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MURINE EMBRYONAL CARCINOMA (P19) CELLS AS A MODEL SYSTEM TO EXPLORE THE BIOLOGY OF THE AMYLOID PRECURSOR PROTEIN

by

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A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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This thesis is dedicated to my family, my parents Jack and Mary, my grandmother May Mack, my sister Jane and brother Paul. Each one has given me support and love I value beyond measure. I thank them for lending sympathetic ears and for never failing to shine a light at the end of my tunnel.

Abstract

In this thesis, the embryonal carcinoma (P19) cell line was stably transfected with the wild-type cDNA for the human amyloid precursor protein 751 isoform ($hAPP_{751}$) to study the consequences of overexpressing this protein on cell activities potentially relevant to the genesis of Alzheimer's disease. Genetic linkage studies have implicated APP as a key mediator of Alzheimer's disease-type neuropathology. A catabolic fragment of APP, the Aβ peptide, is the core protein of the amyloid plaque that is a defining lesion in Alzheimer's disease brain. All inherited risk factors alter the generation and/or aggregation of Aβ and this observation has focussed research efforts on elucidating the toxic properties of this peptide. However, the biology of APP is complex and our understanding of its role in the mature brain is very limited. In using our transfected clones, we found unexpected repression of APP expression when cells were grown as grafts in rat cortex, precluding analysis of how the adjacent brain might respond to chronically elevated levels of surface-expressed APP or of its secreted fragments. APP is highly expressed in neurons vulnerable to Alzheimer's disease neurodegeneration and evaluation of intracellular APP activities may help to determine the basis of their vulnerability. We developed a highly specific monoclonal antibody to a human Aβ epitope for use in high resolution electron microscopy of mature neuroectodermally differentiated P19 clones. We were able to localise APP fragments bearing this epitope and, in so doing, define sites of APP catabolism and reveal organelles spontaneously associated with these fragments. We approached the functional consequences of these intracellular associations by analysing 2 parameters of particular relevance to cell viability, namely oxidative metabolism and signal transduction. APP-transfected clones exhibit altered mitochondrial morphology and autophagy reminiscent of the changes induced, by others, using the oxidative phosphorylation inhibitor cysteamine. In these clones, the mitochondrial membrane potential was decreased by a mechanism distinct from complex I inhibition. In the early post-differentiation period, immunoblotting for the pleiotropic enzyme mitogen-activated protein kinase (MAPK) revealed steady-state hyperphosphorylation coincident with the upregulation of APP. In more mature cultures, MAPK phosphorylation in response to stimulation with acid fibroblast growth factor was attenuated in APP-expressing cells. Strong immunoreactivity was seen in a reticular distribution throughout the soma and in the nucleus in unstimulated cells, with only a moderate increase in intensity and nuclear translocation after growth factor treatment. Thus, our ultrastructure study places APP and its metabolites in a position to influence cellular activities, and we have demonstrated functional consequences to $hAPP_{751}$ overexpression within central nervous system-type cells. APP-associated dysfunction of key components of oxidative metabolism and signal-dependent kinase activity, respectively, find important parallels in the abnormalities described in Alzheimer's disease brain.

Résumé

Afin d'étudier les conséquences de la sur expression de l'isoforme 751 du précurseur de la protéine amyloïde humaine ($hAPP_{751}$) sur les activités cellulaires pouvant être en relation avec la maladie d'Alzheimer, une lignée cellulaire de type carcinome embryonnaire (P19) a été transfectée avec l'ADNc non muté de l'$hAPP_{751}$. Plusieurs études génétiques convergentes suggèrent que l'altération du métabolisme de l'APP, accompagnée du dépôt progressif de son fragment β amyloïde (Aβ) au niveau de plaques, est un événement capital dans la pathogénèse de la maladie d'Alzheimer. Tous les facteurs de risques héréditaires modifient la production et/ou l'agrégation de l'Aβ dirigeant alors les efforts de recherche sur la compréhension des propriétés toxiques de ce peptide. Cependant, la biologie de l'APP est complexe et nos connaissances actuelles sur son rôle dans le cerveau mature sont très limitées. En greffant les clones transfectés dans le cortex de rat, nous avons observé une perte inattendue de l'expression du transgène au cours de son développement ce qui nous empêche de répondre à la question suivante: comment le cerveau répond-il aux taux chroniques élevés de l'APP exprimée en surface ou a ses fragments sécrétés. L'expression de l'APP est très importante dans les neurones sensibles au processus neurodégénératif de la maladie d'Alzheimer. Ainsi, en premier lieu, afin de mieux comprendre cette vulnérabilité, nous avons évalué les effets de l'APP sur les fonctions vitales de la cellule. Pour cela, nous avons développé un anticorps monoclonal hautement spécifique d'un épitope de l'Aβ humain. Cet anticorps a été utilisé en microscopie électronique à haute résolution sur les clones matures neuroectodermaux différenciés (P19). Cette étude nous a permis de localiser les fragments de l'APP portant cette épitope et ainsi nous aider a définir précisément les sites de catabolisme de cette protéine et des différents organelles naturellement associés à ses fragments. Les conséquences fonctionnelles de ces associations intracellulaires ont été évaluées en analysant deux différents paramètres associés à la survie cellulaire, le métabolisme oxydatif et les signaux de transduction. Les clones transfectés présentent des mitochondries dont la morphologie est altérée et une autophagie similaire à celle observée après stimulation par la cystéamine, un inhibiteur de la phosphorylation oxydative. Dans ces clones, le potentiel de membrane mitochondriale est réduit par un mécanisme distinct d'une inhibition du complexe I. Au cours des stades précoces précédant la différenciation, il a été observé, par immunoblotting, une hyperphosphorylation chronique de la "mitogen-activated protein kinase" (MAPK), une enzyme pléiotropique. Cet événement est concomitant à une augmentation de la production de l'APP. Lorsque les cellules sont plus matures, la phosphorylation des MAPKs dans les cellules stimulées par le "acidic fibroblast growth factor" est diminuée dans les cellules exprimant l'APP. Pour les cellules non stimulées, une forte immunoréactivité, distribuée de manière réticulaire, est observée à l'intérieur de tout le soma et dans le noyau. Le traitement par le facteur de croissance entraîne seulement une augmentation très modérée de l'intensité de ce marquage et une faible translocation dans le noyau. Ainsi, d'après nos études ultrastructurales, il apparaît que l'APP et ses métabolites sont en mesure d'influencer les activités cellulaires et nous avons démontré les conséquences fonctionnelles de la sur expression de la $hAPP_{751}$ dans les cellules de type nerveux. Le dysfonctionnement des composantes clefs du métabolisme oxydatif et des kinases dépendantes de ligands (MAPK) montre d'importants parallèles avec les anomalies décrites dans le cerveau des patients atteints de la maladie d'Alzheimer.

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Glossary

Aβ	Amyloid β peptide
aFGF	Acidic fibroblast growth factor
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole
APP	Amyloid precursor protein
ATP	Adenosine 5′-triphosphate
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CaM kinase II	Calcium/calmodulin-dependent protein kinase II
cGMP	3′5′-cyclic monophosphate
CM	Conditioned medium
CMTMR	Chloromethyl-tetramethylrosamine methyl ester
CNS	Central nervous system
DAB	3′3′-diaminobenzidine tetrahydrochloride
DMEM/DME	Dulbecco's modified Eagle's medium
DOPA	Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GAP-43	Growth-associated protein-43
GAPDH	Glyceraldehyde-3-dehydrogenase
GFAP	Glial fibrillary associated protein
GTP	Guanosine 5′-triphosphate
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin
KPI	Kunitz-type protease inhibitor
MAP1,2	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MARK	Microtubule affinity regulating kinase
mtDNA	mitochondrial DNA
MTT	3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide
MPP	1-methyl-4-phenylpyridium
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
N-CAM	Neuronal cell adhesion molecule
NeuN	Neuron-specific nuclear antigen
NF 68,160,200	Neurofilament
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate

NSE	Neuron-specific enolase
pAD	APP-transfected clones
PAGE	Polyacrylamide gel electrophoresis
placZ	Control-transfected clones
PMSF	Phenylmethylsulfonyl fluoride
PBS	Phosphate-buffered saline
PBS+T	Phosphate-buffered saline + 0.2% Triton X-100
PVDF	Polyvinylidene fluoride
RAGE	Receptor for advanced glycation end products
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
TBS	Tris-buffered saline
TBS+T	Tris-buffered saline + 0.1% tween 20
TNT	Transcription/translation
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
RIPA	Radio-immuno precipitation assay
$\Delta\Psi_M$	Mitochondrial membrane potential

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"Discovery consists of seeing what everybody has seen and thinking what nobody has thought." Albert Szent-Györgyi

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Statement of Authorship

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Paper 1/Chapter 4 submitted

Aβ immunoreactivity reveals putative proteolytic compartments and intracellular peptide associations in neuroectodermally differentiated P19 cells expressing human amyloid precursor protein

Susan M. Grant, Adriana Ducantenzeiler, Moshe Szyf, A. Claudio Cuello

Paper 2/Chapter 5 published in NeuroReport 10(1):41-46, 1999

Mitochondrial abnormalities in neuroectodermal cells stably expressing $hAPP_{751}$

Susan M. Grant, Sai L. Shankar, Ruth M.E. Chalmers-Redman, William G. Tatton, Moshe Szyf, A. Claudio Cuello

Paper 3/Chapter 6 submitted

Phosphorylation of mitogen-activated protein kinase is altered in neuroectodermal cells overexpressing the human amyloid precursor protein

Susan M. Grant, Anne Morinville, Dusica Maysinger, Moshe Szyf, A. Claudio Cuello

This thesis is based on the data described in Chapter 3, and in Chapters 4, 5, and 6, which have been submitted for publication. The following statements outline the contributions of each of the authors.

Dr. A. Claudio Cuello: Principle investigator on all the studies described herein, and main intellectual influence in all matters pertaining to the brain and immunocytological techniques.

Dr. Moshe Szyf: Full collaborator on all the studies described herein, and main intellectual influence in all matters pertaining to molecular biology.

Mrs. Adriana Ducatenzeiler: A laboratory technician who contributed her expertise in the preparation and characterisation of the monoclonal antibody McSA1.

Dr. William Tatton and Dr. Ruth Redman-Chalmers: Collaborators in the functional analysis of mitochondria in transfected P19 clones. Contributed to the intellectual content of paper 2/Chapter 5.

Mr. Sai Shankar: Ph.D. candidate in the laboratory of Dr. Tatton. Did all of the data collection and analyses of the mitochondrial membrane potentials.

Dr. Dusica Maysinger: Collaborator in the analysis of MAPK activation in APP-expressing clones. Contributed to the intellectual content of paper 3/Chapter 6 and, in particular, suggested investigating the activation of MAPK family members in the P19 cell lines.

Ms. Anne Morinville: Ph.D. candidate in the laboratory of Dr. Maysinger. Performed Western analyses of cell lysates I prepared during initial studies of ERK, p38, and JUN kinase. Contributed to the intellectual content of paper 3/Chapter 6.

Ms. Susan Grant: Ph.D. candidate in the laboratory of Dr. Cuello. I did all of the cell culture work, all of the *in vivo* work and preparation of brain tissue, and all of the cytochemical and molecular analyses that are presented in Chapters 3 to 6. I prepared and characterised the monoclonal antibody in conjunction with Mrs. Ducatenzeiler, and prepared the cells for final electron microscopy processing by the technician, Mrs. Marie Ballak. I wrote all of the manuscripts.

Chapter I

Introduction

Dementia might be defined as a chronic and progressive state of deteriorated mental function in an otherwise alert individual. According to the Canadian Study of Health and Aging (McDowell 1995), dementia afflicts 2.4% of all Canadians aged 65 to 74, rising to 34.5% at the age of 85. Eight percent of Canadians over the age of 64 are clinically demented and, in the great majority of cases, the diagnosis is Alzheimer's disease. With the growing numbers of long-lived Canadians, and the high social and economic cost of caring for demented individuals, Alzheimer's disease is poised to become a top health care issue in the coming millennium.

Named for the physician who described its histopathological hallmarks in 1907, Alzheimer's disease was slow to be accepted as a pathological entity distinct from normal aging or affective psychiatric disorders. Through the 1950s and 1960s data accumulated that the dementia had an organic basis, that brain lesions were identical in presenile and senile dementias, and that the severity of these lesions correlated with mental status (reviewed in Blessed et al. 1968 and Katzman 1986). The modern era of Alzheimer's disease research began in the 1980s with the molecular analysis of proteins extracted from affected brains.

In fact, Alzheimer's disease brain exhibits a number of abnormalities. The major morphological changes are: cortical atrophy, neuronal loss, neurofibrillary tangles, and neuritic plaque (Katzman 1986). The neuroanatomical evolution of these changes has been detailed (e.g., Arnold et al. 1991; Braak & Braak 1996; Rogers & Morrison 1985). Whereas Aβ amyloid deposits are the earliest lesion and eventually accumulate

throughout the cortex and underlying white matter, the relationship between amyloid burden and disease severity is inconsistent. Neurofibrillary changes, which include intracellular tangles, neuropil threads, and dystrophic neurites investing plaques, develop in a stereotypic anatomical pattern which can be correlated with the progression of dementia. The best correlate to mental status, however, is synaptic attrition (Davies et al. 1987; Terry et al. 1991).

Alzheimer's disease symptomatology is progressive, beginning with memory impairment and gradually including personality changes, problems with language use and, eventually, motor dysfunction. This symptom complex reflects the cell-specific pathology of the disease. Neurons that interconnect the association cortices with the hippocampal formation and associated limbic structures are selectively lost, effectively isolating brain structures required for memory and learning (Hyman et al. 1984). Neurotransmitter changes reflect this pattern of neurodegeneration; hence, there is profound loss of projecting cholinergic neurons, loss of glutamatergic neurons of the corticocortical association fibres and hippocampal pathways, and loss of specific neuropeptidergic (somatostatin, substance P, corticotropin-releasing factor) neurons, with relative sparing of catecholaminergic pathways (Fine 1986; Francis et al. 1993a,b; Guela & Mesulam 1989).

Normal aging brain exhibits many of the changes considered pathognomonic of Alzheimer's disease (reviewed in Mrak et al. 1997). The distinction lies in increasing prevalence with normal aging versus increasing density or severity in Alzheimer's disease. The overlap does suggest, however, that the biology of aging is an important determinant in the development of this disease. A host of other abnormalities have been described in Alzheimer's disease brain including: re-expression of embryonic genes (Kondo et al. 1996); increased Jun and Fos immediate-early gene expression (Anderson

et al. 1994), metabolic dysfunction (reviewed in Blass 1993); Maillard reaction-related protein modifications (Smith et al. 1994); altered activity of signal transduction systems including protein kinase C (Lanius et al. 1997) and G protein-coupled effectors (reviewed in O'Neill et al. 1994); and the presence of inflammatory mediators (Bauer et al. 1991; Walker & McGeer 1992). One of the major challenges of Alzheimer's disease research is to unravel the relationships between these events with the goal of determining their place in the initiation of, propagation of, or response to the disease process.

Current molecular biology-based research in Alzheimer's disease has been spurred by the discovery of genetic risk factors (reviewed in Rubinsztein 1997). Thus, the protein precursor of Aβ was discovered (see Chapter 2), and a small number of kindred with early-onset disease were found to have mutations in this gene that increase production of Aβ40-43. Localisation of the amyloid precursor protein (APP) to chromosome 21 also explained the prevalence of Alzheimer's disease-type pathology in the brain of older Down's syndrome patients (Rumble et al. 1989). A larger number of hereditary Alzheimer's disease mutations were localised to a new gene family, the presenilins 1 and 2 (Levy-Lahad et al. 1995; Sherrington et al. 1995). Inheritance of the apolipoprotein E4 allele was shown to confer risk for the early onset of the disease (Poirier et al. 1993; Saunders et al. 1993; Yoshizawa et al. 1994).

All known genetic factors enhance β amyloid deposition. This observation has led to the "amyloid cascade hypothesis" of Alzheimer's disease that places the Aβ peptide in a pivotal role for the induction of a progressive neuropathology (see Hardy 1997 and Selkoe 1994). However, others (e.g., Roses 1994 and Terry 1996) believe that Aβ amyloid is a consequence, and not a cause, of the disease. To date, introduction of

Aβ into intact brain has failed to replicate Alzheimer's disease pathology, although it has produced some similar changes (see Section 2.6.5).

This thesis seeks to investigate the biology of APP, and its fragments, within a cell model representative of central nervous system (CNS) phenotypes from two perspectives. First, the processing of the holoprotein and intracellular localisation of its cleavage products are explored using a monoclonal antibody generated for this purpose in combination with high resolution electron microscopy. Second, the effects of overexpression of APP on two activities believed to be dysfunctional in Alzheimer's disease brain, namely oxidative metabolism and kinase activation, are examined. The ultrastructural analysis provides valuable context for the functional studies in terms of placing the protein in a position to influence intracellular events. The ultimate aim of this work is to identify cell processes vulnerable to APP interference so that pharmacological means of redressing these changes can be developed.

Chapter 2

The Amyloid Precursor Protein (APP)

2.1 The discovery of APP

The characterisation of APP began with the work of Glenner and Wong (1984a,b) and Masters et al. (1985) more than a decade ago when they isolated and sequenced the 4kD core protein in cerebrovascular amyloid and neuritic plaque. Based on this sequence, the 695 amino acid precursor protein was subsequently cloned by several groups (Goldgaber et al. 1987; Kang et al. 1987; Robakis et al. 1987; Tanzi et al. 1987; Zain et al. 1988) and the APP family immediately expanded with the cloning of the 751 amino acid (Kitaguchi et al. 1988; Ponte et al. 1988; Tanzi et al. 1988), and 770 amino acid isoforms (Kitaguchi et al. 1988). These cDNAs predict a large protein with a single transmembrane domain, a long N-terminal extracellular region and a short cytoplasmic tail (Kang et al. 1987).

The APP locus was mapped to the long arm of chromosome 21 (21q11.2-q21) [Robakis et al. 1987; Tanzi et al. 1987, 1988] and individuals with Down's syndrome (trisomy 21) were confirmed to express higher levels of APP message (Neve et al. 1988; Tanzi et al. 1987). The ~400kb APP gene was partially mapped (Kitaguchi et al. 1988; Lemaire et al. 1989, Yoshikai et al. 1990) and the 5′ promoter (Salbaum et al. 1988) and 3′ untranslated regions (de Sauvage et al. 1992) described. The results of these studies, and the structural motifs imparted by the amino acid sequences, are summarised in Fig. 1.

2.2 The APP family

2.2.1 APP in evolution

APP has been conserved through evolution. Positive high-stringency hybridisation of the human APP clone to rabbit, sheep, hamster, mouse, lobster, *Xenopus* and *Drosophila* DNA was demonstrated early on (Robakis et al. 1987; Tanzi et al. 1987). Indeed, the rat homologue of APP_{695} was shown to be 97% identical to the human protein (Shivers et al. 1988). Comparison of the 173 nucleotides around the Aβ peptide coding region in the DNA of cow, sheep, human, dog, polar bear, guinea pig, pig, rat and mouse allowed Johnstone et al. (1991) to generate a phylogenetic tree showing that species that share amino acid identity with humans through this region are also those species that develop Aβ deposits. Thus, antisera raised against human amyloid cross-react with senile plaque and cerebrovascular amyloid in dog, bear, and primate brain (Podlisny et al. 1991; Selkoe et al. 1987).

2.2.2 APP isoforms

APP is ubiquitously expressed with the highest levels of mRNA found in brain, kidney, heart and spleen, and the lowest level in liver (Tanzi et al. 1987). Nonetheless, considerable diversity is achieved by alternate splicing of the primary transcript so that isoform expression is notably cell-type dependent. Proteins containing sequences encoded by exon 7 (APP_{751} and APP_{770}) predominate in peripheral tissues and non-neuronal cells within the brain; protein lacking exon 7 (APP_{695}) is neuron-specific (LeBlanc et al. 1991; Rohan de Silva et al. 1997).

Within the brain, APP message is highly expressed in, but not confined to, neurons prone to Alzheimer's disease lesions. Thus, it is abundant in pyramidal neurons of cortical layers II, III, V, and VI, in the pyramidal cell layer of all cornu ammonis (CA) fields of the hippocampal formation, and in the Purkinje cells of the cerebellum

(Bahmanyar et al. 1987; Lewis et al. 1988). Detailed immunocytochemical analysis of protein expression in rodent brain confirms and expands these results to include mitral cells of the olfactory bulb, granule cells of the hippocampus, cerebellum, and olfactory bulb and neurons within numerous subcortical structures (Card et al. 1988; Ouimet et al. 1994). Much weaker immunoreactivity is sparsely detected in glial cells (Card et al. 1988).

Eight splice variants of the APP transcript have been identified (reviewed by Sandbrink et al. 1996). The longest, incorporating all exons and named according to amino acid length, is isoform 770. In isoform 751, exon 8 is skipped deleting a stretch of 19 amino acids bearing homology to the MRC OX-2 antigen of thymocytes (Clark et al. 1985). Isoform 714 (Golde et al. 1990) includes exon 8 but omits exon 7, which encodes a 56 amino acid domain with homology to Kunitz-type serine protease inhibitors (KPI domain) [Ponte et al. 1988; Tanzi et al. 1988]. Isoform 695 skips both exons 7 and 8. A series of 4 analogous proteins that lack exon 15, designated L-APPs (isoforms 752, 733, 696, and 677), is expressed in non-neuronal tissues (Sandbrink et al. 1994a). De Sauvage and Octave (1989) have also reported a novel brain transcript in which the sequence for the C-terminal 208 amino acids encompassing the transmembrane and intracellular portions of APP is replaced with 20 amino acids bearing homology to the Alu repeat family.

2.2.3 APP-like genes

APP is part of a gene family. Two groups independently cloned an APP homologue from human libraries (Sprecher et al. 1993; Wasco et al. 1993). Subsequently, Sandbrink et al. (1994b) demonstrated that these clones were transcripts of a single gene (named APLP2) which, like APP, is produced in several isoforms with

alternative splicing of exons equivalent to 7 and 15. Tissue distribution of APP and APLP2 is very similar suggesting that these proteins share a similar function (Sandbrink et al. 1994c; Wasco et al. 1993). APLP2 is 52% identical and 71% similar to APP at the amino acid level with conservation of the major motifs of APP (as depicted in Fig. 1). It differs through the transmembrane region; the Aβ sequence is not conserved (Sprecher et al. 1993;Wasco et al. 1993). The murine homologue of APLP2, named APLP1, was also cloned by Wasco et al. (1992). As well, the *Drosophila* APP gene family member, APPL, has been cloned (Rosen et al. 1989).

2.3 APP expression

2.3.1 Developmental expression

Using isoform-specific antisera in immunoblots of post-natal brain homogenates, Löffler and Huber (1992) demonstrated that APP expression was upregulated in developing rodent CNS with the highest level of expression occurring at postnatal days 10 to 16. The KPI-containing isoforms predominated during this time, which corresponds to a period of dynamic change in the neuronal cytoskeleton and establishment of neural circuitry. Similarly, expression of cell-associated KPI-containing isoforms peaked during the period of rapid axon elongation in the retinofugal pathway of the hamster, and cell-associated APP_{695} and soluble ectodomain forms peaked during end-arbor formation and synaptogenesis (Moya et al. 1994). Immunohistochemical localisation of APP confirmed the appearance of protein in subpopulations of neurons according to a developmentally proscribed pattern of neuronal differentiation (Arai et al. 1994; Ohta et al. 1993; Salbaum & Ruddell 1994). The APP homologue in *Drosophila*

was also shown to be expressed and processed in a stereotypic fashion reflecting a role in neural patterning and function (Torroja et al. 1996).

2.3.2 Expression in experimental neurotoxicity

A number of studies have suggested that the relative abundance of isoforms expressed within the brain is subject to change with injury. Various neurotoxic insults *in vivo* produce an increase in the expression of APP including: excitotoxins (Siman et al. 1989; Solà et al. 1993; Wallace et al. 1993); ischemia (Abe et al. 1991; Kalaria et al. 1993; Ohgami et al. 1992; Stephenson et al. 1992; Tomimoto et al. 1995), and trauma (Chen et al. 1996; Lewén et al. 1995; Otsuka et al. 1991; Scott et al. 1991). The 695 and 751/770 isoforms are differentially regulated by injury with the KPI-containing proteins generally showing the greater and more prolonged response. Whereas this upregulation can occur robustly in reactive, non-neuronal cells (i.e., glia, macrophages, vascular elements) [e.g., Siman et al. 1989; Solà et al. 1993], it has also been demonstrated in affected neurons (e.g., Scott et al. 1991; Stephenson et al. 1992).

Interpretation of these studies is a complex problem given our limited understanding of the biology of APP (reviewed below) and the variety of neuronal systems examined. For example, APP upregulation was observed both in neurons deprived of their target by axotomy (Scott et al. 1991) and in target tissue deprived of afferent input by lesioning or blockade of neurotransmission (Beach et al. 1996; Wallace et al. 1993). Enhanced APP immunoreactivity, as was found in neuronal processes adjacent ischemic lesions, may reflect altered protein trafficking, or attempts at regeneration (Stephenson et al. 1992). It is possible that the APP gene is non-specifically activated because the promoter binds immediate early gene-type transcription factors and heat shock elements activated by stress (Brugg et al. 1995; Dewji & Do 1996; Donnelly

et al. 1990; Lahiri & Nall 1995). However, osmotic stress selectively upregulated APP mRNA and protein in neurons of the supraoptic and paraventricular nucleus concomitant with expression of the immediate-early c-*fos* gene (Palacios et al. 1995). The APP promoter also responds to growth factor-mediated transcriptional activation (Lahiri & Nall 1995) suggesting the possibility that enhanced expression in target tissue follows an increase in local concentrations of neurotrophins due to the loss of functional innervation (Banati et al. 1994; Wallace et al. 1993).

2.3.3 Expression in aging and Alzheimer's disease

Cellular APP content, as measured in peripheral lymphocytes, is positively correlated with age (Pallister et al. 1997). In aged human brain, there is a trend towards a higher ratio of 751/770 to 695 isoforms (Anderson et al. 1989; König et al. 1991; Koo et al. 1990b, Tanaka et al. 1992). Specifically in Alzheimer's disease, the data is most consistent for a relative increase in the proportion of the 770 isoform (Tanaka et al. 1988, 1989; König et al. 1991). There may also be brain region-specific increases in the proportion of KPI-containing isoforms in Alzheimer's disease (Johnson et al. 1990; Neve et al. 1988), although this is not a universal finding (Tanzi et al. 1993). It is noteworthy, however, that APP expression in Alzheimer's disease brain, as in control brain, remains primarily neuronal (Tanzi et al. 1993). In this respect, Alzheimer's disease differs from most acute neurotoxic insults in which glial APP expression is upregulated.

2.4 The processing of APP

APP is a highly processed protein. The Aβ peptide is a minor product of APP metabolism, but its prominence in Alzheimer's disease has made the processing of APP a field of intensive investigation. The following sections review the early data regarding

APP metabolism, and Chapter 4 expands on this subject with work completed for this thesis. Unless otherwise stated, amino acid numbering is based on the 695 isoform.

2.4.1 Post-translational processing

During its transit through the endoplasmic reticulum and Golgi apparatus, APP acquires glycosaminoglycan side chains (Dyrks et al. 1988; Weidemann et al. 1989; reviewed in Breen et al. 1998). Although 2 potential sites near the transmembrane domain exist for N-linked attachment of carbohydrate residues (i.e., Asn-Xaa-Ser/Thr where Xaa cannot be proline), only Asn_{467} appears to be used (Påhlsson et al. 1992). Enzymatic analysis of these residues indicates that the oligosaccharide is of the bi- or tri-antennary complex type with a fucosylated tri-mannosyl core and bisecting *N*-acetyl glucosamine residues (Saito et al. 1995). Sialic acid residues are variably incorporated on the N-linked glycosaminoglycan side chains, apparently in a developmentally-regulated pattern (Saito et al. 1995; Sodeyama et al. 1994). APP is also tyrosine sulphated in the *trans*-Golgi network on one or both of Tyr_{217} and Tyr_{267} (Schubert et al. 1989c; Weidemann et al. 1989). APP can carry O-linked carbohydrate residues which attach to Ser/Thr sites (Weidemann et al. 1989). Pangalos and co-workers (1995) have also described serine-linked chondroitin sulfate glycosaminoglycan attachments to APP (Appican) and APLP2. Appican is found in human brain where, cell cultures studies suggest, it is produced almost exclusively by astrocytes (Shioi et al. 1995).

The post-translational modifications noted above are concentrated in the extracellular portion of APP and are postulated to play a role in APP-mediated cell-cell and cell-matrix interactions (Breen et al. 1998). An additional form of post-translational modification, threonine and serine phosphorylation, occurs within the cytoplasmic domain of APP. The cell cycle-dependent $p34^{cdc2}$ kinase phosphorylates APP at Thr_{668}

with the highest level of phosphorylation occurring at G_2/M when this kinase is maximally active (Suzuki T.et al. 1994). Whether this result is relevant to non-replicating CNS cells is questionable. A kinase that is highly expressed in brain, the proline-directed glycogen synthase kinase -3β, also phosphorylates APP at this residue *in vitro* (Alpin et al. 1996). APP is not a substrate for another multifunctional proline-directed protein kinase expressed in neurons, mitogen-activated protein kinase (Alpin et al. 1996; Suzuki T. et al. 1994). Other, *in vitro*, studies have demonstrated that protein kinase C and calcium/calmodulin-dependent protein kinase II (CaM kinase II) can phosphorylate Ser_{655}, and that CaM kinase II can phosphorylate Thr_{654} (Gandy et al. 1988; Suzuki et al. 1992). The significance of these modifications is unclear. As is discussed below, kinases influence APP processing, but phosphorylation of APP *per se* does not appear to be required (Efthimiopoulos et al. 1994).

2.4.2 Cellular trafficking

APP is subject to polarised trafficking within the widely used model system, Madin-Darby canine kidney cells (e.g., Haass et al. 1994b), and within neurons (e.g., Koo et al. 1990a). In the polarised kidney epithelial cells, APP and its metabolites appeared almost exclusively (90%) in the basolateral compartment. Mutation studies revealed two sorting mechanisms involving distinct pools of APP (Haass et al. 1995). Protein destined for the cell surface was directed to the basolateral surface according to a C-terminus signal involving Tyr_{653} and loss of this motif redirected some of the protein to the apical compartment. However, metabolites of APP continued to be sorted to the basolateral compartment, suggesting there are additional signals in the ectodomain that participate in cell trafficking.

While these results are intriguing, it should be noted that epithelia and neurons do not necessarily use or interpret sorting signals in an identical fashion, and APP trafficking is a case in point (reviewed in Higgins et al. 1997). Most basolaterally trafficked proteins undergo dendritic sorting in neurons, but APP underwent fast anterograde axonal transport (Koo et al. 1990a; Morin et al. 1993), in a process mediated by signals embedded in the *N*-glycosylation domain and in the juxtamembranous Aβ domain (Tienari et al. 1996). Loss of these signals favoured somatodendritic localisation, probably directed by a recessive signal in the C terminus (Tienari et al. 1996). Simons et al. (1995) have demonstrated transcytosis of APP in hippocampal neurons with protein first delivered to the axon and subsequently endocytosed and rerouted to the dendrites.

In addition to sorting of the protein, Tanzi et al. (1993) have reported compartmentalisation of APP message. Along with the expected perinuclear site, mRNA was localised to apical and proximal dendrites and to the axon hillock implying the capacity for translation within specific neuronal compartments. Previously, Denman et al. (1991) had shown that APP message is not translated with maximal efficiency; 70% of APP_{695} mRNA and 50% of $APP_{751/770}$ mRNA in rat brain was associated with polysomes. These two studies, taken together, suggest a rapid response capacity for site-specific expression of APP, and add to the complexity of control exerted by the cell over this protein.

2.4.3 Proteolysis

APP is a substrate for three secretase activities (Fig. 2) [reviewed in Checler 1995]. The majority of APP is directed to the constitutive exocytic pathway in which α-secretase cleaves the protein at the Lys_{612}-Leu_{613} bond to generate a soluble N-terminal ectodomain ($APP_{\alpha S}$) and a membrane embedded C-terminal fragment (Esch et al. 1990;

Kuentzel et al. 1993). The C-terminal fragment is subsequently cleaved within the transmembrane domain by γ-secretase to yield a second secreted fragment, a 3kD peptide referred to as p3 (Busciglio et al. 1993a; Haass et al. 1992b, 1993).

The kinetics of APP metabolism were reported by Kuentzel et al. (1993) working with endogenous $APP_{751/770}$ in human H4 neuroglioma cells. Radioactivity mass balance analysis indicated that 70-80% of synthesised protein was degraded before post-translational processing was complete. Only mature, terminally glycosylated, membrane-embedded APP was a substrate for secretase processing. The half-life of this protein was short with the cleaved ectodomain detected in cell lysates concomitant with the appearance of mature APP. The half-life of appearance of the secreted N-terminal fragment in the extracellular medium was approximately 30 min. This is consistent with an APP half-life of 30 to 60 min reported by others (Oltersdorf et al. 1990; Overly et al. 1991; Weidemann et al. 1989). Cleavage products are detected intracellularly *in vitro* (Kuentzel et al. 1993; Sambamurti et al. 1992) and *in vivo* (Sapirstein et al. 1994; Tokuda et al. 1994), but a portion of APP is delivered intact to the cell surface where it is also subject to α-secretase cleavage (Koo & Squazzo 1994; Lo et al. 1994).

α-Secretase proteolysis is enhanced by neurotransmitters that activate G protein-coupled receptors expressed, endogenously or by transfection, in a variety of cell types (reviewed by Buxbaum & Greengard 1996 and Nitsch & Growdon 1994). These include: M1 and M3 muscarinic receptors (Farber et al. 1995; Nitsch et al. 1992; Slack et al. 1995; Wolf et a. 1995), serotonin 5-HT2a and 5-HT2c receptors (Nitsch et al. 1996), and metabotropic glutamate receptors (Lee et al. 1995). A variety of other stimuli are reported to increase $APP_{\alpha S}$ secretion including: estrogens (Jaffe et al. 1994), interleukin-1 and -1β (Buxbaum et al. 1992; Vasilakos et al. 1994), bradykinin (Nitsch et al. 1993), and thrombin (Davis-Salinas et al. 1994).

Most of these stimuli converge at the level of intracellular signalling molecules and modulation of the processing of APP after receptor activation can be replicated by direct activation of these molecules. Thus, phorbol esters mimic activation of the phospholipase C/protein kinase C cascade to produce an increase in $APP_{\alpha S}$ secretion and, in general, a decrease in Aβ secretion (Buxbaum et al. 1993; Mills et al. 1996; Slack et al. 1995). Mellitin mimics activation of phospholipase A_2 with similar downstream effects on APP metabolism (Emmerling et al. 1993; Nitsch et al. 1997). Increased intracellular calcium may also influence APP processing but the effect appears to be complex, perhaps reflecting activation of proteases as well as participation in signalling cascades (reviewed in Buxbaum & Greengard 1996 and Checler 1995). A central role for mitogen-activated kinase as the ultimate downstream effector of the multiple signal cascades affecting APP processing is suggested by the recent studies of Mills et al. (1997) and Desdouits-Magnen et al. (1998). As noted above, phosphorylation of APP itself does not appear to be involved in regulating α-secretase activity. The mechanisms responsible have yet to be determined.

A minor proteolytic pathway involves cleavage at the Met_{596}-Asp_{597} bond (695 numbering) by β-secretase followed by γ-secretase cleavage of the resulting C-terminal fragment to release the 4kD Aβ peptide (Haass et al. 1992b; Seubert et al. 1992). Although the kinetics of p3 and Aβ peptide generation are similar (Busciglio et al. 1993a), α- and β-secretase activities diverge at the level of the *trans*-Golgi (e.g., McConlogue et al. 1996) and can be discriminated by pharmacologic manipulation and by mutation.

Treatment with ammonium chloride or chloroquine decreases Aβ production (Fuller et al. 1995; Haass et al. 1993; Higaki et al. 1995) implicating an acidic

compartment as the site of its generation. Whether this acidic compartment is the lysosome is controversial. Lysosomal enzyme inhibition increases the lysosomal content of C-terminal fragments bearing an intact Aβ domain leading to speculation that a pool of APP may be trafficked to this organelle for degradation and, in the process, set the stage for Aβ generation (Caporaso et al. 1994; Golde et al. 1992; Haass et al. 1992a; Hayshi et al. 1992; Siman et al. 1993). However, multiple lines of evidence (our own studies included) suggest that β-secretase cleavage occurs within the *trans*-Golgi network or within endosomes, and those C-terminal fragments that escape immediate γ-secretase cleavage are directed to the lysosome for degradation (Dyrks et al. 1993; Gabudza et al. 1994; Haass et al. 1993; Higaki et al. 1995; Kuenetzel et al. 1993; Nordstedt et al. 1993; Yamazaki et al. 1996).

The human Aβ sequence appears to be naturally amyloidogenic, particularly as expressed in human astrocytes and neurons (Busciglio et al. 1993a; Wertkin et al. 1993). When human protein or rodent protein "humanised" by mutation is expressed in rodent neurons, the amount of 4kD peptide secreted is increased relative to that derived from the natural rodent protein (De Strooper et al. 1995). Rare mutations associated with hereditary Alzheimer's disease or cerebral amyloid angiopathy increase Aβ secretion (Cai et al. 1993; Citron et al. 1992; Haass et al. 1994a; Levy et al. 1990). These mutations cluster around the α- or β-secretase cleavage sites, although it is unclear whether the altered amino acids influence enzyme specificity, protein trafficking, or both. The unusual apical secretion of Aβ derived from the Swedish mutant form of APP in Madin-Darby canine kidney cells (Lo et al. 1994), and the demonstration of a unique intracellular pool of Aβ derived from the Swedish mutant APP in COS-1 cells (Martin et al. 1995) suggest that this APP mutant, at least, is sorted aberrantly.

The identity of the α-, β-, and γ-secretases remains unresolved. A number of candidate proteases have been identified that can cleave synthetic peptides at the appropriate sites (reviewed in Checler 1995). As well, protease inhibitors have been useful in classifying the primary activity of the secretase enzymes in intact cell systems (e.g., Citron et al. 1996a). In this regard, inclusion of the Kunitz protease inhibitor domain does not seem to influence proteolytic processing of APP (Ladror et al. 1994).

α-Secretase appears to recognise its cleavage site in relation to distance from the membrane and is relatively resistant to amino acid changes (Maruyama et al. 1991). Conversely, β-secretase activity is sequence sensitive and most amino acid substitutions abolish cleavage (Citron et al. 1995). It should be noted that there is microheterogeneity in the N-terminal amino acids of both Aβ and p3 suggesting laxity in substrate-enzyme recognition or binding (e.g., Busciglio et al. 1993a).

More recently, attention has fallen on the γ-secretase because of the realisation that a longer, more fibrillogenic 42/43 amino acid form of Aβ is the initial peptide to deposit as amyloid in the brain (Lemere et al. 1996). Most Aβ or p3 terminates at Val_{635} but, in 10% of secreted peptides, the cleavage occurred two or three amino acids further C terminal (Asami-Odaka et al. 1995; Citron et al. 1996b). Since the longer forms are generated constitutively at a fixed percentage, any manipulation that increases β-secretase cleavage has the concomitant effect of increasing secretion of Aβ(42/43). As well, certain familial Alzheimer's disease pedigrees are associated with mutations adjacent to the γ-secretase cleavage site. Thus, mutation of Val_{717} (770 numbering) [Suzuki N. et al. 1994; Tamaoka et al. 1994] or Ile_{716} (Eckman et al. 1997) promote production of the longer peptides. Mutations in the presenilin 1 and 2 proteins, which comprise the majority of hereditary Alzheimer's disease, have also been shown to

promote γ-secretase activity at the more C-terminal site (Borchelt et al. 1996; Tomita et al. 1997). There is experimental support for the notion that γ(40) and γ(42/43) are distinct enzymes (Citron et al. 1996b; Klafki et al. 1996a), but their identity and relation to APP trafficking and N-terminal secretase cleavage remain to be resolved.

2.5 The function of APP

Despite its widespread expression, the cellular function of APP is poorly understood. Most work to date has focussed on its role in neuronal differentiation wherein cell surface APP or the soluble APP ectodomain may act in concert with extracellular matrix and cell adhesion molecules to facilitate neurite growth. However, a number of other properties have been ascribed to APP that may affect the function of mature CNS cells. These activities are summarised below. Chapters 5 and 6 describe work, completed for this thesis, that explores the functional consequences of APP overexpression in our model cell system.

2.5.1 Role in neurite growth

The best studied biological role for APP is its ability to promote the morphological changes accompanying neuronal differentiation. APP is a cell surface protein (Jung et al. 1996) being localised to patches at the tips of cell processes and in growth cones of actively elongating neurites of cultured hippocampal neurons [Ferreira et al. 1993; Mattson et al. 1994 (although see critique of Storey et al. 1996a)] and human glioma cells (Kametani et al. 1990). In mature cultured neurons, APP antibodies label axons in a punctate pattern. This APP immunoreactivity co-localises with clathrin (Ferreira et al. 1993; Yamazaki et al. 1997), β1 integrin, (Storey et al. 1996b; Yamazaki et al. 1997), talin (Storey et al. 1996b) and α-adaptin (Yamazaki et al. 1997). APP also

co-localises with β1 integrin in astrocytes (Yamazaki et al. 1997). The co-localisation data have been interpreted to mean that APP is a component of adhesion patches and, through extracellular matrix binding domains on it ectodomain, assists in axonal growth and stabilisation.

APP possesses a high and a low affinity heparin binding site through which it interacts with the heparan sulfate proteoglycans within the extracellular matrix (Multhaup 1994; Multhaup et al. 1994). APP has additionally been shown to bind fibronectin, collagen IV, entactin, and laminin with high affinity (Narindrasorasak et al. 1995). It is interesting to note that extracellular matrix proteins influence the biogenesis of APP *in vitro* (Bronfman et al. 1996; Mönning et al. 1995; Octave et al. 1989). Extracellular adhesion molecules are also a prominent and early component of Alzheimer's disease plaque and angiopathy prompting speculation that aberrent tissue remodelling is involved in the disease process (Eikelenboom et al. 1994; Murtomäki et al. 1992; Snow et al. 1988).

Secreted APP ectodomain accumulated in association with microexudate produced by cultured cells (Klier et al. 1990; Small et al. 1992). Substratum-bound APP, in conjunction with heparan sulfate proteoglycans, was neuritotropic for chick sympathetic neurons and rat hippocampal neurons (Small et al. 1994). A heparinase-insensitive neuritotropic activity was described by Ninomiya et al. (1994), although this was localised to residues 319-335 which Multhaup (1994) had previously found to be a high affinity heparin binding site. Cell-associated APP is also involved in neuritogenesis. Antisense treatment that effectively downregulated APP expression inhibited neurite outgrowth in cortical neurons (Allinquant et al. 1995), and in differentiated PC12 cells (Kibbey et al. 1993). Recent work with primary cultures of astrocytes and neurons from APP-deficient mice elucidated complementary roles for secreted and cell-associated APP

in neuronal development (Perez et al. 1997). Neurons appeared to require cell-associated APP for survival, axogenesis, and arborisation. Astrocyte-derived APP secretory products influenced axon growth, dendrite number and branching.

The mechanism(s) by which APP might accomplish these effects remains to be determined, but is likely to be multifactorial given the complex, cell-type specific processing and developmentally regulated expression of APP. Signal transduction may play a role. Soluble APP stimulated tyrosine phosphorylation of insulin receptor substrate-1 in PC12 cells suboptimally treated with nerve growth factor (NGF), thereby potentiating the neuritotropic effects of NGF (Wallace et al. 1997). Activation of the α subunit of the guanosine triphosphate (GTP)-binding protein G_o increased the number of neurites per neuroblastoma cell (Strittmatter et al. 1994), and membrane-inserted APP is reported to complex with G_o through a C-terminal binding domain (Nishimoto et al. 1993). As well, APP may play a role in transcriptional activation of cytoskeletal proteins, as reported by Ramakrishna et al. (1997) with respect to β-actin gene expression in COS-1 cells. Protease inhibition may be important, both to initial neuronal development and to repair and plasticity in the mature brain. KPI-containing APP_{751} was shown to induce axonal sprouting in the sciatic nerve, perhaps by removing the inhibitory influence of proteases such as thrombin (Alvarez et al. 1992). By enhancing cell adhesion (LeBlanc et al. 1992; Schubert et al. 1989b), APP may contribute to the tensile forces required for the polarised growth of microtubules (Lafont et al. 1993). Alternatively, APP may directly interact with the cytoskeleton. Binding mediated by a C-terminal domain has been demonstrated in cultured neurons (Allinquant et al. 1994).

Examination of APP *in vivo* suggests a role for this protein in synaptogenesis. As noted in Section 2.3.1, APP expression is upregulated in developing neural systems at the time synaptic connections are established (e.g., Moya et al. 1994). Using confocal and

electron microscopy to examine synaptic sites in rodent brain, Schubert et al. (1991) observed that the majority of synapsin-positive structures were also immunoreactive for APP, although the vesicular localisation of these proteins was separate. Most recently, a synaptotrophic effect was demonstrated in mice transgenic for wild-type APP_{695} or APP_{751} (Mucke et al. 1994), or for a familial Alzheimer's disease mutant form of APP_{751} (Wong et al. submitted).

2.5.2 Other activities

While not necessarily divorced from the role APP may play in neuronal differentiation, a number of other activities have been reported for this protein or its N-terminal fragment. The KPI domain of APP is a relatively specific inhibitor of some trypsin-like arginine esterases (Sinha et al. 1990). Indeed, the APP_{751} α-secretase ectodomain is identical to protease nexin 2, an inhibitor of platelet coagulation factor XI_a (Smith et al. 1990) released from its storage site in platelet α-granules in response to activating stimuli (Cole et al. 1990; Li et al. 1994). In addition, a metalloproteinase inhibitor domain capable of inhibiting the matrix-degrading enzyme gelatinase A has been localised to the C-terminal portion of the APP ectodomain (Miyazaki et al. 1993).

Within the cysteine-rich portion of the N-terminus of APP and APLP2 resides a binding site for copper type II (Hesse et al. 1994). APP mediates a redox reaction in which bound copper(II) is reduced to copper(I) [Multhaup et al. 1996]. APP also binds zinc(II) and binding of this ion has been shown to modulate the interaction with heparan sulfate proteoglycans (Multhaup et al.1994).

$APP_{\alpha S}$, at nanomolar concentrations, suppressed action potentials and hyperpolarised cultured hippocampal neurons (Furukawa et al. 1996). Within hippocampal slices, $APP_{\alpha S}$ shifted the frequency dependence for induction of long-term

depression and enhanced long-term potentiation induced by high frequency stimulation (Ishida et al. 1997). Both effects involved guanosine 3',5'-cyclic monophosphate (cGMP)-dependent activation of high-conductance potassium channels that, in turn, modulated calcium influx. Removal of extracellular APP *in vivo* by the intraventricular infusion of N-terminal-directed antibodies impaired performance on passive avoidance tests of memory in 2 separate studies (Doyle et al. 1990; Huber et al. 1993). Moreover, intraventricular administration of an APP peptide comprising residues 319-335 improved performance in learning and memory tests (Roch et al. 1994). Thus, the secreted APP ectodomain appears to positively influence memory formation.

In addition to having a potential modulatory role in calcium-mediated neuronal activation, Mattson and colleagues have described a protective role for $APP_{\alpha S}$ in calcium-mediated neurotoxicity (Barger et al. 1995; Mattson et al. 1993). In these studies, pretreatment of cultured neurons with $APP_{\alpha S}$ stabilised $[Ca^{2+}]_i$ in the face of glutamate activation of N-methyl-D-aspartate (NMDA) receptors or hypoglycemia. Treatment with $APP_{\alpha S}$ evoked a rapid increase in cGMP that, as noted in the electrophysiological studies, mediated the attenuation of $[Ca^{2+}]_i$ (Barger et al. 1995; Mattson et al. 1993).

Secreted APP is reported to be an autocrine factor in the growth regulation of fibroblasts (Saitoh et al. 1989; Schubert et al. 1989a). Nanomolar concentrations of the secreted APP_{751} (but not APP_{695}) ectodomain were mitogenic for Swiss 3T3 fibroblasts (Schubert et al. 1989a). Similarly, low concentrations of $APP_{\alpha S}$ restored growth in human fibroblasts in which endogenous APP was suppressed by an antisense expression vector (Saitoh et al. 1989). No mechanism was explored in these early studies, but $APP_{\alpha S}$ has been shown to activate mitogen-activated protein kinase, which could

influence cell viability and proliferation (e.g., Greenberg et al. 1995). This topic is discussed further in Chapter 6.

Despite its very different topology, membrane-inserted APP was reported to exhibit some properties of a G protein-coupled receptor. The cytoplasmic tail sequence 657-676 bound and activated G_o. This response could be abolished by treatment with pertussis toxin and stimulated by exposure to monoclonal antibody 22C11, which presumably induced dimerisation or comformational changes through binding its N-terminal epitope (Nishimoto et al. 1993; Okamoto et al. 1995). Nishimoto's group have recently expanded on these studies by demonstrating that expression of a familial Alzheimer's disease form of APP in a neuronal-like COS cell clone induced apoptosis, and that this effect involved activation of G_o protein (Giambarella et al. 1997; Yamatsuji et al. 1996). These results, while intriguing, are difficult to reconcile with the apparently normal brain development and function in individuals carrying this mutation. However, toxicity secondary to APP expression has been reported in transfected, neuroectodermally-differentiating P19 cells (Yoshikawa et al. 1992), transfected, differentiating neuroblastoma cells (Maruyama et al. 1994), and fetal trisomy 21 neurons in culture (Busciglio & Yankner 1995). These studies do suggest that overexpression of the holoprotein carries a biological consequence and that commitment to a neuronal phenotype induces the cellular machinery through which APP, or its metabolites, might act adversely. Chapter 5 explores this theme further.

2.6 Activity of Aβ peptide

There is a wealth of information regarding the activity of Aβ *in vitro* and *in vivo* (reviewed by Mattson 1997). However, most of the literature describes the effects of supraphysiological (greater than picomolar) concentrations of peptide, and the relevance

of these results to Alzheimer's disease pathology remains to be established. These data will be briefly summarised below, as will some of the work regarding Aβ aggregation.

2.6.1 Toxicity *in vitro*

Aβ1-40(42), or a peptide comprising residues 25-35, is widely reported to induce apoptosis in a variety of cultured primary and clonal neuronal-type cells (e.g. Estus et al. 1997; Forloni et al. 1993; Le et al. 1995; Li et al. 1996; Loo et al. 1993; Watt et al. 1994; Zhao & Duffy 1996). Necrosis has also been described (Behl et al. 1994a; Gschwind & Huber 1995). In general, these results reflect exposure to 10-100 μM of peptide, concentrations that far exceed those found in cerebrospinal fluid or plasma (Seubert et al. 1992). Sensitivity to Aβ is also notably cell-type dependent. PC12 cells, which are frequently used in these experiments, are very susceptible (Gschwind & Huber 1995; Pike & Cotman 1993, 1995). Variations in phenotype, culture conditions, and age of primary neurons also render direct comparisons difficult. The mechanism(s) of Aβ-induced neurotoxicity has yet to be resolved, but the molecular repertoire of a given cell type may be a major factor. For example, NGF inhibited or enhanced toxicity in Aβ-treated PC12 cells depending on the complement of high and low affinity NGF receptors expressed (Rabizadeh et al. 1994).

Analogous to excitotoxicity, disruption of calcium homeostasis appears to play a role in Aβ neurotoxicity. NMDA channel activation enhanced Aβ toxicity in a substantia nigra/neuroblastoma hybrid cell line and in cultured cortical neurons (Koh et al. 1990; Le et al. 1995). Aβ-Induced toxicity was independent of NMDA receptor activation in two other studies of cultured cortical neurons, however (Brown et al. 1997; Busciglio et al. 1993b). Mattson and colleagues (1992) showed that Aβ enhanced the susceptibility of

human cortical neurons to apoptosis induced by calcium ionophore or glutamate without inducing toxicity itself (Mattson et al. 1992). When calcium influx was attenuated by activation of metabotropic glutamate receptors (Copani et al. 1995), or buffered by calcium-binding proteins (Pike & Cotman 1995), cells were rendered resistant to Aβ-induced apoptosis.

Aβ treatment induced expression of c-Jun and subsequent apoptotic death in cortical neurons (Estus et al. 1997). Conversely, treated γ-aminobutyric acid (GABA)ergic hippocampal neurons did not express c-Jun and were resistant to Aβ (Anderson et al. 1995). These studies, and the demonstration by Paradis et al. (1996) that Aβ induced the down-regulation of the anti-apoptotic protein Bcl-2 in favour of the pro-apoptotic protein Bax, suggest that it is acting through induction of an apoptotic program as opposed to directly damaging cells. Surprisingly, therefore, enhanced expression of Bcl-2 did not inhibit Aβ-induced apoptosis (Behl et al. 1993).

There is considerable experimental data relating oxidative stress to Aβ toxicity. Nitric oxide may serve as a pro-oxidant second messenger in Aβ-exposed cells (Goodwin et al. 1997; Le et al. 1995). Alternatively, Aβ may impair the redox activity of the cell (Shearman et al. 1994) and cause accumulation of mitochondrially-derived hydrogen peroxide (Behl et al. 1994b; Mark et al. 1997). Binding of Aβ to the receptor for advanced glycation end products (RAGE) is reported to induce oxidant stress in neurons and microglia (Yan et al. 1996). Finally, Aβ can generate free radicals in cell-free systems and, thus, has the potential to directly injure membranes and macromolecules (Hensley et al. 1994). Mitochondrial DNA damage (Bozner et al. 1997), activation of the transcription factor NF-κB (Kaltschmidt et al. 1997), activation of glutamine synthetase

and creatine kinase (Hensley et al. 1994), and impairment of Na^+/K^+-ATPase activity (Mark et al. 1997) have all been ascribed to Aβ-mediated oxidative stress.

2.6.2 Toxicity *in vivo*

Data regarding neurotoxicity induced by Aβ in intact brain comes from two experimental paradigms: direct injection (Emre et al. 1992; Frautschy et al. 1991; Games et al. 1992; Giordano et al. 1994; Kowall et al. 1992; Podlisny et al. 1993; Stein-Behrens et al. 1992; Weldon et al. 1998) and transgenic engineering (Games et al. 1995; Holcomb et al. 1998; Hsiao et al. 1995, 1996; Nalbantoglu et al. 1997; Sturchler-Perrat et al. 1997). Neither technique has reproduced the spectrum of Alzheimer's disease-type neurodegeneration. Nonetheless, some aspects of Alzheimer's disease pathology are induced including abnormal phosphorylation of tau, reactive gliosis, microglial activation, Aβ accumulation, and neuritic changes (e.g. Frautschy et al. 1991; Geula et al. 1998; Irizarry et al. 1997; Sturchler-Pierrat et al. 1997). Impaired learning has been demonstrated in some transgenic pedigrees (Holcomb et al. 1998; Hsiao et al. 1996; Nalbantoglu et al. 1997). Work *in vivo* remains stymied by the apparent species- and age-dependent nature of the response (Geula et al. 1998).

2.6.3 Trophic activity

Subnanomolar concentrations of Aβ peptides were mitogenic in PC12 cells (Luo et al. 1996) and Aβ1-28 was reported to enhance the early survival of hippocampal neurons (Whitson et al. 1989). When applied to culture substratum, Aβ promoted attachment and neurite outgrowth in cultured cortical or hippocampal neurons (Koo et al. 1993; Wujek et al. 1996). Low concentrations of peptide have been reported to activate

tyrosine phosphorylation in PC12 cells (Luo et al. 1995, 1996), although whether this functions like tyrosine phosphorylation stimulated by growth factors is unclear. Most reports of Aβ and cellular kinases indicate a toxic interaction (Takashima et al. 1993; Yankner et al. 1990a; Zhang et al. 1994).

2.6.4 Aβ as ligand

Shortly after its discovery, Aβ was proposed to be a peptide ligand based on antibody studies and on similarities between APP and the epidermal growth factor precursor (Allsop et al. 1988). The strongest support for this notion comes from studies in peripheral tissues wherein Aβ modulated substance P activity via serpin receptor complex binding (Joslin et al. 1991; Khalil et al. 1994). Yankner et al. (1990b) reported that both the early neurotrophic and late neurotoxic effects of Aβ in hippocampal cultures involved tachykinin receptors, but these results were not replicated (Rush et al. 1992) and Aβ was shown to interact only weakly with tachykinin receptors despite some amino acid similarities with the true neuropeptide ligands (Kimura & Schubert 1993; Mitsuhashi et al. 1990; Rovero et al. 1992).

2.6.5 Ion-mediated effects

As noted above, Aβ appears to destabilise calcium homeostasis. One mechanism may involve the ability of this hydrophobic peptide to incorporate into lipid structures and form cation-selective channels (Arispe et al. 1993, 1996). Aβ was also reported to interfere with nicotine-invoked calcium influx in PC12 cells (Takenouchi et al. 1994) and potassium-invoked acetylcholine release in hippocampal slices (Kar et al. 1996). Potassium channel dysfunction, identical to that found in fibroblasts from Alzheimer's

disease patients, was induced in normal fibroblasts exposed to nanomolar concentrations of Aβ (Etcheberrigaray et al. 1994).

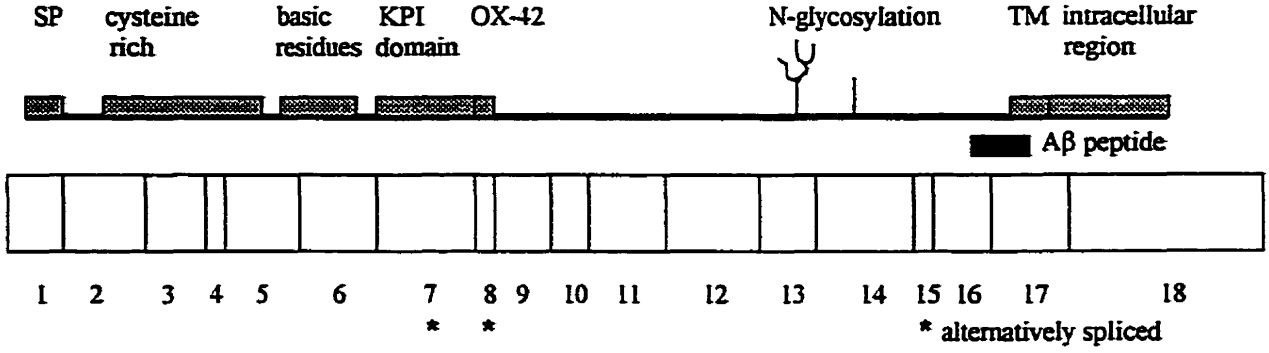
2.6.6 Fibrillogenesis

Aβ toxicity is enhanced by aggregation, and variation in the aggregate content of test solutions has been a confounding factor in much experimental work (discussed in Mattson & Rydel 1992). There are many things that induce Aβ fibrillogenesis and/or aggregation including: low pH (Burdick et al. 1992; Wood et a. 1996); zinc (Esler et al. 1996), time in solution (Burdick et al. 1992; Pike et al. 1991), apolipoprotein E4 (Ma et al. 1994; Sanan et al. 1994; Wisniewski et al. 1994), complement protein C1Q (Webster et al. 1995), heparan sulfate (Kisilevsky et al. 1995), α_1-antichymotrypsin (Ma et al. 1994) and transglutaminase (Dudek & Johnson 1994).

Structural studies have revealed that Aβ fibrils are formed spontaneously through conversion of the α helical peptide into a β-pleated sheet stabilised by its hydrophobic core (residues 17-21) [reviewed in Kisilevsky & Fraser 1997]. The fibrils, 7-10 nm in diameter and of varying lengths, self-assemble into protofilaments that are the basis of amyloid. Aggregation is a 2-step process involving, first, formation of a nucleation centre and, second, propagation of fibril formation. The thermodynamic barrier to nucleation introduces a lag time in the aggregation of Aβ that is much shorter for the 42/43 amino acid form of the peptide. Hence, an abundance of the longer peptide accelerates amyloidosis and may be the basis of the earlier onset of hereditary Alzheimer's disease. Once formed, Aβ amyloid is very resistant to degradation.

Figure 1. Schematic of the motifs found within the APP gene. A. The 18 exons encoding APP_{770} are shown with the corresponding structural motifs deduced from the amino acid sequence. The signal peptide (SP) is followed by a region rich in cysteine residues and, then, by a stretch of sequence which is 45% Asp and Glu residues imparting a highly negative charge to the region. The Kunitz protease inhibitor (KPI) domain and short sequence with homology to the OX-42 antigen are alternately spliced, as is exon 15. Although there are two potential N-glycosylation sites, only one appears to be used. Following the transmembrane (TM) domain is a short cytoplasmic tail. The Aβ peptide derives from exons 16 and 17, and is partially contained within the transmembrane domain. B. The APP promoter lacks a TATA box and is GC-rich – both features of a house-keeping promoter. Two AP 1 binding sites and one region bearing homology to heat shock response elements are present, together with possible CpG methylation (Msp I) sites. C. Two polyadenylation signals within the 3′ untranslated region give rise to two possible mRNA transcripts of 3.2 and 3.4 kB, respectively.

A



B

5′
-400

CTAGCAGCAGTCCTTTATACGACACCC**CCGG**GAGGCCTGCGGGGTCGGA**TGATTCA**AGCTCACGGGGACGAGCA
Msp I AP 1 binding site

GGAGCGCT**CTcGACTtTTCTAG**AGCCTCAGCGTCCTAGGACTCACCTTTCCCTGATCCTGCACCGTCCCTCTCCTGG
heat shock response

CCCCAGACTCTCCCTCCCACTGTTCACGAAGCCCAGGTGGCCGTCGG**CCGG**GGGAGCGGAGGGGGCGCGTGGGGTGC
Msp I

AGGCGGCGCCAAGGCGCTGCACCTGTGGGCGCGGGGCGAGGGCCCCTCC**CCGG**CGCGAGCGGGCGCAGTTCC**CCG**
Msp I Msp I

GATCAGC**TGACTCG**CCTGGCTCTGAGCCCCGCCGCCGCGCTCGGGCTCCGTCAGTTTCCTCG.....
AP 1 binding site mRNA initiation
3′

C

5′ CTGCAGGATGATTGTAGAGAATCATTGCTTATGACATGATCGCTTTCTACACTGTATTAC

ATA**AATAAA**TTA**AATAAA**ATAACCCCGGGCAAGACTTTTCTTTGAAGGATGACTAC
....poly (A) tail (1)

AGACATTAAATAATCGAAGTAATTTTGGGTGGGGAGAAGAGGCAGATTCAATTTTCTTTA

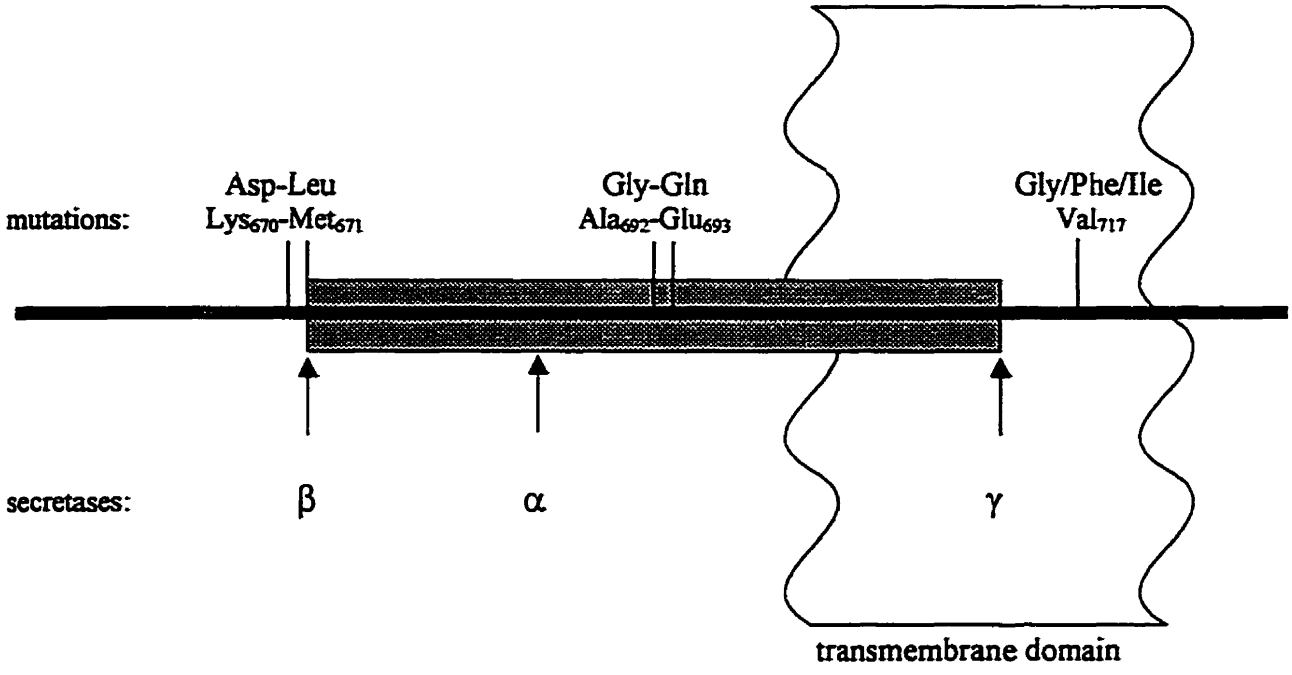
ACCAGTCTGAAGTTTCATTTATGATACAAAAGAAGATGAAAATGGAAGTGGCAATATAAG

GGGATGAGGAAGGCATGCCTGGCAAAACCCTTCTTTTAAGATGTGTCTTCAATTTGTATA

AAATGGTGTTTTCATGTA**AATAAA**TACATTCTTGGAGGAGC 3′
.....poly (A) tail (2)

(1) 3.2 kb mRNA
(2) 3.4 kb mRNA

Figure 2. Schematic of the Aβ-peptide region showing its orientation within the plasma membrane. The sites for cleavage by α-, β-, and γ-secretases are indicated as are the locations of hereditary mutations known to enhance production of amyloidogenic peptide.


mutations:
Asp-Leu
Lys670-Met671
Gly-Gln
Ala692-Glu693
Gly/Phe/Ile
Val717
secretases:
β
α
γ
transmembrane domain

Chapter 3

The Model System

3.1 The P19 cell line

3.1.1 History

Embryonal carcinoma cells are pluripotent stem cells generated through cloning of experimentally induced teratocarcinomas (reviewed in Graham 1977). Michael McBurney (University of Ottawa) produced the P19 cell line in 1982 by grafting 7-day egg cylinders from C3H/He mice to the testis of syngeneic males (McBurney & Rogers 1982). The resulting primary tumour was directly subcloned, and the derived P19 cell line expanded and characterised. P19 cells have a normal male karyotype (40:XY) and, therefore, display normal gene dosage and chromosome stability. When injected into blastocytes (even as a single cell), they produce chimeras of a wide variety of tissues (Rossant & McBurney 1982). However, few live progeny result and these animals typically contain embryonal carcinoma tumours in addition to apparently normal chimeric tissues. Such studies demonstrate the pluripotential nature of P19 cells but, also, suggest that the environment *in vivo* is not sufficient to ensure complete reversion of these cells to normal germ tissue.

P19 cells differentiate poorly under standard culture conditions, but retain the capacity to undergo induced differentiation after repeated passaging *in vitro* (Fig. 3). Chemical induction in conjunction with a shift to growth as aggregates is required to activate a differentiation gene program. Monolayers of undifferentiated cells are replated onto a substrate (e.g., bacterial grade plastic dishes) to which they cannot attach forcing the cells to self-adhere and grow as aggregates or embryoid bodies. In the presence of

10^{-7} M all-*trans* retinoic acid the cells undergo neuroectodermal differentiation (Jones-Villeneuve et al. 1982). In the presence of 0.5-1% v/v dimethyl sulfoxide the cells undergo mesodermal differentiation (McBurney et al. 1982). Retinoic acid treatment in the absence of aggregation, and aggregation in the absence of retinoic acid fail to induce differentiation except for the appearance of a small number of endoderm-like cells (Jones-Villeneuve et al. 1982).

P19 cells have been used extensively to study the molecular events involved in differentiation (e.g., Kranenburg et al. 1995; McCormick et al. 1996) and, in particular, the role of retinoic acid receptors in neuronal development (Jonk et al. 1994; reviewed in Bain et al. 1994). Further discussion of these themes is outside the scope of this thesis; however, the characteristics of neuroectodermal progeny of P19 cells are pertinent to the studies described herein and are reviewed below.

3.1.2 Neuroectodermal progeny

MacPherson and McBurney (1995) have written a comprehensive review of the protocols involved in culturing and differentiating P19 cells, as well as describing the phenotypic characteristics of the neuronal progeny. In addition, the receptor expression and electrical properties of long term neuroectodermal cultures have been described.

Typically, once retinoic acid-treated aggregates are plated onto a tissue culture substrate, a monolayer of fibroblast-like cells migrates out from the aggregate. Within 3 days, neuronal-like cells are seen to extend processes over this monolayer. With increased time in culture the aggregates flatten and astrocytes and the fibroblast-like cells form a confluent carpet over which neurons form an extensive interconnecting network of polarised cell processes. Beginning at 3 days, and becoming established after 1 to 2 weeks, the process-bearing cells become reactive to antisera against a number of neuron-

specific markers including: neural cell adhesion molecule (N-CAM), the neurofilament proteins (NF 68, NF 160, and NF 200), microtubule-associated proteins (tau, MAP 2), neuron-specific enolase (NSE), neuron-specific nuclear antigen (NeuN), growth-associated protein-43 (GAP-43), and the synaptic vesicle proteins synaptophysin, synaptotagmin and syntaxin (Finley et al. 1996; MacPherson & McBurney 1995; Parnas & Linial, 1995; Turetsky et al. 1993).

The neurotransmitter profile of neurons derived from P19 cells closely resembles that of the mammalian neocortex (Staines et al. 1994). The majority of cells (~60%) are GABAergic being immunoreactive for GABA and glutamic acid decarboxylase, and positive for both GABA-transaminase activity and high affinity GABA uptake sites (McBurney et al. 1988). The neuropeptides somatostatin and neuropeptide Y are found in ~20% of neurons. GABAergic neurons are frequently immunoreactive for somatostatin and, occasionally, for neuropeptide Y. Neuropeptide Y and somatostatin themselves co-localise in ~10-20% of neurons positive for either neuropeptide. In very mature cultures (> 4 weeks old), ~20% of neurons become immunoreactive for enkephalin. Cultures are consistently negative for vasoactive intestinal peptide, cholecystokinin, neurotensin, and corticotropin-releasing factor. A very small number of cells (typically <1%) are immunoreactive for calcitonin gene-related peptide, galanin, substance P, tyrosine hydroxylase, dopamine β-hydroxylase, dihydroxyphenylalanine (DOPA) decarboxylase, and serotonin. Similarly, a small number of cells demonstrate positive histochemistry for acetylcholinesterase and for reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent diaphorase activity (Staines et al. 1994).

Choline acetyltransferase activity and evoked acetylcholine release have been reported in P19 neurons (Jones-Villeneuve et al. 1982; McBurney et al. 1988; Parnas & Linial 1995); however, the cholinergic phenotype appears not to be stably or reliably

expressed. Similarly, most investigators find few catecholaminergic cells in retinoic acid-treated P19 cultures, but Sharma and Notter (1988) reported a predominance of catecholaminergic neurons and the absence of neurons reactive to antibodies against choline acetyltransferase and glutamic acid decarboxylase in their study of one P19 cell subline. It would appear that P19 cells can be reliably differentiated to neuronal cells, but that the ultimate phenotype is a matter of, as yet, poorly defined genetic switches.

Concomitant with the expression of proteins for neurotransmitter synthesis, neuroectodermally differentiated P19 cells express receptors and electrical properties supporting synaptic connectivity *in vitro*. Functional $GABA_A$ receptors mediating inhibitory transmission have been described in 7 to 12 day post-retinoic acid cultures (Finley et al. 1996; Reynolds et al. 1996). The predominant synaptic activity, however, is glutamate-mediated excitatory transmission (Finley et al. 1996). Electrical and pharmacological analysis identify these synapses as primarily involving α-amino-3-hydroxy-5-methyl-isoxazole (AMPA)/kainate receptors. Functional NMDA receptors have also been characterised and contribute to excitatory glutamatergic neurotransmission in P19 neurons (Finley et al. 1996; Turetsky et al. 1993). Using calcium influx as the sole end point, Morley and colleagues (1995) also detected functional NMDA receptors in differentiated P19 cells. They found evidence for metabotropic glutamate receptors, but failed to detect AMPA/kainate receptors able to flux calcium. Multiple glutamate receptor subunits are known to be expressed in P19 neurons (Ray & Gottlieb 1993); thus, the ion properties noted above may reflect the particular subunits assembled in these neurons.

While the spotlight has fallen on the neuronal progeny of retinoic acid-treated P19 cells as an experimental paradigm, neuroectodermally differentiated cultures grown in serum-based media contain non-neuronal cells as well. After about 1 week in culture,

glial fibrillary acidic protein (GFAP) immunoreactive astrocytes can be detected with increasing abundance (Jones-Villeneuve et al. 1982). Also present is a fibroblast-like cell that resembles the myofibroblastic cells that are widely found in developing embryos (Rudnicki et al. 1990). Subclones of such cells have demonstrated multiple characteristics of smooth muscle (Blank et al. 1995; Rudnicki et al. 1990). Mature cultures of neuroectodermally differentiated P19 cells have been reported to develop a subpopulation of cells that, by immunocytochemical and functional criteria, resemble microglia (Aizawa et al. 1991). Finally, oligodendrocytes have been subcloned from retinoic acid-treated cultures and also found myelinating host neurons in grafts of differentiated P19 cells *in vivo* (Staines et al. 1996).

3.2 Stably transfected P19 clones

Undifferentiated P19 cells can be efficiently transfected with plasmid DNA and will express the transfected cDNA after differentiation making this cell line particularly suitable for the investigation of protein expression within a neuroectodermal cell context. We took advantage of this property to prepare several clones stably expressing the wild-type 751 amino acid isoform of human APP as a tool to explore APP processing and activity.

3.2.1 Transfection and selection

Transfections were accomplished by calcium phosphate precipitation (Kingston et al. 1996). Plasmid pAD-751 (the gift of K. Beyreuther, Centre for Molecular Biology, Heidelburg), constructed as described in Weidemann et al. (1989), contains the complete coding region plus approximately 200 base pairs of 3′ untranslated sequence of the

human 751 amino acid isoform of APP. Transcription is initiated from the cytomegalovirus promoter/enhancer and polyadenylation of the transcript is directed by the SV40 polyA signal. 20 μg of pAD-751 was co-transfected with 2 μg of the dual reporter/selection plasmid pCMVlacZ II, obtained from the American Tissue Culture Collection (Rockville, MD). pCMVlacZ II encodes the neomycin resistance gene for selection of transfected clones in the presence of the aminoglycoside G 418 (Geneticin®), as well as the bacterial β-galactosidase enzyme for the histochemical detection of transfected cells (Lim & Chae 1989). The latter cDNA is also under the control of the cytomegalovirus promoter. P19 cells were transfected, in parallel, with only the pCMVlacZ II plasmid to generate experimental controls.

Geneticin® 0.6 mg/ml was added to standard culture media to select for clones in which the plasmid DNA had become stably integrated into the host genome. Integration was subsequently confirmed by hybridisation with an internal BamH I fragment of the APP cDNA to extracts of clone genomic DNA cut with BamH I and electrophoresed through a 0.8% agarose gel before transfer to a nylon membrane. Control clones were selected by positive X-gal histochemistry confirming expression of the β-galactosidase protein. Embryonal carcinoma cells, although efficiently transfected, are also prone to inactivation or loss of the transfected DNA (McBurney et al. 1994); hence, clones were maintained in 0.2 mg/ml Geneticin® during routine passaging prior to the start of differentiation.

3.2.2 APP expression in transfected clones *in vitro*

The cytomegalovirus promoter is considered to be a constitutively active, intermediate expressor with demonstrated activity in undifferentiated and neuroectodermally differentiated P19 cells (Fukuchi et al. 1994a). However, cells

derived from teratocarcinomas can exhibit repression of cytomegalovirus promoter activity via the binding of nuclear factors to motifs in the viral DNA (Liu et al. 1994). These nuclear proteins are downregulated with differentiation (Liu et al. 1994) and we, and others (Wu & Adamson 1993), have found that P19 clones become permissive for cytomegalovirus promoted gene expression upon retinoic acid-induced differentiation.

To demonstrate protein expression, 2 week old neuroectodermal cultures were fixed and processed for immunoreactivity toward a monoclonal antibody (clone 22C11) that recognizes an N-terminal epitope conserved between human and rodent APP (Fig. 4). There is moderate immunoreactivity in the control placZ cells, indicative of expression of the endogenous protein, and strong, diffuse immunoreactivity in the cell body and processes of pAD cells.

As discussed in Section 2.4, APP is a highly processed protein. Its metabolic fate is likely to be of importance in the pathophysiological events leading to Alzheimer's disease, as well as being integral to normal APP biology. P19 cells are capable of fully processing and metabolising APP. Hung et al. (1992) reported a pattern of secretory cleavage of the endogenous 695 amino acid protein by neuronal P19 cells that was analogous to their results in primary cultures of hippocampal neurons. Non-differentiated P19 cells stably expressing the 695 or 751 amino acid isoforms of human APP were shown to produce the secreted peptides, p3 and Aβ (both the 40 and the 42 amino acid forms), as well as the corresponding ectodomains (Ho et al. 1996). Expression of the KPI-containing 751 amino acid isoform favoured β-secretase processing in this study; however, whether this is relevant to neuroectodermally differentiated P19 cells is unclear since these cells undergo profound changes in protein expression [including protease expression (e.g., Bolduc et al. 1997; Kobayashi et al. 1996)] with retinoic acid treatment. Conditioned media and lysates of one month old

cultures of our differentiated pAD clones, grown of in the presence of ^{35}S-methionine, revealed fragments immunoreactive to N- and C-terminal specific antisera (see chapter 4). These results suggest that our P19 clones process the transgenic human protein through the expected exocytic pathway.

3.3 APP expression in transfected clones *in vivo*

3.3.1 Grafting APP-expressing clones into rat brain

Grafting in the central nervous system has been studied for some years from the perspective of repopulating damaged brain tissue and of understanding the clues guiding neurotransmitter identity and connectivity in the brain. More recently, grafting has been used as a tool to explore the effects of chronic exposure of targeted brain regions to secreted proteins. Two groups reported specific neuropathological and behavioural effects, respectively, after the injection of PC12 cells transfected with C-terminal fragments of the APP cDNA (Neve et al. 1992; Tate et al. 1992). Cortical atrophy and abnormally phosphorylated tau in the adjacent neocortex were found in 4 month old mice that received hippocampal-cortical injections as newborns (Neve et al. 1992). Adult rats with grafts in the suprachiasmatic nuclei of the hypothalamus exhibited disrupted circadian regulation 4 to 6 weeks after surgery (Tate et al. 1992).

McBurney's group reported that retinoic acid-treated P19 cells successfully implant and complete their neuroectodermal differentiation within adult rat brain (Morassutti et al. 1994). The caveat in using murine cells in a mature rat host is the need for chronic immunosuppression; however, our experience, and that of others (e.g.,

Brundin et al. 1988; Morassutti et al. 1994) indicates that rats tolerate cyclosporin A treatment well.

We chose to inject our APP-expressing clones into the entorhinal cortex. This area of the brain is a consistent and early site of neuropathology in Alzheimer's disease and we reasoned that it may have intrinsic vulnerability to whatever detrimental effects are mediated by APP or its metabolites. Stereotaxic coordinates were chosen with reference to published studies (e.g., Kurumaji & McCulloch 1990; Parent et al. 1994), and to visualisation of injected dye (Fig. 5).

Our protocol was modelled on that of Morassutti et al. (1994). pAD and placZ clones were induced to differentiate as embryoid bodies in the presence of 0.3 μM all-*trans* retinoic acid for 48 h, at which point the aggregates were collected and replated in fresh media with retinoic acid for a further 24 h. To prepare the cells for grafting, the 72 h aggregates were collected, washed once in cold sterile phosphate-buffered saline pH 7.2 plus 0.1 mg/ml calcium chloride, 0.1 mg/ml magnesium chloride, and 0.6% w/v glucose, then resuspended in this wash buffer plus 5% heat-inactivated rat serum. Cells were held on ice prior to injection. By trypan blue exclusion, approximately 60% of cells remained viable after up to 8 hours on ice and were capable of growing as neuroectodermal progeny *in vitro* if returned to culture. Volumes were adjusted to deliver approximately 30,000 cells per injection.

Adult male Sprague Dawley rats (275-300 g) were anesthetised with Equithesin (chloral hydrate 85 mg/kg, sodium pentobarbital 20 mg/kg) and placed in a stereotaxic frame. Three microlitres of prepared cells were injected into the right entorhinal cortex at AP +1.4, L –3.1, and V –7.5 relative to the intra-aural line through a 26G cannula canted 15° medial-lateral and connected to a Hamilton syringe mounted in a Sage perfusion pump. The injection was given over 3 min followed by a 5 min waiting period,

to allow some dispersion of the bolus, and then slow withdrawal of the cannula. Rats were treated post-operatively with buprenorphine (0.1-0.5 mg/kg) and Tribrissen® (trimethoprim 80mg/kg, sulfadiazine 400 mg/kg daily for 3 days). Immunosuppression (cyclosporin A 10 mg/kg daily) was initiated 24 h prior to surgery and continued until the end of the experiment.

After 4 weeks, the rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, the brains post-fixed in the same solution overnight, and then cryoprotected in 20% sucrose in 0.1 M phosphate buffer prior to cryosectioning. Fifty micrometer sections were processed for immunocytochemistry using monoclonal antibodies against NF 160, GFAP, and APP. Adjacent sections from one animal are shown in Figs. 6 through 8.

In Fig. 6, reactive astrocytes delineate the cannula track and graft, but are also found in substantial patches within the body of the graft. The latter cells may be P19-derived glia. Antibody to NF 160 (Fig. 7) stains host processes infiltrating the margins of the graft as well as neuritic elements and, rarely, cell bodies within the graft. Again, the latter immunoreactivity may be P19-derived neurons. These results established that the clones were able to integrate into the entorhinal cortex and survive at least 4 weeks *in vivo* in a cyclosporin A-treated host.

Given the high level of APP expression *in vitro*, it was surprising to find that formic acid pretreatment and high antibody concentrations were necessary to detect APP immunoreactivity in the grafted clone (Fig. 8). β-Galactosidase expression was also difficult to demonstrate by immunocytochemical or histochemical techniques (data not shown). These results may indicate failure of sufficient differentiation to permit cytomegalovirus promoter derepression, or may indicate that some characteristic of the *in vivo* environment induced silencing of the cytomegalovirus promoter. However, the

pattern of immunoreactivity observed within the graft was very interesting. The graft was well vascularised and it was around these vessels that the immunoreactivity was strongest, both in terms of the diffuse deposition characteristic of staining throughout the brain, and in terms of immunoreactive processes.

Investigation of Aβ accumulation was attempted but no immunoreactive material was detected within the graft using two commercially available antisera. However, these antisera were not very active toward plaque in sections of Alzheimer's disease brain which inspired, in part, the undertaking to generate a monoclonal antibody against Aβ, as reported in Chapter 4.

The grafting work was not pursued beyond the pilot study described above. While it raised some very intriguing questions, some of which will be discussed further in Chapter 7, the failure to achieve high expression of APP *in vivo* negated the purpose of the grafts, namely to examine the effect of chronic exposure to cell surface-associated or soluble APP on the entorhinal cortex. The remainder of this thesis focuses on studies undertaken *in vitro*.

The specific objectives of this work are as follows:

1) to investigate the spontaneous subcellular localisation of APP fragments within mature, neuroectodermally differentiated P19 clones.

2) to identify functional changes associated with APP overexpression.

The goal was to test the hypothesis that: **APP and/or its cleavage products act intracellularly to induce functional deficits affecting the long-term viability of central nervous system-type cells.**

Figure 3. Phenotypes of P19 cells prior to and 6 days after neuroectodermal differentiation. A. Live culture of a P19 clone stably transfected with $hAPP_{751}$. B. The same clone after differentiation in retinoic acid. At 6 days, neuronal-type cells (arrows) can be seen growing over fibroblastic- or astrocytic-type cells. This culture was fixed and stained to APP expression. Scale bar represents 200 μm.

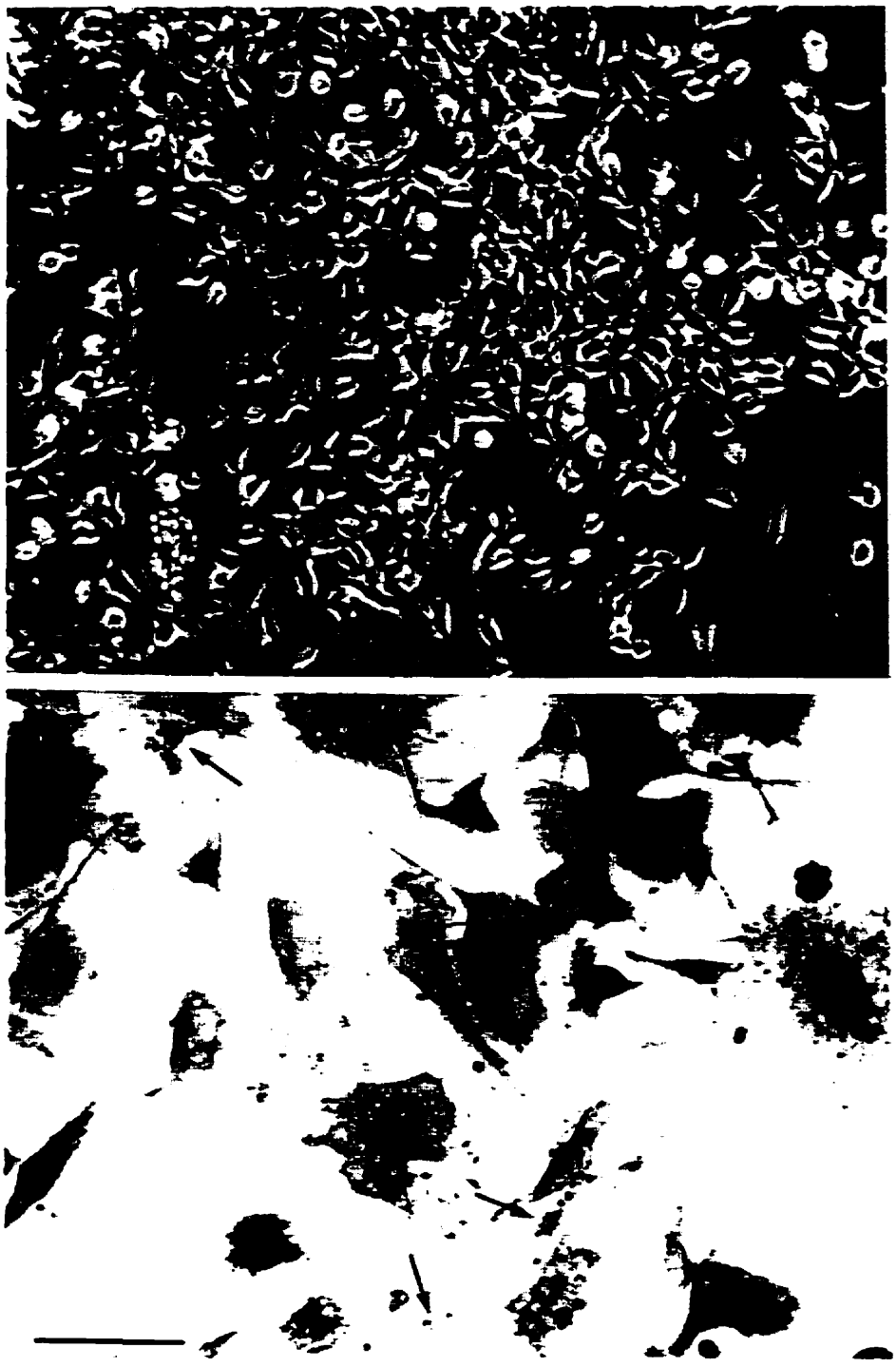


Figure 4. Stably transfected P19 clones were grown for 2 weeks after neuroectodermal differentiation at which time they were fixed and examined for APP expression. mAb 22C11, which recognizes an N-terminal epitope in human and rodent APP, was used to detect expression of endogenous and transgenic protein (see Chapter 4, Methods). A. Control reaction in which primary antibody was omitted. B. Control clone placZ 12. C. APP-transfected clone pAD 28. Scale bar represents 200μm.

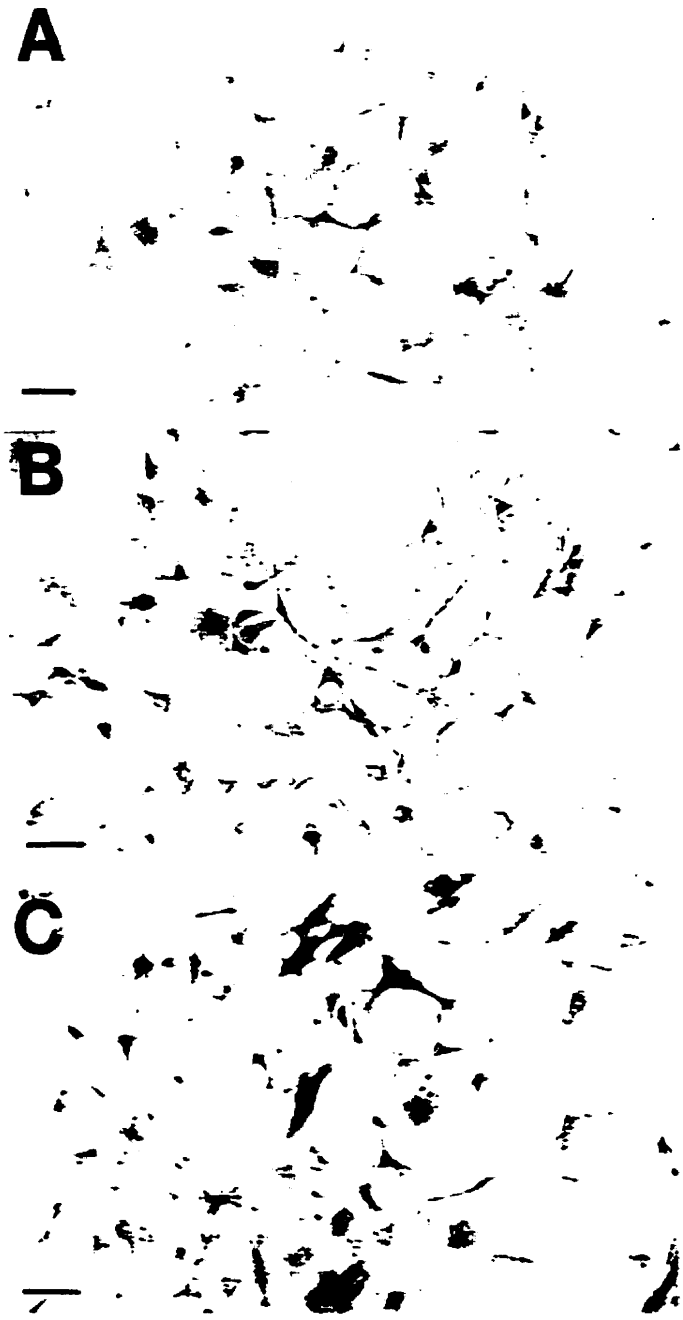

A
B
C

Figure 5. Visualization of the P19 graft site in rat entorhinal cortex. India ink was injected at the coordinates selected: AP +1.4, L –3.1, V –7.5 relative to the intra-aural line with a 15° medial to lateral cant. The brain section was lightly counterstained with neutral red.

Figure 6. Graft of APP-transfected clone pAD 28 4 weeks after implantation in the entorhinal cortex of a mature rat host. 50 μm thick free-floating sections were reacted with a mAb to the astrocyte-specific marker GFAP. A. Intense immunoreactivity persisted along the cannula track and at the margins of the graft. Reactive astrocytes were also present within the graft itself. B. An enlarged view of the immunoreactive cells within the graft. C. An enlarged view of the host astrocytes delineating the graft margin. Scale bar represents 200 μm.

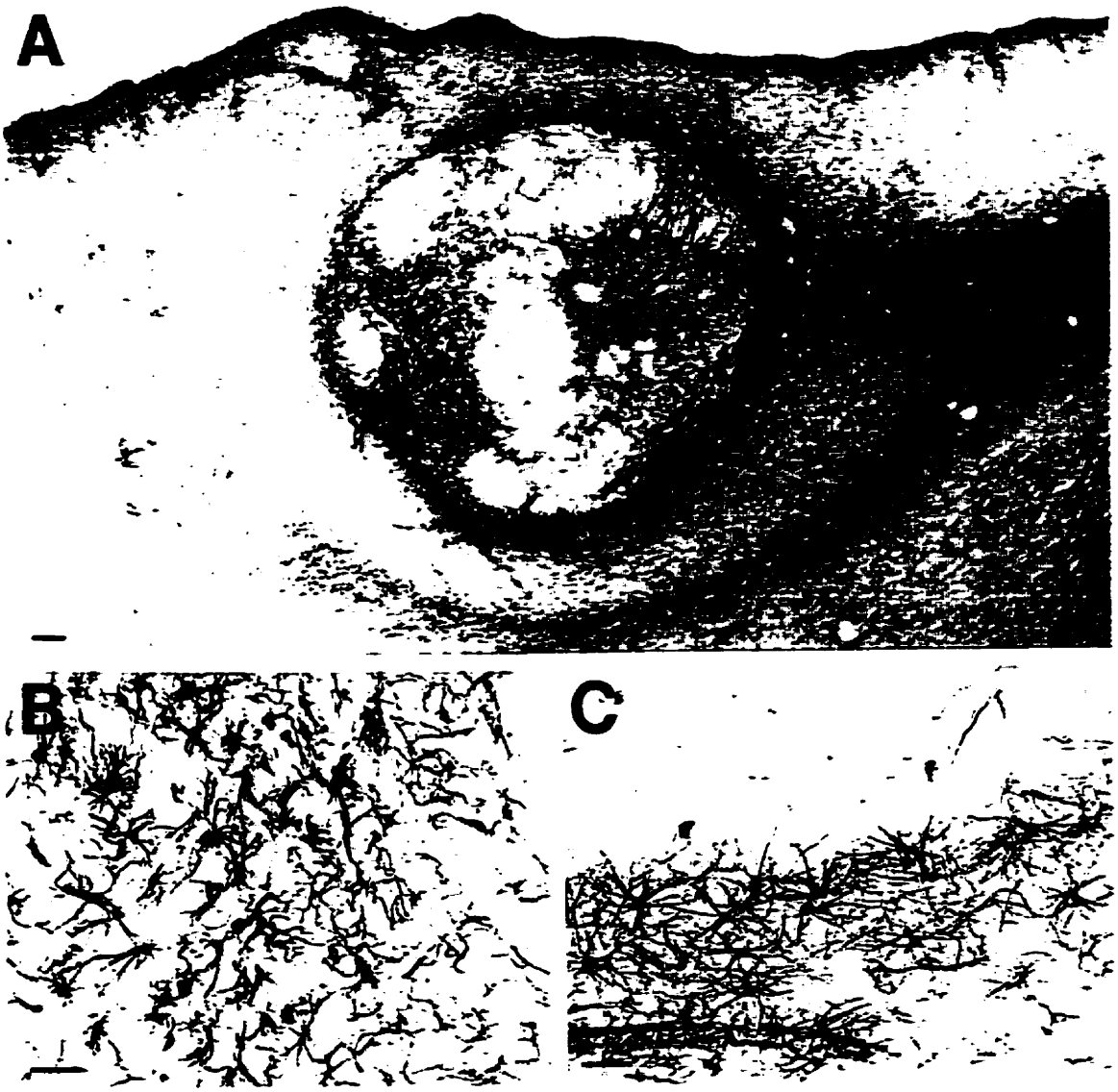

A
B
C

Figure 7. An adjacent section of the pAD 28 graft was stained for the neuron-specific marker NF 160. A. The graft is clearly demarcated within the host tissue. B. There is limited incursion of the host processes into the margin of the graft. C & D. Immunoreactive neuritic elements and occasional cell bodies (arrows) are found within the body of the graft. Scale bar represents 200 μm.

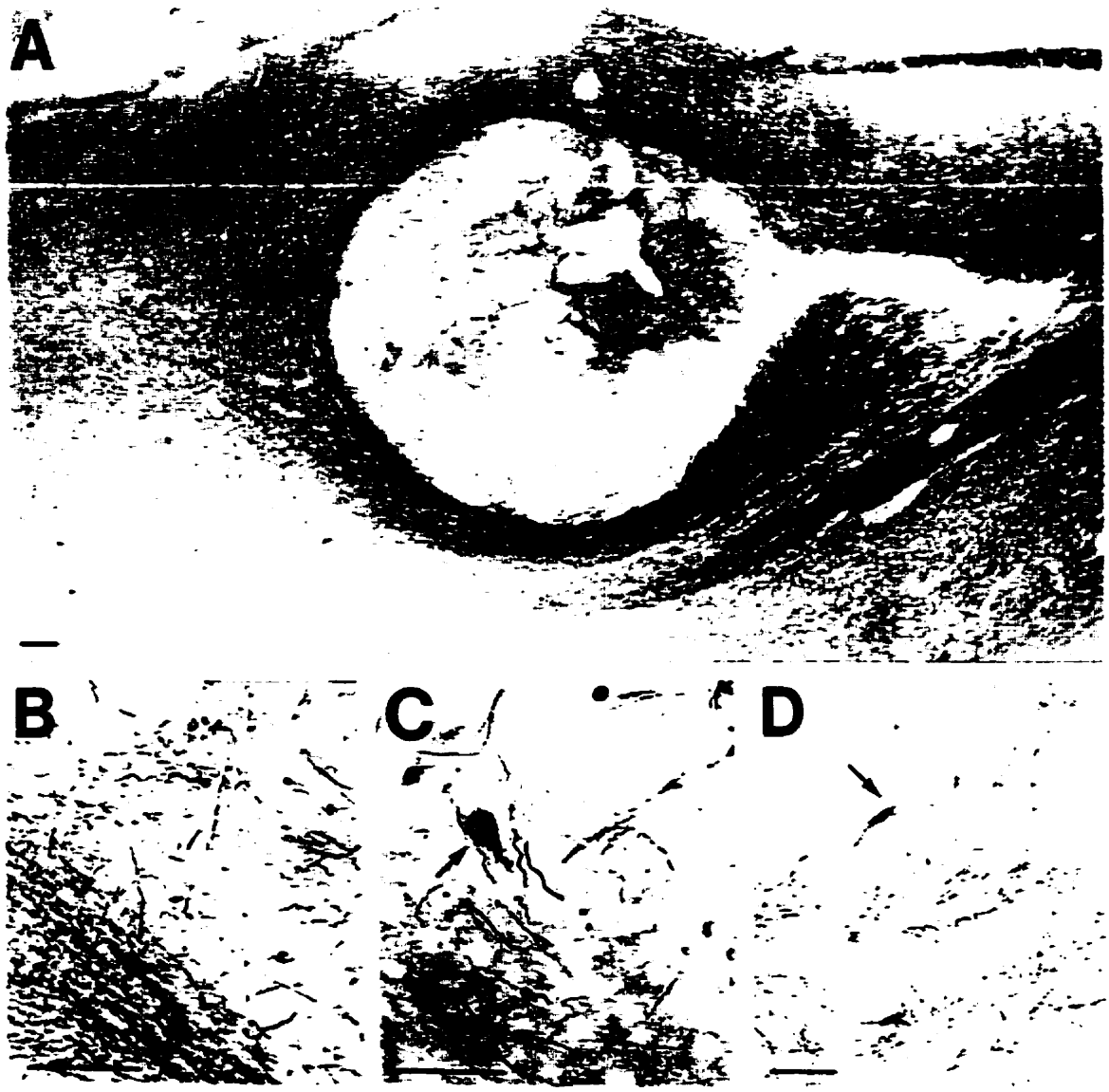
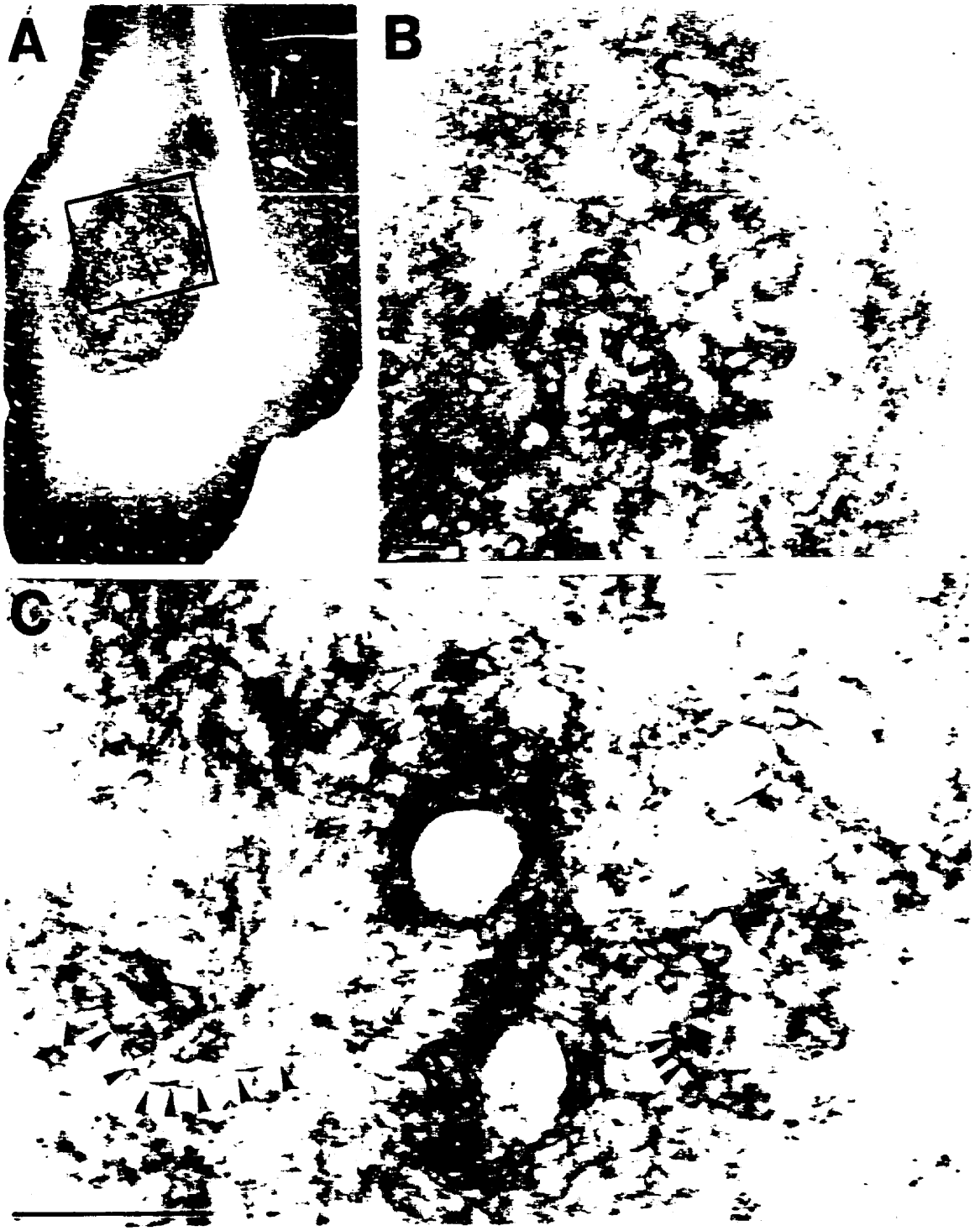

A
B
C
D

Figure 8. Immunoreactivity for APP was revealed in a formic acid-treated section of the pAD 28 graft. A. There is enhanced immunoreactivity within the graft relative to the adjacent host tissue. B. The staining was most intense around vascular elements infiltrating the graft. C. An enlarged view of the graft reveals diffuse cell body staining and immunoreactivity throughout numerous cell processes (outlined with arrowheads). Scale bar represents 200 μm.


A
B
C

Chapter 4

Aβ epitope immunoreactivity reveals putative proteolytic compartments and intracellular protein associations in neuroectodermally differentiated embryonal carcinoma cells expressing the human amyloid precursor protein

Susan M. Grant, Adriana Ducatenzeiler, Moshe Szyf, A. Claudio Cuello

Abstract

Intracellular processing of the amyloid precursor protein (APP) is believed to play a critical role in the development of Alzheimer's disease plaque. The trafficking of this protein has been inferred from numerous biochemical manipulations in a variety of cultured cell systems. We have used high resolution electron microscopy coupled with our specific monoclonal antibody, McSA1, to directly visualize those structures that are spontaneously associated with the Aβ epitope within mature neuroectodermally differentiated embryonal carcinoma (P19) cells. Immunoreactivity was intense in early endosomes. Immunoreactive deposits were also localised within the *trans*-Golgi network and in dilatated rough endoplasmic reticulum (ER), but were not found in lysosomes. Aβ immunoreactivity was associated with microtubules and filaments, with the outer mitochondrial membrane, and with the nuclear envelope. Our results are consistent with the biochemical data relating to β-secretase cleavage but, in addition, provoke speculation that the ER might harbor low-level γ-secretase activity of importance in the setting of retarded APP transit. The potential for intracellular fragments containing the Aβ epitope to engage in physiologically relevant interactions is suggested by our observation of immunoreactivity associated with specific organelles. Thus, our results expand on current data regarding APP trafficking and provoke intriguing questions about APP function.

Introduction

Progressive deposition of insoluble aggregates of the 39 to 43 amino acid β-amyloid (Aβ) peptide, derived from the proteolytic cleavage of the amyloid precursor protein (APP), gives rise to one of the pathological hallmarks of Alzheimer's disease. Although initially discovered in the context of senile accumulations, Aβ is now known to be a low abundance product of normal APP metabolism and, along with the non-amyloidogenic p3 peptide and the N-terminal ectodomains, is a soluble secreted fragment (Haass et al. 1992b; Seubert et al. 1992; Shoji et al. 1992; reviewed in Checler 1995).

The major APP processing pathway involves an N-terminal cleavage within the Aβ domain by an unknown protease activity, termed α-secretase. β-Secretase, the identity of which is also unresolved, preserves the intact Aβ domain within the C-terminal fragment so that subsequent γ-secretase cleavage within the transmembrane domain liberates the 4 kD Aβ peptide. β-Secretase cleavage is increased for APP proteins bearing mutations around either the α- or the β-secretase cleavage site (Cai et al. 1993; Citron et al. 1994; Haass et al. 1994a). Increased production of a longer, more fibrillogenic 42/43 amino acid form of Aβ has been linked to mutations in the APP protein adjacent to the γ-secretase site (Eckman et al. 1997; Suzuki et al. 1994), and to mutations in the presenilin 1 or presenilin 2 proteins (Borchelt et al. 1996; Tomita et al. 1997). Since inheritance of these mutated genes results in early onset Alzheimer's disease, the cellular processing of APP appears to be a critical determinant of this disease.

To study the intracellular processing of APP, we have developed a mAb highly specific to an epitope in the human Aβ sequence and applied it in a high resolution electron microscopic examination of neuroectodermally differentiated P19 cells stably transfected with the 751 amino acid isoform of human APP. After differentiation with retinoic acid, P19 neurons are known to upregulate and process APP in a manner analogous to primary neuronal cells *in vitro* (Hung et al. 1992). Robust APP expression persists in our transfected clones during long term culture providing a useful model to investigate secretase activity and Aβ epitope localisation in mature central nervous system-type cells.

Materials and Methods

Reagents

Unless otherwise specified, all tissue culture reagents were purchased from Gibco BRL (Burlington, Canada), and all laboratory chemicals from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Montréal, Canada). Protease inhibitors were purchased from Boehringer-Mannheim (Laval, Canada)and prepared as 100X frozen stocks. All SDS-PAGE was carried out in a Mini-Protean II gel apparatus using material and methods obtained from BioRad (Mississauga, Canada).

Generation of APP-transfected P19 clones

Murine embryonal carcinoma (P19) cells, obtained from M. McBurney of the University of Ottawa (McBurney & Rogers 1982), were routinely cultured in 10% fetal bovine serum (FBS) [ImmunoCorp, Montréal, Canada] in Dulbeco's modified Eagle's media (DME) supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, and prophylactic antibiotics (0.03 mg/ml kanamycin, 0.2 mg/ml streptomycin, and 0.02 mg/ml neomycin). Neuroectodermal differentiation was induced essentially as described (McBurney et al. 1988). Subconfluent monolayers of cells were dissociated with 10 mM EDTA and replated at 10^6 cells/10 ml media supplemented with 0.3 μM all-*trans* retinoic acid (Sigma) in bacterial grade petri dishes for 4 days with one change of the differentiation media after 48 h. At the end of retinoic acid treatment, the cell aggregates

were mechanically dissociated, plated on tissue culture plastic, and maintained with regular changes of 10% FBS/DME media.

Transfections were accomplished by calcium phosphate precipitation (Kingston et al. 1996). Plasmid pAD-751 (the gift of K. Beyreuther; Weidemann et al. 1989) contains the complete coding sequence plus approximately 200 base pairs of non-coding 3′ sequence of the 751 amino acid isoform of human APP under the transcriptional control of the cytomegalovirus promoter. 20 μg of pAD-751 was co-transfected with 2 μg of the dual reporter/selection plasmid pCMVlacZ II (American Tissue Culture Collection, Rockville, MD), which expresses both β-galactosidase and neomycin resistance under viral promoters. Control cells were transfected with pCMVlacZ II alone. Both APP-transfected clones, termed pAD, and control transfected clones, termed placZ, were selected with and then maintained, during routine passaging, in Geneticin® (Gibco BRL). pAD clones were analyzed by Southern blotting using an internal BamHI fragment of APP as the probe. Activity of the transgenes in selected pAD and placZ clones was confirmed by detection of β-galactosidase activity using X-gal histochemistry on differentiated cells (Lim & Chae 1989).

Generation of mAb McSA1

Synthetic $A\beta_{1-40}$ (Bachem, Torrance, CA) was conjugated to keyhole limpet hemocyanin (Boehringer-Mannheim) with glutaraldehyde and 15 or 25 μg of peptide, emulsified in Freund's adjuvant (Difco, Detroit, MI), was used to immunize Balb/c female mice by multiple site intracutaneous and intraperitoneal injection. All animals

developed antibody responses and one animal in the 15 μg group was selected for subsequent intrasplenic boost and fusion with the non-producing mouse myeloma cell line Sp_2/O.Ag, essentially as described (Cuello & Côté 1993; Köhler & Milstein 1975). Supernatants of the resulting clones were screened for immunoreactivity to senile plaque in sections of frontoparietal cortex from an Alzheimer's disease brain. A highly positive clone was subcloned by limiting dilution and the subclone McSA1 was chosen for subsequent analyses. McSA1, an IgG1, κ chain immunoglobin (Isostrip kit, Boehringer-Mannheim), was prepared as a 25-fold concentrate of hybridoma supernatant (Minicon Concentrator, Amicon, Beverly, MA) for the applications reported herein.

Comparative ELISA

Initial experiments were done to determine the antibody and antigen dilutions to be used in subsequent comparative assays. Microassay plates were coated with antigen dilutions in PBS pH 7.4 and adsorbed overnight at 4°C. Unoccupied binding sites were blocked with 3% BSA in PBS for 1 h at room temperature. Subsequently, McSA1, diluted 1:750 in PBS, was allowed to incubate for 1 h at room temperature. After extensive washing with PBS, wells were incubated for 1 h at room temperature with a HRP-conjugated goat anti-mouse IgG affinity-purified antiserum (Jackson ImmunoResearch, Bio/Can Scientific, Mississauga, Canada) diluted 1:5000 in PBS. Wells were extensively washed with PBS and bound peroxidase activity was detected colorimetrically with 2,2azinobis(3-ethylthiazline)sulfonic acid. Competition assays were performed to map the McSA1 epitope and to determine potential cross-reactivity with other proteins of interest. McSA1 1:750 was mixed with various concentrations of

peptides comprising residues 1-12, 10-20, 12-28, 25-35 (American Peptide Company, Sunnyvale, CA), or 1-40 of the Aβ sequence for 1 h at 37°C before being applied to wells precoated with 300 ng/ml $A\beta_{1-40}$. Similarly, McSA1 1:750 was preincubated for 1 h at 37°C with various concentrations of bovine serum albumin (BSA), human IgG, ubiquitin or human recombinant apolipoprotein E (Calbiochem, La Jolla, CA), or the brain peptides substance P (Peptide Institute Inc., Osaka, Japan) or neurokinin A (Peninsula Laboratories Inc. Belmont, CA) prior to its use in $A\beta_{1-40}$ precoated wells. To determine the specificity of McSA1 for the human peptide, serial dilutions of synthetic human $A\beta_{1-40}$ or rodent $A\beta_{1-40}$ (Quality Controlled Biochemicals Inc., Hopkinton, MA), which differs from the human sequence at residues Gly^5, Phe^{10}, and Arg^{13}, were applied and detected as described above.

Immunocytochemistry

Light Microscopy

Blocks of formalin-fixed frontoparietal cortex from a 64 year old female Alzheimer's disease patient (11 h post-mortem delay) were cryoprotected in 30% sucrose overnight then cut into 50 μm thick sections on a freezing sledge microtome. Sections were permeabilized for 30 min in PBS + 0.2% Triton X-100 (PBS+T) followed by a 15 min incubation in 88% formic acid. After extensive washes, sections were blocked with 3% BSA in PBS for 1 h at room temperature. McSA1 1:1500 in PBS was incubated with the tissue overnight at 4°C. After further washes the sections were incubated with the secondary antibody, HRP-conjugated goat anti-mouse IgG affinity-purified antiserum

(Jackson ImmunoResearch) diluted 1:2000 in PBS, for 1 h at room temperature. Following additional washes, immunoreactivity was detected using 0.06% DAB in the presence of 0.01% hydrogen peroxide. The immunocytochemistry was repeated using McSA1 preincubated with competitor peptides or proteins as described above. To assess the relative affinity of the antibody for its epitope presented either as the cleaved Aβ fragment or as the intact holoprotein, recombinant APP was prepared by *in vitro* transcription/translation (TNT Coupled Reticulocyte Lysate System, Promega, Madison, WI). Dilutions of McSA1 (1:1500, 1:3000, 1:4500 and 1:6000) reaching and exceeding the limit of detection of amyloid plaque were preincubated for 1 h at 37°C with aliquots of the TNT reaction. The detection limit of preincubated antibody was then compared with that of McSA1 alone as described above.

Immunoreactivity in neuroectodermally differentiated P19 clones was also evaluated using McSA1 and an N-terminal anti-APP monoclonal antibody (Anti-Alzheimer precursor protein A4, clone 22C11, Boehringer-Mannheim). At the completion of retinoic acid-induced differentiation, pAD and placZ clones were plated on poly-l-lysine coated coverslips and grown for 2 weeks in 10% FBS/DME media. After 2 brief washes in PBS the cells were fixed for 20 min at room temperature in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 supplemented with 200 mM sucrose. The cells were washed extensively in PBS, blocked for 1 h in 5% horse serum in PBS+T, and incubated overnight with mAbs 22C11 1:300 or McSA1 1:200 in PBS. Staining was completed using the Elite ABC kit (Vector, Burlington, CA) according to the manufacturer's directions with DAB/hydrogen peroxide detection.

Electron microscopy

Mature (1-2 month old) cultures of neuroectodermally differentiated P19 clones were prepared for preembedding immunogold electron microscopy as described with some modifications (Pickel et al. 1993). After several PBS washes, cells were fixed for 30 min at room temperature with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 supplemented with 50 mM sucrose and 0.4 mM calcium chloride. After fixation, cells were incubated with 3 changes of buffer I (1% BSA, 1% FBS, 0.2% saponin, 0.02% sodium azide in PBS) over 10 min. McSA1 1:200 in PBS supplemented with 50 mM sucrose and 0.1% BSA was incubated with the cells overnight at 4°C. To control for non-specific staining, one set of pAD cultures was incubated with dilution buffer only and one set with the mAb 4G8 1:500 (Senetek PLC Senescence Technology, Maryland Heights, MO) [Kim et al., 1990], which recognizes residues 17-24 of the Aβ sequence. Cells were washed extensively with 0.01 M PBS, reblocked for 10 min in buffer II (0.1% gelatin, 0.5% BSA in 0.01M PBS), and then incubated for 2 h at room temperature with 1 nM gold-conjugated goat anti-mouse IgG (British BioCell, Cedar Lane Laboratories, Hornby, Canada) diluted 1:50 in buffer II. After one wash in buffer II and 3 washes in 0.01 M PBS, the cells were refixed for 10 min at room temperature with 2% glutaraldehyde in 0.01 M PBS. Silver intensification of the immunogold deposit was then performed as follows. Cells were washed 3 times in 0.01 M PBS and once in 0.2 M citrate buffer pH 7.4. The silver reagent (IntenS EM Silver Enhancement Kit, Amersham, Oakville, Canada) was prepared according to manufacturer's directions and added to the cells with constant agitation for exactly 10 min. After further washes with 0.2 M citrate buffer followed by 0.1 M phosphate buffer, the cells were dehydrated, osmicated and Epon embedded. Ultrathin sections were cut and counterstained with uranyl acetate and lead citrate. Grids were viewed with a Philips 410 transmission electron microscope.

Immunoprecipitation of APP fragments

Neuroectodermally differentiated clones were plated in 30 mm dishes and grown under conditions identical to those described for the electron microscopy series. After 4 weeks, cell monolayers were washed 3 times with PBS and then metabolically labeled for 16 h with 100 μCi of ^{35}S-methionine (Amersham) in 1 ml of 10% FBS/DME media lacking methionine or cysteine. The conditioned media was collected, chilled, and clarified by centrifugation before being supplemented with CM buffer (final concentrations: 50 mM Tris HCl pH 7.4, 1% Triton X-100, 0.1% SDS, 50 mM sodium orthovanadate, 10 μM leupeptin, 10 mM benzamidine, 2 μg/ml aprotinin, 1 mM PMSF). Monolayers were washed 3 times with PBS then lysed in 500 μL RIPA buffer (50 mM Tris HCl pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 10 mM sodium fluoride, 30 mM sodium pyrophosphate, 50 μM sodium orthovanadate, 10 μM leupeptin, 10 mM benzamidine, 2 μg/ml aprotinin, 1 mM PMSF). The lysates were incubated on ice for 30 min then centrifuged at 15,000 g for 15 min at 4°C. The supernatants were collected for subsequent immunoprecipitation.

Cell lysates and conditioned media were processed in parallel with all procedures carried out on a rocking platform at 4°C, unless otherwise indicated. Samples were precleared by incubation for 3 h with 2 μl of normal rabbit serum and 50 μl of protein A-agarose (Affi-Gel, BioRad). Nonspecifically bound material was removed by brief centrifugation to pellet the agarose and the cleared supernatants were transferred to new tubes. The secreted N-terminal ectodomain of APP was immunoprecipitated from conditioned media with 0.5 μg of mAb 22C11, and the intracellular C-terminal fragments

were precipitated from the cell lysates with 5 µl of a rabbit antiserum generated against the C-terminal of APP (gift of K. Beyreuther). After 2 h, 5 µg of rabbit anti-mouse IgG (Sigma) was added to the conditioned media preparation to facilitate capture of the mouse IgG by protein A. After incubation of both preparations overnight, immune complexes were precipitated with 50 µl of protein A-agarose for 3 h. The agarose was pelleted by brief centrifugations and washed twice in buffer A (10 mM Tris HCl pH 8.0, 150 mM sodium chloride, 0.025% sodium azide) plus 0.1% Triton X-100, once in buffer A without detergent, and once in buffer B (50 mM Tris HCl pH 6.8). The final wash buffer was aspirated and 30 µl of loading buffer was added to the agarose pellet which was immediately boiled for 5 min, cooled, vortexed, and recentrifuged in preparation for gel loading. Precipitated N-terminal fragments were recovered in 2X Lammeli sample buffer and separated in a 10% SDS-PAGE gel. The gel was fixed for 30 min in 5% glutaraldehyde prepared in 0.4 M sodium borate/phosphate buffer, pH 6.2, treated with EN^{3}HANCE (DuPont NEN, Guelph, Canada), dried, and exposed to Kodak X-OMAT film at -80°C for 3 days. Precipitated C-terminal fragments were recovered in Tris/tricine sample buffer and separated in a 16.5% Tris/tricine SDS-PAGE gel according to Klafki et al. (1996b). The proteins were transferred to a 0.1 µm polyvinylidene fluoride membrane (Immobilon P^{SQ}, Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, 10% methanol. The dried membrane was exposed to Hyperfilm-βmax (Amersham) at -80°C for 2 weeks.

Analysis of APP expression

Western Analysis

Monolayers of undifferentiated cells or neuroectodermally differentiated cells at various times after retinoic acid treatment were washed 3 times with PBS, dissociated with 10mM EDTA, and collected by low speed centrifugation. The pellets were incubated on ice with lysis buffer (50 mM Tris HCl pH 7.6, 2% NP-40, 150 mM sodium chloride, 0.01% sodium azide, 10 mM sodium fluoride, 30 mM sodium pyrophosphate, 20 mM iodoacetamide, 50 μM sodium orthovanadate, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM PMSF) for 30 min and then centrifuged at 15,000 g for 15 min at 4°C. Aliquots of the supernatant were analyzed for total protein content by the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce, Rockford, IL) and 15 μg of protein were boiled in 2X Lammeli sample buffer prior to loading in a 7.5% SDS-PAGE gel. Proteins were separated by overnight electrophoresis at 30V at 4°C then transferred to a nitrocellulose membrane (BioRad) in 25 mM Tris, 192 mM glycine, 20% methanol. After blocking overnight with 5% skim milk powder in TBS with 0.1% tween 20 (TBS+T), the membrane was incubated for 1 h at room temperature with a mouse ascites preparation of mAb 22C11 (gift of K. Beyreuther) diluted 1:10,000 in TBS+T. Following extensive washes in TBS+T, the membrane was incubated for 1 h at room temperature with a neat preparation of the bispecific monoclonal antibody McC10 (anti-mouse, anti-HRP; Kenigsberg & Cuello, 1990) preincubated with 5 ng/ml horseradish peroxidase. The membrane was washed extensively in TBS+T before detection of the immunoreactive bands by chemiluminescence (ECL Kit and Hyperfilm-ECL, Amersham) according to the manufacturer's directions.

Northern Analysis

mRNA was isolated from undifferentiated cells and from cells cultured as described above for 4 weeks after retinoic acid treatment by the guanidine thiocyanate method of Chomczynski and Sacchi (1987). 15 μg of total mRNA was separated in a 1.2% formaldehyde agarose gel and transferred to Hybond N+ membranes (Amersham) in 10X SSC. The APP transcript was detected with a 459 bp cDNA fragment labeled with $^{32}\alpha$P-dCTP (Random Primed DNA labeling kit, Boerhinger-Mannheim). The membrane was hybridized overnight at 42°C in 10% dextran sulfate, 50% formamide, 1 M sodium chloride, 1% SDS, 0.2 mg/ml denatured salmon sperm DNA, washed twice for 5 min at room temperature in 2X SSC and once for 20 min at 65°C in 2X SSC, 1% SDS before being exposed to Kodak X-OMAT film at -80°C. Equal loading was confirmed by visualization of 18S rRNA using a $^{32}\gamma$P-GTP-labeled (T4 kinase, United States Biochemical, Cleveland, OH) 24mer oligonucleotide as described (Szyf et al., 1990).

Results

Characterization of mAb McSA1

Antisera and clones were initially screened by immunocytochemistry in Alzheimer's disease brain sections to ensure that the antibody would detect Aβ in a 'native' state. Indeed, McSA1 localises both diffuse and neuritic plaque (Fig. 9A panels *b* and *c*) and cerebrovascular amyloid (data not shown) in formic acid-treated sections of affected cortex. The staining is abolished when the antibody is preincubated with competitor peptide (Fig. 9A panel *a*). McSA1 also recognizes soluble, synthetic Aβ as demonstrated by its activity in ELISAs. Because the original antigen comprised the entire 40 amino acid peptide sequence, we were interested in determining the specific residues involved in antibody recognition. McSA1 was preincubated with 10-fold dilutions of peptides corresponding to overlapping residues of $A\beta_{1-40}$, as well as with the complete peptide. As expected, the original antigen, $A\beta_{1-40}$, effectively competed for binding (Fig. 9B). The peptide encompassing residues 1-12 of Aβ was also an effective competitor; none of the other peptides exhibited any activity in this assay. As well, no cross-reactivity was seen for any of the proteins examined by competition in immunocytochemical or ELISA assays (data not shown).

Since P19 cells express endogenous murine APP after differentiation, it was of interest to determine the cross-reactivity between human and rodent sequences. Each peptide was prepared in serial dilutions and subject to an indirect ELISA with McSA1 (Fig. 10C). Within the sensitivity of the assay, McSA1 is highly specific for the human sequence. This result confirms the epitope mapping data because the amino acid

differences between the two sequences reside in 3 of the first 13 residues. It is likely, therefore, that either or both of Arg^5 and Tyr^{10} in the human Aβ sequence is (are) critical for antibody recognition.

APP expression in transfected P19 clones

APP expression is known to be regulated at multiple levels including by transcriptional activation in response to cellular stress (Solà et al. 1993), by cell-specific alternative splicing (LeBlanc et al. 1991), by alternative polyadenylation and translational regulation (de Sauvage et al. 1992), by complex post-translational processing (Weidemann et al. 1989), and by regulated degradation in the secretory processing pathway (Nitsch & Growdon 1994). APP mRNA could be detected in the pAD clones prior to differentiation, but was greatly upregulated by retinoic acid-induced differentiation. (Fig. 10A, compare lanes 3 and 4). Similarly, APP protein levels were only slightly increased in undifferentiated pAD clones. Neuroectodermal differentiation was permissive for expression of the transgenic protein as well as the endogenous protein, and APP expression remained robust in pAD clones for at least 1 month after terminal differentiation (Fig. 10B). The neuroectodermal progeny of P19 cells include cells with neuronal, glial, and fibroblast-like phenotypes (Jones-Villeneuve et al. 1982). This mix was reflected in the isoforms of endogenous APP expressed in differentiated cells. The evolution of the cultures from predominantly neuronal at 3 days to increasing numbers of maturing, proliferation-capable glial and fibroblastic types after 1 week in culture is mirrored by the shift in intensity from the ~95 kD band corresponding to the 695 amino acid neuronally-expressed isoform to the ~100 and 110-115 kD bands

corresponding to the immature and mature 751 and 770 amino acid isoforms expressed in non-neuronal cells (Fig. 10B lanes 2-4 and 6-8).

Immunocytochemistry was performed on differentiated clones after 2 weeks in culture using 2 different antibodies. The mAb 22C11 recognizes an area between residues 66 and 81 of the N-terminal domain of human and rodent APP, as well as the related protein APLP2 (Chauvet et al. 1997). Compared with controls in which the primary antibody was omitted, weak immunoreactivity was present in the placZ 12 clone (Fig. 10C panels *a* and *b*, respectively) and strong diffuse immunoreactivity was seen in the transfected clone pAD 18 (Fig. 10C panel *c*). As expected, based on the ELISA results, McSA1 did not stain the control clone (Fig. 10 panel *e*) but strong, primarily perinuclear staining was seen in the human APP-expressing clone pAD 34 (Fig. 10C panel *f*).

We found abundant soluble APP α-secretase N-terminal immunoreactive fragments in conditioned media from APP-transfected clones (Fig. 10D). Similarly, immunoprecipitation of cell lysates with C-terminal specific antisera yielded a prominent band of ~11.5 kD likely corresponding to the α-secretase C-terminal fragment (Fig. 10E). Sensitive 2-site ELISAs have demonstrated that P19 cells do generate Aβ peptides (Ho et al. 1996), but our immunoprecipitation studies, albeit performed with a relatively small number of cells, would suggest that β-secretase cleavage remains a minor pathway in P19 cells transfected with wild-type human APP, confirming the fidelity of normal APP processing in overexpressing clones.

Ultrastructural localisation of Aβ immunoreactivity

The McSA1 dilution of 1:200 provided specific labeling of cells transfected with human APP under the more stringent fixation required for preservation of ultrastructural detail. Occasional non-specific reactions were observed in placZ clones at higher concentrations of McSA1. In the absence of primary antibody there was no labeling of any structure (Fig. 11 *a* and *b*). Both mAbs McSA1 and 4G8, specific for non-overlapping epitopes of Aβ, revealed immunoreactive material within dilatated ER, which was present in numerous cells (Fig. 11 *c-h*).

Immunoreactivity was also localised to vesicles in the perinuclear compartment. In the absence of techniques to demonstrate specific biochemical markers, the nature of these vesicles cannot be definitively determined. On morphological criteria, we interpret the vesicles in Fig. 12*a* to represent the *trans*-Golgi network or endosomes/primary lysosomes. The vesicles in Fig. 12*b* are clearly part of the *trans*-Golgi network. In Fig. 12*c*, the immunoreactive material appears to be deposited within the lateral compartment of *trans*-Golgi cisternae.

Although immunoreactivity was commonly found in single deposits, aggregates were the rule in clathrin-coated vesicles located in peripheral regions of occasional cells (Fig. 13). Despite extensive searching, Aβ immunoreactivity could not be found in larger, more centrally located vesicles (prelysosomes) or secondary lysosomes to which endosomes might deliver the peptide for degradation.

Aβ immunoreactivity could be consistently localised to several organelles or cytoskeletal elements as depicted in Figs. 14 and 15. Immunoreactive deposits were frequently found associated with the outer mitochondrial membrane (Fig. 14 *b* and *c*). Immunoreactivity was associated with microtubules which may reflect transport of APP

fragments in secretory vesicular traffic. Vesicles, *per se*, were not seen although it is possible that the silver depositing around the gold-antibody complex obscured smaller structures (Fig. 14 *d*). Filaments, particularly those adjacent to filament bundles, were also labeled with immunoreactive deposits (Fig. 14 *e*). Immunoreactivity was rarely seen within the nucleus or along nuclear membranes in profile. However, when the nuclear membrane was in the plane of section, immunoreactive deposits dotted the surface in amongst the nuclear pores (Fig. 15 and inset).

Discussion

The pathways involved in the processing of APP and the generation of Aβ have been the subject of intensive investigation. Most work to date has relied on biochemical techniques to distinguish functional compartments of particular relevance to α-, β-, or γ-secretase activities. We have used a highly specific mAb coupled with high resolution electron microscopy to directly visualize intracellular compartments where the epitope is revealed, and to localise cell structures with which Aβ immunoreactivity is spontaneously associated. Using this approach, we find Aβ immunoreactivity within the *trans*-Golgi network, early endosomes, and dilatated ER. In addition, immunoreactive deposits are associated with microtubules, filaments, mitochondria, and the nuclear envelope. No staining of lysosomes is seen with our antibody.

It is likely that important differences in the dynamics of protein processing exist between proliferative, clonal cell lines and cells that are committed to a differentiated state. When P19 cells undergo retinoic acid-induced differentiation there are profound morphological changes and *de novo* expression of phenotype-specific proteins including structural proteins (e.g., neurofilament) [Paterno et al. 1997], synthetic enzymes (e.g., glutamic acid decarboxylase) [Staines et al. 1994], receptors (e.g., Trk family members) [Salvatore et al. 1995] and proteases (e.g. convertases) [Bolduc et al. 1997; Kobayashi et al. 1996]. APP is also upregulated in differentiated P19 cells (Yoshikawa et al. 1990) where it has been shown by others to undergo metabolism via α-, β-, and γ-secretase activities (Fukuchi et al. 1996a; Ho et al. 1996). Neuroectodermally differentiated P19 cells are, therefore, a useful model to investigate sites wherein the Aβ epitope is

generated, as per our study, and to assist in the identification of the secretase enzymes and in the study of intracellular APP-protein interactions, both of which are critical issues in Alzheimer's disease research.

The antibody we have generated for this study is highly specific to an epitope in the human Aβ sequence. Several observations lead us to believe that the observed immunoreactivity does not represent the intact holoprotein. No staining of human tissue was seen in the neuropil surrounding amyloid deposits. The staining patterns of mAb 22C11 (APP N-terminal specific) and McSA1 were distinct in our transfected cells (Fig.10C). When limiting dilutions of McSA1 were preincubated with APP prepared by *in vitro* transcription/translation, there was no discernible difference in the sensitivity toward Aβ deposits when compared with non-competed McSA1 (data not shown). At the electron microscopic level, we did not observe staining within the Golgi stacks or at the plasma membrane, two sites of strong immunoreactivity with APP antisera (Culvenor et al. 1995; Palacios et al. 1992; Yamazaki et al. 1993). Other antibodies directed against the region of Aβ that is recognized by McSA1 have also been reported not to recognize the holoprotein (Hyman et al. 1992; McGeer et al. 1992; Wolozin et al. 1992; Wong et al. 1985), a result that may relate to the proximity of this site to the membrane or to folding or post-translational modification of this region within the protein. It is important to note, however, that we cannot determine whether the McSA1 immunoreactivity represents the Aβ peptide only, or N- or C-terminal fragments in which the epitope has been revealed by secretase cleavage.

Aβ immunoreactivity was found in the *trans*-Golgi network, an acidic tubulovesicular compartment believed to serve as a staging area for the exocytic pathway in which the sorting and packaging of proteins are accomplished (Anderson & Pathak

1985; Farquhar & Palade 1981). Another of the post-translational processing functions of the Golgi apparatus is the conversion of proproteins destined for secretion, such as proinsulin, proalbumin, and proopiocortin, to mature forms (Farquhar & Palade, 1981). The responsible proteases are poorly localised and it is believed they encounter their substrates during transit of the precursor through the Golgi complex and become co-packaged into secretory vesicles. When Golgi trafficking is disrupted with brefeldin A (Busciglio et al. 1993a; Haass et al. 1993) or a dominant negative Rab1B mutant (Dugan et al. 1995), both the maturation and cleavage of APP are inhibited indicating that transit through this compartment is a necessary step for the eventual localisation of the protein to the secretase-containing compartments. However, two studies have found that at least a portion of β-secretase activity is resistant to manipulations of *trans*-Golgi function suggesting that exposure to this β-secretase enzyme pool might involve aberrant or alternate sorting processes (Martin et al. 1995; Tienari et al. 1997). It is tempting to suggest that our observation of *trans*-Golgi immunoreactivity might be indicative of that portion of APP trafficked to immediate β-secretase cleavage.

α-Secretase activity occurs in the default exocytic pathway. Constitutive APP cleavage occurs during transit along this pathway with α-secretase fragments reported in extracts of white matter from rodent and human brain (Sapirstein et al. 1994; Tokuda et al. 1994) and within vesicles isolated from human neuroglioma cells (Kuentzel et al. 1993). APP delivered intact to the plasma membrane is also subject to α-secretase cleavage as demonstrated by cell surface labeling techniques (Koo et al. 1996; Lo et al. 1994) and by enhanced p3 and soluble ectodomain secretion for APP mutants incapable of endocytosis (Koo et al. 1996; Koo & Squazzo 1994; Tienari et al. 1997). The immunoreactivity we observed in association with microtubules may reflect secretory

transport of N-terminal α-secretase fragments bearing the McSA1 epitope; our antibody would be unable to detect α-secretase C-terminal fragments or the p3 peptide.

There is convincing evidence that an important site of sequential β- and γ-secretase activity is the early endosome, shortly after internalization of intact cell-surface APP, with subsequent recycling back to the cell surface and Aβ release (Haass et al. 1993; Koo & Squazzo 1994; Peraus et al. 1997). The dense immunoreactivity we observed in some coated vesicles found in peripheral zones, and the demonstration of clathrin-coated pits at the cell membrane, support the interpretation that APP had undergone trafficking to the cell surface followed by endocytosis and β-secretase cleavage. Our results directly support the proposal that the early endosome is an APP proteolytic compartment. It should be noted that the endosomes depicted in Fig. 13, panels b-e, were localised to relatively few cells. As retinoic acid-treated P19 cells mature into a heterogeneous mix of phenotypes, it may be that the propensity for or rate of endocytosis and recycling vary with cell type. The cell depicted in Fig. 13a was more typical of the majority of neuroectodermal progeny. Here Aβ immunoreactivity was found on the rim of a cell surface invagination adjacent to a coated pit in the process of forming. We suggest this photomicrograph depicts the release of APP cleavage products, possibly from an endosome being recycled to the cell surface.

Even in those cells with abundant immunoreactive endosomes, we were unable to localise Aβ immunoreactivity to larger perinuclear vesicles (late endosomes or prelysosomes) or to secondary lysosomes. Whether Aβ is generated in lysosomes is controversial. For example, lysosomatropic agents and lysosomal enzyme inhibitors do not consistently enhance the co-localisation of β-secretase fragments to the lysosomal

compartment (pro: Golde et al. 1992; Siman et al. 1993; con: Busciglio et al. 1993a; Haass et al. 1993; Higaki et al. 1995). With respect to our results, Aβ derived from wild type APP is known to exhibit N-terminal heterogeneity from the use of alternative cleavage sites or from post-cleavage pruning (Citron et al. 1994; Yamaguchi et al. 1998); hence, it is possible that the McSA1 epitope was rapidly lost within the lysosomes.

The recent discovery that presenilin proteins interact specifically with the immature N-glycosylated form of APP indicates that one of the events predisposing to Alzheimer's disease might occur at a very early step in APP processing (Weidemann et al. 1997; Xia et al. 1997). Presenilins 1 and 2 have been localised to the ER (Kovacs et al. 1996), but how their interaction with immature APP in this compartment influences subsequent γ-secretase cleavage has yet to be determined (reviewed by Haass 1997 and Kim & Tanzi 1997).

It was very interesting to find that many of the cells stably expressing APP had extremely dilatated ER reminiscent of cells naturally specialized for production and secretion of a single protein product, e.g., plasma cells or gonadotrophs. The dilatated ER was invariably immunoreactive for Aβ epitopes as demonstrated by two non-overlapping monoclonal antibodies. No deposits were seen in the absence of a primary antibody ruling out non-specific 'sticking' to the proteinacious contents. We speculate that prolonged residence of APP in the ER may facilitate cleavage by a protease that otherwise has low activity toward wild-type APP, thereby changing the topology of the protein with downstream effects on trafficking. The common 'gain of function' effect from the myriad presenilin mutations may, therefore, be to delay ER exit of nascent APP.

In support of this notion, Cook and colleagues (1997) have reported that manipulations which prevent protein transport from the ER have no effect on the intracellular production of $A\beta_{1-42}$, implying that γ(42)-secretase cleavage occurs within the ER. Also consistent with this view, co-transfection of mutant (but not wild-type) presenilin 1 with wild-type APP had the dual effect of increasing production of $A\beta_{1-42}$ and reducing secretion of the α-secretase N-terminal product (Ancolio et al. 1997). It is noteworthy that mutant APP, truncated after the 51st, but not the 40th, amino acid from the Aβ start site, is still a substrate for α- and β-secretase cleavage (Citron et al. 1995). If the scenario we propose is true, it might be possible to identify and selectively inhibit the putative ER-resident γ(42)-secretase, thereby reducing the production of the fibrillogenic Aβ(42/43) peptide and delaying the onset of amyloidosis (Iwatsubo et al. 1994; Kisilevsky & Fraser 1997).

The demonstration of Aβ immunoreactivity associated with specific organelles and cytoskeletal elements was another intriguing aspect of our study. The function of APP has yet to be clearly elucidated (reviewed by Mattson 1997), but most studies have focussed on a role for the holoprotein at the cell surface or of the secreted ectodomain within the extracellular matrix. No role has been ascribed to the p3 peptide and Aβ is generally regarded as a toxic byproduct of APP metabolism. It is notable, therefore, that human neurons (Simons et al. 1996) and astrocytes (Busciglio et al. 1993a) have an intrinsic capacity to catabolize APP in an amyloidogenic manner, and that intracellular Aβ has been found in neuroblastoma cells (Fuller et al. 1995; Xu et al. 1997), neuronally differentiated human teratocarcinoma cells (Cook et al. 1997; Turner et al. 1996; Wertkin et al. 1993), and cultured hippocampal neurons after viral transfer of the APP cDNA (Tienari et al. 1997). In fact, there is an expanding list of proteins interacting with motifs

in the C-terminal of APP including the heterotrimeric G_o protein (Nishimoto et al. 1993), the phosphotyrosine binding domain proteins FE65 and protein X11 (Borg et al. 1996; Guénette et al. 1996; McLoughlin et al. 1996), and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Schulze et al. 1993].

An interaction between the cytoskeleton and APP, apparently mediated through the C terminus, has been found previously (Allinquant et al. 1994; Refolo et al. 1991). The appearance of Aβ immunoreactivity on the nuclear membrane has also been reported before in biopsied human muscle (Zimmermann et al. 1988), APP cDNA-infected Sf9 cells (Currie et al. 1991) and mammalian cells transfected with, respectively, an Aβ minigene (Johnstone et al. 1996) or a C-terminal construct (Maruyama et al. 1990) both lacking a signal peptide. We can draw no conclusions regarding the physiological significance, if any, of these associations except to note that many of the transfected cells analyzed by EM had dysmorphic nuclei (see Fig. 11 *c*).

Whereas spurious associations arising from protein overexpression must always be kept in mind when analyzing transfected models, increased APP expression in individuals with trisomy 21 is invariably associated with Alzheimer's disease-type pathology by mid-life lending support to our approach of examining APP metabolism in aged cultures of overexpressing clones. Importantly, our transfectants expressed wild-type APP in keeping with the fact that the vast majority of Alzheimer's disease patients do not have APP mutations. Higher protein concentrations were a necessary prerequisite of our EM analysis of the products of APP metabolism, but the relative activities of α- and β-secretase in mature, neuroectodermally differentiated P19 cells do not appear to be changed in the face of APP overexpression.

P19 cells transfected with APP have been reported to deteriorate rapidly after neuroectodermal differentiation (Yoshikawa et al. 1992) and, indeed, substantial cell death was observed immediately after retinoic acid treatment in both pAD and placZ clones. However, the cells examined in our studies had been maintained in culture for 1 to 2 months. Enhanced APP expression is compatible with cell viability as the current transgenic models (e.g., Czech et al. 1997; Games et al. 1995; Sturchler-Pierrat et al. 1997) and our own cultures demonstrate. The ability to keep differentiated pAD clones in culture for prolonged periods may, in fact, facilitate the expression of processing changes or protein associations predisposing to Alzheimer's disease-type pathology. For example, in neuronal progeny of differentiated human teratocarcinoma cells, β-secretase cleavage became more pronounced over time (Turner et al. 1996). In this regard our approach may have advantages over those studies employing non-central nervous system-type cells or transient transfections. McSA1 is highly specific for its epitope and permits the direct visualization of potential sites of proteolysis or peptide associations within the cell, although it may not discriminate the context of epitope presentation after initial cleavage of the holoprotein. Nonetheless, the results presented here are a valuable addition to the current biochemical and light microscopic analyses of APP processing, and provoke intriguing questions regarding intracellular interactions of potential significance to APP trafficking and function.

Figure 9. Characterization of mAb McSA1 by immunocytochemistry in Alzheimer's disease cortex and by comparative ELISA. (A) 50 μm formic acid-treated sections of frontoparietal cortex from a 64 year old Alzheimer's disease patient were incubated with McSA1 and Aβ immunoreactive deposits visualized by peroxidase-activated DAB deposition. (a) McSA1 staining was abolished by preincubation with 300 ng/ml of a peptide corresponding to residues 1-12 of the Aβ sequence. (b & c) low and high magnification views reveal the localisation of diffuse and neuritic plaque by McSA1 with negligible staining of the surrounding neuropil. Scale bars represent 100 μm. (B) Competition ELISA in which McSA1 was preincubated with 10-fold dilutions of peptides corresponding to overlapping portions of the original antigen, $A\beta_{1-40}$. (C) Indirect ELISA comparing the reactivity of McSA1 to the human Aβ sequence and the rodent Aβ sequence which differs in 3 of the first 13 residues.

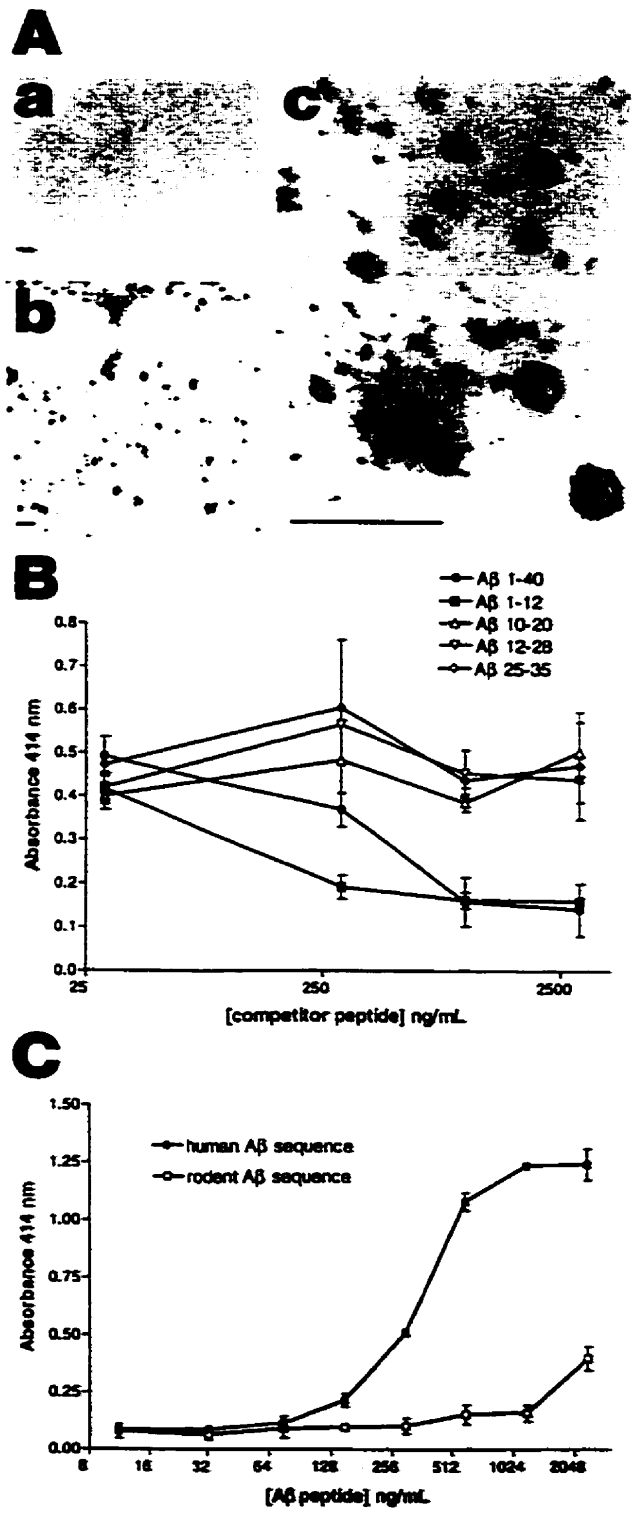

A
a
b
c
B
Aβ 1-40
Aβ 1-12
Aβ 10-20
Aβ 12-28
Aβ 25-35
Absorbance 414 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
25
250
2500
[competitor peptide] ng/mL
C
human Aβ sequence
rodent Aβ sequence
Absorbance 414 nm
0.00
0.25
0.50
0.75
1.00
1.25
1.50
8
16
32
64
128
256
512
1024
2048
[Aβ peptide] ng/mL

Figure 10. Analysis of APP expression in P19 cells stably transfected with the 751 amino acid isoform of human APP (pAD clones), and their transfection controls (placZ clones). (A) Northern analysis of APP mRNA before (lanes 1 & 3) and after (lanes 2 & 4) neuroectodermal differentiation. Rows 1 & 2 are short and long exposures of the same blot to reveal levels of endogenous APP in the control clone. Row 3 is the same blot probed for 18S message. (B) Western analysis of total cell lysates before and after neuroectodermal differentiation. 15 μg of protein were loaded in each lane and detected with mAb 22C11 directed to an N-terminal epitope of APP. Lanes 1 & 5, undifferentiated cells; lanes 2 & 6, differentiated 3 days; lanes 3 & 7, differentiated 9 days; lanes 4 & 8, differentiated 4 weeks. M_r markers are indicated to the left. (C) P19 clones were grown for 2 weeks after neuroectodermal differentiation before being fixed in 4% paraformaldehyde and subject to immunocytochemistry with either mAb 22C11 (a-c) or McSA1 (d-f) coupled with peroxidase-activated DAB deposition. (a & d) pAD clones were incubated in the absence of primary antibody as negative controls. (b & e) placZ clones. (c & f) pAD clones. Scale bar represents 100 μm. (D) placZ and pAD clones grown for 4 weeks after differentiation were metabolically labeled for 16 hours and the conditioned media was immunoprecipitated with mAb 22C11. The immune complexes were resolved by 10% SDS-PAGE and the gels processed for fluorography. (E) 4 Week cultures of differentiated cells were metabolically labeled for 16 hours and the cells lysed in RIPA for immunoprecipitation with rabbit antisera to the C-terminus of APP. Immune complexes were resolved by 16.5% Tris/tricine SDS-PAGE and proteins transferred to a 0.1 μM PVDF membrane before being exposed to Hyperfilm-βmax. M_r markers are indicated to the left.

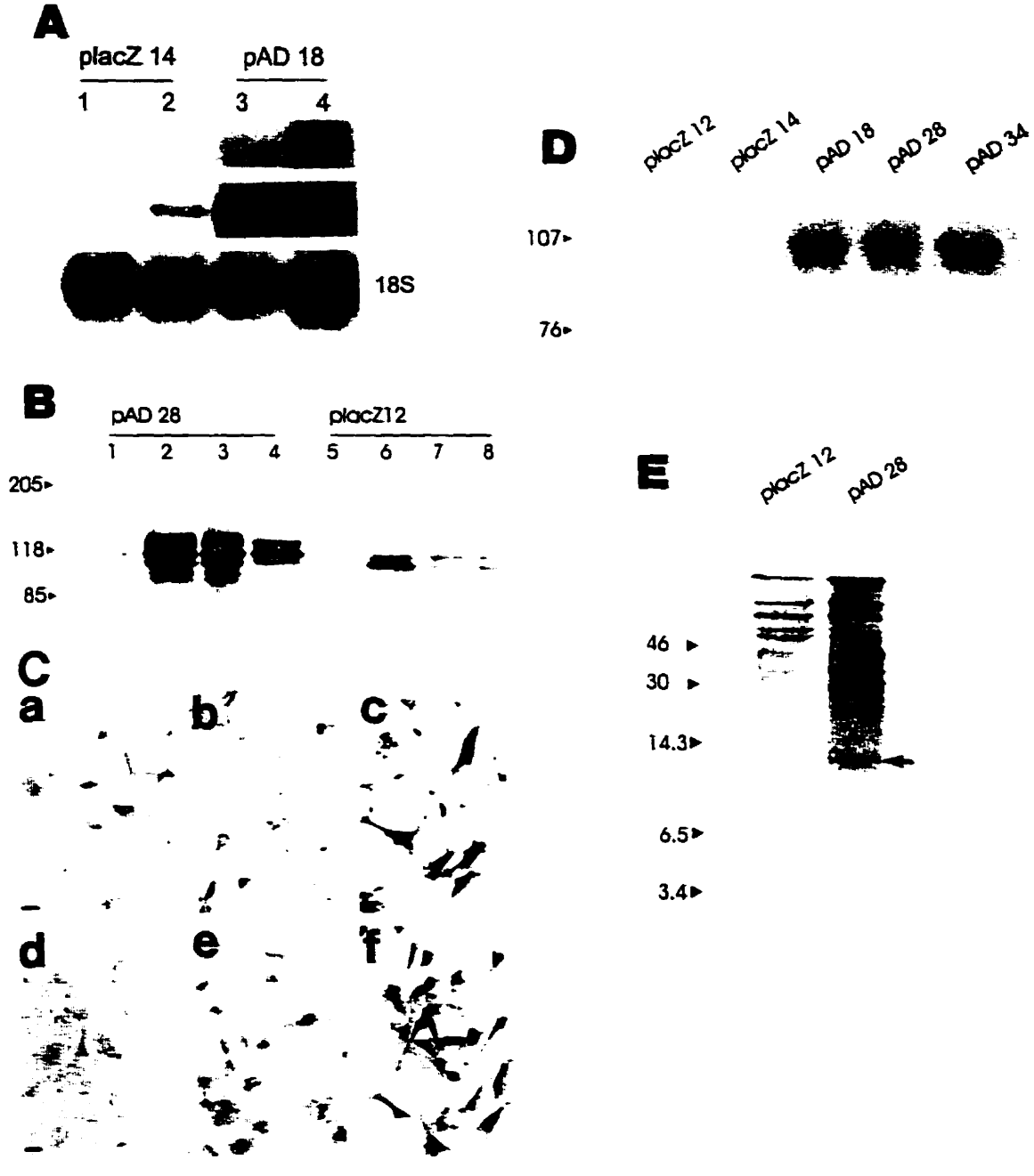

A
placZ 14
pAD 18
1
2
3
4
18S
B
pAD 28
placZ12
1 2 3 4 5 6 7 8
205
118
85
C
a
b
c
d
e
f
D
placZ 12
placZ 14
pAD 18
pAD 28
pAD 34
107
76
E
placZ 12
pAD 28
46
30
14.3
6.5
3.4

Figure 11. Neuroectodermally differentiated P19 clones were maintained in 10% FBS/DME media for 1-2 months prior to processing for silver-intensified immunogold cytochemistry. All photomicrographs are of clone pAD 28. (a & b) Cells incubated in the absence of primary antibody are negative for any staining. (c, d, g) Cells were incubated with mAb 4G8 which recognizes residues 17-24 of the Aβ sequence. (e, f, h) Cells were incubated with McSA1 which recognizes residues 1-12 of the Aβ sequence. Both antibodies stain material within a dilatated structure revealed, at higher magnification (g & h), to be ribosome-studded ER filled with fine electron-lucent fibrillar material. Some of the silver-intensified immunogold deposits are indicated with arrows. Asterisks provide orientation between the paired low and high magnification views of each staining. Scale bars represent 20 μm (a, c, e) or 2 μm (b, d, f, g, h).

c
e
f
g
h

Figure 12. mAb McSA1 localises Aβ immunoreactivity to the trans-Golgi compartment in mature, differentiated pAD 28 cells. Silver-intensified immunogold complexes in vesicles are indicated by arrows (a & b). (c) Immunoreactive deposits are seen within the lateral compartment of cisternae on the *trans* face of a Golgi stack. Scale bar represents 2 μm.

b

Figure 13. Immunoreactivity, often present as multiple deposits, was localised to coated vesicles in mature, neuroectodermal cultures of clone pAD 28. (a) Clathrin coated pits were seen to form along the plasma membrane. An adjacent invagination is immunoreactive. (b) The plasma membrane has been disrupted during processing, but several immunopositive and immunonegative endosomes, were found adjacent to the cell surface. (c-e) Multiple immunopositive endosomes were located in the cell periphery. Scale bar represent 2 μm.

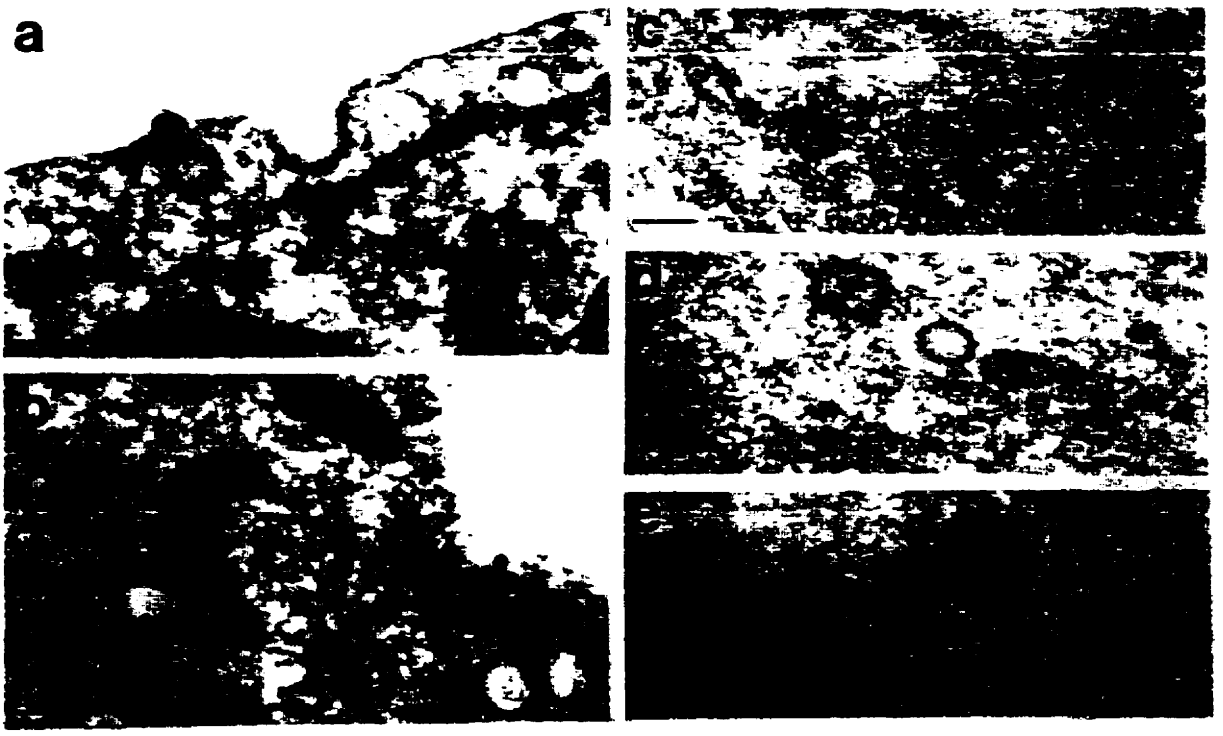

a

Figure 14. McSA1 revealed Aβ immunoreactivity associated with several intracellular structures in mature, neuroectodermally differentiated pAD 28 cells. (a) At low power, immunoreactive deposits were localised to mitochondria (m) and in areas traversed by microtubules (mt) and filaments (f). These associations are highlighted in high magnification views. (b & c) Immunoreactive deposits were associated with the outer mitochondrial membrane. (d) Immunoreactive deposits were located along microtubules and, occasionally, in the associated fibrillar material. (e) Immunoreactive deposits were typically excluded from regions of bundled filaments, but were found associated with adjacent 'loose' filaments. Scale bar represents 2 μm.

a
b
c
d
e

Figure 15. Aβ immunoreactivity was rarely observed in the nucleus of neuroectodermally differentiated pAD 28 cells under the specific staining conditions employed in our study. However, in those few cells with a substantial amount of nuclear membrane in the plane of section, multiple immunoreactive deposits were found over the nuclear envelope. The deposits could be visualized in amongst the nuclear pores (inset of highlighted rectangle). Scale bar represents 2 μm.

Connecting Text

The previous results provided clear evidence that APP fragments were available within specific intracellular compartments of neuroectodermally differentiated cells. We noticed abnormalities in the mitochondria of numerous cells during this study, and were prompted to examine the ultrastructure of clones soon after differentiation, a time when APP expression is greatly upregulated and cell vulnerability may, therefore, be greatest. We observed striking mitochondrial abnormalities in young neuroectodermal progeny. Thus, we decided to investigate whether these mitochondria were functionally impaired.

Chapter 5

Mitochondrial abnormalities in neuroectodermal cells stably expressing $hAPP_{751}$

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Moshe Szyf, A. Claudio Cuello

Abstract

Metabolic hypofunction is a common finding in a number of neurodegenerative diseases, including Alzheimer's disease. The strong linkage between the amyloid precursor protein (APP) and Alzheimer's disease led us to examine whether overexpression of this protein in CNS-type cells had an effect on mitochondria. We found abnormal morphology in mitochondria of the neuroectodermal progeny of P19 cells stably transfected with human APP_{751}. In addition, the mitochondria of APP-transfected clones had a decreased mitochondrial membrane potential. These changes were independent of Aβ toxicity and distinct from complex I inhibition. Our results may have important implications for the earliest events in the pathophysiology of Alzheimer's disease and, by extrapolation, for intervention therapies.

Introduction

Mitochondrial dysfunction has been demonstrated in a number of neurodegenerative diseases, including Alzheimer's disease (Beal et al. 1993). Regional metabolic abnormalities have been documented by positron emission tomography, even in patients with early disease who exhibit only memory impairment (Duara et al. 1986). As well, deficits in specific enzymes catalyzing oxidative phosphorylation have been described in patients with Alzheimer's disease (Chandrasekaran et al. 1994; Gibson et al. 1988; Kish et al. 1992; Parker Jr. et al. 1997). Non-critical inherited mutations in mitochondrial DNA (mtDNA) and/or cumulative mtDNA damage have been suggested to underlie this metabolic dysfunction giving rise to a slowly evolving excitotoxic neurodegeneration (Beal et al. 1993). Recent evidence that the mitochondrial complex IV enzyme cytochrome c plays a key role in programmed cell death (Reed 1997), and growing data regarding the function(s) of the mitochondrial resident Bcl-2 protein family (Reed et al. 1994) suggest that mitochondria mediate cell survival in multiple ways. It was of interest, therefore, to determine how the amyloid precursor protein (APP), which is strongly implicated in the development of Alzheimer's disease (Selkoe 1994) might influence mitochondrial function.

In the present study, we examined mitochondria in neuroectodermally differentiated murine embryonal carcinoma (P19) cells stably expressing the human 751 amino acid isoform of APP. The morphology of these organelles was examined by electron microscopy, and their function was evaluated, indirectly, by measuring the pyridine redox state of the cells and, directly, by measuring the mitochondrial membrane potential.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO) and tissue culture reagents from Gibco BRL (Burlington, Canada), unless otherwise stated.

Cell culture

P19 cells (obtained from M. McBurney, University of Ottawa) were transfected by calcium phosphate precipitation of 20 μg of the APP cDNA plasmid pAD-751 (kindly provided by K. Beyreuther, Centre for Molecular Biology, Heidelberg) and 2 μg of the selection plasmid pCMVlacZ II (American Tissue Culture Collection, Rockville, MD). Clones were selected in 0.6 mg/ml Geneticin® and maintained, during routine passaging, in 0.2 mg/ml Geneticin®. Incorporation and expression of the wild type human 751 amino acid isoform of APP, under the control of the cytomegalovirus promoter, was confirmed by Southern and Northern blotting using a radiolabelled internal fragment of the APP cDNA. Protein expression in differentiated cells was evaluated by Western blotting and by immunocytochemistry (see below). pAD-751-expressing clones are designated pAD; control clones transfected only with pCMVlacZ II are designated placZ.

P19 clones were maintained in Dulbecco's modified Eagle media (high glucose) supplemented with 10% fetal bovine serum,1 mM sodium pyruvate, 2 mM l-glutamine, and prophylactic antibiotics (0.03 mg/ml kanamycin, 0.2 mg/ml streptomycin, 0.02 mg/ml neomycin) in a 37°C, 5% CO_2 atmosphere. Neuroectodermal differentiation was performed as described (McBurney et al. 1988). Subconfluent monolayers were dissociated in 10 mM EDTA and plated in bacterial grade petri dishes at 10^6 cells/10 ml of media supplemented with 0.3 μM all-*trans* retinoic acid. After 4 days, cell aggregates were dissociated and plated on tissue culture substrates.

Light and electron microscopy

At the completion of retinoic acid differentiation, cells were plated on poly-l-lysine coated coverslips (for immunocytochemistry) or tissue culture plastic (for electron microscopy) and maintained in 10% FBS/DMEM media for 3 days. For light microscopy, the coverslips were fixed for 10min at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 supplemented with 50 mM sucrose and 0.4 mM calcium chloride. After nonspecific binding of protein was blocked by a 30min incubation at 22°C in blocking buffer (5% horse serum in PBS + 0.1% tween 20), the primary antibody was applied overnight at 4°C. McSA1, our mouse monoclonal antibody that recognizes an epitope within the N terminal 12 amino acids of human Aβ, was used diluted 1:500 in blocking buffer. The reaction was developed with Vector Elite ABC reagents according to the manufacturer's protocol using 0.06% diaminobenzadine as the chromogen.

For electron microscopy, 3 day old neuroectodermal cultures were collected in 10 mM EDTA, washed with Hank's balanced salt solution, pelleted and fixed for 2 h at 4°C in 3% glutaraldehyde. The cell pellet was post-fixed in osmium, Epon-embedded and processed for electron microscopy with uranyl acetate and lead citrate applied as counterstains. Grids were viewed with a Philips 410 transmission electron microscope.

MTT assay

At the end of retinoic acid treatment the cell aggregates were mechanically dissociated, resuspended in 10% FBS/DMEM media lacking phenol red, plated at 100 μL/well of a 96 well plate and allowed to grow undisturbed for 72 h. For an additional 48 h the cells were grown in media alone, or media supplemented with 10 or 100 μM of

peptide comprising amino acid residues 25-35 of amyloid β (Aβ)-protein, or the reverse sequence peptide Aβ35-25 (American Peptide Co., Sunnyvale, CA). At the end of this treatment, the cells were incubated a further 4 hours in 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml, followed by solubilization overnight at 37°C in 10% SDS. Absorbance of the formazan product was read at 600 nm. Duplicate experiments were performed, in triplicate, on the neuroectodermal progeny of each clone.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_M$) was estimated using the aldehyde-fixable fluorescent probe chloromethyl-tetramethylrosamine methyl ester (CMTMR, Mitotracker Orange, Molecular Probes, Oregon) essentially as described (Wadia et al. 1998). After retinoic acid treatment, cells were plated onto poly-l-lysine coated coverslips and cultured for 3 days in 10% FBS/DMEM media. Cells were incubated in 138 nM CMTMR for 15 min at 37°C and then in dye-free media for 10 min before being fixed on ice in 4% paraformaldehyde for 10 min. A Leica TCD confocal scanning microscope coupled to an argon-krypton laser (Omnichrome, USA) was used to resolve individual mitochondria. Using a pinhole of 20 and an excitation filter wavelength of 488 nm and long-pass emission filter of 590 nm, images were scanned through a 100X oil immersion objective at 512 x 512 x 8 bits per pixel resolution, background offset of -1, and averaged 32 times in bidirectional scan mode. Another series of cells was treated for 6 h with 15 nM rotenone, a complex I inhibitor, prior to incubation with CMTMR and processing as described above. Images were saved in a TIFF format and anaylzed using the Metamorph™ software package. Fluorescence intensity measurements were obtained

using a rectangular region tool of 2 x 2 pixels within 2 regions of each mitochondria for approximately 100 mitochondria per treatment condition. The data were analyzed using nonparametric statistics (Mann-Whitney U test) using the Statistica™ software package.

Results

Based on their robust expression of the $hAPP_{751}$ transgene after neuroectodermal differentiation, 3 pAD clones (18, 28, and 34) were selected for analysis of the effect of this expression on mitochondrial function. They were compared with 2 placZ clones (12 and 14) to control for the nonspecific effects of transfection and clonal selection. The phenotypic evolution of neuroectodermally differentiated P19 cells has been described previously (McBurney et al. 1988). When grown in 10% serum, neuronal, glial, and fibroblastic type cells mature over time with neuronal characteristics appearing first at approximately day 3-4. As depicted in Fig. 16a & b, differentiated pAD cells are strongly immunoreactive for the human Aβ epitope. Examination by electron microscopy revealed striking differences in the morphology of mitochondria in numerous pAD cells that did not exhibit the typical ultrastructural stigmata of apoptosis or necrosis (Behl et al. 1994a). Compared with control placZ clones, which contained mitochondria packed with regular lamellar cristae and a uniformly electron dense matrix, pAD clone mitochondria exhibited several abnormalities (Fig. 16c *vs* 16d). In some instances the mitochondrial matrix was extremely electron dense (Fig. 16e). Frequently, the inner mitochondrial membranes were collapsed into circular bodies leaving large vacant areas within the organelle (Fig. 16d, f). Another common feature of mitochondria from pAD cells was the occurrence of whorling membranous inclusions, sometimes involving adjacent endoplasmic reticulum (Fig. 16f, g).

Whether these mitochondrial ultrastructural abnormalities were associated with functional changes was assessed in 2 ways. The pyridine redox status of the cells was measured by quantifying the enzymatic reduction of MTT to its colored formazan

product (Table I). Hydride ions can be generated outside of the mitochondria (e.g. by NADH-dependent dehydrogenases of the glycolytic pathway) but MTT reduction is frequently used to gauge oxidative phosphorylation activity, often as a surrogate measure of cell viability (Brown et al. 1997; Shearman et al. 1994). There was no difference in basal levels of MTT reduction between placZ and pAD clones assayed between days 3 and 5 after neuroectodermal differentiation. Nor was there a loss of cell viability after exposure to 10 or 100 μM Aβ25-35, a peptide previously reported to be toxic *in vitro* (Behl et al. 1994a; Brown et al. 1997; Shearman et al. 1994).

Mitochondrial function was also assessed by using a potentiometric dye to measure the $\Delta\psi_M$ of individual mitochondria within each of the pAD and placZ clones. Decreased fluorescence intensity was observed in all 3 experimental clones compared with controls. Note the shift in the fluorescence intensity to the left in Fig. 17. Moreover, the magnitude of the decrease in $\Delta\psi_M$ was consistent among the pAD clones. To determine if this change reflected inhibition of the respiratory chain, cells were treated with the complex I inhibitor rotenone prior to measurement of $\Delta\psi_M$. Rotenone treatment produced a decrease in $\Delta\psi_M$ of similar magnitude in both APP-transfected and control-transfected clones (decreases of 13.2 and 24.5% for placZ 12 and 14, respectively, versus 13.3, 9.1, and 15.6% for pAD 18, 28, and 34, respectively).

Discussion

Using direct visualization of mitochondrial morphology and assays of mitochondrial function, we have shown that overexpression of human APP_{751} in neuroectodermally differentiated P19 cells produces distinct abnormalities. Aβ, and a peptide comprising amino acids 25-35 of this protein, are widely reported to induce cell death in neuronal cell lines (Behl et al. 1994a; Shearman et al. 1994) and in primary CNS cultures (reviewed in Mattson 1997). Both apoptosis and necrosis have been reported and either form of cell death invokes aberrant mitochondrial function. P19 cells do process APP to α- and β-secretase cleavage products (Ho et al. 1996) and, thus, pAD clones would be expected to secrete Aβ into their media. However, vulnerability to Aβ is notably cell-type dependent (Brown et al. 1997; Pike & Cotman 1993) and our MTT analyses indicate that neuroectodermally differentiated P19 cells remain viable in the presence of high concentrations of Aβ25-35.

The morphological abnormalities we observed in pAD mitochondria are reminiscent of Gomori bodies, which are astrocytic inclusions homologous to corpora amylacea, a prevalent cytoplasmic inclusion in aged and, particularly, in Alzheimer's disease brain (Brawer et al. 1994; Schipper & Cissé 1995) Gomori bodies can be induced in cultured astrocytes by exposure to the oxidative phosphorylation inhibitor cysteamine. They begin as mitochondria with irregular, swollen, often saccular, cristae and evolve over a period of days into complex autophagosomes (Brawer et al. 1994). The longevity of cysteamine-treated astrocytes parallels our observation that APP-transfected P19 cells remain viable with this degree of mitochondrial abnormality.

Viral-mediated overexpression of $hAPP_{751}$ in cultured human muscle was reported to induce a loss of cytochrome c oxidase staining from otherwise ultrastructurally preserved mitochondria (Askanas et al. 1996). Our observation of a decrease in $\Delta\psi_M$ in all pAD clones is the first report, based on functional analysis, that APP expression *per se* can affect the potential for mitochondria to carry on oxidative phosphorylation.

In concert with a proton gradient established by H^+ pumping, the electrochemical gradient establishes the mechanism for energy transduction and the production of ATP (Harold 1986). These gradients are stable under basal conditions but Loew and co-workers (1993), using high resolution confocal imaging in living neuroblastoma cells, observed that neurite mitochondria undergo spontaneous and reversible decreases in membrane potential. The rotenone data imply that APP (or its metabolites) is not acting as a toxin within complex I of the respiratory chain analogous to the action of the Parkinsonian agent 1-methyl-4-phenylpyridinium (MPP^+) [Bates et al. 1994]. Rather, APP (or its metabolites) might be acting to promote a decrease in $\Delta\psi_M$ by interfering with maintenance of either the proton or the electrochemical gradients, or by stabilizing a population of mitochondria spontaneously shifted to a lower $\Delta\psi_M$. Metabolic heterogeneity between different CNS cell types, different brain regions, and even different neuronal compartments (synaptic versus non-synaptic mitochondria fractions) has been demonstrated (Lai 1992). Moreover, mitochondria isolated from the liver of aged rats, but not of young animals, can be subdivided into distinct populations based on $\Delta\psi_M$ (Hagen et al. 1997). These data provide a possible basis for the brain region selectivity and age dependence of Alzheimer's disease fuelling our speculation that APP, through its action on the mitochondria, might promote a cascade of adverse intracellular

events involving metabolic compromise and dysregulation of mitochondrial protein function leading, ultimately, to the Alzheimer's disease-specific pattern of neuronal dystrophy, eventual cell loss, and reactive gliosis.

Conclusion

Numerous studies have documented defects in oxidative phosphorylation in Alzheimer's disease brain, although how these defects arise and, as importantly, how they induce the specific neuropathology of Alzheimer's disease have not been determined. There is strong data suggesting that APP plays a pivotal role in this disease and we have, indeed, found that overexpression of $hAPP_{751}$ and the accompanying intracellular production of APP proteolytic fragments in neuroectodermally differentiated P19 cells is associated with aberrant mitochondrial morphology and function. At the ultrastructural level, the inner mitochondrial cristae are remodeled, and affected mitochondria appear to undergo an autophagocytic process. Expression of APP and its metabolites is not associated with a global decline in viability as determined by the capacity for MTT reduction. Instead the overexpressing clones are characterized by a decrease in $\Delta\psi_M$. This relative uncoupling of oxidative phosphorylation is not a consequence of complex I toxicity and the mechanism remains to be clarified. These results have important implications for the pathophysiology of Alzheimer's disease which, in turn, might elucidate targets for pharmacotherapy at the very earliest stages of the disease.

Table 1. Redox status based on colourimetric determination in MTT reduction

		% of basal absorbance			
		Aβ25-35		Aβ35-25	
	basal absorbance[a]	10	100μM	10	100μM
placZ 12	0.383 ± 0.035	100	90	129	131
placZ 14	0.273 ± 0.010	110	101	142	169
pAD 18	0.339 ± 0.000	73	74	102	96
pAD 28	0.215 ± 0.008	100	109	110	92
pAD 34	0.227 ± 0.021	128	94	107	116

[a] absorbance at 600 nm ± SEM

Figure 16. Expression of $hAPP_{751}$ in transfected clones and the associated mitochondrial ultrastructural abnormalities 3 days after retinoic acid-induced neuroectodermal differentiation. a & b) APP expression is revealed with a human Aβ epitope-directed monoclonal antibody, McSA1, in APP-transfected clones pAD 34 and pAD 18, respectively. c) Mitochondrial morphology in control-transfected clone placZ 14. d-g) Mitochondrial abnormalities in the APP-transfected clone pAD 28. Membranous whorls encroaching on dysmorphic mitochondria are highlighted with arrows in panels f & g. Scale bars represent 200 μm (a,b), 20 μm (c,d) or 2 μm (e-g, and inserts c & d).

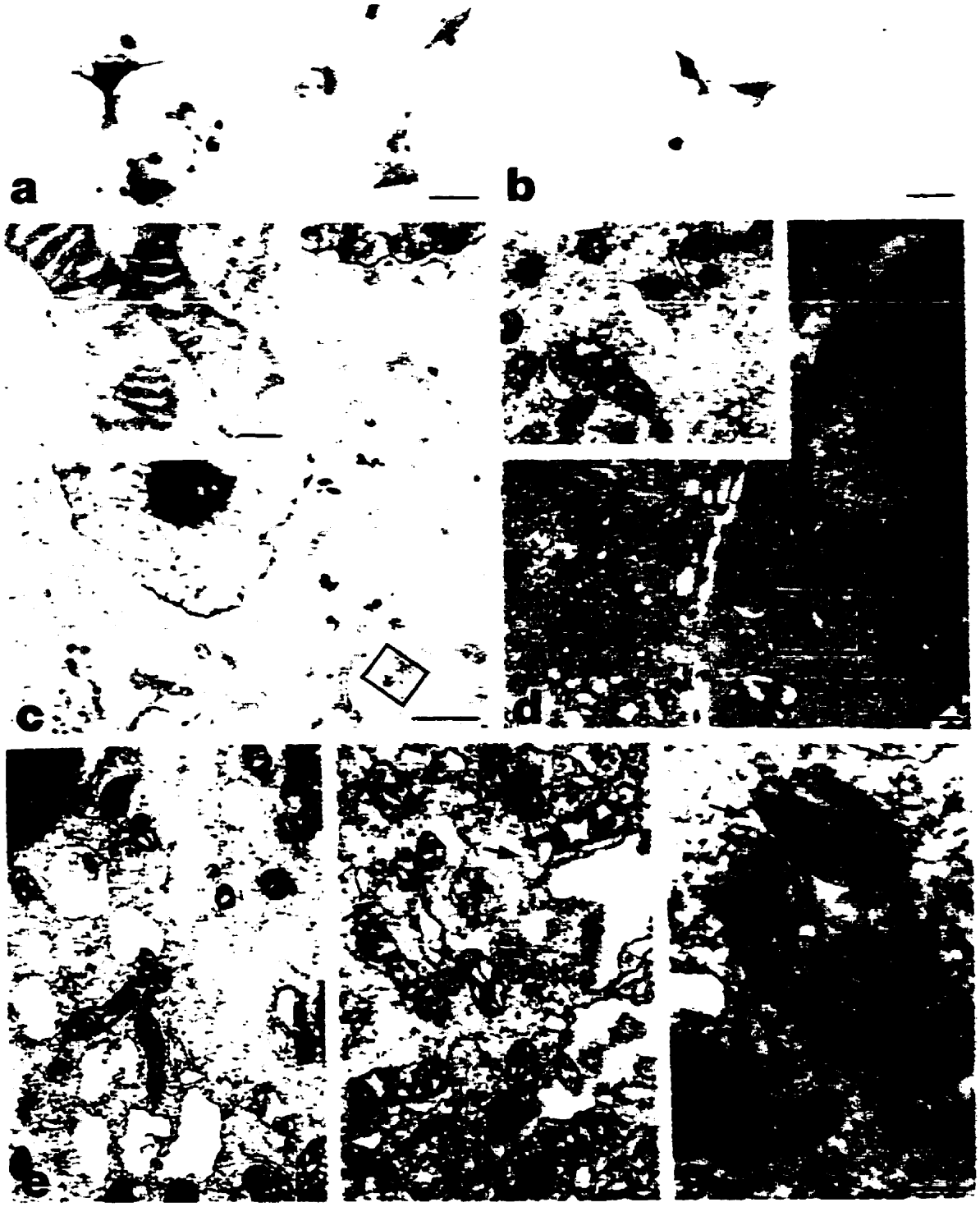
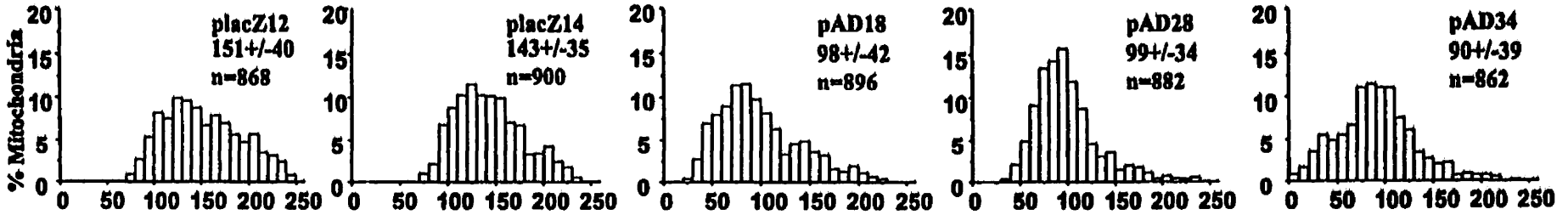
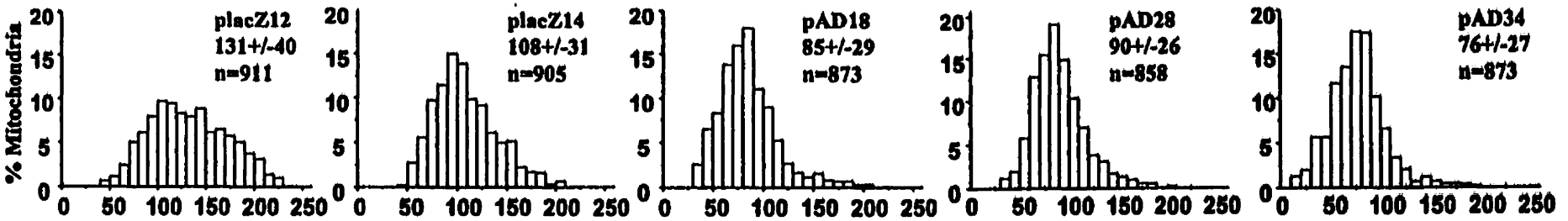

a
b
c
d
e

Figure 17. Frequency distribution of $\Delta\psi_M$, measured as average CMTMR mitochondrial fluorescence intensity, for control- and APP-transfected clones 3 days after neuroectodermal differentiation. Note the shift in $\Delta\psi_M$ towards the left indicating a decrease in $\Delta\psi_M$ in untreated, as well as rotenone treated, pAD clones. The data were analyzed using the Mann-Whitney U nonparametric statistical test. In untreated cultures (top row) significant differences were found between control and untransfected clones (pAD18, pAD28, pAD34; $p<0.01$). Exposure to 5 nM rotenone (bottom row) caused a significant shift to the left for clone placZ12 ($p<0.05$), and for the remaining clones ($p<0.01$).

Untreated (6h)



Rotenone 15nM (6h)



CMTMR Fluorescence In Detector Units (Estimate of $\Delta\Psi_M$)

Connecting Text

During our anaylsis of mitochondrial function, we established that the redox potentials of all 3 day old clones were similar, and that the Aβ postulated to be secreted in the media was unlikely to be inducing cell death. To further investigate the occurrence of cell loss in differentiated, transfected clones, we examined the phosphorylation (and, hence, activation) of members of the mitogen-activated protein kinase family. These proteins, ERK 1 and 2, Jun kinase, and p38, have been identified as important mediators of cell survival. The phosphorylation of $p42^{MAPK}$ (ERK 2) seemed particularly sensitive to APP overexpression; therefore, we investigated its dynamic phosphorylation at early and later times of neuroectodermal growth.

Chapter 6

Phosphorylation of mitogen-activated protein kinase is altered in neuroectodermal cells overexpressing the human amyloid precursor protein 751 isoform

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Abstract

Hyperphosphorylation of the microtubule-associated protein tau is a histopathological hallmark of Alzheimer's disease. However, the aberrant expression or processing of the amyloid precursor protein (APP) is the only known genetic basis for presenile familial Alzheimer's disease, and the molecular connection between APP and tau has been perplexing. Attention has focused on proline-directed serine/threonine kinases as mediating the cytoskeletal modifications of Alzheimer's disease, and we show that overexpression of APP can influence the activation of a candidate kinase, the mitogen-activated protein kinase (MAPK). In murine embryonal carcinoma cells stably transfected with the human 751 isoform of APP, we observed steady-state hyperactivation of $p42^{MAPK}$ concomitant with APP overexpression 3 days after neuroectodermal differentiation. In more mature differentiated cells, immunocytochemical analysis revealed enhanced basal somatic and nuclear immunoreactivity for phosphorylated MAPK coupled with an attenuated phosphorylation response to growth factor stimulation. Our results suggest that APP can influence the MAPK signaling pathway in such a way that the absolute and time-dependent activation required for discrimination of the appropriate downstream response are compromised. Such an effect would have important consequences for the functioning of cells coincidentally expressing both proteins, a situation that occurs in neuronal populations vulnerable to Alzheimer's disease pathology.

Introduction

One of the major histopathological hallmarks of Alzheimer's disease is the neurofibrillary tangle (NFT), an intraneuronal inclusion composed of paired helical filaments derived from abnormally phosphorylated microtubule-associated protein tau. Indeed, a spectrum of cytoskeletal abnormalities can be detected with antibodies to phosphorylated epitopes in tau that includes neuropil threads throughout the affected cortex, swollen dystrophic neurites, and extracellular 'ghost' tangles marking the death of an affected neuron (reviewed by Goedart 1993). NFT density increases in a stereotypic anatomical pattern in Alzheimer's disease that correlates with the progression of dementia (Braak & Braak 1996). However, inherited forms of this disease appear to involve altered amyloid precursor protein (APP) metabolism and no genetic linkage has been made to tau. It is possible, however, that APP or its metabolites influence the dynamic phosphorylation and/or dephosphorylation of tau.

Several proline-directed serine/threonine kinases have been identified that catalyse the addition of phosphate to tau at those sites which are typically non-phosphorylated in adult neurons but that become phosphorylated in Alzheimer's disease. Mitogen-activated protein kinase (MAPK), also known as extracellular signal regulated kinase (ERK), is particularly intriguing because it can phosphorylate tau *in vitro* on sites peculiar to paired helical filament tau (Drewes et al. 1992). Moreover, its topographic distribution in human brain corresponds to neuronal populations vulnerable to NFT development (Arendt et al. 1995; Hyman et al. 1994; Pei et al. 1994; Trojanowski et al. 1993b).

APP expression is high in the same neuron populations (Bahmanyar et al. 1987; Lewis et al. 1988); hence, it seemed pertinent to examine MAPK activation in APP-expressing cells. We have stably transfected the 751 amino acid isoform of human APP

into murine embryonal carcinoma (P19) cells that can be induced, by aggregation in the presence of retinoic acid, to differentiate into neuronal, glial, and fibroblastic (i.e., neuroectodermal) cell types. MAPK phosphorylation was assessed using a phospho-specific MAPK antibody under basal conditions and in response to growth factor stimulation. We show that overexpression of APP in newly differentiated clones is associated with hyperphosphorylation of MAPK, and that more mature cells exhibit an attenuated phosphorylation response to acidic fibroblast growth factor (aFGF) stimulation. Our results are consistent with the notion that aberrant APP expression can be juxtaposed to both defining lesions of Alzheimer's disease: pathological accumulations of Aβ peptide and of hyperphosphorylated tau.

Materials and Methods

Materials

Recombinant human aFGF was purchased from Promega, dissolved in phosphate-buffered saline (PBS) with 1% bovine serum albumin, and stored as frozen aliquots. All-*trans* retinoic acid and heparin were purchased from Sigma. The PhosphoPlus p44/42 MAP Kinase Antibody kit was purchased from New England BioLabs.

Preparation of stably transfected P19 cells

Wild type P19 cells (gift of M. McBurney) were transfected with the complete cDNA of the 751 amino acid isoform of human APP under the control of the cytomegalovirus promoter (gift of K. Beyreuther; Weidemann et al 1989). Cells were co-transfected with the selection plasmid pCMVlacZ II (American Tissue Culture Collection, Rockville, MD). Stably transfected clones, termed pAD, were identified by Southern analysis and expression of the transgene was confirmed by mRNA and protein determinations in neuroectodermally differentiated cells. Control clones, termed placZ, were transfected with pCMVlacZ II only.

Neuroectodermal differentiation

Cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM l-glutamine, and prophylactic antibiotics (0.03 mg/ml kanamycin, 0.2 mg/ml streptomycin, and 0.02 mg/ml neomycin). Undifferentiated clones were maintained in selection media (0.2 mg/ml Geneticin®).

Neuroectodermal differentiation was performed essentially as described (MacPherson & McBurney 1995). Subconfluent monolayers of cells were dissociated in 10 mM EDTA and replated, at 10^6 cells per 10 ml media plus 0.3 μM all-*trans* retinoic acid, in bacterial grade plastic dishes for 3 days. At the end of treatment, the aggregates were mechanically dissociated and plated onto tissue culture plastic.

MAPK phosphorylation

Plates of undifferentiated cells, and cells at various times after differentiation, were placed on ice, washed twice with cold PBS pH 7.4, and incubated for 20 min in cold lysis buffer (50 mM Tris HCl pH 8.0, 137 mM sodium chloride, 1% Nonidet P40, 10% glycerol, 50 μM sodium orthovanadate, 20 mM sodium pyrophosphate, 10 μM leupeptin, 10 mM benzamidine, 2 μg/ml aprotinin, 1 mM phenylmethylsulphonylfluoride). Supernatants were collected after centrifugation of cell lysates at 12,000 g for 15 min at 4°C. Total protein was determined by the bicinchoninic acid method (BCA kit, Pierce) and 25 μg of protein was boiled in 2X sample buffer prior to separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Samples for MAPK detection were separated in 12% gels prepared with low-bis acrylamide (acrylamide:bis ratio of 118.5:1) as described (Mills et al. 1997). Identical samples for APP detection were separated in 7.5% gels prepared with standard bis acrylamide (ratio of 37.5:1).

Immunoblotting was performed as described by the manufacturer. Non-phosphorylated protein was detected with a rabbit antisera generated against a peptide corresponding to residues 345 to 358 of rat $p42^{MAPK}$. The membrane was then stripped by incubation in 62.5 mM Tris HCl pH 6.8, 2% sodium dodecyl sulphate, 100 mM β-

mercaptoethanol at 65°C for 30 min. After thorough washing the membrane was reprobed with rabbit antiserum raised against a peptide corresponding to residues 196 to 209 of human $p44^{MAPK}$ phosphorylated on both threonine 202 and tyrosine 204. A purified mouse monoclonal antibody recognising an N-terminal epitope of human and rodent APP (clone 22C11, Boerhinger-Mannheim) was used to detect APP.

aFGF stimulation

Immunocytochemistry:

At the end of retinoic acid treatment, APP clone pAD 18 and control clone placZ 14 were plated on poly-l-lysine coated cover slips and grown in standard 10% serum media for 12 days. The coverslips were washed twice with DMEM (without additives) and incubated for 15 min at 37°C in assay media alone (DMEM + 1 µg/ml heparin) or in assay media + aFGF 50 ng/ml. At the end of treatment, the cells were washed briefly with PBS and fixed for 15 min at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 with 50 mM sucrose and 0.4 mM calcium chloride. Immunocytochemical detection of phosphorylated MAPK was performed according to the manufacturer's protocol using the Vector ABC reagent and diaminobenzidine as chromogen. The coverslips were cleared in ascending alcohols and xylene, mounted in Entellan® and photographed through a Leitz microscope.

Imunoblotting:

In parallel, 12 day old cultures of placZ 14 and pAD 18 cells were washed in PBS and collected with 1 mM EDTA. The cell suspension was divided into 2 equal aliquots, the cells pelleted and washed twice on ice with PBS before being resuspended in assay media or assay media + 50 ng/ml aFGF. After a 15 min incubation at 37°C, the cells were chilled in an ice water bath, pelleted, and resuspended in 2X electrophoresis sample buffer. Equal volumes of boiled, centrifuged sample were loaded in a 12% low bis gel and the blots processed for MAPK and phospho-MAPK immunoreactivity as described above.

Quantitation of results:

To compensate for differences in protein loading and antibody sensitivity, the intensity of the immunoreactive bands for MAPK and phospho-MAPK were quantified by measuring the density of the autoradiograms with MasterScan Biological Imaging Systems™ scanner and software. The extent of phosphorylation was calculated as intensity of phospho-MAPK divided by intensity of non-phosphorylated MAPK, and expressed as a fold increase in normalised phosphorylated MAPK.

MAPK expression depends on the differentiation state. Hyperphosphorylation of MAPK coincides with APP expression.

All clones demonstrated robust expression of a 42 kDa band, corresponding to $p42^{MAPK}$ (ERK2), prior to differentiation. Neuroectodermal differentiation induced a reduction in total MAPK expression in all the P19 clones relative to that seen in undifferentiated cells; however, MAPK phosphorylation increased (Fig. 18). There was a 4 fold increase in the amount of normalised phospho-MAPK for both of the placZ control clones between the end of retinoic acid treatment (day 0) and day 3 of neuroectodermal growth. By comparison, phospho-MAPK increased 21 fold in pAD 18 cells and 10 fold in pAD 28 cells coincident with the upregulation of APP.

Both undifferentiated and differentiated cells were cultured in the same 10% fetal bovine serum media; hence, the enhanced phosphorylation seen in all clones after differentiation must reflect a change in signal transduction capabilities, presumably in support of phenotypic maturation or function. Apparently APP interacts with this altered capability to enhance phosphorylation of MAPK. The secreted N-terminal APP ectodomain has been reported to activate MAPK in a Ras-dependent fashion in PC12 cells (Greenberg et al. 1994, 1995). Murayama and colleagues (1996) implicated the intact membrane-inserted form of APP in a G_o protein-dependent activation of MAPK. In both cases, the increase in phosphorylation was rapid and declined over time. Ours is the first report that high levels of intracellular APP can cause the steady-state hyperactivation of $p42^{MAPK}$.

P19 cells express predominantly $p42^{MAPK}$ (ERK2)

The neurotransmitter profile of P19-derived neurons resembles that of the mammalian neocortex (Staines et al. 1994) making this cell line an attractive system for studies pertaining to central nervous system-type cells. Because $p42^{MAPK}$ localises to neurons vulnerable to the development of NFT, it was particularly interesting to note that P19 cells express almost exclusively ERK 2. Using rabbit antisera developed against rat ERK 1 (gift of New England Biolabs), only very faint 44 kD bands were detected after prolonged exposure of the blots (data not shown). However, the phospho-specific antibody did reveal a low level of immunoreactive phosphorylated $p44^{MAPK}$ after differentiation (Fig. 18). Interestingly, there was a notable difference in the effect of differentiation on the intensity of the 42 and 44 kD bands such that $p42^{MAPK}$ phosphorylation was enhanced out of proportion to that of $p44^{MAPK}$.

APP overexpression attenuates the response to aFGF

MAPK is known to participate in the signalling cascade activated by ligand binding of tyrosine kinase growth factor receptors. Once phosphorylated, MAPK can interact with cytosolic partners and is also translocated to the nucleus where it causes transcriptional activation of genes to effect a biological response (Force & Bonventre 1998; Robbins et al. 1994). Neurotrophic factors promote the survival, development and regeneration of neurons and, therefore, are of great interest in neurodegenerative diseases (reviewed in Hefti et al. 1989). aFGF expression in mature rat brain is limited to specific neuron populations including those known to be affected by neurodegeneration (Stock et al. 1992; Wilcox & Unnerstall 1991). Moreover, MAPK is known to colocalise with receptors for FGF in rat retinal neurons (Blanquet & Jonet 1996). For these reasons, it was of interest to determine if APP overexpression could influence the response of MAPK to aFGF.

Undifferentiated and retinoic acid-treated P19 cells express receptors for FGFs (Ameerun et al. 1996; Mummery et al. 1993). Exposure to basic FGF (bFGF) induced MAPK phosphorylation in differentiated P19 cells (Ameerun et al. 1996), and both aFGF and bFGF were mitogenic in undifferentiated cells (Mummery et al. 1993). When 12 day old cultures of our P19 clones were treated with aFGF 50 ng/ml, in the presence of heparin, we observed a 10-fold increase in MAPK phosphorylation on immunoblots of control clone placZ 14, but only a 4-fold increase for APP clone pAD 18 (data not shown). Immunohistochemical analysis of the response to aFGF was very revealing (Fig. 19). Most of the reactivity in unstimulated placZ 14 cells was associated with a population of mitotically active cells probably representative of the fibroblast-like cells that develop after retinoic acid treatment (Rudnicki et al. 1990). Larger, spreading cells in these cultures were immunonegative. After 15 min of stimulation with aFGF, both types of cells exhibited intense nuclear and perinuclear immunoreactivity. In contrast, unstimulated pAD 18 cells exhibited strong immunoreactivity in a reticular pattern throughout the perikarya with variable nuclear staining. After aFGF treatment there was moderately enhanced somatic and nuclear staining.

In protocols designed to separate cytoskeletal proteins from the cytosolic fraction, a portion of cellular MAPK copurifies with microtubule-associated protein 2 and tubulin (Morishima-Kawashima 1996; Reszka et al. 1995). APP, too, can bind the cytoskeleton (Allinquant et al. 1994; Refolo et al. 1991), and was reported to directly bind tau (Smith et al. 1995). Recent studies indicate that APP also interacts with proteins bearing homology to the phosphotyrosine binding protein Shc, which is known to participate in tyrosine kinase receptor signalling (Borg et al. 1996; Fiore et al. 1995). It is tempting to speculate that APP might be involved in bringing kinase cascade proteins in proximity to

microtubule-tethered MAPK thereby activating MAPK and influencing the phosphorylation-dependent dynamics of microtubule stability. In this scenario, overactivation of MAPK could be fostered by the abnormal expression or trafficking of APP, as occurs in Down's syndrome or hereditary Alzheimer's disease, facilitating the hyperphosphorylation of tau.

The high level of basal phosphorylation in clone pAD 18 and minimal response to aFGF stimulation suggest that APP overexpression induced a change in both the magnitude and the transience of MAPK phosphorylation. Both parameters are believed to dictate the outcome of MAPK-mediated signalling (Marshall 1995; Sugden & Clerk 1997). If APP can alter these parameters, there may be important consequences for neuronal activities involving MAPK, including long term potentiation (Kornhauser & Greenberg 1997) and synaptic plasticity (Jovanovic et al. 1996).

It is also noteworthy that MAPK has been identified as an important downstream effector in the regulated catabolism of APP (Desdouits-Magnen et al. 1998; Mills et al. 1997). One can conceive of a cycle involving altered catabolism of APP and dysregulation of MAPK activation leading, especially in cells with coincidentally high expression of both proteins, to loss of control over important MAPK-dependent events.

Figure 18. Neuroectodermal differentiation of P19 clones is associated with changes in total MAPK content and in the proportion of phosphorylated MAPK. Undifferentiated (UD) cells, cells at the end of retinoic acid-induced differentiation (0), and cells cultured for 3 days after differentiation (3) were analysed by immunoblotting 25 μg of total protein. A. rabbit antisera to non-phosphorylated MAPK, B. rabbit antisera to MAPK phosphorylated on both threonine 202 and tyrosine 204, C. mouse monoclonal antibody to APP. Control clones: placZ 12 and placZ 14; APP-transfected clones: pAD 18 and pAD 28. Neuroectodermal differentiation induces downregulation of MAPK but enhances its phosphorylation. Overexpression of APP by day 3 is associated with hyperphosphorylation of MAPK in transfected clones. P19 cells express predominantly $p42^{MAPK}$ (ERK 2). After differentiation both MAPKs can be detected by the phospho-specific antisera; however, $p42^{MAPK}$ is the predominant activated protein.

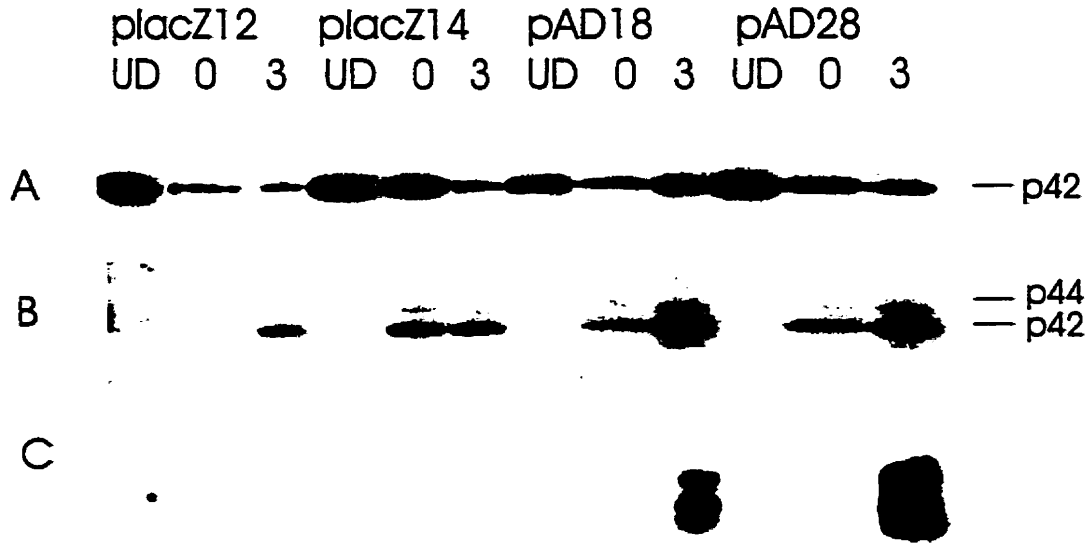
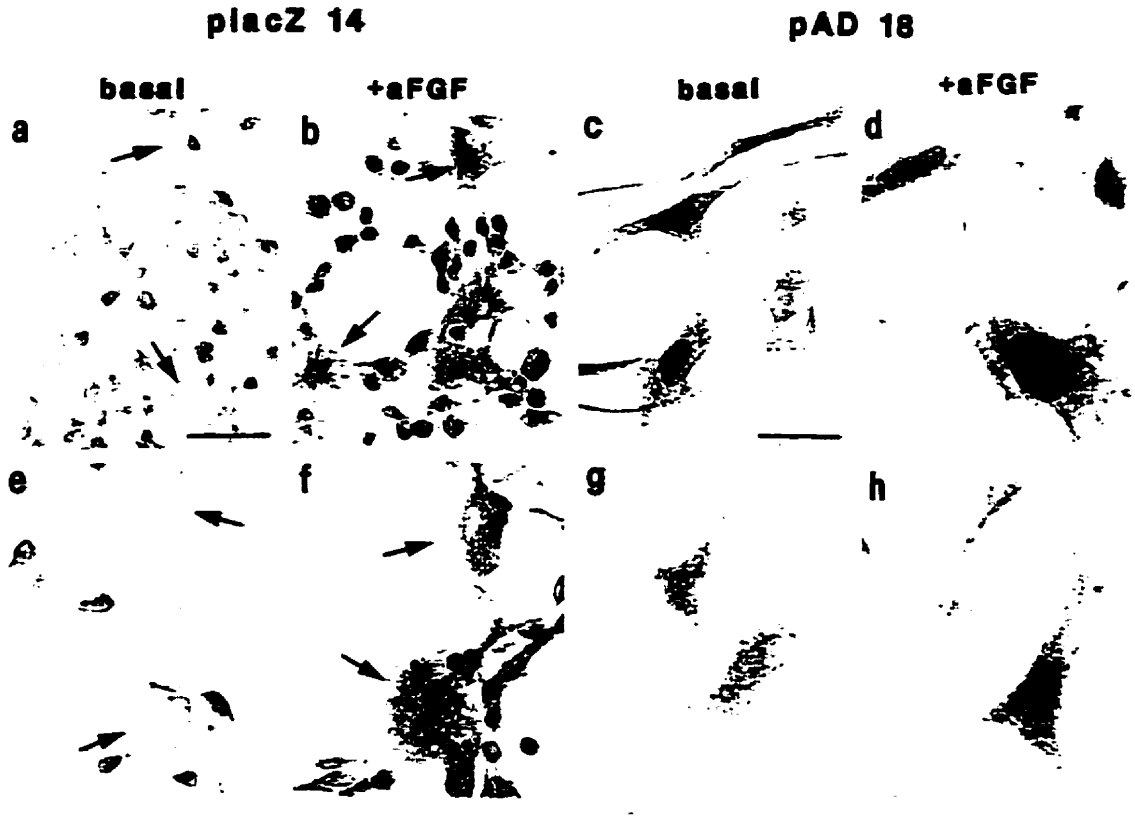

placZ12
placZ14
pAD18
pAD28
UD 0 3 UD 0 3 UD 0 3 UD 0 3
A
B
C
p42
p44
p42

Figure 19. Twelve day old neuroectodermal cultures were exposed to 0 (basal) or 50 ng/ml (+aFGF) of growth factor for 15 min at 37°C before being fixed and processed for immunoreactivity to the phospho-MAPK antisera. Arrows in panels a, b, e, and f indicate more fully differentiated cells in cultures of the control clone placZ 14. Cultures of APP-transfected clone pAD 18 typically contain flattened, process-bearing cells that are immunoreactive for phospho-MAPK before and after aFGF treatment. Scale bar represents 200 μm.


placZ 14
pAD 18
basal
+aFGF
basal
+aFGF
a
b
c
d
e
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g
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Chapter 7

Discussion

That APP has a role to play in Alzheimer's disease seems irrefutable. However, its contribution to the neuropathology is generally viewed in terms of the secretion and extracellular deposition of its Aβ peptide fragment. The data offered in this thesis suggest that, within cells expressing high amounts of APP, key cell functions are compromised potentially inducing a slowly evolving degeneration.

There is an abundance of information concerning the abnormalities observed in post-mortem Alzheimer's disease brain. Numerous studies *in vitro* have attempted to define the molecular basis for these abnormalities. In the following sections I would like to take the opportunity to speculate about how our results might be interpreted within the context of these other investigations. My goal is to identify novel pharmacological targets of particular relevance to early intervention in Alzheimer's disease.

7.1 P19 cells as a model system

Despite the difficulties we encountered with expression of the transgene, P19 cells can be recommended for studies of APP activity in the CNS on several grounds. First, retinoic acid treatment induces an irreversible commitment to differentiation, and the neuroectodermal progeny reflect phenotypes found in mammalian neocortex. Second, P19 cells are capable of fully processing human APP to produce the same cleavage products as are found in human brain. Third, P19 cells do not undergo apoptosis in response to moderate (but supraphysiological) levels of exogenous Aβ. This resistance permits examination of the effects of APP overexpression. Fourth,

differentiated P19 cells can be cultured for many weeks and will maintain expression of the transgene during this time. Long term cultures may be particularly valuable in examining the evolution of the interaction of APP with the intracellular environment.

A difficulty with P19 cells, alluded to in Section 3.1.2, is variability in the relative proportion of astrocytes, fibroblast-like cells, and neurons produced, and in the expression of neurotransmitter phenotype. A clonal effect appears to operate, which is exacerbated by the introduction of foreign DNA. Thus, our stably transfected clones exhibited considerable variability with respect to post-differentiation morphology. It is perhaps even more remarkable, then, that the effect of APP on mitochondrial function and MAPK phosphorylation was consistently observed among the APP-transfected pAD clones relative to the control placZ clones.

All of the studies described herein used neuroectodermal cultures grown in serum-based media. While this media can support at least the initial development of all cell types, it is probably not ideal for the complete differentiation of neurons, in particular. Serum is also a source of variability in cell growth and responsiveness. In hindsight, a better experimental design would have been to use a defined media with minimal serum and added trophic factors to enhance neuronal survival. As well, regular generation of new stably transfected clones would have minimised the confounding influence of prolonged passaging in selection media.

Finally, the pAD clones used for the studies described herein were chosen for their high levels of APP expression. A better approach may have been to look for a correlation between the level of APP expression (i.e., low, medium, and high expressing clones) and the degree of MAPK activation or mitochondrial uncoupling. Demonstration of the results in another cell line would also have been valuable. An alternative source of cells for the analysis of APP overexpression in a CNS context is transgenic embryos.

Primary cultures of specific neuronal populations or of glial cells should be a powerful system to examine the effects reported here in P19 cells, provided the transgene promoter is sufficiently active *in vitro*.

7.2 Lessons from P19 cell grafts

Many factors influence the successful implantation of cells into the brain including: donor and host immunogenetic differences, maturity of donor and host, cavitation of host tissue at the graft site, graft vascularisation, and functional "appropriateness" of the graft phenotype to the host site (Dunnett et al. 1986; Lund et al. 1989). APP expression *per se* does not appear to influence graft survival because grafts from mouse embryos bearing trisomy 16 (the murine equivalent of trisomy 21) persisted for at least one year in syngeneic hosts (Richards et al. 1993). Successful implantation of differentiated P19 and human embryonal carcinoma (NT2N) cells into cyclosporin A-treated rat hosts has been described (Morassutti et al. 1994; Trojanowski et al. 1993a). Fukuchi et al. (1994b) demonstrated expression *in vivo* of an APP C-terminal cDNA using the cytomegalovirus promoter in P19 cells grafted into mouse hippocampus.

Our failure to find the expected overexpression of APP in our 4 week old graft might reflect the attrition of high expressing cells, the persistence of transcriptional repressors inhibiting cytomegalovirus promoter activity, or the selection and expansion *in vivo* of a subclone that had lost or silenced its transgene. An effect of cyclosporin cannot be ruled out, although to date it has not been reported to influence APP production.

It was interesting to note that, at high antibody concentrations, APP immunoreactive material, perhaps reflecting endogenous murine protein, was localised to areas of the graft infiltrated by vascular elements. The blood-brain barrier status after transplantation is a controversial issue; however, in one study of transplanted C6 glioma

cells, extravasation of dye or horseradish peroxidase was extensive in a one week old graft (Bertram et al. 1994). Cerebral vessels are subject to senile changes and vessels in Alzheimer's disease brain have a number of additional abnormalities (reviewed by Kalaria 1996). Although not correlated with functional deficits, it is possible these changes compromise the barrier in aged brain. Brewer and Ashford (1992) reported that rat hippocampal neurons exposed to human serum exhibited an increase in immunofluorescence for four Alzheimer's disease-related antisera: anti-phosphorylated tau, anti-Aβ, anti-MAP 2 and anti-ubiquitin. The increase was greater with serum from old versus young humans, did not occur with fetal bovine serum, and was not observed with anti-neuron specific enolase antisera. It is tempting to suggest that our results reflect a reactive upregulation of endogenous murine APP in response to serum borne factors. The localisation of immunoreactivity within processes makes this a more likely scenario than infiltration of soluble APP ectodomain from rat plasma.

7.3 Intracellular processing of APP

To accomplish our analysis of the intracellular localisation of APP catabolic fragments, we developed a monoclonal antibody highly specific to a human epitope in the Aβ domain. Using this antibody in a preembedding EM protocol, we could visualise immunoreactivity in vesicular compartments that previous biochemical studies had suggested might be sites of α- and β-secretase activity. Ours is the first evidence that does not rely on inhibiting cellular processes to identify the proteolytic compartments.

In addition to confirming a role for the *trans*-Golgi and for endosomes in APP proteolysis, we observed immunoreactivity within dilatated ER. We speculate that this latter finding might be indicative of low level γ-secretase activity in this compartment, and might be the mechanism by which presenilin mutations, through their interaction

with immature forms of APP, promote γ(42)-secretase cleavage. Long-lived trisomy 16 grafts exhibit ultrastructural pathologies that include extended ER and Golgi further suggesting that overexpression of APP taxes the protein processing machinery of the cell. Since all newly synthesised proteins destined for fast axoplasmic transport pass through the Golgi, failure to efficiently process APP may be detrimental to activities dependent on rapid protein transport, as well as possibly being amyloidogenic.

Our EM study also revealed Aβ immunoreactivity spontaneously associated with particular organelles. We observed deposits at the outer mitochondrial membrane, associated with microtubules and filaments, and decorating the nuclear envelope. These observations emphasize that APP metabolites could act from within the cell to affect activities localised to these sites. It cannot be ruled out that these are spurious associations reflecting the overexpression of the precursor protein; however, there are intriguing parallels to other proteins with hitherto established roles within a single cell compartment. The cytosolic enzyme GAPDH has also been localised to the plasma membrane, mitochondria, cytoskeleton, and nucleus. In addition to its known role in glycolysis, a number of non-glycolytic activities have been described (Ishitani et al 1998 and references therein). Bcl 2 has anti-apoptitic activity mediated through its association with the mitochondria. However, it is also found in the ER and nuclear envelope (Krajewski et al. 1993), and has been shown to stabilise the ER Ca^{2+} pool (Distelhorst et al. 1996) and to promote axon regeneration in the CNS (Chen et al.1997).

As described in Chapters 2, 4, and 6, the C terminus of APP is the site of several protein interactions and is subject to dynamic phosphorylation. Conditional expression of the terminal 117 amino acids of APP in human neuroblastoma cells was reported to induce pronounced cytotoxicity. Exposure to conditioned media had no effect suggesting the toxicity involved intracellular mechanisms (Sopher et al. 1994). The Aβ peptide has

at least one intracellular partner, ERAB, which is thought to be a hydroxysteroid dehydrogenase enzyme, and the Aβ-ERAB interaction was shown to mediate toxicity in neuroblastoma cells (Yan et al. 1997). These reports, combined with our ultrastructural data, support the notion that APP or its metabolites might have important intracellular activities of relevance to the viability of mature CNS cells.

7.4 APP and oxidative metabolism

When first characterising our APP transfected clones, we investigated whether overexpression of the protein, and presumably of its Aβ metabolite, resulted in apoptosis (see Section 2.6.1). Fukuchi and colleagues, also working with P19 transfectants, had described both toxicity and enhanced survival with different APP constructs (Fukuchi et al. 1992b, 1996b); hence, the effect of APP on P19 survival was unclear. Both undifferentiated and retinoic acid-treated P19 cells appear to be relatively resistant to Aβ-induced toxicity as determined by MTT assay. However, examination of the ultrastructure in differentiated clones revealed a striking mitochondrial abnormality in cells lacking the nuclear changes typical of apoptosis or necrosis. Our collaborators at the Mt. Sinai School of Medicine determined that APP-expressing clones exhibit a decrease in $\Delta\psi_M$ relative to control transfectants.

Oxidative phosphorylation depends on the proton-motive force generated by proton pumping and establishment of an electrochemical gradient. The decrease in membrane potential implies relative uncoupling of respiration with potentially serious consequences for energy metabolism and Ca^{2+} sequesteration. This reduction in $\Delta\psi_M$ is distinct from the collapse of the membrane potential that accompanies acute cytotoxic insults, but may be particularly relevant to the slowly evolving nature of Alzheimer's disease. Indeed, oxidative defects have been uncovered in other neurodegenerative

disorders including Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (Parker Jr. & Swerdlow 1998; Swerdlow et al. 1998; reviewed in Beal 1996). The neurodegeneration characteristic of each disease may reflect the specific oxidative abnormality; however, post-mortem evidence of free radical damage points to prolonged oxidative stress in each of these disorders (Beal 1996).

Our observations in P19 cells need to be confirmed and expanded with particular reference to the generation of reactive oxygen species, Ca^{2+}-buffering capacity, and ATP status of APP-expressing cells. Preliminary studies in newly differentiated P19 clones indicate that APP overexpression may induce some change in anti-oxidant systems as revealed by increased resistance to high (>500 μM) concentrations of hydrogen peroxide. Conversely, no difference in vulnerability to sodium nitroprusside-induced toxicity was found between APP-transfected and control clones (data not shown). As the following review suggests, the specific mitochondrial changes induced by APP overexpression may be very relevant to the pathophysiology of Alzheimer's disease-type neurodegeneration.

Free intracellular Ca^{2+} plays a pivotal role in the transduction and exocytic response to ligand-mediated signals. Mitochondria are an important Ca^{2+}-buffering organelle that sequesters and releases Ca^{2+} as dictated by the needs of the microenvironment (Simpson & Russell 1998). Agents that cause loss of membrane potential (permeability transition) disrupt mitochondrial Ca^{2+} cycling and induce cell death. It is conceivable that less severe decreases in $\Delta\psi_M$ could produce a more indolent defect. In this regard, human neuroblastoma cybrids transformed with mitochondria from Alzheimer's disease patients exhibit subtle disturbances in Ca^{2+} homeostasis (Sheehan et al. 1997). Even subtle changes, if persistent, may be sufficient to disrupt Ca^{2+}-dependent events in sensitive systems, i.e., cholinergic neurotransmission (Peterson & Gibson 1983).

As discussed in Chapter 5, there is ample antemortem functional and postmortem biochemical evidence for a defect in oxidative metabolism in Alzheimer's disease brain. Acute uncoupling of oxidative phosphorylation promotes the toxic accumulation of reactive oxygen species (Zamzami et al. 1995). Chronic hypometabolism may induce less severe oxidative stress in Alzheimer's disease brain but, over time, oxidative damage to membrane lipids, proteins, and DNA accumulates (Beal et al. 1993). If APP can influence $\Delta\psi_M$ and/or oxidant control systems *in vivo*, the increase in APP expression with aging (Section 2.3.3) may reach a threshold wherein its effect on mitochondrial function, perhaps in combination with patient-specific factors that affect mitochondrial integrity or antioxidant defences, could bring about an Alzheimer's disease-type neuropathology.

It should not be neglected that the effect of APP on $\Delta\psi_M$ may serve a purpose in promoting certain cell functions. For example, both reactive oxygen species and Ca^{2+} can influence differentiation and plasticity (Mattson 1998; Mattson & Barger 1993; Sohal & Allen 1990). In this regard, cortical neurons from trisomy 21 fetuses degenerated, beginning at 1 week in culture, coincident with elevated levels of reactive oxygen species (Busciglio et al. 1995). It is tempting to speculate that higher levels of APP in these cells exacerbated the effects of reactive oxidative species produced normally during neuronal differentiation.

7.5 APP and kinase activation

One of the key puzzles in Alzheimer's disease is understanding the connection between APP/Aβ-associated lesions (plaque) and tau/PHF-associated lesions (neurofibrillary changes). The cytoskeletal changes involve abnormal phosphorylation of neurofilament triplet proteins and, most strikingly, of tau (Grundke-Iqbal et al. 1986;

Talamo et al. 1989; Vickers et al. 1994). Interest has focussed, therefore, on the possible abnormal activity of kinases and/or phosphatases. Our observation that overexpression of APP alters the phosphorylation dynamics of a candidate kinase, MAPK, provides a novel mechanism for reconciling the origin of the two lesions.

It should be noted that MAPK is not the only kinase capable of phosphorylating tau. Microtubule affinity-regulating kinase (MARK) and protein kinase C, for example, also phosphorylate tau in such a way as to affect its binding of microtubules (Combs et al. 1998; Drewes et al. 1995, 1997). It is unknown whether APP exerts any influence over the activity of these kinases.

As discussed in Chapter 6, and further demonstrated in our EM study in Chapter 4, APP associates with the cytoskeleton and this may allow it to mediate protein-protein interactions leading to the activation of microtubule-tethered MAPK. MAPK is also activated by Ca^{2+} and by hydrogen peroxide (Guyton et al. 1996; Luo et al. 1997; Xia et al. 1996); hence, the mitochondrial changes associated with APP overexpression may underlie MAPK hyperphosphorylation, particularly at the 3 day time point of neuroectodermal differentiation when our data were collected. Interestingly, ATP depletion was reported to activate other relevant kinases (a neurofilament kinase and a tau kinase) in PC12 cells (Bush et al. 1995). Because the dephosphorylation of MAPK is an integral part of the kinase signalling mechanism (reviewed in Hunter 1995), it is also possible that APP influences the activity of MAPK phosphatases or their access to phosphorylated MAPK. This is an interesting possibility that has yet to be explored.

Abnormally phosphorylated tau self-assembles into paired helical filaments which resist degradation. Recent work by Del C. Alonso et al. (1997) indicates that paired helical filament tau can sequester normal tau, MAP 1, and MAP 2, and thereby promote microtubule disassembly. Thus, it may be that minor changes in tau

phosphorylation, provided it occurs on residues leading to the persistent paired helical form of the protein, are sufficient to reach a threshold for self-promoting changes in microtubule stability. This slow evolution of tau phosphorylation is in keeping with the idea that APP-associated neuropathology depends on reaching an event threshold.

7.6 Overview and conclusions

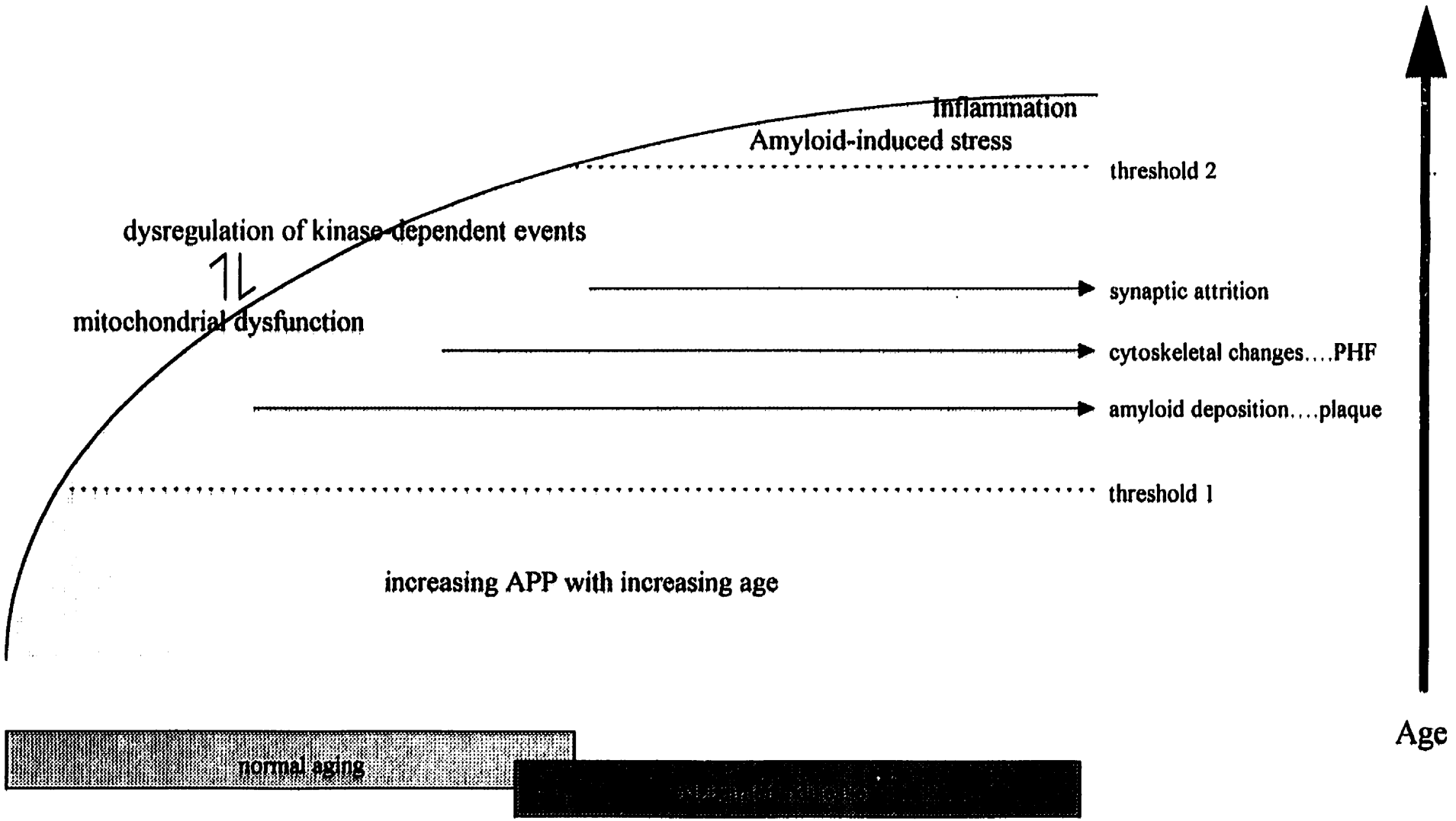
The work completed for this thesis has fulfilled the stated objectives of revealing the intracellular associations of APP catabolic fragments and investigating functional changes, specific to APP-overexpressing cells, that may be particularly relevant to the genesis of the slowly evolving neurodegeneration of Alzheimer's disease.

We have found the following:

- Aβ-epitope-containing fragments were found in *trans*-Golgi vesicles and in endosomes in proximity to the cell surface. Abnormally dilatated ER was identified as a potential site of secretase cleavage.
- These fragments were also spontaneously associated with mitochondria, microtubules, filaments, and the nuclear membrane thereby placing them in a position to interact with organelle-specific factors.
- Overexpression of APP in neuroectodermally differentiated clones was correlated with abnormal mitochondrial morphology and autophagy.
- The mitochondria of differentiated APP-expressing clones exhibited a decrease in mitochondrial membrane potential but remained as sensitive to complex I inhibition as control clones.
- $p42^{MAPK}$ expression became downregulated, but the proportion of phosphorylated protein was increased by neuroectodermal differentiation. Upregulation of transgenic

of the APP effect and by increasing Aβ amyloidosis. Amyloid plaque stimulates reactive changes that place additional demands on compromised neurons and exacerbate the primary defects. As well, plaque is the trigger for an inflammatory reaction that is directly toxic. The age of onset of Alzheimer's disease reflects the time the event threshold is reached, the resilience of an individual's neuronal circuitry, and the rapidity of reaching the secondary trigger point.

Figure 20. A hypothetical model of Alzheimer's disease. APP expression slowly rises with age, eventually reaching a threshold beyond which key cellular activities become dysregulated. Amyloid deposition and cytoskeletal changes begin. Progression to Alzheimer's disease, in certain individuals, occurs as APP expression and, perhaps, Aβ-peptide accumulation exceed the threshold for more global toxic changes, including inflammation. Between the two event horizons, the senile changes of normal aging and the preclinical stages of Alzheimer's disease overlap.


Inflammation
Amyloid-induced stress
threshold 2
dysregulation of kinase-dependent events
mitochondrial dysfunction
synaptic attrition
cytoskeletal changes....PHF
amyloid deposition....plaque
threshold 1
increasing APP with increasing age
Age
normal aging

Chapter 8

Potential therapeutic targets

"The desire to take medicine is perhaps the greatest feature which distinguishes man from animals." Sir William Osler

The therapeutic armamentarium against Alzheimer's disease is very small. Current therapies such as tacrine and donepezil aim to boost remaining neurotransmitter function (Mohr et al. 1994), although the underlying neurodegeneration may not be slowed. Repair of the damaged neuronal circuitry has been proposed (Cuello 1994), but problems of specificity, efficacy, and delivery remain to be solved. Inhibition or even reversal of plaque formation has received considerable attention (Soto et al. 1998) and may prove valuable in combating progression of the later stages of the disease. Our data suggests there are other mechanisms at work in the earlier, indolent stage of the disease that may be amenable to pharmaceutical correction.

The mechanism by which APP induces a decrease in $\Delta\psi_M$ is fertile ground for drug development studies. Among APP's myriad activities (reviewed in Section 2.5.2) ion channel modulation, copper binding, or protease activity may have a role to play. New protein partners are emerging which may interact with APP. In this regard, APP and Bcl 2 were upregulated in an identical fashion in rat retina after lesioning (Chen et al. 1996), and both Bcl 2 and Bcl-x_L have been shown to modulate $\Delta\psi_M$ (Hennet et al. 1993; Vander Heiden et al. 1997). Localization of the mutated protein products of Wilson's disease (Lutsenko & Cooper 1998) and Freidreich ataxia (Campuzano et al. 1997) to mitochondria also point to this organelle as an important site for neurotoxic actions. It

might, therefore, be useful to look for interactions between APP and resident mitochondrial proteins.

It will be important to elucidate the mechanism by which APP alters the phosphorylation dynamics of MAPK. If it involves activation through Ca^{2+} and/or oxidants arising from the uncoupling of respiration, then improving mitochondrial function will be the goal. If the mechanism involves an effect of APP itself on the signalling cascade, then it will be important to determine the proteins involved as a first step to inhibiting the process.

Amelioration of the changes wrought by increased APP may prove of great benefit to individuals genetically at risk for developing Alzheimer's disease, to patients with the very earliest symptoms, and perhaps even to the healthy elderly as a preventative measure in old age.

List of original contributions

1. We generated and characterised a new monoclonal antibody that demonstrates excellent reactivity to amyloid in human brain tissue as well as activity against soluble synthetic peptide in ELISAs. This antibody recognises an epitope within amino acids 1-12 of Aβ and is specific for the human protein.

2. We applied preembedding immunogold electron microscopy to resolve the intracellular associations of APP fragments bearing the Aβ epitope. Immunoreactivity was found in the *trans*-Golgi and in early endosomes confirming published reports of proteolysis in these compartments. Ours is the first report to localise the subcellular generation of APP fragments without interfering in normal cell function.

3. We demonstrated the spontaneous association of Aβ-epitope-containing fragments with mitochondria, microtubules, filaments, and the nuclear envelope. We also directly visualised these fragments within abnormal dilatated ER.

4. Using MTT assays to measure cell death, we determined that P19 cells are relatively resistant to the neurotoxic effects of soluble Aβ.

5. In collaboration with Dr. Tatton and his colleagues: Overexpression of $hAPP_{751}$ in neuroectodermally differentiated cells was found to be associated with a decrease in $\Delta\psi_M$. This effect was distinct from complex I inhibition. Our observation is the first

report that high intracellular levels of APP can produce a discrete functional deficit in oxidative phosphorylation..

6. We observed distinctive morphological abnormalities in mitochondria of APP-overexpressing cells after retinoic acid-induced neuroectodermal differentiation.

7. We established that P19 cells express almost exclusively $p42^{MAPK}$. Using phosphorylation-specific anti-MAPK antisera, we described the effect of neuroectodermal differentiation on MAPK activation

8. We observed dysregulation of MAPK phosphorylation concomitant with APP overexpression. In early differentiated cultures, a sustained activation of MAPK was found. In mature APP-expressing cells, we observed an attenuated response to aFGF stimulation.

"I do not know what I may appear to the world, but to myself I seem to have been only a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Sir Isaac Newton

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