Distribution and regulation of proteins related to neuronal degeneration.

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CHAPTER 2
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SUMMARY

1 Using in situ hybridisation, Tau 1 and Tau 2 gene expression has been studied in adult and neonatal rats in the basal forebrain and hippocampal regions. Tau 1 mRNA levels are significantly higher in the neonatal (gestation day 17) rats, with Tau 2 mRNA levels being significantly higher in the adult rats (**Chapter 3**).

2 Using in situ hybridisation, Tau 1 and Tau 2 gene expression in the basal forebrain and hippocampal regions has been studied in adult rats in response to intraperitoneal administration of the following compounds: ondansetron (100ng/kg), MK801 (1mg/kg), GYKI52466 (30mg/kg), indomethacin (5mg/kg) and aniracetam (10mg/kg). None of the compounds cause a significance change in either Tau1 or Tau 2 mRNA levels after a 24 hour period (**Chapter 3**).

3 Glial cell cultures have been used to study the distribution and expression of NADPH diaphorase expression in response to exposure of the cells to cytokines, excitatory amino acid agonists and proteases. The glial cultures have been characterised by positive staining to glial fibrillary acidic protein and NADPH diaphorase. Points 3-5 summarise the work carried out using these cultures (**Chapter 4**). Lipopolysaccharide (100μg/ml) significantly increases NADPH diaphorase staining. A maximum induction is observed after a 6 hour time period. Interleukin-1 (0.25ng/ml) also significantly increases NADPH diaphorase staining, and again maximum induction occurs after a 6 hour time period. The lipopolysaccharide-induced increase in NADPH diaphorase staining remains unaltered following combined exposure of the cells to lipopolysaccharide (100μg/ml) and the calmodulin anatgonist W7 (400μM).

4\ The excitatory amino acid agonists glutamate (50μM), AMPA (50μM) and NMDA (50μM and 100μM) significantly increase NADPH diaphorase staining. This induction occurs after a 24 hour time period. The inhibitors APV (100μM) and CNQX (100μM) reduce the glutamate and AMPA-induced increase in NADPH diaphorase staining to near control values, again after a 24 hour time period. The glutamate-induced increase in NADPH diaphorase staining is reduced following combined exposure of the cells to glutamate (50μM) and the calmodulin antagonist W7 (400μM)

5\ Exposure of the cells to the proteases α-chymotrypsin (300ng/ml) and trypsin (500ng/ml) increase NADPH diaphorase staining. This induction occurs after a 24 hour time period.This increase is reduced to near control levels following combined exposure of the cells to the proteases and their respective inhibitors chymostatin (100μM) and trypsin inhibitor (1μg/ml), again after a 24 hour time period.

6\ Basal forebrain primary cultures have been used to study the distribution and expression of cytoskeletal proteins and NADPH diaphorase staining following exposure of the cells to nitric oxide, excitatory amino acid agonists and cytokines. The cells have been characterised by positive immunostaining to choline acetyltransferase (ChaT), neuron specific enolase, neurofilament and microtubule-associated protein 2 (MAP2). Points 6-10 summarise the work carried out using these cultures (**Chapter 5**).

7\ Nerve growth factor (200ng/ml) significantly increases ChaT and MAP2 immunoreactivity after a 4 day period, with no significant effect on tau immunostaining. MAP2 gene expression can also be induced following 24 hour exposure to nerve growth factor (200ng/ml). Exposure of the cultures to nerve growth factor (200ng/ml) also significantly increases NADPH diaphorase staining,

and neuron specific enolase immunostaining, after a 24 hour time period. Pretreatment with dexamethasone (1μM) reduces the nerve growth factor-induced increase in MAP2 immunoreactivity to near control values, as does the nitric oxide scavenger haemoglobin (20μM). The calmodulin antagonist W7 (400μM) reduces the nerve growth factor-induced increase in MAP2 immunoreactivity to near control values.

8 Glutamate (50μM) increases NADPH diaphorase staining in the neuronal cultures following exposure over a 24 hour time period. W7 does not alter the glutamate-induced increase. The non-NMDA receptor agonist kainate (10μM) significantly increases MAP2 immunoreactivity and MAP2 gene expression after a 24 hour time period. The compound has no significant effects on tau immunoreactivity. The metabotropic receptor agonist ACPD does not alter tau or MAP2 immunoreactivity.

9 The nitric oxide releasing compounds SNP (50μM), SNAP (50μM) and SIN-1 (50μM) significantly increase MAP2 immunoreactivity after a 24 hour time period. The compounds caused no significant effects on tau or NSE immunoreactivity. The increase in MAP2 immunoreactivity is reduced to near control values following combined exposure of the cells to SNAP (50μM) and haemoglobin (20μM), and, is mimicked by the cyclicGMP analogue 8bromocyclicGMP (20μM). Neither nerve growth factor (200ng/ml) or the nitric oxide releasers cause any detectable effect on neuronal cell morphology.

10 Exposure of the cells to lipopolysaccharide (100μg/ml) and interleukin-1 (0.25ng/ml) causes no significant effect on amyloid precursor protein or cyclooxygenase immunoreactivity after a 24 hour time period.

11 Studies of brain sections taken form two transgenic mice models models had enabled a preliminary investigation into the possible role(s) of amyloid precursor protein within the CNS. Complete removal of the amyloid precursor protein gene results in a dramatic onset of reactive gliosis in mice at 6 months of age compared to age matched control animals. At 13 months of age, the age matched control animals possess a significantly higher level of reactive astrocytes. The calcium-binding protein calbindin is significantly increased in the amyloid precursor protein-null mice at 13 months of age compared to age matched controls, and levels of synaptophysin and MAP2 remain unaltered in the null mice compared to age matched controls. A study of presenilin-1 expression and distribution in the null and wildtype animals reveals membrane bound immunoreactivity using an antibody directed against the N terminus. Immunoreactivity using an antibody directed against the C terminus reveals a cytoplasmic distribution. No difference in presenilin-1 levels occur in the null mice compared to the age matched controls in cortical and hippocampal regions. There is the possibility that the null mice at 6 months of age have an increase in cerebellar presenilin-1 levels compared to age matched control animals. Animals with a mutated α-secretase site on amyloid precursor protein display a marked increase in reactive gliosis at 6 months of age compared to age matched control animals (**Chapter 6**).

12 An initial study of postmortem brain tissue from Alzheimer's Disease patient and age matched control reveals a dramatic increase in reactive gliosis and AT8 immunostaining within the diseased tissue. A significant level of colocalisation between plaques and tangles and presenilin-1 appears in some areas of diseased brain tissue (**Chapter 7**).

Chapter 1

INTRODUCTION.

1.1 Alzheimer's Disease

Alzheimer's Disease was first reported in 1907 by Alois Alzheimer (Alzheimer., 1907). It is now recognised as the fourth leading cause of death in the developed world with an estimated 17-25 million sufferers world-wide. Its profound morbidity has stimulated research into therapeutic strategies aimed at alleviation and ultimately prevention of the disorder. At autopsy, the Alzheimer's Disease brain is characterised by a number of important pathological changes. First, there is a dramatic loss of neurons and synapses in many areas of the central nervous system. Researchers have found a loss of brain tissue in patients to be 30 times as fast as age matched controls. This tissue loss is thought to start in the regions involving higher cognitive function such as the hippocampus, and the disease can be clearly seen as a qualitatively different process from normal ageing (Simic et al.,1997). Secondly, many neurotransmitters are reduced including acetylcholine, noradrenalin, serotonin, dopamine, glutamate and substance P (Davis et al.,1980; Iversen et al.,1983; Kowall and Beal.,1988). This dramatic and global reduction of many neurotransmitters and the profound tissue loss accounts for the broad clinical manifestations of the disease that include memory impairment, hallucinations, paranoia, restlessness, depression, aggression, aphasia, apraxia and personality disorders. Molecular and biochemical techniques as well as the recent development of animal models have contributed greatly in the search for understanding of the pathological processes in the disease. There is now strong reason to hope that drugs directed at the disease neuropathology will be able to greatly reduce or halt the cognitive decline and suffering of the Alzheimer's Disease patient.

1.2 Neuropathology of Alzheimer's Disease

Unique pathological lesions are found in the brains of Alzheimer's Disease patients. Specifically, two microscopic deposits originally described by Alois Alzheimer in 1907 are observed: the neurofibrillary tangle and the senile plaque. As will be discussed in more detail in Section 1.3.1, the classic senile plaque of Alzheimer's Disease is a complex lesion of the cortical neuropil containing several

abnormal elements. It consists of a central deposit of extracellular amyloid fibrils (the core) surrounded by dystrophic neurites, activated microglia and fibrillary astrocytes. As will also be discussed in more detail in Section 1.5.2, the principal structural feature of tangles is the paired helical filament which is comprised mainly of abnormally phosphorylated isoforms of tau protein. Neurofibrillary tangle (NFT) formation and synaptic loss have been correlated with the degree of cognitive deficits in the disease (Arriagada et al.,1992; DeKosky et al.,1990).

The regions most affected by neuron loss, synaptic dysfuntion and, neurofibrillary tangle (NFT) and plaque formation are the basal forebrain cholinergic system, amygdala, hippocampus and neocortex. Dysfunction and death of these neurons leads to reduction in synaptic inputs and deafferentation of targets predominantly in the amygdala, hippocampus and neocortex (Whitehouse et al.,1982; Arnold et al.,1991; Terry et al.,1991). The basal nucleus of Meynert and the diagonal band nucleus provide the major source of cholinergic input to the cerebral cortex and hippocampus, and a reduction in the number of cholinergic neurons in these structures by two thirds has been reported (Arendt et al.,1985; Nagai et al., 1983; Lehman et al.,1980).

The degeneration process in Alzheimer's Disease is a complicated pathway. Over the last ten years, many key experiments have helped to unfold some of the complexities underlying the disease, and there is much scope for further research.

1.3 The amyloid plaque

1.3.1 Introduction

The senile plaque first described by Alois Alzheimer was isolated by Glenner and coworkers (Glenner and Wong., 1984). They found it to be composed of a 4kDa peptide that aggregates into a fibrillar β-pleated structure, and named this peptide amyloid. Amyloid peptide (Aβ) is a 40-42 amino acid residue protein. Aggregated Aβ deposits appear green under polarised or fluorescent light after staining with Congo red. It is this classic staining that has come to characterise amyloid deposits. It has been known since 1968 that the density of senile plaques found in Alzheimer's Diseased brains correlates with the severity of clinical dementia (Blessed et al., 1968). Senile plaques are typically 50μm in diameter and possess a central deposit of extracellular amyloid fibrils (the core) which is surrounded by dystrophic neurites, activated microglia and reactive astrocytes. Two types of plaque have been described. The classic senile or neuritic plaques consist of fibrillar, congophilic Aβ deposits that are associated with dystrophic and degenerating neurites. Due to their β-pleated nature, these plaques stain with Congo red or thioflavin. The second type of plaque are the diffuse or preamyloid plaques. These consist of amorphous, noncongophilic Aβ deposits that are not associated with significant neuronal pathology.

Aβ has been hypothesised to be causally related to Alzheimer's Disease. The 'Aβ hypothesis' states that excess deposition of Aβ in the brain somehow can lead to neurodegenerative changes and eventually to dementia. The evidence is as follows. First, Aβ deposition is the earliest neuropathological marker in Alzheimer's Disease, and also in related disorders such as Down's Syndrome, where it can precede neurofibrillary tangle formation by two to three decades (Mann et al., 1992). Aβ deposition is relatively specific to Alzheimer's and related disorders (Selkoe et al., 1993). Aβ is toxic to cultured neurons and cell lines (Mattson .,1992; Yankner et al.,1990). Finally, as is discussed in Section 1.4.6, mutations in the Aβ precursor APP gene cause early onset familial Alzheimer's Disease. Collectively, these facts strongly implicate Aβ deposition in the neuropathology of the disease. However, there has been much research into the time course of events

that occur in Alzheimer's Disease and a key question is which process triggers the Alzheimer's cascade of events. It has also been argued that only a small percentage of Alzheimer's Disease cases resulting from mutations of APP are attributable to excess Aβ production (Cai et al.,1993; Felsenstein et al.,1993).

1.3.2 Aβ production and aggregation

The production of Aβ is discussed in detail in Section 1.4.4; Aβ has been shown to be a product of normal cellular metabolism and has been detected in media from a wide variety of cultured cells as well as in human cerebrospinal fluid (Haass et al.,1992; Seubert et al.,1992; Shoji et al.,1992; Busciglio et al.,1993). It is possible that abnormal accumulation of Aβ in Alzheimer's Disease results from events that slow down its degradation and removal. The normal mechanism(s) of degradation is not known, although it is thought that proteolysis by extracellular enzymes and /or catabolism following cellular uptake are likely candidates. One possible reason that amyloid is so robust in plaques is that it is resistant to enzymatic or cellular degradation. In Alzheimer's Disease, an altered balance may occur, whereby the ratio of Aβ degradation to synthesis is altered as a result of enhanced Aβ release or decreased Aβ removal.

Aβ aggregates to form insoluble, fibrillar extracellular deposits, the main constituent of plaques. Aβ toxicity requires the peptide to be present in the form of an amyloid aggregate. Indeed, amyloid is a generic term used to describe a class of otherwise unrelated peptides which under physiological conditions aggregate to form insoluble filaments. Many studies have investigated the mechanisms of and factors affecting aggregation, and I will discuss some but not all of the work. Fibril formation has been described as a build up of a one-dimensional crystal with non-uniform growth occurring in planes perpendicular to the axis of elongation (Lansbury et al., 1992). The C-terminus of the amyloid protein has been thought to be crucial to aggregation. Neuritic and cerebrovascular plaques are composed of variants of Aβ differing at their C-termini (Joachim et al.,1988; Prelli et al.,1988). Neuritic plaques contain mainly β(1-42) (Roher et al.,1993). The following are some of the elements thought to effect A β aggregation. First, the aggregation rates and fibril morphologies of

Aβ and related peptides in vitro are thought to be pH-dependent (Terse et al.,1994). Secondly, metal ions have been studied. Ever since the observation that aluminium is present as aluminosilicates in plaques and tangles, (Candy et al.,1986), exposure to the metal has been considered a risk factor for the disease. Assays in vitro have demonstrated that aluminium can cause Aβ(1-40) to aggregate (Kawahara et al.,1994). Finally, free radicals and other covalent modifications of Aβ have been implicated in its aggregation process.

1.3.3 **Inflammatory mechanisms and Aβ**

In addition to the classical neuronal pathology associated with Alzheimer's Disease, there is growing evidence that chronic inflammatory processes contribute to the pathogenesis of the disease. A variety of inflammatory proteins have been found in Alzheimer's post mortem tissue. Numerous immunohistochemical studies have identified proinflammatory cytokines such as interleukin-1 and interleukin-6 (Sheng et al.,1995), complement proteins such as C1q and C4 (Ishii et al.,1992), and acute phase proteins such as α1-chymotrypsin (Abraham et al.,1988) in senile plaques. Evidence suggests that immobilised Aβ can activate the classical complement cascade by an immunoglobulin-dependent pathway through binding to C1q, the first component of the C1 complex(Rogers et al.,1992). Rogers and coworkers also suggested that the Aβ-C1q binding interactions require aggregated rather than monomeric Aβ.

Additional evidence to support a chronic inflammatory state associated with Alzheimer's Disease is the finding that there is an unexpectedly low prevalence of the disease in patients with rheumatoid arthritis (McGeer et al.,1992). This is discussed in more detail in Section 1.10.1.

1.3.4 **Proteins associated with Aβ**

Another possible explanation for the persistence of Aβ within plaques is its association with other plaque components. Cells found in the vicinity of senile deposits release factors such as proteoglycans (Snow et al.,1988; Su et al.,1992), α-chymotrypsin (Pasternack et al.,1989) and apolipoprotein E (Strittmatter et al.,1994; Strittmatter et

al.,1995) that can bind to Aβ. Interestingly, proteoglycans may be of particular relevance to Aβ accumulation since these glycoconjucates are an invariable feature of all amyloidogenic deposits (Snow et al.,1989). Recent work shows that Aβ can be catabolized by microglia and proteoglycans which co-localize with senile plaques and may slow down the degradation of Aβ (Shaffer et al.,1995).

1.4. **Amyloid precursor protein**

1.4.1 **Introduction**

Senile plaques can be found in the brain of normal elderly patients but accumulate greatly in subjects with Alzheimer's Disease. This extracellular deposit is composed mainly of Aβ. As mentioned previously, Aβ is derived from a precursor, the amyloid precursor protein (APP). APP possesses the structure of a transmembrane protein, with the Aβ sequence partly embedded in the membrane domain. The structural organisation of APP is shown in Figure 1.

1.4.2**IsoformsofAPP**

Several APP isoforms can be generated by alternative splicing of a single gene located on chromosome 21. The three major isoforms of APP are those of 695, 751 and 770 amino acids. The 751 and 770 isoforms contain an N-terminal Kunitz protease inhibitor domain and are expressed in both neural and non-neural tissues (Kitaguchi et al ., 1988; Ponte et al., 1988; Tanzi et al., 1988). The 695 amino acid isoform is expressed at high levels in neurons, where it undergoes retrograde and anterograde axonal transport, and transcytosis (Koo et al,. 1990; Yamazaki et al., 1995).

After synthesis, APP undergoes post-translational modifications that include signal peptide removal, N-glycosylation, sulphation of tyrosine residues, phosphorylation and proteolytic cleavage (Ghiso et al.,1989; Buxbaum et al.,1990; Oltersdorf et al., 1990; Dyrks et al., 1988). The cleavage processes are discussed in further detail in section 1.4.4.

1.4.3 **APP-like proteins**

Two other mammalian proteins that are highly homologous to APP have been cloned: APLP1 and APLP2 (Wasco et al., 1992; Slunt etal., 1994). This suggests that APP is a member of a multigene family.

Several lines of evidence suggest that APP and its metabolism play an important role in the pathogenesis of Alzheimer's Disease, particularly the occurrence of familial forms of Alzheimer's Disease that are linked to mutations in the APP gene located on chromosome 21, (Clark and Goate.,1993), and to a lesser extent, the fact that Down's Syndrome , which is due to trisomy 21, comprises Alzheimer's-like pathology including the deposition of Aβ.Cells which produce a mutant from of APP (APPΔNL) , recently linked to familial Alzheimer's Disease, secrete six times more βA4 into the medium than wildtype cells (1993, Citron et al., 1992). It has also been demonstrated that cells producing a mutant APP (mutant form of APP717 protein) release a larger proportion of the longer from of Aβ (41 amino acids) which possesses a greater ability to form amyloid fibrils than the shorter form (Suzuki et al., 1994). It has also been shown that some forms of familial Alzheimer's Disease are linked to a variety of these genes and these are discussed in Section 1.9.

1.4.4 **Aβ generation from APP**

APP is the only member of its gene family that contains the Aβ domain and can thus give rise to the amyloidogenic derivative. The precise mechanism of Aβ generation has been the focus of many studies and it is thought that several pathways exist. The first pathway results in the generation of non-amyloidogenic products. As shown in Figure 1, several cleavages can occur in the vicinity of the Aβ sequence. The breakdown elicited by α-secretase occurs within the A β sequence thus preventing the formation of amyloidogenic products.This enzyme generates an N-terminal fragment APP and its C-terminal counterpart of 10-11kDa which remains embedded in the membrane (Esch et al., 1990;Anderson et al., 1991; Sisodia et al., 1992; Seubert et al., 1993). The 10-11kDa counterpart (sometimes referred to as p10) may undergo an additional cleavage leading to the formation of p3 and its complementary product p7 (Haass et al., 1992; Busciglio et al., 1993). Thus the α-secretase pathway precludes the formation of APP derivatives bearing potentially amyloidogenic properties. It should be noted at this stage that other minor non-amyloidogenic cleavages have been described such as that reported in monkey fibroblast CV-1 cells (Zhong et al., 1994).

All of the potentially amyloidogenic products generated from APP contain the full Aβ sequence. Two main pathways generate amyloidogenic products. The first involves a β secretase enzyme. This generates C-terminal products that contain the whole Aβ sequence near or at their N-terminus (Haass et al., 1992). The N-terminal fragment that derives from β secretase cleavage has been detected as a secretion product of human mixed brain cells (Seubert et al., 1993). The second amyloidogenic-generating pathway involves γ secretase, and results in the production of APP species lengthened at their C-terminus by Aβ. This pathway has been demonstrated in neuronal PC12 cells and in human kidney cells (Abderson et al., 1994). However, this generation has been debated as a study performed with control and Alzheimer's Disease brains failed to detect such secretory soluble derivatives containing a complete C-terminal Aβ sequence (Pasternack et al., 1992). The formation of Aβ requires the sequential action of β and γ secretases.

Several studies have looked at possible candidates for the secretases involved in Aβ production. Cathepsins G and B have been postulated as β secretase candidates (Sahasrabudhe et al., 1993; Dreyer et al., 1994). In addition, Sahasrabudhe and coworkers (Sahasrabudhe et al.,1994) also demonstrated that α-chymotrypsin could display β secretase-like activity. Schonlein and coworkers have reported on the purification and characterisation of a metalloprotease from human brain (Schonlein et al.,1994). The multicatalytic proteinase complex appears to display a β secretase-like activity (Ishiura et al., 1989) but can also hydrolyze model peptides thought to behave as γ secretase substrates (Mundy et al., 1994).

Two more possible pathways of Aβ generation exist. In the endosomal-lysosomal pathway, some cell surface APPs are reinternalised (Haass et al., 1992), and cleaved at the N-terminus of the βA4 sequence by β secretase to produce potentially amyloidogenic C-terminal fragments (Golde et al., 1992), and subsequently cleaved by χ secretase to release soluble 4kDa Aβ (Koo et al., 1994). The identification of APP within clathrin coated vehicles in PC12 cells is consistent with utilisation of this pathway (Nordstedt et al., 1993). However, it is unclear whether a direct targeting pathway to the endosomal/lysosomal system without trafficking via the cell surface exists (Sinha et al., 1992). It has recently been shown that signals

within the cytoplasmic domain mediate APP internalisation (Koo et al., 1994) In addition to this reinternalised pathway, a pathway that targets APP from the trans-Golgi network directly to endosomes and lysosomes is thought to exist (Haass et al., 1993).

1.4.5 **Physiological roles of APP**

The physiological role of APP is not well understood, despite many studies. The secreted form of APP (APPs) can function as an autocrine factor to stimulate cell proliferation (Saitoh et al., 1989), and can promote cell-substratum adhesion possibly through an interaction with laminin (Kibbey et al., 1993). APPs also appears to mediate the neurite outgrowth response of PC12 cells to nerve growth factor (Milward et al., 1992). APPs containing a region homologous to the Kunitz type protease inhibitor sequence are thought to have a role in the regulation of extracellular protease activity (Oltersdorf et al., 1989) and are endocytosed by the low density lipoprotein receptor protein (Kounnas et al., 1995). A possible in vivo function of APP has been suggested by the discovery that APP is a very potent inhibitor of the complexes involved in the regulation of the coagulation cascade (Smith et al., 1990, Van Nostrand et al., 1990a; Van Nostrand et al.,1990b). The regulation of APP binding to collagen type1 by heparin and the mapping of the binding sites of APP to collagen type1 has recently been demonstrated (Beher et al., 1996).

1.4.6 **APP mutations**

Many interesting studies have been carried out investigating the effects of mutation to the APP gene on the processing of Aβ. Several familial forms of Alzheimer's Disease have been described that are linked to various mutations located on the APP gene, and these are discussed in Section 1.9. A vast literature covers the effects of APP mutations, most notably a Swedish kindred develops a Alzheimer's Disease phenotype that results in over production of Aβ and a decreased production of non amyloidogenic p3 (Citron et al.,1992). Several mutations thought to be responsible for early onset familial Alzheimer's Disease have been identified on APP near the C terminal end of the Aβ sequence. However, these mutations are not thought to

greatly alter the overall production of Aβ (Tanzi et al.,1991a; Tanzi et al.,1991b; Mullan and Crawford., 1993).

1.4.7 **Transgenic models**

Alzheimer's Disease is a uniquely human disease and for this reason the lack of an animal model has hampered research. Recently, several transgenic animal models with mutated APP have been developed and these are discussed in more detail in Chapter 6.

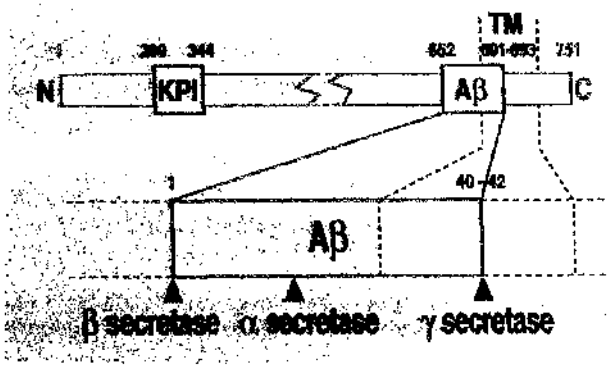


Figure 1 **The structural organisation of APP.**

The drawing represents the structural organisation of APP_{751}. Kunitz protease inhibitor (KPI) and Aβ sequences are boxed. Dashed lines represent the transmembrane (TM).

Illustration taken from Checler., 1995, Figure 1.

1.5 Microtubule-Associated Proteins

1.5.1 Introduction

Brain microtubules contain a variety of microtubule-associated proteins (MAPs) and tubulin. The posttranslational modifications of both tubulin and MAPs are believed to be important in the regulation of assembly of microtubules (Goedert et al.,1993). MAPs are characterised by their ability to promote the assembly and stability of microtubules in axons and dendrites, suggesting a major role in the determination of neuronal morphology and plasticity. Microtubules undergo extensive growth and rearrangement in nerve cells, especially during periods of neurite outgrowth. Several brain MAPs have been identified that differ in their composition and activity. The two major MAPs are MAP2 and tau. MAP2 consists of high molecular weight species (MAP2a and MAP2b) and a lower molecular weight species (MAP2c). The family of low molecular weight MAPs is represented by six isoforms of tau. MAP2 is discussed in detail later. It is worth also mentioning two other structural MAPs :MAP1b and MAP4. MAP1b is believed to cross link microtubules (Noble et al.,1989). Glia also express MAP1b (Diaz-Nido et al.,1989). MAP4 is abundant in a variety of cells including oligodendrocytes (Parysek et al.,1984) and contains microtubule binding domains homologous to those of MAP2 and tau (Olmsted et al.,1986).

1.5.2 Tau Protein

Neurofibrillary tangles (NFTs) and senile plaques are the characteristic hallmarks of Alzheimer's Disease. NFTs constitute the intraneuronal deposits. They are found in the cell bodies and apical dendrites, and in the abnormal neurites that are associated with amyloid plaques. NFTs develop in those nerve cells that undergo degeneration in Alzheimer's Disease. These lesions contain abnormal paired helical filaments (PHFs) as their main fibrous component. PHFs consist of two strands of subunits which twist around each other in a helical fashion. Their insolubility enables them to survive the deaths of the affected cells and remain as 'ghost tangles' that accumulate in the neuropil. These are then engulfed by astrocytes and degraded. Using

immunochemical and biochemical methods it has been shown that NFTs are composed of the microtubule-associated protein tau in an abnormal state of phosphorylation (Delacourte et al.,1986; Goedert et al.,1988; Grundke-Iqbal et al.,1986a; Grundke-Iqbal et al.,1986b; Kosik et al.,1986; Wischik et al.,1988).

Tau is thought to be one of the earliest markers of Alzheimer's Disease. Tau protein in the disease is distinct from normal brain tau in the following ways: it is aggregated into PHFs which colocalise into NFTs. These can be visualised in postmortem tissue immunohistochemically. Tau in the disease is modified in several ways e.g. by phosphorylation, proteolysis and ubiquitination. During the disease process, abnormal tau is thought to spread in a characteristic spatial and temporal sequence, starting in selected neurons in the entorhinal region. It has recently been suggested that senile plaques contain a binding site for tau that is related to its interaction in promoting Aβ aggregation (Smith et al.,1995).

Tau proteins can bind to tubulin and have been shown to play a role in the stabilisation of microtubules in neurons (Matus., 1994). This ability to interact with microtubules is modulated by their phosphorylation state as will be discussed later. Tau has been shown to be capable of influencing the following: net microtubule assembly, the rate of neurite elongation and neuritic stability (Esmaeli-Azad ertal.,1994). Tau has been shown to increase the rate of polymerisation of individual microtubules and slows their rate of depolymerisation (Drechel et al.,1992). Microtubules stabilised by tau, MAP2c or MAP1b are more stable in response to depolymerising agents (Takemura et al.,1992). Projections of certain MAPs may restrict the flexibility of microtubules (Matus., 1994), and projections of certain MAPs from the microtubule surface may promote bundling (Kanai et al.,1989).

1.5.2.1 **Isoforms of Tau protein**

Tau is present throughout the nervous system (Binder et al.,1985). Early studies reported that tau in the adult brain consisted of four to six polypeptides (Cleveland et al.,1977) , while in the foetus, only one tau protein band was found (Couchie et al.,1985). In 1988, Goedert and colleagues identified a 352-amino acid residue of

human tau (Goedert et al.,1988). At this point, all previously reported tau sequences contained, in their carboxy-terminal halves, a characteristic region of three tandem repeats of 31 or 32 amino acids, each displaying a Pro-Gly-Gly-Gly motif. It was proposed that these repeat regions represented the tubulin-binding region of tau (Lee et al.,1991). Goedert and colleagues then isolated cDNA clones encoding an isoform of tau differing from the previously reported tau sequences by the presence of an additional repeat of 31 amino acids giving four rather than three repeat regions in its carboxy-terminal half (Goedert et al.,1989). Goedert and colleagues developed probes derived from cDNA clones encoding the three (named tau type 1) and four (named tau type 2) repeat tau isoforms. They found mRNA recognised by both types of probe in all areas of the human adult brain examined. However, type 1 was abundant in the fetal brain, with type 2 virtually undetectable. Type 1 and type 2 mRNAs were detected in pyramidal cells in the cerebral cortex. In the hippocampus, type1 mRNA was found in pyramidal and granule cells while type 2 was detected in most but not all of the pyramidal cells and absent from the granule cells. These results proved that the expression of tau protein mRNA is both stage and cell specific.

In further studies, Goedert and colleagues reported the sequences of additional isoforms of human tau protein, which differed from the previous forms by the presence of either zero, one or two insertions of 29 amino acids in their amino-terminal region. Full length cDNA clones showed that the insertions occurred in combination with the three and four tandem repeat regions (Goedert et al.,1989). Together with the two isoforms previously described, this gave a total of six human tau isoforms ranging between 352 and 441 amino acids in length, and derived from a single gene by alternative splicing (Goedert et al, 1989). The present nomenclature for the six isoforms of human tau is as follows: three (τ3, τ3S, τ3L) or four (τ4, τ4S, τ4L) tandem repeat regions and the presence of zero (τ3 and τ4), one (τ3S and τ4S) or two (τ3L and τ4L) N-terminal 29 amino acid inserts (Singh et al.,1996). All six isoforms have been reported to be present in a hyperphosphorylated state in PHFs (Grundke-Iqbal et al.,1986; Kopke et al.,1993; Goedert et al.,1992).

The functional significance of having six different isoforms is still unclear. Differences in the distribution between white and grey

matter and ability to aggregate (Butler et al.,1986) have been reported. The molecular geneity of tau is thought to be due to both mRNA processing and post-translational modifcation (Butler at al.,1986).

1.5.2.2 **Tau Localisation**

In the normal brain, tau is localised in the axons and is thus more abundant in white matter than in grey matter (Matus et al.,1981; Binder et al.,1985). In Alzheimer's Disease, and under conditions of neurodegenerative disorder, phosphorylated tau-species are also found in the somatodendritic compartment (Kowall et al.,1987). In a recent study, Janke and colleagues found tau isoforms differing in their tubulin binding characteristics are differentially distributed in subcellular neuronal compartments (Janke et al.,1996).

1.5.2.3 **Tau Phosphorylation**

The phosphorylation state of tau changes during neurodevelopment and highly phosphorylated tau accumulates in the PHFs of Alzheimer's Disease. Normal fetal tau is also phosphorylated and shares common epitopes with PHF tau (Goedert et al.,1989; Kosik et al.,1989). Tau has been shown to be abnormally phosphorylated and aggregated into PHFs in affected neurons (Grundke-Iqbal et al.,1986a and 1986b; Iqbal et al.,1989; Lee et al 1991). The abnormal phosphorylation of tau results in the loss of its microtubule assembly and stability activity (Iqbal et al.,1986; Alonso et al.,1994). Abnormal phosphorylation of tau also results in PHF resistance to proteolysis (Grundke-Iqbal et al.,1988). It is thought that in Alzheimer's Disease, a reactivation of the fetal state of tau protein occurs in selected neurons (Kondratick and Vandre., 1996).

Phosphorylation Sites:

Studies have identified a number of specific phosphorylation sites in normal human fetal and adult tau as well as in PHF-tau isolated from postmortem brain samples (Hasagewa et al.,1992; Watanabe et al.,1993). These initial studies showed that the following residues are phosphorylated in PHF tau and fetal tau, but not in

normal adult tau: Thr-181, Ser-199, Ser-202, Thr-231, Ser-235, Ser-236, Ser-396 and Ser-404. In another set of studies, with the exception of Ser-202 and Ser-396, these sites were found to be partially phosphorylated in normal adult tau (Goedert et al.,1994). Goedert and colleagues also showed that in PHF tau these sites are abnormally phosphorylated, and is hyperphosphorylated. Hyperphosphorylated tau , i.e that which is fully phosphorylated at Ser-202 and Ser-396, is also known as A68 (Goedert et al.,1993). It has been shown that many of the sites are phosphorylated in tau proteins in the fetal brain, but that fetal tau proteins are preferentially dephosphorylated shortly after birth and remain dephosphorylated even when fetal tau is converted into adult tau (Kanemaru et al.,1992 and Bramblett et al.,1993). In developing rat brain, it has been shown that fetal tau is phosphorylated from embryonic day 18 until postnatal day 11. After this time, the level and phosphorylation state of tau dramatically decreases and five adult isoforms appear (Mawal-Dewan et al.,1994). It should be noted that since these and other studies investigating phosphorylation sites in human brain tau are done using postmortem brain slices, there is the risk that rapid dephosphorylation of tau by phosphatases that remain active during the postmortem interval may have occurred. It is now thought that 21 different phosphorylation sites exist in PHF tau (Morishima-Kawashima et al.,1995).

A clear difference in distribution between tau proteins phosphorylated to different states has been seen in cultured rat neurons. Tau proteins phosphorylated at Ser-202 (recognised by the antibody AT8) were found concentrated in the axonal portion that is close to the cell body, while tau proteins that carry an epitope that contains dephosphorylated Ser-202 (recognised by an antibody to Tau1), are enriched in the distal axon and the growth cone (Rebhan et al.,1995). It should be noted at this stage that the rat brain is especially useful for studies on the regulation of tau phosphorylation during development, because regions of the tau molecule are 100% conserved between rats and humans, and because the development of human and rat tau isoforms are very similar (Brion et al.,1993 and Kosik et al.,1989).

Phosphatases/kinases

The finding that PHF-tau is hyperphosphorylated almost equally at Ser/Thr-Pro and Ser/Thr-nonPro sites suggests that proline-directed protein kinases, (PDPKs), and non-proline-directed protein kinases,(non-PDPKs), are involved in the hyperphosphorylation process (Morishima-Kawashima et al.,1995). Several kinases have been shown to phosphorylate tau in vitro, and other studies have shown that protein phosphatases are able to dephosphorylate tau in vitro. An understanding of how the phosphorylation and dephosphorylation of tau is regulated will provide important insights into the pathogenesis of NFTs.

The PDPKs glycogen synthase kinase 3α and 3β have been shown to phosphorylate tau at Ser 202 and Ser-396 (Latimer et al.,1995; Lovestone et al.,1994), and these findings have implicated glycogen synthase kinase (GSK), a neuronal kinase, as being important in the regulation of normal cytoskeletal structure and function and possibly in the neurodegeneration of Alzheimer's Disease. It has recently been demonstrated that, in intact mammalian cells, tau highly phosphorylated in the presence of GSK loses the property of microtubule stabilisation. This suggests that regulation of tau phosphorylation by this enzyme may be important in cytoskeletal modulation during neurodevelopment, and this effect may be lost in neurodegeneration (Lovestone et al.,1996). Other kinases shown to phosphorylate tau at various sites are the PDPKs MAP kinase, cdk5 and cdk2 kinases. (Goedert et al.,1994). The non-PDPKs shown to phosphorylate tau include A-kinase, CaM kinase 2 and CK-1 (Singh et al.,1995; Singh et al.,1996). It has recently been suggested that in tau hyperphosphorylation in Alzheimer's Disease brain, cdk5-catalyzed phosphorylation may serve to upregulate the activities of c-kinase and CK-1 (Sengupta et al.,1997).

Arendt and colleagues have shown that chronic infusion into rat brain of okadaic acid, a specific inhibitor of serine/threonine protein phosphatases 1 and 2A, results in memory impairment accompanied by a PHF-like phosphorylation of tau preen and the formation of Aβ-containing structures in the grey and white matters (Arendt et al.,1995). It has been suggested that a defect in protein phosphatase could be the cause of the abnormal hyperphosphorylation of tau. Gong and coworkers found that Alzheimer's Disease phosphorylated

tau in brain extracts was mainly dephosphorylated by protein phosphatase 2A and 2B and to a lesser extent by protein phosphatase 1, and that this tau phosphatase activity was significantly reduced in the disease (Gong et al.,1995). It has recently been shown that in neurons, phosphorylase kinase may be one of the kinases that participate in the phosphorylation of tau (Paudel.,1997).

It has been suggested that the different tau isoforms may be present at different states of phosphorylation in PHFs (Singh et al.,1996). It is still unknown whether the 6 tau isoforms possess all of the 21 possible phosphorylation sites, and how differently phosphorylated isoforms are aggregated to form the PHF structure.

Implications

The path of events in Alzheimer's Disease is still unclear, i.e. whether tau is first hyperphosphorylated, which induces its dissociation from microtubules and leads to their collapse, or whether microtubule collapse initiates a newly dissociated tau which then becomes hyperphosphorylated. An imbalance between kinases and phosphatases could result in the hyperphosphorylation of tau protein. As discussed earlier, phosphorylation of tau has been shown to prevent proper association of the protein with microtubule. However, it has also been shown that rapid hyperphosphorylation of tau occurs after colchicine-induced microtubule collapse (Mattson et al.,1990). The full cycle of events remains to be elucidated.

1.5.3 **MAP2**

MAP2 and tau contain common sequences in tubulin binding sites, and interact with the C terminal of tubulin that is on the surface of the tubules (Serrano et al.,1984). As well as MAP2 and tau, three other structural MAPs exist. These are MAP1a and 1b, and MAP4, which is an isoform of MAP2c (Lewis et al.,1988; Doll et al.,1993; Kindler et al.,1990). These variants arise by alternative splicing from the same gene (Kindler et al.,1990; Goedert et al.,1991). MAP2c consists of the N and C terminal domains of MAP (2a/b) joined together and lacking the intervening sequence. The C-terminal segment contains a proline-rich region followed by three or four conserved microtubule binding regions. MAP2c is expressed in

developing mammalian brain and is down regulated during brain maturation. It has recently been shown that MAP2c is involved in functionally specific interactions with microtubules and microfilaments (Cunningham et al.,1997). MAP(2a/b) is more prominent in the adult brain (Riederer and Matus.,1985). MAP2c has a widespread distribution in the brain being found in neuronal cell bodies, dendrite, axons and glial cells (Tucker et al.,1990). In contrast, MAP2(a/b) is selectively localised in the dendrites and neuronal cell bodies (Caceres et al.,1984; Huber et al.,1984). In addition to its well known association with microtubules, MAP2 is also colocalised with actin, microfilaments, membrane organelles and dendritic spines (Caceres et al.,1983; Morales et al.,1989). Recently a novel low molecular weight isoform of MAP2c has been discovered. It has been detected in cell cultures containing glial cells but the specific cell types that express it have yet to be elucidated (Ferhat et al.,1994; Doll et al.,1993). In this study MAP2(a/b) is refered to as MAP2.

1.5.3.1 *MAP2 Phosphorylation*

MAP2 is phosphorylated by MAP kinase. Part of the phosphorylation occurs at the C-terminal of the molecule, which contains the microtubule binding domain. Phosphorylation of MAP2 has been shown to cause microtubule disassembly (Burns et al.,1988; Murphy et al.,1983). Completely dephosphorylated MAP2 cannot interact with microtubules and phosphorylation also controls the association of MAP2 with actin microfilaments (Brugg et al.,1991; Selden et al.,1983). MAP2c is more highly phosphorylated in the developing rat brain, whereas MAP2b is more extensively phosphorylated in the adult brain (Sanchez et al.,1995).

1.6 Nitric Oxide

1.6.1 Introduction

Nitric oxide (NO) was first recognised as a messenger molecule in the CNS in 1988 (Garthwaite et al.,1988). It is the unstable intercellular molecule that had been hypothesised a year earlier (Garthwaite J. and Garthwaite G.,1987) to mediate the increased levels of cyclicGMP (cGMP) following activation of glutamate receptor subtypes, particularly those of the NMDA subtype. As will be discussed in more detail later, the enzyme responsible for synthesising NO, nitric oxide synthase (NOS), has been purified, cloned and expressed (Bredt et al.,1991).

1.6.2 Nitric Oxide Functions

NO has been shown to have many diverse roles. It is an important biological messenger involved in vasomotor control, vascular homeostatis, and important neuronal and immunological functions (Moncada and Higgins.,1993). Besides its role as endothelial-derived relaxing factor (Furchgott.,1990; Moncada et al.,1991) and a mediator of macrophage cytotoxicity (Hibbs et al.,1987; Marletta et al.,1989), NO appears to be a neuronal messenger in the brain and peripheral nervous system, and has been found to satisfy many criteria of a neurotransmitter (Garthwaite.,1991; Snyder and Bredt.,1991). NO is a free radical that binds to the iron-sulphur centres of enzymes and by doing this interferes with the function of these enzymes. NO also reacts with oxygen, superoxide and transition metals, and each of the products of these reactions supports further reactions at other sites (Stamler.,1994). The reaction with superoxide anions forms peroxinitrite anions that decompose to yield highly damaging hydroxyl free radicals and nitrogen dioxide (Beckman et al.,1990). NO is a relatively stable compound that can easily cross cell membranes and has the potential to travel significant distances in tissue before reacting with a target. In neuronal cultures, acute exposure to high concentrations of NO by over activating NMDA receptors or, by addition of NO-releasing compounds such as SNP and SNAP, results in neuronal cell death (Dawson et al.,1993;

Dawson and Snyder.,1994). During focal cerebral ischaemia, concentrations of NO may rise very rapidly to levels toxic to neurons (Malinski et al.,1993). NO is responsible for endothelial relaxing factor activity regulating blood pressure. As mentioned previously, stimulation of NMDA receptors has been shown to induce the release of NO which then diffuses to neighbouring cells and amongst other reactions activates guanylate cyclase to raise intracellular cGMP content (Garthwaite et al.,1988; Knowles et al.,1989). NO has been reported to be involved in the induction and maintenance of LTP particularly in the dentate gyrus (Bohme et al.,1991; Izumi et al.,1992; Mizutani et al.,1993). In cell lines , NO has been shown to modulate gene expression (Haby et al.,1994; Peunova et al., 1993), and in hippocampal granule cells NO alters proenkephalin and prodynorphin gene expression (Johnston and Morris., 1994).

1.6.3 NADPH diaphorase

As mentioned previously, NO is produced by NOS, an enzyme that is thought to be present in discrete populations of neurons but not in resting glial cells (Bredt et al.,1991). NOS can be demonstrated conveniently by the histochemical technique for nicotinamide adenine dinucleotide phosphate diaphorase (NADPH diaphorase) staining, and has been shown to be identical to NADPH diaphorase, NOS is localised to the same discrete neuronal populations in the brain that stain for NADPH diaphorase (Dawson et al.,1991; Hope et al.,1991). Dawson and coworkers illustrated NOS catalytic activity to be similar to NADPH diaphorase staining using human kidney cells transfected with NOS cDNA, and showed NOS and NADPH diaphorase staining in the same proportions as in neurons (Dawson et al.,1991). Despite the important roles of NO, a relatively small population of neurons in the brain (except in the cerebellum) contain a high level of NOS. As discussed later, non-neuronal sources of NO exist in the brain, as illustrated by the fact that cultured astrocytes express a form of NOS.

1.6.4 Nitric oxide and Alzheimer's Disease

Until recently, little research has focused on a possible role for microglial cells in Aβ-mediated neuronal degeneration in Alzheimer's Disease. The differences in the morphology of diffuse plaques, which lack reactive glial cells, and the neuritic plaques surrounded by reactive glial cells favours some from of glial involvement in neuritic plaque development. It has been shown that microglia are induced to release NO by Aβ alone and by the synergistic action of Aβ and interferon-χ (Goodwin et al.,1995). It is possible that Aβ-induced activation of microglia, and subsequent NO release is one mechanism of neurotoxicity in Alzheimer's Disease. An increased number of NADPH-diaphorase stained neurons within the substantia innominata of Alzheimer's Disease patients has been observed and this further suggests a role for NO in the neurodegeneration in the disease (Benzing and Mufson.,1995). A loss of nitric oxide responsive soluble guanylyl cyclase activity in the temporal cortex of Alzheimer's Disease patients has been reported (Bonkale et al.,1995). It is still not clear whether this loss reflects changes in pathology or specific signal transduction abnormality. Recent evidence suggests that an imbalance of NO and oxygen radicals may mediate the Aβ-induced endothelial damage on endothelial cells and contribute to the pathology of the normal ageing process, as well as the cell damage in Alzheimer's Disease (Sutton et al.,1997).

1.6.5
Nitric oxide synthase

The enzymes of the nitric oxide synthase (NOS) family share the capability for synthesising NO during the conversion of L-arginine to L-citrulline. NOS exists in two principal isoforms, constitutive and inducible, which differ in their calcium dependency and rate of expression. Constitutive NOS is calcium/calmodulin-dependent and produces NO for short periods in response to receptor stimulation. Neuronal (nNOS) and endothelial (eNOS) are both constitutive. Inducible NOS is calcium independent and induced by a variety of cytokines, inhibited by glucocorticoids, and once expressed synthesises NO for extended periods. Macrophage (mNOS) is

inducible, and is localised to macrophages throughout the body but also occurs in the brain as glial NOS (gNOS), microglial cells being modified macrophages.

nNOS is localised to neurons throughout the peripheral and central nervous system. Dinerman and coworkers found both eNOS and nNOS to occur throughout the brain in a wide range of neuronal cell types. Both were found in the cerebellum and olfactory bulb in the same call populations. However in the hippocampus, eNOS was found to be more concentrated in the pyramidal cells than in any other brain area, with nNOS being restricted to occasional interneurons (Dinerman et al.,1994).

The identification of NOS as the enzyme responsible for the neuronal NADPH diaphorase histochemical reaction (Hope et al.,1991), together with the development of antibodies to the purified NOS (Bredt et al.,1990) have made possible detailed anatomical localisation of the neurons generating NO. The cerebellum is thought to contain the highest levels of NOS activity and cGMP (Greenberg et al.,1982) in the brain. In the cerebellar cortex, NADPH diaphorase histochemistry indicate that NOS is present in basket and granule cells, but not in the Purkinje cells (Vincent and Hope., 1992). In the striatum, NADPH diaphorase histochemistry has shown NOS to be selectively localised in the population of medium-sized, aspiny interneurons that also contain somatostatin and neuropeptide Y (Vincent et al.,1983). NOS synthase appears to be absent from the cholinergic interneurons (Vincent and Hope .,1992). In the cerebral cortex and hippocampus, NOS appears to be present in the populations of interneurons but absent from the principal cells. The pyramidal cells of the cerebral cortex do not contain NOS (Vincent and Hope .,1992). As in the striatum, the cortex has a small population of NOS containing interneurons that express somatostain and neuropeptide Y (Vincent et al.,1983). The hippocampus contains interneurons that stain positively for NADPH diaphorase in the pyramidal cell layer, although the pyramidal cells themselves are not stained (Vincent and Kimura .,1992). In the dentate gyrus, positively stained cells are found in the molecular layer, and in particular just beneath the granule layer (Vincent and Hope.,1992). The cell groups identified as NOS-containing thus make useful models for studying the role of NO in the nervous system.

Astrocytes are known to possess a variety of similar properties to macrophages including the capacity to synthesise histocompatibility agents and cytokines (Robbins et al.,1987). Microglial cells present in the brain exhibit macrophage-like properties and these observations led to the study of NOS types in glial cells. Galea and coworkers found cultured CNS glial cells to express an inducible form of NOS similar to that found in macrophages (Galea et al.,1992). This iNOS was expressed following exposure of the glial cultures to the endotoxin lipopolysaccharide (LPS), and shared many properties with inducible mNOS: both are cytoplasmic, dependent upon NADPH and FAD for full activity, are calcium-independent and competitively inhibited by N^G-monomethyl-L-arginine. It was suggested that iNOS in glia may, by generating NO, contribute to the neuronal damage associated with cerebral ischaemia. Whereas cNOS activity can be tightly regulated by changes in intracellular calcium levels (Bredt et al.,1991), the activity of iNOS is calcium-independent and thus once expressed the enzyme can produce NO for extended intervals resulting in potentially toxic levels (Stuehr and Nathan.,1989).

The initial events in the LPS-induced activation of iNOS in macrophages have been shown to involve tyrosine kinase (Weinstein et al.,1992; Glaser et al.,1993). A role for the involvement of tyrosine phosphorylation events during the early process of iNOS induction has also been demonstrated in cultured glial cells (Feinstein et al.,1994).

Glial NO can be toxic to neurons (Choi et al.,1993) and to oligodendrocytes (Mitrovic et al.,1994). An important question is what determines whether a glial cell can be induced and if the expression of NOS can be regulated by endogenous and /or exogenous factors at the level of transcription, translation and/or post-translational modification. Inactivation of endotoxin-induced NOS transcription has been reported in astrocytes pretreated with deactivating cytokines (Simmons and Murphy.,1993), and NO itself has been shown to interfere with the transcriptional induction of NOS (Park et al.,1994). Park and coworkers found that the magnitude and duration of NO production from activated astrocytes in culture may be determined by signals from adjacent neurons and microglial cells (Park et al.,1994).

Another important finding was the discovery that cultured astrocytes express a constitutive form of NOS thought to be similar to endothelial and neuronal NOS, as well as an inducible form of NOS (Ma et al.,1994). In order to serve some important physiological roles of NO such as maintaining the normal mitogenic state of astrocytes, both constitutive and inducible forms are present.

1.7 **Microglia**

1.7.1 **Introduction**

Neuroglia comprise the largest class of non-neuronal cells. Neuroglia can be further categorised as microglia or macroglia, and macroglia are further subdivided into oligodendrocytes and astrocytes (Frederickson., 1992). Astrocytes constitute the main macroglial cell in the CNS, and were hypothesised at the beginning of the century to be important in brain function. Astrocytes in the mature brain can be divided into two broad categories; they are either fibrous, filament-containing cells found mainly in the bundles of nerve fibres or, are protoplasmic with less fibrous material and located in the grey matter surrounding nerve bodies, axons and synapses (Duffy, 1983). A further category of glial cell exists: reactive glia, and this is discussed in section 1.7.2. The cell filaments are composed of glial fibrillary acidic protein (GFAP) and this is used as a marker for astroglia (Bignami and Dahl, 1974). Astrocytes have multiple functions including the mechanical support of other CNS components, neurotransmitter metabolism and participation in repair and regeneration (Duffy, 1983; Pfeiffer et al.,1977). Microglia are sources of growth factors such as transforming growth factor-β, platelet-derived growth factor and basic fibroblast growth factor. This suggests that these cells provide a trophic support system (Shimojo et al.,1991). Secretion of nerve growth factor by activated brain macrophages has also been reported (Mallat et al.,1989).

Glia have well established roles in the nervous system, including the regulation of external potassium and the uptake of the excitatory amino acids aspartate and glutamate which are released by neurons into the extracellular space (Nicholls et al.,1990). Recent evidence suggests that glutamate acting at metabotropic receptors expressed by astrocytes could modulate glial activity evoked by neurotransmitters, and thus influence the ongoing modulation of neurons by astrocytes (Haak et al.,1997).

Cultured astroglia provide a convenient, enriched population of astroglia for study. Cell cultures have well-known advantages as experimental models, and provide readily observable systems that in many cases retain biological properties which parallel those in vivo.

The greatest source of culture model systems to explore CNS function and dysfunction is fetal brain material from rodents. For studies of human adult CNS disorders however, these models have disadvantages; their fetal origin and their derivation from another species. As fetal material, the molecular and genetic machinery of the cells would be expected to be devoted to growth and development and may have little or no susceptibility to the CNS disorder under investigation. Astroglial cultures prepared from fetal material do not mature in culture to the degree that occurs in vivo.

1.7.2 **Reactive Gliosis**

Extensive evidence indicates that microglia are involved in brain function especially in neuropathological conditions (McGeer et al.,1993). Resting microglia are activated in response to injury, infection and inflammation of the nervous system, whereupon they enlarge and extend numerous processes which become packed with intermediate filaments (Bignami and Dahl.,1976; Duffy.,1983). This phenomenon is known as reactive gliosis. Reactive gliosis is seen in AIDS dementia, prion-associated encephalopathies, acute traumatic brain injury and Alzheimer's Disease (Gajdusek.,1990; Beach et al.,1989; Delacourte et al.,1990). The transition of astrocytes from the resting to the activated state seen in reactive gliosis is associated with an increased expression of new molecules not normally detected in resting astroglia, as well as the upregulation of other factors. It remains controversial whether these changes are detrimental or beneficial (Reier et al.,1989). It is possible that the increase in cytoskeletal proteins within the reactive astrocytes could help assist wound repair by stabilising the surrounding tissue (Reier.,1986). However, it has also been suggested that the glial scar may form a barrier that could hinder regenerative processes such as neurite outgrowth (Reier et al.,1983).

A role for reactive astrocytes in tissue regeneration and repair is also supported by their molecular profile. In vivo astrocytes express extracellular matrix molecules such as laminin, proteoglycans and adhesion proteins (Ard and Bunge.,1988). In vitro they are also able to secrete glycosaminoglycans (Johnston-Green et al.,1991). Caggiano and colleagues demonstrated the involvement of eicosanoids

and nitric oxide in the induction of reactive gliosis (Caggiano and Kraig.,1996).

Several studies have investigated the neuroprotective of astrocytes relevant to the pathogenesis of neurodegenerative diseases. The release of basic fibroblast growth factor and IL-6 from astrocytes has been shown to protect hippocampal neurons against glutamate toxicity and protect neurons against hypoxia respectively (Mattson et al.,1990; Maeda et al.,1994).

1.7.3 **Microglia and Alzheimer's Disease**

As has been discussed earlier, Aβ plaques are associated with a number of other proteins including immune process compounds. Reactive astrocytes have also been found in association with amyloid plaques (Selkoe.,1991), raising the possibility that microglia could be involved in senile plaque formation. MacKenzie and colleagues reasserted a specific role for microglia in the formation of neuritic plaques from diffuse plaques (MacKenzie et al.,1995). They found no association between microglial cells and diffuse plaques but a strong association between microglial cells and the formation of neuritic plaques. The initial event in the formation of senile plaques is thought to be the deposition of Aβ protein, and cultured microglia have been shown to synthesise APP and metabolize it in a way favouring the formation of Aβ (Bauer et al.,1991; Haass et al.,1991). Alternatively, it has been suggested that microglia could take up APP from another source such as degenerating neurons and convert it to Aβ (Cummings et al.,1991). Finally, the presence of pre-established amyloid deposits could cause microglia to become activated (MacKenzie et al.,1995).

Studies to date investigating the association between microglia and senile plaques in Alzheimer's Disease have found a generalised increase in microglia in affected areas of the cortex, and accumulation of microglia around senile plaques (Coria et al.,1993; Itagaki et al.,1989). Pike and colleagues demonstrated that aggregated Aβ peptides induce a reactive phenotype in cultured astrocytes that is paralleled by the selective localisation of reactive astrocytes in Alzheimer's Disease (Pike et al.,1994). They suggested that Aβ itself could be a potent stimulus to astrocytes and may significantly contribute to the reactive gliosis characterisitc of Alzheimer's Disease.

1.7.4 **Other compounds involved in microglial function**

Nerve growth factor (NGF) is a neurotrophic protein required for the development and survival of several populations of neurons. In the CNS, NGF is synthesised by certain neurons, but during brain development and under different pathological conditions, there is an increased production of NGF by astrocytes (Bakhit et al.,1991). However, little is known about how this synthesis process is upregulated. It is possible that several NGF-inducing agents act through different pathways. One of them is the signalling network involving the activation of protein kinase C (PKC). It has recently been suggested that phosphatidylcholine-hydrolising phospholipase C induces the synthesis of NGF by glial cells in both a PKC-dependent and PKC-independent manner (Laviada et al.,1995).

Neurotrophin involvement in the development and growth of the CNS has been studied extensively. Neurotrophins participate in multiple developmental processes ranging from survival of neurons (Levi-Montalcini and Angeletti.,1968; Theonen and Barde.,1980; Henderson et al.,1993), to proliferation of oligodendrocyte precursors (Barres et al.,1994) and axon growth (Zhang et al.,1994). Microglia have been shown to be sources of trophic factors known to support development and normal function of the CNS (Elkabes et al.,1996). Microglia are responsive to neurotrophins; both brain derived neurotrophic factor and neurotrophin-3 induce proliferation and phagocytic activity of microglia in vitro (Elkabes et al.,1996).

A role for the neurotrophic cytokine interleukin-1 (IL-1) as a mediator of astrocyte activaiton and growth was first demonstrated by Giulian and colleagues (Guilian and Lachman.,1985). LI-1 has been shown to induce proliferation of astrocytes cultured from neonatal rat brains, and when injected into the brain to induce reactive gliosis (Guilian and Lachman.,1985). Evidence also reports induction of tumour necrosis factor mRNA and IL-1 in astrocytes following treatment with interferon γ and LPS (Chung and Benveniste.,1990). IL-1 is thought to play a role in the pathogenesis of certain human CNS diseases (Lee et al.,1995). High levels of IL-1 are present in the cerebral tissue of Alzheimer's Disease brains (Griffin et al.,1989). The elevated level of IL-1 is thought to be due to overexpression by

activated microglia in the tissue, particularly by those microglia associated with senile plaques (Griffin et al.,1995). Sheng and colleagues reported a positive correlation between the regional distribution of IL-1 positive microglia and tau positive plaques in Alzheimer's Disease patients (Sheng et al.,1995). This further supports a role for IL-1 in the formation of plaques and in the pathogenesis of Alzheimer's Disease.

1.8 Cholinergic system and Alzheimer's Disease

Over the past two decades, evidence has accumulated suggesting that central cholinergic systems are important in the regulation of learning, memory and cognition (Bartus et al.,1985; Bartus and Johnston.,1976). Degeneration of cholinergic neurons in the CNS is one of the critical components contributing to cognitive impairments in Alzheimer's Disease. Neuropathological changes in the disease involve cell loss and degeneration in the neocortex and many neurotransmitter systems, particularly the cholinergic system, are especially vulnerable. The basal nucleus of Meynert and the diagonal band of Broca provide the major source of cholinergic input to the cerebral cortex and the hippocampus (Divac.,1975; Lehman et al.,1980; Mesulam and Van Hoesen.,1976). A reduction in number of the cholinergic neurons by almost two thirds has been reported in Alzheimer's Disease brain tissue (Nagai et al.,1983). However, Pearson and colleagues reported a shrinkage rather than a loss of these neurons in Alzheimer's Disease brain tissue (Pearson et al.,1985). This result agreed with results from lesioning studies (Sofroniew et al.,1987). It is possible that cholinergic neurons projecting from the basal nucleus of Meynert to the neocortex are atrophied rather than lost in Alzheimer's Disease (Allen et al.,1988).

Several biochemical markers of presynaptic cholinergic nerve endings such as choline acetyltransferase show severe depletion in Alzheimer's Disease (Jope et al.,1994). In contrast to this, receptor binding methods have shown that postsynaptic muscarinic receptors are retained in Alzheimer's Disease (Davies and Verth.,1977; Svensson et al.,1992). These findings led to the attempt to treat Alzheimer's Disease patients with cholinergic agonists.

1.9 **Genetics of Alzheimer's Disease**

1.9.1 **Introduction**

Identification of the underlying causes and means of prevention constitute the current objectives of epidemiological research on Alzheimer's Disease. The major new development in this area is the recognition of genes that predispose to the disease. Genetic autosomal dominant forms of Alzheimer's Disease, called familial Alzheimer's Disease (FAD), result in the relatively early onset of the disease. Studies have led to the discovery of four genes linked to the development of early onset FAD. The first is the APP gene on chromosome 21 (Checler et al.,1993), and mutations in this gene have been discussed in Section 1.4.6. The second of these genes is the apolipoprotein E gene located on chromosome 14 (Corder et al.,1993). Two novel genes have recently been identified: presenilin 1 (PS1/S182) on chromosome 14 (Sherrington et al.,1995; Alzheimer's Disease collaborative group.,1995), and presenilin 2 (PS2/STM2) on chromosome 1 (Rogaev et al., Levy-Lehad et al.,1995). The normal biological role (s)of the presenilins are unknown, however it has been suggested that they participate in intracellular transport (Sherrington et al.,1995).

1.9.2 **Risk Factors for Alzheimer's Disease**

As has been previously discussed, the main risk factors of the disease are: increased production of soluble Aβ and its precursor, failure to remove amyloid deposition, and point mutations of the APP gene. Some of the factors that could cause these are discussed below.

Apolipoprotein E (ApoE), a 34kDa lipoprotein, is a major lipoprotein in the central nervous system, and it plays an important role in hippocampal response to injury, dendritic remodelling and synaptogenisis (Piorier et al.,1994). ApoE is encoded from three alleles (ε2, ε3, ε4) giving six genotypes in humans. Several investigations have shown that a specific apoE isoform, Apo ε4 allele, is a risk factor for both early and late onset Alzheimer's Disease (Schmechel et al.,1993; Strittmatter et al.,1995). The coexistence of ApoE and Aβ has been detected in both senile plaques and

neurofibrillary tangles in the brain of Alzheimer's Disease patients (Namba et al.,1991). Recent evidence suggests that the combination of the herpes simplex virus1 and carriage of the ApoE ε4 allele increase the risk of Alzheimer's Disease and together are damaging to the nervous system (Itahaki et al.,1997).

Head injury has been identified as the main environmental risk factor for Alzheimer's Disease, and may act as a risk factor by causing acute deposition of Aβ (Roberts et al.,1994).

Smoking is thought to be protective against the development of Alzheimer's Disease via the effects of nicotine on nicotinic receptors, Aβ toxicity and induction of antioxidant systems (Brenner et al.,1993).

1.10 Clinical treatments for Alzheimer's Disease and other drugs related to the disease and tau protein expression.

1.10.1 Inverse association of non-steroidal anti-inflammatory drugs and Alzheimer's Disease.

Postmortem analysis of Alzheimer's Disease brain tissue has shown the presence of reactive glial cells densely embedded around amyloid plaques (McGeer et al.,1992). Postmortem tissue also revealed the presence of several immunoprotective proteins that are normally absent or expressed at very low levels in the normal brain (McGeer et al.,1989). These observations indicate a possible immune-mediated process in Alzheimer's Disease and first suggested a role for anti-inflammatory agents in treatment of the disorder. Clinical trials of indomethacin in Alzheimer's Disease showed a small improvement in those treated with the drug (Rogers et al.,1993). These findings suggested an inverse relationship between anti-inflammatory compounds and Alzheimer's Disease and this was further supported by the observation that there is a low prevalence of the disease among patients with rheumatoid arthritis (Broe et al.,1990; McGeer et al.,1990). Studies have suggested that non-steroidal anti-inflammatory drugs act as a protective factor for Alzheimer's Disease (Henderson et al.,1992; Rich et al.,1995).

1.10.2 Clinical treatments for Alzheimer's Disease.

Pharmacological treatment of patients with Alzheimer's Disease is becoming more important and a vast number of drugs have been researched in a variety of countries. There is presently no treatment that will arrest the progression of Alzheimer's Disease. However, it has been shown that in the vast majority of clinical trials, the cholinesterase inhibitor tacrine (tetrahydroaminoacridine) is able to induce very beneficial effects in cognition and memory. The number of

drugs investigated as possible candidates for alleviation of the disease is vast and below is a summary of the main groups of drugs.

Three main classes of drug have been researched; cholinesterase inhibitors, nootropics and neuroprotective agents. Tacrine, velnacrine and eptastigimine are cholinesterase inhibitors. A major defect in neurotransmission in Alzheimer's Disease results from loss of cholinergic function. Reduced choline acetyltransferase activity is seen in postmortem Alzheimer's tissue (Sims and Bowen.,1983). Tacrine, a centrally active, reversible cholinesterase inhibitor, has been reported to improve psychometric test performance and to improve global assessment in Alzheimer's Disease patients(Davis et al.,1992). However, some negative results were also seen (Gauthier et al.,1990).

Aniracetam, oxiracetam, and piracetam and pramiracitam are nootropics with potential for the treatment of Alzheimer's Disease. The term nootropic was derived by Giurgea in 1972 to describe compounds that enhance learning and memory. Aniracetam has been shown to improve the condition of elderly patients suffering from slight to moderate mental deterioration (Canonico et al.,1991). Aniracetam has been shown to modulate the AMPA receptor (Martin et al.,1993). However, aniracetam has been reported to be a less than ideal drug; the effect on AMPA receptors may be only weak and most of the drug may be broken down in the stomach (Service.,1994).

The following neuroprotective agents have been tried at clinical trials for the treatment of Alzheimer's Disease:selegiline, acetyl-L-carnitine, gangliosides, idebenone and nimodipine. A major problem in the treatment of the disease is the lack of substantial knowledge about the actual availability of these drugs in the CNS. Inadequate concentrations in the brain can potentially decrease the clinical usefulness of new and existing drugs. Different methods to overcome this problem have been developed but they also have their side effects and problems. Another aspect of treatment which has been investigated is combination therapy, and given the complex nature pathogenesis of Alzheimer's Disease, this therapeutic approach may be the best option.

Recent research has focused on immune mechanisms in the pathogenesis of Alzheimer's Disease. A large number of immune system proteins have been found to be associated with the disease lesions (Toomama et al.,1990; Rogers et al.,1988). For the near

future, consideration of immune-directed therapy of the disease may be another option.

Project Aim:

Neuronal and glial cell cultures are viable models with which to study the effects of various compounds on the expression and regulation of cytoskeletal proteins. One of the main neuropathological markers of Alzheimer's Disease is the presence of tangles, the main constituent of which are paired helical filaments composed of the microtubule associated protein tau in an abnormal state of phosphorylation. The aim of the current work has been to investigate a possible relationship between nerve growth factor, nitric oxide synthase activity and the expression of microtubule associated proteins in neuronal and glial cultures. Evidence has established a relationship between nerve growth factor, nitric oxide and microtubule associated protein expression in PC12 cells and other cell lines (Peunova et al.,1995; Haby et al., 1994), however, the effect of nitric oxide on cell morphology and protein expression has been less well documented in neuronal cells.

Another main neuropathological marker of Alzheimer's Disease is the presence of senile plaques composed primarily of amyloid protein. The development of two transgenic mouse lines in which the amyloid precursor protein gene has been ablated (Zheng et al.,1995) or altered (Moechars et al.,1996) has provided the second main aim of the project: to investigate the relationship between the amyloid precursor protein and morphological and synaptic markers.

Chapter 2

METHODS.

2.1 Cell Culture

2.1.1:Dissociated primary neuronal culture

Basal forebrain neurons were cultured using a modification of the methods of Hartikka and Hefti (1988). Timed pregnant Wistar rats were killed with an overdose of sagatal injected intraperitonealy on gestation day 17 (E17). Embryos were removed and placed in Dulbecco's Minimum Essential Medium.The basal forebrain region was dissected under a microscope. The localisation of the basal forebrain was from the description of fetal brain anatomy by Inagaki et al (1982). Dissected pieces of basal forebrain tissue were transferred into a culture dish containing Minimum Essential Medium. The tissue pieces were minced and washed three times in Minimum Essential Medium. Cells were dissociated in 10ml of a 0.67% trypsin solution made up in sterile saline, at 37 ^{o}C for 15 minutes. The trypsin was inactivated with 1.5ml heat inactivated fetal calf serum (FCS). Following trypsinisation the cells were washed three times in Minimum Essential Medium and spun at 500g in a DAMON\IEC centrifuge for three minutes per wash. The cells were then resuspended in Dulbecco's Modified Eagles Medium (DMEM) supplemented with the following: 5% horse serum, 0.5% FCS, 100 units/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. The tissue was triturated in supplemented DMEM using a pasteur pipette and cells were plated at a density of 180 x 10^3 cells per 1ml (see section 2.1b) onto 8-well multichamber glass slides previously coated with poly-D-lysine (4µg/ml). The cultures were grown in a humidified incubator supplied with 95% oxygen and 5% carbon dioxide. After 24 hours, the medium was replaced with neurobasal medium supplemented with B27 and penicillin-streptomycin. This was necessary to obtain serum-free conditions. Cells were used within 3 days of seeding.

2.1.1b: Cell counting using trypan blue staining

In order to control the number of cells plated, the cell density was determined using trypan blue. This method also enables the amount of cell damage caused by dissociation to be investigated. The method was essentially identical to that of Banker and Goslin (1991). One drop of cell suspension was mixed with an equal volume of a 0.08% trypan blue solution dissolved in HBSS solution. This mixture was placed onto a haemocytometer, and the number of live and dead cells in one small grid was counted.

For the experiments involving investigation of neurite structure, cells were diluted to a density of 110 x 10^3 cells per 1ml.

2.1.2: Glial cell culture

The method for rat glial culture was adapted from Keller et al (1985). Two day old rat pups were killed with an overdose of sagatal injected intraperitonealy, and their brains removed and placed in saline. The meningeal layer was removed under a dissection microscope, and the cortical tissue removed from the cerebrum. Generally, the tissue from two pups was pooled. The tissue was minced and washed three times in sterile saline before trypsinisation in a 0.25% trypsin/EDTA solution at 37°C for 15 minutes. The trypsin was inactivated with 2ml FCS, and the cells then spun at 500g for three minutes and the trypsin decanted off. The cells were then rinsed in F-12 DMEM nutrient mixture supplemented with the following: 20% FCS, 100 units/ml penicillin, 100µg/ml streptomycin, 3mM L-glutamine and 0.25% amphotericin B. Each rinse was followed by centrifugation at 500g for three minutes. The cells were then triturated in F-12 DMEM nutrient mixture using a pasteur pipette, and then dispersed by passing through a 20-gauge needle into 5ml culture flasks. Once seeded onto the flasks, the cultures were incubated for 48 hours in a humidified incubator supplied with 95% oxygen and 5% carbon dioxide. At this stage it was essential to minimise movement of the cells in order to prevent cell clumps. After 48 hours the medium was replaced with F-12 DMEM nutrient mixture similar to previously but containing only 5% FCS. The medium was replaced every two days thereafter. One week after seeding, the cells underwent a process

of secondary culture. This was done in order to eliminate neuronal and oligodendroglial contamination,and to expand the exisiting glial cell population. Cells were trypsinised in a 0.25% trypsin/EDTA solution for three minutes at 37°C. The culture flasks were then shaken firmly to dislodge the attached cells. The trypsin solution was inactivated with 2ml FCS and the cells centrifuged at 500g for three minutes. The cells were then washed in F-12 DMEM-5% FCS and dispersed in 24 ml F-12 DMEM-5% FCS before being seeding onto 24 well plastic culture plates containing glass coverslips. After 24 hours, the plates were shaken gently and the medium replaced. The cells were then ready for treatment.

2.2 Immunohistochemistry

2.2.1: Antibody reaction

Prior to immunohistochemistry,cultures were fixed for 15 minutes in an ice cold 4% formaldehyde solution. The cultures were rinsed three times in 0.1M phosphate-buffered saline (PBS) for 10 minutes and incubated at room temperature for 60 minutes in 0.1M PBS containing 20% normal goat (polyclonal antibodies) or horse (monoclonal antibodies) serum (NS). The cultures were then incubated for 12 hours at 4°C with specific antibodies against in 0.1M PBS with 1% NS, and 0.5% Triton-X. (details of antibody concentrations are in Materials section). After rinsing in PBS three times for 5 minutes per rinse, the cultures were incubated with biotinylated secondary antibody for 60 minutes, rinsed in 0.1M PBS twice for 10 minutes, and then incubated with ABC-conjugated horse radish peroxidase for 60 minutes in accordance with manufacturers instructions. The peroxidase was visualised with 0.1% diaminobenzidine made up in 0.1M phosphate buffer ph 7.2 containing 0.02% hydrogen peroxide and 0.05% nickel chloride. Slides were then dehydrated in a graded series of alcohols (70%, 90%, 100%%) and mounted with coverslips using DPX.

2.2.2: NADPH diaphorase staining

NADPH-diaphorase staining was performed according to the method of Valtschanoff et al (1992). Tissue culture slides or glass coverslips were fixed for 15 minutes in a 0.2% glutaraldehyde solution in 0.1M phosphate buffer, pH 7.2. Slides were then pre-incubated in 0.1M phosphate buffer containing 0.3% Triton-X-100 for 10 minutes,then transferred to a freshly prepared buffer-Triton solution containing 0.5mg/ml NADPH and a few drops of a 0.2mg/ml nitro blue tetrazolium solution. After incubation at room temperature on a shaker for 10 minutes slides were transferred to an oven at 37 ^{o}C for between 4 and 12 hours. Slides were then rinsed in 0.1M phosphate buffer,dehydrated in a graded series of alcohols and coverslipped with DPX mounting medium. Since the reaction product is slighty soluble in ethanol, rinses in alcohol were short. Within each experiment, all slides received identical dehydration procedures.

2.3 In situ hybridisation

2.3.1: Oligonucleotide Probes

Oligonucleotide probes of unique sequence were synthesised on an Applied Biosystems DNA synthesiser. The probe sequences were as stated in the Materials section.

2.3.2: Cryostat sections

In situ hybridisation was performed according to the method of Wisden and Morris (1992). After treatment, brains were removed and fast-frozen on dry ice. 20μm cryostat sections were cut at the levels of the basal forebrain and hippocampus. The sections were mounted onto sterile slides previously baked at 180 ^{o}C and coated in a 0.01% solution of poly-L-lysine. The sections were then dried at room temperature and fixed in a 4% formaldehyde solution for 15 minutes. The sections were then transferred into a 1x phosphate buffered saline solution (PBS) for 2 minutes and dehydrated in a graded series of alcohols (70%,90%,100%) for 2 minutes each. Sections were stored in 100% alcohol.

0.3pmol oligonucleotide probes(45-mer) were 3'-end labelled with 10pmol of the isotope 5-[α-^{35} S]dATP,specific activity 1300 Ci/mmol.The specific activity of the probes was 1.0 x10^{9} dpm/μg.

For the reaction solution, the following were mixed in an eppendorf tube:

2.2 μl diethylpyrocarbonate(DEPC) treated water
1.25 μl 5x reaction buffer(contains 1M potassium cacodylate,125mm Tris/HCl,1.25mg ml-1 bovine serum albumin,pH6.6)
1.0μl of oligonucleotide at a concentration of 0.3 pmol μl $^{-1}$
1.6 μl [α-35 S] dATP (1300 Ci/mmol)
0.5 μl TdT.

The reagents were mixed carefully using a pipette and incubated in a water bath at 32 ^{o}C for 3-5 hours. The reaction was stopped by addition of 40 μl DEPC-treated water.Sephadex G-25 spin columns

were prepared using 1ml syringes plugged with glass wool. The syringes were filled with a 1:1 solution of Sephadex G-25 powder and Tris/EDTA buffer. This was spun at 500g for 1 minute to obtain the column. 50µl of the reaction mixture was added to the column and spun at 500g for 1 minute to remove unincorporated nucleotides. The eluate was collected in a sterile eppendorf tube. 2 µl of the eluate was analysed by liquid scintillation counting. The counts were in the range of 150 000 to 300 000 dpm /2 µl. 2 µl of 1M dithiothreitol solution made in DEPC-treated water was added to preserve the probe from oxidation. The probes were stored at -20°C until use.

Sections were removed from 100% ethanol and allowed to dry at room temperature. Following this sections could be hybridised with selected probe at 42 °C overnight. In a sterile eppendorf tube the radiolabelled probes were diluted 1:100 in hybridisation buffer. 2 µl 1M DTT was added to the mixture which was then vortexed thoroughly.120 µl of probe/hybridisation buffer was applied to each slide,and slides were then gently covered with parafilm. Air bubbles were removed using blunt ended forceps. A small piece of tissue was saturated with 4xSSC and placed along side the slides in a sealed petridish to maintain humidity. Slides were incubated overnight at 42°C.

After overnight incubation, slides were transferred into coplin jars containing 250ml of prewarmed 1xSSC at 55 °C. The parafilm was gently removed using forceps,and the slides were then washed twice in fresh 1xSSC for 30 minutes at 55 °C with constant agitation. Following this,the slides were transferred into 0.1.xSSC at room temperature for 10 minutes and then dehydrated in graded alcohols (70%, 90%, 100%) for 2 minutes each before air drying.

The sections were then exposed to autoradiographic film at room temperature. Dry slides were attached to paper with tape and exposed to the film for between 4 days to 1 week depending on the probes used and the efficiency of their labelling. Under safe light conditions films were developed for 5 minutes in D19, rinsed in water for 2 minutes, fixed in Unifix for 4 minutes and rinsed in water for 20 minutes.

After film developing, slides were dipped in K5 liquid photographic emulsion. Slides were dipped under safe light in a filtered 1:1 solution of emulsion: distilled water containing 0.5%

glycerol prewarmed to 45 °C. Slides were allowed to dry before being transferred to light-tight slide boxes containing fresh silica gel. The boxes were stored at 4 °C until time of development,usually 5x the duration on film.

Exposed boxes were allowed to come to room temperature. Under safe light, the slides were transferred to glass racks and immersed into 250ml D19 developer at 19°C for 8 minutes. Slides were then rinsed in water and immersed into 250ml of freshly prepared 30% sodium thiosulphate solution for 4 minutes. The slides were then rinsed in water for 15 minutes and then counterstained in 0.2% neutral red solution for 3 minutes and dehydrated in graded alcohols (70%,90%,100%) for 2 minutes. Finally slides were transferred into histoclear for 10 minutes and mounted with glass coverslips using DPX mounting medium.

2.3.3: Cell cultures

The method used for in situ hybridisation of cell cultures was essentially identical to that described for in situ hybridisation of the cryostat sections.

2.4 Experimental design

2.4.1:'In vitro' experimental design

All basal forebrain cultures were treated with drugs for 24 hours or as specified at 37 °C. The drugs were delivered directly to the bathing medium in 10 µl volumes,assuming that the total volume of each chamber on the 8-well multichamber slides was 0.4ml. Drug solutions were administered in 10µl of Hanks Balanced Salt Solution (HBSS), controls received 10µl of HBSS. After the desired time period, the cells were fixed in a 4% formaldehyde solution,rinsed in 1xPBS,dehydrated in ethanol and processed for in situ hybridisation or staining as previously described. In all experiments, at least one well per slide received vehicle treatment for comparison with the other treatment on the same slide.

2.4.2:'In vivo' experimental design

Male Wistar rats weighing between 150 and 200g were injected intraperitonealy with drug dissolved in physiological saline solution or vehicle. At 24 hours postinjection, the rats were killed with an overdose of Sagatal injected intraperitonealy. The brains were removed and 20μm-thick cryostat sections were cut and processed for in situ hybridisation as described previously.

2.5 Haemoglobin solution preparation

This method was identical to Martin et al (1985). Haemoglobin needs sodium dithionite excess to convert met-Hb (Fe 3+ Hb) to oxy-Hb (Fe2+ Hb(O2) 4). Dialysis tubing was boiled in distilled water for 15-20 minutes then rinsed in distilled water. 1290mg of Hb was dissolved in 20ml distilled to give a 1mM solution. 34.82mg of sodium dithionite was dissolved in 100μl of distilled water and added to the Hb solution. The combined solution was was then poured into the dialysis tubing and air bubbles carefully removed. the tubing was placed in distilled water for between 2 and 4 hours. The final 1mM Hb solution was then aliquoted and frozen, to be used within 2 weeks.

2.6 Transgenic models

2.6.1 APP-deficient mice

The production of transgenic mice was performed by Zheng et al (1995) as follows: APP-deficient mice were produced by homologous recombinaton in embryonic stem cells (Zheng et al 1995). To produce mice homozygous for the disrupted gene, cross-matings between heterozygous mice were set up. This resulted in 34% wild type (+/+), 36% heterozygous (+/-) and 24% were homozygous for the mutated APP allele (-/-). The present work studied these transgenic mice at 13 months of age. At 13 months, the mice were killed by sagatal overdose followed by transcardial perfusion with 10% formalin solution. After 48 hours, the brains were removed,

parafin wax embedded and dehydrated through a graded series of alcohols. 10μm sections were cut on a vibratome and mounted onto slides. Sections were taken for immunohistochemistry using specific antibodies against the following: MAP2, synaptophysin, GFAP, APP, calbindin, (see Materials section), as described in Section 2.8.

2.6.2 APP/RK mice

The functional significance of the α-secretase processing of APP was investigated by Moechars and coworkers (Moechars et al.,1996). A mutant APP was created that is partially defective in secretion from three cell types : COS cells, MDCK cells and primary neurons. Expression of this α-secretase mutant in mouse brain (APP/RK mice) resulted in behavioural disturbances, seizures and premature death. The neuropathological changes observed included apoptosis, necrosis and reactive gliosis in the hippocampus, cortex and amygdala (Moechars et al.,1996).

The current study looked at GFAP immunoreactivity in the cortical layers of APP/RK and wildtype mice. The staining method was as described in Section 2.8

2.7 Human postmortem Alzheimer's tissue

Postmortem samples were obtained from Cambridge Brain Bank Laboratories. The control and disease brain tissue had been carefully matched on the basis of age and sex. The diseased tissue was obtained from patients whose cause of death had been determined as Alzheimer's Disease. Brains were fixed by long-term storage in 10% buffered formalin solution. 10μm sections were cut and taken for immunohistochemistry using specific antibodies against the following: MAP2, synaptophysin, GFAP, 6F3D, AT8, S182-C20 and S182-N19 as described in Section 2.8. Double labelling was carried out using the method described in Section 2.9.

2. 8 Immunohistochemistry of tissue sections 2.6 and 2.7.

The method is essentially identical to that described in Section 2.2.1 with the following alterations: To remove wax from the sections and rehydrate, sections were passed through xylene, a series of alcohols (100%, 90%, 70%) and tap water for 2 minutes each. Sections to be treated with antibodies to APP and β amyloid were incubated in 80% formic acid solution for 10 minutes. All sections were incubated in a 0.3% hydrogen peroxide solution for 30 minutes. 0.1M PBS contained 5% NS and 0.3% Triton-X throughout.

2.9 Double Labelling

Tissue sections were passed through xylene and a graded series of alcohols for 2 minutes each. Sections were then incubated for 30 minutes in a 0.3% hydrogen peroxide solution. Sections were then rinsed three times in 0.1M PBS for 10 minutes and incubated for 60 minutes in monoclonal antibody in 0.1M PBS containing 5% normal horse serum and 0.3% Triton-X. The sections were then incubated for 12 hours at 4 oC in the monoclonal antibody in 0.1M PBS containing 5% NS and 0.3% Triton-X. Following secondary antibody treatment and peroxidase visualisation, sections were rinsed in distilled water for 10 minutes, and then incubated in 0.3% hydrogen peroxide solution for 1 hour. A further 10 minute wash in distilled water was then followed by overnight storage in fresh distilled water. Sections were then processed with the polyclonal antibody. The method for this was identical omitting the hour long incubation in 0.3% hydrogen peroxide and using a different visualisation system.

2.10 Radiolabelling

Forebrain and cerebellar section from APP-null and wildtype mice were processed for radiolabelling using an antirabbit ^{35}S secondary antibody to calbindin. The primary calbinidn polyclonal antibody was at a concentration of 1:1000. The steps for the reaction were identical to the process described in Section 2.8 with the

following changes. The ^{35}S-labelled secondary antibody was applied at a concentration of 0.1μCi/ml as described by Blanchard and coworkers for 1 hour (Blanchard et al.,1993). Tissue sections were then washed thoroughly in distilled water for 3x 5 minutes, and dehydrated through a graded series of alcohols (70%, 95% and 100%) and xylene. Sections were then washed in 100% ethanol and allowed to dry. The sections were then exposed to autoradiographic film (β max hyperfilm-Amersham) at room temperature. Dry slides were attached to paper with tape and exposed to film for three days. Under safe light conditions, films were developed for 5 minutes in D19, rinsed in water, fixed in Unifix for 5 minutes and rinsed thoroughly in water

2.11 Image and statistical Analysis

Image analysis quantification of the results was divided into three categories as follows:

1\ Chapter 3 results: to quantify any drug-induced changes in the levels of Tau 1 or Tau 2 mRNA , cell silver grain densities in the basal forebrain and hippocampal CA1 areas were measured using the NIH 'Image'1.52 Macintosh image analysis system following dipping of slides in liquid photographic emulsion. For each region, a total of ten measurements of mean number of pixels occupied by silver grains per unit area were taken.

2\ Chapters 4 and 5: to quantify any drug-induced changes in cultured cell immunoreactivity, mean relative optical density levels per well were measured using the NIH 'Image' 1.52 system. For a single well, a total of between 8 to 12 cell mean optical density levels per well were taken, and the measurements repeated. Background level was assessed by measurement of optical density over an area devoid of cells, and this measurement was subtracted from all values.
Slides dipped in liquid photographic emulsion had measurements of mean number of pixels occupied by silver grains per unit area assessed.

3\ *Chapter 6*: to quantify changes in immunoreactivity in transgenic or wildtype mice, mean optical density values per unit area were measured using the MCID 2 system. Background level was assessed by measurement of optical density over an area devoid of cells, and this measurement was subtracted from all values.

Statistical analysis was as follows:

1\ For glial and neuronal cell culture experiments where multiwell chambers, and thus multiple treatments compared to control values, wre used: analysis of variance ANOVA (one-way stacked) was performed on the data (Minitab). The Dunnett's and Tukey's posthoc tests were then used to assess statistical significance of any drug induced changes, and any differences between specific pairs of treatment respectively.

2\ For the in vivo work, and a relatively simple experimental design where one set of control animals was compared to one set of treated animals, the parametric test, the Student's t test, was used.

3\ For the transgenic work ,where one set of wildtype animals was compared to one set of transgenic animals, and an uneven distribution was apparent, the non-parametric Mann Whitney U test was chosen.

MATERIALS

Antibodies

APP 22c11	2.5mg/ml, rabbit polyclonal	Bochringer Mannheim
AT8	1:10,000, mouse monoclonal	Boerhinger Mannheim
βA4 (6F3D)	1:100, human monoclonal	DAKO
COX2	1:10,000, mouse monoclonal	Transduction Laboratories
calbindin	1:1000, rabbit polyclonal	MRC Labs
ChaT	1:500, mouse monoclonal	Sigma
GFAP	1:1000, rabbit polyclonal	DAKO
MAP2	1:100, mouse monoclonal	Boehringer Mannheim
NSE	1:1000, mouse monoclonal	AFFINITI
synaptophysin	1:100, mouse monoclonal	SIGMA
S182-N19	1:50, goat polyclonal	Santa Cruz Biotech.
S182-C20	1:100, goat polyclonal	Santa Cruz Biotech.
tau	1:500, mouse monoclonal	Sigma

Drugs

- poly-L-lysine, poly-D-lysine, nickel chloride, nitro blue tetrazolium, hydrogen peroxide, β nicotinamide adenine dinucleotide, glycerol, diaminobenzidine, 8bromocyclicGMP, DMSO, DEPC, kainate, NMDA, DTT, EDTA, copper sulphate, ammonium sulphate, glycine, LPS, sodium dithionite, amphotericin B, Hb, indomethacin, PGE2, SNP, W7, SNAP, SIN-1. *SIGMA*

- sodium chloride, sodium dihydrogen orthophosphate, sodium hydrogen phosphate, sodium thiosulphate, sodium citrate, ethanol, sodium acetate, coverslips. *BDH*

- paraformaldehyde, acetic acid, sodium pyrophosphate. *ALDRICH CHEMICAL CO.*

- dextran sulphate, terminal deoxynucleotidyltransferase buffer. *BOERINGER MANNHEIM.*

- [α-^{35}S]dATP. *DUPONT.*

- Dulbecco's modified Eagle's medium, Dulbecco's modified Eagles medium nutrient mixture F12, B27 supplement, heat inactivated fetal calf serum, Hank's balanced salt solution, trypsin, trypsin-EDTA, penicillin-streptomycin, normal horse serum. *GIBCO.*

- ABC complex, mormal goat serum. *VECTOR LABORATORIES.*

- MK801 ondansetron, aniracetam. *RESEARCH BIOCHEMICALS.*

- APV, AMPA, ACPD, CNQX. *TOCRIS NEURAMIN.*

- neutral red. *GURR.*

- GYKI. *Gift from Prof. Stone, University of Glasgow.*

Chemicals

- film, emulsion. *KODAK.*
- DPX mountant. *BDH.*
- histoclear. *NATIONAL DIAGNOSTICS*
- sephadex G25 medium. *PHARMACIA*
- analytical grade alcohol. *ANALAR*
- Triton-X-100, formic acid. *SIGMA*
- silica gel. *FISONS*

Solutions

Hybridisation buffer	50% deionised formamide, 0.02% polyadenylic acid, 10% 4x st saline citrate solution, 5% 0.5M sodium phosphate, 1% sodium pyrophosphate and 10% dextran sulphate. Made up in DEPC-tr water.
1 x standard saline citrate solution (SSC)	0.15M sodium chloride, 0.015M sodium citrate, pH 7.0.
1 x PBS	3.8g NaCl, 4.05ml 1M disodium hydrogen phosphate, 0.95ml 1 sodium dihydrogen phosphate, made up to 500ml with distilled
DEPC water	1ml DEPC per litre of deionised water
Tris-EDTA buffer	10mM Tris, 1mM EDTA in DEPC water
1M DDT	3.09g DTT in 20ml 0.01M sodium acetate, pH 5.2
hydrogen peroxide	300 ml methanol, 3ml hydrogen peroxide
acid-alcohol	0.5% HCl, 70% ethanol

haematoxylin	100% solution (Sigma)
Neutral Red	0.2%, 1ml GAA
Koelle Medium	500mg copper sulphate, 750mg glycine moxed in 750ml of disti water. Add: 74ml of 0.2M acetic acid and 176ml of 0.2M sodiu acetate. Buffer solution to pH 5.0 with sodium hydroxide. Store fridge.
sulphide solution	In fume hood mix: 10ml of 1M acetic acid, 40ml of distilled wa a few drops of ammonium sulphide.

Oligonucleotide probes

MAP2	complementary to the region coding for the N-terminal 15 amino acids common to all MAP2 isoforms (Kindler et al 1990).
tau1	complementary to the nucleotide encoding bases 752 to 909 excluding 796 to 987
tau2	complementary to the nucleotide encoding bases 796 to 788

Chapter 3

Tau gene expression.

Introduction

As has been discussed in Chapter 1 section 1.5.2.1, the six isoforms of tau can be divided into Tau 1 and Tau 2. In the present study, radiolabelled oligonucleotide probes were used to investigate the effects of various compounds related to cognition and/or possible tau modulation on Tau 1 and Tau 2 mRNA expression in adult rats. The basal levels of expression of Tau1 and Tau2 mRNA in adult and neonatal rats was also investigated. In parallel with these experiments, tau immunoreactivity and gene expression levels were studied *in vitro* in primary neuronal basal forebrain cultures, and these results are discussed in Chapter 5.

In the present study, the following compounds were administered intraperitoneally to adult rats:

3.1 *Ondansetron*

Ondansetron is a 5HT-3 receptor antagonist which along with its well documented anti-emetic properties, has been shown to have cognitive enhancing properties, for example by reversing the scopolamine-induced deficit in the mouse dark-light box (Barnes et al.,1990). The compound at a dose of 1mg/kg has recently been shown to ameliorate the performance deficits of the anticholinergic drug scopolamine in mice (Roychoudhury and Kulkarni., 1997). The action of ondansetron in this and other behavioural paradigms has led to the suggestion that the drug may be of some clinical importance in the treatment of cognitive impairment such as in age-associated memory impairment in non-disease states (Crook and Lakin.,1991). However, initial clinical trials with the drug have failed to show any ability to improve cognition (Hall and Ceuoens.,1990).

3.2 *MK801*

MK801 is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist that binds with high affinity to the phencyclidine binding site (Clineschmidt et al.,1982). It has been described as both a neuroprotective agent (Gill et al.,1988; Sato et al.,1988), and a

neurotoxin (Olney et al.,1989). It protects against ischaemic damage (Gill et al.,1988) and is a potent anticonvulsant (Sato et al.,1988). In cell culture, glutamate has been shown to increase tau immunoreactivity (Sindou et al.,1992; Couratier et al.,1995). The effects of glutamate and other excitatory amino acids on cytoskeletal protein immunoreactivity in cell culture is discussed in Chapter 5. In parallel with those results, the use of MK801 enabled investigation of NMDA receptor response to tau gene expression in vivo. MK801 is lipid soluble and thus able to cross the blood brain barrier. Some of the behavioural effects induced by the drug are similar to those of phencyclidine, for example, dysphasia and hallucinations.

3.3 *GYKI52466*

Excessive activation of glutamate receptors is thought to play a crucial role in the pathogenesis of a number of neurological disorders including epilepsy and ischaemia (Choi et al.,1988; Rothman and Olney.,1986). Consequently, glutamate antagonists may be useful as therapeutic agents in such disorders. The selective protective effects of the benzodiazepine compound GYKI52466, and the quinoxaline-dione compound NBQX have been reported in the four vessel occlusion model of transient global ischaemia in rats (Le Peillet et al.,1992). Cerebroprotection by GYKI52466 is thought to be due to blockade of the action of glutamate on postsynaptic non-NMDA receptors; evidence suggests that GYKI52466 is a potent antagonist at AMPA and kainate receptors with little or no action at NMDA receptors (Ouarduoz and Durand.,1991; Moncada et al.,1991). Further evidence suggests that that one possible mechanism by which GYKI52466 produces neuroprotection following transient ischaemia is by prevention of an increase in extracellular glutamate concentration (Arvin et al.,1992).

Glutamate toxicity has been shown to induce a concentration-dependent increase in tau immunoreactivity in primary neuronal cultures (Mattson.,1990; Sindou et al.,1992). The use of GYKI52466 in the present work was to investigate any possible effects of non-NMDA receptor action on tau gene expression in vivo.

3.4 *Indomethacin*

Non steroidal anti-inflammatory drugs (NSAIDS) are among the most widely used therapeutic agents. As has been discussed in 1.10.1, there has recently been evidence suggesting an inverse relationship between NSAID use and onset of Alzheimer's Disease. NSAIDs are thought to reduce the risk of developing the disease (Breitner et al.,1994; McGeer et al.,1992; Rich et al.,1995). NSAIDs inhibit cyclooxygenase, the enzyme responsible for the production of prostaglandins. Ideally, NSAIDs would selectively inhibit the inducible isoform of cyclooxygenase; inhibition of the constitutive form causes the side effects such as gastric and renal damage, and skin reactions. Previous work with indomethacin has used an intraperitoneal dose of 5mg/kg (Bret-Dibat et al.,1994). The plasma half life of the drug is short, around 30 mins in adult humans. A dose of 20mg/kg has been shown to result in 30% mortality in adult rats (Beyer., 1978). In the present study, adult rats were exposed to 5mg/kg indomethacin to investigate any possible effects of the NSAID on Tau1 of Tau2 gene expression.

3.5 *Aniracetam*

Aniracetam is a member of the nootropic class of drug, and has been shown to improve the condition of elderly patients suffering from slight to moderate mental deterioration (Canonico et al.,1991). It is thought that aniracetam may enhance the efficacy but not the potency of AMPA-induced calcium influx in cerebellar granule cells ((Gouliaev and Senning .,1994). Previous work has used doses of between 10 and 300mg/kg intraperitoneally in rats (Consolo et al.,1990; Chopin et al.,1992), and for the present work a dose of 10mg/mg was chosen.

3.6 *In situ hybridisation measurements*

As discussed in Chapter 2 section 2.3, the method for in situ hybridisation in this section of work and also that in Chapter 5, was as described by Morris (1989; 1992). The specificity of the hybridisation protocol is suggested by various pieces of evidence. First, the high stringency conditions employed prevent the hybridisation of the

probes to any other sequences in the tissue. The unique and well documented pattern of Tau1 and Tau2 gene expression in neonatal and adult tissue indicates that these probes are specific. The frequent use of the probes in our laboratory indicates that the hybridisation conditions used allow specific localization of the mRNA sequences to which the probes are complimentary. However, it is worth mentioning the control systems normally employed for in situ hybridisation to prove that hybridisation signals detected represent the authentic complementary mRNA. The controls include hybridisation with a labelled sense oligonucleotide, pretreatment of tissue sections with RNAse A, Northern blots, and the competitive inhibition of the hybridisation signal by excess unlabelled oligonucleotide.

Results analysis was performed by taking careful measurements of the number of pixels occupied by silver grains in defined areas of the basal forebrain and hippocampal CA1 regions. Between ten and fifteen similarly sized areas in each region were marked out. The number of pixels occupied by silver grains within each measured, ensuring that all of the basal forebrain and hippocampal CA1 regions were accounted for, and that no overlap between marked areas had occurred.

Results

3.7 *Tau gene expression in the basal forebrain and hippocampal regions in adult and neonatal rats.*

Following sectioning of the brain regions, slides were taken for in situ hybridisation using probes for Tau1 and Tau2 mRNA. It can be seen that levels of Tau1 are significantly higher in the neonatal rats in both the CA1 and hippocampal regions [Fig. 3.1.1]. Levels of Tau2 were significantly higher in the CA1 and basal forebrain regions in the adult.

3.8 *Effect of a single drug treatment on tau gene expression.*

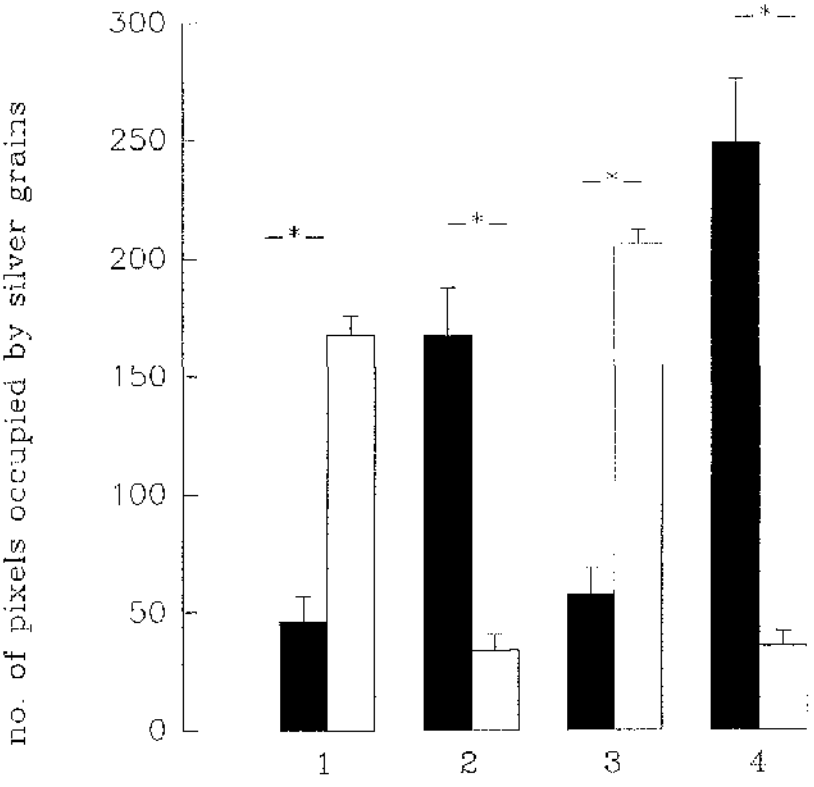
Following intraperitoneal injection of ondansetron (100ng/kg, n=4), MK801 (1mg/kg, n=4), GYKI52466 (30mg/kg, n=4), indomethacin (5mg/kg, n=3), and aniracetam (10mg/kg, n=4) or vehicle (0.9% saline), Tau1 and Tau2 levels were compared in treated and control animals in the basal forebrain and CA1 regions. As can be seen in Fig. 3.2.1, neither Tau1 or Tau2 mRNA levels were significantly altered by a single dose of ondansetron after 24 hours, $p>0.05$ by Student's t test. Fig. 3.2.2 illustrates that a single dose of MK801 did not significantly alter Tau1 or Tau2 levels, again $p>0.05$ by Student's t test. Similarly, a single dose of GYKI53488, indomethacin and aniracetam did not significantly alter Tau1 or Tau levels as illustrated in Figs 3.2.3, 3.2.4 and 3.2.5, $p>0.05$ by Student's t test.

Figure 3.1.1 : Comparison of tau gene expression in the basal forebrain and CA1 hippocampal regions of adult and neonatal rats.

The brains were removed from adult and neonatal (E17) rats, and sections prepared for in situ hybridisation.

Results obtained from autoradiographic films are expressed as mean relative optical density levels ± SEM.

Statistical analysis was carried out using the Student's t-test.
* $p<0.05$.



Data are mean ± SEM, n=4

* $p<0.05$ by two-tailed Student's t test (comparisons indicated by −*−)

1 T1, basal forebrain

2 T2, basal forebrain

3 T1, CA1 hippocampus

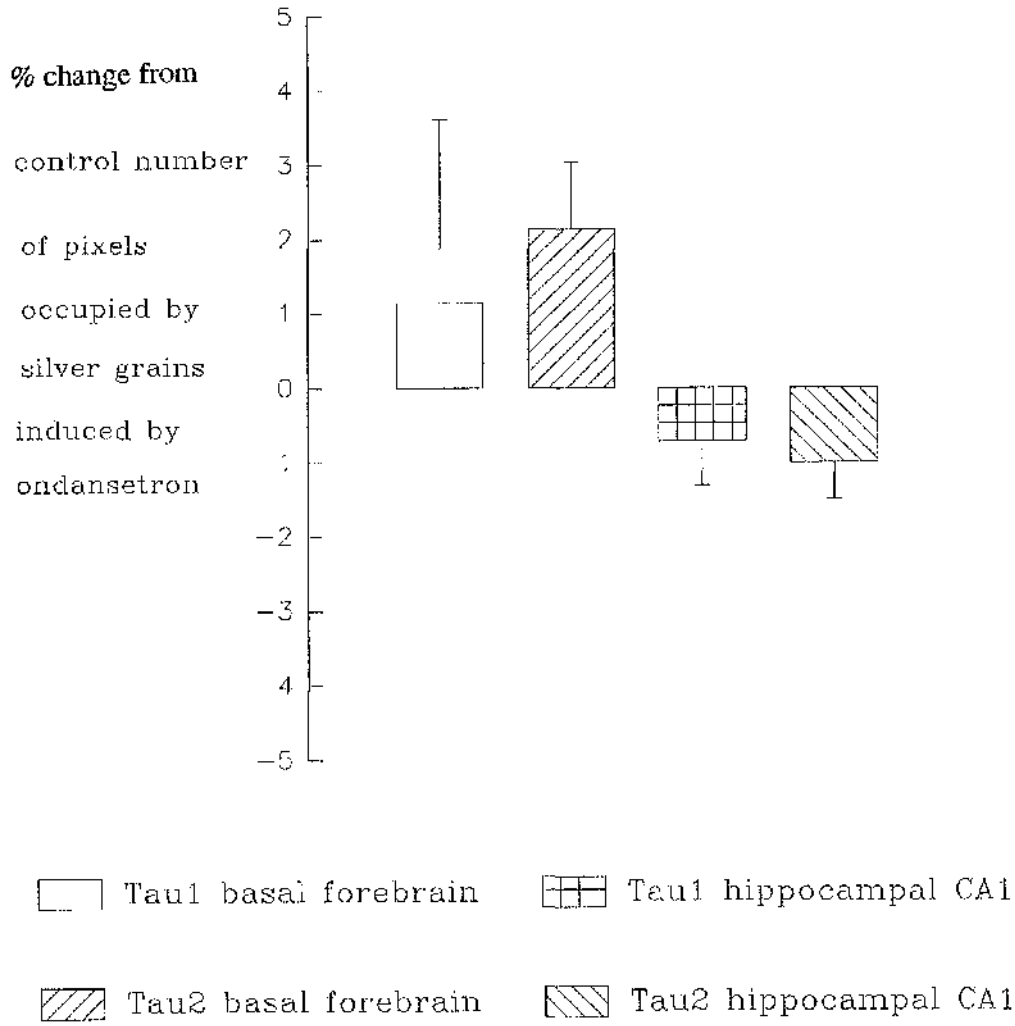
4 T2, CA1 hippocampus

Figure 3.2.1 : Effect of a single dose of ondansetron on tau gene expression in adult rats.

Adult rats were intraperitoneally injected with vehicle (0.9% saline) or ondansetron (100ng/kg). After a time period of 24 hours, animals were killed, their brains removed, and sections processed for in situ hybridisation.

Results obtained from autoradiographic film are expressed as a percentage change of control values.

Statistical analysis was carried out using the one sample t-test. No significant changes were observed.



Data are mean + SEM, n= 4 control, 4 treated.

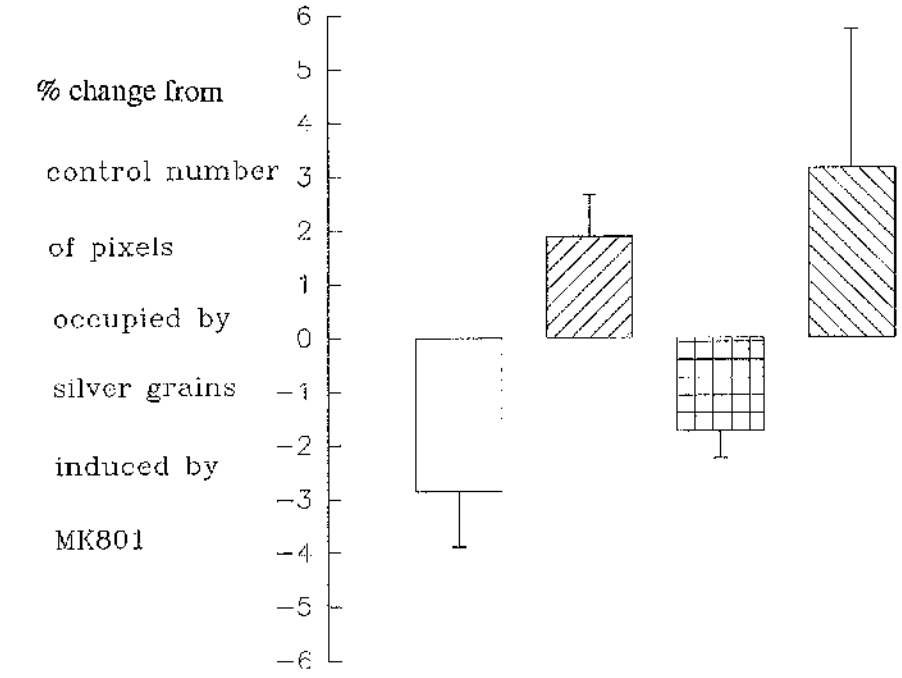
Two tailed P value by one sample t-test $p>0.05$.

Figure 3.2.2 : Effect of a single dose of MK801 on tau gene expression in adult rats.

Adult rats were intraperitoneally injected with vehicle (0.9% saline) or MK801 (1mg/kg). After a time period of 24 hours, animals were killed, their brains removed and sections processed for in situ hybridisation.

Results obtained from autoradiographic film are expressed as a percentage change of control values.

Statistical analysis was carried out using the one sample t-test. No significant changes were observed.



Data are mean ± SEM, n= 4 control, 4 treated.

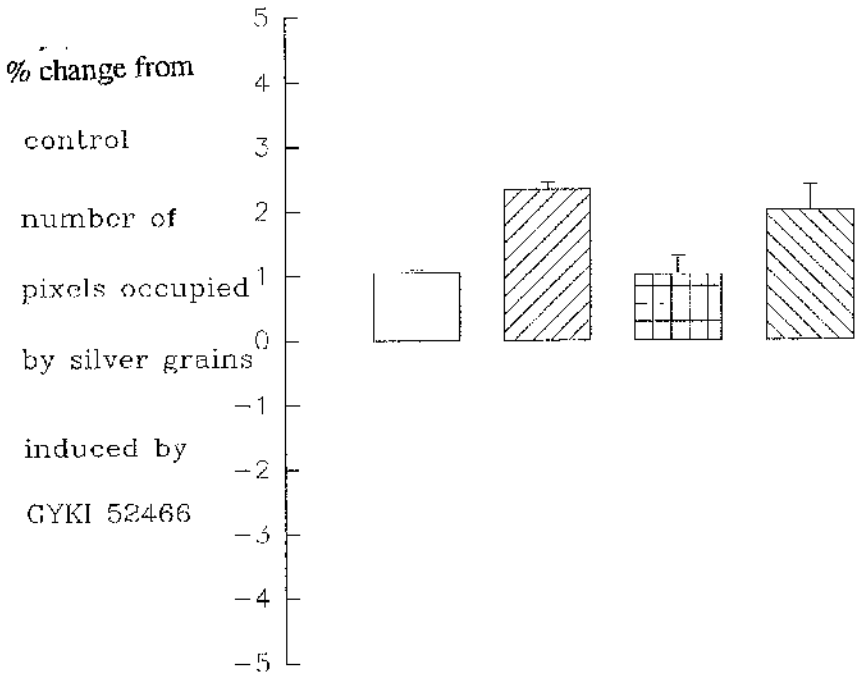
Two −tailed p value by one sample t−test, $p>0.05$.

Figure 3.2.3 : Effect of a single dose of GYKI53488 on tau gene expression in adult rats.

Adult rats were injected intraperitoneally with vehicle (0.9% saline) or GYKI53488 (30mg/kg). After a time period of 24 hours, the animals were killed, their brains removed, and sections processed for in situ hybridisation.

Results obtained from autoradiographic film are expressed as a percentage change of control values.

Statistical analysis was carried out using the one sample t-test. No significant changes were observed.



Data are mean ± SEM, n= 4 control, 4 treated.

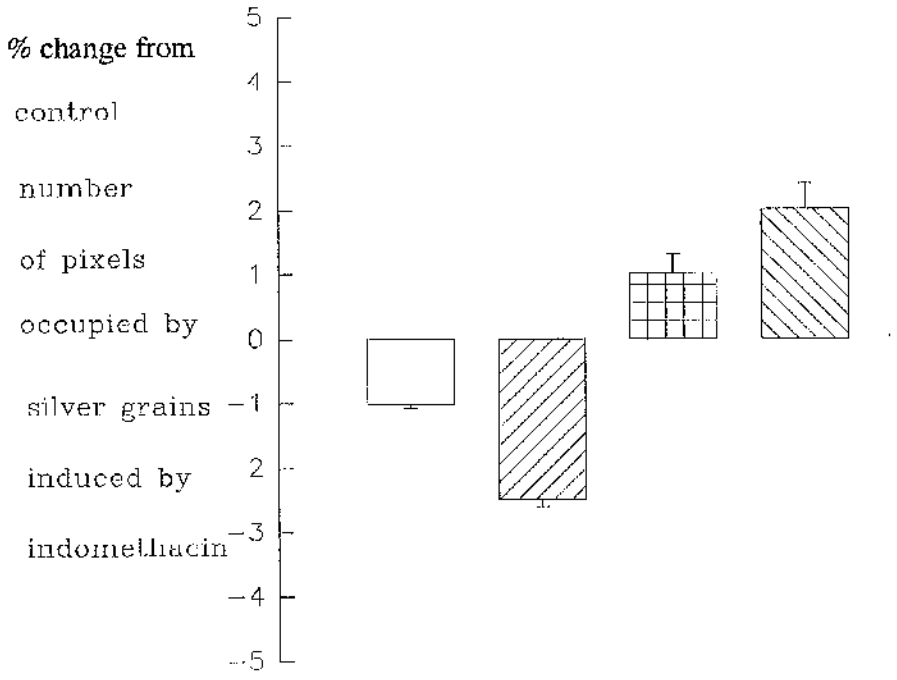
Two tailed P value by one sample t−test, $p>0.05$

Figure 3.2.4 : Effect of a single dose of indomethacin on tau gene expression in adult rats.

Adult rats were injected intraperitoneally with vehicle (0.9% saline) or indomethacin (5mg/kg). After a time period of 24 hours, the animals were killed, their brains removed, and sections processed for in situ hybridisation.

Results obtained from autoradiographic film are expressed as a percentage change of control values.

Statistical analysis was carried out using the one sample t-test. No significant changes were observed.



Data are mean + SEM, n= 4 control, 4 treated.

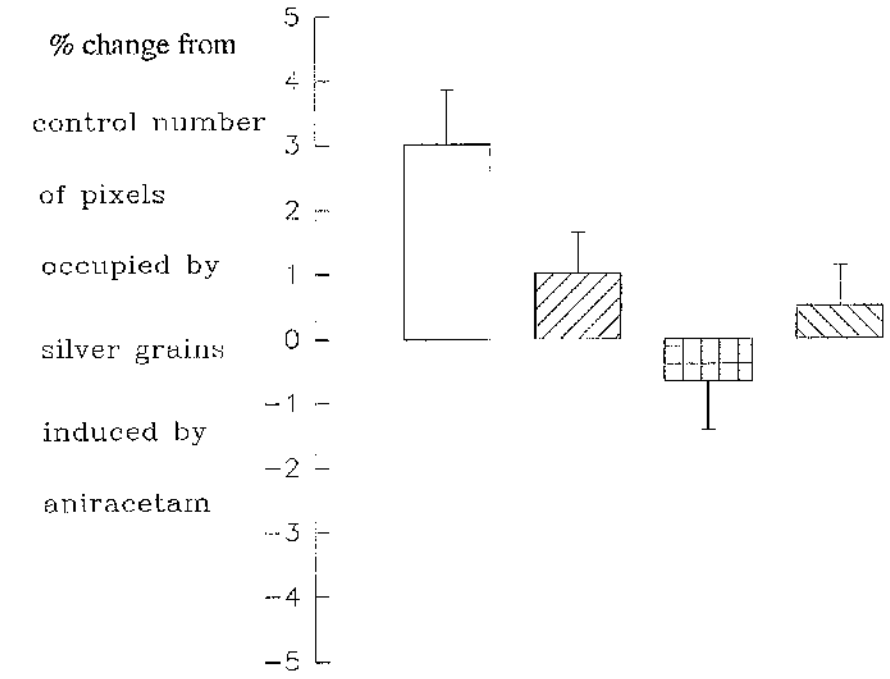
Two tailed P value by one sample t−test,p>0.05

Figure 3.2.5: Effect of a single dose of aniracetam on tau gene expression in adult rats.

Adult rats were injected intraperitoneally with vehicle (0.9% saline) or indomethacin (10mg/kg). After a time period of 24 hours, the animals were killed, their brains removed, and sections processed for in situ hybridisation.

Results obtained from autoradiographic film are expressed as a percentagechange of control values.

Statistical analysis was carried out using the one sample t-test. No significant changes were observed.



Data are mean ± SEM, n= 4 control, 4 treated.

Two tailed P value by one sample t−test, $p>0.05$.

Discussion

3.9 *Tau gene expression in the basal forebrain and CA1 hippocampal regions in adult and neonatal rats.*

Goedert and colleagues have demonstrated the existence of six isoforms of human tau protein ranging from 352 to 441 amino acids in length (Goedert et al.,1989a; Goedert et al.,1989b). Type 1 and Type 2 tau proteins mRNAs are thought to be developmentally regulated. Type 1 mRNA encoding the three repeat isoforms are expressed throughout life, whereas Type 2 transcripts encoding the extra amino acids were absent from the human fetal brain but expressed in the adult brain throughout life. This indicated that tau is a nervous system specific protein, and that tau mRNA is subject to developmentally regulated alternative splicing (Goedert et al.,1989a). A developmental change in mRNA levels for the microtubule associated protein MAP2 has been reported in the rat brain (Garner and Matus.,1988). The levels of transcripts encoding the three and four repeat isoforms of human tau have been shown to be unaltered in the cerebral cortex of Alzheimer's Disease patients (Goedert et al.,1989b; Goedert et al.,1989a). Multiple tau isoforms are found in neurofibrillary tangles, and it is unlikely that the overexpression of Type 1 or Type 2 protein mRNAs causes the formation of paired helical filaments (Goedert et al.,1989b).

A comparison of Type 1 and Type 2 tau protein in neonatal and adult rat brain agrees with previous evidence; Type 1 mRNA is high in the neonatal brain, while Type 2 is abundant in the adult. The juvenile tau consists of a single major component and the transition from this simple pattern to the more complex pattern of 4-6 bands is thought to begin around postnatal day 15 and be completed by postnatal day 35 (Couchie and Nunez.,1985). The neonatal rats used in the present work were at gestation day 17, so as expected very little Type 2 tau was seen. Detailed development studies on tau in the human brain have not been carried out so it is possible that the pattern of expression in embryonic human brain may be similar to that seen in the rat brain. It is thought that stage and cell specific expression of at least some types of tau occurs in the human brain (Goedert et al.,1989a).

3.10 *Effect of single dose intraperitoneal injections on tau gene expression in adult rats.*

None of the drugs used had any effect on tau expression in either the hippocampus or basal forebrain. It is important to discuss a number of considerations that should be made regarding the experimental procedures. First, whether the drugs reached the target sites, and secondly, whether the time points and concentrations used were effective. It would be of interest to repeat the experiments using different time points up to 24 hours. A more stringent experimental procedure would apply a range of concentrations.

Ondansetron:

No visible behavioural effects were observed, and given that no change in Tau1 or Tau2 mRNA levels occurred, it is debatable as to whether the drug reached the target sites.

MK801:

Following the intraperitoneal injections of the drug, the rats displayed a transient sedation phase for approximately 30 minutes. This would suggest that the drug had reached its target, and MK801 is lipid soluble, and thus able to cross the blood brain barrier. Tau1 and Tau2 mRNA levels are thus unlikely to be affected by this dosage.

GYKI52466:

The effects of benzodiazepines on the CNS include reduced anxiety and aggression, and sedation. Approximately 40 minutes after treatment, the rats displayed marked sedation, suggesting that the drug has reached its target site.

Indomethacin:

No behavioural effects were seen following treatment with indomethacin, although with NSAIDS behavioural side effects aren't

normally apparent. No changes in Tau1 or Tau2 mRNA levels were seen, however, perhaps a more thorough experiment would have made use of different time points and doses.

Aniracetam:

The actions of aniracetam in the brain have been debated. The compound is thought to be far from ideal as a drug; its effect on AMPA receptors is thought to be weak and short term, with little of the drug reaching the brain as it is broken down by enzymes and acids in the stomach (Service .,1994). In light of this, perhaps a larger dose of the drug should have been used, and a shorter time point investigated.

Summary

Using in situ hybridisation, the expression of Tau 1 and Tau 2 mRNA in neonatal and adult rats, and, in adult rats in response to intraperitoneal drug injections has been investigated. In agreement with the literature, Tau 1 mRNA levels were higher in the neonatal brain, and Tau 2 mRNA levels higher in the adult brain. Both Tau 1 and Tau 2 mRNA levels remained unaltered following intraperitoneal injection of the listed compounds. The results of these experiments, with the conditions mentioned, suggest that expression of the tau gene does not show a great deal of plasticity. However, it is possible that a shift in expression of one of the other splice variants of tau may go undetected just comparing type1 and type2 tau mRNA levels.

Leading on from these experiments, tau protein and mRNA expression has been investigated *in vitro* using primary dissociated neuronal cultures, and these results are discussed in Chapter 5. In view of the fact that in the neuronal cell culture experiments, cellular levels of the cytoskeletal protein MAP2 change after exposure to excitatory amino acids and nitric oxide releasers with no change in tau, perhaps it would be of interest to repeat these experiments using radiolabelled oligonucleotide probes to monitor MAP2 mRNA levels.

Chapter 4

Regulation and expression of NADPH diaphorase immunostaining in glial cultures.

Introduction

This section of work made use of glial cell cultures. As described in Chapter 2, section 2.1.2, the method of cell culture is based on that of McCarthy and De Vellis (1980). As discussed in the following introductions, excitatory amino acids, interleukin and proteases have been shown to have possible involvement in the neuropathology of Alzheimer's Disease. The aim of the present work was to investigate the effects of these compounds on NADPH diaphorase immunostaining in glial cells in culture.

4.1 *Excitatory Amino Acids*

Introduction

Excitatory amino acids (EAAs), such as glutamate and aspartate, are the major excitatory neurotransmitters in vertebrates. Ionotropic EAA receptors have been classified into three major subtypes based on their differential sensitivity to α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) as selective agonists. Glutamate binds with high affinity to all three subtypes of receptor. More recently, a novel class of EAA receptors was discovered; the metabotropic receptors. These receptors are linked to either stimulation of inositol phospholipid hydrolysis or to inhibition of phospholipase C activation. NMDA, AMPA and kainate receptors can be further subdivided into non-NMDA (AMPA and kainate), and NMDA receptors. The ion channel activated by the NMDA receptor has two distinguishing characteristics: it is permeable to both sodium and calcium and is blocked by magnesium in a voltage-dependent manner. Via an influx of calcium through the channel, cGMP synthesis is stimulated. This stimulation is blocked by magnesium and the selective NMDA receptor antagonist D-(-)-2-amino-5-phosphonovaleric acid (APV).

In the present study, the following compounds have been used to investigate the effects of NMDA and non-NMDA-receptor induced changes in NADPH diaphorase immunoreactivity in glial cell cultures: glutamate, AMPA, an AMPA antagonist- CNQX, NMDA and the selective NMDA receptor antagonist APV.

EAAs and Neurodegeneration

Besides its role as the major excitatory neurotransmitter in the mammalian nervous system, glutamate has been shown to cause degeneration. Lucas and Newhouse first demonstrated the ability of systemically injected glutamate to cause degeneration in the retina 40 years ago (Lucas and Newhouse.,1957). Olney showed that exogenous glutamate causes CNS lesions 12 years later (Olney.,1969). These findings led to the 'excitotoxic' hypothesis of glutamate's action (Olney et al.,1971). Excessive release, abnormal leakage or impaired uptake of glutamate or, altered glutamate receptor sensitivity have all been postulated as possible causes of death in many neuropathological disorders such as Huntington's disease, ischaemia, epilepsy and Alzheimer's Disease.

In addition to the well-established cholinergic deficits, other neurotransmitters are also affected in Alzheimer's Disease such as biogenic amines, amino acids and neuropeptides (Hardy et al.,1985;Gottfries.,1990). In Alzheimer's Disease, cortical and hippocampal pyramidal neurons, which are particularly affected by neurofibrillary tangles and neuronal degeneration, use glutamate as a neurotransmitter (Maragos et al.,1987). Loss of glutaminergic terminals and reduction of glutamate content in these structures have been reported (Cross et al.,1986; Procter et al.,1988; Lowe et al.,1990). A decrease in gluatamic acid levels has been described in the outer molecular layer of the dentate gyrus , the major target of the entorhinal projection to the hippocampus, and in the neocortex in Alzheimer's Disease (Hyman et al.,1987; Hyman et al.,1984). The anatomical distribution of glutamate metabotropic binding sites in comparison to AMPA and kainate binding has been studied in the hippocampal region of Alzheimer's Disease brains, and significantly reduced levels of glutamate metabotropic and AMPA binding sites in the diseased tissue were observed in the CA1 region (Dewar et al.,1991). Taken together these data suggest a role for EAAs in the pathogenesis of Alzheimer's Disease (Maragos et al.,1987). Discrepant changes in the concentration of several amino acids have been reported in postmortem tissue from Alzheimer's Disease patients. Whether these changes correlate with a modification of the neurotransmission of these amino acids remains to be seen. Increased

levels of glutamine and glycine have been observed, and reduced glutamate and taurine contents. (Alom et al.,1991; Pomara et al.,1992). Conflicting data for aspartate levels in the cortex of Alzheimer's Disease patients has been reported (Sasaki et al.,1986; Lowe et al.,1990). The decrease in glutamate levels seen in cortical projections with decreased choline acetyltransferase levels suggests a regulation of glutaminergic cortical neurons by the chilinergic terminals, and evidence supports a role for glutamate in cortical cholinergic terminals (Szerb and Fine., 1989).

A key link between glutamate receptor activation and subsequent neurodegeneration is thought to be agonist-triggered calcium entry. Small subsets of neurons have been found to possess clacium-permeable AMPA/kainate receptor-gated channels and evidence suggests that some of these neurons are sensitive to calcium-dependent AMPA/kainate receptor mediated injury (Ozawa et al.,1991; Brorson et al.,1992; Weiss et al.,1994). Yin and colleagues demonstrated kainate injury to cultured basal forebrain cholinergic neurons to be calcium dependent (Yin et al.,1994). The heightened vulnerability of basal forebrain neurons to kainate toxicity may reflect rapid calcium entry through calcium-permeable AMPA/kainate channels, and there is the possibility that AMPA/kainate receptor-mediated injury may contribute to Alzheimer's Disease neuropathology (Yin et al.,1994).

Astrocytes perform two functions important for maintaining glutamate concentrations in the extracellular space at nontoxic levels. The first is high-affinity glutamate uptake, the second is the conversion of glutamate to glutamine by glutamine synthase. Structural analysis has shown that astrocytes are associated with glutaminergic synapses and that the fine astrocytic processes of these regions are enriched in glutaminergic synthase (Derouiche and Frotscher.,1991). Removal of astrocyes from mixed clutures has been shown to lead to neuronal death in the presence of high glutamate concentrations (Sugiyama et al.,1989; Rosenberg et al.,1992). Astrocytes play a crucial role in the maintenance of the CNS, and injury to astrocytes may promote neurodegeneration directly or indirectly in diseases such as Alzheimer's Disease. Aβ has been shown to increase the secretion of basic fibroblast growth factor and interleukin-1 from astrocytes (Araujo and Cotman.,1992).

Overproduction of these components can result in increased APP and other plaque components thus promoting plaque development.

Evidence has shown that calcium-mediated glutamate toxicity in primary neuronal cultures induces a concentration-dependent increase in tau immunoreactivity (Mattson.,1990; Sindou et al.,1992). Glutamate has been shown to induce a shift from the lowest to the highest molecular weight tau isoform and an acidification of tau proteins (Sautiere et al.,1992). NMDA has been found to induce a significant increase in tau immunoreactivity in rat embryonic neurons, and this is directly related to the onset of neuronal death (Couratier et al.,1995). This immunoreactivity modification is thought to appear under both fast and slow NMDA-receptor mediated toxicity, under magnesium-free conditions and is prevented by NMDA receptor agonists (Couratier et al.,1995). As mentioned earlier, acute NMDA toxicity is associated with an excessive influx of calcium into neurons; in both hippocampal and cerebellar slices NMDA toxicity is abolished when calcium is omitted from the medium (Choi.,1987). Another potential mechanism underlying NMDA toxicity is the production of NO (Dawson et al.,1992). Direct evidence for the involvement of NO in NMDA toxicity was illustrated in a study showing that inhibition of NOS reduces toxicity (Moncada and Higgins.,1991). Calcium can activate extralysosomal proteases, such as calpains, which are involved in cytoskeletal remodelling. It has been shown that calpains can induce proteolysis of tau protein (Johnson et al.,1989). Couratier and colleagues showed that modifications in tau immunoreactivity are related to neuronal depolarisation ,and that both glutamate and sodium-channel coactivation are together needed to, produce changes in tau immunoreactivity (Couratier et al.,1995).

4.2 *Nitric Oxide Toxicity*

As mentioned previously, NO has been implicated in the neurotoxicity associated with glutamate receptor stimulation (Dawson et al.,1990), and this observation has led to speculation that glutamate receptor stimulation and thus NO may be involved in the pathology of neurodegenerative diseases such as Alzheimer's Disease (Meldrum and Garthwaite.,1990). Mitochondrial damage has also been suggested to be important in the pathology of Alzheimer's Disease (Beal et

al.,1993). Increasing evidence suggests that the NO derivative peroxynitrite anion (ONNO-), formed from a reaction between superoxide anion (O2-) and NO, may be responsible for the neurotoxic effects of NO (Lipton et al.,1993; Radi et al.,1991). Bolanos and colleagues suggested that ONOO- may be the agent responsible for the mitochondrial damage seen in astrocytes following induction of NOS (Bolanos et al.,1994). It has recently been shown that neurons are much more vulnerable to damage following exposure to ONOO- than astrocyes, and that ONNO- synthesised and released by astrocytes under trauma may diffuse to neuronal target cells with damaging consequences (Bolanos et al.,1995). This mechanism may explain the the neurotoxicity associated with NO and mitochondrial dysfunction in neurodegenerative diseases.

The use of the calmodulin antagonist W7 in the present work has enabled an investigation into the possible type of NOS involved in any compound-induced change in NADPH diaphorase immunostaining.

4.3 *Proteases*

Proteases are thought to be involved in the neuropathology of Alzheimer's Disease. Serine protcases such as activated complement factors have been found in plaques (Carrell et al.,1986) and, the serine protease inhibitor α1-antichymotrypsin domain is located within APP (McGrogan et al.,1988). As discussed in Chapter 1 Section 1.4.4, studies of the proteolysis of Aβ revealed three distinct pathways. Candidates for the proteases involved in the generation of soluble and potentially amyloidogenic Aβ have been researched. An imbalance between proteases and their inhibitors may play a role in the neuropathology of Alzheimer's Disease.

Thrombin, a serine protease of the blood coagulation system, has been shown to have profound effects on cellular activation in vitro. It is mitogenic for fibroblasts and astrocytes (Cavanaugh et al.,1990), inhibits neurite outgrowth of neuronal cells (Grand et al.,1989) and reverses the stellation of astrocytes (Cavanaugh et al.,1990). Thrombin has also been shown to regulate a number of cellular events in the nervous system (Dihanich et al.,1991), and it is thought that a balance between thrombin and thrombin-like proteinase

on astrocyte surfaces may be crucial for normal astrocyte function (Cavanaugh et al.,1990). The levels of NGF secreted by primary astrocytes are enhanced when cells are cultured in the presence of α-thrombin (Neveu et al.,1993). It is thought that prothrombin synthesised in the brain could constitute an important source of protease able to modulate the expression of NGF. A synergistic effect of nitric oxide synthase and a serine protease has been observed in endothelial cell cultures (Stamler et al.,1992). Serine proteases and their inhibitors have been found in amyloid fibrils (Gollin et al.,1992), and are thought to have an important role in the neuropathology of Alzheimer's Disease.

The present work used the following proteases and inhibitors : α-chymotrypsin and its inhibitor chymostatin, trypsin and trypsin inhibitor.

4.4 *LPS and IL-1*

Microglial cells, the brain resident macrophages, are thought to be involved in most inflammatory and degenerative diseases in the CNS. Like peripheral macrophages, they can be stimulated by lipopolysaccharide to release NO and prostanoids (Chao et al.,1992; Lee et al.,1995). The relationship between LPS and iNOS in glial cells has previously been discussed in detail.

A role for interleukin-1 (IL-1) as a mediator of astrocyte activation and growth was first demonstrated by Giulian and coworkers (Giulian and Lachman.,1985). It was shown to induce proliferation of astrocytes cultured from neonatal rat brains, and when injected into the brain to induce reactive gliosis and enhanced immunoreactivity for GFAP protein. The cytokine is produced by astrocytes and microglia (Giulian et al.,1986), and has been shown to cause the induction of the inducible form of NOS (iNOS) in astrocytes (Lee et al.,1993). Conflicting evidence has emerged concerning the precise role of IL-1. It is now thought that the pattern of astrocyte activation in response to IL-1 is that of a stress response rather than a response to a growth factor (Dinarello.,1994). It is still thought to be a key candidate in the induction of reactive gliosis (Lee et al.,1995). Other cytokines such as interferon-χ are thought to act as priming signals for astrocytes (Lee et al.,1995). Thus, regulation of the IL-1

system may underlie many of the common pathological processes involving reactive gliosis. APP mRNA was found to be enhanced by IL-1 in primary neuronal cultures, and thus may have a role in the neuronal mechanism related to Aβ deposition (Forloni et al.,1992).

As both LPS and IL-1 are well documented inducers of iNOS in cultured glial cells, these compounds were chosen as positive controls in the present work, and used to illustrate an increase in NADPH diaphorase immunostaining.

Results

4.5 *Characterisation of glial cultures.*

In cells cultured from the basal forebrain region of postnatal rats (P2), a high proportion of the cells were identified as glial cells on the basis of their large flat epithelial-like morphology and characteristic positive immunostaining for glial acidic fibrillary protein (GFAP), revealing large unstained nuclei [Fig. 4.1a]. The glial cells were typically 100μm in diameter. A small proportion of neuronal cells was present, clearly distinguishable from the glial cells on the basis of their morphology. To reduce contamination by neuronal cells, the culture flasks were stirred between platings to dislodge the neuronal cells which proved to be less robust than the glial cells. The identity of the neuronal cells was confirmed by in situ hybridisation using an oligonucleotide probe specific for microtubule-associated protein 2 (MAP2) mRNA which is found only in neuronal cell types. After one week in culture, the cells were shaken on an orbital shaker to dislodge oligodendrocytes.

The cultured glial cells stained positive for NADPH diaphorase [Fig. 4.1b]. Processes with stellate-like morphology could be seen with the staining. These flat structures were long (100μm) and had swellings along their length.

Figure 4.1 Characterisation of rat glial culture cells.

Cells were processed for immunocytochemistry using antisera against:

A GFAP **Photograph illustrates glial cell body at high magnification.**

Cells were also processed for:

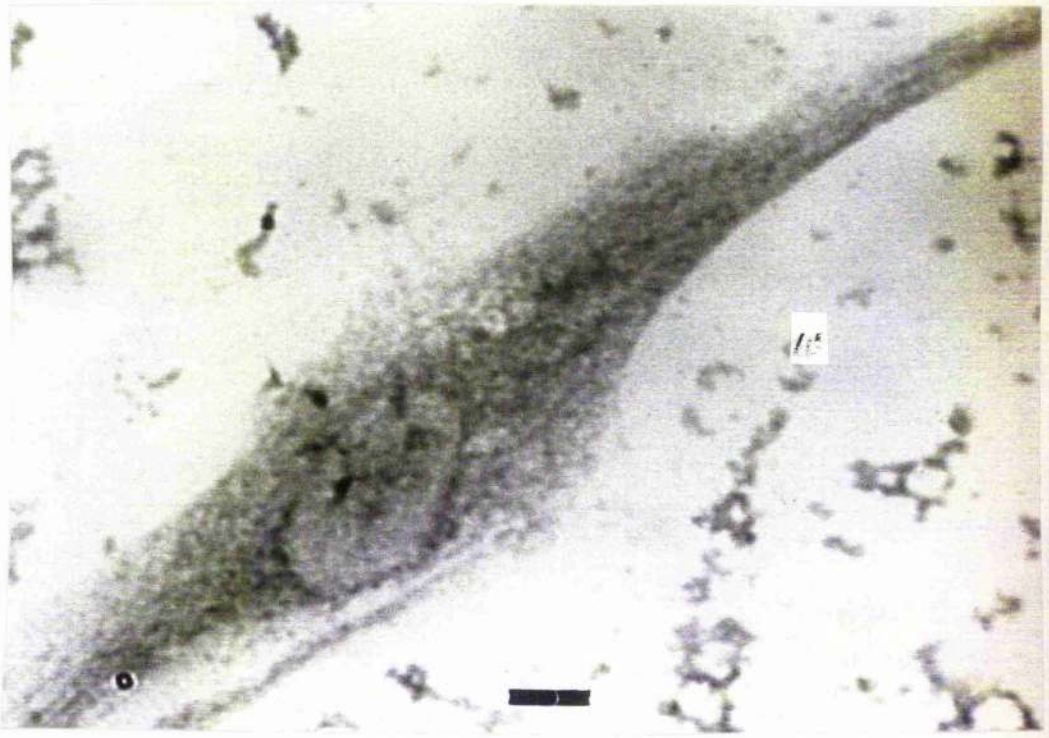
B NADPH diaphorase staining **Photograph illustrates glial cell body at low magnification.**

Scale bar represents:

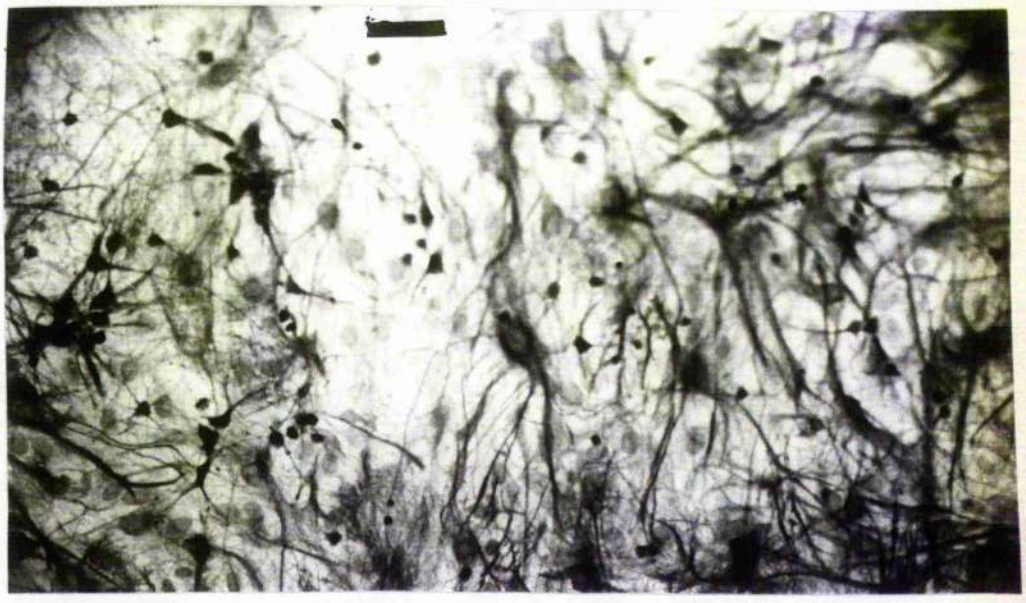
A 5μm.

B 100μm.

A



B



4.6 *Effect of excitatory amino acids on NADPH diaphorase levels in glial cultures.*

Cultures were treated with 50μM glutamate, 50μM AMPA or 50/100μM NMDA made up in HBSS and incorporated directly into the culture medium. Control wells recieved HBSS alone. Cells were exposed to the drugs for a time period of 24 hours. Cells were also treated with 50μM glutamate along with 100μM of the inhibitor APV for 24 hours, and 50μM AMPA plus 100μM of the inhibitor CNQX, also for a time period of 24 hours.

Analysis of variance revealed that treatment with the excitatory amino acids glutamate and AMPA had resulted in a significant increase in NOS levels [Figs 4.2.1, $F(4, 186) = 55.84$, $p<0.001$, $p<0.05$, n = 7 cultures]. NMDA treatment also resulted in a significant increase in NOS levels [Fig. 4.2.2, $F(2,140) = 25.17$, $p<0.001$, $p<0.05$, n = 6 cultures].

Treatment of the cultures with the inhibitors APV(100μM) and CNQX(100μM) reduced the glutamate and AMPA-induced increases in NADPH diaphorase activity to near control levels. The values resulting from simultaneous treatment with glutamate and APV, and AMPA and CNQX for a time period of 24 hours are significantly different from those obtained by agonist alone [Fig. 4.2.1, $p<0.05$ by Tukey's pairwise test.].

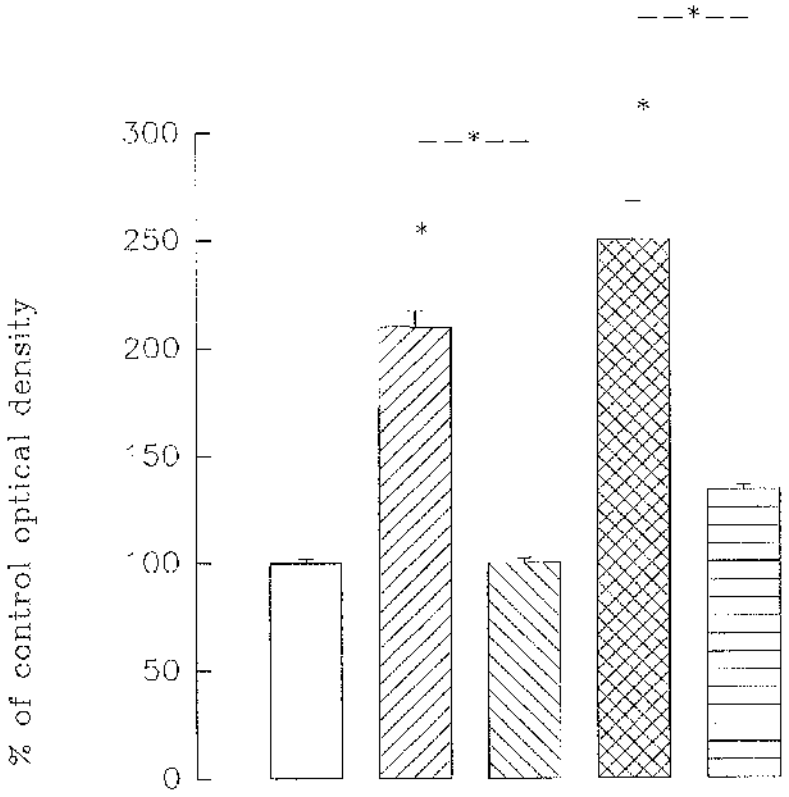
In order to further investigate the involvement of NOS and identify the type of NOS present in the glial cells and increased in the presence of the excitatory amino acids, cultures were exposed to glutamate (50μM) for 24 hours, W7 (400μM) for 4 hours prior to fixation, and the two compounds simultaneously. As shown previously, 50μM glutamate caused an increase in NADPH diaphorase activity. Analysis of variance revealed that Treatment with 400μM W7 had reduced the glutamate-induced increase to near control levels [Fig. 4.2.3, $F(2, 30) = 30.32$, $p<0.001$, $p<0.05$ in Tukey's pairwise test, n = 6 cultures].

Figure 4.2.1 Effect of excitatory amino acids on NADPH diaphorase levels in **postnatal** rat glial culture.

postnatal day 2 (P2) rat glial cultures were treated with 50μM glutamate, 50μM AMPA, coadministration of 50μM glutamate with 100μM APV, orcoadministration of 50μM AMPA with 100μM CNQX in HBSS for 24 hours. Control wells received HBSS alone.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ±SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test, and post-hoc Tukey's pairwise test. *$p<0.05$.



Data are mean ± SEM, n=7 cultures.

ANOVA as follows: $F[4, 186] = 55.84$, $p<0.001$

* $p<0.05$ compared to control by Dunnett's

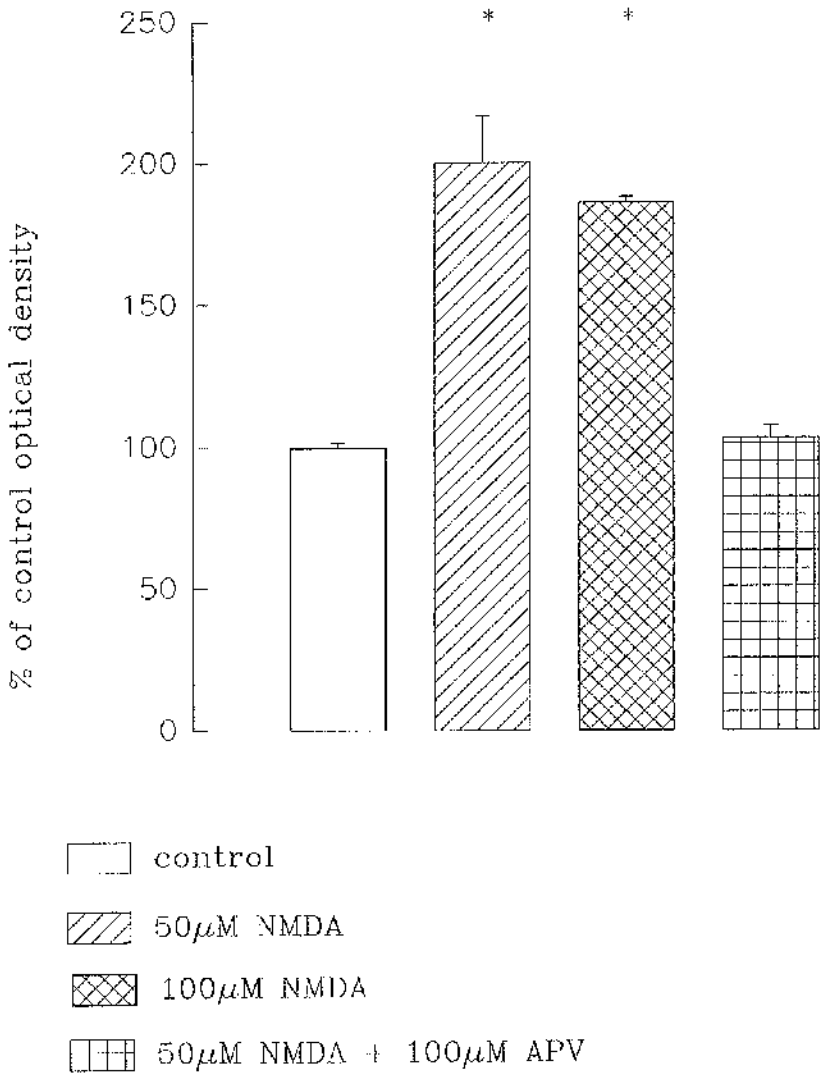
−*− $p<0.05$ by Tukey's pairwise test.

Figure 4.2.2 Effect of excitatory amino acids on NADPH diaphorase levels in postnatal rat glial culture.

Postnatal (P2) rat glial cultures were treated with 50μM and 100μM NMDA, and coadministration of 50μM NMDA plus 100μM APV, all in HBSS for 24 hours. Control wells received HBSS alone.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ±SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test. * $p<0.05$.



Data are mean ± SEM, n=6 cultures.

ANOVA as follows: $F[2, 140] = 25.17$, $p<0.001$.

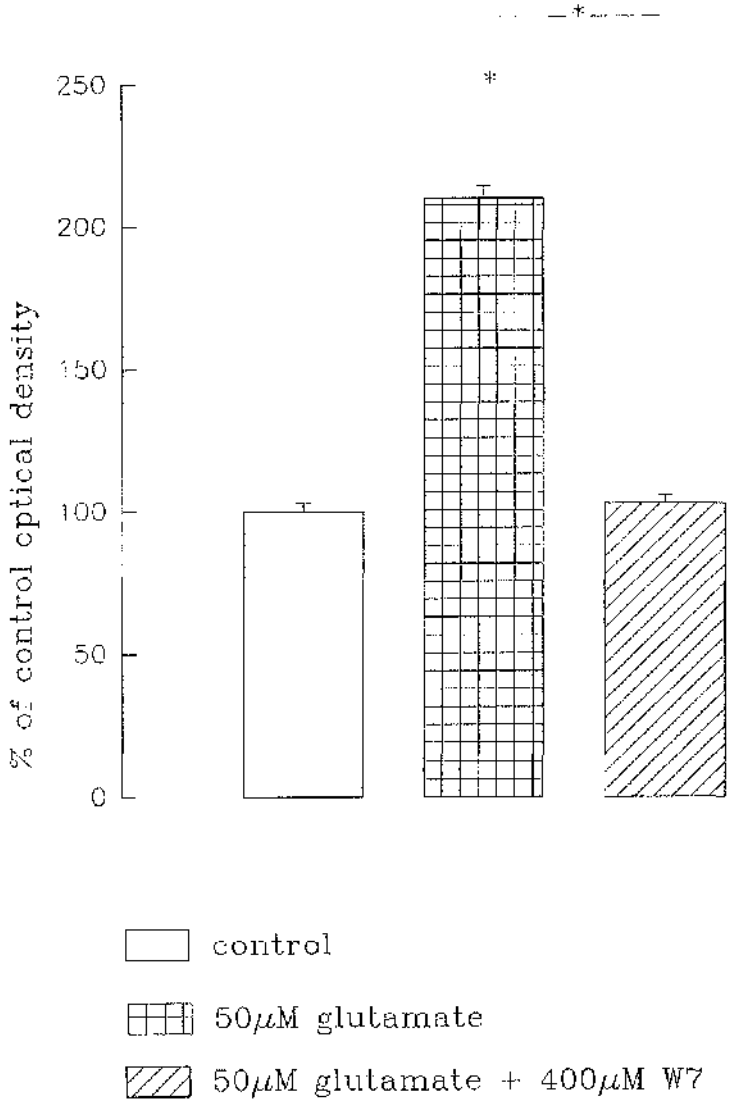
* In Dunnett's $p<0.05$ compared to control.

Figure 4.2.3 Effect of glutamate and W7 on NADPH diaphorase levels in postnatal rat glial culture.

Postnatal (P2) rat glial cultures were treated with 50μM glutamate for 24 hours , 400μM W7 for 4 hours, or coadministration of the two in HBSS. Control wells received HBSS alone.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ±SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test. *$p<0.05$.



Data are mean SEM, n= 6 cultures.

ANOVA as follows: $F[2, 30] = 30.32$, $p<0.001$

* In Dunnett's $p<0.05$ compared to control value.

−*− In Tukey's pairwise test $p<0.05$.

4.7 *Effect of LPS and W7 on NADPH diaphorase activity in glial cultures.*

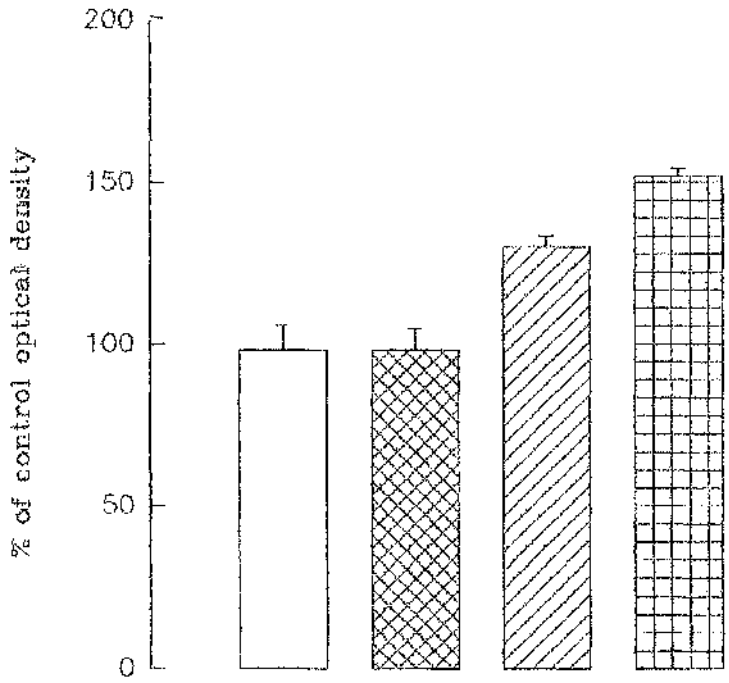
Cell cultures were treated with 100μg/ml of the endotoxin lipopolysaccharide (LPS) for 24 hours and 400μM of the calcium scavenger W7 for 4 hours, or vehicle for 24 hours. Drugs were dissolved in HBSS and incorporated into the culture medium, while control wells recieved HBSS alone. Following this time period, cultures were taken for NADPH diaphorase staining. Treatment with W7 alone did not significantly alter NADPH diaphorase levels [Fig. 4.3.1]. Analysis of variance revealed that treatment with LPS had significantly increased NOS levels , while treatment with both LPS and W7 did not alter the increase in NADPH diaphorase levels seen after treatment with LPS alone [Fig. 4.3.1, $F(3, 63) = 20.19$, $p<0.001$, $p<0.05$, $n = 7$ cultures, $p>0.05$ by Tukey's pairwise test.].

Figure 4.3 Effect of LPS and W7 on NADPH diaphorase levels in postnatal rat glial culture.

Postnatal (P2) rat glial cultures were treated with 100µg/ml LPS for 24 hours , 400µM W7 for 4 hours, or coadministration of the two in HBSS. Control wells received HBSS alone.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ±SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test, and post-hoc Tukey's pairwise test. *$p<0.05$.



control

40μM W7

100μg/ml LPS + 400μMW7

100μg/ml LPS

Data are mean ± SEM, n=7 cultures.

ANOVA as follows: F[3, 63] = 20.19, p<0.001.

* p<0.05 compared to control by Dunnett's test.

---*-- p> 0.05 in Tukey's pairwise test.

4.8 *Time course of LPS and interleukin-1 effect on NADPH diaphorase levels in glial cultures.*

Cultures were treated with 100μg/ml LPS made up in HBSS and incorporated into the medium, or HBSS alone, for 2, 6 and 24 hours. The exposure to 100μg/ml LPS was significantly different from the vehicle by analysis of variance, and the maximum increase in NADPH diaphorase activity occured after a time period of 6 hours. No further increase in levels was observed between 6 and 24 hours [Fig. 4.4.1, ANOVA: $F(3, 80) = 30.27$, $p<0.001$, $p<0.05$, $n = 6$ cultures, $p>0.05$ by Tukey's pairwise test].

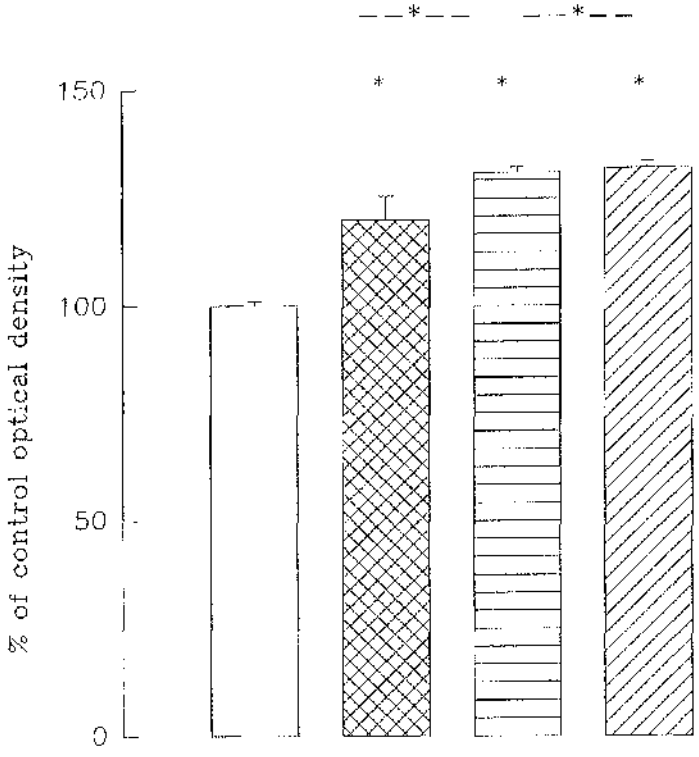
A similar pattern of results occured after exposure to 0.25ng/ml IL-1 over 2, 6 and 24 hours. Again, the drug was made up in HBSS and incorporated into the medium while vehicle wells recieved HBSS alone. The maximum increase in NOS levels occured after 6 hours [Fig. 4.4.2, ANOVA: $F(3,80) = 40.17$, $p>0.001$, $p<0.05$, $n = 6$ cultures, $p>0.05$ by Tukey's pairwise test].

Figure 4.4.1 : Effect of LPS on NOS levels in postnatal rat glial culture - time course.

Cultures were treated with 100µg/ml LPS for time periods of 2, 6 and 24 hours.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ± SEM. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test, and post-hoc Tukey's pairwise test.
*$p<0.05$.



control

100μg ml LPS for 2 hours

100μg/ml LPS for 6 hours

100μg/ml LPS for 24 hours

Data are mean ± SEM, n=6 cultures.

ANOVA as follows: $F[3, 80] = 30.27$, $p<0.001$.

* In Dunnett's $p<0.05$ compared to control.

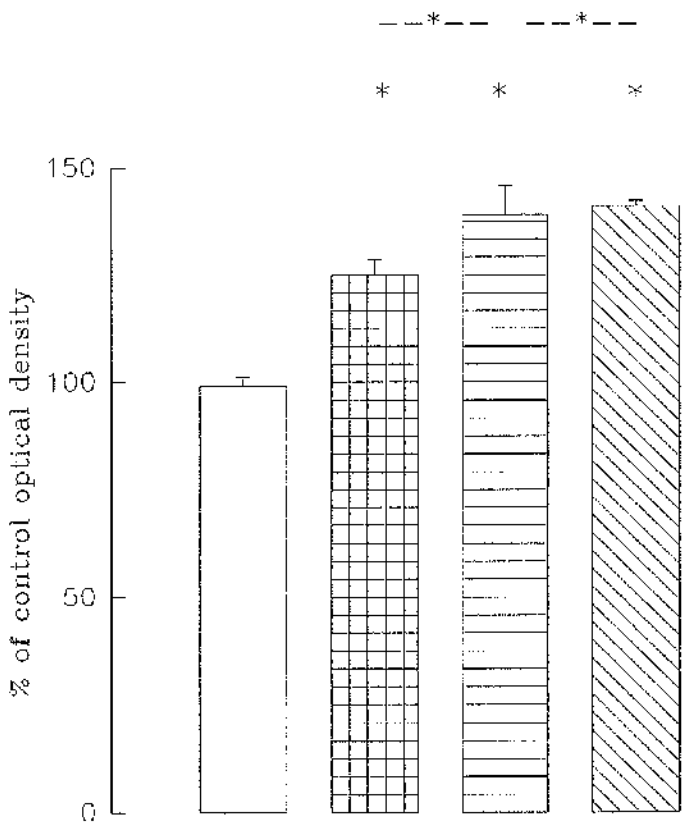
--*-- $p>0.05$ by Tukey's pairwise test.

Figure 4.4.2 : Effect of IL-1 on NOS levels in postnatal rat glial culture - time course.

Cultures were treated with 0.25ng/ml IL-1 for time periods of 2, 6 and 24 hours.

Cells were then taken for NADPH diaphorase staining.

Results are expressed as percentages of control optical density levels ± SEM. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test, and post-hoc Tukey's pairwise test. $p<0.05$.



control

0.25ng/ml IL-1 for 2 hours

0.25ng/ml IL-1 for 6 hours

0.25ng/ml IL-1 for 24 hours

Data are mean ± SEM, n=6 cultures

ANOVA as follows: $F[3, 80] = 40.17$, $p<0.001$.

* In Dunnett's $p<0.05$ compared to control.

--*-- $p>0.05$ by Tukey's pairwise test.

4.9 *Effect of protease and inhibitors on NADPH diaphorase activity in glial cultures.*

The cultures were treated with proteases alone, or protease and inhibitors for a time period of 24 hours. Once again, the compounds were made up in HBSS and incorporated directly into the medium, while the vehicle wells recieved HBSS alone. Exposure to 500ng/ml trypsin resulted in a significant increase in NADPH diaphorase activity, as did exposure to 300ng/ml α-chymotrypsin [Figs 4.5.1 and 4.5.2]. Exposure to 300ng/ml of α-chymotrypsin together with 100μM chymostatin reduced the α-chymotrypsin-induced increase in NADPH diaphorase activity to near control values [Fig.4.5.2, $F(3, 80) = 25.14$, $p<0.001$, $p<0.05$; $p<0.05$ by Tukey's pairwise test, $n = 6$ cultures].

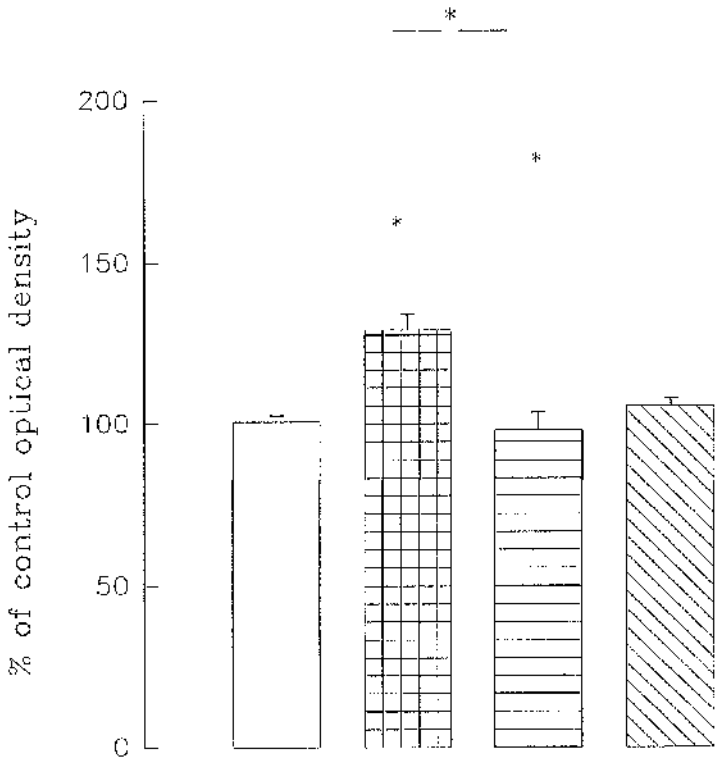
Exposure to 500ng/ml trypsin simultaneously with 1μg/ml trypsin inhibitor resulted in a reduction to near control values [Fig. 4.5.2, $F(3, 80) = 19.99$, $p<0.001$, $p<0.05$; $p<0.05$ by Tukey's pairwise test, $n = 6$ cultures].

Figure 4.5.1 : Effect of proteases and inhibitors on NADPH diaphorase levels in postnatal rat glial culture.

Cultures were treated with 300ng/ml α-chymotrypsin, 100μM chymostatin, coadministration of the two or vehicle. All compounds were administered in HBSS for 24 hours. Vehicle wells recieved HBSS alone.

Cultures were taken for NADPH diaphorase staining.

Results are expressed as a percentage of control optical density levels ± SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test.
* $p<0.05$.



control

300ng/ml chymotrypsin

300ng/ml chymotrypsin + 100μM chymostatin

100μM chymostatin

Data are mean ± SEM, n=8 cultures.

ANOVA as follows: $F=[3, 80] = 25.14$, $p<0.001$

* In Dunnett's $p<0.05$ compared to control value.

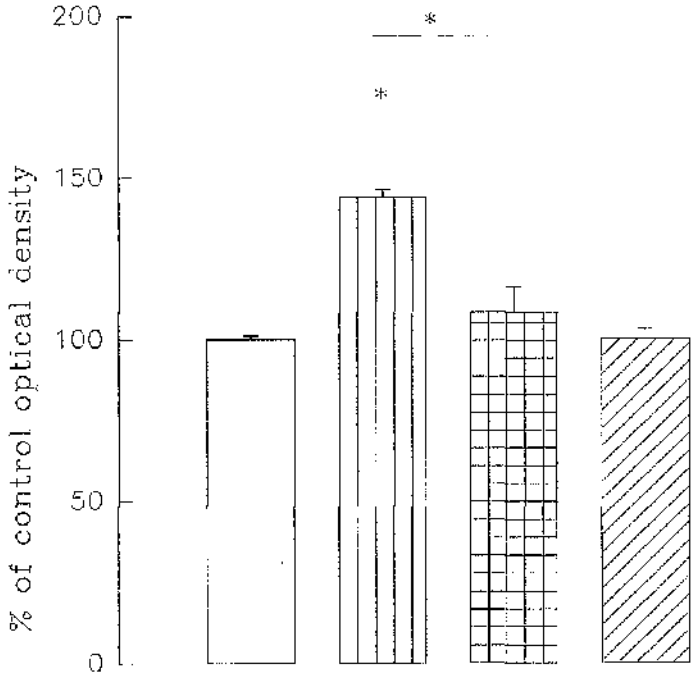
–*– $p<0.05$ by Tukey's pairwise test.

Figure 4.5.2 : Effect of proteases and inhibitors on NADPH diaphorase levels in neonatal rat glial culture.

Cultures were treated with 500ng/ml trypsin, 1µg/ml trypsin inhibitor, coadministration of the two or vehicle. All compounds were administered in HBSS for 24 hours. Vehicle wells recieved HBSS alone.

Cultures were taken for NADPH diaphorase staining.

Results are expressed as a percentage of control optical density levels ± SEM. Significance was determined by one-way ANOVA followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test.
* $p<0.05$.



control

500ng/ml trypsin

500ng/ml trypsin + 1μg/ml trypsin inhibitor

1μg/ml trypsin inhibitor

Data are mean + SEM, n = 6 cultures.

ANOVA as follows: $F[3, 80] = 19.99$, $p<0.001$.

* In Dunnett's $P<0.05$ compared to control value.

–*– In Tukey's pairwise $p<0.05$.

Discussion

4.10 *Glial cell classification*

Glial cells can be divided into the following divisions: astrocytes, oligodendrocytes and microglial. Two discrete populations of astrocytes can de distinguished from one another in culture, based on morphological, biochemical and developmental criteria (Raff et al.,1983.). Type 1 (protoplasmic) astrocytes are flattened and epithelial-like when grown in media containing serum. Under the same conditions, Type 2 (fibrous) astrocytes have rounded somata and long processes (Raff et al.,1983). Cultured protoplasmic astrocytes can be rapidly induced to become stellate, which more closely resembles their appearance in vivo, by treatment with agents that increase intracellular cyclic AMP levels such as β adrenergic agonists and forskolin (Narumi et al.,1978; Bridoux et al.,1986). Serum withdrawal from media is thought to cause astrocytes to convert to stellate morphology (rounded somata from which long processes extend), (Lim et al.,1973). It has been suggested that the ability of serum to inhibit neurite outgrowth in a mouse neuroblastoma cell line is due to serum-derived thrombin (Gurwitz and Cunningham., 1988).

The cells cultured in the present work are Type 1 astrocytes: the cells displayed flat epithelial-like morphology and rounded somata were not present. The method of astrocyte cell culture was that of McCarthy and de Vellis (1980). The use of one to two day old rat pups,and no younger, ensures the absence of viable neurons in the cell suspension obtained form the cerebral cortices. The length of the initial culture period was kept at between 7 and 9 days. Periods shorter than this are thought to be insufficient to obtain successful maturation of astrocytes (McCarthy and de Vellis.,1980). Following this method, after 7 to 9 days in culture, Type 1 astrocytes were separated from oligodendrocytes and Type 2 astrocytes by shaking on an orbital shaker leaving a Type 1 astrocytic culture. It should be noted that while the cultures appeared 90% Type 1 astrocytic, there was a small contamination of microglia, fibroblasts, oligodendrocytes and vascular cells.

4.11 *Effect of LPS and W7 on NADPH diaphorase activity in glial cultures.*

Treatment of the cultures with 100µg/ml of the endotoxin LPS for 24 hours caused a dramatic increase in NADPH diaphorase levels. As mentioned previously, neuronal NADPH diaphorase has been shown to be a nitric oxide synthase (Hope et al.,1991). It is important here to discuss whether this increase in NADPH diaphorase activity induced by exposure to LPS is an increase in NOS levels, and to investigate the type of NOS involved; neuronal, endothelial or inducible. LPS has previously been shown to cause the induction of a calcium-independent NOS in primary glial cultures (Galea et al.,1992; Simmons and Murphy.,1992). Galea and coworkers found the response to LPS to be dose-dependent and maximally effected after 48 hours. Simmons and coworkers exposed the cultures to a higher concentration of LPS (10µg/ml compared to 500ng/ml), and found maximal induction after 12 hours. This induction was found to be completely abolished by the protein synthesis inhibitor cycloheximide. Cultures were exposed to LPS for 24 hours in this set of experiments, and other time points are discussed in Section 4.13. While the concentration used in the present work is considerably higher than in previous studies, 100µg/ml treatment proved to induce the clearest NADPH diaphorase staining. Although a vast literature supports the idea that LPS induces glial NOS in the cultures, and it is more than likely that to be this same type of iNOS in the present work, the NOS type responsible was further investigated using the calmodulin antagonist W7.

The fact that combined exposure of cell cultures to LPS and W7 did not alter the LPS-induced increase in NOS levels indicates that the type of NOS activated by exposure to LPS is calmodulin-independent. In light of recent evidence, W7 appears to no longer be a distinguishing factor between neuronal and inducible NOS; both enzymes are calmodulin-dependent. LPS has been shown to cause induction of an inducible NOS in glial cells through activation of tyrosine kinase, as illustrated by abolishition with the tyrosine kinase inhibitor genistein (Feinstein et al.,1994). The initial events in the LPS activation of macrophages has also been shown to involve tyrosine kinase (Weinstein et al.,1992). iNOS in macrophages and hepatocytes

have been shown to tightly bind calmodulin like a subunit component without the need for a rise in intracellular calcium (Cho et al.,1992; Evans et al.,1992). It now appears that a similar system occurs with iNOS in glial cells: iNOS tightly binds calmodulin and produces NO even when calcium levels are low or absent (Kitamura et al.,1996; Ruan et al.,1996). LPS is thought to induce iNOS in glial cells by the de novo synthesis of iNOS proteins, as illustrated using the protein synthesis inhibitor cycloheximide (Kitamura et al.,1996). It appears that the current work suggests a calmodulin-independent NOS to be activated in the glial cells by LPS, differing from the idea that iNOS is calmodulin-dependent. Recent work has suggested the presence of differential expression and/or structural diversities of the iNOS gene (Park et al.,1996). Thus, it is possible that exposure of the glial cells to LPS resulted in the induction of an inducible yet calmodulin-independent form of NO that may be structurally different to other forms of iNOS. It is possible that structurally different forms of NOS differ in their calmodulin-dependency. A further experiment to establish whether iNOS is induced by LPS would have involved the use of the iNOS inhibitor dexamethasone. As is discussed in the next section of work, where glutamate exposure results in the increase of a calmodulin-dependent form of NOS, the glial cultures do appear to possess more than one type of NOS enzyme.

4.12 *Effect of excitatory amino acids on NADPH diaphorase activity in glial cultures.*

Exposure of the glial cultures to glutamate, AMPA and NMDA resulted in an increase in NOS levels. This increase was reduced to near control levels following combined exposure to the EAAs with their respective selective antagonists. Thus, it would appear that both non-NMDA and NMDA receptors are involved in the induction of NOS in the cultured glial cells. To investigate the type of NOS involved, the calmodulin antagonist W7 was applied to the cultures. In contrast to the LPS results discussed in section 4.11, W7 reduced the glutamate-induced increase in NOS levels to near control values, suggesting that the NOS involved here is calmodulin-dependent.

A key link between glutamate receptor activation and subsequent neurodegeneration is thought to be agonist-triggered

calcium entry. In neocortical cultures, the widespread damage induced by brief activation of NMDA-type glutamate receptors is calcium dependent and is thought to be triggered by the highly calcium-permeable NMDA receptor-gated channel (Choi., 1988; Mac Dermott et al.,1986). With non-NMDA receptors (AMPA or kainate), which gate with low calcium permeability, much longer exposure durations are required to induce widespread cortical neurodegeneration (Koh et al.,1990). There has been a prevailing view that since glutamate causes neuronal death under the discussed conditions, any changes observed in glial cells are a consequence of neuronal damage (Hertz and Schousboe .,1986)

It would appear that in the present results, the type of NOS induced in glial cells by the excitatory amino acid glutamate differs from that induced by LPS, in that it appears to be calmodulin dependent. Morphological examination of the cells reveals that the same cell populations are being stained by NADPH diaphorase in both sets of experiments. The glutamate-induced induction of NOS is much larger than that caused by LPS exposure, and W7 blocked this larger increase illustrating the effectiveness of the compound in the present cell culture conditions. Perhaps it is not surprising that LPS and glutamate cause induction of what appear to be different types of NOS with different calmodulin dependencies: the two compounds act by differing pathways and perhaps the differing patterns of action trigger differential expression of the iNOS gene, structural diversity of the gene has been suggested (Park et al.,1996). LPS is thought to act via a cytokine receptor resulting in induction of iNOS via tyrosine kinase activation (Kitamura et al.,1996) and, as mentioned above, glutamate through the NMDA receptor causes induction of iNOS via an influx of calcium. As mentioned previously, calmodulin dependency is no longer a disguising factor between neuronal and inducible forms of NOS. Thus, it is possible that the glutamate-induced calmodulin-dependent NOS could be neuronal or iNOS. As discussed in section 4.11, the LPS-stimulated NOS is more than likely to be iNOS: a vast literature has shown this to be the case. The finding that glutamate raises the level of a type of NOS differing in calmodulin dependency from that seen following exposure to LPS perhaps strengthens the argument that glial cells posses several types of NOS differing in calmodulin dependency. Further experiments using specific

radiolabelled oligonucleotide probes to iNOS and neuronal NOS would clarify the type of enzyme present in the cultured cells. Also, use of calcium chelators such as EGTA would distinguish between the two forms of the enzyme: low calcium concentration would block the activity of neuronal NOS, while not altering the activity of iNOS.

4.13 *Time course of LPS and IL-1 on NADPH diaphorase activity in glial cultures.*

Exposure of glial cultures to 100μg/ml LPS or 0.25ng/ml IL-1 provided interesting results. As previously mentioned, Galea and coworkers found the response to LPS to be maximally induced after 48 hours in primary astrocyte cultures (Galea et al.,1992), while Simmons and colleagues, using a higher concentration of LPS, found maximal induction after 12 hours and maintained for 36 hours (Simmons and Murphy.,1992), again in primary astrocyte cultures. However, in the present study, the maximal induction following exposure to LPS or IL-1 occurred after 6 hours, with no further increase after 24 hour exposure, although it should be noted that 24 hours was the maximum time point studied. Possibilities for the type of NOS enzyme involved have been discussed in section 4.11. Thus it appears that the glial cells studied in the present work show induction of NOS in perhaps a shorter time course than previously illustrated. The present shorter time course of induction of NOS agrees with recent work where a rapid induction of NOS gene expression has been shown to occur in the heart during endotoxemia (Bateson et al.,1996). The resulting release of NO is thought to depress cardiac contractile function. Bateson and coworkers looked at the time courses of change in iNOS mRNA levels and enzyme activity in the left ventricular wall of rats following injection of LPS. Within 30 minutes of LPS treatment, a rapid increase in iNOS mRNA occurred which peaked after 3 hours and dropped to near baseline levels after 12 hours. Enzyme activity peaked after 6 hours and dropped to baseline levels after 24 hours. Bateson and coworkers concluded that the LPS-induced rapid stimulation of iNOS activity in the rat heart occurs via an increase in expression of the iNOS gene. It is thought that iNOS transcription is regulated by activation of the immune system regulator nuclear factor kappa B (Goldring et al.,1995). As has been discussed

there is evidence for the presence of structurally different forms of iNOS. It is possible that in the glial cells, induction of an inducible form of NOS, structurally different to previous forms of the enzyme in its calmodulin-dependency, could be induced on a faster scale than other forms of the enzyme. The mechanisms behind this are not clear, and further experiments covering a more expansive range of time points would perhaps give a clearer picture.

4.14 *Effects of proteases and inhibitors on NADPH diaphorase activity in glial cultures.*

The results of several studies support the idea that an imbalance between brain proteases and their inhibitors may play a role in Alzheimer's Disease. Levels of active protease nexin-1 (a proteinase inhibitor) are decreased in Alzheimer's Disease hippocampus to around 15% of the levels in age matched controls (Wagner et al.,1988). Further, amyloid deposits of Alzheimer's brain contain α-1 antichymotrypsin (Abraham et al.,1988), and the amyloid precursor shows sequence homology to Kunitz serine protease inhibitors (Kitaguchi et al.,1988). Cavanaugh and coworkers suggested that a correct balance between thrombin and protease nexin-1 on astrocyte surface may be crucial to normal astrocyte function (Cavanaugh et al.,1990). Further evidence of protease involvement in the pathology of Alzheimer's Disease is as follows. NOS activity has been found to be elevated in brain microvessels in Alzheimer's Disease (Dorheim et al.,1994). α-1 antichymotrypsin is a glial-derived protein associated with senile plaques in Alzheimer's Disease (Abraham et al.,1988). Both αα1-antitrypsin and α1-antichymotrypsin may be functionally involved in the pathogeneis of the lesions of Alzheimer's Disease, and the cells thought to be producing both compounds are astrocytes (Gollin et al.,1992). Recent data suggest that α-1 antichymotrypsin interaction with Aβ(1-40) inhibits fibril formation but does not affect the amyloid peptide toxicity, and that toxicity of Aβ can develop without fibril formation (Aksenova et al.,1996). α-1 antichymotrypsin is capable of accelerating the rate of polymerisation of amyloid fibrils thus enhancing plaque deposition (Ma et al.,1994; Eriksson et al.,1995), and is thought to be an important factor in the pathology of the disease.

The current work clearly demonstrates that the proteases chymotrypsin and trypsin significantly increase NADPH diaphorase levels, with respective inhibitors reducing the levels to near baseline levels. The glial cells stained by the NADPH diaphorase appear to be morphologically of the same population as in previous experiments. At this stage it is not possible to speculate the type of NOS involved. For an understanding of the possible type of NOS involved in the protease-induced increase in NADPH diaphorase immunostaining, these experiments should have been repeated using W7, dexamethasone and radiolabelled oligonucleotide probes.

Summary

Cultured glial cells have been characterised by positive staining to GFAP, and used to investigate NOS activity in response to cytokine, endotoxin, excitatory amino acid agonists and proteases. The glial cells appear to possess several types of NOS differing in their calmodulin dependency, and it is possible that the cells respond to different compounds by induction of structurally different forms of the NOS enzyme.

Following on from this investigation of NOS activity in glial cells, it was of interest to investigate NOS activity, and cytoskeletal protein expression, in primary dissociated neuronal cholinergic cells in culture.

Chapter 5

Regulation and expression of cytoskeletal protein and NADPH diaphorase immunostaining in basal forebrain neuronal cultures.

Introduction

This section of the work made use of primary neuronal basal forebrain cultures. As discussed in section 2.1.1, the method of cell culture was based on the method of Hartika and Hefti (1988). The cultured cells were characterised by immunolabelling for neuron specific enolase, neurofilament, MAP2 and choline acetyl transferase. The cell culture model was then used to investigate changes in cytoskeletal protein immunoreactivity, and NADPH diaphorase levels in the cells in response to exposure to the following compounds: nerve growth factor, nitric oxide-releasing compounds and excitatory amino acid agonists.

5.1 Neuron Specific Enolase

In order to characterise the cell cultures, a specific marker for neuronal cells was required. Enolases are glycolytic enzymes that convert 2-phosphoglycerate into phosphoenolpyruvate. There are three homodimeric enolase enzymes in mammalians composed of the subunits $\alpha\alpha$, $\beta\beta$ and $\gamma\gamma$ (Schmechel and Marangos.,1982; Zomzely-neurath.,1983). In the adult mammalian nervous system two of the isozymes have been found. The non-neuronal enolase (NNE) $\alpha\alpha$ dimer is expressed in glial cells (Schmechel et al.,1978). The $\gamma\gamma$ form is specifically localised to neurons and termed neuron specific enolase (NSE) (Moore and McGregor.,1965; Schmechel et al., 1980). The third isozyme, $\beta\beta$, is found in muscle tissue. NSE is also found in peripheral neuroendocrine tissue, and a variety of tumours including melanoma and carcinoids. Recently, NSE has become of interest to neurologists as an indicator of brain damage.

Several studies have shown NSE to be exclusively present in neurons with the highest concentration in CNS neurons. The highest levels of NSE are found in the cortex and caudate putamen, the enzyme being absent in other cells in the brain such as glial cells (Cicero et al.,1970: Pickel et al.,1976). NSE is soluble and present in the cerebrospinal fluid (CSF). An elevated level of NSE in the CSF been researched as a marker of neuronal damage (Hardemark et al.,1989). CSF levels of NSE had been shown to be reduced in Alzheimer's Disease (Cutler et al.,1986).

5.2 Nerve Growth Factor

Nerve growth factor (NGF) is a member of the polypeptide gene family, the neurotrophins, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 (Barde et al.,1982; Ip et al.,1992). NGF, BDNF and NT-3 are present in the hippocampus and the cerebral cortex (Leibrock et al.,1989). Considerable evidence indicates that NGF is necessary for the survival and function of cholinergic neurons projecting from the basal forebrain to the hippocampus and frontal cortex (Thoenen and Barde.,1980; Levi-Montalcini and Angeletti.,1986). As the degeneration of these pathways occurs in the neuropathology of Alzheimer's Disease, and several studies have shown a decrease in NGF receptor function and retrograde transport in Alzheimer's Disease, (Kerwin et al.,1991; Kerwin et al.,1992; Mufson et al.,1995), the potential of NGF as a possible therapeutic agent has been researched (Saffran.,1992). Evidence shows that in the central nervous system, NGF is produced by certain neurons (Gall et al.,1991), astrocytes (Furukawa et al.,1986) and microglia (Mallet et al.,1989).

Numerous studies have illustrated the protective effects of NGF. It has been shown to prevent apoptopic cell death in injured central cholinergic neurons (Wilcox et al.,1995), to protect striatal cholinergic neurons against quinolinic acid excitotoxicity in the rat neostriatum (Perez-Navarro et al.,1994), and to act as a trophic factor on intact and injured basal forebrain cholinergic neurons (Koliatsos et al.,1994). In addition to its function as a neurotrophin, NGF has also been shown to act as a mediator of microglial function.

Two cell surface NGF receptors have been characterised on the basis of binding affinity and signal transduction properties: the rapidly dissociating p^{75} low affinity NGF receptor (Johnson et al.,1986), and the slowing dissociating p^{140} TrkA tyrosine kinase receptor (Kaplan et al.,1991). The function of the low affinity receptor in still unclear, while several lines of evidence show that the activation of the p^{140} TrkA receptor is necessary to elicit NGF responses (Green et al.,1986; Hempstead et al.,1992; Ibanez et al.,1992).

The rat pheochromocytoma PC12 cell line is an NGF-responsive model system that has been useful for studying the

et al.,1991). The function of the low affinity receptor in still unclear, while several lines of evidence show that the activation of the p^{140} TrkA receptor is necessary to elicit NGF responses (Green et al.,1986; Hempstead et al.,1992; Ibanez et al.,1992).

The rat pheochromocytoma PC12 cell line is an NGF-responsive model system that has been useful for studying the mechanisms of NGF actions (Greene and Tischler.,1976). PC12 cells proliferate in serum-containing medium, and when exposed to NGF, withdraw from the cell cycle and gradually adopt the many characteristics of mature sympathetic neurons (Greene and Tischler.,1982). The most dramatic aspect of this is the extension of long axon-like neurites. The processes involved in this neurite extension have been extensively researched, and the expression of microtubule-associated proteins and protein kinases monitored. The induction of MAP1 and tau in response to NGF has been shown to promote microtubule assembly and these factors are regulators of neurite outgrowth in PC12 cells (Drubin et al.,1985; Sano et al.,1990; Teng et al.,1993). Mechanisms behind neurite outgrowth have been less extensively studied in primary dissociated cultures, and it was of interest to investigate cytoskeletal protein expression, cell morphology and neurite outgrowth in response to NGF in basal forebrain neuronal cells.

5.3 Cyclooxygenases

Cyclooxygenase catalyses the first two steps in the biosynthesis of prostanoids from arachidonic acid (the formation of prostaglandin G_2 and its subsequent reduction to prostaglandin H_2), and is the target of non-steroidal anti-inflammatory drugs (NSAIDS) such as indomethacin. Administration of NSAIDs is thought to retard the onset of Alzheimer's Disease (Rich et al.,1995). Two isoforms exist: constitutive (Cox1) and inducible (Cox2). Most currently available NSAIDs inhibit both isoforms but it is thought that selective Cox2 inhibition may reduce unwanted side effects such as gastric and renal damage. It is thought that prostanoids produced by the Cox1 isoform are involved in the maintenance of cellular physiology, whereas those produced by Cox2 are involved in the inflammatory process (Simmons et al.,1992; Mitchell et al.,1993).

The mechanism underlying the inverse relationship between NSAIDS and onset of Alzheimer's Disease has not been established, and an attractive possibility is that it involves a prevention of development of plaques and tangles. In parallel with the in vivo work looking at the effect of indomethacin treatment on tau gene expression, the cell cultures were treated with prostaglandins, and any changes in APP and COX2 expression noted.

Results

5.4 *Characterisation of basal forebrain neuronal cultures.*

In cells cultured from the basal forebrain region of embryonic rats (E17), a large proportion of the cells can be clearly characterised as cholinergic neuronal cells. The cells were typically around 25μm in diameter with many extensive neurites. Neurite length was on average 10-15μm. The final plating dilution of the cultures proved to be vital to the maintenance of the cells. A dilution giving an even distribution of cells with a significant degree of cell-cell contact provided the optimum cell survival. Cells and neurite characteristics were easier to analyse if the cells were not clumped together. The dilutions used are discussed in the methods chapter. Cells showed positive immunostaining for choline acetyltransferase (ChaT), a marker for cholinergic cells [Fig. 5.1a]. Positive immunostaining was also seen for the microtubule-associated protein MAP2, which is found only in neuronal cell types [Fig. 5.1b]. The glycolytic enzyme neuron specific enolase (NSE) also stained positively in the cultures, further characterising them as neuronal cells [Fig. 5.1c]. Positive immunostaining was also seen with neurofilament [Fig. 5.1d]. The high survival rate of the cells, and the use of multiple well plates for cell growth, made the neuronal cell cultures a useful and productive experimental model.

Figure 5.1 : Characterisation of neuronal basal forebrain cultures.

Cells were processed for immunocytochemistry using antisera against the following:

A choline acetyltransferase

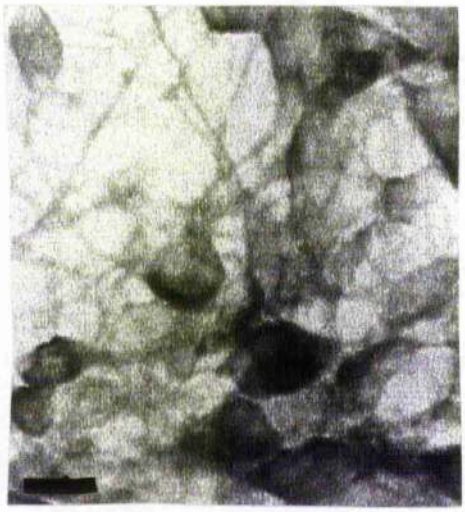
B MAP2

C neuron specific enolase

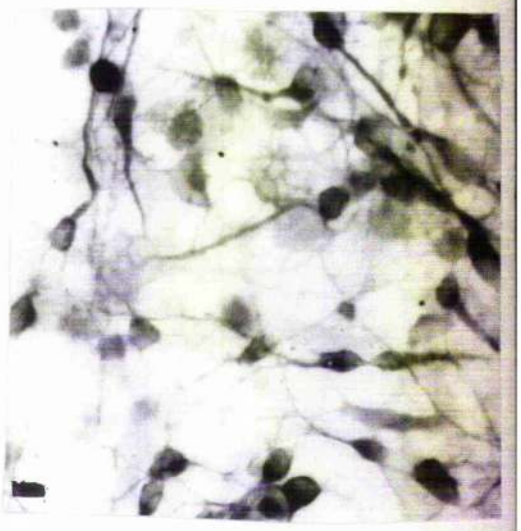
D neurofilament

Scale bar represents 25μm.

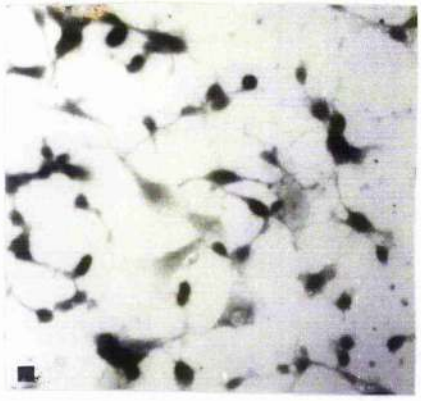
A



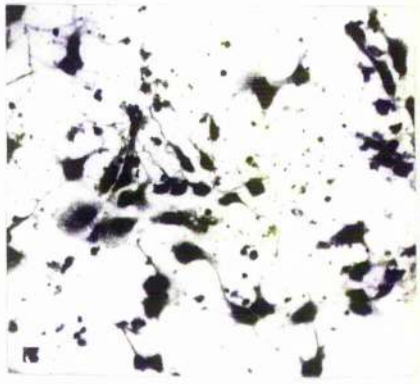
B



C



D



5.5 *NGF effects on ChaT, tau and MAP2 expression in neuronal cultures.*

Cells were treated with nerve growth factor (NGF) at a concentration of 200ng/ml for 4 days. The NGF was made up in the culture growing medium and added directly to the culture at each medium change over the 4 day time period. Control wells received medium alone. As can be seen in Fig. 5.2.1, exposure to NGF resulted in a dramatic increase in ChAT and MAP2 immunoreactivity, with no significant change in tau immunoreactivity.

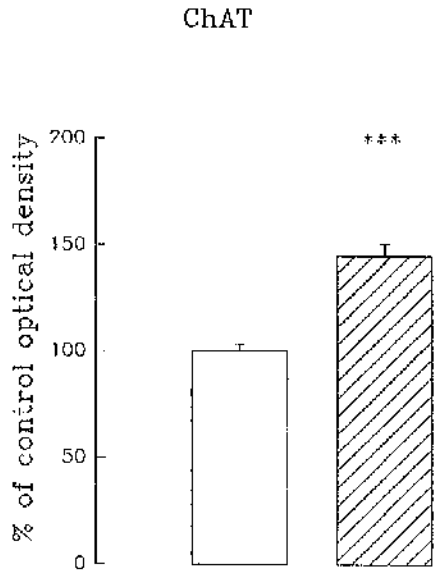
As the effect of NGF on MAP2 was so dramatic, and the ChaT immunostaining was for cell characterisation purposes, it was decided to further investigate the MAP2 result. Cells were exposed to 200ng/ml NGF for time periods of 24 hours, 4 days and 1 week. Again, the NGF was made up in the culture medium, and added each time the medium was changed. Control wells received medium alone. The maximum rise in MAP2 immunostaining induced by NGF occurred after 4 days, with no further increase seen after 1 week [Fig. 5.2.2, $F(3.80) = 20.17$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. The same experiment was repeated by in situ hybridisation using an oligonucleotide probe specific for MAP2 mRNA. Exposure to NGF resulted in an increase in MAP2 gene expression which continued to rise following 4 days and 1 week treatment as shown in Fig. 5.2.3 [$F(3, 80) = 15.41$, $p<0.001$, $p<0.05$, $n = 4$ cultures].

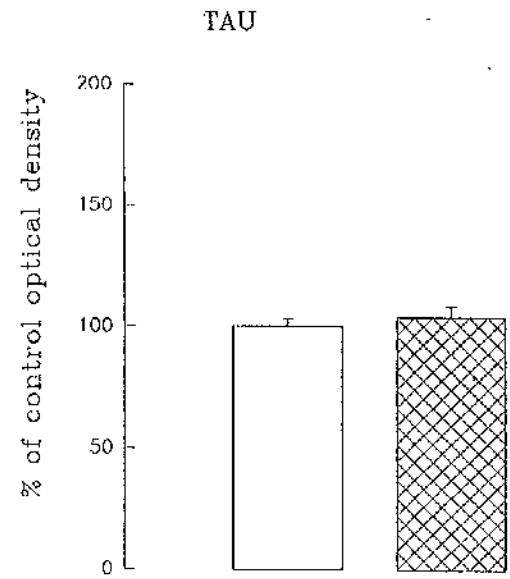
Figure 5.2.1 : Effect of NGF on MAP2, tau and ChaT immunoreactivity in neuronal basal forebrain cultures.

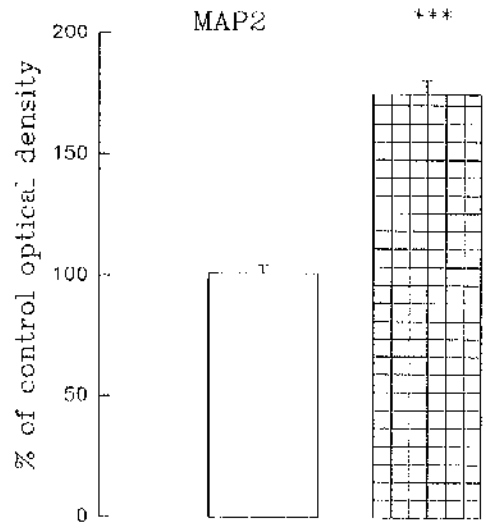
NGF was added to the cultures at a concentration of 200ng/ml for 4 days. Control wells received culture medium alone. After processing for immnunostaining with antisera against MAP2, tau and ChaT, optical density levels were measured.

Results are expressed as a percentage of control optical density level ± SEM.

Significance was measured by Mann Whitney U test. *** $p<0.001$.







Data are mean + SEM

n = 6 cultures.

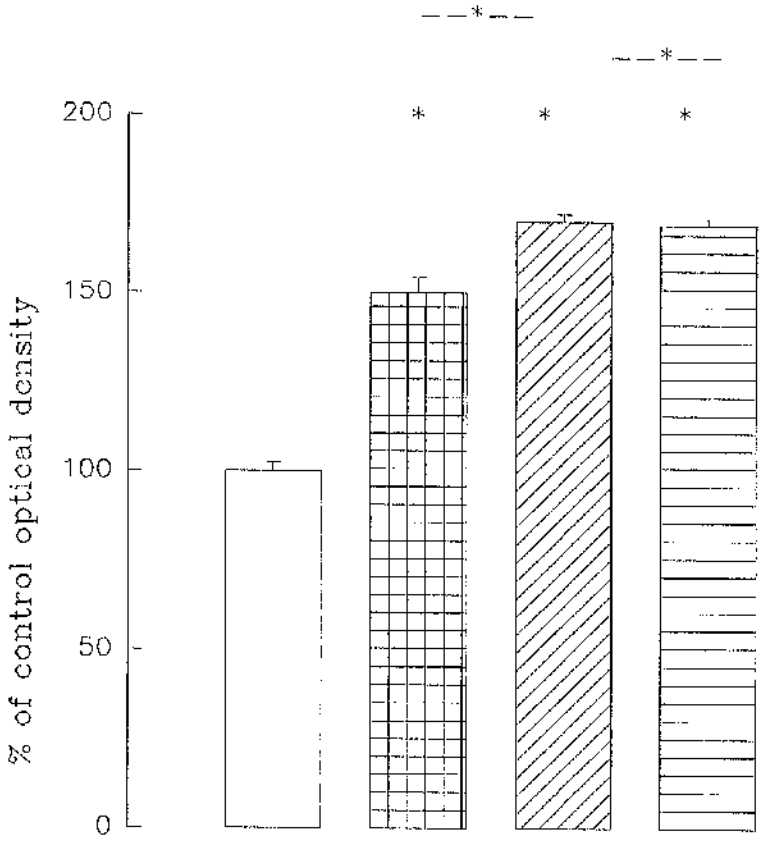
*** $p<0.001$ compared to control values by Mann Whitney U test.

Figure 5.2.2 : Effect of NGF on MAP2 immunoreactivity in neuronal basal forebrain cultures - time course.

Cultures were treated with 200ng/ml NGF for time periods of 24 hours, 4 days and 1 week. Control wells recieved culture medium alone.Cells were then processed for immunostaining using antisera against MAP2.

Results are expressed as a percentage of control optical density levels ± SEM.

Significance was measured using one-way ANOVA and post-hoc Dunnett's test. * $p<0.05$.



control
200ng/ml NGF- 24 hours
200ng/ml NGF- 4 days
200ng/ml NGF- 1 week

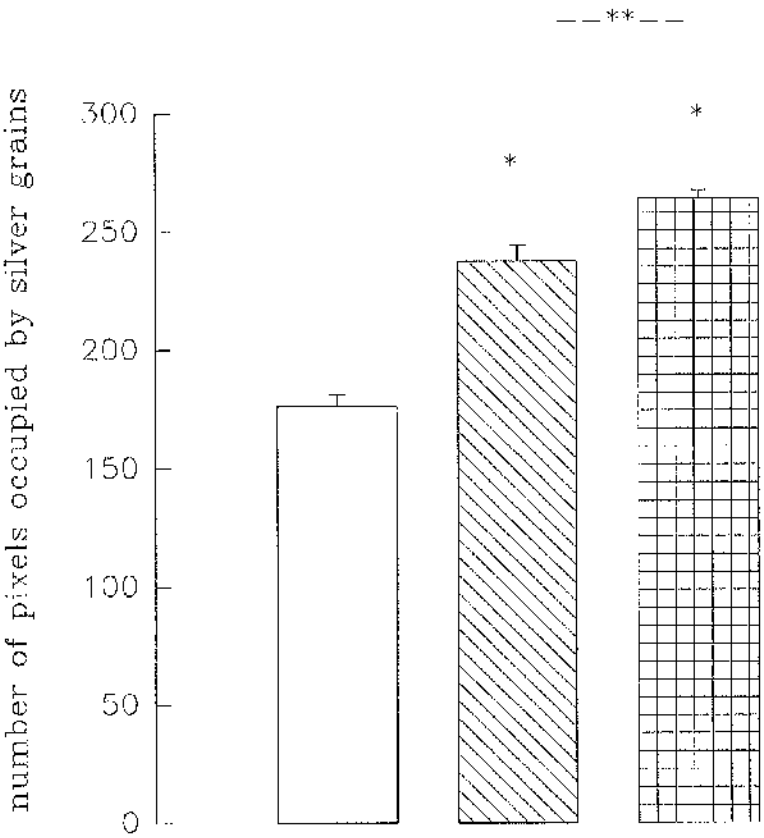
Data are mean SEM, n=6 cultures.
ANOVA as follows: $F[3, 80] = 20.17$, $p<0.001$
* In Dunnett's $p<0.05$ compared to control.
--*--In Tukey's pairwise test $p>0.05$.

Figure 5.2.3 : Effect of NGF on MAP2 mRNA levels in neuronal basal forebrain cultures - time course.

Cultures were treated with 200ng/ml NGF for time periods of 4 days and 1 week. Control wells received culture medium alone.Cells were then processed for in situ hybridisation.

Results, obtained from emulsion-dipped slides, are expressed as mean number of pixels occupied by silver grains ± SEM.

Significance was measured using one-way ANOVA and post-hoc Dunnett's test. * $p<0.05$.



control
200ng/ml NGF 4 days
200ng/ml NGF 1 week

Data are mean ± SEM values, n = 6 cultures.

ANOVA as follows: $F[3, 80] = 15.41$, $p<0.001$
* In Dunnett's $p<0.05$ compared to control.
--**-- In Tukey's pairwise test, $p<0.05$.

5.6 *Effect of NGF on NADPH diaphorase activity in neuronal cultures.*

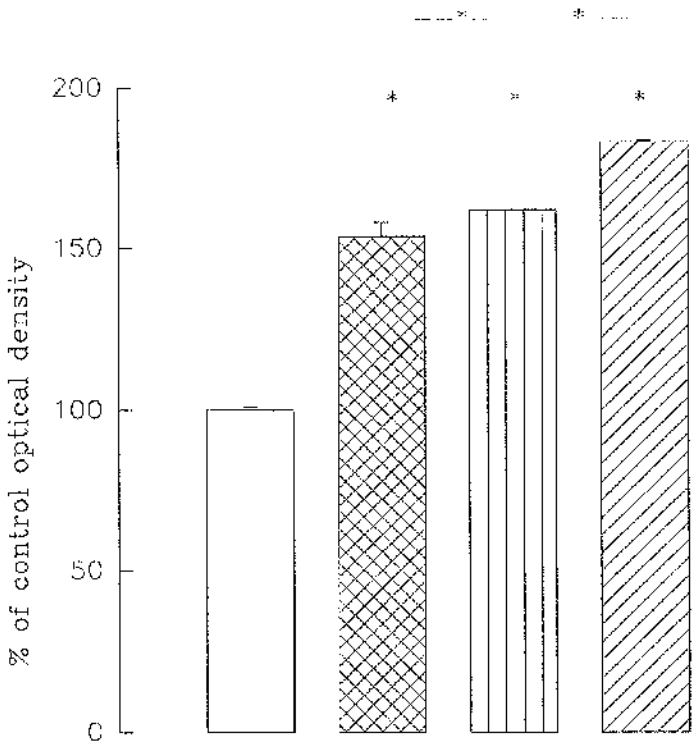
To investigate the possible role of NGF in regulating NOS levels, we treated cells with 200ng/ml of NGF for time periods of 24 hours, 30 hours and 4 days. The addition of NGF was as previously described. Cultures were then stained to detect NADPH diaphorase activity. Exposure to NGF resulted in an increase in NADPH diaphorase levels after 24 hours [Fig. 5.3.1, $F(4, 100) = 40.17$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. The effect of NGF was maximum after 24 hours with no significant increase in NADPH diaphorase activity after longer exposure times [Fig. 5.3.1, by Tukey's $p>0.05$].

Figure 5.3.1 : Effect of NGF on NADPH diaphorase levels in neuronal basal forebrain cultures.

Cultures were again treated with 200ng/ml NGF or vehicle. Treatment was for the following time periods: 24 hours, 30 hours and 4 days. Cells were then processed for NADPH diaphorase staining.

Results are expressed as a percentage of control optical density levels ± SEM.

Statistical analysis was carried out using one-way ANOVA followed by post-hoc Dunnett's, and post-hoc Tukey's. *$p<0.05$.



control

200ng/ml NGF for 24 hours

200ng/ml NGF for 30 hours

200ng/ml NGF for 4 days

Data are mean ± SEM, n=6 cultures.

ANOVA as follows: $F_{[4, 100]} = 40.17$, $p<0.001$.

* In Dunnett's $p<0.05$ compared to control.

−−*−− In Tukey's pairwise test $p>0.05$.

5.7 *Effect of NGF on cytoskeletal protein and glycolytic enzyme immunostaining.*

To examine further the effects of NGF on cytoskeletal proteins and glycolytic enzymes, we pre-treated the cultures with dexamethasone at a concentration of 1μM (Radomski et al ., 1990). Dexamethasone was made up in HBSS and 20% DMSO,and incorporated directly into the culture medium 24 hours prior to the NGF addition. Control wells recieved HBSS and 20% DMSO. NGF was added at 200ng/ml, again made up in the culture medium. Immunostaining for tau, MAP2 and NSE followed. As shown previously, NGF dramatically increased MAP2 immuostaining [Fig. 5.4.1, $F(3, 82) = 21.15$, $p<0.001$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. NGF also increased NSE immunostaining [Fig. 5.4.1, $F(3,81) = 25.24$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. No change in tau immunostaining occurred. Pretreatment with 1μM dexamethasone alone did not alter immunostaining. The NGF-induced increase in MAP2 immunostaining was reduced to near control levels by Pretreatment with dexamethasone [Fig. 5.4.1].

As the actions of dexamethasone are varied, and involve more than iNOS inhibition alone, we repeated this experiment using the nitric oxide scavenger haemoglobin (Hb). 20μM of Hb was added to the cultures in HBSS (Martin et al ., 1985). NGF was again used at a concentration of 200ng/ml added directly to the culture medium. Hb reduced the NGF-induced increase in MAP2 immunostaining to near control value [Fig. 5.4.2, $F(4, 110) = 21.16$, $p<0.001$, $p<0.05$, $n = 6$ cultures].

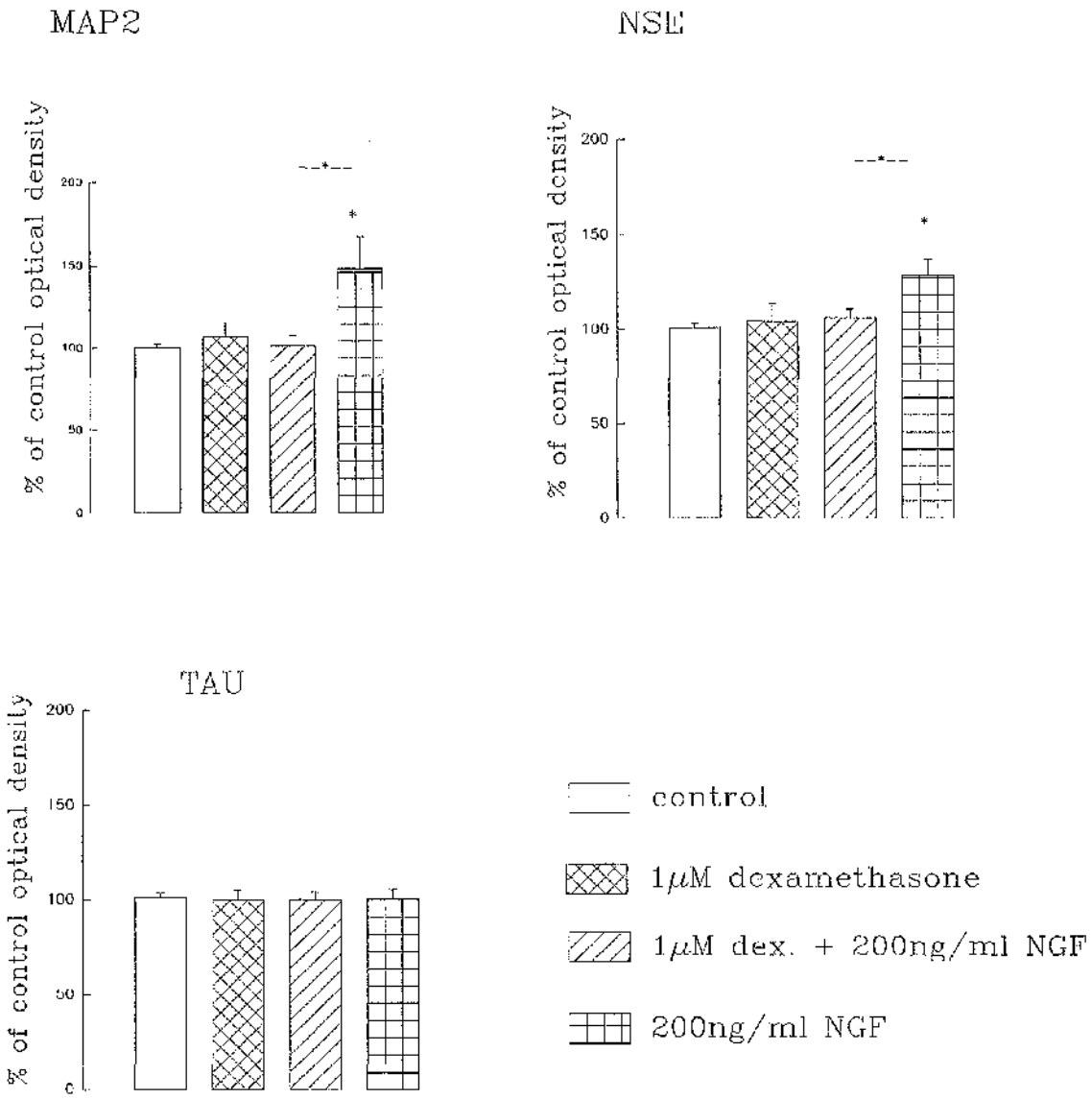
The calmodulin antagonist W7 was then used to investigate the possible type of NOS involved in the increase in MAP2 immunoreactivity. Cultures were again exposed to 200ng/ml NGF for 24 hours. W7 (400μM) was applied to the cells 4 hours prior to fixation. All compounds were made up in culture medium, and control wells received medium alone. W7 reduced the NGF-induced increase in MAP2 immunoreactivity to near control levels [Fig. 5.4.3, $F(4, 161)= 111.17$, $p<0.001$, $p<0.05$, $n= 4$ cultures].

Figure 5.4.1 : Effect of NGF and dexamethasone on MAP2, tau and NSE immunoreactivity in neuronal basal forebrain cultures.

Cultures were pretreated for 24 hours with 1μM dexamthasone in HBSS or vehicle (HBSS alone). Addition of either 200ng/ml NGF to dexamethasone and vehicle pretreated wells, or vehicle, for 24 hours followed. Cells were then processed for MAP2 immuonstaining.

Results are expressed as a percentage of control optical density levels ± SEM.

Significance was analysed byone-way ANOVA followed by post-hoc Dunnett's test. *$p<0.05$.



Data are mean ± SEM, n= 6 cultures.

ANOVA as follows:

MAP2 $F[3, 82] = 21.15$, $p<0.001$

NSE $F[3, 81] = 25.24$, $p<0.001$

TAU $F[2, 44] = 6.55$, $p>0.05$

* In Dunnett's $p < 0.05$ compared to control value.

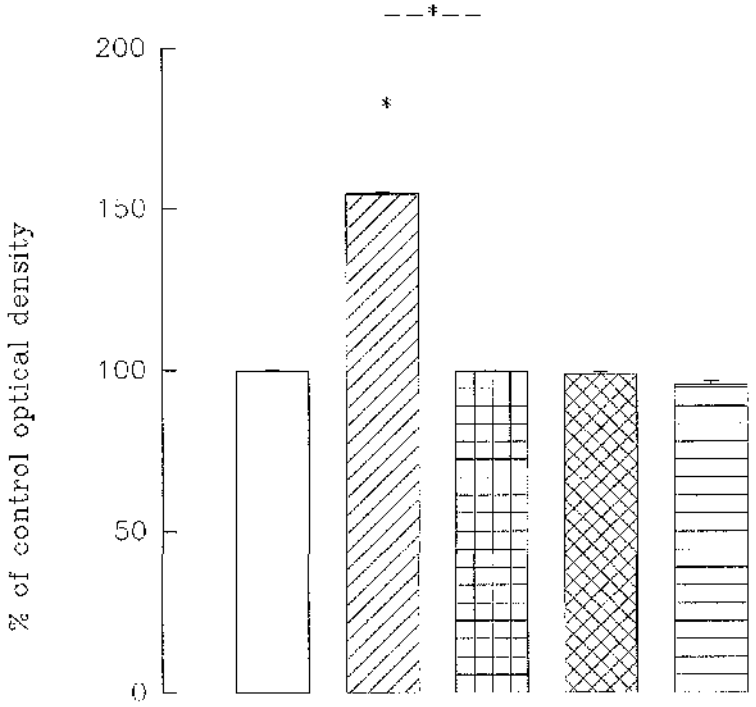
$-*-$ $p<0.05$ in Tukey's pairwise test.

Figure 5.4.2 : Effect of NGF, dexamethasone and Hb on MAP2 immunoreactivity in neuronal basal forebrain cultures.

Cultures were pretreated for 24 hours with 1μM dexamethasone in HBSS or vehicle (HBSS alone). Pretreated wells then recieved vehicle, 200ng/ml NGF, 20μM Hb or coadministration for 24 hours followed. Compounds were administered in HBSS, vehicle received HBSS alone. Cells were then processed for MAP2 immunostaining.

Results are expressed as a percentage of control optical density levels ± SEM.

One-way ANOVA, followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test, was carried out. *$p<0.05$.



control

200ng/ml NGF+ 1μM dexamethasone

200ng/ml NGF

200ng/ml NGF + 20μM Hb

20μM Hb

Data are mean ±SEM, n=7 cultures.

ANOVA as follows: $F[4, 110] = 21.16$, $p<0.001$

* $p<0.05$ compared to control by Dunnett's.

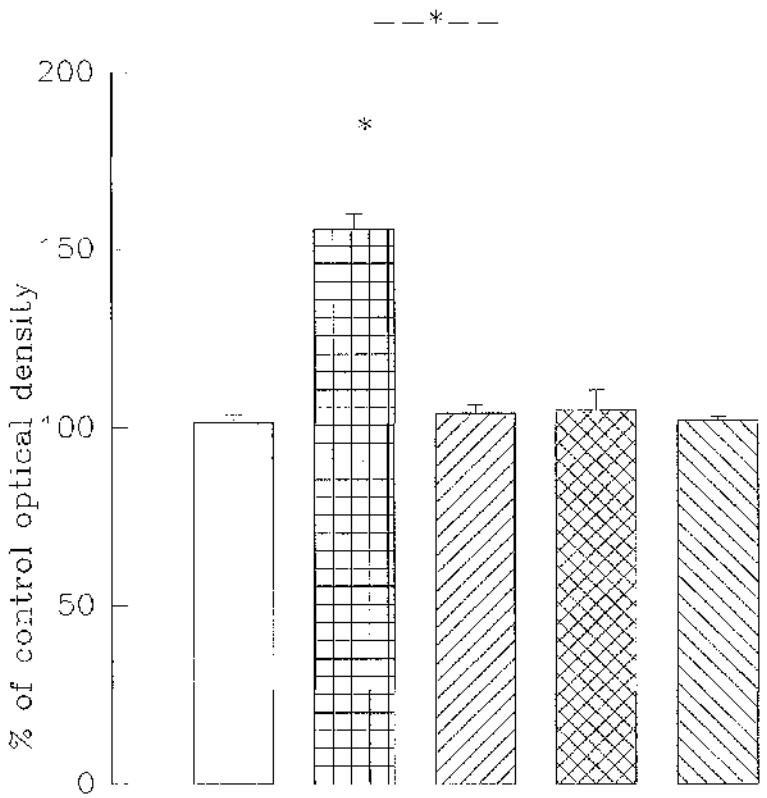
–*– $p<0.05$ by Tukey's pairwise test.

Figure 5.4.3: Effect of NGF, dexamethasone and W7 on MAP2 immunoreactivity in neuronal basal forebrain cultures.

Cultures were pretreated for 24 hours with 1μM dexamethasone in HBSS or vehicle (HBSS alone). Pretreated wells then received vehicle, 200ng/ml NGF for 24 hours followed. W7 (400μM) treatment was for 4 hours prior to cell fixation. Compounds were administered in HBSS, vehicle recieved HBSS alone. Cells were then processed for MAP2 immunostaining.

Results are expressed as a percentage of control optical density levels ± SEM.

One-way ANOVA, followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test, was carried out. *$p<0.05$.



control

200ng/ml NGF

200ng/ml NGF+ 1μM dex.

200ng/ml NGF+ 400μM W7

400μM W7

Data are mean ± SEM, n= 4 cultures.

ANOVA as follows: F[4, 161] =111.17

*p<0.001 compared to control by Dunnett's.

--*-- P<0.05 by Tukey's pairwise test.

5.8 *Effect of glutamate on NADPH diaphorase activity.*

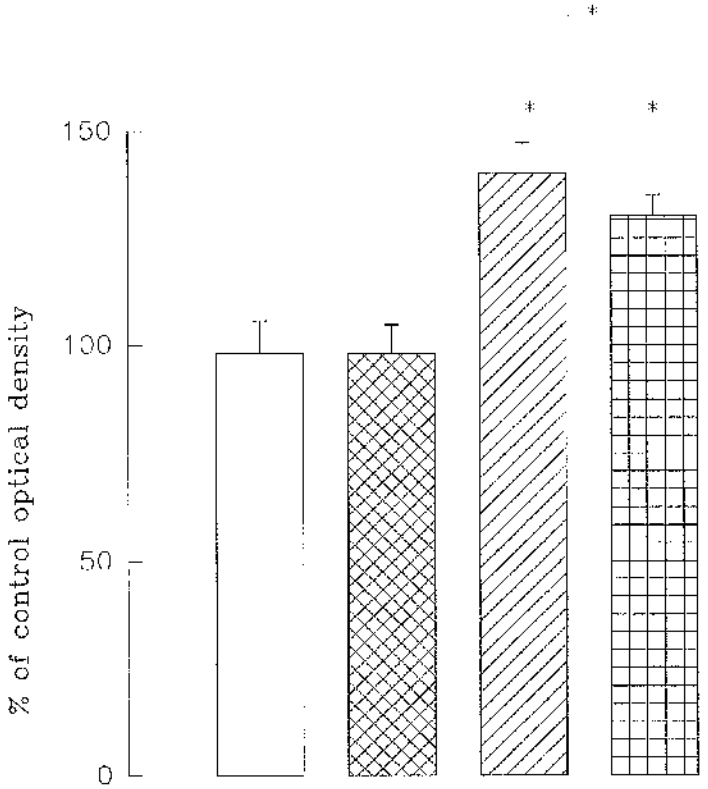
Cells were exposed to 50μM glutamate for a time period of 24 hours. The compound was made up in HBSS and incorporated into the culture medium. Vehicle recieved HBSS alone. Glutamate caused a dramatic increase in NADPH diaphorase activity as illustrated in Fig. 5.5.1 [$F(3, 63) = 20.19$, $p<0.001$, $p<0.05$, $n = 7$ cultures]. Cultures were also treated with 50μM glutamate for 24 hours and 400μM W7, added 4 hours prior to tissue fixation. Exposure to glutamate and W7 did not alter the increase caused by glutamate alone, [$p > 0.05$ by Tukey's pairwise test].

Figure 5.5.1 : Effect of glutamate and W7 on NADPH diaphorase levels in neuronal basal forebrain cultures.

Cultures were treated with 50μM glutamate in HBSS or vehicle (HBSS alone) for 24 hours. 4 hours prior to fixation, cultures were exposed to 400μM W7 or vehicle.

Results are expressed as a percentage of control optical density levels ± SEM.

Significance was analysed by one-way ANOVA followed by post-hoc Dunnett's test. *$p<0.05$.



control

40μM W7

50μM glutamate + 400μMW7

50μM glutamate

Data are mean ± SEM, n=7 cultures.

ANOVA as follows: $F[3, 63] = 20.19$, $p<0.001$.

* $p<0.05$ compared to control by Dunnett's test.
--*-- $p>$ 0.05 in Tukey's pairwise test.

5.9 *Effect of excitatory amino acid agonists on cytoskeletal protein expression.*

Cells were exposed to the non-NMDA agonist kainate (1μM and 10μM) for 24 hours. The metabotropic receptor agonist ACPD was added at concentrations of 10μM and 50μM, also for a time period of 24 hours. Exposure to kainate significantly increased MAP2 immunostaining at both concentrations [Fig. 5.6.1a, $F(2, 60)$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. However, no further increase in MAP2 immunostaining was seen at the higher concentration of 10μM of the compound, [$p>0.05$ by Tukey's pairwise test]. No changes in MAP2 immunoreactivity occurred after exposure to ACPD [Fig. 5.6.1b]. Neither kainate or ACPD altered tau immunoreactivity [Fig. 5.6.2a and b].

The effect of kainate was further investigated by in situ hybridisation using an oligonucleotide probe specific for MAP2 mRNA. Kainate significantly increased MAP2 gene expression, again with no further increase seen at the higher concentration of 10μM [Fig. 5.6.3, $F(2, 60) = 60.17$, $p<0.001$, $p<0.05$], $p>0.05$ by Tukey's pairwise test.

Figure 5.6.1 : Effect of ACPD and kainate on MAP2 immunoreactivity in neuronal basal forebrain cultures.

a Cultures were exposed to 1μM and 10μM kainate in HBSS or vehicle (HBSS alone) for a time period of 24 hours.

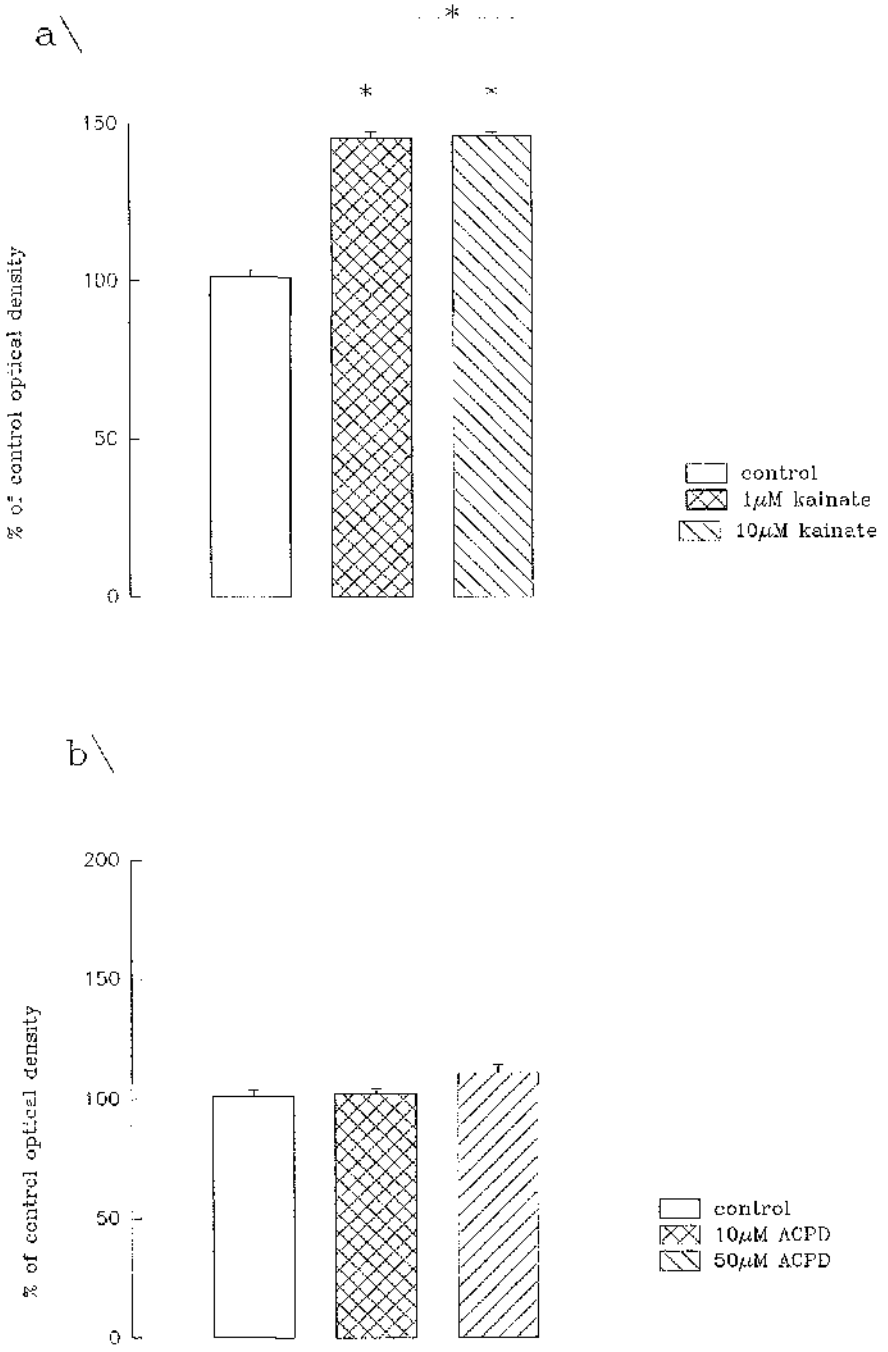
Results are expressed as a percentage of control optical density levels ± SEM.

Significance was analysed by one-way ANOVA followed by Dunnett's post hoc test.. * $p<0.05$.

b Cultures were exposed to 10μM and 50μM ACPD in HBSS or vehicle (HBSS alone) for a time period of 24 hours.

Results are expressed as a percentage of control optical density levels ± SEM.

By one-way ANOVA, no statistically significant difference occured between drug and vehicle treated wells.



Data are mean ± SEM, n= 6 cultures.

ANOVA as follows: kainate $F[2, 60]= 10.18$, $p<0.001$.
ACPD: $F[2, 44]= 5.16$, $p>0.05$.

* In Dunnett's $p<0.05$ compared to control value.
$--^{*}--$ $p>0.05$ in Tukey's pairwise test

Figure 5.6.2 : Effect of ACPD and kainate on tau immunoreactivity in neuronal basal forebrain cultures.

a Cultures were exposed to 1μM and 10μM kainate in HBSS or vehicle (HBSS alone) for a time period of 24 hours.

Results are expressed as a percentage of control optical density levels ± SEM.

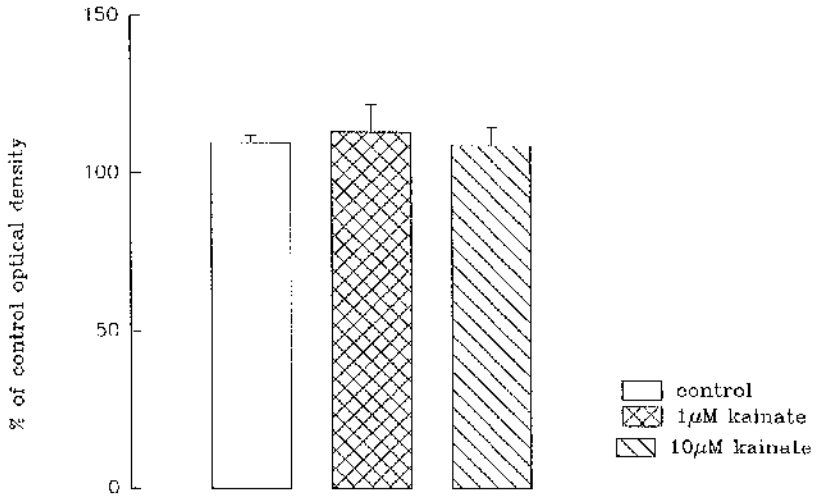
By one-way ANOVA, no statistically significant difference occured between drug and vehicle treated wells.

b Cultures were exposed to 10μM and 50μM ACPD in HBSS or vehicle (HBSS alone) for a time period of 24 hours.

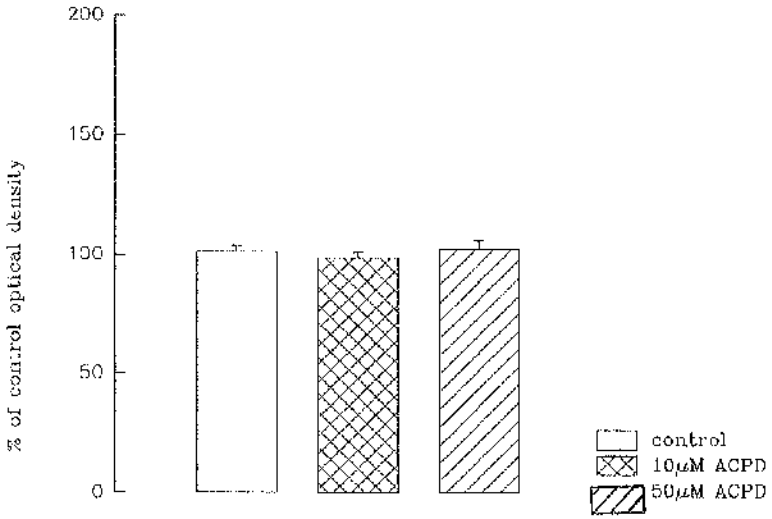
Results are expressed as a percentage of control optical density levels ± SEM.

By one-way ANOVA, no statistically significant difference occured between drug and vehicle treated wells.

a\



b\



Data are mean ± SEM, n= 6 cultures.

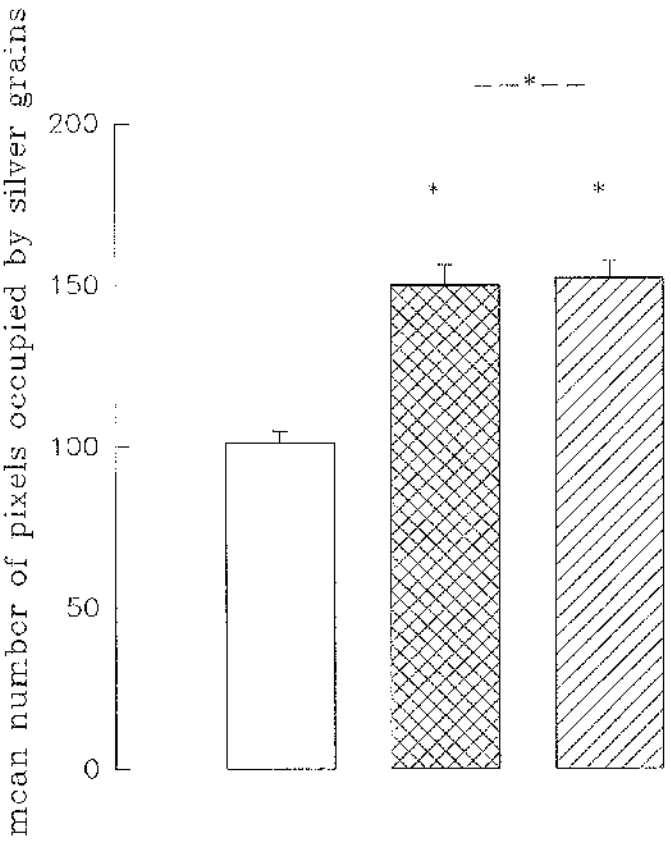
By one-way ANOVA: $p>0.05$.

Figure 5.6.3 : Effect of kainate on MAP2 mRNA levels in neuronal basal forebrain cultures.

Cultures were exposed to 1μM and 10μM kainate in HBSS or vehicle (HBSS alone) for a time period of 24 hours. Cells were then processed for in situ hybridisaiton.

Results, obtained form emulsion-dipped slides, are expressed as mean number of pixels occupied by silver grains ± SEM.

Significance was analysed by one-way ANOVA and post hoc Dunnett's test. * $p<0.05$.



Data are mean ± SEM, n = 6 cultures.

ANOVA as follows: $F\ [2, 60] = 60.17$, $p<0.001$

* In Dunnett's $p<0.05$ compared to control.
--*-- $p>0.05$ by Tukey's pairwise test.

5.10 *Effect of nitric oxide releasing compounds on cytoskeletal protein expression.*

To investigate the effects of NO on cytoskeletal protein expression, cultures were exposed to the NO-releasers SNP, SNAP and SIN-1for 24 hours. The compounds were made up in HBSS and incorporated directly into the medium. Care was taken to make up the compounds immediately prior to use, and to protect them from excessive light, as they are light-sensitive. Control wells received HBSS alone. As shown in Fig. 5.7.1, by ANOVA, 50μM SNP, 50μM SNAP and 50μM SIN-1 all significantly increased MAP2 immunostaining after 24 hours [$F(5, 120) = 79.81$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. Treatment for 24 hours with 20μM of the NO scavenger Hb plus 50μM SNAP reduced the SNAP-induced increase in MAP2 immunoreactivity to near control levels [Fig. 5.7.1]. None of the NO-releasing compounds caused a change in either tau or NSE immunostaining [Fig. 5.7.1].

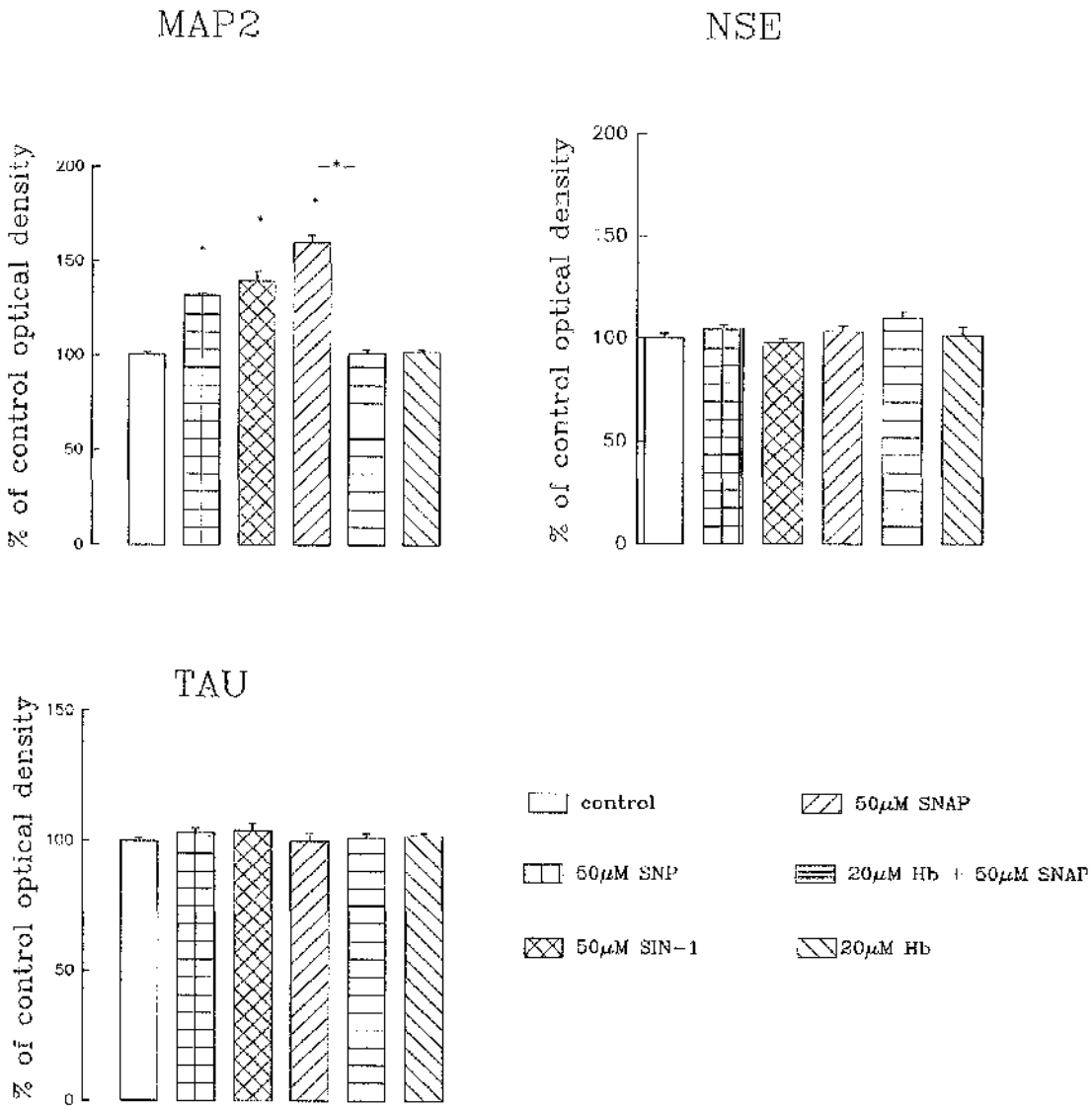
Treatment with the cyclicGMP analogue 8bromocyclicGMP resulted in an increase in MAP2 immunostaining. 8bromocyclicGMP was again made up in HBSS and added directly to the medium. Control wells recieved HBSS alone. By analysis of variance, it can be seen that the concentrations of 20μM and 200μM significantly increased MAP2 immunoreactivity [Fig. 5.7.2, $F(5, 120)= 79.81$, $p<0.001$, $p<0.05$, $n = 6$ cultures]. There was no significant rise in MAP2 levels after exposure to the higher concentration relative to the lower concentration of 8bromocyclicGMP, [$p>0.05$ by Tukey's pairwise test].

Figure 5.7.1 : Effect of nitric oxide releasers on MAP2, NSE and tau immunoreactivity in neuronal basal forebrain cultures.

Cultures were treated with 50μM SNP, 50μM SNAP, 50μM SIN-1, 20μM Hb or vehicle for 24 hours. Cultures were also treated with coadministration of 20μM Hb together with 50μM SNAP for 24 hours. Cultures were then taken for immunostaining to MAP2 , NSE and tau.

Results are expressed as a percentage of control optical density ± SEM.

Significance was analysed using one-way ANOVA followed by post-hoc Dunnett's test and post-hoc Tukey's pairwise test. *$p<0.05$.



Data are mean ± SEM, n= 6 cultures.

ANOVA as follows: MAP2 $F[5, 120]= 79.81$, $p<0.001$.

* In Dunnett's $p<0.05$ compared to control value.

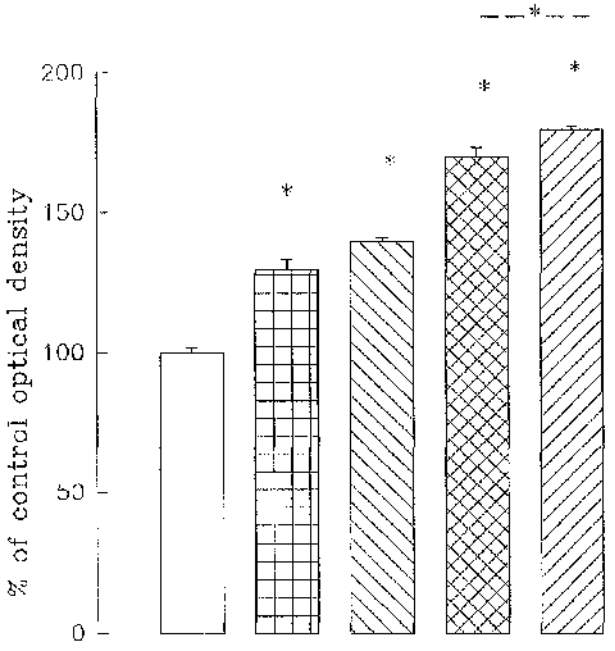
−*− In Tukey's pairwise test $p>0.05$.
No significance for NSE or tau.

Figure 5.7.2 : Effect of SNP and 8bromocyclicGMP on MAP2 immunoreactivity in neuronal basal forebrain cultures.

Cultures were treated with 10μM and 50μM SNP, or 20μM and 200μM 8bromocyclicGMP or vehicle (HBSS alone) for 24 hours.

Results are expressed as a percentage of control optical density level ± SEM.

Statistical analysis was by one-way ANOVA followed by post-hoc Dunnett's test. *$p<0.05$.



Data are mean ± SEM, n = 6 cultures.

ANOVA as follows: $F[4, 110] = 11.95$, $p<0.001$.

* In Dunnett's $p<0.05$ compared to control value.

—*— $p>0.05$ in Tukey's pairwise test.

5.11 *Effect of NGF and NO-releasers on neurite morphology.*

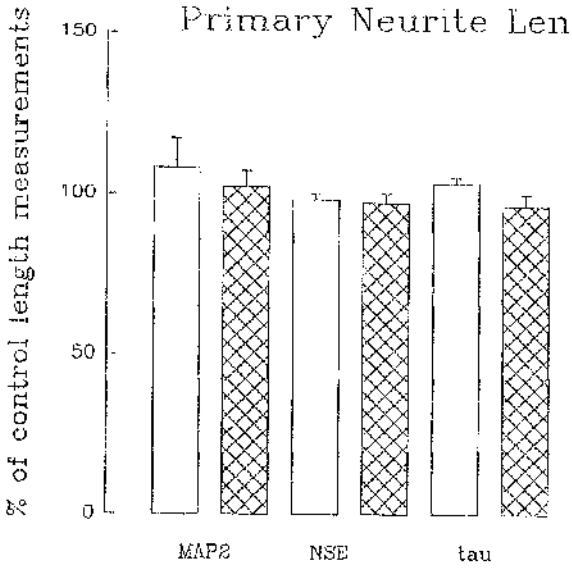
In order to obtain the most accurate assessment of neurite morphology in the neuronal cultures, cells were grown at a further dilution of 20 X 10^3 in order to obtain accurate measurements of the neurite characteristics. Neurites with clear cell origin and length were measured. Cultures were exposed to 50μM SNP, 50μM SNAP and 200 ng/ml NGF for a time period of 24 hours. The NO-releasers were made up in HBSS and incorporated into the medium, vehicle receiving HBSS only. NGF was added directly in the culture medium., vehicle receiving medium only. None of the compounds had an effect on primary neurite length or neurite staining intensity [Figs 5.8.1.and 5.8.2].

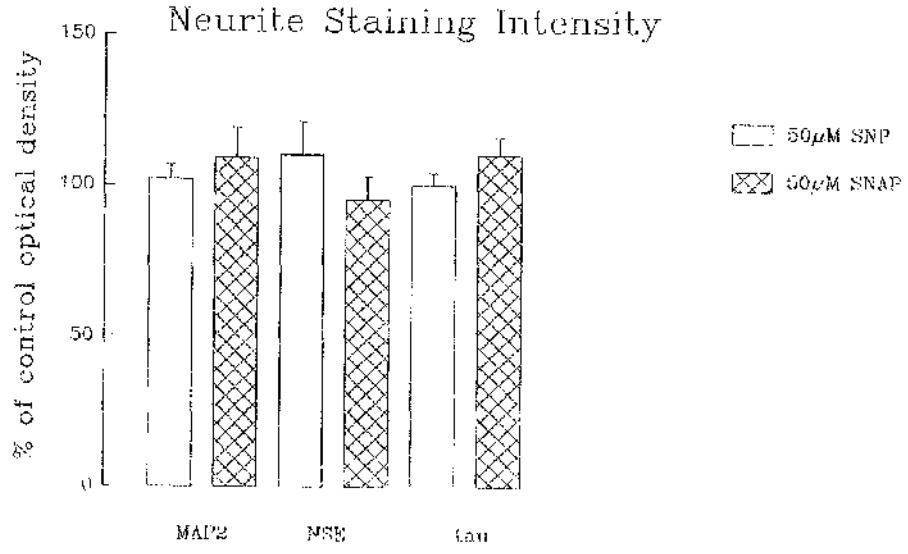
Figure 5.8.1 : Effect of nitric oxide releasers on neurite characteristics in neuronal basal forebrain cultures.

Cultures were treated with 50μM SNP, 50μM SNAP, or vehicle, for 24 hours. Cultures were then processed for immunostaining to MAP2, tau and NSE.

The measurements of primary neurite growths were taken from single neurites with clearly defined lengths and cell origin. Due to the lesser cell number in these experiments, the result of a more dilute cell suspension, single neurites were clearly visible. Results are expressed as a percentage of control optical density or neurite outgrowth measurement ± SEM.

Statistical analysis was by one-way ANOVA: no significant changes were observed.





Data are mean SEM. n=5 cultures

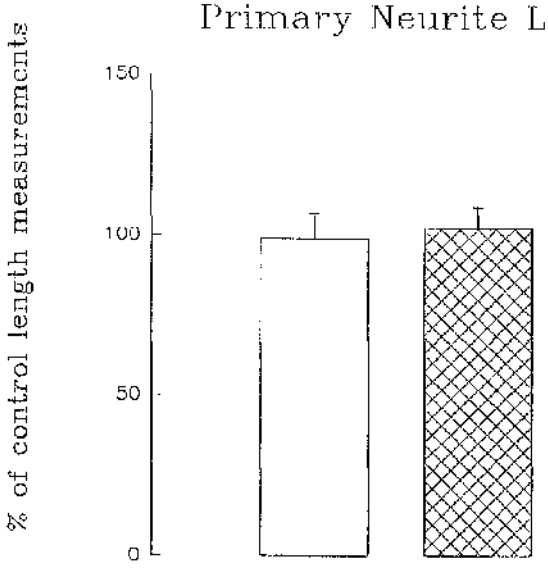
ANOVA: $p>0.05$

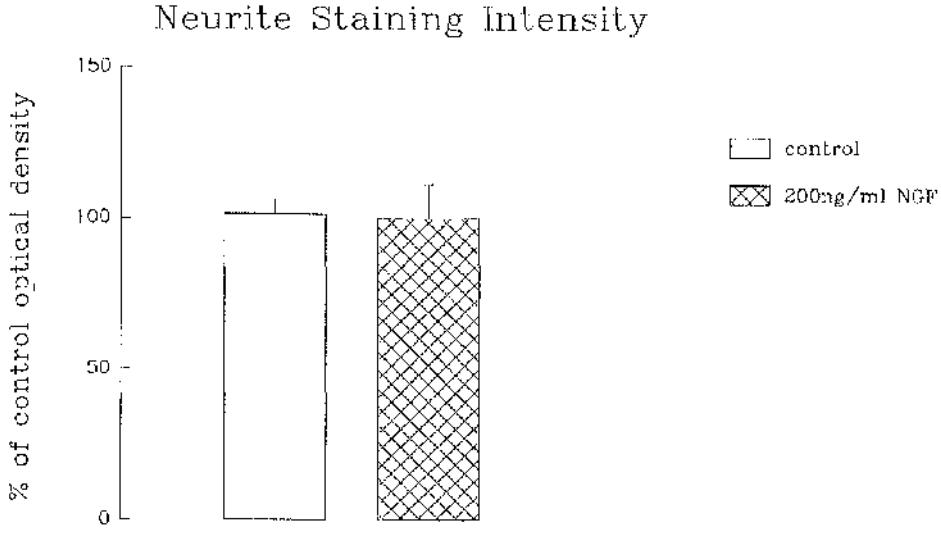
Figure 5.8.2 : Effect of NGF on neurite characteristics in neuronal basal forebrain cultures.

Cultures were treated with 200ng/ml NGF or vehicle for 4 days. Cultures were then processed for immunostaining to NSE.

Results are expressed as a percentage of control optical density or neurite outgrowth measurement ± SEM.

Statistical analysis was by one-way ANOVA: no significant changes were observed.





Data are mean SEM, n=5 cultures.

ANOVA: $p>0.05$.

5.12 *Effect of LPS and IL-1 on APP and COX2 immunostaining in neuronal cultures.*

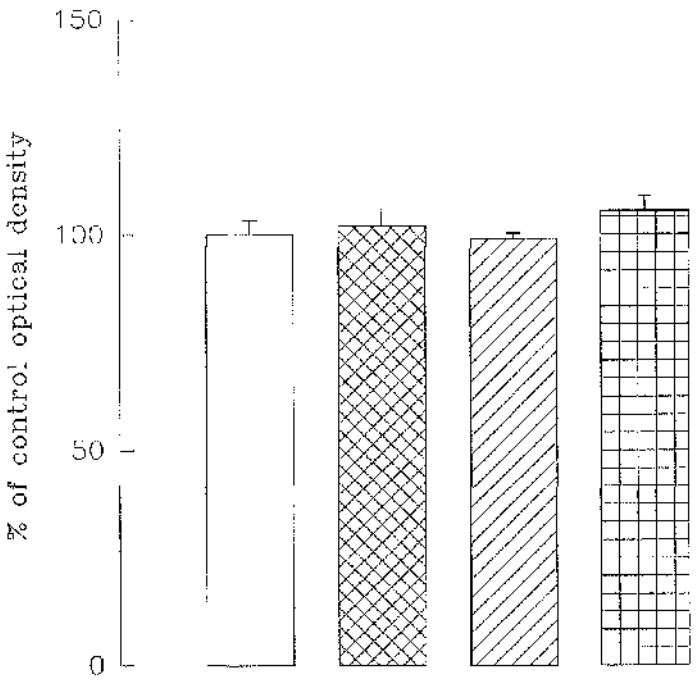
Cultures were exposed to 100μg/ml LPS and 0.25ng/ml IL-1, or coadministration for 24 hours. No significant changes occurred in either APP or COX2 immunostaining [Figs 5.9.1 and 5.9.3 respectively]. Following in situ hybridisation, no changes in APP gene expression occurred after exposure to IL-1 or LPS [Fig. 5.9.2]. PGE2 also did not induce a significant change in APP or COX2 immunostaining levels as shown in Fig. 5.9.4.

Figure 5.9.1 : Effect of LPS and IL-1 on APP immunoreactivity in neuronal basal forebrain cultures.

Cultures were treated with 100μg/ml LPS, 0.25ng/ml IL-1, vehicle (HBSS alone), or coadministration of 100μg/ml LPS with 0.25ng/ml IL-1. All treatments were for a time period of 24 hours. cultures were then taken for immunostaining to APP.

Results are expressed as a percentage of control optical density ± SEM.

Statistical analysis was carried out by ANOVA followed by post-hoc Dunnett's test. No significant differences were observed.



control

100μg/ml LPS

100μg/ml LPS + 0.25ng/ml IL-1

0.25ng/ml IL 1

Data are mean ± SEM, n=6 cultures.

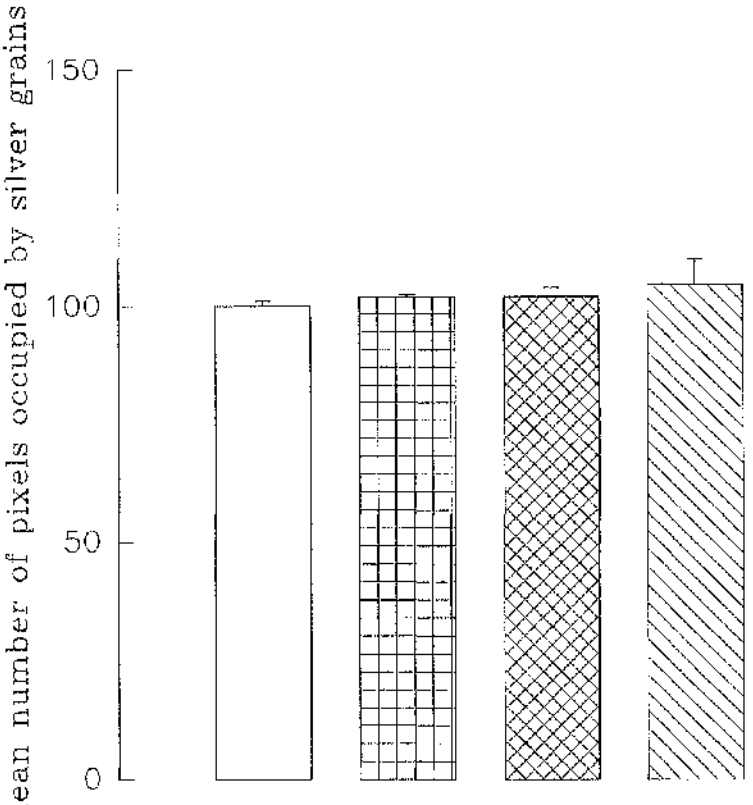
ANOVA: $p>0.05$.

Figure 5.9.2 : Effect of LPS and IL-1 on APP mRNA levels in neuronal basal forebrain cultures.

Cultures were treated with 100μg/ml LPS, 0.25ng/ml IL-1, vehicle (HBSS alone), or coadministration of 100μg/ml LPS with 0.25ng/ml IL-1. All treatments were for a time period of 24 hours. Cultures were then taken for in situ hybridisation.

Results are expressed as mean number of pixels occupied by silver grains ± SEM.

Statistical analysis was carried out by ANOVA followed by post-hoc Dunnett's test. No significant differences were observed.



control
100μg/ml LPS
0.25ng/ml IL-1
100μg/ml LPS + 0.25ng/ml IL-1

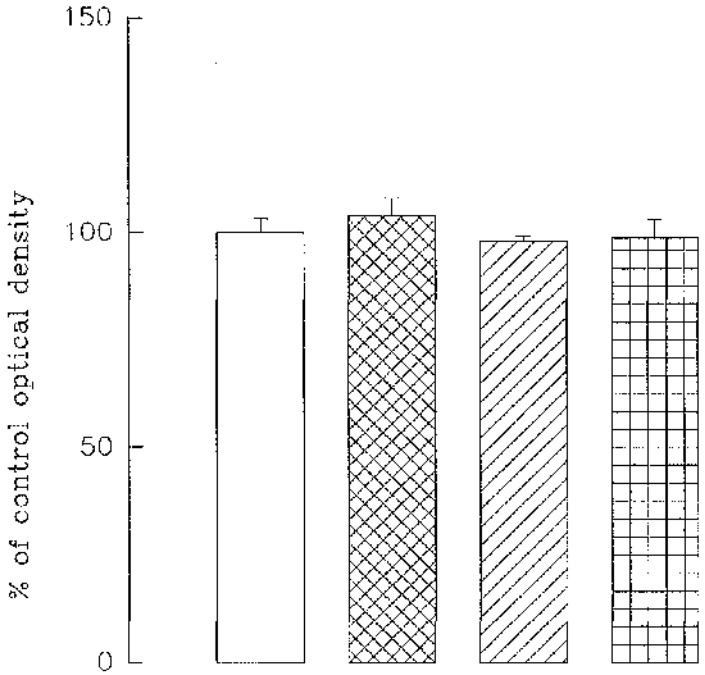
Data are mean ± SEM, n=4 cultures.
ANOVA : $p>0.05$.

Figure 5.9.3 : Effect of LPS and IL-1 on COX 2 immunoreactivity in neuronal basal forebrain cultures.

Cultures were treated with 100μg/ml LPS, 0.25ng/ml IL-1, vehicle (HBSS alone), or coadministration of 100μg/ml LPS with 0.25ng/ml IL-1. All treatments were for a time period of 24 hours. Cultures were then taken for immunostaining.

Results are expressed as a percentage of control optical density ± SEM.

Statistical analysis was carried out by ANOVA followed by post-hoc Dunnett's test. No significant differences were observed.



control

100μg/ml LPS

0.25ng/ml IL-1

100μg/ml LPS + 0.25 ng/ml IL-1

Data are mean ± SEM, n=6 cultures.

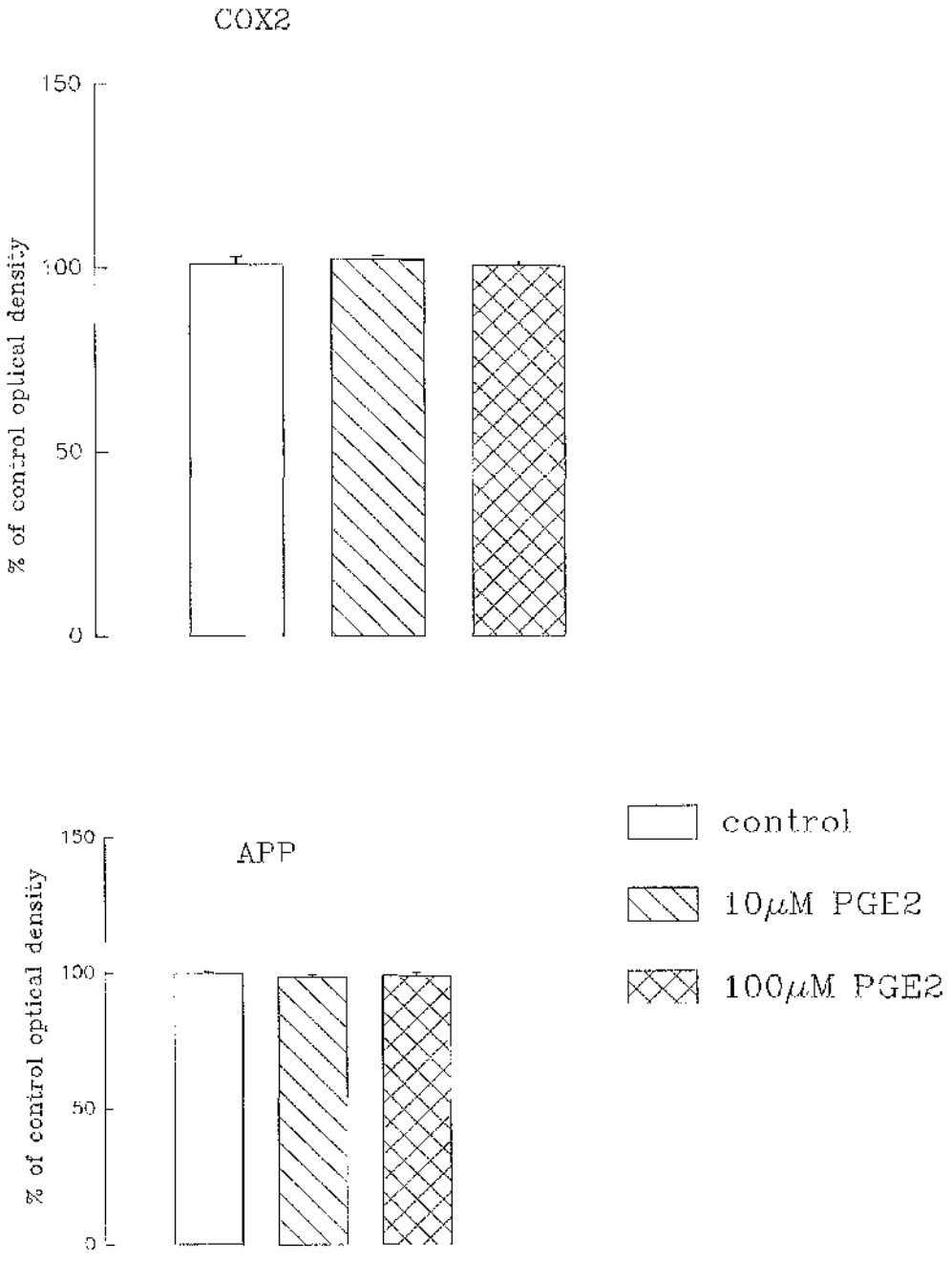
ANOVA : $p>0.05$.

Figure 5.9.4 : Effect of PGE2 on COX 2 and APP immunoreactivity in neuronal basal forebrain cultures.

Cultures were treated with four different concentrations of PGE2: 10μM, 20μM, 100μm and 200μM, or vehicle. All treatments were for a time period of 24 hours. Cultures were then taken for immunostaining.

Results are expressed as a percentage of control optical density ± SEM.

Statistical analysis was carried out by ANOVA followed by post-hoc Dunnett's test. No significant differences were observed.



Data are mean ± SEM, n= 6 cultures.

ANOVA: $p>0.05$

Discussion

5.13a Characterisation of basal forebrain neuronal cultures.

The cells cultured from the basal forebrain region of embryonic rats can be clearly characterised as cholinergic neuronal cells. The cells were typically around 25μm in diameter with many extensive and branching neurites. The average neurite length was 10-15μm. Cells showed positive immunostaining for ChaT; a marker for cholinergic cells. Positive immunostaining was also seen with MAP2, which is only found in neuronal cell types, and NSE, the glycolytic enzyme specific to neuronal cell populations.

It is important at this stage to consider whether the cells grown in culture show any of the phenotypes present in the adult brain basal forebrain. Basal forebrain neurons located in the medial septum, the diagonal band of Broca and nucleus basalis magnocellularis are the main source of cholinergic innervation to the hippocampal formation, cortex and olfactory bulb and have been implicated in cognitive decline associated with Alzheimer's Disease and ageing (Gibbs et al.,1994). The adult basal forebrain shows well documented positive immunoreactivity for ChaT, NSE and MAP2 (Cavicchioli et al.,1991; Gibbs et al.,1989). Thus, the cultured cells used in the present study have a similar phenotype to those cells in the adult basal forebrain in terms of ChaT, NSE, and MAP2 immunoreactivity. However, it should be noted that numerous other properties of the fully intact cholinergic system have not been classified in the cultured cells.

5.13b Cell culture as an experimental model.

Cell cultures have many advantages as experimental models; they provide easily observable systems that often retain biological properties parallel to those observed in vivo. At present, the greatest source of culture model systems to investigate CNS function and dysfunction is fetal brain material from experimental animals, typically rodents. For studies of human CNS disorders these models have two disadvantages: their fetal origin and their source from another species. Also, the experimental animal from which the cultures develop may have little or no natural susceptibility to the disorder being studied.

Also, isolated cells in culture may have many functional differences from the fully intact in vivo situation.

The use of immunohistochemistry and in situ hybridisation to monitor any changes in expression has the advantage that the cell types where any change has occurred can be identified. All of the changes observed occurred in the same population of cells that displayed positive MAP2, ChaT and NSE immunoreactivity, and therefore occurred in the cholinergic neuronal cells.

5.14 Effects of nerve growth factor on ChaT, tau and MAP2 expression in neuronal cultures.

The development and maintenance of the cholinergic neurons innervating the cortex is dependent on the trophic support provided by NGF (Hefti et al.,1985). NGF clearly increases the expression of ChaT and MAP2, but not tau, in the cholinergic cultures after 4 days treatment. Time course treatment with NGF showed a maximal effect on MAP2 immunostaining after 4 days, with no further change after 1 week exposure to NGF. MAP2 gene expression continued to increase after 4 days and 1 week exposure to NGF. A great many experiments document the ability of NGF to support survival, neurite outgrowth and protection of a variety of CNS neurons, most notably the magnocellular cholinergic neurons of the basal forebrain (Gage et al.,1991; Springer.,1988; Wilcock et al.,1995). NGF has been shown to enhance cholinergic function in vitro and in vivo (Higgins and Mufson., 1989; Hefti et al.,1989). Present results agree with past literature demonstrating NGF to selectively increase cholinergic markers in rat basal forebrain in culture (Martinez et al.,1987; Hartikka and Hefti.,1988). The time courses of induction of MAP2 mRNA and protein expression provided curious results. A predicted result would have seen the time course of mRNA increase to be faster than that of the increase in protein expression. However, the mRNA levels for MAP2 continue to rise after 1 week, the protein levels remaining unchanged. It is possible that an increase in mRNA levels precedes the increase in protein levels, or that a regulatory mechanism within the cells may prevent a further rise in MAP2 protein levels, despite the continued increase in gene expression. It would be of

interest to study MAP2 mRNA levels at an early time point of 8 hours and also at a later time point greater than 24 hours.

5.15 Effect of nerve growth factor on NADPH diaphorase levels in neuronal cultures.

NGF causes PC12 cell growth and differentiation. This response to NGF involves a proliferative phase that is followed by growth arrest and differentiation (Greene and Tischler.,1976). The cytostatic effect has recently been shown to be mediated by NO (Peunova et al.,1995). The exposure of neuronal cultures to NGF resulted in an increase in NADPH diaphorase staining after 24 hours. This affect was maximal after 24 hours, with no further increase following 30 hours, or 4days exposure. The NADPH diaphorase stained cells were morphologically of the same population as those positive for MAP2, ChaT and NSE indicating that the cholinergic neuronal cells possess NO activity, although whether this indicates the presence of NOS or, the possible type of NOS involved can not be defined. NOS has been shown to be localised in the same neuronal population as NADPH diaphorase in vivo (Bredt et al.,1991). NADPH diaphorase immunostaining has shown colocalisation with the cholinergic basal forebrain (Pasqualotto and Vincent.,1991), and, colocalisation of NOS and the low affinity nerve growth factor has been demonstrated in the medial septum and diagonal band nuclei (Peng et al.,1994). Neuronal NOS immunoreactivity has been clearly demonstrated in basal forebrain sections (Bredt and Snyder 1991).

The time course of the effect of NGF on NADPH diaphorase immunostaining is similar to that seen previously (Peunova et al.,1995), with a maximal effect seen after 24 hours. Peunova and coworkers suggested that a large proportion of the NGF-induced NOS activity in PC12 cells is calcium-independent especially at the early stages of NGF action (within 24 hours), and may be due to iNOS (Peunova et al.,1995). Different types of NOS may be activated during the different stages of NGF's action on the cell line, starting with induction of a calcium-independent inducible form (Peunova et al.,1995). Whether a similar pattern occurs in neuronal cells involving inducible and neuronal NOS, where basal levels of neuronal NOS rise in the presence of NGF together with the induction of iNOS, can not

be elucidated from the present results. The present results do provide, evidence that NGF induces a form (s) of NOS in neuronal cells. Holtzman and colleagues recently showed that the expression of neuronal NOS in developing basal forebrain cholinergic neurons is regulated by NGF (Holtzman et al.,1996). They raised the possibility that endogenous NGF may regulate regulate neuronal NOS expression in developing basal forebrain cholinergic neurons, and that NGF may act through NO to affect differentiation and maturation of cholinergic neurons. The high affinity nerve growth factor receptor, the p^{140}trkA tyrosine kinase receptor, is thought to be essential and sufficient to elicit NGF responses (Kahle et al.,1994). This receptor has intrinsic tyrosine kinase activity and is capable of evoking cellular neurotrophic responses in the absence of the p^{75} low affinity receptor (Loeb and Greene., 1993). As has been discussed previously, LPS induction of iNOS is thought to be via tyrosine kinase activation (Kitamura et al.,1996). As the actions of NGF are through the high affinity tyrosine kinase receptor, this would argue in favour of the presence of iNOS in the neuronal cells. Further experiments using radiolabelled oligonucleotide probes to nNOS and iNOS would clarify the possible type (s) of NOS present in the neuronal cells, and whether a similar pattern to that seen in PC12 cells where iNOS is induced in the beginning stages of NGF's action (Peunova et al.,1995).

5.16 Effect of nerve growth factor on cytoskeletal proteins and glycolytic enzyme immunostaining in neuronal cultures.

The NGF-induced increase in MAP2 and NSE immunostaining was reduced to near baseline levels following pre-treatment of the cultures with dexamethasone. Dexamethasone is not primarily an inducible NOS inhibitor, the synthetic glucocorticoid is used clinically to suppress the immune response and the processes of inflammation (Parillo and Fauci., 1979). iNOS transcription has recently been shown to be regulated by activation of the immune system regulator nuclear factor kappa B (NFκB) (Goldring et al., 1995). It is thought that inhibition of iNOS gene expression may be due to a decreased availability of NFκB (Colasanti et al., 1995), and dexamethasone may inhibit iNOS gene expression by affecting NFκB activation (Colasanti et al., 1995). As the actions of dexamethasone are varied, a similar

experiment was carried out using the NO scavenger haemoglobin (Hb), focusing on MAP2 immunoreactivity.

Exposure of the cultures to Hb and NGF resulted in MAP2 immunostaining reducing to baseline levels. The results obtained with Hb and dexamethasone suggest that the effect of NGF on MAP2 immunoreactivity may involve NO activity, and may involve iNOS. Further experiments discussed in section 5.19 also provide further evidence for the involvement of NO; the results obtained with NGF are mimicked by the NO-releasers SNP and SNAP. Inducible NOS activity has been demonstrated in other neuronal cell types: LPS-activated granule neurons express iNOS activity (Sato et al.,1996), and an increase in iNOS immunoreactivity is thought to accompany regenerative processes in peripheral ganglia (Magnusson et al.,1996). It has also recently been shown that direct intracerebellar injection of LPS induces in vivo neuronal expression of the iNOS gene (Minegolomb et al.,1996). Thus, it is possible that the present neuronal cells possess iNOS activity.A further experiment using the calmodulin antagonist W7 suggests that the NGF-induced increase in MAP2 immunoreactivity involves a calmodulin-dependent NOS: exposure of the cells to W7 reduced the NGF-induced increase in MAP2 immunoreactivity to near control values. This agrees with the fact that the neuronal form of NOS is a calmodulin-dependent enzyme (Schmidt et al.,1991; Bredt and Snyder., 1990). However, as has been discussed previously, W7 can not be used as a distinguishing compound as both neuronal and inducible NOS are calmodulin-dependent. Recent data shows that glial cells may possess a calcium-insensitive but calmodulin coupling NOS, and that iNOS induced from glial cells is coupled tightly with endogenous calmodulin even in the absence of calcium (Kitamura et al.,1996). The calmodulin-binding region of iNOS has been shown to account for its calcium independence (Ruan et al.,1996). In the present work, inhibition of the NGF-induced increase in MAP2 immunostaining by dexamethasone indicates the presence of an inducible form of NOS in the neuronal cultures, while inhibition with W7 indicates the presence of a calmodulin-dependent form of NOS. As it is more than likely that the neuronal cells contain basal levels of neuronal NOS, it is possible that they also possess inducible NOS activity.

As has been mentioned previously, the conclusive way to elucidate the type (s) of NOS involved in the neuronal and glial cells would be to use specific oligonucleotide probes. Also, calcium involvement has not been investigated in the present work. iNOS is now though to be calcium-independent, thus removal of calcium by using chelators such as EGTA would decrease neuronal NOS activity. Induction of astroglial iNOS has been shown to involve tyrosine kinase activity (Feinstein et al.,1994; Kitamura et al.,1996). A further neuronal culture experiment using the tyrosine kinase inhibitors genistein and herbimycin A would enable any involvement of tyrosine kinase activation in NOS activity to be studied.

An important question unanswered at the present time is whether the differential expression of the iNOS gene reported by Park et al (1996) is due to differences in the calmodulin binding site. As discussed in Chapter 4, the cultured glial cells may possess different types of NOS enzyme differing in calmodulin dependency, and this may also be true of the neuronal cholinergic cells.

5.17 Effect of glutamate on NADPH diaphorase levels in neuronal culture.

One process speculatively linked to the pathogenesis of Alzheimer's Disease has been the neurotoxicity of glutamate (Choi et al.,1988). NMDA and NO have been shown to increase MAP2 gene expression in hippocampal granule cells (Johnston and Morris., 1994). In light of this evidence, the effect of excitatory amino acids on NADPH diaphorase and cytoskeletal immunostaining was investigated. Exposure of the basal forebrain cell cultures to glutamate resulted in a significant increase in NADPH diaphorase staining. Treatment with W7 and glutamate resulted in no change in the glutamate-induced rise in NADPH diaphorase staining. This result is similar to that seen in the glial cultures, where an LPS induced increase in NADPH diaphorase levels appeared to involve a calmodulin-independent NOS; NADPH diaphorase immunostaining remaining unaltered after W7 exposure (Chapter 4). As has been discussed, it is possible that the neuronal cultures, as well as the glial cells, possess various forms of NOS. It may be that the cultured

neuronal cells possess neuronal NOS, iNOS and, differential expression of iNOS with various forms differing in calmodulin dependency. As discussed at the end of section 5.16, experiments to further investigate the different forms of NOS are necessary.

It is important to also discuss discrepancies in NADPH diaphorase immunostaining reported. NADPH-diaphorase histochemistry has been widely used to identify neurons that produce NO (Valtschanoff et al.,1993; Dawson et al., 1992). However, it should be noted that recent studies have noted discrepancies regarding the correlation between NADPH-diaphorase activity and NOS immunoreactivity. Whereas some authors report a complete overlap of both (Decker and Reuss.,1994; Hashikawa et al.,1994; Valtschanoff et al.,1993), others report that NADPH diaphorase histochemistry and nNOS immunohistochemistry do not necessarily label the same neuronal population (Kharazia et al.,1994; Vizzard et al.,1994). These differences may be due to the sensitivity of NADPH diaphorase to paraformaldehyde as a fixative (Spesser and Layes.,1994), or to the existence of enzymes other than NOS displaying NADPH diaphorase activity (Hope et al.,1991). In the present study, great care was taken to ensure identical staining conditions throughout each set of experiments.

5.18 Effect of excitatory amino acids on cytoskeletal protein expression.

Basal forebrain cholinergic neurons have been shown to be selectively vulnerable to non-NMDA receptor mediated neurotoxicity (Weiss et al.,1994). Glutamate has been shown to induce a dose-dependent increase in tau immunoreactivity in primary neuronal cultures (Sindou et al.,1992; Sautiere et al.,1992). In light of this evidence, cultured cells were exposed to the non-NMDA receptor agonist kainate, and the metabotropic receptor agonist ACPD. Kainate significantly increased MAP2 immunoreactivity, with no significant effect on tau immunoreactivity. ACPD had no significant effect on the immunoreactivities of either cytoskeletal protein. This result suggest that the cultures may be sensitive to non-NMDA receptor activated increase in MAP2 immunoreactivity, and an

absence of metabotropic receptor involvement in cytoskeletal protein immunoreactivity. The induction of MAP2 but not tau may indicate a preferential action of kainate on dendritic as opposed to axonal morphology. However, as is discussed in Section 5.20, the NGF-induced increase in MAP2 is not linked to detectable changes in dendritic morphology.

5.19 Effect of NO releasers on cytoskeletal protein expression.

The NO-releasing compounds SNP, SNAP and SIN-1 breakdown in solution releasing NO. All three of these compounds caused an increase in MAP2 immunoreactivity with no effect on tau or NSE levels. This increase in MAP2 levels was reduced to near baseline levels following combined exposure to SNAP and the NO scavenger Hb. The membrane-permeable cyclicGMP analogue, 8bromocyclicGMP, also increased MAP2 immunoreactivity. As discussed previously, NO is released by cells containing NOS following an increase in intracellular calcium levels. NO can then diffuse to affect neighbouring cells, where many of its actions involve the activation of guanylate cyclase which results in increases in intracellular cGMP levels and stimulation of cGMP-dependent protein kinase (Garthwaite et al., 1988; Knowles et al.,1989). Recently, it has been shown that NO stimulates expression of the immediate early genes c-fos and zif/268 in PC12 cells (Haby et al.,1994; Peunova et al.,1993). NO has also been shown to alter proenkephalin, prodynorphin and MAP2 gene expression in hippocampal granule cells (Johnston et al.,1994a; Johnston et al.,1994b). Thus, it is possible that NO may regulate gene expression in those areas of the brain containing NOS activity. As shown in Section 5.15, the neuronal cholinergic cultures contain NOS activity.

Recent evidence has shown that certain drugs which cause NO release in solution can also give rise to non-specific effects unrelated to the release of NO (Butler et al.,1995). Therefore a number of NO-releasing compounds from different structural classes were used. All three compounds increased MAP2 immunoreactivity, and their only common feature is NO release. This, together with the fact that the NO scavenger Hb reduced the increase in MAP2 immunoreactivity

strongly implicates NO in this affect. NO-induced increases in c-fos and zif/268 mRNA levels in PC12 cells have been shown in one case to involve the activation of guanylate cyclase and cGMP-dependent protein kinase (PKG), as mimicked by 8bromocGMP (Haby et al.,1994). Other results implicated synergism with calcium influx following treatment with the calcium ionophore A23187 (Peunova et al.,1993). The present results suggest that NO may exert an induction in MAP2 immunoreactivity through a pathway that involves guanylate cyclase and cGMP-dependent protein kinase, corresponding with the results seen in the cell line. However, further experiments using the cGMP phosphodiesterase inhibitor MY5445 and the selective PKG inhibitor KT5823 would strengthen this theory.

It is important at this stage to discuss the main antibodies used in the experiments. As has been discussed, tau immunoreactivity has remained unaltered, with dramatic effects being observed in MAP2 immunoreactivity. The tau antibody is a mouse monoclonal antibody which reacts with all isoforms of tau in both the phosphorylated and unphosphorylated states. It recognises tau along the microtubules, in axons, somata and ribosomes. The fact that no immunoreactivity changes have occurred, and no alterations in mRNA levels have occurred, indicate no change in synthesis of any isoform. Also any changes in phosphorylation that may have occurred will not have affected the results. The MAP2 antibody is also monoclonal and reacts with all known forms of MAP2: MAP2a, 2b and 2c. It is also a phosphorylation-independent antibody.

5.20 Effect of NGF and NO releasers on neurite morphology.

In view of the fact that NGF and the NO releasing compounds had such a dramatic effect on MAP2 immunoreactivity, and of the well documented effect of NGF on neuronal cell survival and neurite outgrowth, an investigation of neurite morphology was carried out. The use of a more dilute cell suspension in this set of experiments enabled clear measurements of the neurites. Surprisingly NGF did not induce any change in primary neurite length or neurite staining intensity. It had been expected that NGF may cause some change in neurite morphology. It is possible that an undetected change occurred,

and it is worth considering that there may have been a morphological change to the dendritic spines or neurite branching points. Perhaps it was an oversight to have focused on the primary neurite length given that this remains unaltered. A look at further branching points may have given clearer results.

Growth of nerve fibres occurs under three conditions: during development, as regeneration of neurites after lesion and in the differentiated nervous system as a prerequisite of structural plasticity. Neurite growth is regulated on two levels of the neuron: in the cell body, and at the growing tip of axons or dendrites. A variety of factors have been described which influence neurite growth on these different levels, the most extensively studied molecule being NGF (Thoenen and Barde .,1980; Teng and Greene., 1994). The reasons why NGF failed to cause any change in neurite growth in the present study remain unclear.

5.21 Effect of LPS and IL-1 on APP and COX2 immunostaining in neuronal cultures.

Recent studies have suggested a neuroprotective influence for non-steroidal anti-inflammatory drugs (NSAIDS), and a beneficial association between concurrent NSAID use and cognitive functioning in Alzheimer's Disease (Rich et al., 1995). IL-1 is thought to be involved in the neuronal mechanisms underlying Aβ deposition in AD (Forlini et al.,1992). As with NOS, COX2 possesses distinct cytokine-inducible isoforms (iNOS and Cox2 respectively). In the present study, the effect of LPS and IL-1 on APP and COX2 immunoreactivity was investigated. Neither compound or combined exposure to both altered APP or COX2 immunoreactivity. Exposure of the cultures to the prostaglandin PGE2 did not alter COX2 or APP immunoreactivity. Thus it appears that the compounds at the concentrations and time points used have no effect on APP or COX2 expression. Evidence suggests that induction of COX2 by LPS can occur in bovine aortic endothelial cells (Akarasereenont et al.,1994). A similar system does not appear to occur in neuronal cells at the concentrations used. It has recently been shown that induction of COX2 mRNA may involve activation of NMDA receptors in vivo

(Miettinen et al.,1997). In light of this evidence, perhaps more conclusive results would have been obtained using excitatory amino acid agonists and antagonists. Further investigations into the possible function (s) of APP have been carried out using two transgenic APP models, and this is discussed in chapter 6.

Summary:

In the present section of work, cholinergic neuronal cultures have been characterised by positive immunostaining to ChaT, MAP2 and NSE. Using these cultured cells, the distribution and expression of cytoskeletal proteins, and NADPH diaphorase immunostaining have been investigated as summarised below:

1\ Exposure of the cells over a 24 hour period to NGF resulted in a dramatic increases in ChaT, MAP2 and tau immunoreactivity.

2\ The cultured cells appear to possess a type of NOS that is regulated by NGF.

3\ NGF induced an increase in MAP2 and NSE immunoreactivity. This induction of MAP2 expression may be through a neuronal calmodulin-dependent form of NOS.

4\ Exposure of the cells over a 24 hour period to glutamate resulted in the induction of a calmodulin-independent form of NOS.

5\ It is possible that the neuronal cells possess various types of NOS enzyme differing in their calmodulin-dependency.

6\ Exposure of the cells to NO-releasing compounds over a 24 hour period resulted in a dramatic increase in MAP2 expression. It is possible that NO may regulate MAP2 expression through a pathway involving guanylate cyclase and cGMP-dependent protein kinase. The

induction of MAP2 expression by NGF and NO-releasers was not accompanied by any detectable morphological changes.

The next stage of work has focused on APP function. An investigation into the distribution and regulation of cytoskeletal and other proteins in response to APP removal and mutation has been carried out.

Chapter 6

Immunohistochemical study of morphological and synaptic markers in two mutant mouse models.

INTRODUCTION

This section of work made use of two transgenic mice models. The aims were as follows: to investigate glial fibrillary acidic protein (GFAP), synaptophysin, MAP2, the calcium binding protein calbindin D28k, and presenilin immunoreactivity in two transgenic mice models: one with deleted amyloid precursor protein (APP-null), and another with a mutated α-secretase cleavage site on the APP gene (APP/RK). Changes in immunostaining observed have been discussed in relation to possible physiological role (s) of APP.

6.1 *Transgenic Models*

The lack of a suitable animal model for Alzheimer's Disease has slowed our understanding of the pathogical mechanisms that occur in the development of Alzheimer's Disease. Many research groups have been attempting to generate transgenic rodent models of Alzheimer's Disease to determine if these mice show the neuropathological hallmarks of the disease and demonstrate memory and learning deficiencies. The neuropathological hallmarks of Alzheimer's Disease include proteinaceous deposits known as plaques and neurofibrillary tangles, as well as neuronal and synaptic loss. The related processes of neurodegeneration and synaptic loss are the key substrates of Alzheimer's Disease. An ideal animal model of the disease would develop each of the pathological symptoms of the disease as a function of age.

The majority of animal models for Alzheimer's Disease have focused on manipulation of the APP gene. Prior to 1995, animals transgenic for APP had failed to show extensive Alzheimer's Disease-type neuropathology (Quon et al ., 1991, Pearson et al ., 1993). Transgenic mice have been produced that express various forms of the human APP gene (Price and Sisodia, 1994). Recently, Games et al reported the production of transgenic mice that express high levels of human mutant APP, (with valine at residue 717 substituted by phenyalanine), and progressively develop many of the pathological hallmarks of the disease including numerous thioflavin-positive amyloid deposits, neuritic plaques, synaptic loss and reactive gliosis (Games et al., 1995).

The present study has focused on the following two transgenic models:

1\ APP-null mice:

A complete deficiency of APP in mice was generated by homologous recombination in embryonic stem cells (Zheng et al.,1995). The homozygous APP-deficient mice weighed 15-20% less than age-matched wild type controls, and demonstrated premature mortality, seizures and behavioural abnormalities such as aggression and hostility. At 14 weeks of age, four out of six animals showed reactive gliosis in the hippocampus, striatum and cortex (Zheng et al ., 1995). Synaptic loss was observed especially in the dentate gyrus and LTP impairment has also been reported.

A complete removal of the APP gene may allow insight into the function of APP in AD, and serve as a valuable strain for the possible insertion of the human APP gene. In the present study, brain sections taken from the APP-null mice have been compared with sections from age-matched wildtype animals, focusing on the following: reactive gliosis levels have been investigated by quantification of GFAP immunoreactivity, quantification of calbindin immunoreactivity has been measured using both immunohistochemistry and radiolabelling, and, a comparison of MAP2 and synaptophysin immunoreactivity has also been carried out.

2\ APP/RK mutant mice

The proteolysis step of APP secretion, mediated by an as yet unknown proteinase named α secretase, is an intrinsic and essential step in the mechanism of APP secretion. Whereas the known clinical mutations in APP affect the β and γ secretase sites, no natural mutations have been described at the α secretase site. The functional importance of the α-secretase processing of APP was examined by Moechars and coworkers (Moechars et al.,1996). Introducing a double mutation in the α secretase region by replacing two basic residues with two acidic residues gave a APP/RK mutant. This mutant was secreted at a reduced rate from three different cell types: COS cells, MDCK cells and primary neurons (De Strooper et al.,1993). Expression of this α-secretase mutant in the brain of transgenic mice

resulted in behavioural disturbances, seizures and premature death. The neuropathological changes observed included apoptosis, necrosis and reactive gliosis in the hippocampus, cortex and amygdala (Moechars et al.,1996). The results demonstrated the severe neurotoxicity resulting from incorrect α secretase processing of APP in the CNS.

In the present study, it was fortunate to obtain brain sections from these transgenic mice and examine GFAP immunostaining in APP/RK and age matched wildtype animals.

6.2 *Calcium binding proteins*

An uncontrolled sustained elevation of free calcium ions is toxic to neurons and may eventually lead to cell death (Siesjo et al ., 1985). Neurons possess several control mechanisms that prevent such an overload of calcium. Besides storage in cellular granules and extrusion of calcium by pumps and ionic exchange (Rasmussen et al ., 1990), neurons contain specific proteins such as parvalbumin and calbindin D-28k which transiently bind calcium ions (Celio et al ., 1990). Evidence suggests that dysfunction of calcium homeostasis occurs during ageing. An increased calcium concentration in whole brain homogenates (Michaelis et al ., 1992), and an elevated concentration of free calcium ions in synaptosomes and dorsal root ganglion cells have been demonstrated in aged rats (Kirischuk et al ., 1992). Calbindin D-28k plays an important role in the buffering system which protects tissue from calcium-induced cellular injury (Tao et al .,1996a) and calbindin D-28k has been shown to be expressed in the hippocampus of AD brains (Tao et al ., 1996b). The protein has also been shown to be expressed in a number of forebrain areas implicated in learning and memory (Persechini et al., 1989, Baimbridge et al ., 1992, Heizmann et al ., 1992). Calbindin D-28k levels have been shown to be reduced in the hippocampus and cerebral cortex of AD patients (Ichimiya et al., 1988). The influx of calcium into the postsynaptic neuron is likely to be an important event in memory formation, and it is thought that calbindin D28k may have a role in the activation of calcium-dependent intracellular signaling pathways underlying memory function. Transgenic mice deficient in calbindin

D28k show impairments in learning paradigms and fail to maintain long term potentiation (Molinari et al.,1996).

In the present study, calbindin immunoreactivity levels were quantified by immunohistochemistry and radiolabelling in order to establish any effect removal of the APP gene has on calcium binding protein levels.

6.3 *Presenilins*

As discussed in Chapter 1 section 1.9.1, two novel genes, presenilins 1 and 2, have been linked to the development of early onset Alzheimer's Disease. The presence of six to nine hydrophobic domains indicates that presenilins 1 and 2 (PS-1, PS-2) share a serpentine topology which produces one large and several small hydrophobic loops (Kovacs et al.,1996). The mechanism by which the early onset familial Alzheimer's Disease (FAD)-associated mutations exert their effect is unknown. Around two separate causative mutations in 52 individual pedigrees linked to chromosome 14 have been identified on PS1 (S182), and two mutations have been detected in PS2 (Wasco et al.,1995; Sorbi et al.,1995; Tanahashi et al.,1995; Cruts et al.,1995).

PS1 and PS2 proteins show 67% identity and have homology to Caenorhabiditis elegans, indicating that the PS genes are conserved during evolution (Levitan and Greenwald.,1995). Although the role(s) of the presenilins have not been discovered, several predictions have been made as to their biological role based on their structures. These include possible roles as receptor molecules, ion channel or membrane structural proteins or protein transporters (Levitan and Greenwald.,1995; Sherrington et al.,1995). PS2 has also recently been implicated in apoptosis (Vito et al.,1996).

PS1 immunostaining has been found intracellularly in the perikarya of cells, mostly neurons, appearing as thick granules resembling large vesicles (Moussaoui et al.,1996). The gene product for PS1 has been found in neurons in human, rat and mouse CNS as a membrane bound protein of 48kDa, with dendritic staining stronger than axonal staining (Elder et al.,1996). PS1 mRNA has also been seen to be widely expressed in non-neural tissues including the heart, liver and lung (Sherrington et al.,1995). PS1 expression has been

found to be predominant in the hippocampus and cerebral, i.e. in those areas of the brain that are most susceptible to plaque formation (Cribbs et al.,1996).

The present work has examined the effect of APP deletion on PS-1 expression in various areas of the brain. Using antibodies directed against the N and C termini of PS-1, the pattern of immunostaining in APP-null and age matched control animals has been investigated.

Results

6.4 *GFAP immunoreactivity in brain sections of APP-null and wild type animals.*

GFAP-immunostained coronal sections through the hippocampus, corpus callosum and cortex from wildtype (wt) and APP-null mice (-/-) at 13 months of age were examined for reactive gliosis. (all antibodies used in this section of work are described in the Materials section p52). As can be clearly seen in Figs 6.1.1 and 6.1.2, a larger degree of GFAP immunoreactivity occurred in the wt animals. The wt animals show marked GFAP immunoreactivity: astrocytes with enlarged cell bodies and thicker processes staining heavily for GFAP. In the hippocampus, extensive GFAP immunoreactivity was observed in the CA1 region, and dentate gyrus especially the granule cell layer. GFAP immunoreactivity was seen throughout the cortical layers. Surprisingly, the APP-null animals displayed a low level of GFAP immunoreactivity principally localised in the hippocampus, corpus callosum and outer cortical layer. GFAP immunoreactivity was investigated quantitatively, and the numbers confirmed the photographic observations, as shown in Fig. 6.1.3. By the Mann-Whitney U-test, the wt animals show a dramatically higher GFAP optical density [Fig.6.1.3, $p<0.001$, $n = 4$ animals]. GFAP immunostaining was also significantly higher in the corpus callosum of the wt animals [Fig. 6.1.4, $p<0.001$ by Mann Whitney U test, $n = 4$]. Cell counts for cortical layers 2-3 were also significantly higher for the wt animals [Fig. 6.1.5, $p<0.001$ by Mann Whitney U test, $n = 4$].

Figure 6.1.1 (top photographs): GFAP immunostaining in the cortex of APP-null and wildtype mice.

Photomicrographs illustrate cortical sections from the brains of :

a APP-null mice and
b wildtype mice processed for immunohistochemistry using an antibody against glial fibrillary acidic protein (GFAP).

Scale bar represents 25μm.

Figure 6.1.2 (bottom) : GFAP immunostaining in the hippocampus of APP-null and wild type mice.

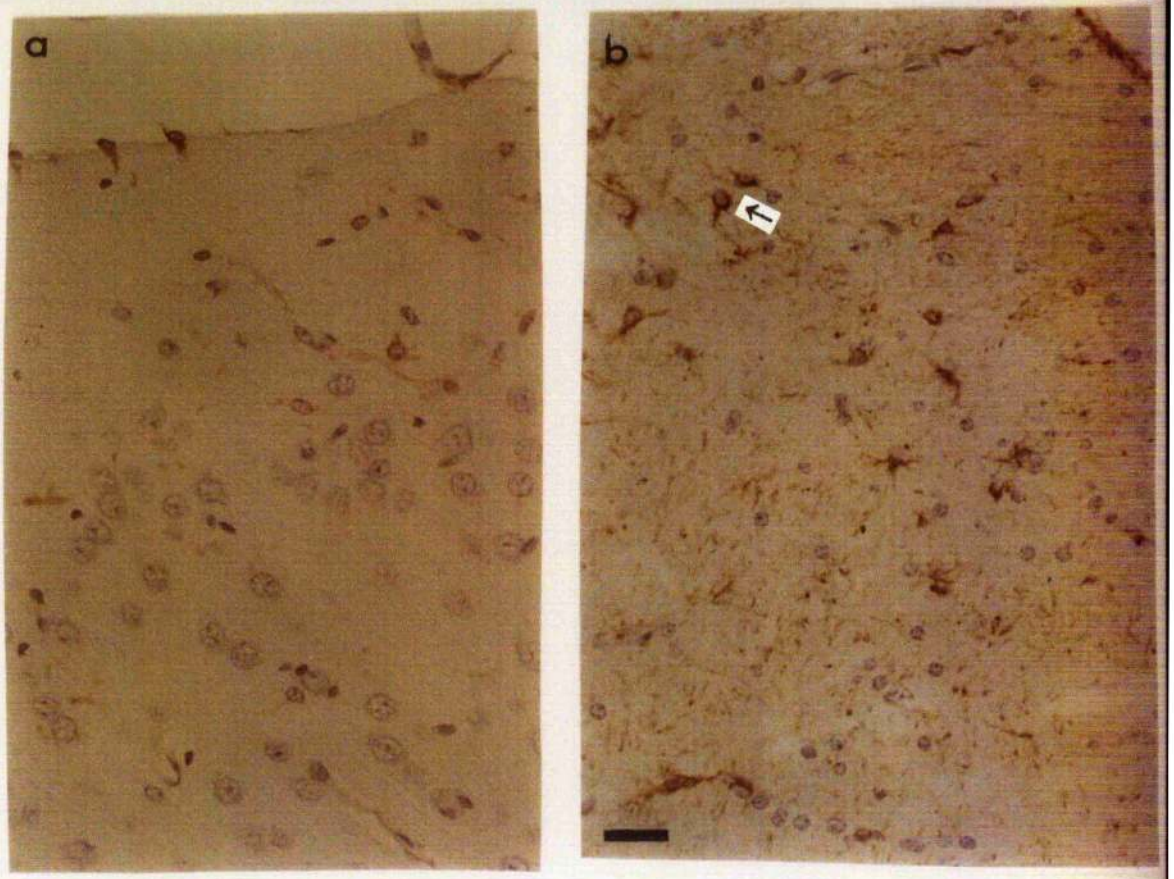
Photomicrographs illustrate hippocampal sections from the brains of

a APP-null mice and
b wildtype mice processed for immunohistochemistry using an antibody against glial fibrillary acidic protein (GFAP).

The photomicrographs show the granule cell layer, dentate gyrus and hilus.

Scale bar represents 25μm.

arrow indicate reactive GFAP positive glial cells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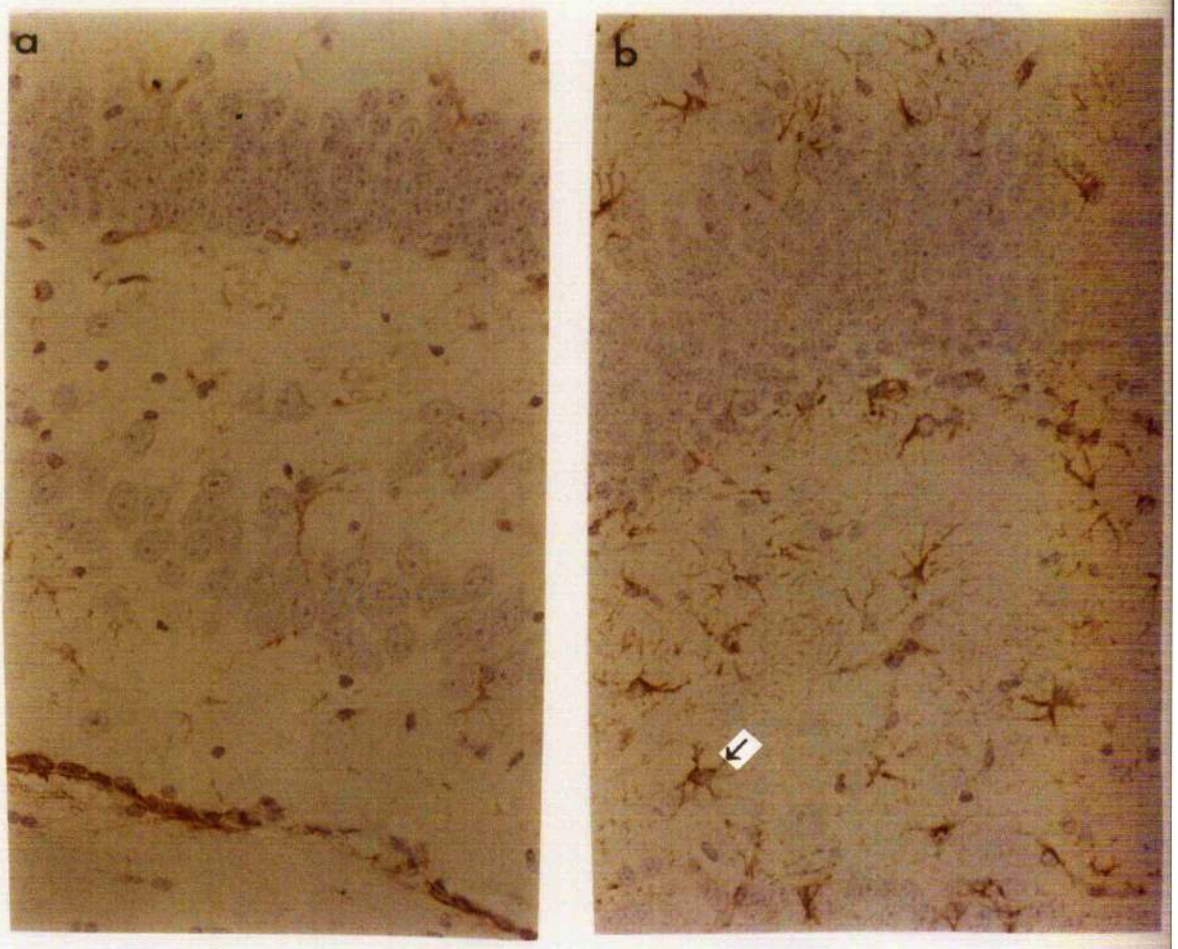
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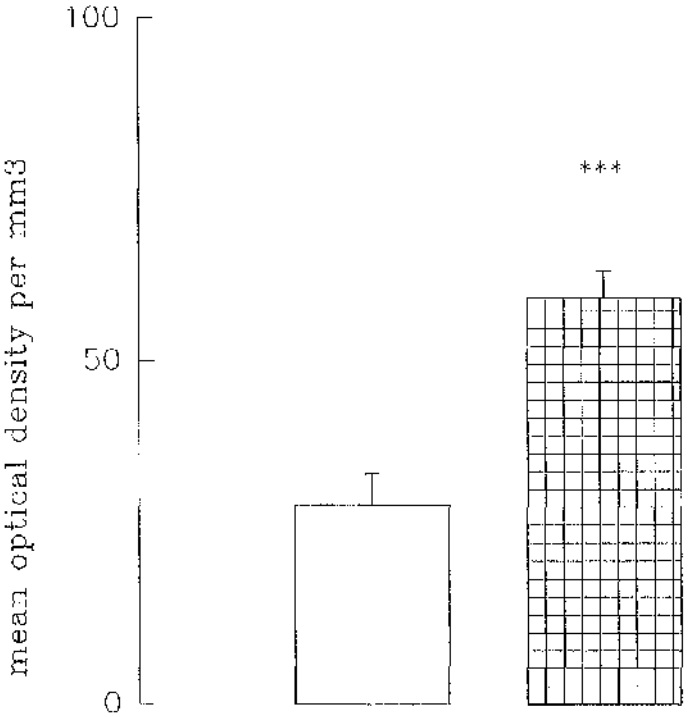
Figure 6.1.3 : Quantification of GFAP immunostaining in APP-null and wild type mice in the hippocampus.

Following processing for immunohistochemistry using antisera against GFAP in the hippocampal region, mean optical density levels of GFAP staining intensity were quantified.

Results are expressed as mean optical density levels per mm^3 ± SEM.

Statistical analysis was by Mann Whitney U test. *** $p<0.001$.



APP null mice

wild type mice

Data are mean ± SEM, n= 4 null, 4 wt.

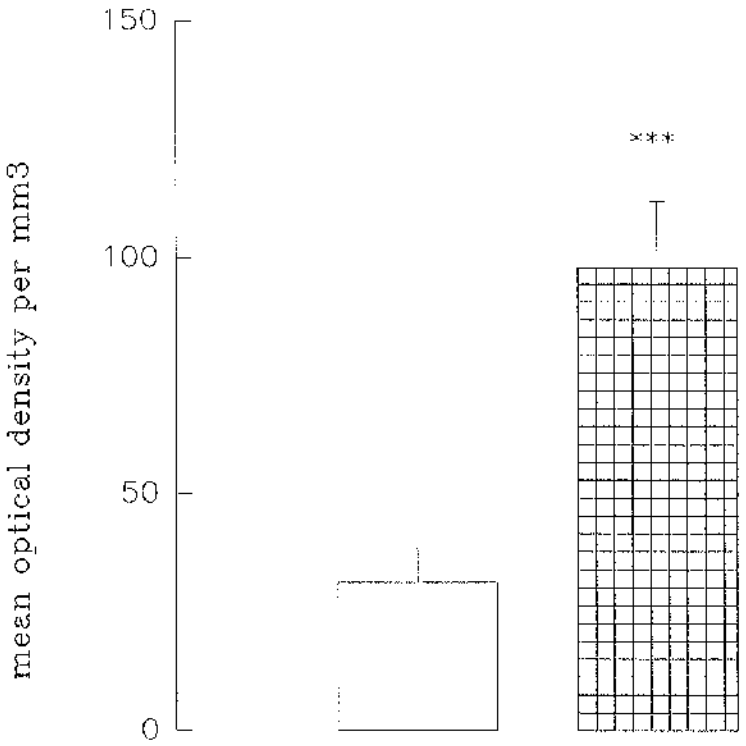
*** $p<0.001$ compared to null value by Mann Whitney U test.

Figure 6.1.4 : Quantification of GFAP immunostaining in APP-null and wild type mice in the corpus callosum.

Following processing for immunohistochemistry using antisera against GFAP, mean optical density levels of GFAP staining intensity were quantified.

Results are expressed as mean optical density levels per $mm^3 \pm$ SEM.

Statistical analysis was by Mann Whitney U test. *** $p<0.001$.



APP null mice

wild type mice

Data are mean ± SEM. n= 4 wild type, 4 null mice.

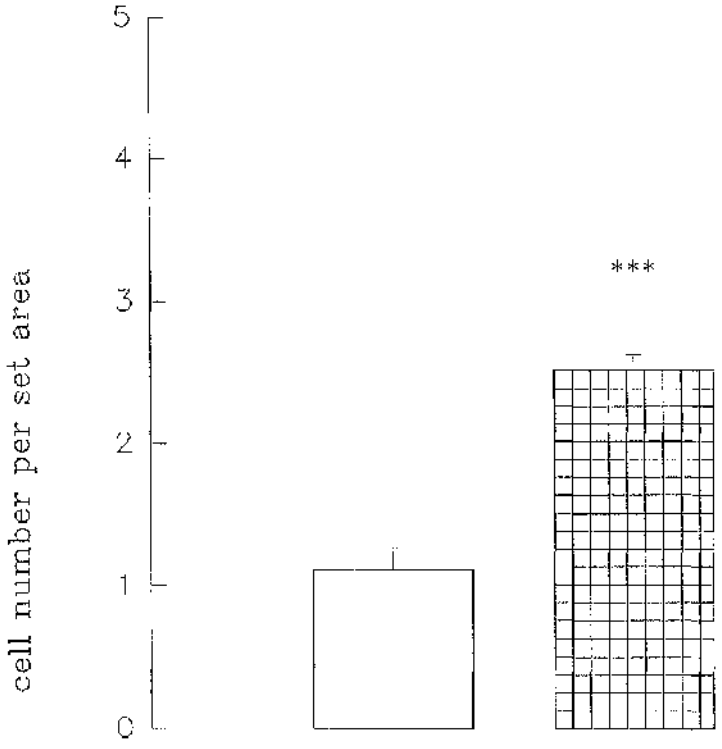
*** $p<0.001$ compared to null values by.
Mann Whitney U test.

Figure 6.1.5 : Reactive astrocyte cell count of the outer cortical layer (layer I) in APP-null and wild type mice.

Cell counts were taken in the outer cortical layers following immunohistochemistry of cortical sections using antisera against GFAP.

Results are expressed as an average reactive astrocyte cell count ± SEM.

Statistical analysis was carried out using the Mann Whitney U test. *** $p<0.001$.



APP null mice

wild type mice

Data are mean ± SEM. n= 4 wildtype, 4 APP null mice.

*** $p<0.001$ compared to null value by Mann Whitney U test.

6.5 *Calbindin D28k immunoreactivity in brain sections of APP-null and wild type animals.*

As clearly illustrated in Figs. 6.2.1 and 6.2.2, calbindin D28k immunoreactivity is higher in the APP-null mice compared to the wt animals in the entorhinal and cerebral cortices. These observations were confirmed by immunoradiolabelling, as described in Chapter 2 section 2.10, of coronal and cerebellar sections [Figs.6.2.3 and 6.2.4 respectively]. The highest level of calbindin D28k labelling can be seen in the corpus callosum. Immunoreactivity was quantitatively measured in three regions: the entorhinal cortex, CA1 pyramidal cells and frontal cortex. Calbindin D28k immunoradiolabelling was significantly higher in the APP-null animals compared to wt mice in all three regions [Fig. 6.2.5, $p<0.05$, $n = 4$ for all three regions]. An important consideration at this stage is whether the increased immunolabelling and radiolabelling represents an increase in immunoreactivity, an increase in the number of pyramidal cells containing calbindin D28k protein, or both. From the photographs in Figs. 6.2.1 and 6.2.42 it appears that an increase in the number of pyramidal cells containing calbindin has occurred in the APP-null animals. However, a more accurate assessment would have been obtained by cell counts.

Figure 6.2.1 : Calbindin D28k immunostaining in cortical sections from APP-null and wild type mice.

Cortical sections from
a APP-null mice and
b wildtype mice were processed for immunohistochemistry using antisera against calbindin.

The photomicrographs show cortical layers I-III.

Scale bar represent 100µm.

I

II

III

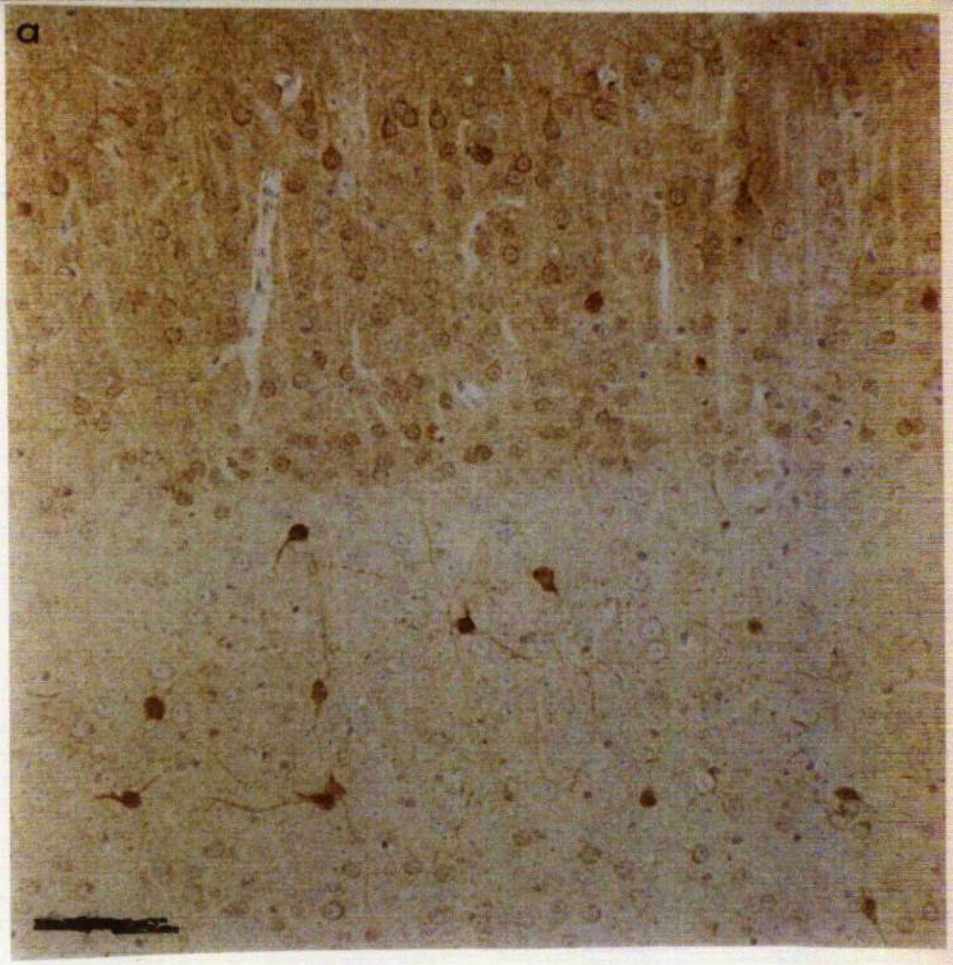
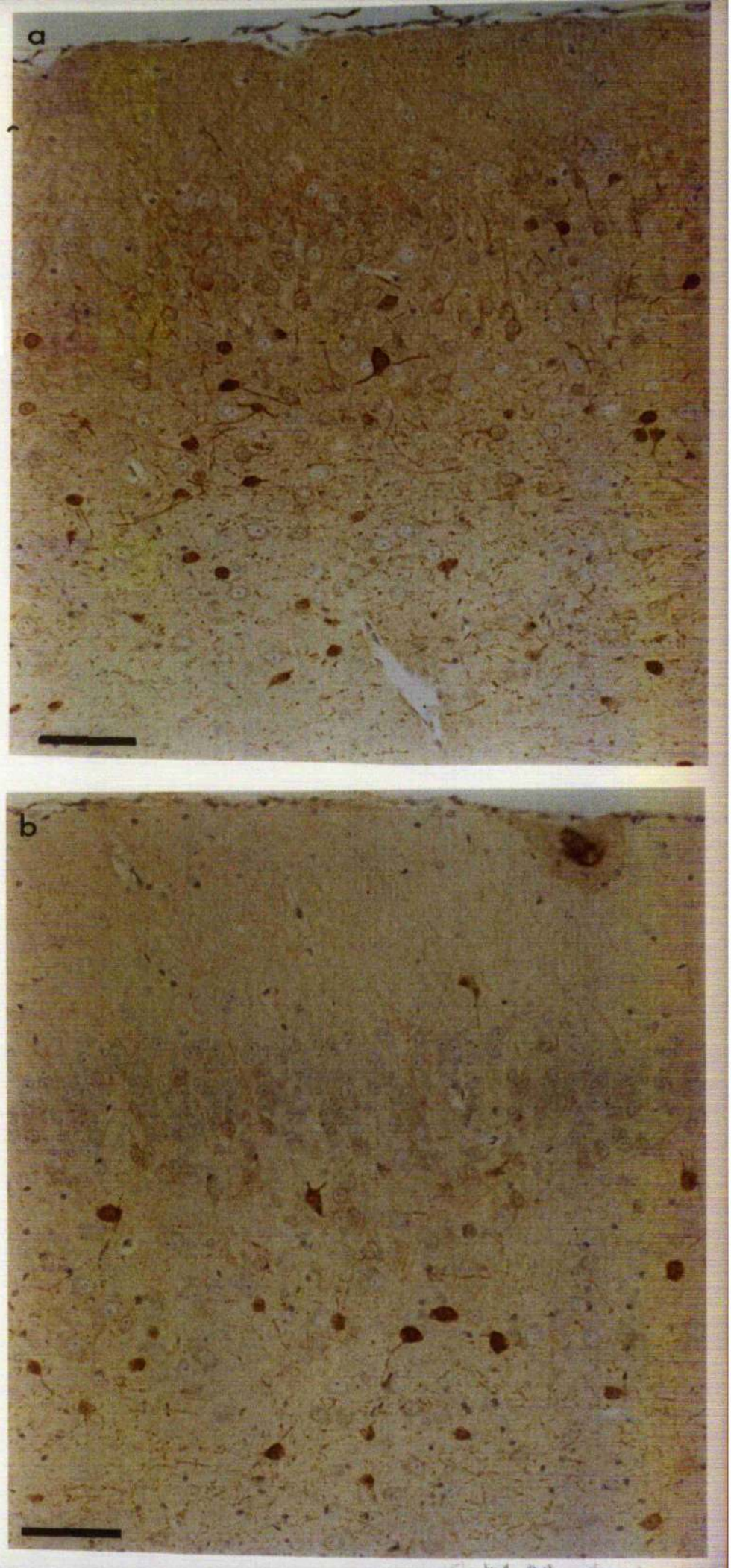


Figure 6.2.2 : Calbindin D28k immunostaining in entorhinal cortical sections from APP-null and wild type mice.

Entorhinal cortical sections from
a APP-null mice and
b wildtype mice were processed for immunohistochemistry using antisera against calbindin.

The photomicrographs show cortical layers I-III.

Scale bar represent 100μm.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II
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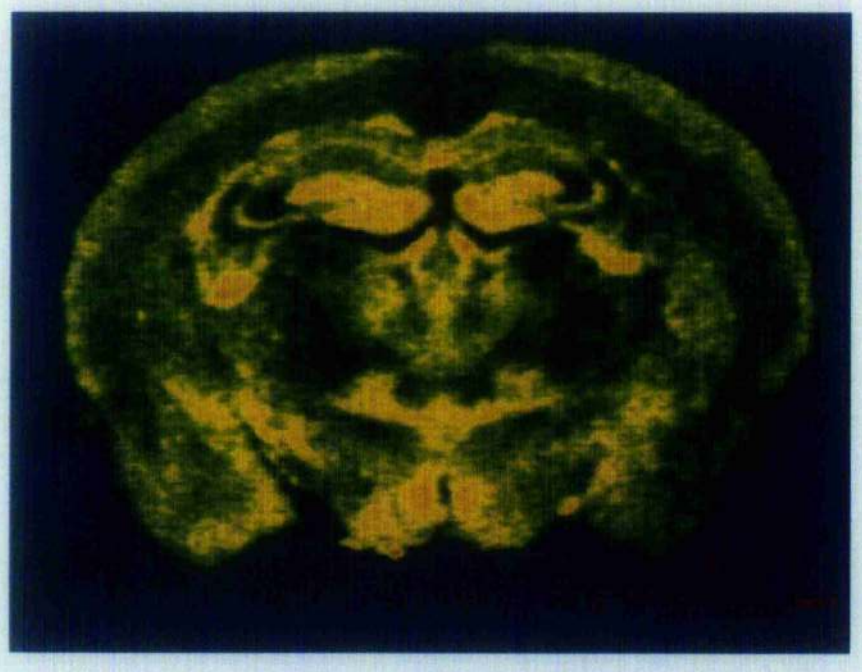
Figure 6.2.3 : Calbindin D28k radioimmunolabelling of coronal sections from APP-null and wildtype mice.

Radiolabelling was carried out using a polyclonal primary antibody to calbindin, and a ^{35}S labelled secondary antibody. Photomicrographs illustrate labelling intensities in APP-null and wildtype animals.

Immuno-radiolabelling for Calbindin protein in coronal section of mouse.

Wild Type



APP null

Increasing immunoreactivity

Figure 6.2.4: Calbindin radioimmunolabelling of cerebellar sections from APP-null mice at 6 months of age , and wildtype mice.

Radiolabelling was carried out using a polyclonal primary antibody to calbindin, and a ^{35}S labelled secondary antibody. Photomicrographs illustrate labelling intensities in APP-null and wildtype animals.

Immuno-radiolabelling for Calbindin protein in coronal section of mouse.

Wild Type

APP null

Increasing immunoreactivity

Figure 6.2.5 : Quantification of calbindin D28k radioimmunolabelled sections from APP-null and wildtype mice.

Radiolabelled sections were quantified in the following areas:

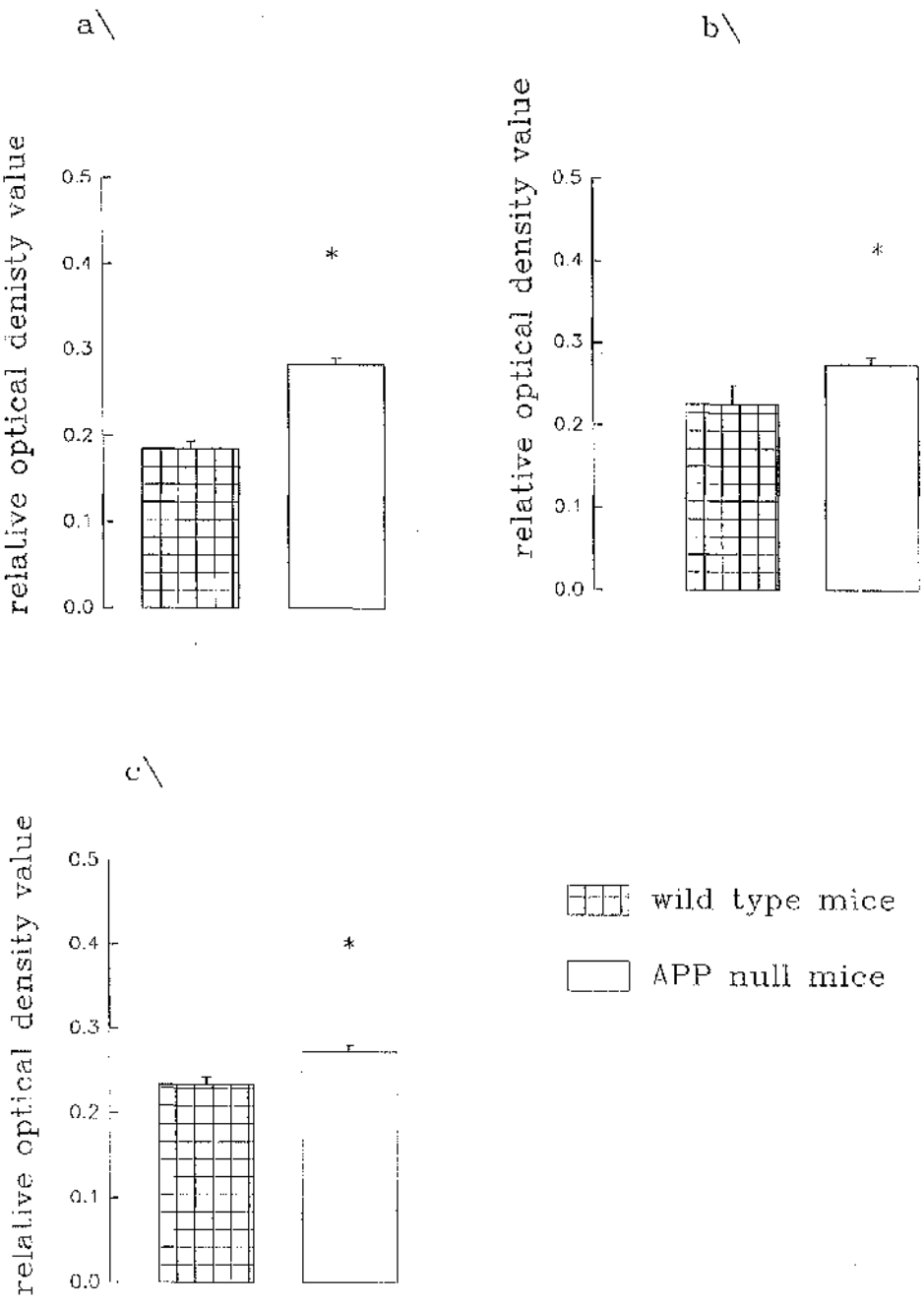
a CA1

b entorhinal cortex

c frontal cortex.

Results are expressed as relative optical density values ± SEM.

Statistical analysis was carried out using Mann Whitney U test. *** $p<0.001$.



Data are mean ± SEM n= 4 wild type, 4 APP null mice.

* $p<0.05$ compared to null value by Mann Whitney U test.

6.6 *MAP2 and synaptophysin immunoreactivity in brain sections of APP-null and wild type animals.*

As can be seen in Fig. 6.3.1, an initial observation of synaptophysin immunostaining in the cortex reveals no difference in synaptophysin immunostaining between APP-null and wt animals.

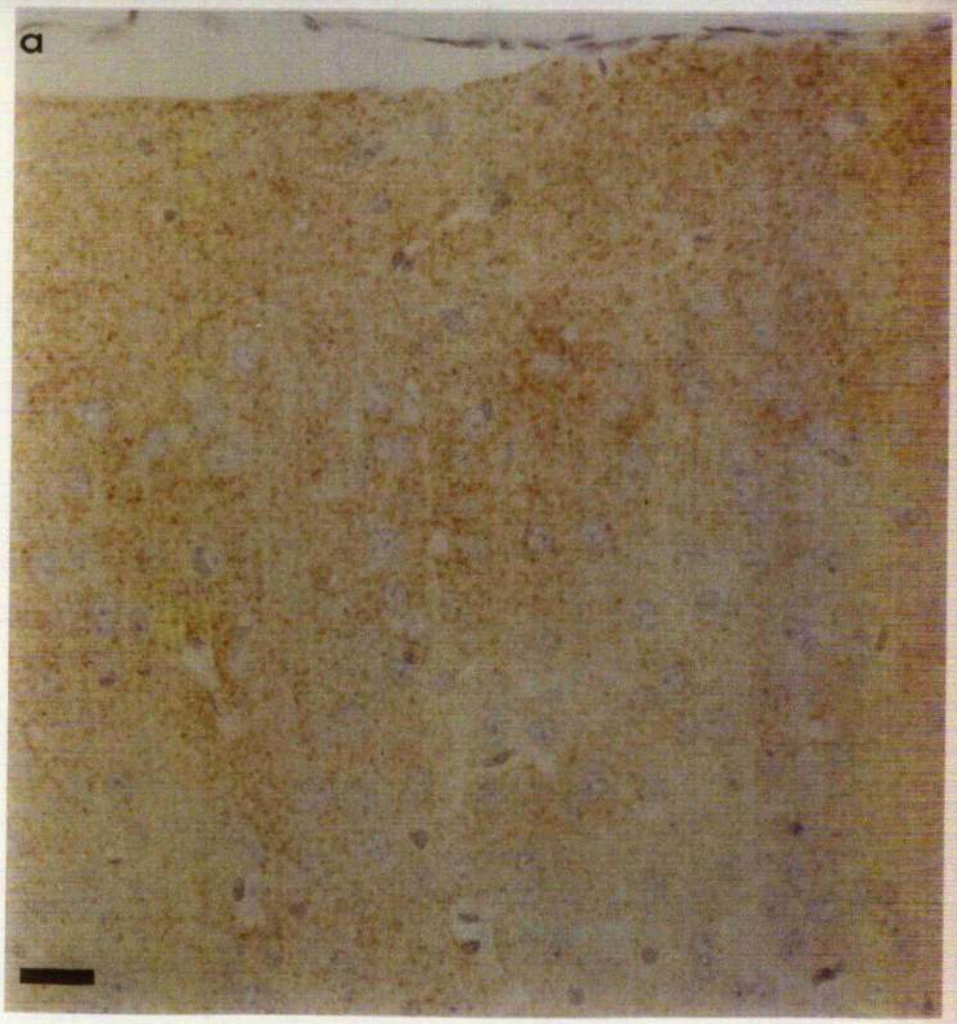
MAP2 immunostaining was also no different in APP-null and wt animals (data not shown).

Figure 6.3.1 : Synaptophysin immunostaining in cortex of APP-null and wildtype mice.

Photomicrographs illustrate synaptophysin immunostaining in
a APP-null mice and
b wildtype mice cortical sections.

The photomicrographs show cortical layers I-III.

Scale bar represents 50μm.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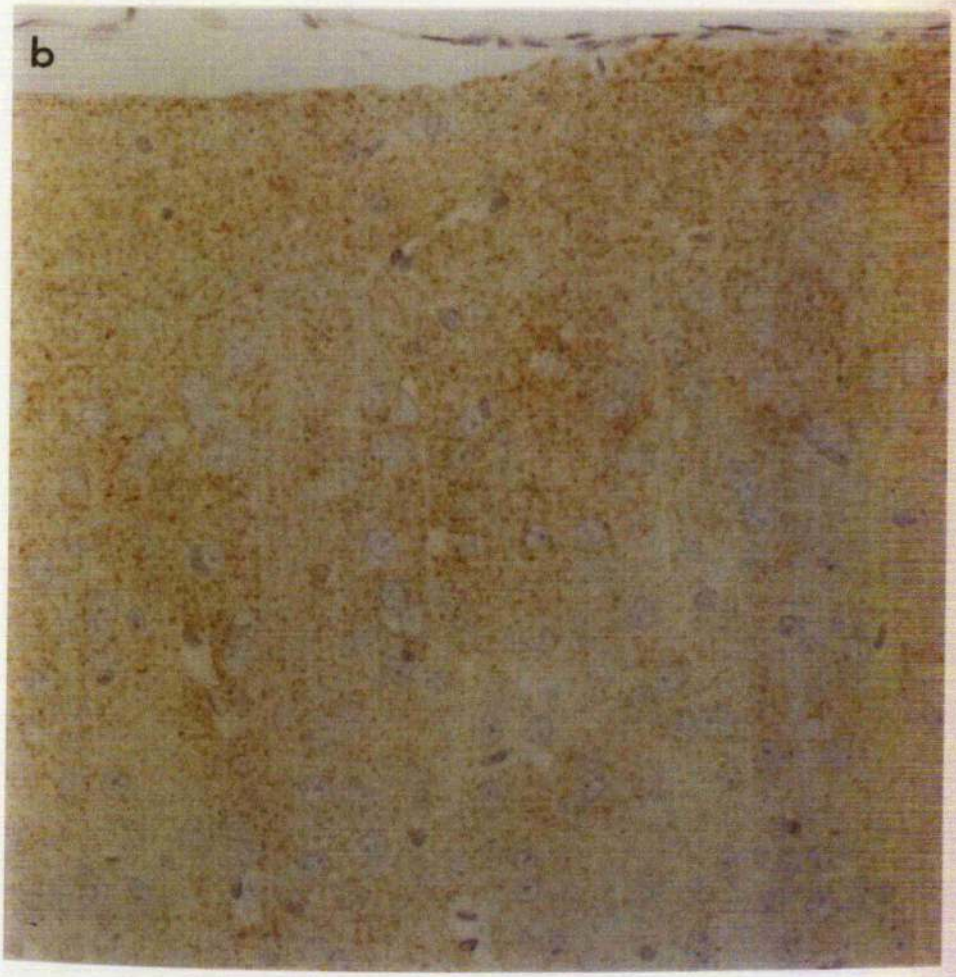
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6.7 *PS-1(S182) immunoreactivity in brain sections of APP-null and wild type animals.*

The pattern of distribution of S182 N-19 and S182 C-20 was investigated in tissue sections from the brains of APP-null and wt mice. The pattern of distribution was similar to that described for APP in rats (Mita et al ., 1989-to get). Interestingly, S182 antibody staining was observed not only in those regions of the brain that display Alzheimer's Disease pathology such as the cortex and hippocampus, but also in the striatum and cerebellum. The N-terminal S182 antibody staining appeared to be more abundant than the C-terminal and had a membrane-bound distribution. The C-terminal S182 appeared to be mostly cytoplasmic [Figs. 6.4.1 and 6.4.2]. This initial observation illustrated no difference in PS-1 immunoreactivity between APP-null and wild type animals.

Figure 6.4.1 (top photographs): S182-N19 immunostaining in the cortex of APP-null and wild type mice.

Photomicrographs illustrate cortical sections from
a APP-null mice and
b wild type mice processed for immunohistochemistry using an antibody against S182-N-19.

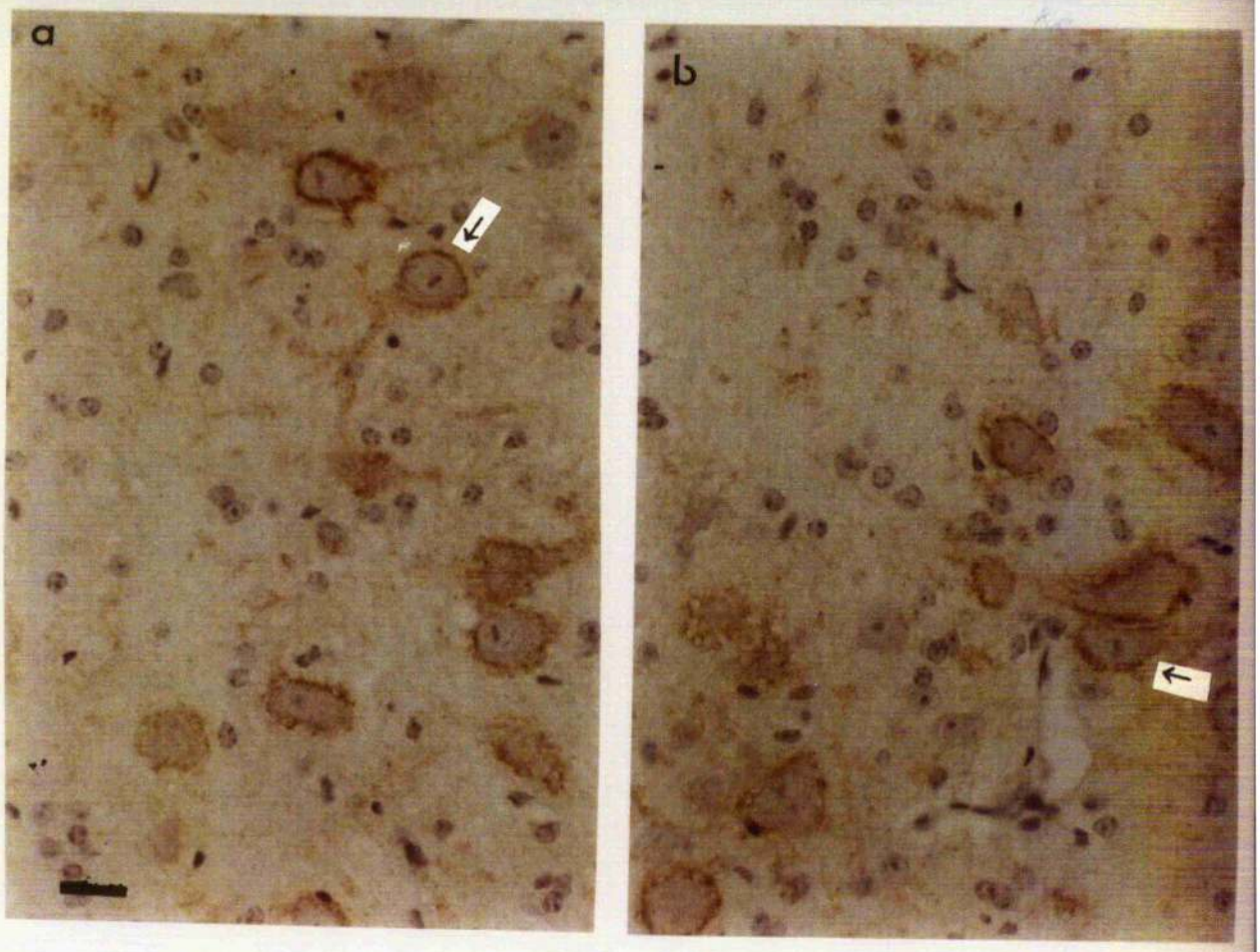
Scale bar represents 25µm.

Figure 6.4.2 (bottom photographs): S182-C-20 immunostaining in the cortex of APP-null and wild type mice.

Photomicrographs illustrate cortical sections from
a APP-null mice and
b wild type mice processed for immunohistochemistry using an antibody against S182-C-20.

Scale bar represents 25µm.

arrows indicate S182 positive neurons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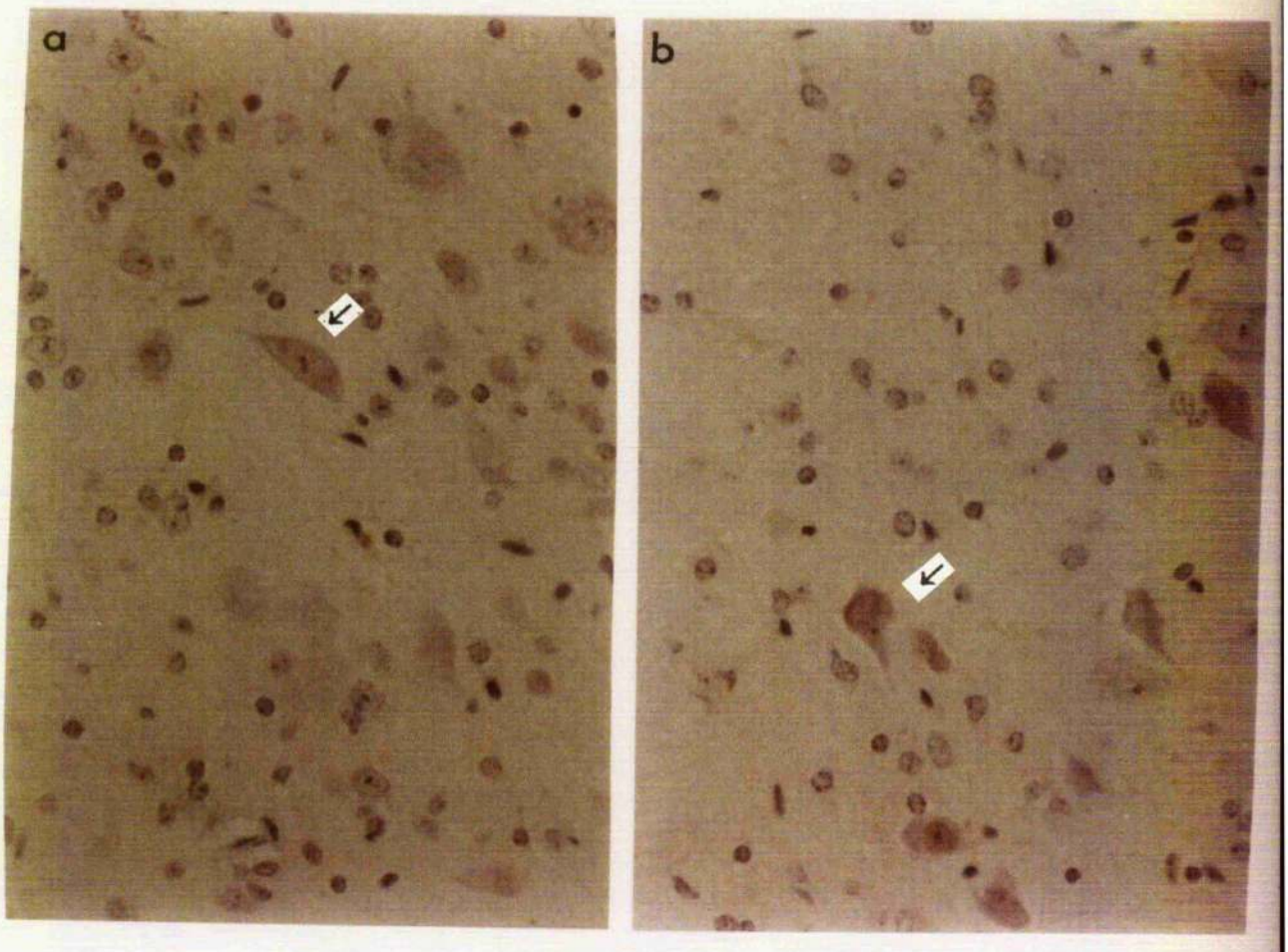

a
b

6.8 *GFAP immunoreactivity in brain sections of APP/RK and wild type animals.*

GFAP immunoreactivity was markedly higher in the 6 month old APP/RK mice compared to age matched wild type animals. As seen in the APP-null and wt mice, the reactive astrocytes had enlarged processes and reactive cell bodies. Reactive astrocytes were observed throughout the cortical layers and hippocampal region. The corpus callosum was also very reactive. Figs. 6.5.1 and 6.5.2 illustrate the dramatic increase in reactive gliosis in the APP/RK mice in the cortical and hippocampal regions. The observations were analysed quantitatively, and GFAP immunostaining was proved to be significantly higher in the hippocampus and corpus callosum [Figs. 6.5.3 and 6.5.4, $p<0.001$ by Mann Whitney U test, $n = 4$ for both regions]. Cell counting in the cortical layers revealed a higher count throughout layers 2-3 in the APP/RK mice compared to wt animals [Fig. 6.5.5, $p<0.001$ by Mann Whitney U test, $n = 4$].

Figure 6.5.1 (top photographs) : GFAP immunostaining in the hippocampal region of APP/RK and wildtype mice.

Sections from the hippocampal region of APP/RK and wildtype mice were processed for immunohistochemistry using antisera against GFAP.

Photomicrographs illustrate the reactive astrocytes in the dentate gyrus and hilus of:

a APP/RK mice
b wildtype mice

Scale bar represents 25µm.

Figure 6.5.2 (bottom): GFAP immunostaining in the cortex of APP/RK and wildtype mice.

Sections from the cortex of APP/RKand wildtype mice were processed for immuonhistochemistry using antisera against GFAP.

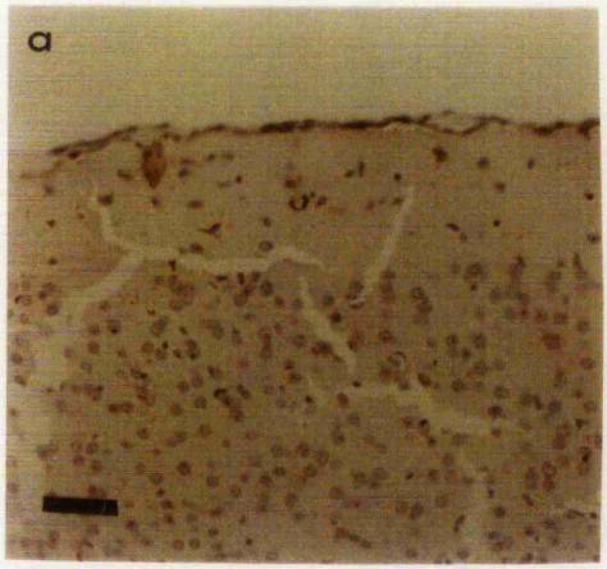
Photomicrographs illustrate the reactive astrocytes in:

a APP/RK mice
b wildtype mice

Scale bar represents 25µm.


a


b


a

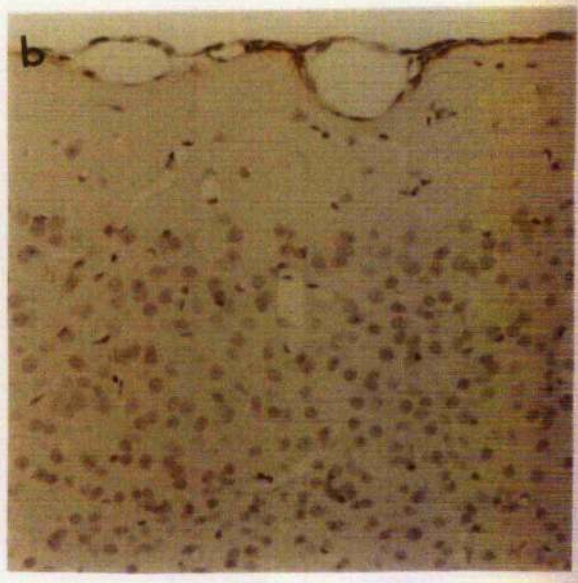
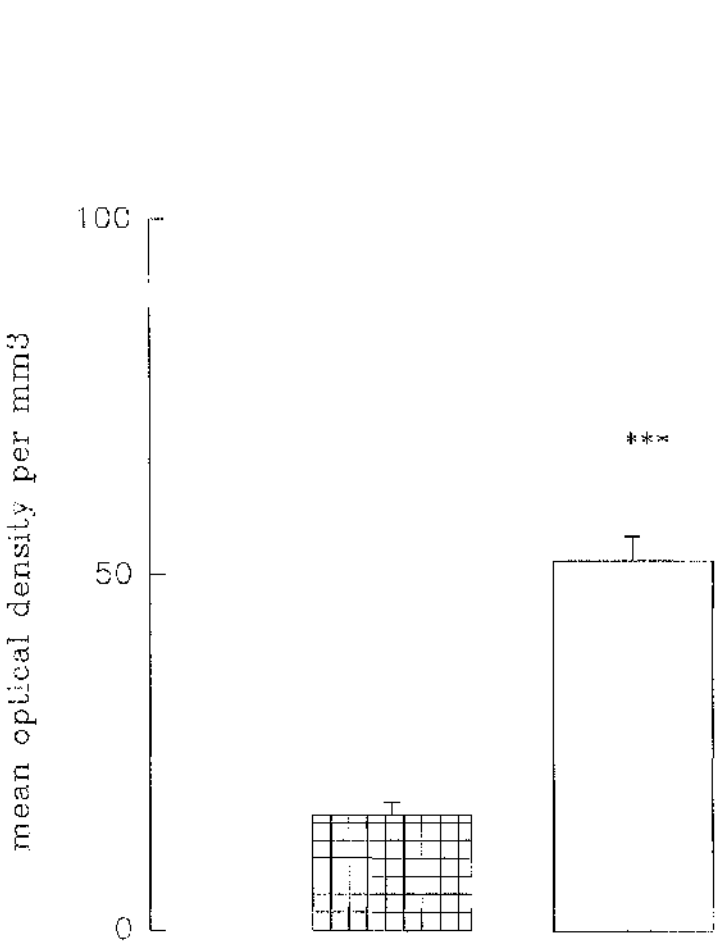

b

Figure 6.5.3 : Quantification of GFAP immunostaining in the hippocampal region of APP/RK and wild type mice.

Following processing for immunohistochemistry using antisera against GFAP, mean optical density levels in the dentate gyrus were quantified.

Results are expressed as mean optical density levels of GFAP staining intensity per $mm^3 \pm$ SEM.

Statistical analysis was by Mann Whitney U test. *** $p<0.001$.



wild type mice

APP/RK mice

Data are mean ± SEM, n= 4 wild type, 4 APP/RK.

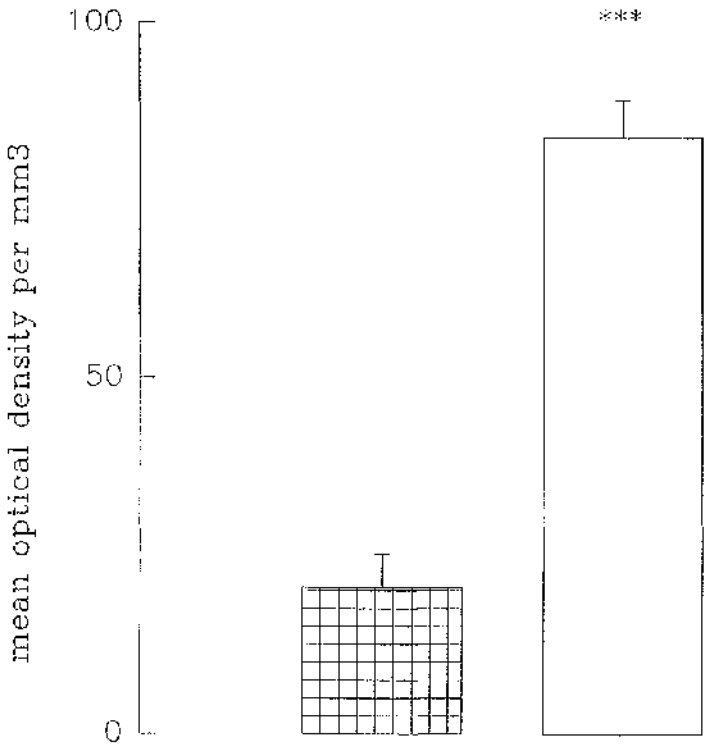
*** P<0.001 compared to wildtype values by Mann Whitney U test.

Figure 6.5.4 : Quantification of GFAP immunostaining in the corpus callosum of APP/RK and wild type mice.

Following processing for immunohistochemistry using antisera against GFAP, mean optical density levels in the corpus callosum were quantified.

Results are expressed as mean optical density levels of GFAP staining intensity per $mm^3 \pm$ SEM.

Statistical analysis was by Mann Whitney U test. *** $p<0.001$.



wild type mice

APP/RK mice

Data are mean ± SEM. n= 4 wild type, 4 APP/RK mice

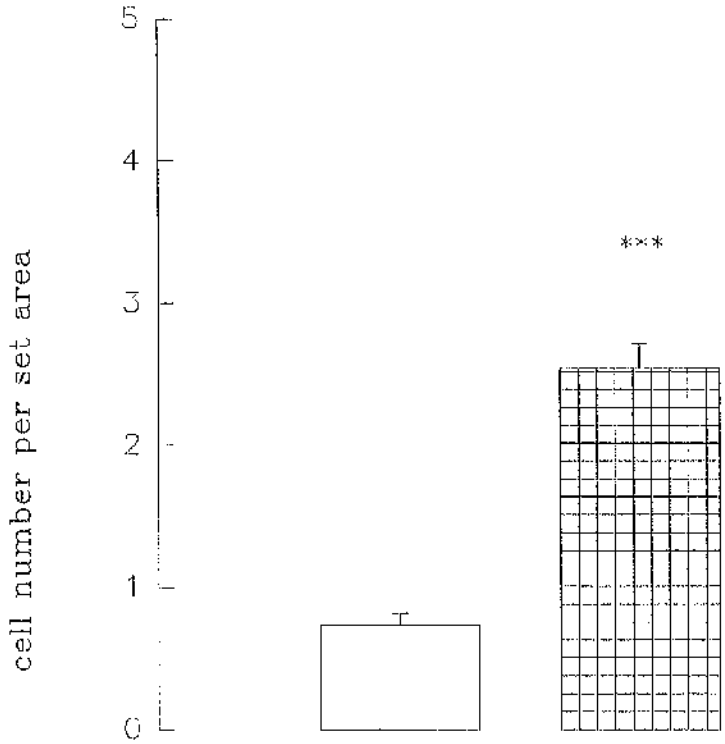
*** $p<0.001$ compared to wild type values by Mann Whitney U test.

Figure 6.5.5 : Reactive astrocyte cell count of the outer cortical layer (layer I) in APP/RK and wild type mice.

Cell counts were taken in the outer cortical layers following immunohistochemistry of cortical sections using antisera against GFAP.

Results are expressed as an average reactive astrocyte cell count ± SEM.

Statistical analysis was carried out using the Mann Whitney U test. *** $p<0.001$.



wild type mice

APP/RK mice

Data are mean ± SEM, n= 4 wildtype,4 APP/RK mice.

*** p<0.001 compared to wild type
by Mann Whitney U test.

Discussion

6.9 *GFAP immunoreactivity in APP-null and wild type brain sections.*

In view of the fact that four out of six APP-null mice displayed reactive gliosis at 14 weeks of age (Zheng et al.,1995), the results obtained in the present study for GFAP immunoreactivity were surprising. The wild type animals showed marked reactive gliosis: astrocytes with enlarged cell bodies and thicker processes stained heavily for GFAP in the hippocampus and through the superficial layers of the cortex. The intense astrocytic reaction was absent in the APP-null mice. Reactive gliosis occurs in many disease states, in response to neuronal injury (Eddleston and Mucke.,1993), and in normal ageing brains (Norton et al.,1992; Dickson et al.,1993). Thus, the observed reactive gliosis seen in the wild type animals may be due to the normal ageing process. The mice were at 13 months of age, and taking into account the average longevity of mice to be 18-20 months, 13 months would classify as fairly aged. An interesting further study would be to compare GFAP immunoreactivity levels in 13 month and younger aged animals, to investigate the effect of ageing on GFAP levels. It is also possible that reactive gliosis may have peaked in the APP-null mice after 14 weeks, and somehow desensitised during the period between 14 weeks and 13 months. The mechanisms underlying reactive gliosis are still unclear, although it is thought to be a tissue-based mechanism, with changes in eicosanoid and nitric oxide levels mechanisms by which glial structure may be altered (Caggiano et al.,1996).

Abnormal regulation and processing of APP may contribute to Alzheimer's Disease. Five different mutations have been identified in the human APP gene, linking the gene to early onset familial Alzheimer's Disease (Mullan et al.,1993). By developing a transgenic model of complete removal of the APP gene, it is possible to investigate the possible physiological roles of APP in the brain and the relevance of this role in the mechanisms that lead to the development of Alzheimer's Disease. Prior to this model, complete removal of the gene had not been examined. Muller and coworkers developed mice with a disrupted APP gene. These animals were severely impaired in

spatial learning and exploratory behaviour, and showed increased incidence of agenesis in the corpus callosum (Muller et al.,1994). No reactive gliosis was reported in these animals, so it is possible that a partial retention of function in the APP gene prevented the development of reactive gliosis. The fact that the APP-null animals showed marked reactive gliosis at 14 weeks but not at 13 months could be due to an accelerated process which had somehow stabilised by 13 months. Further work on these animals between the ages of 14 weeks and 13 months is clearly necessary. There is the future possibility that these animals will be used for the insertion of a human APP FAD gene to generate a mouse model of Alzheimer's Disease, and to investigate the effects of mutations in the FAD gene on protein expression and regulation.

6.10 *Calbindin D28k immunoreactivity in APP-null and wild type brain sections.*

Calbindin D28k plays an important role in the buffering system which protects tissue from calcium-induced cellular injury (Tao et al.,1996a). It is expressed in the hippocampus of Alzheimer's Disease brains (Tao et al.,1996b). The results from the current study show an increase in calbindin D28k in the superficial frontal cortical layer, superficial entorhinal cortical layer and CA1 in the APP-null mice compared to age matched control animals. Removal of the APP gene may have resulted in a potential calcium-induced toxicity which was controlled by increased levels of calbindin D-28k buffering system. This observation may suggest an interesting link between APP function and calbindin activity. It is important, however, to address the issue of whether the observed increase in calbindin immunoreactivity is the result in an increase in the number of cells, or an increase in the calbindin labelling intensity within the cells. An initial observation of the stained sections suggests an increase in cell number as well as cell staining intensity, although quantification by cell counting is necessary to verify this. An interesting possibility is the de novo synthesis of calbindin protein within pyramidal cells in response to removal of the APP gene.

6.11 *MAP2 and synaptophysin immunoreactivity in APP-null and wild type brain sections.*

Severe synaptic loss occurs throughout the brain in Alzheimer's Disease, and it has been shown that the severity of synaptic loss correlates with the degree of cognitive impairment to a substantially better degree than either plaques or tangles (Terry et al.,1991). Synaptophysin and MAP2 immunoreactivity remained unaltered in the APP-null animals compared to wildtype animals. It should be noted however that the synaptophysin and MAP2 studies were initial observations and no quantification was performed. No alteration in synaptophysin immunoreactivity levels could be due to the fact that the APP-null animals underwent regular and extensive behavioural testing throughout their 13 months. Regular stimuli may have helped to maintain synaptic function that may have been altered by removal of the APP gene. A further study to investigate in more detail the effect of removal of the APP gene on synaptic formation would use immunostaining for synaptosomal associated proteins such as SNAP-25. SNAP-25 is a 25kD synaptosomal associated protein that is highly expressed in murine hippocampus, being localised mainly in axons and axon terminals (Branks and Wilson., 1986; Oyler et al.,1989). Expression of this protein has been found to be strongly induced during late stages of axonal growth, and during the onset of synapse formation (Catsicas et al.,1991; Oyler et al.,1991; Osen-Sand et al.,1993).

MAP2 immunoreactivity remained unchanged in the APP-null animals compared to wild type animals. Thus, MAP2 may have no connection with APP and thus levels were no altered by removal of the APP gene.

As discussed in section 6.1, GFAP levels in the wildtype animals changed dramatically, and this could be due to the normal ageing process. It would be of interest to compare synaptophysin and MAP2 immunoreactivity levels in 13 month and younger wildtype animals to see if the ageing processes alters either.

6.12 *Presenilin-1 immunoreactivity in APP null and wild type brain sections.*

The pattern of distribution of PS-1 (S182) in both APP-null and wild type animals was similar to that seen in rats (Mita et al.,1989). PS-1 was observed not only in those regions of the brain that display Alzheimer's Disease pathology, but also in the striatum and cerebellum. The cerebellum shows Aβ plaques though of the diffuse type. N-terminal immunoreactivity was stronger than that of C-terminal PS-1, and had a membrane-bound distribution. The C-terminal PS-1 appeared to be mostly cytoplasmic. The PS-1 immunoreactivity appeared to be present mostly in neurons, with a lack of immunoreactivity observed in blood vessels or white matter. The levels of N- and C-terminal PS-1 remained unchanged between APP-null and wildtype animals. However, an initial study of cerebellar tissue from the APP-null mice at 6 months of age indicated a possible increase of N- and C- terminal PS-1 compared to that observed in age-matched controls (photographs not shown). This preliminary study requires further investigation, and there is the interesting possibility that PS-1 may have a synaptic role and that an increase in expression may compensate for the lack of APP. Recent results suggest that alteration in the processing of APP may have considerable physiological effects on synaptic plasticity (Nalbantoglu et al., 1997). It is thought that PS-1 exists in small quantities as a full length protein of 43-47 kDa, but instead is rapidly processed into an endoproteolytic fragments. Proteolysis results in the generation of two fragments: a 25-38kDa N terminal fragment, and a 16-19kDa C terminal fragment (Thunakaran et al.,1996; Podlisny et al.,1997). The significance of the two fragments remains to be elucidated, and it is not known whether one fragment has a more active role than the other.

The relationship between presenilins and APP metabolism is still unclear. Present evidence is as follows. In increased secretion of the amyloidogenic Aβ 1-42 peptide in cultured fibroblasts from individuals in early onset familial Alzheimer's Disease (FAD) pedigrees with PS1/PS2 mutations has been observed (Scheuner et al.,1996; Lemere et al.,1996). Recent evidence has demonstrated that PS-1 mutations may alter the trafficking or processing of APP

stimulating the activity of γ secretase thus resulting increased production of $A\beta_{1-42}$ (Citron et al.,1997). Increased Aβ production has been reported in fibroblasts from an FAD patient who has a mutation in the PS-1 gene (Martins et al.,1995). Key current questions are whether the presenilins interact directly with APP, or whether the effects of the presenilin mutations are mediated through a unique and indirect pathway. Recent work has suggested the possibility that mutant PS-1 can directly induce changes in tau phosphorylation in neurons in vivo (Giannakopoulos et al.,1997). PS-1 is thought to have a neuroprotective role, and in Alzheimer's Disease low cellular expression of the protein may be associated with increased neuronal loss and NFT formation (Giannakopoulos et al.,1997). The formation of stable complexes between PS-2 and APP has recently been observed in COS cells, suggesting a possible participation of presenilins in intracellular processing of APP (Weidemann et al.,1997). It seems likely that a final common pathway in the pathogenesis of sporadic and genetic forms of Alzheimer's Disease exists, and a central question is how the presenilins mutations cause the typical neuropathology. Recent evidence suggests that PS-1 is required for the correct formation of the axial skeleton, normal neurogenesis and neuronal survival (Shen et al.,1997).

The present study of brain sections from APP-null transgenic mice has enabled a preliminary investigation into the possible actions of APP, and a look at the pattern of distribution of N and C terminal PS-1 in mice brain sections. The search for possible roles of APP continues, and recent evidence has shown that human APP can protect neurons against chronic and acute excitotoxic insults *in vivo*, and that impairments of neuroprotective human APP could contribute to neurodegeneration (Masliah et al.,1997).

6.13 *GFAP immunoreactivity in APP/RK and wild type brain sections.*

The marked increase in GFAP immunoreactivity seen in the APP/RK animals at 6 months of age agrees with the reported data for Moechars (Moechars et al.,1995; Moechars et al.,1996). The astrocytes appeared strongly reactive with enlarged cell bodies and thicker processes. The 6 month wild type animals showed little

reactive gliosis, unlike the more aged mice discussed in section 6.9 that showed marked reactive gliosis.

The mutation of the APP gene at the α-secretase site appears to have resulted in the onset of reactive gliosis in these animals. This onset of reactive gliosis, along with the previously reported severe behavioural deficits, and neuropathological changes including necrosis and apoptosis, (Moechars et al., 1995; Moechars et al.,1996), demonstrate the severe cellular neurotoxicity resulting from incorrect α-secretase processing of APP in the CNS.

It is worth discussing the two transgenic animals investigated in this study, as well as other studies using transgenic models. LaFerla and coworkers developed a transgenic mice that overexpress the Aβ peptide intracellularly (LaFerla et al.,1995). The phenotypic traits observed in these animals were similar to those seen by Moechars and colleagues. This suggests a possibility that cell bound APP/RK fragments could lead to intracellular Aβ peptide , thus resulting in a situation similar to that seen in the LaFerla model where Aβ peptide is produced intracellularly. This would then imply that Aβ peptide produced intracellularly causes neurodegeneration. This in comparison with extracellularly produced Aβ peptide requires further investigation. It has been suggested that the normal and pathological physiology of APP in the brain includes more than production of Aβ peptide (Moechars et al.,1996). It is of interest that the APP-null mice at 14 weeks demonstrated several phenotypic characteristics in common with the APP/RK mice (Zheng et al.,1995). The absence or suppression of an unknown function of APP will explain this similarity and further research is necessary.

Summary

Studies of brain sections taken from two transgenic mice models has enabled a preliminary investigation into the possible actions of APP within the CNS. Complete removal of the APP gene has been previously shown to result in a dramatic onset of reactive gliosis at 14 weeks (Zheng et al.,1995) which, as shown in the present work, stabilises by 13 months by an as yet unknown mechanism.

Calbindin immunoreactivity increases in the 13 month APP-null animals, suggesting an interesting link between APP and calcium buffering. There is also the possibility of a link between APP and PS-1: an initial investigation of the APP-null mice at 6 months of age suggests an increase of cerebellar PS-1 compared to age-matched controls animals. Mutation of the α-secretase site on APP results in severe neuronal degeneration.

Having investigated protein levels in the transgenic mice, it was of great interest to study the pattern of distribution of presenilin and GFAP in postmortem human Alzheimer's Disease brain tissue.

Chapter 7

Immunohistochemical study of presenilin-1 and glial fibrillary acidic protein expression in human Alzheimer's Disease postmortem brain tissue.

Introduction

The previous sections of work have focused on cytoskeletal protein immunoreactivity and gene expression, particularly of those proteins involved in the pathogenesis of Alzheimer's Disease. As the theory behind the project has also focused on various aspects of the disease pathology, it was of great interest to be able to examine postmortem Alzheimer's Disease brain tissue. While this was only a preliminary study, several interesting observations have been made.
The postmortem tissue was obtained from the Cambridge Brain Bank Laboratories. Immunostaining of the following was carried out on the diseased and age matched control tissue sections : GFAP, PS-1 and AT8.

7.1 *AT8 antibody:*

Numerous antibodies have been developed which show selective specificity for Alzheimer's Disease pathology, including AT3, AT8, AT10 (Novak et al.,1991; Mercken et al.,1992; Yen et al.,1987). The AT3 antibody recognises both pathological and normal structures, and has been shown to label NFTs in Alzheimer tissue as well as the normal axonal distribution of tau in non-diseased tissue (Novak et al.,1991). Similarly, the AT10 antibody reacts with normal tau as well as that in the diseased state (Yen et al.,1987). Perhaps the most commonly used monoclonal antibody is AT8 (Mercken et al.,1992). This antibody has been shown to positively stain for NFTs and senile plaques, recognising the PHF epitopes with no cross-reactivity with normal tau (Mercken et al.,1992). In the present study, sections of postmortem Alzheimer's Disease brain tissue were stained using the AT8 antibody.

7.2 *Presenilin-1:*

As has been discussed in more detail in Chapter 6, immunohistochemical studies have revealed PS-1 to be present in neuritic plaques in sporadic Alzheimer's Disease brains, suggesting that this protein may be involved in the development of the disease pathology (Wisniewski et al., 1995). In situ hybridisation studies

revealed that both PS-1 and PS-2 are expressed at the highest levels in the hippocampus and cerebellum (Kovacs et al., 1996; Suzuki et al., 1996; Cribbs et al., 1996). PS-1 and PS-2 mRNA levels have been found to be considerably higher in neurons, with lower levels in glial cells (Lee et al., 1996). The in situ hybridisation of Lee et al also revealed PS-1 to be expressed in somatodendritic and neuropil compartments of neurons in the neocortex and hippocampal formation. In the present study, it was of interest to carry out double immunolabelling of AT8 and PS-1 in postmortem Alzheimer's Disease brain sections.

Results

7.3 *Comparison of Alzheimer's Disease and age matched control brain sections.*

The slide-mounted brain sections from the Alzheimer's Disease patients appear noticeably smaller than the age-matched control tissue sections, with greatly enlarged ventricles. In the diseased tissue, the areas most affected by tissue loss are the entorhinal and frontal cortices. Perhaps the most striking initial observation was the degree of tissue loss between patients, some showing very little remaining entorhinal matter, and large portions of the cerebral cortex engulfed by expanded ventricles.

7.4 *GFAP immunostaining in Alzheimer's and control brain sections.*

As with the transgenic models, GFAP immunostaining proved interesting in the human sections. A dramatic difference between patterns of immunoreactivity was observed. As can clearly be seen in Fig. 7.1a, reactive astrocytes occur throughout the cortical layers in Alzheimer's tissue. While reactive cells are confined to the outer layer in the control tissue, (7.1b), in the diseased tissue, the reactive cells are more numerous and are present deep in the cortex. The outer layer is fiercely reactive, the cells having greatly thickened processes.

Figure 7.1 : GFAP immunostaining in the cortex of Alzheimer's Disease and control postmortem tissue.

Sections from the cortex of postmortem Alzheimer's and control tissue were processed for immunohistochemistry using antisera against GFAP. Photomicrographs illustrate reactive gliosis in

a Alzheimer's tissue
b control tissue.

Scale bar represents 100 μm.


a


b

7.5 *AT8 and S182 (PS-1)-AT8 double labelling in Alzheimer's and control brain sections.*

Figures 7.2 shows AT8 (a) and S182-AT8 (b and c) double labelling in the CA3, subiculum and CA2 regions of Alzheimer's Disease tissue respectively. AT8 immunoreactivity illustrates plaques, tangles and proteinaceous deposits throughout the three regions. In this particular patient, the most striking pathology is seen in the subiculum. Colocalisation of S182 with plaques and tangles can clearly be seen in the subiculum and CA2 areas. S182 immunoreactivity is strongest in the large hippocampal pyramidal cells.

Figure 7.2 : S182 N-19 and AT8 labelling in Alzheimer's Disease postmortem tissue.

a Tissue sections processed for immunocytochemistry using antisera against AT8 in postmortem tissue from the CA3 region. Arrow indicates tangle, smaller proteinaceous deposits surround.

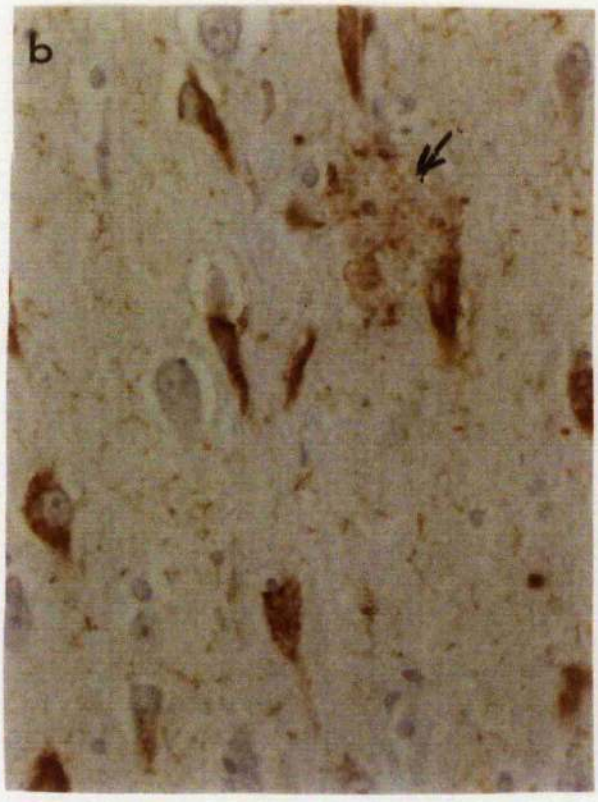
Tissue sections were processed for double labelling using antisera against S182 -N19 (purple staining) and AT8 (dark brown staining) in postmortem tissue from the following regions:

b subiculum, arrow indicates large plaque and proteinaceous deposit. Numerous flame shaped tangles surround.

c CA2, arrow indicates doubled labelled granule cell.

Scale bar represents 50 μm.


a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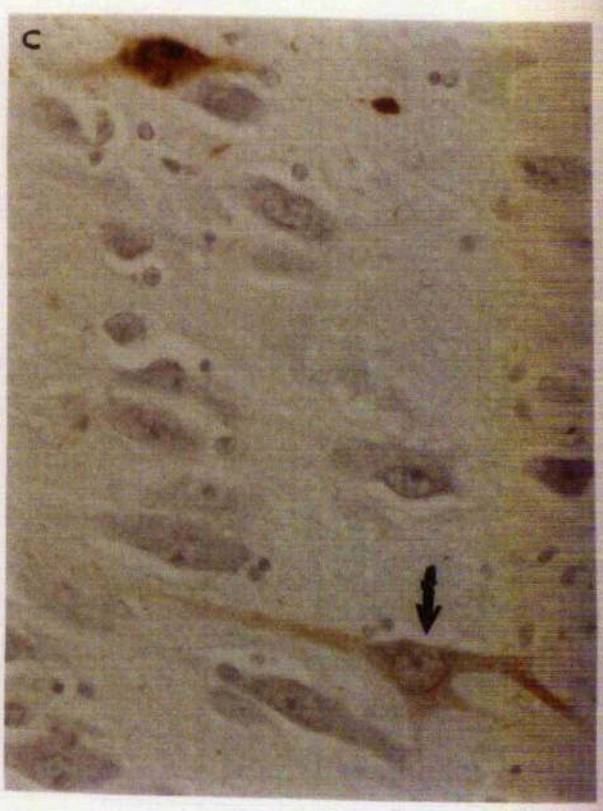
c

Discussion

Comparison of the brain sections from Alzheimer's and age matched control tissue, reveals a dramatic atrophy in the diseased brain tissue. The lateral ventricle is enlarged, and the hippocampus shrunken, with a large space between the hippocampus and the inferior horn of the temporal cortex.

7.6 *Reactive gliosis in Alzheimer's Disease brain tissue.*

As was expected, a marked increase in GFAP immunoreactivity was evident in the Alzheimer's Disease tissue where reactive gliosis is seen throughout all cortical layers. Reactive astrocytes are also seen in the control tissue but to a lesser extent. In both the Alzheimer's and control tissue, the most striking reactive gliosis was seen in the dentate gyrus, CA1 and CA3 areas. As discussed previously, reactive gliosis occurs in non disease states as part of the ageing process (Nirton et al.,1992; Dickson et al.,1993).

7.7 *AT8 and S182 immunostaining in Alzheimer's Disease tissue.*

The AT8 antibody stains for Alzheimer's pathology. As expected, immunoreactivity was stronger in the diseased tissue, revealing positive NFTs and senile plaques. NFTs of 30μm diameter are seen in the subiculum, entorhinal and cerebral cortices. Large plaques of around 60μm in diameter are seen in the subiculum, and small proteinaceous deposits are evident throughout the diseased tissue. Immunostaining of the senile plaques revealed negative amyloid cores surrounded by positive neurites.

PS-1 (S182) distribution in the human sections was again similar for N and C terminal antibodies. Both were widely distributed in the temporal lobe, entorhinal cortex and hippocampal regions, and predominant in the hippocampus and cerebral cortex, i.e, those areas most susceptible to plaque formation, as reported previously by Cribbs and coworkers (Cribbs et al.,1996). The observed PS-1 immunostaining was consistent with previous evidence reporting PS-1 immunostaining intracellularly in neurons appearing as thick granules resembling large vesicles (Moussaoui et al.,1996). N and C terminal

immunoreactivity was possibly at a lower level in the dentate gyrus than in the CA fields although this initial observation is without quantification. PS-1 distribution appears to be essentially within the neuronal population of the brain, with a lack of immunoreactivity seen in the blood vessels and white matter glial cells. This contrasts with APP which has been shown to be expressed ubiquitously in all cell types of the human brain (Tanzi et al.,1993; Hyman et al.,1993), although other groups have observed lower but significant levels of PS-1 immunoreactivity in white matter glial cells (Lee et al.,1996).

Defining the cellular and subcellular localisation pattern of PS-1, determining whether it is present in the pathological hallmarks of Alzheimer's Disease such as the neuritic plaques or neurofibrillary tangles, is expected to provide insights into the pathophysiological function of this important protein. Double staining with S182 N-19 and AT8 illustrated some colocalisation of PS-1 within plaques and tangles in the hippocampal and entorhinal cortical areas, however, other areas such as the cerebral cortex did not illustrate this colocalisation. The reason for this difference in areas showing colocalisation is unclear, although differences in severity of neurodegeneration between regions may account for the difference. Several similarities in the expression profile of PS-1 and APP have been described. First, both genes are ubiquitously expressed, and the pattern of expression of the two is similar (Cribbs et al.,1996; Mita et al.,1989). It has been suggested that an overlapping PS-1 immunoreactivity with Alzheimer's Disease pathology and APP expression may be the result of altered levels of PS-1 expression within affected areas. Recent evidence has shown that PS-1 protein does not appear to be a primary constituent of amyloid plaques (Weber et al.,1997). However, PS-1 immunoreactivity has been found in the neuritic component of senile plaques as well as in neurofibrillary tangles (Levey et al.,1997). As discussed in section 6.12, the mechanism by which presenilin mutations cause typical Alzheimer's Disease pathology remains to be elucidated. Recent evidence suggest that presenilins may be involved in the pathogenesis of sporadic as well as familial Alzheimer's Disease (Takami et al.,1997). Localisation of PS-1 immunoreactivity in both familial and sporadic Alzheimer's Disease suggests that genetically heterogenous forms of the disease

share a common pathophysiology involving the PS-1 protein (Levey et al.,1997).

Summary

It was of great interest to perform an initial study of GFAP and presenilin immunoreactivity in human post mortem Alzheimer's Disease tissue. The current study investigated pathology in brain sections from one patient, and clearer results would obviously be obtained by looking at tissue from several patients. As expected, a greater degree of GFAP immunoreactivity was observed in the diseased tissue, with numerous reactive astrocytes present. Colocalisation of PS-1 protein and plaques and tangles has been reported by some, but not all, research groups. In the present study, overlap of PS-1 within plaques and tangles was observed in some but not all areas. It is possible that intracellular PS-1 somehow becomes extracellularly bound to Aβ and PHF tau. The mechanisms behind this, and the reasons why it occurs in shown but not all cases, remain to be elucidated.

Chapter 8

GENERAL DISCUSSION.

This work was undertaken to investigate the distribution and expression of proteins related to neuronal degeneration. Using cell culture models, we have focused on changes in cytoskeletal protein expression and cell morphology. In addition, two mutant mouse models have been used to study the distribution of morphological and synaptic markers.

Chapter 3 has dealt with tau gene expression in the basal forebrain and hippocampal regions of adult and neonatal rats (gestation day 17). A comparison of Tau type1 and Tau type 2 mRNA distribution in adult and neonatal rat brain agrees with previous reports: Tau 1 mRNA levels are higher in the neonatal rat brain, Tau 2 mRNA levels higher in the adult rat brain. Type 1 and type 2 tau proteins are thought to be developmentally regulated, with type 2 transcripts being absent from the fetal brain but expressed in the adult brain (Goedert et al.,1988a; Goedert et al.,1989b). The transition from the simple type 1 to a more complex pattern of type 2 in the rat brain begins around postnatal day 15, and is completed by postnatal day 35 (Couchie and Nunez., 1985). The present results are consistent with this: the neonates at gestation day 17 showed very little type 2 tau mRNA in the basal forebrain and hippocampal regions.

Intraperitoneal administration of the following compounds to adult rats did not alter Tau 1 or Tau 2 mRNA levels in either the basal forebrain or hippocampus after a 24 hour time period: ondansetron, MK801, GYKI52466, indomethacin, aniracetam. Thus, from these experiments it appears that expression of the tau gene does not show a great deal of plasticity. However, the compounds were administered at only one dose, and over a single time period. Future investigations into tau gene plasticity would involve several different drug concentrations and administration time points. In light of the fact that the *in vitro* experiments covered in chapter 5 illustrated dramatic induction of MAP2 protein and mRNA levels in response to a wide

variety of compounds, it would be of interest to repeat these experiments to monitor MAP2 mRNA levels.

NADPH diaphorase has been shown to be a nitric oxide synthase (Hope et al.,1991), and nitric oxide synthase (NOS) can be demonstrated conveniently by the histochemical technique for NADPH diaphorase immunostaining (Dawson et al.,1991). Chapters 4 and 5 have looked at the distribution and regulation of NADPH diaphorase immunostaining in glial and neuronal primary cultures. The endotoxin LPS has previously been shown to cause induction of a calcium-dependent inducible NOS in primary glial cultures (Galea et al.,1992; Simmons and Murphy.,1992). The cytokine interleukin-1 has also been shown to cause induction of iNOS in glial cells (Lee et al.,1995). In agreement with this, the glial cultures in the present work illustrated increased NADPH diaphorase immunostaining in response to exposure to LPS, and also in response to interleukin-1. As there is a vast literature to support the induction of iNOS by LPS and interleukin-1, the increase in NADPH diaphorase immunostaining in the present work has been taken as an increase in NOS activity. Use of the calmodulin antagonist W7 illustrated that the type of NOS induced in response to LPS is a calmodulin-independent form. Inducible NOS (iNOS) in macrophages and hepatocytes has been shown to tightly bind calmodulin (Cho et al.,1992; Evans et al.,1992). Recent evidence suggests that a similar system occurs in glial cells: iNOS tightly binds calmodulin and produces NO even when calcium levels are low or absent (Kitamura et al.,1996; Ruan et al.,1996). The enzyme induced in the present results in response to LPS is more than likely to be iNOS given that a vast number of studies have shown this, however, the NOS in the present work is calmodulin-independent. Recent work has suggested the presence of differential expression and /or structural diversities of the iNOS gene (Park et al.,1996). It is possible that the glial cells possess several forms of iNOS differing in their calmodulin dependency. It would be of interest to discover

whether the difference in the structure of the enzymes is in the calmodulin binding site. Exposure of the glial cells to glutamate resulted in the induction of a calmodulin-dependent NOS, further indication that the glial cells possess more than one type of NOS enzyme, and different forms of the enzyme could be activated in response to different compounds. Future work using radiolabelled oligonucleotide probes to monitor iNOS and neuronal NOS mRNA levels would give a clearer insight into the type (s) of NOS present in the glial cells.

The neuronal cells also appear to possess several different types of NOS again differing in their calmodulin dependency. Nerve growth factor (NGF) has been shown to induce a form of NOS in the neuronal cells. It has recently been suggested that the expression of neuronal NOS in developing basal forebrain cholinergic neurons is regulated by NGF (Holtzman et al.,1996). The high affinity NGF receptor, the p140 trkA receptor, is thought to be essential and sufficient to elicit NGF responses (Kahle et al.,1994). LPS induction of iNOS is thought to be via tyrosine kinase activation (Kitamura et al.,1996). The NGF-induced increase in MAP2 protein levels in the neuronal cells has been shown to be through a calmodulin-dependent form of NOS. In contrast, glutamate exposure resulted in the induction of a calmodulin-independent form of NOS. Thus, it is possible that the neuronal cells possess both basal levels of neuronal NOS and form (s) of iNOS differing in calmodulin-dependency, similar to the situation seen in the glial cells. It is likely that different forms of NOS are activated in response to different compounds acting through different pathways. As with the glial cells, future work using radiolabelled oligonucleotide probes to monitor iNOS and neuronal NOS mRNA levels would clarify the results presented.

Chapter also focused on the distribution and regulation of the cytoskeletal proteins MAP2 and tau in neuronal cultured cells. NO has been previously shown to stimulate the expression of the immediate

early genes c-fos and zif/268 in PC12 cells (Haby et al.,1994). NO has also been shown to alter proenkephalin, prodynorphin and MAP2 gene expression in hippocampal granule cells (Johnston et al.,1994a; Johnston et al., 1994b). Thus it is possible that NO may regulate gene expression in those areas of the brain that contain NOS activity. The present results suggest that NO may exert an induction in MAP2 immunoreactivity through a pathway that involves guanylate cyclase and cGMP-dependent protein kinase, and the neuronal cells may thus respond to NO in a similar manner to hippocampal granule cells and PC12 cells. Further work using specific inhibitors such as the cGMP phosphodiesterase inhibitor MY5445 and the cGMP dependent protein kinase inhibitor KT5823 would clarify the pathway involved in NO-stimulated MAP2 induction. Despite the dramatic effect of NO on MAP2 protein expression, no detectable effect of NO on neuronal cell morphology was observed. However, it is possible that an alteration to the dendritic spines or neurite branching points may have occurred and had not been detected with the antibodies used.

Recent studies have suggested a neuroprotective influence for NSAIDS and a beneficial association between NSAID use and cognitive functioning in Alzheimer's Disease (Rich et al.,1995). The target of the NSAIDS is cyclooxygenase (COX). Evidence suggests that induction of COX2 by LPS can occur in bovine aortic endothelial cells. In the neuronal cell however, exposure of the neuronal cells to interleukin-1 and LPS failed to alter COX protein or gene expression. Amyloid precursor protein (APP) protein and gene expression levels remained unaltered following exposure to LPS and interleukin-1. Chapter 6 covers a further investigation into the role (s) of APP.

The use of two transgenic mice models, one with the APP gene completely removed, the other with a mutated α-secretase site on the APP gene, has enabled a preliminary investigation into the possible actions of APP within the CNS. Complete removal of the APP gene results in a marked increase of reactive gliosis within most areas of the

the brain at 6 months of age (Zheng et al.,1995). The present work suggests that this induction of reactive gliosis normalises by 13 months of age, by an as yet unknown mechanism. The age matched wildtype mice showed a marked induction of reactive gliosis, especially in the corpus callosum and hippocampus, compared to the APP-null mice. It is possible that this was due to the normal ageing process; as reactive gliosis has been shown to occur in normal ageing brains (Norton et al.,1992; Dickson et al.,1993). Calbindin immunoreactivity has been shown to increase in the APP null mice at 13 months of age compared to age matched control animals. Thus suggests an interesting link between APP and calcium; it is possible that removal of the APP gene resulted in a potential calcium-induced toxicity which was controlled by increased levels of calbindin. The relationship between APP metabolism and presenilin function is unclear. The distribution of PS-1 in the mice was similar to that seen in rats (Mita et al.,1989), and no difference in pattern of distribution or immunoreactivity was observed in the APP-null mice in comparison to age matched control animals in the cortical and hippocampal regions. However, an initial study of cerebellar sections from the APP-null mice at 6 months of age suggest a possible increase of PS-1 in the null animals compared to controls. There is the interesting possibility that PS-1 may have a synaptic role, and recent results suggest that an alteration in the processing of APP may have considerable effects on synaptic plasticity (Nalbantoglu et al.,1996). The distribution of N and C terminal PS-1 has been compared in the APP null and age matched control animals. N terminal PS-1 has been shown to have a membrane-bound distribution, the C terminal appears to be mostly cytoplasmic. It is thought that PS-1 exists in small quantities as a full length protein, and is rapidly processed into an array of stable N and C terminal endoproteolytic fragments (Thunakaran et al.,1996; Podlisny et al.,1997). Further studies on the APP-null mice, especially between the ages of 6 and 13 months would

enable a further understanding of the roles of APP within the CNS. The mutation of the α-secretase site appears to have resulted in the onset of reactive gliosis, agreeing whit the initial report of Moechars (Moechars et al.,1996). This onset of reactive gliosis, along with the previously reported severe behavioural deficits and neuropathological changes demonstrate the severe cellular neurotoxicity resulting from incorrect α-secretase processing.

Chapter 7 has looked at neuropathology of Alzheimer's Disease in human postmortem brain tissue. A large degree of reactive gliosis was evident in the diseased brain throughout the cortical layers, corpus callosum and hippocampus. PS-1 distribution was throughout the temporal lobe, entorhinal cortex and hippocampus, and was consistent whit previous reports of PS-1 immunostaining intracellularly in neurons appearing as thick granules (Moussaoui et al.,1996). Defining the cellular and subcellular localisation pattern of PS-1 is expected to provide insights into pathophysiological function of the protein. Differing evidence exists regarding the presence of PS-1 in the pathological hallmarks of Alzheimer's Disease such as neuritic plaques and neurofibrillary tangles. Colocalisation of PS-1 protein with some plaques and tangles was observed in some but not all regions in the present study. A further study looking at several different patients would enable a clearer picture to be observed. The mechanism by which presenilin mutations cause Alzheimer's Disease pathology and their link to APP is of current research importance.

It seems likely that for the future, the development of further transgenic models will enable the relationship between presenilins, the amyloid generation pathway, the development of plaques and tangles and synaptic loss to clarified. It is hoped that transgenic models may provide a preclinical model for testing therapeutic drugs.

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