Regulation of expression and cellular release of acetylcholinesterase

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

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August 2013

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapters 3 and 4 are based on work from jointly authored publications, namely Hicks, D. A., Makova, N. Z., Nalivaeva, N. N. & Turner, A. J. 2013. Characterisation of acetylcholinesterase release from neuronal cells. *Chem Biol Interact,* **203,** 302-308 and Hicks DA, Makova NZ, Gough M, Parkin ET, Nalivaeva NN, Turner AJ (2013) The amyloid precursor protein represses transcription of acetylcholinesterase in neuronal cell lines. *J Biol Chem. Accepted.* All of the data in these papers that are present in this thesis are attributable to me. The contribution of other authors was in the form of preliminary work (N. Z. Makova), DNA constructs (M. Gough, E. T. Parkin) or editorial work (N. N. Nalivaeva and A. J. Turner).

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Publications

Hicks DA, Makova NZ, Gough M, Parkin ET, Nalivaeva NN, Turner AJ (2013) The amyloid precursor protein represses transcription of acetylcholinesterase in neuronal cell lines. *J Biol Chem*. **286,** 26039-26051

Hicks DA, Makova NZ, Nalivaeva NN, Turner AJ (2013) Characterisation of acetylcholinesterase release from neuronal cells. *Chem Biol Interact*. **203**, 302-308

Neelov IM, Klajnert B, Makova NZ, Hicks D, Pearson H, Vlasov GP, Ilyash MY, Vasilev DS, Dubrovskaya NM, Tumanova NL, Zhuravin IA, Turner AJ, Nalivaeva NN (2012) Molecular properties of lysine dendrimers and their interactions with Aβ peptides and neuronal cells. *Curr Med Chem*. **20**,134-143

Hicks DA, Nalivaeva NN, Turner AJ. (2012) Lipid rafts and Alzheimer's disease: protein-lipid interactions and perturbation of signaling. *Front Physiol*. **3**,189

Hicks D, John D, Makova NZ, Henderson Z, Nalivaeva NN, Turner AJ (2011) Membrane targeting, shedding and protein interactions of brain acetylcholinesterase. *J Neurochem*. **116**,742-746

Abstract

Acetylcholinesterase (AChE) is a hydrolytic enzyme which has been linked to the pathological progression of the neurodegenerative disease, Alzheimer's disease (AD). AD is thought to be driven by the toxic amyloid-β (Aβ) peptide, which derives from proteolytic cleavage of amyloid precursor protein (APP). Here, release of AChE from two neuronal cell lines, SN56 and SH-SY5Y, was investigated and found to be driven by at least two distinct pathways, shedding and exocytosis. The former was found to be mediated by an unknown metalloprotease, sensitive to the inhibitor batimastat. Shedding was also found to be dependent on the action of protein disulphide isomerase. The cellular release of AChE was potentiated by agonism of muscarinic acetylcholine receptors (mAChRs) by carbachol. This process was found to derive, in part, from transcriptional upregulation of AChE by mAChRs, likely involving the Egr-1 transcription factor.

Subsequent work established, for the first time, a mechanistic link between APP and regulation of AChE expression. Over-expression of APP in neuronal cell lines led to reductions of AChE mRNA, protein and catalytic activity. Assessment of other cholinergic genes revealed repression, by APP, of the membrane anchor of AChE, PRiMA, but no changes in mRNA levels of butyrylcholinesterase or the high affinity choline transporter, CHT. This regulatory relationship between APP and AChE was confirmed when knockdown of APP in wild type SN56 cells resulted in a significant increase in AChE mRNA. The ability of APP to repress AChE transcription was shown to be independent of proteolytic processing of the former, as inhibition of each of the secretase enzymes responsible for APP proteolysis had no effect on AChE activity. However, APP-mediated repression of AChE was dependent on the N-terminal, extracellular E1 domain and specifically the copper-binding domain within. Deletion of these domains completely ablated the ability of APP to effect transcriptional repression of AChE.

These studies have implications for greater understanding of the role of the cholinergic system and AChE in the pathological progression of AD. This work further elucidates a physiological role for APP, the perturbation of which may contribute to neurodegeneration.

Contents

Table of Figures

Results II

<u>Abbreviations</u>

α-Bgt: α-bungarotoxin
Aβ: amyloid beta peptide
ACE (2): angiotensin converting enzyme (2)
Acetyl CoA: acetyl coenzyme A
ACh: acetylcholine
AChE: acetylcholinesterase
AD: Alzheimer's disease
ADAM: a disintegrin and metalloprotease
Akt: aka protein kinase B
AICD: amyloid precursor protein intracellular domain
AMPA: 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
APLP: amyloid precursor protein-like protein
ApoE: apolipoprotein E
Arc: activity-regulated cytoskeleton-associated protein
Arf6: ADP-ribosylation factor 6
ATCh I: acetylthiocholine iodide
APP: amyloid precursor protein
APS: ammonium persulphate
BACE-1: β-site APP cleaving enzyme 1
BDNF: brain-derived neurotrophic factor
BCA: bicinchoninic acid
BCh: butyrylcholine
BChE: butyrylcholinesterase
BFCN: basal forebrain cholinergic neuron
BiP: binding immunoglobulin protein (aka GRP78 or HSPA5)
BSA: bovine serum albumin
C.elegans: *Caenorhabditis elegans*
CaMKII: calcium/ calmodulin-dependent kinase II
Cdc2: cell division control protein 2 homolog
Cdk5: cyclin-dependent kinase 5
ChAT: choline acetyltransferase
ChE: cholinesterase
CHT: high affinity choline transporter
CNS: central nervous system
CRAC: cholesterol recognition/ interaction amino acid consensus
CREB: cAMP response element (CRE) binding protein

CBP: CREB binding protein
CSF: cerebro-spinal fluid
CTF: C-terminal fragment
CuBD: copper binding domain
Dab1: disabled-1
DAG: diacylglycerol
DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
(H/V) DBB: horizontal/ vertical diagonal band of Broca
DMEM: Dulbecco's Modified Eagle Medium
DMEM F-12: Dulbecco's Modified Eagle Medium and Ham's F-12 (1:1)
DMSO: dimethyl sulphoxide
DTNB: 5-5`-dithiobis (2-nitro) benzoic acid
DYRK1A: dual specificity tyrosine-phosphorylation-regulated kinase 1A
ECL: electrochemiluminescence
EGCG: epigallocatechin-3-gallate
EDTA: ethylenediaminetetraacetic acid
EGFR: epidermal growth factor receptor
Egr-1: early growth response protein 1
Endo H: endoglycosidase H
ER (AD): endoplasmic reticulum (associated degradation)
ERK: extracellular signal-regulated kinase
Ets: E-twenty six
FAK: focal adhesion kinase
FBS: foetal bovine serum
FGF: fibroblast growth factor
FHB: four-helix bundle
FRET: Förster resonance energy transfer
GABA: γ-amino butyric acid
GFLD: growth factor-like domain
GPCR: G-protein coupled receptor
GSK-3β: glycogen synthase kinase 3β
GWAS: genome-wide association study
HAT: histone acetyltransferase
HDAC: histone deacetylase
HEK: human embryonic kidney
HMG CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
HRP: horseradish peroxidase
HSPG: heparin sulphate proteoglycan

IDE:	insulin degrading enzyme
IL:	interleukin
ITG:	integrin
IP3:	inositol trisphosphate
JNK:	jun N-terminal kinase
JIP:	JNK-interacting protein
KPI:	Kunitz protease inhibitor
LB:	Lennox B
LRP1:	low density lipoprotein receptor-related protein 1
LTCC:	L-type calcium channel
LTP:	long term potentiation
mAChR:	muscarinic acetylcholine receptor
MAPK:	mitogen-associated protein kinase
MCI:	mild cognitive impairment
miR:	microRNA
MMP:	matrix metalloprotease
MWCO:	molecular weight cut-off
nAChR:	nicotinic acetylcholine receptor
NBM:	nucleus basalis of Meynert
NEP:	neprilysin
NF-κB:	nuclear factor κB
NFAT:	nuclear factor of activated T-cells
NGF:	nerve growth factor
NMDA:	N-methyl D-aspartate
NT-3, -4:	neurotrophin-3, -4
dNTP:	deoxynucleotide triphosphate
Iso-OMPA:	tetraisopropyl pyrophosphamide
P75^{NTR}:	p75 neurotrophin receptor
PAS:	peripheral anionic site
PBS:	phosphate buffered saline
PDI:	protein disulphide isomerase
PI3K:	phosphatidylinositol 3-kinase
PICALM:	phosphatidylinositol binding clathrin assembly protein
PIP$_2$:	phosphatidylinositol 4,5-bisphosphate
PKC:	protein kinase C
PL (A, C, D):	phospholipase (A, C, D)
PMA:	phorbol 12-myristate 13-acetate
PRAD:	proline-rich attachment domain
PRiMA:	proline-rich membrane anchor
PrP:	prion protein
PS:	presenilin
PSD-95	post-synaptic density 95

PVDF:	polyvinylidene difluoride
qPCR:	quantitative polymerase chain reaction
RAGE:	receptor for advanced glycation end products
RER:	rough endoplasmic reticulum
RIP:	regulated intramembrane proteolysis
RPMI-1640:	Roswell Park Memorial Institute- 1640
RTK:	receptor tyrosine kinase
RT-PCR:	reverse transcriptase polymerase chain reaction
sAPPα/ β:	soluble APPα/ β
SAP:	serum response factor accessory protein
SDS	sodium dodecyl sulphate
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA:	sarco/endoplasmic reticulum Ca^{2+}-ATPase
siRNA:	small interfering RNA
SNARE:	soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor
SOC:	super optimal broth with catabolite repression
SREBP:	steroid response element binding protein
SRE:	serum response element
SRF:	serum response factor
TACE:	tumour necrosis factor α converting enzyme
TBS:	Tris- buffered saline
TBST:	Tris-buffered saline + 1% Tween-20
TEMED:	tetramethylethylenediamine
TGN:	trans-Golgi network
TF:	transcription factor
Trk:	tropomyosin receptor kinase
UTR:	untranslated region
VAChT:	vesicular acetylcholine transporter
WAT:	tryptophan amphiphilic tetramerisation domain

Mouse models
Tg2567/ APP$_{Swe}$: APP$_{695}$ with double mutations at Lys, Met 670/671Asn, Leu
3xTg AD: APP mutant as above, plus tau P301L and PS1 M146V

Chapter 1

Introduction

Chapter 1: Introduction

1.1 The cholinergic system

1.1.1 Brain regions and development

Cholinergic neurons of the central nervous system (CNS) can be separated into projection neurons and interneurons. Those falling into the former category are largely located in the forebrain and upper brainstem. Those in the latter category represent neurons in the hippocampus, cerebral cortex, hypothalamus and nucleus accumbens (Schliebs and Arendt, 2006), although the area of densest cholinergic innervation is the striatum (Mesulam et al., 1992).
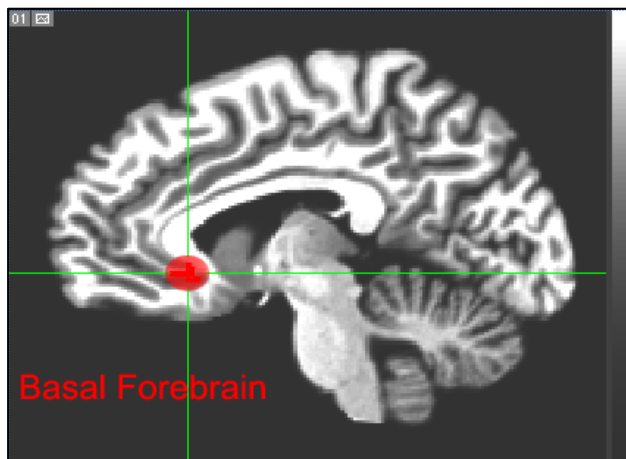
Figure 1.1: location of the basal forebrain
An fMRI image of a human brain with the location of the basal forebrain indicated.
Image taken from:
http://en.wikipedia.org/wiki/File:Basal_Foreb rain_Monakhos_2007.png*, June 30th, 2013*

The basal forebrain cholinergic system (see Fig. 1.1 for location) is a conglomeration of several distinct brain regions. It comprises the medial septal nucleus, horizontal and vertical diagonal bands of Broca (HDBB and VDBB, respectively) and the nucleus basalis of Meynert (NBM) (Auld et al., 2002, Schliebs and Arendt, 2006). Cholinergic efferents from this brain region project throughout the cortex, for example, medial septal nucleus axons project to the hippocampus (Gaykema et al., 1990, Auld et al., 2002, Bruel-Jungerman et al., 2010), which is of critical importance in learning and memory (Scoville and Milner, 1957, O'Keefe and Dostrovsky, 1971, Gil-Bea et al., 2010). In addition, there are significant non-cholinergic neurons, most notably GABAergic interneurons, i.e. those with γ-amino butyric acid (GABA) as a neurotransmitter, making contact with the cholinergic neurons (Sarter and Bruno, 2002, Schliebs and Arendt, 2006).

In the cholinergic system, the key neurotransmitter is acetylcholine (ACh), the main function of which, in the developed brain, is to act as a chemical messenger across

cholinergic synapses (Dale, 1914, Prado et al., 2002). However, there is a well-documented expression of ACh and its receptors in non-neuronal tissues (Wessler et al., 2003). Supporting the notion of functional roles aside from neurontransmission, ACh, with its receptors, precedes synaptogenesis in development (Bruel-Jungerman et al., 2010). It can act as a morphological signalling molecule, working to guide the development of the mammalian cortex. This is through the control of neuronal differentiation and synaptogensis, creating a network to support complex cognitive functions in the developed brain. Although this process is incompletely understood, ACh is believed to be capable of promoting a beneficial milieu for neuronal development. In this way, ACh potentiates the growth of hippocampal neuronal progenitors (Van Kampen and Eckman, 2009) and the proliferation and differentiation of embryonic stem cells (Landgraf et al., 2010). It is thought that perturbation of these processes is likely to contribute to or exacerbate neurological disorders (Campbell et al., 2010). Indeed, transgenic mice expressing an inactive form of the enzyme that hydrolyses ACh, acetylcholinesterase (AChE), display increased ACh levels and proliferation of progenitors in the subgranular zone (Cohen et al., 2008). Furthermore, in the developed brain, ACh acts as an important modulator of cortical signalling (Berger-Sweeney, 2003, Bruel-Jungerman et al., 2010).

Furthermore, in addition to ACh, recent work suggests that development of the cholinergic phenotype in neurons is dependent on expression of the transcription factor (TF) NKx2-1. Data suggest that this TF acts in prenatal brain development, but also in maintenance of this phenotype postnatally. Specific targeting of this TF leads to neuronal degeneration (Magno et al., 2012).

1.1.2 Function

The brain cholinergic system is involved with a wide variety of processes. These include modulating cerebral blood flow, cortical activity and the sleep-wake cycle. In addition, of great significance is its involvement in cognitive function and neuronal plasticity (Schliebs and Arendt, 2006, Schliebs and Arendt, 2011).

The basal forebrain cholinergic system has been demonstrated to play vital roles in memory, informational processing and attention (Baxter and Chiba, 1999, Deiana et al., 2011, Dumas and Newhouse, 2011) and hence these functions are disturbed in some

neurodegenerative conditions which result in loss of cholinergic neurons (Bartus et al., 1982, Sarter and Bruno, 1999). Furthermore, in healthy ageing, there is increased cholinergic recruitment to these brain areas to maintain the aforementioned cognitive functions. However, in a condition such as Alzheimer's disease, this functional compensation is not possible, which results in cognitive degeneration (Dumas and Newhouse, 2011).

Cholinergic neurons, with their origins in the basal forebrain, project to the hippocampus (Gaykema et al., 1990, Auld et al., 2002). As mentioned, the hippocampus plays an important role in episodic memory (Scoville and Milner, 1957) and also spatial awareness (O'Keefe and Dostrovsky, 1971). In this way, hippocampal learning and memory function are highly dependent on the cholinergic system (Bruel-Jungerman et al., 2010, Drever et al., 2011), demonstrated by the learning impairments seen in instances of cholinergic dysfunction (Bartus et al., 1982, Bruel-Jungerman et al., 2010). Conversely, cholinergic enhancement, such as after treatment with the AChE inhibitor donepezil, improves performance in learning tasks (Rokem and Silver, 2013).

Selective lesions of cholinergic neurons induce down-regulation of the proteins Arc and brain-derived neurotrophic factor (BDNF) in the hippocampus. It was subsequently observed that these proteins were essential for hippocampal spatial memory acquisition, i.e. registering information about the environment and spatial orientation within it. The authors showed that pharmacological normalisation of Arc and BDNF levels restored memory performance (Gil-Bea et al., 2010). Furthermore, ACh has been linked to the process of spatial memory acquisition (Deiana et al., 2011). This is an example of a way in which ACh exerts a modulatory effect in plasticity, facilitating long term potentiation (LTP) (Drever et al., 2011), an inter-neuronal signalling phenomenon thought to underlie learning and memory processes (Cooke and Bliss, 2006). This is mediated through various acetylcholine receptors (AChRs), initiating downstream signalling pathways (Drever et al., 2011). Recent data have linked ACh to signalling functions outside the cholinergic system, whereby reducing ACh release causes increased sensitivity to dopaminergic agonists in the striatum (Guzman et al., 2012).

In addition, the cholinergic system has been recently shown to modulate cellular bioenergetics and energy metabolism. This was suggested to be effected though potentiation of glycolysis and oxidative phosphorylation (Lu et al., 2012).

1.1.3 Choline acetyltransferase and the cholinergic synapse

In general, at a chemical synapse (see Fig 1.2), a neurotransmitter, stored in a vesicle, will be released into the synaptic cleft by exocytosis from the presynaptic neuron. The neurotransmitter will traverse the synapse and bind its cognate receptor on the postsynaptic cell membrane, initiating downstream signalling cascades (Germann, 2005).

In cholinergic neurons, the neurotransmitter was shown to be ACh (Dale, 1914), which is synthesised by choline acetyltransferase (ChAT) (Nachmansohn, 1943, Oda, 1999), from choline and acetyl coenzyme A (acetyl CoA), the latter deriving from glucose metabolism. ChAT is a globular protein, existing predominantly in a soluble form, although a minority of approximately 10% is membrane bound. Post-synthesis, ACh is transported into vesicles by the vesicular acetylcholine transporter (VAChT). The co-localisation of the genes for ChAT and VAChT (the latter lies within the first intron of the former) and their consequent co-regulation has led to this gene region being referred to as the cholinergic locus (Erickson et al., 1994, Oda, 1999, Prado et al., 2002). Regulation and function of ChAT and VAChT have been previously comprehensively reviewed (Prado et al., 2002). Interestingly, new data have revealed ChAT to be regulated at an epigenetic level, with its expression, at least in part, modulated by histone acetylation (Aizawa et al., 2012).

Arrival of an action potential in the presynaptic neuron causes opening of voltage-gated calcium channels. This results in rapid increases in intracellular Ca^{2+} levels, with the Ca^{2+} ions binding synaptotagmins. The synaptotagmins, with their complexin co-factor, bind the SNARE proteins that are critical in exocytic processes (Sudhof, 2012).

Figure 1.2: schematic of a cholinergic synapse
1. The neurotransmitter ACh is synthesised from acetyl CoA and choline by ChAT.
2. ACh is subsequently loaded into synaptic vesicles by the transporter VAChT. **3.**
On arrival of an action potential, the stored ACh is released into the synapse by
exocytosis. **4.** ACh binds and activates receptors on both pre- and post-synaptic
membranes, the nAChRs and the mAChRs. **5.** ACh is then hydrolysed by AChE,
releasing acetate and choline. **6.** The choline is transported into the pre-synaptic
neurons by CHT. **7.** This choline, in addition to acetyl CoA, is used for synthesis of
ACh.

Hence, ACh-containing vesicles dock and fuse with the cell membrane, releasing ACh
into the synaptic cleft. It is this exocytic event that is targeted by botulinum toxin
(Burgen et al., 1949) and potentiated by latrotoxins from spiders of the *Latrodectus*
genus (Sudhof, 2001). ACh subsequently traverses the synapse and binds its cognate
receptors on the post-synaptic membrane, either ionotropic nicotinic acetylcholine
receptors (nAChRs) or G-protein linked muscarinic acetylcholine receptors (Germann,

2005). The nAChRs are ligand-gated cation channels (Albuquerque et al., 2009), whereas mAChRs are G-protein coupled receptors (GPCRs), which initiate intracellular signalling cascades (Eglen, 2006).

Regulation of ACh action is key, as over-activation of cholinergic receptors can result in death, as evidenced by nerve agents such as sarin and VX (Abu-Qare and Abou-Donia, 2002, Joosen et al., 2008). Regulation of synaptic ACh signalling is the main role of AChE and under some circumstances, butyrylcholinesterase (BChE) (Massoulie et al., 1993, Darvesh et al., 2003, Giacobini, 2003). These cholinesterase (ChE) enzymes hydrolyse ACh in the synaptic cleft, thereby terminating neurotransmission (Massoulie et al., 1993, Germann, 2005). It is this hydrolytic process that is targeted by cholinesterase inhibitors in some neurodegenerative conditions, with the aim of boosting ACh signalling (Greig et al., 2013).

The hydrolysis of ACh releases choline and acetate (Massoulie et al., 1993), after which the choline is transported into the presynaptic terminal by the sodium dependent high affinity choline transporter (CHT) (Sarter and Parikh, 2005). Subsequently, the recycled choline is re-incorporated into ACh and loaded into synaptic vesicles by VAChT, awaiting further action potentials (Prado et al., 2002).

1.1.4   The nicotinic acetylcholine receptors (nAChRs)

The nAChRs are ligand-gated, cation permeable ionotropic receptors (Albuquerque et al., 2009). They are functionally implicated in attention, cognition (Levin and Simon, 1998), mood, emotion (Shytle et al., 2002) and also cell survival (Utsumi et al., 2004, Del Barrio et al., 2011) and plasticity (Lendvai et al., 2013). Given the plethora of functions in which nAChRs are involved, it is perhaps not surprising that their dysfunction has been linked to a number of diseases, such as AD, Parkinson's disease, schizophrenia, attention deficit/ hyperactivity disorder and chronic pain (Hurst et al., 2012).

There is expression of 10 nAChR subunits in mammalian neuronal systems, specifically α2 to α7, α9, and β2 to β4. These subunits are capable for forming a range of homomeric and heteromeric pentamers (Unwin, 2005), each with different pharmacological properties (Dajas-Bailador and Wonnacott, 2004, Albuquerque et al.,

2009). One example of this is channel Ca^{2+} permeability (Seguela et al., 1993), which is highest in α7 homomers (Castro and Albuquerque, 1995, Dajas-Bailador and Wonnacott, 2004). Each subunit has a conserved extracellular domain of approximately 200 residues, followed by three transmembrane domains, a cytoplasmic loop exhibiting size and sequence variability and finally a fourth transmembrane domain with a variable extracellular carboxyl region (Albuquerque et al., 2009). Agonists, such as ACh or nicotine, interact with the neurotransmitter binding sites. This event causes a conformational change, which travels towards the channel gate, resulting in its opening (Aldea et al., 2010). These subunits together form a membrane spanning pentamer with a small central pore (Unwin, 2005).

In brain, nAChR subunits have a distinct mRNA expression pattern (Whiteaker et al., 2000b, Gotti and Clementi, 2004). Radio-ligands have been frequently used to map nAChR distribution in CNS, such as [^{3}H] nicotine and [^{125}I] epibatidine (Whiteaker et al., 2000a), but also agents selective for specific subtypes, including methyllycaconitine (MLA) α-bungarotoxin(α-BGT) from krait venom (Whiteaker et al., 1999, Whiteaker et al., 2000b, Jones et al., 2004) and, more recently, α-conotoxins (McIntosh et al., 1999, Terlau and Olivera, 2004, Janes, 2005, Kasheverov et al., 2009, Armishaw et al., 2010).

Calcium signalling is of critical importance in nAChR function, as the primary influx is augmented by Ca^{2+} from voltage-operated calcium channels and from intracellular stores (Dajas-Bailador and Wonnacott, 2004). Many of the functions of nAChRs derive from this transmembrane ion flux and consequent alterations in electrical excitability, which may lead to electrical firing (Dajas-Bailador and Wonnacott, 2004, Albuquerque et al., 2009, Yakel, 2013). However, the Ca^{2+} also undertakes several signalling roles, such as mitogen-activated protein kinase (MAPK) cascade activation and activation of the cAMP-response element binding protein (CREB) TF (Nakayama et al., 2001, Dajas-Bailador et al., 2002). Consequently, there have been significant reported effects on gene expression, with a cDNA microarray showing significant alterations in more than 163 genes after 1 day of nicotine treatment (Dunckley and Lukas, 2006). Structure, function and signalling of nAChRs are vast fields, and have been previously comprehensively reviewed (Dajas-Bailador and Wonnacott, 2004, Albuquerque et al., 2009, Hurst et al., 2012, Yakel, 2013).

1.1.4.1 The α7 nAChR

Through the prism of AD, the α7 nAChR has the closest links with pathology (Wang et al., 2000, Hurst et al., 2012), perhaps because of its strong links with cognition (Levin and Simon, 1998, Levin et al., 2009). It is especially abundant in the hippocampal areas, shown by electron microscopy of α7 nAChRs at hippocampal synapses (Fabian-Fine et al., 2001) and through α-Bgt probes (Seguela et al., 1993, Mielke and Mealing, 2009). In addition, α7 nAChRs have been shown to play a key role in neurogenesis, enhancing the survival of adult born neurons in the dentate gyrus. In the absence of nAChRs, few of these neurons survive, although survival is stimulated by donepezil, boosting cholinergic signalling (Campbell et al., 2010). It has also been suggested that chronic treatment with nAChR agonists can increase receptor numbers (Christensen et al., 2010) and consequently promote neuronal activation and LTP (Soderman et al., 2010, Thomsen et al., 2010). A further role in neuronal maintenance has recently been demonstrated by the deleterious effects on dendritic spines in α7 knockout mice (Morley and Mervis, 2013). In addition, α7 nAChRs seem to play an important role in regulating the machinery of exocytosis involved in ACh release (Parikh et al., 2010). Recent work has implicated α7 nAChRs in control of the duration of release, although not the amplitude. In this way, these receptors are able to modulate cholinergic neurotransmission, with the added implication that they may play a role in modulating glutamatergic signalling (Parikh et al., 2010).

Similarly, through nAChR activation, a number of beneficial effects have been ascribed to nicotine. For example, nicotine treatment has been shown to increase mRNA levels and immunoreactivity of nerve growth factor (NGF) and its receptor TrkA in cortex and hippocampus (Formaggio et al., 2010). Given the roles played by NGF in synaptic plasticity and neuroprotection (Aloe et al., 2012), it is possible that nAChR agonism may have beneficial effects.

Furthermore, α7 nAChRs are localised in and functionally dependent on lipid rafts (Bruses et al., 2001). Reductions in membrane levels of sphingomyelin and cholesterol slow desensitisation kinetics and increase agonist affinity, indicating a role for membrane lipid composition in modulation of α7 nAChR function (Colon-Saez and Yakel, 2011). Lipid rafts are small cell membrane microdomains, enriched in cholesterol and sphingomyelin (Simons and Ikonen, 1997), which have been linked to

neurodegenerative disease in a number of ways (Cordy et al., 2006, Hicks et al., 2012). A possible reason for this association has been suggested from an analysis of the α7 nAChR proteome. Pulldown with α-Bgt coated beads and subsequent analysis by mass spectrometry revealed 55 interacting proteins, one of which is BASP1/NAP-22, a cholesterol binding protein which may have involvement in any lipid raft associations of α7 nAChR. This study further confirmed roles for α7 nAChR in neurite outgrowth and maintenance through its interacting partners, including α-catenin 2, homer 1 and neuromodulin (Paulo et al., 2009).

1.1.5   The muscarinic acetylcholine receptors (mAChRs)

Muscarinic acetylcholine receptors are class I seven-transmembrane G-protein coupled receptors (GPCR), of which there are five subtypes, denoted M1 to M5. The proteins are encoded by intron-less genes, which display great inter-species sequence similarity (Eglen, 2006). Signalling through mAChRs involves heterotrimeric G proteins, existing as G_{α} and $G_{\beta\gamma}$ subunits (Mizuno and Itoh, 2009). Due to interactions with different G proteins, downstream signalling is mediated differentially between the receptors, with M2 and M4 coupled to $G_{\alpha\ i/o}$ and M1, M3 and M5 coupled to $G_{\alpha\ q/11}$ (Eglen, 2006). As with nAChRs (Parikh et al., 2010), mAChRs, specifically M1, interact with other neurotransmitter systems, as it has been shown to inhibit noradrenaline release (Roberts et al., 2001).

Binding of ACh to mAChRs and consequent receptor activation leads to the release of GDP bound to G_{α}. Its subsequent replacement with GTP leads to the generation of two distinct entities, G_{α}-GTP and $G_{\beta\gamma}$ (Mizuno and Itoh, 2009). At this point, signals are transmitted to downstream effector molecules, the nature of the signalling pathway being governed by the identity of the G_{α} subunit. M1, M3 and M5 receptors signal through G_q family members, namely $G_{\alpha q}$ and $G_{\alpha 11}$ (Eglen, 2006, Mizuno and Itoh, 2009). The G_{α} subunit subsequently activates phospholipase C-β (PLC-β), resulting in hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to IP3 and DAG. The signal is further propagated by the release of intracellular Ca^{2+} and activation of protein kinase C (PKC) (Rhee, 2001, Mizuno and Itoh, 2009). However, mAChRs have been strongly linked to a number of different signalling molecules, including phospholipase D, phospholipase A_2, adenylyl cyclase (von der Kammer et al., 2001) and Ras (Igishi and Gutkind, 1998). To regulate signalling, the intrinsic GTPase activity of G_{α} then

hydrolyses GTP and the inactive G_α-GDP can re-associate with $G_{\beta\gamma}$, ready for the next receptor activation event (Mizuno and Itoh, 2009).

Although mAChRs have important roles in post-synaptic cholinergic signalling in the developed brain they, like nAChRs, have been shown to have key developmental functions (Eglen, 2006). Their expression occurs as early as 14 days in rats and precedes synaptogenesis and neurotransmission, indicative of a role in regulation of neural cell proliferation and differentiation (Schlumpf et al., 1991).

1.1.5.1 The M1 mAChR

The M1 mAChR is most highly expressed in basal forebrain regions (Wess, 2004) and on this receptor our work has been focused. The phenotype of knockout mice has given an indication of its functions. Mice deficient in M1 mAChRs show a hyperactive phenotype, combined with reduced performance in learning and memory tasks and an almost complete abolition of MAPK signalling downstream of M1 (Wess, 2004).

M1 mAChR has been demonstrated to have key functions in learning, memory and induction of LTP through modulation of synaptic excitability of hippocampal neurons (Berkeley et al., 2001, Eglen, 2006). The effects on LTP derive from activation of extracellular signal-regulated kinase (ERK), which can be mimicked by the mAChR agonist carbachol, and lead to phosphorylation and activation of TFs Elk-1 and c-fos (Hodge et al., 1998, Berkeley et al., 2001, O'Donnell et al., 2012). It had previously been shown that 1,2-diacylglycerol was a key mediator in the induction of c-fos in response to carbachol stimulation of SH-SY5Y neuroblastoma cells (Larsson et al., 1994). Although Ca^{2+} chelation attenuates activation of ERK by carbachol treatment, it is not abolished, as is the case with N-methyl D-aspartate (NMDA) receptor signalling. This is indicative of a second, Ca^{2+}-independent pathway linking mAChR agonism to ERK activation (Rosenblum et al., 2000).

1.1.5.2 Gene regulation

Signalling downstream of mAChRs has been linked to regulation of a number of immediate-early genes, such as those in the Jun, Fos and Egr families, but also transcriptional regulators such as NGFIB and Etr101 (von der Kammer et al., 1998, von der Kammer et al., 2001).

1.1.5.2.1 Egr family proteins

Egr family proteins are in the Cys_2His_2 class of zinc finger TFs (Beckmann and Wilce, 1997) and bind DNA in a sequence-specific manner (O'Donovan et al., 1999, von der Kammer et al., 2001). Depending on the target promoter, this binding can result in transcriptional activation or repression (von der Kammer et al., 2001). Functionally, Egr-1 is involved in mediating the response to growth factors and its knockout potentiated tumour development (Gitenay and Baron, 2009). Egr-1 is also linked to the regulation of specific genes, one of which is AChE (Nitsch et al., 1998).

Activation of mAChRs has been shown to regulate levels of Egr-1, -2, -3 and -4 at protein and mRNA levels, though the major target appears to be Egr-1 (Nitsch et al., 1998, von der Kammer et al., 2001), most often through the MAPK cascade (outlined in Fig 1.3) (Gitenay and Baron, 2009). This signalling is blocked by atropine, an mAChR antagonist (Cushny, 1910) and the Ca^{2+} chelator EGTA, but potentiated by phorbol-12-myristate-13-acetate (PMA), a DAG analogue. Further confirming a role for Ca^{2+} in the signalling pathway, expression is induced by Ca^{2+} ionophores and Ca^{2+} channel agonists (Beckmann and Wilce, 1997). The process is unaffected by forskolin, which activates cAMP production, indicating that cAMP is not involved in this process (von der Kammer et al., 1998). Ca^{2+} signalling can cause activation of CREB and products of PLC activity can potentiate binding of the serum response factor (SRF) to serum reponse elements (SREs) in immediate early genes. Four SREs and CRE-like sites are located upstream of the Egr-1 gene. In addition, Egr expression is also thought to be coupled to NMDA receptors, L-type Ca^{2+} channels and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptors (Beckmann and Wilce, 1997).
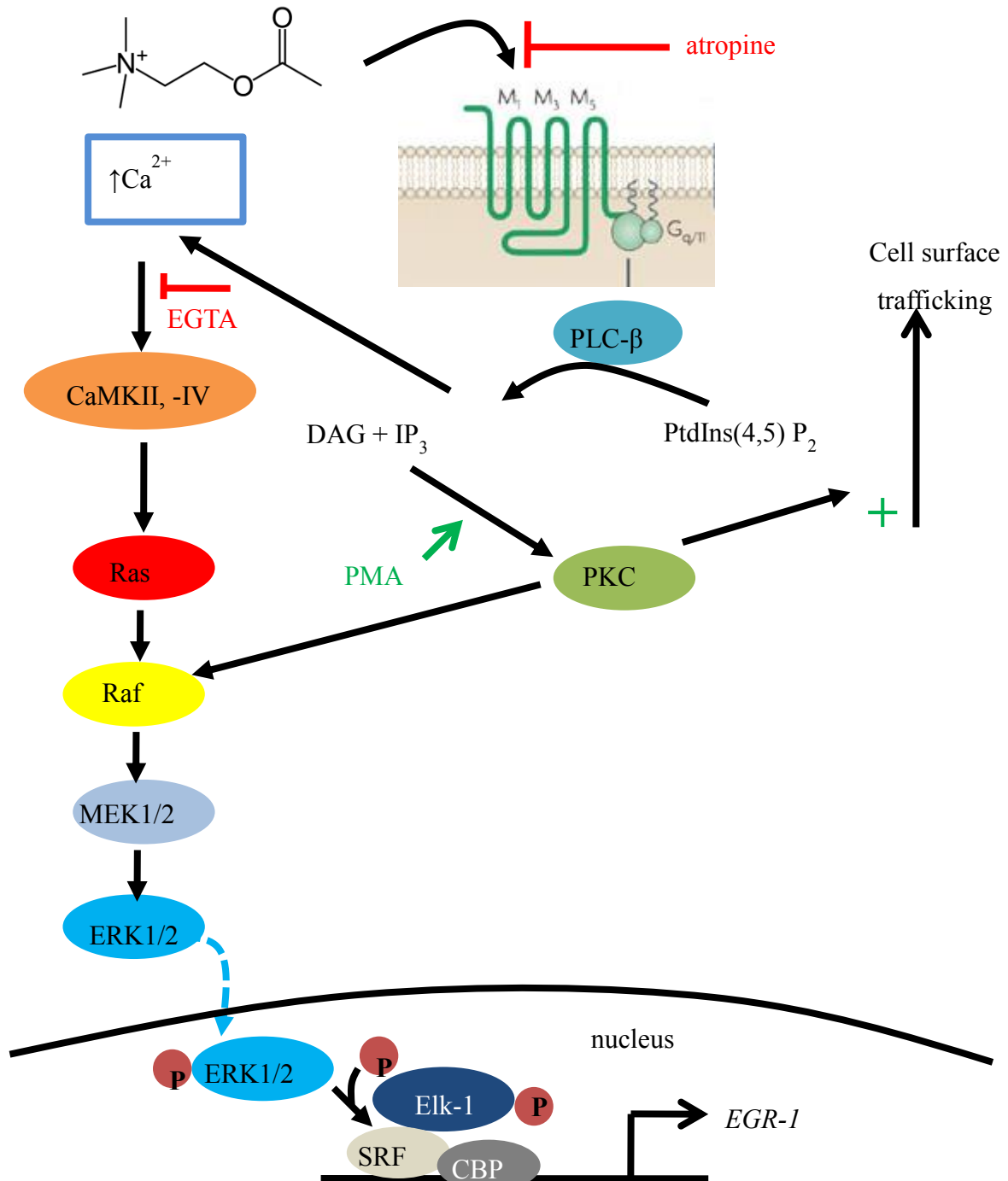
Figure 1.3: putative signalling pathway downstream of mAChR activation
Overview of a putative signalling cascade whereby mAChR activation results in activation of ERK, Elk-1 and subsequent transcriptional activation of Egr-1. Initially, ACh binds a mAChR, which results in activation of the GPCR and release of the G_α subunit. Signalling cascades through PLC-β, which hydrolyses phosphoinositides to generate DAG and IP3. Consequent increases in intracellular Ca2+ activate CaMKII and –IV which initiates the phosphorylation and activates a sequence of proteins, including Ras, culminating in activation of ERK, which in turn activates Elk-1. The latter recruits co-activators and binds target DNA-binding sequences, such as the SRE upstream of Egr-1. Binding of this transcriptional activatory complex to the Egr-1 promoter increases its gene transcription, which ultimately results in transcriptional changes in Egr-1 target genes, such as AChE.

1.1.5.2.3 Elk-1 as a regulatory TF

Egr-1 activation, and hence regulation of AChE, has been closely linked to the TF, Elk-1. It has been proposed that the aforementioned MAPK activation leads to activation of transcription factors of the Elk-1 and SAP-1/-2 families (Hodge et al., 1998, Gitenay and Baron, 2009, Yang et al., 2013). Elk-1 then forms a complex with CREB-binding protein (CBP) and SRF, which binds and activates the Egr-1 promoter. Recently, though, a novel nuclear factor κB (NF-κB) dependent pathway was discovered (Gitenay and Baron, 2009).

Elk-1 is a member of the E-twenty six (Ets) family of TFs, which has key involvement in many biological processes (Laudet et al., 1999, Besnard et al., 2011). Elk-1 has a four domain structure, denoted A-D. Of these, the A domain is the DNA-binding domain, while the D domain comprises the consensus binding sequence for MAPKs (Marais et al., 1993). DNA-binding is usually accomplished in a complex with SRF (Herrera et al., 1989), binding SREs in such genes as *c-fos* and *egr-1* (Tsai-Morris et al., 1988, Treisman et al., 1992). Phosphorylation and activation of Elk-1 can occur on nine residues in the C domain, of which Ser383 and Ser389 are critical for transcriptional activity (Janknecht et al., 1994, Gille et al., 1995, Cruzalegui et al., 1999). Elk-1 phosphorylation can recruit co-activators including CBP, p300 or Srb mediator, the latter providing a link to RNA Pol II (Janknecht and Nordheim, 1996, Boyer et al., 1999). Although potentiated by kinases, Elk-1 signalling is inhibited by phosphatases such as protein phosphatase 2B (PP2B) (Sugimoto et al., 1997, Tian and Karin, 1999).

1.1.6   Trophic factors and receptors

The neurotrophin family has four members, nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and NT-4. They all bind the p75 pan-neurotrophin receptor (p75^{NTR}) with nM affinity and bind cognate tropomyosin receptor kinases (Trks) with pM affinity. NGF binds TrkA, BDNF and NT-4 binds TrkB and NT-3 binds TrkC. Signalling through Trks leads to promotion and maintenance of synaptic contacts between hippocampal/cortical neurons and basal forebrain cholinergic neurons (BFCNs) (Allen et al., 2012) and also suppression of apoptosis through activation of the phosphatidylinositol 3-kinase (PI3K)/ Akt pathway (Blochl and Blochl, 2007). LTP is a key prerequisite for memory formation and BDNF signalling through TrkB plays a vital

role in its maintenance. Consequently, any perturbations in either NGF, BDNF or their receptors can lead to memory dysfunction (Allen et al., 2012).

1.1.6.1 Nerve growth factor (NGF)

Of the neurotrophins, NGF was the first to be discovered (Cohen et al., 1954) and subsequent analysis revealed high inter-species sequence similarity (Hallbook, 1999). Deriving from pro-NGF, it exerts its function through binding to the tropomyosin receptor kinase A (TrkA), a receptor tyrosine kinase (RTK) (Kaplan et al., 1991, Klein et al., 1991). NGF is also capable of binding the p75^{NTR}, which initiates apoptotic signalling cascades in the absence of TrkA (Huang and Reichardt, 2003), through activation of jun N-terminal kinase (JNK) and NF-κB (Hamanoue et al., 1999, Miller and Kaplan, 2001). Interestingly, at low concentrations (10-50pM), the AD-linked amyloid precursor protein (APP) can potentiate NGF signalling in a synergistic fashion (Wallace et al., 1997).

Given the crucial role played by NGF in maintenance of BFCNs (Aloe et al., 2012, Bruno and Cuello, 2012), it has been suggested that age-related cognitive decline may result from disruption in NGF signalling. One example of this is the effect of reactive oxygen species (ROS), which can negatively impact NGF signalling as peroxynitration renders NGF biologically inactive (Jonnala and Buccafusco, 2001, Bruno and Cuello, 2012). Although evidence for a decrease in total NGF in ageing is not compelling (Larkfors et al., 1987, Katoh-Semba et al., 1998), an increase in matrix metalloprotease-9 (MMP9) has been observed in cognitive impairment (Bruno et al., 2009), with MMP9 being the major degradative enzyme of NGF (Bruno et al., 2009). As mentioned, signalling is though the ligand-receptor axis, so perturbations in TrkA function would generate the same phenotype as reduced NGF. This was shown by RNA$_i$ mediated knockdown of TrkA, which impaired attentional processes in aged rats and reduced the ability of neurons to release ACh (Parikh et al., 2012).

1.1.6.2 p75 pan-neurotrophin receptor (p75^{NTR})

Whereas Trks have well-defined trophic roles, p75^{NTR} has a range of functions from trophism to cell death. The signalling outcome can, in part, be driven by binding of co-receptors, with NgR and Lingo1 acting to prevent p75^{NTR} activation and sortilin directing the receptor towards initiating apoptotic signalling (Nykjaer et al., 2004,

Blochl and Blochl, 2007). Further regulation of its function may involve proteolytic cleavage of the receptor, requiring a metalloprotease and also the γ-secretase complex involved in the cleavage of APP and Notch. The extent of receptor proteolysis may regulate formation of the receptor complex comprising p75^{NTR} and TrkRs (Jung et al., 2003).

It is likely that p75^{NTR} exerts an inhibitory role on the trophic status of BFCNs and in this way modulates hippocampal function. Knockout of p75^{NTR} in a mouse model led to improved spatial learning and increased hippocampal ChAT activity. BFCNs also display increases in size and number which, in addition to the increased ACh synthesis, potentiates cholinergic signalling (Barrett et al., 2010).

Similarly to nAChRs (Bruses et al., 2001), cell membrane composition may exert a regulatory effect on p75^{NTR} through interaction with the lipid microdomain protein, cavelolin (Bilderback et al., 1999). Stimulation of p75^{NTR} by NGF or the AD-linked Aβ peptide leads to increased intracellular ceramide, which can inhibit the pro-survival Ras/ERK and PI3K pathways (Brann et al., 2002). Furthermore, several proteins which bind the intracellular region of p75^{NTR} have been linked to apoptosis (Blochl and Blochl, 2007, Arevalo et al., 2009). These signalling pathways appear to converge on activation of JNK, p53 and caspases (Blochl and Blochl, 2007). Kainic acid has been shown to increase p75^{NTR} expression and data show up to 70% BFCN cell death 5 days after kainic acid injection (Dechant and Barde, 2002).

1.2   The cholinesterases

1.2.1   Acetylcholinesterase (AChE)

The principal function of AChE is as a hydrolytic enzyme, displaying a prototypical α/β hydrolase fold in its protein structure (Ollis et al., 1992, Massoulie et al., 1993), with a twelve-stranded β-sheet surrounded by fourteen α-helices (Dvir et al., 2010). AChE is principally located in the synaptic cleft and at the neuromuscular junction where it cleaves ACh to generate acetate and choline (Fig. 1.2) with such efficiency that hydrolysis of ACh is limited only by the rate of diffusion (Quinn, 1987, Colletier et al., 2006). It hydrolyses a range of substrates over a wide range of magnitudes, with a k_{cat} for ACh in excess of $10^8 M^{-1} s^{-1}$, but a k_{cat} of $4 \times 10^3 M^{-1} s^{-1}$ for the amide analogue

acetylazacholine (Quinn, 1987). The precise catalytic mechanism for AChE has previously been reviewed in some detail (Taylor and Radic, 1994).

1.2.1.1 Enzyme topology and the active site

The active site of AChE follows a fixed consensus topology of its catalytic triad, specifically a nucleophile (Ser, Asp, Cys), His and an acidic residue (Asp, Glu) (Massoulie et al., 1993), which, in *Torpedo* is typically represented by Ser200, His400 and Glu327 (Colletier et al., 2006, Dvir et al., 2010). It was subsequently shown that it is the active site Ser that reacts with organophosphate agents (MacPhee-Quigley et al., 1985). The active site residues appear to be redundant for synthesis and trafficking, as mutation of all active site residues did not impair secretion or protein immunoreactivity (Shafferman et al., 1992). The active site is located at the bottom of a deep, narrow 20Å gorge lined with aromatic residues (Silman and Sussman, 2008, Dvir et al., 2010). That it appears too narrow for substrate entry is indicative of a degree of conformational flexibility (Harel et al., 1992, Massoulie et al., 2008). There are two substrate binding sites, the acylation (A) site, at the bottom of the gorge, and the peripheral (P) site, at the top. As established by site-directed mutagenesis and inhibitor binding studies, the three main residues in the peripheral anionic site (PAS) are Trp279, Tyr70 and Asp72 (Colletier et al., 2006, Silman and Sussman, 2008). Mechanistically, ACh transiently binds the PAS as the first stage in the catalytic process (Dvir et al., 2010). Ligands at the A site inhibit all catalytic activity, whereas binders at the P site such as fasciculin, propidium and thioflavin T (ThT) only interfere with hydrolysis of some substrates (Auletta et al., 2010). There are a number of active-site targeting AChE inhibitors, some of which, like donepezil and rivastigmine, are in clinical use as AD treatments (Azevedo Marques et al., 2011, Greig et al., 2013).

1.2.1.2 Transcription and alternative splicing

AChE is transcribed from a 6kb gene with multiple transcription initiation sites (Massoulie et al., 1993, Bronicki and Jasmin, 2012). The promoter contains a number of regulatory elements, including binding sites for Sp-1, Egr-1 and AP2 TFs (Getman et al., 1995, Nitsch et al., 1998), in addition to several heat shock elements, resulting in the induction of AChE transcription after heat shock (Chen et al., 2010b). Additionally, AChE expression has been suggested to be regulated by differentiation (Li et al., 1993).

Upwards of 90% of human genes undergo alternative splicing and AChE is no exception (Fig. 1.4). Alternative splicing of a single AChE gene in the 5` region leads to the generation of isoforms with tissue-specific expression patterns in brain, muscle and erythropoietic tissue (Li et al., 1993, Taylor et al., 1993). The brain isoform, for example, was shown to use a more upstream transcriptional start site (Li et al., 1993). Alternative transcript variants have been identified in exon 1, of which there are four in humans, denoted hE1a-d. These variations in the 5` regulatory region of AChE generated different 5` UTRs, the function of which is currently unclear. However, human exon 1d (hE1d) (and mE1e in mouse) has been shown to encode N-terminally extended AChE variants (Meshorer et al., 2004, Bronicki and Jasmin, 2012), which have been linked to apoptosis (Toiber et al., 2009, Bronicki and Jasmin, 2012), such as that induced by hyperglycaemia in retinoblastoma cells (Masha'our et al., 2012).

Figure 1.4: generation of multiple mRNA species from a single gene
AChE is present as a single gene in mammals, but multiple AChE isoforms exist. These species, denoted R, H and T, are generated by alternative splicing at the mRNA level.
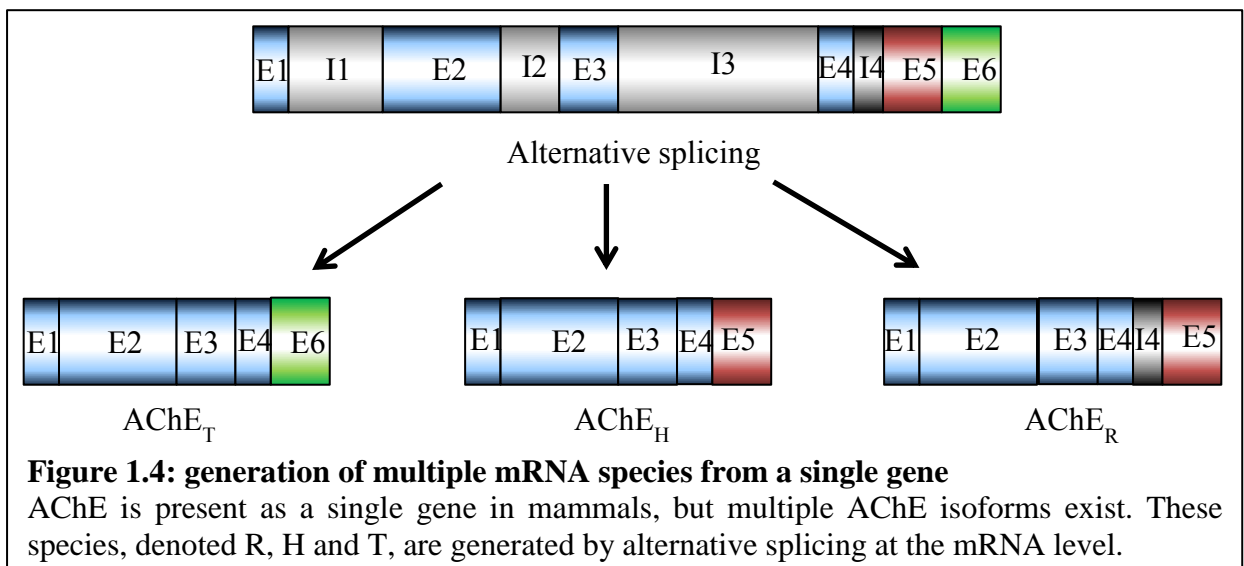
AChE pre-mRNA is also subjected to alternative splicing at the 3` end. This leads to the generation of three transcripts, encoding readthrough ($AChE_R$), hydrophobic ($AChE_H$) and synaptic ($AChE_T$). $AChE_H$ exists as glycosylphosphatidylinositol (GPI)-anchored dimers, localised to erythrocytes (Massoulie et al., 2005). In neurons though, alternative splicing produces either $AChE_T$ or $AChE_R$, depending on whether the 5` donor site downstream of E4 splices to the acceptor site upstream of E5 or E6. The synaptic $AChE_T$ is generated by splicing to the distal E6 splice site, incorporating E6 into the mRNA (Massoulie et al., 1993, Bronicki and Jasmin, 2012). Little is known about this process, but the identity of the splicing factors may play a role in pathway determination, with SC35 apparently favouring $AChE_R$ and ASF/ SF2 favouring $AChE_T$ (Meshorer et al., 2004). However, although $AChE_T$ usually predominates, cell stress

promotes upregulation of $AChE_R$ (Soreq and Seidman, 2001, Shaked et al., 2008). Further downstream from this splicing point, the 3` UTR is involved in mRNA stability as the RNA-binding protein HuD/ nELAV4 binds AU-rich elements and enhances AChE mRNA stability (Deschenes-Furry et al., 2003).

1.2.1.3 Protein synthesis and posttranslational modification

AChE is synthesised at the level of the rough endoplasmic reticulum (RER), after which it is translocated into the ER lumen for signal peptide cleavage. Although first synthesised as inactive precursors, the enzyme acquires activity in the ER. In this key organelle, there is evidence for involvement of chaperones such as BiP in the folding of AChE molecules. Furthermore, glycans are added in the ER and subsequently re-modelled as the enzyme progresses to the Golgi apparatus (Massoulie et al., 1993). AChE has three N-glycosylation sites and contains approximately 10-15% carbohydrate. Correct glycosylation appears to have a major impact on its trafficking as mutation of Asn to Gln reduced secretion of AChE by 80% (one site mutated) or 99% (all sites) (Velan et al., 1993). Non-glycosylated recombinant AChE was also shown to be cleared from the circulation more quickly, implicating the role glycosylation in protein in stability (Mendelson et al., 1998). AChE has been shown to be a protein for which posttranslational modifications are of great importance (Nalivaeva and Turner, 2001). There have been reports of an inactive pool of AChE, which remains intracellular and EndoH sensitive, i.e. glycan-free (Fernandez et al., 1996, Massoulie et al., 2008). It has been suggested that this catalytically inactive pool may be involved in mediating non-hydrolytic functions of AChE (Massoulie et al., 2008). Active AChE, however, is trafficked to the cell surface and the blockade of this process by colchicine treatment indicates microtubule involvement (Lucas and Kreutzberg, 1985). Once at the plasma membrane, the half-life of AChE residence there has been indicated to be 50 hours (Rotundo and Fambrough, 1980).

In a typical neuron, AChE staining reveals localisation to the RER, Golgi apparatus, nuclear envelope and also the plasma membrane of soma and dendrites (Henderson, 1989). Recently, a novel AChE species has been identified, a 55kDa protein with nuclear localisation (Santos et al., 2007), the upregulation of which has been linked to apoptosis (Xie et al., 2011). However, it has previously been demonstrated that AChE

displays marked disparities between localisation patterns in neuronal and non-neuronal cells (Thullbery et al., 2005)

1.2.1.4 The t peptide
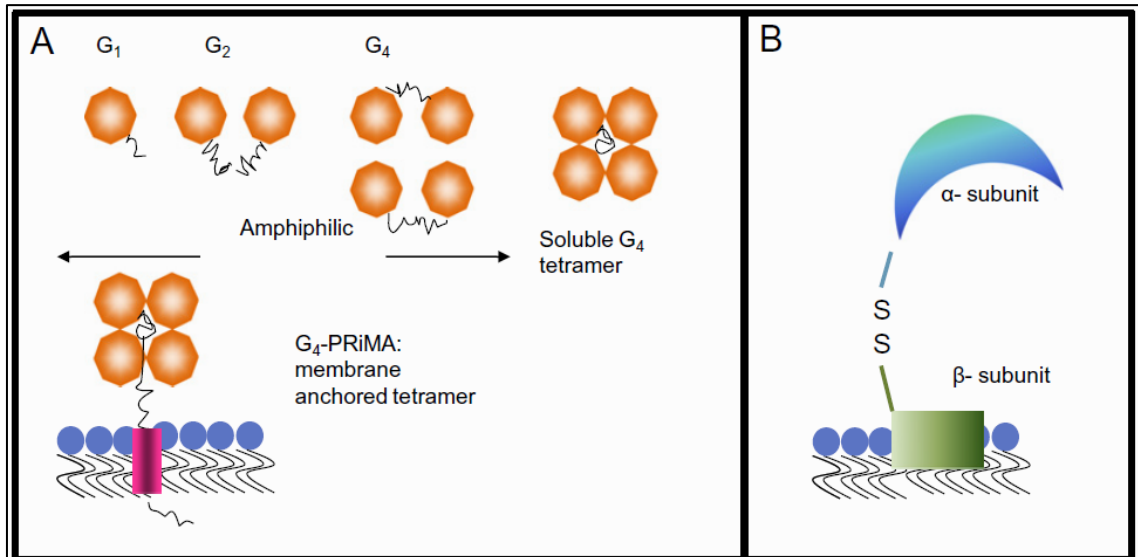
Figure 1.5: topology of membrane-bound AChE and TSH receptor
A. AChE and BChE are very similar with regard to subunit assembly, this figure being representative of the process in the nervous system. The G_4 tetramer is assembled starting with the G_1 monomer, which dimerises to form G_2. The G_2 dimer then itself homo-dimerises to form the G_4 tetramer, which can be either soluble or membrane bound via PRiMA. **B.** The thyroid stimulating hormone receptor (TSHR) has a similar topology. The membrane bound TSHR β- subunit is analogous to PRiMA, while the membrane anchored α- subunit, able to be shed, is equivalent to AChE. Furthermore, the disulphide bonds between the two components are similar in both systems. Taken from Hicks *et al.* (2013).

The AChE t peptide is present at the C-terminus of $AChE_T$, a region of importance in posttranslational modification (Coussen et al., 2001, Nalivaeva and Turner, 2001). It can induce intracellular degradation of AChE, a process blocked when the t peptide is occluded, such as when it is bound to PRiMA, its proline rich membrane anchor. The degradation of unbound AChE occurs via ER-associated degradation (ERAD) and can be blocked by proteasome inhibition (Falasca et al., 2005). The t peptide has been noted to share sequence similarity with the ER retention motif, KDEL (CSDL in AChE). This would ensure retention of AChE subunits at the ER until they are PRiMA associated. Supporting this, mutants containing only the catalytic site are processed and trafficked much more quickly than wild-type AChE (Falasca et al., 2005). The t peptide also plays

a key role in the assembly of higher order AChE species. AChE exists as a monomer (G_1), dimer (G_2) and tetramer (G_4) and it is $AChE_T$ tetramers that are localised to synapses (Inestrosa and Perelman, 1989, Fernandez et al., 1996, Dvir et al., 2010), as two pairs of linked dimers (Gennari et al., 1987). A key domain in the $AChE_T$ C-terminus is the proline-rich attachment domain (PRAD), which is the region through which attachment to PRiMA is mediated (Massoulie et al., 2008). Subunits are linked together by C-terminal disulphide linkages and another disulphide bond links the tetramer to its PRiMA (Vigny et al., 1979, Roberts et al., 1991, Perrier et al., 2002).

1.2.1.5 Non-catalytic functions

Recognition of non-catalytic functions of AChE has existed for some time (Greenfield et al., 1988, Soreq and Seidman, 2001, Massoulie et al., 2008). Such roles were posited given the multiplicity of AChE transcripts from a single gene and its pre-synaptogenesis expression pattern, being present in areas lacking ACh or ChAT (Robertson et al., 1988, Brimijoin and Hammond, 1996, Grisaru et al., 1999). Combined with high ChE expression in the embryonic nervous system (Small et al., 1996), this was considered to be indicative of a developmental role (Massoulie et al., 1993, Falugi and Aluigi, 2012). One example of this is that AChE is found in *Drosophila* photoreceptor cells before any functional neuronal connections are present (Wolfgang and Forte, 1989). Further developmental roles ascribed to AChE include neurite elongation and adherence and also synaptogenesis (Paraoanu and Layer, 2008, Bronicki and Jasmin, 2012, Falugi and Aluigi, 2012). To support this, AChE was shown to increase neurite outgrowth in cultured neurons, with this effect dependent on Ca^{2+} influx through LTCCs. This phenomenon is sensitive to PAS blockers, but not agents targeting the active site (Grisaru et al., 1999, Soreq and Seidman, 2001, Day and Greenfield, 2002, Sperling et al., 2012). Two hypotheses were suggested to explain the neurite outgrowth mediated by AChE. The first involved initiation of intracellular signalling cascades, while the second focused on the role of AChE as an adhesion protein. The hypothesis was that AChE, through its adhesion properties, would facilitate adhesion of other proteins involved in neurite outgrowth, such as laminin-1 (Halliday and Greenfield, 2011, Sperling et al., 2012).

However, despite these roles in neurite outgrowth and that in ACh hydrolysis, AChE^{-/-} mice, though compromised, are viable (Xie et al., 2000, Camp et al., 2010). It is thought

that the phenotype of AChE$^{-/-}$ mice is ameliorated by regulation of ACh levels by BChE (Li et al., 2000, Hartmann et al., 2007). There are significant morphological perturbations in synapses in AChE$^{-/-}$ mice, such as fragmented synaptic termini (Adler et al., 2011). Similarly, mutation of the *Drosophila* AChE orthologue, Ace, results in reduced dendritic branch length and synaptic termini. This was phenocopied by chronic administration of AChE inhibitors, suggesting that genetic or pharmacological targeting of Ace can result in morphological synaptic alterations (Kim et al., 2011).

1.2.1.6 Apoptosis

As mentioned previously, AChE has been linked to apoptosis (Soreq et al., 1994, Zhang et al., 2002, Zhang and Greenberg, 2012) and AChE infusion into rat brain mediated a range of deleterious processes, including astrocyte hypertrophy, neuronal loss and behavioural defects (Chacon et al., 2003). Furthermore, antisense oligonucleotides targeting AChE increased cell count and cell proliferation, while suppressing apoptosis-induced DNA damage (Jiang and Zhang, 2008). Recent data have also linked AChE to apoptosis via induction of Ca^{2+} influx (Day and Greenfield, 2003, Zhu et al., 2007a). Increases in intracellular Ca^{2+} can occur during apoptosis and this can increase AChE mRNA stability. In addition, AChE promoter activity is responsive to Ca^{2+} dependent proteins such as calpain and calcineurin and also nuclear factor of activated T-cells (NFAT) (Zhu et al., 2007b, Jiang and Zhang, 2008). Pro-apoptotic signalling molecule JNK has also been linked to AChE, as both proteins were suggested to be key agents in apoptosis induced by H_2O_2 (Zhang et al., 2008, Xie et al., 2011). Data have shown that AChE$_T$ is capable of mediating cell death, while AChE$_R$ is a positive regulator of cell proliferation (Jiang and Zhang, 2008). Also, targeting of AChE may have an anti-inflammatory effect, as a microRNA (Ambros, 2004), miR132, has been stated to reduce inflammation by repressing AChE (Shaked et al., 2009).

Short peptides derived from the C-terminus of AChE (T14 and T30) have been shown to displace α-Bgt from receptors and bind α7 nAChRs with pM to nM affinity. In this way, AChE C-terminal peptides were shown to be able to induce expression of α7 nAChRs (Bond et al., 2009). One outcome of this and the consequent increased Ca^{2+} flux was activation of NMDA receptors and LTCCs, which in turn caused CaMKII activation, ROS generation and caspase activation (Day and Greenfield, 2003). To reconcile these emerging disparities in the function of AChE, Bond *et al.* suggested that

the effects of AChE may be concentration dependent. In their study, although T14 had deleterious effects at higher concentrations, at lower concentrations, they saw a promotion of neurite outgrowth (Greenfield et al., 2008, Bond et al., 2009). It was suggested subsequently that this binding event may represent an ACh-independent signalling pathway between AChE and α7 nAChR (Halliday and Greenfield, 2011).

During AChE mediated apoptosis, a change in enzyme localisation is observed, moving towards and into the nucleus. As AChE does not have a recognised nuclear localisation signal, this may be indicative of further interactions with proteins capable of nuclear trafficking (Santos et al., 2007, Halliday and Greenfield, 2011).

1.2.1.7 Cellular release of AChE

The earliest observations of cellular AChE release saw this phenomenon in adrenal gland (Chubb and Smith, 1975) and AChE was also discovered in secretory vesicles (Gratzl et al., 1981) but subsequently AChE was shown to be spontaneously released from hippocampus, cerebellum (Appleyard, 1987, Appleyard et al., 1988), caudate nuclei and substantia nigra (Greenfield et al., 1983, Llinas and Greenfield, 1987). In addition, this release was potentiated by the mAChR agonist, carbachol and blocked by atropine (Appleyard, 1987, Nalivaeva and Turner, 1999). It has been suggested that secreted AChE derives from an intracellular pool, representing about 60-70% of total cellular AChE, of which 30% is secreted per hour (Rotundo and Fambrough, 1980).

The mechanisms behind AChE release await comprehensive elucidation, although several authors have suggested a multiplicity of pathways involved, notably Schweitzer, who suggested a constitutive and a regulated pathway, the latter being Ca^{2+}-dependent pathway (Schweitzer, 1993). This involvement of Ca^{2+} is suggestive of an exocytic pathway, as Ca^{2+} plays a critical role in exocytosis, as mentioned previously for neurotransmitter release (Morgan et al., 2005). Data also indicated a possible proteolytic event (Stieger and Brodbeck, 1985, Bon and Massoulie, 1997) and a reduction in AChE release after treatment with metalloprotease inhibitors further confirmed that proteolysis may be involved in at least one of these pathways (Nalivaeva and Turner, 1999). However, this paper made reference to the amyloid precursor protein (APP) as a possible analogous release process (Nalivaeva and Turner, 1999). APP is a transmembrane protein (Weidemann et al., 1989) and indeed proteolytic cleavage of

transmembrane proteins is widely seen (Hooper et al., 1997). However, AChE does not fit the paradigm of a transmembrane protein, as it is attached to the membrane by an entirely separate protein, PRiMA (Perrier et al., 2002). One example of a similar system is the human thyrotropin receptor, which has two subunits, one membrane spanning and the other anchored to it via disulphide bonds (Couet et al., 1996a) and disulphide bonds also link AChE to PRiMA (Fig. 1.5) (Perrier et al., 2002, Dobbertin et al., 2009). Couet *et al.* were able to show that the release of the thyrotropin receptor ectodomain was dependent on the combined actions of a metalloprotease and protein disulphide isomerase (Couet et al., 1996a, Couet et al., 1996b).

1.2.2 Butyrylcholinesterase (BChE)

1.2.2.1 Structure and sequence

BChE, like AChE, can catalyse the hydrolysis of a wide variety of choline and non-choline esters (Darvesh et al., 2003, Giacobini, 2003). The catalytic form of BChE, like AChE, is a tetramer. The tetramerisation process is critically dependent on the C-terminal 50 amino acids in BChE, which, like AChE, form a four-helix bundle (Blong et al., 1997). The two enzymes also have sequence (50-55%) and structural similarity, as BChE has a central β-sheet, surrounded by α-helices. However, given the architecture of their respective active sites, BChE has different reactivity with substrates and inhibitors (Radic et al., 1993). Interestingly, mutation of Phe295 and Phe297 in AChE can increase BTCh hydrolysis, while reducing hydrolysis of ATCh, implicating these residues in determining substrate specificity (Vellom et al., 1993). BChE is able to hydrolyse larger substrates, such as BCh *in vitro* and accommodate larger inhibitors such as iso-OMPA (Saxena et al., 1997b, Xu et al., 2008). BChE is more heavily glycosylated than AChE, having nine N-glycosylation sites, to three in AChE (Saxena et al., 1997a). There is significant sequence similarity in the C-terminal region and the t peptides of the respective enzymes are very similar. Given the presence of the t peptide in BChE, it is able to associate with PRiMA and it is these PRiMA-linked BChE tetramers that predominate (Saez-Valero et al., 1993, Johnson and Moore, 2012).

1.2.2.2 Transcription and expression pattern

There are notable differences in expression patterns between AChE and BChE. As indicated, AChE derives from a single gene, but there are multiple splice variants

(Massoulie et al., 1993). However, BChE, although encoded by one gene, is only present in one isoform, $BChE_T$. AChE is mostly expressed at synapses and neuromuscular junctions, yet BChE is expressed in liver, lungs, heart, intestinal muscosa, brain and plasma, the latter being notably low in AChE (Brimijoin and Hammond, 1988, Jbilo et al., 1994). In brain, the expression of AChE is predominantly neuronal, while the majority of BChE is localised to glial cells, although it is expressed in specific neuronal populations, such as in hippocampus, thalamus and amygdala (Darvesh et al., 1998, Darvesh and Hopkins, 2003). Immunohistochemical staining has revealed BChE to strongly co-localise with ChAT and in specific, BChE rich neuronal sub-populations, ACh levels are shown to be under the control of BChE (Darvesh et al., 1998, Johnson and Moore, 2012).

1.2.2.3 Function

The function of BChE is somewhat unclear, as BCh is not present in mammalian nervous systems and deficient individuals are mostly asymptomatic (Li et al., 2008, Johnson and Moore, 2012). It has been suggested to have a scavenging role in detoxification (Massoulie et al., 1993), a notion supported by the role of BChE in the breakdown of the anaesthetic suxamethonium (Jensen and Viby-Mogensen, 1995, Darvesh et al., 2003) and also cocaine, acetylsalicylate and heroin (Darvesh et al., 2003). A further function of BChE may be through a compensatory mechanism, whereby it can cleave ACh in the absence of AChE (Mesulam et al., 2002, Hartmann et al., 2007) and a counter-regulation between the two ChEs has been reported, with increased AChE as a response to BChE knockdown (Bodur and Layer, 2011).

The viability of the $AChE^{-/-}$ mouse has been previously reported (Li et al., 2000, Xie et al., 2000, Camp et al., 2010) and has been proposed that the compensatory action of BChE is critical (Li et al., 2000, Lockridge et al., 2003), as a double AChE/ BChE knockout is lethal. However, in model systems lacking BChE, such as *Danio rerio*, AChE mutatations are lethal (Darvesh et al., 2003, Johnson and Moore, 2012). However, in the case of $BChE^{-/-}$ models, no severe phenotype is seen and there are no deleterious effects on neurite outgrowth as is seen to be the case with $AChE^{-/-}$ mice (Li et al., 2006, Duysen et al., 2007, Johnson and Moore, 2012).

1.2.3 The proline rich membrane anchor (PRiMA)

1.2.3.1 Attachment

AChE has long been known to have a membrane anchor, initially referred to as the P subunit (Gennari and Brodbeck, 1985, Gennari et al., 1987, Inestrosa et al., 1987), but later identified as PRiMA in neurons, a type I integral membrane protein (Perrier et al., 2002) and ColQ at the neuromuscular junction (Noureddine et al., 2008). Similarly to AChE, the PRiMA gene can be alternatively spliced, generating PRiMA I and II, with the former being most functionally important (Noureddine et al., 2007, Leung et al., 2009). Its expression is exclusively cholinergic, co-localising with AChE in BFCNs, while being absent from neighbouring GABAergic neurons and substantia nigra dopaminergic neurons, which express high levels of AChE (Henderson et al., 2010). As with AChE, BChE can associate with PRiMA (Saez-Valero et al., 1993), in part due to the high sequence similarity between AChE and BChE in the t peptide. The ChE-PRiMA interaction is mediated between the tryptophan amphiphilic tetramerisation (WAT) domain in the AChE t peptide and a PRAD in PRiMA. The WAT domain is encoded by exon 6 and is therefore only present in $AChE_T$ (Dobbertin et al., 2009) and $AChE_T\Delta E5$, E6 mutants are catalytically active, but are unable to associate with PRiMA (Dobbertin et al., 2009). Four α-helical t-peptides form a coiled coil structure around the PRAD, with disulphide bonds stabilising this quaternary interaction and forming a four helix bundle (Heider and Brodbeck, 1992, Liao et al., 1993, Simon et al., 1998, Dvir et al., 2004).

1.2.3.2 Trafficking and regulation

The t peptide has been demonstrated to be critical for tetramerisation as AChEΔt mutants only form monomers (Chen et al., 2011b). In addition, PRiMA expression is also vital for tetramerisation as, in its absence, mostly dimers are formed (Dobbertin et al., 2009, Chen et al., 2010a). In addition to the essential role of PRiMA in AChE trafficking to the cell surface (Chen et al., 2011b), the structure of PRiMA, and specifically its fatty acid acylation, is suggestive of a role in localising AChE to lipid rafts (Xie et al., 2009, Xie et al., 2010b). PRiMA also has a CRAC cholesterol binding domain, which may be vital in targeting AChE to the cholesterol-rich lipid rafts (Xie et al., 2010b).

Although AChE and BChE are heavily glycosylated, the elimination of the glycosylation sites does not affect the tetramerisation process. This may be because none of the glycosylation sites are localised near the t peptide. However, these mutations severely affect trafficking as the PRiMA-linked G_4 tetramers are retained in the ER (Choi et al., 2008, Chen et al., 2011a, Chen et al., 2011b). It has been suggested that there is a parallel regulation of AChE and PRiMA by cAMP, as the cAMP signalling cascade induces expression of AChE and PRiMA transcripts, leading to increased abundance of PRiMA-linked G_4 AChE (Choi et al., 2008). Similarly, signalling through MAPK and Raf has been strongly implicated in the regulation of PRiMA expression (Xie et al., 2009).

1.2.3.3 The PRiMA knockout mouse

Although PRiMA has a key role in directing AChE oligomerisation and trafficking (Chen et al., 2011b), the CNS is able to adapt to its absence. PRiMA knockout mice are indistinguishable from wild type mice in terms of weight, body temperature and ventilation, while displaying only a mild behavioural phenotype. In the mutant mice, AChE is mainly retained in neurons and hence extracellular ACh levels are very high. To accommodate this, a global CNS reduction in mAChRs is seen, although not nAChRs (Farar et al., 2012), in addition to increases in BChE (Farar et al., 2013).

1.3 The amyloid precursor protein (APP)

1.3.1 Topology

The amyloid precursor protein (APP) is a type I integral membrane protein (Weidemann et al., 1989). It has three isoforms (APP_{695}, APP_{751} and APP_{770}), generated by differential splicing of exon 7 and 8 (Tanzi et al., 1987, Sandbrink et al., 1996), a process altered by aging (Sandbrink et al., 1994, Beyreuther et al., 1996). Exon 7 is homologous to protease inhibitors of the Kunitz type (KPI domain), while exon 8 is related to the MRC OX-2 antigen in thymocytes (Kitaguchi et al., 1988, Sandbrink et al., 1996).

Figure 1.6: schematic representation of APP$_{695}$
The amyloid precursor protein is a multi-domain protein, with key domains growth factor like domain (GFLD), copper-binding domain (CuBD), E1, E2, Aβ and AICD indicated. The YENTPY motif in the C-terminal region encompasses residues 682-687 in APP$_{695}$, which are crucial in interaction of APP with intracellular binding partners. The full length protein can be cleaved either sequentially by α- and γ-secretases, at the sites indicated, yielding the sAPPα fragment and AICD. Alternatively, APP can be cleaved sequentially by β- and γ-secretases, yielding sAPPβ, Aβ and functional AICD.

APP$_{695}$ lacks both KPI and OX-2 domains, while APP$_{751}$ only lacks the OX-2 domain (Henriques et al., 2007). Regulatory mechanisms specific to each isoform have been suggested (Henriques et al., 2007). In terms of distribution, APP mRNA is expressed in almost every tissue, where only the isoform ratio differs (Araki et al., 1991). It is APP$_{695}$ that predominates in neurons (Gralle and Ferreira, 2007) and APP mRNA represents 0.2% of the total mRNA in these cells (Beyreuther et al., 1996). Although not the focus

of this work, APP has been ascribed a number of functions in non-neuronal tissues, such as keratinocytes (Nalivaeva and Turner, 2013, Puig and Combs, 2013).

APP is a multi-domain protein (Fig. 1.6) and a number of these domains have key functional roles. In the N-terminal region is the E1 domain, containing a growth factor-like domain (GFLD) and copper-binding domain (CuBD), to which a number of interacting proteins bind (Barnham et al., 2003, Kong et al., 2008, Rice et al., 2013), such as integrins (Young-Pearse et al., 2008). The more C-terminal regions of the ectodomain include an acidic rich domain, the Kunitz Protease Inhibitor (KPI) domain (APP751 and APP770), OX-2 domain (APP770) (Aydin et al., 2012) and the most C-terminal region of the holoprotein is referred to as the amyloid precursor protein intracellular domain (AICD), through which a number of signalling pathways and transcriptional regulatory events are mediated (Leissring et al., 2002, Chang and Suh, 2010, Beckett et al., 2012, Pardossi-Piquard and Checler, 2012). Its protease inhibitory role aside, the function of the KPI insert is unclear, although it has been shown to be essential for the ability of APP to act as a mitogen for 3T3 cells in culture (Schubert et al., 1989).

1.3.2 Regulation of expression

APP expression is known to be regulated by a number of cytokines, such as interleukin-1 (IL-1) (Goldgaber et al., 1989), but also trophic factors, such as NGF and fibroblast growth factor (FGF) (Wion et al., 1988). At a transcriptional level, binding sites for a number of TFs have been found, Sp1 and AP-1 among them (Beyreuther et al., 1996). More recently, levels of APP transcription have been shown to be regulated by signalling through AICD (von Rotz et al., 2004) and NF-κB (Chami et al., 2012).

1.3.3 Proteolysis

There are two proteolytic pathways of APP processing, though they operate differently in neuronal and non-neuronal tissues (Delvaux et al., 2012). In neurons, amyloidogenic processing is the minor pathway and involves sequential cleavage by β-secretase, BACE-1, and the γ- secretase complex, both aspartic proteases (Anderson et al., 1992, Vassar et al., 1999, Bergmans and De Strooper, 2010). This ultimately releases Aβ, responsible in large part for the pathogenesis of AD (Hardy and Higgins, 1992), although it does have physiological roles (Pearson and Peers, 2006, Arevalo et al., 2009,

Chasseigneaux and Allinquant, 2011). The second, non-amyloidogenic, pathway involves α-secretase cleavage of APP. This cleavage occurs between Lys16 and Leu17, within the Aβ region (Esch et al., 1990, Allinson et al., 2003). This precludes deposition of Aβ. There is also the release of the large, soluble ectodomain, the neuroprotective sAPPα, which is shed from the cell surface (Allinson et al., 2004).

Furthermore, sAPPα has been detected not only in the brain, but in the CSF (Palmert et al., 1989). Both pathways result in generation of AICD, which can act as a transcriptional regulator (Cao and Sudhof, 2001, Leissring et al., 2002, Chang and Suh, 2010, Schettini et al., 2010), although there remains some controversy (Hebert et al., 2006). This release of a transcriptionally active intracellular domain is analogous to the processing of Notch and steroid response element binding protein (SREBP), a process called regulated intramembrane proteolysis (RIP) (Schroeter et al., 1998, De Strooper et al., 1999, Brown et al., 2000).
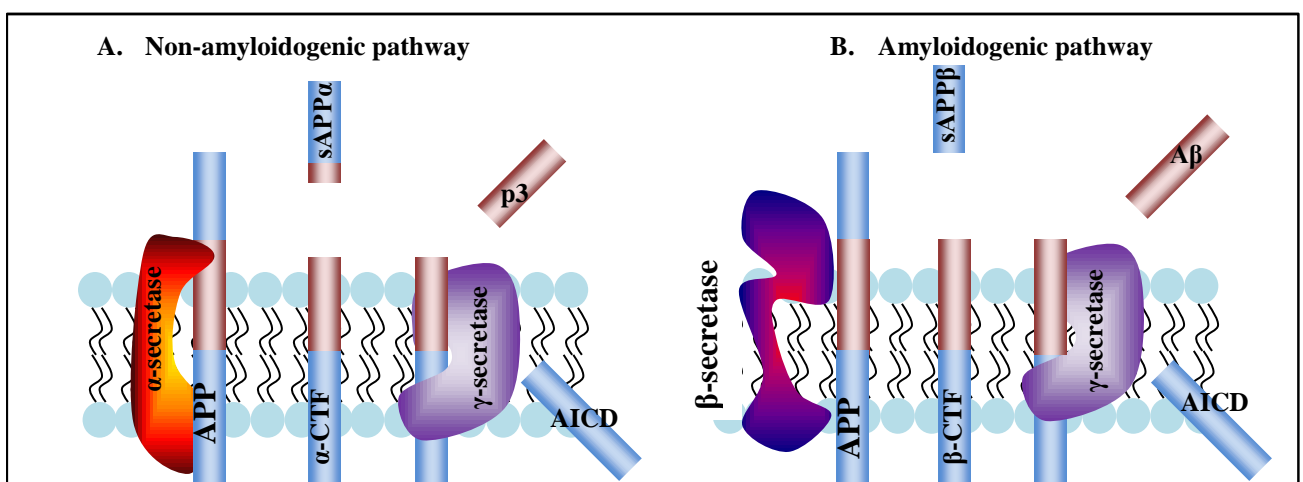
Figure 1.7: schematic of APP processing

A. In the non-amyloidogenic pathway, the α-secretase complex cleaves APP between Lys16 and Leu17. This is believed to involve the ADAMs family of enzymes, specifically ADAM9, ADAM10 and ADAM17. As a consequence, sAPPα is released into the extracellular milieu. The membrane-bound α-CTF stub is then cleaved by the γ-secretase complex (presenilins, nicastrin, Aph-1 and Pen-2). This cleavage releases a small p3 fragment outside the cell and the intracellular domain (AICD) is released into the cytosol, where it has been reported to act as a transcriptional regulator. In the figure, the Aβ region of APP is in red and α-secretase cleavage occurs within this region, hence Aβ cannot be produced in this pathway.

B. In the amyloidogenic pathway, APP is instead cleaved by the β-secretase complex, which releases sAPPβ. As with non-amyloidogenic cleavage, the membrane bound stub (here β-CTF) is cleaved by the γ-secretase complex, which again releases AICD into the cytosol. However, the most significant consequence of this pathway is the extracellular release of Aβ.

In the physiological situation, Aβ can be degraded by a wide range of amyloid degrading enzymes, such as neprilysin (NEP) and insulin degrading enzyme (IDE) (Carson and Turner, 2002, Turner et al., 2004), both shown to clear brain amyloid *in vivo* (Liu et al., 2011). As perturbation of amyloid degradation is a possible route to pathology, manipulation of these enzymes has been suggested as a possible therapeutic avenue (Nalivaeva et al., 2008, Nalivaeva et al., 2012).

1.3.3.1 Regulation of processing

APP processing has been shown to be regulated by a wide variety of receptors, such as 5HT and the epidermal growth factor receptor (EGFR) and also biomolecules, including bradykinin, steroid hormones and glutamate (Racchi and Govoni, 2003). It has also been shown that signalling through mAChRs can enhance non-amyloidogenic processing, increasing release of sAPPα (Nitsch et al., 1992, Farber et al., 1995). This process is dependent on PKCα and ERK, but not PLC (Canet-Aviles et al., 2002). This may explain the ability of AChE inhibitors to positively regulate non-amyloidogenic processing of APP (Racchi et al., 2001, Peng et al., 2007). These inhibitors cause increased ACh levels, which may activate mAChRs. Conversely, signalling through NF-κB activates transcription of BACE-1 and γ-secretase components, consequently promoting amyloidogenic processing (Chami et al., 2012). AICD is also able to upregulate BACE-1 expression, exhibiting a positive feedback effect, as AICD is generated via BACE-1 cleavage (Chang and Suh, 2010).

1.3.3.2 The secretases

α-, β- and γ- secretases were terms devised to represent an activity that was known to exist, but not which enzymes performed the cleavage. At the time, the secretases were agents known to proteolytically cleave APP, but specific detail was elusive. It was later found that BACE-1 was responsible for β-secretase activity (Vassar et al., 1999) and that a complex of presenilin, nicastrin, Aph-1 and Pen-2 had γ- secretase activity (Lichtenthaler et al., 1999, Yu et al., 2000). The α- secretase cleavage was found to be mediated by zinc metalloproteases of the a disintegrin and metalloprotease (ADAM) family (Lammich et al., 1999, Lichtenthaler, 2010). ADAM10 appears to be the constitutive α-secretase in primary neurons, with RNAi knockdown of ADAMs 9 and 17 having no effect on sAPPα release. However, ADAM17 acts as a regulated sheddase, cleaving APP after agonist treatment. For example, it was shown that PMA stimulation

of ADAM17 increased sAPPα release (Kuhn et al., 2010). Further investigation has implicated ADAM9 as a regulator of ADAM10 and not directly involved in APP processing as ADAM9 was shown to cleave the ADAM10 CTF, which accumulated in knockout mice (Cisse et al., 2005, Tousseyn et al., 2009). This finding was recapitulated for cleavage of the prion protein (PrP) as ADAM9 was unable to cleave the protein without co-expression of ADAM10 (Taylor et al., 2009). The ADAMs have a wide number of substrates, including angiotensin-converting enzyme (ACE) (Ehlers et al., 1991, Allinson et al., 2004), ACE2 (Lambert et al., 2005) and each other (Parkin and Harris, 2009). The ADAMs have been reviewed in greater detail elsewhere, but it is of note that different cell lines express different combinations of ADAMs (Edwards et al., 2008, Gooz, 2010, Gough et al., 2011). Hence the shedding enzyme for a particular protein may, in some cases, be dependent on cell type (Allinson et al., 2003, Edwards et al., 2008).

It has been suggested that the amyloidogenic pathway is intracellular (Chyung et al., 1997), dependent on endocytosis of APP and hence its YENTPY internalisation motif. In light of this, Tyr682 has been suggested to have a key role in Aβ formation (Barbagallo et al., 2010). The site of Aβ production has been suggested to be the trans-Golgi network (TGN), with APP being trafficked from the endosomes to the TGN (Choy et al., 2012)

Although this represents the canonical APP processing sequence, recent work has implicated other enzymes in APP cleavage. These enzymes, such as cathepsin B (Asai et al., 2011) or meprin (Jefferson et al., 2011), likely represent minor processing pathways.

1.3.4 Trafficking and localisation

Synthesised in the ER, APP is subsequently trafficked to the plasma membrane via the Golgi apparatus, where significant posttranslational modifications occur. Modification is via O- and N-glycosylation, tyrosine sulphation and phosphorylation. In fact, only an estimated 10% of APP molecules reach the cell surface, most being localised to the Golgi apparatus and TGN (Thinakaran and Koo, 2008, Haass et al., 2012). At the cell surface, APP may be shed by α-secretase or it will undergo YENTPY-dependent

endocytosis and delivery to endosomes (Lai et al., 1995). The fate of APP at this point is either a recycling to the cell surface, or lysosomal degradation (Haass et al., 1992).

In terms of membrane localisation, APP has been suggested, in part, to be localised to lipid rafts (Parkin et al., 1999). It has recently been suggested that this is mediated by the ability of the APP transmembrane domain to bind cholesterol, which is enriched in lipid rafts (Barrett et al., 2012). It has been suggested that this lipid rafts localisation promotes amyloidogenic processing (Cordy et al., 2003, Ehehalt et al., 2003, Vetrivel and Thinakaran, 2010), and indeed, increased levels of plasma membrane cholesterol enhance this pathway (Beel et al., 2010). This may derive from lipid raft promoting co-localisation between APP and enzymes of the amyloidogenic pathway, BACE-1 (Benjannet et al., 2001, Hattori et al., 2006) and the γ-secretase complex (Hur et al., 2008). GPI-anchorage of BACE-1, ensuring constitutive lipid raft localisation, has been shown to promote amyloidogenic cleavage of APP (Cordy et al., 2003). Conversely, the α-secretases are localised in non-raft regions of the plasma membrane (Beel et al., 2010), although if targeted to lipid rafts, they can compete with BACE-1 for APP cleavage and a reduction in amyloidogenic processing is seen (Harris et al., 2009).

1.3.5 Function

1.3.5.1 The APP holoprotein

Little is known about the function of full length APP, but its structure, such as its juxtamembrane tri-lysine motif, has led to suggestions that it may act as a receptor (Kang et al., 1987). Cell culture experiments have consistently shown a function for APP as an adhesion molecule, adhering to other cells or extracellular matrix components (Khalifa et al., 2010). Furthermore, key roles for APP have been outlined in neurogenesis (Lazarov and Demars, 2012) synaptogenesis (Tyan et al., 2012) regulation of synaptic activity (Octave et al., 2013), response to ischaemia (Nalivaeva and Turner, 2013) and axon arborisation. This is supported by high levels of APP expression in regions undergoing synaptic modifications and APP may have a role in the maintenance of cholinergic synapses (Isacson et al., 2002, Gralle and Ferreira, 2007, Westmark, 2013). This role in adhesion may be mediated by an Arg-His-Asp-Ser tetrapeptide motif, which has sequence similarity to the prototypical integrin binding motif, RGD. This motif likely has interacting roles with extracellular matrix proteins and heparan

sulphate proteoglycans (HSPGs) (Zheng and Koo, 2011). Several of the functional roles of APP in adhesion have been attributed to dimerisation (Soba et al., 2005, Khalifa et al., 2010), a process shown to be dependent on the N-terminal E1 domain (Isbert et al., 2011).

Some functional roles of APP have been elucidated from knockout mouse models (Korte et al., 2011, Aydin et al., 2012). APP$^{-/-}$ mice have been shown to be viable and fertile, although they do exhibit reduced brain and body weight. Behaviourally, the mutant mice show reduced exploratory and locomotor activity as wells as defects in LTP. These models have revealed a possible compensatory role for the APP-family member, amyloid precursor protein-like protein 2 (APLP2). Viability of these mice is contingent on APLP2 expression, as even APP$^{-/-}$, APLP1$^{-/-}$ mice are viable. However, knockout of APP and APLP2 is lethal (Wang et al., 2005, Zheng and Koo, 2011, Aydin et al., 2012). Overall, these mouse models have displayed essential roles for APP in development of CNS and PNS, synaptogenesis, synaptic plasticity (Aydin et al., 2012, Caldwell et al., 2013) and nerve regeneration (Kotulska et al., 2010). Paradoxically though, APP can also display inhibitory effects on neurite outgrowth (Billnitzer et al., 2012), perhaps through its binding to the DR6 death receptor (Nikolaev et al., 2009), which implicates APP in having a regulatory role in neuron development.

In terms of signalling, APP is capable of inducing a subset of neuroprotective genes. This results in down-regulation of c-jun and SPARC/ osteonectin, but also activation of the PI3K/ Akt pathway. This potentiates anti-apoptotic, pro-survival effects, such as the blockade of JNK signalling (Kogel et al., 2005).

1.3.5.2 sAPPα

Trophic and neuroprotective functions have been ascribed to the soluble derivatives of APP for many years (Araki et al., 1991, Mattson et al., 1993, Smith-Swintosky et al., 1994). One example is the work of Araki *et al.*, who showed increased neuronal survival and neurite outgrowth in cortical culture treated with sAPPα (Araki et al., 1991). Structurally, sAPPα has six domains, denoted D1-D6. The neuroprotective properties of this soluble ectodomain are dependent on D1 and D6, possibly through binding to HSPGs (Corrigan et al., 2011). It has been suggested that this trophic activity may in part derive from activation of a membrane-bound guanylate cyclase and the

consequent regulation of Ca^{2+} and neuroprotection mediated by cGMP (Barger et al., 1995, Barger and Mattson, 1995). Recent work has also implicated Egr-1 as a TF, downstream of MAPK, through which sAPPs can enhance neurite outgrowth (Chasseigneaux et al., 2011). Overall, sAPPα has been implicated in a range of cellular processes, including neurite outgrowth, metal homeostasis, cell proliferation and induction of LTP (Chasseigneaux and Allinquant, 2011).

1.3.5.3 sAPPβ

On the other hand, many of these functions are apparently absent in sAPPβ. The protective effects of this fragment against glucose deprivation and excitotoxicity are 50-100 fold less potent than sAPPα (Furukawa et al., 1996, Chasseigneaux and Allinquant, 2011). SAPPβ is capable, like sAPPα, of promoting axonal growth (Chasseigneaux et al., 2011), but can potentiate axon pruning due its association with the death receptor, DR6 (Nikolaev et al., 2009). Soluble fragments of APP have recently been shown to drive neural differentiation of human embryonic stem cells and sAPPβ was particularly potent in this regard (Freude et al., 2011). It has also been shown that sAPPβ is active in gene regulation and it has been specifically linked to regulation of Klotho and transthyretin (TTR) expression (Li et al., 2010).

1.3.6 The amyloid precursor protein intracellular domain (AICD) and C-terminal signalling

Soluble AICD, liberated from the non-amyloidogenic cleavage of APP, via α- and γ-secretases, is released into the cytosol and rapidly degraded (Cupers et al., 2001). This is largely mediated by insulin-degrading enzyme (IDE) (Edbauer et al., 2002) and, in general, may represent a mechanism for regulation of AICD function. Given that AICD is degraded in the presence of IDE inhibitors, an alternative pathways for degradation is likely (Buoso et al., 2012), such as by caspases and the proteasome (Chang and Suh, 2010, Beckett et al., 2012). However, AICD generated via β- and γ-secretase cleavage has been shown to be functionally active as a transcriptional regulator (Goodger et al., 2009, Belyaev et al., 2010, Flammang et al., 2012). This process is apparently mediated via the binding of a number of interacting proteins, thought to number in excess of twenty (Beckett et al., 2012).

The binding of these binding partners is thought to depend on the phosphorylation status of the two Tyr residues in the C-terminal YENTPY motif. This phosphorylation can be potentiated by NGF, as Tyr682 has been shown to be phosphorylated by the NGF receptor, TrkA (Matrone et al., 2011). Indeed, mutation of Tyr682 has significant deleterious effects on NGF/ TrkA signalling, resulting in reductions in dendritic spine density and memory impairments (Matrone et al., 2012).

The importance of phosphorylation (Tamayev et al., 2009) has been shown by the key roles played by phosphotyrosine binding domains (PTBs) in AICD-interacting partners such as X11/ Mint (Borg et al., 1996, Ando et al., 2001, Swistowski et al., 2009) and Disabled 1 (Dab1) (Homayouni et al., 1999). These binding partners may also act as a scaffold, such as the ability of Mint3 to recruit TA2 and YAP (Swistowski et al., 2009) Further, the ^{667}VTPEER^{672} motif contains T668, which can be phosphorylated by enzymes including cdk5, cdc2 and GSK-3β. Its phosphorylation status has been suggested to regulate binding of the Fe65 adaptor protein to the APP C-terminal ^{682}YENTPY^{687} motif (Fiore et al., 1995, Ando et al., 2001, McLoughlin and Miller, 2008). This is of importance, because not only does Fe65 binding target AICD to the nucleus (Cao and Sudhof, 2001, Cao and Sudhof, 2004), the complex can mediate effects on cell motility (Guenette, 2002) and brain development (Guenette et al., 2006).

Arguably the best-studied of the AICD target genes is neprilysin (NEP) (Pardossi-Piquard et al., 2005), a metalloprotease with the ability to degrade Aβ (Carson and Turner, 2002) and therefore suggested as a possible therapeutic target in AD (Nalivaeva et al., 2008, Nalivaeva et al., 2012). After the initial study by Pardossi-Piquard *et al.*, subsequent work was able to show that AICD was able to bind the NEP promoter and activate its transcription through an epigenetic mechanism involving displacement of histone deacetylases (HDACs) from the NEP promoter (Belyaev et al., 2009). This process of transcriptional regulation also seems to require binding AICD-interacting partners Fe65 and Tip60 (Cao and Sudhof, 2001), where Fe65 stabilises AICD and directs its nuclear translocation, whereas Tip60 is capable of chromatin modification (Kimberly et al., 2001). AICD, though, is capable of regulation a number of target genes, including epidermal growth factor receptor (EGFR) (Zhang et al., 2007), low density lipoprotein receptor- related protein 1 (LRP1) (Liu et al., 2007), GSK-3β (Kim et al., 2003) and aquaporin-1 (Huysseune et al., 2009).

However, despite this ostensibly beneficial role of AICD signalling, it has also been closely linked to toxicity and cell death (Borquez and Gonzalez-Billault, 2011). This may derive from a Tip60 dependent enhancement of p53 activity (Alves da Costa et al., 2006, Chang and Suh, 2010), or induction of GSK-3β expression (Kim et al., 2003). Supporting a neurotoxic role of AICD, it has been shown to impair adult hippocampal neurogenesis in an age-dependent manner. This can result in memory impairment, such as that involved in spatial learning. In an AICD transgenic mouse, impaired neurogenesis was observed at three months of age, linked to activation of stress-kinase pathways and upregulation of pro-inflammatory cytokines (Ghosal et al., 2010). Furthermore, AICD has been linked to the down-regulation of ApoJ/ clusterin. This results in an accumulation of misfolded proteins in the ER, followed by increased expression of ER chaperones, decreased protein synthesis and increased ERAD. The effect of these cellular responses can be activation of apoptotic pathways (Kogel et al., 2011). However, this area remains particularly controversial, as a recent study showed AICD to be trophic rather than toxic, potentiating neurite outgrowth (Zhou et al., 2012).

1.4 Alzheimer's disease (AD)

AD is essentially a disease of cognition, with defects in episodic and spatial memory particularly common. However, symptoms are not limited to memory and can include irritability, jealousy, personality changes and aggression (Selkoe, 2001). The pathological hallmarks in the brains of AD sufferers are extracellular plaques formed from aggregated Aβ and some intracellular proteins (Alzheimer et al., 1995, Selkoe, 2001, Friedrich et al., 2010) and neurofibrillary tangles consisting of hyper-phosphorylated tau protein (Nukina and Ihara, 1986, LaFerla and Oddo, 2005). It is a progressive neurodegenerative condition deriving from neuronal cell death, primarily in the basal forebrain (Whitehouse et al., 1981, Coyle et al., 1983), with a variable age of symptomatic onset. AD is caused by death of mature neurons, but altered proliferation, migration and differentiation of neuronal precursor cells is also contributory (Colon, 1973, Terry et al., 1981, Donovan et al., 2006, Crews and Masliah, 2010).

1.4.1 A historical perspective

According to a recent Swedish study, there are currently 35 million dementia sufferers worldwide, with an estimated cost, in 2010, of nearly £400bn (Wimo et al., 2013). However, the genesis of AD research was 106 years ago when Alois Alzheimer detailed a patient with significant personality changes, in whose post-mortem brain he discovered the plaques which are now considered to be a pathological hallmark of AD (Alzheimer et al., 1995). However, decades passed before the protein constituent of these plaques was identified as Aβ, deriving from APP (Glenner and Wong, 1984a, Glenner and Wong, 1984b). At a similar time, the characteristic neurofibrillary tangles present in AD brains were identified as being composed of the microtubule-associated protein, tau (Grundke-Iqbal et al., 1986a, Wolozin et al., 1986). It was also suggested at this point that the phosphorylation status of tau may be linked to disease pathology (Grundke-Iqbal et al., 1986b). Soon after this, APP was genetically linked to AD (Tanzi et al., 1987) and missense mutations were discovered, which were strongly linked to familial, early onset AD (Chartier-Harlin et al., 1991, Goate et al., 1991, Murrell et al., 1991). Subsequent papers added mechanistic insight, with mutations in APP shown to cause overproduction of Aβ (Citron et al., 1992), such as the Swedish mutation of Lys670, Met671 to Asn670, Lys671 (Mullan et al., 1992, Haass et al., 1995). However, a recent Ala673Thr mutant has recently been identified, which is protective against AD (Jonsson et al., 2012). Using much of this early data, Hardy and Higgins formalised the amyloid cascade hypothesis, which is still a dominant hypothesis in the field to this day. This hypothesis places Aβ at the top of a pathological cascade, its production leading to Ca^{2+} dyshomeostasis, tau hyper-phosphorylation and cell death (Hardy and Allsop, 1991, Selkoe, 1991, Hardy and Higgins, 1992). This hypothesis (Fig. 1.8) still retains significant prominence, despite ongoing debate as to its validity (Hardy and Selkoe, 2002, Hardy, 2009, Teich and Arancio, 2012).

Figure 1.8: the amyloid cascade hypothesis
A schematic of the amyloid cascade hypothesis, whereby amyloidogenic cleavage of APP generates Aβ monomers. These monomers are capable of aggregation, forming toxic oligomers and the plaques which represent one of the pathological hallmarks of AD. Oligomers mediate pathology in a number of ways, leading synaptic dysfunction and neuronal loss but also increases in tau phosphorylation, which similarly contribute to neuronal loss. Both of these processes have been shown to be potentiated by inflammation.

1.4.2 The toxic effects of Aβ

1.4.2.1 Synaptic dysfunction

In the post-mortem brains of AD patients, Aβ appears in senile plaques and hence these were thought to be the pathological forms of Aβ (Selkoe, 2001). However, work done a decade after the publication of the amyloid cascade hypothesis showed that smaller oligomers were likely the most toxic form of Aβ (Walsh et al., 2002, Cleary et al., 2005, Wilcox et al., 2011), although the precise species responsible remains unclear (Haass, 2010). The oligomers also correlate much better with synaptic dysfunction and cognitive defects than do the plaques (Sakono and Zako, 2010). The second proteolytic cleavage step in the amyloidogenic pathway can generate two main isoforms of Aβ, namely $A\beta_{1-40}$ and $A\beta_{1-42}$, although modified forms of Aβ do exist, such as pyroglutamated Aβ (Nussbaum et al., 2012). It is thought that the latter is more toxic

because it is more prone to aggregation and oligomer formation (Jarrett et al., 1993, Ballard et al., 2011) and that an alteration in the ratio of these Aβ species may drive AD pathology (Kuperstein et al., 2010). This synaptic function mediated by small assemblies of Aβ may cause synaptic loss through down-regulation of synaptic proteins such as syntaxin-1 and SNAP-25, which are involved in vesicle exocytosis (Clare et al., 2010) and scaffold proteins such as Shank (Roselli et al., 2009) and post-synaptic density 95 (PSD95) (Gylys et al., 2004). However, despite this, it has been suggested that Aβ monomers may be neuroprotective (Giuffrida et al., 2009), acting to modulate ion channels at physiological concentrations (Pearson and Peers, 2006).

1.4.2.2 Calcium dyshomeostasis

A plethora of damaging functions has been ascribed to Aβ in AD, but one of the best characterised is the Aβ-mediated disruption of Ca^{2+} homeostasis, formalised in the calcium hypothesis of AD (Berridge, 2011). This states that aberrant APP metabolism (i.e. decreased α-secretase (Epis et al., 2010); increased Aβ production or decreased degradation) leads to enhancement of cellular Ca^{2+} influx through the actions of Aβ oligomers, perhaps mediated through the cellular prion protein (Berridge, 2011), which Aβ is known to bind (Lauren et al., 2009, Gimbel et al., 2010) or LRP1 (Fuentealba et al., 2010). Aβ aggregates may form pores independently (Caughey and Lansbury, 2003, Randall et al., 2010), or potentiate Ca^{2+} influx through receptors such as NMDARs. It has been suggested that AICD may be involved through its regulation of the expression of key Ca^{2+} signalling proteins, such as the ryanodine receptor (RyR), calbindin and the sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) (Leissring et al., 2002, Stutzmann et al., 2006, Berridge, 2011). Ultimately, this remodelling of Ca^{2+} signalling can disrupt synaptic plasticity and LTP, while inducing neuronal cell death (Shirwany et al., 2007, Querfurth and LaFerla, 2010, Berridge, 2011, Kawahara et al., 2011).

1.4.2.3 Further pathological drivers

Aβ has also been linked to suppression of the proteasome, which may lead to accumulation of misfolded proteins (Christie et al., 1999). Over-activation of cell surface receptors can also be mediated by Aβ, with glutamate receptors (e.g. NMDAR, mGluR5) particularly targeted (Shankar et al., 2007, Crews and Masliah, 2010). Glutamate receptors have been linked to synaptic and neuronal plasticity and also

learning and memory. Hence, any perturbation of their function or signalling pathways could contribute to the AD phenotype (Dinamarca et al., 2013). In addition to glutamate receptors, Aβ is capable of binding a wide range of cell surface receptors, including GM1 ganglioside (Yamamoto et al., 2007), TrkA (Bulbarelli et al., 2009), insulin receptor (Zhao et al., 2008) and Frizzled receptor (Magdesian et al., 2008), interfering with neurotrophin and insulin signalling (Sakono and Zako, 2010), while Frizzled activation potentiates GSK-3β activity and tau phosphorylation (Magdesian et al., 2008). Other cell surface binding partners can facilitate Aβ internalisation, such as nAChR, LDLR and receptor for glycation end products (RAGE) (Yan et al., 1996, Wang et al., 2000, Yamada et al., 2008, Sakono and Zako, 2010). Once internalised, Aβ is involved in a number of intraneuronal pathological events (Billings et al., 2005, LaFerla et al., 2007).

Aβ is further able to potently generate reactive oxygen and nitrogen species (ROS and RNS) which interfere with mitochondrial function (Hirai et al., 2001, Hauptmann et al., 2006). Production of toxic aldehydes after membrane lipid peroxidation inhibits key mitochondrial enzymes (Keller et al., 1997, Humphries and Szweda, 1998). Further, electron transport, ATP production and oxygen consumption are all negatively affected while the mitochondrial membrane potential is also impaired. Increased abundance of the superoxide radical leads to H_2O_2-mediated oxidative stress, which leads to mitochondrial release of cytochrome c (Caspersen et al., 2005, Querfurth and LaFerla, 2010), an event which initiates an apoptotic cascade (Liu et al., 1996).

Other biological processes linked to AD include perturbations in cholesterol metabolism, inflammation and increased expression of heat shock proteins in response to misfolded proteins (Podtelezhnikov et al., 2011). In order to elucidate significant pathways which may be involved in AD, Podtelezhnikov *et al.*, used transcriptomic analysis of normal aged and AD patients. Though thousands of genes differed in their expression, disturbances in key pathways were identified, namely cell cycle regulation, lipid metabolism and axon guidance pathways (Podtelezhnikov et al., 2011). AD is also linked to oxidative stress, impaired protein folding in the ER and deficiencies in autophagic and lysosomal clearance of damaged proteins (Pimplikar et al., 2010, Querfurth and LaFerla, 2010).

1.4.3 Tau

Tau is a microtubule associated protein and plays important roles in neuronal vesicular trafficking (Weingarten et al., 1975). Its phosphorylation and aggregation in AD can impair these processes, having a deleterious effect on neuronal viability (Billingsley and Kincaid, 1997). Its phosphorylation is stated to be triggered by Aβ (Hardy and Allsop, 1991, Hardy and Higgins, 1992, De Felice et al., 2008) and a number of kinases have been linked to these pathological phosphorylation events, such as GSK-3β (Hanger et al., 1992), ERK2 (Reynolds et al., 2000), dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) (Woods et al., 2001) and cyclin-dependent kinase 5 (cdk5) (Baumann et al., 1993). Although tau mutations appear in other forms of dementia, such as corticobasal degeneration and frontotemporal dementia (Morris et al., 1999), none have been identified in AD (Ballard et al., 2011).

1.4.4 Genetics of AD

Genetic loci for familial AD (FAD) have been identified, such as mutations in *APP* (Goate et al., 1991) and the γ-secretase subunits *PSEN1* and *PSEN2* (Schellenberg et al., 1992, Levy-Lahad et al., 1995), with the presenilin mutations able to promote generation of $Aβ_{1-42}$ (Fang et al., 2006). This said, it has been suggested that mutations in genes for APP and the presenilins can have pathological effects independent of Aβ (Pimplikar et al., 2010). Although mutations give a defined disease aetiology, only 5% of patients have FAD, the remainder exhibiting a sporadic form of the disease of mostly unknown aetiology (Ballard et al., 2011), although AD model animals mostly over-express mutant APP and/or PS-1. However, risk genes have been identified and genome-wide association studies (GWAS) continue to add more. The prototypical AD risk gene is *APOE* (apolipoprotein E), with the ε4 allele conferring a 3-10 fold increase in risk (Corder et al., 1993, Saunders et al., 1993). A recent GWAS identified further risk loci at the genes encoding clusterin and phosphatidylinositol binding clathrin assembly protein (PICALM) (Harold et al., 2009). However, outside of these genes, the risk ratios are increasingly modest, approximately 1.2 (Ballard et al., 2011). It is also possible that epigenetic modifications may play a part in the disease as the chromatin-remodelling sirtuins are downregulated in AD, which have roles in energy homeostasis and apoptosis (Chouliaras et al., 2010).

It is possible that the myriad of genetic susceptibility loci identified in GWAS studies merely confer vulnerability to AD but that the pathological process is initiated by one or more environmental factors. Epidemiological studies have been able to identify some common AD environmental risk factors, such as hypertension, smoking, Type II diabetes, traumatic brain injury and body weight (Ballard et al., 2011, Mayeux and Stern, 2012). In addition, such things as diet, cognitive function and physical activity can all be protective against disease onset (Mayeux and Stern, 2012).

1.4.5 AD therapeutics

Therapeutic avenues under investigation are multifarious, yet this has not currently been translated into clinically efficacious treatments, with ChE inhibitors (rivastigmine, galantamine and donepezil) the only widely available treatment in the UK. The NMDA receptor antagonist memantine is no longer recommended for use as a front line monotherapy, with its use limited to severe AD patients or those for whom ChE inhibitors are ineffective or contraindicated (Farrimond et al., 2012). However, ChE inhibitors do not represent a cure, rather a therapy with short- to medium-term moderate efficacy (Birks, 2006). Drugs have also been used to target the neuropsychiatric symptoms of AD, such as antipsychotics (e.g. risperidone, olanzapine), anticonvulsants (carbamazepine) and antidepressants (e.g. citalopram). Evidence suggests these drugs may have some efficacy in targeting behavioural symptoms (Ballard et al., 2011), but they are not disease-modifying agents.

There are a number of disease modifying approaches in clinical trials (Tayeb et al., 2012), including development of GSK3 inhibitors, tau aggregation inhibitors and zinc modulators. The agents that are either in phase III trials, or have recently completed trials are all amyloid-centric approaches. These include the γ-secretase inhibitors such as semagacestat, which recently failed in Phase III trials (Samson, 2010) and anti-Aβ antibodies such as bapineuzumab and solinezumab have also been unsuccessful (Wilcock, 2010, Karran, 2012). Although the anti-aggregation inhibitors tramisprosate failed in Phase III (Aisen et al., 2011), encouraging results have recently been published using a retro-inverso peptide to block Aβ aggregation (Parthsarathy et al., 2013). It has been suggested that pre-symptomatic biomarkers may pave the way to earlier intervention in AD, thus significantly increasing the probabilities of therapeutic efficacy (Selkoe, 2012).

1.5 The cholinergic system in AD

Significant changes are seen in the cholinergic system during the progress of AD. For example, there is widespread neuronal shrinkage and loss in the basal forebrain cholinergic system, suggesting a selective vulnerability of these neurons (Whitehouse et al., 1981, Coyle et al., 1983, Grothe et al., 2010). However, cholinergic neurons in the striatum and brainstem are either spared or only affected later in the disease (Kar and Quirion, 2004).These disturbances in BFCNs are recapitulated in the Tg2576 mouse model of AD (Apelt et al., 2002) and BFCN lesions result in deficits in motor learning and increased amyloid pathology (Conner et al., 2003, Laursen et al., 2012). Early reports of cholinergic dysfunction led to the formalisation of the cholinergic hypothesis of AD, stating that the cognitive roles played by BFCNs meant that any disturbance of their function in disease would lead to cognitive decline, such as that displayed in AD (Bartus et al., 1982, Francis et al., 1999, Contestabile, 2011).

1.5.1 Changes in cholinergic markers

Furthermore, there are significant changes in cholinergic proteins in AD, such as AChE (Talesa, 2001), which shows a global decrease in brain, preferentially in the G_4 form (Atack et al., 1983, Bierer et al., 1995, Rinne et al., 2003). AChE has been shown to be decreased as early as the onset of mild cognitive impairment (MCI) (Haense et al., 2011). In plasma, G_4 AChE is also reduced, but overall increases in AChE are seen, deriving from increased G_1 and G_2 AChE (Garcia-Ayllon et al., 2010). Further, AChE displays altered glycosylation patterns prior to amyloid deposition (Saez-Valero et al., 1999, Fodero et al., 2002), which has been shown to be induced by the PS1-A246E mutant (Silveyra et al., 2008) The inflammatory phenotype associated with AD (Akiyama et al., 2000) is capable of modulating the cholinergic system, as the cytokine interleukin-1 can activate AChE expression (Schliebs, 2005, Schliebs et al., 2006).

Analysis of cholinergic markers in Tg2576 AD mice showed significant decreases in hippocampal high-affinity choline uptake and reductions in cortical nAChRs, M1 mAChRs and M2 mAChRs (Apelt et al., 2002). These data broadly concur with those from human AD brains, which showed reduced nAChR and M2 mAChR binding in cortex and hippocampus (Araujo et al., 1988), in addition to reduced ChAT levels (Araujo et al., 1988, Bierer et al., 1995, Sihver et al., 1999, Van Dam et al., 2005).

However, Araujo *et al.* were unable to show any changes in M1 mAChR levels (Araujo et al., 1988). The suggested downregulation of M1 mAChRs has led to suggestions that its agonism may provide therapeutic benefit in AD (Fisher, 2011) and, indeed, this approach can counteract BFCN loss in a cell proliferation model (Van Kampen and Eckman, 2009). Reduced ACh release is characteristic of AD (Hoshi et al., 1997, Isacson et al., 2002) and may derive, in part, from reductions in ChAT (Araujo et al., 1988, Bierer et al., 1995). However Hoshi *et al.*, did not find any perturbation in ChAT activity in primary neurons treated with Aβ. They suggested that any deficiencies in ACh released were caused by insufficient acetyl CoA, in turn caused by Aβ-induced inhibition of pyruvate dehydrogenase (Hoshi et al., 1997). This is compounded by a high energy demand, which results in BFCNs using acetyl CoA as an energy source, exacerbating any shortages in acetyl CoA required for ACh synthesis (Schliebs and Arendt, 2006).

1.5.2 The role of acetylcholinesterase in AD

$AChE_T$ has been shown to be capable of inducing amyloid fibril formation, independently of its active site (Inestrosa et al., 1996), whereas this is not seen with BChE, which has been shown to attenuate fibril formation (Inestrosa et al., 1996, Diamant et al., 2006). Although this initial work was *in vitro*, it has been subsequently corroborated *in vivo* (Rees et al., 2003, Reyes et al., 2004). This process is said to be mediated via the AChE C-terminus, which shares sequence similarity with Aβ and has a propensity for fibrillisation and may therefore nucleate Aβ aggregation (Cottingham et al., 2002, Greenfield and Vaux, 2002, Jean et al., 2008). It has been suggested that these fibrillogenic AChE C-terminal peptides may be generated by cleavage of AChE by IDE or NEP (Jean et al., 2007). Furthermore, AChE is commonly localised to senile plaques in AD, forming a stable complex with the amyloid fibrils (Mesulam et al., 1987, Alvarez et al., 1997). Plaque-associated $AChE_T$ displays different kinetic parameters from the free enzyme (Mesulam et al., 1987, Geula and Mesulam, 1989), with alterations in k_m and V_{max}, plus a greater resistance to low pH. Further modifications include a resistance to inhibition, with AChE inhibitors such as propidium, tacrine and edrophonium displaying substantial increases in IC_{50}. These complexes have also been shown to potentiate neurotoxicity to a greater extent than Aβ alone (Alvarez et al., 1998, Reyes et al., 2004). This is mediated through increased intracellular Ca^{2+} and irreversible loss of mitochondrial membrane potential (Dinamarca et al., 2010) and *in*

vivo, astrocyte reactivity and neuronal loss (Reyes et al., 2004). AChE, possibly independently of Aβ, is also capable of activating an apoptotic cascade through activation of GSK-3 and Bax, followed by apoptosome formation and activation of capases 3 and 9 (Greenberg et al., 2010).

As these pro-aggregation processes are not mediated through the AChE active site (Inestrosa et al., 1996), inhibitors which target this region have no effect, although AChE-induced Aβ aggregation can be inhibited by agents targeting the PAS, such as propidium (Bartolini et al., 2003) and monoclonal antibodies (Reyes et al., 1997). Cognisant of this, medicinal chemists are generating new compounds which target the active site and the PAS in an effort to block the hydrolytic and pro-aggregation effects of AChE (Belluti et al., 2011). The synthetic agent IDN5076 has been shown to release AChE from plaques and reduce overall plaque number, consequently decreasing memory impairments in APP-PS1 AD mice (Dinamarca et al., 2008, Carvajal and Inestrosa, 2011).

AChE was also shown to be increased in a streptozotocin-induced model of AD, which features progressive learning and memory defects. The symptoms of streptozotocin treatment can be ameliorated by epigallocatechin-3-gallate (ECGC), an antioxidant polyphenol. The effects of ECGC include a reduction in AChE activity to control levels (Biasibetti et al., 2012). AChE expression is also thought to be induced by apoptosis (Zhang et al., 2002) and also Aβ, the latter possibly through increased intracellular Ca^{2+} (Sberna et al., 1997, Sberna et al., 1998, Small, 2011) and this up-regulation of AChE has been suggested to be mediated by α7 nAChR. The induction is potentiated by α7 nAChR agonists choline and nicotine and inhibited by α-Bgt and MLA (Fodero et al., 2004). It is possible, though, that this Aβ-mediated increase in AChE levels derives from reduced protein degradation (Hu et al., 2003).

Furthermore, deletion of an AChE allele was shown to be protective against Aβ toxicity and the effects of the muscarinic antagonist scopolamine (Espallergues et al., 2008). Concurring with this work, Fu *et al.* showed that administration of an antisense oligonucleotide targeting AChE helped preserve cognitive functions in mice challenged with intracerebrovascular administration of Aβ (Fu et al., 2005).

Although negative cellular effects have been associated with $AChE_T$, such as promotion of apoptosis, $AChE_R$ (readthrough) has been suggested to be protective against Aβ toxicity. Perhaps through competition with $AChE_T$, $AChE_R$ is able to reduce Aβ oligomer formation and ablate Aβ toxicity to cultured cells. In addition, $AChE_R$/ APP_{Swe} mice show far fewer pathological hallmarks of AD than do APP_{Swe} mice. Furthermore, immunoblot analysis of human AD hippocampi revealed substantial decreases in $AChE_R$, which may drive AD pathology (Berson et al., 2008).

1.5.3 Disruption of trophic support

Aβ may further affect the cholinergic system by disrupting the trophic support offered by neurotrophins such as NGF and BDNF (Auld et al., 2002, Schliebs and Arendt, 2011). There is a recognised disruption in NGF signalling in AD, with possible causes suggested to be dysfunctional NGF transport or down-regulation of TrkA (Auld et al., 2002), with the former observed in 3xTg AD mice mice (Perez et al., 2011). This is particularly important as NGF has been shown to modulate APP levels in PC12 cells (Mackinnon et al., 2012). A role for NGF in AD is supported by the anti-NGF mouse model. Although NGF mutations are embryonic lethal, anti-NGF monoclonal antibodies can be used to target NGF, resulting in a ≥ 50% decrease in NGF levels. Phenotypically, these mice display amyloid deposits, neurofibrillary tangles and significant widespread neuronal loss, coupled to cholinergic deficits and impaired performance in memory tasks (Capsoni et al., 2000). It has been suggested that reduced maturation of NGF may contribute to AD. The conversion of proNGF to NGF is mediated by plasmin, the deficit of which in AD brain can lead to proNGF accumulation (Cuello et al., 2009), suggested to mediate apoptosis through $p75^{NTR}$ (Fombonne et al., 2009). In addition, the degradative enzyme MMP-9 is increased in human cortex, leading to greater breakdown of NGF (Bruno et al., 2009, Cuello et al., 2009). A novel peptide-based drug, cerebrolysin, has been stated to restore the proNGF/ mNGF balance (Ubhi et al., 2012) and has been shown to be efficacious in restoring cognitive function in vascular dementia patients (Chen et al., 2013).

Similar, in 3xTg AD mice, significant alterations are seen in the NGF signalling pathway. In these mice, the cholinergic system is the first to show disruption, manifesting in a 23% reduction in BFCNs. Cortical proNGF was increased, although

not in hippocampus and TrkA was down-regulated in both cortex and hippocampus, yet p75^{NTR} was found to be unchanged (Perez et al., 2011).

1.5.4 Interactions between Aβ and α7 nAChRs

It is generally accepted that Aβ is capable of binding α7 nAChRs with high affinity (Wang et al., 2000, Fodero et al., 2004), although there remains some dispute with the suggestion that, by altering the packing of lipids within the plasma membrane, Aβ influences receptor and channel function indirectly (Small et al., 2007). An interaction between Aβ and α7 nAChR may, however, mediate Aβ-induced suppression of LTP (Li et al., 2011). It is likely that Tyr188 in α7 nAChR plays an important functional role, as its mutation reduces Aβ- and ACh-mediated receptor activation, although it had no effect on nicotine agonism (Tong et al., 2011). It is possible that Aβ is an endogenous ligand for this receptor (Barrantes et al., 2010, Hernandez and Dineley, 2012) and that Aβ can induce Ca^{2+} influx through its interaction with α7 nAChR, which has been suggested to activate GSK-3β and potentiate tau phosphorylation. This promotion of tau phosphorylation was shown to be Ca^{2+}-dependent as it was completely blocked by the Ca^{2+} channel blocker, nifedipine (Hu et al., 2008). Tau phosphorylation and ionic flux through NMDA receptors and α7 nAChR have recently been shown to be reduced in brain slices after blockade of Aβ binding to α7 nAChR by the agent S24795 (Wang, 2010). In addition, a new $α_7β_2$ receptor was described relatively recently, which has been shown to be particularly sensitive to Aβ (Liu et al., 2009).

Moderate to low (pM-nM) concentrations of Aβ or short exposure times appear not to lead to permanent changes in α7 nAChR expression (Barrantes et al., 2010), although α7 nAChR is upregulated in AD brains (Counts et al., 2007) and BFCNs generated from human ES cells (Wicklund et al., 2010). However, there are opposing findings, suggesting that nAChR levels remain relatively unchanged, but defects in trafficking and turnover may decrease the effective levels (Sihver et al., 1999, Jurgensen and Ferreira, 2009). Conversely, raised concentrations or exposure times can lead to dysregulation of signalling through α7 nAChR and perturbations in ERK/ MAPK and CREB signalling. As α7 nAChRs have been linked to lipid raft localisation (Bruses et al., 2001) and amyloidogenic processing is thought to occur predominantly in lipid rafts (Cordy et al., 2003, Ehehalt et al., 2003), lipid rafts have been suggested to represent a platform which promotes the interaction between Aβ and α7 nAChRs (Khan et al.,

2010). Accumulation of intraneuronal Aβ may be potentiated by high affinity binding to α7 nAChR and subsequent endocytosis.

Furthermore, deletion of α7 nAChR in mice has been reported to protect from dysfunction in synaptic integrity. These mice expressed APP and Aβ to similar levels to wild type mice, yet they were more able to solve cognitive challenges (Dziewczapolski et al., 2009).Yet, other groups have shown opposing findings, that α7 nAChR deletion leads to accelerated cognitive decline, with selective increases $Aβ_{1-42}$ relative to $Aβ_{1-40}$ (Hernandez et al., 2010).

Given its role in cognition (Lendvai et al., 2013), agonism of α7 nAChR has been suggested as a therapeutic avenue in AD as its currents are suppressed in the disease (Newhouse et al., 2001, Soderman et al., 2010, He et al., 2012). However, other studies have shown nicotine administration to exacerbate tau phosphorylation (Oddo et al., 2005, Deng et al., 2010). The AChE inhibitor galantamine showed improved results in cognitive performace, although it is not clear whether these effects derived from AChE inhibition or from the role of galantamine as a positive modulator of nAChRs (Gron et al., 2006, Pakaski and Kalman, 2008). Further work has implicated nicotine, through nAChRs, as being able to positively modulate non-amyloidogenic processing of APP (Lahiri et al., 2002)

1.5.5 Aβ-induced cell death mediated by p75^{NTR}

Another AD-linked receptor is p75^{NTR}, which can bind neurotrophins, but also binds $Aβ_{1-42}$, with FRET analysis revealing an interaction between $Aβ_{1-42}$ oligomers and the p75^{NTR} extracellular domain (Knowles et al., 2009). Its expression is highly restricted, with predominant localisation in BFCNs (Fombonne et al., 2009). The subsequent signalling can initiate apoptosis and promote Aβ production through modulation of APP processing. Similarly, $Aβ_{1-42}$ has the ability to increase p75^{NTR} expression, creating a positive feedback loop (Sotthibundhu et al., 2008, Coulson et al., 2009, Chakravarthy et al., 2010). Aβ can also block poly-ubiquitination of p75^{NTR}, the ubiquitination being potentiated by TRAF6 and p62, with these proteins inhibiting cell death (Geetha et al., 2012). Aβ- activated p75^{NTR} is capable of effecting p53-mediated cell death, through p38 MAPK and JNK signalling, with possible involvement of NF-κB (Costantini et al.,

2005, Knowles et al., 2009). Indeed in p75^{NTR} deficient mice, there is a marked reduction in Aβ-induced cell death (Coulson et al., 2009).

1.6 Aims

It is clear that AChE has important roles to play in physiology and pathology, particularly in relation to AD (Inestrosa et al., 2008, Halliday and Greenfield, 2011, Hicks et al., 2011). The aim of the work presented herein is to further explore key areas of AChE biology, concentrating on the nexus between AChE and neurodegeneration. However, more specifically, the objectives of this thesis are investigation of:

- SN56 cells as an appropriate model for the study of ChE biology
- Cellular release of AChE and elucidation the extent to which shedding and/or exocytic processes may contribute to this
- The mechanism behind any shedding process and the proteases involved therein
- The control of AChE expression by mAChR agonism and elucidation of the signalling intermediates involved
- The regulation of AChE expression by APP and the mechanism by which this is effected

Chapter 2

Experimental Procedures

Chapter 2: Experimental procedures

2.1 Materials

2.1.1 Cell lines

The SN56 cell line (kindly provided by Prof. A. Szutowicz, Medical University of Gdańsk, Poland) was originally developed as an murine immortalised septum-derived cholinergic cell line, hybridised with neuroblastoma cells. Cells express a neuronal and cholinergic phenotype like that of septal cells of the basal forebrain (Hammond et al., 1990).

SH-SY5Y neuroblastoma cells (donated by Dr J. L. Biedler (Sloan-Kettering Institute, NY, USA) are thrice-cloned from the human SK-N-SH cell line. These cells have an adrenergic phenotype and express high levels of dopamine β- hydroxylase and GABA, although the cells do express AChE, ChAT and BChE (Biedler et al., 1978). SH-SY5Y cells over-expressing the human APP_{695} isoform were kindly provided by Dr. I. J. Whitehouse (University of Leeds).

The NB7 human neuroblastoma cell line (a gift from Dr V. Kidd (St Jude Children's Research Hospital, Memphis, TN, USA) displays an adrenergic phenotype, again expressing high levels of dopamine β- hydroxylase and also tyrosine hydroxylase (Thiele, 1998). NB7 cells also express high levels of NEP, APP and Fe65 (Belyaev et al., 2009).

The non-neuronal mouse fibroblast cell line GD25 (a gift from Prof. S. Johansson, Uppsala University, Sweden) was also cultured. These cells lack expression of integrin β1, with the integrin β1 re-expressed in the GD25β1 line (Fassler et al., 1995).

2.1.2 Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM), DMEM F-12, Roswell Park Memorial Institute (RPMI) 1640, penicillin/ streptomycin, L-glutamine and trypsin-EDTA were purchased from Lonza (Slough, Berkshire, United Kingdom). Non-essential amino acids were from Sigma-Aldrich (Gillingham, Dorset, UK). OptiMEM Reduced Serum

Medium was from Gibco (Life Technologies, Paisley, Renfrewshire, UK) and Lipofectamine was similarly from Life Technologies.

2.1.3 Inhibitors and activators

Carbachol and muscarine were purchased from Sigma-Aldrich, as were bacitracin, wortmannin, tunicamycin, Exo1, 1, 10-phenanthroline, atropine and sodium valproate. Batimastat, GM6001, β-IV and DAPT were from Calbiochem (Merck Chemicals, Nottingham, UK) and EDTA was from Gibco.

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Target	Host	Dilution	Source
ACE2	Goat	1:1000	R & D System, Abingdon, Oxfordshire , UK
AChE	Mouse	1:200	Millipore, Watford, Hertfordshire, UK
AChE	Goat	1:500	Santa Cruz Biotechnology, CA, USA
β-actin	Mouse	1:10000	Sigma-Aldrich
ADAM10	Rabbit	1:5000	From Prof. W. Annaert, University of Leuven, Leuven, Netherlands
ADAM17	Rabbit	1:2000	Calbiochem
Akt	Rabbit	1:2000	EnoGene Biotech Co, distributed through Source BioScience Life Sciences, Nottingham, UK
pAkt (T308)	Rabbit	1:1000	Cell Signaling Technology, distributed through New England Biolabs (NEB), Hitchin, Hertfordshire, UK
pAkt (S473)	Rabbit	1:500	BioLegend, San Diego, CA, USA
APP (22C11)	Mouse	1:2000	Millipore
APP (α-CTF)	Rabbit	1:2000	Sigma-Aldrich
sAPPβ	Rabbit	1:250	Signet Laboratories, Dedham, MA, USA
Choline Transporter (SLC5A7)	Rabbit	1:1000	Aviva Systems Biology, San Diego, CA, USA
FAK	Rabbit	1:1000	Santa Cruz Biotechnology

Integrin β1	Rabbit	1:1000	Cell Signaling Technology
p42/p44 MAPK	Rabbit	1:1000	Cell Signaling Technology
Phospho p42/p44 MAPK	Rabbit	1:1000	Cell Signaling Technology
PRiMA	Rabbit	1:500	From Prof. K. Tsim, Hong Kong University of Science and Technology, Hong Kong
PTEN	Rabbit	1:1000	Cell Signaling Technology
PDI (RL90)	Rat	10µg/ml	Novus Biologicals, Cambridge, UK

2.1.4.2 Other antibodies

In addition, a control antibody (IgG3 isotype control; R & D Systems) was used with the PDI-blocking RL90 antibody. Furthermore, horseradish peroxidase (HRP)-conjugated secondary antibodies were used for Western blotting: anti-mouse, anti-rabbit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and anti-goat (Life Technologies).

2.1.5 Primers

2.1.5.1 Reverse transcriptase PCR (RT-PCR)

Target	**Species**	**Sequence**
ADAM9	Human	F: CTTGCTGCGAAGGAAGTACC R: CACACAAAGCATTCCCAGTG
ADAM9	Mouse	F: GCCAGAACAGCAAAGCCTAC R: ACCAAGCTGGAAATCCACAC
ADAM10	Human	F: TCCCCTTGCAACGATTTTAG R: CGTCTCATGTGTCCCATTTG
ADAM10	Mouse	F: AGCAACATCTGGGGACAAAC R: TTGCATATCCCTTCCTTTGC
ADAM12	Human	F: TCAGCACGTGTTCTGGTCTC R: ACACTTCCACGCCTACCAAC
ADAM12	Mouse	F: GTGGCAAAGACTCCAAGAGC

		R: GTCTGTGCTTCCTCCAAAGC
ADAM17	Human	F: TCATTGACCAGCTGAGCATC
		R: TTCTCAAACCCATCCTCGTC
ADAM17	Mouse	F: AGAAGGATGCTTGGGATGTG
		R: TCCTCCTTGGTCCTCATTTG
α7 nAChR	Human	F: ATCGATGTACGCTGGTTTCC
		R: ACTGCAGCACGATCACTGTC
α7 nAChR	Mouse	F: CATTCCACACCAACGTCTTG
		R: TGAGCACACAAGGAATGAGC
AChE	Human	F: GCTCAGCAGTACGTTAGTCTGG
		R: TGCTGTAGTGGTCGAACTGG
AChE	Mouse	F: GTGGGCATGCACATACTGTC
		R: AGTAGGAGCCCTCGTCCTTC
APP	Human	F: AAGAAGCCGATGATGACGAG
		R: TTCTCATCCCCAGGTGTCTC
APP	Mouse	F: TGATCTACGAGCGCATGAAC
		R: ATGTTTGTCAGCCCAGAACC
BChE	Human	F: ATTTCATATGCCCTGCCTTG
		R: CCACTCCCATTCTGCTTCAT
BChE	Mouse	F: TTACAACCAAGACCGGAAGG
		R: CTGGGAAGGCTTGGTCTATG
ChAT	Human	F: GGGGAAATGGAAGAGAGAGG
		R: TGCTCCTCAGACACCAAGTG
ChAT	Mouse	F: TTCTCATCATACCGGCTTCC
		R: AACGATTCGCTCCATTCAAG
M1 mAChR	Human	F: CCGCTACTTCTCCGTGACTC
		R: GTGCTCGGTTCTCTGTCTCC
M1 mAChR	Mouse	F: AGCAGCAGCTCAGAGAGGTC
		R: TGTATTTGGGGAGCTTTTGG
M3 mAChR	Human	F: CCGAGACGAGAGCCATCTAC
		R: TCTCCTTGACCAGGGACATC
M3 mAChR	Mouse	F: TGCTGGTGATCAGCTTTGAC
		R: GTTTTCTGCTTCCGCTTCTG

2.1.5.2 Real time quantitative PCR (qPCR)

Target	Species	Sequence
AChE	Human	F: TTCCTCCCCAAATTGCTCAG
		R: TCCAGTGCACCATGTAGGAG
AChE	Mouse	F: TTTCTCCCCAAATTGCTCAG
		R: TCCAGTGCACCATGTAGGAG
BChE	Mouse	F: TTACAACCAAGACCGGAAGG
		R: GTTGTGCATAGGGGATACCG
BiP	Mouse	F: TGCAGCAGGACATCAAGTTC
		R: TTTCTTCTGGGGCAAATGTC
CHT	Mouse	F: ATATGGGCTGCATGGAAAAC
		R: CACCAACCAACAAACCAATG
Egr-1	Human	F: GACCGCAGAGTCTTTTCCTG
		R: AGCGGCCAGTATAGGTGATG
Elk-1	Human	F: AATGGCCACATCATCTCCTG
		R: GGTCTTGTTCTTGCGTAGCC
GAPDH	Human	F: CAATGACCCCTTCATTGACC
		R: GACAAGCTTCCCGTTCTCAG
NEP	Human	F: CCTGGAGATTCATAATGGATCTTG
		R: AAAGGGCCTTGCGCAAAG
PRiMA	Mouse	F: ATCATTGTCGCTGTGGTCTG
		R: GGTGCCATTCTCATCCTTTC
SRF	Human	F: GCCACTGGCTTTGAAGAGAC
		R: CAGGTTGGTGACTGTGAACG
U6	Mouse	F: CTCGCTTCGGACAGCACA
		R: AACGCTTCACGAATTTGCGT

All primers were from Sigma-Aldrich.

2.1.6 Plasmids

The plasmids used in this project encoded as follows (pIRESHyg vector, unless stated otherwise): APP$_{695}$ (from Dr. A. R. Whyteside, University of Leeds), APP$_{695}$ Y682G, APP$_{695}$ Y687G, APP$_{695}$ Y682G + Y687G double mutant, APP$_{695}$ H147A + H149A + H151A triple mutant (from Mallory Gough and Dr. E. T. Parkin, Lancaster University,

UK) and APP$_{695}\Delta$E1 (pLBCX vector, c-myc, 6xHis tag (from Prof. C. U. Pietrzik, Department of Pathobiochemistry, University Medical Center of the Johannes Gutenberg University Mainz, Germany).

2.2 Methods

2.2.1 Cell culture and treatment

2.2.1.1 Cell culture

SN56 and GD25 cells were cultured in DMEM, SH-SY5Y cells were cultured in DMEM F-12 and NB7 cells were grown in RPMI1640. All media were supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/ streptomycin (5000U/ml stock) and 2 mM L-glutamine. For SH-SY5Y cells, 1% (v/v) non-essential amino acids (from 100x stock) were added and these cells were not used beyond 20 passages. For transfected cells, either 150 µg/ml hygromycin B (wild type APP$_{695}$ or APP$_{695}$ with tyrosine or histidine mutations) (Life Technologies), 10 µg/ml blasticidin S (APPΔE1) (Life Technologies) or 10 µM puromycin (GD25β1) (Sigma-Aldrich) were added. All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Medium was changed every other day and at 80-95% confluence, cells were sub-cultured using trypsin-EDTA (200 mg/L) treatment for 2-5 min, followed by addition to new flasks with fresh media.

2.2.1.2 Freezing and recovery

At 80-90% confluence, cells were harvested by trypsin-EDTA treatment and centrifuged at 405 x g, followed by re-suspension in normal growth medium containing 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich). The cells were divided into 1ml aliquots and frozen in cryo vials (Nunc, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) at -80°C, using a cryo-cooler to reduce temperature at 1°C/ min. For long term storage, cells were transferred to liquid nitrogen.

For recovery, cells were rapidly thawed in a 37°C water bath, followed by addition to fresh growth medium in a 1:9 (v/v) ratio. Cells were then cultured as described above.

2.2.1.3 Transformation of competent bacteria

JM109 competent bacterial cells (Agilent Technologies, Stockport, Cheshire, UK) were incubated with 50 ng plasmid DNA on ice for 30 min, followed by heat shock for 45 s at 42°C. After returning the bacteria to ice for 2 min, 50 µl of the bacteria-DNA solution was added to 200 µl SOC (Super Optimal broth with Catabolite repression) medium (Life Technologies). This solution was shaken for 1 h at 37°C (200 rpm), spread on ampicillin (100 µg/ml) agar plates and bacteria cultured overnight at 37°C. A single colony was subsequently picked and used to inoculate ampicillin-containing Lennox B (LB) medium, followed by overnight incubation at 37°C on an orbital shaker (300 rpm). Plasmid DNA was extracted as per the manufacturer's instructions using the Plasmid Maxi Kit (Qiagen, Crawley, West Sussex, UK) and checked for APP expression by polymerase chain reaction then further verified by sequencing (GATC Biotech, Konstanz, Germany).

2.2.1.4 Transfection

Plasmid DNA (5 µg) was incubated with Lipofectamine in OptiMEM (ratio of the latter two components, 1:18 (v/v)) for 20min at room temperature. Further OptiMEM was added (to a volume of 5ml for a T-75 flask) to the transfection mix. Subsequently, cells at 50-80% confluence were washed and incubated in the transfection mix for 4 h. Normal culture medium, lacking antibiotics (i.e. penicillin/ streptomycin), was then added in a 1:1 ratio with the existing transfection mix. In the case of transient transfections, cells were lysed 24 h post-transfection.

For siRNA transfections, wild type SN56 cells were transfected with either 25 nM siRNA targeting APP (SmartPOOL, Dharmacon, Thermo Fisher Scientific) or a scrambled sequence (siRNA negative control, Ambion, Life Technologies) at an equivalent concentration using Lipofectamine (Life Technologies). Initially, siRNA was incubated with OptiMEM (1:100 (v/v) and, separately, Lipofectamine was incubated with OptiMEM (1:70 (v/v)) for 5 min at room temperature. These separate solutions were subsequently mixed and incubated for 20 min at room temperature. Normal culture medium was then added (without antibiotics) at a ratio of 4:1 (v/v). Cells at 60-80% confluence were washed an incubated in this transfection mix for 6 h, after which they were washed, incubated in OptiMEM for 24 h and then harvested.

2.2.1.5 Generation of a stable cell line

SN56 cells were transfected as described for transient transfection in 2.2.1.4. However, at 24 h post-transfection, the medium was changed to normal culture medium (without antibiotics). At 48 h post-transfection, cells were passaged 1/50 and grown in normal culture medium (including antibiotics) with the addition of the appropriate selective antibiotics. This was generally 300 µg/ml hygromycin B, the only exception to this being the APPΔE1-expressing cells which were grown in 20 µg/ml blasticidin S. Cells were allowed to grow to approximately 80% confluence before being sub-cultured into fresh selective media. This process was repeated before cells were lysed and over-expression of APP verified by Western blot. After this initial selection phase, the concentrations of the selective antibiotics were halved.

2.2.1.6 Cell treatments

According to the experimental paradigms, cells were treated at 80-90% confluence, which involved washing in OptiMEM, followed by incubation in OptiMEM containing either a pharmacological agent of interest or an equivalent volume of vehicle as control. Concentrations of pharmacological agents and the incubation times varied and are comprehensively detailed in the following chapters. Post-incubation, media samples and/or cell lysates were prepared for subsequent assays.

2.2.2 Sample preparation

2.2.2.1 Preparation of media samples

Media samples were extracted and centrifuged (2400 x g, 5 minutes, 4°C) to remove cell debris. Media samples were then added to a 6 ml 10kDa MWCO Spin X- UF 20 Concentrator (Corning Life Sciences, Amsterdam, The Netherlands). This was followed by centrifugation (2400 x g, 4°C) until the volume reached 0.75- 1.0 ml. At this point the media samples were centrifuged (10,000 x g, 10 min) in a new Eppendorf tube.

2.2.2.2 Preparation of cell lysates

Cells at ≥ 80% confluence or after pharmacological treatment, as indicated above and in subsequent chapters, were washed twice in ice cold phosphate-buffered saline (PBS) and harvested in 10 ml PBS. Cells were pelleted at 2700 x g for 5 min (4°C) and re-suspended in 6 x volume of lysis buffer (50mM Tris-HCl (pH 7.4) with 1% Triton X-

100 and 0.5% sodium deoxycholate) with a 21G needle and syringe. Lysis was performed for 30 min on ice followed by centrifugation at 2700 x g for 5 min to clarify the lysates. Supernatants were collected for assays.

2.2.3 Protein assays

2.2.3.1 Determination of protein concentration

Protein concentration was determined using the bicinconinic acid (BCA) method (Smith et al., 1985). In brief, samples (either medium or lysate) were diluted with water in a 96 well plate to a total volume of 10µl. These samples were incubated for 30min at 37ºC with the BCA solution (BCA with 2% (v/v) copper (II) sulphate). Post-incubation, absorbance at 562nm was measured using a plate reader (Fluostar Omega, BMG LabTech, Aylesbury, Buckinghamshire, UK). Sample concentration was determined using bovine serum albumin (BSA) as a standard at concentrations from 0-1 mg/ml. All reagents were purchased from Sigma-Aldrich.

2.2.3.2 SDS-PAGE

Samples (40 µg unless stated otherwise) were diluted with 5x sodium dodecyl sulphate (SDS) sample buffer (0.25 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.5% (w/v) bromophenol blue and 10% (v/v) β-mercaptoethanol) and boiled at 95°C for 5 min. Gels used had 10% acrylamide content, unless otherwise stated (0.375 M Tris-HCl pH 8.8, 33.5% (v/v) bis-acrylamide (from 30% stock), 0.2% (w/v) ammonium persulphate (APS) and 0.08% (v/v) tetramethylethylenediamine (TEMED). Samples, run alongside Precision Plus protein markers (BioRad, Hemel Hempstead, Hertfordshire, UK), were separated by electrophoresis at 30 mA for 90 min.

2.2.3.3 Western blotting

After SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Hybond-P, GE Healthcare) in a blot module running for 65 min at 30 V. Blots were rinsed with Tris-buffered saline (TBS) and incubated for 2 h in blocking solution (5% (w/v) milk power, 2% (w/v) BSA in TBS + 1% (v/v) Tween-20 (TBST)). The blots were then incubated overnight in primary antibody (5% (w/v) milk powder, primary antibody as indicated in TBS). Blots were washed 4 x 10 min with TBST before addition of secondary antibody (HRP-conjugated anti-IgG; 5% (w/v) milk

powder, 1:2000 (anti-mouse, anti-rabbit) or 1:5000 (anti-goat) antibody in TBST) for 1h, followed by 4 x 10 min washes with TBST. Protein bands were visualised by chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL, USA or ECL Prime Western Blotting Detection Reagent, GE Healthcare) on photographic film (Amersham Hyperfilm ECL, GE Healthcare).

2.2.3.4 Stripping and re-probing blots

Membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% (v/v) SDS and 0.4% (v/v) β-mercaptoethanol) for 30 min at 50°C, followed by 4 x 10 min washes with TBST. After this, membranes were incubated in blocking solution and the protocol performed as described in 2.2.3.3.

2.2.3.5 Enzyme-linked immunosorbent assay (ELISA)

An ELISA kit (Life Technologies) was used to determine the relative abundance of phosphorylated focal adhesion kinase (pFAK; pY397) in total lysates. The protocol was performed as per the manufacturer's instructions. In brief, 50 µg of total lysates was added to a microplate and the volume made up to 50 µl with the provided diluent buffer. Subsequently, pY397 FAK detection antibody was added and the microplate was incubated for 3 h at room temperature. The microplate wells were subsequently washed and anti-rabbit IgG HRP was added for 30 min at room temperature. The microplate wells were again washed and provided stabilised chromogen was added for 30 min at room temperature. Next, an equal volume of stop solution was added and the absorbance was read at 450 nm using a plate reader (Fluostar Omega).

2.2.3.6 Ellman's cholinesterase assay

This assay was based on the classical method (Ellman et al., 1961) with some modifications. For the enzyme assay, in 96 well plates, the Ellman reaction mixture contained 0.4mM 5-5`-dithiobis (2-nitro) benzoic acid (DTNB, Sigma Aldrich), 0.2 mM (medium) or 0.35 mM (lysate) tetraisopropyl pyrophosphamide (iso-OMPA, Sigma Aldrich; BChE inhibitor) or an equivalent concentration of BW248C51 (Sigma Aldrich; AChE inhibitor); 1mM acetylthiocholine iodide (ATCh I, Sigma Aldrich), media sample (optimal volume was 20 µl for medium, 5 µl for lysates based on T-75 culture flasks) and made up to 125 µl with H_2O. All concentrations are final concentrations in the reaction mixture. Solutions were made up in Ellman's phosphate buffer (1:4,

200mM $NaH_2PO_4.2H_2O$: 200 mM Na_2HPO_4, adjusted to pH 7.4). After preincubation with iso-OMPA or BW248C51 for 15 min at 37°C with water and sample to allow binding of the inhibitors with the enzymes, DTNB and ATCh I, were added to the samples to initiate the reaction. Absorbance changes from production of the 2-nitro-5-thiobenzoate (NTB^{2-}) anion were measured using a plate reader (412 nm) (Fluostar Omega). Type IV AChE from *Electrophorus electricus* (diluted 1:12500) (Sigma Aldrich) was used as a positive control in the assays. Absolute values for product formation were calculated from the absorbance values using a millimolar molecular extinction coefficient of 14.64.

2.2.4 Nucleic acid assays

2.2.4.1 Isolation of RNA

RNA was extracted from cells using the RNeasy kit (Qiagen), following the manufacturer's instructions. In brief, cells at $\geq 80\%$ confluence or after pharmacological treatment as indicated above were washed twice in ice cold phosphate-buffered saline (PBS) and harvested in 10 ml PBS. Cells were pelleted at 2700 x g for 5 min (4°C). Cell pellets were re-suspended in lysis buffer RLT (containing 1% (v/v) β-mercaptoethanol) and an equal volume of 70% ethanol added. The resultant solution was added to an RNeasy spin column and centrifuged at 8000 x g for 15 s. The column was subsequently washed with buffers RW1 and RPE as indicated by the manufacturer. RNA was eluted in 50 µl RNase free water and concentration determined by spectrophotometry, reading absorbance at 260 nm.

2.2.4.2 Synthesis of cDNA

cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania). In brief, a 20 µl reaction volume consisted of 1 µg RNA (plus H_2O to 10 µl total), 1 µl oligo (dT) primer, 4 µl reaction buffer, 1 µl RiboLock RNase inhibitor, 2 µl dNTPs and 2 µl reverse transcriptase. The reaction mixture was incubated for 60 min at 42°C, then 5 min at 70°C in a PTC-200 Peltier Thermal Cycler (MJ Research, USA).

2.2.4.3 Reverse transcriptase PCR (RT-PCR)

Specific DNA was amplified in a 20 µl reaction mixture containing: 3 µl dNTP solution (made from equal volumes of 10 mM solutions of individual dNTPs (Promega, Southampton, Hampshire, UK)), 500 nM forward primer, 500 nM reverse primer, 2 µl reaction buffer and 0.5 µl *Taq* DNA polymerase (latter two reagents both from NEB). The reaction volume was made up to 19 µl with H_2O and 1 µl cDNA (equivalent to 50ng) was added. Conditions were as follows: 94°C (5 min), 60°C (30 s), 68°C (50 s), for 35 cycles, then 68°C (10 min), using a PTC-200 Peltier Thermal Cycler.

2.2.4.4 Agarose gel electrophoresis

Amplified DNA incubated with 6x loading buffer (NEB, 2.5% Ficoll 400, 1 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, and 0.015% bromophenol blue, pH 8.0) was loaded on 1% agarose gels with 50 µg ethidium bromide and run for 45 min at 100 V with a 100 bp ladder (NEB). DNA bands were visualised on the Molecular Imager Gel Doc XR System with the Quantity One 4.6.1 programme (BioRad).

2.2.4.5 Quantitative real time PCR (qPCR)

Reactions were performed in triplicate and contained 1 µM forward primer, 1 µM reverse primer, 50 ng cDNA, 10 µl iQ SYBR Green SuperMix (BioRad) and made up to 20 µl with H_2O. Conditions were as follows: one cycle at 95°C (10 min) and then 95°C (10 s), 60°C (15 s) and 72°C (20 s) for 40 cycles on a Rotor-Gene 6000 (Corbett Life Sciences, Cambridge, UK). Transcript abundance was calculated relative to the reference genes U6 or GAPDH, the latter being used in SH-SY5Y only (Hoerndli et al., 2004).

2.2.5 Statistical analysis

All statistical analysis was performed using an unpaired, 2-tailed Student's t-test (Microsoft Excel 2007). Cells treated with pharmacological agents or transfected with DNA constructs were only compared against control, not each other, therefore the t-test is applicable in this work. All error bars displayed are ± SEM. Statistical significance is defined as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). Each n number refers to data derived from an independent cell culture and each immunoreactive band on a Western blot refers to an independent cell culture.

Chapter 3

Results I

Investigation of the mechanism of acetylcholinesterase release from neuronal cells

Chapter 3: Investigation of the mechanism of acetylcholinesterase release from neuronal cells

3.1 Introduction

That AChE can be secreted from cells has been known for nearly 40 years (Chubb and Smith, 1975). This initial study found that K^+ ion treatment increased secretion of AChE from adrenal gland and that this was significantly reduced on omission of Ca^{2+} ions. Later work confirmed these findings and further characterised AChE secretion, revealing both basal and regulated secretion in rat hippocampus (Appleyard, 1987, Appleyard et al., 1988). Subsequently, AChE release was found to be influence by a range of factors, including the ACh analogue carbachol (Appleyard, 1987), locomotor activity (Jones and Greenfield, 1991) and a range of other pharmacological agents (Greenfield, 1991). The role of this secreted form of AChE remains unclear to this day, but even at the time of this initial research, it was noted that AChE was secreted in non-cholinergic areas of the brain, implicating roles beyond the termination of cholinergic neurotransmission (Greenfield, 1991, Appleyard, 1992). A scheme for secretion of AChE in PC12 cells was subsequently put forward by Schweitzer (Schweitzer, 1993). This work was in agreement with the earlier works cited and detailed a system whereby AChE was constitutively secreted (unstimulated secretion) via secretory vesicles, but that there was a second pathway, which was regulated (stimulated secretion) and was Ca^{2+}-dependent. It was suggested that the soluble G_4 tetramer would be secreted via both pathways, whereas secretion of the G_2 form would be exclusive to the latter (Schweitzer, 1993).

However, this does not explain the cellular release of the membrane-anchored, hydrophobic-tailed G_4 species (Massoulie et al., 1993). This species has long been known to have a membrane anchor by which it is associated with the plasma membrane (Gennari et al., 1987, Inestrosa et al., 1987), subsequently identified as PRiMA (Perrier et al., 2002). The notion that this AChE species may be released via pathways distinct from those suggested by Schweitzer (Schweitzer, 1993) led to the suggestion of the involvement of "sheddases" or secretases (Nalivaeva and Turner, 1999). Shedding is a common cellular process, usually involving proteolytic cleavage of a target plasma membrane protein, resulting in release of a soluble ectodomain (Brown et al., 2000). Many physiologically important proteins are shed (Hooper et al., 1997), including APP

(Vassar et al., 1999, Allinson et al., 2004, Kuhn et al., 2010), ACE (Hooper and Turner, 1988, Ehlers et al., 1991, Allinson et al., 2004) and ACE2 (Lambert et al., 2005). Reduction of the cellular release of a protein caused by membrane metalloprotease inhibition is indicative of sheddase involvement, as suggested for AChE (Nalivaeva and Turner, 1999).

This paper also showed an increase in AChE release upon treatment of SH-SY5Y cells with carbachol, a mAChR agonist (Nalivaeva and Turner, 1999), which is in agreement with the earlier findings of Appleyard and Smith, who observed the same phenomenon in rat hippocampus (Appleyard, 1987). Agonism of mAChRs has also been shown to promote shedding of the soluble ectodomain of APP, sAPPα (Nitsch et al., 1992, Canet-Aviles et al., 2002). However, in the case of AChE, it is possible that mAChRs are regulating AChE on a transcriptional level, increasing levels of cellular and secreted AChE, in addition to promoting shedding itself (Nitsch et al., 1998, von der Kammer et al., 2001).

In this work, the cholinergic SN56 cell line has been employed. Since its generation as a hybrid cell line between murine septal neurons and neuroblastoma cells (Hammond et al., 1990), the SN56 cell line has mostly been used as a model to characterise cholinergic neurons. Initially, work focused on the response of cholinergic markers to differentiating agents such as dibutyryl cAMP (Blusztajn et al., 1992), retinoic acid (Pedersen et al., 1995) or a combination of the two (Berse and Blusztajn, 1995, Jankowska et al., 1997). Subsequent work investigated the response of SN56 cells to NGF (Berse et al., 1999, Szutowicz et al., 2004), metals such as zinc (Ronowska et al., 2007) and also to characterise VAChT activity (Barbosa et al., 1999) and calcium waves (Hettiarachchi et al., 2010). In addition, SN56 cells have also been used in the AD field, initially exploring the effects of Aβ on ACh synthesis (Pedersen et al., 1996, Pedersen and Blusztajn, 1997), but also investigating Aβ and tau toxicity (Le et al., 1997), with more recent work concentrating on neurotrophin receptors in AD (Madziar et al., 2003) and the response of neuronal cells to Aβ oligomers (Heinitz et al., 2006, Joerchel et al., 2008).

3.1.1 Aims

The key objectives in this study were to characterise the release of AChE from the SN56 cell line. The foci of investigation were exocytosis and shedding, assessing the relative contribution of each to AChE release. In the case of shedding, the identity of the protease or proteases responsible was sought, via a number of pharmacological treatments. The effect of mAChR activation on AChE release and transcription was also assessed. The final focus was delineation of the cellular signalling pathway linking mAChR agonism with transcriptional upregulation of AChE.

3.2 Optimisation of Ellman's assay and assessment of activity and mRNA of key cholinergic proteins in the SN56 cell line

In this study, significant use of Ellman's assay has been made for cholinesterase activity (Ellman et al., 1961). It was therefore important to characterise the assay under the conditions in which it would be employed and confirm its suitability using the cell lines and amounts of AChE that we intended. As the SN56 cell line was the principal model for this work, AChE presence was first confirmed in culture media samples. Western blot analysis of media samples from three independent sets of cultured cells showed strong immunoreactivity with a commercial anti-AChE antibody (Santa Cruz) (Fig. 3.1A, top panel). The molecular weight was approximately 65kDa, which corresponds to the molecular weight of the AChE monomer. As the AChE tetramer is assembled using disulphide bonds, use of β-mercaptoethanol in the sample buffer for gel electrophoresis will result in dissociation to the component monomers seen in the figure. The identity of the immunoreactive bands was further confirmed by use of a second commercial antibody to AChE (Millipore) (Fig 3.1A, bottom panel), which resulted in immunoreactive bands at the same molecular weight as the Santa Cruz antibody.
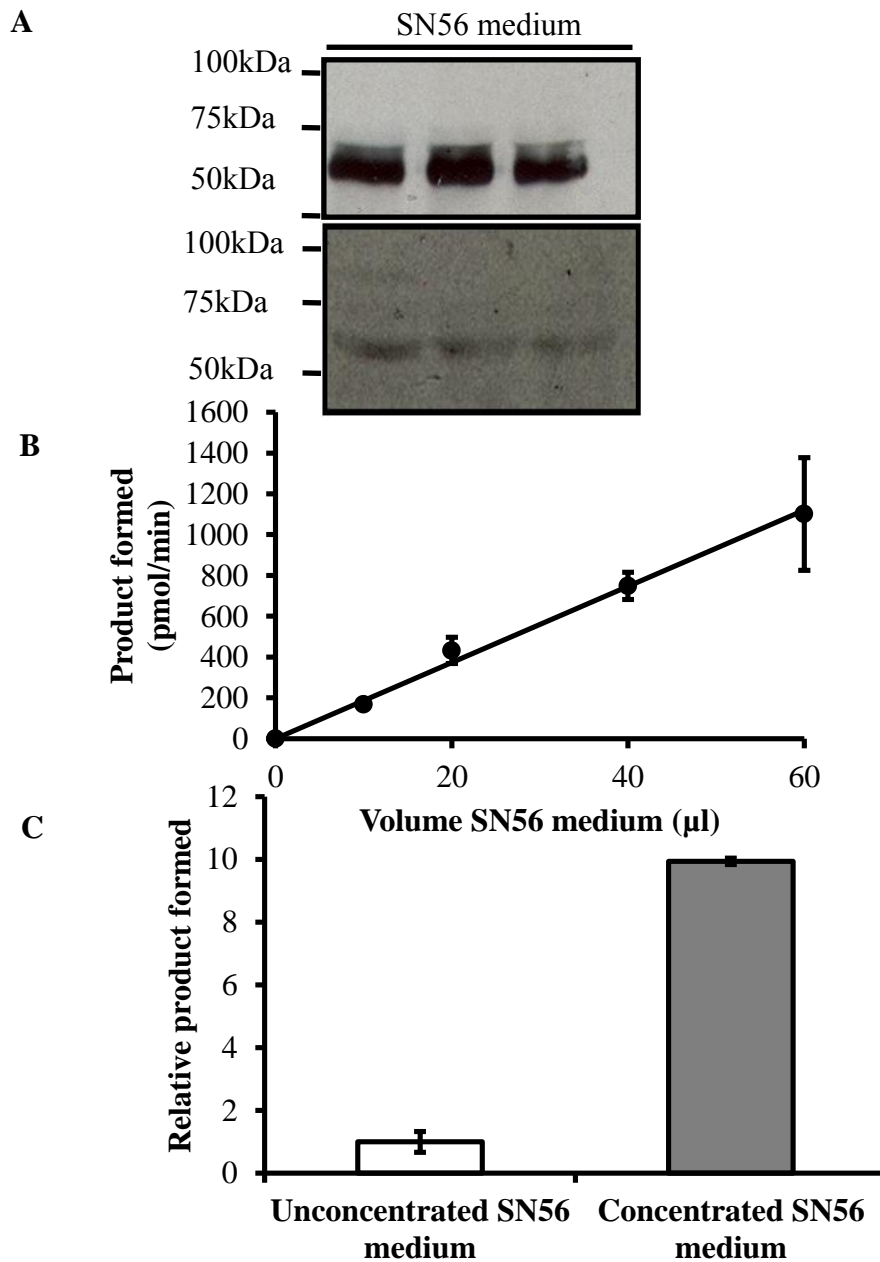
Figure 3.1: optimisation of Ellman's assay
SN56 cells were cultured as described (see section 2.2.1.1) and then incubated in OptiMEM for 24h. **A.** Representative Western blot for AChE. Media samples were spin-concentrated and 20μg protein was run on an SDS-PAGE gel, followed by Western blotting for AChE, with antibodies from Santa Cruz (top panel, 1:500) and Millipore (bottom panel, 1:500) **B.** Concentrated SN56 media samples were assayed for AChE activity over a range of sample volumes, using Ellman's assay (n=3). **C.** SN56 media samples were assayed for AChE activity using Ellman's assay, either pre- or post-concentration (n=3).

That the relationship between AChE protein and activity was directly proportional over the range expected in subsequent assays was ensured. This was important if AChE activity was to be used as a correlate of AChE presence. Using media sample volume as a correlate of AChE protein, a directly proportional relationship between sample volume and AChE activity was shown (Fig 3.1B). This was important in demonstrating the suitability of Ellman's assay for use in our assays. Finally, AChE activity was assayed in un-concentrated SN56 media samples and although AChE activity was detectable, it was at a low level. Centrifugation of these media samples in a Vivaspin column enriched AChE activity approximately ten-fold (Fig 3.1C). Consequently, it was decided that for future assays media would be spin-concentrated before assays.

3.2.1 Cholinesterase activity

In this work, two neuronal cell lines, namely the SN56 (Hammond et al., 1990) and SH-SY5Y neuroblastoma lines were used (Biedler et al., 1978). The former is a mouse hybridoma cell line, generated by the fusion of mouse septal neurons with neuroblastoma cells, with the resultant SN56 cells displaying a cholinergic phenotype (Hammond et al., 1990).The SH-SY5Y cell line is both widely used and well characterised, yet use of the SN56 line is relatively sparse.

The SN56 cell line was further characterised in terms of its cholinesterase activity and thus its suitability as a model system in our assays was assessed. SN56 cells were compared against SH-SY5Y and the neuroblastoma NB7 cell line. The latter display an adrenergic phenotype, expressing high levels of dopamine β- hydroxylase and also tyrosine hydroxylase (Thiele, 1998). NB7 cells also express high levels of APP, Fe65 and NEP (Belyaev et al., 2009). To compare model system suitability, AChE and BChE activities were compared in media samples between the cell lines (Fig. 3.2). To do this, iso-OMPA was used to inhibit BChE and BW248C51 was used to inhibit AChE and in all cases activity was assessed through production of the 2-nitro-5-thiobenzoate anion.

In SN56 and SH-SY5Y cells, AChE predominated relative to BChE to a large extent in media samples, with ratios of 5:1 whereas in NB7 cells the converse was seen, BChE predominated with a ratio of 3:2. In media, total cholinesterase activity was equivalent in SH-SY5Y and NB7 cells, whereas it was twofold higher in SN56 cells (Fig. 3.2). These findings were also recapitulated in membrane fractions (Hicks et al., 2013).

Figure 3.2: distribution of ChEs in neuronal cell lines
NB7, SH-SY5Y and SN56 cell lines were cultured in OptiMEM for 24h, media samples were taken and assayed for ChE activity using Ellman's assay. In all assays, iso-OMPA was used to inhibit BChE and BW248C51 was used to inhibit AChE.
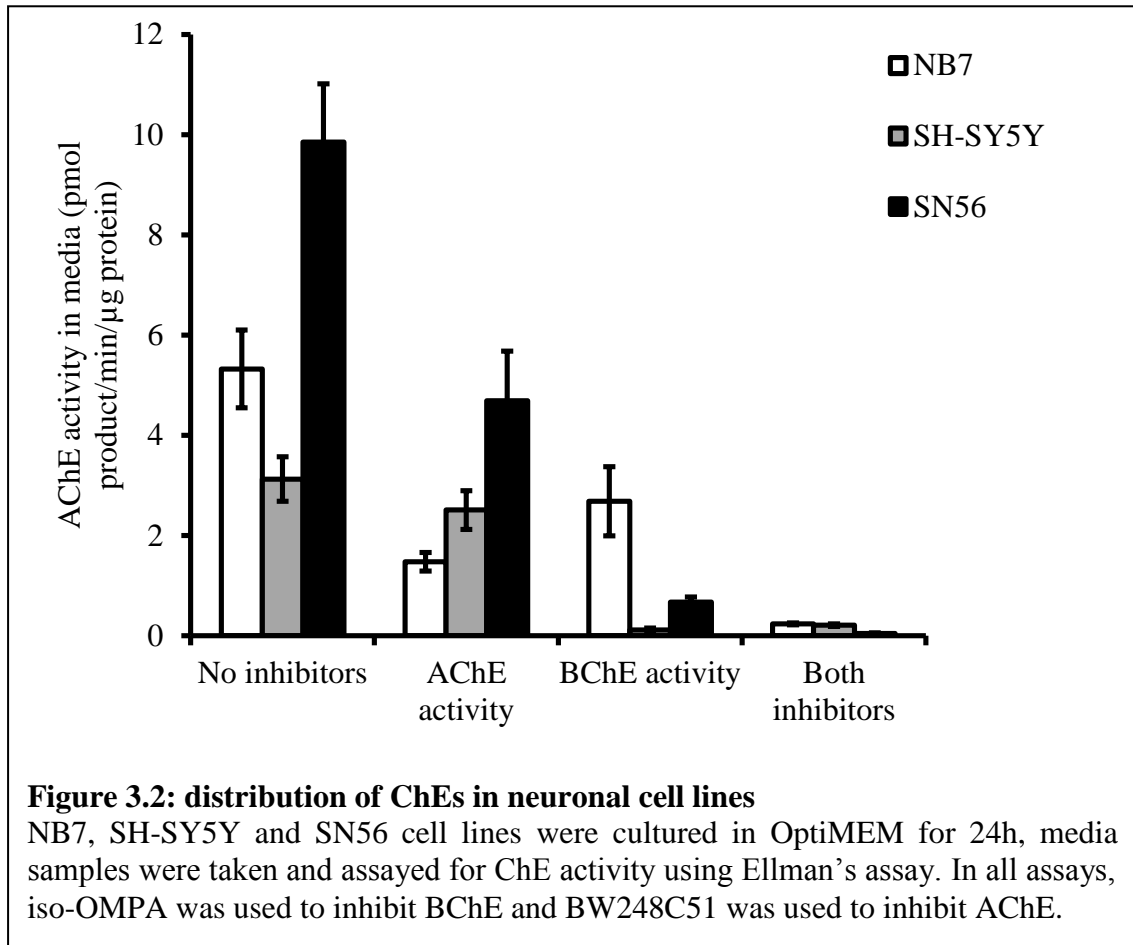
3.2.2 mRNA levels of AD-related and cholinergic genes

After investigation of the relative ChE activities in media samples from SN56 and SH-SY5Y cells, these cell lines were further characterised, with specific foci on candidate sheddases and cholinergic genes. Shedding is a widespread phenomenon, but ADAMs represent a family of proteases involved in a large number of shedding processes. It has also been suggested that AChE is shed by ADAMs, in a manner similar to the non-amyloidogenic processing of APP (Nalivaeva and Turner, 1999). Consequently, the initial investigation focused on the expression of those ADAMs which have been linked to APP proteolysis, namely ADAMs 9, 10 and 17. The mRNA transcript levels of ADAM12 were also investigated, as it is also a recognised sheddase (Edwards et al., 2008). Use of RT-PCR revealed SN56 and SH-SY5Y cells to express all of the ADAMs investigated (Fig. 3.3).

Figure 3.3: comparison of mRNA levels of key AD- and cholinergic-linked genes in SH-SY5Y and SN56 cells
Representative RT-PCR images for key mRNA transcripts. SN56 and SH-SY5Y cells were cultured as described (section 2.2.1.1) until $\geq$ 80% confluence. RNA was extracted and cDNA synthesised. DNA was amplified using RT-PCR and primers specific to the genes indicated. The PCR products were resolved on a 1% agarose gel, using EtBr staining.

As SN56 cells were used as a model of cholinergic neurons, it was important to verify expression of cholinergic markers in these cells. SH-SY5Y cells are very widely used in neuroscience research. Therefore, SN56 cells were compared against SH-SY5Y, the latter representing a very common alternative neuronal model, although not of cholinergic origin. Of those genes investigated, both cell lines expressed AChE, but also two AChRs, specifically the α7 nAChR and M3 mAChR. However, some differences were observed, insofar as ChAT mRNA was detected in SN56 cells, but not in SH-SY5Y. Conversely, M1 mAChR transcripts were found in SH-SY5Y, but not in

SN56 cells (Fig. 3.3). The mRNA for BChE or PRiMA was not detected in either cell line (data not shown), likely due to the sensitivity of the RT-PCR process.

3.2.3 Protein levels of cholinergic and AD-linked proteins

In order to further characterise these cell lines, this time at the protein level, ADAMs and cholinergic proteins were investigated. ADAMs 10 and 17 have been implicated as the major sheddases involved in APP processing (Allinson et al., 2004, Kuhn et al., 2010). Western blot analysis of SH-SY5Y and SN56 cell lines revealed two major immunoreactive bands when probed with an anti-ADAM10 antibody. The molecular weights were consistent with the ADAM10 zymogen precursor (at 80kDa) and also the furin-cleaved active form of the enzyme (at 60kDa). There was a large disparity in expression of ADAM10 between the two cell lines. Although faint immunoreactive bands were visible for SH-SY5Y cells, ADAM10 was expressed to a substantially higher level in SN56 cells (Fig. 3.4). This relationship was mirrored at the mRNA level (Fig. 3.3). Analysis of ADAM17 at the protein level also revealed differences between the two cell lines. Again, two immunoreactive bands were detected, representative of the precursor (at 120kDa) and active forms of the enzyme (at 90kDa). The expression levels of the pro-form did not differ greatly between the cell lines, but the active form of ADAM17 was much more abundant in SN56 cells.

Furthermore, Western blot analysis revealed a single major immunoreactive band at 90kDa for AChE. There was no visible difference in band intensity and hence protein expression between the two cell lines.

Expression of APP was also investigated. APP is a key AD protein and has been suggested to be involved in the regulation of AChE expression through its intracellular domain (Bimonte et al., 2004). Both cell lines were shown to express APP, albeit at a much greater level in SH-SY5Y cells. The immunoblots revealed a single major band at 120kDa in both cell lines, likely representing APP_{695}, which significantly predominates in neurons (Gralle and Ferreira, 2007).

Figure 3.4: comparison of protein levels of key AD- and cholinergic-linked genes in SH-SY5Y and SN56 cells
Representative Western blots for key proteins. SN56 and SH-SY5Y cells were cultured as described (section 2.2.1.1) until ≥ 80% confluence. Lysates were generated as described (section 2.2.2.2) and 35µg protein was run on an 8% SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted for the proteins indicated.

Finally, two other cholinergic proteins were investigated. The high-affinity choline transporter (CHT, SLC2A7, 70kDa) is responsible for re-uptake of choline into presynaptic terminals. It was shown to be expressed at much higher levels in SN56 cells, relative to SH-SY5Y cells. PRiMA, the membrane anchor of AChE, was also assessed by immunoblot, which showed PRiMA protein expression in SN56 cells, but not in SH-SY5Y cells, with an immunoreactive band at approximately 90kDa.

3.2.4 Assessment of relative cholinesterase activities and distribution in a non-neuronal cell line

After investigation of ChE activity in three neuronal cell lines, SN56, SH-SY5Y and NB7, a non-neuronal cell line was investigated, the mouse fibroblast cell line, GD25. The rationale for their use will be explained further in chapter 4. These cells are null for β1 integrin (ITGB1) (Fassler et al., 1995) and we also used GD25β1 cells, with ITGB1 re-expressed. The results seen for AChE were strikingly different from those in neuronal cell lines. AChE activity in the media samples were of a similar magnitude to the neuronal cell lines, yet AChE was only detectable at minimal levels in lysates, several orders of magnitude lower than in neuronal cell lines. However, AChE mRNA is present in these cells (see chapter 4). The expression of ITGB1 did not affect the AChE activity in media samples, suggesting that ITGB1 knockout in GD25 cells is not the cause of the dramatic disparities seen between these cells and neuronal cell lines. Furthermore, no BChE activity was detectable in either lysate or medium.

Figure 3.5: comparison of AChE and BChE activity and distribution thereof between lysates and cell media samples in a non-neuronal cell line GD25 and GD25β1 cells were cultured as described (section 2.2.1.1) until ≥ 80% confluence. Cells were lysed and media samples extracted. Both lysates and media samples were assessed for AChE and BChE activity using Ellman's assay (n=9).

3.3 Investigating the functional AChE unit effecting hydrolysis of ATCh I

Higher order AChE species are known to be associated via disulphide bonds in the C-terminal regions of the individual subunits (Dvir et al., 2004, Dvir et al., 2010). It follows, then, that use of an agent acting to disrupt these bonds would consequently disrupt the formation of AChE tetramers. In this study, DTNB has been used, which binds free thiol groups (Ellman, 1959) and hence prevents formation of higher order AChE species, or causes their dissociation once formed. This allows assessment of the AChE unit responsible for hydrolysis in the assays, by recording the effects of the disruption of AChE tetramers. The efficacy of this approach is shown by the increase in the presence of AChE monomers following tetramer disassembly, as detected by Western blot (Fig. 3.6A, 65kDa). After this confirmation, the effects of disruption of AChE tetramers on AChE activity in medium were assessed. Assaying AChE activity using Ellman's assay showed a significant decrease of 50% in AChE activity in media samples from SN56 cells upon treatment of 100μM DTNB (Fig. 3.6B). Although DTNB is present in the Ellman's reaction mixture, addition of a further 100μM DTNB to the reaction mixture simultaneously with a BChE inhibitor, iso-OMPA, had no effect on AChE activity (data not shown).

Figure 3.6: assessing AChE activity after disruption of disulphide bonds
SN56 cells were cultured as described (section 2.2.1.1) until ≥ 80% confluence. Cells were then incubated for 24h in OptiMEM containing 100μM DTNB or an equivalent volume of solvent (control). Media samples were taken, spin concentrated and **A.** run on an SDS-PAGE gel, followed by Western blotting for AChE, with antibodies from Santa Cruz. A representative Western blot is shown. **B.** assayed for AChE activity in cell medium using Ellman's assay (n=14, p= 2 x 10^{-6}).

3.4 Investigating the cellular release of AChE from SN56 cells

3.4.1 Time course of AChE release

As AChE is demonstrably a secreted protein (Figs. 3.1 and 3.2), the mechanism by which AChE is released from the cell lines was subsequent investigated. Initially, the release of AChE into the culture medium over a 30 hour period was explored, assaying media samples and lysates at nine time points, specifically 15, 30 and 45 min and 1, 2, 4, 6, 24 and 30 h.

Figure 3.7: changes in AChE activity in lysates and media samples over a 30h period
SN56 cells at ≥80% confluence were incubated in OptiMEM for 15, 30, 45min or for 1, 2, 4, 6, 24 or 30h. At each time point, medium was collected, cells harvested and lysates were generated as described (section 2.2.2.2). Lysates and media samples were then analysed for AChE using Ellman's assay (n=6).
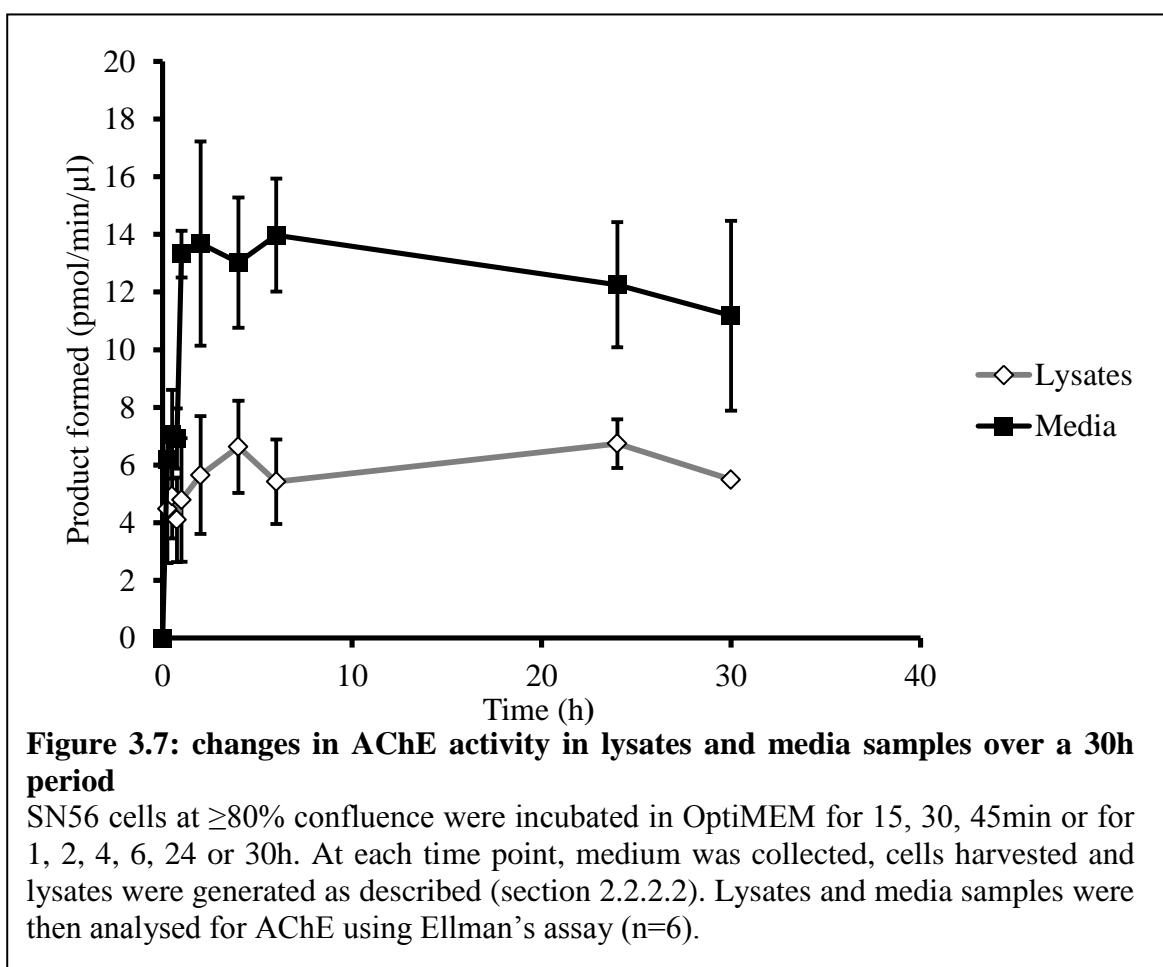
For the 0h time point with media samples, OptiMEM alone was measured and had no measurable AChE activity. Hence at 0h, the AChE activity of the medium is 0pmol product formed/min/µl sample. However, such was the rate of AChE release from SN56 cells, the maximal rate of enzyme activity could be obtained after 1 h of culture. After this early zenith, the AChE activity in media samples obtained at subsequent time points did not increase and a plateau was observed between 1 h and 30 h (Fig. 3.7). Analysis of lysates revealed no marked changes in AChE activity over the 30 h timespan of the

experiments (Fig 3.7). Although the activity in media samples was higher, the culture medium was spin concentrated and accounting for this gives an approximate lysate: medium activity ratio of 5:1 at 1 h.

3.4.2 Pharmacological inhibition of AChE release

To ascertain the nature of the enzymes responsible for the putative shedding of AChE, a range of protease inhibitors were used. As sheddases are predominantly metalloproteases (Hooper et al., 1997), EDTA was used. EDTA chelates metal ions and therefore acts as a *de facto* metalloprotease inhibitor. Incubation of SN56 cells with 1mM EDTA resulted in a significant (50%), decrease in AChE activity in media samples (Fig. 3.8A). Further, it also caused a significant increase in BChE activity in the media (data not shown). Further investigation revealed the shedding process to be sensitive to the hydroxamate metalloprotease inhibitor, batimastat. This inhibitor is more specific than EDTA, targeting matrix metalloproteases (MMPs) and ADAMs with particular potency. Incubation of SN56 cells with batimastat resulted in a decrease of AChE activity in the media of similar magnitude to the EDTA treatment, i.e. in the 40-50% range (Fig. 3.8A).
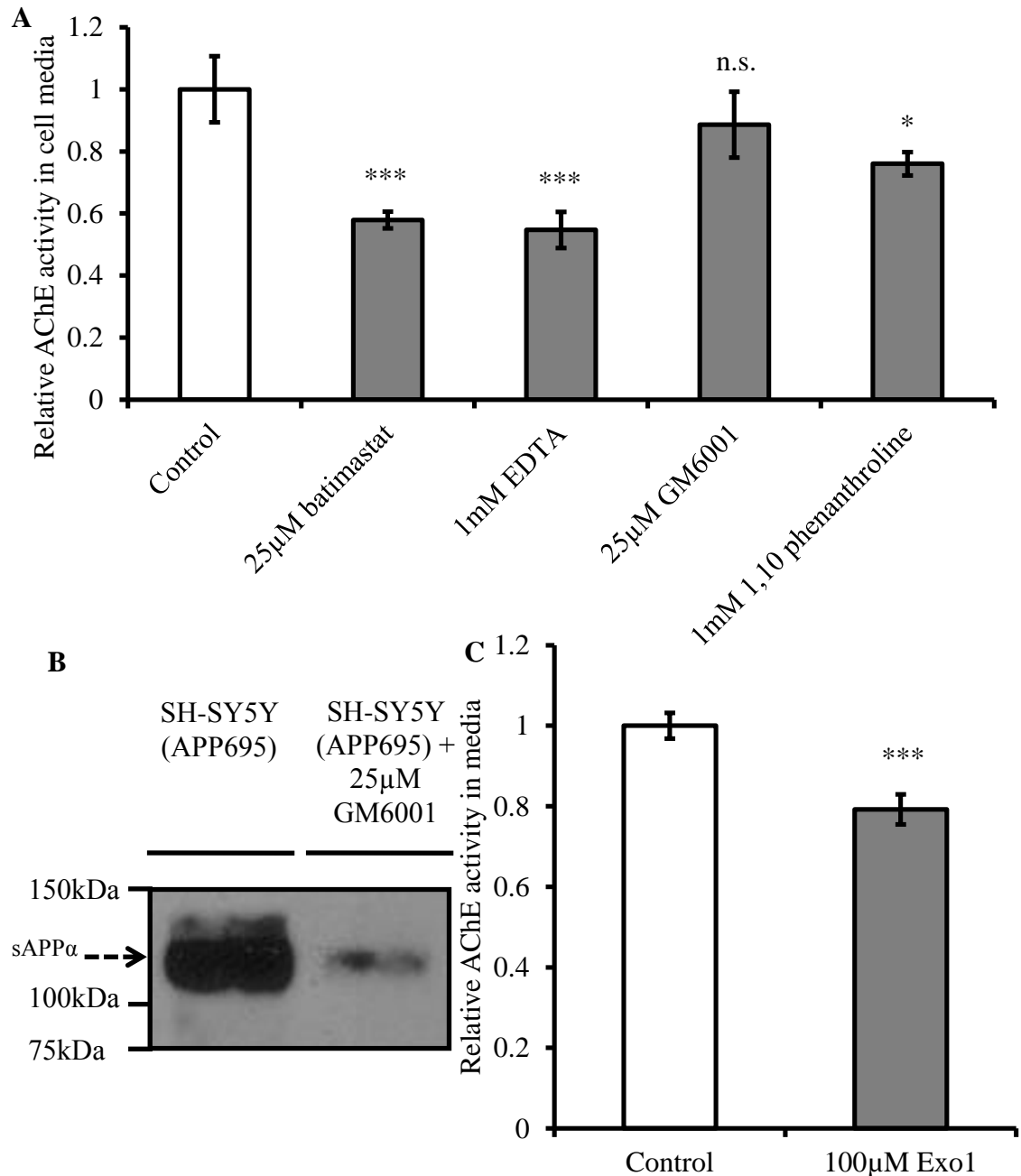
Figure 3.8: pharmacological inhibition of AChE release in SN56 cell
A. SN56 cells were cultured as described in Methods and Materials and incubated for 24 h with OptiMEM containing either DMSO (control) or 25μM batimastat (n=9, p=0.00017), or 1mM EDTA (n=12, p=1.89 x 10^{-6}), or 25μM GM6001 (n=11, p=0.2). Cells were also incubated for 4h with 1,10 phenanthroline (n=6, p=0.016) as indicated. Media samples were then extracted and assayed for AChE activity with Ellman's assay. **B.** Representative Western Blot for sAPPα in SH-SY5Y (APP$_{695}$) cells, either control or after 24 h 25μM GM6001 treatment. **C.** SN56 cells were cultured as described in Methods and Materials and incubated for 24 h with OptiMEM containing DMSO or 100μM Exo1 as indicated. Media samples were then extracted and assayed for AChE activity with Ellman's assay (n=11, p=0.0002).

GM6001, a metalloprotease inhibitor with a broadly similar specificity profile to batimastat, was then used, which did not affect AChE release (Fig. 3.8A). The final metalloprotease inhibitor used was 1,10 phenanthroline, another cation chelator. Incubation of SN56 cells with 1mM 1,10 phenanthroline for 4h resulted in a significant (30%) decrease in AChE release.

Given the failure of GM6001 to block AChE release, the functionality of GM6001 was checked. The α-secretase responsible, in part, for the non-amyloidogenic processing of APP is known to be sensitive to GM6001 (Oh et al., 2004, Delarasse et al., 2011). Inhibition of α-secretase prevents generation of the soluble sAPPα ectodomain, which can be detected by Western blot. SAPPα levels in SH-SY5Y (APP$_{695}$) with or without GM6001 were then compared. Upon treatment with GM6001, a substantial decrease in sAPPα immunoreactivity at 120kDa in media samples was observed (Fig. 3.8B).

As has been previously suggested (Schweitzer, 1993), exocytosis may also play an important role in the secretion of AChE from the cell. In order to investigate this, Exo1 was used, which is a Golgi ARF6 (ADP-Ribosylation Factor) GTPase activator (Feng et al., 2003). Exo1 acts to inhibit exocytosis and treatment of SN56 cells resulted in a 25% decrease in AChE release into the culture medium (Fig. 3.8C).

The vast majority of shedding processes involve cleavage of a membrane bound protein by one or more proteolytic enzymes (Hooper et al., 1997). Examples of this are many, but AChE does not fit this paradigm. It is not a membrane spanning protein, nor is it GPI-anchored in neuronal cells, as is the case for proteins such as the prion protein, which is shed by phospholipase activity (Stahl et al., 1987). It is anchored to the membrane by a second protein, namely PRiMA (Perrier et al., 2002), the only roles of which seem to be as a biosynthetic chaperone and membrane anchor for AChE tetramers (Perrier et al., 2002, Xie et al., 2010a, Chen et al., 2011b). A literature search revealed that the thyroid stimulating hormone receptor (TSHR) and its cleavage and secretion bear considerable similarity to the release of AChE. Both processes involve a membrane anchor (PRiMA for AChE; the β-subunit for TSHR) to which the other component (AChE in PRiMA-AChE; the α-subunit for TSHR) is disulphide bonded. Work dating back over fifteen years elucidated the mechanism for TSHR shedding,

whereby TSHR ectodomain release is dependent on both a thiol reductase and metalloprotease (Couet et al., 1996a, Couet et al., 1996b), and so this paradigm was applied to this study.

Figure 3.9: pharmacological inhibition of AChE shedding in SN56 cells by targeting disulphide bonds
A. SN56 cells were cultured as described (section 2.2.1.1) and incubated for 24 h with OptiMEM containing DMSO or 25µM batimastat (n=8, p=7.6 x 10^{-5}), 1mM bacitracin (n=7, p=0.00066), or a combination thereof (n=8, p=0.0036), as indicated. **B**. SN56 cells were incubated for 24h with 10µg/ml IgG (control) or 10µg/ml RL90 anti-PDI antibody (n=5, p=0.02). Media samples were then extracted and assayed for AChE activity with Ellman's assay.

Bacitracin is routinely used to inhibit thiol isomerases (Dickerhof et al., 2011) and has been previously used to investigate the role of thiol isomerases in shedding of TSHR (Couet et al., 1996a). Incubation of SN56 cells with bacitracin resulted in a significant (40%) decrease in AChE activity in the media samples (Fig. 3.9A). This was approximately equivalent to the decreases seen with either batimastat (Fig. 3.9A) or EDTA (Fig 3.8A). Co-incubation of cells with bacitracin and batimastat did not show significantly greater inhibition than with either agent separately (Fig. 3.9A). In order to identify the thiol isomerase participating in the shedding process, we used the RL90 antibody against PDI (Couet et al., 1996a, Lahav et al., 2002), which also blocks release of TSHR (Couet et al., 1996a). Treatment of SN56 cells with 10μg/ ml RL90 resulted in a significant decrease in shed AChE, by approximately 20% (Fig. 3.9B).

3.5 Effects of mAChR agonism on AChE activity and mRNA

Muscarinic acetylcholine receptors (mAChRs) have been linked to the shedding of a number of proteins, such as APP (Nitsch et al., 1992, Nitsch et al., 1993, Canet-Aviles et al., 2002). It has been suggested that signalling is through protein kinase C (Slack et al., 1993, Canet-Aviles et al., 2002), which most closely associates this process with the G_q heterotrimeric G protein subunit and hence M1 and M3 mAChRs (Lanzafame et al., 2003, Eglen, 2006). In order to investigate mAChR involvement in AChE shedding in SH-SY5Y and SN56 cells, mAChR agonists muscarine and carbachol were used in addition to the antagonist, atropine (Cushny, 1910, Higuchi et al., 1981, Meyer et al., 1982).

Treatment of SH-SY5Y cells with the ACh analogue carbachol resulted in a significant increase in AChE activity in the culture medium. In order to specifically analyse mAChRs, the cells were incubated with muscarine, to activate mAChRs, but which does not affect nAChRs. This treatment resulted in a significant increase in AChE activity in the SH-SY5Y media samples of similar magnitude to that evoked by carbachol treatment, namely 50-60%. Furthermore, the effects of carbachol could be completely ablated by pre-incubation with the selective mAChR antagonist, atropine (Fig. 3.10A).

Figure 3.10: pharmacological activation of mAChRs and effects on AChE
A. SH-SY5Y cells were cultured as described (section 2.2.1.1) and incubated for 24h with OptiMEM containing DMSO, 10μM muscarine (n=9, p=0.014), or 25μM carbachol (p=0.021) (+/- 15 min pre-incubation with 10μM atropine, p=0.0016) as indicated (n=6 for all groups, unless indicated).
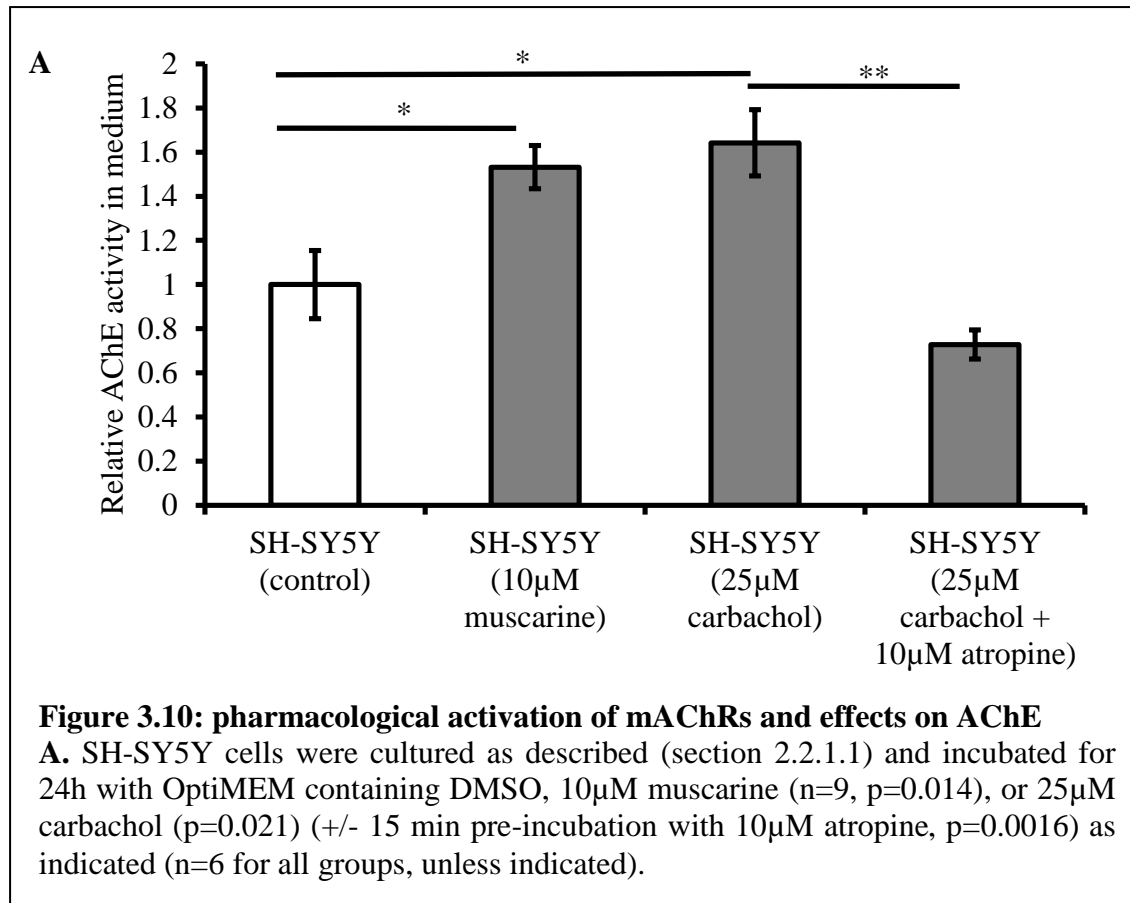
After having shown significant increases in AChE release upon carbachol and muscarine treatment, AChE mRNA levels were assessed after treatment with the former. Treatment of SH-SY5Y cells with 25μM carbachol for 24h resulted in a significant increase in AChE mRNA transcripts, of approximately 80% (Fig. 3.11A). Previous reports have linked Egr family transcription factors as effectors of mAChR regulation of gene expression (von der Kammer et al., 1998, von der Kammer et al., 2001).

Levels of Egr-1 mRNA and also of the associated proteins Elk-1 and serum response factor (SRF) (Besnard et al., 2011) were therefore assessed in SH-SY5Y cells. Although Egr-1 mRNA levels were significantly elevated by 180% after carbachol treatment, no changes in mRNA levels of Elk-1 or SRF were observed (Fig. 3.11A). However, given that these mediators may be regulated by phosphorylation, rather than through expression, activation of ERK was examined after carbachol treatment, a kinase shown to target and phosphorylate Elk-1 (Davis et al., 2000).
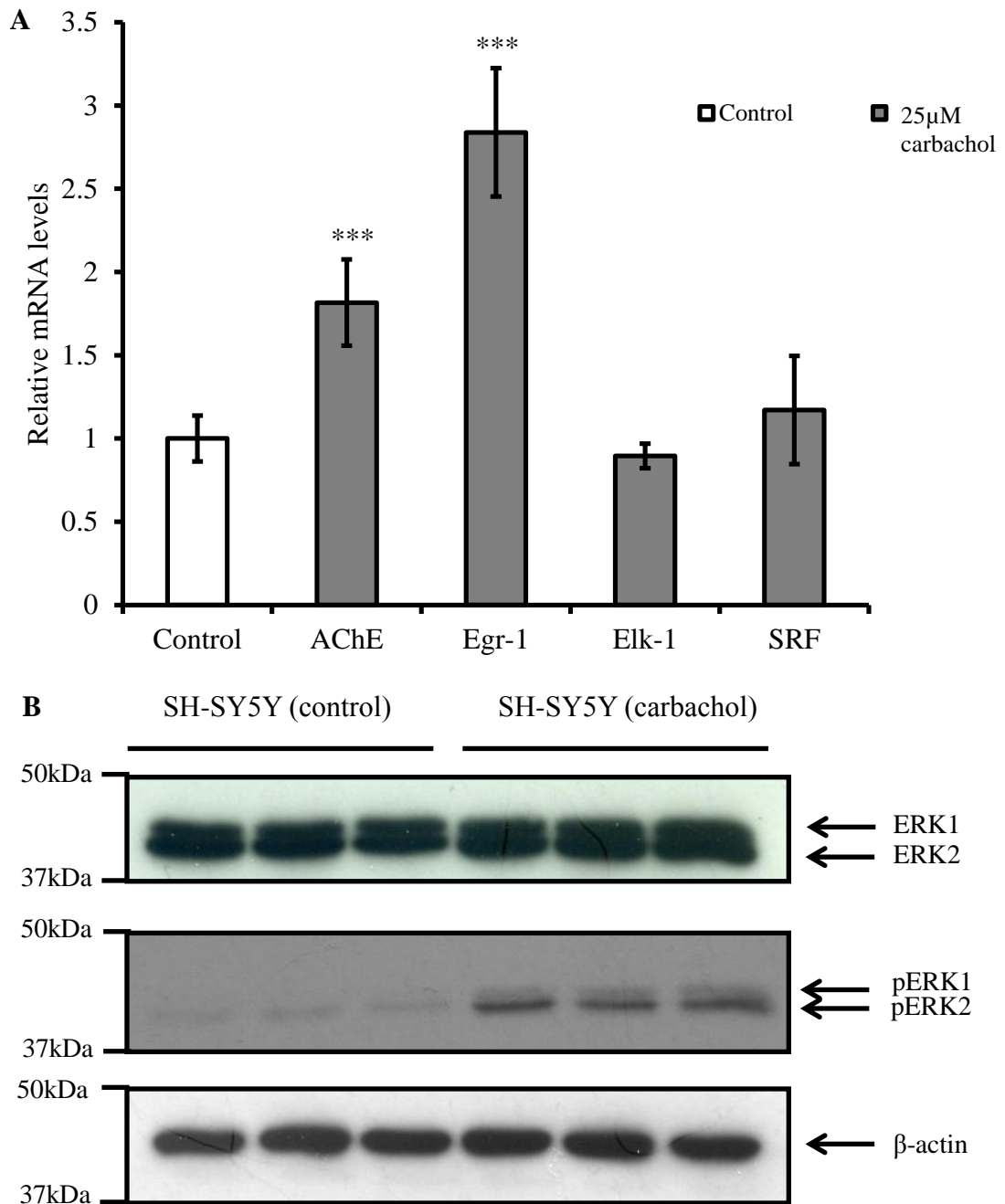
Figure 3.11: carbachol treatment and transcriptional analysis of AChE and associated transcription factors and ERK phosphorylation in SH-SY5Y cells
A. qPCR analysis of relative changes in mRNA levels of AChE (n=9, p=0.0002), Egr-1 (n=9, p=1.7 x 10^{-6}), Elk-1 (n=9, p=0.38) and SRF (n=9, p=0.62) in SH-SY5Y cells after treatment with 25μM carbachol for 24h. **B.** Western blot analysis of SH-SY5Y cell lysates after 25μM carbachol treatment for 24h. Immunoreactivity represents ERK1/2 (top panel), phospho-ERK1/2 (middle panel) and β-actin (bottom panel).

Using Western blot, a significant increase in phospho-ERK immunoreactivity was detected, with no corresponding changes in total ERK. Although increased phosphorylation of ERK1 and 2 was observed, the most marked increase in phosphorylation is of ERK2 (Fig. 3.11B).

3.6 Discussion

3.6.1 Characterisation of the cholinergic phenotypes of neuronal and non-neuronal cell lines

In this work, several cell lines were characterised, examining heterogeneity therein and searching for the most relevant model of AChE release. SN56 and SH-SY5Y cells were shown to share several similarities: in the predominance of AChE over BChE and the relatively similar levels of BChE activity. The key difference between SN56 and SH-SY5Y cells was the far greater AChE activity in SN56 cells, seen in lysates and in media samples. On the contrary, NB7 cells were shown to differ from SN56 and SH-SY5Y cells in their relative levels of ChE activity, with BChE predominating.

Of the neuronal cell lines tested, SN56 cells showed many of the characteristics that would be expected of cholinergic neurons, i.e. high AChE activity relative to low, but measurable BChE activity. However, SH-SY5Y cells also exhibited many of the same features as SN56, in terms of ChE distribution and relative ChE activities. Hence, these cells may also be of use in further investigations. However, NB7 cells showed different characteristics to the other neuronal cell lines thus indicating that it is not an appropriate model for ChE research.

Characterisation was also extended to incorporate a non-neuronal cell line, the mouse fibroblast cell line, GD25. Striking differences were found between these cells and the neuronal cell lines. To a greater or lesser extent, all the neuronal cell lines tested showed measurable BChE activtity in lysates and media. However, in GD25 cells, no acetylthiocholine hydrolysis was observed in the presence of a potent AChE inhibitor. This is indicative of a total lack of BChE activity, both in lysates and media samples. Similarly, in total lysates of GD25 cells, AChE activity was not detectable. However, in conditioned media samples, AChE activity was detected at comparable levels to the neuronal cell lines. From this it was inferred that in the GD25 cells, the catalytically

active AChE tetramer is not trafficked in the same way as in neuronal cell lines. In the latter, it is likely that much of the ChE activity in lysates derives from membrane anchored tetramers. It seems from this investigation that this is not occurring in GD25 cells and a catalytically active molecule is only formed post-release from the cell. GD25 cells are null for ITGB1, so to confirm that the findings did not derive from this, we also used GD25β1 cells, which recapitulated the findings from the GD25 cells.

Thus, a non-neuronal cell line selected for this study was shown to have dramatic differences from all the neuronal cell lines tested. This would suggest that care should be taken when interpreting data from non-neuronal cell lines. Non-neuronal cell lines enjoy a pervasive popularity in neuroscience research, such as HEK and HeLa lines, and the data here and that of others (Belyaev et al., 2010) indicate that they should be used with great caution.

As stated, SN56 and SH-SY5Y cells share similarities in relative ChE expression and distribution. The expression of several putative sheddases and other cholinergic markers was subsequently assessed. Expression in SN56 of a number of other key cholinergic proteins has been verified: AChE, BChE, ChAT, CHT and PRiMA. These findings are important in confirmation of SN56 as a cholinergic cell line as described elsewhere (Hammond et al., 1990). This validation confirms that SN56 are a representative model of forebrain cholinergic neurons and therefore are an appropriate model for our research. Their phenotype has been compared to the common neuroblastoma cell line SH-SY5Y, with which SN56 cells share a number of important similarities. However, there are a number of differences, such as low expression of ChAT, CHT and PRiMA in SH-SY5Y cells, which confirms that they are not cholinergic . Similarly, although both cell lines express mRNA transcripts for ADAM9, 10, 12 and 17, protein analysis revealed substantially higher levels of active sheddases in SN56 cells. These data suggest that SN56 are the pre-eminent cellular model of the cell type most affected in AD. However, there are enough similarities between the two cell lines to suggest that SH-SY5Y would represent a reasonable model if there was a valid reason for excluding the use of SN56.

3.6.2 Analysis of AChE release reveals rapid attainment of maximal AChE acitivity in media samples

The rate of cellular release of AChE over a 30 h time period was further assessed. The OptiMEM medium in which the cells were grown contained no detectable AChE activity. However, already after 1 h of culturing the cells, the AChE activity had reached its maximal level. After a rapid increase during the first hour of culture, the catalytic rate of the enzyme seen in media samples at subsequent time points did not increase. Although these data do not reveal the mechanism behind this substantial increase within the first hour, it is likely due, at least in part, to the high expression of possible sheddases in this cell line. Furthermore, the attainment of a plateau in AChE activity is indicative of a tightly controlled equilibrium between cellular and extracellular AChE. No significant changes were seen in AChE activity in total lysates over the 30h timespan of the assay. This, and the short time over which the increase in AChE activity was observed in the medium, is indicative of a rapid mobilisation of catalytically inactive intracellular AChE. This paradigm has been previously documented in the literature (Fernandez et al., 1996).

It is possible that ACh serves as a sensor of AChE activity in the medium, so when AChE falls, ACh levels rise substantially. In this case, high levels of ACh and consequent receptor activation may initiate a signalling pathway to increase cellular AChE release. As AChE has been removed from the medium in the experiment performed here, it is possible that the rapid and substantial increases in AChE activity in the culture medium are driven by ACh signalling.

3.6.3 Pharmacological treatment reveals metalloprotease involvement in AChE release

Directed by other studies investigating shedding of other proteins, the possible role of metalloproteases in AChE shedding was initially probed. Other pathways for AChE release have previously been described, but of these, only a shedding-type event would necessitate protease involvement.

Significant reductions in AChE activity in media samples after EDTA treatment strongly suggested the existence of a shedding process and also the involvement of a metalloprotease. This concurs with an earlier report whereby pronase treatment of liposome surface-bound AChE resulted in release of 60-65% of the AChE (Stieger and

Brodbeck, 1985). The broad-spectrum nature of EDTA prevented any conclusions about the identity of the protease. The response to EDTA treatment was mimicked by treatment with batimastat, a more selective metalloprotease inhibitor. Analysis of batimastat targets showed that the metalloprotease was a member of either the ADAM or MMP families. A significant finding was the metalloprotease's resistance to a second metalloprotease inhibitor, GM6001. This inhibitor and batimastat have a majority of targets in common, although GM6001 has been shown to have relatively very low potency towards ADAM12 (Oh et al., 2004), uniquely among ADAMs. Other common sheddases, such as ADAMs 9, 10, 17 and MMP9 are targeted by both batimastat and GM6001 with nanomolar K_d values. The literature reports ADAM12 to be exquisitely sensitive to 1,10-phenthanthroline (Oh et al., 2004), which was able to effect a 25% decrease in AChE medium activity after only 4 h. However, it is clear that the results of this treatment are only suggestive of ADAM12 involvement, rather than confirmatory.

Over thirty years ago, Bon and Massoulié first suggested that the hydrophobic anchor of AChE was susceptible proteolysis by pronase and proteinase K (Bon and Massoulie, 1980). Subsequently, Fuentes and Inestrosa documented the proteinase K-mediated release of hydrophilic AChE from its membrane-bound amphiphilic form (Fuentes and Inestrosa, 1992). Treatment of *Torpedo* AChE with proteinase K revealed a band of almost identical molecular weight to that seen after detergent solubilisation. Furthermore, after proteinase K treatment, AChE was unable to reconstitute into liposomes, suggestive of a loss of its membrane anchor (Stieger and Brodbeck, 1985). SDS-PAGE analysis of AChE from human caudate nucleus revealed a heavy monomer and a light monomer. Proteinase K treatment of the former resulted in two SDS-PAGE bands, one at the same molecular weight as the light monomer and another corresponding to the hydrophobic membrane anchor (Gennari et al., 1987), now known to be PRiMA (Perrier et al., 2002). Corresponding to these findings, Liao *et al.* generated a monoclonal antibody against PRiMA, the binding of which was abolished by treatment with proteinase K or dithiothreitol (Liao et al., 1993). These data indicate a protease sensitive-site by which AChE is released from the cell surface. It is thought that the cleavage site is in PRiMA, as proteinase K treatment of bovine brain AChE generated a 13kDa fragment derived from PRiMA (Fuentes et al., 1988).

As outlined previously, it is likely that shedding is not the only mechanism for AChE release. This is mentioned in the literature (Greenfield et al., 1983, Appleyard et al., 1988, Schweitzer, 1993), but is supported with our own data, with the three effective inhibitors only returning a 50% decrease in AChE activity in medium. The involvement of exocytosis was therefore probed, using an inhibitor targeting Arf6-mediated exocytosis, Exo1 (Feng et al., 2003). This again showed a significant decrease in cellular release of AChE, although over the same time period, the decrease was more modest relative to metalloprotease inhibitor use. This may show that shedding is a predominant process, but only one sub-type of exocytosis was targeted, so it is possible that multiple exocytic pathways are involved in AChE release.

3.6.4 Further pharmacological treatment show a role for protein disulphide isomerase in AChE shedding

As described previously, AChE shedding does not precisely mirror processes in the vast majority of other cases of protein shedding. The majority of proteins that are cleaved are integral membrane proteins, often with release of a soluble ectodomain (Hooper et al., 1997). This paradigm does not apply here, as we are dealing with two proteins, AChE and PRiMA. This does, however, show significant similarities with the TSHR and its α- and β- subunits (Couet et al., 1996a, Couet et al., 1996b). In the case of AChE, the cleavage site is currently unknown and, indeed, whether AChE or PRiMA is cleaved.

Use of bacitracin to target cell surface thiol isomerases (Dickerhof et al., 2011) resulted in a significant decrease in AChE activity in the medium. This decrease was of a similar magnitude to that seen with batimastat and, indeed, no additive effects were seen after co-incubation of the two inhibitors. This is indicative of a two-step process, where inhibition of either one of the steps has the same effect of inhibition of both. However, although the effects of bacitracin are consistent with thiol isomerase involvement, its non-specific effects confound definitive conclusions. Further work involved use of the blocking antibody RL90, targeting cell surface PDI. Use of this antibody resulted in a modest, but significant decrease in AChE activity in the medium.

It has already been widely shown that the AChE C-terminus is capable of disulphide bond formation (Bon and Massoulie, 1997, Perrier et al., 2002, Dvir et al., 2004, Dvir et al., 2010). Indeed, it appears likely that it is these bonds that keep the AChE tetramer

assembled, as their disruption with DTNB causes tetramer dissociation. The data presented here concur that disulphide bonds are also formed between AChE and PRiMA.

Given the data presented here and those previously published (Gennari et al., 1987, Fuentes et al., 1988), it seems likely that a two-step process is required to release AChE from PRiMA, namely cleavage of PRiMA and reduction of the disulphide bonds between AChE and PRiMA. It is possible that these two steps must occur in a particular order, such as is outlined for TSHR, whereby reduction of the disulphide bond causes a conformational change, making the target molecule more susceptible to metalloprotease cleavage (Couet et al., 1996a). This ensures that metalloprotease cleavage cannot occur without the disulphide bond being reduced first, perhaps an extra control mechanism.

3.6.5 Acetylcholinesterase is modulated by muscarinic acetylcholine receptor agonism

The ACh analogue carbachol was used to assess the effects of AChR agonism on AChE release. Treatment of SH-SY5Y cells with carbachol resulted in a significant increase in AChE activity in media samples. This is indicative of a role of AChR in modulating AChE release. This being the case, this supports the previous hypothesis that AChRs are involved in a feedback loop, whereby falling AChE results in increased ACh levels, which in turn increases AChE release from the cell. The exclusive role of mAChRs in this process was also confirmed. Firstly, muscarine was used, a selective mAChR agonist, which was able to recapitulate the increases in medium AChE activity seen after carbachol treatment. This would indicate that mAChR agonism is sufficient to cause the increases in AChE release, suggesting no role for nAChRs. Furthermore, pre-incubation of cells with atropine, a mAChR antagonist, prior to carbachol treatment, ablated any increases in AChE activity detected in the medium, indicating that blockade of mAChRs entirely inhibits the effects of carbachol, confirming previously published data (Cushny, 1910).

Previous work has shown mAChR agonism to have a role in promoting shedding of sAPPα (Nitsch et al., 1992, Canet-Aviles et al., 2002) and a PKC-dependent mechanism has been suggested (Slack et al., 1993). It is likely, then, that carbachol treatment is activating a non-specific pathway, involving increased trafficking of a range of proteins to the cell surface, including AChE and APP. This would account for the increased

abundance in media samples of AChE and sAPPα, respectively. Another feature of this system is that it would be quicker to respond to stimuli than a system dependent on gene expression changes. The rapid way in which cells can respond to removal of AChE from the medium has previously been shown. Increased trafficking of AChE to the cell surface, under mAChR control, would offer a suitably rapid mechanism to redress the equilibrium. Furthermore, it is possible that this increased trafficking may also affect those enzymes involved in AChE shedding. Finally, mAChR signalling has been strongly linked to increases in cellular Ca^{2+} levels, which would promote the Ca^{2+}-dependent exocytic pathways that have been outlined by previous studies (Schweitzer, 1993).

The role of mAChR agonism on AChE gene expression was also investigated. This confirmed that mAChRs have a transcriptional regulatory role, concurring with previous work (Nitsch et al., 1998, von der Kammer et al., 2001). To this end, the levels of Egr-1 mRNA were also assayed in response to the carbachol stimulus and, in line with previous work (von der Kammer et al., 1998, Mayer et al., 2008), Egr-1 was found to be similarly increased at the mRNA level, but to a far greater extent, the increase reaching 180%. Given the presence of a serum response element (SRE) in the 5` flanking region of Egr-1 (Tsai-Morris et al., 1988), mRNA levels of serum response factor (SRF) were also investigated. However, qPCR analysis did not show any changes in SRF after carbachol treatment. Further analysis of Elk-1 (Besnard et al., 2011), which has been shown to be an SRF cofactor (Treisman et al., 1992, Hill et al., 1993) linked to regulation of Egr-1 through a mechanism indistinguishable from mAChR signalling (Mayer et al., 2008) demonstrated no changes with carbachol treatment. Although Elk-1 and SRF were not increased at the mRNA level, this does not discount increased binding to the Egr-1 promoter due to phosphorylation. Indeed, increased phosphorylation of ERK after mAChR activation was confirmed, concurring with previous work (Berkeley et al., 2001). This activation of ERK may be responsible for a putative activation of Elk-1 or similar TFs, as Elk-1 is a target of ERK (Davis et al., 2000).

3.7 Conclusion

In search of a model cell line to investigate AChE release, several cell lines have been assessed for relative AChE and BChE activities, namely neuronal SN56, SH-SY5Y,

NB7 and non-neuronal GD25. This revealed the non-neuronal GD25 cells to display substantially different enzyme expression ratios compared to the neuronal cell lines. Of these, SN56 and SH-SY5Y showed similar expression ratios of the ChEs with predominance of AChE, while, by contrast, NB7 cells predominantly expressed BChE. Subsequent phenotypic analysis revealed SN56 cells to express a wide range of cholinergic marker proteins, indicating them to be an appropriate model for this study.

Analysis of AChE release revealed an Arf6-dependent exocytic component and also a shedding event, the latter mediated by a batimastat-sensitive metalloprotease. The efficacy of this proteolytic event is dependent on the concomitant reduction of disulphide bonds between PRiMA and AChE, mediated by PDI.

Expression and release of AChE were found to be modulated by mAChR agonism, with carbachol treatment found to increase AChE transcription and also that of the Egr-1 TF, although no changes were observed in mRNA levels of related TFs Elk-1 or SRF.

Chapter 4

Results II

The regulation of acetylcholinesterase by the amyloid precursor protein

Chapter 4: the regulation of acetylcholinesterase by the amyloid precursor protein

4.1 Introduction

The amyloid precursor protein (APP) and acetylcholinesterase (AChE) are both multi-faceted proteins with a wide range of functions. While they are both linked with growth and development processes, especially in the brain (Gralle and Ferreira, 2007, Halliday and Greenfield, 2011, Zheng and Koo, 2011), they are also closely linked to the causative pathological mechanisms of AD. APP is the precursor of the $A\beta$ peptide, which is suggested to be one of the most important pathological agents in AD (Hardy and Higgins, 1992), with its oligomers considered the most toxic (Haass and Selkoe, 2007, Walsh and Selkoe, 2007). AChE has also been linked to the disease pathogenesis by exacerbating amyloid fibril formation and toxicity (Inestrosa et al., 1996, Alvarez et al., 1997, Rees et al., 2003, Dinamarca et al., 2010) and is the main target of clinically available AD drugs (Greig et al., 2013).

AChE is a key protein in the modulation of cholinergic signalling, which occurs in several systems in the brain, with the basal forebrain system being robustly linked to AD (Coyle et al., 1983). The individual subunits of AChE can associate with each other, forming both dimers and tetramers. The Proline Rich Membrane Anchor (PRiMA) is a 20kDa protein responsible for both AChE tetramerisation and its anchorage to the membrane in neuronal cells. This is a crucial role as tetramers of $AChE_T$ form the functional units at cholinergic synapses (Perrier et al., 2002, Dvir et al., 2010, Henderson et al., 2010, Hicks et al., 2011). Immunofluoresence studies have shown strong co-localisation between AChE and PRiMA in cholinergic neurons, but no localisation of PRiMA in either dopaminergic or GABAergic neurons (Henderson et al., 2010). PRiMA is one of a number of key cholinergic proteins that have been previously been shown to be expressed in SN56 cells (Henderson et al., 2010). Another ChE is the second mammalian cholinesterase, BChE. Although the role of BChE is much less clear than AChE, it is able to hydrolyse ACh and this, plus reports of compensatory increases in expression of BChE as AChE is reduced (Hartmann et al., 2007), mean it should be considered alongside any investigation of AChE. Finally, CHT is another cholinergic protein whose role is in the recycling of choline into the presynaptic terminal. Choline is present in the synapse after ACh hydrolysis and its subsequent re-uptake allows synthesis of new ACh molecules (Sarter and Parikh, 2005).

Proteolyic cleavage of APP results in generation of the APP intracellular domain (AICD), which can act as a transcriptional regulator (Cao and Sudhof, 2001, Kim et al., 2003, Pardossi-Piquard et al., 2005, Belyaev et al., 2009, Schettini et al., 2010). Functional AICD is mostly formed in the amyloidogenic pathway, predominantly from the APP_{695} isoform (Goodger et al., 2009, Belyaev et al., 2010, Flammang et al., 2012). Important residues in APP_{695} for transcriptional regulation are Y682 and Y687, which represent the N- and C-terminal residues of the YENPTY motif in the cytoplasmic domain of APP (Aydin et al., 2012). Although the APP C-terminus is the predominant region for protein-protein interactions, other regions are also involved, e.g. via the extracellular E1 region with reelin (Hoe et al., 2009) and fibulin-1 (Ohsawa et al., 2001), integrin β1 (Young-Pearse et al., 2008, Rice et al., 2013) and also in dimerisation of APP (Isbert et al., 2011). This region comprises two sub-domains, namely a growth factor-like domain (GFLD) and a copper-binding domain (CuBD), the latter playing roles in metal homeostasis, dimerisation and potentially, protein-protein interactions (Barnham et al., 2003, Kong et al., 2008, Weaver et al., 2008, Noda et al., 2013).

It has been widely shown that the cholinergic system can modulate APP trafficking and cleavage, insofar as mAChR agonism promotes non-amyloidogenic processing of APP (Nitsch et al., 1992, Slack et al., 1993, Canet-Aviles et al., 2002, Zimmermann et al., 2004). However, nearly a decade ago, Bimonte *et al.* reported a possible bidirectional relationship, whereby APP may modulate AChE at a transcriptional level. Briefly, this work investigated the effects of mutation of the *feh-1* (an Fe65 orthologue) gene in *C. elegans* on its two AChE genes, *ace-1* and *ace-2*. The authors reported that mutation of the Fe65 orthologue resulted in decreased expression of the *ace-1* and *ace-2* genes. Although they did not perform any experiments directly investigating APP, their discussion made mention of the link between APP and Fe65 signalling and hence whether this regulatory pathway may be involved in AD. It was ultimately this idea that was the starting point for the work described here as well as previous work investigating the regulation of other proteins by AICD.

4.1.1 Aims

The main goal of this study was to investigate whether APP_{695} regulates AChE expression in neuronal cell lines. The concept of the cholinergic system regulating APP

has been well documented (Nitsch et al., 1992, Canet-Aviles et al., 2002), but data about APP regulation of the cholinergic system are sparse. To address this issue a number of stable cell lines over-expressing APP_{695} were generated. As a relationship between APP and AChE was found, the work developed to elucidate the mechanism behind the regulation of AChE. Furthermore, AChE is only one of several cholinergic marker proteins, so whether APP was regulating AChE only, or if it was involved in a more global regulation of the cholinergic system through its component genes was investigated.

4.2 Generation of stable cell lines over-expressing APP_{695}

In order to ascertain whether APP regulated APP, neuronal cell lines over-expressing APP_{695} were utilised, which is the predominant isoform in neurons and therefore the most appropriate to the work. SH-SY5Y (APP_{695}) cells were a kind gift from Dr. I. J. Whitehouse (University of Leeds) and their over-expression of APP_{695} was confirmed and published (Belyaev et al., 2010) and further validated (data not shown). However, a SN56 stable cell line over-expressing APP_{695} was also generated.

There is a substantial increase in APP immunoreactivity in the APP_{695}-transfected cell lines (Fig. 4.1A) and this is recapitulated at the mRNA level (Fig. 4.1B), indicative of successful generation of an SN56 cell line over-expressing $APP_{695.}$ Immunoreactivity for mature APP_{695} is visible at 110kDa.

Figure 4.1: generation and analysis of APP expression in SN56 (APP$_{695}$) cell line
SN56 cells were cultured as described (section 2.2.1.1) until 50-80% confluence. They were then transfected with the APP$_{695}$ construct using Lipofectamine 2000 in OptiMEM. Normal medium was added 4-6h after transfection, and medium was replaced 24h post transfection. At 48h, cells were subcultured (1:50) and henceforth cultured in hygromycin B for selection. **A.** After several passages, lysates were made as described and protein samples were separated by SDS-PAGE, followed by Western blotting, probing for APP and subsequently β-actin. **B.** RNA was extracted from the transfected cells and cDNA synthesised. Conventional PCR was performed as described, using primers specific to APP and GAPDH. Amplified DNA was separated on a 1% agarose gel containing EtBr and bands were visualised with UV light using Gel Doc XR.

4.3 Effects of modulation of APP expression levels on modulation of AChE mRNA, protein and catalytic activity

4.3.1 APP over-expression

After successful generation of a stable cell line expressing APP$_{695}$, the effects of this on AChE were investigated. The catalytic AChE activity in total cell lysates was initially explored and found to be reduced by approximately 80% (Fig. 4.2A). Further investigation showed this to be seen also at the protein level (Fig. 4.2B). There were intense immunoreactive bands for AChE at 65kDa in SN56 (mock) cells, yet no

corresponding bands in those SN56 cells over-expressing APP_{695}. Similarly, this relationship was seen at the mRNA level as AChE mRNA transcripts are reduced by approximately 90% in those cells over-expressing APP_{695}. Assessment of AChE activity, protein and mRNA in SH-SY5Y (APP_{695}) cells resulted in broadly similar findings, emphasising that this effect was not cell line or species specific. The decreases in AChE mRNA, protein and activity, while significant, were far less marked, at approximately 50% (Fig. 4.2D, E, F).
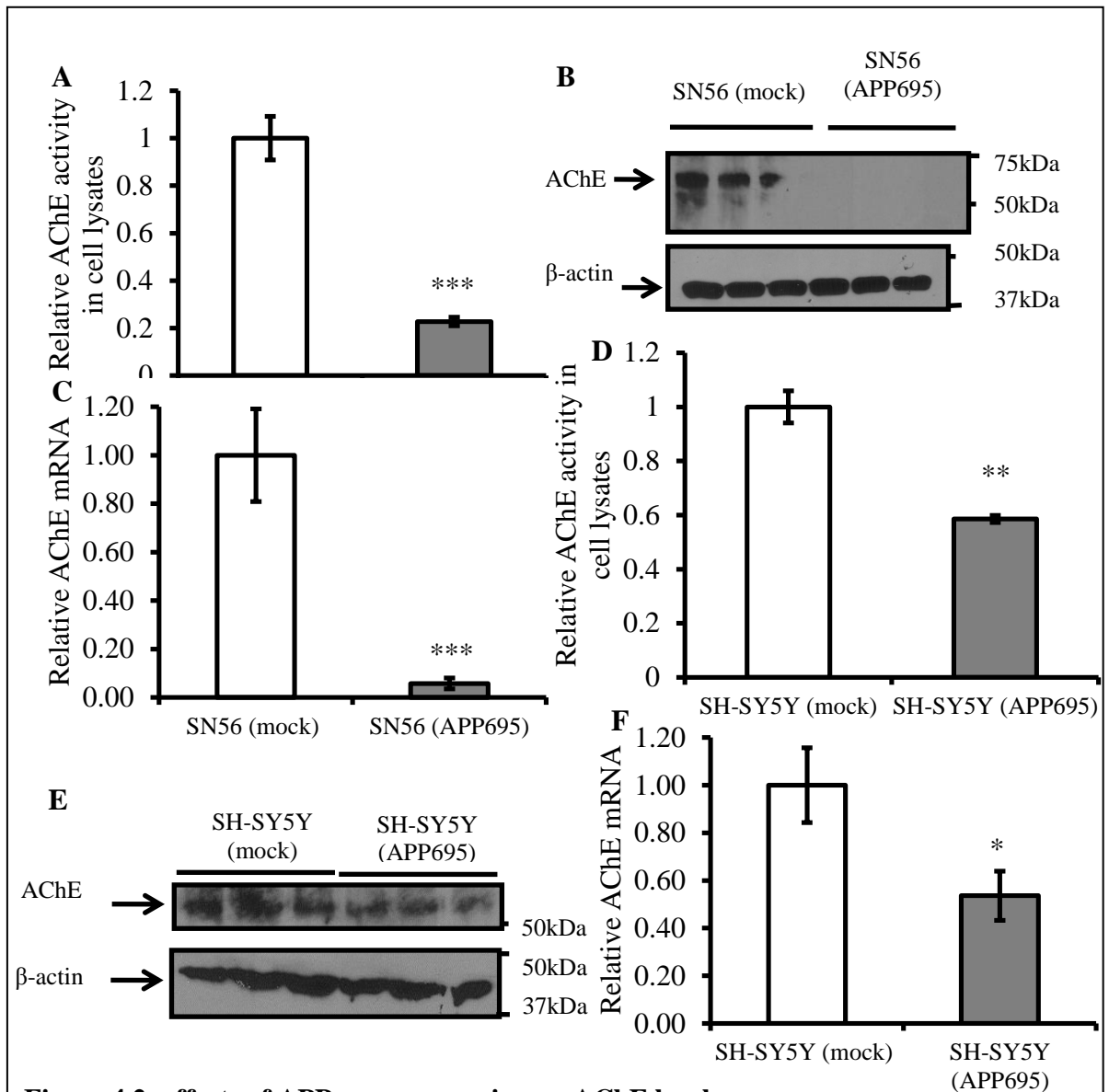
Figure 4.2: effects of APP over-expression on AChE levels
SN56 cells were cultured as indicated previously in Experimental Procedures. **A.** Cell lysates were made as indicated therein and AChE activity was assessed using Ellman's assay (n=6, p < 0.001). **B.** The lysates were made as described and protein samples were separated by SDS-PAGE, followed by Western blotting, probing for AChE and subsequently β-actin. **C.** RNA was extracted from the transfected cells and cDNA synthesised. mRNA transcripts were assessed by qPCR using primers specific for AChE (n=6, p=0.001). These experiments were repeated in SH-SY5Y cells (**D** (n=9, p=0.01)**, E, F** (n=6, p=0.02)).

Whether this reduction in AChE extended to AChE activity in the medium was investigated. As has been shown previously, there are a number of mechanisms operating to regulate the release of AChE from the cell. In media samples, significant reductions of approximately 70% in AChE activity were observed in APP over-expressing cells (Fig. 4.3A).

Figure 4.3: effects of APP$_{695}$ over-expression on medium AChE activity and of transient APP$_{695}$ over-expression
A. SN56 (mock) and SN56 (APP695) cells were cultured as described (section 2.2.1.1) until ≥80% confluence. Cells were incubated in OptiMEM for 24h, after which medium was extracted and assayed for AChE activity using Ellman's assay (n=6, p=0.03) **B.** SN56 cells were cultured as described until 50-80% confluence. They were then transfected with the APP$_{695}$ construct using Lipofectamine 2000 in OptiMEM. Normal medium was added 4-6h after transfection, and medium was replaced 24h post transfection. At 48h, cells were lysed and protein samples were separated by SDS-PAGE, followed by Western blotting, probing for APP and subsequently β-actin. **C.** Lysates were subsequently assayed for AChE activity using Ellman's assay (n=3, p=0.005). **D**. RNA was extracted from transiently transfected and cDNA synthesised. mRNA transcripts were assessed by qPCR using primers specific for AChE (n=5, p=0.01).

To examine possible artifactual effects of a stable over-expression system, the effects of transient transfection of APP$_{695}$ were compared. This approach entailed limiting the time for which cells were incubated with the DNA construct to 48h. At this time, the cells

were lysed and assayed. Western blotting confirmed substantial increases in APP protein levels at 120kDa in the transfected cells (Fig. 4.3B). Furthermore, significant decreases were again seen in AChE activity in total cell lysates. The reduction, though, was not as great as that seen in the stable cell lines, here approximately 50% (Fig. 4.3C). As the effects of APP were on a transcriptional level, mRNA levels of AChE were assessed. The decreases seen here were of the same magnitudes as those in AChE activity, again approximately 50% (Fig. 4.3D).

4.3.2 APP knockdown with siRNA

To further validate the effect of APP expression levels on AChE, the effects of siRNA knockdown of endogenous APP in wild type SN56 cells on AChE mRNA levels were examined. For this, SmartPool siRNA was used, comprising four different siRNA duplexes targeting the same gene.

Figure 4.4: effects of APP knockdown on AChE mRNA
Wild type SN56 cells were transfected with either 25 nM siRNA targeting APP or a scrambled sequence at an equivalent concentration using Lipofectamine The transfection medium was replaced with OptiMEM after 6-8 h and cells harvested after 24 h. **A.** APP knockdown was verified by Western blot and **B.** AChE mRNA levels were assayed by qPCR (scramble: n=7, p=0.43; siRNA: n=9, p=0.03).

After transfection, APP knockdown was verifed by Western blot (Fig. 4.4A). At 120kDa, this showed a substantive decrease in APP protein levels in those cells transfected with the siRNA, but cells transfected with a scrambled negative control oligonucleotide showed no change in APP immunoreactivity relative to control. The effects of the siRNA treatment on AChE mRNA levels were then investigated. Transfection with the scrambled negative control duplex had no effect on AChE mRNA, but siRNA-mediated knockdown of APP resulted in a nearly 90% increase in AChE mRNA (Fig. 4.4B).

4.4 Analysis of possible secretase involvement in the APP-mediated transcriptional repression of AChE
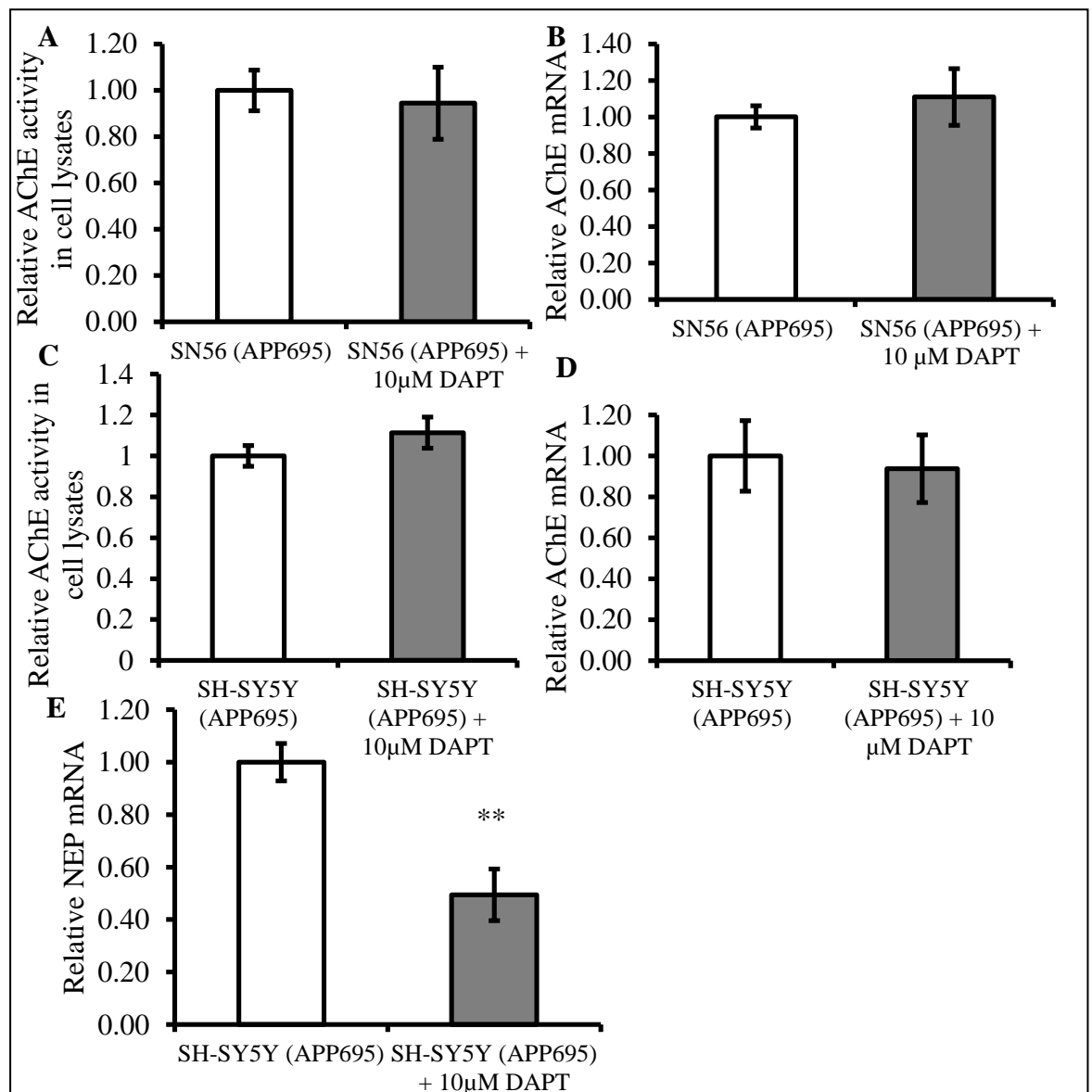
Figure 4.5: assessment of the involvement of γ-secretase in repression of AChE
A. AChE activity in cell lysates from SN56 (APP695) and SN56 (APP695) treated with 10μM DAPT for 24h (n=6, p = 0.41) and also **B.** in SH-SY5Y cells (n=6, p=0.26) **C.** qPCR analysis of AChE mRNA transcripts with same treatment in SN56 cells (n=6, p=0.54) and **D.** also in SH-SY5Y cells (n=6, p=0.55). **E.** qPCR for NEP in SH-SY5Y (APP695) cells, either control or 24h 10μM DAPT (n=5, p=0.001). Statistical significance is defined as follows: $p \leq 0.01$ (**).

Given the prominence that has been afforded AICD in gene regulation (Muller et al., 2008, Chang and Suh, 2010, Beckett et al., 2012), the AICD-dependence of the APP-mediated repression of AChE was explored. To do this, the γ-secretase inhibitor DAPT was used to block AICD production from its precursor. SN56 (APP_{695}) cells were treated with the inhibitor to assess if it would reverse the repression of AChE effected by APP. DAPT treatment did not result in any change in AChE activity or mRNA levels (Fig. 4.5A and B). These experiments were repeated in SH-SY5Y (APP_{695}) cells and these data recapitulated those generated from the SN56 (APP_{695}) cells (Fig. 4.5C and D). However, it was important to validate the efficacy of the DAPT. In order to do this, its effect on mRNA levels of the AICD-regulated gene, NEP, was investigated (Pardossi-Piquard et al., 2005, Belyaev et al., 2009). This resulted in a significant decrease in NEP mRNA, approximating 50% (Fig. 4.5E).

Figure 4.6: Effects of α- and β-secretase inhibition on AChE activity
AChE activity in cell lysates from SN56 (APP695) treated with **A.** 25μM GM6001 for 24h (n=6, p =0.78) or 1μM β-IV for 24h (n=6, p=0.52). **B.** AChE activity in cell lysates from SH-SY5Y (APP695) cells (n=7; p=0.83 (GM6001), p=0.51(β-IV)). **C.** Inhibitor efficacy was confirmed by Western blot for sAPPα in cell culture medium from SH-SY5Y (APP695) cells, either control or after 24h 25μM GM6001 treatment or **D.** for sAPPβ in medium from SH-SY5Y (APP695) cells after 24h 1μM β-IV treatment.

Although γ-secretase-mediated release of AICD is the best characterised pathway by which APP can modulate gene expression, some literature has implicated the soluble ectodomains in intracellular signalling and gene regulation (Li et al., 2010, Chasseigneaux et al., 2011). To address this question, SN56 (APP_{695}) cells were treated with GM6001 or β-IV, potent inhibitors of α- and β- secretases, respectively.

As with γ-secretase inhibition, there was no change in AChE activity in SN56 (APP_{695}) cells upon inhibition of α- or β-secretases, hence precluding formation of the sAPPα and sAPPβ ectodomains, respectively (Fig. 4.6A). Similarly, repeating these experiments in SH-SY5Y (APP_{695}) cells resulted in no significant changes in AChE activity (Fig. 4.7B). To confirm efficacy of the inhibitors, Western blots were performed for the soluble ectodomains of APP. Treatment with GM6001 under the conditions employed resulted in a significant decrease in sAPPα immunoreactivity in media samples and equally, treatment with β-IV caused a dramatic decrease in levels of sAPPβ in the medium. Both soluble fragment exhibited immunoreactivity at approximately 110kDa (Fig. 4.6C and D).

4.5 Investigation of the effects of introduction of mutations and deletions in APP

The binding of some APP interacting partners is independent of γ-secretase cleavage, such as Fe65, Dab1 and Numb, with the majority of these interacting proteins binding in the C-terminal region (Aydin et al., 2012). In APP_{695}, two of the key residues involved in C-terminal protein-protein interactions are Tyr682 and Tyr687. In order to investigate the possible role of these residues in regulation of AChE, three mutants of APP_{695} were generated. Two of these constructs were point mutants (Y682G and Y687G, respectively), while the third was a double mutant, in which both tyrosine residues were mutated to glycine residues (Y682G, Y687G; denoted YYGG). Stable SN56 cell lines expressing each of these constructs were generated and over-expression verified by Western blot, with immunoreactivity at approximately 120kDa (Fig. 4.7A and B). After this, the effect of the over-expression of these APP mutants was examined. Expression of each of the constructs resulted in a significant decrease in AChE activity in cell lysates (Fig. 4.7C), as also seen with wild type APP (Fig. 4.2A). Further, AChE mRNA levels were assessed in these cell lines and, again, the mutants were able to recapitulate the effects of wild type APP. APP_{695}, with mutations at Y682, Y687 or both together, was still able to effect the transcriptional repression of AChE (Fig. 4.7D).

Figure 4.7: effect of APP$_{695}$ mutagenesis and over-expression on AChE activity and mRNA

Western blot for APP (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells SN56 cells over-expressing mutant APP695, specifically **A.** Y682G and Y687G and **B.** the Y682G,Y687G double mutant (YYGG). **C.** AChE activity in SN56 (mock) and SN56 (mutant APP695) cell lysates (Y682G: n=6, p < 0.001; Y687G: n=6, p=0.02; YYGG: n=6, p < 0.001). **D.** qPCR analysis of AChE mRNA transcripts (Y682G: n=6, p=0.01; Y687G: n=6, p=0.005; YYGG: n=6, p=0.009). Statistical significance is defined as follows: p ≤ 0.05 (*), p ≤ 0.01 (**) or p ≤ 0.001 (***).

After the lack of effect of mutation of key C-terminal residues, an extracellular domain of APP was examined as a possible mediator of the regulatory process, exploring the outcome of deleting the E1 domain from APP$_{695}$. A stable cell line (SN56 APP$_{695}$ΔE1) was generated and expression of the APPΔE1 construct was verified by Western blot, the size of the immunoreactive product band being consistent with wild type APP lacking this domain, at 90kDa (Fig. 4.8A). The SN56 APPΔE1 cell line was assayed for AChE activity and no difference was observed in AChE activity between mock and APP transfected cells (Fig. 4.8B). Similarly, qPCR analysis of AChE transcripts revealed no significant difference between mock cells and cells over-expressing APPΔE1 (Fig. 4.8C). As with the wild type APP$_{695}$, the acute effects of over-expression were investigated. SN56 cells were transiently transfected with the APPΔE1 construct and expression validated by Western blot, with immunoreactivity at 90kDa (Fig. 4.8D).
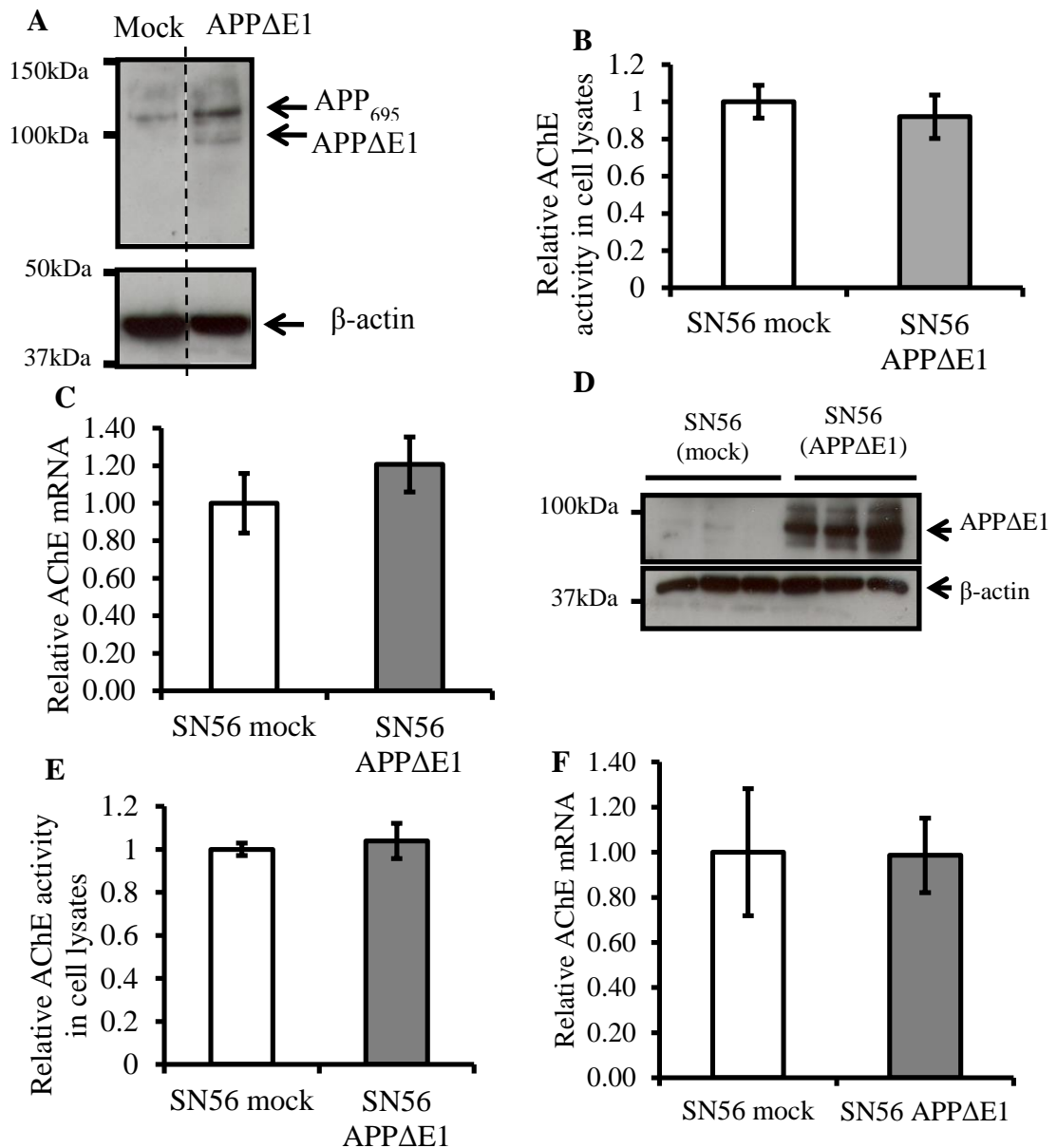
Figure 4.8: effects of over-expression of APPΔE1 on AChE
A. Western blot for APP (1:1000 anti-CTF) (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells and SN56 cells over-expressing APPΔE1. The vertical dashed line on the blot indicates alignment of samples from distal lanes run on the same immunoblot. **B.** AChE activity in SN56 (mock) and SN56 (APPΔE1) cell lysates (n=27, p=0.51). **C.** qPCR analysis of AChE mRNA transcripts (n=9, p=0.33). **D.** Western blot for APP (1:1000 anti-CTF) (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells and cells transiently transfected with APPΔE1. **E.** AChE activity in SN56 (mock) and SN56 (transient APPΔE1) cell lysates (n=9, p=1.0). **F.** qPCR analysis of AChE mRNA transcripts in SN56 (mock) and SN56 (transient APPΔE1) cells (n=6, p=0.96).

As with the stable cell lines, we assayed AChE activity and subsequently utilised qPCR to quantify mRNA levels of AChE. After transient transfection with APPΔE1 neither activity nor mRNA was altered (Fig. 4.8E and F).

As the E1 domain of APP has been shown to be responsible for mediating the transcriptional repression of APP, further investigation was focused on the mechanism by which this is achieved. To ascertain a putative E1 sub-domain which may be involved in mediating transcriptional repression of AChE, a CuBD mutant was generated, mutating three critical metal-binding His residues to Ala.

Figure 4.9: effects on AChE of over-expression of APPΔCuBD
A. Western blot for APP (1:2000 22C11) (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells and SN56 cells over-expressing APPΔCuBD. **B.** AChE activity in SN56 (mock) and SN56 (APPΔE1) cell lysates (n=12, p=0.53). **C.** qPCR analysis of AChE mRNA transcripts (n=9, p=0.27).
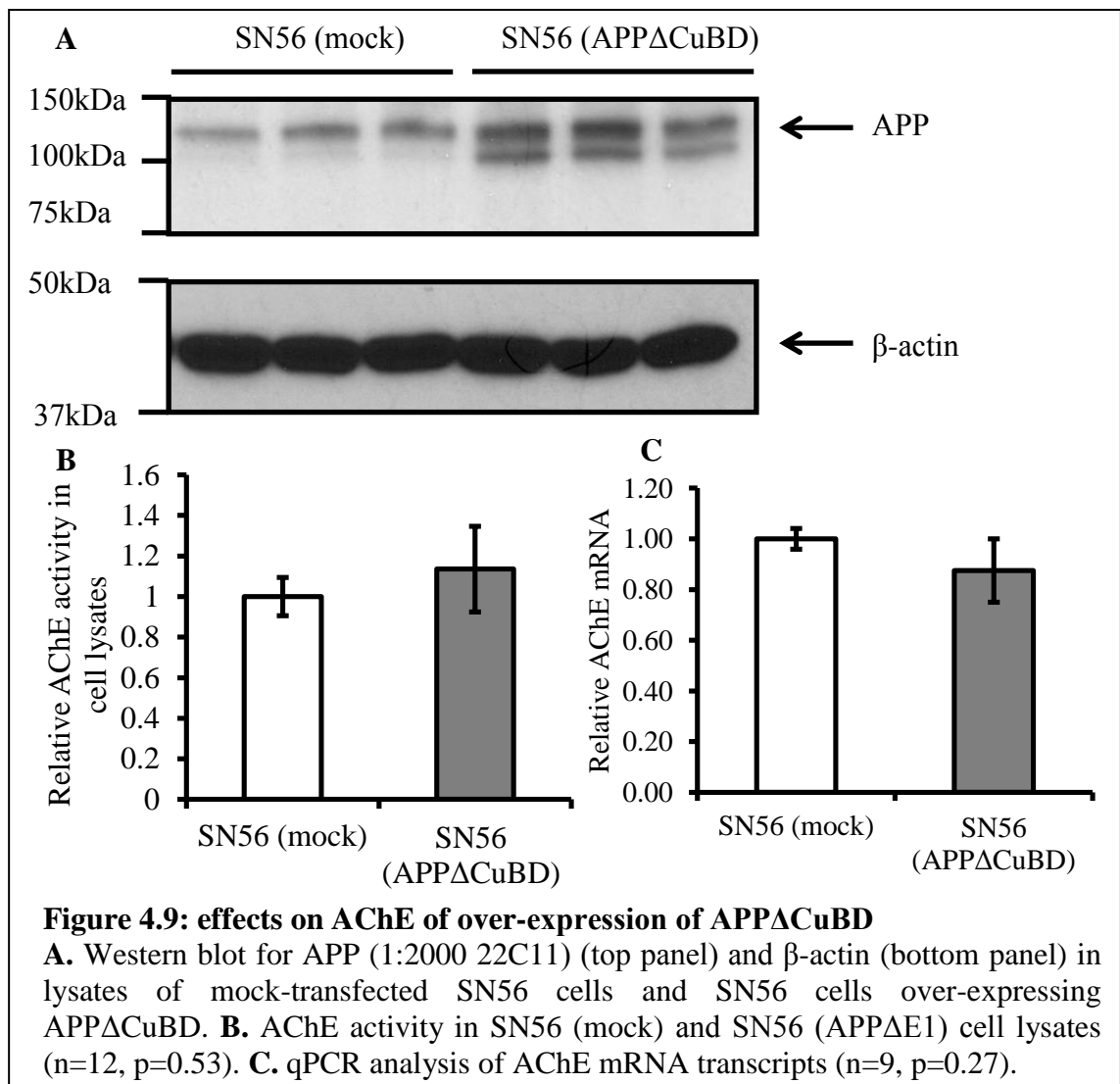
This APP construct was over-expressed in SN56 cells (Fig. 4.9A) to assess whether mutation of the CuBD would compromise the ability of APP to repress the transcription of AChE. Western blots showed an APP-immunoreactive band at 120kDa. As with expression of the ΔE1 construct, the SN56 (APPΔCuBD) cells did not show any decreases in AChE activity in cell lysates (Fig. 4.9B). Similarly, at an mRNA level, qPCR did not show any changes upon over-expression of the APPΔCuBD construct Fig. 4.9C).

4.6 Integrins as putative APP N-terminal interacting partners in the regulatory process

Several N-terminal APP binding partners have been reported, including integrin β1 (ITGB1) (Young-Pearse et al., 2008, Rice et al., 2013) and integrin α5 (ITGA5) (Yamazaki et al., 1997). Most of the APP binding partners listed are adhesion proteins and many do not have any defined intracellular signalling activity. However, integrins are transmembrane proteins with an intracellular NPXY motif (Calderwood et al., 2003), downstream of which intracellular signalling is better defined than any other candidate APP N-terminal ligands.

Clarke *et al.* (2012) have identified a binding partner for the aforementioned integrins, outlining a paradigm where a membrane-anchored protein could bind integrins and subsequently initiate intracellular signalling cascades through focal adhesion kinase (FAK) and Akt. The membrane protein, angiotensin-converting enzyme 2 (ACE2) was able to bind ITGB1 and ITGA5 (Clarke et al., 2012), both of which co-localise with APP. Although ACE2 is not expressed in SN56 cells (data not shown), a plasmid encoding human ACE2 was used to see if it could mimic the effects of APP, thereby implicating integrins as the agents through which APP is mediating transcriptional repression. ACE2 was transiently over-expressed in SN56 cells and successful over-expression verified by Western blot and substantial increases of ACE2 immunoreactivity could be seen at 90kDa (Fig. 4.10A).

A

SN56 (mock)　SN56 (ACE2)

150kDa

← ACE2

75kDa
50kDa

37kDa

← β-actin

B

Relative AChE activity in lysates

1.2
1
0.8
0.6
0.4
0.2
0

***

SN56
(mock)

SN56
(ACE2)

C

Relative AChE mRNA

1.20
1.00
0.80
0.60
0.40
0.20
0.00

**

SN56 (mock)　SN56 (ACE 2)

Figure 4.10: effects of ACE2 over-expression on AChE activity and mRNA levels.
SN56 cells were transiently transfected with ACE2 and **A.** over-expression was verified by Western blot. **B.** AChE activity was assayed using Ellman's assay (n=6, p=0.0003) and **C.** AChE mRNA levels were assessed by qPCR (n=6, p=0.004).

Ellman's assay was subsequently employed to record the impact of ACE2 over-expression on AChE activity. In SN56 cell lysates, AChE activity was reduced to a level commensurate with over-expression of APP (Fig. 4.10B). Similarly, analysis of AChE mRNA levels revealed a significant decrease after transient over-expression of ACE2 (Fig. 4.10C).

As a consequence of these data and that of Clarke *et al.* (2012), a putative role for integrin signalling downstream of APP binding was investigated. To be a convincing interacting partner, ITGB1 must be expressed in wild type SH-SY5Y and SN56 cells and this expression was confirmed by Western blot (130kDa, Fig. 4.11A). In order to undertake further studies, GD25 cells were used, which are a mouse cell line null for ITGB1. Their ITGB1- expressing counterparts, GD25β1 cells, were also used, initially confirming ITGB1 expression levels in both cell lines by Western blot (Fig 4.11B). APP_{695} was transiently expressed in both cell lines and the successful transfection was verified by Western blot (120kDa, Fig. 4.11C and D). AChE mRNA levels were then analysed in these cells by qPCR (Fig. 4.11E). In GD25 and GD25β1 cells, an approximately 30% decrease in AChE mRNA was observed. After statistical analysis, these decreases in mRNA fell short of statistical significance (p=0.08 and 0.11).

However, no differences between those cells null for ITGB1 and those cells expressing ITGB1 were detected.
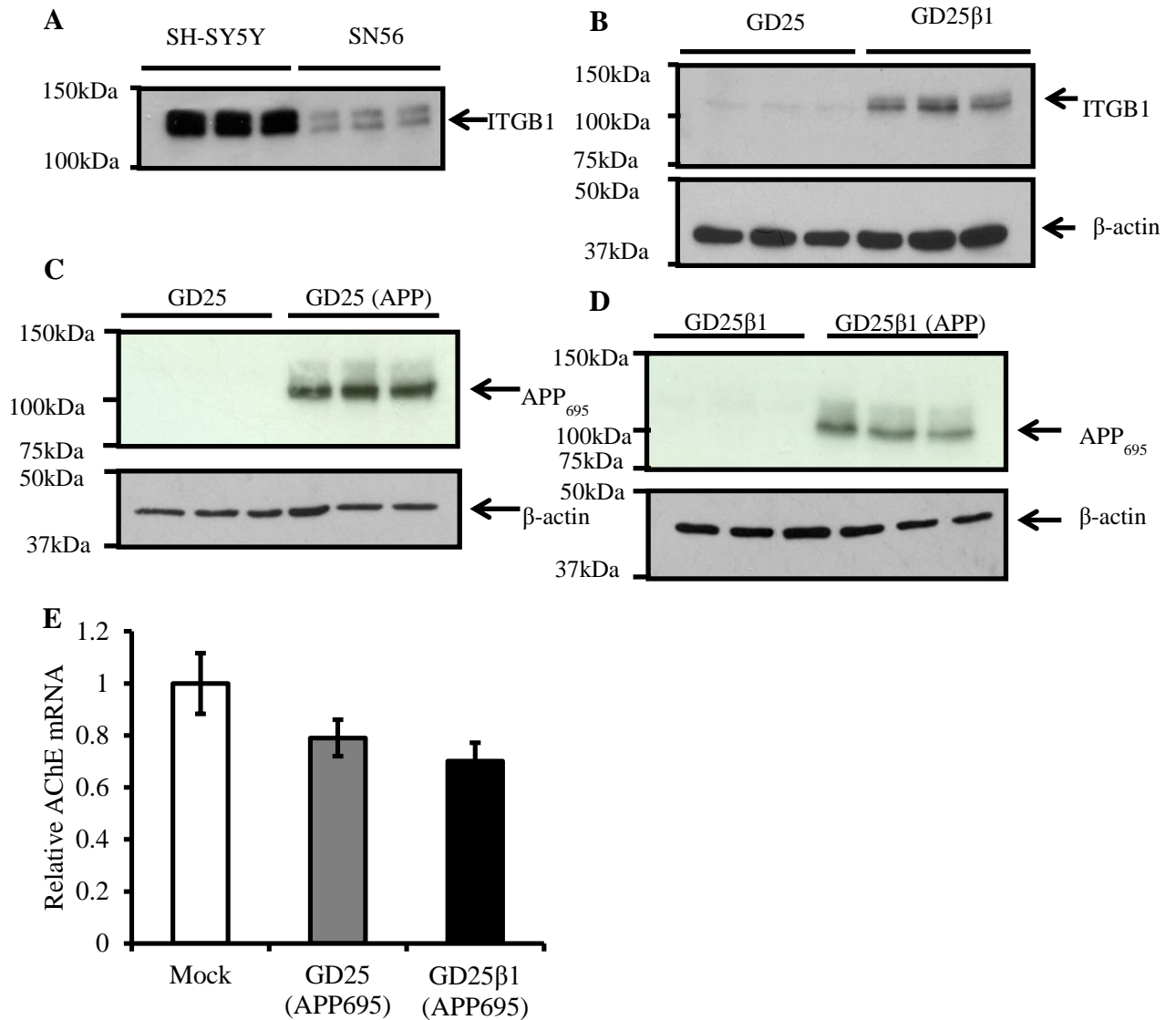
Figure 4.11: over-expression of APP$_{695}$ in GD25 and GD25β1 cells and consequent investigation of AChE mRNA levels
Western blot analysis was used to assess expression of ITGB1 in **A.** SH-SY5Y and SN56 cells and **B.** in GD25 and GD25β1 cells. **C and D.** GD25 and GD25β1cells were transiently transfected with APP$_{695}$ and over-expression was confirmed by Western blot. **E.** RNA was subsequently extracted from these cells and qPCR was used to assess AChE mRNA levels (GD25: n=9, p=0.08; GD25β1: n=9, p=0.11)

4.7 Cell signalling downstream of APP

Far less has been published on repression of AChE than its activation, although one report shows induction of AChE by H_2O_2, a process which could be blocked by activation of Akt (Xie et al., 2011).

Given these data, the centrality of Akt in a number of signalling pathways and data linking its phosphorylation and activation to integrin signalling (Clarke et al., 2012), a possible role of Akt in the signalling process was explored. By Western blot, levels of total Akt and phospho-Akt between SN56 (mock) and SN56 (APP$_{695}$) cells were compared.

Figure 4.12: investigation of putative signalling pathways downstream of APP$_{695}$
A. Western blot analysis was used to assess expression of Akt, phospho-Akt (T308) and PTEN in SN56 (mock) and SN56 (APP$_{695}$) cells. **B.** SN56 (APP$_{695}$) cells were treated with wortmannin (250nM for 4h (n=6, p=0.77; or 1μM for 1h (n=6, p=0.35)) and AChE activity assayed by Ellman's assay. **C.** Relative levels of pFAK were quantified by ELISA in SN56 (mock) and SN56 (APP$_{695}$) cells (n=6, p=0.62).

105

The data showed an increase in protein levels of total Akt and marked increases in levels of phospho-Akt in the SN56 (APP_{695}) cells were detected, with immunoreactivity at approximately 65kDa for Akt and phospho-Akt (Fig. 4.12A, pAkt antibody a kind gift from Dr. I. C. Wood, University of Leeds). However, this increase was specific to the Thr308 phosphorylation site, as no immunoreactivity for Akt phosphorylated at Ser473 was observed (data not shown).

As a consequence of this putative role for Akt in mediating the transcriptional repression of AChE in this system, signalling molecules that have been shown to modulate Akt phosphorylation were investigated, namely focal adhesion kinas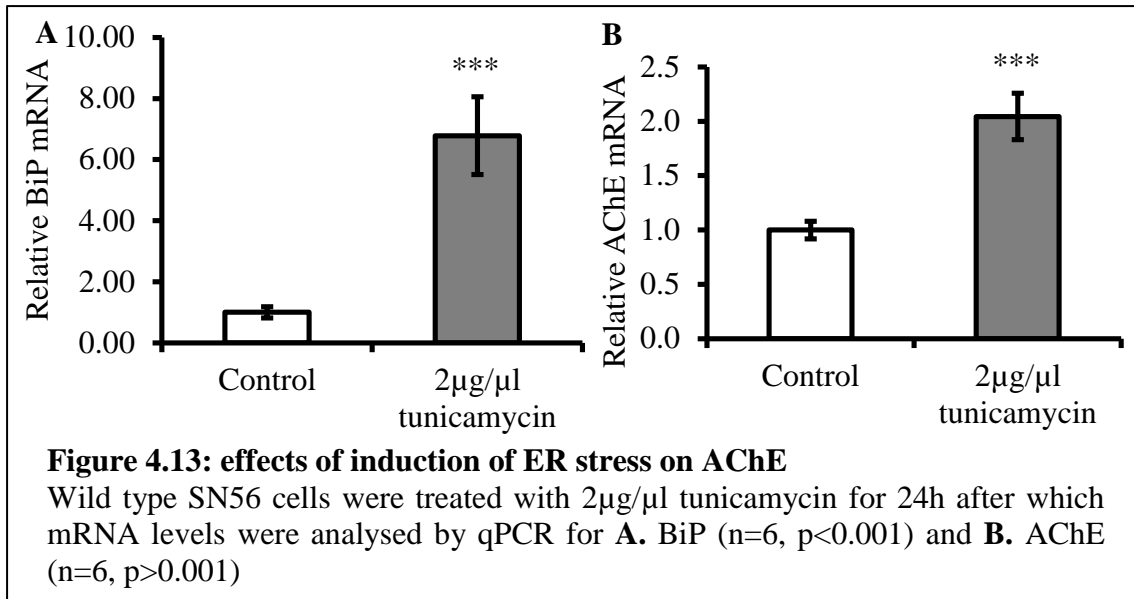e (FAK) and phospho-FAK, PTEN and phosphatidylinositol-3-kinase (PI3K). There were no observable differences in PTEN protein levels between the cell lines (55kDa, Fig. 4.12A). PI3K activity can be inhibited by treatment with wortmannin. SN56 (APP_{695}) cells were treated with wortmannin on the premise that increased AChE activity after treatment would implicate PI3K in the signalling pathway under investigation. However, at two different concentrations of wortmannin, no changes in AChE activity were detected (Fig. 4.12B). As shown by Clarke *et al.*, phosphorylation and activation of FAK is capable of initiating a signalling cascade that can modulate Akt activity (Clarke et al., 2012). Levels of phosphorylated FAK (pFAK) were analysed by ELISA in SN56 (mock) and SN56 (APP_{695}), no changes in pFAK were observed between these two cell lines (Fig. 4.12C).

4.8 Assessment of the specificity of the regulation of AChE

Although over-expression is a widely used technique in molecular biology, it does involve expression of large amounts of protein, which could potentially stress the translational machinery, such as the endoplasmic reticulum. This could result in non-specific effects, confounding the data. To address this potential issue, the widely used reagent tunicamycin was used to induce ER stress in wild type SN56 cells. To monitor the induction of ER stress, the chaperone BiP was used as a marker.

Figure 4.13: effects of induction of ER stress on AChE
Wild type SN56 cells were treated with 2µg/µl tunicamycin for 24h after which mRNA levels were analysed by qPCR for **A.** BiP (n=6, p<0.001) and **B.** AChE (n=6, p>0.001)

Treatment of SN56 cells with tunicamycin induced ER stress as evidenced by the marker BiP, the transcripts of which increased approximately sevenfold (Fig. 4.13A). Under these treatment conditions, AChE transcripts approximately doubled (Fig. 4.13B). In addition, transient transfection of neither ACE2 nor APP caused an increase in BiP mRNA, with the latter causing a significant decrease (data not shown).

4.9 The role of histone acetylation in the regulation of AChE

Possible epigenetic modifications were subsequently assessed as the root cause for the transcriptional repression observed. Epigenetic changes have been linked to regulation of gene expression for a large number of genes. One such gene, NEP, has been extensively studied in our laboratory. The NEP gene is regulated by APP and this regulation is in part mediated by AICD binding to the NEP promoter (Pardossi-Piquard et al., 2005, Belyaev et al., 2009). Acetylation of the histone protein component of chromatin is generally linked to activation of transcription, with the level of acetylation maintained by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Ooi and Wood, 2008). In the NEP paradigm, AICD displaces HDACs from the gene promoter, increasing both histone acetylation and gene transcription (Belyaev et al., 2009, Belyaev et al., 2010). Whether this was the mechanism by which APP was able to exert transcriptional repression of AChE was investigated. To do this, a previously employed non-specific HDAC inhibitor, sodium valproate, was used (Gottlicher et al., 2001).

Figure 4.14: effects of HDAC inhibition on AChE activity
SN56 (APP$_{695}$) cells were cultured as described (section 2.2.1.1) and treated for 48h with 10μM sodium valproate. Cells were subsequently lysed and AChE activity measured by Ellman's assay (n=9, p=0.99)
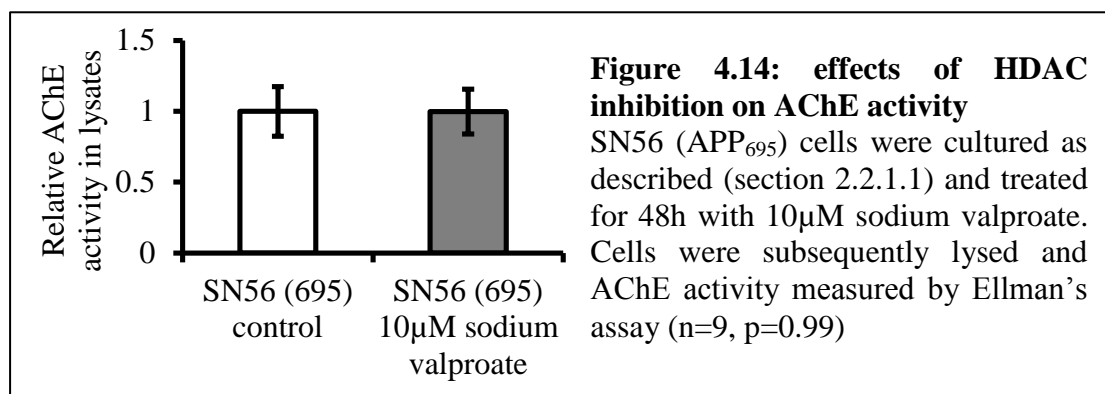
Treatment of SN56 (APP$_{695}$) cells with sodium valproate did not result in any significant changes in AChE activity (Fig. 4.14).

4.10 The effect of APP$_{695}$ over-expression on other genes of the cholinergic system

In the paradigm of gene regulation by AICD, this pathway is responsible for the modulation of many genes (Chang and Suh, 2010, Aydin et al., 2012). In light of this, the effects of APP$_{695}$ over-expression on three other cholinergic genes were investigated. Putative changes in the mRNA levels of the hydrolytic enzyme BChE, the membrane anchor of AChE, PRiMA and finally the high affinity choline transporter (CHT) were probed.

SN56 cells over-expressing APP$_{695}$ exhibited no differences in mRNA levels of either BChE or CHT (Fig. 4.15A and B). However, analysis of PRiMA mRNA levels revealed a significant decrease in the transfected cells, approximately 50% (Fig. 4.15C). PRiMA mRNA levels in SN56 cells over-expressing the ΔE1 mutant of APP were also investigated. The relationship between APP and PRiMA was seen to recapitulate that of AChE. While wild type APP$_{695}$ had a significant effect on PRiMA transcripts, deletion of the E1 domain completely ablated this (Fig. 4.15D).

Fig. 4.15: qPCR analysis of cholinergic gene expression after APP$_{695}$ over-expression

QPCR analysis of mRNA transcripts in SN56 (mock) and SN56 (APP$_{695}$) cells, assaying **A.** BChE (n=6, p=0.51), **B.** CHT (n=9, p=0.99) and **C.** PRiMA transcripts (n=7, p < 0.001). **D.** qPCR analysis of mRNA in SN56 (mock) and SN56 (APPΔE1) cells (n=6, p=0.4).
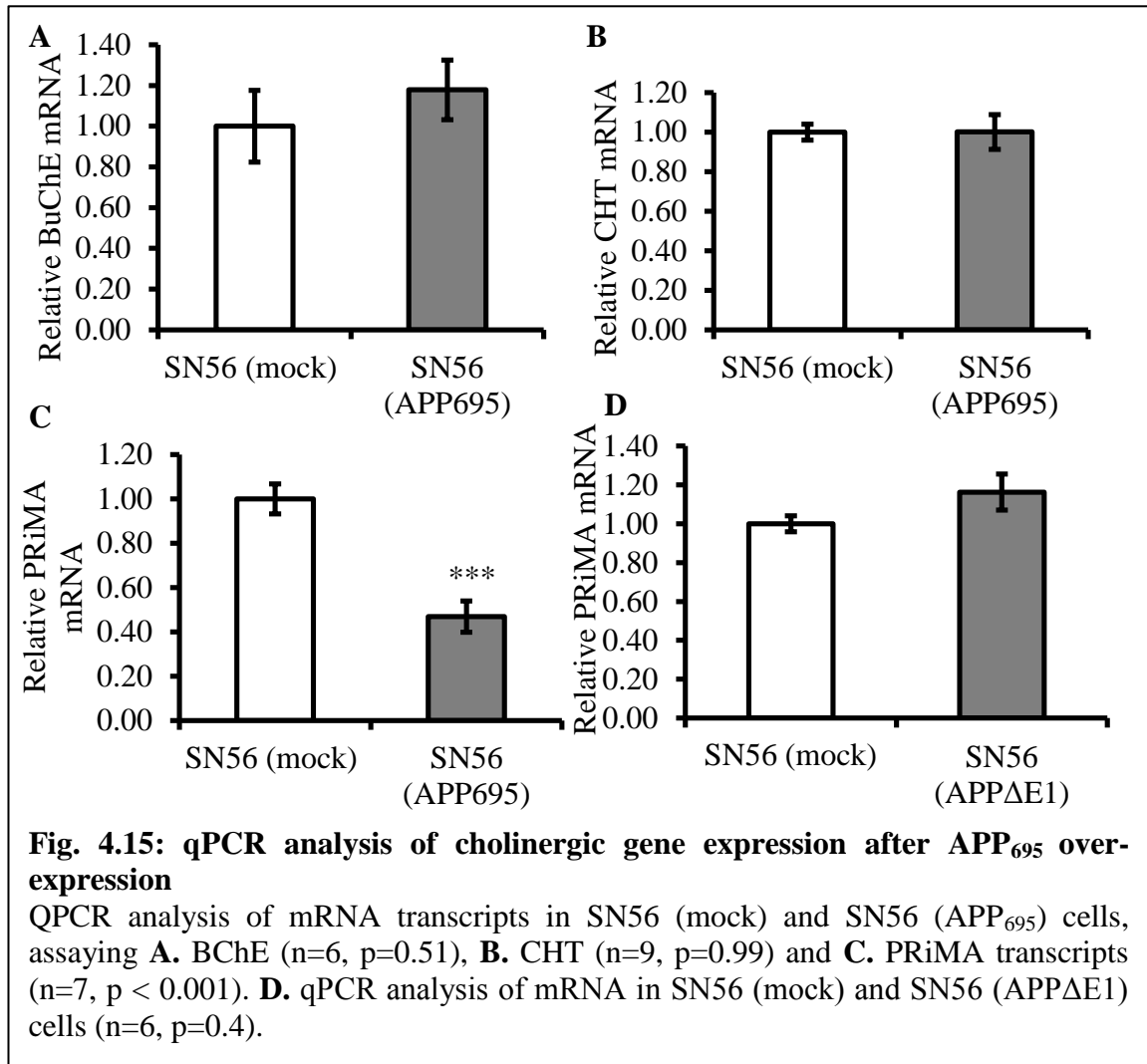
4.11 Discussion

4.11.1 Over-expressed and endogenous APP both mediate transcriptional repression of AChE

A significant part of the rationale for the genesis of these studies derived from the work of Bimonte *et al.*, who described activatory effects on AChE of an Fe65 orthologue in *C.Elegans* (Bimonte et al., 2004). They linked this finding to APP through its intracellular domain, the studies of which were still in their infancy at that time. The data shown here do not support a role for AICD, which is not to say Fe65 does not activate AChE, just that it would be unlikely that APP initiates this process. It is possible, however, that this discrepancy derives from species differences. For example,

Fe65 expression is substantially higher in NB7 cells compared to SH-SY5Y cells (Belyaev et al., 2009), yet AChE activity is significantly lower in the former.

4.11.2 Transcriptional repression of AChE is independent of secretase action

In terms of transcriptional regulation of specific genes by APP, the majority of its target genes are regulated by AICD. A significant number of targets genes have been ascribed to AICD, several of which are strongly linked to AD (Chang and Suh, 2010, Beckett et al., 2012). However, the demonstrable dispensability of γ-secretase in the work described here indicated that the regulatory relationship seen does not follow the AICD paradigm. Similarly, α- and β- secretases were shown to be dispensable, implicating the APP holoprotein in transcriptional repression of a specific gene, AChE. This represents a novel notion, whereby APP can regulate specific genes independently of its cleavage. However, although target genes in this paradigm currently represent a small cadre, very recent work from Pierrot *et al.* has indicated that HMG CoA reductase is also a gene target of the APP holoprotein, with APP again mediating repression (Pierrot et al., 2013).

4.11.3 The copper-binding domain in the E1 region of APP is critical for transcriptional repression of AChE

Experiments were performed to identify the specific region(s) of APP that mediated the transcriptional repression of AChE. Initially, guided by the literature, work focused on the intracellular C-terminus of APP. A number of key interacting partners bind here, with these processes not dependent on γ-secretase cleavage of APP (Aydin et al., 2012). These proteins mediate a variety of signalling processes and include Fe65 (Fiore et al., 1995); Dab1 (Homayouni et al., 1999); Numb (Roncarati et al., 2002); the JNK-interacting protein, JIP1b (Taru et al., 2002, Inomata et al., 2003); X11/ Mint (Borg et al., 1996) and also SH2-binding proteins such as Grb2 (Zhou et al., 2004). Grb2 aside, these interactions are mediated through phospho-tyrosine binding (PTB) domains in the interacting proteins. These binding events are centred on the YENTPY motif in the APP C-terminal tail, with the key binding residues being the two tyrosine residues Y682 and Y687 (Aydin et al., 2012). To elucidate any role for these residues in the transcriptional repression of AChE, mutant constructs were generated, mutating the two tyrosines to glycines, both individually (Y682G and Y687G) and together (YYGG). However, over-expression of these mutant APP constructs resulted in decreases in AChE activity and

mRNA commensurate with those seen with wild type APP$_{695}$. This finding represented an initial indication that the APP C-terminus was not involved in the transcriptional repression of AChE. Similarly, Pierrot and colleagues were able to identify a juxtamembrane GxxxG motif as being critical for the APP-mediated repression of HMG CoA in their system, independent of the C-terminus (Pierrot et al., 2013).

As a consequence of this, an APPΔE1 construct was employed to further investigate the possible involvement of the N-terminal E1 domain in AChE repression (see Fig. 1.6 for schematic of APP). Although the E1 domain is large, its deletion does not affect the trafficking or localisation of the mutant APP (Kaden et al., 2009). Unlike the C-terminal region, the E1 domain had not been linked to regulation of any specific genes. However, a number of putative interacting partners have been discovered (Aydin et al., 2012, Rice et al., 2013), one of which may represent part of a signalling pathway resulting in repression of AChE. The data showed that E1 deletion ablated the repressive effect exerted on AChE by APP. Although the E1 domain is present in the soluble ectodomains, sAPPα and sAPPβ, the cleavage step responsible for their generation is not required in this paradigm. However, given the large size of the E1 domain, it was important to characterise the specific region required for APP to repress AChE transcription. There are two sub-domains in E1, a growth factor-like domain (GFLD) and the CuBD. The latter comprises three key histidine residues, which were mutated to alanines to generate an APPΔCuBD construct. This construct has been shown to traffic in the same way as wild type APP$_{695}$ and undergoes proteolysis in a very similar manner, although sAPPβ levels are slightly elevated (E. T. Parkin and M. Gough, personal communication). As with the APPΔE1 construct, the APPΔCuBD construct was unable to exert any effect on AChE, either at the activity or mRNA level. From these data, a critical importance of the His triad comprising the CuBD in the repression of AChE was inferred.

The E1 domain has been linked with dimerisation (Soba et al., 2005, Isbert et al., 2011) and indeed the CuBD has been specifically tied to this process (Noda et al., 2013). It is possible, therefore, that the derivation of AChE repression is APP dimerisation and, indeed, APP and APLPs are frequently cited in APP interactome studies (Bai et al., 2008, Soler-Lopez et al., 2011, Kohli et al., 2012). However, no specific genes have been linked to the process of dimerisation and a recent review indicated that, to date, the

main functions of APP dimerisation were cellular adhesion and intercellular interactions (Khalifa et al., 2010).

The number of interacting partners of the APP N-terminus continues to grow (Aydin et al., 2012, Rice et al., 2013). Given their breadth and that defined intracellular signalling pathways have been ascribed to at least some of these interacting proteins, this surely represents a more convincing mechanism by which APP might initiate a signalling cascade culminating in the transcriptional repression of AChE.

4.11.4 Integrins represent possible mediators of APP transcriptional repression of AChE

Reported N-terminal APP binding partners include fibulin-1, reelin, F-spondin, Lingo-1, contactin 2, pancortins 1 and 3, integrin β1 (ITGB1) and integrin α5 (ITGA5) (Yamazaki et al., 1997) (Young-Pearse et al., 2008, Aydin et al., 2012, Rice et al., 2013). Of these, ITGB1 is capable of binding copper binding domains (Weaver et al., 2008), although this has not been directly shown for APP. A necessary requirement of a binding partner is that the interacting protein must be able to transduce intracellular signals which ultimately result in transcriptional repression of AChE. Most of the APP binding partners listed are adhesion proteins and many do not have any defined intracellular signalling activity. However, integrins do have defined intracellular signalling activity (Schwartz, 1992, Calderwood et al., 2003), modulating such proteins as focal adhesion kinase and Akt (Schaller et al., 1992, Clarke et al., 2012).

Clarke *et al.* were able to show binding of ACE2 to ITGB1 and ITGA5, followed by subsequent initiation of signalling cascades through FAK (Clarke et al., 2012). Given that these integrins were of interest due to their being APP interacting partners, ACE2 was over-expressed in SN56 cells and it was found that this could recapitulate the effects of APP over-expression. This is not a non-specific effect as the transient transfection of ACE2 and APP did not show any increases in cell stress, as evidenced by qPCR analysis of the ER chaperone and cell stress marker BiP. This clearly shows that the commonality between the effects of APP and ACE2 over-expression did not derive from the over-expression protocol itself. Furthermore, specific induction of cell stress using tunicamycin resulted in an increase in AChE mRNA levels, not the decrease seen with APP over-expression.

In light of a putative common signalling pathway, a possible role for ITGB1 in the APP-mediated repression of AChE was investigated. To do this, ITGB1-null GD25 cells (Fassler et al., 1995) and their ITGB1-expressing counterparts, GD25β1, were used. Although decreases in AChE mRNA of approximately 30% were seen, there was no difference between those cells null for ITGB1 and those expressing ITGB1. Although it is possible that this finding derives from a cell line specificity of this APP-AChE regulatory relationship, an effect of APP_{695} over-expression was seen, from which it was inferred that it was unlikely that ITGB1 had a significant role in the transcriptional repression of AChE by APP. However, Clarke *et al.* showed co-immunoprecipitation of ACE2 with ITGA5 (Clarke et al., 2012) and furthermore, ITGA5 has been shown to interact with APP (Yamazaki et al., 1997). Given that ITGA5 is a binding partner of ACE2 and APP, the elusive nature of common interacting proteins and the well characterised downstream integrin signalling pathways, ITGA5 remains a possible candidate as the first protein involved in the regulatory pathway described here.

4.11.5 Phosphorylation of Akt may represent a key step in the signalling cascade linking APP to transcriptional repression of APP

There is significantly more literature concerning the activation of AChE than its repression, often linking its elevation to apoptosis (Zhang et al., 2002, Masha'our et al., 2012, Zhang et al., 2013). However, one paper, examining the response of AChE to H_2O_2 treatment, reported that these H_2O_2- induced increases could be blocked by over-expression of Akt (Xie et al., 2011). Given this finding and links between integrin signalling and Akt activation (Khwaja et al., 1997, Clarke et al., 2012), the levels of total Akt and two phospho-forms (T308 and S473) were investigated. Recapitulating some of the findings of Clarke *et al.*, a marked increase in total Akt upon stable over-expression of APP_{695} in SN56 cells was found. Concomitant increases in phospho-Akt were found but only at the T308 phospho-epitope, with no immunoreactivity found at S473. Much of this increase in active phospho-Akt derived from the observed increase in total Akt, but the data indicate the possibility of an increased proportion of phospho-Akt also. These data, therefore, showed that APP over-expression can increase Akt levels and hence Akt signalling, which may be of central importance in the transcriptional repression of AChE.

Given the implication of Akt in the pathway by which APP can exert transcriptional repression of AChE, related proteins were investigated. PI3K is closely linked to Akt signalling (Khwaja et al., 1997, King et al., 1997), yet use of the PI3K inhibitor, wortmannin, had no effect on AChE activity. This may derive from the fact that much of the increase in Akt is due to increased total Akt, rather than the increased phosphorylation with which PI3K would be closely involved. PTEN was also investigated, which has been identified as having the ability to repress Akt signalling (Stambolic et al., 1998). No changes in PTEN protein levels were observed, but this does not unequivocally exclude a role for PTEN as its function may be dependent on phosphorylation. FAK has been linked to integrin signalling and increases in Akt (Schaller et al., 1992, Clarke et al., 2012) and so an ELISA was used to investigate phospho-FAK (pY397), but no difference between SN56 (mock) and SN56 (APP$_{695}$) cells were observed. However, this does not exclude a role for FAK in this signalling pathway. The data showed that Y397 is not involved, but FAK has four other Tyr residues, in addition to four Thr and twelve Ser residues (Grigera et al., 2005), any of which may act as an interacting site for a binding partner.

4.11.6 APP does not repress AChE through HDAC-mediated histone deacetylation

Although many genes are regulated by binding of activating and repressive TFs to promoter regions (Levine and Manley, 1989), regulation of a growing list of genes is known to be epigenetic, involving histone modifications such as acetylation and methylation (Ooi and Wood, 2008). A number of genes germane to this investigation have been shown to be regulated at an epigenetic level, namely NEP (Belyaev et al., 2009, Belyaev et al., 2010), BDNF (Walker et al., 2013), ChAT (Aizawa et al., 2012) and AChE itself (Sailaja et al., 2012). Whether increased HDAC activity may be responsible for the observed transcriptional repression was investigated, using a well-defined, broad-spectrum HDAC inhibitor, sodium valproate (Gottlicher et al., 2001, Nalivaeva et al., 2009). Although the data suggest HDAC involvement is unlikely, such is the smorgasbord of possible epigenetic modifications (Ooi and Wood, 2008), epigenetics mechanisms remain a possible root of AChE repression.

4.11.7 APP is not a global regulator of the cholinergic system in neuronal cell lines

After confirming a regulatory role of APP in modulating AChE expression, it was hypothesised that APP may regulate the several genes encoding cholinergic system proteins, rather than just one gene. To address this, we investigated the genes encoding three key cholinergic proteins. BChE is the other mammalian cholinesterase and although its function remains unclear, it has been shown that its regulation is linked to levels of AChE (Mesulam et al., 2002, Darvesh et al., 2003, Lockridge et al., 2003). However, we did not observe any differences in BChE mRNA levels after over-expression of APP_{695} and consequent down-regulation of AChE. It is entirely possible, though, that any BChE-centric compensatory mechanisms derive from alterations in trafficking or shedding, not expression.
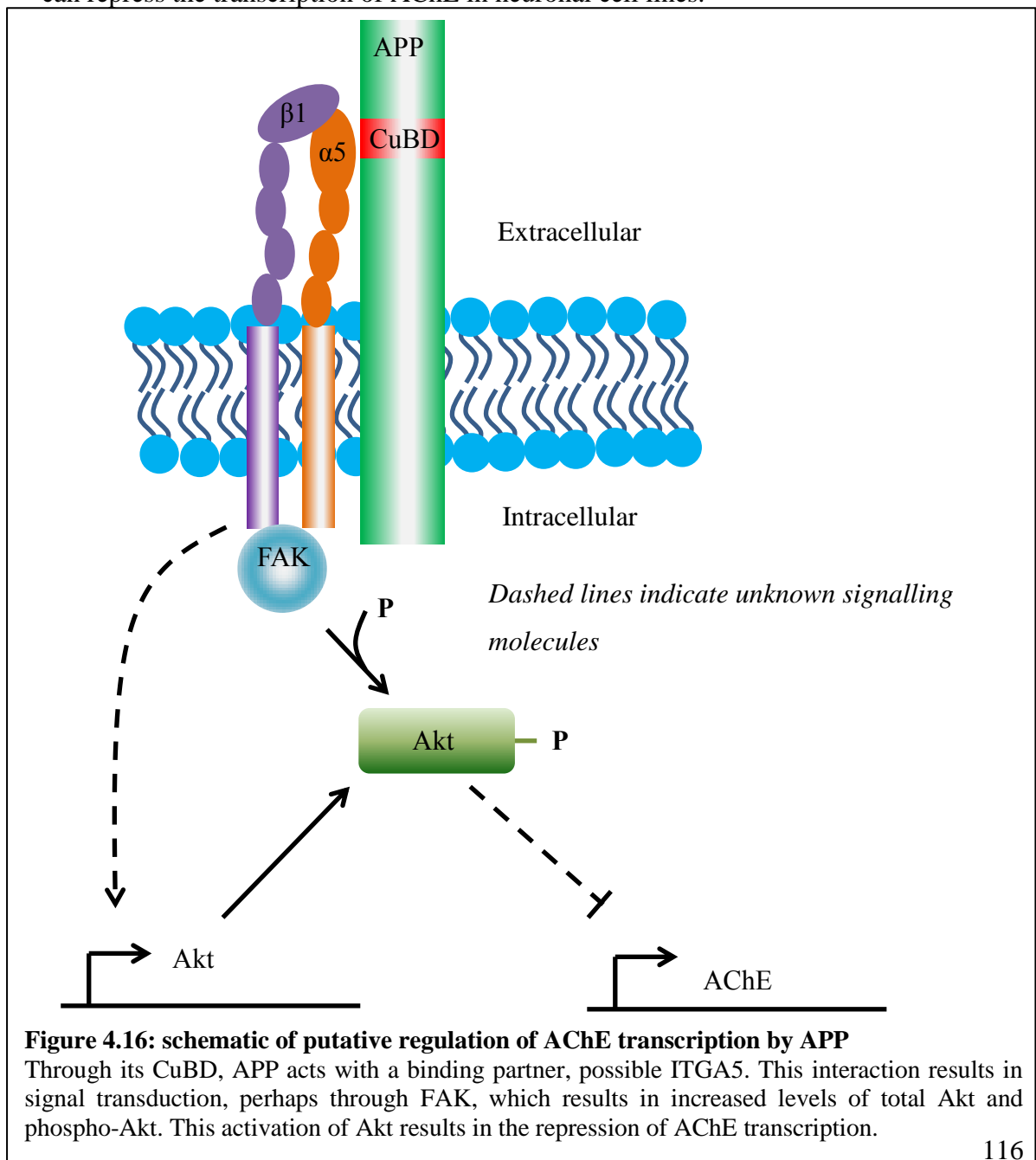
The mRNA levels of the choline transporter, CHT, in SN56 (mock) and SN56 (APP_{695}) cells were also investigated. CHT, responsible for transporting choline into the pre-synaptic terminal after ACh hydrolysis (Sarter and Parikh, 2005), has been shown to co-localise with APP, although this was not observed in SN56 cells (Wang et al., 2007). Similarly to BChE, there were no differences in CHT mRNA levels between the two cell lines.

The final gene under investigation was that encoding the membrane anchor of AChE, PRiMA (Perrier et al., 2002). In this case, a significant decrease in PRiMA mRNA was observed in the SN56 (APP_{695}) cells as compared to the mock transfected cells. Like AChE, APPΔE1 was unable to recapitulate the reductions in mRNA observed after transfection with wild type APP_{695}. It is possible that PRiMA is targeted independently by this pathway and indeed both PRiMA and AChE have a CREB binding site in their promoter regions, but PRiMA lacks the Egr-1 site present in AChE (Getman et al., 1995, Perry et al., 2004). However, it may be considered unlikely that two genes whose expression must necessarily be tightly coupled would be targeted independently by this APP-mediated pathway. The lack of regulation of two other significant cholinergic genes and the disparate chromosomal locations of AChE and PRiMA (Getman et al., 1992, Perrier et al., 2002) also support this. Given the role of PRiMA as the chaperone and membrane anchor of AChE (Perrier et al., 2002, Xie et al., 2010a, Hicks et al., 2011), it is likely that their expression levels are tightly coupled. This derives from the fact that AChE cannot be mobilised without PRiMA, so any increase in AChE

shedding, such as that derived from mAChR agonism (Hicks et al., 2013), will also require increased levels of the membrane anchor. Given the main role of AChE is as a hydrolytic enzyme and the consequent need for rapid changes in its cell surface expression, this would suggest that the expression of PRiMA is exquisitely responsive to AChE levels. This, then, proffers an alternative hypothesis, namely that AChE is the only target of APP-mediated repression and that PRiMA responds because of the marked changes in AChE levels, not because it is independently targeted by APP.

4.12 Conclusion

In this work, a novel regulatory pathway has been outlined (Fig. 4.16), whereby APP_{695} can repress the transcription of AChE in neuronal cell lines.

Figure 4.16: schematic of putative regulation of AChE transcription by APP
Through its CuBD, APP acts with a binding partner, possible ITGA5. This interaction results in signal transduction, perhaps through FAK, which results in increased levels of total Akt and phospho-Akt. This activation of Akt results in the repression of AChE transcription.

116

Consequently, there are significant decreases in AChE protein levels and catalytic activity. This pathway is independent of cleavage of APP by any secretase enzymes, excluding a role for AICD in this system. The critical region in APP has been identified as being the CuBD, located in the N-terminal E1 region. The signalling pathway downstream of APP likely involves a cell surface binding partner, perhaps an integrin and may involve activation of Akt, which has been shown to block increases in AChE expression (Xie et al., 2011). Finally, the possibility of regulation of the cholinergic locus by APP was explored, but ultimately the data showed AChE and PRiMA to be the only down-regulated genes. This paradigm may represent the genesis of a burgeoning field of investigation, as evidenced by this work and a recent paper implicating APP in the secretase-independent regulation of lipid metabolism through its down-regulation of HMG CoA reductase (Pierrot et al., 2013).

Chapter 5

Discussion

Chapter 5: Discussion

5.1 The cholinergic hypothesis of AD

The cholinergic hypothesis contends that cholinergic dysfunction is the primary driver of the cognitive dysfunction observed in AD (Bartus et al., 1982). The work outlined here posits hypotheses as to how perturbations in the cholinergic system may lead to cell death, or drive amyloid pathology. Firstly, this work has shown that APP can repress AChE transcription in neuronal cell lines, which is independent of its cleavage. This is the first example of the APP holoprotein regulating a specific gene, rather than one of its fragments. It is clear that a multi-protein signalling pathway links APP and AChE, so any disruption of this would increase AChE expression, leading to increased ACh hydrolysis and possible apoptosis. Any dysregulation in the localisation or trafficking of APP would disrupt AChE repression and may then lead to the cholinergic perturbations seen in AD. In this case, APP would be involved, but it would be independent of the function or formation of Aβ.

Furthermore, cell death may be caused by stress-induced upregulation of AChE, although Aβ can also induce cell stress (Querfurth and LaFerla, 2010). Investigating such a possibility in this work, the ER stressor tunicamycin was shown to increase AChE mRNA, confirming the work of others (Greenberg et al., 2010). Thus any induction of ER stress could mediate apoptosis through AChE, perhaps in part explaining the cholinergic vulnerability in AD. Heightened AChE would necessarily lead to reduced signalling through mAChRs, which have been shown to positively regulate non-amyloidogenic processing of APP (Nitsch et al., 1992). In this case, up-regulation of AChE would precede increased amyloidogenic processing of APP, but would ultimately be the causative factor.

The work here, then, has outlined two paradigms whereby selective death of cholinergic neuronal cells may occur. These processes are independent of Aβ and thus represent supporting mechanism for the cholinergic hypothesis.

5.2 SN56 cells represent a robust model for the study of Alzheimer's disease

As the range of models available for the study of AD continues to increase, the debate concerning which are the most appropriate shows no sign of abating (Gotz and Ittner,

2008, Gotz and Gotz, 2009, Lee and Han, 2013). Cell culture of immortalised cell lines is a popular investigative tool and, indeed, it is one employed in this study. However, a criticism of this method is questionable similarity to human neurons, which, for the most part, do not divide. Another issue is the phenotypic similarity, as cell lines may express different gene cohorts from primary cells. While primary cells are better representations of neurons or glia *in vivo*, it is still an *in vitro* approach. This itself has drawn criticism, because AD is a disease of multi-factorial aetiology and therefore it has been suggested that culture of isolated cells cannot represent accurately an intact organ, in this case the brain (Horrobin, 2003, Lindsay, 2003). Model organisms are widely used *in vivo*, with greater emphasis being placed on the use of organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Gotz and Ittner, 2008, Costa et al., 2011, Munoz-Lobato et al., 2013).

There is undoubtedly an extent to which a parochial attitude towards *in vitro* cell culture models has pervaded the field (Horrobin, 2003). However, it seems clear that each model system brings its own set of advantages and disadvantages. In light of this, it becomes a case of selecting the most appropriate model for the project at hand, whether that be *C. elegans* or *Mus musculus.* In this work, SN56 cells have been demonstrated to express key cholinergic proteins and AChE, BChE, CHT, ChAT and PRiMA have been investigated, as well as several AChRs. Expression of these genes was compared to the SH-SY5Y cell line and on this basis, SN56 cells represent a model system that is closer in phenotype to basal forebrain cholinergic neurons (BFCNs). This neuronal subpopulation is of key importance in AD (Schliebs, 2005, Schliebs and Arendt, 2011), as cholinergic efferents project to a wide range of brain regions, hence BFCNs are involved in such functions as memory and information processing (Auld et al., 2002). These neurons have already been shown to be selectively targeted in the early stages of AD pathology (Whitehouse et al., 1981, Bartus et al., 1982, Coyle et al., 1983, Francis et al., 1999) and so research on neurodegeneration and neuroprotection in AD should focus on BFCNs to a greater extent. As has been shown through gene and protein analysis, SN56 therefore represent a good model for study of BFCNs. SH-SY5Y cells have also been shown to be a possible model system, although their phenotypic similarity to BFCNs is lower than SN56 cells.

5.3 Identification of the AChE sheddase may lead to a novel therapeutic for AD

It is over twenty years since the amyloid cascade hypothesis was first formalised (Hardy and Higgins, 1992). Since then, debate over the centrality of Aβ in the pathology of AD has continued (Hardy, 2009, Karran et al., 2011, Teich and Arancio, 2012). However, Aβ is still a prominent therapeutic target (Golde et al., 2011), as evidenced by the recent, but thus far unsuccessful, therapeutic strategies of γ-secretase inhibition and immunotherapy (De Strooper et al., 2010, Samson, 2010, Karran, 2012). Although amyloid plaques are considered a pathological hallmark, it is thought to be Aβ oligomers that disrupt LTP and lead to synaptic dysfunction (Walsh et al., 2002, Cleary et al., 2005, Sakono and Zako, 2010, Benilova et al., 2012). The formation of these oligomers is necessarily dependent on aggregation of Aβ monomers and hence this step has been considered as a therapeutic target. For example, this was the therapeutic target of tramiprosate, marketed as Alzhemed, which ultimately failed to show clinical benefit (Aisen et al., 2011). However, encouraging support of this target has recently come from a collaboration generating a peptide which prevents Aβ aggregation and then using this *in vivo*, in AD transgenic (APP$_{Swe}$/ PS1ΔE9) mice. Beneficial readouts in features such as neurogenesis, inflammation and amyloid deposition were subsequently observed (Parthsarathy et al., 2013).

The validity of the amyloid cascade hypothesis still remains to be confirmed or rejected. That mutations in either APP or the presenilins can lead to early-onset AD affirms a clear role for APP in AD. The clustering of these mutations around the β- and γ-secretase sites is indicative that Aβ production is of central importance in the progress of AD (Karran et al., 2011). In addition, a recent AD-protective mutation at the β-secretase site resulted in a 40% reduction in amyloid peptides (Jonsson et al., 2012). Although recent amyloid-targeting clinical trials have shown disappointing results, this may result not from addressing the wrong target, merely lacking an efficacious way in which to modulate the target (Karran et al., 2011, Karran, 2012). Perhaps this derives in part from the inability of trialled therapies to target amyloid deposited before treatment (Auld et al., 2002, Karran et al., 2011), but a lack of understanding of the mechanisms leading to neuronal loss is also responsible.

There are a large number of mouse models available for AD research (Oddo et al., 2003, Gotz and Ittner, 2008, Lee and Han, 2013), yet although they re-capitulate amyloid

deposition and tau pathology, neuronal loss is not seen (Auld et al., 2002). This is a demonstration of how a knowledge gap between amyloid and cholinergic cell death has confounded efforts to generate a representative AD mouse model. Therefore, the description here of a novel link between APP and the cholinergic system may lead to a fuller understanding of the mechanisms underlying the death of cholinergic neurons.

It has been shown that AChE has a putative role in Aβ aggregation (Inestrosa et al., 2008). Firstly, AChE was shown to localise with Aβ plaques and subsequently shown to be actively involved in the promotion of aggregation (Mesulam et al., 1987, Alvarez et al., 1997). The AChE C-terminus has been suggested to be critical for this process, as there is significant sequence similarity between the AChE C-terminal region and Aβ (Greenfield and Vaux, 2002). In this way, AChE is able to act as a heterogeneous nucleus or "seed", for amyloid aggregation. However, this C-terminus is not available for seeding aggregation when AChE is in its membrane bound form, as the AChE C-terminus is bound to PRiMA (Gennari et al., 1987, Perrier et al., 2002, Dvir et al., 2004). Logically, then, AChE can only promote aggregation of Aβ when it is released into the extracellular milieu. Possible pathways by which AChE is released from neuronal cell lines have been described, noting an important role of proteolytic shedding (Greenfield et al., 1983, Schweitzer, 1993, Hicks et al., 2013). A key role in this shedding process for a batimastat-sensitive metalloprotease has been demonstrated here, so this may represent a therapeutic target for AD research. Were AChE shedding to be at least partially blocked, this may help reduce Aβ aggregation. In this way, the data here may contribute to the discovery of new avenues of therapeutic investigation.

When targeting AChE as a therapeutic strategy, the stoichiometry of AChE and the inhibitor is critical. It is well known that acute and potent irreversible inhibition of AChE, such as by organophosphates (e.g. sarin and VX), causes death (Abu-Qare and Abou-Donia, 2002, Joosen et al., 2008). However, the dominant existing therapies for AD serve to validate the safety and feasibility of targeting AChE if the stoichiometry of inhibitor to AChE is correct. These therapies, including donepezil, galantamine and rivastigmine (Greig et al., 2013), reversibly inhibit the catalytic activity of AChE with a good safety profile (Mehta et al., 2012), so it would be possible to block AChE shedding without safety concerns. Furthermore, even if AChE shedding was completely blocked, it has already been shown that AChE can be released by exocytosis, so there

would be some AChE still released. It remains to be seen whether this would be able to compensate for significant reductions in AChE shedding. A paradigm has been outlined whereby AChE is shed in a two-step mechanism, requiring PDI and a metalloprotease (Hicks et al., 2013), likely from the ADAM or MMP family. Targeting either of these is likely to have a number of off target effects, as they are both involved in a number of other cellular processes (Edwards et al., 2008, van Goor et al., 2009). Thus, targeting both of these with lower concentrations of inhibitor may reduce off target effects, but have a synergistic effect in targeting the shedding of AChE.

5.4 M1 mAChR agonism leads to AChE upregulation and Egr-1 activation: implications in therapy

The cholinergic hypothesis states that cholinergic hypofunction, especially in BFCNs, can lead to the cognitive defects seen in AD (Bartus et al., 1982, Francis et al., 1999). This is the rationale behind the current AChE inhibitors in clinical use. Their aim is to reduce AChE hydrolysis of ACh, hence potentiating cholinergic signalling with the purpose of reversing the cholinergic deficit. Although this approach has brought some success, the benefits seen are modest (Birks, 2006). A related approach involves mAChR agonism, aiming to potentiate pathways with reduced activity due to low ACh levels and restore normal mAChR function, perturbed in AD (Fisher, 2011). Other stated benefits include increased non-amyloidogenic processing of APP, increasing sAPPα (Nitsch et al., 1992), and cleavage of the prion protein (PrP^c) (Cisse et al., 2005), believed to be a receptor for Aβ oligomers (Lauren et al., 2009, Gimbel et al., 2010). A comprehensive case reviewing the benefits of M1 mAChR agonists has been outlined previously (Fisher, 2011). While it is unlikely that this approach is necessarily flawed, the results here do suggest a note of caution. The results here indicate that the off target effects of such an approach are likely to be considerable. ERK activation and consequent involvement of at least one, if not more, Ets family TFs (Besnard et al., 2011), must be considered when appraising this therapy. However, one concern is the transcriptional activation of AChE by Egr-1 binding that is seen after mAChR activation. This will surely work against any efforts to restore cholinergic signalling and it must be assessed as to whether the risks of AChE activation outweigh the other benefits conferred by mAChR agonism.

5.5 A novel regulatory function for APP

The normal physiological function of APP is one of the overlooked enigmas of AD research. Although the roles of APP in cell adhesion are often mediated via protein-protein interactions (Young-Pearse et al., 2008, Khalifa et al., 2010, Rice et al., 2013), functions of APP can also be inferred from the genes which it has been shown to regulate. For example, APP can regulate amyloid metabolism through its modulation of expression of specific genes, i.e. NEP and itself (von Rotz et al., 2004, Pardossi-Piquard et al., 2005), while a pro-apoptotic role has been suggested through its regulation of p53 (Alves da Costa et al., 2006). However, all of these genes are regulated through the C-terminus and the data presented here are the first to show regulation of a specific gene by the APP E1 domain and the CuBD within. Furthermore, this represents a novel and solid link between APP and regulation of the cholinergic system. It is becoming increasingly apparent that APP is capable of gene regulation through several of its domains, as outlined by Pierrot *et al.*, who were able to confirm a role for APP in lipid metabolism. Their work showed that a juxtamembrane GxxxG motif, independent of γ-secretase cleavage, reduced expression of HMG CoA reductase, a cholesterol biosynthetic enzyme (Pierrot et al., 2013). At present it is unclear if either the CuBD or GxxxG regulates a cadre of genes, like AICD, or whether regulation is limited to AChE/ PRiMA and HMG CoA reductase, respectively. However, both works strongly suggest two novel areas in which the APP holoprotein is functionally important.

5.6 APP may mediate neuroprotection through its transcriptional repression of AChE

There are clearly beneficial effects to be derived from AChE repression in the disease process. The most obvious of these is the decreased hydrolysis of ACh that is the desired outcome of the predominant current therapeutic strategies. This reduction in AChE catalytic activity, then, is likely to confer therapeutic benefit. However, pharmaceutical inhibition of AChE still allows the non-catalytic activities of AChE, such as promoting Aβ aggregation. In this respect, a reduction in AChE expression may be more beneficial than small molecule targeting of the active site.

Furthermore, AChE has a number of pro-apoptotic functions, so APP may have a role in modulating these. AChE has been shown to be elevated by a number of apoptotic stimuli and recently a scheme was suggested for a mechanism by which AChE might be

directly involved in cell death (Greenberg et al., 2010, Xie et al., 2011, Zhang and Greenberg, 2012). Hence it is possible that APP may act in a neuroprotective manner by modulating these pro-apoptotic pathways. Although generally AChE is decreased in AD (Atack et al., 1983), it is clear that significant amounts of this derive from death of AChE-expressing cells (Whitehouse et al., 1981, Coyle et al., 1983), rather than down-regulation of AChE. There is some evidence of increased plasma AChE in AD, indeed it was subsequently posited as a biomarker (Garcia-Ayllon et al., 2010). However, there is not a wide evidence base for this as yet and indeed it is not clear which AChE species are involved in apoptosis. There was a recent suggestion that N-terminally extended AChE species were the main pathological agents (Toiber et al., 2008, Toiber et al., 2009), so, in searching for increased AChE in AD, it is not always clear which species are involved.

Just as it is unclear if increased AChE in AD is a significant pathological driver, it is unclear if the APP-mediated repression of AChE is disturbed in the disease. However, were there to be perturbation of the repression of AChE, there would necessarily be negative outcomes which may contribute to the progression of the disease. In light of this, the data reported here represent a major step in elucidating the precise links between APP and AChE.

5.7 Future Perspectives

5.7.1 Deriving therapeutic benefit from inhibition of AChE shedding

As has been outlined previously, there may be therapeutic benefit in blocking AChE shedding, as this is likely to be a critical step in its promotion of Aβ aggregation. However, this approach is contingent on identifying the metalloprotease involved. The methodology employed has narrowed the candidate field from the entire proteome to a small subset of metalloproteases. The displayed sensitivity to batimastat, but resistance to GM6001 creates another criterion by which candidate enzymes can be filtered.

However, this approach does not offer specificity, as the metalloprotease inhibitors that were used are relatively broad spectrum. In this search for a specific proteolytic enzyme, the work of Oh *et al.* identified GM6001 as being approximately 1000-fold less potent in targeting ADAM12 than ADAMs 9, 10 and 17 (Oh et al., 2004). This presents

ADAM12 as a possible AChE sheddase and so a future approach, in the absence of a selective inhibitor, would be to use siRNA in an attempt to phenocopy the effects of batimastat. That is to say, if transfection with ADAM12 siRNA is able to reduce AChE shedding, this would be an indication that it is involved in cellular AChE shedding.

A clear requirement in searching for this metalloprotease is to be more systematic and develop a high throughput screen allowing the employment of RNA$_i$ libraries to assay the effects on AChE release of knockdown of candidate metalloproteases. The work reported here has limited the pool of candidate enzymes to approximately 20, so this approach is eminently feasible.

Following identification of the metalloprotease, small molecules can be designed for its inhibition, if they do not already exist. Given the likelihood of this protease being a member of a family of enzymes, specificity of inhibition is key. Given the small number of structural differences between batimastat and GM6001, the thienylthiomethyl group present in batimastat, but not in GM6001, is a good candidate as a functional group capable of inhibiting ChE shedding. Given this information, it is quite possible to design a small molecule compound library centring around this functional group. Screening of this library and assessment of the ability of different compounds to block AChE shedding may result in discovery of a useful lead compound. Following this, *in vivo* validation of the compound's efficacy is a long-term, but ultimately important, goal.

5.7.2 Understanding the mechanism by which APP targets the transcription of AChE
Although we have outlined a novel gene regulatory pathway by which APP can repress transcription of AChE, further work is needed to build on these findings. The first step is to identify the APP binding partner mediating the signalling pathway, a protein suggested to belong to the integrin family. There are likely to be a number of intracellular mediators in this paradigm and the involvement of Akt has been suggested, although there are likely to be several other proteins involved, both upstream and downstream of the putative role of Akt. One of the most important steps is to elucidate how this pathway operates in the nucleus. Our data indicate that epigenetic modification is unlikely, although we have only investigated acetylation of histones. In light of this, transcriptional repression of AChE may derive from binding of the repressive AP-2 TF,

or inhibition of binding of activatory TFs, such as Egr-1 or Sp1, all of which have binding sites in the AChE promoter (Getman et al., 1995).

Full elucidation of this signalling pathway will allow us to understand the extent to which it is perturbed in AD. Any element of this signalling pathway may be disturbed, leading to an undesirable increase in AChE. However, given that this pathway likely involves many molecules with multiple actions and targets (e.g. integrins, Akt, Egr-1), specifically targeting this pathway for therapeutic benefit will prove challenging.

Greater benefit could be derived from two other avenues of investigation. Firstly, we have tentatively suggested that this pathway may indicate a neuroprotective role for APP. It would be interesting to investigate whether APP could block the increases in AChE seen upon cellular exposure to certain apoptotic stimuli, such as H_2O_2 (Xie et al., 2011). Were this to be possible, further investigation of the effects of cell viability would surely be warranted.

Secondly, over the past five to ten years, the prevailing zeitgeist concerning APP has been investigation of the functions of AICD (Muller et al., 2008, Belyaev et al., 2009, Chang and Suh, 2010, Schettini et al., 2010, Beckett et al., 2012, Pardossi-Piquard and Checler, 2012). The number of AICD target genes has progressively grown and now two new regulatory pathways have been elucidated, involving the CuBD (the present work) and also the juxtamembrane GxxxG motif (Pierrot et al., 2013). Further investigation of these regions and any further possible target genes is likely to generate important information concerning putative physiological functions of APP. Although linked to AD many years ago, the normal physiological functions of APP have largely lacked clear definition. In this way, identification of further regulated genes and processes will be of significant benefit in AD research.

5.8 Conclusion

In summary, the importance of SN56 cells as a representative model of BFCNs has been underscored, which makes them of particular utility in the investigation of AD. Analysis of ChE activity and expression of several cholinergic markers showed these cells to have a cholinergic phenotype. SN56 cells were subsequently compared to SH-SY5Y cells, the latter being shown to similarly express some cholinergic markers, like SN56

cells, but to lack expression of ChAT, CHT and PRiMA. Finally, in model systems, there is no absolute hierarchy of usefulness. There are relative benefits and shortcomings of each model and hence the model should be selected on the aims of the project or multiple models tested.

The data presented here showed the AChE shedding process to be mediated by a batimastat-sensitive metalloprotease, with AChE release in this pathway dependent on the activity of PDI. An exocytic, Arf6-dependent component to AChE release was also observed. Regarding therapeutic avenues, the possible benefits of targeting the shedding of AChE have been discussed and how this may reduce formation of Aβ oligomers.

Muscarinic agonism was shown to increase shedding of AChE and also increased its transcription. This is likely mediated by phosphorylation and activation of ERK and upregulation of Egr-1 expression, the latter a known AChE-activatory TF. Given these data, while not dismissing the approach, concerns have been highlighted with mAChR agonism as an AD therapy, namely widespread off-target actions and also the transcriptional activation of AChE, which may confound any beneficial effects.

This work also outlined the transcriptional repression of AChE mediated by APP_{695}, likely through its CuBD. While PRiMA was similarly repressed, no changes in BChE or CHT mRNA were seen. While this pathway awaits full elucidation, it was inferred from our data than an APP N-terminal binding partner mediated AChE repression through upregulation of Akt. There are cellular benefits from the reduced AChE activity seen after APP_{695} over-expression, notably the decreased esterase activity. However, there may be more global effects of AChE repression, given its multifarious non-catalytic roles, especially in apoptosis. Disturbance of this regulatory pathway may contribute to pathological progression in the disease state.

Two predominant areas for further work based on these data have been outlined. The identification of the AChE sheddase may lead to small molecules to target AChE shedding. Inhibition of this process may reduce Aβ aggregation in AD. Secondly, fuller elucidation of the mechanism by which APP can repress AChE may reveal whether perturbation of this relationship is involved in AD. Further investigation of putative

target genes mediated by the CuBD will likely add to the currently sparse pool of information about the physiological functions of APP.

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