, identifying the cellular receptor for secreted forms of APP will help explain how APP functions. Knowledge of this receptor will give understanding of the signalling pathways that are activated by APP, and also the types of cells in which APP functions.

4.2.4 Effect of APP on levels of PIPs

The results presented in Section 3.3.2 show that the binding of APP-E1 to cells increased the level of both PI(4,5)P₂ and PI(3,4,5)P₃ on the cell surface. This increase in PI(4,5)P₂ and PI(3,4,5)P₃ was localised to neurons but not to glia. As APP bound to the cell surface of neurons but not glia, this provides strong evidence that APP interacts with PIP on the cell surface of neurons. PIPs are key signalling lipids and many cellular signalling pathways such as the PI3K/Akt pathway and the Ca²⁺ pathway involve changes in the level of PIPs (Di Paolo and

De Camilli, 2006). Therefore, it is possible that the APP-E1-induced increase in the level of cell-surface PIP may influence a signal transduction pathway. However, it must be borne in mind that to date the role of cell-surface PIPs in cell signalling is unknown.

In some cellular signalling pathways, such as the PI3K/Akt pathway, signalling is mediated through an increase in levels of PI(3,4,5)P₃ (Andjelkovic et al., 1997). Therefore, the possibility that APP-E1 could activate the PI3K/Akt pathway was investigated by assessing the effect of APP-E1 on Akt phosphorylation in primary cortical cultures. This experiment did not find an effect of APP-E1 on Akt phosphorylation after two hours APP-E1 treatment. Although it is possible that APP-E1 could induce a very rapid transient activation of the PI3K pathway, which is not observed after two hours, the experimental conditions that were used were sufficient to observe an increase in Akt phosphorylation in the positive control treatment (insulin). Therefore, the results suggest that the observed increase in PIP immunoreactivity on the cell surface does not cause activation of the PI3K/Akt pathway by APP-E1.

A more likely explanation for the increase in the level of cell surface PI(3,4,5)P₃ and the increase in the level of cell surface PI(4,5)P₂ is that APP-E1 binding to cells sequesters PI(3,4,5)P₃ and PI(4,5)P₂ on the cell surface. There are reports that PI(4,5)P₂ binding proteins can induce trans-bilayer redistribution of lipids across the membrane (Bucki et al., 2000). Further, PIP-binding proteins have been suggested to laterally cluster PIPs in lipid bilayers by binding to them (Gambhir et al., 2004; McLaughlin and Murray, 2005). So, it is possible that the presence of a PIP-binding protein (APP-E1) stabilises clusters of PIP to some extent, which

may explain the increased levels of cell-surface PI(4,5)P₂ and PI(3,4,5)P₃ that were observed in this study.

A redistribution of PIPs at the cell surface could also occur independently of a change in the total level of PIPs. This would be consistent with data from the UPLC-measurement of total PIP levels in response to sAPP α treatment (Section 3.3.1). The results of the UPLC-MS indicated that sAPP α did not affect levels of total PIPs. However, as the UPLC-MS approach was unable to measure changes in PI(3,4,5)P₃, this method was not suitable for detecting the increase in cell-surface PI(3,4,5)P₃ observed using the antibody and biosensor.

In summary, the observation that APP binding to cells affects the levels of cell-surface PIP provides strong evidence that APP interacts with PIP on the cell surface. These data suggest that APP-E1 increases the level of PIPs on the cell surface, which could have a biological consequence. Further investigation is required to determine whether the observed effects of APP-E1 on cell surface PIP have a resultant effect on cellular physiology.

4.2.5 Other possible roles for the PIP-binding domain in APP

The biological role of the PIP-binding domain in APP is not yet clear. However, it is not known what roles PIPs play on the cell surface. Many studies of PIPs in an intracellular context suggest that PIPs are involved in the targeting of proteins to membranes (Lemmon, 2008; Balla, 2005). Therefore, it is quite likely that APP binding to PIP is involved in its targeting to the correct cellular location. A study has reported that some plant pathogens can use cell surface PIP to gain entry to cells (Kale et al., 2010). Therefore, it is possible that sAPP α binding to cell surface PIP could result in endocytosis of the sAPP α . The experiments in this thesis did not investigate whether sAPP α or APP-E1 was internalised after binding to the cell surface, however endocytosis of sAPP α has been previously reported to occur (Tikkanen, et al., 2002)

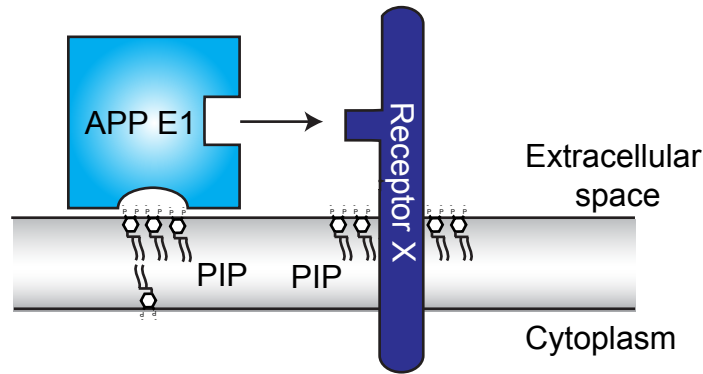
Importantly, there are also some studies that suggest PIPs may affect the biology of APP. Recently, APP trafficking has been demonstrated to be dependent on PI(3)P (Morel, et al., 2013). In addition, it has been known for some time that sAPP α and A β secretion are reduced by PI3K inhibition (Petanceska et al., 1999). Therefore, the E1-PIP-binding domain in APP could also play a role in the trafficking and processing of APP. APP could also potentially interact with PIP inside the cell, where there is a significant pool of APP (Yamazaki et al., 1996; Koo et al., 1996).

4.2.6 Hypothetical suggestions for roles of the lipid-binding domain in APP

This study identified a novel PIP-binding domain in the E1 region of APP. However, determining the biological role of this domain will require further investigation. The data in this study demonstrate that APP binding to PIP alone is not sufficient to explain the cell-surface binding of APP as there is at least one other binding site on the cell surface. However, APP is able to alter cell surface PIPs demonstrating that APP and PIP interact on the cell surface. Therefore, the results suggest that the APP PIP-binding domain could elicit another biological effect.

Some speculative suggestions for role of the PIP-binding domain in APP function are provided in Fig. 4.2. In the first hypothetical model, PIP binding by APP aids the interaction of APP with its receptor by tethering APP to the membrane (Fig. 4.2A). This kind of co-incidence detection is used by many PIP-binding proteins to increase the specificity of binding interactions to particular locations (Carlton and Cullen, 2005). The PIP-binding domain could therefore be involved in targeting sAPP α to regions of cells where the receptor is present. Alternatively, PIP binding could promote a conformational change in APP that allows interaction with the receptor (Jackson et al., 2010). Activation of this receptor might theoretically elicit a trophic effect of APP.

Model A. PIP binding by APP aids the interaction of APP with its receptor



Model B. PIP binding by APP results in propagation of signalling

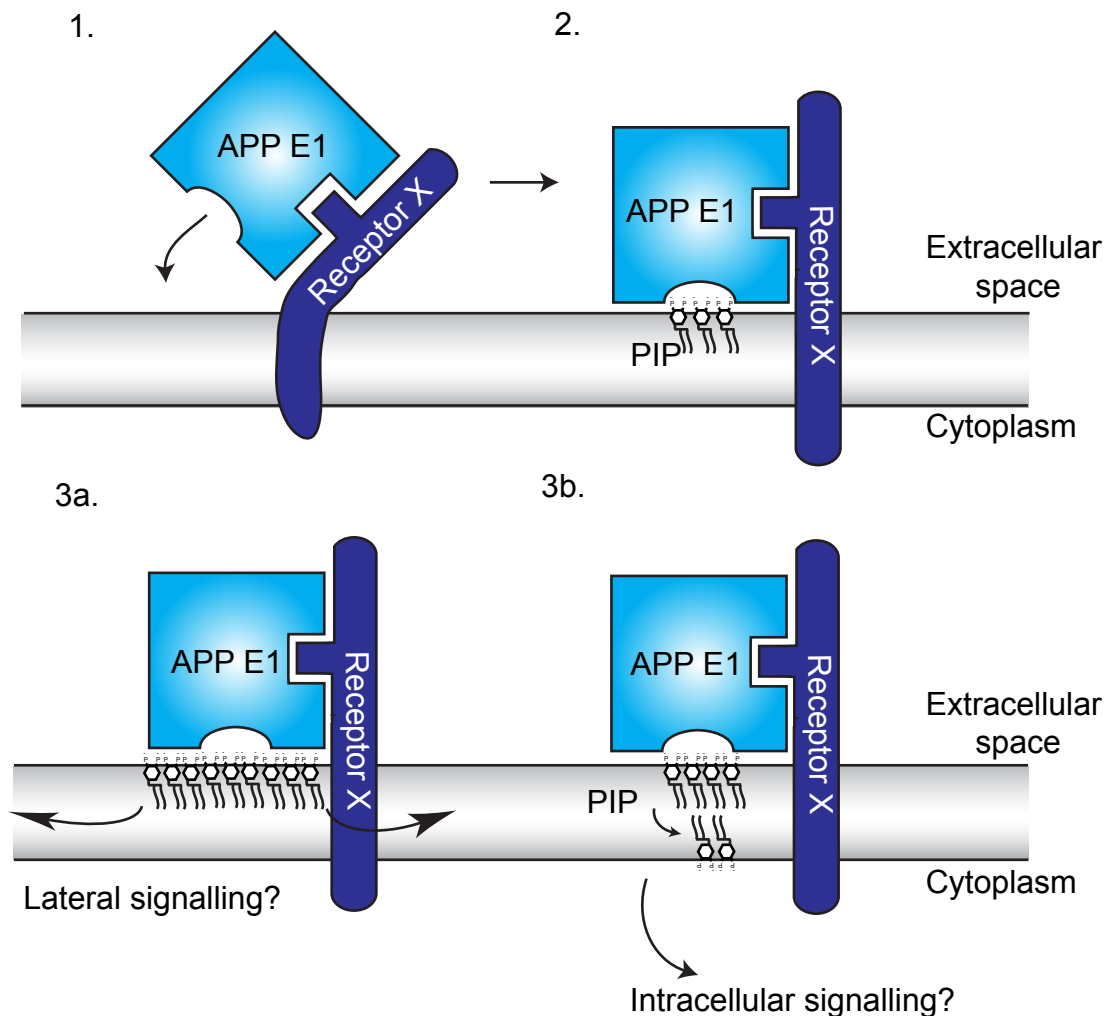


Figure 4.2. Hypothetical models of biological roles for the APP E1 PIP-binding domain. Panel A: The PIP-binding domain may aid the binding of APP to its high-affinity receptor (receptor X). Targeting APP to PIP microdomains could facilitate interactions with a higher affinity receptor present in these PIP domains. Panel B: The APP E1 PIP-binding domain may be involved in the propagation of a signalling event. In the hypothetical model, APP binding to its high-affinity receptor sequesters PIP on the cell surface. This could result in the recruitment of other PIP-binding proteins, resulting in signalling. Propagation of PIP signals could occur either laterally (Panel 3a), or if PIP is able to “flip” from the extracellular leaflet into the intracellular leaflet, trans-bilayer signalling could occur (Panel 3b).

In the second hypothetical model, APP binds to its receptor, and sequesters cell-surface PIP (Fig. 4.2B). This could result in the recruitment of other PIP-binding proteins, resulting in signalling. This type of process could be involved in the mechanism of activation of the receptor and therefore elicit a trophic effect by APP. Importantly, propagation of PIP signals could occur either laterally, or if PIP is able to “flip” from the extracellular leaflet into the intracellular leaflet (van Meer, 2011), trans-bilayer signalling could occur. Hypothetically, outer leaflet to inner leaflet PIP signalling could have important biological implications, for example transmission of a signal to stimulate endocytosis, which is known to involve the assembly of a number of PIP-binding proteins on the cytosolic side of the membrane (Frere et al., 2012).

Determining the function of the PIP-binding domain in APP will be challenging, as the exact function of APP is unclear at this point. However, investigation into the function of the PIP-binding domain may also shed light on aspects of the physiological roles of APP. To investigate some of the hypothetical models above, a possible experimental approach would be to produce cells or animals expressing mutant APP with a non-functional PIP-binding domain. This approach has been used to convincingly demonstrate that the APP intracellular domain controls the trafficking of APP (Perez et al., 1999). Once non-PIP binding APP mutants are produced, the effect of the mutations on a number of aspects of APP biology could be investigated. Trafficking, expression, processing and effects on cellular proliferation could be used as a starting point for further investigation. Additionally, if the PIP-binding domain has an unexpected function, this would be apparent in the mutant cells and could be characterised.

4.3 Final conclusions

The experiments presented in this thesis demonstrate the existence of a novel PIP-binding site in the E1 domain of APP. PIP lipids were found to be present on the surface of cells, and secreted forms of APP localise to these domains. This study increases our understanding of the way APP interacts with the surface of cells, by demonstrating APP interacts with PIPs on the cell surface. Also, this study increases our understanding of the normal composition of the surface of cells by demonstrating the presence of cell surface PIPs. Importantly, many extracellular proteins could potentially interact with cell-surface PIPs, which may have broad implications for many aspects of biology.

These data support the possibility that APP could act in a signalling interaction, possibly by modulating levels of PIPs, which are lipids that are known to be involved in many cellular signalling pathways. The study also provides evidence that sAPP α is likely to function as a paracrine factor, and that the E1 domain is important in this process. This was demonstrated by the experiments that showed the isolated E1 domain could selectively bind to neurons. The PIP-binding domain may contribute to this process, but the data also implicate the presence of a specific high-affinity receptor for the APP E1 domain. This receptor is only expressed on neurons in primary murine hippocampal cultures, and is not likely to be a heparan sulfate glycosaminoglycan. Identifying this receptor will provide a significant advance for our understanding of how APP functions, by determining the direct actions of APP. Understanding of the normal function of APP will provide insight into the biochemical processes that are disrupted in AD, and also into the normal function of the brain.

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Appendices

Appendix I. Materials

Material	Supplier
30% Acrylamide/Bis Solution	Bio Rad Laboratories Pty. Ltd, Gladesville, Australia
37:4 PI (LM-1502)	Avanti Polar Lipids Inc. Alabaster, USA.
37:4 PI(3,4,5)P ₃ (LM-1906)	Avanti Polar Lipids Inc. Alabaster, USA.
37:4 PI(3,4)P ₂ (LM-1903)	Avanti Polar Lipids Inc. Alabaster, USA.
37:4 PI(4,5)P ₂ (LM-1904)	Avanti Polar Lipids Inc. Alabaster, USA.
37:4 PI(4)P (LM-1900)	Avanti Polar Lipids Inc. Alabaster, USA.
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (NA-HEPES)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
APP N-terminal domain recombinant protein corresponding to APP amino acids 18 – 286 with an C-terminal 6xHis tag (APP-E1)	ProSci Inc., Poway, USA
sAPP α * recombinant protein (APP-695 isoform residues 18-612) with a N-terminal 6xHis tag	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Acetonitrile (Lichrosolv grade)	Merck Australia Pty. Ltd, Kilsyth, Australia
AlamarBlue® reagent	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
B27 supplement	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Bio Rad DC protein assay kit	Bio Rad Laboratories Pty. Ltd, Gladesville, Australia
Calcium chloride	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Chloroform	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
cOmplete ultra protease inhibitor tablets	Roche Diagnostics Australia Pty. Ltd. Castle Hill, Australia
Dako mounting medium	Dako Australia Pty. Ltd. Campbellfield, Australia.
Deoxyribonuclease-1	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Dextrose	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
diC8PI(4,5)P ₂	Echelon Bioscience Inc., Salt Lake City, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Disodium hydrogen phosphate	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Dulbecco's modified Eagle's medium (DMEM)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Ethylamine	British Drug Houses Ltd., Poole, UK

Appendix I. Materials

Material	Supplier
Fatty-acid free bovine serum albumin (BSA)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Fetal bovine serum (FBS)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
GlutaMAX TM	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Glycerol	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Glycine	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Hanks Balanced salt solution (HBSS)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Human recombinant basic fibroblast growth factor (FGF2)	Peprotech Inc., Rocky Hill, USA
Human recombinant epidermal growth factor (EGF)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Hybond C Extra nitrocellulose membrane	GE Healthcare Pty. Ltd., Rydalmere, Australia
Hydrochloric Acid (37%)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Igepal CA-630	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Immobilon chemiluminescent substrate	Millipore Australia Pty. Ltd, Kilsyth, Australia
Magnesium chloride	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Membrane Lipid Strips TM	Echelon Bioscience Inc., Salt Lake City, USA
Methanol (HPLC grade)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Neurobasal medium	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Nitric Acid	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Non-fat dry milk powder	Woolworths Ltd., Bella Vista, Australia.
Papain	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Paraformaldehyde	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Penicillin/streptomycin	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
PhosStop phosphatase inhibitor tablets	Roche Diagnostics Australia Pty. Ltd. Castle Hill, Australia
PI(3,4,5)P ₃ Biosensor (recombinant GST-tagged pleckstrin homology domain of general receptor for phosphoinositides-1)*	Echelon Bioscience Inc., Salt Lake City, USA
PI(3)P biosensor (recombinant GST-tagged PX domain of p40 _{phox})*	Echelon Bioscience Inc., Salt Lake City, USA
PI(4,5)P ₂ biosensor (recombinant GST tagged pleckstrin homology domain of phospholipase C- δ 1)*	Echelon Bioscience Inc., Salt Lake City, USA

Appendix I. Materials

Material	Supplier
PI(4)P biosensor (recombinant GST-tagged SidC 3C domain)*	Echelon Bioscience Inc., Salt Lake City, USA
PIP Strips TM	Echelon Bioscience Inc., Salt Lake City, USA
Poly-L-Lysine	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Polyvinylidene fluoride membrane (PVDF)	Bio Rad Laboratories Pty. Ltd, Gladesville, Australia
Porcine mucosal heparin	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Sodium chloride	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Sodium deoxycholate	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Sodium phosphate monobasic dihydrate	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
SphingoStrips TM	Echelon Bioscience Inc., Salt Lake City, USA
Tris	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Triton X-100	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Trypan Blue	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
TrypLE TM	Life Technologies Australia Pty. Ltd., Mulgrave, Australia.
Tween-20	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
Wortmannin DMSO solution	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia
β-Mercaptoethanol	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia

* During the course of the study, it was observed that these recombinant proteins are unstable, and will lose the ability to bind to PIP over time. For best results, aliquots should be flash frozen in liquid nitrogen, stored at -80°C, and only thawed once.

Appendix II. Cell culture media, buffers and solutions

Cell culture media	
Neurobasal plating medium	Neurobasal medium supplemented with 10% v/v FBS, 2 % v/v B27 supplement, 1 mM GlutaMAX TM , 100 U mL ⁻¹ penicillin and 100 µg mL ⁻¹ streptomycin
Neurobasal maintenance medium	Neurobasal medium supplemented with 2 % v/v B27 supplement, 1 mM Glutamax TM , 100 U mL ⁻¹ penicillin and 100 µg mL ⁻¹ streptomycin.
Neurosphere proliferation medium	DMEM supplemented with 2% B27, 100 U mL ⁻¹ penicillin, 100 U mL ⁻¹ streptomycin, 20 ng mL ⁻¹ human FGF2 and human EGF 20 ng mL ⁻¹
Buffers and solutions	
Phosphate buffered saline (PBS)	154 mM NaCl, 8 mM NaH ₂ PO ₄ , 2 mM Na ₂ HPO ₄ , pH 7.3
Imaging buffer	125 mM NaCl, 5 mM KCL, 10 mM dextrose, 1 mM MgCl ₂ , 2 mM CaCl ₂ , 10 mM Na-HEPES, 2% B27 supplement, pH 7.5.
Tris-buffered saline with tween (TBS-T; protein-lipid overlay assay)	150 mM NaCl, 50 mM Tris-base, pH 7.5, 0.1% Tween-20.
TBS-T (western blotting)	150 mM NaCl, 25 mM Tris-base, pH 8, 0.2% Tween-20
SDS-PAGE running buffer	25 mM Tris, 192 mM Glycine, 0.1% SDS
Laemmli sample buffer	10% β-mercaptoethanol, 2% SDS, 50 mM Tris pH 6.8, 10% glycerol
Cell lysis buffer	150 mM NaCl, 50 mM Tris, 0.5% w/v sodium-deoxycholate, 1% v/v Igepal-CA630, 0.1% SDS, pH 7.4
Electroblotting buffer	25 mM Tris, 192 mM Glycine, 20% v/v Ethanol

Appendix III. Primary antibodies used in the study

Antigen [clone]	Type	Species	Application	Dilution	Supplier (Catalogue No.)
6xHis	Polyclonal (ChIP Grade)	Rabbit	ICC PLO	1:1000 1:3000	Abcam Ltd., Cambridge, UK. (ab9108)
APP (a.a.66-81) [22C11]	Monoclonal IgG	Mouse	PLO	1:1000	Millipore Australia Pty. Ltd, Kilsyth, Australia. (MAB348)
APP/A β (a.a.1-16 of A β) [6E10]	Monoclonal IgG1	Mouse	PLO WB	1:1000 1:2000	Covance Pty. Ltd., North Ryde, Australia. (SIG-39300)
GAPDH	Polyclonal	Rabbit	WB	1:20,000	Millipore Australia Pty. Ltd, Kilsyth, Australia. (ABS16)
GFAP	Polyclonal	Rabbit	ICC	1:1000	Millipore Australia Pty. Ltd, Kilsyth, Australia. (AB5804)
GST [GST-R 6G9]	Monoclonal IgG	Rat	ICC	1:500	Sigma-Aldrich Pty. Ltd., Castle Hill, Australia. (SAB4200055)
Heparan Sulfate [F58-10E4] epitope	Monoclonal IgM	Mouse	ICC	1:250	Seikagaku Corporation Tokyo, Japan. (370255)
MAP2	Polyclonal	Rabbit	ICC	1:1000	Millipore Australia Pty. Ltd, Kilsyth Australia. (AB5622)
PanAkt [C67E7]	Monoclonal IgG	Rabbit	WB	1:1000	Cell Signalling Technology Inc. Boston, USA (4691)
PhosphoAkt-Ser473 [D9E]	Monoclonal IgG	Rabbit	WB	1:1000	Cell Signalling Technology Inc. Boston, USA (4060)
PI(3,4,5)P ₃	Monoclonal IgM	Mouse	ICC	1:500	Echelon Bioscience Inc., Salt Lake City, USA. (Z-P345)
PI(3,5)P ₂	Monoclonal IgM	Mouse	ICC	1:500	Echelon Bioscience Inc., Salt Lake City, USA. (Z-P035)
PI(3)P	Monoclonal IgM	Mouse	ICC	1:500	Echelon Bioscience Inc., Salt Lake City, USA. (Z-P003)
PI(4,5)P ₂	Monoclonal IgM	Mouse	ICC	1:500	Echelon Bioscience Inc., Salt Lake City, USA. (Z-P045)
PI(4)P	Monoclonal IgM	Mouse	ICC	1:500	Echelon Bioscience Inc., Salt Lake City, USA. (Z-P004)

Abbreviations: PLO – protein-lipid overlay assay, ICC – immunocytochemistry, WB – western blot, a.a. – amino acids. Monoclonal antibody clones are indicated in square brackets. Antibody dilutions refer to 1 mg mL⁻¹ stocks

Appendix IV. Secondary antibodies used in the study

Antibody	Conjugate	Application	Dilution	Supplier (Catalogue No.)
Goat anti-mouse IgG	Alexa-594	ICC	1:1000	Life Technologies Australia Pty. Ltd., Mulgrave, Australia. (A-11037)
Goat anti-mouse IgG	Alexa-488	ICC	1:1000	Life Technologies Australia Pty. Ltd., Mulgrave, Australia. (A-11029)
Goat anti-mouse IgM	Alexa-594	ICC	1:1000	Life Technologies Australia Pty. Ltd., Mulgrave, Australia. (A-21044)
Goat anti-rat IgG	Alexa-594	ICC	1:1000	Life Technologies Australia Pty. Ltd., Mulgrave, Australia. (A-11007)
Goat anti rabbit IgG	Alexa-488	ICC	1:1000	Life Technologies Australia Pty. Ltd., Mulgrave, Australia. (A-11034)
Goat anti rabbit	HRP	PLO WB	1:1000	Dako Australia Pty. Ltd., Campbellfield, Australia. (P044801-2)
Goat anti-mouse	HRP	PLO WB	1:5000 1:5000	Dako Australia Pty. Ltd., Campbellfield, Australia. (P044701-2)

Abbreviations: HRP - Horse-radish peroxidase. PLO – protein-lipid overlay assay, ICC – immunocytochemistry, WB – western blot, Ig – Immunoglobulin, Antibody dilutions refer to 1 mg mL⁻¹ stocks.

