STUDIES ON THE AMYLOID PRECURSOR PROTEIN: IMPLICATIONS FOR ALZHEIMER'S DISEASE

Thesis submitted for the degree of Doctor of Philosophy, in the Faculty of Science of the University of London

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ABSTRACT

It is believed that the progressive accumulation and deposition of beta-amyloid peptide (β/A4) in Alzheimer's disease (AD), results from one or several errors in the normal metabolism of its precursor, amyloid precursor protein (APP).

Using antibody 22C11 raised to the N-terminus of APP, amyloid precursor protein-like immunoreactivity (APPLIR) was determined by Western blotting in a soluble and two membrane fractions of human brain. Species of APPLIR present were characterised using antibodies 7H5, 10D5 and DA1 which recognise motifs of APP other than the N-terminal sequence. Total soluble APPLIR was found to be higher in AD, which may reflect reduced non-amyloidogenic α-secretory processing of APP. Given that choline acetyltransferase activity was also lower in AD when comparing the same samples, this result may be related to cholinergic hypoactivity. Multiple regression analysis, used to investigate correlations of cortical cell parameters with APPLIR concentrations, indicated that pyramidal neurones may represent the major source of APP in brain and that secretory APP processing possibly depends on cortical pyramidal neurone activity. Furthermore, measurements of secreted APPLIR concentration in ventricular cerebrospinal fluid of patients receiving lithium, hypothesised to attenuate the signal transduction cascade linking receptor activation to secretory processing of APP, and those drugs capable of reducing cortical excitatory transmission e.g. anticholinergic drugs, were found to be reduced.

When comparing human brain enzyme preparations thought to contain the candidate β/A4-generating protease β-secretase, enzyme activity was found to be lower in AD temporal cortex when compared to controls. Thus alternative, potentially amyloidogenic pathways may be favoured in AD e.g. the endosomes/lysosomes. In energy perturbed PC12 cells, observations following treatment with the lysosomotropic agent chloroquine suggested that endosomal/lysosomal processing of APP may be favoured with sparing of secretory cleavage.

Finally, the first monoclonal antibody (3B11) to the APP homologue, amyloid precursor-like protein 2 (APLP2) was prepared and characterised. Observations suggested that APLP2 may be abundant in brain membranes, which may highlight an important role for this non-amyloidogenic species. Development of 3B11 makes it possible to examine APLP2 in preparations containing APP.

In conclusion, there appears to be a relationship between cortical pyramidal neurone hypoactivity, cholinergic pathology and reduced non-amyloidogenic processing of APP. Lowered rates of β-secretase cleavage of APP may also be a feature of AD. Mechanism(s) favouring mismetabolism of APP are unknown. However, perturbed energy metabolism may be implicated. Possible therapeutic interventions based on pharmacological approaches being developed for AD (e.g. enhancing cholinergic transmission) have been suggested to beneficially affect the metabolism of APP by favouring α-secretory processing which cannot lead to β/A4 deposition.
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<th>Description</th>
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<tr>
<td>1,4,5-IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindole-acetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin; 5-hydroxytryptamine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACT</td>
<td>$\alpha$-antichymotrypsin</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>APLP</td>
<td>Amyloid precursor-like protein</td>
</tr>
<tr>
<td>APLP1</td>
<td>Amyloid precursor-like protein 1</td>
</tr>
<tr>
<td>APLP2</td>
<td>Amyloid precursor-like protein 2</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>APP-CSPG</td>
<td>Chondroitin sulfate proteoglycan form of APP</td>
</tr>
<tr>
<td>APPL</td>
<td>Amyloid protein precursor-like</td>
</tr>
<tr>
<td>APP/PN-II</td>
<td>Secreted extracytoplasmic portion of APP/protease nexin-II</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>$\beta$/A4</td>
<td>Beta-amyloid protein</td>
</tr>
<tr>
<td>BPB</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>[Ca$^{2+}$]i</td>
<td>Intracellular Ca$^{2+}$ concentration</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide $M$-chlorophenylhydrazone</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CS-GAG</td>
<td>Chondroitin sulfate glycosaminoglycan chain</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIW</td>
<td>Distilled de-ionised water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco's MEM/Nutrient Mix F12 (1:1)</td>
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<tr>
<td>DRN</td>
<td>Dorsal raphé nucleus</td>
</tr>
<tr>
<td>DS</td>
<td>Down's syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer's disease</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GABA</td>
<td>y-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HD</td>
<td>High buoyant density</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>KPI</td>
<td>Kunitz-type protease inhibitor</td>
</tr>
<tr>
<td>L-APP</td>
<td>Leukocyte-derived APP</td>
</tr>
<tr>
<td>LCSF</td>
<td>Lumbar cerebrospinal fluid</td>
</tr>
<tr>
<td>LD</td>
<td>Low buoyant density</td>
</tr>
<tr>
<td>mACHR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenalin</td>
</tr>
<tr>
<td>nbM</td>
<td>Nucleus basalis of Meynert</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPXY</td>
<td>Asn-Pro-Xxx-Tyr consensus motif</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFK-1</td>
<td>6-phosphofructo-1-kinase</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylflouride</td>
</tr>
<tr>
<td>RSMP</td>
<td>Rat sperm membrane protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>VCSF</td>
<td>Ventricular cerebrospinal fluid</td>
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Corrigenda
CHAPTER 1

1.0 INTRODUCTION

1.1 DEMENTIA

1.1.1 Definition

The term dementia is applied to an organic brain syndrome characterised by a general impairment of intellect, memory and personality, with no impairment of consciousness. The neuropsychological manifestations of dementia include combinations of impairments of memory, language, visuospatial skills, abstraction, calculation, judgement and executive functions. Non-cognitive behavioural symptoms may also be present and include personality changes, delusions, hallucinations, depression, mania, anxiety, aggression, purposeless hyperactivity, and altered sexual behaviour (Katzman, 1986).

1.1.2 Forms of dementia

There are many forms of dementia syndrome, the most common being dementia of the Alzheimer type (Alzheimer's disease, AD) and vascular or multi-infarct dementia. Aetiologies of dementia include a variety of degenerative, metabolic, infective or toxic processes. For a list of putative causes of dementia see Table 1.1. It should be noted that the symptoms of dementia may be simulated by other psychiatric syndromes including depression, delirium, neurosis and personality disorder and functional psychoses. The concept of pseudodementia was originally proposed to draw attention to this fact (Kiloh, 1961), although it has recently become almost synonymous with depressive presentations.

1.2 ALZHEIMER'S DISEASE

In 1907 Alois Alzheimer reported the case of a 51 year old woman who died after a 5-6 year illness characterised by dementia, dysphasia, and psychosis (Alzheimer, 1907). Clinically and histologically this case was significant because of its close
### Table 1.1 POSSIBLE AETIOLOGIES OF DEMENTIA

#### Degenerative
- Dementia of Alzheimer type
- Lewy body dementia
- Parkinson's disease
- Parkinsonism-ALS-dementia complex of Guam
- Progressive supranuclear palsy
- Motor neurone disease
- Non-Alzheimer dementia of frontal lobe type
- Pick's disease
- Huntington's disease
- Multiple sclerosis
- Idiopathic thalamic degeneration

#### Vascular
- Multi-infarct dementia
- Lacunar state
- Binswanger's disease
- Amyloid angiopathy

#### Post neurological insult
- Dementia pugilistica
- Following open or closed head injury
- Cerebral anoxia
- Carbon monoxide poisoning
- Subarachnoid haemorrhage

#### Infective
- AIDS-dementia complex
- Creutzfeldt-Jakob disease
- Gerstmann-Sträussler syndrome
- Herpes simplex encephalitis
- Post bacterial or fungal meningitis/encephalitis
- Neurosyphilis

#### Toxic
- Alcohol related dementia
- Heavy metal poisoning

#### Space occupying lesions
- Chronic subdural haematoma
- Primary or metastatic intracranial tumor

#### Metabolic/endocrine
- Hypothyroidism
- Vitamin B₁₂ deficiency
- Folate deficiency
- Wilson's disease (disorder of metal metabolism)
- Mitochondrial/ carbohydrate/lysosomal disorders

#### Miscellaneous
- Normal pressure hydrocephalus
- Epilepsy
- Systemic lupus erythematosus (SLE)
- Cerebral sarcoidosis
resemblance to senile dementia, a condition well known at the time, but only in persons at, or, after the age of 65. Recognising the clinical and neuropathological similarities between the pre-senile disorder (onset of symptoms before the age of 65), and the more common senile condition, Alzheimer believed the two to be the same. However, the term Alzheimer's disease was applied to describe dementia occurring in patients with pre-senile onset only. Therefore, it was usual practise to recognise senile dementia and pre-senile dementia as two closely related, but clinically distinct entities.

This concept was altered however, after landmark studies in the late sixties (Roth et al., 1966; Blessed et al., 1968), when it became apparent that most senile dementia was not the result of benign senescent forgetfulness (memory defects associated with normal ageing), or a phenomenon secondary to some other condition such as cerebral atherosclerosis, but rather was the result of the same degenerative brain lesions that Alois Alzheimer described as pre-senile dementia in 1907.

1.2.1 Incidence

As the most common cause of dementia today, AD accounts for approximately 50% of all cases of dementia (Tomlinson and Corsellis, 1984), and is the fourth or fifth most common cause of death after heart disease, cancer and stroke (Post, 1994). At present, an estimated 400,000 people in the UK and 2.5-3 million people in the USA are affected by this disorder, which although considered predominantly a disease of old age, is not confined just to the elderly population. Approximately 18,000 of those affected in the UK are below retirement age and some have symptoms as early as their fourth decade (Rossor, 1993). The median duration of the disease is 7-10 years from diagnosis, and death is most commonly due to bronchopneumonia (Rossor, 1993).

Since a diagnosis of AD is still based largely on clinical criteria (see 1.2.2), estimates of prevalence can show degrees of variation because of differences in how dementia is defined, the method of measurement, and the composition of the population in which dementia is to be measured. Roughly 3-5% of individuals over the age of 60-65 years, and up to 48% of individuals over 85 years can be diagnosed as having probable AD (Evans, 1990; see also 1.2.2). Prevalence rates for AD are thought to double every 4.5 years between ages 60 and 95 (Jorm and Korten, 1987).

The increasing incidence of AD, is primarily due to the demographic shift towards a growing proportion of old people in the population, and as our society ages,
the long-term medical, social and economic implications are becoming a matter of wide concern. Now recognised as a leading public health problem for the 21st century, the cost of medical and nursing care, social services and caregiver time in the United States has been estimated at $113.2 billion for 1991 alone and in the UK, the total cost of all NHS, community and non-NHS care for 1992 has been estimated at £1.4 million (Gray, 1994).

1.2.2 Diagnosis

At present there are no valid biological markers for AD, therefore on the basis of an appropriate clinical picture of progressive dementia supported by laboratory findings that exclude other aetiologies of dementia, a diagnosis of probable AD can be made. Definite AD however, can only be confirmed with certainty following histological examination of the brain post mortem or more rarely by cerebral biopsy.

Neuropathologically, AD is characterised by an abundance of extracellular amyloid deposits and intraneuronal neurofibrillary changes (Wisniewski et al., 1985; see also 1.2.4.2 and 1.2.4.4), and for a definite diagnosis confirmation of the presence, and in some cases the quantity, of these hallmarks in neocortical regions other than the hippocampus and entorhinal cortex, regions which are often affected in elderly non-demented cases (Davies et al., 1988; Mann et al., 1990a) is required (Khachaturian, 1985; Mirra et al., 1991).

To set valid guidelines for diagnosis, standardised criteria have been published which provide levels of probability of diagnosis. The current clinical criteria for dementia and AD include the Mini-Mental State Examination (Folstein et al., 1975), the Blessed Information-Memory-Concentration test (Blessed et al., 1968) and those established by the American Psychiatric Association in the Diagnostic and Statistical Manual, revised third edition (1987), the International Classification of Diseases (1992), the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (1984; 1992). For review see Farlow, (1994).
1.2.3 Clinical symptoms and signs

1.2.3.1 Cognitive symptoms

Most descriptions of AD correctly emphasise cognitive impairment as a key feature of the condition (American Psychiatric Association, 1980; McKhann et al., 1984; Medical Research Council, 1987b). A wide variety of cognitive symptoms occur at different times during the course of AD. Commonly these include not only impairments in memory, but also disturbances in other non-memory cognitive domains such as language use, perception, the ability to learn skills, solve problems, think abstractly and make judgements (Katzman, 1986).

In the early stages of the illness, the symptoms are often vague and diffuse and develop insidiously. However, memory loss is the cardinal and most common presenting complaint. Memory disturbances include both poor recall and impaired recognition. Language ability is frequently normal early in AD, although reduced overall conversational output and difficulty finding words may be noted. Slight impairment of visuospatial functioning (misplacing objects or getting lost) and concentration may also occur, and the patient is often unable to cope with complex situations. Motor skill learning, however is preserved, but calculation (including skills such as handling money), and judgement are also affected relatively early. As the disease progresses, the symptomatology is dominated by the cardinal cortical disturbances of dysphasia, dyspraxia (difficulty dressing or operating appliances), dysgnosia (failure to recognise family members or spouses) and increasingly compromised visuospatial disturbance. Memory disturbances worsen, however emotional functioning, personality and social behaviour are relatively spared and may remain intact long after memory has been lost. Towards the final stages of the disease, all mental functions, including the emotional ones, are severely impaired. Motor skills are lost and eventually language becomes obviously non-fluent and terminally the patient may be reduced to a state of near-mutism.

1.2.3.2 Non-cognitive symptoms

The non-cognitive symptoms of AD, encompass a variety of psychopathological and behavioural changes. Abnormal behaviours reported include wandering (Hope and Fairburn, 1990), urinary incontinence (Rabins et al., 1982), sleep disturbances (Teri et
aggression (Ware et al., 1990) and sexual disinhibition (Kumar et al., 1988). Wandering and urinary incontinence are major problems for both patients and carers and are often predictors of institutionalisation (O'Donnell et al., 1992). However aggression is the behavioural symptom which causes most distress to carers, both at home and in hospital. The majority of aggressive acts are directed towards other people or objects, in the form of verbal aggression, as well as physically aggressive acts such as hitting (Ware et al., 1990), with very few patients engaging in self-directed aggressive acts, such as hair-pulling, biting and cutting (Burns et al., 1990b; Burns et al., 1990a).

Documented psychopathological symptoms include disturbances of mood, such as anxiety, major depression and mania (Lopez et al., 1990; Pearlson et al., 1990). Approximately 40% of patients also experience delusions during the course of their illness (Deutsch et al., 1991). Often occurring in the early to middle stages of the disease, delusions frequently lead to aggressive behaviour. Hallucinations, are also well recognised, but less prevalent, with the majority of studies reporting hallucinations in about 25% of cases (Deutsch et al., 1991).

1.2.4 Neuropathology

1.2.4.1 Overview

Atrophy of the brain is a common finding in AD. This is prominent, and although usually widespread, tends to affect circumscribed-temporal and parietal areas most noticeably, with less frontal lobe atrophy. The primary sensory areas are relatively spared (Brun, 1983; Tomlinson and Corsellis, 1984; Esiri et al., 1986; Esiri et al., 1990).

When examined at post mortem the brain can be small, weighing 60-80% of the normal, and if cut coronally, the appearance of atrophy is enhanced by widening of the sulci, thinning of the cortical ribbon and moderate to severe dilation of the lateral and third ventricles (Damasio et al., 1983). Since brain weight normally depends on a patients age and sex, the severity of cerebral atrophy is often assessed by comparing brain volume with cranial capacity (Hubbard and Anderson, 1981) or by measuring either cortical thickness (Terry et al., 1981; Mann et al., 1985) or length (Duyckaerts et al., 1985). Reduction in cortical area may result from a decrease in the length rather
than in width of the cortex, indicating loss of columns of cells and fibres perpendicular to the pial surface.

Microscopically, AD is characterised by a number of specific histological features, in particular the two classic hallmarks of the disease, neurofibrillary tangles and senile plaques. Other pathological alterations seen in the brain of AD patients include neuropil threads, Hirano bodies, granulovacuolar degeneration and amyloid angiopathy. It is not within the scope of this thesis to review these latter alterations, however the two classic hallmarks of AD will be reviewed in detail in the following sections (see 1.2.4.2 and 1.2.4.4). The role of beta-amyloid protein (B/A4), a principle component of senile plaques, and its precursor molecule, amyloid precursor protein (APP) in the aetiology of AD will also be reviewed (see 1.2.5 and 1.3).

1.2.4.2 Neurofibrillary pathology

The intraneuronal deposits found in the brains of AD patients are commonly described as neurofibrillary lesions (as reviewed, Goedert, 1993). There are a number of types of these lesions: neurofibrillary tangles (NFT), found predominantly in large pyramidal neurones and apical dendrites, and neuropil threads found in distal dendrites. Intraneuronal fibrillary lesions are also found in the abnormal neurites associated with neuritic plaques. Ultrastructurally, all three lesions contain abnormal paired helical filaments (PHF) as their major components, and a number of straight filaments as a minor fibrous component. Neurofibrillary lesions develop in cells which undergo degeneration in AD, and their relative insolubility enables them to survive after the death of the associated neurone. This gives rise to the characteristic, extracellular ghost tangles which accumulate in the neuropil. The temporal mechanisms leading to the formation of neurofibrillary lesions is poorly understood, however unlike senile plaques (see 1.2.4.4), these lesions seem to be associated with neurones which degenerate in AD (cortical pyramidal neurones; see 1.2.6), and correlations have been described between tangle count, dementia and neocortical synapse number (Terry et al., 1991).

1.2.4.3 Paired helical filaments and tau protein

PHF are a common feature of intraneuronal lesions. Current evidence suggests that they are made entirely of the microtubule associated protein, tau (Brion et al., 1985), in an abnormally phosphorylated state. Ultrastructurally PHF have been shown
to consist of two strands of subunits twisted around each other in a helical fashion (Wischik et al., 1985), and can be isolated from tangle fragments or dispersed filaments (Wischik et al., 1988b; Greenberg and Davies, 1990). These two types of PHF have tau epitopes in common, but differ in their solubility in strong denaturing agents, and sensitivity to proteases (Goedert et al., 1992b; Goedert, 1993). Straight filaments also contain PHF, which also share tau epitopes with the former types (Goedert, 1993). Tau isotopes associated with these filaments have been termed PHF-tau (Greenberg and Davies, 1990).

In the nervous system, tau is one of two major microtubule-associated proteins. Encoded in humans by a single gene on chromosome 17 (Neve et al., 1986), multiple isoforms of tau are produced in a developmentally regulated manner by alternative splicing of mRNA (Goedert et al., 1988). Six human tau isoforms have been described, ranging in length from 352 to 441 amino acids and differing by the presence or absence of three inserts (Goedert et al., 1988). All isoforms contain three or four tandem repeats of 31 or 32 amino acids, the repeat regions contain 18-amino acid microtubule-binding elements (effective in promoting microtubule assembly, Lee et al., 1989), that are separated by flexible linker sequences which do not bind to tubulin (Butner and Kirschner, 1991). Tau is known to be a potent promotor of tubulin polymerisation in vitro (Cleveland et al., 1977); binding of tau to microtubules would seem to reduce their dynamic instability. Tau appears to have a similar function in vivo, since microinjection into fibroblasts has been shown to increase microtubule mass and resistance to depolymerising agents. Thus, tau function appears to be necessary for normal neuronal function and maintenance of transport in neurites (Kosik and Greenberg, 1994), as well as stabilisation of axonal morphology (Goedert, 1993). The characteristics of PHF-tau have been shown to differ from those of normal or recombinant tau in a number of ways, it has a higher apparent molecular weight (Greenberg and Davies, 1990; Goedert et al., 1992b), it is highly insoluble (Delacourte et al., 1990) and although tau normally co-purifies with microtubules, PHF-tau does not. Since no differences in the levels of expression of mRNAs corresponding to the different isoforms of tau have been detected in AD compared with age matched controls (Morrison, 1993), abnormal forms of tau protein that exist in AD are proposed to arise from post-translational modification. One such modification thought to account for these abnormal features and thus be essential for PHF assembly is excessive
phosphorylation of tau (Wolozin et al., 1986; Greenberg and Davies, 1990; Greenberg et al., 1992), which results in a characteristic shift in electrophoretic mobility of tau when run on polyacrylamide gels. Indeed, a set of three tau bands, referred to initially as A68 proteins (Wolozin et al., 1986), found to be present in regions with abundant neurofibrillary pathology, with an apparent molecular mass of 55-69 kDa, (compared with 45-65 kDa for normal tau proteins) has been found to contain highly phosphorylated species of all six normal human tau isoforms (Brion et al., 1991; Goedert et al., 1992b). Although eight possible phosphorylation sites have been proposed on PHF-tau (tau in normal mature brain is phosphorylated at two or three sites; Brion et al., 1993), biochemical and immunochemical analysis of PHF has identified only a few specific phosphorylation sites (Goedert et al., 1993). These sites are on serine/threonine/proline residues mainly located outside the tandem repeats which correspond to the microtubule-binding region (as reviewed, Goedert, 1993). Although phosphorylation of tau negatively regulates its ability to bind to microtubules, little is known about the relative contributions made by individual phosphorylation sites (Goedert et al., 1992b). Following phosphorylation, the reduced affinity of tau for microtubules may account for its self aggregation into PHF, together with destabilisation of microtubules and resultant disruption of cell function. These data lend support to the hypothesis that in AD, abnormal phosphorylation of tau gives rise to PHF and tangle formation leading to blockage of intraneuronal transport, thus provoking cell death.

Processes leading to the hyperphosphorylation of tau are unknown. Activation of one or several kinases, reduced activation of phosphatases or a combination of both may alter the phosphorylation state of tau (Goedert, 1993). Activation of protein kinase C (PKC) seems to result in tau phosphorylation, however, this does not give rise to the characteristic mobility shift associated with PHF-tau (personal communication, B.H. Anderton). Additionally, glycogen synthase kinase-3, implicated in the production of PHF-tau (Brion et al., 1993), may be down regulated and inactivated by PKC activation (Goode et al., 1993). A number of other candidate kinases include, serine directed mitogen activated kinase (Drewes et al., 1992), proline directed protein kinase (Vulliet et al., 1992; Paudel et al., 1993), and cAMP-dependent protein kinase (Robertson et al., 1993). In addition, it remains to be seen what role alterations in dephosphorylation of tau proteins by phosphatases might play in the pathogenesis of AD
Protein phosphatase 2-A, appears to be particularly active in the dephosphorylation of tau, previously hyperphosphorylated by kinases shown to produce PHF-tau (Paudel et al., 1993). Thus, treatment of PHF-tau extracted from AD brain dispersed filaments reverted the electrophoretic mobility characteristics of PHF-tau to that of normal tau isoforms. Mechanisms modulating the activity of this phosphatase are currently under investigation. Whether phosphorylation occurs before or after the assembly of tau into PHF and whether or not it is essential for PHF assembly is another unanswered question. Tau protein has been shown to have a tendency to self-associate and form fibrils (Montejo de Garcini et al., 1986) and it has been shown recently that expressed fragments of tau protein, containing the repeated tubulin-binding region, can assemble PHF-like filaments in vitro and in the absence of phosphorylation (Crowther et al., 1992; Wille et al., 1992). To date therefore, proposals that A68 proteins represent a precursor stage in the development of cross-linked insoluble aggregates that constitute the PHF (Greenberg and Davies, 1990; Goedert et al., 1992b) have not been proven. In light of these findings, it is conceivable that the phosphorylation of tau protein in PHF occurs as a secondary process in which kinases act on PHF that have already accumulated within neurones in AD. Kinases have been reported to be associated with PHF prepared by a variety of methods (Vincent and Davies, 1992).

1.2.4.4 Senile plaques and amyloid protein

In AD, senile or neuritic plaques are found in the hippocampus, amygdala, entorhinal cortex, and neocortical association areas (Wisniewski et al., 1989). Ranging in size from 50 to 200 μm (Terry, 1985), these lesions, in classic terms consist of a compacted spherical deposit of extracellular fibrillar beta-amyloid protein (β/A4) surrounded by variable numbers of dystrophic neurites, both axonal and dendritic. Such plaques may also contain activated microglial cells intimately surrounding the core of β/A4 as well as reactive astrocytes around the periphery (Masters et al., 1985a). The fibrils of the plaque core are ultrastructurally distinct from PHF (they are extracellular, unpaired, and approximately 8 nm in diameter).

The term beta-amyloid protein is a generic description applied to a class of otherwise unrelated peptides, which under physiological conditions aggregate to form insoluble filaments about 7-9 nm wide. These peptides are usually derived from a larger
precursor protein (see 1.3), and have in common an antiparallel β-pleated sheet secondary structure, a characteristic which gives rise to the Congo red birefringence and proteolysis resistant characteristics of the classical plaque (Tomlinson and Corsellis, 1984). Masters et al. (1985b) purified plaque core amyloid from AD cortex and demonstrated that the subunit protein in the core had an amino acid composition identical to that of the 4 kDa meningovascular β/4 first purified and sequenced by Glenner & Wong (1984). It is now clear however, that the plaque core amyloid and to a lesser extent the meningovascular amyloid contains a mixture of amyloid peptides (ranging in length from 39-43 residues) rather than a single species (Kang et al., 1987; Miller et al., 1993), and is now becoming apparent that longer β/4 species (β/4_{1-42/43}) may favour deposition in AD because of a higher capacity (compared to shorter species, β/4_{1-40}) to aggregate into fibrils (see 1.2.5).

β/4 is detected throughout the neuropil in a variety of morphological forms. These include large diffuse plaque-like structures without amyloid cores, and mature and burnt-out plaques with dense cores (as reviewed, Selkoe, 1994). Both mature and burnt-out plaques are readily detected using Congo-red, thioflavin or classical silver stains. Diffuse plaques, however are recognised by more sensitive silver staining methods or immunologically using antibodies to β/4 (Yamaguchi et al., 1988). Diffuse plaques do not exhibit cellular interactions with neurones, astrocytes or microglia (Rozemuller et al., 1989), and appear to represent the majority of all neuronal β/4 deposits (Mann, 1994). The relationship between the various types of plaques (if any), is presently unknown. However it has been hypothesised that neuritic plaques develop from preceding diffuse plaques (Yamaguchi et al., 1988). According to this hypothesis, diffuse plaques, over time, condense adopting a more ordered, amyloid-like structure that exhibits weak to moderate staining with amyloid stains and begin to show indications of cellular interactions. These so-called primitive plaques (Ogomori et al., 1989) continue to develop into classic senile or neuritic plaques. The final stage in this sequence is the burnt-out or compact plaque, consisting of a shrunken dense core of amyloid. These plaques are few in number and typically devoid of cellular elements (Probst et al., 1987).
1.2.5 Pathological role of β/A4

Indirect evidence supporting a role for β/A4 in the aetiology of AD came following the discovery that the gene for the β/A4 precursor protein APP, is localised to chromosome 21 (Kang et al., 1987) and is overexpressed in Down's syndrome (DS; due to trisomy 21). DS is often cited as an example of amyloidosis caused by high expression of APP (Rumble et al., 1989), and it has been proposed that β/A4 deposition in AD may also be a consequence of APP overexpression (Cohen et al., 1988; Higgins et al., 1988; Neve et al., 1988; Palmert et al., 1988; Tanzi et al., 1988). It is generally accepted that DS invariably results in the development of AD-like neuropathology by the fourth or fifth decade (Oliver and Holland, 1986). A number of studies have shown that DS progresses in an orderly and sequential manner, whereby diffuse plaques occur during the early stages (Ikeda et al., 1989; Verga et al., 1989), followed some years later by the formation of further neuropathology e.g. NFT, and apparent related decline in cognitive ability, in brains exhibiting mature neuritic plaques (Mann and Esiri, 1989; Mann et al., 1990c). These data lend credence to the idea of plaque evolution (see 1.2.4.4), and by analogy have been used to argue a similar temporal sequence in the appearance of pathology in AD. Indeed, when looking at the apparent distribution of different plaque types within the brain, plaques found in normal aged brains and in relatively spared regions of AD brain (e.g. cerebellum), tend to be of the diffuse type and are not associated with neurodegenerative changes (Joachim et al., 1989; Mann et al., 1990b). In contrast, neuritic plaques are usually found distributed throughout severely affected regions in AD e.g. entorhinal cortex, hippocampus, amygdala and neocortical association areas, and are associated with neurodegenerative change.

Assembly of β/A4 into aggregated fibrils (fibrillogenesis) has been postulated to be a crucial event in transforming benign diffuse plaques into injurious neuritic ones. Mechanisms leading to β/A4 fibril formation are unknown, however, it has been suggested that longer β/A4 species favour fibrillogenesis. Iwatsubo et al (1994) have investigated the deposition of β/A4 in brain using end-specific monoclonal antibodies which recognise different β/A4 species. Application of these antibodies has demonstrated that all plaques, both diffuse and cored, contain β/A41-42/43, while only a subset of plaques (mostly mature) contain β/A41-40. If according to the proposed hypothesis of plaque evolution (see 1.2.4.4), diffuse plaques evolve into mature or
cored plaques, these data strongly imply that the initial β/A4 species deposited in brain tissue, in AD, is β/A4_{1-42/43} rather than β/A4_{1-40}. Microglial cells have been proposed to remove these last two or three amino acids (which strongly promote fibril formation in vitro; Jarrett et al., 1993) and convert β/A4_{1-42/43} into β/A4_{1-40}. Indeed, in young patients with DS double labelling of cortical plaques for β/A4_{1-40} and β/A4_{1-42/43}, as well as for microglial cells, has shown that in contrast to β/A4_{1-40}-positive plaques, diffuse β/A4_{1-42/43}-positive plaques contain few or no microglia. In older DS or AD patients, most β/A4_{1-42/43}-plaques were found to contain microglia as were the much more numerous β/A4_{1-40}-positive plaques (unpublished observation; cited in Mann, 1995). It would seem therefore that the earliest diffuse plaques in the cortex are free from activated microglial cells, although they subsequently appear rapidly and their numbers seem to increase dramatically with time, particularly in cored plaques.

The purpose for this is not clear, however, loss of these last few amino acids from β/A4_{1-42/43} may allow for plaque dissolution. Microglial cells may thus serve vital roles in plaque evolution through promoting dissolution of β/A4. Indeed, there is evidence in AD (Hyman et al., 1993), DS (Hyman et al., 1993; Royston et al., 1994) and aged non-demented individuals (McKenzie, 1994) that the amount of amyloid deposited in the cerebral cortex does not continue to increase throughout the disease course, but stabilises at some point. This may reflect a halt in β/A4 production, or a balancing out between rates of deposition or dissolution. Furthermore, a change from β/A4_{1-42/43} to β/A4_{1-40} may change its level of neurotoxicity. These possible functions for microglia would account for the close anatomical relationship between them and the amyloid fibrils in plaques (Wegiel and Wisniewski, 1990). Microglia may also promote the reactive astrocytosis which surrounds and infiltrates plaque cores (Mann et al., 1992b), through secretion of interleukins (Guilian et al., 1988).

1.2.6 Distribution of neurofibrillary tangles and selective neuronal vulnerability

Quantitative measurement of cell numbers in AD has been complicated by cerebral atrophy (Miller et al., 1980; Hubbard and Anderson, 1981; see also 1.2.4.1), and early studies suggested that nerve cell numbers were unaltered in AD (Tomlinson and Henderson, 1976; Terry et al., 1977; Terry, 1979). However, more recent observations allowing for atrophy by counting cells in columns of cerebral cortex rather
than in individual fields, indicate in AD a significant decrease (approximately 36-46 %) in the number of large pyramidal neurones in circumscribed (temporal and parietal) cortical (in particular layers III and V of the neocortex; Terry et al., 1981; Mountjoy et al., 1983; Hubbard and Anderson, 1985) and hippocampal (CA1) areas (Terry et al., 1981; Mann et al., 1986). Cell loss from the entorhinal region, especially layer II (Hyman et al., 1984) has also been described. Remaining neocortical pyramidal neurones of layer III and V are sites for tangle formation (Pearson et al., 1985; Lewis et al., 1987). Pyramidal neurones form the majority of cortical efferents (Jones, 1984), and such neuronal loss is thought to be primarily responsible for the cognitive deficits observed in AD. This proposal is supported by the findings of several studies where significant correlations between synapse and cell loss in these areas and ratings of dementia have been described (Neary et al., 1986; DeKosky and Scheff, 1990; Terry et al., 1991).

Loss of cholinergic neurones in the nucleus basalis of Meynert (NbM) giving rise to the cholinergic innervation of the cerebral cortex, is also well recognised in AD (Mann and Yates, 1982; Perry et al., 1982; Whitehouse et al., 1982; Nagai et al., 1983; Arendt et al., 1985). Furthermore, regional differences in the extent of cell loss in this region have also been observed, with those parts which project to the most severely involved areas of the cortex showing the greatest pathology (Hardy et al., 1986).

The observations of NFT formation in the NbM (Ishii, 1966) and cholinergic neurites in senile plaques (Struble et al., 1982), may indicate a link between the cholinergic system and the pathological features of AD. Indeed, these findings also complement biochemical data indicative of altered cholinergic function in AD (see 1.2.9.1). The serotonergic nuclei of the brain stem, particularly the dorsal raphé (Yamamoto and Hirano, 1985), and the noradrenergic locus coeruleus (Tomlinson et al., 1981; Marcyniuk et al., 1986) are also sites of cell loss in AD (see 1.2.9.3 and 1.2.9.4 respectively). Like the basal forebrain, neuronal loss in these nuclei appears to be organised topographically (Hardy et al., 1986; Marcyniuk et al., 1986), such that the pathology is greatest in those parts which send fibres to regions severely involved by the pathology of AD, particularly the medial temporal lobe (specifically the hippocampus, entorhinal cortex, amygdala and the parahippocampal gyrus).
Although the mechanism(s) of cell death in AD are unknown, neurones which project to areas exhibiting severe pathology commonly contain NFT. It is apparent that only certain subgroups of pyramidal neurones are prone to tangle formation, specifically the large pyramidal cells in layers III and V. Smaller pyramidal cells in layers II, VI and in the upper part of layer III appear to be resistant to NFT formation, as are the spiny stellate cells and small pyramidal cells in layer IV, as well as all classes of inhibitory interneurones (Pearson et al., 1985). It has been proposed that such selective neuronal vulnerability to tangle formation can be understood in terms of morphology and specific connectivity patterns. Several quantitative analyses of NFT distribution in the neocortex (Pearson et al., 1985; Lewis et al., 1987; Braak et al., 1989; Hof et al., 1990; Hof and Morrison, 1990), hippocampus (Hyman et al., 1986), and amygdala (Brady and Mufson, 1990) have stressed the correlations between the localisation of these lesions and the large pyramidal neurones which give rise to corticocortical and hippocampal projections.

Projections within the neocortex, connecting one area of cortex with another (corticocortical connections) are anatomically complex. At the simplest level, functional columnar circuitry is one of the fundamental characteristics of the neocortex, with all neurones within a narrow strip of cortex being functionally coupled (Mountcastle, 1957). However, corticocortical more usually refers to neurones which interconnect areas of neocortex within the same hemisphere (association fibres), or opposite hemispheres (commissural connections). Almost all corticocortical projections arise from pyramidal neurones in layers III and V. These neurones form the majority of cortical efferents (Jones, 1984), and are targets for early tangle formation in circumscribed (temporal and parietal) areas (Pearson et al., 1985; Lewis et al., 1987). Furthermore, normal functioning of corticocortical activity is implicit to higher cognitive function, and thus there is a clear correlation between cognitive deficit and synapse and cell loss, attributable to loss of corticocortical projections (Neary et al., 1986; DeKosky and Scheff, 1990; Terry et al., 1991).

Hippocampal projections, interconnect the hippocampal formation with the association cortices, entorhinal cortex and basal forebrain (structures crucial to memory), and are severely affected early in the pathology of AD (Hyman et al., 1984; Pearson and Powell, 1989; Hyman et al., 1990). Projections from the hippocampal formation to the entorhinal cortex, amygdala, and neocortical areas originate from the
CA1 field and the subiculum, areas shown consistently to contain numerous NFT (Hirano and Zimmerman, 1962; Hyman et al., 1990; Braak and Braak, 1991). Layers II and V of the entorhinal cortex also display high densities of NFT (Hyman et al., 1984; Hyman et al., 1986; Brady and Mufson, 1991). Indeed, the entorhinal cortex has been identified as a site of severe and early involvement in AD (Hyman et al., 1984; Hyman et al., 1986; Braak and Braak, 1991), and the degeneration of connections to specific nuclei in the amygdala as well as other limbic and cortical association areas has been shown to correlate with high NFT densities in layer V of the entorhinal cortex (Van Hoesen, 1982).

1.2.7 Distribution of senile plaques

Regional and laminar variations in NFT and senile plaque distribution in the neocortex suggest that NFT are more closely associated with the presence of corticocortically projecting neurones (see 1.2.6). While the distribution of plaques correlates with corticocortical terminations, the correlation has been shown not to be as strong as for NFT, suggesting that multiple neuronal systems may contribute to their formation (Pearson et al., 1985; Rogers and Morrison, 1985; Duyckaerts et al., 1986; Lewis et al., 1987; Braak et al., 1989; Hof et al., 1990; Hof and Morrison, 1990). Although less specific, the regional and laminar distribution of senile plaques may still be related with respect to connectivity. Indeed, it has been proposed that senile plaque distribution reflects the degeneration of terminals projecting from neurones that contain NFT (Lewis et al., 1987).

The distribution of senile plaques in the hippocampus also displays a strong correlation with specific projections (Hyman et al., 1990). However, the distribution is somewhat variable, with some zones containing more plaques than others, in particular, layer III of the dentate gyrus, and the superficial layer of the subiculum display high senile plaque densities (Hyman et al., 1990).

1.2.8 Correlates of cognitive impairment

A correlation between the presence of NFT, senile plaques and senile dementia was suggested by Alzheimer in 1907. However the first demonstration that structural changes in the brains of elderly subjects may be related to the severity of cognitive impairment during life was that of Blessed and colleagues (1968), who observed a
correlation between numbers of senile plaques and the severity of cognitive impairment. However, both demented and non-demented patients were included in the correlation thus weakening it.

Subsequent autopsy findings in other studies have indicated that Alzheimer-type lesions are always associated with some level of cognitive impairment in a high proportion of cases (Morris et al., 1991), but several studies have shown that the number of senile plaques may not be a good pathological correlate for the loss of cognitive function. In one such study (Wilcock and Esiri, 1982), plaques were found to correlate poorly with cognitive dysfunction, furthermore, numerous β/A4 plaques have been detected in non-demented elderly cases (Braak and Braak, 1991). Although amyloid deposition is characteristic of AD, it may not therefore be the critical step that leads to cognitive dysfunction (as suggested by Hardy and Allsop, 1991; see also 1.5). Indeed, in studies of patients examined by cerebral biopsy (i.e. in the early to middle stages of the disease), correlations have been found between pyramidal cell loss from the cortex and global ratings of severity of dementia (Neary et al., 1986) and between synapse loss and cognitive impairment (DeKosky and Scheff, 1990).

1.2.9 Neurotransmitter Pathology

Neurotransmitter pathology is discussed here, because this is a factor that seems to influence metabolism of APP. It is important to emphasise that while much APP metabolism takes place in glutamatergic cortical pyramidal neurones (see Chapter 3), the activity of these cells is influenced by other transmitter-receptor interactions.

1.2.9.1 Cholinergic pathology

One of the most consistently described deficits in AD brains is that of the cholinergic system, first described by Bowen et al. (1976) and closely followed and corroborated by other groups (Davies and Maloney, 1976; Perry et al., 1977a; Perry et al., 1977c; Davies, 1979).

Neuropathological studies confirm that there is usually a considerable loss of cholinergic neurones in the nbM (Whitehouse et al., 1982; Nagai et al., 1983) and assay of cholinergic terminals by choline acetyltransferase (ChAT) activity has revealed post mortem reductions in all neocortical areas studied (Bowen et al., 1976; Davies and Maloney, 1976; Perry et al., 1977a; Perry et al., 1977c). Determination of
the integrity of the cholinergic system at earlier stages of the disease by biopsy (Bowen et al., 1982; Sims et al., 1983a), also revealed deficits. Furthermore, the loss of this activity has been correlated with both the severity of dementia (Francis et al., 1985; Neary et al., 1986), and neuropathological changes including senile plaque formation (Perry et al., 1978; Mountjoy et al., 1984) and neuronal loss (Mountjoy et al., 1984) especially of the pyramidal cells (Neary et al., 1986).

Several other parameters of cholinergic function have been found to be reduced in AD. Biochemical analysis of neurosurgical samples has confirmed the presence of impaired acetylcholine (ACh) turnover in the cholinergic synapse, with decreased choline uptake (Sims et al., 1983a) and reduced ACh synthesis (Bowen et al., 1983). In keeping with these biochemical results, studies using acetycholinesterase histochemistry (Henke and Lang, 1983; McGeer et al., 1986) or ChAT immunocytochemistry (Rasmayr et al., 1989) have demonstrated a widespread loss of cortical cholinergic innervation in AD.

With respect to clinical significance, there is little doubt that the cholinergic system is involved in cognitive processes, learning and memory (as reviewed, Hasselmo and Bower, 1993). Thus loss of cholinergic neurones in AD may have a specific role in the pathophysiology of AD (Perry et al., 1978). This assumption is supported by observations that lesions of the nbM deplete the cortex of its cholinergic innervation, and impair learning and memory in a number of animal species (Aigner et al., 1987; Irle and Markowitsch, 1987; Ridley et al., 1992). In humans, cholinergic antagonists such as scopolamine, have been shown to interfere with learning in healthy young adults and to produce memory deficits qualitatively similar to those found in early AD. Furthermore, this disturbance may be reversed by physostigmine, a cholinesterase inhibitor, but not by amphetamine (Drachman and Leavitt, 1974; Bareggi et al., 1982).

On the basis of these and subsequent data (Bartus et al., 1982; Coyle et al., 1983), the cholinergic hypothesis was formulated. This hypothesis suggests that degeneration of cholinergic neurones in the basal forebrain system, with consequent presynaptic deficit in cholinergic projections, (predominantly to the cortex) may be a key feature of AD, thus inviting a parallel with Parkinson's disease, in which nigrostriatal dopaminergic neurones selectively degenerate. Degeneration of cholinergic forebrain neurones may explain the decrease in cholinergic markers in the cortex in AD. However, there is some doubt as to the validity of the view that AD is a primary
disorder of the cholinergic system (Bowen et al., 1977). Degeneration of the nbM appears to be topographic in that more severe degeneration is observed in parts which project to the most severely affected neocortical areas (Adrendt et al., 1985), suggesting that cholinergic pathology may not precede neocortical pathology. This is supported by the work of Sofroniew and Pearson (1985), who observed retrograde degeneration of the nbM following topical cortical application of excitatory amino acids. Furthermore, degeneration of the nbM does not appear to be inevitably linked to AD, but is also seen in a number of other neurological conditions including some where dementia or cortical pathology are not part of the clinical picture e.g. olivopontocerebellar atrophy (Kish et al., 1988). It is therefore apparent that a deficit in cholinergic transmission can explain only part of the cognitive decline in AD.

1.2.9.2 Glutamatergic pathology

Histochemistry for glutamate and its putative synthetic enzyme glutaminase, and retrograde transport of radiolabelled aspartate has provided evidence for a transmitter role of this excitatory amino acid in a number of pathways, including corticocortical association pathways, corticofugal pathways and the major hippocampal pathways (as reviewed; Ottersen, 1991; see also Vincent and McGeer, 1980; Herrling, 1985 and Girault et al., 1986). It is reasonable to assume therefore that pyramidal neurones giving rise to these pathways are glutamatergic, a proposal supported by retrograde tracing techniques using $[^3]H$-D-aspartate (Fonnum and Walaas, 1978; Streit, 1980; Kohler, 1986).

Due to difficulties in separating neurotransmitter glutamate from large metabolic pools and because glutamate concentrations appear to be sensitive to post mortem factors (Procter et al., 1988b), studies of brain glutamate concentrations have provided equivocal evidence regarding the status of glutamatergic pyramidal neurones in AD (Francis et al., 1993b). A number of studies report reduced glutamate levels in the cortex and hippocampus at post mortem (Arai et al., 1985; Hyman et al., 1987; Procter et al., 1988b), while others report no change (Perry et al., 1987). In biopsy material, the potassium-stimulated release of endogenous glutamate and aspartate has been reported to be unaltered in AD compared to controls (Smith et al., 1983), although direct measurement has been inconclusive (Perry et al., 1987; Procter et al., 1988b). In one study of temporal lobe biopsy samples, of the 14 amino acids measured, glutamate
was the only one found to be depleted in AD. Furthermore, loss of glutamate correlated with reduced density of pyramidal neurones in layer III of the neocortex (Lowe et al., 1990). Therefore one interpretation is that the glutamate content of the samples studied has been influenced by loss of pyramidal neurones. Additionally, Hyman et al (1987) reported an 80% reduction in glutamate concentration in the terminal zone of the perforant pathway. Consistent with this, decreased glutamate staining was observed in the molecular layer of the dentate gyrus (Kowall and Beal, 1991), and the same study also reported reduced numbers of glutamate and glutaminase immunoreactive pyramidal neurones in the CA fields of the hippocampus.

Glutamine has been suggested as a likely precursor for the transmitter pool of glutamate (Ottersen, 1991), and in view of this, phosphate-activated glutaminase activity has been assayed as a potential marker of glutamatergic neurones (Donoghue et al., 1985). However when directly measured, phosphate-activated glutaminase activity was found to be unaffected in AD frontal and temporal cortex (Procter et al., 1988a). In contrast, loss of glutaminase immunoreactive cortical neurones has been noted in AD patients (Akiyama et al., 1989; Kowall and Beal, 1991), and in the hippocampus, glutaminase-reactive pyramidal neurones have been shown to contain tangles and exhibit distorted morphology (Kowall and Beal, 1991).

As an alternative to measurement of glutamate concentrations or glutaminase activity, both of which may be sensitive to post mortem factors (Procter et al., 1988b), many studies have employed [3H]-D-aspartate binding to assess presynaptic glutamatergic terminals. Initially binding of [3H]-D-aspartate, was thought to reflect labelling of the high affinity presynaptic glutamate transporter (Cross et al., 1986), and on the basis of depleted [3H]-D-aspartate binding in the cerebral cortex and hippocampus, loss of glutamatergic terminals in AD was suggested (Palmer et al., 1986; Procter et al., 1986; Cross et al., 1987; Cowburn et al., 1988). However, unaltered [3H]-D-aspartate binding has been shown, even in cortical regions known to be severely affected by structural degeneration in AD (Palmer et al., 1986; Cowburn et al., 1988). It is possible that glial proliferation in AD may have confounded the results of these studies, since glial cells also possess high affinity glutamate uptake transporters (Beach et al., 1989). A 40-50% decrease in sodium dependent active uptake of [3H]-D-aspartate into synaptosomes prepared from prompt autopsy AD material was reported by Proctor et al (1988b), although it is unclear whether this represents a
reduction of glutamatergic neurones (the most likely interpretation), or a *peri mortem* effect, as non-demented control tissue with short autopsy was not available. More recently, however, cloning of at least one glutamate transporter suggests that there is little uptake of $[^3H]D$-aspartate by presynaptic terminals (Rothstein *et al.*, 1994). Although far from conclusive, the results of this one study indicate that some reinterpretation of previous data may be necessary.

The lack of a specific marker for presynaptic glutamatergic terminals, has prevented the direct demonstration of a loss of glutamatergic pyramidal neurones in AD. Since there is a marked selective loss of layer III and layer V pyramidal neurones which utilise glutamate as their transmitter, and synaptic loss is estimated at 40 % of elderly control values (Davies *et al.*, 1987; Scheff *et al.*, 1990; Terry *et al.*, 1991), it is probable that there is a substantial presynaptic glutamatergic deficit in AD. There is much evidence linking glutamatergic transmission with learning and memory function in animals (for review see, Hyman *et al.*, 1990). For example, *N*-methyl-*D*-aspartate (NMDA) receptor antagonists have been shown to inhibit the development of long-term potentiation, a process that may underlie some forms of memory (Morris *et al.*, 1986; Collingridge, 1987), and impair learning behaviour in rats (Staubli and Lynch, 1992). Moreover, drugs which may enhance NMDA-mediated transmission (e.g. *D*-cycloserine) appear to facilitate learning behaviour in some species (Monahan *et al.*, 1989), and induce long-term potentiation in others (Collingridge and Singer, 1990). Furthermore, the hippocampal formation, including the entorhinal cortex, a structure with many glutamatergic neurones (Ottersen, 1991) has a pivotal role in neural systems underlying memory (Alkon *et al.*, 1991; Ridley and Baker, 1991). It therefore follows that the loss of glutamatergic function (possibly exacerbated by the cholinergic deficit; see 1.2.9.1) may contribute to cognitive decline in AD.

### 1.2.9.3 Serotonergic pathology

Cell loss and morphological changes in the dorsal raphé nucleus (DRN) are well established in AD (Ishii, 1966; Mann and Yates, 1983; Yamamoto and Hirano, 1985; Chen *et al.*, 1994). However biochemical analysis of serotonin (5-HT) containing neurones in AD indicates that structural pathology of raphé neurones may not necessarily be reflected by reduced serotonergic innervation of the cortex. Studies in both biopsy and *post mortem* tissue taken from AD patients have reported reduced
concentrations of 5-HT and its major metabolite 5-hydroxyindole-acetic acid (5-HIAA) (Bowen et al., 1979; Cross et al., 1983; Gottfries et al., 1983; Arai et al., 1984a; D'Amato et al., 1987; Palmer et al., 1987a; Reinikainen et al., 1988; Burke et al., 1990), in many cortical areas. However, this is not consistent finding, with other studies showing no evidence of a selective reduction in presynaptic 5-HT in AD (Adolfsson et al., 1979; Baker and Reynolds, 1989; Leake et al., 1993). This discrepancy may in part be explained by the inadvertent selection of institutionalised patients. By comparison with patients who have died in a community setting, within an institutionalised group the complex interrelationships between behavioural changes (often determining the need for institutionalisation; see 1.2.3.2), cognitive symptoms (see 1.2.3.1), as well as drug treatment and other confounding factors, such as disease severity, need to be considered. In an attempt to address these issues, brains from 20 consecutive post mortem examinations carried out as part of a larger community based study of AD (where non-cognitive behavioural changes may not be over-represented), were examined for structural and functional markers of serotonergic innervation in two cortical areas (see Chen et al., 1996). Despite a loss of 40% of tryptophan hydroxylase immunoreactive cells in the DRN and a loss of presynaptic uptake sites (as determined by \[^{3}H\]-paroxetine binding) of comparable magnitude in the temporal cortex, concentrations of 5-HT and 5-HIAA were not significantly reduced in either area. Since individual DRN neurones project to several areas of the brain and no significant loss of \[^{3}H\]-paroxetine binding was found in the frontal cortex, this may provide evidence for sprouting of remaining serotonergic innervation in a region less affected by the pathological process of AD. In view of these findings, presynaptic serotonergic activity is possibly maintained in AD. Indeed turnover of transmitter appears to be enhanced in AD patients compared to controls, based on the increased ratio of 5-HIAA to 5-HT in cortical tissue and the nucleus basalis (Sparks et al., 1992).

1.2.9.4 Noradrenergic pathology

A number of studies describe loss of locus coeruleus cell bodies in AD (Mann et al., 1980; Bondareff et al., 1981), with concurrent tangle occurrence and noradrenergic markers in neuritic plaques (Fowler et al., 1992). There is also a corresponding loss of noradrenaline (NA) in some neocortical areas (Cross et al., 1981; Francis et al., 1985; Palmer et al., 1987b), although the concentration of the NA metabolite
3-methoxy-4-hydroxphenylglycol has been reported to be elevated (Gottfries et al., 1983), unaltered (Palmer, 1987) or reduced (Cross et al., 1983). This may arise as a result of the pre-terminal state of AD patients. Deficits in NA do not appear to be correlated with dementia, although they may contribute to the non-cognitive deficits (Fowler et al., 1992).

1.2.9.5 Cortical interneuronal systems

In AD, reductions have been reported in several intrinsic neurotransmitter and neuromodulator systems of the cerebral cortex. A number of studies have shown significant reductions in cortical levels of γ-aminobutyric acid (GABA) and its synthetic enzyme glutamic acid decarboxylase (GAD; Bowen et al., 1976; Perry et al., 1977b; Davies, 1979; Rossor et al., 1980b; Perry et al., 1981), while others have found no change (Davies and Maloney, 1976; Perry et al., 1984). When present, these reductions have been shown to be restricted to very few cortical areas, while the same studies report a widespread loss of cortical ChAT activity in every area studied (Rossor et al., 1980b; Perry et al., 1977b). Loss of GABAergic innervation has also been indicated by reduced levels of GABA re-uptake markers (Hardy et al., 1987; Simpson et al., 1988), and reduced somatostatin immunoreactivity (Davies et al., 1980; Rossor et al., 1980a; McKinney and Richelson, 1984) which is co-localised with GABA in non-pyramidal neurones (Hendry et al., 1984). It should be noted that the assessment of the activity and integrity of GABA containing neurones in post mortem tissue may be affected by epiphenomena e.g. agonal state (Francis et al., 1993b) and thus the interpretation of such studies may be complicated. However, in one careful study where AD and control subjects were matched for the nature of terminal illness, no change in GAD activity was found (Reinikainen et al., 1988). Furthermore, one study of ante mortem biopsy tissue did not show a great loss of GABAergic neurones (Lowe et al., 1988), which suggests that the clinical features and pathological changes in AD brain are not dependent on the loss of GABAergic neurones.

A more widespread loss has been found in the levels of cortical somatostatin (reported loss ranging from 40-80 %; Davies et al., 1980; Ferrier et al., 1983; Wood et al., 1983; Arai et al., 1984b; Tamminga et al., 1987; Kumar et al., 1991; Mazurek and Beal, 1991), the density of somatostatin-positive fibres (Chan-Palay, 1987; Kowall and Beal, 1988), and the number of somatostatin-positive neurones (Chan-Palay, 1987;
Gasper et al., 1989). A few studies, however have found no changes in the levels of cortical somatostatin, or in the number of cortical somatostatin-positive neurones (Candy et al., 1985; Kowall and Beal, 1988). Although more widespread than the loss of GABA, the reductions in somatostatin concentrations have also been found to be restricted to far fewer cortical areas when compared to the loss of cortical cholinergic innervation. In addition, direct comparisons have shown that loss of cortical somatostatin, when present is smaller (40-76 %) than the loss of cortical ChAT activity (50-90 %; Davies et al., 1980; Rossor et al., 1980a; Ferrier et al., 1983).

Levels of a number of other neuropeptides have been investigated in AD cortex and have been shown to display no loss (vasoactive intestinal polypeptide; Rossor et al., 1980b; Perry et al., 1981) or inconsistent loss (substance P and cholecystokinin; Perry et al., 1981; Perry et al., 1981; Beal and Mazurek, 1987).

1.3 AMYLOID PRECURSOR PROTEIN

Molecular cloning studies have shown that β/A4 (see 1.2.4.4) is derived by unidentified proteolytic processes from a 695-770 amino acid membrane bound glycoprotein, designated β-amyloid precursor protein (APP). This precursor of β/A4 is encoded by a single-copy gene on chromosome 21 (Multhaup, 1994), and displays a high degree of evolutionary conservation (Robakis et al., 1987). APP appears to be expressed in virtually all neuronal and non-neuronal mammalian tissues, with the brain and kidney showing the highest levels of expression (Tanzi et al., 1988).

1.3.1 Amyloid precursor protein structure

The APP gene consists of 19 exons which code for a typical N- and O-glycosylated transmembrane protein containing a large extracellular domain, followed by a transmembrane domain and a short cytoplasmic domain (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988). Multiple isoforms of APP are generated by alternative mRNA splicing and major isoforms of 695, 751 and 770 amino acids (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987) have now been identified. Both APP$_{751}$ and APP$_{770}$ contain a 56 amino acid insert (coded for by exon 7) in the extracytoplasmic region with a approximate 50 % homology to the Kunitz family
of serine protease inhibitors. This domain is referred to as the Kunitz-type protease inhibitor domain or KPI domain and APP isoforms containing this insert will be referred to in this thesis as \(\text{APP}_{\text{KPI}}\). The exact role of the KPI motif in the proteolytic processing of APP is unknown. \(\text{APP}_{770}\) has an additional 19 amino acid insert (OX-2 domain; encoded by exon 8) of unknown functional significance, immediately adjacent to the KPI domain.

Other major structural domains present include a 17 residue signal peptide, an N-terminal cysteine rich region, a novel zinc binding motif (Bush et al., 1994), an acid rich domain, 2 heparin binding domains, N- and O-linked carbohydrate attachment sites, and \(\text{G}_{\text{q}}\) and clathrin binding motifs (see Fig. 1.1).

More recently a third alternatively used splice site in the APP gene, involving exon 15 has been identified (Konig et al., 1992). This exon codes for 18 amino acids which precede the \(\beta/\text{A4}\) domain of APP by 16 amino acids. It has been suggested that alternative splicing of exon 15 may influence the pathway leading to the liberation of \(\beta/\text{A4}\) (Konig et al., 1992). APP transcripts excluding exon 15 were originally identified in peripheral leukocytes, and therefore denoted leukocyte-derived APP or L-APP mRNA.

Qualitative studies (Palmert et al., 1989) have suggested that \(\text{APP}_{695}\) is the predominant species of APP protein in brain, apparently consistent with the presence of abundant \(\text{APP}_{695}\) mRNA in this tissue. However, results from a study by Van Nostrand and colleagues (1991) identify \(\text{APP}_{\text{KPI}}\) as the predominant species (see also Webster et al., 1994) indicating that in human brain, APP protein concentrations do not necessarily correspond with APP mRNA levels.

### 1.3.2 Amyloid precursor protein-like proteins

It is now evident that APP is a member of a highly conserved family of related proteins (Wasco et al., 1992; Wasco et al., 1993c; Slunt et al., 1994). A Drosophila transcript has been identified (Rosen et al., 1989) that encodes a protein which demonstrates strong sequence homology with \(\text{APP}_{695}\). The new gene defined by this transcript is the amyloid protein precursor-like or \(\text{APPL}\) gene, and its protein product has been termed \(\text{APPL}\) (Martin-Morris and White, 1990). Although \(\text{APPL}\) bears sequence similarity with APP, the protein also has regions which are highly dissimilar (Sprecher et al., 1993), and therefore differs in overall domain organisation.
The molecule depicted corresponds to APP_{695}. Splicing of the KPI-encoding exon in the indicated position yields APP_{751}, while inclusion of both KPI and OX-2 exons yields APP770. The 17 residue signal peptide is followed by 170 residues with 12 cysteines, within this region is a heparin binding domain and a zinc binding domain. The next domain is largely acidic, consisting of 100 residues, and contains a primary structure motif composed of proline (P), glutamate (E), serine (S) and threonine (T). Then there is a second heparin-binding domain which is coincident with a growth promoting domain, and two N-linked carbohydrate attachment sites. The β/A4 protein is a 39-42 amino acid peptide corresponding to a transmembrane region of APP that extends into the extracellular space. Arrows indicate the α, β and γ-secretase cleavage sites. G_α and clathrin binding motifs are both located within the short cytoplasmic domain. Modified from Robakis, 1994.
recently two mammalian cDNAs one from mouse and one from human brain have been isolated, which encode two amyloid precursor-like proteins (APLPs) termed APLP1 and APLP2 (also called APPH) respectively (Wasco et al., 1992; Sprecher et al., 1993; Wasco et al., 1993b). In addition, a third mammalian cDNA encoding a rat sperm membrane protein (RSMP) has been isolated which shows sequence homology with APP in the transmembrane and cytoplasmic domains (Yan et al., 1990). However, this cDNA isolated is only partial length, and the remainder of the coding sequence for RSMP is currently unavailable for further comparison.

1.3.2.1 APLP1

Structurally APLP1 is predicted to be 42% identical and 64% similar to APP at the amino acid level (Wasco et al., 1992). Thought to resemble a membrane-associated glycoprotein (Wasco et al., 1992), the regional distribution and cellular specificity of APLP1 in rat brain and human hippocampal formation is reported to be virtually identical to that of APP (Wasco et al., 1993d). Northern blot analysis also reveals that in the mouse, APLP1 message is present in a variety of peripheral organs (Wasco et al., 1992). In humans, the gene for APLP1 has been mapped to chromosome 19, in the same general vicinity that has been postulated to contain the late-onset familial AD (FAD) gene defect (Pericak-Vance et al., 1991; see also 1.4.2). The overall conservation of amino acid sequence and domain structure within APP and APLP1 implies that they may share common functions and be processed similarly. APLP1 however, notably lacks sequences which correspond to either the KPI domain or the β/A4 peptide (Sprecher et al., 1993). Subsequently, APLP1 cannot act as a substrate for amyloid formation, however, an alteration in, or the overproduction of APLP1 could affect the overall maturation and metabolism of APP and ultimately the generation of β/A4. Factors or events leading to the up-regulation of APLP1 might also result in increased expression of APP and processing through an amyloidogenic pathway.

1.3.2.2. APLP2

Presently, APLP2 bears the closest structural relationship to APP. It is similar in overall domain structure, with virtually all the identified domains and motifs that characterise APP present. Specifically, an N-terminal cysteine rich region, a
zinc-binding motif, an acid rich domain, at least one N-glycosylation site, a hydrophobic membrane spanning domain, a cytoplasmic domain containing a Go coupling motif, a clathrin binding motif, potential serine/threonine, casein kinase I/II, and tyrosine phosphorylation sites and unlike APLP1, a KPI domain. However like APLP1, APLP2 also lacks the β/α4 domain and therefore cannot act as a substrate for β/α4 deposition in AD.

Several major APLP2 isoforms have been identified that are encoded by alternatively spliced APLP2 transcripts. Both APP and APLP2 mRNA are expressed in brain and peripheral tissues (Wasco et al., 1993b; Slunt et al., 1994), and in situ hybridisation studies have disclosed similarities in the distributions of APP and APLP2 mRNA in mouse (Slunt et al., 1994) and human (Wasco et al., 1993b) central nervous system. In addition, APLP2 has been shown to mature through the same secretory/cleavage pathway as APP (Slunt et al., 1994).

More recently, several APLP2 isoforms encoded by alternatively spliced transcripts have been reported to be post-translationally modified by a chondroitin sulphate glycosaminoglycan chain (CS-GAG; Thinakaran and Sisodia, 1994; Pangalos et al., 1995). Furthermore, APP isoforms that lack sequences encoded by exon 15 (L-APP) are also modified by CS-GAG, whereas APP forms containing the exon 15 are not. It has been suggested that CS-GAG modification of a subset of APP and APLP2 isoforms represents a means of generating functional diversity for these similar proteins. In addition, chondroitin sulphate proteoglycans have been found in and around senile plaques and neurofibrillary tangles, and have as a consequence been suggested to play a role in the pathogenesis of AD (Brittis et al., 1992).

In view of the similarity between APP and APLP2 (APLP2 is predicted to be 52 % identical and 71 % similar to APP^151) the problem of distinguishing these two proteins with the use of antibodies becomes apparent. Recently Sisodia et al (1994) have documented that a wide array of anti-APP antibodies fail to discriminate APP from APLP2. However, to address this issue, a monoclonal antibody (3B11) recognising only APLP2 has now been raised (Chapter 7; see also Webster et al., 1995).
1.3.3 APP processing

1.3.3.1 Constitutive α-secretory pathway

Following synthesis in the endoplasmic reticulum, APP is thought to undergo extensive post-translational modification in the Golgi apparatus, including signal peptide removal, N- and O-linked glycosylation (Refolo et al., 1989; Weidemann et al., 1989), sulphation of tyrosine residues (Weidemann et al., 1989), phosphorylation (Oltersdorf et al., 1990) and addition of glycosaminoglycan side chains to yield a chondroitin sulphate proteoglycan (Shioi et al., 1992). Following maturation, approximately 20% of all newly synthesised full length APP undergoes proteolytic cleavage on the cell surface (Caporaso et al., 1992a), between amino acids Lys 16 and Leu 17 of β/A4 (or residue 688 according to APP770), thus precluding the formation of intact β/A4 and resulting in the release a large, soluble, extracytoplasmic portion protease nexin-II (PN-II; Oltersdorf et al., 1989; Van Nostrand et al., 1989; see also 1.3.4) also referred to as APP₈ (Schubert et al., 1989b; Weidemann et al., 1989; Esch et al., 1990). PN-II or APP₈ has been detected in cerebrospinal fluid (CSF; Palmert et al., 1989), human brain (Moir et al., 1992), serum (Bush et al., 1990; Podlisny et al., 1990), and in the conditioned media of various cells in culture (Weidemann et al., 1989). Since APP is axonally transported (Koo et al., 1990a), it is likely that APP₈ is released from axonal growth cones and axon terminals at synapses. A 10 kDa non-amyloidogenic C-terminal fragment remains associated with the cell following cleavage (Haass et al., 1991; Haass et al., 1993). It has been proposed that the 10 kDa fragment is cleaved further, following the identification of a secreted 3 kDa C-terminal fragment of β/A4 termed p3 into cell culture media and CSF (Haass et al., 1993).

The constitutive secretory pathway for APP is currently designated the α-secretory processing pathway, so-called because the (as yet undiscovered) enzyme which performs this non-amyloidogenic cleavage has been designated α-secretase (Esch et al., 1990; Seubert et al., 1993). Few details are available concerning α-secretase, although it is likely to be a member of a class of enzymes which regulates the shedding of ectodomains from a variety of transmembrane molecules, including growth factor precursors, cell adhesion molecules, receptors and ectoenzymes (Ehlers and Riordan, 1991). These enzymes appear to act primarily at or near the cell surface and to specify cleavage of substrates at a certain distance from the plasma membrane, largely without regard for the primary sequence surrounding the cleavage site (Sisodia, 1992).
1.3.3.2 Endosomal/lysosomal pathway

Studies of APP metabolism in the presence of either brefeldin A or monensin have to date failed to implicate the endoplasmic reticulum as an important site for the discrete proteolytic processing of APP (Caporaso et al., 1992a). Consequently, several groups have undertaken experiments to determine whether acidic compartments of the cell (e.g. endosomes/lysosomes or the trans-Golgi network) are important in APP metabolism. (Cole et al., 1989; Caporaso et al., 1992a; Golde et al., 1992; Haass et al., 1992a).

The possibility of endosomal/lysosomal metabolism of APP is supported by the identification of a conserved Asn-Pro-Xxx-Tyr (NPXY) consensus motif located in the cytoplasmic domain of APP (Chen et al., 1990), which may mediate its re-internalisation from the cell surface via clathrin-coated vesicles. The co-purification of APP with these vesicles has subsequently been demonstrated directly (Nordstedt et al., 1993). In addition, vesicle-neutralising agents such as chloroquine and ammonium chloride, when applied to cultured cells have been associated with greatly enhanced recovery of full-length APP and an array of C-terminal fragments, including non-amyloidogenic and potentially amyloidogenic fragments (Caporaso et al., 1992a; Estus et al., 1992a; Golde et al., 1992; Haass et al., 1992a). β/ A4 peptides however, have not yet been recovered from the lysosomes (Haass et al., 1993) and vesicular neutralisation fails to consistently diminish β/ A4 production in certain cell types (Busciglio et al., 1993), although neutralisation-induced stabilisation of the standard array of potentially amyloidogenic C-terminal fragments appears to be consistent.

Presently, it seems unlikely that β/ A4 is generated in the lysosomes (Haass et al., 1993), although this possibility cannot be ruled out, since β/ A4 may be secreted before it can accumulate in the cell at detectable levels, or it may be produced extracellularly from secreted lysosomally produced amyloidogenic precursors.

1.3.3.3 β-secretory processing of APP and normal cellular processing of β/ A4

Until mid-1992, β/ A4 had generally been described as being an abnormal metabolite of APP. However, this concept became obsolete with the discovery by several groups that a soluble β/ A4 species is detectable in body fluids from various
species, and in the conditioned medium of a wide variety of APP-expressing primary cells and transformed cell lines (Haass et al., 1992b; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). A sensitive sandwich enzyme-linked immunosorbant assay for the peptide has demonstrated that the levels of soluble β/Α4 secreted are in the high picomolar to low nanomolar range (Seubert et al., 1992). The cellular site involved in the N- and C-terminal cleavage responsible for β/Α4 generation has not yet been established, and the consistent inability to recover β/Α4 from cell lysates or from purified vesicles has shifted the focus away from the terminal degradative compartments of the cell such as the lysosomes (see 1.3.3.2). It is thought that β/Α4 may be generated in a compartment distal to the ER, since brefeldin abolishes its production and does not result in its accumulation intracellularly (Haass et al., 1993).

The possibility of heterogeneous cleavage of APP along the constitutive secretory pathway (i.e. cleavage in the pre-cell surface pathway or at the cell surface) has been suggested. At least two secreted APPs-like molecules that do not end at residue 16 of β/Α4 have been identified as components of the APPs pool of cleaved and released APP ectodomains. (Anderson et al., 1992; Seubert et al., 1993). One of these species appears to end at Met596 of APP695 indicating that normal secretion of APP can involve a proteolytic cleavage that creates the N-terminus of β/Α4 (Seubert et al., 1993). The activity responsible for the cleavage of APP between residues Met596 and Asp597 has been designated β-secretase (Seubert et al., 1993) to distinguish it from the originally defined α-secretase.

The cellular mechanisms by which the C-terminus of β/Α4 is generated is still unexplained, since this region of the APP molecule resides within the cell membrane. Gandy et al (1992) suggest this step might involve the trafficking of APP or a potentially amyloidogenic fragment into a multi-vesicular body where vesiculated APP or an APP fragment could be liberated from the bilayer. This is supported by ultrastructural evidence that multi-vesicular bodies are immunoreactive for APP epitopes (Caporaso et al., 1994). Once the cleavage which generates the carboxyl terminus of β/Α4 has occurred (performed by the putative activity γ-secretase), fusion with the plasma membrane of a vesicular body containing intra-luminal β/Α4 could result in the release of β/Α4 into the extracellular space.
1.3.3.4 Candidate APP processing enzymes

The role of non-lysosomal cytosolic Ca\(^{2+}\)-dependent neutral cysteine proteases (calpains) in the processing of APP, has received much attention, in view of their possible participation in a number of Ca\(^{2+}\) mediated neuronal events such as neurite outgrowth, synaptic transmission, long-term synaptic modification, and neuronal degeneration (Lynch and Baudry, 1984; Siman et al., 1987; Siman and Noszek, 1988).

Siman et al (1990) have focused on the processing of APP by calpain 1, and their results indicate that this enzyme may be involved in the normal and perhaps pathological processing of APP. Indirect evidence for calpain involvement in APP processing has come from in vivo experiments carried out by these authors, using intracranial administration of excitatory amino acids. APP levels in the hippocampus were shown to decrease following intraventricular infusion of kainate, a treatment that rapidly activates hippocampal calpain I and selectively decreases the content of calpain substrates (Siman and Noszek, 1988; Siman et al., 1989b). When coupled with other findings from the study, such as the co-localisation of calpain I and APP in the same cells, the extreme sensitivity of APP to the protease in vitro, and the capacity of calpain I to generate a 16 kDa C-terminal fragment of APP likely to contain the entire β/A4 peptide, a role for calpain I in APP processing is strongly suggested. Furthermore, if kainate induced APP loss is the result of increased proteolysis, it suggests a mechanism whereby excitatory neurotransmission may act through calcium influx and calpain activation to control APP catabolism. However, although β/A4 reportedly modifies neuronal sensitivity to excitotoxins by impairing intraneuronal calcium regulation (Mattson et al., 1992), calpain activation has not yet been directly implicated in this, and although previously hypothesised to play a key role in producing neuronal structural damage associated with excitotoxicity (Siman and Noszek, 1988; Siman et al., 1989b), a link between an excitotoxic mechanism and AD has not been well defined.

Given the importance of Ca\(^{2+}\) in the neuronal functions listed above, and because of evidence implicating calpain I in the processing of APP, the metalloproteases should also be considered as potential candidates for APP processing. Nelson & Siman (1989) identified and characterised four Ca\(^{2+}\) dependent enzymes, which on the basis of their inhibitor profiles and the ability of Zn\(^{2+}\) to reconstitute their activities, were found to be members of the metalloprotease class of enzyme. Three of
the brain metalloproteases (designated MP-92, MP-70 and MP-65 according to their apparent molecular masses), have been found to be quite similar to calpain I in regard to the levels of Ca\(^{2+}\) necessary to activate them. By virtue of this, these proteases are considered by the authors as potential candidates for participating in Ca\(^{2+}\) mediated neuronal events and a role in APP catabolism cannot be ruled out. Subsequently, McDermott et al. (1992) have identified a metalloprotease with the specificity to generate the N-terminus of β/A4 (between Met\(_{396}\) and Asp\(_{397}\) of APP\(_{693}\), also the putative β-secretase cleavage site) from its precursor. However, presently there are no strong candidates for β-secretase. More recently it has been claimed that the putative α-secretase may be a metalloprotease, since this enzyme activity does not appear to be inhibited by the addition of general inhibitors of serine- aspartyl- or cysteine-proteases to the media of differentiated PC12 cells (Walsh et al., 1993). Indeed the most recent candidate for α-secretase is the metalloprotease gelatinase A (Miyazaki et al., 1993). This enzyme cleaves synthetic β/A4 at the Lys\(_{165}\)-Leu\(_{17}\) bond, however it cannot be considered a strong candidate for being α-secretase until it has been shown to cleave full-length, membrane-bound APP at β/A4 Lys\(_{165}\)-Leu\(_{17}\).

An α\(_i\)-antichymotrypsin (ACT) binding protein termed clipsin, a descriptive acronym for its identification as a chymotrypsin-like protease has been identified in rat brain (Nelson and Siman, 1990). Given that the two integral plaque components identified to date (β/A4 and ACT) may have functional links with chymotrypsin-like proteases, it has therefore been hypothesised that such enzymes may be involved in the formation of neuritic plaques. Clipsin activity has been reported to preferentially degrade membrane-associated APP (Nelson and Siman, 1990) and to be elevated in AD when compared to controls (Savage and Siman, 1990). However, in a more detailed study, it has been suggested that the increased activity of this enzyme in AD may not contribute to the pathogenesis of the disease but is merely an epiphenomenon, since the increased hydrolysis of a synthetic substrate for chymotrypsin-like proteases, by this enzyme, was found to correlate with not only disease state, but the nature of terminal illness and post mortem delay. In contrast the activity towards three other synthetic substrates, including one that models the N-terminus of β/A4 (the putative β-secretase cleavage site), was found to be independent of post mortem delay (Stratmann et al., 1992). There was however, no difference in activity between AD and control samples.
1.3.3.5 Regulation of APP processing

Recently, receptor-activated APP release has been demonstrated giving rise to the proposal that receptor-controlled processing of APP may be mediated through intracellular activation signals, such as phosphorylation by kinases. Initial evidence came from experiments using human embryonic kidney cells transfected with cDNA encoding for human muscarinic acetylcholine receptor (mAChR) subtypes. These experiments demonstrated that activation of both m1 and m3 AChR with the muscarinic agonist carbachol (a stable acetylcholine analogue) significantly increased the release of APP (Nitsch et al., 1992). These findings have subsequently been confirmed in a rat pheochromocytoma PC12 cell line transfected with the m1 receptor gene, and extended by the observation that stimulation of interleukin-1 (IL-1) receptors also increased the release of APP derivatives (Buxbaum et al., 1992). The increase in receptor activated APP release was found to be unaffected by the translation inhibitor cyclohexamide indicating that pre-existing APP is cleaved and released in response to receptor activation. Concomitantly, levels of cell-associated full-length APP were found to decrease to 20-40% of control levels as a result of receptor stimulation.

These observations indicate that first messengers can activate, via receptor stimulation, a proteolytic processing pathway that cleaves pre-existing APP holoprotein, resulting in the secretion of APP into the extracellular medium. As well as enhancing secretion of APP, mAChR activation has also been shown to increase the secretion of p3, the 3 kDa fragment of β/A4 lacking its N-terminus. Furthermore, increased β/A4 secretion has been shown to be blocked by m1 AChR activation with carbachol (Hung et al., 1993). Based upon the assumption that APP and p3 are derived from proteolytic pathways involving α-secretase cleavage and that β/A4 is a product of β-secretase cleavage (Haass et al., 1993), it has been proposed that receptor activation enhances α-secretase cleavage, while decreasing β-secretase processing (Nitsch and Growden, 1994). As a consequence, amyloidogenic APP processing may be decreased by receptor activation, while non-amyloidogenic processing may be enhanced.

The increase in APP release mediated by mAChR has been shown to be specific to m1 and m3 receptor subtypes, as activation of m2 and m4 subtypes was observed not change APP release (Nitsch et al., 1992). Since activation of bradykinin, 5-HT2, 5-HT1c (Nitsch et al., 1995) and IL-1 (Buxbaum et al., 1992) receptors has also been shown to cause a similar stimulation of APP secretion, it is likely that the second
messenger systems coupled to these receptors accounts for the specificity of the response. Bradykinin, 5-HT₂, 5-HT₁C, m1 and m3 AChR, and IL-1 receptors are all coupled to diacylglycerol (DAG) formation via phospholipase C activation (Fisher et al., 1992), whereas m2 and m4 receptors are primarily coupled to the cAMP signal transduction pathway.

These data have led to the hypothesis that activation of cell surface receptors that generate DAG as part of their signal transduction may stimulate APP₅ secretion and inhibit β/A4 formation. DAG is the physiological activator of PKC, and plays a central role in the regulation of cellular events controlled by PKC. DAG is formed by phospholipid hydrolysis as a result of first messenger-induced stimulation of cell surface receptors that are coupled, via G proteins, to phospholipase C activation (see Nishizuka, 1992, for review). Receptor activated APP₅ secretion has been shown to be blocked by many protein kinase inhibitors including chelerythrine chloride and staurosporine (Nitsch and Growden, 1994), indicating that a protein kinase is involved in the signal transduction cascade that links receptor activation to APP processing. Experiments with fibroblasts transfected with the cDNA encoding for the PKCα isoenzyme clearly indicate that this PKC subtype can stimulate APP₅ secretion (Slack et al., 1993). PKC activation with phorbol esters (Buxbaum et al., 1990) or inhibition of protein phosphatases 1 and 2A with okadaic acid (Buxbaum et al., 1990; Caporaso et al., 1992b) has also been shown to produce a dramatic acceleration of non-amyloidogenic APP degradation. Furthermore, this PKC activated processing can be demonstrated to occur at the expense of amyloidogenic APP processing (Buxbaum et al., 1993; Hung et al., 1993; Jacobsen et al., 1994).

These findings imply a central role for PKC in the signal transduction cascade, coupling cell surface receptor activation to both increased APP₅ secretion and decreased β/A4 formation. Clearly protein phosphorylation plays a critical role in the regulation of APP₅ and β/A4 secretion, however the mechanism by which stimulation or inhibition of intracellular protein phosphorylation regulates the processing of APP remains to be elucidated. Certain cytoplasmic phosphorylation sites on APP have been identified (Gandy et al., 1988; Suzuki et al., 1992), however, analysis of the processing of phosphorylation-site mutant APP molecules has demonstrated that receptor-activated phosphorylation of the APP molecule itself (Gandy et al., 1988; Suzuki et al., 1992) seems unlikely to be necessary for phosphorylation-regulated α-secretory cleavage to
occur, since mutants lacking the phosphorylation sites are still cleaved and secreted in response to PKC activation (Hung and Selkoe, 1994). However it is possible that substrates other than APP, e.g. proteases in APP cleavage, are phosphorylated in response to receptor activation.

Experiments designed to determine the significance of receptor activation for APP processing in the intact brain have also been carried out. Using a hippocampal slice superfusion system for the measurement of APP release in response to neuronal depolarisation, Nitsch et al (1993) have demonstrated that electrical depolarisation enhances APP release from slices twofold within 50 min of stimulation. Concomitantly, electrical depolarisation increased the release of endogenous neurotransmitters including acetylcholine and glutamate three- to fivefold. Increased APP release was found to correlate with increasing stimulation frequencies in the range of 8-18 Hz, which matches the physiological firing frequency of hippocampal pyramidal cells.

These data suggest that APP release from hippocampal slices in vitro may be a function of neuronal activation. Pharmacological blockade of action potential formation with tetrodotoxin was found to inhibit depolarisation induced APP release, demonstrating that action potentials can regulate APP processing in brain tissue (Nitsch et al., 1993). More recently, cholinesterase inhibitors have been shown to significantly increase APP release from superfused cortical slices of rat brain, which suggests that inhibition of cholinesterase having the effect of increasing the levels of synaptic ACh may result in the activation of non-amyloidogenic processing of APP in brain (Mori et al., 1995).

1.3.4 Biological functions of APP

The normal functions of APP are beginning to emerge. Secreted forms of APP are now recognised to be PN-II, a member of a large family of transmembrane proteins that are potent inhibitors of serine proteases (Van Nostrand and Cunningham, 1987; Oltersdorf et al., 1989; Van Nostrand et al., 1989). PN-II may also be the natural factor XIa inhibitor present in platelet alpha-granules (Bush et al., 1990; Smith et al., 1990), suggesting that APP/PN-II may be involved in the regulation of proteases that control the blood coagulation cascade.
With regard to the structure and localisation of APP, it has been suggested that APP may function as a receptor (Kang et al., 1987), although, the only evidence supporting this possibility is the report that APP binds a heterotrimeric G-protein, Go (Nishimoto et al., 1993). However, linkage of APP to a G-protein may reflect a mechanism for routing APP in the cell, since G-proteins play important roles in membrane trafficking (Bomsel and Mostov, 1992). Segments in the extracellular N-terminal portion of APP which specifically bind to collagen, laminin and heparin have also been identified (Multhaup et al., 1992), suggesting a possible biological function in cell adhesion and in cell growth. APP also exists in a chondroitin sulphate proteoglycan form (APP-CSPG; Shioi et al., 1992; Shioi et al., 1993). Although the biological function of APP-CSPG is not known, it has been suggested that the chondroitin sulphate chains inhibit binding of APP to laminin, hence cells may modulate their adhesion to the extracellular matrix by regulating the proteoglycan form of APP (Robakis, 1994).

The presence of APP in platelets and fibroblasts, (cells important for tissue repair following injury), suggests that APP may have a function in cell growth and regeneration. Indeed, secreted forms of APP have been shown to promote autocrine and paracrine regulation of cell proliferation in fibroblasts (Ninomiya et al., 1993b). Based on studies using synthetic peptides and deletion constructs of APP, the apparent determinant of this function has been localised to residues 328-332 of APP, just beyond the point of insertion of the KPI domain suggesting that this function is not isoform specific (Ninomiya et al., 1993a).

With respect to cell growth, APP expression has been shown to correlate well with changes in cellular differentiation or growth. For example, in developing chick brain, APP expression has been shown to increase precisely during the developmental period (E7-E10) when neurite outgrowth is maximal (Small et al., 1992). In PC12 cells, nerve growth factor (NGF) concomitantly stimulates neurite outgrowth and APP expression (Refolo et al., 1989), and in the central nervous system levels of APP expression have also been shown to correlate with NGF-stimulated neurite outgrowth (Clarris et al., 1994). These data further imply roles for APP in developmental plasticity and in the response of the brain to injury. Recent reports have suggested that expression of APP may be readily induced after some form of brain injury (Wallace et al., 1991; Wakita et al., 1992), an observation consistent with the presence of acute
phase elements in the APP gene promotor (Salbaum et al., 1988). In animals enhanced APP immunoreactivity has been observed after experimental concussive head injury (Games et al., 1992), needle stab injury (Otsuka et al., 1991), persistent focal ischaemia (Abe et al., 1991b) and ibotenic or kainic acid injection (Siman et al., 1989a; Shigamatsu et al., 1992). Such an increase in APP immunoreactivity is thought to reflect increased expression of APP, since APP mRNA has been reported to be increased in reactive astrocytes after kainic acid induced neuronal damage (Siman et al., 1989a). Furthermore Kongure and colleagues have demonstrated induction of APP mRNA following focal ischaemia and heat shock (Abe et al., 1991a; Abe et al., 1991b). In humans APP immunoreactivity has been observed to accumulate within axonal swellings around cerebral infarcts (Ohgami et al., 1992), and following head injury, APP immunoreactivity has been shown to be increased in the perikarya of neurones and in neurites (Roberts et al., 1994).

In view of the proposed protective and trophic biological functions of APP, increased expression of this protein following injury may reflect a compensatory mechanism, whereby the brain is required to maintain extracellular concentrations of APP. However, the presence of β/A4 in diffuse plaques has also been reported to be increased in patients following severe head injury, leading the authors to hypothesise that situations leading to increased APP expression may provide sufficient potential for the pathological deposition of β/A4 as a result of APP mismetabolism under conditions of stress (Roberts et al., 1994).

Further support for a role for APP in synaptic plasticity, has come from data demonstrating that secreted forms of APP_{695} and APP_{751} can regulate intracellular Ca^{2+} concentration ([Ca^{2+}]i) and modify [Ca^{2+}]i responses to glutamate (Mattson et al., 1993), an excitatory neurotransmitter implicated in the process of synaptogenesis during development, and in learning and memory in adults. Ca^{2+} is also known to be a major regulator of growth cone behaviour in developing neurones and of long term potentiation at synapses (as reviewed in Mattson, 1992); and APP has been shown to cause a rapid and reversible reduction in the resting [Ca^{2+}]i in neurones (Mattson et al., 1993). In addition, one laboratory has shown that infusion of APP antibodies into the lateral ventricle in rats can impair the performance on tasks that require learning and memory (Huber et al., 1993). Such a function for APP may be evolutionary conserved because mutations of an APPL an APP-like protein (see 1.3.2) involved in learning and
memory in *Drosophila* result in behavioural abnormalities that can be corrected by insertion of the human APP gene into the mutant fly (Luo et al., 1992).

The observation that APPs can regulate \([\text{Ca}^{2+}]_{i}\) is suggestive of a neuroprotective role for APP. Secreted forms of APP_695 and APP_751 have been observed to protect cultured rat hippocampal and human cortical neurones against excitotoxic and hypoglycaemic injury (Mattson et al., 1993). The mechanism of this function is thought to involve attenuation of elevated \([\text{Ca}^{2+}]_{i}\) that mediates glutamate induced excitotoxic injury. Antibody blocking experiments indicate that both the \([\text{Ca}^{2+}]_{i}\)-stabilising and neuroprotective actions of APPs are mediated by a glycosylated region common to all forms of APP that is found adjacent to \(\beta/A4\) (amino acids 444-592 of APP_695; Mattson et al., 1993).

Much less is known about the function of full-length membrane-inserted APP, but recently evidence of neuronal adhesion and neurite-promoting activities of the holoprotein when it is inserted at the cell surface has been presented (Qiu et al., 1993).

### 1.3.5 \(\beta/A4\): Mechanisms of neuronal degeneration

#### 1.3.5.1 Neurotoxicity

To date much of the work on the biological effects of \(\beta/A4\) has been directed towards its toxic activities, based on the assumption that it is a pathological metabolic product. Both \(\beta/A4\) and \(\beta/A4\)-containing APP fragments have also been shown to have neurotoxic potential (Yankner et al., 1990b; Yankner et al., 1990a; Kowall et al., 1991). In a number of studies, treatment of cultured neurones with soluble, synthetic \(\beta/A4\) was observed to directly and/or indirectly induce neurotoxicity (Koh et al., 1990; Yankner et al., 1990a; Copani et al., 1991; Mattson et al., 1992), suggesting potential involvement of \(\beta/A4\) in AD neurodegeneration. In addition, following the *in vivo* injection of soluble \(\beta/A4\) formation of an insoluble deposit and focal pathology was reported (Kowall et al., 1991). A similar result has been observed after injection of plaque cores purified from AD tissue (Frautschy et al., 1991; Frautschy et al., 1992). Furthermore, \(\beta/A4\) has been reported to spontaneously assemble in solution, forming aggregates that share several properties with the insoluble \(\beta/A4\) found in plaques. Subsequently, it has been suggested (Pike et al., 1993) that the neurotoxicity of \(\beta/A4\) *in vitro* is dependent upon the ability of the peptide to form insoluble polymeric aggregates over time (*in vitro* peptide ageing; Pike et al., 1991; Busciglio et al., 1992; Pike et al., 1993).
In support of this possibility, Pike et al (1991) have also reported in a previous study, that aggregated but not soluble β/A4 is associated with neurotoxicity in developing hippocampal cultures.

Exact mechanisms of neuronal degeneration in AD remain unknown. However, the possibility that β/A4 induces the formation of free radicals which mediate cell injury has been suggested by the observation that vitamin E (an antioxidant) protects cultured PC12 cells against β/A4 toxicity (Behl et al., 1992). Furthermore, oxidation promotes and antioxidants prevent aggregation of β/A4 (Dyrks et al., 1988a), suggesting that oxidation may promote conversion of β/A4 from a non-toxic soluble form to an aggregated form.

Altered cellular calcium homeostasis has also been implicated in the mechanism of β/A4 neurotoxicity. In a series of studies on cultured human cortical and rat hippocampal neurones, β/A4 (in the aggregated form) caused a progressive elevation of [Ca$$^{2+}$$]i, and increased [Ca$$^{2+}$$]i responses to excitatory amino acids (Koh et al., 1990; Mattson et al., 1992; Mattson et al., 1993). β/A4 induced neuronal injury under the same conditions was prevented by the removal of extracellular Ca$$^{2+}$$.

Precisely how β/A4 disrupts neuronal [Ca$$^{2+}$$]i is unclear. It seems unlikely that the [Ca$$^{2+}$$]i-destabilising action of β/A4 is mediated by actions at specific receptors, since the process requires aggregation of the peptide, has a slow time course, and no specific binding sites for β/A4 have been detected in neurones. However, β/A4 may have the potential to disrupt the function of neuronal plasma membrane proteins involved in the regulation of [Ca$$^{2+}$$]i. This is based on the observation that β/A4 can form channels that flux Ca$$^{2+}$$ when incorporated into artificial lipid bilayers (Arispe et al., 1993).

Since Ca$$^{2+}$$ ions are thought to participate both in glutamate neurotoxicity (Choi, 1985; Garthwaite and Garthwaite, 1986; Choi, 1987) and in neurite outgrowth (Anglister et al., 1982; Mattson and Kater, 1987) it has be hypothesised that β/A4 may alter Ca$$^{2+}$$ metabolism in such a way as to render cortical neurones more susceptible to insult. Indeed, when the effects of β/A4 on glutamate receptor-mediated neurotoxicity in mature cortical cultures was examined, prolonged exposure to β/A4 was found to increase neuronal vulnerability to excitotoxic damage (Koh et al., 1990). Over-activation of glutamate receptors has been shown to be highly toxic to central neurones, most probably as a result of the opening of cation channels permitting an excessive
The efflux of Na$^+$ and Ca$^{2+}$ (Rothman and Olney, 1986; Choi, 1988). A substantial body of evidence indicates that this neurotoxic mechanism may contribute to neuronal loss in a variety of neurodegenerative conditions, including Alzheimer's disease (Choi, 1988). However, the rapidity of excitotoxic processes and the lack of evidence of elevations in releases excitatory amino acids in the brain in AD (for review, see Francis et al., 1993b) argue against a conventional excitotoxic process in AD.

Perturbed energy metabolism has also been proposed as a mechanism contributing to premature cell death in AD. Consistent with such proposals, various forms of energy deprivation in neuronal culture have been shown to enhance the sensitivity of cells to the toxic effects of glutamate (Novelli et al., 1988), in addition, β/A4 has been shown to increase the vulnerability of neurones that have been weakened by an energy deficit by exacerbating the action of endogenous excitatory amino acid neurotransmitters (Copani et al., 1991). These findings are consistent with earlier hypotheses that normal concentrations of glutamate can become neurotoxic under certain conditions (for review, see Rothman and Olney, 1987).

1.3.5.2 Perturbed energy metabolism in AD

Evidence for alterations in energy metabolism in AD remains far from definitive. In vivo studies of the brain in AD have consistently reported reductions of temporoparietal energy-related metabolism, as assessed from measures of cerebral blood, glucose or oxygen utilisation (Risberg, 1980; Benson et al., 1983; Chase et al., 1984; McGeer et al., 1986; Rapoport et al., 1991). These changes have generally been interpreted as indicating diminished functional activity in the disease affected brain. However, whether these alterations reflect biochemical abnormalities within the regions of reduced metabolic activity, or whether such abnormalities may contribute to the degenerative process remain unresolved. Parallel measurements by computerised tomography (Chawluk et al., 1987) as well as biochemical estimates of tissue loss (Najlerahim and Bowen, 1988) indicate that the reductions can be explained at least partly by focal areas of atrophy in the brain. McGeer et al (1990) have also provided evidence, based on the correlations between the metabolic rate of glucose measured in vivo and enzyme markers determined in post mortem brain for neuronal loss and gliosis in the areas of reduced metabolism.
There is substantial evidence for an intrinsic abnormality of mitochondrial function in AD. In ultrastructural studies, Alzheimer plaques have been found to contain distorted mitochondria (Wisniewski and Terry, 1973). More recently findings have suggested that there is a selective deficit in the azide-sensitive mitochondrial complex IV in the cerebral cortex of AD patients. Furthermore, deficits in oxidative energy metabolism have been shown to accompany normal ageing, and may be related to an age-dependent increase in mitochondrial DNA mutations (Corral-Debrinski et al., 1992; Bowling et al., 1993; Mecocci et al., 1993).

Alterations in a number of enzymes involved in glucose metabolism or energy production have been reported in AD brain samples obtained at autopsy. There are marked decreases in the activities of two mitochondrial multi-enzyme dehydrogenase complexes, the pyruvate dehydrogenase complex and the 2-ketoglutarate dehydrogenase complex (Gibson et al., 1988). The activities of hexokinase (Bowen et al., 1979; Iwangoff et al., 1980) and 6-phosphofructo-1-kinase (PFK-1; Bowen et al., 1979) have also found to be decreased in AD. Most of these enzymes are involved at identified control sites in glycolysis or the oxidative metabolism of pyruvate, and the observed reductions would be expected to reduce the capacity for glucose metabolism. However, it is difficult in these studies to completely rule out effects from post mortem degradation or altered agonal state, especially where the brain is already affected by disease (Sims et al., 1987a). These difficulties are highlighted in one early study of multiple enzymes in post mortem tissue, in which the activity of PFK-1 was found to be reduced to approximately 10% of control values (Bowen et al., 1979). However, this enzyme is particularly susceptible to the effect of post mortem delay, agonal state, and tissue freezing (Bird et al., 1977; Iwangoff et al., 1980), therefore it is difficult to interpret the results with any confidence. Subsequent measurements in biopsy samples from the neocortex have revealed no consistent reductions in the activity of this enzyme in AD (Sims et al., 1987a).

*In vitro* measurements of the oxidation of glucose and pyruvate have not detected reductions in biopsied neocortex from patients with AD (Sims et al., 1987c). Indeed, the production of \(^{14}\text{CO}_2\) from [U-\(^{14}\text{C}\)]glucose was increased rather than decreased in the AD group (Sims et al., 1983b), this coupled with the finding that the adenylate energy charge (ATP + \(\frac{1}{2}\) ADP / [ATP + ADP + AMP]) was unaltered in tissue from patients with AD, is not consistent with the idea of an imbalance between
energy production and utilisation in AD. There was however some loss of adenine nucleotides in the samples from AD patients (ATP content was significantly lower and AMP was also reduced) most likely associated with a selective degeneration of some metabolite-enriched components. In a further study, the maximum oxygen uptake rates, determined in the presence of an uncoupling agent under conditions allowing measurement of the activity of either total or synaptosomal mitochondria in the tissue samples, were found to be unchanged from control values. Thus the changes observed in key enzymes in post mortem brains either were absent in these samples or were insufficient to interfere with metabolic activity under the conditions studied. The metabolic measurements provide evidence that the capacity for glucose and pyruvate metabolism is preserved in AD and seems unlikely to account for the reduced metabolic rates seen in vivo.

It is usual to relate metabolism to the adenylate energy charge, as described above. This measure is often considered to be convenient short hand for expressing relative energy states, however, it ignores non-adenylate energy stores and does not take into account mechanisms for regulation and coupling of energy supply to utilisation. Therefore its application can be misleading, since cell metabolism can respond in various ways to changes in the concentrations of individual nucleotide cofactors, metabolites, and ion fluxes, thus the calculated energy charge may be an oversimplification that reflects inaccurately the true energy state and metabolic activity level of the tissue (Lajtha et al., 1981).

Overall, the evidence to date appears inconclusive with regard to reductions in glucose and energy metabolism observed in vivo in AD. There is however some evidence, although far from conclusive, for changes in metabolism more subtle than a simple overall reduction in metabolic capacity. Thus the increase in $^{14}$CO$_2$ production from [U-$^{14}$C]glucose in biopsied neocortex from AD patients (Sims et al., 1983b), taken together with increases seen in neocortical mitochondrial respiration under conditions producing submaximal activity (Sims et al., 1987c), raises the possibility that mitochondria may be partially uncoupled such that more substrate is metabolised to maintain the same rate of ATP production. These findings are similar to those induced in preparations from animal brain exposed to the mitochondrial uncoupler carbonyl cyanide M-chlorophenylhydrazone (CCCP; Gibson and Blass, 1976). Cultured skin cells from Alzheimer patients have also shown abnormalities that are consistent with
mitochondrial uncoupling and consequent decreased efficiency of conversion of ADP to ATP, including increases in oxidation of radioactive glucose in short-term incubations and in lactic acid production (Sims et al., 1987b). Increased immunocytochemical reactivity with anti-PHF and Alz-50 antibodies has also been observed in fibroblasts from control subjects after treatment with CCCP, suggesting an accumulation of Alzheimer-like antigens under such conditions (Blass et al., 1990). It has been hypothesised that this immunoreactivity may be associated with new epitopes resulting from abnormal post-translational modifications of molecules normally found in fibroblasts, such as abnormal phosphorylations analogous to modifications of neuronal cytoskeletal proteins that have been proposed to occur in AD (Glenner, 1988; Wischik et al., 1988a). In addition, it has been reported that incubation of HeLa cells with CCCP can lead to disorganisation of the cytoskeleton (Maro and Bornens, 1982), and other mitochondrial inhibitors, such as oligomycin that impair ATP synthesis, to the disorganisation of the intermediate filament network in human skin fibroblasts (Klymkowski, 1988).

Thus a variety of metabolic insults appear to lead to increased immunoreactivity with anti-PHF antibodies, and accumulation of Alzheimer-like antigens may be a relatively non-specific response to stress on the cells. It is interesting that ubiquitin, a well documented component of PHF (Glenner, 1988), is a heat-shock protein whose synthesis is stimulated by not only heat shock but also by oxidative and other stresses (Schlesinger, 1986). Furthermore, the promoter for the APP gene also contains a heat-shock element (Salbaum et al., 1988), suggesting that metabolism of APP may also be affected by metabolic stresses. Indeed, release from PC12 cells, of a 60 kDa C-terminal APP fragment containing intact β/A4 has been shown to occur following stress (Baskin et al., 1991), highlighting the importance of possible induction of alternative amyloidogenic processing of APP under such conditions. Furthermore, in one other study, inhibition of oxidative energy metabolism with sodium azide or CCCP was found to increase the proteolysis of APP to the 11.5 kDa derivative by approximately 80 fold, thus raising the possibility that energy-related metabolic stress may alter the metabolism of APP and lead to decreased APP secretion and increased generation of a potentially amyloidogenic derivative (Gabuzda et al., 1994).
1.4 GENETIC LINKAGE STUDIES IN AD

Familial aggregation of AD has been shown by several epidemiological surveys to be an inherited trait with autosomal dominant transmission and age-dependent penetrance (Breitner et al., 1988). Though familial AD (FAD) is rare (< 10% of all AD), the characteristic clinico-pathological features, amyloid plaques, NFT, synaptic and neuronal loss, neurotransmitter deficits and dementia appear to be indistinguishable when FAD is compared with typical non-familial or sporadic AD (as reviewed by Estus et al., 1992a; see also Ghetti et al., 1992; Lantos et al., 1992; Mann et al., 1992a; Cairns et al., 1993 and Kennedy et al., 1993).

1.4.1 The APP gene

Given that one of the neuropathological features of AD is the deposition of β/4, which is derived from APP, initial studies to identify a familial AD gene focused on chromosome 21. In a small proportion of families with early (pre-senile) onset FAD, the disease trait was found to co-segregate with genetic markers clustered on the proximal long arm of chromosome 21 near the APP gene (St.George-Hyslop et al., 1987; Goate et al., 1989). Several early onset FAD-associated mutations in APP have now been identified.

In 1991, a missense mutation causing an amino acid substitution, Val → Ile at position 717 of APP770 within the transmembrane domain, downstream from the C-terminus of the β/4 domain, was identified (Goate et al., 1991). Two additional changes in codon 717 (Val → Phe, Murrell et al., 1991; and Val → Gly, Chartier-Harlin et al., 1991), one at codon 692 (Ala → Gly, Hendricks et al., 1992), and a double mutation at codons 670 (Lys → Asp) and 671 (Met → Leu) at the N-terminus of β/4 have also been identified (Mullan et al., 1992). The codon 692 mutation has been found to segregate either with AD or with a phenotype similar to hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-Dutch; Levy et al., 1990; Van Broeckhoven et al., 1990). In the rare HCHWA-Dutch, there is a premature and severe deposition of β/4 in the microvessels of the brain and meninges, resulting in multiple haemorrhages that are eventually fatal. Patients also develop large numbers of parenchymal β/4 deposits in the cerebral cortex that are indistinguishable from the diffuse plaques of AD and in DS. Why β/4 bearing this mutation, should be
deposited to such an extent in the cerebral microvasculature and why the cortical deposits appear not to progress to full-blown neuritic plaques in these non-demented patients are unanswered questions.

The only mutations that are clearly associated with FAD, are those at codon 717 and the double Swedish mutation at codons 670/671. It has been proposed, that APP mutations lead to enhanced production of β/A4 resulting in eventual deposition of the peptide in the cortex and cerebral vessels. In vitro cell culture studies have shown that the FAD double mutation at codons 670/671, results in a marked increase (approximately five to eightfold) in β/A4 secretion, with the second substitution found to confer most of this effect (Citron et al., 1992; Cai et al., 1993). This increase does not appear to be accompanied by any associated changes in APPs secretion, but the possibility that the release of the shorter secreted APP form ending in Met696 of APP695 (Seubert et al., 1993) is increased has not been ruled out.

Thus APP mutations seem to be responsible for a small percentage (< 10%) of early onset familial cases. These studies show that certain defects on the APP gene are sufficient to induce the clinical and histopathological characteristics of AD, however, the majority of FAD pedigrees are not associated with mutations in APP (Tanzi et al., 1992). Indeed, the majority of pedigrees with a late-onset of FAD (> 65 years), and a subset of pedigrees with pre-senile onset FAD show either weak or negative evidence for co-segregation with genetic markers from chromosome 21 (Tanzi et al., 1987; Van Broeckhoven et al., 1987).

Further genetic linkage studies have provided good evidence for two other FAD susceptibility loci. The first, found to be predominantly associated with late-onset FAD has been mapped to chromosome 19 (see 1.4.2) and the second, mapped to chromosome 14 (see 1.4.3) appears to be the major susceptibility locus in a significant proportion (probably ≥ 70%) of pedigrees with early onset FAD (St.George-Hyslop et al., 1992; Van Broeckhoven et al., 1992). Although FAD susceptibility loci have been identified on three separate loci, there are still a few pedigrees that do not show good evidence for linkage to any of these sites, clearly other loci must exist elsewhere in the genome.
1.4.2 Chromosome 19

1.4.2.1 Apolipoprotein E

Recently, Strittmatter et al. (1993) have identified a small protein, apolipoprotein E (apoE) which is capable of binding to βA4 with high avidity. ApoE, a major apolipoprotein is synthesised by many organs, primarily the liver and is involved in the transport and metabolism of lipids (Mahley, 1988). In the brain it is produced and secreted by astrocytes (Ignatius et al., 1986) and it has been implicated in the growth and repair of the nervous system during development or following injury (Mahley, 1988). It is apparent that apoE plays a prominent role in the redistribution of cholesterol to the neurites for membrane biosynthesis during axon elongation and to the Schwann cells for myelin formation. However, it is possible that the high levels of apoE in the regenerating nerve reflect an additional role, unrelated to lipid transport.

It has been reported that the expression of apoE increases markedly in AD and other chronic neurodegenerative diseases such as Creutzfeldt-Jakob disease and Scrapie (Diedrich et al., 1991). Namba et al. (1991), Wisniewski and Frangione (1992) and Strittmatter et al. (1993) have all shown apoE immunoreactivity extracellularly in senile plaques, and at sites of cerebral vessel congophilic angiopathy, and also intracellularly in NFT. It is of additional interest that the gene for apoE is located on chromosome 19 near the genetic markers showing linkage with late-onset FAD (Pericak-Vance et al., 1991). A number of studies suggest that the apoE type 4 allele (ε4, one of the three common apoE alleles), may be important in the pathogenesis of AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993; Liddell et al., 1994). In these studies, analysis of apoE alleles in AD and controls have demonstrated that the frequency of ε4 is increased in affected members of late-onset FAD pedigrees (Strittmatter et al., 1993; Corder et al., 1993), and in autopsy confirmed and clinically diagnosed cases of sporadic AD (Saunders et al., 1993; Liddell et al., 1994). There is also evidence that brains of patients homozygous for ε4, contain increased amounts of vascular amyloid and increased number and density of amyloid and neuritic plaques compared to those homozygous for ε3 (Schmechel et al., 1993). Furthermore, Houlden et al. (1993) have demonstrated that in Alzheimer patients with APP mutations, the apoE genotype modulates the age of onset.

Regarding the possible action of apoE in the brain, there is histological evidence to suggest that it may act as a pathological chaperone (Wisniewski and Frangione,
1992). This term encompasses a number of proteins which are associated with β/A4 once it forms an amyloid fibril. These proteins are biochemically unrelated and by definition are considered to mediate β-pleated amyloid formation, but are not themselves components of the final fibrils. Strittmatter et al (1993) have reported high avidity binding of apoE to soluble β/A4, which is in accord with the hypothesis that apoE acts as a chaperone for β/A4 aiding its sequestration into plaques. Additionally, this group have reported that binding of apoE to β/A4 is isoform specific, where ε4 binds with greater avidity than ε3 the most common isoform of apoE.

On the basis of these studies, a biochemical role for apoE in the development of AD has been postulated, suggesting that the binding of ε4 to β/A4 may be related to the transition between diffuse, apparently benign β/A4 deposition and neuritic damaging deposits. Since ApoE can be localised to the defining pathological lesions of AD, it is tempting to suggest that apoE may be involved in the formation of these lesions and in particular may be central to a unifying hypothesis regarding the pathological events in AD, i.e. apoE may be the link between amyloid deposition and tau phosphorylation.

Experiments carried out by Strittmatter et al (1994), have shown that the ε3 variant binds tau protein more readily than does the ε4 variant, with subsequent experiments showing that ε3 binds only to non-phosphorylated tau. In AD, tau protein is abnormally phosphorylated resulting in loss of function and aggregation into PHF-tau, ultimately leading to tangle formation. This group speculate that one role of ε3 is to bind to tau making it unavailable for phosphorylation. If ε4 does not do this, over time more tau will become hyperphosphorylated resulting in increased instability of microtubule structures. In view of this, ε4 may not itself be a risk factor for AD, but rather it is a lack of ε3 (or some other isoform e.g.ε2 ) required for normal neuronal function and resistance to neurofibrillary change. However, the ε4 genotype appears to be a major genetic susceptibility risk factor for late-onset FAD and sporadic AD. It should be noted that not all subjects studied carried copies of ε4, a high proportion of people with typical AD pathology are homozygous for ε3. So, this genotype is neither necessary or sufficient to cause AD and therefore other genes or risk factors must be operating in these cases.
1.4.2.2 APLP1

Wasco et al (1992) have identified an APP-like protein, APLP1 (see 1.3.2.1) that maps to chromosome 19 in the same general vicinity that has been postulated to contain the late-onset FAD gene defect (Pericak-Vance et al., 1991; Wasco et al., 1993a). Although APLP1 does not contain the β/A4 sequence and therefore cannot act as a substrate for amyloid formation, the conservation of amino acid sequence and domain structure within APP and APLP1 implies that they may share common functions and be processed similarly. If APP and APLP1 indeed interact with the same sets of enzymes, then an alteration in, or the overproduction of APLP1 could affect the maturation and metabolism of APP and ultimately the generation of β/A4. It is also possible that factors or events leading to the up-regulation of APLP1 might also result in increased expression of APP and processing through an amyloidogenic pathway.

1.4.3 Chromosome 14

As reviewed in 1.4.1, APP mutations account for only a small percentage of early-onset FAD cases. Several groups (Schellenberg et al., 1992; St.George-Hyslop et al., 1992) have discovered genetic linkage to a major FAD locus on chromosome 14 with the linkage appearing to be strongest in the early-onset pedigrees. On this chromosome, a number of candidate genes have been mapped to the implicated region that could be related to some aspect of the known pathology of AD (St.George-Hyslop et al., 1992) including an HSP70 gene family member, the transcriptional regulator cFOS, cathepsin G and α1-antichymotrypsin (ACT). However to date, there is no evidence that the cathepsin G, ACT and cFOS genes are the sites of the FAD mutation on chromosome 14. Analysis of the HSP70 gene is pending.

* See note added, page 67.

1.5 THE AMYLOID CASCADE HYPOTHESIS

Proponents of the amyloid cascade hypothesis, nominate mismetabolism of APP leading to deposition of β/A4 as central and primary events in all cases of AD (Hardy and Allsop, 1991). Following β/A4 deposition, a cascade of other pathological events is set in motion eventually leading to NFT formation, cell loss and clinical presentation (see Fig. 1.2).
Mismetabolism of APP leading to plaque and tangle formation and to cell death has been proposed to represent an early and critical event in the progression of AD (modified from Hardy and Allsop (1991)).
To briefly summarise, support for the amyloidogenic theory of AD is derived from several sources already reviewed in previous sections. These are: (1) Identification of mutations in APP in FAD that are linked to amyloidogenesis (Ashall and Goate, 1994; see also 1.4.1); (2) The association of AD-type neuropathology and symptoms in DS, with overexpression of the APP gene on chromosome 21 (see 1.2.5); and (3) Experimental evidence for β/A4 neurotoxicity and potentiation of excitatory amino acid toxicity (see 1.3.5.1).

An alternative view of the role of APP mismetabolism in AD is based upon observations that APP derivatives such as APP3 have protective and trophic biological function (see 1.3.4). Thus a shift in APP processing in favour of increased β/A4 production and reduced release of APP3 could endanger neurones in two ways: (1) Increased levels of β/A4 would allow accumulation and aggregation of the peptide, in which case neurones exposed to β/A4 would be more vulnerable to insult (see 1.3.5.1); (2) Reduced levels of APP3 would result in the disruption of biological processes normally influenced by APP and its metabolites.

1.6 RATIONALE, HYPOTHESES AND AIMS OF THE STUDIES

1.6.1 Rationale

The preceding discussion highlights the importance of APP in AD, and identifies several reasons for studying the processing of this protein: (1) The principal cell type affected in AD is the cortical pyramidal neurone, thought to be a major source of APP in brain; (2) Neurotransmitters regulate APP processing pathways; (3) There are major glutamatergic and cholinergic deficits in AD; (4) Altered processing of APP may contribute to neurodegeneration in AD.

1.6.2 Hypotheses

The preceding points (1-4) may be formulated into a number of working hypotheses which form the basis for the experimental work to be described in subsequent Chapters.

(1) Hypoactivity of cholinergic function and of glutamatergic cortical pyramidal neurones in AD will reduce secretion of APP3 and favour increased β/A4 production.
In AD potentially amyloidogenic processing of APP is related to increased membrane serine protease activity.

Perturbed energy metabolism in AD contributes to neurodegeneration by altering processing of APP.

1.6.3 Aims of the studies

This thesis addresses three potential events (neurotransmission, enzyme proteolysis and perturbed energy metabolism) which may act to influence the metabolism of APP and therefore contribute, at least, to the spread of AD pathology in the AD brain. (Hereafter, the term APP-like immunoreactivity [APPLIR] will be used when referring to APP, in light of evidence that anti-APP antibodies fail to discriminate APP from APLP2 (Sisodia et al., 1994; Slunt et al., 1994). When these studies were undertaken, antibodies recognising only APLP2 were unavailable, an issue addressed in Chapter 7).

The general aims were:

1. To characterise APPLIR present in fractions prepared from AD brain, and assess relationship(s) with various cortical cell parameters.

2. To investigate a proteolytic activity in AD cortex, with respect to degradation of APPLIR substrate.

4. To assess the effect of perturbed energy metabolism and activation of the key protein kinase, PKC, on the metabolism of APPLIR in PC12 cells.

5. To characterise APPLIR in human CSF (from the ventricles, VCSF) and assess, with respect to cholinergic hypoactivity in AD, the effects of drugs likely to alter metabolism of APPLIR.

6. To characterise a novel APLP2 specific antibody.
NOTE

Two Alzheimer mutations in the novel genes for presenilins 1 and 2 on chromosomes 1 and 14 respectively have now been described (Rogaev E.I et al., Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene, Nature, 376, 775-778; Sherrington R. et al., Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease, Nature, 375, 754-760). These proteins are homologues of the C. elegans sel-12 gene product which interacts with a member of the Notch family of cell surface signalling receptors (Levitan D., Greenwald I., Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene, Nature, 377, 351-354). Thus these mutation suggest that defective intracellular signalling may be a candidate mechanism contributing to AD pathogenesis. Furthermore the Notch family has been shown by genetic studies to be linked to the Drosophila homologue of glycogen synthase kinase-3, an enzyme proposed to be involved in NFT formation (Ruel L. et al., Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch, Nature, 362, 557-560) (see 1.2.4.3). Aberrant regulation of signal transduction in AD may therefore be a mechanism worthy of further investigation.
CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General

Unless otherwise stated, all standard chemicals were of analytical grade. Tris buffer, hydrochloric acid (HCl), glycine, methanol, glacial acetic acid, perchloric acid, calcium chloride (CaCl₂), sodium chloride (NaCl), ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis-β-amino-ethyl ether (EGTA), sodium acetate, sodium carbonate anhydrous, copper sulphate, sodium potassium tartrate, Folin-Ciocalteu's reagent, choline chloride, potassium chloride (KCl), magnesium sulphate (MgSO₄), sodium dihydrogen orthophosphate (Na₂HPO₄), glucose, sucrose, magnesium chloride 6 hydrate (MgCl₂.6H₂O), triethylamine (TEA; HiPerSolv grade), paraformaldehyde were from BDH Ltd. (Poole, Dorset, UK). Bovine serum albumin (BSA), 2-mercaptoethanol, phosphate buffered saline (PBS) tablets, dithiothreitol (DTT), HEPES, n-butanol, polyoxyethylene sorbitan monolaurate (Tween-20), dimethyl sulfoxide (DMSO), Triton X-100, Brij-35, phenylmethylsulphonylflouride (PMSF), phorbol 12,13-dibutyrate, bradykinin, staurosporine, oligomycin, 2-deoxyglucose, N-tosyl-L-lysine chloromethyl ketone, N-tosyl-L-phenylalanine chloromethyl ketone, soya bean trypsin inhibitor, pepstatin, leupeptin, ATP, ADP and AMP, Chelex-100 resin and heparin-agarose column were from Sigma Chemical Co. (Poole, Dorset, UK). Dialysis membrane size 1-8/32" was from Medicell International Ltd. (Liverpool, UK); Centricon-3 and Centricon-10 concentrating tubes were from Amicon Ltd. (Stonehouse, Kent, UK).

2.1.2 Cell culture

Rat pheochromocytoma PC12 cells (ATCC, CRL 1721) were obtained from PHLS Centre for Applied Microbiology & Research (Porton Down, Salisbury, Wilts U.K.). Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine and 4500
mg/ml D-Glucose, but without sodium pyruvate, Dulbecco's MEM/Nutrient Mix F12 (1:1; DMEM/F12) without L-Glutamine, DMEM with L-Glutamine, but without glucose and sodium pyruvate, DMEM/F12 without L-Glutamine and glucose, heat inactivated foetal calf serum (FCS), heat inactivated horse serum and gentamicin (10 mg/ml) were purchased from Gibco/BRL. (Paisley, Scotland, UK). A serum-free media supplement containing insulin (0.5 mg), transferrin (0.5 mg) and sodium selenite (0.5 μg) was purchased from Boehringer Mannheim (Lewis, East Sussex, UK). Bovine dermal collagen Vitrogen 100 (3 mg/ml) was purchased from Imperial Labs (Andover, Hants, UK). Murine 7S nerve growth factor was purchased from Promega (Southampton, Hants, UK). Culture plastics including flasks (25 cm² and 75 cm²), 6-well plates (9.6 cm²/well), 10 ml 'Shortie' pipettes and 0.2 μm-pore filters were from ICN Flow (High Wycombe, Bucks, UK). 10 ml pipettes with and without suction adaptor were from Western Lab Services (Aldershot, Hants UK). 50 ml centrifuge tubes and tubes for cryogenic storage of cells were from NUNC Products Gibco/BRL and trypan blue solution was from Sigma Chemical Co.

2.1.3 Sodium dodecyl sulphate-polyacrylamide electrophoresis

Acrylamide, N,N’-Methylene-bis-acrylamide (Bis-acrylamide), N,N,N’,N’-Tetramethylethlenediamine (TEMED) all electrophoresis grade and glycerol, were purchased from BDH Ltd. Sodium dodecyl sulphate (SDS), Bromophenol blue (BPB), Coomassie brilliant blue R-250, ammonium persulphate (APS), SDS-7B pre-stained molecular weight markers were all from Sigma Chemical Co.

2.1.4 Western blotting

Hybond-ECL Western nitrocellulose membrane, Hyperfilm-ECL (10 x 24 cm) and ECL detection reagents were purchased from Amersham International (Amersham, Bucks, U.K.). Saran wrap was from the Genetic Research Inst. (Dunmow, Essex, UK.)

2.1.5 Antibodies

Amino terminal antibody 22C11, a mouse monoclonal raised against a recombinant fusion protein containing APP695 (epitope mapped to amino acids 60-100,
Weidemann et al., 1989) was obtained from Boehringer-Mannheim; monoclonal mouse anti-β-APP LN27 recognising an epitope within the first 200 amino acids of the APP N-terminus, was raised against recombinant APP_{695} (Arai et al., 1991) and came from Zymed Laboratories (San Francisco, USA); mouse monoclonal 10D5 raised against human β/A4 1-28 (APP_{770} 653-681; epitope mapped to β/A4 1-16, Hyman et al., 1991); mid-region APP affinity-purified rabbit polyclonal anti-5 raised against bacterial construct pBx5 (APP_{770} 444-592; with most reactivity to 444-544, Oltersdorf et al., 1990); mouse monoclonal 7H5 raised against bacterial construct pBx9 (APP_{770} 289-374, Hyman et al., 1991) which recognises the Kunitz protease inhibitor domain were all gifts from Dr Dale Schenk, (Athena Neurosciences, San Francisco, USA.); 3B11, raised against a synthetic peptide corresponding to amino acids 596-609 of APLP2 was produced by Professor N Groome (Oxford Brookes University, Oxford, UK; see also Webster et al., 1995 and Chapter 7); polyclonal antibody D2-1, raised against full length APLP2-751 (Slunt et al., 1994) was a gift from Dr S Sisodia (John Hopkins University School of Medicine, Baltimore, USA); antibody DA1 a rabbit polyclonal raised against synthetic β/A4 1-25, was from Dr D Allsop, (SmithKline Beecham, Harlow, Middlesex, UK); peroxidase-conjugated affinity isolated goat anti-mouse immunoglobulins (IgG), and peroxidase-conjugated goat anti-rabbit IgG were from Dako Ltd. (High Wycombe, Bucks, UK.).

2.1.6 Characterisation of APLP2 antibody

Full length baculovirus expressed APP_{751} (20 μg/ml, Knops et al., 1991) and purified secreted APP_{695} (Esch et al., 1990) were gifts from Dr Dale Schenk, (Athena Neurosciences); COS-1 cells transfected with APP_{770}, APLP2 or empty vector were from Dr R Tanzi (Harvard Medical School, USA); H4 cells transfected with mouse APLP1 (Wasco et al., 1992), human APLP2 or empty vector (Wasco et al., 1993b); Chinese hamster ovary (CHO) cells transfected with mouse APLP2 (Slunt et al., 1994) and a glutathione fusion protein of the 113 amino acid ectodomain of APLP2 (Smith and Johnson, 1988) were all gifts from Dr S Sisodia (John Hopkins University); rat cortical and cerebellar cultures (Dutton et al., 1981) were from Dr B. Pearce (School of Pharmacy, London, UK); and SH-SY5Y cells (Murphy et al., 1991) were a gift from Dr S Anderson (Astra Neuroscience Research Unit, London, UK).
2.1.7 Immunocytochemistry

Vectastain Elite ABC detection system was from Promega, biotinylated rabbit anti-mouse IgG from Dako Ltd., formic acid, citric acid, thimerosal, diaminobenzidine, 3-amino-9-ethyl carbazol (AEC) and 30% hydrogen peroxide ($\text{H}_2\text{O}_2$) were all from Sigma Chemical Co., DPX mountant was from BDH Ltd., haematoxylin, and histoclear were purchased from R. A. Lamb (London, U.K.), Mowiol 4-88 aqueous mountant was from Harco Harlow Chemical Co. (Harlow, Essex, UK).

2.2 METHODS

2.2.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), using the Mini-PROTEAN II dual slab apparatus from Biorad Ltd., USA.

Discontinuous gels consisted of a resolving or separating (lower) gel with a short stacking gel set on top. The differences in pH and composition between these two gels caused the samples to be concentrated into narrow bands before separation occurred during migration through the separating gel. The porosity of a gel was determined by the relative proportion of acrylamide monomer to cross linking agent. The degree of porosity was selected to provide optimal resolution and this was dependent generally upon the molecular weight range of the proteins being studied. However the type of sample was also taken into consideration. For example, separation of APP in cell lysate was enhanced on SDS-7.5% PAGE, but in conditioned culture medium better resolution was obtained by separation on SDS-10% PAGE.

2.2.1.1 Casting of Gels

Gels were cast (1.5 mm thick) in groups of 10 using a Mini-PROTEAN 11 Multi Casting Chamber (Biorad Ltd., USA). The casting chamber was assembled according to the manufacturers instructions. For casting, solutions A-E (see below) were made up in distilled de-ionised water (DIW) and stored at 4°C (solutions A and B) or at room temperature (solutions C and D) for no longer than three months. Solution E was prepared fresh as required.
Solution A - 30 % (w/v) acrylamide monomer / 0.8 % (w/v) bis-acrylamide

Solution B - 3 M Tris-HCl (pH 8.8)

Solution C - 10 % (w/v) SDS

Solution D - 0.4 % (w/v) SDS/0.5 M Tris-HCl (pH 6.8)

Solution E - 10 % (w/v) APS

TEMED - initiator

2.2.1.2 Preparation of separating gels

The required ratios of the above solutions needed to make a single gel of a given percentage are shown in Table 2.1. Solutions except E and TEMED were mixed in a Buchner flask and degassed under vacuum for 15 min with continuous stirring. Initiation of polymerisation followed addition of solution E and TEMED. Gels were poured immediately from the bottom of the casting chamber through a stopcock valve, using a 50 ml syringe, until the level was 22 mm from the top of the smallest plate. 400 \( \mu l \) n-butanol was layered over the top of each gel to exclude air and encourage polymerisation. After gels had set (approximately 45 min), n-butanol was washed off with DIW and excess water absorbed with blotting paper.

2.2.1.3 Preparation of stacking gel

A stacking gel solution was made according to the solution ratios shown in Table 2.2. Degassing was for 7 min as previously described (see 2.2.1.2). Following the addition of solution E and TEMED, the required volume of degassed solution was introduced into the casting chamber on top of the separating gel, to the level of the smallest plate, using a 20 ml syringe. Sample wells were formed by insertion of a plastic comb around which the stacking gel polymerised. The dimensions of the teeth of the comb determined the number and size of the resulting wells and the volume of sample which could subsequently be applied. Routinely 10 well combs were used, allowing a maximum 50 \( \mu l \) (or 100 \( \mu g \) protein) to be loaded. After gels had set
Table 2.1 CASTING OF SEPARATING GELS.

<table>
<thead>
<tr>
<th>% Acrylamide</th>
<th>15</th>
<th>12</th>
<th>10</th>
<th>7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIW (ml)</td>
<td>3.6</td>
<td>4.6</td>
<td>5.25</td>
<td>6.1</td>
</tr>
<tr>
<td>Solution A (ml)</td>
<td>5</td>
<td>4</td>
<td>3.35</td>
<td>2.5</td>
</tr>
<tr>
<td>Solution B (ml)</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Solution C (µl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Solution E (µl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Volumes necessary to make a single gel of a given percentage (common percentages used are shown).

Table 2.2 CASTING OF STACKING GEL.

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>VOLUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (ml)</td>
<td>1.25</td>
</tr>
<tr>
<td>A (ml)</td>
<td>0.65</td>
</tr>
<tr>
<td>E (µl)</td>
<td>25</td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>10</td>
</tr>
</tbody>
</table>

Volumes necessary to make a single stacking gel (3.9 % acrylamide).
(approximately 30 min), the casting clamp was dismantled, excess gel trimmed away and sandwiches stored at 4 °C wrapped in moist tissue surrounded by aluminium foil. Gels were stored for no more than three days prior to use.

2.2.1.4 Gel running conditions

Gels were assembled into tanks according to the manufacturers instructions. Each tank held approximately 800 ml of electrode (running) buffer (25 mM Tris, 190 mM glycine, 0.05 % (w/v) SDS, pH 8.3), prepared by combining 200 ml of 4x concentrated electrode buffer with 600 ml DIW. To ensure good electrical contact, any air bubbles were removed from the bottom of the gels by swirling the lower buffer with a pipette until the bubbles cleared. Samples were loaded into the wells under the electrode buffer with a Hamilton syringe, which was inserted to about 1-2 mm from the well bottom before delivery.

Electrophoresis was carried out at room temperature at 180 V for 10 % gels or 100 V for 7.5 % gels. A run was terminated when the BPB front had reached the bottom of a gel, this was approximately after 45 min for 10 % gels or 1.5 h for 7.5 % gels. Pre-stained SDS-7B molecular weight markers and appropriate reference sample (where indicated) were electrophoresed in wells adjacent to the samples.

2.2.1.5 Coomassie blue staining of polyacrylamide gels

Gels were fixed and stained simultaneously by soaking in 0.25 % (w/v) Coomassie Brilliant Blue R-250, 10 % (v/v) glacial acetic acid and 50 % (v/v) methanol) for 1 h. Destaining was carried out by soaking, with gentle agitation, in repeated changes of destain solution (10 % (v/v) glacial acetic acid and 40 % (v/v) methanol until blue background had disappeared. Finally gels were washed in 10 % (v/v) acetic acid and stored in the same solution prior to drying. Gels were dried using a gel drying kit from Promega.

2.2.2 Western blotting

Western blotting was performed according to Towbin et al (1979), using the Mini Trans-Blot electrophoretic transfer cell from Biorad Ltd., USA. Following electrophoresis gels were equilibrated for 10 min in ice cold transfer buffer (25 mM
Tris pH 8.3, 181 mM glycine and 20\% (v/v) methanol). Nitrocellulose membranes were cut to the dimensions of a gel and labelled for identification and orientation. Cut membranes were then equilibrated by briefly soaking in DIW and then in transfer buffer for at least 10 min. Complete wetting of the membrane was important to ensure proper binding, (abrupt wetting can lead to entrapment of air bubbles in the matrix, which can block transfer of molecules; gloves were always worn when handling membranes). Filter paper (Whatman 3mm, BDH Ltd.) also cut to the dimensions of a gel and fibre pads were saturated by soaking in transfer buffer. Care was taken to avoid trapping air bubbles during the wetting process. A mini Trans-Blot electrode was installed in the buffer chamber (see manufacturers instructions) and the buffer tank filled with ice cold transfer buffer (approximately 400 ml). A cooling unit was prepared in advance, by filling with ice and storing in the freezer until required (see manufacturers instructions).

2.2.2.1 Assembly of transfer sandwich and transfer conditions

For assembly of the transfer sandwich an open gel holder was placed in a shallow tray filled with ice cold transfer buffer, so that the clear panel was flat on the bottom of the tray with the grey panel resting against the side. A pre-soaked fibre pad was placed on the clear panel followed by saturated filter paper and nitrocellulose. An equilibrated gel was placed on top of the membrane making sure no air bubbles were trapped between the two. The sandwich was completed by placing a piece of saturated filter paper on top of the gel, followed by a fibre pad and closing the cassette taking care not to disrupt the gel-membrane contact. The gel cassette was always inserted into the buffer tank with the grey panel facing the grey cathode electrode panel. With the cooling unit in place, the tank was filled with ice cold transfer buffer and transfer was for 1 h 30 min at 100 V.

2.2.2.2 Post-transfer treatment of membranes

After transfer membranes were removed, allowed to air dry, then placed in 25 ml Sterilin tubes (BDH Ltd) ready for immunoblotting. Antibody incubation conditions have been summarised in Table 2.3. All steps were carried out with rotation and tubes were changed, prior to washing, after reaction with primary and secondary antibodies. After final washing, immobilised proteins were detected using the enhanced
Table 2.3 ANTIBODIES & INCUBATION CONDITIONS FOR WESTERN BLOTTING.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Blocking of binding sites</th>
<th>Post block washing</th>
<th>Primary antibody incubation</th>
<th>Post primary antibody wash</th>
<th>Second antibody incubation</th>
<th>Post second antibody wash</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>22C11</td>
<td>Overnight at 4 °C in PBS containing 4 % (w/v) milk powder</td>
<td>N/A</td>
<td>1/250 (0.2 µg/ml)</td>
<td>2 x 10 min &amp; 2 x 5 min (PBS)</td>
<td>Peroxidase-conjugated goat anti-mouse IgG 1/1000 (1 µg/ml) (1 h 30 min)</td>
<td>4 x 10 min &amp; 2 x 5 min</td>
<td>ECL DETECTION (see 2.2.2.2)</td>
</tr>
<tr>
<td>3B11</td>
<td></td>
<td></td>
<td>1/20</td>
<td>2 x 10 min &amp; 1 x 5 min</td>
<td>Peroxidase-conjugated goat anti-mouse IgG 1/1000 (1 µg/ml) (1 h 30 min)</td>
<td>3 x 10 min &amp; 2 x 5 min</td>
<td></td>
</tr>
<tr>
<td>Mouse Serum</td>
<td></td>
<td></td>
<td>2.5 ml undiluted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10D5</td>
<td>As for 22C11 with additional 1 h 30 min at 37 °C in PBS containing 4 % (w/v) milk powder and 0.05 % Tween 20</td>
<td></td>
<td>1/556 (5 µg/ml)</td>
<td>4 x 10 min</td>
<td>Peroxidase-conjugated goat anti-mouse IgG 1/3000 (0.33 µg/ml)</td>
<td>4 x 10 min &amp; 2 x 5 min</td>
<td></td>
</tr>
<tr>
<td>7H5</td>
<td></td>
<td></td>
<td>1/500 (5 µg/ml)</td>
<td>2 x 10 min &amp; 2 x 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA1</td>
<td></td>
<td></td>
<td>1/750 (2 h)</td>
<td>2 x 10 min &amp; 1 x 5 min</td>
<td>Peroxidase-conjugated goat anti-rabbit IgG 1/1500 (0.165 µg/ml)</td>
<td>3 x 10 min &amp; 2 x 5 min</td>
<td></td>
</tr>
</tbody>
</table>

All antibodies were diluted in the appropriate blocking solution at 37 °C for 1 h unless otherwise specified. Washing was with PBS containing 0.05 % Tween 20, and second antibody incubations were carried out at room temperature for 1 h unless otherwise specified.
chemiluminescence (ECL) Western blotting detection system (Amersham International). Following incubation of membranes with equal volumes of ECL reagents A and B (see manufacturers instructions), membranes were exposed to Hyperfilm-ECL (Amersham International) for an appropriate time (dependent on the amount of signal assessed after 5 min exposure). Films were developed using Kodak LX-24 x-ray developer (1 part developer + 3 parts water) for 2 min and fixed with Kodak FX-40 x-ray liquid fixer (1 + 4). Films were washed thoroughly before drying and molecular weight standards marked on films once dry.

2.2.2.3 Quantification of autoradiograms

Autoradiograms were quantified by scanning with a semi-automated scanning densitometer (EDC, Helena Labs.; slit width 3 x 0.3 mm at 610 nm). For the determination of APPLIR in human brain fractions (see Chapter 3, section 3.2), rat APP substrate and a 100 kDa product (see Chapter 4, section 4.2) and human VCSF (Chapter 6, section 6.2), band density (integral of band density as a proportion of integral of the same band in a reference sample) was calculated to give a value in arbitrary units for each protein species. For analysis of APPLIR in PC12 pellets and conditioned medium (Chapter 5, section 5.2), the average density of a band was normalised to the average density of the same band recovered under control conditions (t-statistic) giving a value expressed as a percentage of the control (100 %) for each protein species.

2.2.3 Tissue processing procedures

2.2.3.1 Human

Tissue samples were of two types, conventional or short post mortem examination where tissue was obtained with minimum delay.

2.2.3.1.1 Conventional post mortem subjects

Conventional post mortem delay brains were from both AD subjects, and non-demented control subjects (see Appendix 1). The brains were bisected sagittally, one hemisphere was fixed in 10 % formalin for histological examination and the other frozen at -70 °C until required for study. The frozen hemispheres used were
subsequently warmed to -10 °C and cut into 0.5 cm coronal slices, from which blocks of cortical grey matter and underlying white matter were taken from the appropriate areas for enzyme preparation (see 2.2.6.1.2).

2.2.3.1.2 Short post mortem subjects

Short post mortem delay brains were from AD subjects, and subjects with non-Alzheimer type dementias (see Appendix 2). Post mortem examinations were made within 3.5 h of death. One hemisphere was fixed as above, and the other sliced coronally into 1 cm thick slices and placed into ice cold Krebs-Ringer phosphate buffer (141 mM choline chloride, 5 mM KCl, 1.3 mM MgSO₄, 1.3 mM CaCl₂ and 10 mM Na₂HPO₄, pH 7.4), containing 5 mM glucose and frozen at -70 °C within 4 h of death (Procter et al., 1990) until required for enzyme preparation and extraction of APPLIR (see 2.2.6.1.2 and 2.2.4.1 respectively).

2.2.3.1.3 Histopathological assessments

Clinical and histological assessment indicated that all conventional post mortem delay control subjects used were free of gross psychiatric or neurological disturbance. Subjects diagnosed as having AD underwent standard neuropathological examination of the brain to exclude causes of dementia other than AD, and to determine the nature and extent of any vascular changes. No patient with AD included in the study had vascular disease judged as more than mild. All AD subjects from both series had clinical evidence of global cognitive impairment. In addition senile plaque and neurofibrillary tangle formation in the hippocampus and neocortex was of a severity and distribution indicative of a diagnosis of AD (Wilcock and Esiri, 1982; Medical Research Council, 1987a).

Histopathological examination of subjects with other causes of cognitive impairment (short post mortem delay series only), showed them to include one case each of Pick's disease, vascular dementia, pseudodementia and multisystem degeneration. The remaining two cases had an inconclusive diagnosis as the histopathological features were not characteristic of AD or any other neurodegenerative condition (see Appendix 2).
2.2.3.2 Rat tissue

Rat tissue was obtained after stunning and decapitation, from adult rats (250-300 g males) of the Sprague-Dawley strain and litter mate 4-5 day old pups of the same strain. Adult rat tissue was handled under conditions designed to mimic the treatment of human brain under conventional post mortem conditions. The heads were placed in sealed bags in a water bath at 37 °C that was allowed to cool (2-4 °C/h) towards room temperature for 2 h (Procter et al., 1991).

2.2.4 Distribution of APPLIR in human post mortem brain and correlations of various cellular parameters with APPLIR and APP mRNA concentrations (see Chapter 3)

2.2.4.1 Preparation and analysis of samples for determination of APPLIR

Brain slices from the short post mortem delay series only (Table 2.4) were warmed to -10 °C and blocks of frontal and temporal cortex (areas BA 9 and BA 20 respectively) were taken for the determination of APPLIR. Grey matter with all cortical layers was dissected, on ice, free of meninges and white matter and prepared essentially by the method of Arai et al (1991), to yield a soluble fraction and two membrane fractions of high and low buoyant density.

Approximately 500 mg of tissue was homogenised in 2 vol buffer A (0.1 M HEPES, 0.75 M NaCl, 1 mM EGTA, 1 mM DTT, N-tosyl-L-lysine chloromethyl ketone [1 μg/ml], N-tosyl-L-phenylalanine chloromethyl ketone [1 μg/ml], soya bean trypsin inhibitor [1 μg/ml], pepstatin [1μg/ml] and leupeptin [1 μg/ml], pH 7.0), and centrifuged at 100,000 x g for 1 h. The supernatant (soluble fraction) was removed, divided into 200 μl aliquots and stored at -70 °C; an additional 70 μl aliquot was taken for protein estimation (see 2.2.5). When required, individual aliquots were thawed on ice and mixed with 100 μl 3x concentrated sample buffer (50 mM Tris-HCl, 100 mM DTT, 10 mM EGTA, 2 mM PMSF, 4 % SDS (w/v), 0.1 % (w/v) BPB, 10 % (v/v) glycerol and 1μg/ml each of pepstatin and leupeptin final concentration, pH 6.8). The pellet was resuspended in 10 vol 30 % sucrose in buffer A and centrifuged as above. The resultant pellet (high density (HD) membranes), was dissolved in 2.5 vol 50 mM Tris-HCl, 10 mM EGTA, 4 % SDS (w/v), 10 % (v/v) glycerol, (pH 6.8) containing all
Table 2.4 APPLIR MEASUREMENT IN HUMAN POST MORTEM BRAIN: DEMOGRAPHIC DETAILS OF SHORT POST MORTEM DELAY SUBJECTS STUDIED.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Post mortem delay (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer's disease (n=14)</td>
<td>72</td>
<td>11F;1M</td>
<td>2.4 (1.5-3.5)</td>
</tr>
<tr>
<td>(58-88)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pick's disease</td>
<td>79</td>
<td>F</td>
<td>1.5</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>82</td>
<td>F</td>
<td>1.5</td>
</tr>
<tr>
<td>72</td>
<td>M</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Inconclusive</td>
<td>91</td>
<td>M</td>
<td>2.5</td>
</tr>
<tr>
<td>86</td>
<td>M</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Pseudodementia</td>
<td>72</td>
<td>M</td>
<td>1</td>
</tr>
<tr>
<td>63</td>
<td>F</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Multisystem degeneration</td>
<td>63</td>
<td>F</td>
<td>2</td>
</tr>
<tr>
<td>Frontal lobe dementia</td>
<td>71</td>
<td>F</td>
<td>2</td>
</tr>
</tbody>
</table>

Non-AD demented control subjects are case numbers 1, 5-7, 10, 16, 17, 19 and AD subjects are case numbers 3, 4, 8, 9, 11-15, 18, 21, 23, 25, 26 of Appendix 2. For AD cases, values are mean with range in brackets.
the protease inhibitors of buffer A. DTT, PMSF, and BPB giving final concentrations of 100 mM, 10 mM and 0.1 % (w/v) respectively were added to the dissolved pellet after a 70 μl aliquot had been taken for protein estimation (see 2.2.5). The material that floated on the supernatant (low density (LD) membranes) was recovered as a pellet following centrifugation in 0.32 M sucrose in buffer A and dissolved in 2.5 vol of sample buffer as described above for HD membranes. All fractions were stored at -70 °C until required. On thawing, samples were heated in a boiling water bath (100 °C for 4 min) and triplicate aliquots containing 100 μg protein electrophoresed on 7.5 % SDS-gels (see 2.2.1), along with a reference human brain extract also containing 100 μg of protein and 10 μl SDS-7B molecular weight markers. After transfer electrophoretically to a nitrocellulose membrane (see 2.2.2), APPLIR was determined with antibody 22C11, and the APPLIR species present defined using antibodies 10D5, 7H5 and anti-5 (see Table 2.3 for antibody incubation conditions). Immunoreactive proteins were visualised using an ECL detection system (see 2.2.2.2) and autoradiograms quantified as described in section 2.2.2.3.

2.2.4.2 Statistics

Intercorrelation of variables was performed using Spearman's correlation coefficient (r), the null hypothesis being rejected at P < 0.025 to reduce the chance of false positive results. Group comparisons were made using the Mann-Whitney U test.

2.2.4.3 Correlation of various cellular parameters with APPLIR or APP mRNA concentration

Cerebral cortex from 15 demented patients (short post mortem delay series; Table 2.5), was prepared and species of APPLIR present, identified and quantified as described in 2.2.4.1. Pyramidal neurone and astrocyte counts, total tissue GABA content, sodium dependent d-aspartate uptake, ChAT activity and measurements of APPg and APP Ki mRNA had previously been determined (see, Procter et al., 1994) for the same samples and are displayed in Table 3.2. These values were then used to investigate any intercorrelations with values for APPLIR or APP mRNA concentration.
Table 2.5 DEMOGRAPHIC DETAILS OF SHORT POST MORTEM DELAY SUBJECTS STUDIED TO INVESTIGATE CORRELATIONS OF VARIOUS CELLULAR PARAMETERS WITH APPLIR AND APP mRNA CONCENTRATION.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Post mortem delay (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer's disease (n=9)</td>
<td>69</td>
<td>8F,1M</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>(58-80)</td>
<td></td>
<td>(1.5-3.5)</td>
</tr>
<tr>
<td>Pick's disease</td>
<td>79</td>
<td>F</td>
<td>1.5</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>82</td>
<td>F</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>M</td>
<td>2.5</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>91</td>
<td>M</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>M</td>
<td>1.5</td>
</tr>
<tr>
<td>Pseudodementia</td>
<td>72</td>
<td>M</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>F</td>
<td>2</td>
</tr>
<tr>
<td>Multisystem degeneration</td>
<td>63</td>
<td>F</td>
<td>2</td>
</tr>
</tbody>
</table>

Non-AD demented control subjects are case numbers 1, 5-7, 10, 17 and AD subjects are 3, 4, 8, 9, 11, 12-15 of Appendix 2. For AD cases, values are mean with range in brackets.
2.2.4.4 Statistics

The test of intercorrelations of APPLIR species and APP mRNA with measures of pyramidal neurones, interneurones and astrocytes, was made by multiple regression analysis, using the SPSS-PC+ computer program. Included in the regression analysis were the potentially confounding factors, age, post mortem delay and duration of terminal coma, where appropriate. Correlation of ChAT activity with APP species was examined using Spearman's correlation coefficient ($r_s$), and comparisons between groups were made using the Mann Whitney $U$ test.

2.2.5 Protein determination

The assay was adapted from the method of Lowry et al (1951). Human tissue pellet fractions (7.5 µl; approximately 60 µg protein; see 2.2.4.1) were incubated, in triplicate, in 1 M NaOH (to give a final vol of 0.5 ml) in a water bath at 37 °C with constant shaking for 1 h 30 min until the pellet was dissolved. Supernatant fractions (7.5 µl; approximately 70 µg protein; see 2.2.4.1) and PC12 cell lysate (20 µl; approximately 120 µg protein; see 2.2.4.2) were mixed with an equal volume of 1 M NaOH and incubated in 0.5 M NaOH (final vol 1 ml) as above. After incubation, 0.5 ml DIW was added to all tubes containing 0.5 ml 1 M NaOH to give a final concentration of 0.5 M NaOH in all tubes. An appropriate standard curve (7.5-200 µg/ml) was constructed using a BSA standard (fraction V, dissolved in 0.5 M NaOH to give 1 mg/ml). After incubation, the standards, samples and blanks were then treated simultaneously with 5 ml Reagent X (2 % (w/v) sodium carbonate, 0.01 % (w/v) copper sulphate and 0.02 % (w/v) sodium potassium tartrate). Colour development was initiated by addition of 0.5 ml Folin-Ciocalteu's reagent (diluted 1:1 v/v with DIW). Tubes were vortexed and allowed to stand at room temperature for 45 min before reading the optical density of each tube at 660 nm using a Cecil spectrophotometer (2000 series; Cecil Instruments Ltd, Cambridge, UK.).
2.2.6 Comparison of a membrane serine protease activity prepared from human and rat brain with respect to degradation of APPLIR extracted from rat cortical membranes (see Chapter 4)

2.2.6.1 Enzyme preparations

2.2.6.1.1 Rat enzyme

A serine protease activity was prepared from rat tissue (see 2.2.3.2) as described by Siman et al (1990). All procedures were carried out at 4 °C unless otherwise indicated. Grey matter from adult rats dissected free of white matter, and whole forebrains from 5 day old rats stripped of meninges were homogenised using a motor driven glass/teflon homogeniser assembly (10 passes, setting 4) in 20 volumes 50 mM HEPES, 1 mM EDTA and 1 % Triton X-100 (pH 7.5). The homogenate was left to stand for 30 min with occasional vortexing and was then centrifuged (150,000 x g ) for 1 h in a Europa 65 ultracentrifuge. The pellet was resuspended using an Ultra-Turrax homogeniser assembly (setting 13,500 rpm), to original volume in 1 mM HEPES and 0.1 mM EDTA (pH 7.5), made up in DIW purified by Chelex-100 resin and shaken in a water bath for 1 h at 37 °C. The resuspension was then centrifuged as above. Pellets were resuspended to 10 % original volume in 50 mM HEPES, 1 M MgCl₂, 0.1 % Brij-35 (pH 7.5) and left to stand on ice for 30 min with occasional vortexing. Samples were centrifuged as above for 1 h. The supernatant from this extraction was dialysed against 20 mM HEPES, 0.5 M NaCl, 1 mM EDTA, 0.1 % Brij-35 (pH 7.5) overnight at 4 °C and centrifuged as above to yield enzyme preparations which were frozen (without loss of activity) and stored at -70 °C for subsequent use in the assay described in section 2.2.6.3 (routinely within 1 month of preparation).

2.2.6.1.2 Human enzyme

Human brains were of two types, those with short or conventional post mortem delays (see Tables 2.6 and 2.7). Preparations were from the superior frontal cortex (BA 9) and parietal lobe (BA 39/40 or BA 7, conventional and short post mortem delay series) and middle temporal gyrus (BA 21, short post mortem series only). There was no selection of cases by disease severity. Age, sex, drug treatment, and mode of death
Table 2.6 SERINE PROTEASE ACTIVITY: DEMOGRAPHIC DETAILS OF SHORT POST MORTEM DELAY SUBJECTS STUDIED.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Alzheimer's Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 6</td>
<td>n = 9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>mean</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>63-91</td>
</tr>
<tr>
<td>Gender</td>
<td>3F;3M</td>
<td>8F;1M</td>
</tr>
<tr>
<td>Terminal Coma (h)</td>
<td>mean</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>0-240</td>
</tr>
<tr>
<td>Post mortem delay (h)</td>
<td>mean</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>1-2.5</td>
</tr>
</tbody>
</table>

Non-AD demented control subjects are case numbers 1, 5-7, 10, 17 and AD subjects are case numbers 3, 4, 8, 9, 11-15 of Appendix 2. For non-AD demented controls and AD cases, values are mean with age in brackets.
## Table 2.7 SERINE PROTEASE ACTIVITY: DEMOGRAPHIC DETAILS OF CONVENTIONAL POST MORTEM CONTROL AND AD SUBJECTS STUDIED.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Alzheimer's Disease (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>77</td>
<td>80</td>
</tr>
<tr>
<td>(range)</td>
<td>48-85</td>
<td>73-88</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4F;4M</td>
<td>6F;2M</td>
</tr>
<tr>
<td><strong>Agonal state</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sudden</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>protracted</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>unknown</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Post mortem delay (h)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>(range)</td>
<td>24-53</td>
<td>11-57</td>
</tr>
</tbody>
</table>

Control subjects are case numbers 1-8 and AD subjects are case numbers 9-16 of Appendix 1. For control and AD subjects, values are mean with range in brackets.
(agonal state) were all recorded where this information was available (see Appendices 1 and 2).

Enzyme was prepared essentially by the method of Nelson and Siman (1990). Blocks of cortical grey matter and underlying white matter were thawed and 1.2 g of grey matter from all cortical layers was dissected free of white matter on ice, stripped of meninges and blood vessels, homogenised and centrifuged as described above. The pellet was resuspended in 16 vol 1 mM HEPES, 0.1 mM EDTA (pH 7.5), made up in DIW purified by Chelex-100 resin and incubated in a shaking water bath at 37 °C for 1 h. Samples were centrifuged (as above), the resultant pellet resuspended in 1 vol 50 mM HEPES, 1 M MgCl₂, 0.1 % Brij-35 (pH 7.5) and left to stand for 30 min with occasional vortexing. After centrifugation (as previous), the supernatant from this extraction was dialysed and centrifuged as above to yield enzyme preparations.

2.2.6.2 APP substrate preparation

APP was extracted from adult rat cortical membranes, by a method similar to that of Siman et al (1990). After stunning and decapitation, 1.25 g of cortex was dissected free of blood vessels, meninges and white matter, homogenised using a motor driven glass/teflon homogeniser assembly (10 passes, setting 4) in 10 vol buffer B (20 mM Tris-HCl, 5 mM 2-mercaptoethanol, 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, 50 µM pepstatin, 50 µM leupeptin, 100µM PMSF, pH 7.4) and centrifuged (14,000 x g; 20 min at 4 °C). The resultant pellet was resuspended and washed twice in the same buffer, resuspended in 10 vol DIW containing 50 µM leupeptin and 50 µM pepstatin and left (with occasional vortexing) for 1 h at 2 °C. After centrifugation (31,000 x g; 45 min at 4 °C), the supernatant was mixed with 0.1 vol buffer C (100 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, 0.5 mM pepstatin, 0.5 mM leupeptin, pH 7.4) and re-circulated (1 h) over a heparin-agarose column equilibrated with the same buffer. The column was then washed sequentially with buffer D (10 mM Tris-HCl, pH 7.6) and buffer D containing 0.25 M NaCl and the substrate finally eluted from the column with buffer D containing 0.5 M NaCl and concentrated using Centricon-3 concentrating tubes to yield a suitable volume for assay (approximately 100 µl)
2.2.6.3 Assay conditions

5 μl of enzyme preparation (containing extract equivalent to 1 mg original wet weight of tissue) and 7.5 μl of rat APP substrate (containing extract equivalent to 13 mg original wet weight of tissue) were incubated in triplicate for 2 h at 37 °C, in the presence of 7.6 mM CaCl₂ or 2 mM PMSF where indicated. Reactions were stopped with the addition of 25 μl modified Laemmli sample buffer (25 mM Tris-HCl, 5 mM EGTA, 50 mM DTT, 1 mM PMSF, 0.05 % BPB, 2 % SDS, 5 % glycerol, pH 6.8), followed by heating in a boiling water bath for 4 min. Following separation (25 μl of each sample) by 7.5 % discontinuous SDS-PAGE (see 2.2.1), APPLIR was analysed by Western blotting using antibody 22C11 (see 2.2.2 and Table 2.3). Pre-stained SDS 7B molecular weight markers (10 μl) and a reference human brain extract (25 μl) were also run adjacent to the samples on each gel and autoradiograms quantified as described in section 2.2.2.3

2.2.6.4 Statistics

Group means were compared using Student's t test or Mann-Whitney U test as appropriate. Intercorrelation of variables was tested by Spearman's rank correlation coefficient (r).

2.2.7 Does perturbed energy metabolism alter the balance of non-amyloidogenic and amyloidogenic processing of APPLIR in PC12 cells? (see Chapter 5)

2.2.7.1 Cell culture

2.2.7.1.1 Culture conditions

All solutions and media were obtained as sterile solutions if possible. Sterilisation of solutions, if necessary, was by autoclaving or by filtering through a 0.2 μm-pore filter. Cell stocks were maintained in 75 cm² plastic culture flasks coated with collagen (see 2.2.7.1.2), in DMEM containing 6 % FCS (v/v), 6 % horse serum (v/v) and 1 % gentamicin (v/v) at 35 °C in humidified CO₂ and air (5:95; see Greene and Tischler, 1976), until required for experiments. All cells at this stage were grown in the absence of NGF and media was renewed every 3-4 days. At confluency, cells were
dislodged mechanically by gentle flow of incubation medium and re-seeded into freshly coated flasks usually at a 1:5 ratio.

2.2.7.1.2 Preparation of collagen films on cell culture surfaces

10 ml of chilled Vitrogen 100 Collagen solution was mixed well with 500 ml sterile DIW. The surfaces to be coated were covered with this solution to a depth of 1-2 mm (10 ml for 75 cm² culture flasks, or 3 ml for a 9.6 cm² cell culture well) and incubated at 37 °C for 1 h 30 min, to promote gelation. After rinsing with sterile DIW, films were either used immediately for cell culture or allowed to dry and stored in the cell culture hood for future use.

2.2.7.2 Perturbation of cell metabolism

PC12 cell monolayers were detached as described in section 2.2.7.1.1, and cell suspensions pooled and spun (3,500 x g; 7 min) to give a single pellet. The pellet was washed twice to remove any traces of serum by centrifugation (as above) in serum-free DMEM/F12 nutrient mix containing a serum supplement (insulin [5 μg/ml], transferrin [5 μg/ml] and sodium selenite [5 ng/ml]) and 1 % gentamicin (v/v). The final pellet was resuspended in the same serum-free medium using a syringe and 19 gauge needle, followed by vortexing. 100 μl of the final suspension was taken for cell counting (see 2.2.7.3) and cells plated at 7 x 10⁶ cells/well onto collagen coated 35 mm 6 well plates. Cells were maintained in 3 ml serum-free medium, and NGF treatment (400 ng/ml) began after 24 h and continued for 3 days. NGF (10 ng/μl) was dissolved in PBS containing 0.1 % BSA and stored at -70 °C for up to 6 months without loss of activity (personal communication Dr P Dougherty, UMDS, Guy’s Campus, London, U.K.).

Metabolism was perturbed as previously described (Reynolds et al., 1982) by treatment of cells for 3 h (in the presence of NGF; 400 ng/ml) with 5 mM 2-deoxyglucose and 0.2 μg/ml oligomycin, in 3 ml glucose- and pyruvate-free DMEM/F12 nutrient mix containing a serum supplement (as described above). Chloroquine (50 mM) where added, was present through out the incubation period, with bradykinin (500 nM) and phorbol 12,13-dibutyrate (100 nM) where indicated, present for 1 h post energy perturbation, in fresh glucose- and pyruvate-free DMEM/F12. Each condition was performed in triplicate which was considered to represent an experimental n of 1. Controls were parallel cultures not treated with these
agents in the presence or absence of perturbed energy. The effect of the PKC inhibitor staurosporine (1 μM) was also studied on cultures incubated with and without phorbol 12,13-dibutyrate (as described above). Following incubation, cells were separated from the incubation medium by centrifugation (3,500 x g; 7 min), and resuspended in 1 ml cold PBS. 450 μl aliquots of uniform cell suspension were taken twice, and centrifuged (as previous) to give two equal cell pellets for determination of nucleotides by HPLC (see 2.2.7.4) and analysis of APPLIR (see 2.2.7.2.1). The remaining 100 μl of cell suspension was used to determine cell viability post energy perturbation (see 2.2.7.3). Conditioned medium was stored at -20 °C until required for concentration and analysis of APPLIR (see 2.2.7.2.2).

2.2.7.2.1 Analysis of APPLIR in cell pellets

Pellets were resuspended in 300 μl of modified Laemelli sample buffer (50 mM Tris-HCL, pH 6.8, 10 mM EGTA, 2 mM PMSF, 4 % (w/v) SDS, 10 % (v/v) glycerol), sonicated (10 sec, low power, setting 3; MSE sonicator, USA) and centrifuged (12,000 x g; 15 min at 4 °C in a Beckman J2-21 centrifuge). Following removal of 70 μl of supernatant for total protein determination (see 2.2.5), DTT and BPB were added to give final concentrations of 100 mM and 0.1% (w/v) respectively and samples stored (-70 °C) until required. On thawing, samples were heated in a boiling water bath (100 °C for 4 min) and aliquots containing 100 μg protein were electrophoresed on 7.5 % SDS-gels along with 10 μl pre-stained molecular weight markers (see 2.2.1). After electrophoretic transfer (see 2.2.2.1), blots were probed with antibodies 22C11 and DA1 (see Table 2.3 for antibody incubation conditions) and immunoreactive proteins were visualised using an ECL detection system (see 2.2.2.2). Autoradiograms were scanned and quantified as described in section 2.2.2.3.

2.2.7.2.2 Analysis of APPLIR in conditioned medium

Conditioned medium was thawed and 2.5 ml concentrated using Centricon-10 concentrators to a final concentration of 400 μl, by centrifugation at 2,400 x g for approximately 80 min at 4 °C (Beckman J2-21 centrifuge). 4x concentrated modified Laemmli sample buffer, DTT and BPB were added to give final concentrations as above and samples stored at -70 °C until required. On thawing, samples were heated in a boiling water bath (100 °C for 4 min) and 50 μl aliquots electrophoresed on 10 %
polyacrylamide gels along with 10 μl pre-stained molecular weight markers (section 2.2.1). After electrophoretic transfer (section 2.2.2.1), blots were probed with antibodies 22C11, DA1 and 7H5 (see Table 2.3 for antibody incubation conditions) and immunoreactive proteins were visualised using an ECL detection system. (see 2.2.2.2) For scanning and quantification of autoradiograms see 2.2.2.3.

2.2.7.3 Cell counting and determination of cell viability

Trypan blue stain was used to determine total cell counts and viable cell numbers. 100 μl of a uniform cell suspension (see 2.2.7.2) was diluted with 100 μl PBS and 10 μl trypan blue stain (1 % w/v in PBS), mixed thoroughly and allowed to stand for 5-15 min. With the cover slip in place a small amount of trypan blue-cell suspension was transferred to the haemocytometer using a Hamilton syringe by carefully touching the edge of the cover-slip with the needle of the syringe and allowing each chamber to fill by capillary action. All the cells (non-viable cells stain blue) in the 1 mm centre square and four 1 mm corner squares were counted. Each square of the haemocytometer represented a total volume of $10^4$ cm$^3$. Since 1 cm$^2$ is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and total number of cells) was determined using the following calculations:

$$\text{CELLS PER ML} = \text{average count per square} \times \text{dilution factor} \times 10^4$$

$$\text{TOTAL CELLS} = \text{cells per ml} \times \text{original volume from which 100 μl sample of cell suspension was taken from}$$

$$\% \text{CELL VIABILITY} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

2.2.7.4 Determination of ATP, ADP and AMP

For extraction of nucleotides all solutions used were at 4 °C. Cell pellets (see 2.2.7.2) were washed in PBS (3,500 x g for 7 min; Eppendorf mini-centrifuge), resuspended in 200 μl DIW and 100 μl (10 % v/v) perchloric acid, vortexed and left to stand on ice for 10 min. Following centrifugation (11,000 x g for 10 min; Eppendorf mini-centrifuge) acid extracts were neutralised by addition of 250 μl cold 1 M K$_2$HPO$_4$
and neutralised extracts then centrifuged as above. Supernatants were removed for assay of ATP, ADP and AMP immediately. Stock solutions (100 mM) for each nucleotide (ATP, ADP and AMP) were prepared in 85 mM TEA and stored at -70 °C. Before use, these were diluted in 85 mM TEA to make a mixed standard containing 10 mM of each nucleotide. HPLC of extracted nucleotides was kindly performed by Dr S Davies (Institute of Neurology, London, UK). Briefly, nucleotides were separated using an isocratic ion pair reverse method modified from Perret et al (1991). Using Beckman System Gold High Performance Liquid Chromatography System from Beckman RIIC (High Wycombe, Bucks, UK), separation of nucleotides was performed at 30 °C with a Hicrom S5OD2 column (250 mm x 4.6 mm, Hicrom Ltd., Reading, Berkshire, UK), and UV detection at 254 nm. The mobile phase consisted of 85 mM TEA, pH 6.0, containing 2 % methanol and flow rate was 1 ml/min. Nucleotide concentrations were calculated by integration of peak areas. Assignment of nucleotide peaks and calculation of absolute concentration was obtained by comparison to external standards of known nucleotide composition and concentration.

2.2.7.5 Statistics

Group means were compared using Student's t test.

2.2.8 Can drugs which affect neurotransmitter function alter the processing of APPLIR in human brain? (see Chapter 6)

2.2.8.1 Samples

Ventricular cerebral spinal fluid (VCSF) samples were obtained during perioperative air encephalography from 85 patients undergoing stereotactic psychosurgery for intractable depression (Pangalos et al., 1992). This procedure necessitates removal of the ventricular fluid from the patient in order to introduce air for a ventriculogram, enabling the appropriate stereotactic calculations to be made prior to surgery. The patients were of mean age 49 (± 10 SD, range 26-71) and comprised 23 males and 62 females, and were categorised according to the Research Diagnostic Criteria (Spitzer et al., 1978) as Major Depressive Disorder (unipolar depression; 52 %), Bipolar Disorder (42 %) and Obsessive Compulsive Disorder (6 %). Drug treatments received in the two weeks prior to the operation were recorded and classified into 5 categories (Pangalos et al., 1992): antidepressants antipsychotics, tranquillisers,
lithium salts, as listed in sections 4.3; 4.2.1 and 4.2.2; 4.1; 4.2.3 respectively, of the British National Formulary (Joint Formulary Committee, 1990), and other psychotropic drugs. For the purpose of this study, patients were divided into four groups according to treatment with lithium or antidepressants (see Table 2.8 and Appendix 3 for demographic details).

2.2.8.2 Preparation and analysis of VCSF

VCSF samples stored at -70 °C were thawed and 100 μl aliquots centrifuged (15,400 x g; 10 min at 4 °C; Koolspin) to remove any debris and mixed with 20 μl of 50 mM Tris-HCl containing 4 % SDS, 10 mM EGTA and 10 % glycerol (pH 6.8); 4 μl 50 mM PMSF; 15.5 μl 1 M DTT containing 1 % BPB and 5 μl glycerol. After 4 min at 100 °C, 50 μl of each sample (containing 35 μl original VCSF) was electrophoresed on 10 % SDS-gels (see 2.2.1), along with pre-stained molecular weight markers (10 μl) and a reference VCSF sample (50 μl). After Western blotting (see 2.2.2), samples were probed with antibodies 22C11, 7H5, anti-5 and 10D5 (refer to Table 2.3 for incubation conditions) to characterise the APPLIR species present. For quantification of autoradiograms see section 2.2.2.3. The values obtained in arbitrary units for each APPLIR protein species were corrected for total VCSF protein (Luxton et al., 1989). Total protein in VCSF was kindly determined by Dr E Thompson (The National Hospital for Nervous Diseases, London, U.K.) using the method of Luxton et al (1989).

2.2.8.3 Statistics

Group means were compared using Chi squared analysis and ANOVA followed by LSD test. Statistical analysis was performed on log transformed data as the original data was not normally distributed.

2.2.9 Production and characterisation of a novel monoclonal antibody (3B11) to Amyloid precursor-like protein 2 (see Chapter 7)

2.2.9.1 Antibody production

The monoclonal antibody 3B11 was produced by Professor N Groome (Oxford Brookes University, Oxford, UK) according to my requirements. Briefly, overlapping
Table 2.8 DEMOGRAPHIC DETAILS OF SUBJECTS USED IN THE STUDY OF APPLIR IN VCSF.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=15)</th>
<th>Antidepressant (n=25)</th>
<th>Lithium (n=7)</th>
<th>Lithium and antidepressant (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) Mean ± SD</td>
<td>49 ± 9</td>
<td>53 ± 13</td>
<td>47 ± 11</td>
<td>48 ± 9</td>
</tr>
<tr>
<td>Gender</td>
<td>9F,6M</td>
<td>18F,7M</td>
<td>4F,3M</td>
<td>31F,7M</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unipolar</td>
<td>7</td>
<td>13</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>Bipolar I</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Bipolar II</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Obsessional</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Antipsychotics</td>
<td>5</td>
<td>13</td>
<td>6</td>
<td>22</td>
</tr>
</tbody>
</table>

Control subjects are case numbers 3, 4, 9, 16, 17, 21, 29, 37, 45, 48, 52, 58, 73, 79, 82, subjects on antidepressant therapy are case numbers 13, 30, 36, 46, 53, 59, 62, 65, 67, 77, 88, 91, 97, subjects on lithium therapy are case numbers 15, 20, 25, 26, 31, 99, 105 and subjects receiving both types of therapy are case numbers 5, 10, 11, 14, 24, 27, 32-34, 38, 44, 47, 54, 55, 57, 60, 61, 64, 67, 69-72, 74, 84, 85, 87, 89, 90, 93-96, 98, 102-104, 108 of Appendix 3.
tetradecapeptide sequences from a 98 amino acid sequence of the ectodomain of APLP2 (aa 566-664; see Fig. 2.1) were synthesised and evaluated for purity using a previously published method (Yon et al., 1994; see also Webster et al., 1995). Female Balb/c mice were primed with a subcutaneous injection of human BCG vaccine (Lachmann et al., 1995), followed by three subcutaneous booster immunisations of peptide-tuberculin conjugate in Freund's incomplete adjuvant at three week intervals. Groups of three mice each received injections of two overlapping peptide conjugates e.g. B4 and C4 (see Fig. 2.1). After 10 days the mice were bled and screened by electrophoresis and Western blotting to identify high responders (see 2.2.9.2). Selected high responding mice received intraperitoneal and intravenous boosts of peptide-tuberculin conjugate on each of the two days prior to spleen removal. Spleens were removed aseptically, and splenocytes fused with Sp2 myeloma cells as previously described (Galfre and Milstein, 1995). The resulting hybridomas were suspended and diluted, then plated out into 96 well tissue culture plates. After 10-14 days, supernatant was taken from each well and tested for specificity by ELISA using plates coated with appropriate peptides (in this case B4 and C4). Those hybridomas giving strong positive results were expanded and re-tested by ELISA and Western blotting (see 2.2.9.2.2). The hybridoma clone designated 3B11 was subcloned to confirm monoclonality, and the immunoglobulin class of the antibody determined using an Immuno Type Kit (Sigma Chemical Co.).

2.2.9.2 Selection of high responding mice and identification of positive hybridoma supernatants

2.2.9.2.1 Samples

Supernatant and high density membrane fractions of human frontal cortex were prepared for SDS-PAGE (see 2.2.4.1) and equal volumes mixed. Lumbar CSF (LCSF) was obtained from two patients who had undergone cerebral biopsy leading to histological diagnoses of AD (subject A) and frontal lobe dementia (subject B) (Francis et al., 1993b), and VCSF from two patients (case numbers 5 and 8 of Appendix 3) with bipolar depression undergoing psychosurgery. All were prepared for electrophoresis as described in section 2.2.8.2. PC12 cells were grown in the presence or absence of NGF, metabolic inhibitors or bradykinin (see 2.2.7.2) and cell lysate and conditioned
Amino acids shown in bold are identical in APLP2 and APP\textsubscript{751}. Solid lines indicate overlapping tetradecapeptide sequences used to inoculate mice for antibody generation. Mice inoculated with both B4 and C4 were subsequently used for hybridoma generation and monoclonal antibody (3B11; see 2.2.9.1) production.
medium prepared for SDS-PAGE (see 2.2.7.2.1 and 2.2.7.2.2). Rat cerebellar and cortical cultures (section 2.1.6) were prepared from 7 day old and embryonic (E17) rats respectively, and grown according to the method of Dutton et al (1981). After 7 or 12 days respectively in vitro, cells were exposed to 500 μM glutamate in Krebs-Ringer phosphate buffer (141 mM choline chloride, 5 mM KCl, 1.3 mM MgSO₄, 1.3 mM CaCl₂ and 10 mM Na₂HPO₄, pH 7.4) for 30 min, and placed in 1.5 ml fresh buffer for a further 24 h. Buffer from both culture types was concentrated 2.5 times using Centricon-10 concentrators, by centrifugation at 2,400 x g for approximately 30 min at 4 °C (Beckman J2-21) and cell supernatant obtained by freezing and thawing cells in DIW followed by centrifugation (11,000 x g; 10 min at 4 °C). For SDS-PAGE both buffer and cell supernatant were mixed with concentrated sample buffer, DTT and BPB to allow for dilution (see 2.2.7.2.1 and 2.2.7.2.2). The following all gifts (see 2.1.6) were prepared for SDS-PAGE as described in section 2.2.7.2.1: SH-SY5Y neuroblastoma cells, H4 human neuroglioma cells stably transfected with mouse APLP1 or human APLP2, a glutathione fusion protein of the 113 amino acid ectodomain of APLP2, COS-1 cells transiently transfected with mouse APLP2-751 cDNA, purified secreted APP_{695} from transfected 293 cells, baculovirus expressed APP_{751}, and CHO cells transfected with mouse APLP2.

2.2.9.2.2 SDS-PAGE and Western blotting

Pooled human frontal cortex, supernatant and high density membrane fractions (100 μg protein), human LCSF as well as VCSF (equivalent to 14 and 35 μl original volume respectively), baculovirus expressed APP_{751} and purified secreted APP_{695} (each 1 μg protein), PC12 cell lysate (100 μg protein) and PC12 conditioned medium corresponding to approximately (0.26 ml original volume), cortical cell supernatant corresponding to approximately 2 x 10⁵ cells) and buffer from incubation of primary cultures (corresponding to 77 μl original volume) were separated on 7.5 % SDS-polyacrylamide gels (see 2.2.1) and electrophoretically transferred to nitrocellulose membranes (see 2.2.2). Membranes were probed with undiluted mouse sera (to identify high responders) or hybridoma supernatant (to identify positive clones; refer to Table 2.3 for incubation conditions).
2.2.9.3 Peptide absorption of antibody

10 µg/ml of peptide B4 or C4 (obtained from Professor N Groome) was dissolved in 3B11 hybridoma supernatant, mixed well and shaken for 3 h at room temperature or overnight at 4 °C. After mixing, the solution was spun (5000 x g; 10 min at 4 °C) and the supernatant transferred to a labelled tube ready for Western blotting alongside original antibody solution using known positive controls (see 2.2.9.2.2).

2.2.9.4 Immunocytochemistry

2.2.9.4.1 Immunostaining of paraffin embedded sections

Immunostaining was carried out on human post mortem brain material from four patients with histologically diagnosed AD that had been briefly fixed (1 h) in 10 % formalin and embedded in paraffin wax. Serial sections (a gift from Dr F Sherriff, Neuropathology Department, Radcliffe Infirmary, Oxford, UK), were de-waxed in histoclear (2 x 10 min), taken to 100 % alcohol and endogenous peroxidase activity blocked by incubation in 1 % hydrogen peroxide (H₂O₂) in methanol for 30 min. Sections were then subjected to microwave antigen retrieval (Sherriff et al., 1994) before immunocytochemistry with 3B11 or LN27. Briefly, the sections were brought to boil in 0.01 M citrate buffer (pH 6.0) in a coplin jar in a microwave oven (Deltaware), allowed to cool for 5 min, reboiled, then allowed to cool for 20 min. For β/A4 detection with antibody 10D5, the sections were treated with 90 % formic acid for 5 min instead of microwaving. All sections were washed in Tris buffered saline (TBS; pH 7.6) containing 0.2 % Tween 20 (TBS/tween) and incubated for 30 min in FCS (1:20). After blocking, sections were incubated with 3B11 (1:5), LN27 (1:200) or 10D5 (1:200) for 3 h and rinsed in TBS/tween. All antibodies were diluted in TBS/tween. Sections were rinsed as above, incubated for a further 30 min in biotinylated rabbit anti-mouse IgG (1:200) and rinsed again. An ABC detection system was used and peroxidase activity revealed with 0.025 % (w/v) diaminobenzidine and 0.25 % H₂O₂ in TBS for 10 min. Sections were washed in tap water, counterstained lightly with haematoxylin (approximately 5 seconds then washed in tap water) and dehydrated through alcohols and histoclear and mounted in DPX.
2.2.9.4.2 Immunostaining of free floating sections

After stunning and decapitation brains were removed from two adult rats (250-300 g males) of the Sprague-Dawley strain and immersion fixed in 4 % (w/v) paraformaldehyde for 24 h and then cryoprotected in 15 % sucrose (w/v) for 2-4 weeks at 4 °C. Human post mortem brain material was derived from a collection of post mortem tissue from the Neuropathology Department at the Radcliffe Infirmary, Oxford. Case 1040/94 was a clinically schizophrenic male aged 41 y, case Du 1194/94 was a clinically and histologically normal female aged 81 y, and case 1154/94 was a female patient age unknown with a clinical history of dementia and a diagnosis of AD was confirmed following histological examination. Tissue from these patients was briefly fixed and cryoprotected as described above. 50 µm sections of rat and human brain were cut on a freezing microtome (Litz kryomat) and stored in PBS containing 0.01 % thiomersal at 4 °C. For immunostaining of near adjacent sections with 3B11, D2-1 and LN27 microwave antigen retrieval was performed as described in section 2.2.9.4.1. For β/A4 detection with antibody 10D5, the sections were treated with 90 % formic acid for 5 min instead of microwaving. All subsequent steps were performed on an orbital shaker in 7 ml Sterilin bijou bottles (BDH Merck Ltd.). Endogenous peroxidase activity was blocked by pre-treatment of sections with 3 % (v/v) H2O2 in DIW for 20-30 min at room temperature. After washing (DIW; 2 x 5 min), and incubation in PBS containing 2 % (v/v) FCS (2 h, at room temperature), sections were incubated with 3B11 (1/750), LN27 (1/750), 10D5 (1/1000) and D2-1 (1/5000). All antibodies were diluted in PBS containing 0.3 % (v/v) triton and 1 % (v/v) FCS and incubation was for 3 days at 4 °C. After washing in PBS (2 x 5 min), sections were incubated with 0.5 % (v/v) of appropriate biotinylated antibody in PBS containing 0.05 % (v/v) triton and 1 % (v/v) FCS for 2 h at room temperature. For detection, sections were incubated in 1 % ABC (Vectastain kit; made up at least 30 min before use according to manufacturers instructions) in PBS containing 0.05 % (v/v) triton and 1 % (v/v) FCS for 90 min. Peroxidase activity was visualised using 1.7 mM AEC in acetate buffer (0.1 M, pH 5.2) containing 0.1 % H2O2. Sections were washed in DIW to stop AEC colour development and mounted on glass slides in Mowiol 4-88 aqueous mountant.
CHAPTER 3

3.0 DISTRIBUTION OF APP-LIKE IMMUNOREACTIVITY IN HUMAN POST MORTEM BRAIN AND RELATIONSHIP WITH VARIOUS CORTICAL CELL PARAMETERS.

3.1 INTRODUCTION

The finding of alternative APP mRNAs has led to speculations that in AD, changes in the ratios of APP isoforms with and without KPI domains may result in alterations of APP processing that favour the formation of β/44 (Price et al., 1989; Tanzi et al., 1989). However, recent investigations of the differential expression of APP mRNAs have yielded disparate results. RNA blotting studies have shown decreased levels of APP_695 mRNA (Johnson et al., 1988; Neve et al., 1988), as well as increased levels of APP_770 mRNA in the brains of individuals with AD as compared with controls (Tanaka et al., 1988). These findings suggest that in individuals with AD, APP_KPI may be the predominant isoform. In contrast, in situ hybridisation experiments have demonstrated a selective increase in APP_695 expression in subcortical neurones, but not in cortical neurones (Palmert et al., 1988).

Since these studies have used different methodologies to measure APP mRNA levels and because direct comparisons of levels of individual transcripts were not performed, it is not possible to resolve these discrepancies readily. However, using an approach allowing direct analysis of relative levels of APP_695 and APP_770/751 mRNAs within single RNA samples isolated from different brain regions in human controls, subjects with AD and non-human aged primates, the expression of APP transcripts was found not to be selectively altered in AD cortex. Although an age-associated change in expression was clearly demonstrated, the differential expression of APP transcripts did not correlate with the deposition of β/44 in cases of AD and aged monkeys (Koo et al., 1990b). It should be noted that in all of these studies mRNA levels were determined in post mortem brain tissue, which is vulnerable to pre mortem variables as well as artefacts introduced by post mortem degradation which may explain some of the discrepancies.
In view of such inconclusive and contradictory findings, a number of groups have undertaken to determine whether ageing or AD might be associated with alterations in the amounts of individual isoforms of APP (Rumble et al., 1989; Arai et al., 1991; Nordstedt et al., 1991). In one of these, a detailed study describing APP in fractions of human brain (Arai et al., 1991), no alterations were described and this was also found when unfractionated brain was studied (Nordstedt et al., 1991). However, as was the case with the mRNA studies, these groups used conventional post mortem tissue and therefore the data may not be definitive because of problems inherent in studying human post mortem tissue.

It was therefore considered important to investigate the distribution of APPLIR in a series of fractions prepared from brains collected specially from AD and other demented patients, using techniques designed to minimise autolysis (see 2.2.3.1.2). This study did not include non-demented control subjects as it was almost impossible to obtain such brains under similar autopsy conditions. Therefore, given the rigorous standard for comparison, the group of other dementias (see Appendix 2) was considered to represent a more appropriate control (Procter et al., 1990). A soluble fraction and two membrane fractions of high (HD) and low (LD) buoyant density respectively were prepared and APPLIR characterised (see 2.2.4.1) and quantified (see 2.2.2.3).

Using a subset of these patients (see Table 2.5) the study was extended to investigate the relationship between APPLIR concentrations and values determined previously for various biochemical and morphometric indices including sodium dependent D-aspartate uptake (Procter et al., 1988b) and total tissue GABA concentration (Lowe et al., 1988; biochemical indices of glutamatergic pyramidal cells and interneurones respectively), ChAT activity (a measure of the terminals of cholinergic neurones), as well as levels of APP\textsubscript{695} and APP\textsubscript{KPI} mRNA (Harrison et al., 1994) and pyramidal cell and astrocyte counts (see 3.2.2).

3.2 RESULTS

3.2.1 Distribution of APPLIR in human brain

As shown in Fig. 3.1, antibody 22C11 identified three major protein bands in the HD (lane 2) and LD (lane 3) membrane-associated fractions. They had a molecular
Fractions illustrated are HD membranes (except as indicated; S, soluble fraction; L, LD membranes). 22C11 identified 3 major bands in soluble fractions (lanes 1 and 4), HD and LD membranes (lanes 2, 3 and 5). Open arrow, arrowhead, arrowhead with asterisk and filled arrow indicate bands 1-4 respectively. Band 2 in HD membranes is prominent with all antibodies except 10D5 (lanes 15-19). This was independent of diagnosis and cortical region based on an analysis of 15 cases (9 with AD), illustrated here by frontal cortex of an AD case (lanes 1-3, 6, 8, 11, 15), Pick's disease (9, 16, 22), pseudodementia (17), multisystem degeneration (10), different AD cases (12, 14) and temporal cortex of Pick's disease (4, 5, 7, 13, 21) and different AD cases (18-20, 23). Band 3 reacted strongly with all antibodies except 7H5 (lanes 20-23).
mass of approximately 100, 120 and 140 kDa respectively. In contrast, the soluble proteins detected in the cytosolic fractions (Fig. 3.1, lanes 1 and 4) had approximate molecular masses of 95, 100 and 120 kDa. All four bands have previously been described in human cerebral cortex (Arai et al., 1991; Nordstedt et al., 1991; Moir et al., 1992), and for the purpose of this study will be numbered 1-4 in order of descending molecular weight. Based on their reactivity with antibodies 22C11, 10D5, anti-5 and 7H5, bands 1 and 2 appear to represent APP\textsubscript{KPI}. However, in HD membranes, band 2 reacted weakly with 10D5 (Fig. 3.1, lanes 15-19) indicating that APP\textsubscript{KPI} may not be abundant in this fraction. The APP homologue APLP2 may therefore be enriched in HD membranes. Antibodies 22C11 and 7H5 will recognise both APP and APLP2 since the identified domains and motifs that characterise APP are predicted to occur in APLP2, including the KPI domain and a similar N-terminal. In contrast 10D5 will not identify APLP2, since it lacks the β/A4 domain. Band 3 reacted strongly with all antibodies except 7H5 and was therefore considered to represent the non-KPI containing isoform APP\textsubscript{695} (Fig. 3.1, lanes 20-23). Band 4 reacted only with 22C11 and anti-5 and is predicted to be a degradation product of the same non-KPI containing isoform.

Of the various demographic features and peri mortem epiphenomena (i.e. age, sex, post mortem, coma or illness duration, drug status; see Table 2.4 and Appendix 2) and duration of storage at -70 °C, only few showed associations with any of the protein measurements. All APPLIR species in LD membranes from the temporal cortex of all cases was found to be affected by terminal coma, $r_s = -0.78$ ($P = 0.002$, band 1), $r_s = -0.79$ ($P = 0.001$, band 2) and $r_s = -0.79$ ($P = 0.001$, band 3). This relationship was also found for the AD cases alone $r_s = -0.91$ ($P = 0.005$, for both bands 1 and 3), and for the frontal cortex of all cases $r_s = -0.69$ ($P = 0.01$, band 3). The duration of storage affected the determination of APPLIR in LD membranes from frontal cortex of all cases, $r_s = 0.65$ ($P = 0.009$, band 1) and $r_s = 0.61$ ($P = 0.016$, band 2), and in the soluble fraction of frontal cortex from AD patients $r_s = -0.65$ ($P = 0.012$, band 4); however, this factor was matched for the groups. Generally, the APPLIR species measured showed positive correlations with other species in the same fraction. For example, bands 2 and 3 in the soluble fraction from the frontal cortex of all cases were correlated ($r_s = 0.77$, $P = 0.001$). In contrast, only one significant intercorrelation was observed between any APPLIR species measured between fractions. This was between
bands 1 (HD membranes) and 3 (soluble fraction) from the frontal cortex of all cases ($r_s = 0.49, P = 0.02$). This absence of intercorrelation may suggest that APPLIR in the soluble and membrane fractions exists in separate pools and no appreciable mixing takes place during the preparative procedures.

Band 4 of the soluble fraction was higher in frontal cortex of AD brain compared with those with other diagnoses (see Table 3.1). This band reacted only with 22C11 and not 7H5 (see Fig 3.1), and, therefore may represent degradation of APP$_{695}$. Table 3.1 also shows that only two of the other bands unaffected by epiphenomena were significantly different between the groups. These differences were in the temporal cortex, with band 3 in both the soluble and HD fractions being higher and lower respectively, in AD. These bands in comparison with other bands in the equivalent fractions, reacted strongly with antibodies 10D5 and anti-5 as well as 22C11 but weakly with 7H5 (see Fig. 3.1). Band 3 in both the soluble and HD fractions therefore had the characteristics of APP$_{695}$. Another band in the soluble fraction (band 2) was insensitive to epiphenomena and reacted strongly with all antibodies (see Fig. 3.1); therefore this had the characteristics of APP$_{KPI}$ as did band 1 in the HD membranes (see Fig. 3.1).

3.2.2 Correlations of various cortical cell parameters with APPLIR and APP mRNA concentrations

Additional data determined previously for the patients examined in this study (see Table 2.5) has been presented in Table 3.2. The patients with AD showed a loss of cholinergic activity and aspartate uptake when compared to patients with other causes of dementia (see Table 3.2). There were no other significant differences between the AD group and the other cases (Procter et al., 1988a). The potentially confounding factors of age, post mortem delay and duration of terminal coma showed significant correlations with various values of APPLIR in the different brain preparations and with mRNA (see Table 3.3). ChAT activity was unaffected by age (frontal cortex, $r_s = 0.32$; temporal cortex, $r_s = 0.06$), post mortem delay (frontal cortex, $r_s = 0.38$; temporal cortex, $r_s = 0.03$) and coma (frontal cortex, $r_s = 0.38$; temporal cortex, $r_s = 0.36$). Astrocyte numbers (Table 3.2) did not correlate with any measure of APP or its mRNA (see Table 3.3; Fig. 3.2 B, D and F). Measures of APP and APP mRNA showed correlations with neuronal markers; the interneurone index, i.e. concentration of GABA (see Table 3.2), was correlated with only APP$_{695}$ (Table 3.3; Fig. 3.2 C), and the
Table 3.1 APPLIR DETERMINED WITH ANTIBODY 22C11 IN NEOCORTICAL FRACTIONS PREPARED FROM TWO CATEGORIES OF PATIENTS WITH CLINICAL DEMENTIA.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Frontal cortex</th>
<th>Temporal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD</td>
<td>Others</td>
</tr>
<tr>
<td><strong>Soluble</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 2 (APP&lt;sub&gt;69&lt;/sub&gt;)</td>
<td>1262 ± 170</td>
<td>1284 ± 294</td>
</tr>
<tr>
<td>Band 3 (APP&lt;sub&gt;69&lt;/sub&gt;)</td>
<td>2282 ± 248</td>
<td>1889 ± 244</td>
</tr>
<tr>
<td>Band 4 (APP&lt;sub&gt;69&lt;/sub&gt;)</td>
<td>403 ± 88&lt;sup&gt;f&lt;/sup&gt;</td>
<td>127 ± 62</td>
</tr>
<tr>
<td>Total</td>
<td>3946 ± 453</td>
<td>3301 ± 502</td>
</tr>
<tr>
<td><strong>HD membranes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 1 (APP&lt;sub&gt;69&lt;/sub&gt;)</td>
<td>436 ± 45</td>
<td>465 ± 51</td>
</tr>
<tr>
<td>Band 2 (APlP2)</td>
<td>808 ± 102</td>
<td>862 ± 79</td>
</tr>
<tr>
<td>Band 3 (APP&lt;sub&gt;69&lt;/sub&gt;)</td>
<td>296 ± 65</td>
<td>349 ± 39</td>
</tr>
<tr>
<td>Total</td>
<td>1541 ± 177</td>
<td>1677 ± 106</td>
</tr>
<tr>
<td><strong>LD membranes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 1&lt;sup&gt;c&lt;/sup&gt; (?)</td>
<td>253 ± 38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>238 ± 40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Band 2&lt;sup&gt;c&lt;/sup&gt; (?)</td>
<td>561 ± 65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>514 ± 53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Band 3&lt;sup&gt;c&lt;/sup&gt; (?)</td>
<td>203 ± 30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>194 ± 35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>1016 ± 118</td>
<td>945 ± 100</td>
</tr>
</tbody>
</table>

Results, in arbitrary U/mg protein are expressed as mean ± SEM for AD (n=14) and other diagnoses (n=8), except where given (*9-10 subjects, *6 subjects). Superscript <sup>c</sup> identifies bands affected by epiphenomena (see 3.2.1). Superscripts <sup>as</sup> identify differences between AD and other diagnoses (*<sup>d</sup> P < 0.1, *<sup>e</sup> P < 0.05, *<sup>f</sup> P < 0.02, *<sup>g</sup> P < 0.01; Mann-Whitney U test).
Table 3.2 CORTICAL CELL MARKERS AND CHOLINERGIC ACTIVITY.

<table>
<thead>
<tr>
<th></th>
<th>Pyramidal neurone counts (numbers/mm²)</th>
<th>Aspartate uptake (fmol/mg protein/min)</th>
<th>GABA (nmoles/mg protein)</th>
<th>Astrocyte counts (numbers/mm²)</th>
<th>ChAT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frontal cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>195±41</td>
<td>8.4±3.7*</td>
<td>11.2±2.5</td>
<td>194±243</td>
<td>54±39**</td>
</tr>
<tr>
<td>Pick's disease</td>
<td>199</td>
<td>20.5</td>
<td>13.1</td>
<td>249</td>
<td>129</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>187</td>
<td>11.4</td>
<td>13.2</td>
<td>61</td>
<td>111</td>
</tr>
<tr>
<td>Depressive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pseudodementia</td>
<td>289</td>
<td>13.4</td>
<td>12.9</td>
<td>111</td>
<td>188</td>
</tr>
<tr>
<td>Multisystem degeneration</td>
<td>333</td>
<td>12.3</td>
<td>11.4</td>
<td>21</td>
<td>100, 111</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>165, 290</td>
<td>13.5</td>
<td>12.9</td>
<td>10, 121</td>
<td></td>
</tr>
<tr>
<td><strong>Temporal cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>n.d.</td>
<td>7.9±3.4***</td>
<td>12.4±5</td>
<td>227±136</td>
<td>37±30*</td>
</tr>
<tr>
<td>Pick's disease</td>
<td>n.d.</td>
<td>20.2</td>
<td>12.2</td>
<td>360</td>
<td>173</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>n.d.</td>
<td>16.2</td>
<td>21.3</td>
<td>61</td>
<td>135</td>
</tr>
<tr>
<td>Depressive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pseudodementia</td>
<td>n.d.</td>
<td>22</td>
<td>24.3</td>
<td>78</td>
<td>117</td>
</tr>
<tr>
<td>Multisystem degeneration</td>
<td>n.d.</td>
<td>40.6</td>
<td>22.0</td>
<td>158</td>
<td>181</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>n.d.</td>
<td>12.6, 27.5</td>
<td>9.5, 22.6</td>
<td>21, 63</td>
<td>39, 71</td>
</tr>
</tbody>
</table>

*Values for AD are mean ± SD of 9 cases. Where AD cases were significantly different from other dementias are indicated * P < 0.05, **P < 0.005, ***P < 0.001, Mann Whitney U test. Pyramidal neurone and astrocyte counts, aspartate uptake, concentration of GABA and ChAT activity are described in Procter et al. 1994.
Table 3.3 CORRELATIONS OF VARIOUS CORTICAL CELL MARKERS WITH APPLIR AND APP mRNA CONCENTRATIONS BY MULTIPLE REGRESSION ANALYSIS ALLOWING FOR CONFOUNDING FACTORS.

<table>
<thead>
<tr>
<th>Confounding factors</th>
<th>Pyramidal cell indices</th>
<th>GABA</th>
<th>Astrocytes</th>
<th>Age</th>
<th>Post mortem delay</th>
<th>Coma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frontal cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Soluble fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP(_{KPI})</td>
<td>-0.45</td>
<td>-0.33</td>
<td>-0.1</td>
<td>-0.69</td>
<td></td>
<td>n.e.</td>
</tr>
<tr>
<td></td>
<td>(P = 0.036)</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP(_{695}) (A)</td>
<td>-0.36</td>
<td>-0.4</td>
<td>0.08</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP(_{695}) (B)</td>
<td>-0.38</td>
<td>-0.58</td>
<td>-0.35</td>
<td>0.52</td>
<td>-0.69</td>
<td>n.e.</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>(P = 0.013)</td>
<td>n.s.</td>
<td>(P = 0.027)</td>
<td>(P = 0.006)</td>
<td></td>
</tr>
<tr>
<td><strong>Low density membranes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP(_{KPI})</td>
<td>0.49</td>
<td>0.1</td>
<td>0.01</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APLP2</td>
<td>0.65</td>
<td>-0.11</td>
<td>0.25</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(P = 0.040)</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP(_{695})</td>
<td>0.14</td>
<td>0.22</td>
<td>0.13</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.e.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>mRNA determinations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA APP(_{695})</td>
<td>0.62</td>
<td>-0.1</td>
<td>0.16</td>
<td>0.61</td>
<td>n.e.</td>
<td>-0.57</td>
</tr>
<tr>
<td></td>
<td>(P = 0.028)</td>
<td>n.s.</td>
<td>n.s.</td>
<td>(P = 0.033)</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 CORRELATIONS OF VARIOUS CORTICAL CELL MARKERS WITH APPLIR AND APP mRNA CONCENTRATIONS BY
MULTIPLE REGRESSION ANALYSIS ALLOWING FOR CONFOUNDING FACTORS.

<table>
<thead>
<tr>
<th></th>
<th>Pyramidal cell indices</th>
<th>GABA</th>
<th>Astrocytes</th>
<th>Confounding factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA APP&lt;sub&gt;KPI&lt;/sub&gt;</td>
<td>0.37</td>
<td>0.24</td>
<td>-0.05</td>
<td>Age 0.69 n.e.</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>Post mortem delay P = 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coma -0.73</td>
</tr>
</tbody>
</table>

Temporal cortex

**High density membranes**

<table>
<thead>
<tr>
<th></th>
<th>APP&lt;sub&gt;KPI&lt;/sub&gt;</th>
<th>APLP2</th>
<th>APP&lt;sub&gt;695&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.32</td>
<td>0.33</td>
<td>-0.04</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>0.53</td>
<td>0.38</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>0.23</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

**Low density membranes**

<table>
<thead>
<tr>
<th></th>
<th>APP&lt;sub&gt;KPI&lt;/sub&gt;</th>
<th>APLP2</th>
<th>APP&lt;sub&gt;695&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.53</td>
<td>-0.56</td>
<td>-0.15</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>0.44</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are BETA parameter of multivariate regression equation, n.s. indicates where \( P > 0.05 \); n.e. indicates where variable was not entered in the multiple regression equation. Pyramidal cell indices were cell counts in frontal cortex and D-aspartate uptake in temporal cortex. (A) and (B) identify bands with the immunological characteristics of APP<sub>695</sub> (see 3.2.1). There were no significant correlations between cortical cell markers and measures of APP in high density membranes of frontal cortex or soluble fraction of temporal cortex.
Fig. 3.2 CORRELATIONS OF VARIOUS CELLULAR PARAMETERS WITH APPLIR OR APP mRNA CONCENTRATION.

A and B are in soluble fraction of frontal cortex; C and D are in high density membranes of temporal cortex; and E and F are in frontal cortex. APP units are arbitrary units/mg protein. Filled and open squares are AD and non-AD dementias respectively, and linear regression lines are indicated where multiple regression indicates significant correlations (Table 3.3).
pyramidal cell indices showed correlations with measures of APP species and APP mRNA (Table 3.3; Fig. 3.2 A and E).

In the temporal cortex there was significant correlations between ChAT activity and APP$_{695}$ in the soluble fraction ($r_s = -0.57$, $P = 0.027$; Fig. 3.3 A) and in the low density membranes ($r_s = 0.68$, $P = 0.005$). There were also significant correlations between mRNA for APP$_{695}$ and APP$_{KPI}$ and ChAT activity ($r_s = 0.68$, $P = 0.023$; $r_s = 0.69$, $P = 0.006$; Fig. 3.3 B and C; all respectively).

### 3.3 DISCUSSION

This study has examined APPLIR in the cerebral cortex of a group of patients with dementia. Conclusions can be drawn, because potentially confounding factors such as duration of terminal coma, storage time, and post mortem delay have been controlled for, or investigated. Moreover, the brains used were specially collected using techniques designed to minimise artefacts introduced by post mortem degradation.

APPLIR could be resolved into four species. The molecular mass values for species of APP-like immunoreactivity with the three highest masses (140, 120, and 100 kDa) indicate that they may represent full-length APP holoproteins (Dyrks et al., 1988b; Selkoe et al., 1988; Weidemann et al., 1989; Buxbaum et al., 1990; Oltersdorf et al., 1990), although this would require confirmation with an antibody against the C-terminal region of APP. Based on their reactivity with antibodies 22C11, 10D5, anti-5 and 7H5, the following tentative identities have been assigned to these proteins: bands 1 and 2 correspond to APP$_{KPI}$, although the APP homologue APLP2 may be enriched in band 2 (HD membrane fraction); and band 3 corresponds to APP$_{695}$. Band 4 reacted strongly with 22C11 and anti-5 and may represent degradation of APP$_{695}$ or different states of post-translational modification of the protein. The latter interpretation is supported by information obtained from cells transfected with full-length APP isoforms (Weidemann et al., 1989; Oltersdorf et al., 1990).

In the frontal cortex, no significant changes in APP$_{KPI}$ or APP$_{695}$ have been identified when comparing membrane fractions prepared from AD and other (demented) subjects. However, in the temporal cortex, APP$_{695}$ was found to be significantly reduced in AD. The results also indicate that APP$_{KPI}$ may not be abundant in HD membranes (see Table 3.1) and that APLP2 may be enriched in this fraction.
Fig. 3.3 CORRELATION BETWEEN CHOLINE ACTYLTRANSFERASE ACTIVITY AND APPLIR OR APP mRNA CONCENTRATION.

A is temporal cortex, and B and C are frontal cortex. APP units are arbitrary units/mg protein. Filled and open squares are AD and non-AD dementias respectively, and linear regression lines indicate significant correlations (using Spearman's correlation coefficient, $r_s$).
This has now been confirmed (see 8.5) using 3B11 a specific monoclonal antibody to APLP2 (the production and characterisation of 3B11 have been described in Chapter 7).

The main finding however, is of more soluble APLIR in the brains of patients with AD when compared with those from patients with other dementias (see Table 3.1). The soluble fraction is considered to represent primarily the cell cytoplasm/intracellular membranes and therefore to contain full-length APP. Given that ChAT activity was lower in AD when comparing the same samples (Francis et al., 1993a; Procter et al., 1990), this result may reflect decreased efflux from the cerebral cortex, possibly related to cholinergic hypoactivity, present only in AD, leading to reduced α-secretory processing of APP. Confirmation of a higher concentration of full-length APP<sub>695</sub> in the soluble fraction, using an antibody to the C-terminus of the molecule would support this interpretation and clarify further the exact APP species present in the soluble fraction.

Results shown in Table 3.3 (see also Fig. 3.2 E and F) suggest that pyramidal neurones rather than interneurones or astrocytes appear to be the major source of APP in brain, as the amount of mRNA APP<sub>695</sub> detected was correlated with the number of pyramidal neurones rather than these other cells. As discussed above, there appears to be more soluble APLIR in the brains of patients with AD when compared with those from patients with other dementias (see Table 3.1) and multiple regression analysis has identified a relationship between the concentration of soluble APLIR and the number of pyramidal neurones determined for the same samples, whereby the concentration of APP<sub>kpl</sub> was high in the soluble fraction when the number of pyramidal neurones was low (Fig. 3.2 A). Thus a straightforward interpretation of these findings is that when cortical degeneration is high (indexed by loss of pyramidal neurones) excitatory input into the remaining neurones would decrease and almost certainly reduce secretion of APP<sub>s</sub> resulting in the accumulation of full-length APP species inside remaining neurones. Under normal circumstances this full-length APP would undergo constitutive processing through the α-secretase pathway, known to be positively modulated by neuronal depolarisation (Nitsch et al., 1992). It is expected that these events would be exacerbated by reduced receptor-mediated APP<sub>s</sub> secretion linked to cholinergic hypoactivity (Bowen et al., 1992; Buxbaum et al., 1992; Nitsch et al., 1992), such as occurs in AD compared with other causes of dementia, and has been reported for the patients examined in the present study (see Table 3.2). The present finding of high concentrations of APP<sub>695</sub> in the soluble fraction when ChAT activity is low (Fig. 3.3 A)
is compatible with this proposal. Furthermore, reduced non-amyloidogenic processing of APP under these conditions, may favour increased amyloidogenic processing of APP by alternative pathways, since APP processing pathways are thought to compete for the same APP substrate (see 1.3.3.5). This interpretation is supported by the observation that lumbar CSF of patients with evidence of pyramidal cell loss had lower concentrations of soluble APP\textsubscript{kpl} compared to controls (Francis et al., 1993c). However, AD is a progressive condition and post mortem brains with more severe degeneration represent end stages (Lowe et al., 1988); therefore, one other interpretation is that a subpopulation of pyramidal neurones with low amounts of soluble APP species undergo selective degeneration.

In subjects with severe pathology, evidenced by fewer pyramidal neurones or a low index of interneurones, there are also lower concentrations of APP\textsubscript{695} in membrane fractions (see Table 3.1; see also Fig. 3.2 C) and of APP\textsubscript{695} mRNA (see Fig. 3.2 E). This is compatible with the hypothesis that the increase of APP\textsubscript{kpl} in the soluble fraction in subjects with severe pathology (Fig. 3.2 A) may be due to functional changes in APP secretion, although the apparent fall in APP\textsubscript{695} mRNA is itself indicative of impaired transcription and/or synthesis.

This study did not include undemented control subjects, which prevents complete validation of the conclusions drawn and necessitates cautious interpretation of the data. Nonetheless, these studies have identified soluble forms of APP which probably accumulate inside pyramidal neurones, and cell membrane associated forms which appear to decrease with degeneration of these neurones. While the precise nature of the neurotoxic process in AD remains to be elucidated, further work is required to establish whether the intracellular accumulation of soluble APP is toxic or whether the lack of one or both of the secreted forms of APP\textsubscript{s} (Seubert et al., 1993) is detrimental to normal neuronal integrity. Although it is not yet clear which of the APP functions suggested by in vitro experiments (see 1.3.4) represent physiologically important activities in vivo, these proteins may have an array of important biological functions, the loss of which may be involved in the pathogenesis of AD.
CHAPTER 4

4.0 COMPARISON OF A MEMBRANE SERINE PROTEASE ACTIVITY PREPARED FROM HUMAN AND RAT BRAIN WITH RESPECT TO DEGRADATION OF APPLIR EXTRACTED FROM RAT CORTICAL MEMBRANES

4.1 INTRODUCTION

If as suggested in Chapter 3, constitutive processing of APP to yield APPs is reduced in AD, more APP substrate may become available for processing through alternative potentially amyloidogenic pathways. Mechanisms leading to mismetabolism of APP are unknown. However, alterations in protease systems have been proposed to underlie the pathology of AD (Carrell, 1988) in the more common non-familial sporadic cases where mutations in the APP gene have not be identified.

Several studies suggest an increase in the expression of APP isoforms showing protease inhibitory activity (Neve et al., 1988; Tanzi et al., 1988; Johnson et al., 1990), might be related to the formation of β/A4, by altering the activity of proteases normally involved in the metabolism of APP. This is further supported by the finding that deposits of β/A4 have been reported to occur in transgenic mice with altered APP751 expression (Quon et al., 1991). However, the lack of availability of widely accepted, well characterised animal models for the neurodegeneration of AD precludes a critical evaluation of this issue.

Further evidence implicating altered proteolysis of APP in AD included the identification of the serine protease inhibitor α1 - antichymotrypsin, as an integral component of the senile plaque (Abraham et al., 1988). Significantly, the N-terminus of β/A4 is formed through hydrolysis of APP on the carboxyl side of Met96 of APP695, (Kang et al., 1987), a target of chymotrypsin-like proteases (Lorand, 1981), therefore the presence of ACT in senile plaques suggested the involvement of such a protease in the pathology of AD. Indeed, the mRNA encoding this protein is considered to be overexpressed in areas of pathology in AD brain (Abraham et al., 1988), therefore it seems reasonable that ACT might be accumulating in response to the local release of a
chymotrypsin-like protease, in much the same way that ACT expression is induced in the liver by secretion of proteases during inflammation (Katsunuma et al., 1980). Furthermore, if proteases exist whose normal function is to clear soluble β/APP protein before it can be deposited, these enzymes may be inhibited by the presence of ACT in the same vicinity.

As a consequence, much research has been devoted to identifying proteases that might be involved in the formation and deposition of β/APP (see 1.3.3.4). Consequently, a number of studies (Abraham et al., 1991; Ishiura, 1991; McDermott and Gibson, 1991; Small et al., 1991; McDermott et al., 1992; Razzaboni et al., 1992) have identified enzyme activities capable of cleaving appropriate short synthetic peptide substrates at the correct positions. However, demonstrating physiologically relevant cleavage of intact APP at the same peptide bond has proved a more difficult proposition.

Given the importance of demonstrating more physiologically relevant cleavage of intact APP, the present study describes the investigation of clpsin activity (see 1.3.3.4) with respect to the degradation of APP-like immunoreactive substrate extracted from rat cerebral cortical membranes (see 2.2.6.2). Using enzyme preparations from human brain (see 2.2.6.1.2), rat pup and adult rat brain (see 2.2.6.1.1), the hydrolysis of this substrate has been studied using methods described in 2.2.6.3. The results for human tissue have been evaluated in terms of other features of the tissue (APPLIR, as well as numbers of pyramidal neurones and astrocytes, see Chapter 3, Tables 3.1 and 3.2) and demographic characteristics of the subjects such as post mortem delay, duration of terminal coma, and presence/absence of AD (see Tables 2.6 and 2.7).

4.2 RESULTS

Antibody 22C11 identified one major protein band in the substrate fraction prepared from rat cortical membranes. This band had the approximate apparent molecular mass of 116 kDa and was essentially stable with incubation (Fig. 4.1). No APPLIR was observed in any enzyme preparations under the assay conditions investigated here.

Addition of enzyme preparations from rat pup brain caused obvious loss (74%) of substrate immunoreactivity after incubation (see Table 4.1). In contrast, no loss of immunoreactivity was observed in incubations with preparations from adult rat cortex.
Fig. 4.1 REPRESENTATIVE WESTERN BLOTS WITH ANTIBODY 22C11 OF ENZYME INCUBATION MIXTURES AND FRACTIONS OF HUMAN BRAIN.

\[ \begin{align*}
A &= \text{substrate: no incubation; } \\
B &= \text{substrate: incubation (2 h) without enzyme preparation; } \\
C &= \text{substrate: incubation (2 h) with a human enzyme preparation. Note presence of 100 kDa product; } \\
D &= \text{soluble fraction (band of lowest molecular weight appears as a doublet); } \\
E &= \text{high density membranes; } \\
F &= \text{low density membranes. Numbers identify molecular weight markers.}
\end{align*} \]
Table 4.1 COMPARISON OF ACTIVITY OF SERINE PROTEASE PREPARATIONS.

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Immunoreactivity after 2 h incubation</th>
<th>116 kDa substrate (arbitrary units)</th>
<th>100 kDa product (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added (n = 5)</td>
<td></td>
<td>1.38 ± 0.23</td>
<td>0.13 ± 0.09</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pup, no post mortem delay (n = 2)</td>
<td></td>
<td>0.35, 0.38</td>
<td>0.14, 0.10</td>
</tr>
<tr>
<td>Adult, no post mortem delay (n = 5)</td>
<td></td>
<td>1.40 ± 0.41</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>Adult, post mortem delay (2 h; n = 4)</td>
<td></td>
<td>1.24 ± 0.09</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td><strong>AD frontal cortex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short post mortem delay (n = 8)</td>
<td></td>
<td>0.61 ± 0.35</td>
<td>0.61 ± 0.28</td>
</tr>
<tr>
<td>Short post mortem delay + Ca(^2+) (n = 9)</td>
<td></td>
<td>0.76 ± 0.45</td>
<td>0.73 ± 0.35</td>
</tr>
<tr>
<td>Short post mortem delay + PMSF (n = 6)</td>
<td></td>
<td>1.55 ± 0.37</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>Routine post mortem delay (n = 8)</td>
<td></td>
<td>0.16 ± 0.15</td>
<td>0.51 ± 0.18</td>
</tr>
<tr>
<td><strong>AD temporal cortex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short post mortem delay (n = 9)</td>
<td></td>
<td>0.57 ± 0.46</td>
<td>1.21 ± 0.44</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± SD. n = the number of brains except for pup tissue, which were pools (each of 4 brains). Human control values and statistical analysis are given in the text.
(see Table 4.1). Incubation with enzyme preparations from both the frontal and temporal cortex of short post mortem delay (approximately 2 h) AD brains resulted in loss of substrate immunoreactivity. This effect was less marked than with pup enzyme (compare 0.35, 0.38 units for pup brain, with 0.61 ± 0.35 and 0.57 ± 0.46 units for AD brain, frontal and temporal cortex respectively; see also Table 4.1). Only in incubations with human enzyme preparations, did a 100 kDa product accumulate (see Table 4.1). Loss of substrate and accumulation of product was reduced by PMSF and was insensitive to Ca\(^{2+}\) (see Table 4.1).

4.2.1 Influence of post mortem delay and coma duration

Small amounts of substrate immunoreactivity remained after incubation with enzyme from conventional post mortem delay (approximately 38 h) brains. In contrast, enzyme preparations from short post mortem delay samples were unable to degrade substrate so efficiently (compare 0.16 ± 0.15 units conventional post mortem delay samples, with 0.61 ± 0.35 and 0.57 ± 0.46 units short post mortem delay samples, frontal and temporal cortex respectively; see also Table 4.1). These measures of immunoreactivity were not influenced by duration of terminal coma (\(r_s; P > 0.05\)).

4.2.2 Frontal cortex: Influence of AD

Incubations of enzyme preparations from conventional post mortem delay control brains (n = 8) gave mean values for substrate and product of 0.29 ± 0.22 and 0.63 ± 0.41 arbitrary units respectively. These were higher than, but not significantly different from values for the equivalent AD group (0.16 ± 0.15 and 0.51 ± 0.18 units, substrate and product respectively; see also Table 4.1). Alzheimer samples of short post mortem delay gave values for substrate (0.61 ± 0.35 units, see Table 4.1) and product (0.61 ± 0.28 units, see Table 4.1) that were not significantly different from short post mortem delay controls (n = 6), the non-Alzheimer dementia group (mean values were 0.65 ± 0.37 and 1.03 ± 0.64 units for substrate and product respectively). Thus in the frontal cortex there was no evidence in AD of significant changes in protease activity compared to the appropriate controls.
4.2.3 Temporal cortex: Influence of AD

For brains of short post mortem delay, the mean value for substrate following incubation ($0.57 \pm 0.46$ units, Table 4.1) was not significantly different from that for the controls ($0.49 \pm 0.32$ units). However, significantly more product accumulated in the AD group ($1.21 \pm 0.44$ units, Table 4.1) compared with the control group ($0.72 \pm 0.22$ units, $n = 6; P < 0.05$, Student's t test). Another index of enzyme activity was calculated (based on total immunoreactivity of both substrate and product after incubation), which was significantly lower in AD than controls (Fig. 4.2; compare bars on the right).

4.2.4 Relationship to total tissue APPLIR

When comparing total tissue APPLIR for both frontal and temporal cortex (short post mortem delay samples; see Chapter 3 for APPLIR determination and also Table 3.1 for total tissue APPLIR), the APP-like immunoreactive content of both areas was higher, but only reached statistical significance in the temporal cortex (Fig. 4.2; see also Table 3.1). The APPLIR content of the soluble fraction and low density membranes of the AD group did not correlate with any indices of enzyme activity (product formation, loss of substrate or total immunoreactivity of product and substrate). In contrast, the APPLIR of high density membranes from the temporal cortex was correlated with loss of substrate (see Fig. 4.2; inset); this was not a feature of frontal cortex ($r = 0.04, n = 8$).

Enzyme activity of AD samples correlated positively with the number of layer III pyramidal neurones but was not correlated with the number of astrocytes (Fig. 4.3) or pyramidal neurones in layer V ($r = 0.32, n = 8$; see also Table 3.2 for cell counts).

4.3 DISCUSSION

The main finding of this study is that duration of post mortem delay apparently determines the amount of protease extracted from human cerebral membranes. If this study had not examined tissue specially collected with minimum post mortem delay (see 2.2.3.1.2), it is doubtful whether any meaningful results would have been obtained. Consequently, conclusions can be drawn with confidence since the risk from post
Fig. 4.2 SERINE PROTEASE ACTIVITY AND APPLIR IN TEMPORAL CORTEX FROM TWO CATEGORIES OF PATIENTS WITH CLINICAL DEMENTIA.

Mean (± SEM) enzyme activity in arbitrary units (reciprocal of total immunoreactivity of substrate and 100 KDa product after incubation for 2 h) of AD (crosshatched bar; n = 9) and non-AD (dotted bar; n = 6) groups was significantly different (P = 0.023, Student’s t test). Mean (± SEM) total APPLIR in arbitrary units/mg total protein of soluble (SOL) fraction, high density (HD) and low density (LD) membranes of AD (solid bar; n = 9) and non-AD (open bar; n = 6) groups. Soluble APPLIR was significantly different between groups (P = 0.015). Inset: the relationship for the temporal cortex of AD cases between APPLIR and the reciprocal of the amount of substrate after 2 h incubation (enzyme activity in arbitrary units; r = -0.82, P = 0.007, line of best fit for illustration).
Fig. 4.3 CORRELATION BETWEEN SERINE PROTEASE ACTIVITY AND CELL NUMBER IN FRONTAL CORTEX FROM AD SUBJECTS.

Units of enzyme activity are arbitrary (reciprocal of total immunoreactivity of substrate and 100 kDa product after incubation for 2 h) in relation to layer III pyramidal neurone numbers (A; $r_s = 0.79$, $P = 0.019$; line of best fit for illustration) and astrocyte numbers (B; $r_s = 0.21$, not significant; corresponding value for temporal cortex, $r_s = 0.32$, not significant.)
mortem artefact has been reduced. Confounding factors such as post mortem delay and coma duration have also been controlled for, or investigated.

In view of the short post mortem delays, it should be pointed out that it was virtually impossible to obtain brains for normal subjects matched to the AD cases for this factor. Therefore, the group of non-Alzheimer dementias was considered to represent a more appropriate control (Procter et al., 1990).

The enzyme activity studied here prepared from human brain, had the features of the serine protease clipsin, previously described in the brain of rat pups (Nelson and Siman, 1990). It was extracted with a detergent-containing high-ionic buffer, was insensitive to Ca\(^{2+}\), inhibited by PMSF and degraded immunoreactivity with the characteristics of APP. Furthermore, enzyme preparations from human and adult rat brain had distinctive features. A 100 kDa product was found to accumulate when substrate and human enzyme preparations were incubated. This was independent of post mortem delay, brain region and diagnosis. In addition, the formation of this product was specific only to human preparations. In contrast to rat pup preparations, adult rat enzyme showed no evidence of enzyme activity. This may have been due to the presence of an endogenous inhibitor. However, Nelson & Siman (1990) suggest that this enzyme activity may have a circumscribed role, largely constrained to a brief developmental period, since they describe a narrow window of elevated activity surrounding postnatal day 5, which was 12-14 fold higher than levels in day 1 or adult rat brain. These authors speculate that inappropriate expression of this activity outside this developmental period might lead to inappropriate processing of specific substrates such as APP.

This does not appear to be the case in AD brain, since in the frontal cortex there was no evidence in AD of significant changes in protease activity when compared to controls, and in the temporal cortex, the apparent activity was lower in AD when compared with controls. This difference might represent either the presence of more (or increased access to an) inhibitor or a decrease in enzyme expression. Regardless, the lower activity may explain the higher total APPLIR in AD samples compared with controls. Such a conclusion is supported by the inverse relationship between enzyme activity and APPLIR (see Fig. 4.2).

Based on a previous study (Nelson et al., 1993), the enzyme preparations studied here are thought to contain β-secretase, a putative activity that creates the
N-terminus of β/A4 (see 1.6.3.3). If as proposed in Chapter 3 pyramidal neurones represent the major source of APP in brain, β-secretase and much of the full-length APP may be co-localised in pyramidal cell membranes so it is possible that accumulation of β/A4 in AD may the result of increased accessibility of enzyme to substrate, secondary to membrane dysfunction for example (see Bowen et al., 1973; Nixon et al., 1992; Skinner et al., 1993 and also Roth et al., 1995, for review). However, this would need to be studied by other methods.

The most straightforward conclusion to be drawn from the present finding of an association between protease activity and neurone number (see Fig. 4.3), and the comparisons of enzyme activities in AD and control brains, is that increased deposition of β/A4 is not due to increased membrane serine protease (e.g. β-secretase) activity. Previously (Chapter 3) it has been predicted that α-secretory processing of APP may be reduced in AD, thus processing of APP by lysosomes (as reviewed in Estus et al., 1992b and Nixon et al., 1992) may, represent the main source of β/A4 found in AD brain.
CHAPTER 5

5.0 DOES PERTURBED ENERGY METABOLISM ALTER THE BALANCE OF NON-AMYLOIDOGENIC AND AMYLOIDOGENIC PROCESSING OF APP IN PC12 CELLS?

5.1 INTRODUCTION

Perturbed energy metabolism has been proposed to have a role in the pathogenesis of AD, and as reviewed in section 1.3.5.2 there is a consistent, although inconclusive, body of evidence suggesting that impaired energy metabolism may occur in AD.

It was hypothesised at the start of the present experimental work, that perturbed energy metabolism may reduce the release of secreted APP derivatives through the α-secretory processing pathway and favour increased liberation of β/A4. As a consequence, increased concentrations of β/A4 may result in accumulation and aggregation of the peptide, in which case neurones exposed to β/A4 could become more vulnerable to insult (see 1.3.5.1), and reduced concentrations of soluble APP derivatives may place neurones at risk by depriving them of a neuroprotective substance (see 1.3.4).

The present experiments sought to investigate the status of secreted derivatives under a condition of perturbed energy metabolism. To study the effects of perturbed energy metabolism on the secretion of APP, the rat pheochromocytoma cell line designated PC12 was used. Whilst there is some evidence that non-neuronal brain derived cells may synthesise APP in vitro (de Sauvage and Octave, 1989; Quon et al., 1990), the majority of APP in the brain appears to be of neuronal origin (Bahmanyar et al., 1987; Shivers et al., 1988). Therefore PC12 cells were considered to be an appropriate cell line for this study, since they acquire, over a period of days, a sympathetic neurone-like phenotype when treated with NGF (Greene and Tischler, 1976). They also have increased levels of APP expression, thus providing an interesting model neuronal system in which to study the processes controlling APP metabolism. Indeed, there are several reports of altered APP production by PC12 cells in response to various factors (Refolo et al., 1989; Schubert et al., 1989a).
During the course of the present work, it was reported that direct or indirect activation of PKC can increase the \( \alpha \)-secretory processing of APP with sparing of amyloidogenic APP processing (see 1.3.3.5). If perturbed energy metabolism contributes to the pathogenesis of AD by reducing production of APP\(_{\text{S}}\), this potentially damaging phenomena may be overcome by a process that increases protein phosphorylation. One such mechanism is the activation of PKC which may divert APP from amyloidogenic processing to the \( \alpha \)-secretory pathway, thus preventing \( \beta \)/A4 accumulation and possibly maintaining an important physiological function of APP\(_{\text{S}}\).

To study this, cells were energy perturbed and treated with bradykinin and phorbol 12,13-dibutyrate (phorbol ester; see 2.2.7.2), compounds which can increase PKC activity. Another agent, chloroquine, was also used as this has been reported to influence processing of APP by the endosomal/lysosomal system (see 1.3.3.2).

### 5.2 RESULTS

In both cell lysate and conditioned medium, antibody 22C11 recognised two major protein bands with apparent molecular masses of 125 kDa and 116 kDa, originally considered to represent the major APP isoforms APP\(_{\text{KPI}}\) and APP\(_{695}\) respectively (Weidemann et al., 1989). For the purpose of this study, these bands will be referred to as bands 1 and 2, with band 1 representing APP\(_{\text{KPI}}\) and band 2 representing APP\(_{695}\).

Intracellular 22C11 immunoreactivity was significantly increased following treatment of cells for 3 h with chloroquine compared to control (band 1, 145 %; band 2 135 %; \( n = 4 \), both \( P < 0.05 \); Fig. 5.1 A). Immunoblots with antibody DA1 confirm that the increase observed in band 1 was due in part, to APP (band 1, 132%, \( n = 4 \), \( P < 0.05 \); Fig. 5.2). In contrast, treatment for 1 h with bradykinin reduced intracellular 22C11 immunoreactivity (band 1, 90 %; band 2, 92 %; \( n = 15 \), both \( P < 0.05 \); Fig. 5.1 A ). A similar but larger effect was observed following treatment with phorbol ester (band 1, 68 %; band 2, 74 %; \( n = 7 \), both \( P < 0.05 \); Fig. 5.1 A).

Incubation with oligomycin and 2-deoxyglucose for 3 h significantly reduced cellular ATP concentrations to 28 % of control (1.6 ± 0.1 compared to 5.7 ± 0.7 nmoles/mg protein ATP for control cells, \( n = 15 \), \( P < 0.001 \), students t-test), and ADP concentrations to 52 % of control (3.4 ± 0.5 compared to 6.6 ± 0.8 nmoles/mg
Fig. 5.1 REGULATION OF APP PROCESSING IN PC12 CELLS: EFFECT OF CHLOROQUINE, BRADYKININ AND PHORBOL ESTER AS DETERMINED WITH ANTIBODY 22C11.

(A) Cell Lysate, (B) Conditioned Medium. PC12 cells were incubated in the absence or presence of 50 mM chloroquine (3 h), 500 nM bradykinin (1 h) and 100 nM phorbol ester (1 h). Intracellular and secreted APPLIR were then analysed as described in sections 2.2.7.2, 2.2.7.2.1 & 2.2.7.2.2. Within each experiment, incubations were carried out in triplicate, and data were normalised to the average amount of APPLIR recovered under control conditions. The results shown represent means ± SEM of 5, 7 and 15 experiments, *, $P < 0.05$, t-statistic. Open bars indicate band 1 representing APP$_{KPI}$ and crosshatched bars depict band 2 representing APP$_{695}$. 

(A) Cellular Lysate: Chloroquine, Bradykinin, Phorbol Ester. (B) Conditioned Medium: Chloroquine, Bradykinin, Phorbol Ester.
Fig. 5.2 REGULATION OF APP PROCESSING: EFFECT OF CHLOROQUINE IN ENERGY PERTURBED PC12 CELLS AS DETERMINED WITH ANTIBODY DA1.

Energy perturbed PC12 cells were incubated for 3 h in the absence or presence of 50 mM chloroquine. Intracellular APPLIR was then analysed with antibody DA1 as described in sections 2.2.7.2 & 2.2.7.1. Within each experiment, incubations were carried out in triplicate, and data were normalised to the average amount of APP recovered under control conditions. The results shown represent the means ± SEM of 4 or 5 experiments * , P < 0.05, t-statistic. Open bars indicate band 1 representing APP<sub>KPI</sub> and crosshatched bars depict band 2 representing APP<sub>695</sub>. 
protein ADP for control cells, n = 15, P < 0.01). Cellular AMP concentrations were also reduced to 71 % of control (1.5 ± 0.2 compared to 2.1 ± 0.3 nmoles/mg protein AMP for control cells, n = 15, not significantly different P > 0.05), however, the adenylate energy charge was unchanged (0.6 ± 0.04 compared to 0.6 ± 0.03 nmoles/mg protein for control cells (n = 15, not significantly different, P > 0.05). Cell viability as determined by trypan blue exclusion, was not significantly affected by perturbed energy metabolism (69 ± 2.9 % viable cells) compared to control (74 ± 2.4 %, n = 39, P > 0.05). By contrast, intracellular 22C11 immunoreactivity was significantly reduced when compared to controls (band 1, 67 %, n = 13, P < 0.05; band 2, 83 %, n = 13, P < 0.05; Fig 5.3 A). This reduction was restored to the control range by incubation in the presence of chloroquine (band 1, 81 %; band 2, 111 %, n = 6, not significantly different, P > 0.05; Fig 5.3 A) and bradykinin (band 1, 56 %; band 2, 87 %, n = 3, not significantly different, P > 0.05; Fig. 5.3 A). However, treatment with phorbol ester only restored band 2 APPLIR to the control range (compare band 1, 59 %, P < 0.05 and band 2, 84 % not significantly different, P > 0.05, both n = 3; Fig. 5.3 A). In contrast to 22C11 immunoreactivity, DA1 immunoreactivity in cell lysate was unaltered by perturbed metabolism (band 1, 94%; band 2, 85%, n = 5; Fig 5.2) indicating that under these conditions, APP was unaffected.

Incubation of PC12 cells in the presence of chloroquine for 3 h had no apparent effect on the secretion of band 1 (105 %; n = 3), but secretion of band 2 was significantly reduced (71 %; n = 3) compared to control (Fig. 5.1 B, and Fig. 5.4 A, compare lanes 9 and 10). Treatment for 1 h with bradykinin significantly increased secretion of 22C11 immunoreactivity (band 1, 169 %; band 2, 209 %; n = 15; P < 0.05; Fig. 5.1 B, and Fig. 5.4 A, compare lanes 1 and 2). A similar increase was seen following incubation for 1 h with phorbol ester (band 1, 149 %; band 2, 190 %; n = 7 P < 0.05; Fig. 5.1 B and Fig. 5.4 A, compare lanes 1 and 3). Both basal and phorbol ester stimulated APPLIR secretion were attenuated by the protein kinase C inhibitor staurosporine (Fig. 5.5). Antibody DA1 confirmed that in part, the effect of both bradykinin and phorbol ester was due to increased secretion of APP (Fig. 5.4 B, lanes 2 and 3). The presence of KPI containing APP-like species in band 1 after both treatments was confirmed with antibody 7H5 (Fig. 5.4 C, lanes 2 and 3).
Fig. 5.3 REGULATION OF APP PROCESSING IN ENERGY PERTURBED PC12 CELLS: EFFECT OF CHLOROQUINE, BRADYKININ AND PHORBOL ESTER AS DETERMINED WITH ANTIBODY 22C11.

(A) Cell lysate, (B) Conditioned medium. PC12 cells were energy perturbed as described in section 2.2.7.2 and incubated in the absence or presence of 50 mM chloroquine (3 h), 500 nM bradykinin (1 h), 100 nM phorbol ester (1 h). Intracellular and secreted APPLIR were then analysed as described in sections 2.2.7.2, 2.2.7.2.1 & 2.2.7.2.2. Within each experiment, incubations were carried out in triplicate, and data were normalised to the average amount of APPLIR recovered under control conditions. The results shown represent means ± SEM of 13, 6 and 3 experiments, *, P < 0.05, t-statistic. Open bars indicate band 1 representing APP_{KPI}, and crosshatched bars depict band 2 representing APP_{695}. 
Fig. 5.4 REPRESENTATIVE WESTERN BLOTS OF CONDITIONED MEDIUM FROM PC12 CELLS WITH VARIOUS ANTIBODIES.

(A) Antibody 22C11 identified two major bands in conditioned medium from control cells (lanes 1, 7 and 9). Open triangle indicates band 1 representing APP<sub>kpl</sub>, and filled triangle depicts band 2, representing APP<sub>42</sub>. Secretion of 22C11 immunoreactivity was increased following treatment of cells with bradykinin (500 nM, 1 h; compare lanes 1 and 2) or phorbol ester (100 nM, 1 h; compare lanes 1 and 3). Energy perturbation, by treatment of cells with deoxyglucose (5 mM) and oligomycin (0.2 μg/ml) for 3 h, reduced secretion of APP<sub>42</sub> (compare lanes 1 and 4), however this was restored following treatment with bradykinin (500 nM, 1 h; lane 5) or phorbol ester (100 nM, 1 h; lane 6). Treatment of cells with staurosporine (1 mM for 1 h) reduced basal APP<sub>42</sub> secretion (compare lanes 7 and 8), in contrast, treatment with chloroquine (50 mM, 3 h) had no apparent effect on secretion of band 1 APP<sub>42</sub>, although secretion of band 2 APP<sub>42</sub> was reduced (compare lanes 9 and 10). (B) Antibody DA1 recognised the same two bands in conditioned medium from control cells (lane 1). DA1 immunoreactivity was increased following treatment with bradykinin (500 nM, 1 h; lane 2), or phorbol ester (100 nM, 1 h; lane 3). Energy perturbation reduced DA1 immunoreactivity (lane 4). (C) Antibody 7H5 identified 1 major band only in conditioned medium from control cells (lane 1). 7H5 immunoreactivity was increased following treatment with bradykinin (500 nM, 1 h; lane 2) or phorbol ester (100 nm, 1 h; lane 3).
PC12 cells were incubated in the absence or presence of phorbol ester (100 nm), staurosporine (1 mM) or a combination of both for 1 h (see 2.2.7.2), and secreted APPLIR, as determined with antibody 22C11, analysed as described in section 2.2.7.2.2. Within each experiment, incubations were carried out in triplicate, and the data were normalised to the average amount of APPLIR recovered under control conditions. The results shown represent means ± SEM of 9 experiments, *, $P < 0.05$, t-statistic. Open bars indicate band 1 representing APP$_{409}$ and crosshatched bars depict band 2 representing APP$_{695}$. 
In contrast, perturbed energy metabolism significantly reduced secretion of 22C11 immunoreactivity (band 1, 73%; band 2, 69%; n = 9; P < 0.05 compared to control; Fig. 5.3 B; see also Fig. 5.4 A lanes 1 and 4). Immunoblots with antibody DA1 confirmed that this effect was partially due to decreased secretion of APP (Fig. 5.4 B, compare lanes 1 and 4). The effect observed on 22C11 immunoreactivity under conditions of perturbed metabolism, was partially reversed by incubation with chloroquine for 3 h (band 1, 86%; band 2, 91%; n = 5; compared to control; NS, Fig. 5.3 B, and Fig. 5.4 A, lanes 7 and 9) and completely restored to control values by incubation for 1 h with bradykinin (band 1, 94%; band 2, 89%; n = 3; Fig. 5.3 B, and Fig. 5.4 A, compare lanes 4 and 5) or phorbol ester (band 1, 101%; band 2, 91%; n = 3; Fig. 5.3 B and Fig. 5.4 A, compare lanes 4 and 6). Antibody DA1 confirmed an increase in APP secretion under these conditions (Fig. 5.4 B, lanes 2 and 3).

5.3 DISCUSSION

Bradykinin and phorbol ester, known to activate PKC, have been examined for ability to affect APP metabolism. Both were found to significantly increase the secretion of APPLIR, supporting previous studies indicating that mature APP can undergo PKC regulated α-secretory cleavage in PC12 cells e.g. (Buxbaum et al., 1990; Caporaso et al., 1992b; see also 1.3.3 5). In these cells, bradykinin binds with high affinity to the B1-type bradykinin receptor (Pozzan et al., 1986), and one of the earliest events detected during interaction of bradykinin with this receptor, is the hydrolysis of phosphatidylinositol bisphosphate (PIP₂) to DAG and inositol 1,4,5-triphosphate (1,4,5-IP₃) by phospholipase C activation (Yano et al., 1984; Lambert et al., 1986). DAG then activates PKC by translocating cytosolic forms to the plasma membrane. Bradykinin also activates phospholipase A₂ to yield arachidonic acid, which like phorbol ester can activate PKC directly, thus mimicking the effect of receptor stimulation (Burch and Axelrod, 1987). Fig. 5.5 shows that both basal and phorbol ester enhanced APPLIR secretion could be attenuated by the PKC inhibitor staurosporine (Tamaoki et al., 1986; Gescher, 1992), indicating that a protein kinase is involved in the signal transduction pathway leading to secretion of APPLIR. In the absence of an appropriate assay, further studies are clearly needed to investigate the
effect of direct or indirect stimulation of PKC on the production of β/A4. Recently, it has been reported that activation of PKC increases the release of APP, while simultaneously decreasing the release of β/A4 (Buxbaum et al., 1993; Gabuzda et al., 1993; Jacobsen et al., 1994). This indicates that β/A4 production can also be regulated by the phospholipase C-DAG signal transduction pathway. The ability of PKC activation to reduce the formation of β/A4 is probably due to a phosphorylation event(s) diverting mature APP from an alternative amyloidogenic pathway to the α-secretory pathway.

As described in section 1.3.3.2, the majority of APP molecules are thought to be degraded by a chloroquine-sensitive endosomal/lysosomal proteolytic pathway (Caporaso et al., 1992a). Full-length APP has been detected in endosomal/lysosomal preparations and shown to be degraded within this compartment to multiple C-terminal APP fragments. Some of these fragments appear to contain intact β/A4 sequences and thus are potentially amyloidogenic (Estus et al., 1992a; Golde et al., 1992). Indeed, lysosomotropic agents such as chloroquine and ammonium chloride have been shown to inhibit β/A4 production, raising the possibility that an alternative pathway leading to the formation of β/A4 peptide involves acidic intracellular compartments (Estus et al., 1992a; Golde et al., 1992; Haass et al., 1992a).

In this study, treatment of PC 12 cells with the weak base chloroquine, thought to neutralise acidic organelles, resulted in significantly higher amounts of APPRIR recovered in the cell lysate, compared with control cells (Fig. 5.1 A). This confirmed the existence of a chloroquine-sensitive intracellular pathway for APP metabolism (Caporaso et al., 1992a) in this cell line. In view of the inverse relationship between the release of APP and β/A4 formation (see 1.3.3.5), the observations that APP can either undergo PKC-regulated secretion or be degraded in a potentially amyloidogenic, chloroquine-sensitive intracellular compartment, suggest a scheme for the cellular trafficking and proteolytic processing of APP in PC 12 cells. According to this scheme, increased protein phosphorylation (following PKC activation), by accelerating the transit of APP through a secretory pathway, may diminish the amount of substrate available for lysosomal processing and thus generation of β/A4. Such a scheme is consistent with a model in which APP and β/A4 peptide are derived from competing pathways of APP metabolism, and thus the amount of mature APP available for processing may represent the limiting step in the formation of β/A4. The observation
that intracellular APPLIR was significantly reduced following treatment with either bradykinin or phorbol ester (agents which activate PKC; Fig. 5.1 A) supports this proposal. Indeed, if the majority of intracellular APPLIR as determined with the N-terminal APP antibody 22C11 is considered to represent full-length mature APP (although this requires confirmation with an appropriate C-terminal APP antibody), then PKC activation would seem to increase the rate at which this is processed at the same time reducing the intracellular pool of APPLIR available for potentially amyloidogenic processing by an alternative pathway e.g. the endosomes/lysosomes.

In previous chapters, it has been proposed that in AD there may be a shift in APP processing favouring increased liberation of β/α4. Such a change in APP metabolism would almost certainly promote β/α4 deposition and compromise the normal biological function of APPs. The mechanism of such a shift remains unknown, however there does appear to be a relationship between cortical pyramidal neurone hypoactivity, cholinergic dysfunction and reduced non-amyloidogenic α–secretory processing of APP (see Chapter 3). As reviewed in 1.3.5.2, perturbed energy metabolism has been implicated in the pathogenesis of AD and may represent a biochemical mechanism underlying mismetabolism of APP. Using the protocol described in 2.2.7.2, cellular ATP levels were reduced by approximately 70% in perturbed PC12 cells compared with control. This effect was similar to previously published results (Reynolds et al., 1982) and although ATP concentrations were reduced, the adenylate energy charge of the cells remained unchanged. This mimics the situation described in AD biopsy brain (see 1.6.8). Under these conditions, in PC12 cells, both the intracellular pool and secretion of APPLIR was found to be significantly reduced. This may index a shift away from non-amyloidogenic processing towards potentially amyloidogenic pathway(s). In one other study (Gabuzda et al., 1994) inhibition of energy metabolism has been shown to alter the processing of APP i.e. induce a potentially amyloidogenic 11.5 kDa derivative. In addition it has been reported that PC12 cells, when maintained under selected stressful or injurious conditions release increased quantities of a potentially amyloidogenic APP product (Baskin et al., 1991). Therefore such an interpretation may not be without precedent. (Clearly, future studies will need to rule out more generalised reduction of protein synthesis which could also account for the reduced intracellular and secreted APPLIR and address the fate of C-terminal APPLIR derivatives under the conditions studied).
In perturbed cells, chloroquine, (by inhibiting lysosomal processing of APP) was found to restore the intracellular pool of APPLIR to the control range (Fig. 5.3 A, "stress + chloroquine"), however, values did not return, to those observed in non-perturbed cells (Fig. 5.1 A, "chloroquine"). This may represent an overall reduction in the intracellular pool of APPLIR, as a result of enhanced (potentially amyloidogenic) processing, in energy perturbed cells. This interpretation is by no means conclusive. Further investigation is required to rule out other factors which could influence intracellular APPLIR concentrations e.g. attenuated protein synthesis.

Restoration of the intracellular pool of APPLIR by chloroquine, to the control range, in perturbed cells (Fig. 5.3 A, "stress + chloroquine") may be responsible for the observed increase in secretion of APPLIR from the same cells (Fig. 5.3 B, "stress + chloroquine"). Under such conditions, chloroquine may indirectly enhance secretion of soluble APPLIR, by blocking intracellular degradation of mature APPLIR species. Thus causing a redistribution of the intracellular pool of APPLIR in favour of non-amyloidogenic pathway(s). This further highlights the concept that amyloidogenic and non-amyloidogenic APP processing pathways compete for the same substrate. Thus, inhibition of processing through one pathway, may favour processing through another.

Previously, chloroquine has been demonstrated to exert inhibitory effects on the degradation of mature APP holoprotein, but to have no effect on APP\textsubscript{\textit{s}} secretion (Caporaso et al., 1992a). An explanation has been offered in the preceding paragraph to account for the observation that secretion of APPLIR is increased in perturbed chloroquine-treated cells. In perturbed cells, it may be important for survival to maintain APP\textsubscript{\textit{s}} secretion. Thus, it is possible that in the presence of chloroquine, cells are able to utilise any mature APPLIR that becomes available (by inhibition of intracellular degradation) for secretory processing. The observation that chloroquine significantly reduced the secretion of band 2 in control cells (Fig. 5.1 B, "chloroquine") was unexpected. This finding is unlikely to be due to selective proteolytic degradation since secretion of APPLIR has previously been shown to be the same for control and chloroquine-treated cells even after 4 h. After 6 and 8 h, a small decrease in the recovery of secreted APPLIR has been reported, which may be the result of degradation (Caporaso et al., 1992a). However, the decrease in recovery observed after this time was not comparable to that seen in this study, after only 3 h.
5.3.1 Restoration of α-secretory processing of APPLIR in perturbed cells

As discussed above, perturbation of energy metabolism in PC12 cells may alter the metabolism of APP, so that potentially amyloidogenic processing is favoured at the expense of secretory non-amyloidogenic processing. If maintaining secretion of soluble APPLIR is important for normal cell function/survival, then it is important for the APP substrate to be diverted away from potentially amyloidogenic pathway(s). Chloroquine has been shown here to indirectly enhance secretion of soluble APPLIR in perturbed cells (Fig. 5.3 B, "stress + chloroquine"). A more direct approach however, would be to stimulate the signal transduction cascade, thought to regulate the balance of the activities of non-amyloidogenic and amyloidogenic APPLIR processing pathways. PKC is known to play a central role in this signal transduction cascade; and bradykinin and phorbol ester, agents known to stimulate PKC, have been shown in this study (and others; see 1.3.3.5) to rapidly accelerate secretory processing of APPLIR. Furthermore, this has been shown to occur with sparing of β/α4 production (see 1.3.3.5). Treatment of perturbed cells with these agents completely restored the secretion of APPLIR to control values (Fig. 5.3 B, "stress + bradykinin" and "stress + phorbol ester") indicating that under these conditions the mechanism by which soluble APPLIR secretion occurs, remains effective. It would be interesting to establish whether these agents affect the processing of potentially amyloidogenic C-terminal APP derivatives using the same paradigm. Although further studies are required to address this issue, the intracellular pool of APPLIR in perturbed cells did increase during treatment with bradykinin and phorbol ester (Fig. 5.3 A, "stress + bradykinin" and "stress + phorbol ester"). This is suggestive of a shift in the direction of APPLIR processing away from intracellular, potentially amyloidogenic pathway(s), e.g. in the lysosomes, thus providing more substrate for secretory processing.

The observation that the PKC related mechanism of soluble APPLIR secretion remains effective under conditions of perturbed metabolism has important implications for treatment. This is because it is possible that a disturbance of this mechanism may be relevant to the pathogenesis of AD, and particularly to the complex events which lead to the final common pathway of amyloidogenesis. Many components of both APPLIR processing pathways and the signal transduction cascades which regulate them (e.g. APP cleaving proteases, protein kinases and protein phosphatases acting on APP or
APP proteases) may eventually serve as targets for rational drug therapy by anti-amyloidogenic agents (see 8.3.2).

The above discussion provides the most straightforward interpretation of the data presented. However, referring back to an issue already raised in 1.3.2.2, APP is a member of a highly conserved family of related proteins, and antibody 22C11 fails to discriminate APP from APLP2. Therefore, an attempt has been made, to discriminate APP from APLP2 by using the antibody DAI raised against a synthetic peptide sequence in rat APP which corresponds to β/A4 1-25. Since this region is absent in APLP2, DAI will only recognise APP. It was hoped this would strengthen the interpretation by indicating that the effects observed could be attributed to changes in APP. APLP2 is known to lack the β/A4 domain and subsequently cannot act as a substrate for β/A4 formation, thus raising the question of the importance of this protein in the pathogenesis of AD.

DA1 immunoreactivity of cell lysate was insensitive to perturbed energy metabolism (Fig. 5.2, "stress") suggesting that the effect on 22C11 immunoreactivity indexed reduced intracellular APLP2. Thus the changes discussed above may reflect altered APLP2 metabolism. However, it is known that only low concentrations of APLP2 can be recovered in medium after 3 h (see Chapter 7, Fig. 7.1 D), therefore the reduction in secreted 22C11 APLIR can probably be attributed to a change in concentration of APP under these conditions. As a consequence, reduced secretion of APPs may account for the observation that the intracellular pool of APLIR is apparently maintained (Fig. 5.2 "stress").

Other aspects of the work with antibody DA1 as shown in Fig. 5.4, B, confirm that (i) secretory processing of APP is accelerated by treatment with bradykinin and phorbol ester and (ii) APP is processed through a chloroquine-sensitive intracellular pathway. Thus many of the observed changes described in this Chapter would seem to reflect changes in APP metabolism.
CHAPTER 6

6.0 CAN DRUGS WHICH AFFECT NEUROTRANSMITTER FUNCTION ALTER THE PROCESSING OF APP\textsubscript{LIR} IN HUMAN BRAIN?

6.1 INTRODUCTION

In previous chapters, it has been hypothesised that a metabolic imbalance of the relative utilisation of non-amyloidogenic and potentially amyloidogenic APP processing pathway(s), favouring reduced APP\textsubscript{\textgamma} release and increased liberation of \beta/A4 may be a feature of AD. To examine a possible correlation between APP metabolic pathway utilisation and AD, a number of investigators have sought to identify AD-related changes in APP metabolism in CSF. Initially, CSF concentrations of APP\textsubscript{\textgamma} were found to be higher in AD patients than in control subjects (Ghiso et al., 1989; Weidemann et al., 1989; Kitaguchi et al., 1990). However other reports have indicated that CSF concentrations of APP\textsubscript{\textgamma} may be decreased in patients with AD (Palmert et al., 1990; Prior et al., 1990; Henriksson et al., 1991; Clarke et al., 1993). In these studies, a variety of antibodies raised against synthetic peptides, or expression products corresponding to various domains of APP were employed, which may explain the discrepancies. More recent studies using a highly specific and selective monoclonal antibody prepared against purified, native human APP\textsubscript{\textgamma}, have subsequently revealed diminished concentrations of APP\textsubscript{\textgamma} in the CSF from patients with AD and from patients with hereditary cerebral haemorrhage with amyloidosis (HCHWA\textsubscript{I}; see 1.6.1), when compared to age matched controls. Individuals with HCHWA\textsubscript{I} have a point mutation in the gene encoding cystatin C, a cysteine protease inhibitor, which leads to altered processing of APP and deposition of resulting amyloid in the cerebral vessel walls (Ghiso et al., 1986). If as the results described above suggest, a parallel can be drawn between AD patients and individuals afflicted with HCHWA\textsubscript{I}, the lower CSF APP\textsubscript{\textgamma} concentrations in AD may be indicative of excessive proteolysis of variant APP or decreased utilisation of non-amyloidogenic \alpha-secretory processing.

It is now well established that \alpha-secretory processing of APP can be increased by first messenger-induced stimulation of cell surface receptors coupled via G protein to phospholipase C activation (see 1.3.3.5). Thus dysfunctional neurotransmission may
contribute to the pathogenesis of AD by altering the metabolism of APP in such a way as to compromise the normal biological function of APP$\alpha$ and promote $\beta$/A4 formation, a possibility supported by evidence of deficits in cholinergic and/or glutamatergic neurotransmission in AD (see 1.2.9.1, 1.2.9.2 and also Chapter 3).

By extension then, it may be hypothesised that APP$\alpha$ secretion into CSF, can be modulated by pharmacological manipulation of the signal transduction cascade linking receptor activation to APP metabolism. According to this hypothesis, treatment of affective disorders with drugs such as lithium, hypothesised to attenuate stimulated phosphoinositide-mediated signal transduction and those drugs with anticholinergic or other neuronal activity inhibiting properties (i.e. drugs capable of reducing cortical excitatory transmission) such as antidepressants, would be associated with lower APP$\alpha$ concentrations in CSF. The effect therefore of therapy with these drugs, on indices of APP$\alpha$ secretion in rare samples of human ventricular CSF (VCSF; Pangalos et al., 1992) has been studied here. For comparison, the effect of lithium treatment on APP$\alpha$ secretion from PC12 cells is also described.

6.2 RESULTS

6.2.1 Effect of lithium and/or antidepressant therapy on APPLIR concentration in VCSF

In VCSF, antibody 22C11 directed to the extracellular N-terminal region of APP identified two major forms of APPLIR, with approximate molecular weights of 125 kDa and 116 kDa. These bands are thought to represent secretory forms of APP derived from APP$\alpha$ and APP$\alpha$$\alpha$ respectively (Fig. 6.1). An antiserum to the KPI domain of APP (7H5), showed a clear reaction with the 125 kDa protein which was therefore considered to correspond to the secreted APP$\alpha$$. The 116 kDa band showed no immunoreactivity with 7H5 and was therefore considered to correspond to the secreted form of APP$\alpha$$\alpha$ (Fig. 6.1). Both bands have previously been described in CSF (Palmert et al., 1989) and in the culture medium of PC12 cells (Refolo et al., 1989). It should be noted that the 125 kDa band did not react strongly with antibody 10D5 (Fig. 6.1). It is therefore possible that the APP homologue APLP2 is present in greater quantities in this band. The identity of proteins between approximately 58 and 100 kDa
Fig. 6.1 WESTERN BLOTS OF APPLIR IN VCSF: RESULTS WITH ANTIBODIES ANTI-5 AND 10D5 COMPARED WITH REPRESENTATIVE BLOTS USING ANTIBODIES 22C11 AND 7H5.

Lane numbers identify nature of drug treatment: Lanes 1, 5 and 9, control; 2, 6 and 10, antidepressant, 3, 7 and 11, lithium; 4, 8 and 12, antidepressant and lithium. Blots in this figure were exposed for a sufficient length of time to reveal trace immunoreactivity present in some samples. Note that of the two bands recognised by 22C11, anti-5 and 10D5 (solid arrows), only one of the highest apparent molecular mass reacted strongly with 7H5 (open arrows).
could not be established since these bands were not consistently recognised by all the antibodies.

The mean VCSF APP^{pi} value determined with 22C11 (Table 6.1) for patients receiving both lithium and antidepressants was found to be significantly lower than that of patients receiving neither. This was not the case for APP_{695}, although the differences in values between control and combination therapy was only slightly smaller in magnitude. Antibodies anti-5, 10D5 and 7H5 confirmed the direction of changes seen with 22C11 (Fig. 6.1). The four treatment groups did not show any significant differences in age, sex, diagnostic group, or treatment with drugs of other categories (Chi squared analysis, $P > 0.05$) or total CSF protein (ANOVA $P > 0.05$).

6.2.2 Effect of lithium treatment on secretion of APPLIR from PC12 cells

In conditioned medium, antibody 22C11 recognised two major protein bands with approximate molecular masses of 125 kDa and 116 kDa, considered to represent major APP isoforms APP^{pi} and APP_{695} respectively (see 5.2). Treatment for 1 h with lithium (1.5 mM) significantly increased secretion of 22C11 immunoreactivity (APP^{pi}, 150%; APP_{695}, 158%; $n = 8$; $P < 0.05$; Fig. 6.2). A similar increase was also seen following incubation for 1 h with bradykinin (APP^{pi}, 169%; APP_{695}, 209%; $n = 15$; $P < 0.05$; Fig. 6.2). The effect of treatment with a combination of lithium and bradykinin was to increase further the secretion of APP_{s} (APP^{pi}, 230%; APP_{695}, 266%; $n = 6$; $P < 0.05$; Fig. 6.2).

6.3 DISCUSSION

This study has shown that treatment with drugs considered to interfere with phosphoinositide turnover (lithium salts) or with those with anticholinergic or other neocortical neuronal activity inhibiting properties such as antidepressants, can bring about differences in the secretion of soluble APPLIR into VCSF. Patients receiving both lithium and antidepressants had significantly less APP^{pi} in their VCSF, than patients receiving neither. This was not the case for APP_{695} although the difference in values between control and combination therapy was only slightly smaller in magnitude. In contrast, treatment of PC12 cells with therapeutic concentrations of lithium significantly increased secretion of soluble APPLIR (Fig. 6.2). This result was
Table 6.1 EFFECTS OF DRUG TREATMENT ON ANTIBODY 22C11 IMMUNOREACTIVE SPECIES IN VCSF.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n</th>
<th>KPI</th>
<th>695</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>33 ± 7</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Antidepressant</td>
<td>25</td>
<td>23 ± 6</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Lithium</td>
<td>7</td>
<td>24 ± 6</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Lithium and antidepressant</td>
<td>38</td>
<td>16 ± 3*</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Units are arbitrary units/mg protein. *, mean significantly different from control patient group (patients not receiving lithium or antidepressant but a drug from at least one other category; see Table 2.8 and Appendix 3). ANOVA F probability = 0.0486 followed by LSD test ($P < 0.05$). Statistical analysis performed on log transformed data as the original data was not normally distributed.
PC12 cells were incubated in the absence or presence of bradykinin (500 nM), lithium (1.5 mM) or a combination of both for 1 h (see 2.2.7.2), and secreted APPLIR, as determined with antibody 22C11, analysed as described in 2.2.7.2.2. Within each experiment, incubations were carried out in triplicate, and data were normalised to the average amount of APPLIR recovered under control conditions. The results shown represent means ± SEM of 8, 15 and 6 experiments, *, P < 0.05, t-statistic. **Open bars indicate APP$_{KPI}$ and crosshatched bars depict APP$_{695}$.**
unexpected, in view of the proposal that treatment with lithium should attenuate stimulated phosphoinositide-mediated signal transduction, and thus reduce secretory processing of APPLIR. Such a discrepancy may possibly be explained by differences in the length of time of treatment. It was thought that PC12 cells were unlikely to survive in millimolar lithium-containing media for long periods of time, thus, they only received lithium acutely (1 h). In contrast, when given to patients as prophalaxis or treatment for mania and/or depression, therapeutic blood concentrations of lithium (0.5-1.5 mM) need to be maintained, which can only be achieved by long-term treatment. Therefore the results described here for VCSF may reflect effects of treatment that can be elicited only after chronic administration of lithium in vivo. Another obvious difference between the two paradigms, is species.

The exact psychotherapeutic action of lithium is unknown, however, lithium treatment is known to lead to the depletion of cellular inositol, secondary to its block of inositol monophosphatase (see also Berridge et al., 1989). By trapping inositol in this way, primarily in the form of inositol monophosphates (see Fig. 6.3), it is predicted that less inositol will be available for resynthesis of phosphoinositides, thus leading to attenuation of stimulated phosphoinositide-mediated signal transduction in affected cells. Experimental evidence to support this idea has come from a number of studies on brain slices demonstrating a large accumulation of inositol monophosphates concomitant with a reduction in inositol content after receptor activation in the presence of therapeutic concentrations of lithium (Allison and Stewart, 1971; Allison et al., 1976; Sherman et al., 1981; Sherman et al., 1985). More recently, acutely administered therapeutic concentrations of lithium have been shown to increase levels of second messenger 1,4,5-IP₃ in guinea pig and rabbit brain cortex slices (Lee et al., 1992). Thus acute treatment, by its elevating effect on 1,4,5-IP₃ content and presumably Ca²⁺ levels, may be able to stimulate secretory processing of APP via activation of PKC by Ca²⁺ (see Fig. 6.3). This is only speculation, since it is not known whether the increase in 1,4,5-IP₃ content brought about by lithium treatment is large enough to facilitate release of Ca²⁺ from internal stores. However, it does provide a possible explanation to account for the observation that acute lithium treatment enhanced APPLIR secretion from PC12 cells. In the presence of bradykinin, secretion of soluble APPLIR from lithium treated cells was increased further. Since bradykinin will induce breakdown of phosphoinositides following activation of its receptor, in the presence of lithium, more
In this model, secretion of soluble APP derivatives from PC12 cells was increased by acute (1 h) exposure to lithium (see Fig. 6.2), presumably due to the accumulation of the second messenger 1,4,5-IP_3 (breakdown inhibited by lithium, asterisk) releasing Ca^{2+} from the endoplasmic reticulum to stimulate APP processing by α-secretase, involving activation of protein kinase C. Chronic lithium administration (see Table 6.1) inhibited the resynthesis of phosphoinositides secondary to depletion of cellular inositol (indicated by asterisk), so according to this model and in sequence: PIP_2, DAG and 1,4,5-IP_3 concentration would be decreased and APP processing by α-secretase inhibited as indicated by reduced efflux of soluble APP derivatives into ventricular CSF (see Table 6.2). Under these conditions processing of APP by alternative pathways may be favoured leading to accumulation of β/A4.
inositol may be trapped which may have an additive effect on the accumulation of 1,4,5-IP_3. This may influence secretory processing of APP through activation of PKC following release of Ca^{2+} from internal stores.

At the beginning of this Chapter, it was hypothesised that secretion of APP into CSF may be modulated by pharmacological manipulation of the signal transduction cascade linking receptor activation to APP metabolism. Previous findings in cell lines have demonstrated that secretion of APP into conditioned medium can be increased by cholinomimetic drugs (Nitsch and Growden, 1994), and more recently, cholinesterase inhibitors have been shown to significantly increase APP secretion from superfused cortical slices of rat brain (Mori et al., 1995). However, to date it has not been possible to test this in humans, but, if APP catabolism is unaltered, the major observation of this study that anticholinergic drugs and those drugs which reduce neocortical neuronal activity apparently reduce APP secretion in man supports this tenet, since α-secretory processing of APP appears to be positively modulated by neuronal depolarisation (Nitsch et al., 1993).

Thus a possible role of dysfunctional neurotransmission in AD is indicated as the reciprocal relationship between the secretion of APP and the production of β/A4 favours APP production under normal conditions but may favour β/A4 production where there is loss or interference with cholinergic and/or glutamatergic neurotransmission (Francis et al., 1993b; Francis et al., 1993c; Nitsch and Growden, 1994). Thus lithium and drugs with anticholinergic properties are associated with reduced secretion of APP (see also Clarke et al., 1993). Furthermore, lumbar CSF concentrations of APP have been found to be reduced in patients with AD or non-AD dementia (Francis et al., 1993c) and certain forms of brain APP are higher (indicating reduced secretion; see Chapter 3) in AD. It is also possible that generation of the other cardinal feature of the AD brain, NFT, could be influenced by failure of neurotransmission (Bowen et al., 1994). This hypothesis is supported by the observation that schizophrenics treated with antipsychotic medication with anticholinergic side-effects, had a higher incidence of neurofibrillary pathology than those not receiving such treatment (Wiszewski et al., 1994).
CHAPTER 7

7.0 PRODUCTION AND CHARACTERISATION OF A NOVEL MONOCLONAL ANTIBODY (3B11) TO AMYLOID PRECURSOR-LIKE PROTEIN 2

7.1 INTRODUCTION

As reviewed in 1.3.2, APP is a member of a larger family of proteins that include the amyloid precursor-like proteins (APLPs) APLP1 and APLP2. At present, APLP2 is the best characterised member of the APLPs (see 1.3.2.2), although, its role in AD remains unclear since lacking the β/Α4 domain, APLP2 cannot act as a substrate for β/Α4 formation. It is conceivable however, that an alteration in, or the overproduction of APLP2 could affect the overall maturation and metabolism of APP. In addition, factors or events leading to the up-regulation of APLP2 might also result in increased expression of APP and processing through an amyloidogenic pathway. Thus the importance of understanding any relationship between APP and APLP2, their regulation and functions, is apparent. However, because of the extensive homologies between APP and APLP2, many of the widely utilised antibodies raised against APP (e.g. 22C11) cross-react with APLP2 (Sisodia et al., 1994; Slunt et al., 1994) potentially confounding the interpretations of many immunoochemical and biochemical studies which attempt to characterise APP.

To overcome this problem, a unique monoclonal antibody 3B11 has been raised to a peptide sequence present in APLP2 and not APP (see 2.2.9; see also Webster et al., 1995). This Chapter documents the specificity of 3B11 by immunoprecipitation and Western blot analysis of human cerebrospinal fluid and extracts prepared from human brain. In addition, a variety of different cell lines have been examined under control and experimental conditions for factors that may affect the metabolism of APLP2. Immunocytochemistry has also been used to investigate the anatomical localisation of APLP2 in rat brain as well as in AD and non-AD brain and preliminary data on this has been presented.
7.2 RESULTS

7.2.1 Western blot analysis and characterisation of 3B11

Sera from mice injected with peptide B4/C4 (Fig. 2.1) reacted strongly with two high molecular weight protein bands on Western blots of human brain membrane fractions (Fig. 7.1 A, lane 1). These bands had approximate molecular masses between 120 and 140 kDa and corresponded with bands previously identified with the commercially available N-terminus anti-APP antibody 22C11 (see 3.2.1). Sera from mice injected with any of the other peptides gave either weak or no reactivity with these bands (Fig. 7.2), and sera from uninjected mice failed to react with any protein bands at all (Fig. 7.1 A, lane 2). Resulting hybridoma supernatant obtained following fusion of spleen cells from successfully immunised B4/C4 mice with myelomas, identified bands similar to those recognised by sera from the same mice, but with less non-specific binding (Fig. 7.1 A, lane 3). After cloning and expansion, of positive wells, culture supernatant obtained from a clone designated 3B11, identified two major protein bands in human cortical membranes (Fig. 7.1 A, lane 4) and supernatant fractions (Fig. 7.1 B, lane 1) with approximate molecular masses between 120 and 140 kDa. The same two bands were identified with the polyclonal antibody D2-1 raised against full length APLP2751 (Fig. 7.1 E, lane 1). An example of a negative clone is also shown (Fig. 7.1 A, lane 5). Specific 3B11 immunoreactivity was abolished following incubation of hybridoma supernatant with peptide B4 (Fig. 7.1 B, compare lanes 1 and 2), but not peptide C4. The glutathione fusion protein of the 113 amino acid ectodomain of APLP2 was also detected on Western blots by 3B11 (Fig. 7.1 B, lane 3). However, 3B11 did not detect purified secreted APP695, baculovirus-expressed APP751, or COS-1 cells transfected with APP770 compared with those transfected with empty vector (Fig. 7.1 B, lanes 4, 5, 9 and 10). This was in contrast with antibody 22C11 which did recognise baculovirus expressed APP751 (Fig. 7.1 B, lane 6). 3B11 also identified the increased expression of APLP2 in H4 cells transfected with human APLP2 cDNA (Fig. 7.1 B, compare lanes 7 and 8), and in COS-1 cells transfected with mouse APLP2751 cDNA (Fig. 7.1 B, compare lanes 9 and 11). Antibody D2-1 detected the same bands in COS-1 transfected cells (Fig. 7.1 E, compare lanes 2 and 4). Increased expression of APLP1 in H4 cells transfected with the mouse APLP1 gene was not detected with 3B11.
Fig. 7.1 CHARACTERISATION OF MONOCLONAL ANTIBODY 3B11 (RAISED TO PEPTIDES B4/C4) BY WESTERN BLOT ANALYSIS.

(A) High density membrane fractions from human brain (lanes 1-5) blotted with serum (lanes 1 and 2) or hybridoma supernatant (lanes 3-5) from mice inoculated with B4/C4 peptide (lanes 1, 3 and 4), uninoculated mice (lane 2), selected positive clone (lane 3), expanded positive clone (3B11, lane 4) and negative clone (lane 5). (B) Western blots using monoclonal antibody 3B11 (except lane 6) of human brain supernatant (lanes 1 and 2), glutathione fusion protein of the 113-amino acid ectodomain of APLP2 (lane 3), baculovirus-expressed APP_{751} (lane 4) and purified secreted APP_{695} (lane 5), COS-1 cells transfected with APP_{770} (lane 10), H4 (lane 7) and COS-1 cells (lane 11) transfected with APLP2 cDNA and H4 (lane 8) and COS-1 cells (lane 9) transfected with vector. Baculovirus-expressed APP_{751} was also blotted with monoclonal antibody 22C11 (lane 6). (C) Western blots using monoclonal antibody 3B11 of ventricular CSF (lanes 1 and 2) and lumbar CSF (lanes 3 and 4). (D) Western blots using monoclonal antibody 3B11 of PC12 cell lysate (control, lane 1; energy perturbed, lane 2), conditioned medium (lanes 3-12) from 3-day incubation without NGF (lane 3), with NGF (lanes 4-12) and in addition with bradykinin (lanes 10 and 12) for 3 h (lanes 4, 9 and 10), 6 h (lanes 5, 11 and 12), 12 h (lane 6), 16 h (lane 7) and 72 h (lane 8), medium from cerebellar granule cells (lanes 13 and 14) and cortical cell cultures (lanes 15 and 17) without glutamate (lanes 13 and 15), with glutamate (lanes 14 and 17) and cortical cell supernatant without glutamate (lane 16) and with glutamate (lane 18). (E) Western blots using polyclonal antibody D2-1 of human brain supernatant fraction (lane 1), COS-1 cells transfected with vector (lane 2), with APP_{770} (lane 3) and APLP2 (lane 4).
For production of monoclonal antibody 3B11, groups of three mice each received injections of two overlapping peptide sequences (see Fig. 2.1 and section 2.2.9.1). After 10 days, mice were bled and sera screened by electrophoresis and Western blotting (see 2.2.9.2.1). Sera from mice injected with peptide sequences other than B4/C4, gave either weak (lanes 1-6) or no immunoreactivity (lanes 7 and 8) compared with the same bands identified by sera from mice injected with B4/C4 (Fig. 7.1 A, lane 1).
APLP2 was detected in PC12 cell lysate (Fig. 7.1 D, lane 1) as well as in the cell supernatant of cortical neurones (Fig. 7.1 D, lane 16), as three high molecular mass bands. These bands had approximate molecular masses between 116 and 140 kDa. Two of the bands identified, corresponded with major bands previously identified with antibody 22C11 (see 5.2) Additional lower molecular mass bands were also present in PC12 lysate, which may represent degradation products of APLP2. Under conditions of perturbed energy metabolism, APLP2 immunoreactivity was reduced in PC12 cell lysate when compared with control (Fig. 7.1 D, compare lanes 1 and 2). This was also a feature of 22C11 immunoreactivity (see Fig. 5.3; see also Webster et al., 1993). A single high molecular mass immunoreactive band of approximately 120 kDa was observed in conditioned medium from PC12 cells, cerebellar granule cells and cortical cultures (Fig. 7.1 D, lanes 3, 13 and 15 respectively). The release of APLP2 into conditioned medium of PC12 cells was stimulated by NGF (400 ng/ml, for 3 days; Fig. 7.1 D, compare lanes 3 and 8), in a time dependent manner (Fig. 7.2 D, lanes 4-8). Release of APLP2 immunoreactivity was also stimulated by bradykinin (500 nM for 3 or 6 h; Fig. 7.1 D, compare lanes 9 and 10, and 11 and 12). In contrast, exposure of cerebellar granule and cortical cultures to glutamate (500 μM, for 30 min) resulted in a reduction of APLP2 immunoreactivity in conditioned incubation medium (24 h after treatment; Fig. 7.1 D, compare lanes 13 and 14, and 15 and 17). This was accompanied by reduced APLP2 immunoreactivity in the cell supernatant of cortical neurones (Fig. 7.1 D, compare lanes 16 and 18) and cerebellar granule cells. APLP2 was also present in both human LCSF and VCSF (Fig. 7.1 C, lanes 1-4) as two major bands of approximately 116 and 130 kDa, with an additional lower molecular weight band present at approximately 86 kDa. In one example of LCSF, the highest molecular weight band resolved into a doublet (Fig. 7.1 C, lane 6).

7.2.2 Immunocytochemical localisation of APLP2 in brain

Similar cellular staining patterns were seen in the cortex in both rat (Fig. 7.3) and human brain (Fig. 7.4) with antibodies LN27 (A), 3B11 (B) and D2-1 (C), indicating that in general the pattern of APLP2 labelling resembled that of APP. In these sections, there was clear labelling of pyramidal neurones with all three antibodies. In human dorsal raphé neurones however, there was no 3B11 immunoreactivity (Fig. 7.5 B), thus within the brain there may be regional differences in the distribution of
Near adjacent sections of rat frontal cortex labelled with LN27 (A), 3B11 (B) and D2-1 (C). All three antibodies clearly label pyramidal neurones. Scale bar = 200 μm
Near adjacent sections of non-AD neocortex (see 2.2.9.4.2; case number 1040/94) labelled with LN27 (A), 3B11 (B) and D2-1 (C). All three antibodies clearly label pyramidal neurones. Scale bar = 100 µm
Fig. 7.5 IMMUNOSTAINING OF FREE-FLOATING SECTIONS OF BRAIN STEM FROM A CONTROL SUBJECT.

Near adjacent sections of brain stem (see 2.2.9.4.2; case number 1194/94) showing the dorsal raphé nucleus labelled with LN27 (A) and 3B11 (B). Only LN27 clearly labels neurones. Scale bar = 200 μm.
APP and APLP2. Similar results with 3B11 and D2-1 were also observed in rat brain. Furthermore, in AD cortex, strong labelling by 3B11 was found in dystrophic neurites surrounding neuritic plaques using two different types of section, paraffin embedded (Fig. 7.6 A and B) and free-floating (Fig. 7.7 C). Serial sections of paraffin embedded tissue showed that these same neurites were labelled with the APP-specific antibody 10D5 (Fig. 7.6 C and D) and the antibody LN27 (Fig. 7.6 E and F) which also recognises APP. Similar staining of near adjacent free-floating sections was also observed with these same antibodies and in addition antibody D2-1 (Fig. 7.7).

7.3 DISCUSSION

A monoclonal antibody 3B11 has been successfully raised to APLP2 which does not recognise APP. Since APLP2 has been found to be encoded by several mRNA derived by alternative splicing (Wasco et al., 1993b; Sandbrink et al., 1994; Slunt et al., 1994), the presence of multiple high molecular mass protein bands identified by 3B11 is likely to reflect the existence of APLP2 isoforms analogous to $\text{APP}_p$ and $\text{APP}_g$. Indeed, mRNA (Slunt et al., 1994) and cDNA (Wasco et al., 1993b) encoding APLP2 with and without the KPI domain have been detected. These multiple bands may also reflect post-translational modification, since APLP2 has been shown to be a chondroitin sulphate proteoglycan (see 1.3.2.2). Further studies are required to confirm that the 140 kDa band contains APLP2 with the KPI domain. However, this sequence is very similar to that in $\text{APP}_p$ and existing antibodies are likely to cross-react, although it may be possible in the future to make specific antibodies to test this.

The identification of APLP2 in conditioned medium and CSF suggests that APLP2 may be processed in vitro and in vivo to produce a soluble truncated form of the protein. The control of this secretory pathway appears qualitatively to be similar to that for APP, as both NGF and bradykinin increase the appearance of APLP2 in conditioned medium (Fig. 7.1 D; see also Chapter 5, and Nitsch et al., 1992; Webster et al., 1993). In contrast to 22C11 immunoreactivity (which was easily detectable in medium after 1 h). 3B11 immunoreactivity could be detected in conditioned medium, after 3 days incubation of PC12 cells (absence of NGF; Fig. 7.1, lane 3) or 3 h (presence of NGF, Fig. 7.1, lane 4). Similarly, 3 h treatment of PC12 cells with bradykinin was required, to see clearly, an effect on APLP2 secretion, whereas only 1h
Serial sections of diseased brain (see 2.2.9.4.1) labelled with 3B11 (A and B), 10D5 (C and D) and LN27 (E and F) are shown. Arrows in (C) and (D) identify an extracellular β-amyloid deposit and those in A, B, E and F neurites around the same deposit. (C) Scale bar = 500 μm; (D) Scale bar = 50 μm.
Near adjacent sections of diseased brain (see 2.2.9.4.2; case number 1154/94) labelled with LN27 (A), 10D5 (B), 3B11 (C) and D2-1 (D) identifying extracellular β-amyloid deposits. Scale bar = 200 μm.
was required for 22C11 immunoreactivity (see Chapter 5; and also Webster et al., 1993). The above interpretation is based on the assumption that the antibody 22C11 is detecting mainly APP in conditioned medium; an alternative interpretation is that 3B11 is not as sensitive as 22C11. Follow up studies are needed to resolve this issue. Furthermore, the observation that treatment of cortical neurones and cerebellar granule cells with glutamate reduced APLP2 immunoreactivity in conditioned incubation medium and cell supernatant also requires further investigation. The results of such a study may shed light on mechanisms regulating APLP2 processing. Preliminary results described in this study indicate that APP and APLP2 processing may be stimulated by receptor-linked protein phosphorylation.

Consistent with previous in situ hybridisation studies (Slunt et al., 1994), initial immunocytochemical investigations with antibodies 3B11, LN27 10D5 and D2-1 has demonstrated a similar distribution of APP and APLP2 in the human cerebral cortex. In rat brain, the distribution of APP and APLP2 also appears to be similar. The APP-specific antibody 10D5 could not be used on rat brain, since this antibody is raised against human β/A4 1-28, and there is no equivalent epitope in rat APP. Any differences however, in the distribution of APP and APLP2 would be apparent by comparing 3B11 and LN27 immunostaining. LN27, raised to an epitope within the first 200 amino acids of the APP N-terminus (a region of high homology between APP and APLP2), will recognise both proteins, thus any differences between the staining patterns of 3B11 and LN27 may be attributed to differences in the distribution of APP.

Although APP and APLP2 may share similar distribution patterns within the cortex, this may not be the case for all brain regions. In human dorsal raphe neurones, no APLP2 labelling was observed with either 3B11 (Fig. 7.5 B) or D2-1 indicating that in this region, APLP2 is absent. A similar staining pattern was observed in rat dorsal raphe neurones. These findings are only preliminary and clearly further studies are required to investigate fully the distribution of APLP2 in human and rat brain. The results of such an investigation may shed light on the functional significance of these two proteins, their relationship to each other, and the possible relevance of APLP2 in AD.
CHAPTER EIGHT

8.0 GENERAL DISCUSSION

In the introduction to this thesis (see 1.6.2), a number of hypotheses were advanced concerning aspects of pathology of AD. These will be reconsidered in the light of the experimental data presented.

8.1 HYPOTHESES

8.1.1 Cholinergic dysfunction and hypoactivity of glutamatergic cortical pyramidal neurones in AD will reduce secretion of secreted soluble APPLIR and favour increased 6/A4 production

The study of APPLIR distribution in human post mortem brain, described in Chapter 3, provides evidence that, in AD, when cortical degeneration is high, remaining pyramidal neurones accumulate full-length APPLIR. APPLIR corresponding to APP_{KPI} correlated inversely with pyramidal neurone number (i.e. the APP content was highest in tissue with the least neurones) and APPLIR corresponding to APP_{695} was increased in AD (see Fig. 3.2 A and Table 3.1). Since secretory processing of APP to produce APP_{5} has been shown to be positively modulated by neuronal depolarisation (see 1.3.3.5), this putative accumulation of APP within remaining pyramidal neurones is probably linked to reduced receptor activation consequent upon degeneration of other pyramidal neurones (Francis et al., 1993c). Almost certainly this would be exacerbated in AD by loss of excitatory cholinergic modulation. Indeed in AD, APP_{695} concentrations were found to be higher in the soluble fraction (see 3.3) when ChAT activity (of the same samples) was low. Furthermore, treatment with lithium, a drug hypothesised to attenuate the signal transduction cascade linking receptor activation to APP metabolism (Fig. 6.3), and those drugs with anticholinergic or other neuronal activity inhibiting properties (i.e. drugs capable of reducing cortical excitatory transmission) was associated with lower APPLIR concentrations in VCSF (Chapter 6). Such reduced efflux of APPLIR from the brain may be interpreted to represent reduced α-secretory processing, leading ultimately to reduced secretion of non-amyloidogenic APP derivatives probably from cortical pyramidal neurones which are subject to regulation by the appropriate receptors.
In summary, there appears to be a relationship between cortical pyramidal neurone hypoactivity, cholinergic pathology and reduced \( \alpha \)-secretory processing of APP. If APP\(_5\) and \( \beta / A4 \) are, as has been suggested in other studies (see 1.3.3.5), derived from competing pathways of APP metabolism, then lost neurotransmitter function (see 1.2.9) may contribute to the pathogenesis of AD by shifting APP metabolism in such a way as to compromise the normal biological function of APP\(_5\) (due to reduced secretion) and favour \( \beta / A4 \) formation. Future clinical trials on neurotransmitter related drugs (see 8.3) should provide paradigms for advancing our understanding of this interesting example of a relationship between transmission, protein metabolism and an aspect of neurohistopathology.

8.1.2 A shift in processing of APPLIR in favour of increased \( \beta / A4 \) production is related to increased membrane serine protease activity in AD (a hypothesis proposed by Siman and colleagues)

A membrane serine protease preparation, and candidate activity postulated to be involved in neuritic plaque formation has been studied (Chapter 4). Based on related work using rat brain, (Nelson et al., 1993) this preparation has the potential to generate the \( N \)-terminus of \( \beta / A4 \) and thus may contain the enzyme \( \beta \)-secretase hypothesised to cleave APP at the \( N \)-terminus of \( \beta / A4 \) (Fig. 1.1). Subsequently increased processing by this enzyme activity may be a requirement for senile plaque formation in AD.

In the frontal cortex, there was no evidence in AD of a significant increase in this activity when compared to controls (see 4.2.2). Indeed in the temporal cortex activity was found to be lower in AD. This may index reduced production of \( \beta / A4_{1-40} \), the suspected product of \( \beta \)-secretase activity. The importance of this is that it could also index enhanced formation of the longer \( \beta / A4_{1-42/43} \) species thought to be initially deposited in brain tissue in AD (see 1.2.5), by making more APP available for degradation to the longer species by an alternative pathway. If lowered rates of \( \alpha \)- and \( \beta \)-secretase cleavage of APP are a feature of AD (as suggested in this thesis), then endosomal/lysosomal processing of APP may be favoured, since these pathways are thought to compete for the same APP substrate.

Therefore it is important to test the hypothesis that in AD compared with control, more APP is processed in the endosomal/lysosomal system. Such an inquiry could form the basis of a future study, although it should be emphasised that current
dogma argues that lysosomes are not the source of β/A4 (see 1.3.3.2). More studies are also needed on the activity, designated γ-secretase, that liberates the C-terminus of β/A4. The combined action of the β- and γ-secretases may be responsible for the formation of β/A4_1-40. Thus in AD, it is possible the changes in the affinity of γ-secretase for a differential splice site may represent a mechanism for the production of longer β/A4_1-42/43 species which favour fibrillogenesis.

In conclusion, these findings do not support Siman's hypothesis. It seems that reduced rather than increased processing of APP by a membrane serine protease activity (e.g. β-secretase) may be related to senile plaque formation, by causing an imbalance (together with reduced α-secretase activity) of the relative utilisation of non-amyloidogenic and an alternative amyloidogenic APP processing pathway(s).

8.1.3 Perturbed energy metabolism in AD contributes to neurodegeneration by altering processing of APP\_LIR

The mechanisms underlying AD pathology remain to be elucidated, however perturbed energy metabolism has been studied (see Chapter 5) as a possible biochemical mechanism leading to mismetabolism of APP. Using a cell culture system (PC12 cells) secretion of soluble APP\_LIR was found to be reduced when cellular ATP concentrations were compromised (Figs. 5.3 B and 5.4 A). This is similar to the situation observed in brain in AD (Chapter 3). Furthermore, there was some evidence to suggest that potentially amyloidogenic endosomal/lysosomal processing of APP may be favoured under conditions of perturbed metabolism suggesting that non-amyloidogenic and amyloidogenic APP processing pathways may compete for the same APP substrate. Indeed alterations in metabolism may contribute in some way to the pathology of AD by favouring β/A4 production and reducing the secretion of soluble APP derivatives. Now that there is some evidence to support a role for perturbed energy metabolism, it is important to directly measure the generation of β/A4 peptides in the present experimental paradigm. In a future study, it may be possible to investigate this following the development of a suitable assay (e.g. a sandwich ELISA using antibodies available only to workers in one or two specific companies (D.M. Bowen personal communication).
8.2 POSSIBLE RELEVANCE OF THE FINDINGS FOR THE PATHOGENESIS OF AD

Three potential events (neurotransmission, enzyme proteolysis and perturbed energy metabolism) which may act to influence the processing of APP (Fig. 8.1) have been investigated in this thesis. Based on the observations presented (see 8.1.1-8.1.3), an imbalance in the relative utilisation of non-amyloidogenic and potentially amyloidogenic APP processing pathways has been proposed to be a feature of AD. This is considered to represent a potential mechanism leading to reduced secretion of APP$_{s}$ and enhanced production of longer $\beta$/A$_{41-42}$ species. With respect to the latter, extracellular accumulation and aggregation of longer $\beta$/A$_{4}$ species has been proposed as a critical event in the aetiology of AD, perhaps even an event where the product, $\beta$/A$_{4}$, is neurotoxic (see 1.2.5 and 1.3.5.1). However, excessive $\beta$/A$_{4}$ deposition alone may not be sufficient for the development of AD. Based on observations that APP$_{s}$ has potential neuroprotective and neurotrophic functions (see 1.3.4), it is possible that APP mismetabolism may result in neuronal degeneration by compromising a normal neuroprotective function of APP$_{s}$ (as a consequence of its reduced secretion). For example, if APP$_{s}$ functions as a trophic or maintenance factor in the synapses, an alteration in the processing of APP, and consequently, an alteration of the concentration or activity of APP$_{s}$ in the synapses could lead to the synapse loss and neuronal cell death, as observed in AD.

This study (Chapters 3 and 6) indicates that APP processing may be modified by neurotransmitter pathology such as occurs in AD, in particular cholinergic and glutamatergic deficits (see 1.2.9.1 and 1.2.9.2), although, it seems unlikely that mismetabolism of APP, as a result of these neurotransmitter deficits, acts as a primary initiating mechanism in AD. There is no clear evidence to indicate that the neurotransmitter changes reported in AD precede neuronal dysfunction and cell death. However, it is possible that APP mismetabolism as a result of impaired neurotransmission, caused by some initiating factor e.g. perturbed energy metabolism, may contribute to the progression of AD by exacerbating $\beta$/A$_{4}$ deposition and APP$_{s}$ loss and thus render neurones vulnerable to insult.

With respect to disease progression, deposition of diffuse amyloid is considered one of the first changes to be seen in the brain in AD (see 1.2.4.4 and 1.2.5).
Three known pathways for metabolic processing of APP are illustrated: the \( \alpha \)-secretory-, \( \beta \)-secretory- and endo-lysosomal- pathways. It is hypothesised that increased protein phosphorylation, by accelerating the removal of mature APP via the \( \alpha \)-secretory pathway, diminishes the amount of substrate available for processing via alternative pathways which may generate \( \beta \)/A4 peptide. Altered neurotransmission, enzyme proteolysis or perturbed energy metabolism may influence APP processing and favour \( \beta A4 \) production.
However, the size and shape of the deposits and their distribution pattern exhibit considerable inter-individual variation thus questioning their role in the early stages of AD. This is in contrast with neurofibrillary pathology which is said to follow a stereotyped pattern with regard to affected cell types, cellular layers and brain regions, with little individual variation (see 1.2.7). In view of their contrasting patterns of development, it seems unlikely that neurofibrillary pathology develops as a mere consequence of the neurotoxic action of β/A4. However, it has been proposed that neuritic plaque distribution reflects the degeneration of terminals of neurones that contain NFT (Lewis et al., 1987). Thus, abnormalities in neurotransmission arising in the terminal fields as a result of neurofibrillary pathology may contribute to the progression of AD by altering the balance of non-amyloidogenic and amyloidogenic APP processing in those regions.

8.3 IMPLICATIONS FROM THIS THESIS FOR THERAPEUTIC STRATEGIES FOR AD

8.3.1 Replacing lost neurotransmitter function

The concept of receptor-coupled regulation of APP processing may have a major implication for treatment. As discussed above, deficits in cholinergic and/or glutamatergic transmission, in AD, may shift the post-translational processing of APP in favour of amyloidogenic pathways, thus altering the concentration or activity of APP₅ and enhancing β/A4 production. Specific neurotransmitter systems and the cellular signalling mechanisms involved in the regulation of APP metabolism may therefore be potential targets for drug design to promote non-amyloidogenic APP₅ production and block β/A4 formation. The data presented in this thesis suggest that traditional pharmacological approaches being developed for the symptomatic treatment of AD (e.g. enhancing cholinergic transmission with agonists or acetylcholinesterase inhibitors; Davis et al., 1992) would be expected to increase excitability of cortical pyramidal neurones (which are subject to regulation by the appropriate receptors; Bowen et al., 1995) and beneficially affect the metabolism of APP by favouring the α-secretory processing pathway. Processing of APP through this pathway is likely to maintain the extracellular supply of APP₅ apparently necessary for normal brain function, and cannot lead to senile plaque formation. Thus if β/A4 deposition is central
to the disease progression, *enhanced* α-secretase activity should reduce deposition of β/A4 and negatively influence the development of further pathology and symptoms.

Glutamatergic hypoactivity in AD is almost certainly exacerbated by the inhibitory action (on remaining pyramidal neurones) of GABA interneurones and 5-HT, the latter effect mediated by the 5-HT$_1A$ receptor (Bowen *et al.*, 1995). Therefore, manipulation of the cholinergic system perhaps in combination with an antagonist of the 5-HT$_1A$ receptor induced hyperpolarisation may represent another promising pharmacological approach for normalising glutamatergic transmission and beneficially affecting metabolism of APP (Dijk *et al.*, 1994; Dijk *et al.*, 1995).

Although signal transduction pathways linking receptor activation to APP metabolism are becoming better understood, mechanisms regulating the hyperphosphorylation of tau, leading to formation of PHF-tau and NFT are less well described (see 1.2.4.3). It may be pessimistically speculated that agents which stimulate PKC activity (i.e via the M$_1$ receptor) may lead to PHF formation. However, there is little evidence that this is the case. One kinase implicated in the formation of PHF-tau, glycogen synthase kinase-3, may be down regulated and inactivated by PKC activation (Lovestone *et al.*, 1994). Additionally, findings of a recent study (Davies *et al.*, 1994) suggest that hypoactivity of glutamatergic neurones may be implicated in the aberrant mechanisms of tau hyperphosphorylation, a proposal based on observations that when treated with a high (1 mM) concentration of glutamate, primary cultures of foetal rat cortical neurones contained much lower amounts of hyperphosphorylated tau identified with antibody AT8 (Brion *et al.*, 1993). Thus it may be hypothesised that PKC is pivotal in regulating APP processing and tau phosphorylation. Aberrant regulation of signal transduction involving PKC, may therefore, represent a mechanism underlying the development of both β/A4 and tau pathologies in AD, a possibility so far largely ignored. If this is an important mechanism, there must also be other contributory factors operating in AD since this mechanism cannot explain the temporal and spatial separation of the two pathologies, presently also a limitation of other proposed mechanisms linking β/A4 and NFT formation (Busciglio *et al.*, 1995; Greenberg *et al.*, 1994; Greenberg and Kosik, 1995).

These data provide evidence for the rationale of cholinomimetic based therapy directed at improving the state of pyramidal neurone activation. Such treatment would be expected to ameliorate the glutamatergic hypoactivity associated with loss of
excitatory input to cortical pyramidal neurones, and consequently treat the symptoms of the cognitive deficit in AD, while influencing mechanisms of possible aberrant APP processing and tau hyperphosphorylation, with resultant slowing of the disease process.

### 8.3.2 Other therapeutic approaches

Therapeutic approaches solely aimed at blocking or decreasing the production of \( \beta/\gamma \) have also been proposed for AD. It is anticipated that \( \beta/\gamma \) formation could be halted or decreased by designing inhibitors of enzymes that catalyse the formation of N- or C-termini of \( \beta/\gamma \). Other components of both APP processing pathways and the signal transduction cascades which regulate them (e.g. protein kinases and protein phosphatases acting on APP or APP proteases) may also eventually serve as targets for rational drug therapy by anti-amyloidogenic agents.

However unlike strategies aimed at replacing lost transmitter function, such approaches have limitations as they almost certainly would not reverse cognitive deficits already present. The situation is complex as it has been proposed that plaque deposition seems likely to involve alterations in APP proteolysis that favour production of longer \( \beta/\gamma_{1,42/43} \) peptides, rather than simply an increased production of normal \( \beta/\gamma_{1,40} \). Thus blocking or decreasing the overall production of \( \beta/\gamma \) may have unforeseen consequences, as it is possible that normally produced \( \beta/\gamma \) peptides may have an as yet unidentified important physiological role (see 1.3.5.1). In view of this, targeting \( \beta/\gamma \) producing pathways may not represent an appropriate approach. It may therefore prove useful to design compounds that inhibit the aggregation of \( \beta/\gamma \) since this seems to be important for causing toxicity, based on both in vitro and in vivo studies (see 1.3.5.1). It has been proposed that the binding of apo-\( \epsilon 4 \) to \( \beta/\gamma \) is related to the transition between diffuse and neuritic \( \beta/\gamma \) deposition (see 1.4.2.1). Although the relevance of this binding to AD pathophysiology remains to be established, modulation of these interactions may represent another target for therapeutic intervention. Indeed it is possible to speculate that approaches at cholinergic replacement therapy may affect the expression or secretion of apo-\( \epsilon 4 \) from astrocytes in homozygous individuals for this allele and thus reduce/prevent binding to \( \beta/\gamma \) and its aggregation into plaques. Furthermore, production of apo-\( \epsilon 4 \) may be the link between abnormal \( \beta/\gamma \) deposition and tangle formation, since the \( \epsilon 3 \) allele (absent in homozygous \( \epsilon 4 \) individuals) may stabilise tau preventing hyperphosphorylation and self assembly into PHF (see 1.4.2.1).
8.4 TACRINE: THE STARTING POINT FOR RATIONALE THERAPY?

One anticholinesterase, tetrahydroaminoacridine (THA, tacrine, "Cognex"), has passed the Food and Drug Administration's licensing procedures for use in treating mild to moderate AD. It has also been approved by the authorities in France. However, studies have been equally divided between those reporting benefit for some patients, and those reporting no benefit (Bryne and Arie, 1994). The dose of tacrine varies considerably between studies, and most report high levels of hepatotoxicity and cholinergic side effects. Also, most studies used too few patients to provide the power to prove or disprove the hypothesis. Well designed trials with adequate numbers of subjects have shown that tacrine benefits some patients, and the benefits are expressed either as improvement in the core deficits of AD, or as reduced rate of deterioration (Davis et al., 1992). However, only very few patients benefit greatly. This may be due to many reasons: the dose may be inadequate; larger doses are commonly associated with more side effects and incidence of toxicity, with resultant withdrawal of the subject from the study; also, positive diagnosis of AD cannot be absolute in life: it is likely that a number of patients included in a trial may be suffering from other forms of dementia.

While it is clear that tacrine is not an ideal anticholinesterase inhibitor, with high incidence of toxicity and short duration of action, the results of some trials are encouraging. It is hoped that the next generation of anticholinesterase inhibitors will address these problems (Giacobini et al., 1991). Thus it may be premature and pessimistic to dismiss cholinergic replacement therapy at present, in view of the limitations of available cholinomimetics. In the future, it may transpire that the most effective treatments for AD require polypharmacy. There is substantial evidence that in addition to the cholinergic system, other neurotransmitter systems are impaired in AD, and many believe that these deficiencies attenuate the potential response to cholinergic drugs (Davis and Haroutunian, 1992). Therefore treating several neurotransmitter deficiencies at the same time may enhance the effectiveness of cholinomimetics at lower doses.

The rationale for polypharmacy as a long term therapeutic strategy for AD is compelling: for example, combining growth-promoting compounds designed to prevent synaptic loss or cell death with compounds designed to restore neurotransmitter deficits.
However, polypharmacy also presents difficult methodological problems from the perspective of designing clinical trials and determining efficacy.

8.5 THE FUTURE

Promising transmitter-related drugs (cholinomimetics and 5-HT$_{1A}$ receptor antagonists) that may improve cognitive symptoms in AD and should also beneficially affect the metabolism of APP by favouring non-amyloidogenic processing are under development by the pharmaceutical industry.

Results in Chapters 3 and 7 seem to show that APP$_{KPI}$ may be less abundant than APLP2 in human cortical membranes. (For example, note that the intense band running just ahead of marker 116 in lane 1 of Fig. 7.1 C is apparently only weakly visualised in lanes 15-19 of Fig 3.1). It remains to be established whether the processing of this apparently abundant KPI-containing protein of human brain cortical membranes is stimulated (like APP) by depolarisation and receptor-linked protein phosphorylation (an important issue as the protein should carry out many of the functions of APP without producing $\beta$/A4). The preparation of the monoclonal antibody to APLP2 (see Chapter 7) is the starting point for a detailed study of this type. Other investigations that need to be carried out, using this novel antibody, include those to discover whether changes reported using non-specific APP antibodies (e.g. 22C11) reflect a change in APLP2 rather than in APP.

It is important to discover any effect of these transmitter-related drugs on the signal-transduction-dependent phosphorylation and dephosphorylation of tau protein, in particular, on the aberrant mechanism that leads to the hyperphosphorylation of tau and almost certainly underlies NFT formation, the other pathological hallmark of AD. Such hyperphosphorylation characteristically occurs in the pyramidal neurones and further research is required to provide more compelling support for the theory (based on the hypothesis that hypoactivity of glutamatergic neurones underlies the symptoms of AD; see Francis et al., 1993b) that the number of tangles, like plaques and cognitive impairment, will be reduced by a drug/drugs designed to improve the state of pyramidal neurone activation.

It is by no means certain that such drug(s) will attain therapeutic goals (e.g. delay onset of institutionalisation of patients by extending their ability to function independently). However, a positive outcome is now not inconceivable and treatment
strategies proposed in this thesis (see 8.3) could have a favourable impact on the burden of AD.
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### APPENDIX 1

**DETAILS OF CONVENTIONAL POST MORTEM SUBJECTS STUDIED**

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? Indicates information not available.
### APPENDIX 2

#### DETAILS OF SHORT POST MORTEM SUBJECTS

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### APPENDIX 2

**DETAILS OF SHORT POST MORTEM SUBJECTS**

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</table>
APPENDIX 2

DETAILS OF SHORT POST MORTEM SUBJECTS

? indicates information not available; N.A. indicates not applicable. Superscript letters identify causes of dementia other than AD.

(a) ?AD In all these cases neuropathological examination indicated that the characteristic features of neurodegenerative conditions other than AD were absent, there was however moderately severe arteriosclerosis in the cerebral vessels. Histological features of AD were present, but not to an extent to confidently ascribe the cognitive symptoms to AD alone. In the brain of case 1, senile plaques and neurofibrillary tangles were present in the hippocampus, but were not abundant in the neocortex. This was also the case for cases 17 and 22, which also showed moderately severe amyloid angiopathy.

(b) Pick's Disease This case showed the classical features of Pick's Disease (Tomlinson and Corsellis, 1984), in the absence of any other structural brain abnormality. The brain showed frontal lobe atrophy, with marked nerve cell loss and subcortical gliosis. There were numerous swollen cells containing Pick bodies (Pick cells).

(c) Depressive Pseudodementia This subject had previously had two episodes of Major Depressive Disorder (DSM-3; American Psychiatric Association, 1980), the first some 20 y before the onset of cognitive impairment. On both occasions this had required admission to hospital for treatment with a good recovery and subsequent discharge. the patient's son also suffered from a bipolar affective disorder for which he was receiving lithium treatment. Examination of the brain showed no pathology characteristic of any described neurodegenerative condition, and the brain was judged by the Consultant Neuropathologist, normal.
APPENDIX 2

DETAILS OF PROMPT POST MORTEM SUBJECTS

(d) Multi-System Degeneration  The brain was symmetrically and grossly atrophic especially in the frontal and parietal regions. the corpus callosum was markedly thinned, basal ganglia, hypothalamus, mammillary bodies, substantia nigra, and cerebellum were grossly normal. Microscopically there was loss of pyramidal cells from the cerebral cortex, without a reactive gliosis, but there was subpial gliosis. There were no features suggestive of any recognised neurodegenerative disease, and no evidence of demyelination or cerebral infarction.
### APPENDIX 3

**DETAILS OF SUBJECTS USED IN THE STUDY OF APPLIR IN VCSF**

<table>
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## APPENDIX 3

### DETAILS OF SUBJECTS USED IN THE STUDY OF APPLIR IN VCSF

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APPENDIX 3

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APPENDIX 3

DETAILS OF SUBJECTS USED IN THE STUDY OF APPLIR IN VCSF

? Indicated information not available

For diagnoses and drug treatment, 1 = Yes; 0 = No
ACKNOWLEDGEMENTS

The experimental work described in this thesis was carried out in the Department of Neurochemistry at the Institute of Neurology, 1 Wakefield Street, London. I am grateful to a number of members of the laboratory who have assisted in experimental techniques particularly Gary Stratmann, Konstantinos Vekrellis and Mark Smith. I would also like to thank J. Tracy Alder for her friendship and encouragement.

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Finally I say thank you to my family and friends whose constant bullying was crucial to the completion of this manuscript.