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PROCESSING OF ALZHEIMER'S AMYLOID PRECURSOR PROTEIN IN CULTURED CELLS

By

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Abstract

The deposition in the brain of the 4 kDa beta-amyloid peptide (βA4), from amyloid precursor protein (APP), is a key pathology in Alzheimer's disease (AD). The single APP gene is spliced to give 3 major isoforms. In the majority of body tissues, the most common APP isoforms are APP_{751} and APP_{770}, which both contain a Kunitz protease inhibitor (KPI) domain, APP_{695} is predominant in the brain. APP is processed through several pathways, not all of which lead to βA4 production. Central nervous system (CNS) neurones *in vivo* secrete βA4, which can be detected in the cerebrospinal fluid, though it is unknown why βA4 is deposited in the brain in AD.

NTera2 (NT2) cells derived from a human teratocarcinoma were used as a model of APP processing. Retinoic acid induces these cells to differentiate into a neuronal phenotype (NT2N cells), which has been shown to closely resemble immature human CNS neurones. Both cell types produce high levels of endogenous APP.

Intracellular and secreted APP was studied in both cell types by means of western blotting and immunoprecipitation with a panel of antibodies. It was found that NT2 cells predominantly make and secrete KPI containing APP. NT2N cells make and secrete predominantly APP_{695} though some KPI containing APP is also present.

There is evidence that neurones in the AD brain are in a state of stress, which could increase levels of APP due to a heat shock promotor region in its gene. To investigate this, NT2 cells were subjected to a heat shock, which resulted in increased levels of heat shock protein (HSP) and APP. KPI containing APP predominated, but there was no corresponding increase in secreted APP. Both cell types were also serum deprived, which resulted in little effect on protein production in NT2 stem cells. However, the neuronal cells showed a small increase in intracellular, KPI-containing APP and in HSP. A reduction in overall APP secretion, and cessation of KPI secretion accompanied this.

To further investigate the effects of shock on APP production, mRNA levels in control and serum deprived NT2 and NT2N cells were studied using *in situ* hybridisation. Control NT2 cells contain low levels of APP_{751}, APP_{695} and HSP mRNA, with higher levels of APP_{770} mRNA. After serum deprivation HSP, APP_{751} and APP_{770} mRNA levels all rose significantly, while APP_{695} mRNA levels were unchanged. Control NT2N cells contained high levels of APP_{695} mRNA, lower levels of APP_{751} mRNA, and very low levels of APP_{770} and HSP mRNA. Serum deprivation resulted in unchanged levels of APP_{695} and APP_{770} mRNA, while APP_{751} and HSP levels were increased.

These findings indicate that cellular stress can result in increased levels of APP, specifically APP_{751}, in both neuronal and non-neuronal cells. Increased levels of this isoform have also been reported in AD. Hence cellular stress leads to an increase in an APP isoform implicated in AD, and could also provide an explanation for the increased levels of βA4 in the disease.

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List of Abbreviations

5-HT	5-Hydroxytryptamine
AD	Alzheimer's Disease
AMPA/kainate	α-Amino-3-hydroxy-5-methylisoxazole-4-propionate / kainate
AP	Alkaline Phosphatase
APLP	Amyloid Precursor Like Protein
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
APPs	Secreted APP
AraC	Cytosine Arabinoside
βA4	Beta-Amyloid Peptide
BCA	Bicinchoninic Acid
BCIP	5-Bromo-4-chloro-3-indolyl Phosphate
BSA	Bovine Serum Albumin
CAA	Cerebral Amyloid Angiopathy
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
cDMEM	Complete DMEM (DMEM with added FBS and P/S)
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl Sulfoxide
DTT	DL-Dithiothreitol
E64	trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane

EC	Embryonal Carcinoma
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EOFAD	Early Onset Familial Alzheimer's Disease
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer's Disease
FBS	Foetal Bovine Serum
FDU	Fluorodeoxyuridine
GRP78	Glucose Regulated Protein
HCHWA-D	Hereditary Cerebral Haemorrhage with Amyloidosis of the Dutch Type
HRP	Horseradish Peroxidase
HSE	Heat Shock Element
HSP	Heat Shock Protein
HUVEC	Human Umbilical Vein Endothelial Cell
IL-1	Interleukin-1
kDa	Kilodalton
KPI	Kunitz Protease Inhibitor domain
LOFAD	Late Onset Familial Alzheimer's Disease
mRNA	Messenger RNA
NBT	Nitro Blue Tetrazolium
NFT	Neurofibrillary Tangle
NMDA	*N*-Methyl-D-aspartate
NSAID	Nonsteroidal Anti-Inflammatory Drug
NT2	NTera2 Stem Cells

NT2/D1	NTera2 clone D1
NT2N	NTera2 Neuronal Cells
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffered Saline
PHF	Paired Helical Filament
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PS	Presenilin
PVDF	Polyvinylidene Difluoride
R Buffer	Resolving Gel Buffer
RA	Retinoic Acid
S Buffer	Stacking Gel Buffer
SAD	Sporadic Alzheimer's Disease
SDS	Sodium-dodecylsulphate
SDS-PAGE	Sodium-dodecylsulphate Polyacrylamide Gel Electrophoresis
SP	Senile Plaque
SSC	Standard Sodium Citrate
TBS	TRIS Buffered Saline
TBS-Tw	TBS with 0.01% Tween-20
TE	Trypsin/EDTA
TEMED	N,N,N'N'-Tetramethylethylenediamine
T_i	Incubation Temperature
T_w	Washing Temperature
Tx100	Triton X-100
U	Uridine

Chapter One - Introduction

1.1 ALZHEIMER'S DISEASE

Alois Alzheimer first described Alzheimer's disease (AD) in 1907, in a 52-year-old woman. (Alzheimer, 1907) It is now the most common cause of dementia in the western world. The incidence of AD increases with age, 5% of the population over 65 are affected, rising to 20% of the population over the age of 80. (Katzman, 1988) As modern medicine advances and the average life span increases, AD is becoming an increasing and costly problem. The British government spends millions of pounds each year caring for AD suffers. As there is no cure for AD, and no effective long-term treatments, the cost keeps on rising.

1.1.1 Clinical Presentation

AD presents as a progressive dementia, beginning with a loss of short-term memory and proceeding to involve other cognitive functions. Common complaints of patients or families include forgetfulness about appointments or errands; inability to find the way to an accustomed destination; inability to use money and instruments of daily living, such as a telephone; deterioration in work or homemaking performance; difficulty adapting to changes in the workplace; difficulties in dressing, reading and writing; and inability to recognise previously familiar individuals. (McKhann *et al.* 1984) As the disease progresses language is affected, and in late stages motor function is impaired.

1.1.1.1 Diagnosis

There is no clinical test for AD, pre-mortem diagnosis of the disease relies on observed behavioural changes and is classified according to internationally agreed criteria (such as NINCDS-ADRA and DSM III). (McKhann *et al.* 1984) It principally relies on the exclusion of

other systemic or neurological diseases that could account for the observed cognitive defects. The diagnosis of AD can only be confirmed at autopsy. The disease usually results with death after 5-15 years, commonly due to a complicating factor such as pneumonia.

1.1.2 Pathological Hallmarks

At post-mortem AD diagnosis is confirmed by the presence of neurodegeneration of specific brain areas, as well as neurofibrillary tangles (NFTs) and senile plaques (SPs), the classical pathological hallmarks of AD. (Tomlinson *et al.* 1970) Alzheimer first described NFTs and SPs in 1907. The concentration of NFTs appears to correlate well with the degree of cognitive impairment, though the number of SPs correlates less well. (Wilcock *et al.* 1982) However "amyloid load" which takes into account all amyloid deposits in the grey matter and around blood vessels, does appear to correlate with the severity of dementia. (Braak and Braak, 1990)

1.1.2.1 Neurofibrillary Tangles

Neurofibrillary tangles (NFTs) are abnormal bundles of cytoplasmic fibres in the neuronal cell body. They are one of the classical hallmarks of AD, but are also present in other neurological diseases (e.g. normal ageing, dementia pugilistica, Down's syndrome and Parkinson-dementia of Guam, Esiri *et al.* 1997). NFTs of AD are composed mainly of paired helical filaments (PHFs), approximately 10 nm in diameter, which cross each other at an 80 nm interval. Thus the maximum width of a PHF is 20 nm, and the minimum width 10 nm. (Goldman and Yen, 1986) PHFs are also present in dystrophic neurites.

PHFs are comprised primarily of the microtubule-associated protein tau (Goedert *et al.* 1991), which is in a hyperphosporylated form, (Grundke Iqbal *et al.* 1986) though normal microtubule-associated proteins may also be present. It is interesting to note that NFTs are also immunopositive for ubiquitin, a protein induced by the cellular heat shock response, and other

heat shock proteins. Hyperphosphorylation of proteins is also a component of this response, indicating that NFTs may represent the end point of a failed attempt by affected neurones to respond to a form of cellular stress. (See Section 1.8 for a discussion of the heat shock response and its relevance to AD)

It is possible that the hyperphosphorylation of tau could lead to a destabilisation of the microtubules, resulting in the disruption of intracellular transport and eventual cell death (see Mandelkow and Mandelkow, 1998 for review of the possible role of tau in the pathogenesis of AD).

1.1.2.2 Amyloid

The term amyloid refers to a "pathological proteinaceous substance deposited extracellularly in tissue and most commonly identified by light microscopy as a homogeneous eosinophilic material which stains with alkaline Congo red". (Glenner and Page, 1976) The deposits consist of an accumulation of fibrils, which allow histological identification of amyloid deposits by Congo red birefringence (amyloid deposits appear green under polarised light after Congo red staining). The proteins composing amyloid deposits are in a β-sheet configuration.

1.1.2.2.1 Plaques

Plaques are spherical extracellular protein deposits found in the brains of both normal aged and AD patients. There are four different types; primitive or diffuse plaques which consist of fibrillar amyloid deposits; dense plaques which contain a dense core of aggregated amyloid; neuritic or senile plaques (SPs) which contain a dense amyloid core surrounded by dystrophic neurites and reactive astrocytes; and burnt out plaques, which resemble SPs, but are not associated with dystrophic neurites. (Braak and Braak, 1990) Both diffuse and dense plaques are believed to be

immature SPs, with plaques beginning as diffuse deposits, then progressing to develop the dense core and finally association with dystrophic neurites.

1.1.2.2.2 Cerebral Amyloid Angiopathy (CAA)

CAA is the deposition of amyloid fibrils around the cerebral vasculature, and occurs in normal ageing and other dementias, as well as in AD. (Esiri and Wilcock, 1986) The amyloid deposited around the blood vessels in AD brains has been shown to be identical to that which is found in senile plaques. (Masters *et al.* 1985; Glenner and Wong, 1984)

1.1.2.3 Neurochemical Deficits

The most consistent neurochemical deficit in AD brain is a marked reduction in presynaptic cholinergic markers, notably choline acetyltransferase (ChAT) activity. This is most probably due to the degeneration of cholinergic basal forebrain neurones. This deficit is likely to be secondary to the cortical pathology, caused by retrograde degeneration of the cells of the basal nucleus, which project to areas of the cortex exhibiting heavy pathology. (Pearson and Powell, 1989)

Since postsynaptic markers, such as the M_1 muscarinic receptors, are relatively spared (though they may be partially uncoupled from their second messenger systems), treatment strategies which upregulate the cholinergic system have been attempted. Anti-cholinesterases such as tacrine and donepezil have been used in treatment trials, with moderate cognitive improvement. However, this strategy has several drawbacks: the drugs have a range of deleterious side effects; there are a number of neurochemical deficits (see below) so that treating one in isolation is not wholly effective; and the loss of ChAT is a consequence of pathology, not a causative effect, so the treatment is not effective in the long-term. (Cummings *et al.* 1998)

Other neurochemical deficiencies associated with Alzheimer's disease include depletion of amine neurotransmitter systems and neuropeptides, especially somatostatin and corticotrophin releasing factor. Changes in 5-hydroxytriptamine (5-HT) and noradrenaline appear to be related to behavioural symptoms such as anxiety and depression. As with the cholinergic system, attempts to combat these effects with drugs have not been successful, largely because they interfere with normal compensatory responses. Finally there is a major deficiency in cortical glutamate in AD. (Esiri *et al.* 1997; Cummings *et al.* 1998)

1.1.2.4 Other Pathology

Other pathological abnormalities include synaptic loss, decreased glucose metabolism, reactive gliosis, decreased protein production and phosphorylation with increased stress response proteins such as HSP70 and ubiquitin (see Section 1.8).

1.1.3 Spread of Pathology

The distribution of NFTs and SPs is not uniform throughout the cortex. The spread of neuronal pathology has been shown to correlate with cortico-cortical connectivity (Pearson, 1996; Pearson and Powell, 1989) with the medial temporal lobe being the end and most affected, and the primary sensory and motor areas the least affected. More specifically, the hippocampus, enthorinal cortex, uncal cortex, cortico-medial amygdala and parahippocampal gyrus are the most severely affected. The primary sensory and motor cortex is relatively unaffected, with the intervening association areas of pareito-temporal cortex involved to an intermediate degree. (Esiri *et al.* 1990; Pearson and Powell, 1989). Neurones that project to affected cortical areas contain NFTs, while those receiving from such areas contain SPs. This distribution of pathology has lead to the belief that the spread of AD pathology is dependent on neuroanatomical pathways, though the identification of the agent responsible has yet to be confirmed (see *Section 1.7*).

1.2 BETA-AMYLOID (βA4)

The dense core of SPs consists of an insoluble aggregation of a 4 kDa, 39-43 amino acid peptide, beta-amyloid (βA4) (Masters *et al.* 1985; Glenner and Wong, 1984), derived by proteolytic cleavage of the longer amyloid precursor protein (APP).

1.2.1 Function

βA4 is present in the blood and cerebrospinal fluid (CSF) of non-AD subjects (Shoji *et al.* 1992); it is also secreted from cultured cells under normal conditions. (Haass *et al.* 1992b) The function of βA4 is not well understood, though it has been reported to show neurotrophic effects at low concentration *in vitro*. (Yankner *et al.* 1990) This observation is supported by the reports that both neuronal (Turner *et al.* 1996; Wertkin *et al.* 1993) and glial cells (Morato and Mayor, 1993; Busciglio *et al.* 1993) under normal conditions produce βA4 *in vitro*.

1.2.2 Structure

The amino acid composition of βA4 promotes spontaneous aggregation into amyloid fibrils, with the longer forms ($\beta A4_{1\text{-}42/43}$) showing a markedly enhanced rate of aggregation *in vitro*, compared to that of $\beta A4_{1\text{-}39/40}$. (Pike *et al.* 1991) There is some evidence that the longer forms of βA4 are critically important in AD, and are deposited first in the SPs. (Gravina *et al.* 1995)

1.2.3 Neurotoxic Properties

βA4 forms fibrils when left in solution, in a process known as ageing. These aged solutions of βA4 have some neurotoxic effects. (Ueda *et al.* 1994; Pike *et al.* 1991; Yankner *et al.* 1990; Yankner *et al.* 1989) There are several theories as to the mechanism by which βA4 induces cell death. It has been reported to destabilise calcium homeostasis in neurones, and thus render them vulnerable to excitotoxicity. (Mattson *et al.* 1992; Koh *et al.* 1990)

A second possibility is that βA4 may cause cell death by inducing apoptosis. (LaFerla *et al.* 1995; Loo *et al.* 1993) βA4 down-regulates bcl-2, an anti-apoptotic protein, and increases levels of bar, a protein known to promote cell death. (Paradis *et al.* 1996) Alternatively βA4 may cause cell death via oxidative mechanisms, or by the generation of free radicals. (Hensley *et al.* 1994) Cells exposed to βA4 mount a stress response similar to that seen in oxidative stress. (Davis, 1996)

Another of the pathogenic processes seen in AD is cholinergic dysfunction, and there is some evidence that βA4 may be involved in this process. Non-aged βA4, which is not neurotoxic, has been shown to suppress acetylcholine synthesis in cholinergic neurones. This effect was only seen with solutions of $\beta A4_{(1\text{-}42)}$, not $\beta A4_{(1\text{-}40)}$. (Hoshi *et al.* 1997)

1.3 GENETICS OF AD

In approximately 5 percent of cases AD has a genetic component, and is inherited as an autosomal dominant disease. In many of these cases of familial AD (FAD) the age of onset is earlier than for sporadic AD, in the fifth or sixth decade as opposed to the seventh or eighth decade for sporadic AD. (Selkoe, 1993) Genes on chromosomes 1, 14, 19 and 21 are all implicated in FAD.

1.3.1 Early-Onset Familial AD (EOFAD)

The gene mutations responsible for EOFAD are found on chromosomes 1, 14 and 21 and correspond to presenilin 2, presenilin 1 (see *Section 1.3.1.2*) and amyloid precursor protein.

1.3.1.1 Amyloid Precursor Protein (APP) Mutations

Neuropathological changes, such as those seen in AD, are observed in Downs Syndrome patients. Since these individuals are trisomic for chromosome 21, it was postulated that AD was

caused by duplication of one or more genes on this chromosome. Mapping of the APP gene to a locus on this chromosome made it a possible candidate for EOFAD. (St George-Hyslop *et al.* 1987)

In 1991 a point mutation in the APP gene, changing valine to isoleucine at codon 717, was found to co-segregate with APP. (Goate *et al.* 1991) This was followed by the discovery of two more mutations at this codon, substituting phenyalanine (Murrell *et al.* 1991) or glycine (Chartier-Harlin *et al.* 1991). A double mutation (known as the Swedish double mutation) at codons 670 and 671 (lysine → asparginine and methionine → leucine) was also found to co-segregate with the disease. (Mullan *et al.* 1992)

Two further mutations in the APP gene were identified in a Dutch family displaying cerebral haemorrhage (HCHWA-D), a disease in which amyloid is deposited in the cerebral vasculature, resulting in fatal haemorrhage. Dementia is also a common feature of the disease. The mutations causing HCHWA-D are at codon 693 (glutamate → glutamic acid) (Levy *et al.* 1990) and codon 692 (alanine → glycine) (Hendriks *et al.* 1992).

All these mutations are either within the βA4 sequence, or close to the cleavage sites. These mutations all alter APP processing to make the deposition of βA4 more likely. The mutations at Val^{717} increase the production of the longer forms of βA4, which are more insoluble, (Thinakaran *et al.* 1996; Maruyama *et al.* 1996; Mann *et al.* 1996; Tamaoka *et al.* 1994; Suzuki *et al.* 1994) while the other mutations increase βA4 production by switching APP processing to the β-secretase pathway. (Haass *et al.* 1994; Citron *et al.* 1994; Cai *et al.* 1993; Citron *et al.* 1992) They are all classical gain of misfunction mutations (see *Section 1.5*). However these mutations only account for less than three percent of early onset FAD cases. (Hardy, 1996)

1.3.1.2 Mutations of the Presenilins

In 1995 a mutation in a gene on chromosome 14 was also found to co-segregate with the disease (Sherrington *et al.* 1995), swiftly followed by the finding of a similar gene on chromosome 1. (Levy-Lahad *et al.* 1995a; Levy-Lahad *et al.* 1995b) These genes have been named presenilin 1 and 2, respectively and are highly homologous to each other. Several mutations of presenilin 2 (PS2) and over 50 mutations of presenilin 1 (PS1) have now been identified, together with the APP mutations these account for all cases of early onset FAD. The presenilin mutations are among residues conserved between the two genes and occur in clusters. (See *Section 1.5* for more detail of presenilin functions.)

1.3.2 Late-Onset Familial AD (LOFAD)

1.3.2.1 Apolipoprotein E

Apolipoprotein E (ApoE) expression is increased in AD, and both SPs and NFTs are immunopositive for ApoE. (Namba *et al.* 1991) LOFAD is associated with the ApoE gene on chromosome 19. (Strittmatter *et al.* 1993) ApoE exists in three isoforms, E2, E3 and E4, encoded by alleles ε2, ε3 and ε4, respectively. There is a strong genetic association between the ε4 allele and LOFAD. Possession of the ε4 allele leads to a higher likelihood and an earlier age of onset of LOFAD, with individuals homozygous for that allele most at risk. (Strittmatter *et al.* 1993) The ε2 allele appears to have a protective effect, increasing the age of onset of the disease. (Oyama *et al.* 1995)

It is unknown how ApoE interacts with the pathogenic processes, various alleles may stabilise the βA4 plaques or protect against tangles. It may be that it is solely involved with damage repair, with the ε2 allele being the most effective and the ε4 allele the least. (Hardy, 1996)

1.4 APP

Glenner and Wong first proposed the idea of the involvement of a novel amyloid peptide in AD in 1984. As previously described, this peptide was subsequently isolated and characterised. (Masters *et al.* 1985; Glenner and Wong, 1984) It was predicted that this was derived from a full-length precursor protein (Tanzi *et al.* 1987) and a cDNA clone coding for a protein with 695 residues was isolated from foetal brain tissue and localised to chromosome 21 in 1987. The predicted protein (amyloid precursor protein or APP) resembled a cell surface receptor. (Kang *et al.* 1987) Further screening of peripheral tissue identified an APP mRNA coding for a 751 residue protein, with the 56 amino acid insert closely resembling a Kunitz-type serine protease inhibitor (now known as the KPI region). (Kitaguchi *et al.* 1988; Tanzi *et al.* 1988; Ponte *et al.* 1988)

1.4.1 APP Structure (See Figure 1.1)

APP is a type I transmembrane protein, with a short intracellular C-terminus, and a long, extracellular N-terminus. The βA4 region corresponds to the first 28 amino acids of the extracellular domain, and the adjacent 11-15 amino acids of the transmembrane domain. (Kang *et al.* 1987) It is now known that alternative splicing of the single APP gene on chromosome 21 can give rise to at least 10 protein isoforms. The three most common isoforms are APP_{695} (missing codons 7 and 8), APP_{751} (missing codon 8) and APP_{770}, or full length APP. Codon 7 encodes the KPI region of the protein, while codon 8 encodes a MRC OX-2 domain. (Clark *et al.* 1985) The βA4 peptide is encoded by parts of codons 16 and 17. APP_{770} and APP_{751} are expressed ubiquitously throughout the body, while APP_{695} is found almost exclusively in neurones. (Rohan de Silva *et al.* 1997; Arai *et al.* 1991)

1.4.2 APP Processing (See Figures 1.1 and 1.2)

APP undergoes complex post-translational modifications, including sulphation, phosphorylation, and both N- and O-linked glycosylation. (Weidemann *et al.* 1989) It is co-translationally

translocated into the endoplasmic reticulum (ER) via its signal peptide, then matured via the central secretory pathway. The holoprotein may undergo cleavage at the cell surface, or during trafficking through the ER and Golgi, by one of three secretase activities; or degradation in the endosomal / lysosomal system. (See *Sections 1.4.2.1* and *1.4.2.2*)

1.4.2.1 α-Secretase

The majority of the full-length APP molecules undergo proteolytic cleavage in the ER or Golgi (see below), with only a small percentage reaching the cell surface. A proportion of these molecules undergoes proteolysis by an unidentified protease designated α-secretase. This protease cleaves at amino acid 16 of the βA4 sequence, and therefore cannot lead to βA4 production. (Esch *et al.* 1990) It requires APP to be membrane attached and in an α-helix, and does not appear to be specific for a particular peptide bond, instead cleaving at a set distance from the membrane. (Sisodia, 1992; Esch *et al.* 1990) This secretase activity releases the large, soluble, extracellular domain (referred to as APPsα) from the cell surface. The 83 residue (~10 kDa) C-terminal fragment is retained in the membrane. α-Secretase activity can also occur in late Golgi and trans-Golgi network on the way to the cell surface. (Sambamurti *et al.* 1992)

Alternatively, full-length APP can undergo endocytosis via clathrin-coated vesicles, after which the protein is either trafficked to late endosomes and lysosomes for degradation, or is rapidly recycled within early endosomes to the cell surface. (Selkoe *et al.* 1996; Nordstedt *et al.* 1993; Haass *et al.* 1992) The endosomal/lysosomal pathway leads to the breakdown of APP into many different fragments, some of which contain βA4 which may then be released from the cell. (Estus *et al.* 1992; Golde *et al.* 1992; Haass *et al.* 1992)

1.4.2.2 β- And γ-Secretase

APP also undergoes proteolytic cleavage by a protease termed β-secretase. This enzyme activity cleaves after methionine 671 (APP_{770}), at the amino terminal of βA4. This cleavage releases a truncated APPs molecule, APPsβ, from the cell surface. (Seubert *et al.* 1993; Anderson *et al.* 1992) The remaining 12 kDa membrane attached fragment undergoes cleavage by a protease named γ-secretase within the membrane domain to release soluble βA4 from the cell. This enzyme cleaves at residue 711 or 713 (APP_{770}) to generate $βA4_{1-40}$ or $βA4_{1-42}$. γ-secretase also cleaves the 83 amino acid C-terminal fragment resulting from α-secretase cleavage. This releases the approximately 3 kDa C-terminal fragment of βA4 (designated p3) from the cell. P3 is not amyloidogenic.

β- and γ-secretase activities occur at several sites within the cell. It is assumed that a small proportion of the full-length, cell surface APP undergoes β-secretase cleavage (Anderson *et al.* 1992), and that the resulting 99 residue C-terminal fragment is degraded to βA4, as well as other fragments, in the endosome/lysosome system. A proportion of the APP that undergoes endocytosis from the cell surface is processed by β- and γ-secretase to βA4. This can be shown by radioiodinating cell surface APP and allowing reinternalisation, within 15 minutes radioiodinated βA4 is released from the cell. (Koo and Squazzo, 1994)

There is also evidence that suggests βA4 is generated in the ER and Golgi during early processing of APP. (Cook *et al.* 1997; Hartmann *et al.* 1997) Furthermore, it appears that the longer forms of βA4 ($βA4_{1-42/43}$) are generated in the ER, while $βA4_{1-40}$ is generated in the Golgi. (See *Figure 1.2*)

1.4.3 APP Regulation

APPsα secretion is markedly increased by stimulation of the phospholipase C / protein kinase C (PKC) pathway. (Buxbaum *et al.* 1992; Nitsch *et al.* 1992) It is also enhanced upon electrical stimulation of neurones. (Nitsch *et al.* 1993) There is some evidence that this increase in APPsα secretion decreases the production of βA4 (Hung *et al.* 1993) however this does not appear to be the case in neuronal cells. (Leblanc *et al.* 1998)

Treatment of human umbilical vein endothelial cells (HUVECs), endothelial cells, and neurones with interleukin-1 (IL-1) upregulates APP mRNA production, though it does not have this affect in glial cells. (Lahiri and Nall, 1995; Forloni *et al.* 1992; Goldgaber *et al.* 1989). This IL-1 regulation of APP mRNA appears to occur via a pathway mediated by PKC, (Forloni *et al.* 1992) indicating that this second messenger pathway is important in both APP expression and secretion.

1.4.4 APP Expression

As previously stated, APP_{695} is the predominant isoform found in neuronal cells, while APP_{751} and APP_{770} are the predominant isoforms in glial cells and throughout the other tissues of the body. (Rohan de Silva *et al.* 1997)

1.4.4.1 Changes in APP Expression

Increased levels of APP mRNA and protein may be a normal feature of ageing. Senescent human fibroblasts contain a significant increase in all 3 isoforms of APP when compared to fibroblasts in culture at early passage, and this increase in expression is reflected at the protein level. (Adler *et al.* 1991)

APP mRNA levels are also increased after a number of cellular insults both *in vivo* and *in vitro*, including neurotoxic damage by kainic acid and serum deprivation. (Sudoh *et al.* 1996; Sola *et al.*

1993; Adler *et al.* 1991). Human fibroblasts rendered quiescent by serum deprivation showed increased total levels of APP mRNA when compared to proliferating cells, this increase appeared to consist of increases in all isoforms. (Adler *et al.* 1991) More specifically, it is levels of KPI-containing APP isoforms that are increased. (Sudoh *et al.* 1996; Sola *et al.* 1993) Intracerebroventricular application of kainic acid to rat brain resulted in a large increase in APP_{770} mRNA when examined by *in situ* hybridisation, and a weaker increase in APP_{751} mRNA. (Sola *et al.* 1993) Serum deprivation induced increases in total APP mRNA in rat C6 glioma cells. This increase was entirely due to an increase in APP_{770}, while APP_{695} mRNA levels decreased slightly. Levels of APP_{751} mRNA did not alter. (Sudoh *et al.* 1996)

1.4.4.2 APP Expression in AD

There appears to be an overall increase in APP expression in the brains of individuals with AD. *In situ* hybridisation in the pyramidal neurones of the hippocampus shows this increase in APP mRNA to be largely due to an increase in the KPI-containing APP isoforms. (Johnson *et al.* 1990) Northern blot and ribonuclease protection assays of the frontal cortex of AD patients also show an increase in KPI-containing APP. (Tanaka *et al.* 1989; Tanaka *et al.* 1988) *In situ* hybridisation shows an increase in APP mRNA in the nucleus basalis of Meynert of brains of patients with AD, (Cohen *et al.* 1988) though in this area the increase appears to be in APP_{695}. (Palmert *et al.* 1988) The evidence for an increase in APP mRNA in AD is not unanimous, Harrison *et al.* 1991 found no increase when they compared FAD cases with controls.

The relative amounts of each APP protein have also been measured in control and AD brains. Analysis of the total purified APP protein from one hemisphere of either control or AD brains, by both trypsin inhibition and by reflectance analysis of Western blots, showed KPI-containing APP

protein levels to be significantly increased in AD brain when compared to controls. (Moir *et al.* 1998) Immunohistochemical analysis of the temporal and hippocampal regions showed the increase in APP_{751} / APP_{695} ratio to correlate with the density of SPs in the brain region. The same study also showed that the dystrophic neurites associated with many SPs are immunopositive for KPI-containing APP. (Hyman *et al.* 1992) It has been suggested that expression of the KPI domain reduces α-secretase cleavage and increases βA4 production. (Ho *et al.* 1996)

These data suggest that it is a specific increase in KPI-containing APP isoforms that is a significant event in AD pathology, and that it is possible that APP_{751} is the more important isoform.

1.4.5 Amyloid Precursor like Proteins

There is evidence that APP is one member of a highly conserved family of proteins, with APP-like molecules in evolutionary distant organisms, including *C. elegans* and *Drosophila melanogaster*. Two proteins with a high sequence homology to APP (designated amyloid-precursor-like-proteins 1 and 2, APLP1 and APLP2) have been identified in the mouse brain. (Slunt *et al.* 1994; Wasco *et al.* 1992) Human APLP2 homologues of both APLP2 and, more recently, APLP1 have also been identified. (Lenkkeri *et al.* 1998; Webster *et al.* 1995) APLP2 is present in brain cerebrospinal fluid and conditioned media, and appears to undergo similar processing to APP. (Lyckman *et al.* 1998) APLP1 and 2 are increased in the neurones, blood vessels and dystrophic neurites associated with SPs in AD brain. (McNamara *et al.* 1998) Many APP antibodies cross-react with APLP2 (Webster *et al.* 1995; Slunt *et al.* 1994), though the latter protein does not contain the βA4 sequence and is unlikely to be involved in AD pathology.

1.4.6 APP Functions

The function of APP is unclear, both in the nervous system and in the rest of the body. It is apparent that the secreted derivative, APPsα, and the full length protein have specific properties, with full length APP potentially acting as a receptor, while the truncated form acts on other cells via as yet unidentified receptors.

KPI-containing APP proteins are effective protease inhibitors. (Kitaguchi *et al.* 1988). The secreted form of APP_{751} is identical to protease nexin-II, an inhibitor of factor XIa, and a serine protease involved in the coagulation cascade. (Smith *et al.* 1990) Protease nexin-II is released upon platelet aggregation, suggesting that APP_{751}s could be involved in wound repair. (Van Nostrand *et al.* 1990)

APPsα affects intracellular calcium levels in cultured neurones. (Mattson *et al.* 1993a) Resting calcium levels are lowered, leading to protection against glutamate mediated excitotoxicity. (Mattson *et al.* 1993b) The protein can also activate potassium channels, and so hyperpolarize the neuronal membrane, suppressing action potentials. (Furukawa *et al.* 1996). APPsα can lead to increased synaptic density and associated increased memory retention when infused into rat brains. (Roch *et al.* 1994)

The function of full length APP is, if anything, even less clear. Full length APP_{695} is markedly induced during neuronal differentiation, abundantly expressed in adult neurones, and transported within axons by the fast anterograde system to nerve terminals. (Lyckman *et al.* 1998; Koo *et al.* 1990) The holoprotein may be involved in neurite outgrowth or synaptic formation and maintenance. It may also be involved in cell-cell or cell-matrix interactions, in which it may promote neurite outgrowth. (Koo *et al.* 1993; Ghiso *et al.* 1992) In agreement with the results

obtained when administering APPsα to adult rats, transgenic rats expressing a 1.5 fold increase in APP_{695} over endogenous APP showed an increase in synaptic density. (Mucke *et al.* 1994) However, transgenic mice expressing a 5 fold increase in human APP_{751} exhibited normal learning and memory until 9-10 months of age, when they became impaired in these tasks. (Moran *et al.* 1995)

APP clearly has several roles in the central nervous system, however mice, which lack the APP gene, do not show a dramatic neuronal phenotype. Lack of the gene does not confer early mortality, or an increased morbidity. Young APP null mice exhibit a mild reduction in locomotion, and reduced forelimb grip strength, and deficits in cognitive function and long-term potentiation become evident in an age-dependent manner. (Zheng *et al.* 1996; Zheng *et al.* 1995) Neurones from these mice, cultured at birth, show reduced viability and a reduction in neurite outgrowth. The lack of a vital consequence of APP deletion may be due to the fact the APP homologues compensate for the missing APP proteins. APP and APLP2 have similar turnover kinetics and cell trafficking profiles, indicating that they may have similar roles. (Lyckman *et al.* 1998) This theory is supported by evidence from cells from APP knock-out mice in which APLP2 is up-regulated in response to neurotoxic assault from βA4. (White *et al.* 1998; see Sisodia and Gallagher, 1998 for a review of APP function.)

1.5 PRESENILINS

1.5.1 Properties

Presenilins 1 and 2 are highly homologous proteins with six to eight transmembrane regions. Hence both the N- and C-terminals face the cytoplasmic side of the membrane, as does the large hydrophilic loop between the sixth and seventh transmembrane regions. Cellular levels of the full length proteins are low, due to a constitutive proteolytic cleavage site within this loop. (Li and

Greenwald, 1998) Cleavage generates stable N- and C-terminal fragments, which, once produced, form stable high molecular weight complexes, the levels of which appear to be highly regulated. (Thinakaran *et al.* 1997) The constitutive cleavage occurs in the ER, once formed the fragments are transported to the Golgi. (See Selkoe, 1998 for review)

1.5.2 Functions

PS1 and PS2 are expressed ubiquitously and at comparable levels in most human tissues, including brain. PS1, however is expressed at higher levels in the developing brain, and its expression is very similar to the expression of Notch, a protein important in cell fate and neurogenesis. (Lee *et al.* 1996) Further evidence that PS1 may be involved in the Notch signalling pathway is the fact that it is highly homologous to the *Caenorhabditis elegans* protein SEL-12 (Berezovska *et al.* 1997), a facilitator of LIN-12/Notch signalling during the determination of cell fate in development. (Levitan and Greenwald, 1995) Wild-type human PS1 can rescue the SEL-12 mutant phenotype in *C. elegans*, though most PS1 mutations linked to AD confer only partial functional recovery. (Levitan *et al.* 1996) PS2 may also be involved in this pathway, as its expression is similar to that of PS1 and Notch, although it has also been implicated in apoptosis. (Wolozin *et al.* 1996)

PS1 N- and C-terminal fragments form stable complexes with β-catenin, an important molecule in the Wnt signalling pathway. (Murayama *et al.* 1998; Yu *et al.* 1998) AD causing PS1 mutations decrease the stability of β-catenin in both cultured cells and transgenic mice, also the brains of AD patients bearing such mutations contain a significantly decreased level of β-catenin. (Zhang *et al.* 1998) Loss of β-catenin signalling in neurones leads to increased vulnerability to βA4 induced apoptosis, implying that one way in which PS1 mutations are pathogenic, is by decreasing β-catenin levels and rendering neurones vulnerable to βA4. (Zhang *et al.* 1998)

Other lines of evidence suggest that PS1 mutations lead to mistrafficking of β-catenin, which may also have pathogenic implications for AD. (Nishimura *et al.* 1999)

Deletion of the gene encoding PS1 produces a lethal phenotype characterised by severely disordered somatogenesis and axial skeletal development. Both normal and AD-linked mutant PS1 can rescue this phenotype, indicating that PS1 mutations do not confer loss of function.

1.5.3 The Presenilins and βA4

The AD-linked mutations found in PS1 and PS2 (over 50 to date) are all missense mutations. Cellular and transgenic studies have shown these mutations to lead to an approximate two-fold increase in $\beta A4_{1-42}$. (Citron *et al.* 1997; Mann *et al.* 1996; Duff *et al.* 1996; Scheuner *et al.* 1996) This is reflected in the observation that brains of patients bearing PS1 mutations contain approximately two-fold more $\beta A4_{1-42}$ specific SP, when compared with sporadic AD brains. (Lemere *et al.* 1996)

1.5.3.1 γ-Secretase and the Presenilins

AD causing mutations in the presenilin genes lead to an increase in $\beta A4_{1-42/43}$ production. (Tomita *et al.* 1997; Citron *et al.* 1997; Scheuner *et al.* 1996; Duff *et al.* 1996) There is no associated change in APP expression, and its cleavage by α- and β-secretase does not appear to be affected. Cells from mice lacking PS1 show a 70-80% decrease in βA4 production, together with an increase in both the 83 and 99 residue C-terminal fragments resulting from β-and α-secretase. Together, this evidence indicates that PS1 is crucially involved in γ-secretase cleavage of APP, and that mutations in the presenilins selectively enhance the cleavage of βA4 after residue 42/43.

There is some evidence, based on coprecipitation experiments, that the presenilins and APP form complexes. (Weidemann *et al.* 1997) This may regulate the access of γ-secretase to the peptide bond and so determine the cleavage site. However, other investigators have failed to find presenilin / APP complexes, suggesting that the presenilins may regulate trafficking of either APP or γ-secretase to each other, without interacting directly with APP. (Haass and Selkoe, 1998; see Selkoe, 1998 for a review of presenilin and APP processing).

1.6 HEAD TRAUMA AND AD

As well as gene mutations, a significant risk factor for AD is head trauma. (Gentleman and Roberts, 1992; Graves *et al.* 1990) On examining the brains of patients who survived for between four hours and 2.5 years after a severe brain trauma, cortical βA4 deposits were seen in 30% of the cases. Additionally, APP immunoreactivity was increased in neurones in the vicinity of these deposits. (Roberts *et al.* 1994)

1.6.1 Dementia Pugilistica

Professional boxers often suffer from a dementing syndrome, dementia pugilistica, as a result of repeated blows to the head. This disease is characterised by neuronal loss, cortical NFTs and diffuse βA4 plaques, as well as other symptoms. The NFTs present in dementia pugilistica are indistinguishable form those found in AD brain. (Allsop *et al.* 1990)

Together, these data imply that severe or repeated head injury could induce an increased expression of APP, which in turn could lead to the insoluble βA4 deposits which characterise AD.

1.7 THE INFLAMMATORY RESPONSE AND AD

Although AD is not associated with inflammation, there are many characteristics of the AD brain which are associated with the acute phase immune response, not least of which are the activated microglia found associated with SPs. The SPs themselves are immunopositive for a variety of inflammatory mediators, including acute-phase proteins, cytokines, cell adhesion molecules and virtually the full range of activated classical pathway complement proteins. (Eikelenboom *et al.* 1998; Rogers *et al.* 1993) βA4 can bind the complement protein C1q *in vitro* and so activate the complement pathway. (McGeer and McGeer, 1996) In the presence of interferon-γ βA4 can activate microglia, which may in turn lead to neurone injury. (Meda *et al.* 1995)

While this data does not elucidate the aetiology of AD, it does provide targets for treatment strategies. Patients taking anti-inflammatory drugs on a regular basis (such as rheumatoid arthritis patients) have a lower frequency of AD, indicating that these drugs may have a therapeutic effect in the disease. Initial trails of the nonsteroidal anti-inflammatory (NSAID) drug indomethacin had some positive results. (Rogers *et al.* 1993)

1.8 THE STRESS RESPONSE AND AD

1.8.1 Heat Shock Proteins

All organisms respond to stress by inducing the synthesis of a highly conserved group of proteins called the heat-shock proteins (HSPs). The most well-characterised response is that induced by hyperthermic shock, though these proteins can also by induced by anoxia, ethanol, certain heavy metal ions, and free radicals. (Lindquist and Craig, 1988; Lindquist, 1986) These stress situations initiate abnormal protein configurations or denaturing of proteins. HSPs may bind to these abnormal proteins preventing aggregation, or facilitating a renaturation or repair process. HSPs may also target abnormal proteins for breakdown by proteolysis. (Hamos *et al.* 1991) Prior

induction of HSPs may serve to protect cells against a variety of insults, including periods of ischemia and excitotoxicty. (Chopp, 1993)

Some HSPs are constitutively expressed in a variety of cell types. In these cells the role of HSPs appears to be diverse, including DNA replication, transport of proteins across membranes, binding of proteins in the endoplasmic reticulum and uncoating clathrin-coating vesicles. (Lindquist and Craig, 1988) They may also assist in the folding of newly formed, nascent, proteins. (Hamos *et al.* 1991)

1.8.2 The Stress Response in the AD Brain

There are several indications that neurones in the AD brain may be in a state of stress. Protein production and processing is altered, with a decrease in overall protein synthesis, changes in protein phosphorylation and a disruption of the cytoskeleton. The NFTs contain hyperphosphorylated tau, and have also been shown to contain increased levels of ubiquitin. (Mori *et al.* 1987) This is a highly conserved, ubiquitously expressed protein, which is induced by stress in all cells. (Lindquist and Craig, 1988)

SPs also contain members of the heat shock family of proteins, specifically heat shock proteins 72 and 73, increased levels of these proteins are also found in NFTs. (Perez *et al.* 1991; Hamos *et al.* 1991) Interestingly, HSP72 was found exclusively in SPs and NFTs while another HSP, glucose regulated protein, or GRP78 was increased only in neurones that remain cytologically normal. This could indicate that HSP72 induction is an early response to the formation of abnormal proteins, perhaps targeting them for breakdown, while GRP78 protects cells from the neurotoxic effects of these abnormal proteins. (Chopp, 1993; Hamos *et al.* 1991) HSP mRNA is also increased in the frontal cortex of AD patients, as well as in other neurodegenerative

disorders. (Harrison *et al.* 1993) This increase did not correlate with pre-mortem factors such as pyrexia or coma, supporting the existence of a stress response in AD brains.

1.8.3 APP and the Heat Shock Response

The increase in HSP levels in response to stress is controlled at the transcription level. A transcriptional activator, the heat shock factor, binds to a short highly conserved region of the heat shock gene, known as the heat shock element (HSE). (Sorger, 1991) The promoter region of the APP gene contains a sequence homologous to the HSE, suggesting that APP levels may also be changed in response to cellular stress. (Salbaum *et al.* 1988)

A number of studies into the effect of heat shock and other means of stress on APP levels have been carried out in a variety of cell types, including PC12 neuronal cells, HUVECs and human lymphoblastoid cells. Periods of increased temperature were shown to decrease APP phosphorylation levels, induce the production of additional APP polypeptides (Johnson *et al.* 1993a; Johnson *et al.* 1993b), increase levels of APP mRNA (Ciallella *et al.* 1994; Abe *et al.* 1991), increase total cellular levels of APP, and increase levels of APP secretion (Ciallella *et al.* 1994). In human embryonal carcinoma cells transiently transfected with a region of the APP gene promoter, APP transcription was shown to be stimulated by heat shock, ethanol and sodium arsenite treatment. (Dewji *et al.* 1995) It is also interesting to note that a period of heat shock induced increased phosphorylation of tau in neuronal PC12 cells. (Johnson *et al.* 1993b)

These results suggest that some of the pathological changes seen in AD may be a result of the stress response. If neuronal cells under cytological stress upregulate APP, it is possible that one of the effects of this would be to increase levels of βA4. As βA4 may be neurotoxic, it is reasonable to suppose that cells exposed to increased levels of the peptide would express signs

of being in a state of cellular stress, and as a result increase levels of APP, leading to a vicious circle of increased βA4 production. (See *Section 1.9*)

1.9 βA4 INVOLVEMENT IN AD

As has been previously stated, the spread of AD pathology appears to follow the path of cortical connectivity, suggesting it is propagated by some agent, which is produced by the cortical neurones. (Pearson and Powell, 1989) βA4 is the most popular candidate for this agent, with the "amyloid cascade hypothesis" suggesting that the neurotoxic effects of βA4 are responsible for cell death. (Hardy and Higgins, 1992)

There are many lines of evidence to back up this hypothesis (see *Figure 1.3* for a summary). As previously described, βA4 has neurotoxic properties. APP is upregulated in response to cellular stress of many varieties, and it is reasonable to assume that an increase in APP will lead to an increase in secreted βA4. In this way the pathology could be propagated along cortical pathways, with increased levels of βA4 leading to cellular stress and upregulation of APP, which in turn would lead to increased levels of βA4.

βA4 is critical in the pathogenesis of EOFAD. Mutations in APP, PS1 and PS2 all increase βA4 production, with mutations in the presenilins specifically increasing the more toxic form $\beta A4_{1\text{-}42/43}$. ApoE appears to be involved in βA4 disposal or deposition. (LaDu *et al.* 1994) Patients with one or two ε4 alleles have a significantly higher density of both plaque and vascular βA4 deposits in their brains than other AD patients. (Hyman *et al.* 1995; Schmechel *et al.* 1993)

Head trauma, a major risk factor for AD, increases APP expression, and leads to βA4 deposits in the brain. (Roberts *et al.* 1994) It has also been shown that there is an inflammatory element to

the AD brain, and that this response can be propagated by βA4. These data serve to support the amyloid cascade hypothesis, by showing that increased levels of βA4 lead to increased expression of APP, possibly by several mechanisms. βA4 can activate the complement pathway, leading to increased cytokines such as IL-1, which regulates APP expression and production. The acute phase response leads to increased free radicals, which could be neurotoxic, leading to initiation of the stress response and increased levels of APP. (See *Figure 1.3*)

Since increases in soluble levels of βA4 appear to be pivotal in FAD, it is proposed that βA4 is also increased in sporadic AD (SAD). It is therefore important to study APP processing, to identify events or conditions in the normal brain that could lead to a build up of βA4. As it has been reported that expression of APP_{751} can lead to increases in βA4 production, study of the processing of this APP isoform could lead to greater understanding of the aetiology of SAD.

1.10 METHODS OF STUDYING AD

In 1995 the first successful transgenic mouse model for AD was developed. (Games *et al.* 1995) The animals express a human APP mini-gene, encoding the codon 717, valine to phenylalanine mutation. The animals exhibit AD pathology, and show learning defects. (See Price *et al.* 1998 for a review of null and mutant APP transgenic mice) Transgenic animals for the presenilin mutations have now also been developed. These animals are undoubtedly useful for examining the pathogenesis of FAD, and will be vital in testing new treatment strategies. However, they have told us little about the aetiology and pathogenesis of SAD at present.

The transgenic animals provide a useful tool to identify proteins and processes of potential interest in SAD, but to understand this disease these processes must be examined using the

wild-type proteins, comparing them in control and SAD brain and other tissue. Ways of doing this include the examination of post-mortem human brain tissue, primary cells lines derived from AD and control patients, and the manipulation of established cell lines. All these techniques, however, have their drawbacks.

Human brain tissue, both control and AD, is difficult to obtain, and often there is a long delay between the time of death and the tissue being available to researchers. This post-mortem delay can result in changes in the brain chemistry, leading to misleading and confusing results from biochemical tests.

Cell lines are easier to obtain and work with, but they also have drawbacks. APP expression varies with species, and between tissues within each species. This means that each cell line will express different isoforms of APP and may use different processing pathways. To obtain the best model of the APP expression in the human brain it is, therefore, desirable to work on human neurones. However neurones are post-mitotic, and therefore cannot be kept in continuous culture. It is possible to work with primary cultures of human neurones but these are difficult to obtain and manipulate.

1.11 NTERA2 CELLS

The cell line NTera2 (NT2) is derived from a human teratocarcinoma. Cells from these carcinomas have the appearance of embryonic stem cells. NT2 cells can be induced to differentiate into a number of phenotypes by incubating them with retinoic acid. (Andrews *et al.* 1985; Andrews *et al.* 1984) Among these phenotypes are cells with the characteristics of central nervous system neurones. These cells (NT2N cells) are post-mitotic, and express neuronal markers, such as neurofilament proteins and synaptophysin. (Ackerman *et al.* 1994) After

retinoic acid differentiation the neuronal precursor cells can be selectively plated out, and incubated with mitotic inhibitors, to give cultures of neurones with over ninety-nine percent purity. (Pleasure *et al.* 1992) After one to two weeks in culture the cells form bundles of cell bodies with complex systems of axons and dendrites connecting the bundles. It has been shown that functional synapses form between the cells. NT2 cells are trisomic for several chromosomes, including chromosome 21. In consequence both stem and neuronal cells secrete significant amounts of APP. Previous work on the mRNA of these cells has shown that the stem cells express predominantly APP_{770}, with some APP_{695}; while NT2N cells express predominantly APP_{695}, with some APP_{751}.

These criteria make NT2 and NT2N cells a good model for studying at least one of the processes implicated in the pathology of AD. They are easy to culture and manipulate. They are human neuronal cells, and therefore are a better model of the human brain than any other cell line. They express endogenous APP, without the need to transfect the cells and possibly disrupt the normal chemical pathways within the cells. Also the neuronal cells express the same APP isoforms as found in the brain.

1.12 HYPOTHESES

The work presented in this thesis was designed to test the following hypotheses:

NTera2 neuronal cells make a good *in vitro* model of human CNS neurones with regard to the expression and processing of APP. Upon differentiation from the stem cell to the neuronal phenotype, APP expression will change and alternative processing pathways for the protein will be activated. In contrast to the stem cells, neuronal cells will possess amyloidogenic means of APP breakdown.

NTera2 neuronal and stem cells will respond to cellular stress in the same manner as human and animal cell lines previously tested. Cellular stress will induce a "heat-shock response", as a result of which APP, specifically the KPI-containing isoforms, will be upregulated. As a component of this response APP processing pathways may be altered.

1.13 AIMS OF THE WORK PRESENTED IN THIS THESIS

- To develop a method of producing NTera2 neuronal cells
- To characterise the APP expression and production in NTera2 stem and neuronal cells
- To develop a method of subjecting NTera2 stem and neuronal cells to cellular stress
- To investigate the effects of this stress on APP expression and processing

Figure 1.1 APP STRUCTURE AND CLEAVAGE SITES

The diagram shows the processing of APP_{770}. APP_{751} and APP_{695} are processed by the same secretase activities, though the secreted product lacks either the OX-2 domain in the case of APP_{751}, or both the OX-2 and KPI domains in the case of APP_{695}.

a) Full length APP_{770} (not to scale)

There is a 17 amino acid signal sequence at the N-terminal. APP_{770} contains both the 56 amino acid KPI region (coded for on codon 7) and the 19 amino acid MRC OX-2 domain (coded for on codon 8). The βA4 sequence (encoded by parts of codons 16 and 17) occupies amino acids 671-713, the last 14 of which are part of the transmembrane domain (the membrane is represented by the hatched box)

b) α-secretase cleavage

α-secretase cleaves between amino acids 16 and 17 of βA4 to release the large extra-cellular domain from the cell. The resulting 83 amino acid C-terminal may then be cleaved by γ-secretase to generate p3.

c) β-secretase cleavage

β-secretase cleaves at the N-terminus of βA4, releasing a truncated APPs from the cell. The 99 amino acid C-terminal fragment is then cleaved by γ-secretase to release βA4.

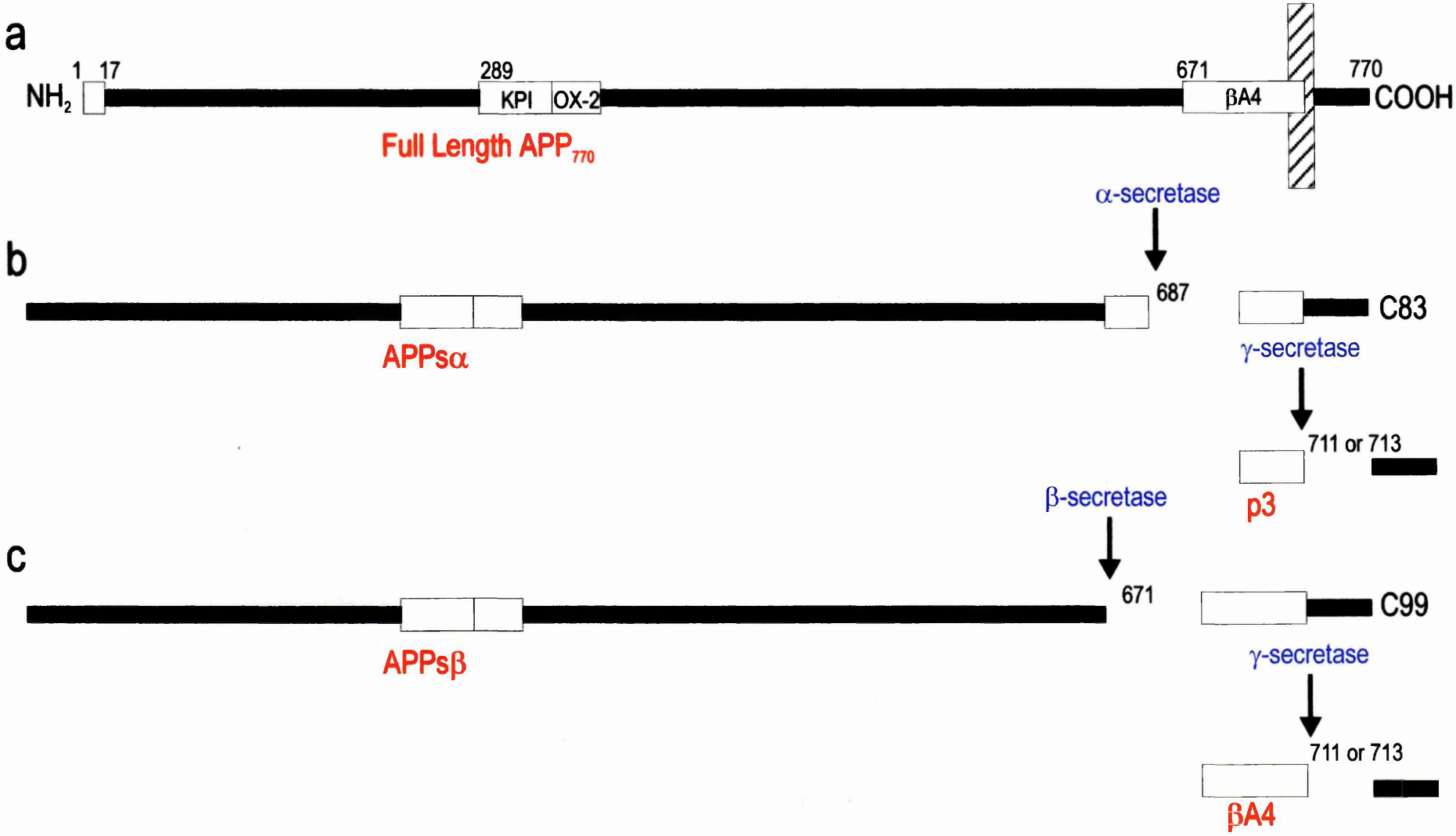

a
1
17
289
671
770
NH2
KPI
OX-2
βA4
COOH
Full Length APP770
α-secretase
b
687
C83
APPsα
γ-secretase
711 or 713
p3
β-secretase
c
671
C99
APPsβ
γ-secretase
711 or 713
βA4

Figure 1.2 CELLULAR PROCESSING OF APP AND POTENTIAL SITES OF βA4 PRODUCTION

APP is matured through the central secretion pathway, with a proportion of the full length protein that reaches the cell surface being cleaved by either α- or β-secretase. The resulting C-terminal fragments are re-internalised via clathrin coated vesicles to early endosomes, where they may be cleaved by γ-secretase to generate p3 and βA4, which are then released from the cell. Alternatively, the C-terminal fragments may be degraded in late endosomes and lysosomes, a process that may also generate βA4.

A proportion of the full length, cell surface APP is also re-internalised in clathrin coated vesicles. This protein may be recycled to the cell surface, cleaved in the early endosomes by any of the 3 secretase activities, or degraded in the endosomal / lysosomal pathway.

Finally, a proportion of APP is cleaved by β- and γ-secretase as it matures through the ER and Golgi. In this system, the ER is the major site of $\beta A4_{1\text{-}42/43}$ production, while the majority of $\beta A4_{1\text{-}40}$ is produced in the Golgi.

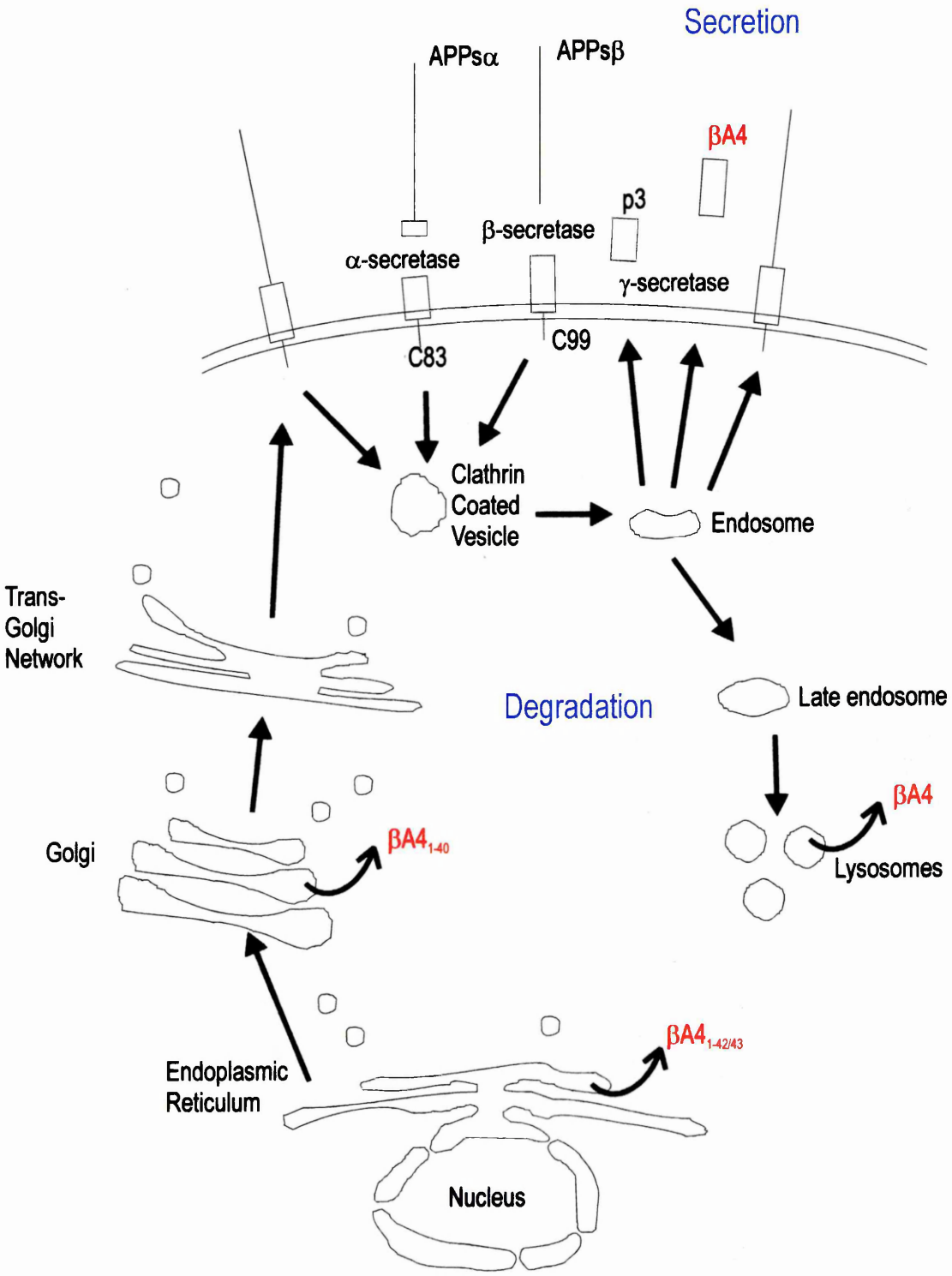
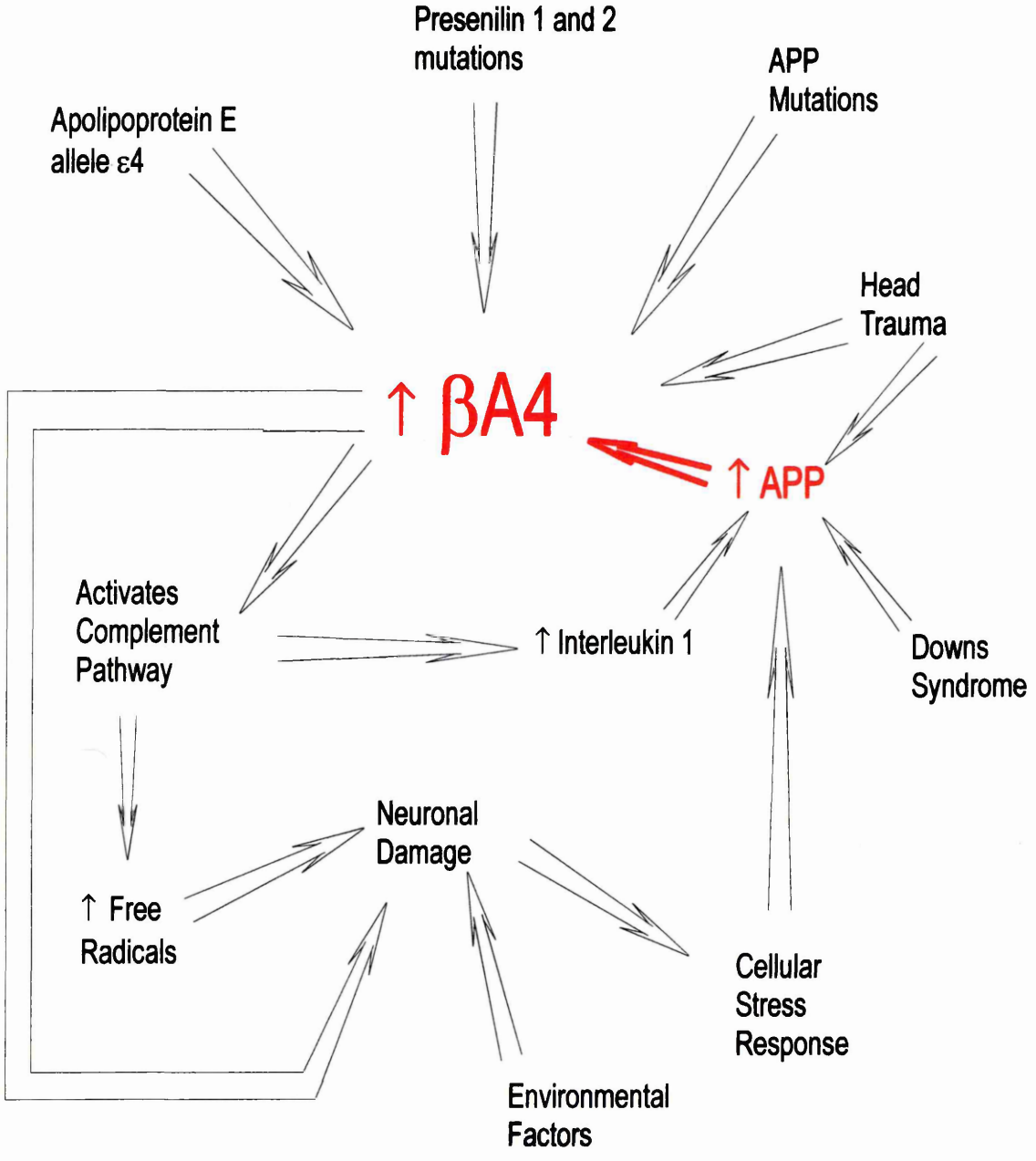

Secretion
APPsα
APPsβ
βA4
p3
β-secretase
α-secretase
γ-secretase
C83
C99
Clathrin
Coated
Vesicle
Endosome
Trans-
Golgi
Network
Degradation
Late endosome
βA4
Lysosomes
Golgi
βA4 1-40
Endoplasmic
Reticulum
βA4 1-42/43
Nucleus

Figure 1.3 FACTORS WHICH COULD LEAD TO INCREASED βA4 IN THE AD BRAIN

A schematic diagram to show the possible causes of increased APP and βA4 in the AD brain, and the "vicious circle" nature of these increases, providing support for the amyloid cascade hypothesis.


Presenilin 1 and 2 mutations
APP Mutations
Apolipoprotein E allele ε4
Head Trauma
↑ βA4
↑ APP
Activates Complement Pathway
↑ Interleukin 1
Downs Syndrome
Neuronal Damage
↑ Free Radicals
Cellular Stress Response
Environmental Factors

Chapter Two - Materials And Methods

Appendix A contains details of the solutions used and suppliers.

2.1 CELL CULTURE

NTera2/clone D1 cells were obtained from Prof. P. Andrews of the University of Sheffield.

2.1.1 NT2 Stem Cells

NT2 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% heat inactivated foetal bovine serum (FBS) and penicillin/streptomycin (P/S) at 37°C, 10% CO_2 / 90% air in a humid environment. Confluent cultures were split 1:3 (resulting in approximately 4 x 10^6 cells seeded per T75 flask (Falcon)) using a non-enzymatic salt solution (cell dissociation solution) or by dislodging with glass beads (Andrews *et al.* 1985). Trypsin/EDTA (TE) was not used as it may promote stem cell differentiation.

The beads (BDH) were 3 mm in diameter and had been treated overnight with concentrated hydrochloric acid (HCl), neutralised with concentrated sodium hydroxide (NaOH), washed overnight with tap water, and finally rinsed with dH_2O. The beads were then decanted into test tubes with foil caps and autoclaved in batches immediately prior to use. Half the media was decanted from the flask and 10-20 sterile glass beads added. The beads were rolled over the surface of the flask to dislodge the cells. The resulting cell suspension was then pipetted into fresh culture flasks and fresh complete media (cDMEM) added.

Cell viability was assessed visually the day following the cell split. Healthy NT2 cells adhere to the culture vessel, while dead or damaged cells detach and are visible floating in the media. Cell viability was invariably greater than 95%.

2.1.2 NT2N Cells

Details of the method of neuronal differentiation and purification are to be found in *Chapter 3.* Briefly, 3.5 x 10^5 NT2 cells were seeded in a T75 flask (Falcon) and fed weekly with cDMEM containing 10^{-5} M retinoic acid (RA) for 24 days. The cultures were then dislodged with TE and split 1:3 into cDMEM containing mitotic inhibitors (cDMEM+I). These cultures were fed twice weekly with cDMEM+I for 14 days. After this time the neuronal cells could be seen as phase bright cell bundles lying on top of a layer of non-neuronal cells, these cell bundles could be detached by shaking the culture vessel. Once in suspension the cells can be transferred to culture vessels pre-treated with poly-D-lysine and Matrigel. Although at this point it was not possible to obtain accurate cell counts, each experiment was carried out on a single batch of NT2N cells, resulting in comparable numbers of cells per flask.

Cultures of NT2N cells were fed weekly with cDMEM and maintained in 10% CO_2 / 90% air in a humid environment. Cell viability was assessed visually on the day following the replating, and twice weekly thereafter. Healthy NT2N cells elaborate cell processes, and both processes and cell bodies adhere to the culture vessel. Non-viable cells do not elaborate processes, and ultimately the cell bodies detach from the culture vessel. Any cultures with less than 90% viability were discarded.

2.1.3 Coating Flasks/Slides

2.1.3.1 Poly-D-Lysine

One hundred μg/ml stock was diluted prior to use dilute 1:10 with sterile dH_2O. Approximately 50% of the usual medium volume was added to the vessel (enough to cover bottom) and left for at least 2 hours. The fluid was then aspirated off and the vessel allowed to air dry.

2.1.3.2 Matrigel

Twenty µl of Matrigel diluted 1:36 with DMEM was added to the poly-D-lysine treated vessel and spread evenly over the surface with a fire polished and shaped Pasteur pipette. Excess liquid was aspirated off and the vessel allowed to air dry. This step was repeated immediately prior to seeding the cells.

2.1.4 Cell Photography

Unless otherwise stated, all cells were viewed and photographed using an Olympus CK2 microscope, using the x20 objective lens. Photographs were taken using a Polaroid Micro SLR camera loaded with Polaroid 667 film.

2.2 PREPARATION OF CELL SOLUBLE AND MEMBRANE PROTEINS

Media was aspirated from a T25 culture flask and 10 ml DMEM was added to wash the cells. Cells were removed by mechanical means (shaking in the case of neurones, scraping with glass beads or a disposable cell scraper in the case of stem cells) and the cell suspension centrifuged at 170 g (Baird & Tatlock auto bench centrifuge) for 10 minutes to pellet cells. From this point, all solutions and cell pellets or suspensions were kept on ice. The DMEM was aspirated and cells resuspended in homogenising buffer plus the following protease inhibitors: leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF). Unless otherwise stated cells from one T75, or 3 T25 flasks were homogenised in 1.0 ml buffer. The cell suspension was passed through 0.1 mm diameter capillary tubing 20 times, this process homogenised the cells by shearing. The homogenate was centrifuged at 100 g (Baird & Tatlock auto bench centrifuge) for 10 minutes to remove unbroken cells and nuclei, and then the supernatant was taken and centrifuged at 50,000 g (Sorval T21) for 1 hour. The supernatant was collected and used as the soluble fraction, the pellet of membrane proteins was resuspended in half the original volume of homogenising buffer with inhibitors and used as the membrane fraction.

The protein concentration of the sample was determined by a Bicinchoninic Acid (BCA) protein assay (see 2.5), and the samples mixed with an equal volume of sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer before running on an acrylamide gel.

2.3 PREPARATION OF WHOLE CELL LYSATES

The cells were harvested from a T25 flask by either tapping the culture vessel (NT2N) or by scraping with glass beads (NT2). Centrifuging at 100 g (Baird and Tatlock auto bench centrifuge) pelleted the cells, and the supernatant was collected. The cell pellet was washed in 5 ml DMEM and re-centrifuged. The supernatant was aspirated and the cell pellet dissolved in TRIS buffered saline (TBS) containing 0.1% sodium-dodecylsulphate (SDS) (unless otherwise indicated the cells from 1 T25 flask were solubilised in 100 µl TBS). The samples were heated to 60°C for 10 minutes and allowed to cool before Triton X-100 (Tx100) was added to a final concentration of 0.8% to neutralise the SDS, and the protein concentration of the sample determined by a BCA protein assay (see 2.5). The sample was mixed with an equal volume of SDS-PAGE sample buffer before running on an acrylamide gel. For cells grown in a 12 well plate, the conditioned media was collected from each well and the cells washed with 1 ml DMEM. 100 µl TBS containing 0.1% SDS was added directly to each well. The resulting samples were then pooled and treated as above.

2.4 PREPARATION OF MEDIA SAMPLES

Cell cultures were washed with approximately 5 ml DMEM per T25 flask and fresh cDMEM added (3 ml per T25 flask for 24 hour collections, 5 ml per T25 for 7 day samples). Cells were incubated for the required length of time, then the conditioned media decanted into tubes and stored at 4°C.

The samples were analysed by either SDS-PAGE (see *2.7*) followed by western blotting (see *2.10*) or by immunoprecipitation (see *2.11*) followed by SDS-PAGE and western blotting. Prior to analysis, the proteins in each sample were concentrated as follows. Samples comprising of conditioned cDMEM were concentrated by ammonium sulphate fractionation as described below (see *2.4.1*). Conditioned Optimem samples could not be treated in this way, as the total protein concentration was too low, so they were concentrated by ultra-centrifugation (see *2.4.2*). After concentration all samples were stored at -20°C.

Ammonium Sulphate Fractionation

Three hundred mg ammonium sulphate was added to 1 ml of media sample (Wertkin *et al.* 1993). This was mixed at room temperature for 1 hour, before being centrifuged at 600 g (Fisher Scientific force 7 microfuge) for 10 minutes. The supernatant was aspirated and the pellet resuspended in 100 µl TBS (resulting in a 10-fold concentration).

2.4.2 Ultra-Centrifugation

This was achieved using a centricon-10 (Amicon) with a 10 kDa molecular weight cut off point. Two ml of sample was added to the top chamber and spun at 5000 g (Sorval T21) until only 200 µl remained (approximately 1 hour), resulting in a 10-fold concentration.

2.5 BCA PROTEIN ASSAY

2.5.1 Introduction

Measurement of protein concentration was based on the biuret reaction. The disadvantage of some methods (such as the Lowry method) is that they cannot be used in the presence of non-ionic detergents and some buffer salts, which form insoluble precipitates with the Folin-Ciocalteau reagent. The BCA assay uses bicinchoninic acid to monitor the Cu^{1+} formed during the biuret

reaction. This can be used in the presence of 0.1 M TRIS or 1.0% SDS. (See Smith *et al.* 1985 for a full table of interfering compounds).

2.5.2 Method

A kit was purchased from Sigma and the recommended protocol followed. BCA reagent was prepared by mixing bicinchoninic acid solution with 4% copper sulphate solution in the ratio 50:1. Serial dilutions of the unknown samples were prepared along with a range of bovine serum albumin (BSA) standards from 0 to 1.0 mg/ml. 10 μl of each sample or standard was added to three wells of a 96 well plate and 200 μl BCA reagent added. The plate was incubated at 37°C for 30 minutes, allowed to cool to room temperature, then the optical density read at 570 nm with a multiscan plate reader (Labsystems).

A standard curve was plotted using a linear regression and the protein concentrations of the unknown samples calculated using Microsoft Excel. BSA standards were prepared in all experimental solutions used (see *Figures 2.1 and 2.2*). All solutions, except sample buffer with dithiothreitol (DTT), gave linear results with the BSA standards, therefore standards were routinely prepared in the same solution as the sample to be tested.

2.6 PREPARATION OF MONKEY BRAIN EXTRACT

As monkey brain is a rich source of APP, and this APP shows high homology with human APP, samples of this material were used as positive controls during western blotting and immunoprecipitations. Frontal cortex of monkey (*Macaca fasicularis*) brain, previously stored at -20°C (source: University of Washington Primate Centre, Seattle, Washington), was homogenised in 10 volumes of homogenising buffer, with protease inhibitors added as previously described (see 2.2). The homogenate was centrifuged at 170 g (Baird & Tatlock auto bench centrifuge) for 10 minutes to remove unbroken cells and nuclei, then the supernatant was taken and centrifuged

at 50,000 g (Sorval T21) for 1 hour. The supernatant was collected and used as the soluble fraction, the pellet of membrane proteins was resuspended in half the original volume of homogenising buffer with inhibitors, and this was then used as the membrane fraction. These stocks were stored at -20°C.

2.7 SODIUM-DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Samples were mixed with a buffer containing SDS and either 2-mercaptoethanol or DTT and heated to 90°C for 2 minutes to denature the proteins. The samples were then analysed on an acrylamide gel using the BioRad "mini-gel" system. All gels were 0.75 mm in thickness and 7.5% acrylamide, unless otherwise stated. Samples were loaded using either a 10 or 15 well comb. In the case of immunoprecipitation results or media samples 20 μl total volume was loaded. Immunoprecipitation results gave insufficient sample to determine the protein concentration, and the different protein concentrations of cDMEM and Optimem media did not allow direct protein concentration comparison. For cell samples the protein concentration of the sample was determined and either 5 or 10 μg total protein loaded (as indicated in the results section). In initial experiments, cell samples were loaded using the same volume in each lane, without correcting for protein concentration. The protein concentration was determined after the gels were run, but unfortunately insufficient of the sample remained for the gels to be duplicated with protein loading corrected. The gel was run in a TRIS/glycine buffer at a constant voltage, usually 100 V. (Adapted from Laemmli, 1970).

2.8 TRICINE GELS

The SDS-PAGE method of protein separation does not resolve proteins below 10 kDa molecular weight. To overcome this, a method using TRICINE in the running buffer was developed (Schagger and Von Jagow, 1987). Use of urea in the resolving gel instead of glycerol also

improves the resolution of low molecular weight proteins. By this method it was possible to clearly separate proteins with molecular weights as low as 4 kDa. As before, proteins were denatured in sample buffer, before being analysed on a 12% acrylamide, 0.75 mm mini-gel. Gels were run at a constant voltage of 80 V, and the gel tank was placed in a bowl of ice to prevent overheating of the gel.

2.9 PROTEIN TRANSFER TO MEMBRANE

Proteins were transferred to a nitro-cellulose (0.45 μ pore size; Amersham) or polyvinylidene difluoride (PVDF) (Bio-Rad) membrane in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% methanol, adjusted to pH 10.5 with sodium hydroxide (NaOH). (Adapted from Matsudaira, 1987).

2.9.1 Method

The gel was immersed in transfer buffer for about 5 minutes, as was the membrane (PVDF was pre-wet in methanol before immersing in buffer). Gel and filter were sandwiched together, with a pre-wet piece of filter paper on either side, ensuring that all air bubbles were excluded. The resulting sandwich was surrounded with foam pads and placed in a BioRad mini-gel transfer apparatus, ensuring that the gel was closest to the cathode. A frozen cooling pack was inserted into the tank, and the tank filled with buffer. The protein transfer was run for 40-60 minutes at 0.2 A constant current. After transfer the membrane was rinsed in TBS and stored in TBS at 4°C for up to 7 days, or wrapped in cling film and stored at -20°C.

2.10 WESTERN BLOTTING

2.10.1 Method

Membranes were blocked in blotto, a 5% solution of non-fat milk protein in TBS (see *Appendix A*), for 30 minutes at room temperature or overnight at 4°C. Blots were incubated with primary

antibody for 2 hours at room temperature, washed with three 5 minute washes in TBS with added Tween-20 (TBS-Tw), and incubated with secondary antibody for a further hour. Blots were then washed with one 15 minute and two 5 minute with TBS-Tw, plus a final 5 minute wash with TBS (for enhanced chemiluminescence, ECL) or alkaline phosphatase (AP) buffer and developed. All antibodies were diluted in blotto. Primary antibody concentrations are indicated in results, secondary antibodies were used at a concentration of 1:2000 unless otherwise stated.

2.10.2 Development

Antibodies conjugated to horseradish peroxidase (HRP) were visualised using ECL (Amersham), with the reagents prepared according to the manufacturers' instructions. Equal volumes of reagents 1 and 2 were mixed to give a final volume of 8 ml per blot. The blots were incubated in this solution for 5 minutes, before being transferred to a dark room where they were wrapped in cling film and exposed to x-ray film (X-OMAT, XAR-5, Kodak) for between 30 seconds and 30 minutes. The film was then developed using standard protocols (Kodak).

Antibodies conjugated to AP were visualised with nitro blue tetrazolium (NBT) / bromo-chloro-indolyl phosphate (BCIP) in AP buffer. Equal volumes of NBT and BCIP solutions were mixed to a total volume of 5 ml per blot. This was then added to the blot and incubated for up to one hour, with fresh NBT/BCIP solution applied after 30 minutes if necessary.

Table 2.1 Specificity And Source of Primary Antibodies Used in This Thesis

Antibody	Type	Specificity	Full Length or Secreted Protein	Source
22C11	Mouse monoclonal	APP_{695}, APP_{751}, APP_{770}, APLP2	Both	Boehringer Mannheim
993**	Rabbit polyclonal	APP_{751}, APP_{770}	Both	Produced in the lab
3B11	Mouse monoclonal	APLP2	Both	Kindly donated by M-T Webster
6E10	Mouse monoclonal	APP_{695}, APP_{751}, APP_{770}	Secreted only	Seneteck
EH4	Mouse monoclonal	Designed to recognise APP_{695}, APP_{751}, APP_{770}	Secreted only	Produced in the lab
FC8*	Mouse monoclonal	Designed to recognise APP_{695}, APP_{751}, APP_{770}	Secreted only	Produced in the lab
HD11*	Mouse monoclonal	Designed to recognise APP_{695}, APP_{751}, APP_{770}	Both	Produced in the lab
HE1*	Mouse monoclonal	Designed to recognise APP_{695}, APP_{751}, APP_{770}	Both	Produced in the lab

Table 2.1 (cont.) Specificity and Source of Primary Antibodies Used in This Thesis

Antibody	Type	Specificity	Full Length or Secreted Protein	Source
1032**	Rabbit polyclonal	βA4	N/A	Produced in the lab
58617**	Rabbit polyclonal	βA4	N/A	Produced in the lab
58618**	Rabbit polyclonal	βA4	N/A	Produced in the lab
6F/3D	Mouse monoclonal	βA4	N/A	Produced in the lab
HSP70	Mouse monoclonal	Constitutive and inducible forms of HSP70	N/A	Sigma

** these antibodies were produced and characterised as part of the investigations carried out in this thesis, and are further discussed in* Section 4.4.

*** these antibodies were produced and characterised by Dr D Parkinson prior to the work carried out in this thesis*

2.10.3 Determination of Molecular Weight

Pre-stained molecular weight markers (Sigma) were run on the SDS-PAGE / TRIS-TRICINE gels along with the samples. After western blotting the resulting x-ray film was lined up with the original nitro-cellulose or PVDF membrane and scanned (Umax Astra 1200S) into the Kodak

Digital Science 1D imaging program. Based on the mobility of the pre-stained standards, the program calculated the molecular weight of bands obtained by western blotting. The pre-stained markers used are detailed in *Tables 2.3 and 2.4.*

Table 2.3 Details of Secondary Antibodies Used in This Thesis

Antibody	Conjugate	Host Animal	Source
Anti-Mouse IgG	Alkaline Phosphatase	Goat	Sigma
Anti-Mouse IgG	Horseradish Peroxidase	Sheep	Boehringer Mannheim
Anti-Rabbit IgG	Horseradish Peroxidase	Sheep	Boehringer Mannheim

Table 2.2 Pre-stained Molecular Weight Markers Used For SDS-PAGE

Protein	Molecular Weight (kDa)
Triosephosphate Isomerase	26.6
Lactic Dehydrogenase	36.5
Ovalbumin	45.0
Pyruvate Kinase	58.0
Fructose-6-phosphate Kinase	84.0
β-Galatosidase	116.0

Table 2.4 Pre-stained Molecular Weight Markers Used For TRIS-TRICINE Gels

Polypeptide	Molecular Weight (kDa)
Triosephosphate Isomerase	26.6
Myoglobin	17.0
a-Lactalbumin	14.2
Aprotinin	6.5
Insulin Chain B, Oxidised	3.5
Bradykinin	1.1

2.11 IMMUNOPRECIPITATIONS

2.11.1 Introduction

Using immunoprecipitation it is possible to selectively concentrate a specific protein from a mixture. This has a threefold benefit; it can be used to concentrate a protein which is at a very low concentration; to pick out one particular protein from a sample which contains a high total protein concentration; or to separate one protein from a sample which contains many proteins of similar molecular weight to the protein of interest.

Primary antibody is added to the sample, which binds to the protein of interest. Agarose beads with specific proteins bonded to them are then added. The proteins on the beads bind to the antibody in the antibody-antigen complex. Centrifugation is used to pellet the beads, bringing the antibody-antigen complex with them. The supernatant is aspirated and the proteins eluted from the beads in SDS-PAGE sample buffer, prior to analysis.

There are several different agarose beads available for this procedure, each with a different protein bound to them, and tests were carried out to determine which type of bead was best for each antibody. In the case of rabbit antibodies agarose beads bound to protein A were used. Several different beads were tested for mouse antibodies including protein A, protein G and goat-anti-mouse-IgG antibodies. Protein A did not bind the mouse antibodies, while both protein G and the goat-anti-mouse-IgG beads did bind. However when samples recovered using protein G were analysed on a western blot proteins bands could not be distinguished due to smearing of the sample. (See *Figure 2.3)* This was found to be related to the secondary antibody used in the immunoblot and presumably was due to some protein G remaining in the sample and non-specifically binding to the secondary antibody. Therefore protein A beads were used for rabbit antibodies, and goat-anti-mouse-IgG beads for mouse antibodies.

2.11.2 Method

Samples were diluted in immunoprecipitation buffer if necessary. Unless otherwise stated, all samples were denatured with 0.1% SDS and heated to 60°C for 10 minutes. The sample was then cooled, centrifuged at 600 g (Fisher Scientific Force 7 microfuge) and Tx100 was added to 0.8%, volume/volume. The sample was then vortexed and the centrifugation repeated. Media samples were buffered with TRIS.HCl pH 7.4 to a final concentration of 15 mM. Primary antibody was added at concentrations stated and samples were mixed at room temperature for 2 hours. 50 µl of the appropriate agarose bead slurry (diluted to 50% with dH_2O) was added and the samples mixed for a further hour.

Tubes were centrifuged at 2000 rpm (Baird & Tatlock auto bench centrifuge) for 5 minutes and supernatant aspirated. One ml of TBS was added, and the tubes were mixed for 5 minutes to

wash the beads. The tubes were then re-centrifuged, the supernatant aspirated, and the beads eluted with SDS-PAGE sample buffer. Immunoprecipitates from 1 ml of initial media or cell sample were eluted in 100 µl of 1x SDS-PAGE sample buffer, resulting in a 10x concentration.

2.12 SERIAL IMMUNOPRECIPITATIONS

2.12.1 Introduction

When using monoclonal antibodies to analyse a protein sample it is important to be aware of any cross-reactivity of the antibody. This can either be between different isoforms of the same protein, or between two or more similar proteins. While this can be overcome by using a different antibody for each protein or isoform this is not always possible due to experimental and financial constraints. One way to overcome the problem is to use a series of immunoprecipitation steps to clear any unwanted proteins from the sample, then analysing the remaining proteins of interest. For example, most commercially available antibodies to APP also react with APLP2 (Slunt *et al.* 1994), which could give misleading results. To overcome this problem an antibody (3B11, Webster *et al.* 1995) which is specific for APLP2 was obtained. Using serial immunoprecipitations media samples were first cleared of APLP2 using 3B11, following which immunoprecipitation steps with the commercial APP antibodies that would yield only APP, were carried out.

In a similar way, serial immunoprecipitations can be used to separate different isoforms of a protein, or different cleavage products of a full-length precursor protein.

2.12.2 Method

The initial method follows that of standard immunoprecipitations with the sample and antibody being incubated for 1 hour at room temperature. However, after pelleting beads the supernatant

was decanted into a clean tube and a further antibody added. The beads were washed and eluted as previously, while the second antibody incubated with the sample. This process was repeated as above for each antibody step.

2.13 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs were used to test the specificity of monoclonal antibodies. Ninety-six well plates (Nunc Maxisorb, flat bottomed wells) were prepared by adding to each well 100 μl of a 5 μg / ml solution of the peptide antigen or BSA in phosphate buffered saline (PBS) (resulting in 500 ng of peptide per well). The plate was incubated at 37°C for 1 hour then washed 3 times with dH_2O. The plate was blocked with 100 μl per well of a 1 mg / ml solution of BSA in PBS, incubated at room temperature for 1 hour.

Cell culture supernatants containing the monoclonal antibodies to be tested were mixed with an equal volume of 1 mg / ml BSA in PBS and 100 μl of this solution added to each appropriate well, at least 3 wells were used for each peptide / antibody combination. The plate was incubated at room temperature for 2 hours, then washed 3 times with TBS.

A 1:2000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) was prepared in 1 mg / ml BSA in TBS and 100 μl added to each well. The plate was incubated at room temperature for 1 hour, then washed 3 times with TBS. 10 mM p-nitrophenyl phosphate was prepared in AP buffer, and 100 μl per well added to the plate. After 15 minutes the reaction was stopped with 100 μl per well 2 M sodium hydroxide, and the optical density of each well read on a plate reader at 405 nm. The results were analysed using Microsoft Excel.

2.14 ANTIBODY CONCENTRATION ON A PROTEIN G COLUMN

1 ml of protein G bound agarose beads was added to a 0.7 cm diameter disposable mini-column (Bio-Rad). 5 ml binding buffer was added and pumped through to pack the beads. Hybridoma supernatant containing the antibody was then pumped through the column at a flow rate of 0.5 ml / min. The beads were washed with 5 ml binding buffer, then eluted with 0.1 M glycine pH 2.6. 1 ml fractions were collected and immediately neutralised with 250 μl 0.5 M TRIS pH 8. The antibody containing fraction was identified by determining the optical density at 260 and 280 nm using a Genequant II. The protein concentration of the fraction was also calculated at this time.

2.15 IN SITU *HYBRIDISATION*

2.15.1 Fixation of Cells

NT2 and NT2N cells were grown on coverslips, pre-coated with poly-D-lysine and Matrigel in the case of NT2N cells (see *Section 2.1.3*). Cells were incubated with cDMEM or Optimem (1 ml per coverslip) for 24 hours, then washed in DMEM and fixed by immersion in chilled (-20°C) acetone for 5 minutes. The coverslips were allowed to air dry before being mounted onto glass microscope slides ready for *in situ* hybridisation.

2.15.2 Labelling of Oligonucleotide Probes

All probes used have been previously shown by the Pearson lab to be specific for the mRNA required (see *Table 2.5* for sequences and specificity). The method is based on that described by Heath *et al.* 1996. The oligonucleotide probes were 3' tail labelled with [^{35}S] dATP using a commercially available kit (NEN/DuPont oligonucleotide 3' labelling system). Radiolabelled probe was separated from any unincorporated nucleotides by chromatography using a nucleic acid purification cartridge ("NENSORB", NEN/DuPont). The amount of radioactivity incorporated into the probe was measure on a scintillation counter, and the volume of probe containing 10^6 counts

per minute was calculated. Mr M Sanders carried out this work; the author performed all other procedures.

Table 2.5 Probe Sequences

Probe	Length	Sequence	Reference	T_i	T_w
APP_{695}	30	5' CTG CTG TTG TAG GAA CTC GAA CCA CCT CCT 3'	(Ponte *et al.* 1988)	27°C	52.5°C
APP_{751}	60	5' CAT CAG GGG TAC TGG CTG CTG TTG TAG GAA TGG CGC TGC CAC ACA CGG CCA TGC AGT ACT 3'	(Kitaguchi *et al.* 1988)	41.6°C	54.4°C
APP_{770}	57	5' GTT TAA CAG GAT CTC GGG CAA GAG GTT CCT GGG TAG TCT TGA AAC TTT GGG ACA 3'	(Kitaguchi *et al.* 1988)	33.6°C	56.1°C
HSP	30	5' CGA TCT CCT TCA TCT TGG TCA GCA CCA TGG 3'	(Shimizu *et al.* 1999)	28°C	54°C

2.15.3 In Situ *Hybridisation Procedure*

The slides to be hybridised were placed on sheets of chromatography paper soaked in 4x standard sodium citrate (SSC) buffer. Labelled probe was mixed with hybridisation buffer to give 10^6 counts in 100 μl and this volume was applied to each slide. Slides were then incubated at the optimum incubation temperature (T_i) for each probe (see *Table 2.6*) overnight.

Table 2.6 Washing Temperatures for Oligonucleotide Probes

Probe	Length (bases)	T_i	T_w
APP_{695}	30	27°C	52.5°C
APP_{751}	60	41.6°C	54.4°C
APP_{770}	57	33.6°C	56.1°C
HSP	30	28°C	54°C

T_i - incubation temperature

T_w - washing temperature

APP - Amyloid Precursor Protein

HSP - Heat Shock Protein

Slides were then washed in 1x SSC at room temperature, followed by four 15 minute washes in 1x SSC at the optimum washing temperature (T_w) for each probe (see *Table 2.4*). This was followed by a wash in 1x SSC at room temperature for 1 hour and a final rinse in dH_2O. Slides were allowed to air dry.

2.15.4 Autoradiography

Slides were dipped in Ilford photographic emulsion to examine cellular distribution of mRNA. Slides were dipped in liquid emulsion at 40°C, placed on a cold plate for 15 minutes to allow the emulsion to gel, then allowed to air dry for 3-4 hours. The slides were placed in lightproof boxes and placed at 4°C for an exposure time of 7 weeks. All the above procedures were carried out in the dark.

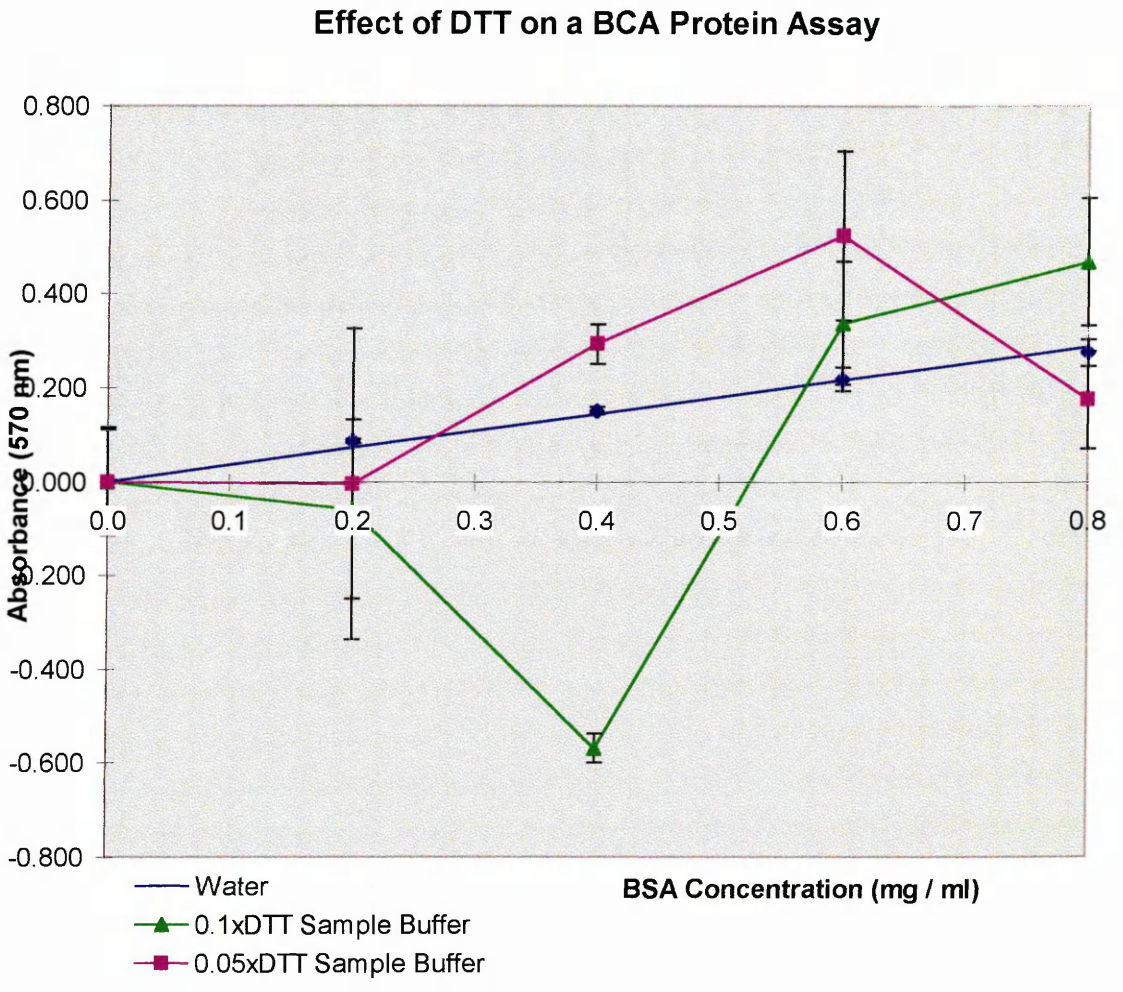
2.15.5 Development and Quantification.

Slides were developed in Phenisol for 2 minutes, washed in 2% acetic acid for 30 seconds, fixed in 30% sodium thiosulphate for 5 minutes and rinsed in running water for 20 minutes. They were allowed to air dry before being counterstained with 0.1% Cresyl fast violet for 1 minute and washed in distilled water. The slide were then dehydrated in serial alcohols (70%, 80%, 90% and 100% - 1 minute in each) before clearing in xylene for at least 5 minutes and mounted with DPX

Image analysis was performed using a Seescan image analyser system (Seescan Instruments, Cambridge). Individual cells were highlighted and the total area of the cell calculated, along with the area covered with silver grains. The mean size of grain was measured, allowing the mean number of grains per cell to be calculated. Fifty randomly selected cells were counted for each experimental procedure and probe. The background level of grains was calculated by counting grains over 5, randomly selected and non-cell containing, areas of each slide equivalent in size to the average cell size for that slide. These counts were averaged, and subtracted from cell grain counts for each slide. The non-parametric Mann Whitney U test was used to compare the mean number of grains per cell for each probe (Levene's test for equality of variance showed the results to have a non-normal distribution).

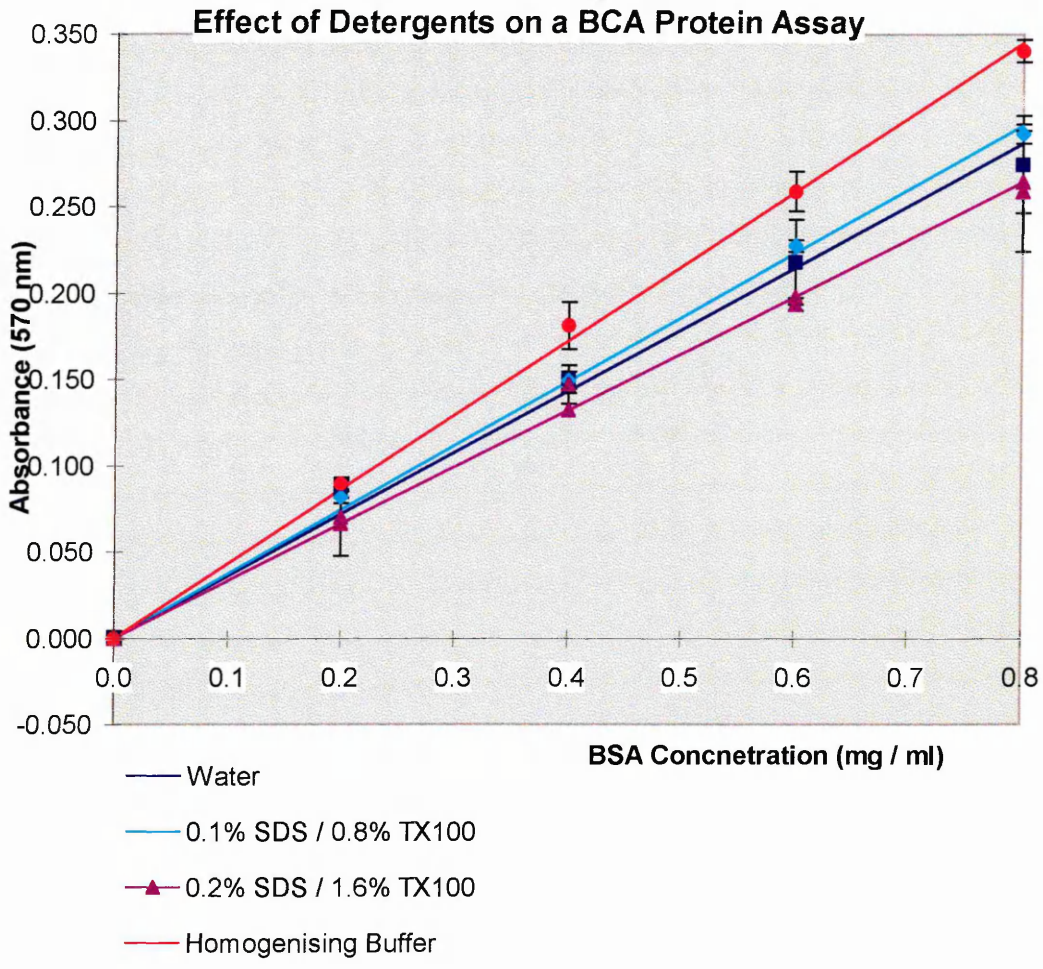
Photographs of the stained cells were taken using an Olympus BX60 microscope and the x40 objective lens. Images were captured using a JVC 3CCD colour video camera and Neotech Image Grabber PC software.

Figure 2.1 DITHIOTHREITOL (DTT) SAMPLE BUFFER INTERFERES WITH A BCA PROTEIN ASSAY



BSA standards were prepared in either dH_2O or DTT sample buffer diluted 1:10 or 1:20 with dH_2O. A BCA protein assay was carried out as described. BSA in dH_2O gives a linear result with increasing concentrations, while even the 1:20 dilution of DTT buffer gives very erratic readings.

Figure 2.2 BCA PROTEIN ASSAYS CAN BE CARRIED OUT IN THE PRESENCE OF SDS/TX100 OR HOMOGENISING BUFFER



BSA standards were prepared in either dH_2O, SDS / TX100 or homogenising buffer, at concentrations used in preparation of cell samples. A BCA protein assay was carried out as described. All solutions give linear results with increasing concentrations of BSA.

Figure 2.3 COMPARISON OF EFFECTIVENESS OF PROTEIN A, PROTEIN G AND GOAT-ANTI-MOUSE-IgG BEADS AT RECOVERING MOUSE IgG

Monoclonal antibody 22C11 or TBS was added to 0.5 ml TBS, and recovered with agarose beads bound to the appropriate protein. The samples were analysed on a 7.5% acrylamide gel and transferred to nitro-cellulose membrane. The membrane was then immunoblotted using a HRP-conjugated anti-mouse IgG secondary antibody only. The blot was developed using ECL.

Lane 1 22C11 and goat-anti-mouse-IgG beads

Lane 2 goat-anti-mouse-IgG beads only

Lane 3 22C11 and Protein G beads

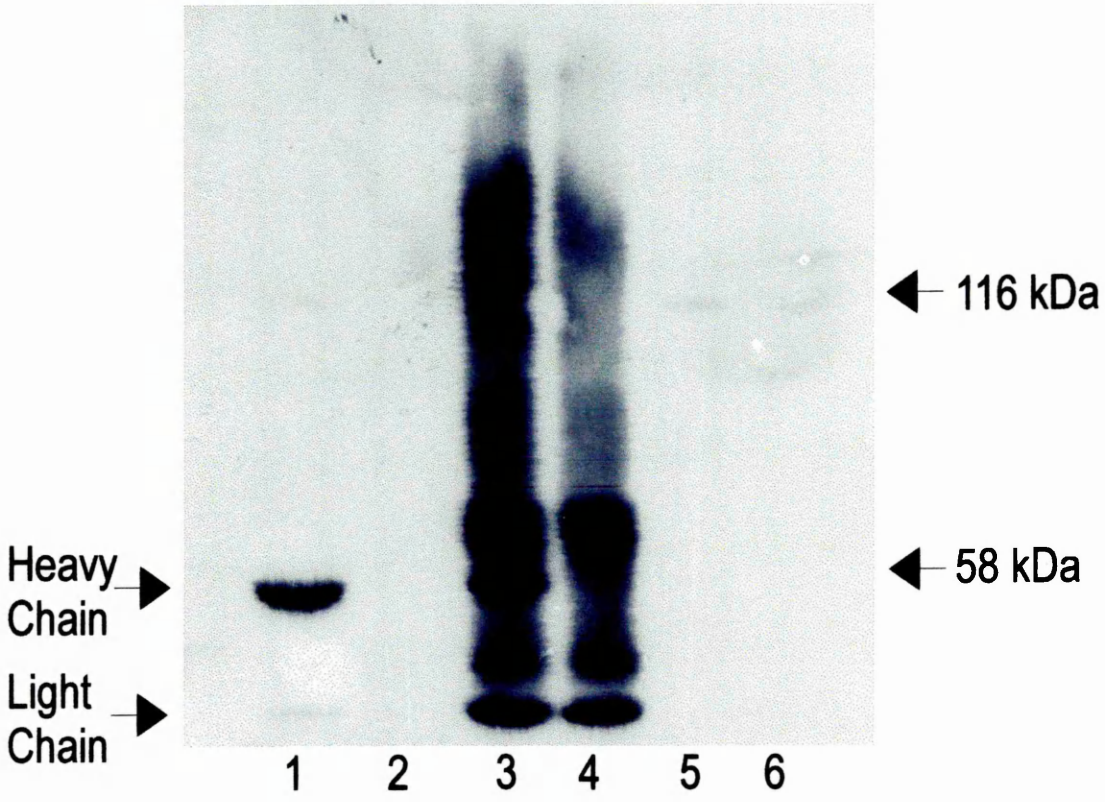
Lane 4 Protein G beads only

Lane 5 22C11 and Protein A beads

Lane 6 Protein A beads only

Figures to the right of the blot indicate the mobility of molecular weight markers.

As can be seen in lanes 3 and 4, the use of Protein G beads leads to smearing of the immunoblot, even after no immunoprecipitation has occurred.


116 kDa
58 kDa
Heavy Chain
Light Chain
1
2
3
4
5
6

Chapter Three - Production Of NTera2 Neurones

3.1 INTRODUCTION

3.1.1 NTera2-Clone D1

The Tera2 cell line was derived from a lung metastasis of a 22 year old Caucasian man with a primary testicular germ cell tumour. Low passage Tera2 cells (first characterised in Andrews *et al.* 1984) exhibit a modal 61,XY karyotype with a range of 59 to 63 chromosomes. They contain single, normal X and Y chromosomes, and are disomic for chromosomes 4, 5, 6, 8, 10, 13, 14, 15, 18 and 19. They are trisomic for chromosomes 3, 7, 9 and 11, while 80% of the cells are trisomic for chromosomes 20, 21 and 22 and 60% trisomic for chromosome 16.

The NTera2 cell line was cloned from these cells and exhibits a similar karyotype. Specifically, clone NTera2/D1 (NT2/D1) has a modal 63,XY karyotype, (range 54-63) and exhibits many of the morphologic and biochemical features of other established human embryonal carcinoma (EC) cell lines. NTera2 (NT2) cells do not resemble Tera2 cultures and probably arose from a minor population of EC cells. When injected into athymic mice NT2/D1 cells form tumours containing a variety of somatic tissues including neural elements. *In vitro* the cells must be maintained at high density to maintain their EC characteristics. (Andrews *et al.* 1984)

3.1.2 Differentiation Of NT2/D1 Cells

Like many murine EC cell lines, NT2/D1 cells are pluripotent (Andrews *et al.* 1984). When treated with 10^{-5} M all-trans-retinoic-acid (RA) for seven days, immunoreactivity of an embryonal cell surface marker, SSEA-3, decreases (Andrews, 1984). After one day of RA treatment, followed by replating, most cells undergo morphological differentiation. After seven days less

than 20% of cells are SSEA-3 positive. Among the differentiated cells is a population which exhibit a neurone-like morphology. Treatment with RA for as few as 7 days is enough to induce the presence of these cells, though the cells must be cultured for a further 7-14 days before the neuronal precursor cells are evident. (Andrews, 1984) Although several other compounds can induce this differentiation, none are as effective as RA at non-cytotoxic concentrations (Andrews *et al.* 1986). Unlike the EC stem cells and other differentiated cells, these neurone-like cells react with tetanus toxin and exhibit the presence of neurofilament 200 by immunoreactivity (Andrews, 1984). Furthermore, after 2 weeks of RA treatment, the neurone-like cells express all three classes of neurofilament protein, and do not express vimentin filaments (found in some neurones, glial and other cells) or glial filaments. Although NT2 stem cells express cytokeratin filament (an EC cell intermediate filament) the neurone-like cells do not (Lee and Andrews, 1986).

3.1.3 NT2N Cells Appear To Be Embryonic CNS Neurones

Pure cultures of the neurone-like cells (NT2N cells) can be obtained by selective replating and treatment of cultures with mitotic inhibitors (see *3.2*; Pleasure *et al.* 1992). These pure cultures have been extensively studied in an attempt to characterise them. The cells are motile, clumping together to form cellular aggregates, and are post-mitotic. They do not incorporate bromodeoxyuridine, and are not affected by mitotic inhibitors. Using immunocytochemistry, it has been shown that NT2N cells express several neurone specific markers, summarised in *Table 3.1*. Most of the proteins listed are expressed in embryonic forms or levels.

Table 3.1 Neuronal Markers Expressed By NT2N Cells

(adapted from Pleasure et al. 1992)

Neuronal marker	*Function*	*Expressed by NT2N cells*
NF-L	Neurofilament sub-units	✓
NF-M		✓
NF-H		✓
NF-66	CNS specific neurofilament sub-unit	✓
peripherin	PNS specific neurofilament sub-unit	✗
MAP1A	Cytoskeletal proteins	✓
MAP1B		✓
MAP2		✓
Tau		✓
110 kDa tau isoform	PNS specific	✗
NCAM	Neuronal cell adhesion molecule	✓
GAP-43	Growth associated protein, found in growth cones	✓
Synaptophysin	Markers for secretory activity	✓
Chromogranin		✓

NF-L, NF-M, NF-H, NF-66 - Neurofilament protein L, M, H, 66

MAP1A, MAP1B, MAP2 - Microtubule Associated Protein 1A, 1B, 2

NCAM - Neuronal Cell Adhesion Molecule

GAP-43 - Growth Associated Protein 43

NT2N cells exhibit a neurone-like morphology, elaborating processes which fall into two classes: ones that are thick at the base and taper progressively from the cell body, and a second which are thin and untapering. The distribution of cytoskeletal elements confirms that these are dendrites and axons, respectively (Pleasure *et al.* 1992).

3.1.4 NT2N Cells Express Functional Neurotransmitter Receptors

NT2N cells express at least two types of glutamate receptor, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate / kainate (AMPA/kainate or non-NMDA) receptors (Younkin *et al.* 1993). Patch-clamping experiments show that both of these receptors respond to glutamate, and this response can be abolished by application of a combination of NMDA and non-NMDA antagonists. NT2N cells also exhibit glutamate excitotoxicity; 1 mM glutamate added to the culture media for 15 minutes compromises membrane integrity and impairs mitochondrial function of 6 week old NT2N cell cultures. It has no effect on NT2 stem cells or 2 week old neuronal cultures (Younkin *et al.* 1993).

There is some evidence that NT2N cell cultures contain at least 20% cholinergic neurones (Zeller and Strauss, 1995). NT2N cells express muscarinic acetylcholine receptors, primarily the m_3 subtype, which is linked to the phospholipase C signal transduction pathway. They also contain all the elements of the muscarinic / phospholipase C signal transduction pathway, and it has been shown that in response to the agonist carbachol, 1,2-diacyl-*sn*-glycerol (DAG), *myo*-inositol-1,4,5-trisphophate ($Ins(1,4,5)P_3$) and intracellular calcium (Ca^{2+}) levels all rise (Wolf *et al.* 1995).

3.1.5 NTera2 Cells Produce APP

As a consequence of being trisomic for chromosome 21, NT2 and NT2N cells produce readily detectable quantities of APP (Wertkin *et al.* 1993). There is a change in APP expression after

NT2 cells are induced to differentiate into the neuronal phenotype, with NT2 stem cells expressing predominantly APP_{751} and APP_{770}, while the NT2N cells express predominantly APP_{695} (Ackerman *et al.* 1994; Wertkin *et al.* 1993).

3.1.6 Summary of Introduction

In summary, NT2N cells are post-mitotic and exhibit a neuronal morphology with axons and dendrites. They have receptors that respond to neurotransmitters, and the proteins expressed indicate that the cells most closely resemble embryonic CNS neurones. Since the cells are human in origin, they potentially provide a good model for studying normal neuronal function, and also processes leading to neurodegenerative diseases. Naturally, obtaining pure cultures of these cells is of paramount importance. The work discussed in this chapter describes different techniques tried in order to produce the highest yield of pure neurones after RA differentiation.

3.2 CELL CULTURE METHODS

The cell culture methods used and the results obtained will be presented in combination.

3.2.1 Maintenance of Stem Cell Cultures

Stem cell cultures of NT2 cells were maintained as described in *Section 2.1.*

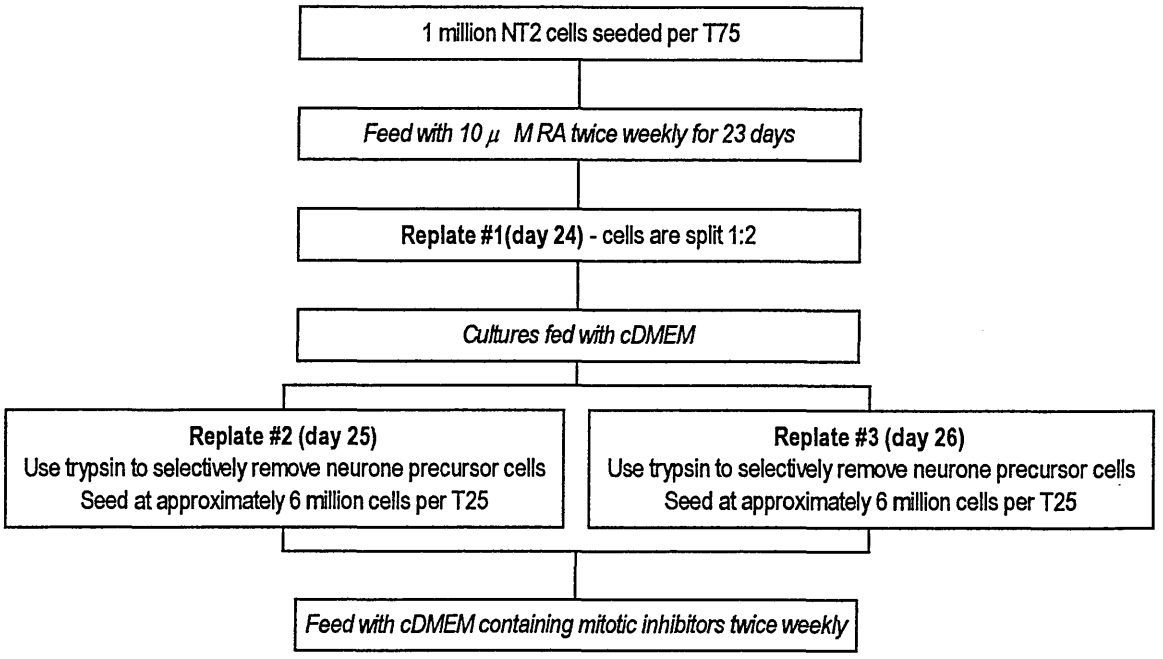
3.2.2 Neuronal Differentiation and Purification

3.2.2.1 Differentiation (See **Figure 3.1***)*

Prof. Peter Andrews of Sheffield University (adapted from Pleasure et al. 1992) recommended the initial method used for neuronal purification. The procedure is detailed in full in *Appendix C.* In brief, cells were seeded at a density of 10^6 cells per T75 and fed with 10^{-5} M RA for 24 days. On day 25 the cells were split into media without RA to allow neurone precursor cells to settle on the top of the cell layer (replate #1). On the next 2 days the cell cultures were treated with TE to

remove selectively these precursor cells (replates #2 and #3). The precursors were replated at a density of approximately 6 x 10^6 cells per T25 flask and fed with cDMEM containing mitotic inhibitors (1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine and 10 μM uridine).

Figure 3.1 Original Protocol For Obtaining Neuronal Cultures



3.2.2.2 Purification

Although the selective replating of neuronal precursors provided enriched cultures of NT2N cells, there were also other cells present. These cells did not resemble NT2 stem cells, being much larger, and formed a monolayer beneath the neuronal cells. The neuronal precursors migrated across this monolayer to form cell aggregates. After 7-10 days in culture, multiple processes could be seen interconnecting these cell clusters. Some of these processes also appeared to be forming connections with the non-neuronal monolayer (see *Figure 3.2*).

Treatment with mitotic inhibitors was continued until all the non-neuronal cells died, leaving only the mature NT2N cells. Cultures were used as neurones when judged pure by eye; several fields

per flask were viewed at x10 magnification with a phase contrast microscope, if no non-neuronal cells were seen the cultures were assumed pure. This purification step could take several weeks, and varied greatly from batch to batch of cultures, with some cultures reaching a pure state in 7-10 days, while other cultures contained contaminating cells for up to 4 weeks. It was also problematic, as the NT2N cells did not survive for more then a few days when the cultures were pure, tending to detach from the culture vessel, and so had to be used immediately. To try and improve the rate of purification, and of neuronal survival, several alternative methods were tried.

3.2.3 Alternative Methods

3.2.3.1 Increased Neuronal Survival Rate.

Other sources have reported the influence of non-neuronal cells on neurone survival rate (Pleasure *et al.* 1992). Following the protocol laid out in this paper for pre-treating culture flasks before seeding out neurone precursors was found greatly to improve the length of time neurone cultures could be kept. Flasks were treated with one coat of poly-D-lysine and two coats of Matrigel (see *Section 2.1.3* for details). This encouraged neurite outgrowth, and also prolonged the survival of contaminating non-neuronal cells.

3.2.3.2 Increased Levels of Neuronal Purification

After replate #1, neuronal precursor cells could be seen as phase bright cells lying on top of multiple layers of smaller cells. Although the aim of replates #2 and #3 was to remove as many of the neuronal precursors as possible, complete removal was never achieved. If the remaining cells were continued in culture, fed with cDMEM, maturation of the NT2N cells took place. As in pure cultures the cells formed aggregates and elaborated processes, but due to the lack of mitotic inhibitors these neuronal cells were quickly overgrown by non-neuronal ones. To combat this, mitotic inhibitors were added to the media immediately after replate #1. This prevented NT2N

cells being overgrown by contaminating ones, while allowing them to mature and elaborate processes.

After 2 weeks culture in the presence of mitotic inhibitors, the NT2N cells had formed cellular aggregates and processes, and were loosely attached to a monolayer of non-neuronal cells. Tapping of the culture vessel was sufficient to remove selectively the neurones, which were then replated into flasks pre-treated with poly-D-lysine and Matrigel and fed with cDMEM. After 3 to 5 days in culture the neurones had adhered to the vessel and recommenced neurite outgrowth. The cells were ready to use after 7 to 10 days in culture and could be maintained for 2 to 3 months. The level of purity of cultures obtained by this level was much higher than other methods (compare neurones in *Figure 3.2a* with those in *Figure 3.3*), though on occasion contaminating cells were still present. The removal of these was attempted by reintroducing 10 μM fluorodeoxyuridine and 10 μM uridine, but this had the effect of killing all cells in culture, so impure cultures were discarded.

3.2.3.3 Improved Neuronal Yield

To maximise neuronal yield and replate efficiency, RA cultures were split at different densities at replate #1, from 1:1 to 1:6. The purpose of this split was to allow neuronal precursors to settle on top of the other cells present. It was postulated that a split to a lower cell density would allow a greater number of precursors to migrate and therefore increase the neuronal yield.

As expected splits of 1:1 and 1:2 resulted in a higher number of non-neuronal cells which decreased the replate efficiency of neurones. However, splits of 1:5 or 1:6 resulted in decreased neuronal survival rate. Therefore a split of 1:3 was chosen as the most efficient for both neurone survival and replate ability (see *Figure 3.4*).

The refined method of neuronal differentiation and purification which was adopted for subsequent studies is outlined in *Table 3.2* below, and detailed in *Appendix B*.

Table 3.2 Improved Protocol for Neuronal Differentiation

Day	Procedure	Culture Media
0	Split a confluent T25 flask of stem cells 1:10	cDMEM with 10^{-5} M RA
7, 14, 21	Feed cells	cDMEM with 10^{-5} M RA
24	Split cells 1:3	cDMEM with mitotic inhibitors (cDMEM+I)
28	Feed cells	cDMEM+I
31	Feed cells	cDMEM
35	Feed cells	cDMEM
38	Mechanically dislodge neuronal cells and replate onto culture vessels coated with poly-D-lysine and Matrigel	cDMEM
Every 7 days	Feed neuronal cells	cDMEM

3.3 SUMMARY

If NT2N cells *in vitro* are to be used as a model for human CNS neurones *in vivo*, standard methods of differentiation and purification are essential. As has been reported previously, differentiation of NT2 cells to NT2N cells using RA is a terminal differentiation; NT2N cells maintain their neuronal morphology for many months in culture in the absence of RA. However, it is not known if the length of time cells are cultured in RA affects the eventual phenotype. Seven days is enough to result in eventual neuronal characteristics (Andrews, 1984), though published

protocols use 21-28 days culture with RA (Prof. Andrews personal communication; Pleasure *et al.* 1992). NT2N cells developed this way have been characterised for many neuronal markers (Wolf *et al.* 1995; Ackerman *et al.* 1994; Younkin *et al.* 1993; Pleasure *et al.* 1992; Lee and Andrews, 1986) and appear to be embryonic CNS neurones, but it is vital to remember that these are cultures derived from a tumour cell line, not primary neuronal cultures. Variable neuronal phenotype may occur due to the variable karyotype of the NT2 stem cells. Culture conditions of the NT2 stem cells must be rigorously monitored to prevent spontaneous differentiation, which may also lead to variable neuronal phenotype. An awareness of the effect of the concentration of RA and the length of time it is present in the culture is also important. To ensure that results obtained from NT2N cells are comparable to those expected from *in vivo* studies, a consistent method for preparing the cultures should be used.

Similarly the purity of the NT2N cell cultures is of utmost importance. Though NT2 and NT2N cells have been extensively studied, little work has been done on the phenotype of the non-neuronal or "background" cells present in NT2N cultures. As discussed in this chapter and elsewhere (Pleasure *et al.* 1992), these cells promote the survival of neurones, and so can be assumed to provide support, either nutritional, biochemical or mechanical, to the neuronal cells. During the course of this work, it was discovered that these cells also secrete some proteins (specifically APP, discussed further in *Chapter 4*). In view of this fact, if NT2N cells are to be used in biochemical secretion or metabolism studies, the presence of non-neuronal cells must be eliminated to prevent confounding of the results.

The work presented in this chapter describes an improved protocol for the differentiation and purification of NT2N cells from NT2 stem cells. This method ensures that all NT2N cells will be comparable in terms of RA effects, and by eliminating all trace of non-neuronal cells it is possible

to be confident that the results obtained are from a cell which is the closest model of a human CNS neurone available at the present time.

Figure 3.2 NT2 STEM CELLS AND NEURONAL CELLS

(a) NT2 stem cells, passage 36. Magnification x100.

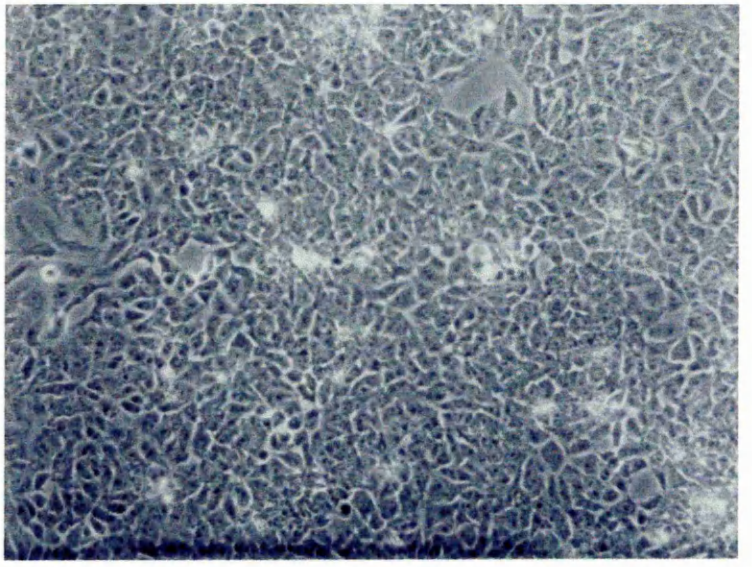
Cells form a continuous monolayer.

Cells were maintained in cDMEM, and split 1:3 when confluent using scraping.

(b) NT2N cells, purified using the initial method recommended by Prof. Andrews. Magnification x1000.

The neuronal cell bodies form phase bright aggregates. They can be seen to be sitting on top of a monolayer of non-neuronal cells, which are not NT2 stem cells. The neuronal cells have elaborated processes that seem to connect with both the cell types present.

a) NT2 STEM CELLS



b) NT2N NEURONAL CELLS

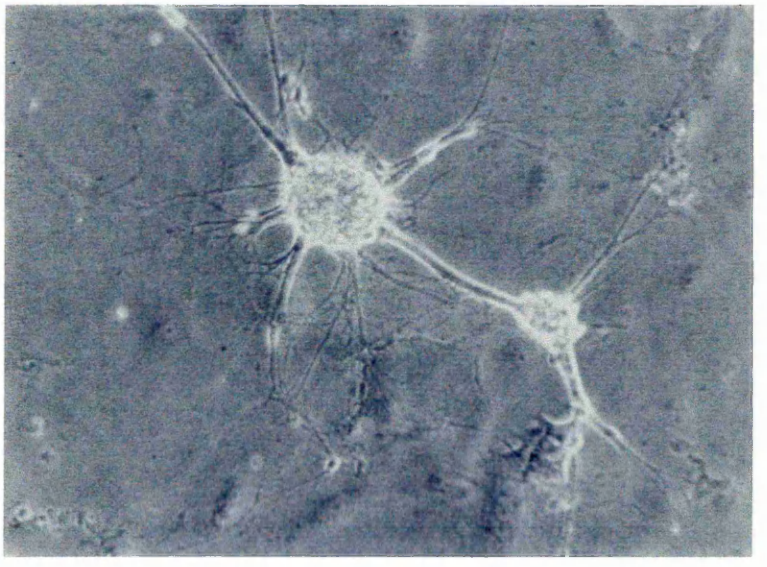


Figure 3.3 INCREASED PURIFICATION LEVELS ARE OBTAINED BY ALTERING THE PROTOCOL USED FOR REPLATING THE NEURONAL PRECURSOR CELLS

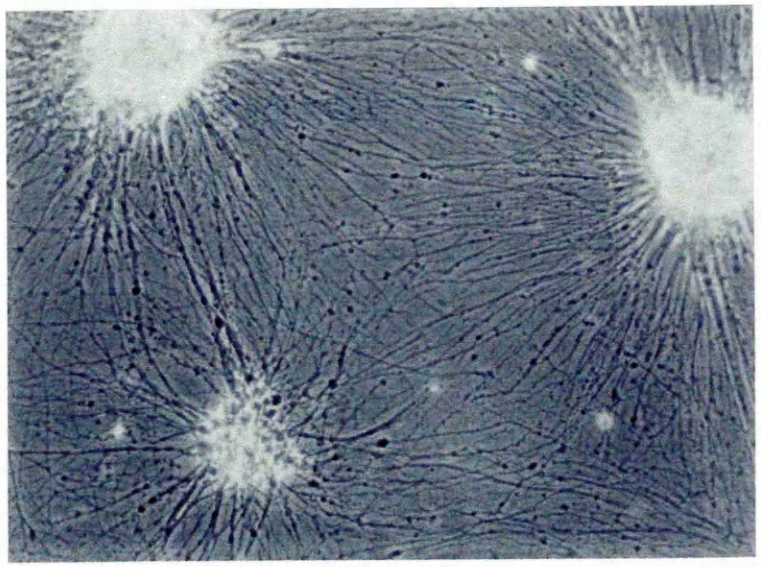
By allowing the neuronal precursors to mature before replating onto plastic coated with poly-D-lysine and Matrigel it is possible to replate only NT2N cells, resulting in an absence of background cells. As previously, the cell bodies form phase bright aggregates and elaborate processes which interconnect with each other and with these aggregates. The cultures shown are all from different batches of neurones, as can be seen the level of purification is the same for each, the only difference is in the size of the cell body aggregates. The cultures shown are also representative of cells after different lengths of time in culture.

(a) batch 010497P35 (after 2 weeks in culture)

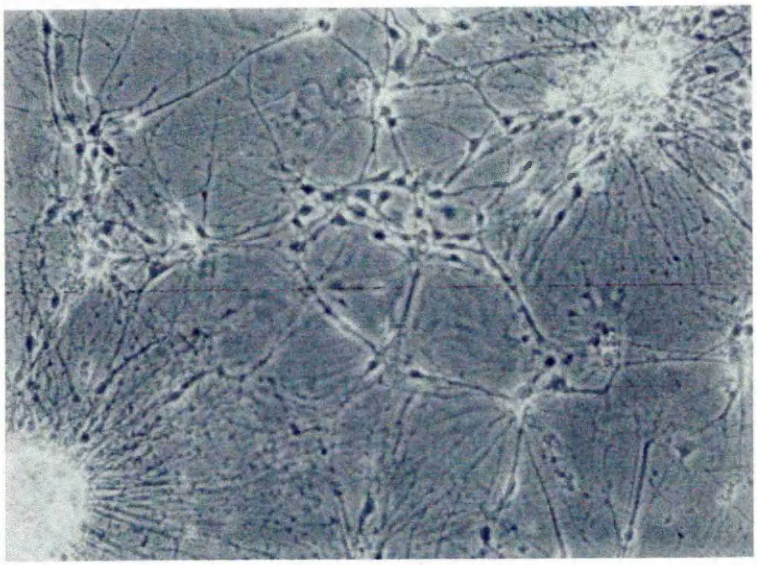
(b) batch 240397P44 (after 3 weeks in culture)

(c) batch 130297P38 (after 9 weeks in culture)

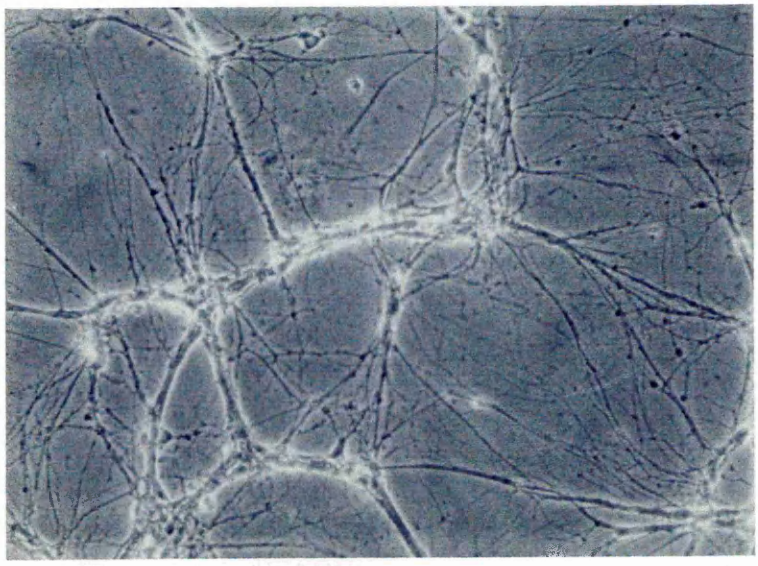
All photographs are at x1000 magnification.



a



b



c

Figure 3.4 EFFECT OF SPLIT RATIO ON RA-TREATED NT2 CELLS

Cells were treated with RA for 25 days, then split using TE and cultured in the presence of mitotic inhibitors for 10 days. The neuronal precursor cells can be seen as small phase bright cells on the top of the other cellular layers (examples indicated by arrowheads). They have already begun to elaborate processes. Magnification x500.

(a) 1:2 split

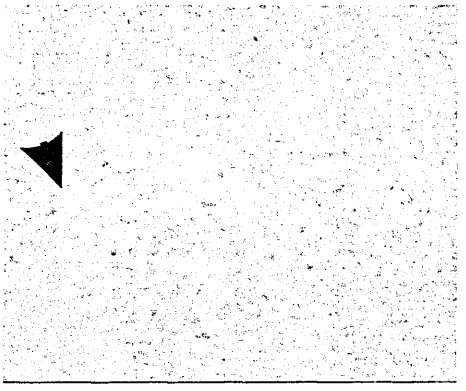
(b) 1:3 split

(c) 1:4 split

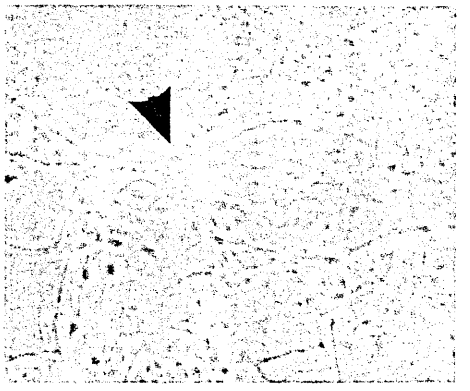
(d) 1:5 split

(e) 1:6 split

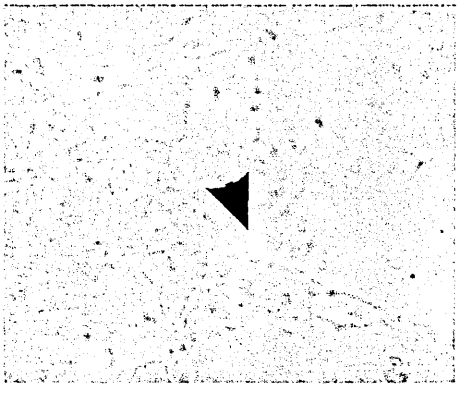
a) 1:2



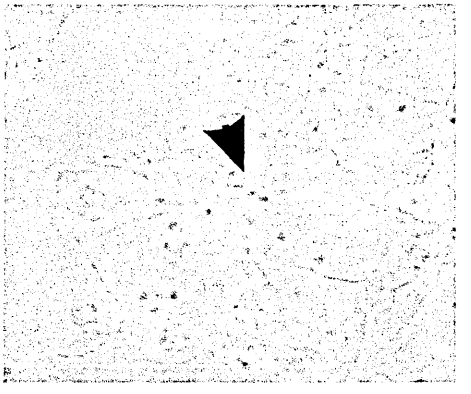
b) 1:3



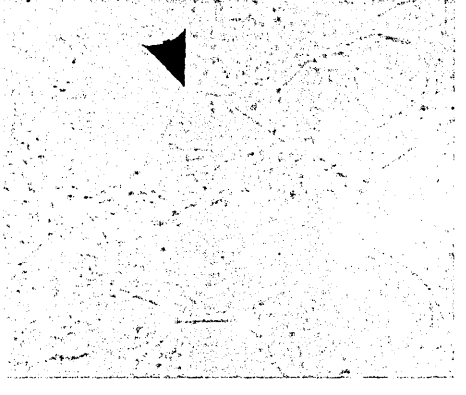
c) 1:4



d) 1:5



e) 1:6



Chapter Four - Characterisation Of APP Production And Processing In NTera2 Stem Cells And Neurones

4.1 INTRODUCTION

As discussed in *Chapter 3*, NT2 and NT2N cells potentially provide a valuable system for investigating human neurodegenerative diseases. The stem cells are easy to maintain in large numbers in culture, while the neuronal cells are one of the best models of human CNS neurones currently available for *in vitro* research. They have an additional advantage for researchers wishing to study AD, as a result of being trisomic for chromosome 21 (Andrews *et al.* 1984). Both NT2 and NT2N cells produce an easily detectable amount of endogenous APP (Wertkin *et al.* 1993).

APP production and processing was studied in both cell types, to obtain an understanding of what changes in processing of APP may accompany neuronal differentiation. APP has previously been studied in NT2 and NT2N cells at both the mRNA and the protein level. However there is some inconsistency in the results. RT-PCR studies have shown that both cell types contain all three APP isoforms, but that NT2N cells contain predominantly APP_{695} mRNA, while stem cells contain predominantly APP_{751} and APP_{770} mRNA (Ackerman *et al.* 1994; Wertkin *et al.* 1993). PCR studies of cDNA libraries produced from the cells found APP_{695} in both libraries, while APP_{770} existed solely in the stem cell library and APP_{751} solely in the neuronal library (Ackerman *et al.* 1994).

Protein studies on cell lysates have shown NT2 cells to contain predominantly APP_{751} and / or APP_{770}, with a trace of APP_{695} while the neuronal cells contain only APP_{695} (Wertkin *et al.* 1993).

The same study reported that NT2 cells secrete $APP_{751/770}$ while NT2N cells secrete only APP_{695}, though two bands were present on the immunoblot, the larger one was attributed to APP secretion from contaminating cells. However, these results may be misleading, as the study concerned used only the monoclonal antibody LN21, which recognises all isoforms of APP and which may also cross react with APLP2. Identification of APP isoforms was based on size alone, which could be problematic as immature and fully glycosylated forms of APP run at different sizes on a gel, with mature APP_{695} being the same weight as immature APP_{751} and APP_{770}. Another reason for these apparently contradictory results could be due to spontaneous differentiation of stem cells due to alterations in culturing conditions, leading to a variation in neuronal phenotype.

APP isoforms expressed by NT2 and NT2N cells grown in this laboratory were analysed using immunoblotting. A number of antibodies were used to distinguish between the different isoforms of APP, as well as APLP2. Intracellular and secreted proteins were examined in both cell types. Once the expression pattern of APP in the cells was established, processing pathways were examined, to investigate any differences in amyloidogenic and non-amyloidogenic processing between stem cells and neurones.

4.2 METHODS

4.2.1 APP Production and Secretion

NT2 and NT2N cells were cultured as described in *Chapters 2* and *3*. For each experiment, one T75 flask of each cell type was incubated with 12 ml fresh culture media for 24 hours, then the conditioned media was collected and concentrated using ammonium sulphate fractionation. Cell pellets were collected, resuspended in 250 µl homogenising buffer with protease inhibitors and homogenised to give soluble and membrane protein fractions as described. Samples were

separated on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane prior to analysis by western blotting.

4.2.2 Antibodies

Samples were examined by immunoblotting with several antibodies as detailed in *Table 4.1*. The antibodies were used at the concentrations or dilutions indicated in *Table 4.4* and incubated at room temperature for 2 hours. The blots were then incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour, and developed using ECL.

Table 4.1 Antibodies Used In Immunoblotting

Antibody	Type	Epitope	APP isoforms detected	Source
22C11	Mouse monoclonal	12 amino acids at the NH_2 terminus of APP	APP_{695}, APP_{751} APP_{770}, APLP2	Boehringer Mannheim
993	Rabbit polyclonal	Within the KPI domain	APP_{751}, APP_{770}	Produced in the lab
3B11	Mouse monoclonal	Within amino acids 598-617 of APLP2	APLP2	Kindly donated by M-T Webster
6E10	Mouse monoclonal	Within amino acids 1-28 of the βA4 domain of APP	APP_{695}, APP_{751} APP_{770}, not APLP2 or APPsβ	Seneteck

Table 4.2 Working Concentration of Antibodies

Antibody	Dilution	Final Concentration
22C11	1:100	0.5 μg / ml
993	1:500	Diluted from immune serum
3B11	1:70	Diluted from cell culture supernatant
6E10	1:100	Diluted from cell culture supernatant

4.2.3 Contaminating Cells

An NT2N cell culture containing a monolayer of non-neuronal cells was incubated in culture media, 12 ml cDMEM, in a T75 culture vessel, for 24 hours. The conditioned media was collected, and the neuronal cells removed by shaking the culture vessel. The remaining background cells were incubated in 12 ml fresh culture media for a further 24 hours, after which the media was collected and the cells discarded. Conditioned media from both periods, along with unconditioned cDMEM was concentrated 10-fold using ammonium sulphate fractionate, separated on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane. This was then immunoblotted with antibodies 22C11 and 993.

4.2.4 Monoclonal Antibody Production

4.2.4.1 Western Blotting

The 100 most positive clones from each fusion were further tested for the ability to western blot, and to immunoprecipitate APP. Monkey cerebral cortex, soluble extract was run on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane as a source of APP for testing of western blotting capabilities. Cell culture supernatants were mixed with an equal volume of blotto and incubated with the blot for 2 hours. The blots were then incubated with an AP-conjugated

secondary antibody and developed with NBT/BCIP. Control blots were incubated with 0.5 μg / ml 22C11 and developed in the same way.

4.2.4.2 Immunoprecipitation

For immunoprecipitation experiments, 200 μl of the monkey brain extract was mixed with 600 μl immunoprecipitation buffer. 200 μl of the cell culture supernatant was added and the mixture incubated at room temperature for 2 hours. 100 μl of a 50% solution of goat anti-mouse IgG bound agarose beads was added and incubated for a further hour. The beads were eluted in 50 μl SDS-PAGE sample buffer, and 20 μl of each sample run on a 7% SDS-PAGE gel. The gel was transferred to nitro-cellulose membrane and western blotted with 0.5 μg / ml 22C11. The blot was developed using chromogenic techniques.

4.2.5 Amyloidogenic Cleavage of APP from NT2 and NT2N Cells

A T75 flask of each NT2 and NT2N cells was incubated with 12 ml cDMEM for 24 hours. The media was collected, along with two control samples of unconditioned cDMEM. One ml of each sample was subjected to a serial immunoprecipitation procedure as described in *Section 2.12.* The first control sample was incubated with 40 μl 22C11 (2 μg), the second with 40 μl 6E10 (cell culture supernatant). The test samples were incubated with a series of 3 repetitions of 40 μl 6E10, followed by a final immunoprecipitation step with 40 μl 22C11. The resulting proteins were eluted with 50 μl SDS-PAGE sample buffer, 20 μl of each sample was loaded onto a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane.

Duplicate gels were run, with both being transferred to nitro-cellulose membrane. The first membrane was western blotted with 6E10 and the second with 22C11 (each antibody was at a 1:100 dilution, primary antibody incubated for 2 hours). Blots were developed using ECL.

4.2.6 Detection of βA4

Solutions of synthetic $\beta A4_{1-40}$ (Sigma) were prepared in dH_2O at various concentrations, and mixed with SDS-PAGE sample buffer. 20 μl of each dilution was loaded on two 12% TRIS-TRICINE gels. One gel was transferred to nitro-cellulose membrane, the other was transferred to PVDF. Each membrane was cut in half and all four membranes were western blotted using 6E10 at a dilution of 1:100 for 2 hours. One half of each membrane was then incubated with an anti-mouse IgG secondary antibody conjugated to HRP, the other half with an anti-mouse IgG secondary antibody conjugated to AP. Each membrane was incubated for 1 hour at room temperature. The blots incubated with the HRP-conjugated antibody were then incubated with ECL solution and exposed to X-ray film, while the blots incubated with the AP-conjugated antibody were incubated with NBT/BCIP solution for one hour.

4.2.7 βA4 Immunoprecipitations

Several antibodies, detailed in *Table 4.3* were tested for the ability to immunoprecipitate βA4 out of solution. Synthetic βA4 solutions were prepared at a concentration of 200 ng / ml in a solution of 1 mg / ml BSA in TBS. 0.5 ml of this solution was incubated with each antibody to be tested for 2 hours at room temperature, the resulting antibody/peptide complex was then recovered using agarose beads. The beads were eluted in 25 μl SDS-PAGE sample buffer, and 20 μl of this was run on a 12% TRIS-TRICINE gel, as described previously. The gel was then transferred to nitro-cellulose membrane and immunoblotted with antibody 6E10, again as described previously.

Table 4.3 Antibodies to βA4

Antibody	Type	Epitope	Source
6E10	Mouse monoclonal	Within amino acids 1-28 of βA4	Commercial, Seneteck
1032*	Rabbit polyclonal	Within amino acids 1-19 of βA4	Produced in the lab
58617*	Rabbit polyclonal	Within amino acids 1-9 of βA4	Produced in the lab
58618*	Rabbit polyclonal	Within amino acids 1-9 of βA4	Produced in the lab
6F/3D	Mouse monoclonal	Within amino acids 8-17 of βA4	Commercial, Dako

** These antibodies were produced and characterised by Dr D Parkinson before the onset of the work described in this thesis.*

4.2.8 Method

NT2 cells were grown to confluence in a 12 well plate. Media from each well was collected and pooled, to give 12 ml of 3 day conditioned media, and the cells were then harvested using the method in *Section 2.3.* NT2N cells and media from a T25 culture flask were collected and the cells processed as described in *Section 2.3.*

One hundred μl of each cell sample and 1 ml of each media sample was immunoprecipitated with 100 μl of antibody 6E10 overnight, as described in *Section 2.11.* The antibody/antigen complex

was recovered using 100 μl of agarose bound anti-mouse-IgG, and the beads were eluted in 100 μl SDS-PAGE sample buffer.

20 μl of each sample was loaded onto each of a 7% SDS-PAGE gel, and a 12% TRIS-TRICINE gel. Both gels were run as previously described, and then transferred to nitro-cellulose membrane. Each nitro-cellulose membrane was immunoblotted with antibody 6E10 and developed using ECL as described previously.

4.2.9 Immunoprecipitation of Synthetic βA4 from Conditioned Media

100 ng βA4 was added to 0.5 ml unconditioned cDMEM and to media conditioned by NT2 cells for 24 hours. The samples were incubated with 40 μl 6E10 as previously described, the antibody was recovered by addition of 50 μl anti-mouse-IgG agarose beads, and the beads eluted in 50 μl SDS-PAGE sample buffer. 20 μl of each sample was separated on a 12% acrylamide TRIS-TRICINE gel, which was transferred to nitro-cellulose membrane and immunoblotted with 6E10 as before.

4.3 APP PRODUCTION AND SECRETION

NTera2 stem and neuronal cells were examined by the methods details in *Section 4.2.1* to characterise the production and secretion of APP by each cell type.

4.3.1 Results

The first task was to assess APLP2 production and secretion by NT2 and NT2N cells, this was examined using the specific monoclonal antibody 3B11. Using this antibody, it was not possible to detect any APLP2 in the NT2N cells, nor was any of this protein detected in the media from these cells. A single band of approximately 100 kDa was detected in the membrane fraction of NT2 cells, but not in the soluble fraction or in secreted proteins (see *Figure 4.1a)*. From these results

it was determined that NT2N cells do not make detectable amounts of APLP2, and that, while NT2 cells do make a detectable amount of the protein, it is not secreted at detectable levels. Therefore any proteins detected in the media of either cell type with antibodies 22C11 or 993 are APP isoforms.

Antibody 22C11 detected 3 bands, of approximately 115, 128 and 137 kDa, in both soluble and membrane fractions of NT2 cells (see *Figure 4.1b*). The staining was more intense in the membrane fraction, and of this, the two larger bands were predominant. A single band of approximately 128 kDa was detected in the media of these cells. Immunoblotting with antibody 993 detected two bands of approximately 128 and 137 kDa in the soluble and membrane fractions, and a single band of 128 kDa in the media (see *Figure 4.1c*).

NT2N cells did not contain detectable amounts of APP in the soluble fraction of the cellular proteins, but 2 bands were identified with antibody 22C11 in the membrane fraction (see *Figure 4.1b*). The major band was approximately 113 kDa, with a band at 130 kDa also visible. Both of these bands were also detectable in the media from these cells. Antibody 993 did not detect any intracellular APP, but detected a single band of approximately 130 kDa in the media from the cells (see *Figure 4.1c*).

4.3.2 Conclusion

From these data it was concluded that NT2 cells make predominantly APP_{751} and / or APP_{770}, which is secreted (see *Table 4.4*). A small amount of APP_{695} is produced, but could not be detected in the media. As expected, the majority of intracellular APP is membrane bound. The two bands detected by antibody 993 in the cellular fractions of the cells probably represent

immature and mature, fully glycosylated, forms of APP_{751} and APP_{770}, as it is not possible to separate the two isoforms on a gel.

Table 4.4 Molecular Weights Of APP Isoforms In NT2 Cells, Along With The Antibodies Detecting Them

	$APP_{751/770}$			APP_{695}		
	Immature	**Mature**	**Soluble**	**Immature**	**Mature**	**Soluble**
Size (kDa)	*128*	*137*	*128*	*115*	*128*	*N/A*
22C11	✓	✓	✓	✓	✓	✓
993	✓	✓	✓	✗	✗	✗
6E10	✗	✗	✓	✗	✗	✓
3B11	✗	✗	✗	✗	✗	✗

NT2N cells make predominantly APP695, which is secreted (see *Table 4.5*). One or both of the KPI-containing APP isoforms is also made and secreted, and since this isoform is not detected in the cellular proteins, it may be assumed that is has a rapid turnover within the cell. NT2N cells do not make or secrete a detectable amount of APLP2, while NT2 cells make only a small amount, which is membrane associated and does not appear to be secreted at detectable levels.

Table 4.5 Molecular Weights Of APP Isoforms In NT2N Cells, Along With The Antibodies Detecting Them

	$APP_{751/770}$			APP_{695}		
	Immature	**Mature**	**Soluble**	**Immature**	**Mature**	**Soluble**
Size (kDa)	*130*	*N/A*	*130*	*113*	*130*	*113*
22C11	✓	✓	✓	✓	✓	✓
993	✓	✓	✓	✗	✗	✗
6E10	✗	✗	✓	✗	✗	✓
3B11	✗	✗	✗	✗	✗	✗

4.4 CONTAMINATING CELLS

As described in *Chapter 3* cultures of NT2N cells may also contain non-neuronal "background" cells. These cells appear to provide support for the neuronal cells (see *Sections 3.2.2.2 and 3.2.3.1*) and may contribute to secreted proteins found in the media of these cultures. Other researchers have attributed KPI-containing APP isoforms found in neuronal cultures to contamination from these cells (Wertkin *et al.* 1993). In order to determine if these cells do secrete APP, media from mixed cultures, and from cultures of background cells only, was analysed, as described in *Section 4.2.3.*

4.4.1 Results

A faint band of approximately 135 kDa was detected in unconditioned media, this is presumably due to the presence of bovine APP in the FBS added to the media (see *Figure 4.2a*). This band was also present, at a greater density in the conditioned media from the background cells only.

Media from the mixed cell culture also contained this band, along with another of approximately 115 kDa.

Antibody 993 detected a single band of 135 kDa in all three media samples (see *Figure 4.2b*). This was faintest in the unconditioned media sample, and strongest in the media from the mixed cell population.

4.4.2 Conclusion

This data shows that any APP_{695} detected in the media of mixed cell populations can be attributed to the presence of neuronal cells. However, the same cannot be said for any KPI-containing isoforms detected. The background cells secrete an appreciable amount of KPI-containing APP, though it is not possible to say which isoform exactly. However the KPI-containing band was more intense in media from the mixed cell population than from the background cells alone, indicating that the neuronal cells do secrete one or more of those isoforms. As a result of this finding, any NT2N culture that contained contaminating cells was discarded, and all experiments were performed on cultures that contained only neuronal cells.

4.5 DESIGN AND TESTING OF MONOCLONAL ANTIBODIES

4.5.1 β-Secretase Specific Antibody

In order to differentiate between amyloidogenic and non-amyloidogenic pathways of APP secretion, it was decided to attempt to produce a monoclonal antibody that was specific for APPsβ. The aim was to produce an antibody that would recognise only soluble APP that had been cleaved at the amino terminus of βA4, i.e. by β-secretase, and not APPsα. It was predicted that as a result of this, the proportion of secreted APP that had undergone amyloidogenic cleavage could be determined.

4.5.1.1 Antibody Design and Production

The sequence of APP_{770} was retrieved from the Swisspro database, at the European Bioinformatics Institute site on the Internet, and the required peptide sequence identified. The peptide (peptide 303, see *Figure 4.3*) was synthesised by Research Genetics, USA and once received was conjugated to BSA using glutaraldehyde. This protein antigen was then sent to Sheffield Hybridomas, where it was injected into mice that were later screened for antibody production. The strongest responding mouse was identified and the spleen removed, homogenised and the cells fused with a myeloma cell line. The hybridoma cells were then cloned out and each clone tested for the production of the relevant monoclonal antibody. This initial testing was by ELISA (immunisation, fusion and initial testing carried out by Sheffield Hybridomas). Two fusions were carried out, resulting in approximately 1000 clones to be tested.

4.5.1.2 Further Testing

Clones were tested against peptides 303 and 304 (see *Figure 4.3*) to identify an antibody that was specific for the cleavage product. All positively testing clones reacted with both peptides, indicating that no antibody was specific for APPsβ. Since the antigen chosen is APP specific, and not found in APLP2 the antibodies were cloned further to produce an APP specific antibody. Clones identified as positive by Sheffield Hybridomas were passed on to this lab for further screening, as described in *Sections 4.2.4.1* and *4.2.4.2*

4.5.1.3 Results

After these tests, 2 clones, EH4 and FC8 appeared to immunoprecipitate APP successfully out of a solution of proteins extracted from monkey frontal cortex (see *Figure 4.4a*), though neither reacted with APP on an immunoblot. However, when the immunoprecipitation results were western blotted with the anti-mouse IgG secondary antibody only, a band at approximately 130 kDa was detected. Since this band ran at the same weight as APP it was not possible to

differentiate between the two proteins. Attempts to purify the antibodies on a Protein G column, as described in *Section 2.14*, were unsuccessful, as the non-specific band was still present after purification (see *Figure 4.4b*)

4.5.2 α-Secretase Specific Antibody

An alternative strategy to study amyloid production is to use antibodies that recognise epitopes within the βA4 sequence. To this end a further antibody was developed, against an epitope in the first 12 amino acids of βA4, immediately C-terminal to the β-secretase cleavage site. This antibody was intended to be used to differentiate between full length APP and APLP2, and between APPsα and APPsβ. APLP2 does not contain the βA4 sequence, therefore the antibody produced would be APP specific. Also, since β-secretase cleaves APP after the amino-terminus of βA4, the antibody would recognise secreted APP that had been cleaved by α-secretase only.

The peptide antigen (peptide 305, see *Figure 4.3*) was synthesised and the antibodies produced by Sheffield Hybridomas as previously. Hybridomas were screened by ELISA for secretion of a relevant antibody and positively testing clones were further tested for western blotting and immunoprecipitation capabilities, as before. The clones were graded according to performance, and the most promising ones identified for further cloning. As previously, 2 fusions were carried out, resulting in approximately 1000 initial clones, of which approximately 150 were further tested by western blotting and immunoprecipitation. This resulted in 10 clones chosen for re-cloning.

Of these, 2 clones were successfully re-cloned, HE1 and HD11. These were used to immunoprecipitate full-length secreted APP from the conditioned media of NT2 cells. This was not successful, despite initial positive results when immunoprecipitating APP from monkey brain

extract. To ensure that the clones were still secreting the relevant antibody, both antibodies were tested on an ELISA, as described in *Section 2.13.*

Each antibody was tested against several peptides, one positive, and several negative controls. The results showed that each antibody recognised almost any peptide bound to BSA with glutaraldehyde (see *Figure 4.5*). They appeared to be specific for the linking region of the antigen, not the specific peptide, or the BSA sequence.

4.6 AMYLOIDOGENIC CLEAVAGE OF APP FROM NT2 AND NT2N CELLS

Following the failure to produce antibodies specific for individual secretion activities, a commercial antibody, 6E10, became available. This antibody recognises an epitope in amino acids 1-28 of the βA4 domain of APP and, therefore, will only react with secreted APP which has been cleaved by α-secretase, not β-secretase cleaved APP, or secreted APLP2. This antibody was used, in conjunction with antibody 22C11, in serial immunoprecipitation experiments, to determine what proportion of secreted APP was processed via amyloidogenic pathways.

4.6.1 Results (See Figure 4.6*)*

No APP was detected by immunoprecipitation with 6E10 in the control sample. A faint band was visible after immunoprecipitation with 22C11 (compare lanes *1* and *2* in *Figure 4.6a* and *b*), this is probably due to bovine APP present in the FBS added to the complete media.

Immunoprecipitations with 6E10 from NT2 conditioned media detected 2 bands of approximately 110 and 135 kDa, with the larger band predominant. Both bands decreased in intensity with each successive immunoprecipitation step (see *Figure 4.6a* and *b* lanes *4-6*). Immunoprecipitation with 22C11 also resulted in these two bands, but as they were detected with both 22C11 and 6E10 on western blotting, it is not possible to say whether these bands are due to APPsα which was not

completely cleared by the 6E10 immunoprecipitation steps, or if they indicate the presence of APPsβ. If lane *7* is compared on *Figure 4.6a* and *b*, it can be seen that the intensity of the 2 bands is similar on each immunoblot, which may indicate that they are due to the presence of APPsα, and that a further immunoprecipitation step with 6E10 may remove them.

Immunoprecipitations with 6E10 from NT2N conditioned media gave similar results, recovering 2 APP bands of 115 and 140 kDa, though in this case the smaller band was predominant (see *Figure 4.6a* and *b*, lanes *9-11*). When immunoblotted with 22C11 the final immunoprecipitation step also resulted in these two bands. However, these bands were not detected after immunoblotting with 6E10 (compare lane *12*, *Figure 4.6* and *b*). This indicates that the 6E10 immunoprecipitation steps did clear all APPsα, and the protein recovered by 22C11 was APPsβ.

4.6.2 Conclusions

Both NT2 and NT2N cells secrete APP, which can be detected by immunoprecipitation with antibodies 22C11 and 6E10. It seems likely that all secreted APP from NT2 cells is a result of non-amyloidogenic cleavage, and that the majority of APP secreted by NT2N cells is also derived from this pathway. However, a small proportion of APP appears to undergo amyloidogenic breakdown in NT2N cells. Although the presence of APPsβ is not conclusive proof that the neuronal cells make βA4, it is an indication that at least one of the secretase activities required for this, is present and functional in these cells. This result agrees with results from other research groups, which have detected secreted and intracellular βA4 in the neuronal cells, but not in the stem cells (Wertkin *et al.* 1993).

4.7 DETECTION OF βA4

The results of the serial immunoprecipitation studies indicated that NT2N cells may produce βA4. Using pulse chase labelling methods other groups have shown that the cells do make βA4, and that most of this is secreted into the media (Wertkin *et al.* 1993). Work was carried out to see if the neurones produced by the methods described in this work also secrete βA4.

4.7.1 Preliminary Studies

4.7.1.1 Detection of βA4

Initial studies were carried out to see if a peptide as small as the 4 kDa βA4 peptide could be resolved on a gel, and to discover what sensitivity could be achieved by western blotting, as described in *Section 4.2.6.*

On the nitro-cellulose membrane the βA4 was seen as a tight band running at approximately 4 kDa (see *Figure 4.7*). The system was sensitive enough to detect the smallest amount of βA4 used, 50 ng, and comparable results were obtained with both the ECL and the chromogenic techniques. However, the βA4 did not appear to bind to the PVDF membrane as it could not be detected with either ECL or chromogenic means.

4.7.1.2 βA4 Immunoprecipitations

Several antibodies were tested for the ability to immunoprecipitate βA4 out of solution, as described in *Section 4.2.7.* As can be seen in *Figure 4.8*, the only antibody that successfully recovered βA4 was 6E10. However if lane 1, which contains a control of 50 ng of βA4, is compared with lane 2, which would contain 80 ng of βA4 if there was 100% recovery, it can be seen that the actual recovery level is less than 50 %.

4.7.2 βA4 Immunoprecipitation From NT2 and NT2N Cells and Media

NT2 and NT2N cell and media samples were tested by immunoprecipitation for the presence of βA4, as described in *Section 4.2.8.* βA4 was not detected in any of the immunoprecipitations, though synthetic βA4 that was run as a positive control, was detected on the western blot (see *Figure 4.9a*). Full length APP was detected in both cell and media samples (see *Figure 4.9b*), indicating that 6E10 will immunoprecipitate APP under the conditions used.

4.7.2.1 Conclusion

It is not possible to detect intracellular βA4 using the techniques described. This is unsurprising, since NT2 cells have not previously been shown to make βA4, and while the NT2N cells do produce some βA4, it is rapidly secreted after its formation. (Wertkin *et al.* 1993)

It was not possible to immunoprecipitate βA4 from the media of either cell type, not even from media that had been conditioned by NT2N cells for 7 days. This is more surprising, as the previous study showed NT2N cells continually to secrete the peptide. The lack of success of the present study may be due to two factors; either the levels of βA4 are too low to be detected by the methods used, or the presence of full length APP in the media from cultured cells interferes with the immunoprecipitation at the antibody concentration used.

4.7.3 Immunoprecipitation of Synthetic βA4 from Conditioned Media

In order to test the hypothesis that 6E10 cannot immunoprecipitate βA4 from conditioned media, either due to the chemical composition of the media, or to the fact that any full length APP present saturates the antibody, synthetic βA4 was added to conditioned and unconditioned media, as described in *Section 4.2.9.*

βA4 was recovered from both media samples, though when compared with the control sample of 50 ng βA4 run on the gel it is possible to see that there is less than 50% recovery (see *Figure 4.10* compare lanes *2* and *3* with lane *1*)

4.7.4 βA4 Conclusions

These studies show that it is possible to separate synthetic βA4 on a TRICINE gel, to detect it on a western blot, and to immunoprecipitate it from the conditioned media of NT2 cells. However, it was not possible to detect endogenous βA4, either in whole cell extract or in conditioned media of NT2N cells. Since the techniques have been proved to work, the lack of success in detecting endogenous βA4 may be attributed to the very low concentrations present. To detect it techniques with greater sensitivity, which were not available in the laboratory, must be used.

4.8 CONCLUSION

Using SDS-PAGE and western blotting techniques it is possible to identify the APP isoforms expressed by both NT2 and NT2N cells. These techniques can differentiate between APP and APLP2, and can identify whether the APP produced is KPI-containing or APP_{695}. It is also possible to look at intracellular APP and determine whether it is membrane bound or soluble. Secreted APP can be collected, and after concentration, it can also be analysed by SDS-PAGE and western blotting to determine which isoforms are secreted from each cell type.

The cleavage pathways used by each cell type can also be investigated. Using serial immunoprecipitation from conditioned media it is possible to determine what proportion of the secreted APP is processed via amyloidogenic or non-amyloidogenic pathways. 6E10 can be used to clear the media of APPsα, or non-amyloidogenic APP, and any remaining secreted APP, which must be APPsβ, is immunoprecipitated by 22C11.

Although this study was not able to detect endogenous βA4 either in whole cell extracts of NT2N cells, or in the conditioned media from these cells, it did show that it is possible to resolve and detect βA4 using a TRIS-TRICINE gel system, transfer to nitro-cellulose membrane, and western blotting. It is also possible to immunoprecipitate synthetic βA4 from conditioned cell culture media, showing that the presence of APPs does not interfere with the immunoprecipitation system. Further work needs to be done to increase the sensitivity of these methods, either by concentrating the βA4 in the conditioned media by gel filtration chromatography or by pulsing the cells with radioactivity to label the βA4 and increase detection sensitivity. Nevertheless, as previously stated, the serial immunoprecipitation techniques developed did allow the APP processing pathways in NT2 and NT2N cells to be studied.

Figure 4.1 APP PRODUCTION AND SECRETION BY NT2 AND NT2N CELLS

Cell samples were processed to give soluble and membrane proteins, samples of conditioned media were concentrated 10-fold. All samples were mixed with SDS-PAGE sample buffer and 20 μl of each loaded on 7% SDS-PAGE gels, which were then transferred to nitro-cellulose membranes and immunoblotted with antibodies 3B11, 22C11 and 993.

Lane	Cells	Fraction	Total Protein Loaded
1	NT2	Soluble	12 μg
2		Membrane	9 μg
3		Media	5-fold final concentration*
4	NT2N	Soluble	5 μg
5		Membrane	4 μg
6		Media	5-fold final concentration*

** Approximately 800 μg total protein, the majority of which derives from the FBS*

Figures to the right of the blot indicate the mobility of molecular weight markers.

Only the membrane fraction of NT2 cells contains APLP2. NT2 cells contain 3 major APP bands, at 115, 128 and 137 kDa, and secrete the 128 kDa form. NT2N cells contain a major band of 113 kDa, with a second band at 130 kDa. Both of these forms are secreted, but only the larger one is detected by 993, indicating that the smaller protein is APP_{695}.

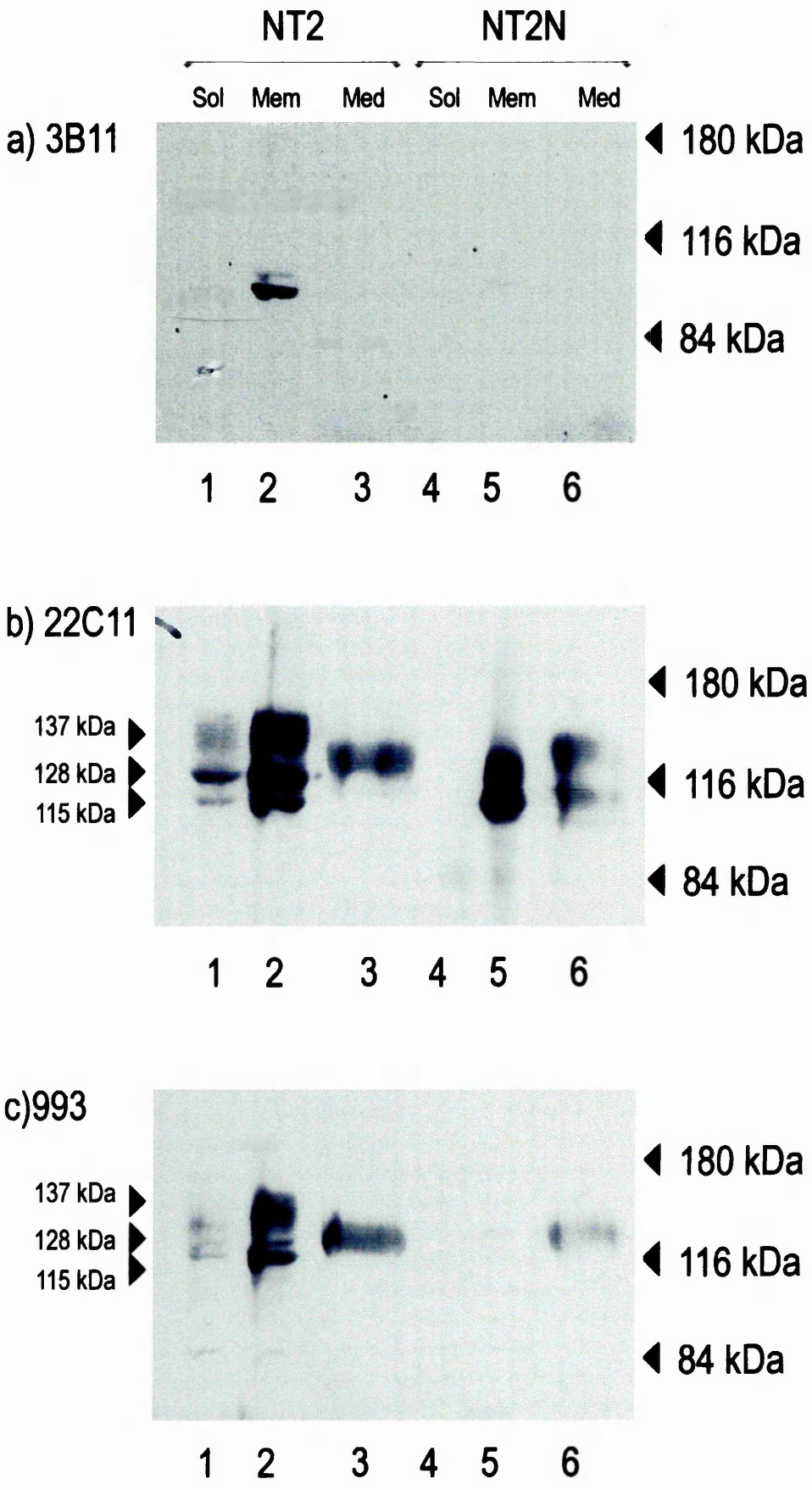

NT2
NT2N
Sol
Mem
Med
Sol
Mem
Med
a) 3B11
180 kDa
116 kDa
84 kDa
1 2 3 4 5 6
b) 22C11
137 kDa
128 kDa
115 kDa
180 kDa
116 kDa
84 kDa
1 2 3 4 5 6
c)993
137 kDa
128 kDa
115 kDa
180 kDa
116 kDa
84 kDa
1 2 3 4 5 6

Figure 4.2 APP SECRETION BY CELLS PRESENT AFTER RA INDUCED DIFFERENTIATION OF NT2 CELL CULTURES

Media samples from a cell culture containing neuronal and background cells, and from a population containing only the background cells, along with unconditioned cDMEM were concentrated 10-fold. 20 μl of each sample was loaded onto 7% SDS-PAGE gels. The gels were transferred to nitro-cellulose membrane and immunoblotted with antibodies 22C11 and 993.

Lane 1 Media from non-neuronal cells

Lane 2 Media from the mixed cell population

Lane 3 Unconditioned cDMEM

Figures to the right of the blot indicate the mobility of molecular weight markers.

The mixed cell population secretes 2 APP forms, of 113 and 130 kDa, however, by comparing the secretion from the background cells only, it can be seen that the 113 kDa form of APP is secreted by the neuronal cells only, while the 130 kDa form is secreted by both cell types.

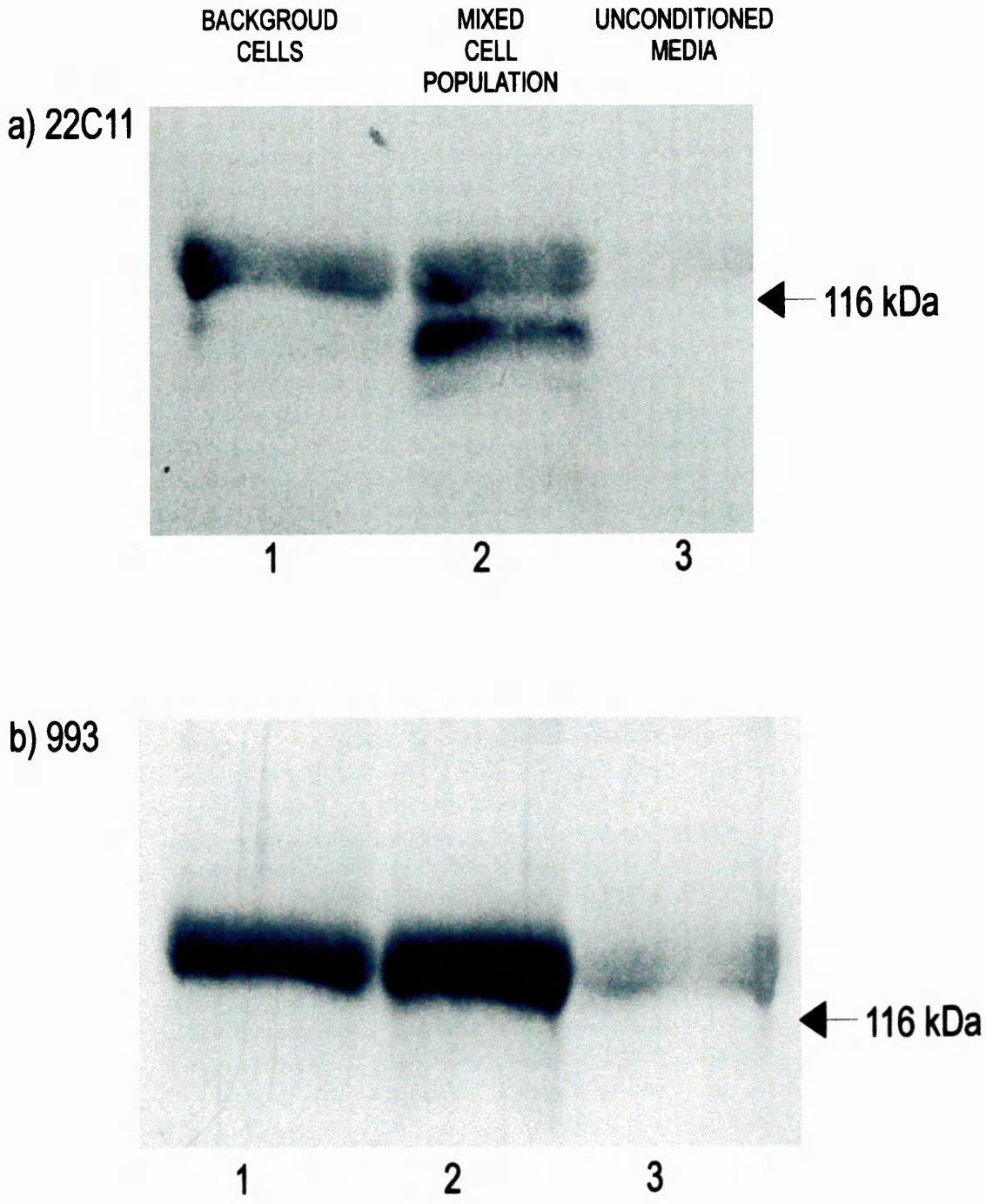
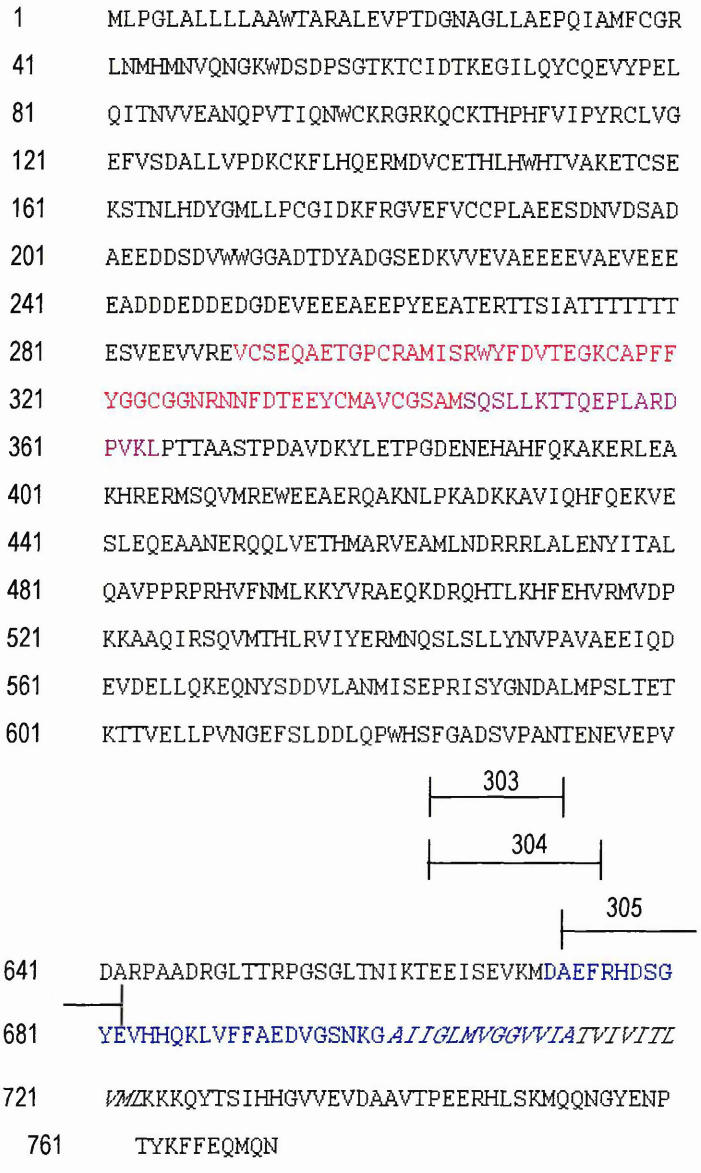

BACKGROUD CELLS
MIXED CELL POPULATION
UNCONDITIONED MEDIA
a) 22C11
116 kDa
1
2
3
b) 993
116 kDa
1
2
3

Figure 4.3 APP770 SEQUENCE AND ANTIBODY RECOGNITION SITES

Amino acid sequence of APP_{770}, with lines to indicate the peptide antigens synthesised for the use of antibody production and testing.



Red	KPI domain
Purple	OX2 domain
Blue	beta-amyloid region
Italic	transmembrane domain

Figure 4.4 IMMUNOPRECIPITATIONS WITH FC8 AND EH4

a) Cell culture supernatant from each clone was used to immunoprecipitate APP from monkey brain. The samples were separated on a 7% SDS-PAGE gel, and immunoblotted with 22C11. The blot was developed using NBT/BCIP.

Lane 1 EH4

Lane 2 FC8

Lane 3 Monkey brain extract, positive control for the immunoblot

b) Increasing amounts of purified FC8 was incubated with monkey brain extract, as described. The samples were separated on a 10% gel, and immunoblotted using an anti-mouse IgG secondary antibody conjugated to AP, only. The blot was developed using NBT/BCIP.

Lane 1 5 μl FC8

Lane 2 10 μl FC8

Lane 3 20 μl FC8

Lane 4 50 μl FC8

Figures to the right of the blot indicate the mobility of molecular weight markers.

The initial blot shows a band running at the same weight as APP, but as can be seen in the second blot, the anti-mouse-IgG antibody detects this band only, indicating that it is not APP. The band is present even after purifying the cell culture supernatant on a protein G column.

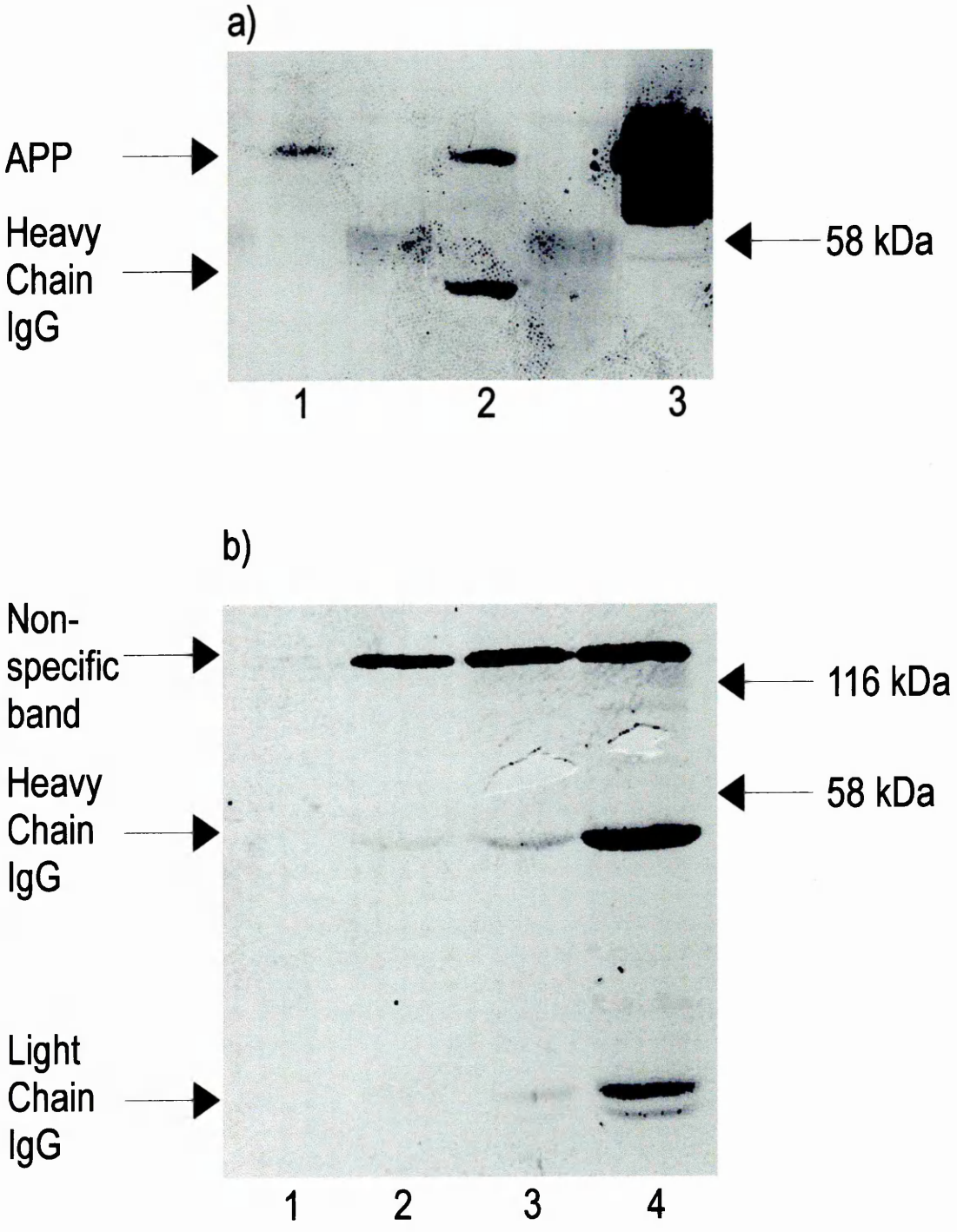
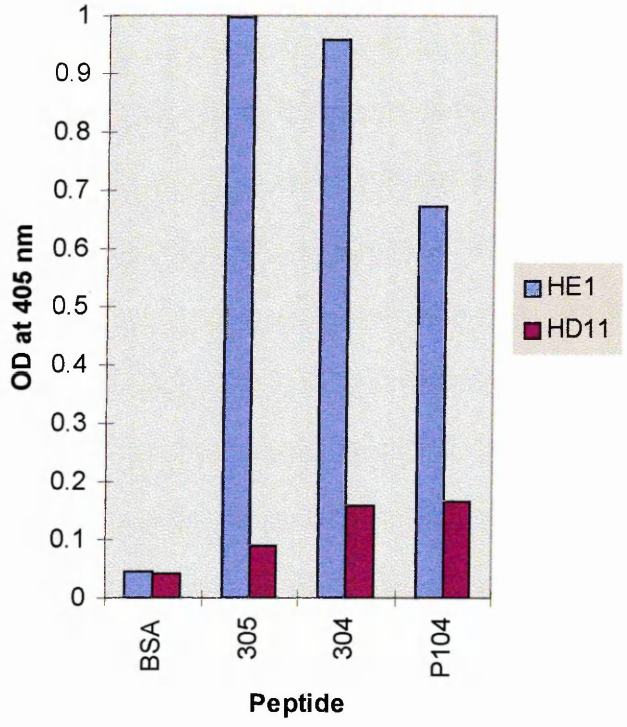

a)
APP
Heavy Chain IgG
58 kDa
1
2
3
b)
Non-specific band
Heavy Chain IgG
Light Chain IgG
116 kDa
58 kDa
1
2
3
4

Figure 4.5 ELISA SHOWING THAT HE1 AND HD11 REACT WITH THE CROSS LINKER



Peptide 1	BSA blank	
Peptide 2	305, positive control	DAEFRHDSGYEV
Peptide 3	304, negative control	EEISEVKMDA
Peptide 4	P104, negative control	CYFQNAQMSEDNHL

All peptides were conjugated to BSA using glutaraldehyde. Although neither antibody recognises BSA only, both recognise all 3 of the peptide antigens, indicating that they may recognise the cross-linking region.

Figure 4.6 NT2N CELLS SECRETE APP VIA BOTH AMYLOIDOGENIC AND NON-AMYLOIDOGENIC PATHWAYS, NT2 CELLS USE ONLY NON-AMYLOIDOGENIC ONES

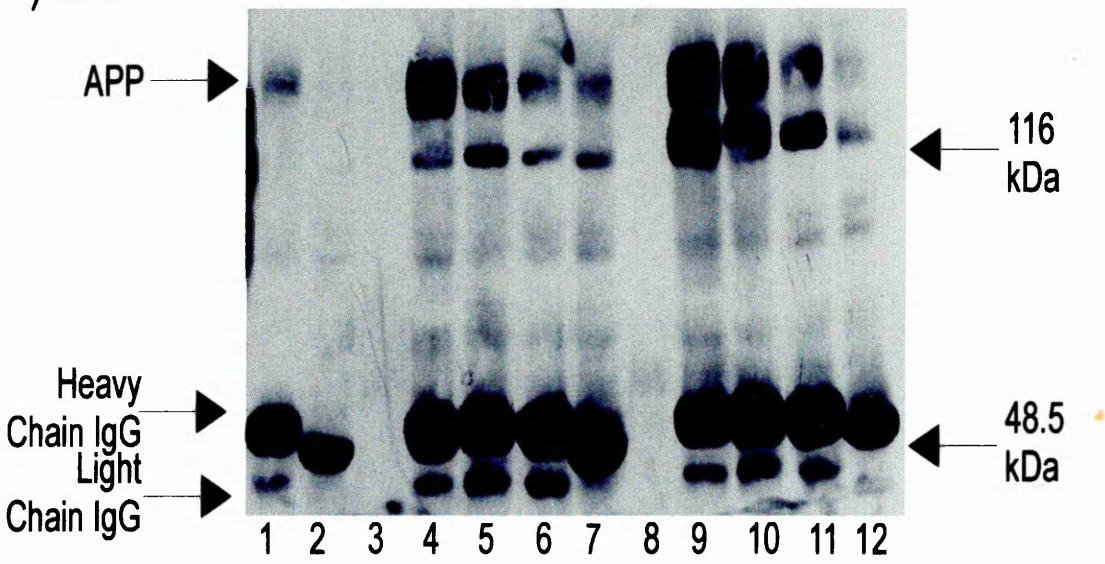
APP was immunoprecipitated from NT2 and NT2N conditioned media, using serial immunoprecipitations with 6E10 and 22C11. Control immunoprecipitations with 22C11 or 6E10 were performed on unconditioned media. The samples were run on 7% SDS-PAGE gels that were transferred to nitro-cellulose membranes and blotted with 22C11 or 6E10.

Lane	Media	Antibody
1	Unconditioned control cDMEM	22C11
2		6E10
3	Blank	
4	NT2 conditioned cDMEM	6E10 #1
5		6E10 #2
6		6E10 #3
7		22C11
8	Blank	
9	NT2N conditioned cDMEM	6E10 #1
10		6E10 #2
11		6E10 #3
12		22C11

Figures to the right of the blot indicate the mobility of molecular weight markers.

NT2 cells appear to secrete only APPsα, detected by both 22C11 and 6E10, while NT2N cells appear to secrete an appreciable amount of APPsβ (compare lane *12* in *a* and *b*).

a) 22C11



b)6E10

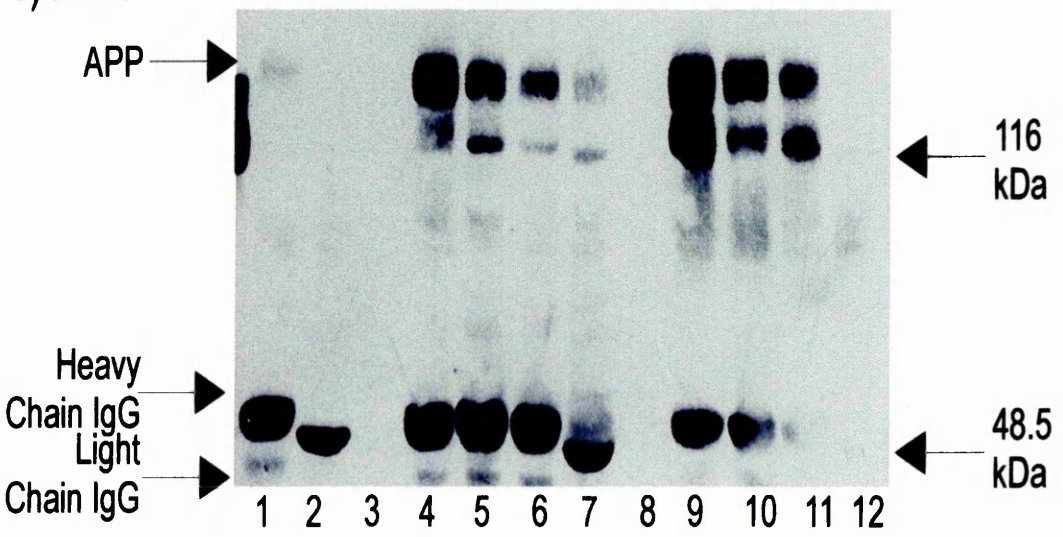


Figure 4.7 DETECTION OF SYNTHETIC bA4 ON A WESTERN BLOT

Solutions of various concentrations of synthetic $\beta A4_{1-40}$ were prepared and run on a 12% TRIS-TRICINE gel. The gel was transferred to nitro-cellulose membrane and immunoblotted with 6E10. The blot was developed using ECL.

Lane 1	50 ng $\beta A4_{1-40}$
Lane 2	100 ng $\beta A4_{1-40}$
Lane 3	150 ng $\beta A4_{1-40}$
Lane 4	200 ng $\beta A4_{1-40}$
Lane 5	250 ng $\beta A4_{1-40}$

Figures to the right of the blot indicate the mobility of molecular weight markers.

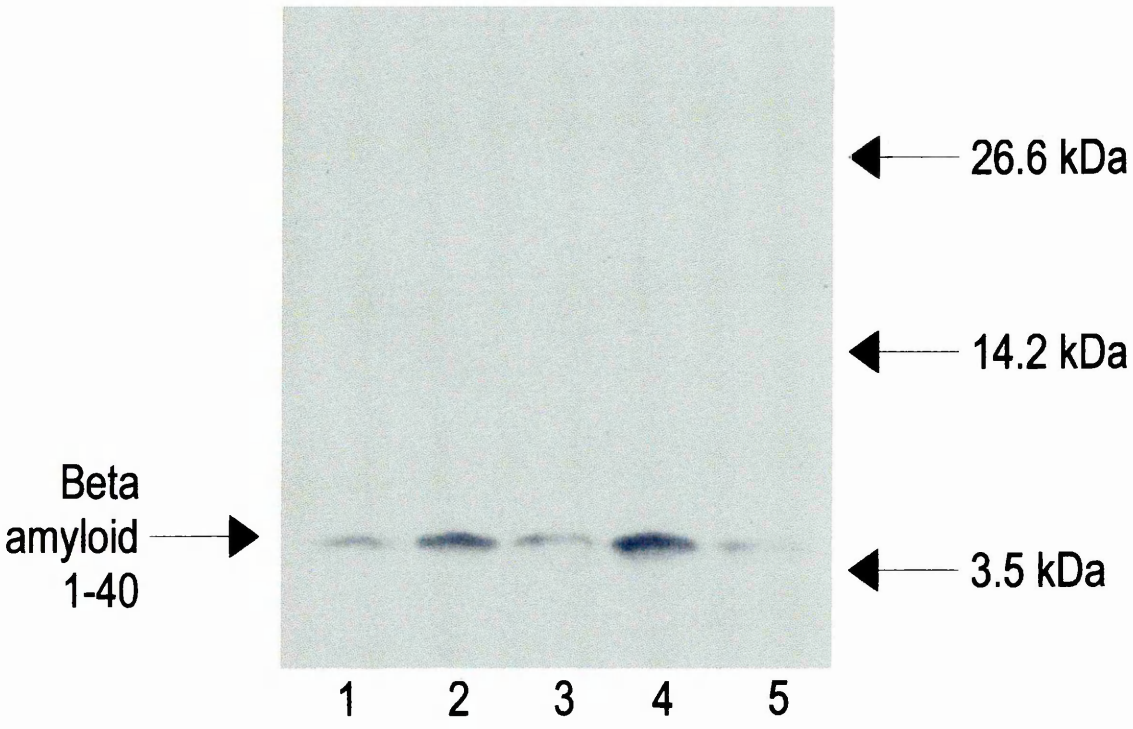

26.6 kDa
14.2 kDa
Beta
amyloid
1-40
3.5 kDa
1
2
3
4
5

Figure 4.8 IMMUOPRECIPITATION OF SYNTHETIC βA4 USING SEVERAL ANTIBODIES

Synthetic βA4 in a 1 mg / ml solution of BSA in TBS was incubated with 50 μg of various antibodies for 2 hours at room temperature. The antibody complex was recovered with agarose beads that were eluted in of SDS-PAGE sample buffer. Samples were separated on a 12.5% TRIS-TRICINE gel, transferred to nitro-cellulose membrane and blotted with 6E10.

Lane	Antibody	Epitope
1	No immunoprecipitation, 100 ng βA4	
2	6E10	Within amino acids 1-28 βA4
3	1032	Within amino acids 1-19 βA4
4	58617	Within amino acids 1-9 βA4
5	58618	Within amino acids 1-9 βA4
6	6F/3D	Within amino acids 8-17 βA4

Figures to the right of the blot indicate the mobility of molecular weight markers.

Only antibody 6E10 successfully recovered the βA4 peptide.

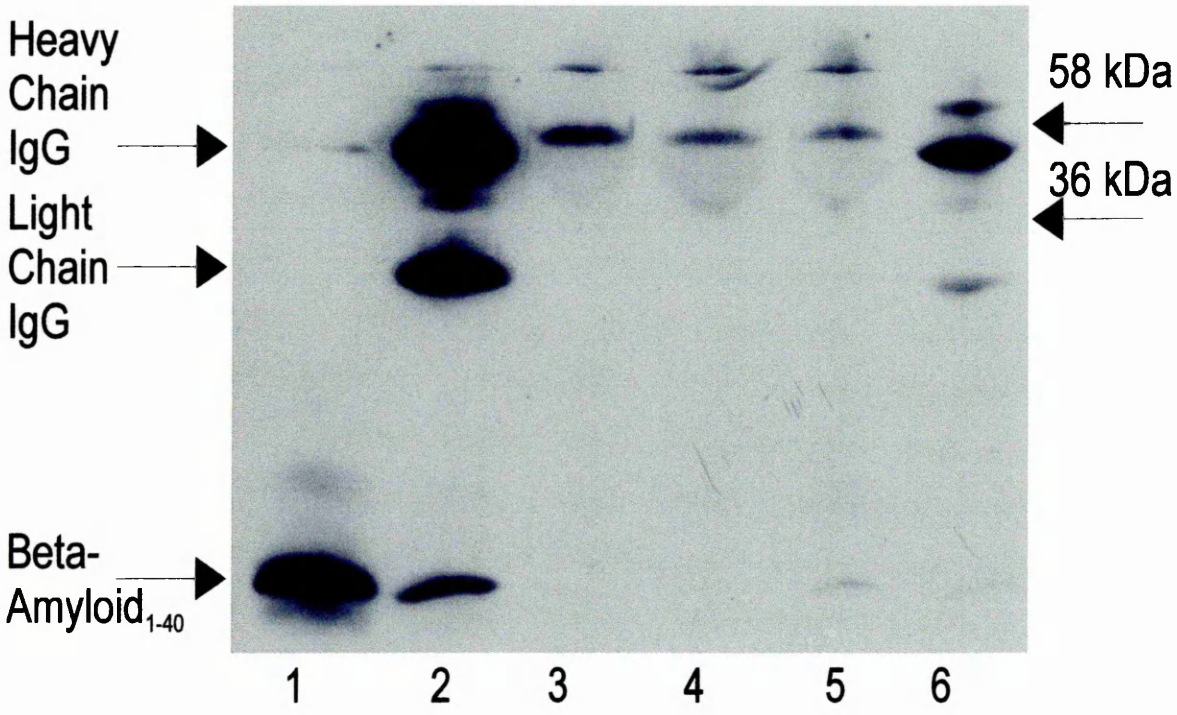

Heavy Chain IgG
Light Chain IgG
Beta-Amyloid1-40
58 kDa
36 kDa
1
2
3
4
5
6

Figure 4.9 βA4 CANNOT BE IMMUNOPRECIPITATED FROM WHOLE CELL EXTRACTS, OR FROM THE CONDITIONED MEDIA OF NT2N CELLS

6E10 was used to immunoprecipitate βA4 from whole cell extracts, and conditioned media. The samples were separated on either a 12% acrylamide TRIS-TRICINE, or a 7% SDS-PAGE gel. The gels were transferred to nitro-cellulose membrane and immunoblotted with 6E10.

a) **12% acrylamide TRIS-TRICINE gel**

Lane 1 50 ng synthetic βA4

Lane 2 100 ng synthetic βA4

Lane 3 Immunoprecipitation from NT2 whole cell extracts

Lane 4 Immunoprecipitation from NT2N whole cell extracts

Lane 5 Immunoprecipitation from 7 day conditioned NT2N media

b) **7% acrylamide SDS-PAGE gel**

Lane 1 Immunoprecipitation from NT2 whole cell extracts

Lane 2 Immunoprecipitation from NT2N whole cell extracts

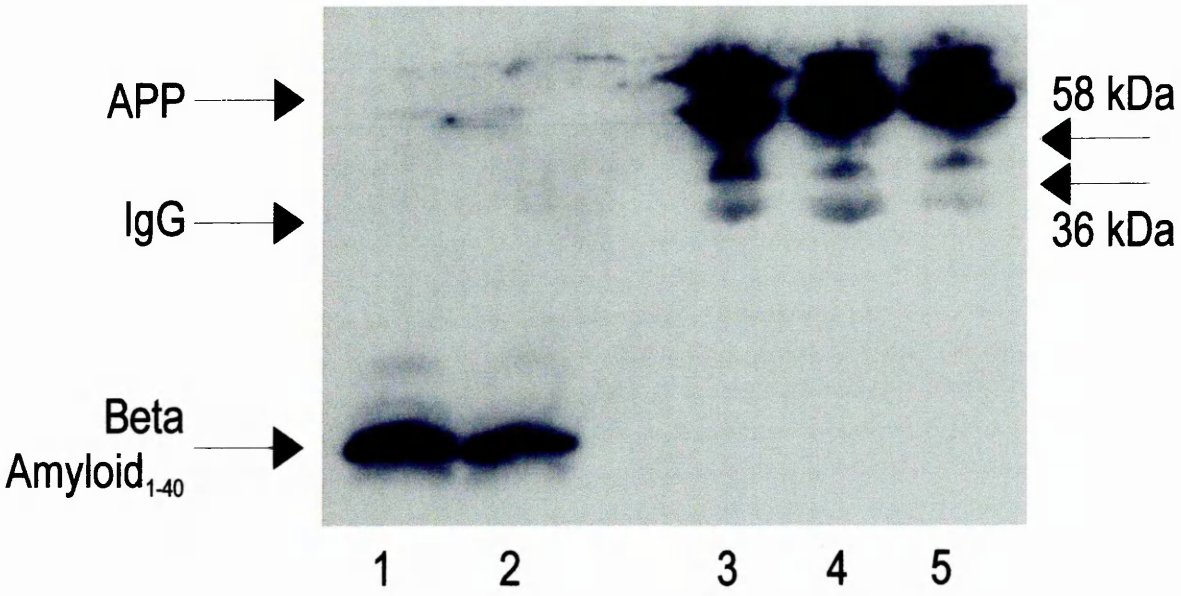
Lane 3 Immunoprecipitation from 3 day conditioned NT2 media

Lane 4 Immunoprecipitation from 7 day conditioned NT2N media

Figures to the right of the blot indicate the mobility of molecular weight markers.

Endogenous βA4 could not be detected in any of the samples using this method.

a) 12% tris-tricine gel



b) 7% SDS-PAGE gel

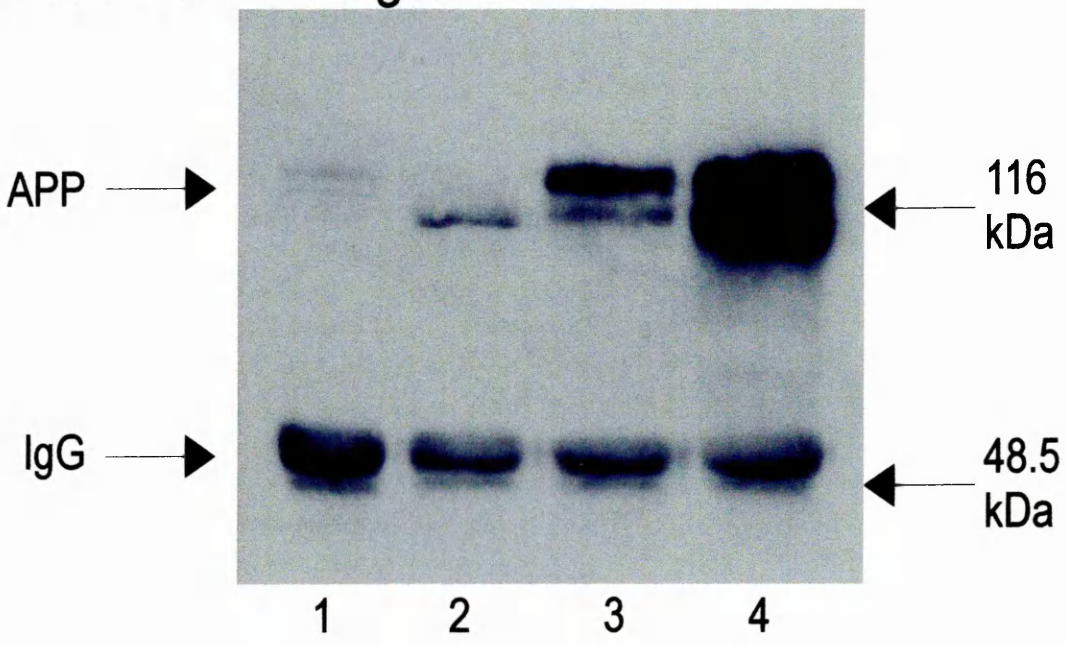


Figure 4.10 IT IS POSSIBLE TO IMMUNOPRECIPITATE SYNTHETIC bA4 FROM CONDITIONED MEDIA

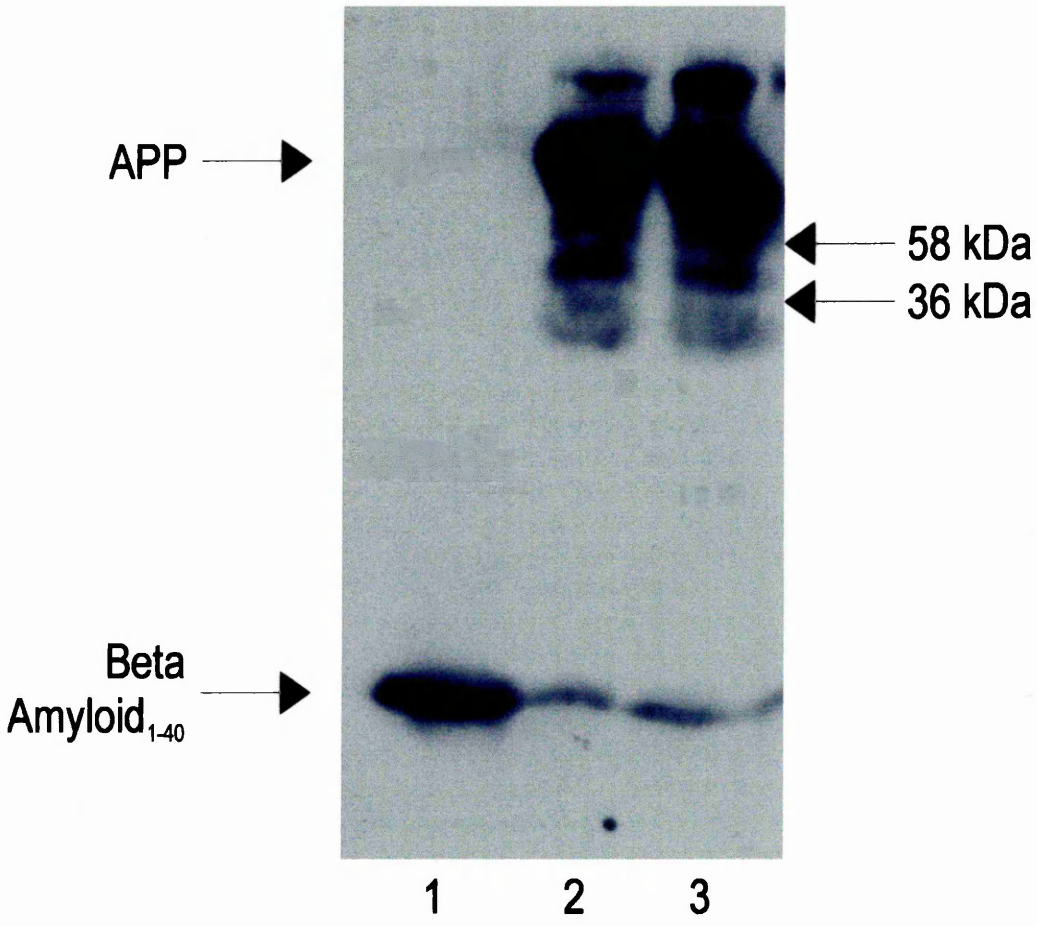
Synthetic βA4 was added to unconditioned cDMEM or 24 hour conditioned NT2 media. Immunoprecipitation with 6E10 was used to recover the peptide. Samples were separated on a 12% acrylamide TRIS-TRICINE gel, which was transferred to nitro-cellulose membrane and immunoblotted with 6E10 as before.

Lane 1 50 ng βA4

Lane 2 synthetic βA4 immunoprecipitated from unconditioned DMEM

Lane 3 synthetic βA4 immunoprecipitated from 24 hour NT2 conditioned DMEM

Figures to the right of the blot indicate the mobility of molecular weight markers.


APP
58 kDa
36 kDa
Beta
Amyloid1-40
1
2
3

Chapter Five - Effects Of Heat Shock On NTera2 Stem Cells And Neurones

5.1 INTRODUCTION

As discussed in the introduction, there are several indications that neurones in the AD brain may be in a state of stress. Protein production and processing is altered, with a decrease in overall protein synthesis and changes in protein phosphorylation, there is also a disruption of the cytoskeleton. Levels of ubiquitin and associated proteins are increased (Wang *et al.* 1991), and heat shock proteins 72 and 73 have been found in both senile plaques and neurofibrillary tangles (Hamos *et al.* 1991). Since APP contains a heat shock element in the promoter region of its gene (Salbaum *et al.* 1988), a number of studies have been done to investigate the effects of heat shock or other metabolic stresses on the expression of APP. These studies have included research on various cell types, and have investigated APP in several ways.

Research on glioma cell lines transfected with APP fusion protein constructs, indicated that heat shock may lead to mis-compartmentalisation of APP (Pappolla *et al.* 1995). The fusion proteins were tagged with SEAP, a secreted derivative of placental alkaline phosphatase. After a heat shock for 30 minutes at 44°C and recovery at 37°C, more than 90% of the cells had accumulated the fusion protein in their cytoplasm, in contrast the control transfected cells did not show alkaline phosphatase activity when examined by immunocytochemistry,

Heat shock also induces changes in APP processing, in PC12 cells that had been treated with nerve growth factor to induce a neuronal phenotype (Johnson *et al.* 1993a). A 30 minute

incubation at 45°C induced the production of two additional APP polypeptides, of approximately 95 and 120 kDa, which were not present in control cells. While levels of constitutively expressed APP did not change, there was an overall rise in APP levels due to the production of these new polypeptides. There was also a 70% decrease in the phosphorylation levels of APP, compared with only a 20% decrease in protein phosphorylation levels overall.

Heat shock induces changes in APP mRNA, also. Human lymphoblastoid cells lines, from normal subjects were subjected to a 42°C heat shock for 30 minutes, then allowed to recover at 37°C for various lengths of time (Abe *et al.* 1991). When examined by northern blot there was a 40-60% increase in APP mRNA at 3 and 8 hours recovery, which had returned to normal levels by 48 hours recovery. The probe used detected all isoforms of APP mRNA as a single band, so there was no indication as to whether the increase was isoform specific.

Secreted APP is also affected by heat stress (Ciallella *et al.* 1994). Human umbilical vein endothelium cells (HUVECs) were subjected to 42°C for 15 to 30 minutes, then allowed to recover at 37°C. Northern blotting showed that APP mRNA was increased at all time points up to 24 hours recovery, and was greatest (an approximately seven-fold increase) after 4 hours recovery. Western blotting of conditioned media also showed an increase in secreted APP at all time points, though this was greatest after only one hour recovery. Intracellular APP also increased, as seen by immunocytochemistry, in this case the increase was greatest at 12 hours recovery.

As neurones are post-mitotic, protecting themselves from stress is, perhaps, more crucial than for other cell types which can regenerate cell numbers. There is some evidence that different cell

types in the brain respond to stress differently (Satoh and Kim, 1994), after heat-shock experiments on primary cell cultures of human foetal neurones, astrocytes, microglia and oligodendrocytes, the pattern of induction of the 72 kDa heat shock protein (HSP72) varied for each cell type. Western blots showed that the protein was highest in the astrocytes and microglia after 8 hours recovery at 37°C, while it peaked in the neurones after 24 hours recovery. Immunocytochemistry showed that only a percentage of each cell type showed HSP72 immunoreactivity.

For the reasons described previously, NT2 stem cells and neurones may provide a good system in which to study APP processing. Both cell types produce and secrete easily detectable quantities of APP, and there is a shift in the pattern of APP expression on differentiation of the stem cells to the neuronal phenotype. If APP is involved in the heat shock response, and it is an element of this which triggers some or all of the pathology of AD, then a human CNS neuronal cell line is one of the best models in which to study the phenomenon.

A previous study has looked at APP expression by heat-stressed NT2 cells (Dewji *et al.* 1995). However, the work used cells transiently transfected with a fragment of the APP gene promoter cloned into a luciferase expression vector, and not endogenous proteins, or indeed the full length APP gene. Also the experiments looked only at transfected stem cells, not neuronal cells, and as previously stated, there may be a different response from the two phenotypes.

5.2 PRELIMINARY EXPERIMENTS

The stress response of cells in culture is cell type specific . There is no standard heat-stress protocol, temperatures ranging from 42-45°C have been used, and the length of time the cells are exposed to the high temperature can vary from 15 minutes to several hours. The method of heat-

shock can vary too, some research groups used CO_2 incubators set at the higher temperature, while others used sealed culture vessels placed in a water bath.

Induction of expression of HSP72 is usually used as a marker of heat shock. This is a member of the 70 kDa family of heat shock proteins, and while it is constitutively expressed at low levels in a number of cells types, its expression is massively increased in response to several types of stress (Satoh and Kim, 1995). Studies were carried out to determine a protocol that would successfully give a stress response in NT2 cells, using the induction of HSP70 as a marker for stress.

5.2.1 Initial Method

T25 flasks of NT2 and NT2N cells were washed with 5 ml DMEM then fed with 5 ml cDMEM, which had been pre-warmed to the stress temperature in a 10% CO_2 incubator to equilibrate the CO_2 levels. The flasks were then placed in the stress environment, for experiments that took place in a CO_2 incubator the caps of the culture vessels were left loose, for experiments in the absence of CO_2 the caps were sealed. After the shock period the media was collected and the cells either harvested, or fed with fresh cDMEM pre-warmed to 37°C, and returned to the 37°C incubator to allow a recovery period. The conditioned media was collected and the cells harvested at the times stated in the results section. The cells were processed to give whole cell lysates, which were then analysed on a SDS-PAGE gel, transferred to nitro-cellulose membrane and immunoblotted with an antibody to the HSP70 family of heat shock proteins (Sigma) which recognises both the constitutive and inducible forms of HSP70 (concentration 1:5000, 1.9 μg antibody). The blot was developed using ECL.

5.2.2 Preliminary Results

Initial experiments took place at 42°C in a 10% CO_2 incubator. Stress periods of 30 and 60 minutes had no effect on HSP70 production in NT2 stem cells, however, even the shorter time was enough to kill the neuronal cells. Following this, all heat shock work was performed on NT2 stem cells only.

Two further protocols tried were a 30-minute incubation at 42°C without CO_2, which had no effect on HSP70 levels, and a one-hour incubation in a water bath at 45°C, which killed all the cells.

A group doing research into the expression of HSP70 through the cell cycle in several mammalian cell lines used a heat stress of 7.5 hours at 42°C (Hang and Fox, 1996). NT2 cells were placed at 42°C in a 10% CO_2 incubator for 8 hours, after which time they were harvested and the media collected. After western blotting the whole cells extracts from heat stressed and control cells, with the antibody to HSP70, a single band of approximately 68 kDa was visible in both lanes, but an additional band at approximately 85 kDa was visible in the heat shocked cells (see *Figure 5.1*).

Table 5.1 Heat Shock Protocols Attempted

Method	Temperature	Environment	CO_2 Level	Time	Effect on NT2 cells	Effect on NT2N cells
1	42°C	Incubator	10 %	30 min.	None	Killed all cells
2	42°C	Incubator	10 %	60 min.	None	N/A
3	42°C	Incubator	0 %	30 min.	None	N/A
4	45°C	Water Bath	10 %	60 min.	Killed all cells	N/A
5 - Final	*42°C*	*Incubator*	*10 %*	*8 hours*	*Increased HSP70*	*N/A*

5.3 EFFECTS OF HEAT SHOCK ON HSP70 PRODUCTION IN NT2 CELLS

5.3.1 Final Method

T25 flasks of NT2 cells were washed with 5 ml DMEM then fed with 5 ml cDMEM, which had been pre-warmed to 42° temperature in a water bath. The flasks were then placed in an incubator at 42° and 10% CO_2, with the caps of the culture vessels were left loose. The flasks were left in the incubator for 8 hours, after which the media was collected and the cells either harvested, or fed with fresh cDMEM pre-warmed to 37°C, and returned to the 37°C incubator to allow a recovery period of up to 24 hours. The conditioned media was collected and the cells harvested at the times stated in the results section. The cells were processed to give whole cell lysates, which were then analysed on a 7% SDS-PAGE gel, transferred to nitro-cellulose

membrane and immunoblotted with various antibodies, as described previously (*Sections 2.3, 2.7, 2.9, 2.10* and *5.2.1* and *Tables 4.3* and *4.4*)

5.3.2 Results

After the heat stress treatment described in *Section 5.3.1*, the NT2 cell cultures were only slightly less confluent than controls, though there were more dead cells floating in the culture media. The morphology of the cells changed after heat-shock (see *Figure 5.2*), with heat-stressed cells appearing to display cytoplasmic projections, they also appeared to be less flatly adherent to the culture vessel than control cells. When immunoblotted with the antibody against HSP70, control cells contained 2 bands, at approximately 67 and 83 kDa. Immediately after heat shocking, cells also contained these two bands. However, after 4 hours recovery at 37°C, a third band was detected in heat shocked cells, at approximately 78 kDa (see *Figure 5.3*). This band was present in cells up to 24 hours after the heat stress treatment, and never detected in control cells.

5.4 EFFECTS OF HEAT SHOCK ON APP PRODUCTION

5.4.1 Intracellular APP

NT2 cells were heat shocked as described above (*Section 5.3.1*), and allowed to recover at 37°C for up to 24 hours. Cells were collected at 0, 4, 14, 19 and 24 hours recovery, and the cell pellets were processed to give whole cell lysates. These were analysed on a SDS-PAGE gel that was transferred to nitro-cellulose membrane and western-blotted with antibodies 22C11 and 993.

Antibody 22C11 detected 2 major bands in the cell extracts at all time points, of approximately 128 and 137 kDa, with a minor band at 115 kDa (see *Figure 5.4a*). In the control cells the higher molecular weight band was predominant. The heat shocked cells harvested without allowing recovery at 37°C resembled the control cells. However, after 4 hours at 37°C there was an

increase in the lower weight molecular band. This band was still dominant at 14 hours recovery, but had returned to the control level by 19 hours recovery. There was a slight decrease in intensity of the higher molecular weight band after 19 hours recovery, and this band was still diminished at 24 hours recovery.

A similar result was obtained when the cell extracts were immunoblotted with 993 (see *Figure 5.4b*), indicating that these changes are due to changes in KPI containing APP isoforms.

5.4.2 Secreted APP

The experiment was repeated, with cell and conditioned media samples collected from control and heat shocked cells after 0, 4, 16, and 24 hours recovery at 37°C. The cell samples were processed and western blotted as described above. The media samples were concentrated 10-fold using ammonium sulphate fractionation, mixed with SDS-PAGE sample buffer and the proteins separated on a 7% acrylamide gel. The gel was transferred to nitro-cellulose membrane and western blotted with 22C11 and 993.

The cell extracts gave the same result as previously, with 2 bands of approximately 128 and 137 kDa in all samples. The lower molecular weight band was increased at 4 hours post heat shock in the stressed cells, but had returned to normal levels after 16 hours recovery. The higher molecular weight band was slightly decreased at 16 and 24 hours recovery. Both antibodies gave comparable results.

Western blots of the media samples resulted in one band of approximately 128 kDa (see *Figure 5.5*). There was only a slight difference between control and heat shocked samples. There was a slight decrease in secretion of APP at 16 hours post heat shock from the stressed cells, and a

slight increase from the same cells after 24 hours recovery. Western blotting with antibodies 22C11 and 993 gave identical results, indicating that all changes are in KPI-containing isoforms.

5.5 CONCLUSION

A technique has been developed for inducing a shock response in NT2 cells. This response is confirmed by the induction of HSP72 in the cells. This protocol of shocking the cells by a period of heat shock, followed by a recovery period at 37°C has an effect on the APP production and processing by the cells.

Although at the time point immediately after the heat shock treatment, there is no difference in intracellular APP between control and stressed cells, after a 4 hour recovery period there is an increase in APP in the stressed cells. Overall APP is increased, with a specific increase in the smaller form of APP, this is most likely to be KPI-containing APP which is not fully glycosylated. After 16 hours of recovery the higher molecular weight form of APP is decreased in the stressed cells. This could be due to the secretion of previously formed mature APP, which is not replaced.

The overall increase in intracellular APP is not mirrored by a concurrent increase in secreted APP. Indeed, there is a slight decrease in secretion from stressed cells.

Figure 5.1 AN 8 HOUR HEAT SHOCK AT 42°C INDUCES THE EXPRESSION OF A NEW HSP70 PROTEIN IN NT2 STEM CELLS

Whole cell lysates of control and heat shocked cells were analysed on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane. The nitro-cellulose membrane was immunoblotted with an antibody to the HSP70 family of heat shock proteins (Sigma).

Lane 1 Control Cells

Lane 2 Heat Shocked Cells

5 ng total protein loaded per lane

Figures to the right of the blot indicate the mobility of molecular weight markers.

Control cells express a protein of approximately 68 kDa, which is also present in the heat shocked cells. However, these cells also exhibit the presence of a second band, of approximately 85 kDa.

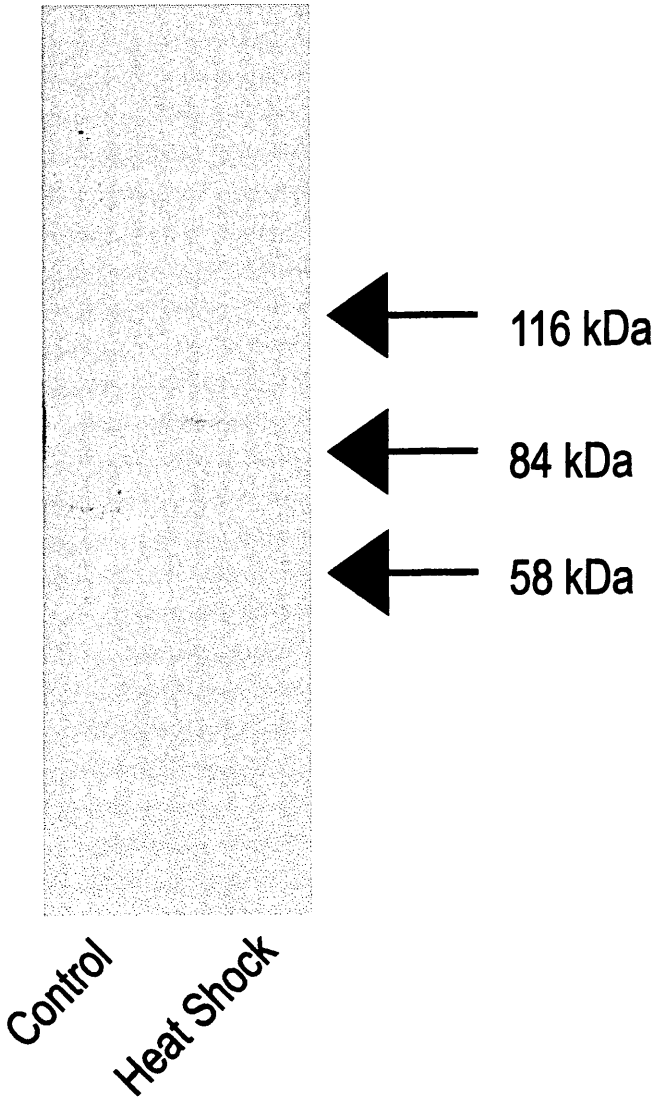

116 kDa
84 kDa
58 kDa
Control
Heat Shock

Figure 5.2 HEAT SHOCK INDUCES SLIGHT CHANGES IN MORPHOLOGY OF NT2 STEM CELLS

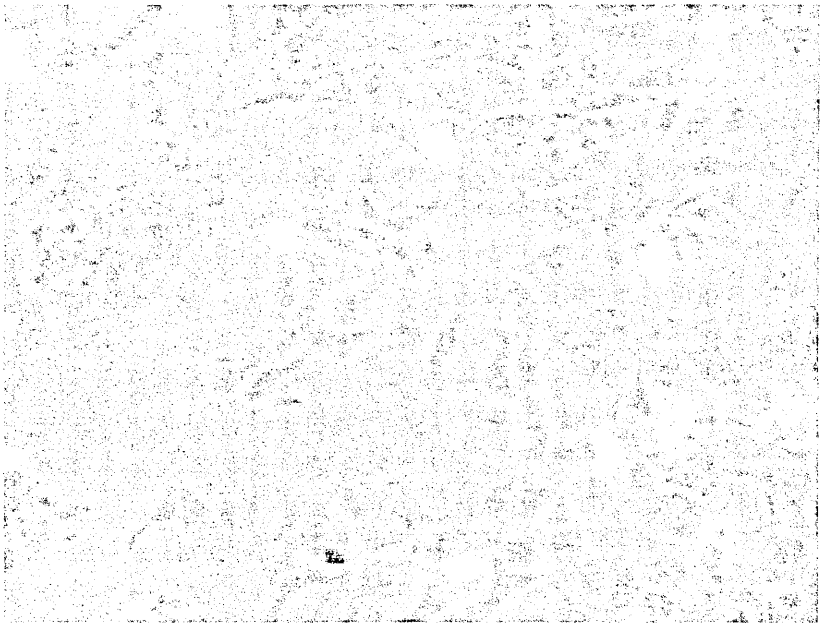
NT2 stem cell cultures were incubated at 37°C (control) or 42°C (heat shock) for 8 hours, then cultures photographed. Magnification x2000.

a Control cells

b Heat shocked cells

The heat shocked culture was slightly less confluent than the control culture, with a small proportion of cells exhibiting an elongated morphology and a number of processes (indicated by arrowheads).

a) Control Cells



b) Heat Shocked Cells

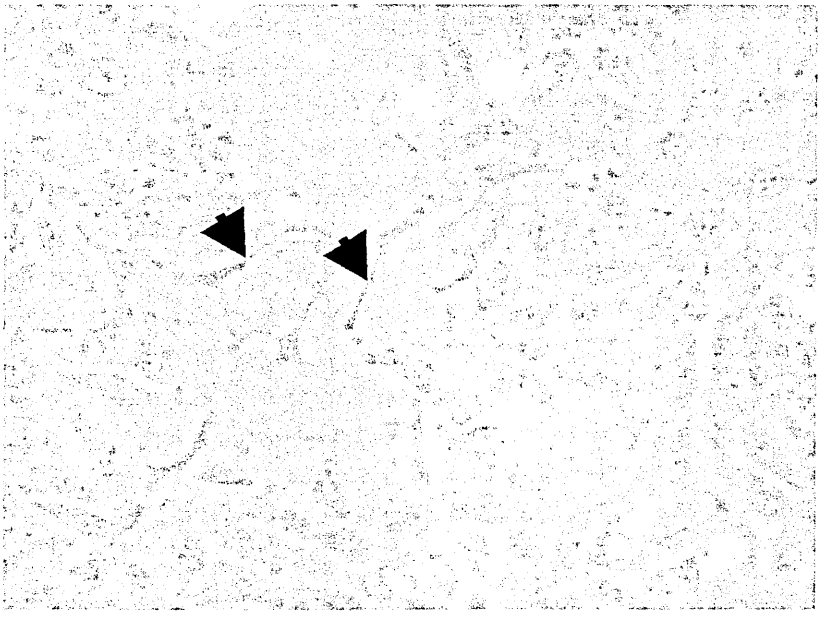


Figure 5.3 RECOVERY AT 37°C AFTER HEAT SHOCK ALLOWS NT2 STEM CELLS TO EXPRESS A THIRD PROTEIN IN THE HSP70 FAMILY

Whole cell lysates of control and heat shocked cells were analysed on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane. The nitro-cellulose membrane was immunoblotted with an antibody to the HSP70 family of heat shock proteins (Sigma).

Lane	Cells	Recovery Time
1	Control	0 hours
2		4 hours
3		14 hours
4		19 hours
5		24 hours
6	Blank	
7	Heat shocked	0 hours
8		4 hours
9		14 hours
10		19 hours
11		24 hours

10 ng total protein loaded per lane

Figures to the right of the blot indicate the mobility of molecular weight markers.

Both control and heat shocked cells express 2 proteins at approximately 68 and 85 kDa, but after 4 hours recovery at 37°C the heat shocked cells express a third protein of the HSP70 family of approximately 78 kDa.

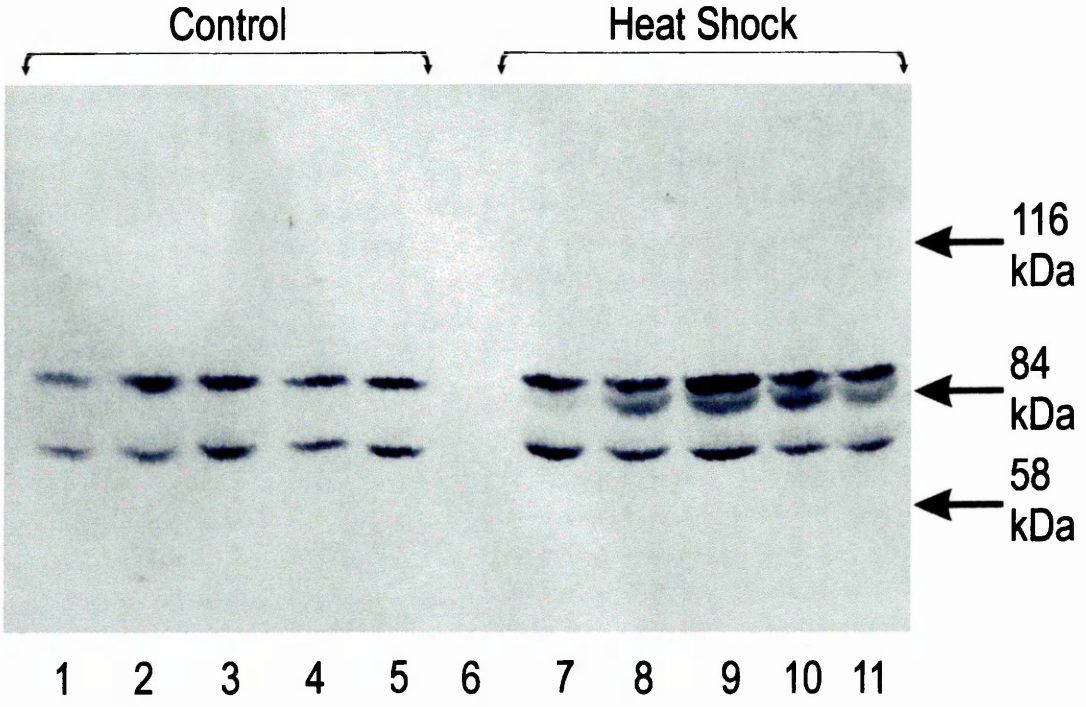

Control
Heat Shock
116 kDa
84 kDa
58 kDa
1 2 3 4 5 6 7 8 9 10 11

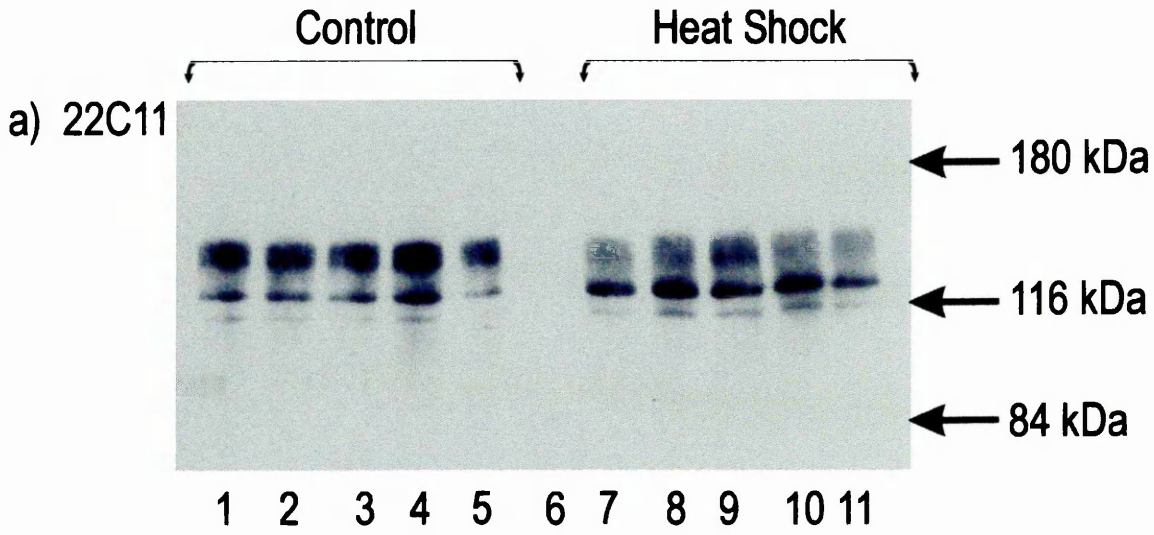
Figure 5.4 HEAT SHOCK INDUCES CHANGES IN APP PROCESSING IN NT2 STEM CELLS

Whole cell lysates of control and heat shocked cells were analysed on a 7% SDS-PAGE gel and transferred to nitro-cellulose membrane. The nitro-cellulose membrane was immunoblotted with antibodies 22C11 (a) and 993 (b).

Lane	Cells	Recovery Time
1	Control	0 hours
2		4 hours
3		14 hours
4		19 hours
5		24 hours
6	Blank	
7	Heat shocked	0 hours
8		4 hours
9		14 hours
10		19 hours
11		24 hours

10 ng total protein loaded per lane. Figures to the right of the blot indicate the mobility of molecular weight markers.

Heat shock induces an increase in the 128 kDa APP isoform that peaks at 4 hours recovery. There is a decrease in the 137 kDa isoform after 19 hours recovery, and this isoform is still diminished at 24 hours recovery. This change is seen with both antibodies, indicating that the changes are in KPI-containing APP isoforms.


Control
Heat Shock
a) 22C11
180 kDa
116 kDa
84 kDa
1 2 3 4 5 6 7 8 9 10 11

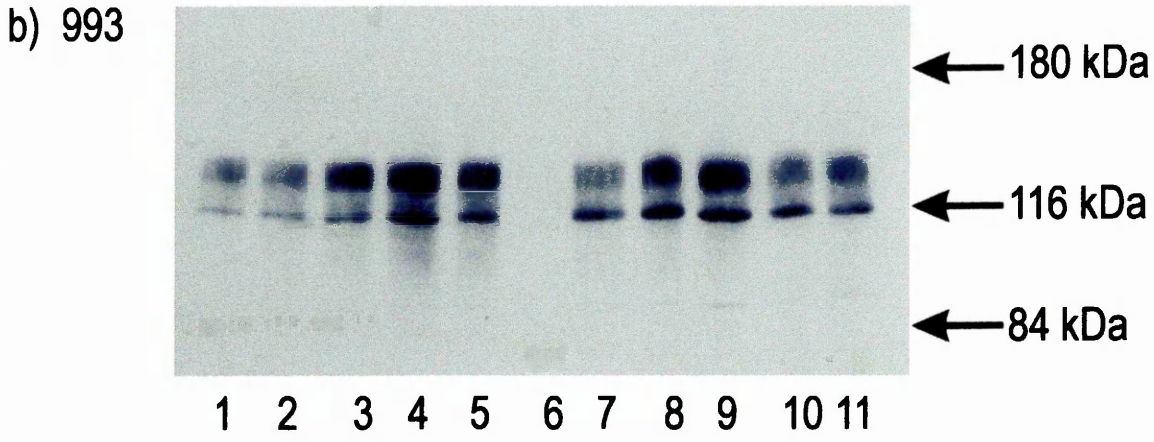

b) 993
180 kDa
116 kDa
84 kDa
1 2 3 4 5 6 7 8 9 10 11

Figure 5.5 HEAT SHOCK INDUCES ONLY SMALL CHANGES IN SECRETED APP FROM NT2 STEM CELLS

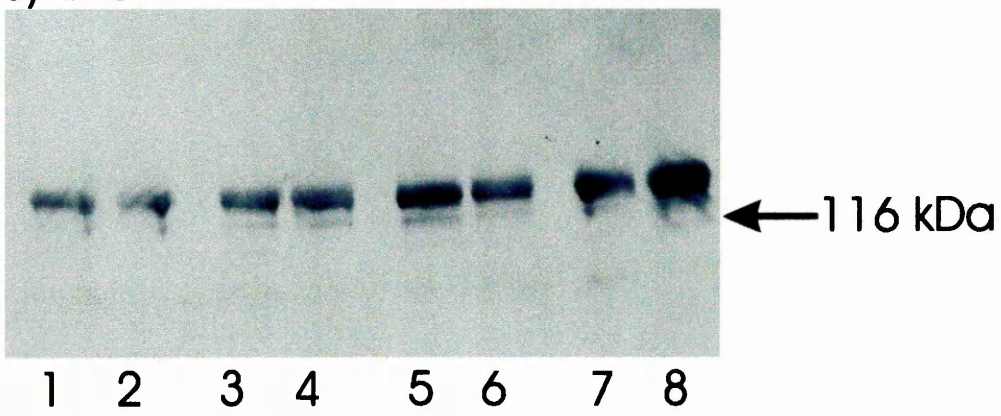
Conditioned media from control and heat shocked cells was concentrated 10-fold, mixed with SDS-PAGE sample buffer, and analysed on a 7% SDS-PAGE gel. The gel was transferred to nitro-cellulose membrane and immunoblotted with antibodies 22C11 (a) and 993 (b).

Lane	Cells	Recovery Time
1	Control	0 hours
2	Heat shocked	
3	Control	4 hours
4	Heat shocked	
5	Control	16 hours
6	Heat shocked	
7	Control	24 hours
8	Heat shocked	

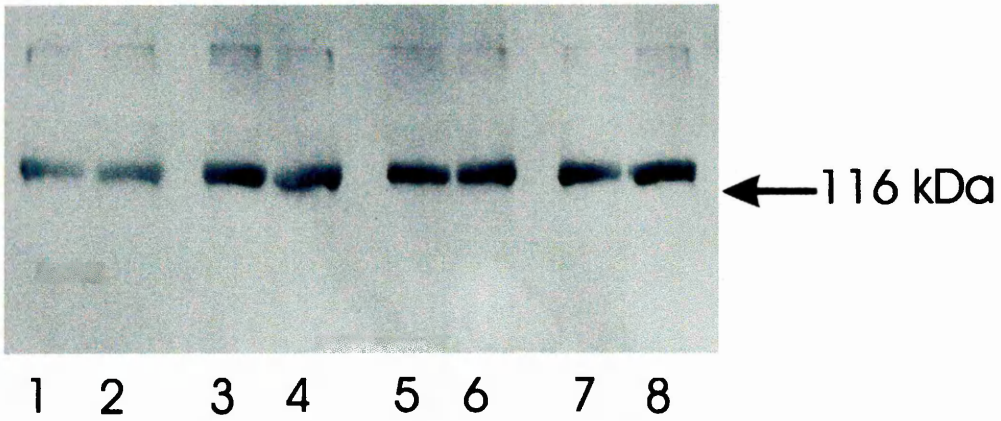
Figures to the right of the blot indicate the mobility of molecular weight markers.

There is little change in APP secretion from NT2 cells following heat shock.

a) 22C11



b) 993



Chapter Six - Effects Of Optimem On NTera2 Stem Cells And Neurones

6.1 INTRODUCTION

As described in *Chapter 5*, attempts to induce a stress response by subjecting NT2N cells to a period of heat shock were not successful, all experiments resulted in the clusters of cell bodies detaching from the culture vessel. The cells did not re-attach after up to 24 hours recovery, and appeared to have died. However, a period of heat shock is not the only way to induce the stress response in cells. Animal studies have shown HSP72 induction in response to hypoxia, ischemia and traumatic injury (Satoh and Kim, 1994). Treatment of cell cultures with cytotoxins such as ethanol and sodium arsenite has been used to induce a stress response in NT2 cells (Dewji *et al.* 1995). There is also some evidence that a lack of nutrients can induce a heat shock response (Lindquist, 1986).

During initial experiments to characterise NT2 and NT2N cells for this work, the cell cultures were incubated with Optimem for 24 hours prior to sample collection. Optimem is an alternative, reduced serum, nutrient media for cell culture. Prior to its addition to the cell cultures L-glutamine was added, but FBS was not. This serum-free media was used to enable any proteins secreted from the cells to be detected without the interference from the bovine proteins present in the FBS. However, during the course of these experiments it was observed that the morphology of the NT2 cells changed after incubation in Optimem. The cultures were less confluent than comparable cultures that remained in cDMEM, and the cells appeared to be rounded up from the culture vessel, when compared with control cells. A number of these cells appeared to have extended cellular processes (see *Figure 6.1*).

This observation was similar to that seen after the NT2 cells had been subjected to heat shock (compare *Figures 5.2* and *6.1*). These results lead to the hypothesis that incubation in Optimem induced a stress response in NT2 cells, therefore it was decided to examine the production of HSP70 in cells which had undergone treatment with Optimem.

6.2 METHOD

T25 flasks of NT2 and NT2N cells were incubated with Optimem for 24 hours. The conditioned media was collected and concentrated by ultra-centrifugation to give a 10-fold final concentration. The cell pellets were processed to give soluble and membrane proteins, as described in *Chapter 2*. Protein concentrations of the cell samples were determined using a BCA assay, after which the samples were mixed with SDS-PAGE sample buffer and separated on a 7% acrylamide gel. The gel was transferred to nitro-cellulose membrane, which was immunoblotted using antibodies to APP and the HSP70 family of heat shock proteins as described previously (See *Section 5.3.1*).

6.3 EFFECTS OF OPTIMEM ON HSP70 PRODUCTION IN NT2 AND NT2N CELLS

Western blotting with an antibody which recognises the HSP70 family of heat shock proteins (Sigma) gave two bands of approximately 67 and 83 kDa in the soluble compartment of NT2 cells. There was no change in band intensity between cells incubated with Optimem, and control cells incubated in cDMEM (see *Figure 6.2*).

Immunoblotting of control NT2N cells resulted in a single faint band of approximately 83 kDa in the soluble compartment. Cells that had been incubated with Optimem also contained this band in the soluble compartment, however it was much more intense. A second band of approximately 67 kDa was also present in the soluble fraction of these cells (see *Figure 6.2*).

6.4 EFFECTS OF OPTIMEM ON INTRACELLULAR APP (SEE FIGURE 6.3)

6.4.1 NT2 Cells

NT2 cells which had been incubated in Optimem contained the same bands as the control cells when immunoblotted with 22C11 (see *Figure 6.3a*), i.e. 3 bands, of approximately 115, 128 and 137 kDa, in both soluble and membrane fractions. Again the staining was more intense in the membrane fraction, and of this, the two larger bands were predominant. On immunoblotting with antibody 993 only the 128 and 137 kDa bands were present, in both the control and Optimem treated cells, confirming these bands as KPI-containing APP (i.e. APP_{751} and/or APP_{770}).

Equal volumes of each sample were loaded onto the gel, resulting in a different protein concentration in each lane (see the legend to Figure 6.3 for exact amounts). This makes it difficult to compare the band intensity of each lane directly. However, using Kodak imaging software it was possible to quantify the bands and normalise for the amount of protein loaded. This confirmed that there was no change in APP production in Optimem treated cells when compared to controls (data not shown).

Table 6.1 Quantification of Western Blot Shown in Figure 6.3b - NT2 Data Only

Lane	Treatment	Fraction	MW (kDa)	Protein (μg)	Intensity	% Control
1	Optimem	Soluble	115	8	5823	85
			128	8	16901	96
			137	8	22846	90
2		Membrane	115	13	81258	108
			128	13	113744	90
			137	13	181606	98
5	FBS	Soluble	115	12	10227	100
			128	12	39407	100
			137	12	38017	100
6		Membrane	115	9	52283	100
			128	9	87246	100
			137	9	127484	100

The data in the column "% Control" was derived using the following calculation:

$$\frac{\text{Band Intensity} \times \dfrac{\text{Amount of Protein loaded in FBS Lane}}{\text{Amount of Protein Loaded}}}{\text{Intensity of Band in FBS Lane}} \times 100\%$$

6.4.2 NT2N Cells

Optimem treatment did have an effect on APP production in NT2N cells, however. When immunoblotted with antibody 22C11 the soluble fraction of cells incubated with Optimem

contained a band of approximately 113 kDa, this was not present in the soluble fraction of control cells (compare lanes *3* and *7* in *Figure 6.3a*). This band was not detected with antibody 993, indicating that it was APP_{695} (compare lane *3* in *Figures 6.3a* and *b*). Antibody 22C11 detected the same bands in the membrane fractions of both the control and Optimem treated NT2N cells, i.e. a major band at approximately 113 kDa, with a band at 130 kDa also visible (see Figure 6.3a, lanes 4 and 8). As with the NT2 stem cells, the different amount of protein loaded in each lane makes direct comparison difficult, but quantification showed that there was an increase in both the 113 and the 130 kDa band, on immunoblotting with antibody 22C11, after treatment with Optimem (see *Table 6.1*).

Table 6.2 Quantification of Western Blot Shown in Figure 6.3a - NT2N Data Only

Lane	Treatment	Fraction	MW (kDa)	Protein (μg)	Intensity	% Control
3	Optimem	Soluble	113	3	21227	N/A
4		Membrane	113	3	74283	143
			130	3	97427	155
7	FBS	Soluble	113	5	0	N/A
8		Membrane	113	4	69085	100
			130	4	84014	100

The data in the column "% Control" was derived using the following calculation:

$$\text{Band Intensity} \times \frac{\text{Amount of Protein loaded in FBS Lane}}{\text{Amount of Protein Loaded}} \Big/ \text{Intensity of Band in FBS Lane} \times 100\%$$

Antibody 993 detected a faint band of approximately 130 kDa in the membrane fraction of Optimem treated NT2N cells, this band was not visible in control cells (compare lanes *4* and *8* in *Figure 6.3b*). Together with the increase in the 130 kDa band seen with antibody 22C11, this result indicates that the production of a KPI-containing APP isoform is increased with Optimem treatment. Antibody 993 did not detect any APP in the soluble fraction of either the Optimem treated, or control, cells (lanes *3* and *7* in *Figure 6.3b*).

6.5 EFFECTS OF OPTIMEM ON SECRETED APP (SEE FIGURE 6.4*)*

Unconditioned media samples, i.e. media which had not been exposed to cells, were concentrated, separated by SDS-PAGE and immunoblotted along with the conditioned media samples. As discussed in *Chapter 4* (see *Section 4.3.2*) the FBS added to DMEM contains

bovine APP, recognised by both antibodies 22C11 and 993 (lane *1* in *Figure 6.4a* and *b*). Optimem is a reduced serum media, with no added FBS, and therefore contains no components recognised by either antibody on an immunoblot (data not shown).

Incubation with Optimem did not appear to affect APP secretion by NT2 stem cells. A single band of approximately 128 kDa was detected in the media of both the control and the Optimem-incubated NT2 cells. This band was detected with both antibodies 22C11 and 993 indicating that it was derived from a KPI containing isoform of APP (see lanes *2* and *3*, *Figure 6.4a* and *b*).

Media from control NT2N cells contained 2 bands of approximately 113 kDa and 130 kDa when immunoblotted with antibody 22C11 (see *Figure 6.4a*, lane *5*). Only the 130 kDa band was detected with antibody 993 (see *Figure 6.4b*, lane *5*). Conditioned Optimem from these cells contained only the band at 113 kDa when immunoblotted with antibody 22C11. This band was not detected by antibody 993, indicating that these cells secrete only APP derived from APP_{695} (compare lane 4 in *Figures 6.4a* and *b*). Quantification of the blot shown in *Figure 6.4b* confirms that NT2N cells cultured in cDMEM do secrete a KPI-containing APP isoform(s), as the band in lane 5 (NT2N conditioned cDMEM) is almost twice the intensity of the band in lane 1 (unconditioned cDMEM) (see *Table 6.2*)

Table 6.3 Quantification of the Immunoblot Shown in Figure 6.4b (Blotted With Antibody 993)

Lane	Cell	Media	MW (kDa)	Intensity	% Control
1	none	cDMEM	128	39069	100
2	NT2	Optimem	128	87890	N/A
3	NT2	cDMEM	128	104215	267
4	NT2N	Optimem	N/A	0	N/A
5	NT2N	cDMEM	130	72369	185

The data in the column "% Control" was derived using the following calculation:

$$\frac{\text{Band Intensity}}{\text{Unconditioned Media Band Intensity}} \times 100\%$$

6.6 CONCLUSION

Although due to experimental problems, it was not possible to induce a stress response in the NT2N cells by a period of heat shock, the results in this chapter show that an alternative protocol was successful. Incubating cells with serum free Optimem for 24 hours induced the production of HSP72 in the neuronal cells, though it did not have the same effect on the NT2 stem cells.

The stress period had a profound effect on the processing of APP in the NT2N cells. There was an overall increase in intracellular APP, including increases in APP_{695} in both the soluble and membrane fractions, along with an increase in membrane bound KPI-containing isoforms. However, this increase is not accompanied by an increase in APP secretion. Indeed, there is a decrease in the secretion of KPI-containing isoforms to below the level of detection.

Optimem does not appear to have such a profound effect on NT2 cells. Although the morphology of the cells changes, there is no increase in HSP70 production, and there are only slight changes in APP processing. This may be because the stem cells require a recovery period following the

stress before the changes in protein production become apparent. An alternative explanation is that the stem cells are more resistant to stress conditions than the neuronal cells. This theory is backed up by their tolerance of short periods of heat shock, as described in *Chapter 5*.

A more direct comparison of the effects of cDMEM and Optimem on APP processing could be carried out using a western blot with equal amount of protein loaded in each lane. However, small sample volumes prevented repeat gels to be prepared which had been corrected for protein loading. Due to time and financial constraints it was not possible to repeat the entire experiment, therefore the results presented in this chapter have to be analysed with a degree of caution. Nevertheless, it is possible to conclude that incubation in Optimem does induce a stress response in NT2N cells. It is also apparent that either as a component of this response, or as a result of it, APP processing is altered in these cells. The preliminary results presented here indicate that the major effect is on KPI-containing APP isoforms, with an increase in production and concurrent decrease in secretion, though further experiments would have to be carried out to confirm this.

Figure 6.1 24 HOURS INCUBATION WITH OPTIMEM INDUCES CHANGES IN THE MORPHOLOGY OF NT2 STEM CELLS

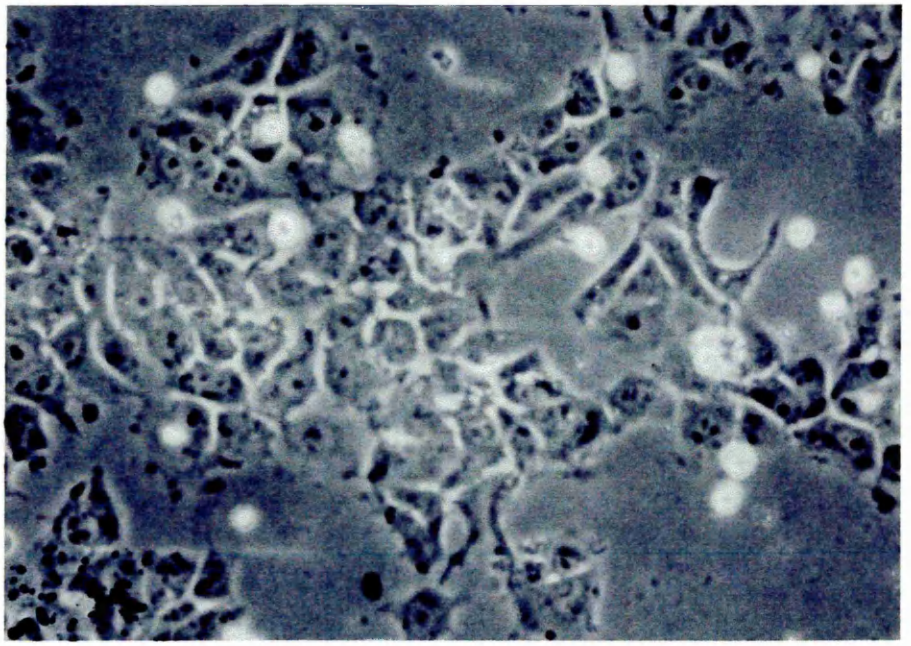
NT2 stem cells were incubated with Optimem for 24 hours; a parallel control culture was incubated with fresh cDMEM for the same time period. After the 24 hour period both cultures were photographed. Magnification x2000.

a control cells

b cells after incubation with Optimem

After the incubation with Optimem the culture is less confluent than controls, with many of the cells appearing to extend cellar processes. When viewed under the phase contrast microscope, the cell bodies in Optimem incubated cultures appeared to be more spherical and less flatly adherent to the culture vessel than controls.

a) Control Cells



b) Optimem Treated Cells

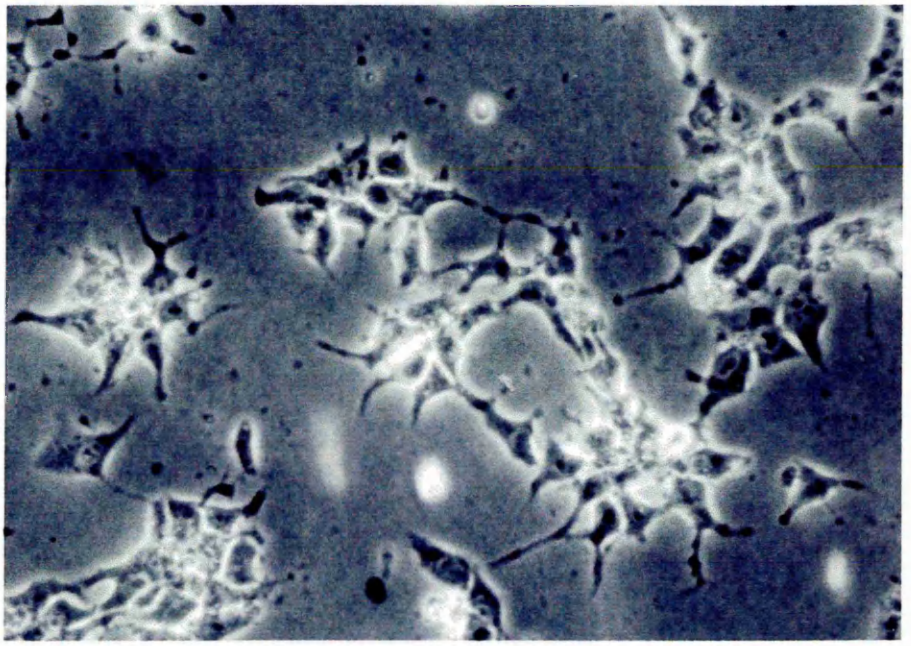


Figure 6.2 A 24 HOUR INCUBATION IN OPTIMEM INDUCES A CHANGE IN HSP70 EXPRESSION IN NT2N CELLS, THOUGH NOT IN NT2 CELLS

NT2 and NT2N cells were incubated with Optimem or cDMEM for 24 hours. After the 24 hour period all cultures were harvested and processed to give soluble and membrane protein fractions. These were analysed on a 7% SDS-PAGE gel, transferred to nitro-cellulose membrane and immunoblotted with an antibody to the HSP70 family of heat shock proteins (Sigma). The blot was developed using chromogenic methods.

Lane	Cells	Treatment	Fraction
1	NT2	Control	Soluble
2			Membrane
3		Optimem	Soluble
4			Membrane
5	NT2N	Control	Soluble
6			Membrane
7		Optimem	Soluble
8			Membrane

5 μg total protein loaded per lane

Figures to the right of the blot indicate the mobility of molecular weight markers.

NT2 cells contain two bands of approximately 67 and 83 kDa in the soluble compartment, there is no change in band intensity between control and Optimem treated cells. NT2N control cells contain only the 67 kDa band in the soluble compartment. Optimem treated cells also contain this band, but it is much more intense. A second band of approximately 83 kDa is also present in the soluble proteins of these cells.

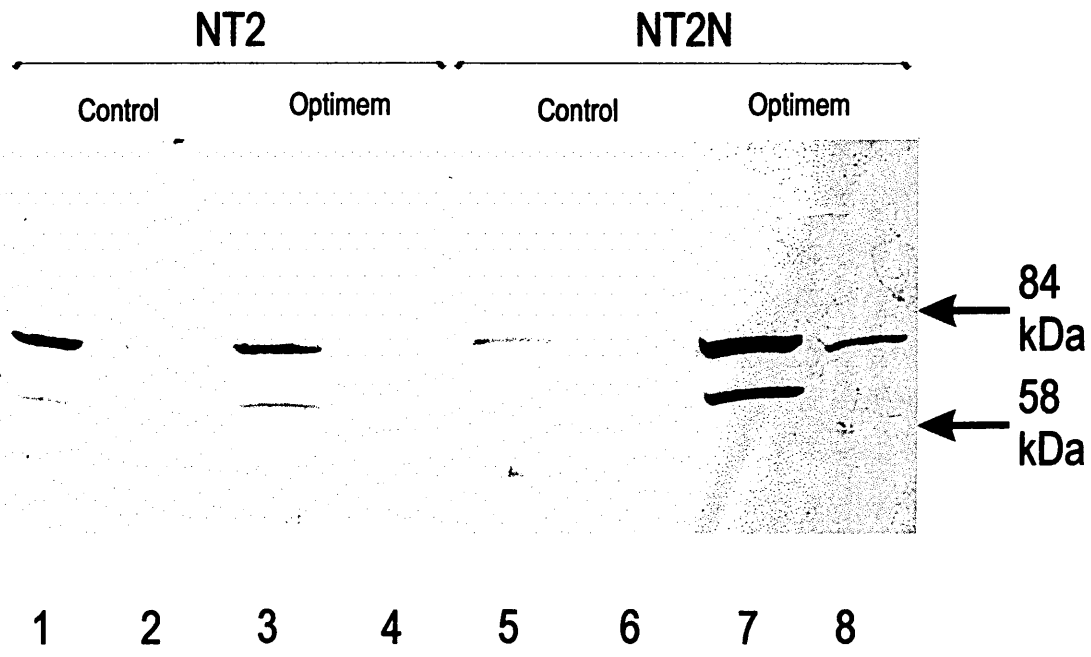

NT2
NT2N
Control
Optimem
Control
Optimem
84
kDa
58
kDa
1
2
3
4
5
6
7
8

Figure 6.3 OPTIMEM TREATMENT INDUCES CHANGES IN APP EXPRESSION IN BOTH NT2 AND NT2N CELLS

Optimem treated and control cells were processed to give soluble and membrane protein fractions, these were separated on a 7% SDS-PAGE gel, transferred to nitro-cellulose membrane and immunoblotted with antibodies 22C11 (a) and 993 (b).

Lane	Treatment	Cells	Fraction	Total Protein Loaded
1	Optimem	NT2	Soluble	8 μg
2			Membrane	13 μg
3		NT2N	Soluble	3 μg
4			Membrane	3 μg
5	Control	NT2	Soluble	12 μg
6			Membrane	9 μg
7		NT2N	Soluble	5 μg
8			Membrane	4 μg

Figures to the right of the blot indicate the mobility of molecular weight markers.

Treatment with Optimem induces an increase in APP in the soluble fraction of NT2N cells, seen in *a* and not *b*, indicating that it is an increase in APP_{695}. The Optimem treatment also induces an increase in membrane bound APP in these cells, seen in both *a* and *b*, indicating that it is an increase in KPI-containing isoforms.

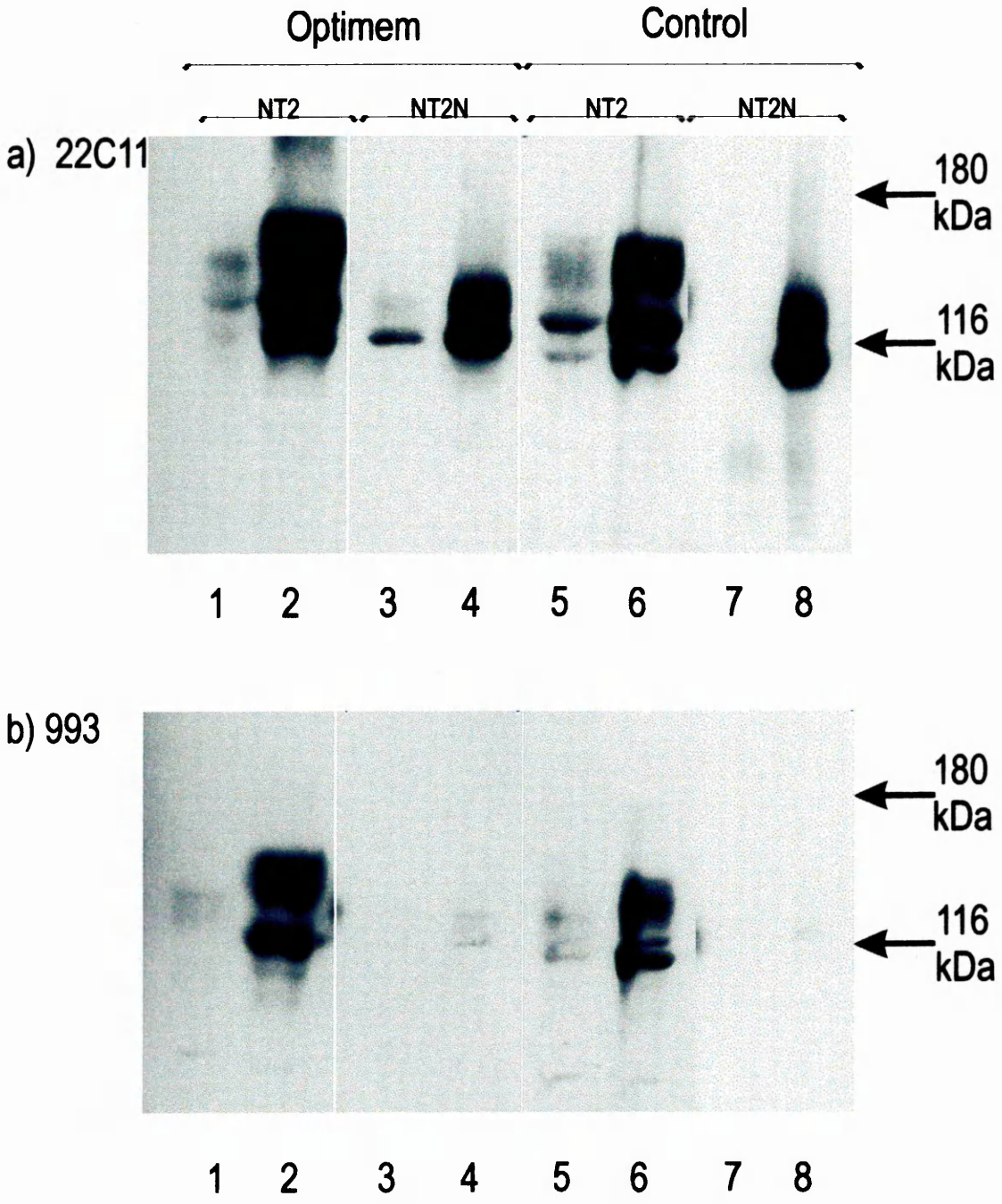

Optimem
Control
NT2
NT2N
NT2
NT2N
a) 22C11
180 kDa
116 kDa
1 2 3 4 5 6 7 8
b) 993
180 kDa
116 kDa
1 2 3 4 5 6 7 8

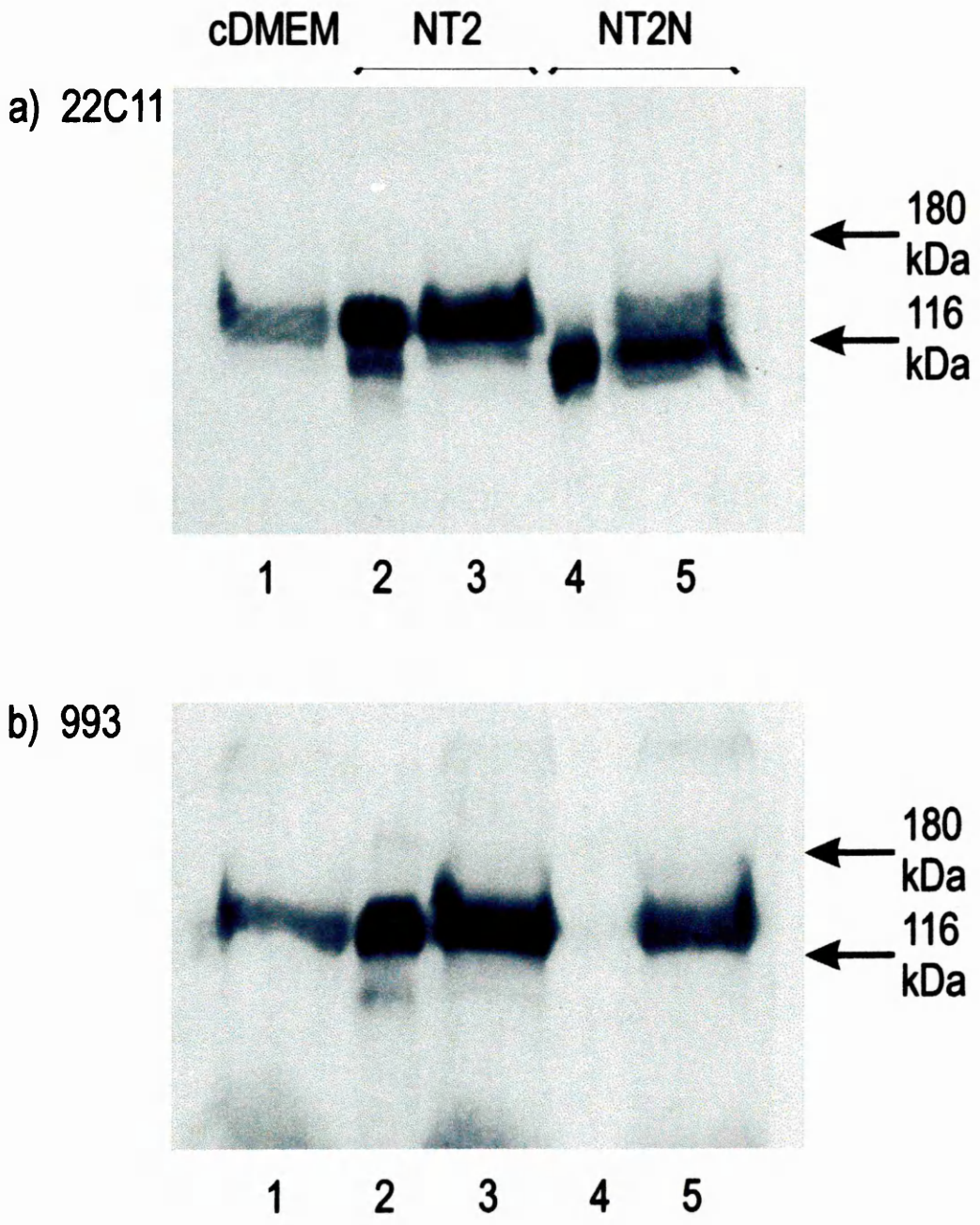
Figure 6.4 TREATMENT WITH OPTIMEM INDUCES PROFOUND CHANGES IN SECRETED APP FROM NT2N CELLS

Conditioned cDMEM and Optimem from NT2 and NT2N cells, along with unconditioned control cDMEM, was concentrated 10-fold, mixed with SDS-PAGE sample buffer and analysed on a 7% SDS-PAGE gel. The gel was transferred to nitro-cellulose membrane and immunoblotted with 22C11 (a) and 993 (b).

Lane 1	Control cDMEM, not exposed to cells
Lane 2	NT2 conditioned Optimem
Lane 3	NT2 conditioned cDMEM
Lane 4	NT2N conditioned Optimem
Lane 5	NT2N conditioned cDMEM

Figures to the right of the blot indicate the mobility of molecular weight markers.

Incubation with Optimem does not appear to affect APP secretion from NT2 cells. NT2N cells after Optimem treatment reduce the secretion of APP, to below the level of detection in the case of the KPI-containing isoforms.


cDMEM
NT2
NT2N
a) 22C11
180 kDa
116 kDa
1 2 3 4 5
b) 993
180 kDa
116 kDa
1 2 3 4 5

Chapter Seven - In Situ Hybridisation

7.1 INTRODUCTION

The work described in the previous chapters has shown that subjecting NT2 and NT2N cells to stressful conditions has an effect on the processing of APP. In the case of both cell types, stress increases the production of APP, though this appears to be retained inside the cells, as there is not an accompanying increase in APP secretion. Since the APP increase is detected with antibodies 22C11 and 993 in both cell types, it would appear to be due to an increase in production of KPI-containing APP isoforms.

Using the antibodies available, it is not possible to distinguish between APP_{751} and APP_{770} by Western blotting. For this reason, it was decided to examine the effects of stress on APP mRNA production, as probes are available for all three major APP isoforms.

7.2 METHOD (SEE SECTION 2.15*)*

Cells were grown on glass coverslips and treated as described in *Section 2.15.1.* Three coverslips of each cell type, and each treatment, were labelled with probes against APP_{695}, APP_{751}, APP_{770} and the inducible form of HSP70 (see *Table 7.1* for probe sequences), giving a total of 12 coverslips per probe. The coverslips, attached to glass microscope slides, were dipped in photographic emulsion for autoradiography, and left to develop for 7 weeks.

Table 7.1 Probe Sequences

Probe	Length	Sequence	Reference	T_i	T_w
APP_{695}	30	5' CTG CTG TTG TAG GAA CTC GAA CCA CCT CCT 3'	(Ponte *et al.* 1988)	27°C	52.5°C
APP_{751}	60	5' CAT CAG GGG TAC TGG CTG CTG TTG TAG GAA TGG CGC TGC CAC ACA CGG CCA TGC AGT ACT 3'	(Kitaguchi *et al.* 1988)	41.6°C	54.4°C
APP_{770}	57	5' GTT TAA CAG GAT CTC GGG CAA GAG GTT CCT GGG TAG TCT TGA AAC TTT GGG ACA 3'	(Kitaguchi *et al.* 1988)	33.6°C	56.1°C
HSP	30	5' CGA TCT CCT TCA TCT TGG TCA GCA CCA TGG 3'	(Shimizu *et al.* 1999)	28°C	54°C

After 7 weeks the slides were developed the cells counter stained with Cresyl violet, and the cells examined by image analysis. Grain counts were performed for up to 50 cells per experimental procedure and probe. Image analysis was performed using a Seescan image analyser system (Seescan Instruments, Cambridge).

7.3 RESULTS

7.3.1 NT2 Cells (See Figure 7.3*)*

Treatment of NT2 cells with Optimem for 24 hours resulted in significant changes in mRNA expression (see *Table 7.2*). There was a 14-fold increase in expression of HSP mRNA, indicating

that the treatment had induced a stress response in the cells. There was no change in APP_{695} mRNA, but there was a 12-fold increase in APP_{751} and a 2-fold increase in APP_{770} mRNA. Levene's test for equality of variance showed the results to have a non-normal distribution, so the non-parametric Mann Whitney U test was used to compare the means. These increases proved to be significant at the $p<0.0001$ level. This data is shown graphically in *Figure 7.1.*

Table 7.2 Expression of APP mRNAs In NT2 Control and Stressed Cells

	Control (cDMEM) cells			Optimem cells		
	N	Mean Grain Count per Cell	SE	N	Mean Grain Count per Cell	SE
APP_{695}	50	6.59	1.24	50	4.36	0.83
APP_{751}	50	9.63	3.55	50	118.35**	10.23
APP_{770}	50	174.65	15.10	49	314.64**	25.47
HSP70	50	1.82	0.73	50	25.47**	4.46

*** p<0.0001 (Mann Whitney U test)*

7.3.2 NT2N Cells (See Figure 7.4*)*

Treatment of NT2N cells with Optimem for 24 hours also resulted in changes in mRNA expression (see *Table 7.3*). There was no significant change in HSP70, APP_{695} or APP_{770} mRNA, but there was a 0.65-fold increase in APP_{751} mRNA, which was significant at the $p<0.05$ level when tested by the Mann Whitney U test. This data is shown graphically in *Figure 7.2.*

Table 7.3 Expression of APP mRNAs in NT2N Control and Stressed Cells

	Control (cDMEM) cells			Optimem cells		
	N	Mean Grain Count per Cell	SE	N	Mean Grain Count per Cell	SE
APP_{695}	38	143.30	13.79	42	177.55	31.44
APP_{751}	32	81.75	15.83	43	135.35*	17.71
APP_{770}	50	157.51	23.55	49	174.41	34.53
HSP70	44	51.70	7.99	45	86.60	13.41

** $p<0.05$ (Mann Whitney U test)*

7.4 CONCLUSION

These results provide further information about the APP expression of both NT2 and NT2N cells under normal conditions. NT2 cells produce mainly APP_{770} mRNA, with very low levels of APP_{751} and APP_{695}. NT2N cells appear to make high levels of both APP_{695} and APP_{770} mRNA, with a moderate amount of APP_{751}. These results disagree with some previous studies, which found NT2 stem cells to produce APP_{770} and APP_{751} in similar quantities, while the NT2N neuronal cells produced only very low levels of these two isoforms (Ackerman *et al.* 1994; Wertkin *et al.* 1993). The data presented in this chapter is supported by the work presented in *Chapter 4*, which showed NT2N cells to secrete KPI-containing APP. These results also show that overall NT2N cells make greater amounts of APP mRNA than NT2 cells.

These data show that treatment with Optimem for 24 hours is sufficient to induce a stress response in the NT2 cells. This appears to contradict the protein data, which indicated that

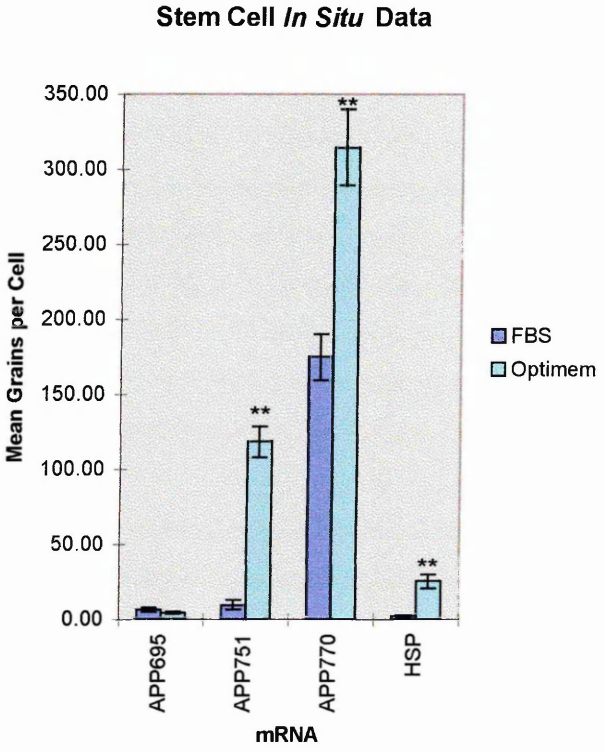
HSP72 protein levels were not elevated in these cells. However, as discussed in *Chapter 6*, it may be that the cells require a recovery period under normal conditions before the HSP72 protein is actually expressed. Alternatively there could be high levels of turnover of HSP72 protein within the cells, so that cells collected at different time points within the 24 hour Optimem incubation period may have different levels of HSP72 protein. To clarify this point further work should be done with altered incubation lengths, and recovery time in cDMEM.

In NT2 stem cells the effects of the stress period on APP mRNA levels is dramatic. There is an overall, highly significant, increase in APP mRNA, but this is splice specific. The greatest increase is in APP_{751} mRNA, with an increase in APP_{770} mRNA and no change in APP_{695} mRNA levels.

Optimem treatment does not have a significant effect on HSP70 mRNA levels in NT2N cells. This is surprising, considering the marked increase in HSP72 protein levels in these cells after the same treatment. There are three possible explanations for the lack of statistical significance of the result; the first possibility is that Optimem treatment does not affect HSP70 mRNA levels in the neuronal cells, which may indicate that the cells are not actually stressed. This seems unlikely, due to the effect the treatment has on the HSP70 family protein levels, and also the effect on APP mRNA levels, discussed below. The lack of significance may be due to the sample size, if 100 cells had been analysed the result may have reached significant status, however, lack of time and resources made this impossible. The third explanation is that the "control" culture conditions induced a stress response in the "control" cells. This is supported by the high grain count for HSP70 mRNA in these cells (a mean of 51.7 compared to 1.82 for the control NT2 cells).

As for the NT2 cells, the effects of the stress response on APP mRNA are significant. Again there is an overall increase, and again this is splice specific. There is no change in APP_{695} or APP_{770} mRNA, but APP_{751} mRNA is increased 0.67-fold, which is significant at the $p<0.05$ level. This increase is of the same proportion as the increase in HSP70.

Figure 7.1 TREATMENT WITH OPTIMEM INCREASES PRODUCTION OF HSP70 mRNA AND ALTERS PRODUCTION OF APP mRNAs IN NT2 STEM CELLS

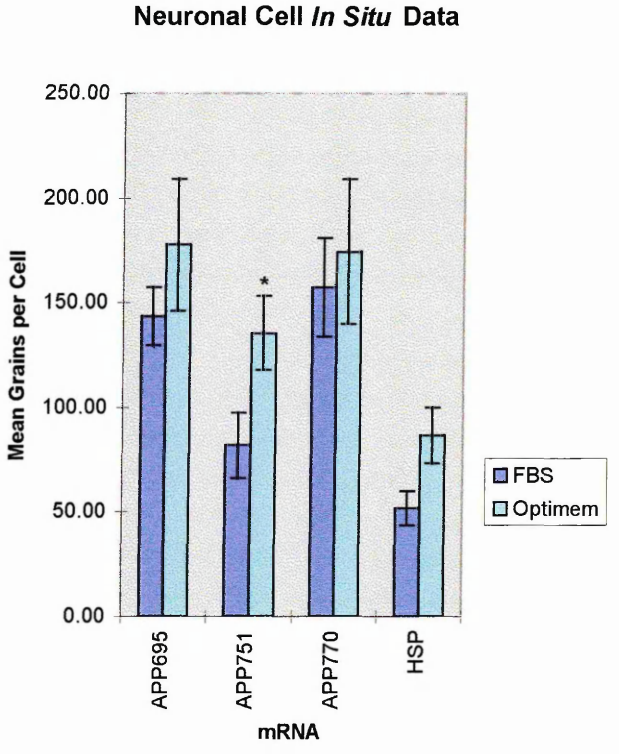


NT2 cells were grown on coverslips, treated with Optimem for 24 hours, fixed, and incubated with probes to HSP70, APP_{695}, APP_{751} and APP_{770} mRNAs

The data shows a highly significant increase in HSP70, APP_{751} and APP_{770} mRNAs after incubation with Optimem.

(** $p<0.0001$, Mann Whitney U)

Figure 7.2 TREATMENT WITH OPTIMEM INCREASES PRODUCTION OF HSP70 mRNA AND ALTERS PRODUCTION OF APP mRNAs IN NT2N CELLS



NT2N cells were grown on coverslips, treated with Optimem for 24 hours, fixed, and incubated with probes to HSP70, APP_{695}, APP_{751} and APP_{770} mRNAs

The data shows a significant increase in APP_{751} mRNA after incubation with Optimem.

(* $p<0.05$, Mann Whitney U)

Figure 7.3 OPTIMEM TREATED NT2 STEM CELLS SHOW AN UPREGULATION OF APP AND HSP mRNAs

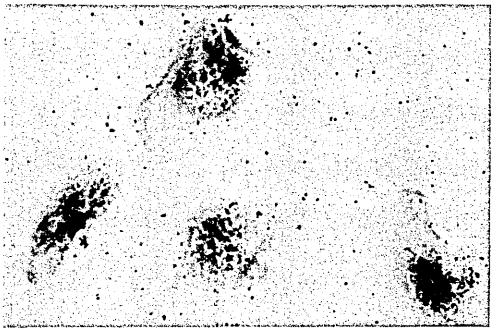
NT2 cells were grown on coverslips, incubated with either cDMEM (control) or Optimem for 24 hours, fixed, and incubated with probes to HSP70, APP_{695}, APP_{751} and APP_{770}.

The cells make predominantly APP_{770} under control conditions (compare ai) bi) and ci)). Optimem treatment upregulates production of APP_{751} mRNA (compare bi) and bii)); APP_{770} mRNA (compare ci) and cii)) and HSP70 mRNA (compare di) with dii)).

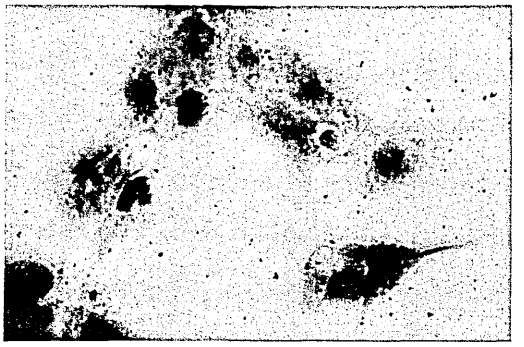
Magnification x5000

a) APP695

i) Control

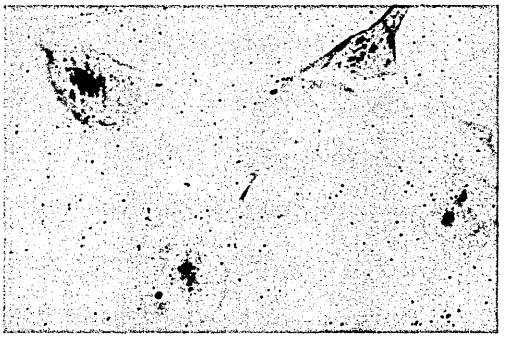


ii) Optimem-stressed

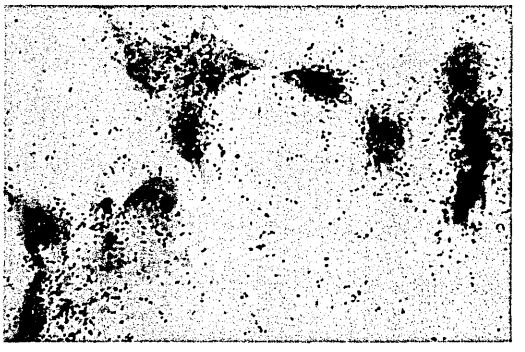


b) APP751

i) Control

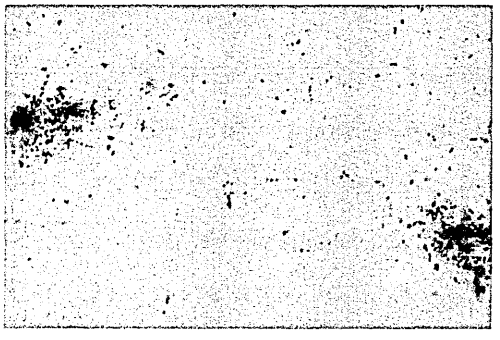


ii) Optimem-stressed

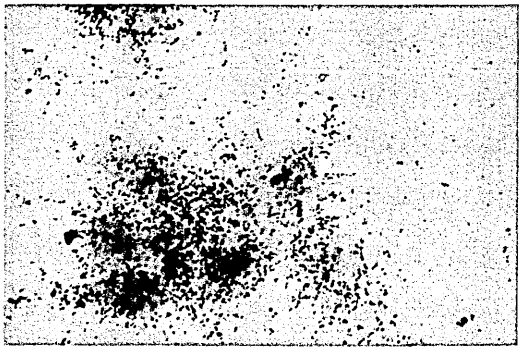


c) APP770

i) Control

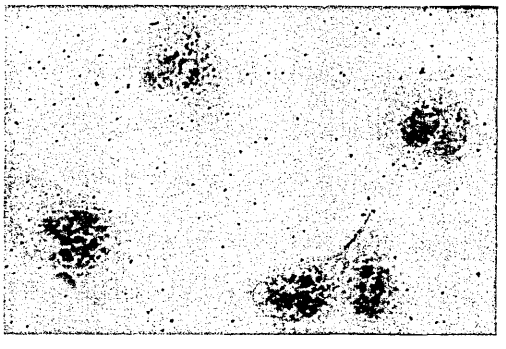


ii) Optimem-stressed



d)HSP

i) Control



ii) Optimem-stressed

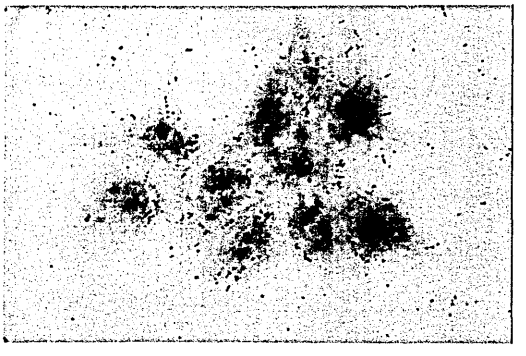


Figure 7.4 OPTIMEM TREATED NT2N NEURONAL CELLS SHOW AN UPREGULATION OF APP_{751} mRNA

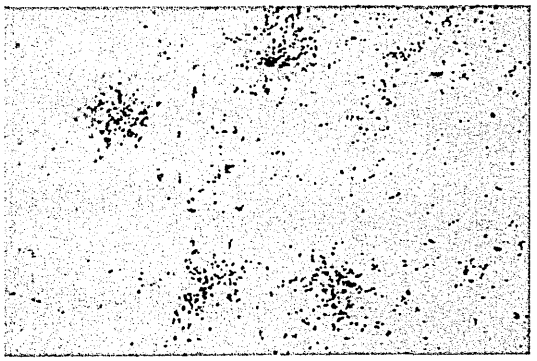
NT2N cells were grown on coverslips, incubated with either cDMEM (control) or Optimem for 24 hours, fixed, and incubated with probes to HSP70, APP_{695}, APP_{751} and APP_{770}.

The cells make predominantly all APP isoforms under control conditions, with APP_{695} and APP_{770} being the more abundant (compare ai) bi) and ci)). Optimem treatment significantly upregulates production of APP_{751} mRNA (compare bi) and bii)).

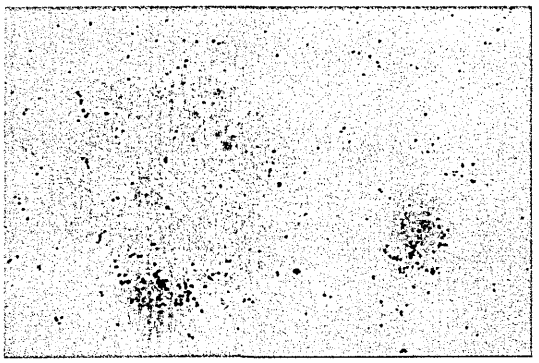
Magnification x5000

a) APP695

i) Control

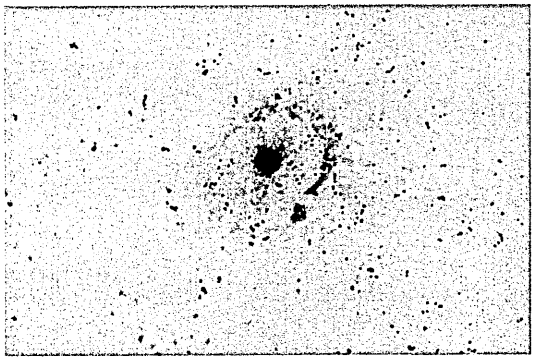


ii) Optimem-stressed

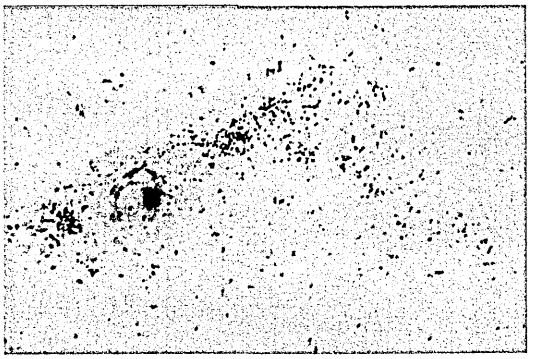


b) APP751

i) Control

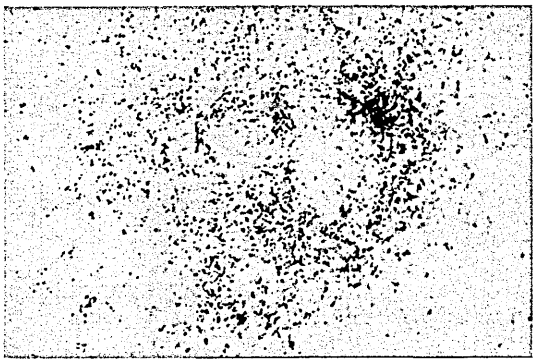


ii) Optimem-stressed

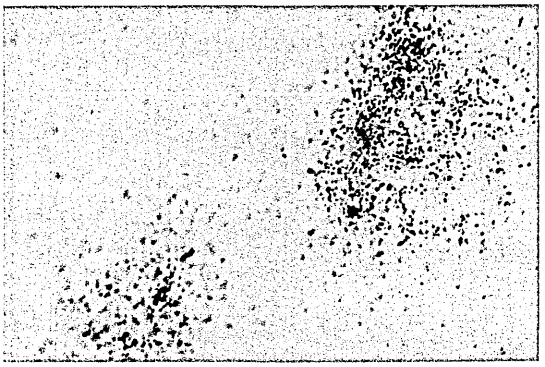


c) APP770

i) Control

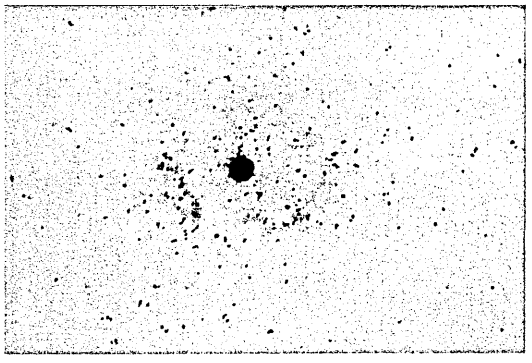


ii) Optimem-stressed

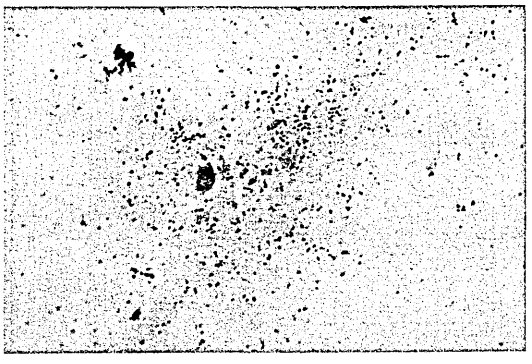


d)HSP

i) Control



ii) Optimem-stressed



Chapter Eight Discussion

8.1 INTRODUCTION

The deposition of βA4 in the brain is one of the key features of AD. Gene mutations in the APP, PS1 and PS2 genes, which are associated with the early onset form of familial AD, all lead to either increased total production βA4 (Haass *et al.* 1994; Citron *et al.* 1994; Cai *et al.* 1993; Citron *et al.* 1992), or an increased proportion of $\beta A4_{1\text{-}42/43}$. (Citron *et al.* 1997; Scheuner *et al.* 1996; Duff *et al.* 1996; Maruyama *et al.* 1996) From this evidence it seems likely that the onset of sporadic AD is also associated with increased βA4 production. Since βA4 is formed from the proteolytic cleavage of APP, any event which increases APP production, or changes APP processing, could potentially increase βA4 production.

The promoter region of the APP gene contains a heat shock element, therefore it is likely that conditions which cause cells to undergo the stress response could lead to an up-regulation of APP. There is also evidence that neurones in the AD brain are in a state of stress. (Harrison *et. al* 1993) These data lead to the hypothesis tested in this thesis; that conditions which induce a stress response in cells result in an increase in production of APP protein, and that this in turn leads to an increase in production of either total, or longer length forms of, βA4. Due to the apparent neurotoxicity of βA4, any increase in this peptide could perpetuate the stress response in the cells, leading to a "vicious circle" of APP and βA4 production.

The primary objective of this work was to examine the processing of APP in NTera2 stem and neuronal cells, both under control conditions and in conditions that are stressful to the cells. Immunoprecipitation and western blotting techniques, using a panel of antibodies, were used to

differentiate between the various different APP isoforms, and to determine whether the APP processing was potentially amyloidogenic or not. Evidence of cellular stress was sought by examination of the induction of HSP72 protein production by the cells. *In situ* hybridisation was also used to examine mRNA levels in order to confirm the protein findings, and to differentiate between the two KPI containing APP isoforms.

8.2 APP PROCESSING IN NT2 AND NT2N CELLS

From the immunoblot data in *Chapter 4* it was concluded that NT2 cells make predominantly APP_{751} and / or APP_{770}, with a small amount of APP_{695} also being produced. The *in situ* data presented in *Chapter 7* indicates that the predominant KPI-containing isoform is APP_{770}. As expected, the majority of intracellular APP is membrane bound. The KPI containing APP isoform(s) is easily detected in the conditioned media of the cells, but the smaller isoform, APP_{695} appears to be secreted at levels below the detection of the methods used. (See *Figures 4.1 and 7.1*).

NT2N cells make predominantly APP_{695}, which is secreted. One or both of the KPI-containing APP isoforms is also made and secreted, and since this isoform is not detected in the cellular proteins, it may be assumed that is has a rapid turnover within the cell. The *in situ* data indicates that the KPI-containing isoform is APP_{770}. (See *Figures 4.1 and 7.2*)

These data confirm the findings of other groups that differentiation of NT2 cells to NT2N cells, using retinoic acid, alters the expression and processing of APP isoforms (Ackerman *et al.* 1994; Wertkin *et al.* 1993). However, in contrast to previous reports, both cell types appear to make at least two, if not all three of the major APP isoforms. The change is in the relative proportions of the isoforms, both inside the cells, and in the conditioned media. As the cells differentiate the

major APP isoform switches from being KPI-containing, to being APP_{695}, the most abundant isoform in the brain. However, the neuronal cells continue to produce and secrete the KPI-containing isoforms. This evidence reinforces the hypothesis that these cells provide a good *in vitro* model of human CNS neurones in the brain, as all three isoforms are produced in similar relative amounts *in vivo* in the human brain. (Rohan de Silva *et al.* 1997)

In the experiments presented in this thesis, soluble and membrane associated APP proteins run at similar mobilities on acrylamide gels. This is surprising, as one would expect proteins present in the soluble fraction to have been cleaved, and therefore be smaller then the membrane associated forms. There are two possible explanations for this. The first is that there is incomplete separation of the fractions during the preparation of the samples. The proteins detected in the "soluble" fraction are actually membrane associated, and more rigorous separation techniques would result in all the cellular APP appearing in the membrane fraction. The second possibility is that the proteins are cleaved immediately after glycosylation in the Golgi, resulting in proteins that are shorter in length but larger in weight than the membrane bound proteins. This is possible, as APP undergoes both N-linked glycosylation, which occurs in the endoplasmic reticulum (ER), and O-linked glycosylation, which occurs in the Golgi complex. There is evidence to suggest that β-secretase cleavage can occur in both the ER and the Golgi. (Cook *et al.* 1997)

APP is one member of a family of proteins, including APLP1 and 2, as discussed in the introduction (see *Section 1.4.5* and Lyckman *et al.* 1998). Although neither of these proteins contain the βA4 sequence, and so are unlikely to be involved with the pathology of AD, they are highly homologous to APP, and many APP antibodies cross-react with APLP2. (Slunt *et al.* 1994) This fact means that care must be taken when interpreting data obtained using these antibodies. As demonstrated by the immunoblot data in *Chapter 4,* NT2N cells do not make or secrete a

detectable amount of APLP2, while NT2 cells make only a small amount, which is membrane associated, and does not appear to be secreted at detectable levels. As a result of this finding, it is possible to be confident that any changes in protein production detected by western blot are changes in APP and not APLP2.

8.2.1 APP Processing Pathways in NT2 and NT2N Cells

Both NT2 and NT2N cells secrete APP, which can be detected by western blotting of the conditioned media from the cells. Using serial immunoprecipitations with antibodies 22C11 and 6E10 it was possible to distinguish between amyloidogenic and non-amyloidogenic cleavage pathways. 6E10 removed APPsα from the media, and any remaining APPsβ was detected using 22C11. Although 6E10 would also recognise any APP which had been cleaved by γ-secretase, it is generally assumed that this enzyme activity occurs after β-secretase cleavage (Cook *et al.* 1997; Hartmann *et al.* 1997), so that the secreted APP species which begins at the amino-terminus of APP and ends at the carboxyl-terminus of βA4 does not exist. Therefore it is assumed that any APP detected by 6E10 has been cleaved by α-secretase and is non-amyloidogenic.

It seems likely that all secreted APP from NT2 cells is a result of non-amyloidogenic cleavage, and that the majority of APP secreted by NT2N cells is also derived from this pathway (see *Section 4.5.2* and *Figure 4.6*). However, a small proportion of APP appears to undergo amyloidogenic breakdown in NT2N cells. All immunoprecipitation steps detected APP of molecular weights 115 and 140 kDa, and the relative intensity of the two bands did not change. From previous results it was apparent that the 115 kDa APP was APP_{695} and the 140 kDa one or both of the KPI-containing isoforms (see *Figure 4.1*), therefore it appears that the amyloidogenic cleavage of APP in NT2N cells is not isoform specific. It appears that a small percentage of the

total APP produced by NT2N cells is processed via amyloidogenic pathways, not just a percentage of APP_{695}.

Although the presence of APPsβ is not conclusive proof that the neuronal cells make βA4, it is an indication that at least one of the secretase activities required for this is present and functional in these cells. This result agrees with results from other research groups, which have detected secreted and intracellular βA4 in the neuronal cells, but not in the stem cells (Wertkin *et al.* 1993). The results reported here show that βA4 in the NT2N cells is likely to be derived from all APP isoforms.

The methods used in this thesis were not able to detect either intracellular or secreted βA4 from either the stem cells or the neuronal cells. It is likely that this is due to problems with the sensitivity of the detection methods used, as other groups have reported the presence of βA4 both in the conditioned media of NT2N cells, and accumulated within the cells (Wertkin *et al.* 1993). In that instance the βA4 was detected by radioactive labelling, which was not attempted in the course of the work presented in this thesis. Although radioactive labelling may be the easiest way with which to detect βA4, the aim of this work was to compare NT2 and NT2N cells under normal and stressed conditions, the procedures required for the introduction of a radiolabelled compound would add another unknown quantity to that investigation. Prior to the introduction of the radiolabelled compound cells must be starved of an essential amino acid, and subsequently must be fed with an excess of that amino acid to flush out the radiolabelled compound. This step of depriving the cells of an essential nutrient may well have induced a stress response in the cells, and so the "normal" conditions would be compromised.

8.3 EFFECTS OF STRESS ON NT2 STEM CELLS

8.3.1 Effects on HSP70 Protein and mRNA

During the course of this work attempts were made to induce a stress response in NT2 stem cells by two different means. Firstly by exposing the cells to a temperature of 42°C for 8 hours, followed by recovery at 37°C for 24 hours. Secondly the cells were stressed by incubating them in serum-free Optimem media. Intracellular and secreted APP, as well as intracellular proteins of the HSP70 family were examined after both treatment strategies. APP and HSP70 mRNA were also examined after the Optimem treatment.

The heat shock treatment induced the production of an approximately 78 kDa protein, which reacted with an antibody to the HSP70 family of heat shock proteins (see *Figure 5.3*). This protein was never detected in control cells, nor was it present in cells immediately after the incubation at 42°C. It was present by 4 hours recovery time at 37°C and remained detectable throughout the remaining 24 hour recovery period.

The antibody used to detect HSP70 was obtained from Sigma (Product No. H-5147) and recognises both the constitutive (HSP73) and inducible (HSP72) forms of human HSP70. (Information taken from Sigma data sheet) There are at least ten recognised members of the human HSP70 family (Voellmy *et al.* 1985; Harrison *et al.* 1986; Harrison *et al.* 1987; Leung *et al.* 1992; Fathallah *et al* 1993; Bonnycastle *et al.* 1994; Tavaria *et al.* 1995) and the functions of many remain unclear. It is not known how many of these the antibody used would react with. The HSP70 antibody detected a band of 83 kDa in control NT2 and NT2N cells, which was up-regulated by NT2N cells after Optimem treatment; a band of 67 kDa was also detected in control NT2 cells, though this was only present in NT2N cells after Optimem treatment. An additional

band at 78 kDa was present in NT2 cells after heat shock treatment, this band was never detected in NT2N cells (see *Table 8.1*). Although the identity of these various isoforms is not clear, the fact that there was an upregulation of some or all of them in treated cells was taken as an indication that the cells had undergone a stress response. Since it was not the object of the work described in this thesis to determine HSP70 expression in NT2 and NT2N cells, the matter was not investigated further.

Table 8.1 HSP70 Expression in Control and Stressed NT2 and NT2N Cells

HSP70 – detected molecular weight.	Present in: Control NT2	Heat-Shocked NT2	Optimem-Treated NT2	Control NT2N	Optimem-Treated NT2N
67 kDa	✓	✓✓	✓✓	✗	✓
78 kDa	✗	✓	✗	✗	✗
83 kDa	✓✓	✓✓	✓✓	✓	✓✓✓

✗ *Not detectable*

✓ *Present, the number of ticks indicates the relative proportions*

Incubation with Optimem did not induce any changes in expression of the HSP70 family of proteins in the NT2 stem cells, though there was a highly significant increase in HSP70 mRNA in these cells when compared with controls (see *Figures 6.2* and *7.1*). There are two possible explanations for these seemingly contradictory results.

The first is that the protein and mRNA studies were carried out on different sets of cells, at different time points. It is possible that for some reason the cells that were taken for proteins studies were more resistant to the Optimem incubation than those taken for mRNA studies. This

resistance could be due to the passage number of the cells, as NTera2 cells have a varying karyotype, and may be induced to differentiate by many different culture conditions. To eliminate the effects of any spontaneous differentiation as much as possible, all experiments were performed on cells with passage numbers between 30 and 40. Another possibility is small inconsistencies between the two batches of Optimem used rendered the second batch less stressful to the cells. This explanation seems unlikely, due to the fact that both sets of cells which had undergone Optimem incubation appeared stressed when viewed under a microscope (see *Figure 6.1*). They were less confluent than control cells and a number of them appeared to extend cellular processes.

The second explanation for the inability of Optimem treatment to induce changes in HSP70 protein production involves the recovery period of the cells, and the production and turnover rate of the protein. The induced protein was not present in heat-shocked cells immediately after the period of high temperature, it did not appear until the cells had had a recovery period of 4 hours (see *Figure 5.3*). The cells that were treated with Optimem did not undergo a recovery period, but were harvested immediately after the Optimem incubation. It is possible that if the cells had been re-fed with the control media, and left at 37°C for a period of time, there would be production of the 78 kDa HSP70 protein. From the *in situ* data it is apparent that Optimem treatment does have an effect on the levels of HSP70 mRNA, it is possible that the NT2 cells only begin to translate that mRNA when it has accumulated to a certain level within the cell. *In situ* data on the heat-shocked cells would help to clarify this point, if it were true one would expect the cells that had zero recovery time to have significantly increased levels of HSP70 mRNA, despite having normal levels of HSP70 protein. One of the documented responses to cellular stress in numerous cell types an overall decrease in protein production. It is possible that the translation of the HSP70 mRNA to HSP70 protein cannot occur while the cells are still in the stressful environment.

It is not apparent why incubation with Optimem should have any effect on HSP70 protein or mRNA production. Optimem is marketed by Gibco life science as a reduced serum medium. It is used routinely by other laboratories to culture NT2 stem and neuronal cells, though in those cases FBS is added to the media (Pleasure *et al.* 1992). Since the exact composition of Optimem is confidential information, it is only possible to make theories as to why it induces a stress response in NT2 cells, and also in NT2N cells, as discussed below. Optimem has added growth factors and trace elements, any one of which may be in some way toxic to NT2 stem and neuronal cells. Optimem also contains sodium pyruvate, which is not present in the normal DMEM used to culture NT2 cells. However, the most likely explanation is that the stress response is caused, not by some component of the media, but by the absence of FBS. A lack of essential nutrients is one of the conditions which will induce a stress response in many cell types (Lindquist, 1986). There may be a compound in FBS which is essential for the healthy growth of NT2 and NT2N cells which is not compensated for in the growth factor enriched Optimem media.

The HSP70 protein data from the heat-shocked cells, and the HSP70 mRNA data from the Optimem treated cells proves that the cells are under stressful conditions. Since the primary objective of this work is to investigate APP production and processing under normal and stressful conditions, the reason Optimem induces the stress response is not as important as the effects of that stress on APP processing.

8.3.2 Effects on APP Production

Heat-shock induces an overall increase in APP in NT2 stem cells (see *Figure 5.4*). This increase is not immediately apparent, cells immediately after heat-shock contain the same amount of APP as control cells. The increase in APP is not apparent until after 4 hours recovery at 37°C. This

delay in the effects of heat-shock on APP production mirrors that of the production of the induced form of HSP70 protein, and may in some way support the suggestion that NT2 stem cells cannot up-regulate protein production whilst still under stressful conditions.

There is a specific increase in the smaller of the 2 major isoforms of APP found in these cells. This is a KPI-containing form, though it is not possible to say if it is APP_{751} or APP_{770} as both isoforms are recognised by the anti-KPI antibody 993, and the two are not separated on a 7% SDS-PAGE gel. It is most likely to be immature APP that is not fully glycosylated, which would indicate that the protein is being retained in the ER. This could lead to an increase in production of $\beta A4_{1-42}$ as there is evidence to suggest that this form of the βA4 peptide is produced in the ER. (Cook *et al.* 1997) The *in situ* data on Optimem treated cells would indicate that this increase is due primarily to an increase in APP_{751} as that isoform showed proportionally the largest increase in mRNA levels, a 12-fold increase compared to control cells. APP_{770} mRNA levels also increased 3-fold compared to control cells (see *Figure 7.1*).

As with HSP70 protein, Optimem incubation had little effect on APP protein production. Experimental cells contained the same isoforms of APP as controls, and they were present in the same proportions.

The heat-shock induced increase in intracellular APP is not mirrored by a concurrent increase in secreted APP. Indeed, there is a slight decrease in secretion from stressed cells (see *Figure 5.5*). The implications of this finding are discussed in *Section 8.5.*

To summarise, stress induces NT2 stem cells to increase production of APP. There is an overall increase in intracellular KPI-containing APP, which is retained inside the cells as there is no

increase in APP secretion. There is no increase in either APP_{695} protein or mRNA. There is, however an increase in APP_{770} mRNA, and a large increase in APP_{751} mRNA. This indicates that the up-regulation of APP production is splice specific, if the increase in production was not splice specific one would expect all APP isoforms to be increased to the same extent, or that the predominant isoform in control cells (APP_{770}) would be increased the most. However, as already stated, this is not the case in NT2 stem cells. As discussed in Chapter One (see *Section 1.4.4.2*) there is a specific upregulation of APP_{751}, an isoform implicated in the pathology of AD. (Hyman *et al.* 1992; Moir *et al.* 1998; Johnson *et al.* 1990; Tanaka *et al.* 1989; Tanaka *et al.* 1988). Furthermore, the protein appears to be retained in the ER, the site of production of the longer forms of βA4 (Cook *et al.* 1997), which are more likely to form the insoluble amyloid deposits typical of the disease. (Gravina *et al.* 1995)

8.4 EFFECTS OF STRESS ON NT2N NEURONAL CELLS

NT2N cells were stressed by incubating them in serum free Optimem for 24 hours. As discussed above, the mechanism by which Optimem causes a stress response is not known, however, Optimem treatment induced the production of an 83 kDa band that reacted with antibodies to HSP70. This band was not present in control cells, therefore it was assumed that cells containing this band were shocked.

8.4.1 Effects on HSP70 Protein and mRNA

Control NT2N cells contained a single band of approximately 83 kDa when immunoblotted with an antibody to HSP70 protein. Cells incubated in serum free Optimem media also contained this band, though it was much more intense. These cells contained, in addition, an approximately 67 kDa band, which was not present in controls (see *Figure 6.2*). However, the results of *in situ* experiments on HSP70 mRNA in control and Optimem treated NT2N cells were not consistent

with these results. There was no significant increase in HSP70 mRNA in the stressed cells (see *Figure* 7.2).

These results are a reversal of the NT2 stem cells results, in which Optimem treatment did not alter HSP70 protein production, but massively increased mRNA levels. This may be due to different transcription pathways or differing responses to stress by the two cell types. It could be that the stem cells have a high HSP70 protein turnover rate, resulting in very little protein present in the cell at one time, despite the presence of high levels of mRNA. In contrast the neuronal cells may have a much lower HSP70 protein turnover rate, resulting in large increases in intracellular protein from a small increase in mRNA levels.

Other possible explanations for the apparently contradictory HSP70 protein and mRNA results in Optimem treated NT2N cells were discussed in *Chapter 7*. There is an approximately 60% increase in HSP70 mRNA in the stressed cells, though this is not a statistically significant increase. This could be due to a small sample size, including more cells in the analysis may have led to a significant result. The other, very probable, explanation is that the "control" cells used in the *in situ* experiment were already in a state of shock. These cells had high levels of HSP70 mRNA (a mean of 51.7 grains per cell, in contrast to the control stem cells that contained a mean of 1.82 grains per cell). As for the NT2 stem cells, the protein studies and the mRNA studies were carried out on two different sets of cells, so the HSP70 protein levels in the *in situ* cells are not known. If this theory is correct, one would expect to have seen increased levels of HSP70 protein in the FBS treated cells.

8.4.2 Effects on APP Production and Processing

The period of stress had a profound effect on the processing of APP in the NT2N cells. There is an overall increase in intracellular APP in stressed cells when compared with controls (compare lanes 4 and 8 in *Figure 6.3a*). This increase is in all APP isoforms, western blotting the cells with antibodies 22C11 and 993 produces the same increase in APP in the shocked cells when compared to the controls. The *in situ* data indicates that the increase in APP protein is mostly due to an increase in APP_{751}, there is a 67% increase in the mRNA for that isoform, an increase that is significant at the $p<0.05$ level (see *Figure 7.2*). There are no significant changes in levels of APP_{695} or APP_{770} mRNA, which seems to contradict the protein data, which shows a clear increase in all isoforms. However, as discussed in section *8.4.1* there are several possible explanations for the apparent contradictory nature of the 2 sets of results.

The most profound effect of the stress period was on APP secretion from NT2N cells. Stressed cells showed an overall decrease in APP secretion, with a reduction of secretion of KPI-containing isoforms to below the limits of detection (see *Figure 6.4*).

In summary, stressed NT2N cells show an overall increase in APP protein production, with a distinct increase in production of KPI-containing isoforms. A decrease in APP secretion, and specifically a loss of secretion of KPI-containing isoforms accompany this increase in production. The *in situ* data shows a significant increase in APP_{751} mRNA, indicating that this isoform may be responsible for most of the observed changes.

8.5 SUMMARY (*SEE* TABLE 8.2 *and* TABLE 8.3)

Conditions of cellular stress affect APP production and processing in both NT2 and NT2N cells. Both heat-shocked stem cells, and Optimem treated neurones show an increase in APP

production that is not accompanied by an increase in secretion, indeed there seems to be a drop in APP secretion. Western blotting indicates that the increase in protein production is due to a specific increase in KPI-containing APP, and mRNA studies indicate that in both cell types it is specifically APP_{751} that is affected.

Although each cell type produces all three APP isoforms, they produce them in different ratios, and appear to process them through different pathways. However, both cell types show the same changes in APP production in response to stress, an increase in APP_{751}. In neither cell type is this the predominant isoform under normal conditions, therefore it's increase is not due to a general up-regulation in APP production, since in that case it would be expected that all isoforms to be increased to the same degree, or the most predominant isoform under normal conditions to show the greatest increase after stress.

Table 8.2 Expression of APP Isoforms By Control NT2 and NT2N Cells

		APP_{695}	APP_{KPI}	APP_{751}	APP_{770}
NT2 stem cells	Intracellular	✓	✓✓✓	N/A	N/A
	Secreted	✗	✓✓✓	N/A	N/A
	mRNA	✓	N/A	✓	✓✓✓
NT2N neuronal cells	Intracellular	✓✓✓	✓	N/A	N/A
	Secreted	✓✓✓	✓	N/A	N/A
	mRNA	✓✓	N/A	✓	✓✓

Western blotting cannot differentiate between APP_{751} and APP_{770}, therefore protein data is presented together as APP_{KPI}

✗ *Not detectable*

✓ *Present, the number of ticks indicates the relative proportions*

Accompanying this splice specific increase in APP_{751} is a decrease in APP secretion. In the case of the neuronal cells there is no detected secretion of APP_{770} or APP_{751} at all under stress conditions. If the APP is not secreted, it is more likely to be broken down within the cell, in which case there is a very strong possibility that there would be an increase in production of βA4 in response to cellular stress, as α-secretase cleavage occurs predominantly at the cell surface (Boseman Roberts *et al.* 1994; Sisodia, 1992) while β- and γ-secretase cleavage occurs in the ER or Golgi (Cook *et al.* 1997; Hartmann *et al.* 1997). A proportion of APP which reaches the cell surface is not cleaved by a-secretase, but is re-internalised and broken down in the endosome / lysosome pathway. (Koo and Squazzo, 1994) It is not known if any particular APP isoform is more likely to be processed in an amyloidogenic fashion. The data from serial

immunoprecipitation experiments presented here indicates that all isoforms are processed through amyloidogenic and non-amyloidogenic pathways in the same proportion. However, it would be interesting to see if that was still the case after shock treatment. It is possible that the high levels of APP_{751} would force more of it to be processed via amyloidogenic pathways.

Table 8.3 Changes in Protein and mRNA Expression In NT2 And NT2N Cells After Stress

		APP_{695}	APP_{KPI}	APP_{751}	APP_{770}	HSP70
NT2 stem cells	Intracellular	↔	↑	N/A	N/A	↑
	Secreted	↔	↔	N/A	N/A	N/A
	mRNA	↔	N/A	↑	↑	↑
NT2N neuronal cells	Intracellular	↓	↑	N/A	N/A	↑
	Secreted	↔	↓	N/A	N/A	N/A
	mRNA	↔	N/A	↑	↔	↔

↔ *No Change*

↑ *Increase*

↓ *Decrease*

Western blotting cannot differentiate between APP_{751} and APP_{770}, therefore protein data is presented together as APP_{KPI}

The results presented here are based on the heat-shock results for the stem cells, and the Optimem results for the neuronal cells.

8.6 IMPLICATIONS FOR ALZHEIMER'S DISEASE

As discussed in the introduction, *Chapter 1*, there are several indications that neurones in the AD brain are under cellular stress. The work presented in this thesis shows that neurones in this condition increase the production of APP, and will most likely also increase production of βA4. As βA4 may be neurotoxic, it is possible that an increase in the production of the peptide could cause neurones in the surrounding area of the brain to suffer conditions of cellular stress, thus increasing APP levels. In this way the pathology could spread through the brain. This theory is known as the amyloid cascade hypothesis. (Hardy and Higgins, 1992)

Neurones under stress specifically increase levels of APP_{751} and there are several reports that this isoform is increased in the AD brain. (Hyman *et al.* 1992). In a mouse model of chronic inflammation APP_{751} was increased in areas of neuronal degradation (Brugg *et al.* 1995). It has also been shown that APP_{751} to APP_{695} ratio in human, dog and rat brain parallels the ability of each brain to form senile plaques. The relative amounts of APP_{751} in different brain areas also mirrors the amount of AD pathology found in each area (Anderson *et al.* 1989).

Previous work carried out in the Pearson laboratory also implicates APP_{751} in the processes of cellular stress, and maybe in the pathogenesis of AD. Immunohistochemistry on pathologically confirmed post-mortem AD brain tissue shows that these brains accumulate APP_{751} when compared to age-matched control brains. In the temporal cortex, pyramidal cells in layer V showed the highest immunoreactivity, these cells are also the most vulnerable to NFTs. In the visual cortex, which contains fewer NFTs there was less APP_{751} staining. Plaques that were immunopositive for βA4 also reacted with the KPI antibody, which may indicate that the dystrophic neurites contain APP_{751}. (Prof. R.C.A. Pearson, personal communication) In a study very similar to the one described in this thesis, Dr Shepherd investigated relative levels of APP

proteins and mRNAs in control and heat-shocked human astrocytes. (Shepherd, 1998) Human astrocytes that have been subjected to a period of heat shock show significantly increased levels of APP_{751} mRNA, together with an intracellular accumulation of KPI-containing APP. As with the NT2N cells, these astrocytes show a decrease in secreted KPI-containing APP following the period of heat shock.

Put together, these findings indicate that APP_{751} may be the APP isoform which is upregulated in response to abnormal cellular conditions, and a change in the relative amounts of this isoform may lead to changes in APP processing and βA4 deposition.

Although these findings do not attempt to explain the initial trigger of cellular shock at the beginning of the disease progression, they do indicate a means by which the pathology could spread through the brain, and also explain the accumulation of extracellular βA4. The initial process could be an increase in βA4 levels, specifically the more neurotoxic $βA4_{1-42}$. This increase could be due to genetic factors, such as PS1 or PS2 mutations, or a mutation in the APP gene, all of which either increase total βA4 levels, or increase the $βA4_{1-42}$:$βA4_{1-40}$ ratios. Alternatively the increase in βA4 could be due to some, as yet unknown, physiological or environmental conditions. There is also the possibility that the initial cause of cellular stress is not due to βA4, but that the effect of the stress is an increase in βA4 levels, which in turn propagates the cellular stress.

8.7 FUTURE WORK

8.7.1 Detection of βA4

As stated above, the data presented in this thesis shows that human, neuronal cells respond to stress by increasing production of APP_{751}, and that this excess protein is not secreted, but retained in the cell. With the current understanding of APP processing pathways, and specifically the sites of βA4 production (see Selkoe, 1998 for a review), it can be assumed that an increase in intracellular APP will lead to an increase in βA4 production. However, to confirm this, it would be necessary to establish a technique of detecting βA4 in both the NT2N cells, and the conditioned media.

Unfortunately, attempts to detect βA4 by western blotting were not successful. Due to constraints of time and finances it was not possible to try alternative techniques of detection. However, methods that may be tried include using immunocytochemistry to identify intracellular βA4. Secreted βA4 may be detected by first isolating it from the conditioned media, using affinity or gel-filtration chromatography, in this way concentrating the peptide before analysis on an SDS-PAGE gel and western blotting. Many published studies use a sandwich ELISA method to detect βA4 in conditioned media or biological fluids (Haass *et al.* 1992b; Seubert *et al.* 1992b), however the antibodies used are not commercially available.

8.7.2 The Fate of the Increased APP

To investigate the time course involved in the events following cellular stress, it would be necessary to allow the cells a recovery period after the stress event. Protein and mRNA data, both intracellular and secreted, would indicate how long the upregulation of APP lasts, and would also indicate whether the increased intracellular protein is eventually secreted. It would also be

interesting to investigate the cellular location of APP following cellular stress. It may be retained in the ER or Golgi, where it seems likely it would be cleaved to form βA4. Alternatively it may be transported to the axon, where it may be involved in processes to minimise damage.

In situ data on heat shocked stem cells would confirm that the increase in APP seen is an increase in APP_{751}, which would confirm that this increase is a general response to cellular stress, and is not neurone specific.

8.7.3 The Method of Optimem Stress

The theory that the stress is caused not by some factor in the Optimem media, but by the absence of FBS could be tested by repeating the experiment using DMEM with no added FBS as the experimental media. If the stress response is due to the lack of essential nutrients the results from FBS deprived cells should be the same as those from the cells treated with Optimem.

8.8 CONCLUSION

The work presented in this thesis demonstrates that human neuronal cells, expressing endogenous APP show an isoform specific increase of APP_{751} in response to cellular stress. This increase in mRNA and intracellular protein is not accompanied by an increase in secretion. The work on NT2 stem cells, as well as work by Dr Shepherd, which found heat-shocked human astrocytes accumulate intracellular KPI-containing APP (Shepherd, 1998); indicates that this response is not neurone specific.

These data show that APP is an important molecule in the cellular response to stress, and indicate that the different isoforms of APP may have different roles. The fact that APP_{751} does not appear to be secreted as effectively after cellular stress provides an insight into one of the means by which βA4 could accumulate in SAD. It is possible that continued cellular insult would

lead to increased βA4 production, which may in turn lead to cellular stress. It is also possible that the upregulation of APP_{751}, and its accumulation within the cells could be the trigger that begins the "amyloid cascade.

References

Abe, K., St George-Hyslop, P.H., Tanzi, R.E. and Kogure, K. (1991) Induction of amyloid precursor protein messenger-RNA after heat- shock in cultured human lymphoblastoid-cells. *Neuroscience Letters* **125**, 169-171.

Ackerman, S.L., Knowles, B.B. and Andrews, P.W. (1994) Gene-regulation during neuronal and nonneuronal differentiation of NTera2 human teratocarcinoma-derived stem-cells. *Molecular Brain Research* **25**, 157-162.

Adler, M.J., Coronel, C., Shelton, E., Seegmiller, J.E. and Dewji, N.N. (1991) Increased gene expression of Alzheimer disease β-amyloid precursor protein in senescent cultured fibroblasts. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **88**, 16-20.

Allsop, D., Haga, S., Bruton, C., Ishii, T. and Roberts, G.W. (1990) Neurofibrillary tangles in some cases of dementia-pugilistica share antigens with amyloid β-protein of Alzheimer's-disease. *American Journal Of Pathology* **136**, 255-260.

Alzheimer, A. (1907) A characteristic disease of the cerebral cortex. *Allgemeine Zeitschrift fur Psychiatrie und Psychisch-Gerichtliche Medizin* **LXIV**, 146-148.

Anderson, J.P., Refolo, L.M., Wallace, W., Mehta, P., Krishnamurthi, M., Gotlib, J., Bierer, L., Haroutunian, V., Perl, D. and Robakis, N.K. (1989) Differential brain expression of the Alzheimer's amyloid precursor protein. *EMBO Journal* **8**, 3627-3632.

Anderson, J.P., Chen, Y., Kim, K.S. and Robakis, N.K. (1992) An alternative secretase cleavage produces soluble Alzheimer amyloid precursor protein containing a potentially amyloidogenic sequence. *Journal Of Neurochemistry* **59**, 2328-2331.

Andrews, P.W. (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Developmental Biology* **103**, 285-293.

Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C. and Fogh, J. (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell-line Tera-2 - differentiation *in vivo* and *in vitro*. *Laboratory Investigation* **50**, 147-162.

Andrews, P.W., Damjanov, I., Simon, D. and Dignazio, M. (1985) A pluripotent human stem-cell clone isolated from the Tera-2 teratocarcinoma line lacks antigens SSEA-3 and SSEA-4 *in vitro*, but expresses these antigens when grown as a xenograft tumor. *Differentiation* **29**, 127-135.

Andrews, P.W., Gonczol, E., Plotkin, S.A., Dignazio, M. and Oosterhuis, J.W. (1986) Differentiation of Tera-2 human embryonal carcinoma-cells into neurons and HCMV permissive cells - induction by agents other than retinoic acid. *Differentiation* **31**, 119-126.

Arai, H., Lee, V.M.Y., Messinger, M.L., Greenberg, B.D., Lowery, D.E. and Trojanowski, J.Q. (1991) Expression patterns of β-amyloid precursor protein (β-APP) in neural and nonneural human tissues from Alzheimer's-disease and control subjects. *Annals Of Neurology* **30**, 686-693.

Berezovska, O., Xia, M.Q., Page, K., Wasco, W., Tanzi, R.E. and Hyman, B.T. (1997) Developmental regulation of presenilin mRNA expression parallels Notch expression. *Journal Of Neuropathology And Experimental Neurology* **56**, 40-44.

Bonnycastle, L.L.C., Yu, C.-E., Hunt, C.R., Trask, B.J., Clancy, K.P., Weber, J.L., Patterson, D. and Schellenberg, G.D. (1994) Cloning, sequencing, and mapping of the human chromosome 14 heat shock protein gene (HSPA2) *Genomics* **23** 85-93

Boseman Roberts, S., Ripellino, J.A., Ingalls, K.M., Robakis, N.K. and Felsenstein, K.M. (1994) Non-amyloidogenic cleavage of the β-amyloid precursor protein by an integral membrane metalloendopeptidase. *Journal Of Biological Chemistry* **269**, 3111-3116.

Braak, H. and Braak, E. (1990) Alzheimer's-disease - striatal amyloid deposits and neurofibrillary changes. *Journal Of Neuropathology And Experimental Neurology* **49**, 215-224.

Brugg, B., Dubreuil, Y.L., Huber, G., Wollman, E.E., Delhayebouchaud, N. and Mariani, J. (1995) Inflammatory processes induce β-amyloid precursor protein- changes in mouse-brain. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 3032-3035.

Busciglio, J., Gabuzda, D.H., Matsudaira, P. and Yankner, B.A. (1993) Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 2092-2096.

Buxbaum, J.D., Oishi, M., Chen, H.I., Pinkaskramarski, R., Jaffe, E.A., Gandy, S.E. and Greengard, P. (1992) Cholinergic agonists and interleukin-1 regulate processing and secretion of the Alzheimer beta/a4 amyloid protein-precursor. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 10075-10078.

Cai, X.D., Golde, T.E. and Younkin, S.G. (1993) Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science* **259**, 514-516.

Chartier-Harlin, M.C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. and Mullan, M. (1991) Early-onset Alzheimer's-disease caused by mutations at codon-717 of the beta-amyloid precursor protein gene. *Nature* **353**, 844-846.

Chopp, M. (1993) The roles of heat-shock proteins and immediate early genes in central-nervous-system normal function and pathology. *Current Opinion In Neurology And Neurosurgery* **6**, 6-10.

Ciallella, J.R., Rangnekar, V.V. and McGillis, J.P. (1994) Heat-shock alters Alzheimer's beta-amyloid precursor protein expression in human endothelial-cells. *Journal Of Neuroscience Research* **37**, 769-776.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I. and Selkoe, D.J. (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's-disease increases beta-protein production. *Nature* **360**, 672-674.

Citron, M., Vigo-Pelfrey, C., Teplow, D.B., Miller, C. and Schenk, D. (1994) Excessive production of amyloid β-protein by peripheral cells of symptomatic and presymptomatic patients carrying the

Swedish familial Alzheimer disease mutation. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 11993-11997.

Citron, M., Westaway, D., Xia, W.M., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., Hyslop, P.S. and Selkoe, D.J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42- residue amyloid beta-protein in both transfected cells and transgenic mice. *Nature Medicine* **3**, 67-72.

Clark, M.J., Gagnon, J., Williams, A.F. and Barclay, A.N. (1985) MRC OX-2 antigen - a lymphoid neuronal membrane glycoprotein with a structure like a single immunoglobulin light chain. *EMBO Journal* **4**, 113-118.

Cohen, M.L., Golde, T.E., Usiak, M.F., Younkin, L.H. and Younkin, S.G. (1988) *In situ* hybridization of nucleus basalis neurons shows increased beta- amyloid messenger-RNA in Alzheimer-disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **85**, 1227-1231.

Cook, D.G., Forman, M.S., Sung, J.C., Leight, S., Kolson, D.L., Iwatsubo, T., Lee, V.M.Y. and Doms, R.W. (1997) Alzheimer's Aβ(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nature Medicine* **3**, 1021-1023.

Cummings, J.I., Vinters, V., Cole, G.C. and Khachaturian, Z.S. (1998) Alzheimer's disease - Etiologies, pathophysiology, cognitive reserve, and treatment opportunities. *Neurology* **51**, S2-S17.

Davis, J.B. (1996) Oxidative mechanisms in beta-amyloid cytotoxicity. *Neurodegeneration* **5**, 441-444.

Dewji, N.N., Do, C. and Bayney, R.M. (1995) Transcriptional activation of Alzheimer's beta-amyloid precursor protein gene by stress. *Molecular Brain Research* **33**, 245-253.

Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.M., Pereztur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M.N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J. and Younkin, S. (1996) Increased amyloid-beta-42(43) in brains of mice expressing mutant presenilin-1. *Nature* **383**, 710-713.

Eikelenboom, P., Rozemuller, J.M. and van Muiswinkel, F.L. (1998) Inflammation and Alzheimer's disease: relationships between pathogenic mechanisms and clinical expression. *Experimental Neurology* **154**, 89-98.

Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) Cleavage of Amyloid β Peptide During Constitutive Processing of Its Precursor. *Science* **248**, 1122-1224

Esiri, M.M., Pearson, R.C.A., Steele, J.E., Bowen, D.M. and Powell, T.P.S. (1990) A quantitative study of the neurofibrillary tangles and the choline-acetyltransferase activity in the cerebral-cortex and the amygdala in Alzheimer's-disease. *Journal Of Neurology Neurosurgery And Psychiatry* **53**, 161-165.

Esiri, M.M., Hyman, B.T., Beyreuther, K. and Masters, C.L. (1997) Ageing and dementia. In: Graham, D.I. and Lantos, P.L. (Eds.) *Greenfields Neuropathology*, 6th edn. pp. 153-213. Arnold

Esiri, M.M. and Wilcock, G.K. (1986) Cerebral amyloid angiopathy in dementia and old-age. *Journal Of Neurology Neurosurgery And Psychiatry* **49**, 1221-1226.

Estus, S., Golde, T.E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X.M., Tabira, T., Greenberg, B.D. and Younkin, S.G. (1992) Potentially amyloidogenic, carboxyl-terminal derivatives of the amyloid protein-precursor. *Science* **255**, 726-728.

Fathallah, D.M., Cherif, D., Dellagi, K. and Arnaout, M.A. (1993) Molecular cloning of a novel human hsp 70 from a B cell line and its assignment to chromosome 5. *Journal of Immunology* **151,** 810-813.

Forloni, G., Demicheli, F., Giorgi, S., Bendotti, C. and Angeretti, N. (1992) Expression of amyloid precursor protein messenger-RNAs in endothelial, neuronal and glial-cells - modulation by interleukin-1. *Molecular Brain Research* **16**, 128-134.

Furukawa, K., Barger, S.W., Blalock, E.M. and Mattson, M.P. (1996) Activation of K+ channels and suppression of neuronal-activity by secreted beta-amyloid-precursor protein. *Nature* **379**, 74-78.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnsonwood, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, I., Little, S., Masliah, E., McConlogue, L., Montoyazavala, M.,

Mucke, L., Paganini, L., Penniman, E., Power, M., Schenk, D., Seubert, P., Snyder, B., Soriano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B. and Zhao, J. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* **373**, 523-527.

Gentleman, S. and Roberts, G. (1992) Risk-factors in Alzheimer's-disease. *British Medical Journal* **304**, 118-119.

Ghiso, J., Rostagno, A., Gardella, J.E., Liem, L., Gorevic, P.D. and Frangione, B. (1992) A 109-amino-acid *C*-terminal fragment of Alzheimer's disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion. *Biochemical Journal* **288**, 1052-1059.

Glenner, G.G. and Page, D.L. (1976) Amyloid, amyloidosis, and amyloidogenesis. *International Review of Experimental Pathology* **1**, 1-92.

Glenner, G.G. and Wong, C.W. (1984) Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical And Biophysical Research Communications* **120**, 885-890.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M. and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's-disease. *Nature* **349**, 704-706.

Goedert, M., Crowther, R.A. and Garner, C.C. (1991) Molecular characterization of microtubule-associated proteins- tau and MAP2. *Trends In Neurosciences* **14**, 193-199.

Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. and Younkin, S.G. (1992) Processing of the amyloid protein-precursor to potentially amyloidogenic derivatives. *Science* **255**, 728-730.

Goldgaber, D., Harris, H.W., Hla, T., Maciag, T., Donnelly, R.J., Jacobsen, J.S., Vitek, M.P. and Gajdusek, D.C. (1989) Interleukin-1 regulates synthesis of amyloid beta-protein precursor messenger-RNA in human-endothelial cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **86**, 7606-7610.

Goldman, J.E. and Yen, S.H. (1986) Cytoskeletal protein abnormalities in neurodegenerative diseases. *Annals Of Neurology* **19**, 209-223.

Graves, A.B., White, E., Koepsell, T.D., Reifler, B.V., Vanbelle, G., Larson, E.B. and Raskind, M. (1990) The association between head trauma and Alzheimer's-disease. *American Journal Of Epidemiology* **131**, 491-501.

Gravina, S.A., Ho, L., Eckman, C.B., Long, K.E., Otvos, L., Younkin, L.H., Suzuki, N. and Younkin, S.G. (1995) Amyloid β protein (Aβ) in Alzheimer's disease brain. *Journal Of Biological Chemistry* **270**, 7013-7016.

Grundke Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Abnormal phosphorylation of the microtubule-associated protein- tau (τ) in Alzheimer cytoskeletal

pathology. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **83**, 4913-4917.

Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. and Selkoe, D.J. (1992a) Targeting of cell-surface beta-amyloid precursor protein to lysosomes - alternative processing into amyloid-bearing fragments. *Nature* **357**, 500-503.

Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B. and Selkoe, D.J. (1992b) Amyloid beta-peptide is produced by cultured-cells during normal metabolism. *Nature* **359**, 322-325.

Haass, C., Hung, A.Y., Selkoe, D.J. and Teplow, D.B. (1994) Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid β-protein precursor. *Journal Of Biological Chemistry* **269**, 17741-17748.

Haass, C. and Selkoe, D.J. (1998) Alzheimer's disease - a technical KO of amyloid-beta peptide. *Nature* **391**, 339-340.

Hamos, J.E., Oblas, B., Pulaskisalo, D., Welch, W.J., Bole, D.G. and Drachman, D.A. (1991) Expression of heat-shock proteins in Alzheimer's-disease. *Neurology* **41**, 345-350.

Hang, H. and Fox, M.H. (1996) Levels of 70-kDa heat shock protein through the cell cycle in several mammalian cell lines. *Cytometry* **25**, 367-373.

Hardy, J. (1996) New insights into the genetics of Alzheimer's-disease. *Annals Of Medicine* **28**, 255-258.

Hardy, J.A. and Higgins, G.A. (1992) Alzheimer's-disease - the amyloid cascade hypothesis. *Science* **256**, 184-185.

Harrison, G.S., Morimoto, R., Kao, F.-T., Chu, E.H.Y., Wu, B.J. and Drabkin, H. (1986) Chromosomal location of human genes encoding the major heat shock protein HSP70. *American Journal of Human Genetics* **39**, A157.

Harrison, G.S., Drabkin, H.A., Kao, F.-T., Hartz, J.,Hart, I., Chu, E.H.Y., Wu, B.J. and Morimoto, R.I (1987) Chromosomal location of human genes encoding major heat-shock protein HSP70. *Somatic Cell Molecular Genetics* **13**, 119-130.

Harrison, P.J., Barton, A.J. and Pearson, R.C.A. (1991) Expression of amyloid beta-protein precursor mRNAs in familial Alzheimer's disease. *Neuroreport* **2**, 153-154.

Harrison, P.J., Proctor, A.W., Exworthy, T., Roberts, G.W., Najlerahim, A., Barton, A.J. and Pearson, R.C.A. (1993) Heat shock protein (hsx70) mRNA expression in human brain: effects of neurodegenerative disease and agonal state. *Neuropathology and Applied Neurobiology* **19** 10-21

Hartmann, T., Bieger, S.C., Bruhl, B., Tienari, P.J., Ida, N., Allsop, D., Roberts, G.W., Masters, C.L., Dotti, C.G., Unsicker, K. and Beyreuther, K. (1997) Distinct sites of intracellular production for Alzheimer's disease Aβ40/42 amyloid peptides. *Nature Medicine* **3**, 1016-1020.

Heath, P.R., Sanders, M.W. and Pearson, R.C.A. (1996) Oligonucleotide methods with ^{35}S. In: *In Situ Techniques for the brain*, pp. 45-47. Chichester: John Wiley and Sons Ltd

Hendriks, L., van Duijn, C.M., Cras, P., Cruts, M., Van Hul, W., van Harskamp, F., Warren, A., McInnis, M.G., Antonarakis, S.E., Martin, J.J., Hofman, A. and Van Broeckhoven, C. (1992) Presenile-dementia and cerebral-hemorrhage linked to a mutation at codon-692 of the β-amyloid precursor protein gene. *Nature Genetics* **1**, 218-221.

Hensley, K., Carney, J.M., Mattson, M.P., Aksenova, M., Harris, M., Wu, J.F., Floyd, R.A. and Butterfield, D.A. (1994) A model for β-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: Relevance to Alzheimer disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 3270-3274.

Ho, L.B., Fukuchi, K. and Younkin, S.G. (1996) The alternatively spliced Kunitz protease inhibitor domain alters amyloid beta protein precursor processing and amyloid beta protein production in cultured cells. *Journal Of Biological Chemistry* **271**, 30929-30934.

Hoshi, M., Takashima, A., Murayama, M., Yasutake, K., Yoshida, N., Ishiguro, K., Hoshino, T. and Imahori, K. (1997) Nontoxic amyloid beta peptide (1-42) suppresses acetylcholine synthesis - possible role in cholinergic dysfunction in Alzheimer's disease. *Journal Of Biological Chemistry* **272**, 2038-2041.

Hung, A.Y., Haass, C., Nitsch, R.M., Qui, W.Q., Citron, M., Wurtman, R.J., Growdon, J.H. and Selkoe, D.J. (1993) Activation of protein kinase C inhibits cellular production of the amyloid β-protein. *Journal Of Biological Chemistry* **268**, 22959-22962.

Hyman, B.T., Tanzi, R.E., Marzloff, K., Barbour, R. and Schenk, D. (1992) Kunitz protease inhibitor-containing amyloid-beta protein- precursor immunoreactivity in Alzheimer's-disease. *Journal Of Neuropathology And Experimental Neurology* **51**, 76-83.

Hyman, B.T., West, H.L., Rebeck, G.W., Buldyrev, S.V., Mantegna, R.N., Ukleja, M., Havlin, S. and Stanley, H.E. (1995) Quantitative-analysis of senile plaques in Alzheimer-disease - observation of log-normal size distribution and molecular epidemiology of differences associated with Apolipoprotein-E genotype and trisomy-21 (Down-Syndrome). *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 3586-3590.

Johnson, G., Refolo, L.M., Merril, C.R. and Wallace, W. (1993a) Altered expression and phosphorylation of amyloid precursor protein in heat-shocked neuronal PC12 cells. *Molecular Brain Research* **19**, 140-148.

Johnson, G., Refolo, L.M. and Wallace, W. (1993b) Heat-shocked neuronal PC12 cells reveal Alzheimer's-disease - associated alterations in amyloid precursor protein and tau. *Annals Of The New York Academy Of Sciences* **695**, 194-197.

Johnson, S.A., McNeill, T., Cordell, B. and Finch, C.E. (1990) Relation of neuronal APP-751/APP-695 mRNA ratio and neuritic plaque density in Alzheimer's disease. *Science* **248**, 854-857.

Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K. and Mullerhill, B. (1987) The precursor of Alzheimer's-disease amyloid-A4 protein resembles a cell-surface receptor. *Nature* **325**, 733-736.

Katzman, R. (1988) Alzheimer's-disease as an age-dependent disorder. *Ciba Foundation Symposium* **134**, 69-85.

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. and Ito, H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* **331**, 530-532.

Koh, J.Y., Yang, L.L. and Cotman, C.W. (1990) Beta-amyloid protein increases the vulnerability of cultured cortical-neurons to excitotoxic damage. *Brain Research* **533**, 315-320.

Koo, E.H., Sisodia, S.S., Archer, D.R., Martin, L.J., Weidemann, A., Beyreuther, K., Fischer, P., Masters, C.L. and Price, D.L. (1990) Precursor of amyloid protein in Alzheimer-disease undergoes fast anterograde axonal-transport. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **87**, 1561-1565.

Koo, E.H., Park, L. and Selkoe, D.J. (1993) Amyloid β-protein as a substrate interacts with extracellular matrix to promote neurite outgrowth. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 4748-4752.

Koo, E.H. and Squazzo, S.L. (1994) Evidence that production and release of amyloid β-protein involves the endocytic pathway. *Journal Of Biological Chemistry* **269**, 17386-17389.

LaDu, M.J., Falduto, M.T., Manelli, A.M., Reardon, C.A., Getz, G.S. and Frail, D.E. (1994) Isoform-specific binding of Apolipoprotein E to β-amyloid. *Journal Of Biological Chemistry* **269**, 23403-23406.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LaFerla, F.M., Tinkle, B.T., Bieberich, C.J., Hauderschild, C.C. and Jay, G. (1995) The Alzheimer's Aβ peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nature Genetics* **9**, 21-29.

Lahiri, D.K. and Nall, C. (1995) Promoter activity of the gene encoding the beta-amyloid precursor protein is up-regulated by growth factors, phorbol ester, retinoic acid and interleukin-1. *Molecular Brain Research* **32**, 233-240.

Leblanc, A.C., Koutroumanis, M. and Goodyer, C.G. (1998) Protein kinase C activation increases release of secreted amyloid precursor protein without decreasing A beta production in human primary neuron cultures. *Journal Of Neuroscience* **18**, 2907-2913.

Lee, M.K., Slunt, H.H., Martin, L.J., Thinakaran, G., Kim, G., Gandy, S.E., Seeger, M., Koo, E., Price, D.L. and Sisodia, S.S. (1996) Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. *Journal Of Neuroscience* **16**, 7513-7525.

Lee, V.M.Y. and Andrews, P.W. (1986) Differentiation of NTera-2 clonal human embryonal carcinoma- cells into neurons involves the induction of all 3 neurofilament proteins. *Journal Of Neuroscience* **6**, 514-521.

Lemere, C.A., Lopera, F., Kosik, K.S., Lendon, C.L., Ossa, J., Saido, T.C., Yamaguchi, H., Ruiz, A., Martinez, A., Madrigal, L., Hincapie, L., Arango, J.C.L., Anthony, D.C., Koo, E.H., Goate, A.M., Selkoe, D.J. and Arango, J.C.V. (1996) The E280A presenilin-1 Alzheimer mutation produces increased A-beta-42 deposition and severe cerebellar pathology. *Nature Medicine* **2**, 1146-1150.

Lenkkeri, U., Kestila, M., Lamerdin, J., McCready, P., Adamson, A., Olsen, A. and Tryggvason, K. (1998) Structure of the human amyloid-precursor-like protein gene APLP1 at 19q13.1. *Human Genetics* **102**, 192-196.

Leung, T.K.C., Hall, C., Rajendran, M., Spurr, N.K. and Lim, L. (1992) The human hear-shock genes HSPA6 and HSPA7 are both expressed and localize to chromosome 1 *Genomics* **12**, 74-79.

Levitan, D., Doyle, T.G., Brousseau, D., Lee, M.K., Thinakaran, G., Slunt, H.H., Sisodia, S.S. and Greenwald, I. (1996) Assessment of normal and mutant human presenilin function in *Caenorhabditis-elegans. Proceedings Of The National Academy Of Sciences Of The United States Of America* **93**, 14940-14944.

Levitan, D. and Greenwald, I. (1995) Facilitation of LIN-12-mediated signalling by sel-12, a *Caenorhabditis-elegans* S182 Alzheimer's-disease gene. *Nature* **377**, 351-354.

Levy, E., Carman, M.D., Fernandezmadrid, I.J., Power, M.D., Lieberburg, I., Vanduinen, S.G., Bots, G.T.A.M., Luyendijk, W. and Frangione, B. (1990) Mutation of the Alzheimer's-disease amyloid gene in hereditary cerebral-hemorrhage, Dutch type. *Science* **248**, 1124-1126.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., Crowley, A.C., Fu, Y.H., Guenette, S.Y., Galas, D., Nemens, E., Wijsman, E.M., Bird, T.D., Schellenberg, G.D. and Tanzi, R.E. (1995a) Candidate gene for the chromosome-1 familial Alzheimer's-disease locus. *Science* **269**, 973-977.

Levy-Lahad, E., Wijsman, E.M., Nemens, E., Anderson, L., Goddard, K.A.B., Weber, J.L., Bird, T.D. and Schellenberg, G.D. (1995b) A familial Alzheimer's-disease locus on chromosome-1. *Science* **269**, 970-973.

Li, X. and Greenwald, I. (1998) Additional evidence for an eight-transmembrane-domain topology for *Caenorhabditis elegans* and human presenilins. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **95**, 7109-7114.

Lindquist, S. (1986) The heat-shock response. *Annual Review Of Biochemistry* **55**, 1151-1191.

Lindquist, S. and Craig, E.A. (1988) The heat-shock proteins. *Annual Review Of Genetics* **22**, 631-677.

Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J. and Cotman, C.W. (1993) Apoptosis is induced by beta-amyloid in cultured central-nervous-system neurons. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 7951-7955.

Lyckman, A.W., Confaloni, A.M., Thinakaran, G., Sisodia, S.S. and Moya, K.L. (1998) Post-translational processing and turnover kinetics of presynaptically targeted amyloid precursor

superfamily proteins in the central nervous system. *Journal Of Biological Chemistry* **273**, 11100-11106.

Mandelkow, E. and Mandelkow, E. (1998) Tau in Alzheimer's disease. *Trends in Cell Biology* **8**, 425-427.

Mann, D.M.A., Iwatsubo, T., Cairns, N.J., Lantos, P.L., Nochlin, D., Sumi, S.M., Bird, T.D., Poorkaj, P., Hardy, J., Hutton, M., Prihar, G., Crook, R., Rossor, M.N. and Haltia, M. (1996a) Amyloid-beta protein (a-beta) deposition in chromosome 14-linked Alzheimer's-disease - predominance of a-beta(42(43)). *Annals Of Neurology* **40**, 149-156.

Mann, D.M.A., Iwatsubo, T., Ihara, Y., Cairns, N.J., Lantos, P.L., Bogdanovic, N., Lannfelt, L., Winblad, B., Maat Schieman, M.L.C. and Rossor, M.N. (1996b) Predominant deposition of amyloid-beta(42(43)) in plaques in cases of Alzheimer's disease and hereditary cerebral hemorrhage associated with mutations in the amyloid precursor protein gene. *American Journal Of Pathology* **148**, 1257-1266.

Maruyama, K., Tomita, T., Shinozaki, K., Kume, H., Asada, H., Saido, T.C., Ishiura, S., Iwatsubo, T. and Obata, K. (1996) Familial Alzheimer's-disease-linked mutations at val(717) of amyloid precursor protein are specific for the increased secretion of A-beta- 42(43). *Biochemical And Biophysical Research Communications* **227**, 730-735.

Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L. and Beyreuther, K. (1985) Amyloid plaque core protein in Alzheimer-disease and Down Syndrome. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **82**, 4245-4249.

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *Journal Of Biological Chemistry* **262**, 10035-10038.

Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I. and Rydel, R.E. (1992) Beta-amyloid peptides destabilize calcium homeostasis and render human cortical-neurons vulnerable to excitotoxicity. *Journal Of Neuroscience* **12**, 376-389.

Mattson, M.P., Barger, S.W., Cheng, B., Lieberburg, I., Smith Swintosky, V.L. and Rydel, R.E. (1993a) Beta-amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's-disease. *Trends In Neurosciences* **16**, 409-414.

Mattson, M.P., Cheng, B., Culwell, A.R., Esch, F.S., Lieberburg, I. and Rydel, R.E. (1993b) Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. *Neuron* **10**, 243-254.

McGeer, P.L. and McGeer, E.G. (1996) Antiinflammatory drugs in the fight against Alzheimer's-disease. *Annals Of The New York Academy Of Sciences* **777**, 213-220.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M. (1984) Clinical-diagnosis of Alzheimer's-disease - report of the NINCDS-ADRDA work group under the auspices of department-of-health-and- human- services task-force on Alzheimer's-disease. *Neurology* **34**, 939-944.

McNamara, M.J., Ruff, C.T., Wasco, W., Tanzi, R.E., Thinakaran, G. and Hyman, B.T. (1998) Immunohistochemical and *in situ* analysis of amyloid precursor- like protein-1 and amyloid precursor-like protein-2 expression in Alzheimer disease and aged control brains. *Brain Research* **804**, 45-51.

Meda, L., Cassatella, M.A., Szendrei, G.I., Otvas, L., Baron, P., Villalba, M., Ferrari, D. and Rossi, F. (1995) Activation of microglial cells by β-amyloid protein and interferon gamme. *Nature* **374**, 647-650.

Moir, R.D., Lynch, T., Bush, A.I., Whyte, S., Henry, A., Portbury, S., Multhaup, G., Small, D.H., Tanzi, R.E., Beyreuther, K. and Master, C.L. (1998) Relative incerase in Alzheimer's disease of s oluble forms of cerebral A-beta amyloid protein precursor containing the Kunitz protease inhibitory domain. *Journal Of Biological Chemistry* **273**, 5013-5019.

Moran, P.M., Higgins, L.S., Cordell, B. and Moser, P.C. (1995) Age-related learning-deficits in transgenic mice expressing the 751- amino acid isoform of human beta-amyloid precursor protein. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 5341-5345.

Morato, E. and Mayor, F. (1993) Production of the Alzheimer beta-amyloid peptide by C6 glioma-cells. *Febs Letters* **336**, 275-278.

Mori, H., Kondo, J. and Ihara, Y. (1987) Ubiquitin is a component of paired helical filaments in Alzheimer's- disease. *Science* **235**, 1641-1644.

Mucke, L., Masliah, E., Johnson, W.B., Ruppe, M.D., Alford, M., Rockenstein, E.M., Forsspetter, S., Pietropaolo, M., Mallory, M. and Abraham, C.R. (1994) Synaptotrophic effects of human amyloid-beta protein precursors in the cortex of transgenic mice. *Brain Research* **666**, 151-167.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B. and Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's-disease in the APP gene at the N-terminus of beta-amyloid. *Nature Genetics* **1**, 345-347.

Murayama, M., Tanaka, S., Palacino, J., Murayama, O., Honda, T., Sun, X., Yasutake, K., Nihonmatsu, N., Wolozin, B. and Takashima, A. (1998) Direct association of presenilin-1 with β-catenin. *Febs Letters* **433**, 73-77.

Murrell, J., Farlow, M., Ghetti, B. and Benson, M.D. (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's-disease. *Science* **254**, 97-99.

Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E. and Ikeda, K. (1991) Apolipoprotein-E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's-disease and Kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Research* **541**, 163-166.

Nishimura, M., Yu, G., Levesque, G., Zhang, D., Ruel, L., Chen, F., Milman, P., Holmes, E., Liang, Y., Kawarai, T., Jo, E., Supala, A., Rogaev, E., Xu, D., Janus, C., Levesque, L., Bi, Q., Duthie, M., Rozmahel, R., Mattila, K., Lannfelt, L., Westaway, D., Mount, H.T.J., Woodgett, J., Fraser, P.E. and St George-Hyslop, P. (1999) Presenilin mutations associated with Alzheimer disease cause defective intracellular trafficking of β-catenin, a component of the presenilin protein complex. *Nature Medicine* **5**, 164-169.

Nitsch, R.M., Slack, B.E., Wurtman, R.J. and Growdon, J.H. (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine-receptors. *Science* **258**, 304-307.

Nitsch, R.M., Farber, S.A., Growdon, J.H. and Wurtman, R.J. (1993) Release of amyloid β-protein precursor derivatives by electrical depolarization of rat hippocampal slices. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 5191-5193.

Nordstedt, C., Caporaso, G.L., Thyberg, J., Gandy, S.E. and Greengard, P. (1993) Identification of the Alzheimer β/A4 amyloid precursor protein in clathrin-coated vesicles purified from PC12 cells. *Journal Of Biological Chemistry* **268**, 608-612.

Oyama, F., Shimada, H., Oyama, R. and Ihara, Y. (1995) Apolipoprotein E genotype, Alzheimer's pathologies and related gene expression in the aged population. *Molecular Brain Research* **29**, 92-98.

Palmert, M.R., Golde, T.E., Cohen, M.L., Kovacs, D.M., Tanzi, R.E., Gusella, J.F., Usiak, M.F., Younkin, L.H. and Younkin, S.G. (1988) Amyloid protein-precursor messenger-RNAs - differential expression in Alzheimer's-disease. *Science* **241**, 1080-1084.

Pappolla, M.A., Sambamurti, K., Efthimiopoulos, S., Refolo, L., Omar, R.A. and Robakis, N.K. (1995) Heat-shock induces abnormalities in the cellular-distribution of amyloid precursor protein (APP) and APP fusion proteins. *Neuroscience Letters* **192**, 105-108.

Paradis, E., Douillard, H., Koutroumanis, M., Goodyer, C. and Leblanc, A. (1996) Amyloid beta peptide of Alzheimer's disease downregulates bcl-2 and upregulates bax expression in human neurons. *Journal Of Neuroscience* **16**, 7533-7539.

Pearson, R.C.A. (1996) Cortical connections and the pathology of Alzheimer's disease. *Neurodegeneration* **5**, 429-434.

Pearson, R.C.A. and Powell, T.P.S. (1989) The Neuroanatomy of Alzheimer's Disease. *Reviews In The Neurosciences* **2**, 101-122.

Perez, N., Sugar, J., Charya, S., Johnson, G., Merril, C., Bierer, L., Perl, D., Haroutunian, V. and Wallace, W. (1991) Increased synthesis and accumulation of heat-shock 70 proteins in Alzheimer's-disease. *Molecular Brain Research* **11**, 249-254.

Pike, C.J., Walencewicz, A.J., Glabe, C.G. and Cotman, C.W. (1991) Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. *European Journal Of Pharmacology-Molecular Pharmacology Section* **207**, 367-368.

Pleasure, S.J., Page, C. and Lee, V.M.Y. (1992) Pure, postmitotic, polarized human neurons derived from NTera-2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *Journal Of Neuroscience* **12**, 1802-1815.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F. and Cordell, B. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature* **331**, 525-527.

Price, D.L., Tanzi, R.E., Borchelt, D.R. and Sisodia, S.S. (1998) Alzheimer's disease: genetic studies and transgenic models. *Annual Review Of Genetics* **32**, 461-493.

Roberts, G.W., Gentleman, S.M., Lynch, A., Murray, L., Landon, M. and Graham, D.I. (1994) Beta-amyloid protein deposition in the brain after severe head- injury - implications for the pathogenesis of Alzheimer's-disease. *Journal Of Neurology Neurosurgery And Psychiatry* **57**, 419-425.

Roch, J.M., Masliah, E., Rochlevecq, A.C., Sundsmo, M.P., Otero, D.A.C., Veinbergs, I. and Saitoh, T. (1994) Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/a4 protein-precursor. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 7450-7454.

Rogers, J., Kirby, L.C., Hempelman, S.R., Berry, D.L., McGeer, P.L., Kaszniak, A.W., Zalinski, J., Cofield, M., Mansukhani, L., Willson, P. and Kogan, F. (1993) Clinical-trial of indomethacin in Alzheimer's-disease. *Neurology* **43**, 1609-1611.

Rohan de Silva, H.A., Jen, A., Wickenden, C., Jen, L.S., Wilkinson, S.L. and Patel, A.J. (1997) Cell-specific expression of beta-amyloid precursor protein isoform mRNAs and proteins in neurons and astrocytes. *Molecular Brain Research* **47**, 147-156.

Salbaum, J.M., Weidemann, A., Lemaire, H.G., Masters, C.L. and Beyreuther, K. (1988) The promoter of Alzheimer's disease amyloid A4 precursor gene. *EMBO Journal* **7**, 2807-2813.

Sambamurti, K., Shioi, J., Anderson, J.P., Pappolla, M.A. and Robakis, N.K. (1992) Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. *Journal Of Neuroscience Research* **33**, 319-329.

Satoh, J. and Kim, S.U. (1994) Hsp72 induction by heat-stress in human neurons and glial-cells in culture. *Brain Research* **653**, 243-250.

Satoh, J.I. and Kim, S.U. (1995) Constitutive and inducible expression of heat-shock protein hsp72 in oligodendrocytes in culture. *Neuroreport* **6**, 1081-1084.

Schagger, H. and Von Jagow, G. (1987) Tricine sodium-dodecylsulphate polyacrylamide-gel electrophoresis for the separation of proteins in the range from 1-kDa to 100-kDa. *Analytical Biochemistry* **166**, 368-379.

Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D. and Younkin, S. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's-disease is increased *in-vivo* by the presenilin-1 and presenilin-2 and APP mutations linked to familial Alzheimer's-disease. *Nature Medicine* **2**, 864-870.

Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Crain, B.J., Hulette, C.M., Joo, S.H., Pericak-Vance, M.A., Goldgaber, D. and Roses, A.D. (1993) Increased amyloid beta-peptide deposition in cerebral-cortex as a consequence of apolipoprotein-E genotype in late-onset

Alzheimer- disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 9649-9653.

Selkoe, D.J. (1993) Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's-disease. *Trends In Neurosciences* **16**, 403-409.

Selkoe, D.J., Yamazaki, T., Citron, M., Podlisny, M.B., Koo, E.H., Teplow, D.B. and Haass, C. (1996) The role of APP processing and trafficking pathways in the formation of amyloid beta-protein. *Annals Of The New York Academy Of Sciences* **777**, 57-64.

Selkoe, D.J. (1998) The cell biology of β-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends in Cell Biology* **8**, 447-453.

Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I. and Schenk, D. (1992) Isolation and quantification of soluble Alzheimer's beta-peptide from biological-fluids. *Nature* **359**, 325-327.

Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., Lieberburg, I. and Schenk, D.B. (1993) Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. *Nature* **361**, 260-263.

Shepherd, C.L. (1998) Astrocytic involvement in pathological processes relevant to Alzheimer's disease. University of Sheffield. PhD.

Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., Rohan de Silva, H.A., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M. and St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's-disease. *Nature* **375**, 754-760.

Shimizu, S., Nomura, K., Ujigara, M., Demura, H. (1999) An additional exon of stress-inducible heat shock protein 70 gene (HSP70-1). *Biochemical and Biophysical Research Communications* **257**, 193-198.

Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.D., McKay, D.M., Tintner, R., Frangione, B. and Younkin, S.G. (1992) Production of the Alzheimer amyloid-beta protein by normal proteolytic processing. *Science* **258**, 126-129.

Sisodia, S.S. (1992) Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 6075-6079.

Sisodia, S.S. and Gallagher, M. (1998) A role for the β-amyloid precursor protein in memory? *Proceedings Of The National Academy Of Sciences Of The United States Of America* **95**, 12074-12076.

Slunt, H.H., Thinakaran, G., Von Koch, C., Lo, A.C.Y., Tanzi, R.E. and Sisodia, S.S. (1994) Expression of a ubiquitous, cross-reactive homolog of the mouse beta- amyloid precursor protein (APP). *Journal Of Biological Chemistry* **269**, 2637-2644.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Smith, R.P., Higuchi, D.A. and Broze, G.J. (1990) Platelet coagulation factor XI_a-inhibitor, a form of Alzheimer amyloid precursor protein. *Science* **248**, 1126-1128.

Sola, C., Garcialadona, F.J., Mengod, G., Probst, A., Frey, P. and Palacios, J.M. (1993) Increased levels of the Kunitz protease inhibitor-containing beta-APP messenger-RNAs in rat-brain following neurotoxic damage. *Molecular Brain Research* **17**, 41-52.

Sorger, P.K. (1991) Heat-shock factor and the heat-shock response. *Cell* **65**, 363-366.

St George-Hyslop, P.H., Tanzi, R.E., Polinsky, R.J., Haines, J.L., Nee, L., Watkins, P.C., Myers, R.H., Feldman, R.G., Pollen, D., Drachman, D., Growdon, J., Bruni, A., Foncin, J.F., Salmon, D., Frommelt, P., Amaducci, L., Sorbi, S., Piacentini, S., Stewart, G.D., Hobbs, W.J., Conneally, P.M. and Gusella, J.F. (1987) The genetic-defect causing familial Alzheimer's-disease maps on chromosome-21. *Science* **235**, 885-890.

Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S. and Roses, A.D. (1993) Apolipoprotein-E - high-avidity binding to beta-amyloid and

increased frequency of type-4 allele in late-onset familial Alzheimer-disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 1977-1981.

Sudoh, S., Kawakami, H. and Nakamura, S. (1996) Serum deprivation alters the expression and the splicing at exons 7, 8 and 15 of the beta-amyloid precursor protein in the C6 glioma cell line. *Molecular Brain Research* **39**, 12-22.

Suzuki, N., Cheung, T.T., Cai, X.D., Odaka, A., Otvos, L., Eckman, C., Golde, T.E. and Younkin, S.G. (1994) An increased percentage of long amyloid-beta protein secreted by familial amyloid-beta protein-precursor (beta-APP(717)) mutants. *Science* **264**, 1336-1340.

Tamaoka, A., Odaka, A., Ishibashi, Y., Usami, M., Sahara, N., Suzuki, N., Nukina, N., Mizusawa, H., Shoji, S., Kanazawa, I. and Mori, H. (1994) APP717 Missense mutation affects the ratio of amyloid β protein species (Aβ1-42/43 and Aβ1-40) in familial Alzheimer's disease brain. *Journal Of Biological Chemistry* **269**, 32721-32724.

Tanaka, S., Nakamura, S., Ueda, K., Kameyama, M., Shiojiri, S., Takahashi, Y., Kitaguchi, N. and Ito, H. (1988) 3 types of amyloid protein-precursor messenger-RNA in human- brain - their differential expression in Alzheimer's-disease. *Biochemical And Biophysical Research Communications* **157**, 472-479.

Tanaka, S., Shiojiri, S., Takahashi, Y., Kitaguchi, N., Ito, H., Kameyama, M., Kimura, J., Nakamura, S. and Ueda, K. (1989) Tissue-specific expression of 3 types of beta-protein precursor messenger-RNA -enhancement of protease inhibitor-harboring types in Alzheimer's-disease brain. *Biochemical And Biophysical Research Communications* **165**, 1406-1414.

Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.P., St George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M. and Neve, R.L. (1987) Amyloid beta-protein gene - cDNA, messenger-RNA distribution, and genetic-linkage near the Alzheimer locus. *Science* **235**, 880-884.

Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villakomaroff, L., Gusella, J.F. and Neve, R.L. (1988) Protease inhibitor domain encoded by an amyloid protein- precursor messenger-RNA associated with Alzheimer's-disease. *Nature* **331**, 528-530.

Tavair, M., Gabiele, T., Anderson, R.L., Mirault, M.-E., Baker, E., Sutherland, G. and Kola, I. (1995) Localization of the gene encoding the human heat shock cognate protein, HSP73, to chromosomes 11. *Genomics* **29**, 266-268.

Thinakaran, G., Teplow, D.B., Siman, R., Greenberg, B. and Sisodia, S.S. (1996) Metabolism of the Swedish amyloid precursor protein variant in neuro2a (N2A) cells -evidence that cleavage at the beta-secretase site occurs in the Golgi-apparatus. *Journal Of Biological Chemistry* **271**, 9390-9397.

Thinakaran, G., Harris, C.L., Rattovitski, T., Davenport, F., Slunt, H.H., Price, D.L., Borchelt, D., R., and Sisodia, S.S. (1997) Evidence that levels of presenilins (PS1 and PS2) are co-ordinately regulated by competition for limiting cellular factors. *Journal of Biological Chemistry* **272**, 28415-28422

Tomita, T., Maruyama, K., Saido, T.C., Kume, H., Shinozaki, K., Tokuhiro, S., Capell, A., Walter, J., Grunberg, J., Haass, C., Iwatsubo, T. and Obata, K. (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid beta protein ending at the 42nd (or 43rd) residue. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **94**, 2025-2030.

Tomlinson, B.E., Blessed, G. and Roth, M. (1970) Observations on the brains of demented old people. *Journal Of Neurological Science* **11**, 205-242.

Turner, R.S., Suzuki, N., Chyung, A.S.C., Younkin, S.G. and Lee, V.M.Y. (1996) Amyloids beta(40) and beta(42) are generated intracellularly in cultured human neurons and their secretion increases with maturation. *Journal Of Biological Chemistry* **271**, 8966-8970.

Ueda, K., Fukui, Y. and Kageyama, H. (1994) Amyloid β protein-induced neuronal cell death: neurotoxic properties of aggregated amyloid β protein. *Brain Research* **639**, 240-24.

Van Nostrand, W.E., Schmaier, A.H., Farrow, J.S. and Cunningham, D.D. (1990) Protease nexin-II (amyloid beta-protein precursor) - a platel*et al*pha-granule protein. *Science* **248**, 745-748.

Voellmy, R., Ahmed, A., Schiller, P., Bromley, P. and Runnger, D. (1985) Isolation and functional analysis of a human 70,000-dalton heat shock protein gene segment. *Proceedings of the National Academy of Sciences of the United States Of America* **82**, 4949-4953

Wang, G.P., Khatoon, S., Iqbal, K. and Grundke-Iqbal, I. (1991) Brain ubiquitin is markedly elevated in Alzheimer-disease. *Brain Research* **566**, 146-151.

Wasco, W., Bupp, K., Magendantz, M., Gusella, J.F., Tanzi, R.E. and Solomon, F. (1992) Identification of a mouse-brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid-beta-protein precursor. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 10758-10762.

Webster, M.T., Groome, N., Francis, P.T., Pearce, B.R., Sherriff, F.E., Thinakaran, G., Felsenstein, K.M., Wasco, W., Tanzi, R.E. and Bowen, D.M. (1995) A novel protein, amyloid precursor-like protein-2, is present in human brain, cerebrospinal-fluid and conditioned media. *Biochemical Journal* **310**, 95-99.

Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L. and Beyreuther, K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's-disease A4 amyloid protein. *Cell* **57**, 115-126.

Weidemann, A., Paliga, K., Durrwang, U., Czech, C., Evin, G., Masters, C.L. and Beyreuther, K. (1997) Formation of stable complexes between two Alzheimer's disease gene products: presenilin-2 and beta-amyloid precursor protein. *Nature Medicine* **3**, 328-332.

Wertkin, A.M., Turner, R.S., Pleasure, S.J., Golde, T.E., Younkin, S.G., Trojanowski, J.Q. and Lee, V.M.Y. (1993) Human neurons derived from a teratocarcinoma cell-line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 9513-9517.

White, A.R., Zheng, H., Galatis, D., Maher, F., Hesse, L., Multhaup, G., Beyreuther, K., Masters, C.L. and Cappai, R. (1998) Survival of cultured neurons from amyloid precursor protein knock-out mice against Alzheimer's amyloid-beta toxicity and oxidative stress. *Journal Of Neuroscience* **18**, 6207-6217.

Wilcock, G.K., Esiri, M.M., Bowen, D.M. and Smith, C.C.T. (1982) Alzheimer's-disease - correlation of cortical choline-acetyltransferase activity with the severity of dementia and histological abnormalities. *Journal Of The Neurological Sciences* **57**, 407-417.

Wolf, B.A., Wertkin, A.M., Jolly, Y.C., Yasuda, R.P., Wolfe, B.B., Konrad, R.J., Manning, D., Ravi, S., Williamson, J.R. and Lee, V.M.Y. (1995) Muscarinic regulation of Alzheimer's-disease amyloid precursor protein secretion and amyloid beta-protein production in human neuronal NT2N cells. *Journal Of Biological Chemistry* **270**, 4916-4922.

Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J.K., Lacana, E., Sunderland, T., Zhao, B.Y., Kusiak, J.W., Wasco, V. and Dadamio, L. (1996) Participation of presenilin-2 in apoptosis - enhanced basal activity conferred by an Alzheimer mutation. *Science* **274**, 1710-1713.

Yankner, B.A., Dawes, L.R., Fisher, S., Villakomaroff, L., Ostergranite, M.L. and Neve, R.L. (1989) Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's-disease. *Science* **245**, 417-420.

Yankner, B.A., Duffy, L.K. and Kirschner, D.A. (1990) Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science* **250**, 279-282.

Younkin, D.P., Tang, C.M., Hardy, M., Reddy, U.R., Shi, Q.Y., Pleasure, S.J., Lee, V.M.Y. and Pleasure, D. (1993) Inducible expression of neuronal glutamate receptor channels in the NT2 human cell-line. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 2174-2178.

Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D., Levesque, L., Rogaev, E., Xu, D., Liang, Y., Duthie, M., St George-Hyslop, P.H. and Fraser, P.E. (1998) The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains β-catenin. *Journal Of Biological Chemistry* **273**, 16470-16475.

Zeller, M. and Strauss, W.L. (1995) Retinoic acid induces cholinergic differentiation of NTera-2 human embryonal carcinoma-cells. *International Journal Of Developmental Neuroscience* **13**, 437-445.

Zhang, Z., Hartmann, H., Do, V.M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P., De Strooper, B., He, X. and Yankner, B.A. (1998) Destabilization of β-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. *Nature* **395**, 698-702.

Zheng, H., Jiang, M.H., Trumbauer, M.E., Sirinathsinghji, D.J.S., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., Stevens, K.A., Slunt, H.H., Sisodia, S.S., Chen, H.Y. and Vanderploeg, L.H.T. (1995) Beta-amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor-activity. *Cell* **81**, 525-531.

Zheng, H., Jiang, M., Trumbauer, M.E., Hopkins, R., Sirinathsinghji, D.J.S., Stevens, K.A., Conner, M.W., Slunt, H.H., Sisodia, S.S., Chen, H.Y. and Vanderploeg, L.H.T. (1996) Mice deficient for the amyloid precursor protein gene. *Annals Of The New York Academy Of Sciences* **777**, 421-426.

Appendix A - Reagents

The recipes for solutions are presented in the same order as the methods laid out in *Chapter 2*. The index contains names of protocols, as well as individual solutions. All reagents were obtained from Sigma unless otherwise stated.

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0.1 M EDTA, 204

0.1 M Sodium Acetate Buffer (pH 3.5), 205

0.1% Cresyl Fast Violet, 205

0.2 M Phosphate Buffer, 204

100% Deionized Formamide, 203

20x SSC, 203

50x Denhart's Solution, 203

Alkaline Phosphatase Buffer, 202

Anode Buffer (Tricine), 200

ANTIBODY CONCENTRATION ON A PROTEIN G COLUMN, 202

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AraC. *See* Cytosine Arabinoside

BCA PROTEIN ASSAY, 197

BCA Reagent, 197

BCIP. *See* Bromo-chloro-indolyl Phosphate Solution

Binding Buffer, 202

Blotto, 201

Bromo-chloro-indolyl Phosphate Solution, 202

Cathode Buffer (Tricine), 200

cDMEM. *See* Complete DMEM

cDMEM +I, 196

CDS. *See* Cell Dissociation Solution

CELL CULTURE, 194

Cell Dissociation Solution, 195

Complete DMEM, 195

Coomassie Brilliant Blue Stain, 200

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Dulbecco's Modified Eagles Medium, 194

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FDU. *See* Fluorodeoxyuridine

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P/S. *See* Penicillin and Streptomycin

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RA. *See* Retinoic Acid

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SDS-PAGE. *See* SODIUM-DODECYLSULPHATE

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ELECTROPHORESIS
SODIUM-DODECYLSULPHATE
POLYACRYLAMIDE GEL
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Stacking Gel (Tricine), 201
Stacking Gel Buffer, 198

TBS. *See* TRIS Buffered Saline
TBS-Tw. *See* TBS-Tween 20
TBS-Tween 20, 202
TE. *See* Trypsin/Ethylenediaminetetraacetic Acid
Transfer Buffer, 201
TRICINE GELS, 200
TRIS Buffered Saline, 201
Trypsin/Ethylenediaminetetraacetic Acid, 195

U. *See* Uridine
Uridine, 196

WESTERN BLOTTING, 201

CELL CULTURE

All solutions obtained from Gibco Life Sciences

Dulbecco's Modified Eagles Medium (DMEM)

High glucose (4.5 g/l)

Without sodium pyruvate

With "Glutamax II"

Stored at +4°C

Foetal Bovine Serum (FBS)

Heat inactivated at 56°C for 30 minutes

Stored in 25 ml aliquots at -20°C

Penicillin and Streptomycin (P/S)

5000 IU/ml penicillin and 5000 μg/ml streptomycin

Stored in 5 ml aliquots at -20°C

Use at 1:100 dilution to give 50 IU/ml penicillin and 50 μg/ml streptomycin.

Complete DMEM (cDMEM)

500 ml DMEM

50 ml FBS (heat inactivated)

5 ml P/S

Trypsin/Ethylenediaminetetraacetic Acid (EDTA) (TE)

0.5 mg/ml trypsin, 0.2 mg/ml (0.7 mM) EDTA in modified Puck's saline A

Stored in 10 ml aliquots at -20°C .

Cell Dissociation Solution (CDS)(Sigma)

A non-enzymatic salt solution prepared in Hank's balanced salt solution without calcium and magnesium

Retinoic Acid (RA) (Sigma)

Light sensitive, handle in dimmed light and wrap tubes in use in foil.

Stock is 10 mg/ml in dimethyl sulfoxide (DMSO), 1 ml aliquots stored at -70°C.

Prepare working stock by diluting to 3 mg/ml with DMSO (10 mM).

Store working stock wrapped in foil at 4°C.

Use at 1:1000 (10^{-5} M final concentration).

Cytosine Arabinoside (AraC) (Sigma)

Stock is 1 mM, dissolve 1.2 mg in 5 ml dH_2O.

Filter sterilise, store in 150 µl aliquots at -20°C.

Use at 1:1000.

Fluorodeoxyuridine (FDU) (Sigma)

Stock is 1 mM, dissolve 12.3 mg in 50 ml dH_2O .

Filter sterilise, store in 1.5 ml aliquots at -20°C.

Use at 1:100.

Uridine (U) (Sigma)

Stock is 1 mM, dissolve 12.2 mg in 50 ml dH_2O.

Filter sterilise, store in 1.5 ml aliquots at -20°C.

Use at 1:100.

1x cDMEM +I

cDMEM (100 ml)

+ 1 µM AraC (100 µl 1 mM stock)

+ 10 µM FDU (1 ml 1 mM stock)

+ 10 µM U (1 ml 1 mM stock)

Poly-D-Lysine (Sigma)

Stock is 100 µg/ml, dissolve 5 mg in 50 ml sterile dH_2O.

Store in 1.5 ml aliquots at -20°C.

Dilute 1:10 with sterile dH_2O before use.

Matrigel (Collaborative research)

Forms gel when warm, so keep cool. Thaw overnight at +4°C, dispense in 0.5 ml aliquots, store at -20°C.

To use, thaw overnight at +4°C, dilute 1:36 with DMEM, store for up to 2 weeks at +4°C.

PREPARATION OF CELL SOLUBLE AND MEMBRANE PROTEINS

Homogenising Buffer

10 mM TRIS.HCl pH 7.8

1 mM EDTA.Na_2

1 mM Sodium chloride (NaCl)

1 mM Potassium chloride (KCl)

Protease Inhibitors

100 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol. Use at 1:100 (1 mM final concentration)

1 mg / ml leupeptin. Use at 1:100 (10 μg / ml final concentration)

2 mg / ml trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64). Use at 1:100 (20 μg / ml final concentration)

5 mg / ml pepstatin A. Use at 1:500 (10 μg / ml final concentration)

BCA PROTEIN ASSAY

BCA Reagent

20 ml Bicinchoninic acid solution

400 μl 4% Copper Sulphate ($CuSO_4$)

SODIUM-DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Resolving Gel Buffer (4xR Buffer)

1.5M TRIS.HCl pH 8.8

(18.2 g TRIS for 100 ml, + HCl to pH 8.8)

Stacking Gel Buffer (4xS Buffer)

0.5M TRIS.HCl pH 6.8

(6 g TRIS for 100 ml, + HCl to pH 6.8)

Electrode Buffer

6 g TRIS

28.8 g glycine

2 g SDS

2 l dH_2O

Sample Buffer (DTT)

(store at -20°C)

308 mg Dithiothreitol (DTT)

2 ml 10% SDS

3 ml 60% glycerol

5 ml 4x S buffer

0.5 ml 2 mg/ml bromophenol blue

Sample Buffer (2ME)

100 mg SDS

1 ml 2-mercaptoethanol

4 ml 60% glycerol

5 ml 4xS buffer

0.5 ml 2 mg/ml bromophenol blue

Resolving Gel

(recipe for two mini-gels)

4 ml 2x acrylamide solution

2 ml 60% glycerol

2 ml 4xR buffer

80 µl 10% SDS

80 µl 5% N,N,N',N'-tetramethylethylenediamine (TEMED)

80 µl 5% ammonium persulphate (APS)

(Load 3.4 ml per gel, overlay with 160µl dH_2O, allow to polymerise for >1 hour.)

Stacking Gel

(recipe for two mini-gel stacks)

2 ml 8% acrylamide (30% acrylamide in dH2O)

1 ml 60% glycerol

1 ml 4xS buffer

(use 300 µl to wash top of each gel and then add to remaining 3.4 ml:)

34 µl 10 % SDS

34 µl 5% TEMED

34 µl 5% APS

Fix

10% acetic acid, 50% methanol

Coomassie Brilliant Blue (CBB) Stain

0.05% w/v CBB R-250 in 10% acetic acid

(0.15 g CBB, dissolve in 270 ml water by stirring for 1 hour, filter, add 30 ml glacial acetic acid)

Destain

5% acetic acid.

TRICINE GELS

Anode Buffer (upper)

0.2 M TRIS pH 8.9

(24.2 g TRIS for 1 l dH_2O, + HCl to pH 8.9)

Cathode Buffer (lower)

0.1% SDS, 0.1M TRIS, 0.1 M Tricine pH 8.25

(1 g SDS, 12.1 g TRIS, 17.9 g Tricine for 1 l, no need to adjust pH)

Gel Buffer (3x)

3M TRIS pH 8.25

(36.3 g TRIS for 100 ml)

Sample Buffer

Laemmli sample buffer with 0.02% Serva Blue G

(0.5% Serva Blue G in 10% acetic acid, add 1 µl to 25 µl sample buffer)

Resolving Gel

3 ml 3x acrylamide solution

3 ml gel buffer

3.2 g urea + dH_2O to 9 ml (or 3 ml 60% glycerol)

mix

+ 90 µl each 10% SDS, 5% TEMED, 5% APS

Stacking Gel

2 ml 4x acrylamide solution (to give final concentration of 4%)

1 ml 3x gel buffer

1 ml 60% glycerol

mix

+ 40 µl each 10% SDS, 5% TEMED, 5% APS

PROTEIN TRANSFER TO MEMBRANE

Transfer Buffer

10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS) pH 10.3 containing 10% methanol

(2.2 g CAPS.Na dissolved in 900 ml dH_2O, adjust to pH 10.3 with 2 M NaOH, then add 100 ml MeOH, stir for about 10 minutes)

WESTERN BLOTTING

TRIS Buffered Saline (TBS)

15 mM TRIS.HCl pH 7.4

140 mM NaCl

Blotto

TBS with 5% bovine milk protein, 0.05% Tween-20

(2.5 g dried skimmed milk, 50 ml TBS, 250 µl 10% Tween-20)

TBS-Tween 20 (TBS-Tw)

TBS + 0.01% Tween-20 (200 ml TBS + 200 µl 10% Tween-20)

Alkaline Phosphatase (AP) Buffer

100 mM TRIS.HCl

100 mM NaCl

5 mM Magnesium chloride ($MgCl_2$)

pH 9.5

Nitro Blue Tetrazolium (NBT) Solution

0.6 mg/ml in AP buffer

(6 mg NBT, dissolve in 300 µl MeOH, add 9.7 ml AP buffer)

Bromo-chloro-indolyl Phosphate (BCIP) Solution

0.3 mg/ml in AP buffer

IMMUNOPRECIPITATIONS

Immunoprecipitation Buffer

50 mM TRIS.HCl pH 7.5

150 mM Sodium Chloride

1 mM EDTA

ANTIBODY CONCENTRATION ON A PROTEIN G COLUMN

Binding Buffer

0.01 M Sodium Phosphate

0.15 M Sodium Chloride

0.01 M EDTA

IN SITU HYBRIDISATION

DEPC-Water

1 ml Diethyl pyrocarbonate (DEPC)

1 l dH_2O

Combine and leave covered to stand for at least 3 hours

Autoclave to destroy DEPC

20x SSC

175.3 g NaCl

88.2 g Sodium citrate

800 ml DEPC-treated water

pH to 7.0 with 1 M NaOH

Make up to 1 l with DEPC-treated water

50x Denhart's Solution

5 g Ficoll

5 g Polyvinylpyrrolidone (PVP)

5 g Bovine serum albumin (BSA)

500 ml DEPC-treated dH_2O

Filter through 0.2 μm filter, aliquot and store at -20°C

Deionized Formamide

5 g BioRad AG 501-X8

50 ml Formamide

Stir for 30 minutes at room temperature. Filter twice with Whatman No. 1, aliquot and store at -20°C until needed

Denatured Herring/Salmon Sperm

100 mg Herring/salmon sperm DNA

10 ml DEPC-treated water

Stir for 2-4 hours with heating if necessary. Shear by sonification for 60 seconds prior to boiling for 10 minutes. Cool rapidly on ice, aliquot (1 ml) and store at -20°C. Before use, boil for 5 minutes and cool rapidly.

0.1 M EDTA

37.22 g EDTA

800 ml DEPC-treated dH_2O

Stir and add 10 M NaOH to assist dissolution. pH to 8.0 with solid NaOH (approximately 20g).

Aliquot at -20°C

0.2 M Phosphate Buffer

A 27.6 g Sodium dihydrogen orthophosphate (NaH_2PO_4)

1 l DEPC-treated dH_2O

B 28.4 g NaH_2PO_4

1 l DEPC-treated dH_2O

Combine 1 part A to 4 parts B to produce the stock solution; store at 4°C

Hybridisation Buffer

10 ml 20x SSC

1 ml 50x Denhart's solution

0.5 ml 0.5 M EDTA

1 ml Denatured herring/salmon sperm

5 mg yeast tRNA

5 mg PolyA

25 ml Deionized formamide

5 g Dextran sulphate

Make up to 50 ml with 20 mM Phosphate buffer. Aliquot and store at -20°C

0.1 M Sodium Acetate Buffer (pH 3.5)

13 ml 0.1 M sodium acetate

87 ml 0.1 M acetic acid

0.1 % Cresyl Fast Violet

1 g Cresyl fast violet, make up to 1 l with dH_2O.

Appendix B - Neurone Protocol

TO PRODUCE >99% NEURONE CULTURES

Day Number	Example	Procedure
1	Friday	Split confluent T25 of stem cells 1:10, use non-enzymatic means of detaching from flask and replate in T25 flasks. Make up to 5 ml media per flask with cDMEM, add RA to 10 μM.
8, 15, 22	Friday	feed with cDMEM plus 10 μM RA.
25	Monday	split 1:3 - wash with 5 ml DMEM, add 2 ml TE, place in incubator for 5-10 min, inactivate with 8 ml cDMEM. Spin to pellet cells, re-suspend in 12 ml 1X cDMEM + I, transfer to T75.
28	Thursday	feed with 12 ml 1X cDMEM + I
32	Monday	feed with 12 ml cDMEM (no inhibitors)
35	Thursday	feed with 12 ml cDMEM (no inhibitors)
39	Monday	feed with 10 ml cDMEM, dislodge neurones by tapping flask 5 times per side, collect cell suspension and add to coated vessels, do not spin. 1 T75 can be replated to 6 chamber slides or 1 12 well plate or 2 T25 flasks
43	Friday	Feed with cDMEM

Feed every 7 days

NB - Stratagene suggest feeding replated neurone cultures with cDMEM containing fluorodeoxyuridine and uridine to remove any contaminating cells, when I have tried this all cells died, including neurones.

LABELLING

Stem cells are labelled with passage number and date of split.

When set up in retinoic acid each flask is given a code in the form DDMMYYPXX, where DDMMYY is the date, and PXX the passage number of the stem cells. Flasks are labelled "RA".

Appendix C - Original Neurone Protocol

Retinoic Acid Differentiation

Aspirate media from a confluent flask of NT2 stem cells.

Add 2 ml TE and incubate until cells are in suspension, inactivate TE by addition of 10 ml cDMEM.

Spin at 1000 rpm for 10 minutes to pellet cells, aspirate supernatant and resuspend cells in cDMEM containing 10^{-5} M RA.

Perform a cell count and seed cells at a density of 10^6 cells per T75 flask, add cDMEM containing RA to a final volume of 12 ml per T75.

Feed every 7 days with 12 ml cDMEM with RA.

Replate #1

On day 24 after seeding.

Aspirate media from culture flask and add 5 ml TE, incubate until all cells are in suspension.

Inactivate TE by addition of 10 ml cDMEM.

Spin at 1000 rpm for 10 minutes to pellet cells, aspirate supernatant and resuspend cells in 30 ml cDMEM.

Transfer to a T165 flask and return to incubator (1:2 split)

Replate #2

On day 25 after seeding.

Aspirate media from T165 flask and add 2 ml TE.

Observe cells under phase contrast microscope and gently agitate flask to remove neuronal precursor cells, these cells are larger and more phase-bright than non-neuronal cells, and are found on the top of the cell layers.

Once all precursors are in suspension, inactivate trypsin immediately with 10 ml cDMEM.

Combine precursors from 2 or more T165 flasks and spin at 1000 rpm for 10 min to pellet precursors.

Meanwhile re-feed T165 with 30 ml cDMEM and return to incubator.

Resuspend cell pellet in 10 ml cDMEM + I.

Perform cell count and seed cells at a density of approximately 6 x 10^6 cells per T25 flask.

Add cDMEM + I to a total volume of 5 ml per T25 and place in incubator.

Replate #3

On day 26 after seeding.

Repeat protocol for Replate #2, discard T165 flasks after this replate.

Neuronal Purification

Feed neuronal cultures with 5 ml cDMEM + I twice weekly.