REGULATION OF BETA-AMYLOID SECRETION *IN VITRO* THROUGH P-GLYCOPROTEIN

by

Fred Chiu-lai Lam

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Department of Neuroscience

The University of British Columbia
Vancouver, Canada

Date Aug. 9, 2002

Abstract

The ~4 kDa β-amyloid (Aβ) peptide is believed to be central to the pathogenesis of Alzheimer's disease (AD). Aβ is formed by cleavage of the amyloid precursor protein (APP) through what is known as the amyloidogenic pathway. An alternate non-amyloidogenic pathway cleaves within the Aβ sequence of APP to form a secreted soluble fragment called APP_S. Initial experiments studying involvement of second messenger pathways in catabolism of APP led to the unexpected observation that the antiprogestin mefipristone (RU486) increased the secretion of APP_S from rat pheochromocytoma (PC12) cells. The effect was both rapid and potent, manifesting within 15 minutes of treating cells with RU486 at nanomolar concentrations. This led to the hypothesis that RU486 was acting through steroid hormone receptors to cause upregulation of APP_S secretion. However, treatment of mouse (E82) fibroblasts devoid of steroid hormone receptors with RU486 still increased APP_S secretion, suggesting that RU486 was acting through a steroid receptor-independent mechanism. RU486 is also a known antagonist of p-glycoprotein (p-gp), a member of the ATP-binding cassette (ABC) transporter superfamily. P-gp is a transporter of many structurally unrelated lipophilic substrates, most noted for its ability to confer the multidrug resistance phenomenon in cancerous cells. Treatment of human carcinoma (KB-3.1) cells expressing low levels of p-gp with RU486 did not lead to an increase in APP_S secretion, however, the effect was restored in KB-T10 cells which overexpressed p-gp. The ability of p-gp inhibitors to increase APP_S secretion was also seen in rat primary cortical cultures.

The ability of p-gp inhibitors to increase APP_S secretion led to a new hypothesis that inhibition of p-gp decreased Aβ release. Transient transfection of HEK293 cells which were stably transfected with a Swedish familial double mutation of APP (K269sw) with a plasmid that encoded human p-gp under the influence of a retroviral promoter (pHaMDR1) increased Aβ secretion approximately 2-fold above control untransfectants. When these cells were treated with the p-gp inhibitors RU486 and RU49953, Aβ secretion was decreased in a dose-dependent manner. Binding studies using synthetic Aβ peptides and reconstituted, purified hamster p-gp vesicles also showed competitive substrate kinetics between Aβ and known p-gp substrates. This was followed by demonstrating direct transport of Aβ through these vesicles in an ATP- and p-gp-dependent manner. These data suggest that Aβ is a *bona fide* p-gp substrate.

APP is expressed throughout the body and thus is the production of Aβ. P-gp, however, has a limited expression profile and while being enriched at the blood-brain barrier, its expression within the central nervous system is low. Given the overlapping substrate profiles of several ABC transporters, it is hypothesized that there may exist a brain-expressed ABC transporter that is able to transport Aβ. Several known ABC transporters are expressed in the brain. Generation of an expression profile of brain-expressed ABC transporters in the AD brain may allow for identification of a brain-expressed Aβ transporter, with the hypothesis that increased expression of a brain-expressed Aβ transporter may lead to increased amyloid burden. Using quantitative competitive RT-PCR, the goal is to generate an expression profile of genes in normal aged versus AD brain. Data show an approximately 3-fold increase in gene expression of

ABCC5 in Alzheimer frontal cortex compared to age-matched controls. Efforts to create expression profiles for four other ABC transporters are underway in hopes of providing candidate transporters for functional analysis in the future such that a solid bridge between ABC transporters and AD may be forged.

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Aβ	β-amyloid peptide
$A\beta_{1-40}$	β-amyloid terminating at amino acid 40
$A\beta_{1-42}$	β-amyloid terminating at amino acid 42
$A\beta_{x-40}$	N-terminal truncated Aβ terminating at amino acid 40
$A\beta_{x-42}$	N-terminal truncated Aβ terminating at amino acid 42
$A\beta_{17-x}$	C-terminal truncated Aβ starting at amino acid 17
AchEI	acetylcholinesterase inhibitor
AD	Alzheimer's Disease
APLP	amyloid precursor like protein
ApoE	apolipoprotein E
APP	amyloid precursor protein
APP_S	N-terminal cleavage product of APP
$APP_{S\alpha}$	N-terminal α-secretase cleavage product of APP
$APP_{S\beta}$	N-terminal β-secretase cleavage product of APP
AuxB1	Chinese Hamster Ovary cells with low levels of p-glycoprotein
BACE	beta-site APP cleaving enzyme
BBB	blood-brain barrier
C99	C-terminal β-secretase cleavage product of APP
CHO	Chinese Hamster Ovary cells
CH^RB30	Chinese Hamster Ovary cells enriched in p-glycoprotein
CNS	central nervous system
CTF	C-terminal fragment of APP
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAD	familial Alzheimer's Disease
HEK293	human embryonic kidney 293 cells
KPI	Kunitz Protease Inhibitor
LRP	low density lipoprotein receptor
LTP	long term potentiation
MIANS	2-(4-maleimidoanilino)-napthalene-6-sulfonic acid
NGF	nerve growth factor
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
QCRT-PCR	quantitative competitive reverse transcriptase-polymerase chain reaction
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

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I. INTRODUCTION

Part One – Alzheimer's Disease and the Amyloid Hypothesis

Alzheimer's disease (AD) is the most common cause of senile dementia, with two characteristic histopathological hallmarks: extracellular neuritic plaques and intracellular neurofibrillary tangles (Alzheimer 1907a, b). Efforts to tease out the components of the neuritic plaques and tangles have led to the discovery of key proteins that are believed to be involved in the disease process. Advances in molecular genetics have allowed for manipulation and study of certain genes that have established strong pathological links leading to the development of transgenic animal models that recapitulate the plaques and tangles with some neurological deficits. Evidence now supports that the pathophysiology of AD is complex, involving but not limited, to aberrant endogenous neuronal protein processing, inflammatory mechanisms, microvascular events, and environmental factors (Selkoe, 2001, Lukiw and Bazan, 2000), creating a scenario of multifactorial mechanisms that contribute to the present understanding of AD.

The Neuritic Plaque

Alzheimer's Disease (AD) is characterized by extracellular depositions of insoluble amyloid plaques in the brain along with intracellular neurofibrillary tangles. The discovery of congophilic amyloid plaques in the AD brain (Glenner and Wong, 1984) led to the cloning of the amyloid precursor protein (APP) (Kang et al., 1987). This ignited a plethora of research into the involvement of β-amyloid (Aβ) in AD, converging into the so-called "beta-amyloid cascade hypothesis" in AD. Explicitly stated, this hypothesis places Aβ as the central molecule in the etiology of AD. Accumulation of Aβ in the brain triggers a cascade of toxic mechanisms leading to plaque formation and cell death. Support that Aβ is involved in the pathogenesis of AD comes from several findings, including genetic linkage studies, transgenic animal models of AD, and *in vitro* studies revealing neurotoxic properties of Aβ.

Neuritic plaques can be found dispersed throughout the cortex, and can largely be classified into two categories: core versus diffuse. Core plaques are composed of several constituents, in particular an amyloid focus, and dystrophic neurites which can be found both within the core and immediately surrounding the plaque (Dickson, 1997). Associated with these plaques are activated microglia, and reactive astrocytes. Much of the amyloid is made up of the longer, more hydrophobic $A\beta_{1-42}$ isoform; however, the more abundantly produced $A\beta_{1-40}$ isoform can also be found interspersed with $A\beta_{1-42}$. On the other hand, diffuse plaques are composed mostly of $A\beta_{17-42}$, are dispersed throughout areas of the brain that are not typically associated with AD (i.e. thalamus, cerebellum), and lack a compact focus. Attention has been given to these diffuse plaques as being

precursors of neuritic plaques. This hypothesis is supported through animal model studies and immunohistochemical studies of patients with Down's syndrome, who have an extra copy of the gene which gives rise to Aβ (Lemere et al., 1996).

The β-amyloid Peptide and Its Precursor

The ~4kDa β-amyloid (Aβ) peptide is cleaved from the larger amyloid precursor protein (APP). Aβ was originally isolated from meningeal arteries of AD and Down's patients (Glenner and Wong, 1984a; Glenner and Wong, 1984b) and further sequencing studies led to the cloning and localization of the APP gene on chromosome 21 (Kang et al., 1987). The gene contains 18 exons spanning over 170 kb with exons 16 and 17 encoding the 40-43 amino acid Aβ sequence (Goldgaber et al., 1987; Yoshikai et al., 1990; Tanzi et al., 1992). APP is a type I transmembrane glycoprotein that is ubiquitously expressed throughout the body and exists in several different isoforms ranging between 110-140 kDa (Selkoe et al., 1988). Alternative splicing and posttranslational modifications yield three major isoforms of 695, 751, and 770 residues (Oltersdorf et al., 1990; Weidemann et al., 1989). While the two larger isoforms contain an extracellular Kunitz serine protease inhibitor domain (Smith et al., 1990), the 695 isoform is most highly expressed in neurons while the 751/770 isoforms are found in higher abundance in nonneural tissues (Neve et al., 1988).

Cleavage of APP by α-secretase

APP can be cleaved proteolytically at various sites to liberate both amyloidogenic and non-amyloidogenic fragments by *secretase* enzymes (Figure 1). Amyloidogenic fragments are those which contain an intact Aβ sequence, thus having the potential to be cleaved subsequently to form Aβ. Conversely, non-amyloidogenic products are those that either do not contain the Aβ sequence, or have been cleaved within the sequence to preclude Aβ formation. The majority of membrane-associated APP is cleaved by α-secretase between amino acids 612 and 613 (based on the 695 sequence), corresponding to residues 16-17 of the Aβ sequence, yielding a soluble secreted fragment called $APP_{S\alpha}$ and a membrane-associated 83 residue C-terminal fragment. Cleavage within the Aβ sequence precludes amyloid formation and thus this has been named the non-amyloidogenic pathway of APP processing. Several members of the ADAM family (*a* *d*isintegrin *a*nd *m*etalloprotease-family) of proteases, namely ADAM10, ADAM17, and TACE, exhibit α-secretase activity (Lammich et al., 1999; Koike et al., 1999 Buxbaum et al., 1998), while earlier work showed that a chymotryptic-like activity in proteosomes also contribute to α-secretase cleavage (Marambaud et al., 1996, 1997). This suggests that there may be more than one enzyme that is capable of α-secretase cleavage. However, in situ hybridization of ADAM10, ADAM17, and APP in the brain by Marcinkiewicz and Seidah (2000) showed colocalization of ADAM10 and APP and weak overlap with ADAM17, suggesting that ADAM10 has a higher likelihood for being a brain α-secretase.

Cleavage of APP by β-secretase

Amyloid formation begins with cleavage of APP by β-secretase, which cleaves N-terminal to the α-secretase site, at residue 1 of the Aβ sequence (Benjannet et al., 2001). This releases a slightly more truncated secretory fragment called $APP_{S\beta}$ and a larger membrane-bound C-terminal fragment (CTF) called C99, containing an intact Aβ sequence. There is much controversy as to where β-secretase activity is compartmentalized along the intracellular secretory pathway of APP but recent cloning and identification of two β-secretases, called BACE enzymes, as membrane-anchored aspartyl proteases will hopefully shed more light on this issue.

The proteases BACE (*b*eta-site *A*PP *c*leaving *e*nzyme, Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yar et al., 1999; and Lin et al., 2000), and its homolog BACE2 (Yan et al., 1999; Solans et al., 2000; Farsan et al., 2000), are type I transmembrane proteins. The BACE gene is found on chromosome 11q-23-24 while BACE2 is located on chromosome 21q22 within the Down's syndrome critical region (Yan et al., 1999). Subcellular and tissue localization show that BACE is highly expressed in brain and has low expression in peripheral tissues (Vassar et al., 1999) while the converse is true for BACE2 (Bennett et al., 2000; Farzan et al., 1999). BACE is expressed in the brain with highest activity in the Golgi, trans-Golgi, secretory vesicles, and endosomes. These findings are in line with the body of evidence indicating that Aβ is most likely formed in acidic compartments of the secretory pathway (reviewed by Nixon et al., 2000).

Cleavage of APP by BACE yields C-terminal fragments starting at Asp-1 and Glu-11 of the Aβ sequence (Vassar et al., 1999). BACE2 also cleaves at Asp-1 but has higher activity at Phe-19 and Phe-20 (Farzan et al., 2000). Both BACE and BACE2 are proproteins with a prodomain that is cleaved intracellularly to yield mature active enzymes. The BACE gene encodes a 501 amino acid protein containing a 21 residue N-terminal signal peptide sequence followed by a 22-45 residue proprotein domain (Vassar et al., 1999). A large luminal domain (residues 46-460) followed by a single transmembrane spanning segment (residues 461-477) ending in a short cytoplamsic carboxy terminal (residues 478-501) defines the type I classification for this protein. It is now known that at least for BACE, a family of subtilisin-like proprotein convertases are involved in proprotein cleavage, which most likely takes place in a late-Golgi compartment after post-translational modification of proBACE in the endoplasmic reticulum system (ER) (Benjannet et al., 2001; Bennett et al., 2000; Creemers et al., 2001; Pinnix et al., 2001). Cleavage of the proprotein, termed proBACE, occurs at the RLPR↓ motif, yielding a mature BACE starting at Glu-46 (Vassar et al., 1999). Thus far, the proprotein convertase, furin, seems to be a strong candidate processing enzyme for BACE (Bennett et al., 2001; Benjannett et al., 2001). Controversy surrounds the ability of other members of the convertase family to cleave BACE, as different labs have shown conflicting data regarding the cleavage abilities of certain convertases (Benjannet et al., 2001; Creemers et al., 2001; Pinnix et al., 2001). Furthermore, proBACE itself has β-secretase activity (Creemers et al., 2001; Benjannet et al., 2001; Shi et al., 2001), although much lower than that of mature BACE (Benjannet et al., 2001), begging the question of whether or not proprotein cleavage is even a required event. However, data

showing highest β-secretase activity in a soluble form of BACE suggest that processing is required for efficient cleavage (Benjannet et al., 2001). This is complemented by data showing that the processing of the propeptide domain facilitates proper folding of mature BACE (Shi et al., 2001). Less is known regarding the processing of BACE2; however, one article suggests that BACE2 undergoes autocatalytic processing of its prodomain (Hussain et al., 2001).

Apart from proprotein processing, BACE also undergoes alternative splicing, followed by complex post-translational glycosylation, and phosphorylation. Three splice variants (BACE-I-432, BACE-I-457, and BACE-I-476) have been found so far in the human brain (Tanahashi and Tabira, 2001) and one in the pancreas (Bodendorf et al., 2001). Alternative splicing occurs in the luminal domain of BACE, yielding deletions of 25 (BACE-I-476), 44 (BACE-I-457), and 69 (BACE-I-432) amino acids in the three isoforms. Of these, BACE-I-457 and BACE-I-476 demonstrate weakened β-secretase activity compared to full length BACE (Tanahashi and Tabira, 2001). The pancreatic variant lacks 44 amino acid residues in between the catalytic domain and is deficient in β-secretase activity (Bodendorf et al., 2001). Complex N-glycosylation occurs in the ER (Capell et al., 2000) prior to proprotein cleavage. Cleavage of the proprotein domain is required for efficient shuttling of proBACE through the ER (Benjannett et al., 2001) while phosphorylation of a serine residue at position 498 by casein kinase I regulates the intracellular trafficking of BACE through the secretory pathway (Walter et al., 2001). It thus appears that BACE fits the profile of a β-secretase candidate. That Aβ production and β-secretase activity are markedly decreased in BACE knock-out mice suggests that

BACE is the primary brain β-secretase and thus represents a good target for AD therapy (Roberds et al., 2001).

Cleavage of APP by γ-secretase

The final step in formation of Aβ is the cleavage of the CTF of APP by γ-secretase. Both the CTFs, C83 and C99, are substrates for γ-secretase. Cleavage of C99 yields Aβ peptides of x-40/42, while C83 cleavage yields the soluble fragment p3 (Figure 1). The more soluble $A\beta_{1-40}$ isoform is the most abundant form of Aβ that is produced, while the fibrillar, plaque-forming longer $A\beta_{1-42}$ comprises ~10% of total Aβ (Younkin, 1995). The cleavage site for γ-secretase is unique in that it is situated within the membrane (Figure 1). This suggests that γ-secretase is a membrane-bound protein with a transmembrane active site. The majority of γ-secretase activity is isolated in intracellular acidic compartments, most likely the trans-golgi network (Tomita et al., 1998; Xu et al., 1997) or the endosomal/lysosomal pathway (Peraus et al., 1997; Chyung et al., 1997; Cook et al., 1997; Koo et al., 1994). With the cloning of β-secretase and strong evidence for the ADAM-10 being α-secretase, γ-secretase remains the last piece of the puzzle in the catabolic processing of APP.

Evidence suggests that two transmembrane proteins called the presenilins (PS1 and PS2; Wolfe and Haass, 2001) are involved in γ-secretase cleavage. PS1 is found on chromosome 14 (Sherrington et al., 1995), PS2 on chromosome 1 (Levy-Lahad et al., 1995). Over 75 PS1 and 3 PS2 point mutations make up the majority of the cases of early onset familial Alzheimer's disease (FAD) (Russo et al., 2000; Hardy and Crook,

2001; Wolfe, 2001). Presenilins were first implicated in γ-secretase activity when it was found that familial PS mutations increased the ratio of $A\beta_{1-42}/A\beta_{1-40}$ in transgenic mice and in mutation carrying cell lines (Citron et al., 1997; Borchelt et al., 1996). This was followed by the finding that PS1 knockout mice had reduced levels of γ-secretase activity without altering APP maturation or cellular distribution (De Strooper et al., 1998). More directly, it was also found that presenilins were required for γ-secretase activity *in vitro* (Zhang et al., 2000b; Herrerman et al., 2000). Cross-breeding transgenic mice harbouring FAD mutations in the APP gene with PS1 mutant mice also created an accelerated model of AD, with formation of $A\beta_{1-42}$ plaques as early as 3 months of age (Holcomb et al., 1998). These data suggest that presenilins are able to alter the substrate specificity of γ-secretase.

Presenilins are transmembrane proteins localized mainly in the ER and to a less extent in the Golgi (Kovacs et al., 1996; Walter et al., 1996; De Strooper et al., 1997; Annaert et al., 1999). While much debate has existed regarding the topographic arrangement of presenilins, it is generally accepted that presenilins contain eight transmembrane domains with a large luminal loop between domains 6 and 7 (Doan et al., 1996; Hardy 1997; Li and Greenwald 1998) while some have suggested a six to seven transmembrane topology (Dewj and Singer, 1997; Lehmann et al., 1997). Presenilin holoproteins are rapidly cleaved to form N-terminal and C-terminal fragments (Podlinsy et al., 1997; Ratovitski et al., 1997; Thinakaran et al., 1996) which associate to form high molecular weight complexes. Cleavage occurs at the luminal loop between transmembrane domains 6 and 7, and is dependent on two aspartate residues within those

domains (Wolfe, 2001). Formation of these complexes is believed to impart full function to these proteins (Capell et al., 1998; Yu et al., 1998).

Controversy surrounds the nature of the presenilins in γ-secretase activity. Interpretation of available data suggests that presenilins may be involved in direct regulation of γ-secretase or they may in fact be γ-secretases themselves (Xia et al., 1997). Support for this comes from studies showing the ability to coprecipitate presenilin with APP in cell culture (Xia et al., 1997; Weidemann et al., 1997), and more specifically with the C99 fragment of APP (Verdile et al., 2000; Xia et al., 2000); however, this has been challenged by one report indicating an inability to detect such an interaction (Thinakaran et al., 1998). Furthermore, both APP and PS1 are colocalized to the ER and Golgi (De Strooper et al., 1997, Kovacz et al., 1996), compartments of γ-secretase activity. Two aspartate residues in the sixth and seventh transmembrane helices of PS1 are responsible for γ-secretase activity and Aβ formation (Wolfe et al., 1999; Kimberly et al., 2000). The observation that several transition state γ-secretase inhibitors and novel peptidomimetics bind directly to PS1 (Li et al., 2000a; Seiffert et al., 2000) have also strengthened the direct connection between presenilins and γ-secretase.

Several different scenarios have also been proposed for presenilin's involvement in γ-secretase cleavage. One hypothesis suggests that presenilins are part of a heteromeric complex containing γ-secretase, possibly acting as a chaperone or cofactor. This has been supported by several findings showing PS1 in a γ-secretase activity-containing macromolecular complex with heterologous molecules including β-catenin,

nicastrin, and APP (Capell et al., 1998; Yu et al., 1998; Yu et al., 2000; Li et al., 2000b). In addition, data showing that PS1 has effects in protein trafficking (Naruse et al., 1998) may indicate a role in shuttling APP through the various compartments of the secretory pathway while the assembly of the γ-secretase multimeric complex takes place. The observation that production of both $A\beta_{1-40}$ and $A\beta_{1-42}$ is nearly abolished when C99 is trapped in the ER indicates that PS1, at least in that intracellular compartment, does not have γ-secretase activity (Maltese et al., 2001). Whether this is due to the lack of a critical component of the γ-secretase complex in the ER, or the necessity of PS1 maturation for its activity after trafficking through the ER, remains to be investigated.

The development of transition state analogue γ-secretase inhibitors based on the C-terminal sequence of Aβ supports the hypothesis that γ-secretase is an aspartyl protease (Wolfe et al., 1998, 1999b). That PS1 contains two aspartate residues that are required for γ-secretase activity would seemingly argue for PS1 to be γ-secretase; however, sequence analysis of PS1 does not show any consensus sequences to known protease domains (Saftig et al., 1999). While strong evidence supports an integrated role for presenilins in γ-secretase cleavage and Aβ production, unequivocal data showing that presenilins are indeed the enzymes themselves remain lacking.

Cleavage of C99 by γ-secretase yields Aβ peptides that vary in length between 39-43 amino acids long. The majority of Aβ peptides formed in the brain are $A\beta_{1-40/42}$, suggesting that there are two distinct γ-secretases responsible for producing the two different peptides (Klafki et al., 1996; Citron et al., 1996; Figueiredo-Pereira et al., 1999).

The observation that the production of $A\beta_{1-42}$ preferentially occurs in the ER while that of $A\beta_{1-40}$ occurs in the Golgi (Hartmann et al., 1997) may also suggest that there are either two separate γ-secretases in those two compartments, or that the different environments of the ER and Golgi favour selective cleavages. An alternate hypothesis suggests that a single γ-secretase with relatively non-specific proteolytic specificity cleaves C99 at different positions at its C-terminal that is dependent on substrate positioning at the membrane (Murphy et al., 1999). Work with peptide inhibitors that specifically inhibit either $A\beta_{1-40}$ or $A\beta_{1-42}$ formation also suggests that there is a single γ-secretase that generates the different length Aβ peptides (Durkin et al., 1999). It is doubtful that these issues will be fully addressed without the definitive identification of γ-secretase.

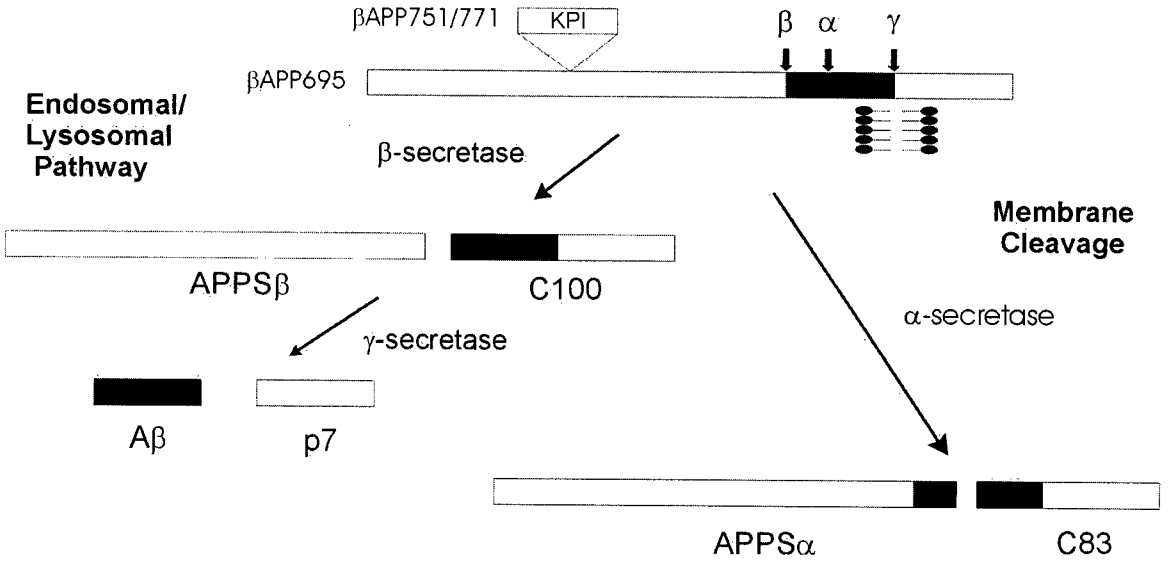

Figure 1. Secretory processing of APP. APP is cleaved by three secretase enzymes at different sites of the secretory pathway. Cleavage by α-secretase precludes Aβ formation and is considered a non-amyloidogenic event, whereas sequential cleavage by β- and γ-secretases produce Aβ and is therefore considered amyloidogenic. The C-terminal fragment C83 can be further cleaved by γ-secretase to form the secreted fragment p3. Cleavage of APP by β-secretase forms the C-terminal fragment C99, containing the intact Aβ sequence (dark bar). Subsequent cleavage by γ-secretase leads to Aβ formation. This is termed the amyloidogenic pathway and is believed to occur in the late secretory, endosomal/lysosomal acid compartments.

The Genetics of Alzheimer's Disease

The majority of cases of AD are sporadic, with unidentified mechanisms of disease onset. Roughly 5-10% of cases have a familial genetic component (reviewed by Selkoe, 2001); however, extensive studies into the small cluster of familial AD (FAD) cases have proven fruitful in understanding the mechanisms of AD. Cloning and polymorphism analyses of the APP gene revealed that several different forms of FAD contained APP point mutations on chromosome 21 (Hardy, 1997). Individuals inherited this mutated gene in an autosomal dominant fashion and often developed early onset AD before age 65. In particular, mutations in the APP gene clustered around putative sites of secretase cleavage, and resulted in rapid accumulation of extracellular Aβ (Selkoe, 2001). This fueled the beta amyloid cascade hypothesis of AD, which was further supported by the observation that trisomy 21 Down's syndrome patients inevitably developed AD-like symptoms in their third decade of life, most likely due to a gene dosage effect (Rumble et al., 1989).

Missense mutations in the APP gene are in fact quite rare. By far, most cases of FAD have been linked to mutations in the presenilins. To date, there have been identified as many as 75 point mutations in PS1 and 3 in PS2 (Wolfe, 2001). Presenilin mutations lead to early onset AD before age 50. Transgenic and gene expression studies show that all mutations lead specifically to increased secretion of $A\beta_{1-42}$, further placing Aβ in the forefront of AD pathogenesis.

While mutations in the APP and PS1/2 genes contribute to early onset forms of AD, increased risk of late onset AD (age 65 or older) has been linked to the apolipoprotein (ApoE) ε4 allele (Strittmatter et al., 1993). ApoE was first implicated in AD following its localization with neuritic plaques (Strittmatter et al., 1993; Namba et al., 1991) and the observation that AD patients carrying the ε4 allele contained more amyloid than those of other ApoE alleles (Rebeck et al., 1993; Berr et al., 1994). The prevalence of AD (45-60% of a cohort with AD) increases in individuals who are either heterozygous or homozygous for the ε4 allele compared to the control population (Rebeck et al., 1993; Chapman et al., 2001). ApoE is normally found complexed with lipoproteins and mediates transport of lipids and cholesterol into cells via binding with the low density lipoprotein receptor (LRP) or the low density lipoprotein receptor-related protein/α2-macroglobulin receptor (Bradley et al., 2000). The mechanisms by which ApoE4 imparts an increased AD risk are unclear. Some data suggest that ApoE and LRP play a protective role in the brain by sequestering and clearing extracellular Aβ (Van Uden et al., 2000; Bradley et al., 2000; Ulery et al., 2000). This process is attenuated in ε4/ε4 homozygous individuals and may increase the brain Aβ burden in the elderly. Other studies show that truncated ApoE4 fragments induce the formation of neurofibrillary tangles in neurons, suggesting that there may be a toxic property to these fragments (Huang et al., 2001). However, there are individuals who are ε4 homozygotes who do not develop AD, leading to the conclusion that having the ApoE4 allele increases the risk, but does not invariantly lead to developing AD.

Functions of APP and Its Derivatives

APP and APP_S

Numerous studies have shown several putative functions for APP and its derivatives. *In vitro* studies identified the neuroprotective and neurotrophic abilities of $APP_{S\alpha}$ (Mattson et al., 1993). The 751/770 isoforms of APP are able to inhibit certain serine proteases, including trypsin and chymotrypsin (Sinha et al., 1990), as well factor XIa (Smith et al., 1990), due to the presence of a Kunitz protease inhibitor (KPI) motif in the ectodomain of these isoforms.

Initial characterization of APP as a membrane-spanning glycoprotein suggested that APP played a role in cell adhesion due to shared similar staining patterns to other cell adhesion molecules on neuronal surfaces (Shivers et al., 1988; Schubert et al., 1989) as well as its synaptic localization (Schubert et al, 1991; Storey et al., 1996). Subsequent studies demonstrated that APP was also involved in cell-cell (Qiu et al., 1995) and cell-substrate adhesion (Breen et al., 1991; Chen & Yankner, 1991), as seen in the *Drosophila* model, where overexpression of APP led to a blistered wing phenotype, suggesting that excessive APP led to a change in the cell-cell adhesion between the layers of epithelial cells during development (Fossgreen et al., 1998). A similar function has been observed with Aβ, where both Aβ and C99 show interactions with elements of the extracellular matrix such as fibronectin and laminin, supporting neurite outgrowth (Ghiso et al., 1992; Koo et al., 1993).

Studies have shown that APP may also be involved in memory and long-term potentiation (LTP). APP695 and APP751 at low picrogram doses display nootropic effects and are able to overcome scopolamine-induced amnesia in animal models (Meziane et al., 1998), while addition of APP_S to hippocampal slices altered the electrophysiological properties of both LTP and long-term depression. Transgenic mice overexpressing the familial Swedish double mutation of APP displayed impairments in LTP which were age-dependent and were accompanied by spatial memory deficits in the absence of significant reductions in the number of synapses and cell death (Chapman et al., 1999). The ability of the Swedish mutation to markedly increase Aβ secretion and decrease APP_S secretion (Citron et al., 1996) thus suggests that the decrease in APP_S may impair synaptic plasticity. This has been supported by studies that have shown that both Aβ and the CTF shorten LTP duration and the amplitude of excitatory postsynaptic potentials (Cullen et al., 1996, 1997; Lambert et al., 1998). The essential role of APP in learning and memory is confounded however, by the fact that APP knockouts show no evidence of neuronal loss (Phinney et al., 1999) or marked changes in behaviour or cognitive ability (Zheng et al., 1995; Perez et al., 1996; Tremml et al., 1998; and Dawson et al., 1999). This may be explained through the compensatory mechanisms of APP-like proteins APLP-1 and APLP-2 (Wasco et al., 1992; Sandbrink et al., 1994), which may be able to substitute for APP. Post-natal death in double knockouts of both APP and APLP-2 shows that both proteins also play key roles in development (VonKoch et al., 1997).

A large body of evidence suggests that APP is also neuroprotective and neurotrophic. Most noted is the ability of APP_S to rapidly stabilise intracellular Ca^{2+}

levels when added to cells in culture (Mattson et al., 1993). This neuroprotective effect of APP_S is evident in its beneficial actions against a host of toxins and mechanisms whereby neurotoxicity can occur. These include increased intracellular Ca^{2+} and induction of free radical species by Aβ (Goodman and Mattson, 1994); glutamate-induced ischemia and excitotoxicity (Mattson, 1994; Mattson et al., 1993); kainate-induced seizures (Steinbach et al., 1998); and N-methyl-D-aspartate-dependent neuronal death (Furukawa et al., 1996).

The neurotrophic effects of APP_S have been demonstrated by transfection of APP cDNA in APP-null neuronal cells, resulting in increased neurite outgrowth (Jin et al., 1994; Ohsawa et al., 1997), specifically dependent on an RERMS amino acid sequence of APP. Antibodies against APP are able to block nerve growth factor (NGF)-induced neurite outgrowth (Clarris et al., 1995) and subsequent studies have shown that this may be due in part to the ability of APP_S to potentiate the effects of nerve growth factor by the activation of the extracellular signal-regulated kinases ERK-1 and -2 via a PI_3 kinase pathway (Wallace et al., 1997a, b). Furthermore, $APP_{S\alpha}$ has been shown to activate the antiapoptotic factor NF-κB (reviewed in Mattson and Camandola 2001; Barger and Mattson, 1996), and may act in synergy with the ability of NGF to activate NF-κB, possibly through the insulin signaling pathway (Luo et al., 2001). Treatment of cultured cortical neurons with antisense oligonucleotides to APP significantly decreased neurite outgrowth (Allinquant et al., 1995), while antibodies to residues 596-612 (1-16 of the Aβ sequence) inhibited cell-substratum adhesion and neurite retraction (Chen and Yankner,

1991). Taken together, this strongly supports a role for APP in neurite outgrowth and salvage.

Due to the predicted membrane-spanning structure of APP, it was initially hypothesized that it acts as a cell surface receptor (Kang et al., 1987). To date, no ligands of APP have been identified; however, numerous cytosolic proteins have been identified which interact with the C-terminal of APP and may be involved in intracellular signalling. The earliest of such findings was the observation that APP interacted with the growth cone G protein (G_o), leading to the subsequent binding of GTP-γS (Nishimoto et al., 1993), which was increased by treament with an N-terminal APP antibody 22C11, and increased GTPase turnover. Other APP-interacting proteins include: the adaptor proteins Fe65 and X11 (reviewed by Russo et al., 1998); APP-BP1, believed to be involved in signal transduction (Chow et al., 1996); a protein homologue of the UV-damaged DNA-binding protein, which may serve as a regulator of gene transcription (Watanabe et al., 1999); and the microtubule binding protein PAT1 (Zheng et al., 1998), which may be involved in APP trafficking.

The Aβ Peptide

Before discussing the role of Aβ, it is necessary to first describe the nature of this peptide. Following its production, Aβ can undergo several modifications including truncation of its N- and C-terminals (Masters et al., 1985; Kang et al., 1987; Mori et al, 1992; Miller et al., 1993; Roher et al., 1993; Naslund et al., 1994; Prelli et al., 1988), probably through the action of amino-, carboxy-, and endopeptidases (Wang et al., 1996). The majority of brain Aβ is $Aβ_{1-40}$ (Mori et al., 1992), while the longer $Aβ_{1-42}$ is mainly

found in neuritic plaques (Miller et al., 1993; Roher et al., 1993). Diffuse plaques contain primarily truncated $A\beta_{17-42}$ (Gowing et al., 1994). Other than these fragments, Aβ peptides starting at residues 2, 3, 4, 5, 6, 8, 9, 10, and 17 have been detected in amyloid plaque cores, evidence that these peptides undergo considerable post-translational modifications (Kuo et al., 2001). A small percentage of Aβ species have also been isolated beginning at Glu11, suggestive of the population of Aβ that is formed by the alternate cleavage by BACE at Glu11 instead of Asp1 (Vassar et al., 1999). Species of $A\beta_{17-X}$ have also been isolated from cultured cell media (Haass et al., 1992).

The sequence of the Aβ peptide largely dictates its properties in solution. Two hydrophobic domains (residues 17-21 and 29-42) impart aggregating properties. In particular, residues 1-16, 17-22, and 29-40 (42) are crucial for Aβ-Aβ interactions (Festy et al., 2001). At low concentrations, Aβ exists as a soluble peptide, but has an affinity to aggregate to form β-pleated sheets when left in solution (Kirschner et al., 1987; Halverson et al., 1990; Inouye et al., 1993). Hydrophobic residues 17-21 and 29-40 (42) contribute to the β-sheet forming regions of the peptide. This spontaneous aggregation is most likely due to the peptide's natural desire to reach the most stable thermodynamic state (McLaurin et al., 2000). In vivo aggregation involves a complex process of peptide modification and interactions with heterologous proteins that ultimately form the neuritic plaque (McLaurin et al., 2000). It is hypothesized that aggregates of $A\beta_{1-42}$ form nuclei and promote 'seeding' of the more soluble $A\beta_{1-40}$ peptide to form fibrils (Jarret et al., 1993a; Ward et al., 2000; Tjenberg et al., 1999; Jackson Huang et al., 2000; El-Agnaf et al., 2000; Festy et al., 2001; Murphy and Pallitto, 2000). The C-terminal of Aβ is critical

in this process as $A\beta_{1-42}$ aggregates much faster than $A\beta_{1-40}$, due in part to the two extra non-polar C-terminal residues (Jarrett et al., 1993b).

Low doses of Aβ have been shown to be neuroprotective, but exhibit neurotoxicity in the micromolar range (Yankner et al., 1990). Soluble Aβ is mitogenic, an activity dependent specifically on residues 25-35 (Whitson et al., 1989; Eckert et al., 1995). While these studies support a neuroprotective role for Aβ, there is a large body of evidence indicating that Aβ is damaging to the nervous system, both in vitro (Pike et al., 1993; Lorenzo and Yankner, 1994) and in vivo (Giovannelli et al., 1998). Increased Aβ deposits are found in sites in the AD brain where most neurodegeneration occurs (Hensley et al., 1995). In FAD, mutations in the APP gene lead to increased secretion of Aβ, in particular $A\beta_{1-42}$, with subsequent exacerbation of disease symptoms (Selkoe, 2001). Injection of $A\beta_{1-42}$ fibrils into the brains of mice that harbour mutations in the neurofibrillary tangle-forming tau protein accelerate tangle formation (Götz et al., 2001). An increase in tangle formation was also seen in double transgenic mice that harboured both APP and tau mutations (Lewis et al., 2001). These findings suggest that the damaging effects of Aβ may be many-fold, partly due to both the toxicity of Aβ itself, as well as the peptide's ability to enhance tauopathy in the brain.

Aβ toxicity is associated with increased intracellular Ca^{2+} and excitotoxicity (Coughlan and Breen, 2000; Joseph and Han, 1992). The increase in Ca^{2+} has been postulated to be due to several possible mechanisms, including: the ability of Aβ to form Ca^{2+}-permeable ion channels (Rhee et al., 1998; Ueda et al., 1997; Price et al., 1998); inhibition of the Na/K-ATPase leading to subsequent elevations in Ca^{2+} (Mark et al.,

1995); and upstream formation of reactive oxygen species (ROS) which ultimately disrupt Ca^{2+} balance (reviewed by Miranda et al., 2000; Mark et al., 1995).

A number of cellular alterations that occur in AD have been linked to the formation of ROS, and accumulating evidence suggests that Aβ plays a pivotal role in the formation of ROS and subsequent cell damage. Initial experiments showed that Aβ increases peroxide levels in cell culture (Behl et al., 1994), and the peroxides can be converted to superoxide radicals via dismutase enzymes, causing membrane lipid peroxidation and cell death. Evidence indicates that this process is copper- or iron-dependent (Huang et al., 1999a, 1999b), and may represent an ongoing process in the AD brain whereby increased reduction of copper by amyloid fibrils leads to concomitant increased free radicals and oxidative cell death (Opazo et al., 2000; Ruiz et al., 1999; White et al., 1999). Studies have shown altered cell electrophysiology and neurotoxicity via treatment with Aβ protofibrils, metastable intermediates in amyloid fibril formation (Hartley et al., 1999), and synaptoxicity in the absence of plaque formation in mice harbouring FAD APP mutations (Mucke et al., 2000), supporting the evolving theory that toxicity may occur before the formation of amyloid aggregates. There is a postulation that it is the process of Aβ fibril formation that causes cytotoxicity rather than the fibril itself. This is supported by observations that overaggregated Aβ is not neurotoxic (Mattson et al., 1995; Mattson et al., 1996) and that fresh soluble oligomer solutions of $Aβ_{1-42}$ and $Aβ_{1-40}$ are toxic in vitro (El-Agnaf et al., 2000). This accumulated body of work suggests that preventing Aβ accumulation in the brain may be a viable avenue of treatment for AD. Phase II clinical trials are now underway to test a novel Aβ vaccine following studies showing that immunization of APP transgenic mice with synthetic Aβ

peptides dramatically reduced plaque accumulation (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000; Marwick, 2000). While further testing needs to be done to determine the effects of this vaccination on cognition and memory, it nonetheless offers a novel avenue of therapy against an increased Aβ burden in the AD brain.

The C99 Fragment

Does neurotoxicity depend on the formation of Aβ? Fukuchi et al. (1992, 1993) demonstrated selective toxicity in neuroblastoma cultures that overexpressed the C-terminal fragment of APP (C99) containing the intact Aβ sequence. C99 transgenic mice were found to have profound neuronal and synaptic degeneration in their hippocampi (Neve et al., 1996; Oster-Granite et al., 1996) as well as impaired learning and memory (Nalbantoglu et al., 1997). The neurotoxic nature of the C99 fragment may be due in part to its ability to alter intracellular Ca^{2+} homeostasis, as is evident in stably transfected PC12 cells (Pascale et al., 1999), via inhibition of the Mg^{2+}/Ca^{2+}-exchanger (Kim et al., 1998, 1999). It has recently been shown that the CTF is able to induce nitric oxide production in astrocytes via the MAP kinase signaling cascade, activate the transcription factor NFκ-B (Bach et al., 2001), as well as increase the proinflammatory mediators tumour necrosis factor and matrix metalloproteinase-9 production in conjunction with interferon-γ (Chong et al., 2001), adding yet further mechanisms of injury in the AD brain.

Neuroinflammation and Alzheimer's Disease

The natural course of progression for AD can extend for well over a decade. During that time, it is hypothesized that a plethora of toxic and damaging mechanisms are at work. One hypothesis that has come to the forefront is the notion that the brain is under siege by a host of inflammatory events, all of which lead ultimately to neurodegeneration. Initially, the view that the brain was susceptible to inflammation was held in low regard, given the traditional belief that this was an "immune-priveleged" organ, being protected by the selective blood-brain barrier (reviewed by Akiyama et al., 2000). We now know that this is not the case. Pioneering work by the McGeers (1988, 1989) and colleagues raised the scenario of an inflammatory process in the brain that occurred in the absence of lymphocytic infiltration, driven by activation of the classical complement cascade as well as the alternative antibody-dependent pathway (McGeer and McGeer, 1998). Several molecules in the brain can trigger the inflammatory cascade. Among these, both Aβ and neurofibrillary tangles can stimulate the complement cascade via direct binding of the C1q protein (Afagh et al., 1996; Rogers et al., 1998). Fibrillar Aβ can be opsonized by C3b (Bradt et al., 1998) with the notion that neurons within the near vicinity may also become opsonized and exposed to attack by inflammatory mediators, microglia, and astrocytes, victims of the innocent bystander effect (McGeer et al., 1989). Semi-quantitative studies of AD brain have shown an upregulation of complement proteins (Yasojima et al., 1999), cytokines, and chemokines. The mechanisms by which Aβ stimulates neuroinflammation are gradually becoming understood (Practico and Trojanowski, 2000; Krause and Clark, 2001; Akiyama et al., 2000), consisting of crosstalk between different classes of inflammatory mediators

(O'Barr and Cooper, 2000; Lukiw and Bazan, 2000; Fassbender et al., 2000). This has led to studies using conventional anti-inflammatories in an attempt to thwart the effects of Aβ (Akiyama et al., 2000; Combs et al., 2000). In particular, the proposed usage of selective cyclo-oxygenase inhibitors to treat clinical AD will be put to the test in determining the treatment efficacy of this class of drugs.

Early experiments investigating signal transduction mechanisms in APP catabolism led to the serendipidous finding that p-glycoprotein, a member of the ATP-binding cassette (ABC) superfamily of transporters, regulated APP processing. This initial finding led to the work outlined in the following chapters. At this point, it is thus appropriate to introduce and review the field of ABC transporters.

Part Two – ABC Transporters and Models of Disease

The ATP binding cassette (ABC) transporter superfamily is one of the largest families of proteins (Dassa and Bouige, 2001; Higgins, 2001). With now over 200 members and increasing, transporters within this family regulate the selective passage of a wide variety of molecules across membranes, from sugars and peptides, to ions and drugs. ABC transporters are so named for their highly conserved ATP-binding cassette motif or nucleotide binding domains, associated with the cytoplasmic side of the membrane (Figure 2). This signature domain consists of three identifiable motifs, the Walker A and Walker B motifs which are commonly found in ATPases (Walker et al., 1982), and a linker hinge region (Schneider et al., 1998). Distinction between members of this superfamily and other ATP-binding proteins is dependent on the criteria that this entire domain be conserved. ABC transporters are composed of four basic structural subunits: two hydrophobic, integral membrane domains, thought to be involved in substrate transport, and two hydrophilic ATP-binding domains (Figure 2, Hyde et al., 1990). Hydrolysis of ATP allows for directional transport of substrate through the transporter, often against a concentration gradient.

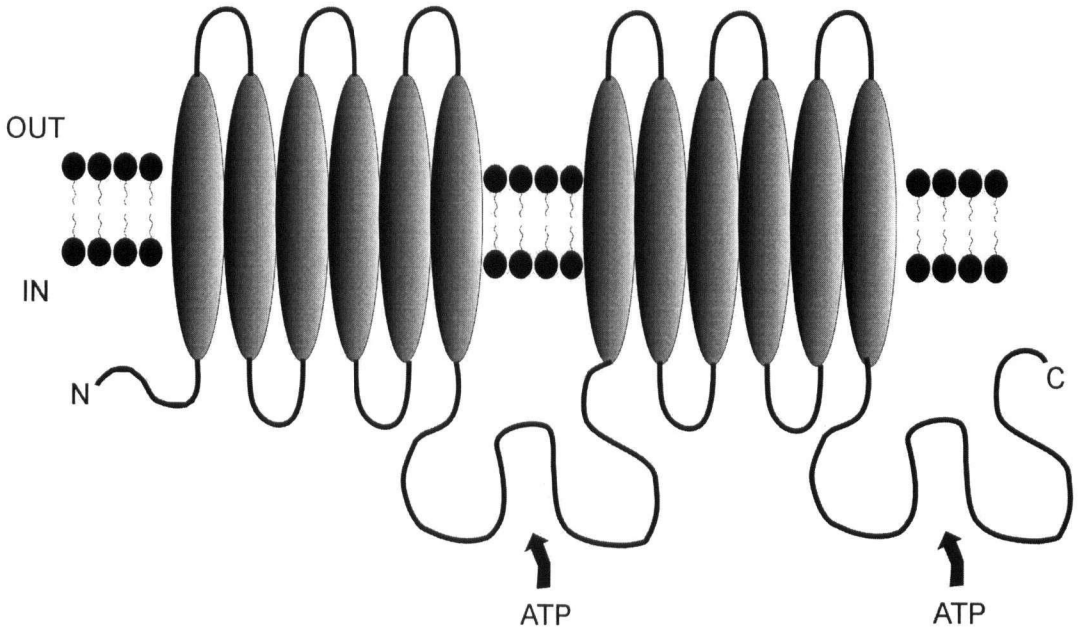

Figure 2. Representative core structure of an ABC transporter. Schematic illustrates the four structural domains of a typical ABC transporter: two transmembrane domains of twelve α-helices, and two ATP binding sites. This model is also representative of the structural organization of p-glycoprotein.

P-glycoprotein (MDR1)

P-glycoprotein (p-gp) is best known for imparting the multi-drug resistant phenotype when overexpressed in tumours (van Tellingen, 2001). Cloned in 1976 by Juliano and Ling, this 170 kDa ABC transporter confers resistance by actively pumping drugs out of the cell (Kartner et al., 1983). P-gp (P for permeability) is the gene product of *MDR1* (Chen et al., 1986). A homologous transporter, MDR3 (van der Bliek and Borst, 1989), is thought to have been spawned through a duplication event of a common ancestor gene (van de Vrie et al., 1998). Only p-gp confers multidrug resistance (Udea et al., 1987, Gros et al., 1988), while MDR3 is involved in bile and phospholipid excretion in hepatocytes (Smith et al., 1998). However, one report has shown that MDR3 is able to weakly transport a subset of drugs that are also substrates of p-gp (eg. Digoxin, paclitaxel; Smith et al., 2000). In mice, three genes *mdr1a* (*mdr3*), *mdr1b* (*mdr1*), and *mdr2* give rise to p-gp-like proteins; however, only *mdr1a and mdr1b* cause multidrug resistance, while *mdr2* is the murine ortholog of human *MDR3* (Gros et al., 1986a, b; Devault and Gros, 1990; Raymond et al., 1990; Croop et al., 1987). For clarity throughout this thesis, the term p-gp will be used in reference only to the human *MDR1* gene product.

Phylogenetically, p-gp is grouped into Class 1 ABC transporters, those which have fused ABC and transmembrane (TM) domains, under the DPL family of *d*rug, *p*eptide, and *l*ipid exporters (Dassa and Bouige, 2000). Structurally, p-gp consists of two symmetrically fused units $(TM\text{-}ABC)^2$ sharing 43% sequence homology between the two halves, characteristic to all p-gp-like eukaryotic systems (Figure 2). Other members in

this family involved in human disease, to name a few, include: mitochondrial transporters ABC6 and ABC7, implicated in X-linked sideroblastic anemia and ataxia (Allikmets et al., 1999); antigen presenting TAP1 and 2 (Beck et al., 1992); the CFTR protein involved in cystic fibrosis (Riordan et al., 1992); and the MRP subfamily of multidrug resistance associated proteins (Borst et al., 1999; and Hipfner et al., 1999). With the cloning of several new members of the ABC family, the issue of appropriate nomenclature for these transporters has led to a new system of classification (Dean et al., 2001). Under the HUGO system of classifying ABC transporters (http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html), p-gp has been given the symbol ABCB1 and the alias PGY1/MDR. As this thesis focuses mainly on p-gp, while respecting the HUGO nomenclature, the term p-gp will be used throughout for ease of discussion.

Pharmacologically, p-gp is a transporter of structually unrelated molecules all sharing the property of being highly lipophilic (Ford and Hait, 1990). Broadly speaking, these agents can be classified as those that are transported by p-gp, and those that are capable of modulating p-gp function. The identification of agents which are able to modulate, and essentially inhibit, p-gp function has been central in reversal of the multidrug resistance phenomenon (Kirshna and Mayer, 2000). The ability of these drugs to block p-gp and chemosensitize resistant cells has allowed for more effective treatment of tumours using chemotherapy. With respect to the central nervous system, this is of great importance, as p-gp is richly expressed at the blood-brain barrier (Kusuhara and Sugiyama, 2001a, b).

In general, it is now believed that p-gp and its relatives act as sentinels in removing toxic substances from cells. This protective role has been shown in *mdr1a* knockout mice (Schinkel et al., 1994). These mice lack functional *mdr1a* expression in brain capillaries and were found to have increased sensitivity to the neurotoxic effects of the anthelminthic pesticide, invermectin, and vinblastine (Schinkel et al., 1994). Studies using the p-gp modulators PSC 833 and SDZ 280-466 in normal mice showed increased susceptibility to the toxic effects of invermectin and hypersensitivity to cyclosporin A, further demonstrating the *in vivo* protection of p-gp at the blood-brain barrier (Didier and Loor, 1995). Over the years; however, studies have indicated that p-gp has other physiological roles as well. These have included roles in cholesterol transport biosynthesis, and esterification (Luker et al., 1999; Metherall et al., 1996; and Debry et al., 1997); possible immunological involvement (Gupta, 1992; Chong, 1993); and regulation of cell death and differentiation (Johnstone et al, 2000). Although the mechanisms of how p-gp is involved in these events are largely unknown, it poses the question of the promiscuity of p-gp in the maintenance of cell function and the homeostasis of the organism.

Multidrug Resistance-associated Protein (MRP)

In terms of multidrug resistance, the MRP subfamily of ABC transporters is the second group of ABC proteins that were identified as being involved in this phenomenon. MRP1 (ABCC1) was the first member of this subfamily to be identified in association with a non-p-gp-mediated multidrug resistance phenotype in a human lung cancer cell line (Cole et al., 1992). Since then, several new members of MRP1-like proteins have

been identified, designated MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), and MRP6 (ABCC6) (Kool et al., 1997). MRP1 has been shown to transport a spectrum of organic anions, many of which are conjugated to glutathione, glucuronide, or sulfate, in the process of toxin elimination (Keppler et al., 1997). It is overexpressed in tumour cell lines selected using several different chemotherapeutic drugs and shares some substrate similarities with p-gp (Hipfner et al., 1999). MRP2 and MRP3 share similar substrate profiles with MRP1 (Keppler et al., 1997; Hirohashi et al., 1999) and MRP2 is also implicated in persistent hyperbilirubinemia and the Dubin-Johnson syndrome (Wada et al., 1998). MRP4 and 5 have been shown to confer resistance to nucleosides and may be involved in cGMP secretion (Schuetz et al., 1999; Wijnholds et al., 2000). MRP6 has been implicated in the connective tissue disorder pseudoxanthoma elasticum (Le Saux et al., 2000).

Structurally, MRP is distinct from p-gp although phylogenetically they are also Class 1 ABC transporters, under the subfamily of organic anion and conjugate drug exporters (Dassa and Bouige, 2001). Apart from having the $(TM\text{-}ABC)^2$ topology, most MRP family members have a large hydrophobic N-terminal domain predicted to have four to six transmembrane helices (Tusnady et al., 1997), except for MRP4 and MRP5, which lack this domain. Members of the MRP family have a broad tissue distribution. With respect to the brain, MRP1, MRP4, and particularly MRP5, have been found to be expressed at the blood-brain barrier (Zhang et al., 2000a).

ABC2 (ABCA2)

A novel brain-expressed ABC transporter has been cloned showing localization to lysosomes and the Golgi apparatus in the cell bodies of oligodendrocytes of rat brain (Zhou et al., 2001). ABCA2 is one of the largest known ABC transporters, encoding for a 2436 amino acid polypeptide bearing the features of a full ABC transporter (Kaminski et al., 2001). While full functional characterization still needs to be carried out, preliminary studies suggest that ABCA2 is involved in sterol regulation in macrophages (Kaminski et al., 2001). Histochemistry in the rat brain demonstrates wide spread cortical expression, with moderate levels in the dentate gyrus and the brain stem (Zhao et al., 2000). It will be of considerable interest to follow future studies regarding the physiological functions of ABCA2 in the human brain with respect to possible involvement in AD pathology.

Summary

This two-part introduction forms the framework of the hypotheses and experiments that are laid out in this thesis. Both the AD and the ABC transporter fields are meccas of intense research activity. The ever-growing body of evidence that contributes to the understanding of the pathologic mechanisms in AD emphasizes the likelihood that several different events act in conjunction to bring about the neurodegenerative and cognitive deficits of the disease. In a different arena, the ABC transporter field is booming from the cloning efforts and the identification of novel transporters with yet unknown functions. The hypotheses in this thesis are formulated in

an attempt to form a bridge between these two fields. The experiments and data shown provide novel evidence in support of the hypothesis that ABC transporters are involved in the physiological regulation of APP catabolism and cellular Aβ release.

II. P-glycoprotein Inhibitors Regulate APP Catabolism

INTRODUCTION

Cleavage of APP to its various fragments is highly regulated. It is generally accepted that the amyloid-forming pathway of APP processing is mutually exclusive of the non-amyloidogenic α-secretase cleavage of APP (Figure 1; Selkoe 2001, Coughlan and Breen, 2000). Various neurotransmitters, acting on first and second messenger pathways are known to up-regulate secretion of $APP_{S\alpha}$, precluding Aβ formation (reviewed by Mills and Reiner, 1999). Whilst the mechanisms of action of these processes still remain largely unknown, from a therapeutic standpoint, identifying agents and cellular pathways which affect formation and secretion of toxic Aβ has value in terms of developing viable anti-amyloid therapies.

It is well known that transient activation of the second messenger PKC pathway upregulates $APP_{S\alpha}$ secretion and decreases Aβ formation (Mills and Reiner, 1999). Work by Mills et al. has now shown strong evidence indicating that several upstream messenger pathways converge on the MAP Kinase cascade to increase APP_S secretion whilst decreasing Aβ secretion (Mills et al., 1997). In an effort to tease out the various downstream effectors of PKC-dependent APP catabolism, rat PC12 cells which were stably transfected with a dominant negative *ras* construct which was under the control of a dexamethasone-inducible promoter (DN1rasPC12 cells; Kremer et al., 1991) were used to study phorbol ester stimulation of APP_S secretion. These cells allowed for the manipulation of the ras signaling pathway in the study of regulated APP catabolism. In

these experiments, the glucocorticoid receptor agonist dexamethasone was used to induce the expression of a dominant negative ras phenotype. To antagonize the actions of dexamethasone, the glucocorticoid and progesterone antagonist mifepristone (RU486) was also used to treat cells. When RU486 was added to PC12 cells alone, an increase in APP_S secretion was observed. This led to the hypothesis that RU486 was acting through the steroid hormone receptor pathway to regulate APP catabolism. Follow-up experiments showed that this was not the case. A search of the literature on actions of RU486 revealed that, apart from its well-known anti-steroidal functions, it was also a p-gp inhibitor (Gruol et al., 1994). A new hypothesis was struck postulating that inhibition of p-gp regulates APP catabolism.

MATERIALS AND METHODS

Cell Culture

Rat pheochromocytoma PC12 cells were purchased from the American Tissue Culture Collection (ATCC) and maintained in Dulbecco's Modified Minimum Essential Media (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco) and 10% horse serum (Gibco) at 37°C, 5% CO_2. PC12 cells stably transfected with a dominant negative *ras* gene (DN1rasPC12 cells; Kremer et al., 1991) under the control of a dexamethasone-inducible MMTV promoter were obtained as a generous gift from Dr. Simon Halegoua (State University of New York at Stony Brook) and maintained similarly to PC12 cells. Mouse fibroblast E82 cells deficient in glucocorticoid and progesterone receptors (Housley et al., 1989) were generous gifts from Dr. Mark Danielson and maintained in DMEM supplemented with 5% FBS. Human carcinoma KB-3.1 cells expressing low levels of p-glycoprotein (Shen et al. 1986) were obtained from Dr. Ira Pastan (NIH) and maintained in DMEM supplemented with 10% FBS.

Cortical Cell Cultures and Drug Exposures

Timed pregnant Sprague-Dawley rats were anesthetized with halothane at 18 days of gestation and the cerebral cortex was removed from rat embryos and dissociated using a method previously described (Murphy and Baraban, 1990), with the exception that the plating medium L-cystine concentration was supplemented to 300 μM. Tissue was dissected and stored in Hanks buffer (4°C). Following dissection, Hank's buffer was aspirated and the brain tissue was dissociated by mild trituration in DMEM containing N1 suppluments (Bottenstein et al., 1980) plus 10% FBS. The resultant single cell

suspension was plated on to 60mm petri dishes (Falcon) at a density of 1.0×10^6 cells/plate. Cultures were maintained for 17 days before use in experimentsas outlined by Fiore et al. (1993) with minor modifications. In brief, prior to drug treatment, cells were washed once with warm phosphate buffered saline (PBS, Sigma) and 1 mL of DMEM containing the appropriate drug was added to the cells for 30 minutes before harvesting for APP_S detection.

Drug Exposures in Cell Lines

Cells were plated onto 60 mm petri dishes (Falcon) at a density of 1.0×10^6 cells/plate 24 hours prior to the experiment to allow for adherence and maturation. Drugs suspended in either dimethyl sulfoxide (DMSO 0.001% v/v, Sigma), or double distilled water were appropriately diluted in DMEM. Cells were washed once with warm PBS and 1 mL of drug-containing DMEM was added to the cells. Plates were returned to the 37°C incubator for 15 minutes before the medium was harvested. The 1 mL of conditioned medium was harvested into 1.5 mL Eppendorf tubes containing protease inhibitor cocktail and placed on ice for further processing. To harvest the cells, 50 μL of cell lysis buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate supplemented with protease inhibitors was added to the cells and left to incubate at 4°C for 15 minutes at which time the cells were scraped off the bottom of the petri dish and harvested into Eppendorf tubes.

Measurement of APP

The conditioned medium containing secreted APP_S was spun at 14,000 rpm on a table top centrifuge for 10 minutes at 4°C to pellet unwanted cellular debris. The resulting medium was desalted in a centrifuge tube (Millipore) containing a nitrocellulose filter with a 30,000 Da cut-off, and precipitated in a speed vacuum. The pellet was resuspended in 20 μL of Laemmli sample buffer for SDS-PAGE. Cell lysates were spun at 14,000 rpm at 4°C for 15 minutes to remove any detergent insoluble materials. 2 μL of the supernatant was removed for protein quantification using the bicichoninic acid (BCA) protein assay (Pierce). To detect secreted APP_S in the conditioned medium, samples normalized to 150 μg of cellular protein were resolved on 10% polyacrylamide gels and western blotting using the monoclonal antibodies 22C11 (Boehringer Manneheim) or WO-2 (a kind gift from Dr. Konrad Beyreuther) was performed to probe for APP_S and $APP_{S\alpha}$, respectively. Bands were detected using ECL (Amersham). Cellular APP in the cell lysate was detected as described above.

Quantitation of APP_S

Blots were subjected to densitometry using Molecular Dynamics image analysis software. Densitometric measurements were performed in the linear range as determined by standard dilution curves of secreted cellular proteins. Optical density values are reported as % control. Each trial (n) represents individual experiments performed on different cells plated separately and completely repeated on at least three separate occasions. Analysis of variance followed by a Dunnett's post-hoc analysis was used to determine the significance of observed differences.

RESULTS

RU486 Increases APP_S Secretion in Cell Culture

RU486 increased APP_S secretion in DN1rasPC12 cells after a 15 minute drug treatment (Figure 3). Cells were treated with either carbachol or phorbol myristic acid (PMA) as a control to ensure that they were responsive to second messenger regulated APP catabolism. The same increase in APP_S was observed in wild type PC12 cells using 1 μM RU486 (Figure 4). As RU486 is a classical steroid hormone receptor antagonist (Baulieu, 1991), the initial hypothesis was that this increase in APP_S secretion was due to the steroidal effects of RU486. To disprove this hypothesis, mouse fibroblast E82 cells that were deficient in glucocorticoid, progesterone, and mineralocorticoid receptors (Housley et al., 1989) were treated with RU486. Again, RU486 increased APP_S from E82 cells after a 15minute drug exposure (Figure 5), indicating that this was probably not a steroid hormone receptor dependent event.

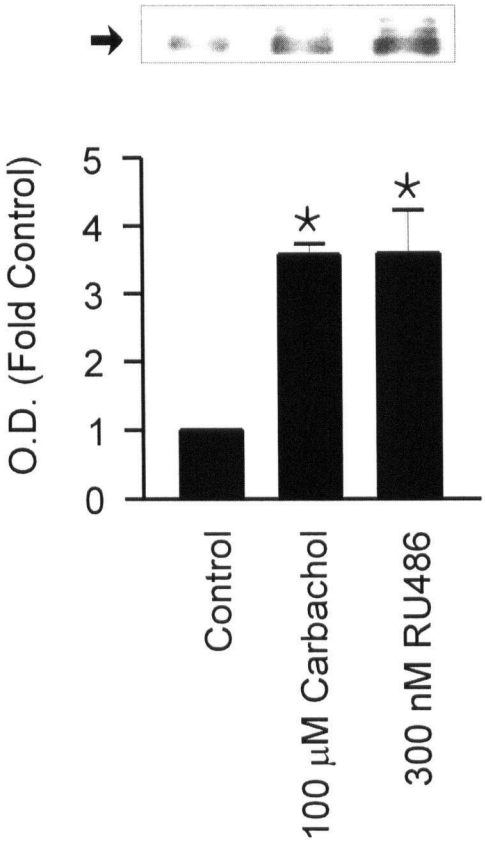

Figure 3. RU486 increases APP_S secretion in DN1rasPC12 cells. DN1rasPC12 cells were treated separately with either drug vehicle alone (Control), 100 μM Carbachol, or 300 nM RU486 for 15 minutes, at 37°C. Media was harvested immediately and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently (* = $p < 0.01$). APP_S fragments were detected using the monoclonal antibody 22C11.

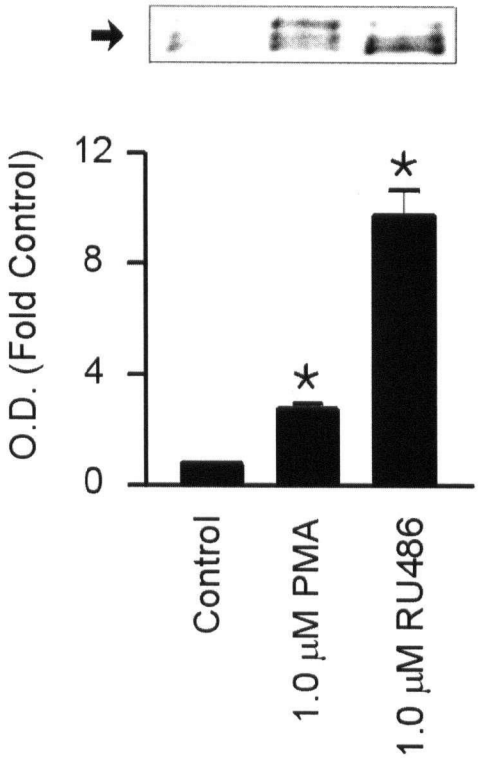

Figure 4. RU486 increases APP_S secretion in wild-type PC12 cells. Wild-type PC12 cells were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 1.0 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently (* = $p < 0.01$). APP_S fragments were detected using the monoclonal antibody 22C11.

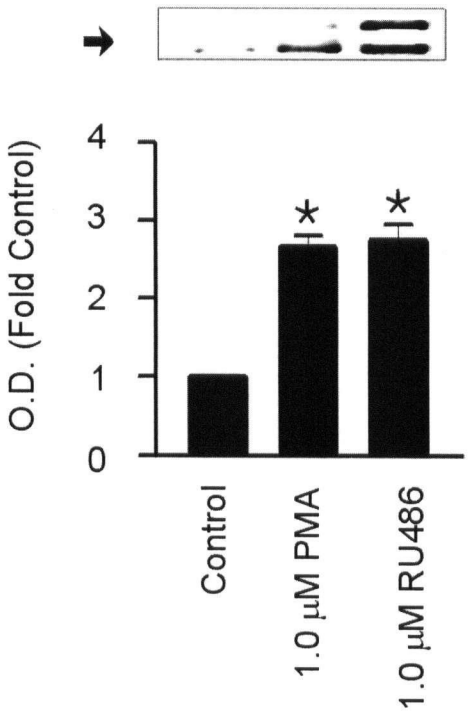

Figure 5. RU486 increases APP_S secretion in E82 mouse fibroblast cells. E82 cells were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 1.0 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently (* = $p < 0.01$). APP_S fragments were detected using the monoclonal antibody 22C11.

RU486 Increases APP_S Secretion in KB-3.1and HEK293 Cells Transiently Transfected with the pHaMDR1/A Construct

Apart from being a steroid hormone receptor antagonist, RU486 is also a well characterized antagonist of p-gp (Gruol et al, 1994). This information led to the revised hypothesis that RU486 increased APP_S secretion due to its interaction with p-gp. To test this hypothesis, human carcinoma KB-3.1 cells which were deficient in p-gp (Ueda et al., 1987), were treated with 0.1 μM RU486. RU486 did not increase APP_S secretion in KB-3.1 cells (Figure 6). Interestingly, PMA did not increase APP_S secretion in these cells either. Next, using the calcium phosphate method of transfection as described in Chen and Okayama (1987), a plasmid encoding human *MDR1 (pHaMDR1)* was transiently transfected into KB-3.1 and HEK293 cells followed by treatment with RU486. Transfected KB-3.1 cells (Figure 7) and HEK293 cells (Figure 8) showed increased APP_S secretion with both PMA and RU486 treatment after a 15 minute exposure. HEK293 cells were probed with the monoclonal antibody WO-2 against the N-terminal Aβ sequence, thus allowing for detection of $APP_{S\alpha}$ in the conditioned medium. Results using WO-2 in HEK293 cells demonstrated that RU846 increased $APP_{S\alpha}$. This was reproduced under slightly different conditions in KB-T10 cells. KB-T10 cells are derived from KB-3.1 cells that have been placed under the chronic selection pressure of colchicine, resulting in endogenous overexpression of p-gp (Ueda et al., 1987). Again, RU486 increased APP_S secretion KB-T10 cells (Figure 9), indicating that this effect was p-gp-dependent.

P-gp Inhibitors Increase APPs Secretion in Mixed Neuronal Culture

P-gp inhibitors were added to primary neuronal cultures to see if this phenomenon was generalizable in the brain. Mixed primary rat neuronal cultures were treated separately with two known p-gp antagonists, cyclosporine A (CsA) and RU4993 (Twentyman, 1992; Marsaud et al, 1998). RU49953 is an RU486 analogue that is more selective for p-gp and does not interact with steroid hormone receptors (Marsuad et al., 1998). Treatment of neuronal cultures with 1.0 μM CsA (Figure 10) or 0.1 μM RU49953 (Figure 11) for 30 minutes significantly increased APP_S secretion.

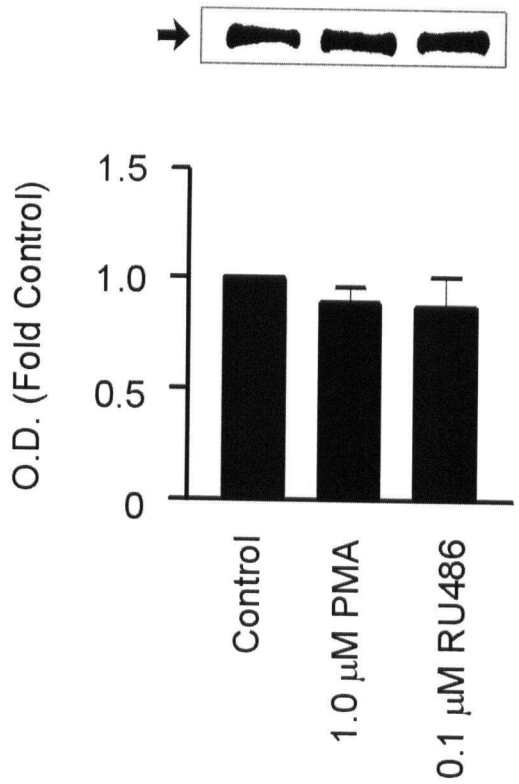

Figure 6. RU486 and PMA do not increase APP_S secretion in KB3.1 cells. KB3.1 cells were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 0.1 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody 22C11.

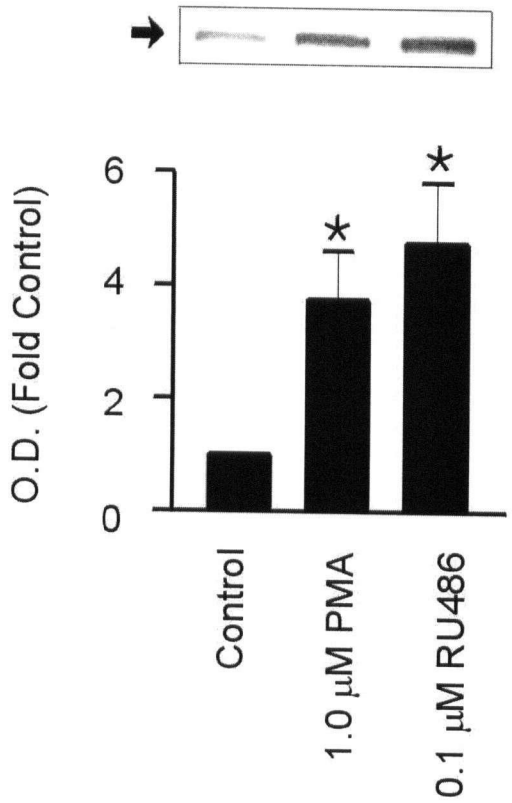

Figure 7. RU486 and PMA increase APP_S secretion in KB3.1 cells transiently transfected with the pHaMDR1/A construct. KB3.1 cells that were transiently transfected with a plasmid encoding human MDR1 were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 0.1 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody 22C11.

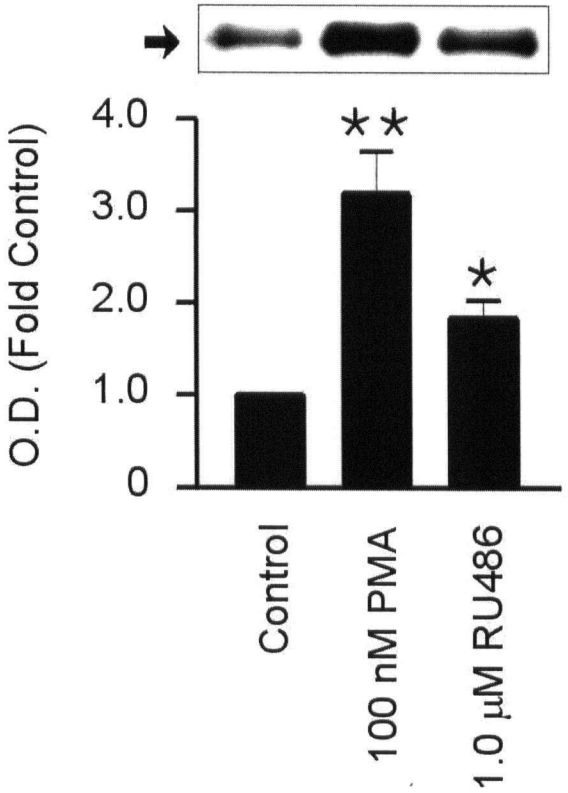

Figure 8. RU486 and PMA increase $APP_{S\alpha}$ secretion in HEK293 cells transiently transfected with the pHaMDR1/A construct. HEK293 cells that were transiently transfected with a plasmid encoding human MDR1 were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 0.1 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody WO-2.

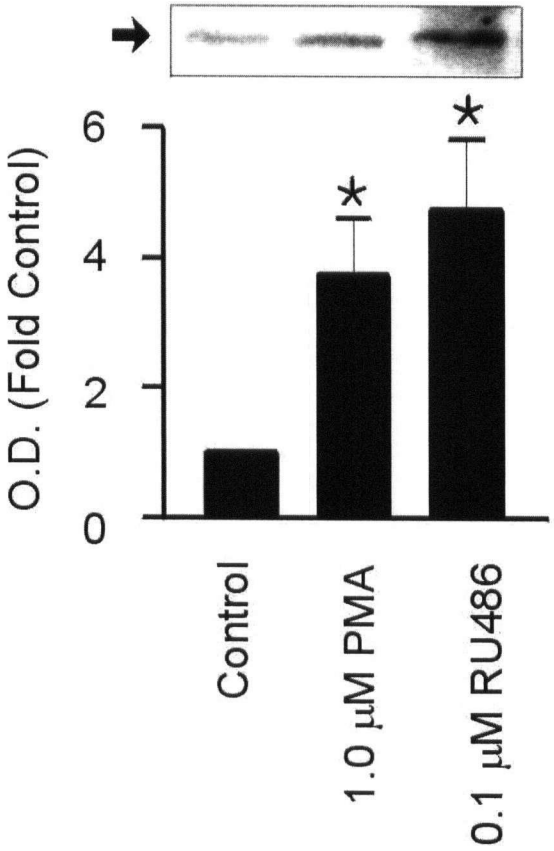

Figure 9. RU486 and PMA increase APP_S secretion in KB-T10 cells which overexpress p-gp. KB-T10 cells were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 0.1 μM RU486 for 15 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 3 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody 22C11.

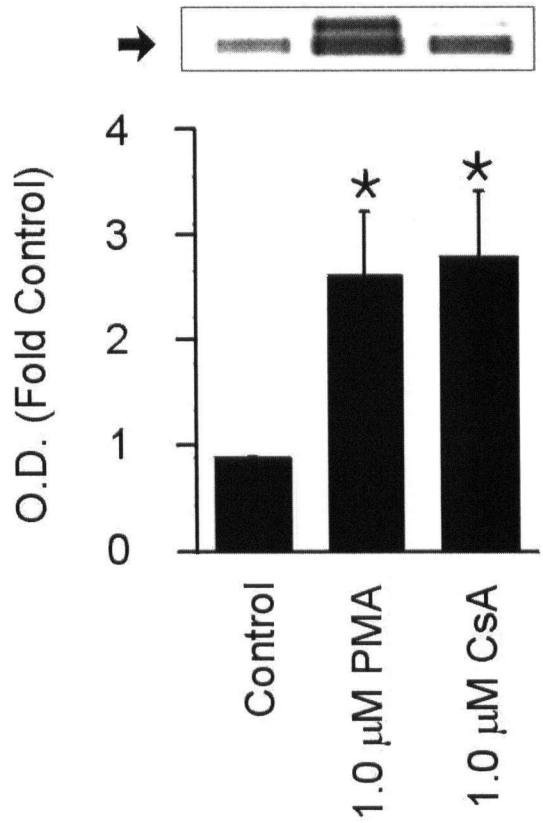

Figure 10. Cyclosporin A increases APP_S secretion in primary mixed rat cortical cultures. Rat primary cortical cultures were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 1.0 μM Cyclosporin A (CsA) for 30 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 4 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody 22C11.

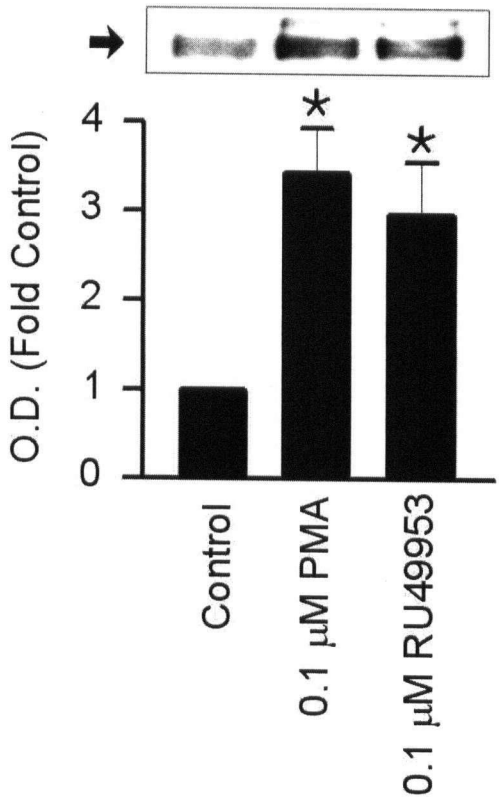

Figure 11. RU49953 increases APP_S secretion in primary mixed rat cortical cultures. Rat primary cortical cultures were treated separately with drug vehicle (Control), 1.0 μM phorbol-12-myristate (PMA), or 0.1 μM RU49953 for 30 minutes, at 37°C. Media was harvested after the exposure period and processed for detection of APP_S as described in Materials and Methods. Representative western blot shows APP_S secretion in the medium following treatment. Arrow denotes APP_S at ~ 97 kDa. Results are means ± SEM of 4 separate experiments performed independently. APP_S fragments were detected using the monoclonal antibody 22C11.

DISCUSSION

This study demonstrates the novel finding that known p-gp inhibitors increase APP_S secretion within a rapid time frame. The mechanism by which RU486 increases APP_S secretion from cells is unknown. It is possible that p-gp, or a substrate of p-gp, allosterically modifies one or more of the enzymes involved in APP cleavage. Evidence now suggests that exogenous Aβ has a down regulatory effect on $APP_{S\alpha}$ secretion (Gitter et al., 2000). That p-gp is a transporter of lipophilic substrates immediately led to the hypothesis that p-gp was an Aβ transporter, the study of which is the focus of the following chapter. Following this hypothesis, it is plausible to postulate that inhibition of p-gp leads to an elevation of intracellular Aβ, resulting in feedback inhibition of either β or γ secretase activity, thereby providing additional substrate for α-secretase cleavage. The notion that ABC transporters are able to regulate heterologous proteins has been previously postulated (Higgins, 1995; Valverde et al., 1992); however, in the absence of the purified secretase enzymes, it is not possible to address these issues at this time.

The observation that RU486 does not increase APP_S secretion in KB-3.1 cells, which express very low levels of p-gp, strengthens the hypothesis that this mechanism is p-gp-dependent (Figure 6). This is further supported by the restoration of the increase via transient transfection with the pHaMDR1/A plasmid which encodes human MDR1 (Figure 7), as well as the ability of RU486 to increase APP_S secretion in p-gp-overexpressing KB-T10 cells (Figure 9). To ensure that signaling systems in the cells were intact, I included an extra treatment with the PKC activator phorbol myristic acid (PMA) in all experiments. It is well known that PMA increases APP_S secretion in cell

lines and neurons (Mills and Reiner, 1999). However, it was consistently observed that PMA was unable to increase APP_S secretion in KB-3.1 cells and that this effect was restored following transient transfection of MDR1. I also observed that PMA was able to increase APP_S secretion from KB-T10 cells, selected from its parental KB-3.1 cells under colchicine selection to overexpress MDR1 (Figure 9). These results imply that PMA upregulation of APP_S secretion is p-gp-dependent. As p-gp is known to be phosphorylated by PKC, it can be hypothesized that the ability of PMA to increase APP_S secretion may be through PKC phosphorylation of p-gp. There is controversy regarding this issue. Data show both up-regulation (Castro et al., 1999) and down-regulation (Idriss et al., 2000; Miller et al., 1998; Tsuruoka et al., 2001) of p-gp activity following phorbol ester treatment. Given this conflicting evidence, it is difficult to interpret my findings mechanistically at the present time.

That p-gp inhibitors increase APP_S secretion in mixed neurons is also of interest (Figures 10 and 11). While p-gp is richly expressed at the blood-brain barrier (Pardridge et al., 1997), there is no documented data showing that it is expressed in neurons. This may imply that there may be a brain-expressed MDR-like ABC transporter which is also capable of modifying APP catabolism. Furthermore, as immunosuppressants such as CsA and FK506 block p-gp (Demeule et al., 1997) and have neuroprotective properties (Snyder et al., 1998), it may be hypothesized that at least part of this neuroprotection results from increased production of neurotrophic APP_S fragments.

In summary, the results in this chapter show a novel method of regulating APP catabolism by p-gp inhibitors. From a therapeutic standpoint, finding agents which enhance the non-amyloidogenic pathway of APP catabolism may help in curbing the brain amyloid burden. As Aβ is the central molecule of interest in the beta-amyloid cascade hypothesis, the next step is to test directly the hypothesis that p-gp inhibitors decrease Aβ secretion.

III. Evidence that Aβ is a Substrate for P-gycoprotein

INTRODUCTION

The mechanism by which Aβ is secreted from cells is unknown. Evidence suggests that Aβ is formed by secretase cleavage, most likely in the endosomal/lysosomal compartment, and then released into the extracellular milieu (Sinha and Lieberburg, 1999; Cook et al., 1997; Peraus et al., 1997; and Xu et al., 1997). Unlike the cleavage of APP by α-secretase, which is thought to be a plasma membrane-associated event leading to direct extracellular release of APP_S, the hydrophobic sequence of Aβ imparts a lipophilic quality to this peptide. This makes it more probable that Aβ is partitioned within the lipid bilayer prior to its release from cells and requires a mechanism of expulsion other than simple diffusion.

The indirect evidence showing an increase in APP_S secretion following treatment of cells with p-gp inhibitors led to several hypotheses. The first hypothesis, considering the mutual exclusive nature of APP_S and Aβ formation, states that p-gp inhibitors concommitantly decrease Aβ secretion. Secondly, p-gp is a transporter of a wide variety of lipophilic substrates. Aβ is a lipophilic molecule, the second hypothesis is that p-gp is an Aβ transporter. The data that was generated from these hypotheses have since been published (Lam et al., 2001).

MATERIALS AND METHODS

Cell Culture and Transfections

HEK293 cells stably transfected with the gene encoding the Swedish APP695 double mutation (K269 cells; a gracious gift from Dr. Dennis Selkoe, Harvard Medical School) were maintained in DMEM supplemented with 10% FBS and 20 μg/mL geneticin (Gibco). Wild type HEK293 cells (a gift from Dr. Lynn Raymond, University of British Columbia) were maintained in DMEM supplemented with 10% FBS. Cells were transfected using either the calcium phosphate method of transfection as described by Chen & Okayama (1987) or Lipofectamine (Gibco). Transfection efficiency was assessed by β-galactosidase staining of cells transfected with the β-galacotosidase gene. Calcium phosphate transfections consistently resulted in over 80% of cells stained with β-gal while Lipofectamine transfections resulted in over 90% of cells stained. Cells were transfected with constructs encoding for human MDR1 (pHaMDR1/A), human MRP1 (a gift from Drs. Roger Deeley and Susan Cole) and/or a plasmid encoding the Swedish APP695 double mutation (under the influence of a CMV promoter, obtained as a gift from Active Pass Pharmaceuticals, Vancouver, BC). Drug exposures were performed as described in the Materials and Methods section in Section II.

Quantification of Aβ in Culture Media

Following the drug exposure, the conditioned media was spun at 14,000 rpm for 5 minutes at 4°C to remove cellular materials. To each 1 mL of conditioned medium, 4 mg of bovine serum albumin was added to increase the total protein content for ease of protein precipitation. Total protein in the media was then precipitated using the classical

trichloroacetic acid precipitation method. The resulting pellet was then resuspended in 20 μL of Laemmli buffer for SDS-PAGE. Cellular proteins were quantified using the BCA assay (Pierce). To resolve Aβ, normalized samples were loaded onto a 16.5% tris-tricine polyacrylamide gel prepared according to Klafki et al. (1996) with the minor modification of excluding Coomasie Blue G-250 from the resolving gel. Proteins were then transferred onto 0.2 μm nitrocellulose as described by Ida et al. (1996) and the membrane was then heated for 5 minutes in boiling PBS. This heating was used to increase the affinity of the monoclonal antibodies used to probe the blot for Aβ (Ida et al., 1996). Aβ was detected using either WO-2 (a gift from Dr. Konrad Beyreuther) or 6E10 (Senetek Inc.), two monoclonal antibodies which recognize N-terminal epitopes of the Aβ sequence. Bands were visualized using ECL (Amersham). Quantification of Aβ was performed using densitometry as described in Section II.

Western Blots of Cellular APP and P-gp

To detect cellular proteins, 100 μg of cell lysate was loaded onto 7.5% polyacrylamide gels and subjected to western blotting. To detect cellular APP, blots were probed with the N-terminal monoclonal antibody 22C11 (Boehringer Manneheim). P-gp was detected using the monoclonal antibody C219 (ID Labs. Inc.) which recognizes an intracellular epitope of p-gp. Bands were visualized using ECL.

β-amyloid peptide binding to purified hamster mdr1

Binding of Aβ peptides to mdr1 was studied using fluorescence quenching, as described previously for peptides and drug substrates (Liu and Sharom, 1996, Sharom et

al., 1998b, 1999). Briefly, highly purified whole cell plasma membranes enriched in mdr1reconstituted into vesicles were generated from Chinese Hamster Ovary B30 cells that overexpressed hamster mdr1 under colchicine selection. and labeled with MIANS were titrated with human synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ (RBI), and quenching of the fluorescence emission at 420 nm was monitored. The dissociation constant K_d was estimated by fitting the data to an equation describing interaction with a single class of binding site.

Modulation of mdr1 ATPase activity and drug transport by β-amyloid peptides

The ATPase activity of mdr1 in plasma membrane vesicles derived from the multidrug-resistant cell line CH^RB30 was measured as described previously (Sharom et al., 1995b) in the presence of increasing concentrations of $A\beta_{1-40}$ or $A\beta_{1-42}$. ATP-dependent uptake of [^{3}H]-colchicine into CH^RB30 plasma membrane vesicles was determined by rapid filtration as outlined earlier (Sharom et al., 1995a and 1998b) in the presence of increasing concentrations of $A\beta_{1-40}$ or $A\beta_{1-42}$. Colchicine uptake was calculated as percent control relative to that measured in the absence of Aβ, and the peptide concentration causing 50% inhibition of uptake, D_m, was estimated using the median effect equation (DiDiodato and Sharom, 1997).

Direct transport of β-amyloid peptide across mdr1 membrane vesicles

Inside-out membrane vesicles were prepared from the wild-type AuxB1 Chinese Hamster Ovary cell line (CH^RAuxB1) and its colchicine-selected mdr1 over-expressing progeny B30 Chinese Hamster Ovary cells (CH^RB30) as described (Juliano and Ling,

1976, Shapiro and Ling, 1995). To allow for Aβ incorporation into the vesicle membrane, 100 nM of either synthetic human $A\beta_{1-40}$ or $A\beta_{1-42}$ (RBI) was added to a 70 μl suspension of vesicles and allowed to equilibrate at 37°C for 15 minutes. Unincorporated Aβ was excluded by passage of the vesicles through a BioGel P-6 size exclusion column (BioRad). To activate transport, Na_4ATP (Sigma) at a final concentration of 1.5 mM was added to the vesicles and the reaction allowed to proceed for 15 minutes at 37°C. Vesicles were then ruptured using five cycles of rapid freeze-thaw in liquid nitrogen and subjected to ultracentrifugation at 100,000*g* for 20 minutes. The supernatant containing intravesicular Aβ was harvested and TCA precipitated as described above for Aβ detection. The pellet containing membrane-bound Aβ was directly resuspended in Laemmli buffer. Both membrane-bound and intravesicular Aβ were subjected to Western blot analysis using the 6E10 antibody as described above.

RESULTS

Transient Transfection with pHAMDR1/A increases Aβ Secretion

In order to study the cellular interaction between Aβ and p-gp, HEK293 cells which were stably transfected with APP695 harbouring the Swedish double mutation (K269 cells) were transiently transfected with pHaMDR1/A using the calcium phosphate method. K269 cells secrete approximately 8-fold more Aβ than wild type HEK cells (Citron et al., 1996) making for ease of Aβ detection in the culture medium. Cells that were transfected with pHaMDR1 showed an increase in Aβ secretion compared to mock and untransfected controls (Figure 12a). This experiment was repeated in a slightly different way by cotransfecting pHaMDR1/A and pCDN3.1APP695sw (encoding the Swedish double mutation of APP695) into wild type HEK293 cells using the gentler Lipofectamine method of transfection. Aβ secretion also increased in HEK293 cells cotransfected with *MDR1* and *APP695sw* (Figure 12b). Transfection with *MDR1* did not result in statistically significant increases in expression of p-gp as detected by the antibody C219, but cellular levels of APP were consistently increased in association with the increase in extracellular Aβ levels (Figure 12c). Neither the increase in Aβ secretion nor the increase in cellular APP levels was observed in cells that were transfected with *MRP1*, an ABC transporter with very low homology to p-gp (Figure 12b, c). From these experiments, it was impossible to determine whether the observed increases in extracellular Aβ after transfection of pHaMDR1/A were due to increases in Aβ secretion or the availability of additional substrate in the form of increased cell-associated APP. For these reasons, other methods were employed to address further the hypothesis that p-gp is an Aβ efflux pump.

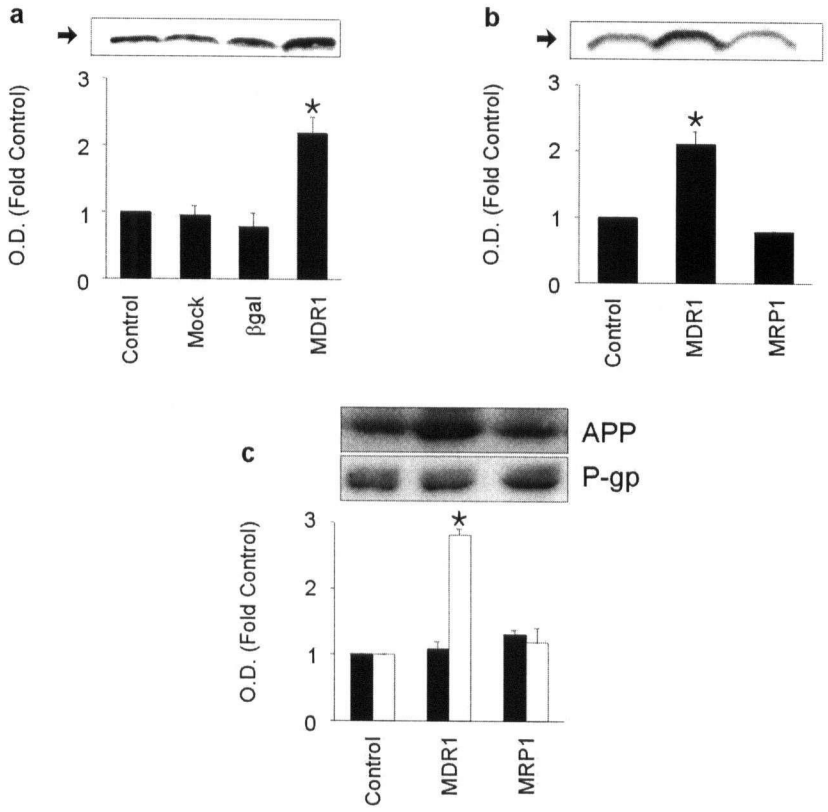

Figure 12. Transient transfection of human *pHaMDR1* increases Aβ secretion. (a) K269sw cells were either not transfected (Control), mock transfected using calcium phosphate precipitation without plasmid (Mock), or with either 10 μg β-galactosidase (βgal) or 10 μg human *MDR1* (MDR1). Forty-eight hours after transfection, the medium was changed and secreted Aβ was measured 1 hour later. Transfection with *pHaMDR1* significantly increased basal Aβ secretion approximately 2-fold above control (n=3, * $p<0.01$). Western blotting was performed using the monoclonal antibody WO-2. Arrow indicates Aβ at approximately 4 kDa. (b) HEK293 cells were either not transfected (Control), or transiently co-transfected using Lipofectamine with 3 μg APP695sw and either 3 μg human *pHaMDR1* (MDR) or 3 μg *pCDNA3.1MRP1* (MRP1) and Aβ measured in the extracellular medium. Western blot detection of Aβ was performed using the 6E10 monoclonal antibody. Again, *MDR1* significantly increased Aβ secretion by ~2-fold over control while MRP1 did not show significant change (n=3, *$p<0.01$). (c) Total APP and P-gp from cellular extracts of transfections. APP was detected using 22C11. P-gp was detected using C219. In cells transfected with *pHaMDR1*, total cellular APP levels were increased (black bars; n=3, *$p<0.01$), while no increases were observed in cells transfected with *pCDNA3.1MRP1*. Transfection with *pHaMDR1* did not result in significant increases in cellular p-gp (white bars; n=3).

The P-glycoprotein Inhibitors RU486 and RU49953 Decrease Aβ Secretion

Pharmacological treatment of K269sw cells transiently transfected with pHaMDR1/A with the p-gp inhibitors RU486 (Gruol *et al.*, 1994) and RU49953 (Marsaud *et al.*, 1998) significantly decreased Aβ secretion compared to cells treated with vehicle, after a 15 minute drug exposure (Figure 13a, b). The 15 minute time frame was chosen to avoid any possible effects of RU486 on gene expression via glucocorticoid or progesterone receptors (Wehling, 1994). Both RU486 (Figure 13a) and RU49953 (Figure 13b) significantly decreased Aβ secretion from control.

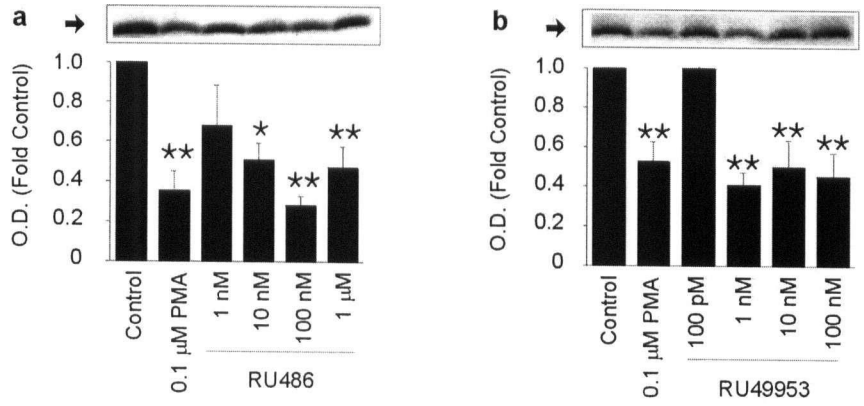

Figure 13. Inhibition of p-glycoprotein reduces Aβ secretion. K269sw cells were transiently transfected with human *MDR1* as in Fig. 12 and exposed to (a) RU486 or (b) RU49953, at varying doses for 15 minutes. Both RU486 (n=6) and RU49953 (n=6) significantly decreased Aβ secretion in these (*$p<0.05$, **$p<0.01$). Aβ was detected using the WO-2 monoclonal antibody.

Competitive Substrate Binding Data Supports Aβ as a Bona fide Substrate of P-glycoprotein

The hypothesis that Aβ might bind p-glycoprotein *in vitro* was next tested using highly purified hamster mdr1 reconstituted into vesicles. Binding of a wide variety of mdr1 substrates, including drugs, modulators, and cyclic and linear peptides, can be quantified by fluorescence quenching of highly purified protein labeled with the fluorophore MIANS at two conserved Cys residues within the Walker A motifs of the protein's nucleotide binding domains (Sharom *et al.* 1998a). Titration of MIANS-labeled mdr1 with synthetic peptides encoding either human $A\beta_{1-40}$ or $A\beta_{1-42}$ resulted in saturable quenching of MIANS fluorescence, suggesting that both peptides interact directly with the transporter (Figure 14a). The binding affinities (K_d) as determined from two independent quenching titrations on different batches of mdr1 were 12.5 ± 1.0 μM and 6.7 ± 1.0 μM for $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively. Precedent for binding (Sharom *et al.* 1998a) and secretion (Sharom *et al.* 1996) of peptides by mdr1 exists, with most peptide substrates having K_d values similar to that exhibited by Aβ.

Bona fide mdr1 substrates are generally capable of competing for transport with other substrates in both plasma membrane and proteoliposome systems (Doige & Sharom, 1992; Sharom *et al.* 1993). To test the hypothesis that Aβ is capable of competing with established mdr1 substrates, the ability of Aβ to alter ATP-dependent uptake of [^{3}H]-colchicine into plasma membrane vesicles derived from colchicine selected, mdr1 overexpressing Chinese hamster ovary CH^RB30 cells was tested. Both $A\beta_{1-40}$ and $A\beta_{1-42}$ competed effectively with [^{3}H]-colchicine for transport, with the

concentration required for 50% inhibition of drug uptake, D_m, estimated to be 27 μM for $A\beta_{1-40}$, and 22 μM for $A\beta_{1-42}$ (Figure 14b).

The ability of mdr1 to transport substrates is dependent upon hydrolysis of ATP, and substrates for transport often stimulate ATPase activity. To test the hypothesis that Aβ peptides might stimulate mdr1 ATPase activity, $A\beta_{1-40}$ and $A\beta_{1-42}$ were added to plasma membrane vesicles derived from CH^RB30 cells and the resultant ATPase activity measured (Figure 14c). Both $A\beta_{1-40}$ and $A\beta_{1-42}$ stimulated ATPase activity; $A\beta_{1-40}$ increased ATPase activity by 100% at a concentration of 50 μM (half-maximal stimulation at 17 μM), whereas in the case of $A\beta_{1-42}$, stimulation of ~40% was observed at 50 μM (half-maximal stimulation at 2 μM). Taken together, these data define Aβ as a *bona fide* mdr1 substrate.

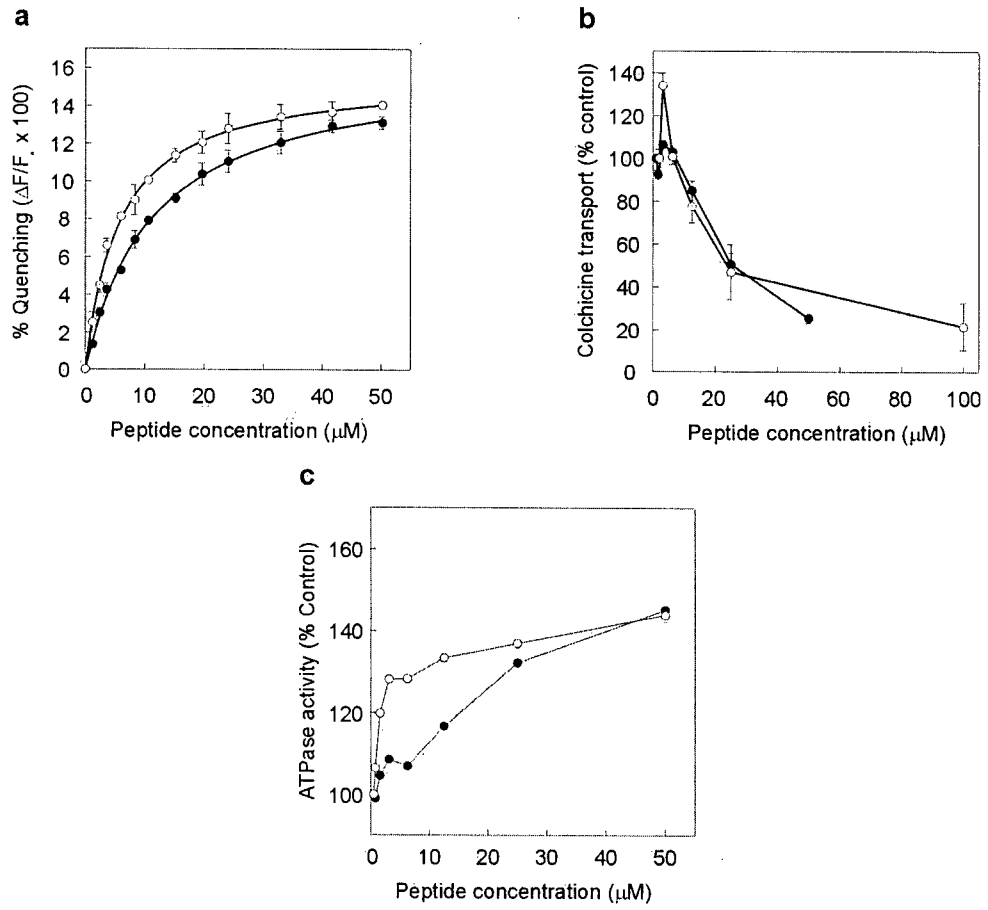

Figure 14. (a) Binding of Aβ peptides to MIANS-labeled p-glycoprotein results in fluorescence quenching. Highly purified MIANS-labeled p-glycoprotein (50 μg/ml) was titrated with increasing concentrations of peptides $A\beta_{1-40}$ (•) and $A\beta_{1-42}$ (o). The percent quenching of the fluorescence emission at 420 nm ($\Delta F/F_0$ x 100) was calculated relative to MIANS-labeled p-glycoprotein in the absence of peptides. The quenching data (shown by the symbols, means ± range, n=3) were fitted to an equation describing interaction of the peptides with a single binding site, as indicated by the continuous line. (b) Aβ peptides block p-glycoprotein-mediated drug transport. Equilibrium uptake of [^{3}H]-colchicine into CH^RB30 plasma membrane vesicles was determined at 22°C in the presence of 1 mM ATP and a regenerating system, and increasing concentrations of $A\beta_{1-40}$ (•) and $A\beta_{1-42}$ (o) peptides. Data are presented as percent of control ATP-dependent [^{3}H]-colchicine uptake in the absence of peptide. (means ± SEM, n=3). (c) Aβ peptides stimulate p-glycoprotein ATPase activity. CH^RB30 plasma membrane vesicles were assayed for Mg^{2+}-dependent ATPase activity in the presence of increasing concentrations of $A\beta_{1-40}$ (•) and $A\beta_{1-42}$ (o). Data are presented as a percentage of control ATPase activity measured in the absence of peptides (means ± SEM, n=3). Where error bars are not visible, they are contained within the symbols.

Direct Transport of Aβ Through P-glycoprotein

To test the hypothesis that an ABC transporter can transport Aβ, an *in vitro* assay in which Aβ transport across the membrane could be directly measured was developed. For these experiments, vesicles prepared from CH^RB30 cells were used (Juliano & Ling, 1996; Shapiro & Ling, 1995). During reconstitution of these vesicles, mdr1 proteins are incorporated in both the normal configuration and in an inside-out configuration; addition of ATP to the external medium selectively activates mdr1 in the inside-out orientation with its ATPase binding sites on the outside of the vesicle, thus allowing for transport of substrates from the outside into the lumen of the vesicles. In order to reconstruct the physiological association of Aβ with the membrane, synthetic human Aβ peptides were incorporated into these vesicles. Since the sequence of human and rodent Aβ differs, antibodies specific to human Aβ selectively measure transport of the synthetic human Aβ across these membranes and do not detect endogenous rodent Aβ in the vesicle membrane. Vesicles were incubated with either $A\beta_{1-40}$ or $A\beta_{1-42}$ (100nM) for 15 minutes at 37°C and any free unbound Aβ in the solution was removed by passage through a size-exclusion column (Figure 15d). ATP was then added to the solution, incubated at 37°C for 15 minutes to activate mdr1 and the membrane and intravesicular fractions were separated and Aβ levels measured using western blot analysis.

A significant decrease in membrane-bound Aβ with a corresponding increase in intravesicular Aβ was observed in the presence of Na_4ATP (Figure 15a,e). In contrast, vesicles treated with the non-hydrolysable ATP analogue AMP-PNP showed no significant changes in either membrane-bound or lumenal Aβ (Figure 15b, e),

demonstrating that transport of Aβ was energy dependent. Transport was also dependent upon overexpression of mdr1, as no detectable changes in Aβ content were observed in either the membrane or the intravesicular compartments when the experiment was carried out using vesicles prepared from the parental AuxB1 cells which are not enriched in hamster mdr1 (Figure 15c, e). Taken together, these data provide strong evidence that mdr1 is an Aβ transporter.

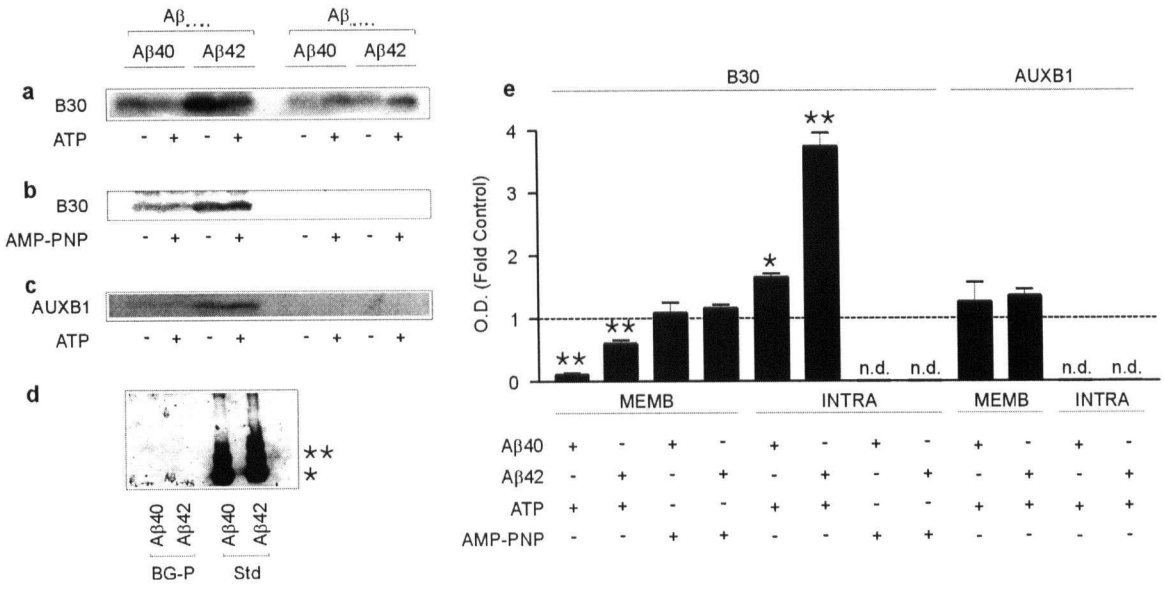

Figure 15. P-glycoprotein mediates transport of Aβ peptides in an ATP-dependent manner. (a) B30 vesicles enriched in hamster class I p-glycoprotein transports pre-inserted synthetic human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides in an ATP-dependent manner (n=3). In this and subsequent panels, western blots using the 6E10 antibody show levels of membrane-bound Aβ peptides ($A\beta_{MEMB}$) and their corresponding levels in the interior of the vesicle ($A\beta_{INTRA}$) before and after addition of nucleotide. (b) The non-hydrolysable ATP analogue, AMP-PNP, does not stimulate transport of Aβ into B30 vesicles (n=2). (c) ATP-dependent transport is also absent in p-glycoprotein deficient AuxB1 vesicles (n=3). No Aβ was detectable within B30 and AuxB1 vesicles treated with AMP-PNP or ATP, respectively. (d) Overexposed western blot of synthetic Aβ peptides spun through a Biogel-P6 size exclusion column (BG-P) compared to standards (Std). 100 nM Aβ standards develop an intense signal while eluant collected from solution containing 100 nM Aβ spun through BioGel-P6 columns show no detectable signal even after overexposure of the blot to ECL film, showing complete binding of Aβ by the column. Molecular weight markers are shown on the left of the figure (Markers). Single asterisk indicates monomeric Aβ at ~4 kDa; a double asterisk indicates Aβ dimers at ~8 kDa. Aβ was detected using the W0-2 monoclonal antibody. (e) Quantification of direct transport assay results. Average O.D. values are normalized to their respective controls (dashed line, $*p<0.05$ and $**p<0.01$). MEMB represents membrane-bound Aβ, INTRA represents Aβ in the vesicle interior, and n.d. represents non-detectable Aβ signal.

DISCUSSION

The events involved in the production of Aβ are increasingly being understood. The process begins with cleavage of APP by the recently identified enzyme β-secretase (Vasser *et al.* 1999; Lin *et al.* 2000), yielding an extracellular fragment known as $APP_{S\beta}$ which is simply shed into the extracellular space (Mills & Reiner, 1999). The remaining 99 amino acid COOH-terminal fragment (C99) consists of 28 charged amino acids on the extracellular side of the membrane, 23 hydrophobic amino acids which presumably traverse the membrane as an α-helix, and 52 charged amino acids constituting the intracellular domain of the polypeptide. The Aβ peptide is produced following cleavage of C99 *within* the membrane (Brown *et al.* 2000) by an enzyme known as γ-secretase [which appears to be identical to the presenilins (Wolfe *et al.* 1999; Lin et al., 2000)]. The resulting 40 and 42 amino acid versions of Aβ are amphipathic, consisting of 28 charged amino acids and either 12 or 14 hydrophobic amino acids (for $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, respectively). The hydrophobic nature of Aβ is consistent with data indicating that the peptide has limited solubility in aqueous solutions (Terzi *et al.* 1995) with a preference for electrostatic binding to the membrane bilayer (Terzi *et al.* 1997). These observations suggest that constitutive release of Aβ from cells may be an active process, and the data in this chapter demonstrating that Aβ secretion can occur through p-gp leads to the hypothesis that other ABC transporters can act as Aβ efflux pumps.

How might an ABC transporter such as p-gp act as an Aβ efflux pump? One model is based upon the so-called vacuum-cleaner hypothesis (Gottesman & Pastan,

1993), in which the ABC transporter draws the Aβ peptide laterally from within the membrane and moves it from the energetically favorable environment of the lipid bilayer into the aqueous environment of the extracellular space. A related model involves the transporter acting as a flippase (Higgins & Gottesman, 1992), either moving the peptide from the inner to the outer leaflet of the membrane or locally altering membrane lipid composition such that the peptide detaches. These observations are not only relevant to the molecular basis of Aβ secretion, they may also be applicable to the mechanism by which amphipathic peptides, proteins lacking signal sequences, or lipid-modified proteins detach from biological membranes (Ambudkar *et al.* 1999; Kuchler *et al.* 1992; Yakushi *et al.* 2000).

This chapter's data provide the first description of a regulated process by which the Aβ peptide is released from membranes. Two aspects of these findings are of relevance to Alzheimer's disease. The first is that Aβ is unlikely to aggregate while attached to the membrane, as the hydrophobic amino acids in the peptide's COOH tail would be shielded by their association with the lipid bilayer. Thus, detachment of Aβ from the membrane represents a critical change in the biophysical properties of Aβ, and is likely to be a prerequisite to the aggregation events which are thought to be at the core of the pathology. The second observation of note is that p-gp is expressed at high levels at the lumenal surface of cerebrovascular endothelial cells (Cordon-Cardo *et al.* 1989), and perhaps at the end-feet processes of astrocytes (Pardridge *et al.* 1997). Thus, changes in p-gp function and/or expression might alter the clearance of Aβ from within the brain, and may even contribute to cerebrovascular amyloid angiopathy. Of greater importance

to the development of Alzheimer therapeutics is the observation that cells throughout the body constitutively produce and release Aβ, yet p-gp is only expressed in a limited number of tissues, and is essentially undetectable in neurons (Fojo *et al.*, 1987). Given the substrate promiscuity between members of the ABC transporter superfamily (Ford & Hait, 1990), it is likely that other brain-expressed ABC transporters are capable of sustaining Aβ efflux. Identifying such neuronal Aβ efflux pumps may open new avenues for ameliorating the Aβ burden in the Alzheimer brain.

IV. Quantifying Expression of Brain-expressed ABC Transporters in the Alzheimer Brain

INTRODUCTION

Efforts to clone the human genome and advances in molecular biology have led to exponential databasing of human genomic sequences. With respect to human ABC transporters, 48 have been cloned to date (Dean et al., 2001). Several of these are associated with diseases including cystic fibrosis, Zellweger's syndrome, Dubin-Johnson's syndrome, and X-linked adrenoleukodystrophy (Dassa and Bouige, 2001). As more is known regarding the function of these transporters, it seems prophetic but reasonable to postulate that this superfamily plays a central role in membrane-associated transport.

Of particular interest to this thesis is the profile of brain-expressed ABC transporters. The data in previous chapters thus far have implicated an ABC transporter in Aβ secretion. As Aβ is largely secreted from neurons in the brain, the obvious question to ask is whether or not a neuronal Aβ ABC transporter exists. There is evidence showing expression of p-gp in blood-brain barrier endothelial cells as well as the end-feet of astrocytes (Pardridge et al., 1997), but not in neurons (Fojo et al., 1987). The task, therefore, is to identify a brain Aβ ABC transporter.

This final chapter describes the methods and approaches used in the quantitative-competitive RT-PCR (QCRT-PCR) approach of screening changes in gene expression of

ABC transporters in both control and AD brains. Two methods are outlined: the traditional agarose gel-based QCRT-PCR protocol, and a capillary electrophoretic method using the Agilent 2100 Bioanalyzer (Hewlett Packard). The data presented in this chapter represent work in progress. QCRT-PCR results presented here were obtained for the ABC transporter MRP5 (ABCC5), an ABC transporter that was first characterized in a human lung carcinoma cell line which shows strong brain expression, and is also expressed at the blood-brain barrier (Suzuki et al., 1997; Zhang et al., 2000a). Preliminary data for four other ABC transporters are also shown using the Agilent technology, demonstrating its sensitivity and efficiency over the traditional methods.

MATERIALS AND METHODS

RNA Extraction

Alzheimer and age-matched control frontal cortex tissues were obtained from Dr. Steven Kish (University of Toronto) and stored at –80°C until ready for use. Total RNA was extracted from the tissues using the Qiagen RNeasy RNA extraction kit according to the manufacturer's protocol. RNA was eluted in RNase-free water and stored at –80°C.

Subcloning of ABC transporters

RNA competitors were synthesized to the following brain-expressed ABC transporters: ABC2 (ABCA2; Vulevic et al., 2001), BCRP (ABCG2; Maliepaard et al.2001), PMP69 (ABCD4; Holzinger et al., 1998), MDR1 (ABCB1), and MRP5 (ABCC5; Suzuki et al., 1997). For ease of discussion, the HUGO nomenclature will be used throughout when referring to the transporters. Brain expression was initially determined by performing gene-specific RT-PCR on total brain RNA (Invitrogen). PCR primers were designed to the different ABC transporters using MacVector6.0 software and synthesized by Geneset Inc. Gene-specific RT-PCR was then performed using the Access RT-PCR System (Promega) to test for purity of PCR products. To obtain PCR products, total brain RNA (Invitrogen) was used as a template. Single band PCR products of expected sizes were resolved onto agarose gels, excised, and reamplified. The reamplified product was gel extracted, subcloned into the pGEM-T Easy TA cloning plasmid (Invitrogen), and transformed into JM109 E.coli bacteria. Transformants were plated onto AIX (ampicillin, IPTG, X-gal) plates for blue/white colony selection.

Positives were picked and grown up in 5 mL of LB for plasmid extraction using the Qiagen Mini-prep kit. EcoRI was used to excise the cloned PCR product to confirm positive cloning. Plasmids with inserts were then sequenced to confirm identity of the cloned fragment using a Leicor sequencer.

Synthesis of Competitor Constructs

To create the internal competitor for competitive RT-PCR, methods were utilized so that the resulting competitor sequence either lacked an internal deletion of several base pairs or contained an additional 80 mer oligonucleotide fragment with the sequence 5'-ATGACCATGATTACGCCAAGATGACCATGATTACGCCAAGATGACCATGA TTACGCCAAGATGACCATGATTACGCCAAG-3'. Deletions were created by excising a fragment ranging from 70-90 nucleotides within the cloned sequence and religating the plasmid. Insertion constructs containing the synthetic oligonucleotide sequence were made by linearization of the plasmid followed by ligation of the insert. All final constructs were sequenced to confirm correct subcloning.

Synthesis of the RNA Competitor Templates

RNA transcripts of the wild type and competitor sequences were prepared by linearizing the plasmid followed by *in vitro* transcription with either SP6 or T7 RNA polymerases as detailed in the Invitrogen Riboprobe *in vitro* transcription kit. Unwanted DNA template was degraded using DNase I. The linearized RNA transcripts were extracted using the RNeasy RNA extraction kit from Qiagen. Transcripts were visualized

on urea formamide agarose gels to assure purity. Stocks were stored at –80°C in RNase-free water.

Quantitative Competitive RT-PCR

All reactions were performed using the one-step Access RT-PCR system (Promega). Five nanograms of total frontal cortex RNA extracted from the brains of normal control age-matched or AD frontal cortices were used in each RT-PCR reaction. A reaction that did not contain reverse transcriptase was included in each run to control for contamination of DNA in the sample, which would be detectable in the PCR reaction. 0, 1000, 5000, 10,000, 50,000, 100,000, and 500, 000 copies of competitor RNA were added to sequential 50 μL reactions. RT-PCR was performed using the following protocol:

First Strand cDNA Synthesis

- 48°C for 45 minutes — reverse transcription
- 94°C for 2 minutes — RT inactivation and denaturation

Second Strand cDNA Synthesis and PCR Amplification

- 40 cycles — 94°C for 30 seconds — denaturation
  60°C for 1 minute — annealing
  68°C for 1 minute — extension
- 1 cycle — 68°C for 7 minutes — final extension
- 1 cycle — 4°C — soak

30 μL of each reaction was resolved onto a 1% Metaphor agarose gel and then imaged.

Quantification of Bands

Densitometric measurements of ethidium bromide-stained agarose gels were performed using the Eagle Eye software (Stratagene). Log of the ratios of competitor to target densities were plotted versus log of copy number of competitor. Linear regression analysis was performed to calculate the equivalence point at which the ratio of target to competitor was equal to 1, indicating the amount of target transcript that was in the sample.

Statistical Analysis of QCRT-PCR Data

ABCC5 copy numbers in each reaction were obtained from each trial through linear regression analysis of the data. Values for the averages ± standard error of the means were calculated for the three normal and three Alzheimer brain samples. To determine significance between normal and AD brain, an unpaired t-test was employed.

Using the Agilent 2100 Bioanalyzer for QCRT-PCR

Hewlett Packard recently developed an automated capillary agarose gel electrophoresis system, the Agilent 2100 Bioanalyzer, for quantitating minute amounts of PCR product. This machine uses spectrophotometry to resolve and measure the amount of DNA in as little as 1 μL from a PCR reaction. RT-PCT reactions were set-up as described above. One microlitre from each reaction was loaded onto the agarose gel microchip and the PCR products were then sized, quantitated, and visualized on the computer using their software. Purity of bands were analyzed using spectrophometry.

The Agilent 2100 Bioanalyzer was used to characterize QC-RTPCR for the following ABC transporters: ABCA2, ABCG2, ABCD4, ABCB1.

RESULTS

MRP5 (ABCC5) Expression is Increased in Alzheimer Frontal Contex Compared to Age-matched Control using Quantitative competitive RT-PCR

QCRT-PCR of three normal age-matched frontal cortex and three AD samples revealed that there was an increase in gene expression of ABCC5 in the AD brain (Figure 17). Table 1 lists the specifics of the brains as documented by Dr. Steven Kish's brain bank, age of the donor, and time of harvest post mortem.

Table 1.

Sample	Brain Bank #	Age (yrs.)	Post mortem Time of Harvest (hrs.)
Control 1	975	77	12
Control 2	1050	75	12
Control 3	1028	78	10
Alzheimer 1	725	77	8.5
Alzheimer 2	695	77	14
Alzheimer 3	255	78	21

Results show that control normal frontal cortex contained 32,817 ± 5,877 copies of ABCC5 transcript per 5ng total RNA. Alzheimer frontal cortex contained 109,786 ± 17,907 copies of ABCC5 transcript per 5ng total RNA, approximately 3 fold higher expression levels of ABCC5 RNA ($p = 0.0150$). Representative agarose gels of the RT-PCR reactions showing the target transcript (368 bp; top bands) versus increasing copy numbers of competitor RNA transcripts (312 bp; bottom bands) and linear regression analysis of results are shown for Control (Figure 16) and Alzheimer (Figure 17) samples.

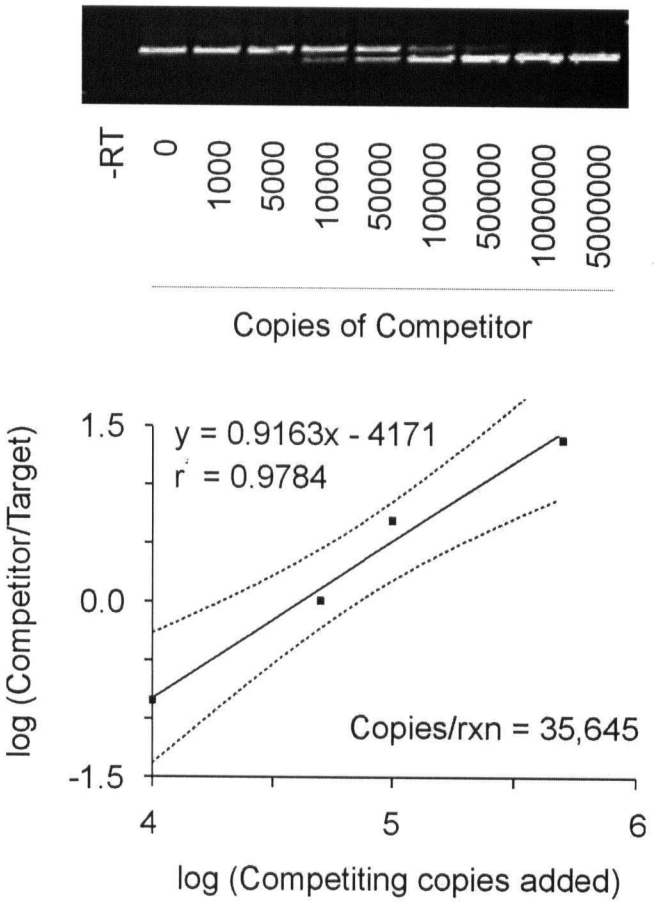

Figure 16. Quantitative competitive RT-PCR of ABCC5 (MRP5) RNA in normal aged frontal cortex. Representative agarose image of electrophoretically resolved, ethidium bromide-stained RT-PCR products derived from wild-type ABCC5 (upper band, 368 bp) and known amounts of competitor template (lower band, 312 bp). Each lane represents an RT-PCR reaction using 5 ng total frontal cortex RNA and a known copy number of competitor (amounts are labeled below the appropriate lanes). The lane labeled –RT is a negative control reaction that did not contain reverse transcriptase to ensure that samples were not contaminated with DNA. Representative logarithmic graph of log (competitor/target) vs. log (copy numbers of competitor) with linear regression analysis.

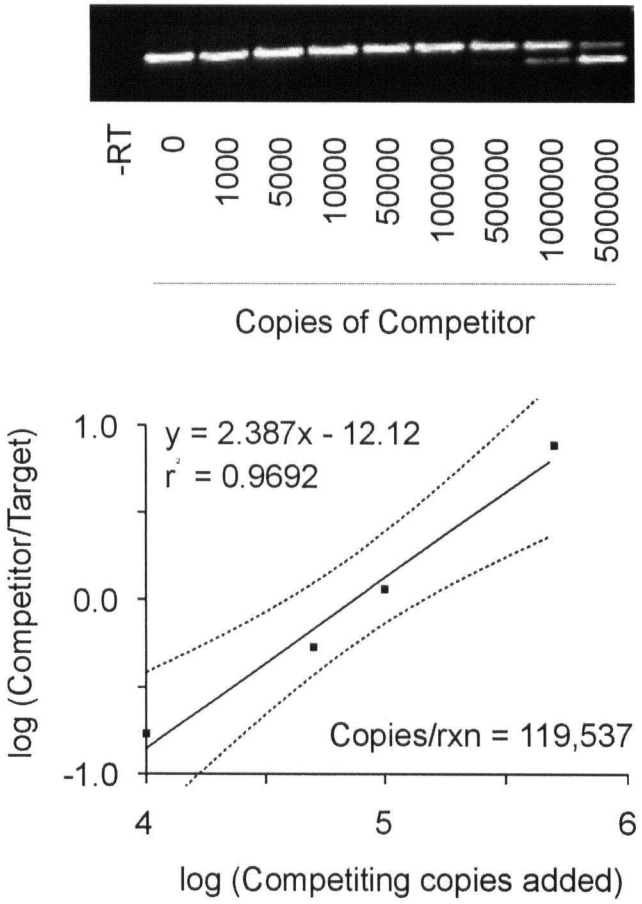

Figure 17. Quantitative competitive RT-PCR of ABCC5 (MRP5) RNA in Alzheimer frontal cortex. Representative agarose image of electrophoretically resolved, ethidium bromide-stained RT-PCR products derived from wild-type ABCC5 (upper band, 368 bp) and known amounts of competitor template (lower band, 312 bp). Each lane represents an RT-PCR reaction using 5 ng total frontal cortex RNA and a known copy number of competitor (amounts are labeled below the appropriate lanes). The lane labeled –RT is a negative control reaction that did not contain reverse transcriptase to ensure that samples were not contaminated with DNA. Representative logarithmic graph of log (competitor/target) vs. log (copy numbers of competitor) with linear regression analysis.

Agilent 2100 Bioanalyzer is sensitive at detecting RT-PCR products of ABC Transporters

Initial analysis of RT-PCR products using the Agilent Bioanalyzer demonstrated that it is very sensitive at detecting RT-PCR products (Figure 18). Capillary electrophoresis of 1 μL of each 50 μL RT-PCR reaction showed a single clean band of expected size for both wild-type and competitor templates of each ABC transporter. Spectrophotometric analysis also showed that there was only one PCR product in each reaction, evident by the single detected peak in each sample, demonstrating the purity and fidelity of the PCR reaction. The outlying peaks are the standard markers that are included in each run of the microchip. Preliminary results show that this equipment is sensitive, requiring only 1 μL of each reaction for visualization of bands. This equipment is also efficient, obtaining qualititative and quantitative data within 30 minutes.

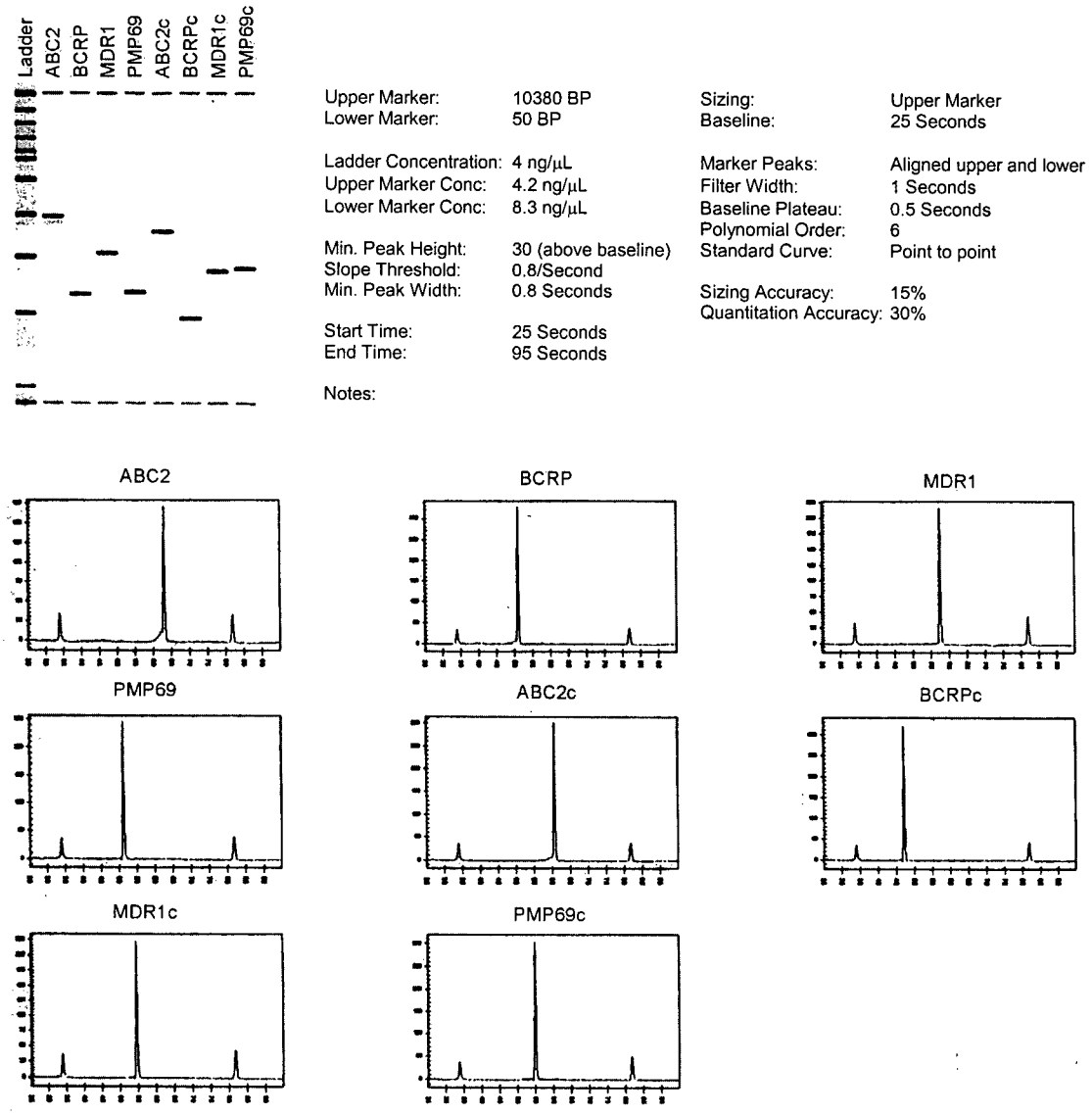

Figure 18. RT-PCR analysis of wild-type and competitor transcripts of ABC2 (ABCA2), BCRP (ABCG2), PMP69 (ABCD4), and MDR1 (ABCB1) using the Agilent 2100 Bioanalyzer. Capillary electrophoresis of 1 µL of each 50 µL RT-PCR reaction using 5 ng of total RNA from Alzheimer frontal cortex (Invitrogen) shows a single band of expected size for both wild-type and competitor templates. Extreme top and bottom bands are standard markers. Lanes of wild-type templates are labeled according to the ABC transporter of interest. Lanes of competitor templates are labeled with a subscript 'c' following the name of the transporter. Graphs are fluorescence measurements of RT-PCR samples plotted against time (seconds) per electrophoresis. A single peak for each sample indicates the presence of a single PCR product. Outlying peaks are due to the presence of the standard markers.

DISCUSSION

QCRT-PCR was developed over a decade ago, capitalizing on the sensitivity of the PCR reaction to amplify minute amounts of sample DNA or RNA (Gilliland et al., 1990; Becker-André et al., 1989; Piatak et al., 1993). This allowed for efficient usage of precious samples, unlike the conventional methods of northern and southern blotting which usually require micrograms of DNA or RNA. Unlike competitive PCR, QCRT-PCR begins quantitation right from the onset of first strand cDNA synthesis during the reverse transcriptase (RT) step by utilizing known amounts of an *RNA* competitor transcript. This method allows for control of any discrepancies in the synthesis of cDNA in the RT reaction (Tsai and Wiltbank, 1996). In contrast, competitive PCR introduces a *cDNA* competitor in the subsequent PCR step. While reliable, this latter method does not control for infidelities or errors in the RT reaction, which may greatly affect product amplification during the PCR step (Zimmermann and Mannhalter, 1996). This discrepancy is alleviated with use of an RNA competitor (Souazé et al., 1996; Auboeuf and Vidal, 1997).

The one-step Access RT-PCR system (Promega) was used to eliminate unnecessary steps between the RT and PCR reactions that would otherwise occur in conventional two-step methods of first performing RT on RNA and then adding DNA polymerase and primer pairs to the reaction for PCR. Another advantage to the Access RT-PCR system is that it utilizes a gene specific approach whereby one primer pair is used throughout both the RT and PCR reactions to detect and amplify only the gene of interest. This is superior to other RT protocols that utilize random hexamer primers or

oligo-dT to create a cDNA pool because this gene specific approach eliminates the random uncontrolled nature of the RT reaction of the former two methods. In a given trial of QCRT-PCR, a fixed amount of sample RNA of interest (the target) is titrated with increasing increments of a known amount of internal competitor RNA. This competitor sequence lacks an internal deletion of several base pairs, allowing for differentiation from the wild-type product on the agarose gel. As well, this method is quantitative because the same primer pair is used to amplify both the gene of interest and the internal competitor, thus eliminating discrepancies between efficiency of annealing and extension of different PCR primers, as is the case when a heterogeneous house-keeping gene (i.e. GAPDH) is used as a marker in semi-quantitative RT-PCR.

QCRT-PCR showed a significant three-fold increase in the number of ABCC5 transcripts in Alzheimer brain compared to normal age-matched controls. ABCC5 is expressed at the blood-brain barrier (BBB; Zhang et al., 2000a), confers resistance to nucleoside analogues and may be involved in cGMP secretion (Jedlitschky et al., 2000; Wijnholds et al., 2000). Studies on aging and its effects on the BBB show that levels of many transport proteins are decreased with aging, including the hexose, amine, and monocarboxylic acid transport systems; however, nothing is known regarding the nucleoside transport system with age (Mooradian, 1994). To the best of my knowledge, this is the first report of an increase in the expression of a putative nucleoside transporter in the AD brain.

One hypothesis for this increase may be due to induction of expression by an increased production of a putative substrate such as cGMP. Cyclic GMP can be produced by several mechanisms, one of which is through upregulation of guanylyl cyclase by nitric oxide (NO, reviewed by Law et al., 2001). NO is synthesized by nitric oxide synthase (NOS), through conversion of L-arginine. There are three isoforms of NOS, two that are constitutively expressed in the brain and endothelial cells, termed nNOS and eNOS, respectively (Bredt and Snyder, 1990; Marsden et al., 1992), and one that is expressed in macrophages only after induction by endotoxins and cytokines, termed iNOS (Stuehr et al., 1991; Xie et al, 1992). It is now known that both nNOS and eNOS are found in other tissues and that constitutive NOS can become inducible and vice versa (Law et al., 2001).

NOS activity and expression can be regulated by several mechanisms. With relevance to AD and neurotoxicity, these include increased glutamate release (Noda et al., 1999), increased Aβ production (Hurst and Barrette, 1989), activation of microglia (Boje and Aurora, 1992), and disruption of Ca^{2+} homeostasis (Schmidt et al., 1992). Evidence has shown that increased NO production is damaging to cells via different mechanisms, including radical formation (Eliasson et al., 1999), production of reactive oxygen species (Beckman et al., 1994), DNA damage leading to cellular ATP depletion (Ha et al., 1999), and mitochondrial dysfunction (Dawson et al., 1992; Gros et al., 1995). In AD, it has been shown that Aβ enhances glutamate excitotoxicity through NO (Noda et al., 1999, Le et al., 1995). Activation of microglia and production of inflammatory mediatiors such as TNF-α also induce NO synthesis (Colton et al., 1994). While NO can be neurotoxic, it may also be neuroprotective, particularly in endothelial cells, to inhibit

platelet aggregation, block leukocyte adhesion, and inhibit Ca^{2+} influx through N-methyl-D-aspartate receptors (Choi, 1993), although no direct evidence of this protection has been shown in AD. Rather, studies have shown an overexpression of both eNOS and iNOS in AD microvasculature (Dorheim et al., 1994), and their overexpression in brain microvessels cultured from AD brain have been shown to cause neurotoxicity *in vitro* (Grammas et al., 1999, Grammas 2000). ABCC5 has been shown to be expressed in endothelial cells (Zhang et al., 2000a). I thus hypothesize that the increased production of NO in brain endothelium leads to increased cGMP production that induces increased expression of ABCC5 in AD brain. Increased ABCC5 expression due to increased substrate availability are in line with the nature of other ABC transporters such as p-gp and MRP1. Increased eNOS in AD microvessels may also lead to increased cGMP production. Taken together, this may be a reasonable hypothesis to explain my findings.

Preliminary data using the Agilent Bioanalyzer demonstrates that this piece of equipment is sensitive and efficient in analyzing RT-PCR results (Figure 18). The advantages of this system over the conventional resolution of bands on an agarose gel are many-fold. First, the Bioanalyzer technique uses a fluorescent DNA labeling system to visualize PCR products versus ethidium bromide, which is carcinogenic and requires visualization under ultraviolet light, which is also harmful. Second, only 1 μL from the PCR reaction is required to visualize products, which leaves plenty of reaction for use in other applications such as subcloning of the PCR product. Third, analysis is rapid; each run takes only 30 minutes from loading of samples onto the microchip to quantitation of product in each sample. Fourth, loading the microchip is less cumbersome than loading

samples onto an agarose gel, where loss of sample can sometimes occur during loading which may skew the results.

The flipside is that this piece of equipment has never been used for QCRT-PCR (personal communication, technical support representative, Agilent Technologies). Initial trials of QCRT-PCR have yielded spurious results using the Bioanalyzer where, for example, extra bands will appear in some lanes or either the competitor or wild-type band will disappear (results not shown). When the remainder of the PCR reactions were resolved using conventional agarose gels, the spurious bands were not present and both the expected wild-type and competitor bands were present (results not shown). These are the hurdles that need to be overcome before the Agilent Bioanalyzer can be considered reliable for QCRT-PCR. Nonetheless, the sensitivity and efficiency of this machine are worthy of further troubleshooting.

In terms of current technology available for screening the genome, the use of microarray chips are becoming increasingly popular in the analysis of levels of gene expression in tissues. DNA molecules are bound to a surface at specific locations and labeled RNA or DNA probes are then hybridized to the template. Levels of the DNA of interest are then quantified. The ability to deposit nucleic acids at high densities onto these 'gene chips' can allow for plating of 250,000 different oligonucleotide probes or 10,000 different cDNAs per square centimeter, allowing for large-scale data acquisition in a short period of time (Lipshutz et al., 1999, Bowtell, 1999; Lockhart and Winzeler, 2000). Microarrays can detect 3 to 10 copies of mRNA per cell in cell lines and simple

tissues, and is thus highly sensitive (Schena et al., 1996; Borwn and Botstein, 1999; Lockhart and Winzeler, 2000). In terms of the CNS, one problem that can arise is that the heterogeneity of the brain can lead to dilution of cell-type-specific species by the presence of multiple cell types (Geschwind, 2000; Luo and Geschwind, 2001); thus, if the goal is to assess cell-specific changes in expression, a technique such as single-cell PCR may be more desirable (Lockhart and Winzeler, 2000).

In summary, the data in this chapter represent preliminary characterization of what will ultimately become a screening profile for brain-expressed ABC transporters in control and AD brain. This is a direct reflection of the present state of science in the age of genomics and proteomics, where the traditional approach of small-scale experiments resulting in characterization of a molecular sequence of known function or relevance has become replaced with large-scale cloning and gene sequencing, followed then by computational translation to determine a function, as aptly put by Cobb and colleagues (2001). Indeed, it is hopeful that finding genes that have regulated levels of expression in the AD brain will allow for further understanding of the molecular mechanisms involved in this disease.

V. General Discussion

Regulation of APP Processing at the Cell Membrane

A summary of the data in the previous chapters demonstrates that inhibition of p-gp leads to increased APP_S secretion and decreased Aβ release (Chapters II and III). This effect is both rapid (manifest after 15 minutes of drug treatment) and potent (nanomolar amounts of inhibitors decrease Aβ release). To postulate on how p-gp alters APP processing, it is necessary to first review the current understanding of cellular APP trafficking.

After its translation, APP is inserted into the ER and shuttled to the Golgi where it is post-translationally modified, then inserted into the plasma membrane. Membrane APP is then constitutively internalized via clathrin-coated vesicles and recycled through the endosomal/lysosomal pathway (Nordstedt et al., 1993; Koo and Squazzo, 1994). Cleavage of APP by the various secretases appears to be compartment-specific. The majority of APP is cleaved by α-secretase (Figure 1) and evidence demonstrates that this most likely occurs at the plasma membrane or in a late secretory pathway (Sisodia, 1992). It has been known for some time that α-secretase activity can be upregulated by PKC (Mills and Reiner, 1999). Findings demonstrating that TACE and ADAM-10 are candidate α-secretases have allowed for further characterization of this cleavage event (Buxbaum et al., 1998; Lammich et al., 1999). PKC-regulated α-secretase cleavage has been shown to be, in part, due to the activity of TACE (Buxbaum et al., 1998) and this activity can be localized to a trans-golgi compartment (Skovronsky et al., 2000), while

unregulated constitutive α-secretase cleavage of APP may be due to membrane-surface cleavage by ADAM-10 (Lammich et al., 1999). Amyloidogenic cleavage of APP by β-secretase (Figure 1) has also been shown to occur in a late secretory compartment (Gandy, 2000). Skovronsky and colleagues (2000) have postulated that the ability of PKC to increase $APP_{S\alpha}$ secretion while decreasing Aβ secretion may be due to competition of α- and β-secretases for APP in the trans golgi, with increased α-secretase cleavage.

Much debate continues regarding the compartment(s) in which Aβ is produced, with even more controversy surrounding the locations of the formation of $A\beta_{1-40}$ and $A\beta_{1-42}$. Several studies suggest that the ER is the major compartment for generation of $A\beta_{1-42}$ (Hartmann et al., 1997; Wild-Bode et al., 1997; Cook et al., 1997; Skovronsky et al., 1998; Greenfield et al., 1999); however one study has shown that retention of C100 in the ER prevents generation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ (Maltese et al., 2001). Pulse-chase experiments have also shown that the majority of C100 fragments are generated from fully glycosylated molecules of APP (Haass et al., 1995; Weidemann et al., 1989) and that Aβ formation is dependent on endocytosis of APP (Perez et al., 1999). This argues for a membrane localized site rather than an early secretory pathway compartment of Aβ production. $A\beta_{1-40/x-40}$ peptides have been shown to be formed exclusively in the trans-golgi where they are packaged for secretion (Greenfield et al., 1999). In the same study, it was shown that a fraction of $A\beta_{x-42}$ remains in an insoluble state in the ER while $A\beta_{1-42/x-42}$ peptides formed in the trans-golgi are packaged for secretion. This is in line with the notion that two separate intracellular pools of Aβ peptides exist: those which are

formed in the early secretory pathway and are not secreted, and a separate population that is formed in the later secretory pathways which constitutes the secreted fraction. Isolation of detergent-insoluble intracellular pools of Aβ peptides from cells has further strengthened this observation (Lee et al., 1998; Morishima-Kawashima and Ihara, 1998; Turner et al., 1996; Skovronsky et al., 1998).

Data presented in chapter III suggest that p-gp is an Aβ transporter. Inhibition of p-gp leads to decreased Aβ secretion and increased APP_S secretion. This can be hypothesized to occur via a two-fold mechanism. First, decreased Aβ secretion is due to inhibition of its transporter. This goes on the premise that the lipophilicity of the Aβ sequence favours its retention in the plasma membrane before release into the extracellular milieu. Real-time insertion of Aβ monomers into brain lipid extracts has been shown by Yip and McLaurin (2001), demonstrating the preference of Aβ for a lipid environment. Second, inhibiting p-gp may lead to an accumulation of Aβ. It has been shown that prolonged incubation of APP-transfected cells with $Aβ_{1-42}$ leads to intracellular accumulation of C-terminal APP fragments without significant alteration of $APP_{Sα}$ secretion (Yang et al., 1995, 1999). $Aβ_{1-42}$ also has a tendency to become rapidly internalized into lysosomes where it becomes resistant to degradation (Burdick et al., 1997; Ida et al., 1996) and may act to alter normal trafficking and protein interactions (Hartmann, 1999). I hypothesize that inhibition of p-gp leads to intracellular accumulation of Aβ which in turn inhibits membrane β- or γ-secretase activity via enzyme product inhibition. This leads to an increased availability of membrane APP for cleavage by α-secretase, leading to increases in $APP_{Sα}$ secretion. As substrate-enzyme

kinetics studies have yet to be done on the candidate secretases, it will be of interest to see whether this hypothesis rings true.

P-glycoprotein inhibitors as a treatment of amyloidosis

Evidence in chapter III shows that Aβ peptides are *bona fide* p-gp substrates and that Aβ transport across membranes is p-gp-dependent. As such, inhibition of p-gp may represent an avenue of treatment against accumulation of extracellular Aβ. But is this necessarily wise? Whilst there is consensus in the field that preventing extracellular accumulation of Aβ ameliorates its neurotoxic effects, there is also increasing evidence that intracellular accumulation of Aβ may be equally damaging. Inhibiting p-gp may lead to increased accumulation of intracellular Aβ. Studies now show that intraneuronal accumulations of Aβ precede development of amyloid plaques in Down syndrome and in APP and PS-1 double transgenics (Gyure et al., 2001; Wirths et al., 2001), suggesting that this may be an early trigger for neurodegeneration. Accumulated $A\beta_{1-42}$ in endosomes is stable and resistant to degradation (Lee et al., 1998; Morishima-Kawashima and Ihara, 1998; Tuner et al., 1996; Skovronsky et al., 1998), and has been shown to perturb the integrity of lysosome membranes, leading to leakage of ROS and neuronal death (Ditaranto et al., 2001). Oxidative stress in turn induces further accumulation of intracellular Aβ and thus forms a vicious cycle of cytotoxicity (Misonou et al., 2000; Paola et al., 2000). As such, which is the lesser of two evils: preventing extracellular Aβ deposition or preventing intracellular accumulation of Aβ? If the ultimate goal of anti-amyloid therapy is total exacerbation of the ill-effects of Aβ, then both evils should be dealt with with equal ferocity.

It is perhaps fitting at this time to discuss p-gp and its function at the BBB. P-gp is an integral part of the BBB, being expressed luminally on the capillary endothelium (Cordon-Cardo et al., 1989; Thiebaut et al., 1987), although one report has shown conflicting evidence of expression on the end-feet of astrocytes (Pardridge et al, 1997). The role of p-gp in keeping toxins out of the central nervous system was revealed in mdr1a-/- knock-out mice that lacked the murine ortholog of p-gp. These mice demonstrated increased susceptibility to the neurotoxic effects of the antihelminthic invermectin (Schinkel et al., 1994). Several studies show that the BBB is a site of bi-directional Aβ transport (Zlokovic et al., 1993; Saitoh et al., 1996; Martel et al., 1996; Poduslo et al., 1997, 1999; Strazielle et al., 2000), and that this transport is receptor-mediated (Zlokovic et al., 1996; Poduslo et al., 1999; Shibata et al., 2000). Furthermore, $A\beta_{1-42}$ in particular accumulates in the brain microvessels, meningeal vessels, and choroid plexus in AD, is believed to affect the integrity of the BBB, and promotes cerebral amyloid angiopathy (Roher et al., 1993; Kalaria et al., 1996; Mackic et al, 1998). P-gp may have a physiological role in clearance of Aβ from the CSF, brain, or endothelial cells, thus preventing unwanted accumulation of Aβ. This may play an interesting part in the recent findings that inocculation of mice with synthetic Aβ peptides and Aβ monoclonal antibodies decreases plaque pathology in Alzheimer mouse models (Morgan et al., 2000; Janus et al., 2000; Weiner et al., 2000; DeMattos et al., 2001; Bard et al., 2000). DeMattos and colleagues (2001) found that Aβ antibodies injected intravenously drastically increased plasma levels of Aβ, and proposed that the peripheral antibodies acted as an 'Aβ sink' to clear CNS Aβ through the BBB. If p-gp is acting to pump Aβ out into the circulation as a means of clearance, inhibiting BBB p-gp may

unwittingly abolish this mechanism. In the aging and demented brain, physiological changes occur at the BBB, leading to changes in levels of various proteins and transporters, including APP (Mooradia, 1994; Premkumar and Kalaria, 1996). It would be of interest to know whether or not levels of p-gp are increased in AD brain microvessels as a protective response against increased Aβ insult, or decreased as a result of degenerative mechanisms, contributing to development of cerebral vascular amyloid angiopathy, breakdown of the BBB, and decreased Aβ clearance, thus exacerbating the effects of amyloid in the brain.

The frontiers of Alzheimer Disease therapy

Do p-gp inhibitors have a place in the treatment of AD? In light of the mass demand for a preventative if not curative treatment for AD and neurodegeneration in general, several avenues of potential therapies have evolved, many of which focus on eradicating the ill effects of Aβ. Modulation of an Aβ transporter should, in theory, add to the beneficial effects of ameliorating extracellular Aβ accumulation; however, as discussed above, there appears now to exist a fine balance between the detriments of keeping Aβ within the cell versus allowing its natural release. The question, therefore, is this: Is the aging brain more effective at dealing with extracellular versus intracellular Aβ accumulation?

Extracellular Aβ is cleared via several receptor-mediated mechanisms, including forming complexes with ApoE and J (Cole and Ard, 2000), as well as uptake via scavenger receptors (Husemann et al., 2001). The observations that insulin-degrading

enzyme (Qiu et al., 1998), endothelin-converting enzyme (Eckman et al., 2001), and neprilysin (Iwata et al., 2001), are endogenous Aβ degrading enzymes also suggest that several mechanisms of protection reside in the CNS. The point has been raised that endothelin-converting enzyme inhibitors may be possible antihypertensives (Gray and Webb, 1996), but administration of these agents to people with AD may exacerbate extracellular Aβ accumulation. Preventing extracellular Aβ fibrillogenesis and aggregation are also viable avenues of therapy. Aβ congeners (Gordon et al., 2001), nitrophenol compounds (Felice et al., 2001), and β-sheet breaker peptides (Sigurdsson et al., 2000), have been found to inhibit assembly of Aβ fibrils as well as impart some neuroprotective effects *in vitro*. All of the above, along with the impressive plaque-abolishing effects of the amyloid vaccines and antibody treatments (Morgan et al., 2000; Janus et al., 2000; Weiner et al., 2000; DeMattos et al., 2001; Bard et al., 2000), in line with the finding that Aβ antisense oligonucleotides improve learning and memory in an aged mouse model (Banks et al., 2001), are attractive avenues of attacking extracellular amyloid deposition.

Interest has also been given to dealing with production of Aβ. With the identification of BACE enzymes as candidate β-secretases and the notion that presenilins may be γ-secretases, development of agents which inhibit these enzymes, thus effectively abolishing Aβ production, is of considerable interest. Even before the definitive identification of γ-secretase, transition-state peptide 'γ-secretase inhibitors' have already been synthesized (Petit et al., 2001; Shearman et al., 2000; Li et al., 2000; Seiffert et al., 2000) that show effective inhibition of γ-secretase activity in cells. BACE inhibitors are

also being developed (Marcinkeviciene et al., 2001) and are of considerable medical and commercial interest. The novel finding that common cholesterol-altering statin drugs, simvastatin and lovastatin, potently reduce both intra- and extracellular Aβ also opens yet another avenue of anti-amyloid therapy (Fassbender et al., 2001). However, there appears to be conflicting reports of their ability to decrease the prevalence of AD and dementia in the aging population who are already long-term users of these drugs (Wolozin et al., 2000; Libow et al., 2000; Lesser et al., 2001; Jick et al., 2000). Of interest is that statins have been found to be potent p-gp inhibitors, in particular simvastatin and lovastatin (Wang et al., 2001), raising the question of whether or not they are acting, in part, on a neuronal or brain-expressed p-gp-like Aβ ABC transporter to decrease extracellular Aβ levels.

While the above methods of Aβ modulation are still early in development, several existing AD therapies should not be dismissed. In particular, it should be recognized that to date, acetylcholinesterase inhibitors (AchEI) are still the only drugs approved by the Food and Drug Administration for the treatment of AD (reviewed by Giacobini, 2000). This therapy was a direct result of the 'cholinergic hypothesis' stating that a 'serious loss of cholinergic function in the CNS contributed significantly to the cognitive symptoms associated with AD and advanced age' (reviewed by Bartus, 2001; Bartus et al., 1982). Early studies demonstrated that cholinergic blockade could mimic some of the cognitive deficits of AD patients (Davis et al., 1981; Smith and Swash, 1978). This led to later studies demonstrating a reduction of brain acetylcholine activity due to loss of cholinergic neurons in the nucleus basalis and projection neurons to the hippocampus and

medial temperal region (reviewed by Guela, 1998). Based on the premise that inhibition of acetylcholinesterase, which degrades acetylcholine at the synaptic cleft, would salvage cholinergic neural transmission in these brain regions, several generations of this class of drugs have undergone rigorous clinical trials. Tacrine was the first AchEI to enter the market and, due to its high levels of hepatotoxicity, has fallen out of favour (Farlow et al., 1992; Knapp et al., 1994). Currently, Donepezil and rivastigmine (Exelon) have largely replaced the use of Tacrine. Donepezil is superior in its single dosing regimen and tolerability, however, it has not been found to be more effective than Tacrine (Rogers et al., 1998), although it has been shown to have long-term benefits (Doody et al., 2001). As well, AchEIs have several unwanted parasympathetic side-effects, as such, it appears that the preference of Exelon for AchE in the CNS may make it the preferred drug of choice in this category (Enz et al., 1991). Furthermore, trials using Exelon have shown the greatest cognitive improvement, however, its common cholinergic side-effects make for difficult prolonged therapy (Corey-Bloom et al., 1998). Yet another AchEI, galantamine, received approval in the United States in February, 2001. This agent has mixed functions, both as an AchEI as well as being a modulator of nicotinic receptors, and has been shown to improve cognition when administered during the early stages of the disease (Coyle and Kershaw, 2001). As such, AchEIs represent effective dementia treatment during early to mid stages of AD; however, they are ineffective at curbing disease progression and do not address the problem of neurodegeneration (Cutler and Sramek, 2000).

Studies demonstrating inflammatory processes in the AD brain have prompted studies using anti-inflammatories as a treatment for AD. To this end, data on the use of agents such as Ibuprofen, other non-steroidal anti-inflammatories, and steroidal anti-inflammatories have not been favourable (Rogers et al., 1993; Aisen et al., 2000; Scharf et al., 1999). However, basic science studies into the mechanisms of these drugs show beneficial effects in terms of suppressing plaque pathology and neuroinflammatory mediators in the brain, as well as down regulating amyloidogenic APP products (Lim et al., 2000; Asanuma et al., 2001; Lee and Wurtmann, 2000). Furthermore, selective COX-2 inhibitors (rofecoxib and celecoxib) may have a place in treating brain inflammation, as COX-2 is highly expressed in the AD brain (Ho et al., 1999). As such, while early trials with non-selective anti-inflammatories may have yielded poor results, future trials with more specific agents may prove fruitful.

Along the lines of anti-inflammatories are the class of anti-oxidants, consisting of agents such as vitamins C and E, and estrogen. There is evidence that oxidative stress occurs in AD as well as in other neurodegenerative diseases as well (Gilgun-Sherki et al., 2001). The effects of anti-oxidants prevent production of radical species which perturb cell membranes, leading to the vicious cycle of cell death and excitotoxicity. Vitamin E taken at 2000 IU/day has been shown to delay institutionalization and severe dementia (Sano et al., 1997). Similar results have also been obtained for vitamin C (Morris et al., 1998). Estrogen has been brought to the forefront in this class due in part to its documented anti-oxidant properties, as well as its neuroprotective and neurotrophic abilities (Lee and McEwen, 2001). In particular, a plethora of work showing protection

against Aβ toxicity and antiapoptotic properties has arisen (Kim et al., 2001; Hosoda et al., 2001; Gursoy et al., 2001; Honda et al., 2001; Zhang et al., 2001). With a large population of post-menopausal women on hormonal replacement therapy, this would be an ideal cohort to conduct long-term clinical trials on the efficacy of estrogens in AD (van Amelsvoort et al., 2001).

Where does this leave the current status of AD therapy? Like many diseases, there is a trend to have a 'flavour of the month'. Currently, emerging anti-amyloid therapies prove to be en vogue. However, it is eminently clear that AD is a multifactorial disease with several intertwining mechanisms occurring simultaneously. As such, the future of AD therapeutics will most likely involve combination therapy consisting of secretase inhibitors, anti-oxidants, anti-fibrillogenics, anti-inflammatories, possibly ABC transporter inhibitors, and so forth, to encompass a more complete picture of the disease. Is such regimen feasible? Remembering that the majority of the target population may already be on several medications to treat age-related disorders, the concept that many seniors will become chronic 'pill-poppers' is not favourable. Perhaps preventative treatment i.e. Aβ vaccination, in the young should also be instigated. Whilst the task is daunting, much progress has been made in finding a treatment for AD.

The ABC's of the Amyloid Cascade Hypothesis

This thesis poses several questions, yet unanswered, which may or may not be relevent to the understanding of the beta-amyloid cascade hypothesis. First, is there in fact a brain-expressed Aβ transporter? Second, does this transporter play a role in AD? If so, third, does modulation of this transporter's activity curb the effects of the beta-amyloid cascade? With the published data available to date, the most attractive candidate for a brain-expressed Aβ ABC transporter is ABCA2 (Kaminski et al., 2001) as it is also a full-transporter with the p-gp-like quality of imparting drug resistance (Dean et al., 2001). These are some of the questions that need to be answered in order to establish a strong link between ABC transporters and AD.

In the meantime, is the controversy surrounding the beta-amyloid cascade hypothesis close to resolution? Opponents of the hypothesis have argued that plaque formation does not correlate with dementia and neurodegeneration. It is becoming more apparent that the toxic effects of Aβ are not plaque-dependent. The presence of stable soluble oligomers of Aβ and intracellular accumulation of these species may be the early triggers of oxidative damage and neuronal death (Ditaranto et al, 2001). Accumulation of intraneuronal Aβ prior to plaque formation in mutant APP and PS-1 double transgenic mice suggests that the neuritic plaque may be the final product of a defeated neuron and not the cause of the death itself (Wirths et al., 2001). Furthermore, studies demonstrating the toxic properties of C-terminal fragments of APP suggest that the pathway leading to Aβ formation itself is detrimental. The hindrance of completely understanding the

pathogenesis of AD, in part, arises due to the lack of an appropriate animal model. Although transgenic mice bearing FAD mutations show evidence of plaques and mild cognitive deficits, they do not recapitulate other aspects of the disease (Selkoe, 2001). In particular, their lack of characteristic behavioural deficits despite having a large brain amyloid burden has been fuel for βaptist opponents (Joseph et al., 2001). Undoubtedly, the task of generating a replica of such a multifactorial disease as AD is daunting and the inadequacies of working in a non-human model of disease have been an ongoing debate. Nonetheless, much has been revealed from work using transgenic models regarding aberrant APP processing. Controversy is a good thing, as it spurns complacency. It will be interesting to follow the future directions of this field in terms of fully understanding how Aβ and other molecules lead to the development of AD.

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