SIGNAL TRANSDUCTION IN ALZHEIMER'S DISEASE: AN INVESTIGATION OF G-PROTEIN LEVELS AND FUNCTIONAL ACTIVITY

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A thesis submitted for the Degree of Doctor of Philosophy
to the Faculty of Medicine, University of Glasgow

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ACKNOWLEDGEMENTS

Throughout my project I was forever amazed at the consideration and help given to me by the staff at the Wellcome surgical institute I can never thank them enough.

I would like to express my gratitude to my supervisors Prof. J. McCulloch and Prof. J.T. Knowler for their support, guidance and patience. The biochemical expertise of John and the extensive neuroscientific expertise of Jim has provided me with an invaluable education. Each has demanded scientific rigor while at the the same time allowed me sufficient independence to develop as a scientist.

During this project I had the pleasure of working closely with Brian M Ross whom I now value as a close friend. I would like to thank Brian for his many contribution. Brian was responsible for the development of the adenylate cyclase assay and the $G_{\alpha s}$ immunoblotting techniques. He performed many of the analyses of these parameters which are reported in this thesis. I would like to thank Fiona Inglis for her surgical expertise in lesioning the medial septal nucleus. To Brian and Fiona thanks for the scintillating discussion. Many thanks to Prof Graham and the staff at the department of Neurosurgery for their pathological assessment of the human material. Many thanks to Prof. D. Graham and Lindsay Dover for their help with the histological assessment of the medial septal lesion study, and to Magaret Roberts for the assistance with the ChAT assay. I would also like to thank Debbie Dewar and Sue Browne for the brain dissections and, together with Mhairi Macrae, for their valuable discussions. Thanks to Graeme Milligan for providing the G-protein antibodies and for his invaluable advice throughout this project.
I am extremely grateful to Prof. A.M. Harper for is continual interest in the project and my career and for supplying the excellent facilities at the Wellcome. Thanks to Prof T.G. Cooke for allowing me to use the photographic and printing facilities.

Thanks to all at the Wellcome Institute especially Lindsay Graham, Margaret Stewart, Gordon Littlejohn, Dave Love, Marion Steele, Michael Dunne, Haley Jane Dingwall, Joan Stewart, Mairi Law, Anne-marie Colquhoun, Christine Stirton, Morag Findlay and all the animal nurses.

A special thanks to Karen Horsburgh for all her help, to my brother Brian (truly the best man), to Gerry (smartie pants) Costley and to Peter Mohan.

Finally, I would like to express my deepest and most special thanks to my wife Annemarie. She has been fantastic throughout this voyage. Words are not enough.

To,

Annemarie and Sean (and baby), Mum and Dad, Jimmy and Ellen and Robert.

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ABSTRACT

Alzheimer's disease is a debilitating neurological disorder which has been predicted to afflict as much as 10 percent of the aged population over 85 years. The disease is characterised by the presence of senile plaques and neurofibrillary tangles, and by widespread neurochemical alterations throughout the brain. The initial event in neurochemical transmission involves the interaction of neurotransmitters with specific receptors. In the majority of cases the modulation of cellular activities by receptor activation is controlled by guanine nucleotide binding proteins (G-proteins). G-proteins are located on the intracellular membrane and respond to receptor stimulation to modulate a variety of signal transduction pathways. Thus, effective therapies targeted at the neurochemical deficits in Alzheimer's disease requires G-protein function to be maintained. This thesis examined the integrity of G-protein mediated signal transduction activities in Alzheimer's disease.

The levels of five G-proteins α subunits $G_{\alpha o}$,$G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$, and the $G\beta$ subunits were examined by semiquantative immunoblot analysis. The functional status of G-proteins was assessed by measuring the activities of the intrinsic GTPase action associated with G-proteins. Additionally, the effector molecule adenylate cyclase was assayed. To determine whether G-proteins could be reliably examined in human postmortem brain material, these parameters were examined for the effect of prolonged intervals between death and the freezing of brain tissue (postmortem interval). The G-protein levels were not significantly altered by prolonged intervals between death and the freezing of brain tissue. At a postmortem interval of 24 hours however, the basal and stimulated GTPase activity was significantly reduced compared to fresh tissue. In this thesis the investigation of the effect of Alzheimer's disease on G-proteins was conducted on control and diseased subjects that were closely matched for age and postmortem intervals.

The distribution of G-protein levels and adenylate cyclase activity was examined in five regions (frontal cortex, hippocampus, striatum, cerebellum and pons) of aged control human brain. In general, the level of $G_{\alpha o}$, $G_{\alpha i}1$, and $G_{\alpha i}2$ are higher in the frontal cortex and hippocampus compared to the cerebellum pons and striatum. However, $G_{\alpha s}L$, $G_{\alpha s}S$ were found to be most abundant in the cerebellum. Basal adenylate cyclase activity was not heterogeneously distributed although the magnitude of stimulation of adenylate cyclase by fluoroaluminate varied significantly between regions. The pattern of G-protein distribution and basal adenylate cyclase activity exhibited an inverse relationship. This suggested that the basal activity in human brain is influenced by the level of $G_{\alpha i}$ and the ratio of $G_{\alpha s}L/G_{\alpha s}S$ species. Within the striatum a third G-protein was detected with the antisera to $G_{\alpha s}$. This protein may be the putative G-protein G_{olf}.

The effect of partial cholinergic denervation of the septal/hippocampal pathway on the activities of G-proteins was examined in the rat hippocampus. Rats were lesioned at the medial septal which induced a significant reduction of choline acetyl transferase activity in the hippocampus. There was no significant reduction in the level of G-proteins. Functionally, the activity of GTPase and its response to muscarinic and GABAergic activity was preserved in lesioned animals. The effector enzyme adenylate cyclase was not significantly altered in the hippocampus of lesioned animals when compared to sham lesioned controls. These findings suggests that following partial denervation of the septal/hippocampus pathway, the remaining hippocampal neurons are capable of an adaptive response to maintain the functional components of the adenylate cyclase signal transduction pathway.

The levels of G-protein α subunits were found to be robust in Alzheimer's disease. However, the level of the $G\beta$ subunit was significantly reduced (by 57%) in the frontal cortex of Alzheimer's diseased samples compared to controls but was preserved in the hippocampus. The ratio of the $G_{\alpha s}$ subtypes ($G_{\alpha s}L/G_{\alpha s}S$) was significantly altered in Alzheimer's disease

frontal cortex compared to controls. There was no significant alteration in the ratio of $G_{\alpha s}L/G_{\alpha s}S$ measured in Alzheimer's diseased hippocampus when compared to controls. The basal adenylate cyclase activity was significantly reduced (by 45%) in Alzheimer's diseased frontal cortex compared to control samples but was preserved in the hippocampus of Alzheimer's disease subjects. The basal GTPase activity was reduced by 25% in Alzheimer's disease frontal cortex when compared to control levels ($P<0.05$). The basal GTPase was also reduced 27% in the hippocampus of Alzheimer's disease samples but failed to achieve statistical significance ($P=0.051$). The magnitude of stimulation of GTPase by carbachol and baclofen was similar for control and Alzheimer's disease frontal cortex and hippocampus preparations.

These findings indicate that although there is no gross alteration in the level of G-proteins, the functional activity of the G-protein controlled adenylate cyclase transduction pathway is compromised in Alzheimer's disease. This may be due to a breakdown in the synchrony between the inhibitory and stimulatory G-protein regulation of adenylate cyclase activity. This is supported by a positive correlation in the levels of the $G_{\alpha i}$ subunits and GTPase activity in the control frontal cortex and hippocampus which was not evident in Alzheimer's disease. Thus, although in this study the coupling of G-proteins to receptors were found to be preserved, the functional responsiveness of G-proteins, as indicated by the GTPase data, is altered in Alzheimer's disease. These findings are pertinent to the development of therapeutic agents designed to alleviate neurochemical deficits associated with Alzheimer's disease.

INTRODUCTION

1. Guanine nucleotide binding proteins (G-proteins) and signal transduction.

1. 1. The discovery of G-proteins.

The discovery of G-proteins originated in the 1950s when Sutherland and co-workers described the involvement of adenosine triphosphate (ATP) in receptor modulation of glycogen metabolism in the liver (Sutherland & Cori, 1951; Rall et al 1956). They identified an enzyme (adenylate cyclase) which catalysed the formation of cyclic adenosine mono phosphate (cAMP) from ATP. Two decades later, Rodbell and co-workers (1971), discovered that guanine nucleotides had the capacity to modulate the receptor stimulation cAMP production by hepatic adenylate cyclase activity. This was the first indication that a protein which binds guanine nucleotides can influence the ability of ligands to modulate cAMP. Greater insight into the role of guanine nucleotides in cell signalling was achieved with the development of GTP analogues such as guanosine 5' (β,γ-imino) triphosphate (Gpp(NH)p), and guanosine 5' (γ-thiotriphosphate) (GTPγS), which are poorly hydrolysed by nucleoside triphosphatases. Specifically, these compounds demonstrated that in frog and avian erythrocytes, the activation of adenylate cyclase by adrenaline is deactivated with a concomitant hydrolysis of GTP and that the duration of stimulation was enhanced with the analogues (Spiegal & Aurbach, 1974). In addition to the effect of GTP on adenylate cyclase activity, a modulatory effect of GTP on phototransduction was described for photon activation of cyclic GMP phosphodiesterase in the rod cone (Wheeler & Bitensky, 1977) thereby indicating that the GTP influence may be a common mechanism employed by diverse cellular transduction systems.

Final confirmation that a specific protein was involved in these transduction processes was

achieved with the purification of GTP binding proteins described for the adenylate cyclase system (Kuhn 1980), and for the phototransduction system (Godchaux & Zimmerman, 1979). The proteins isolated from these systems are now referred to as $G_{\alpha s}$ (stimulatory) and $G_{\alpha i}$ (inhibitory) for the adenylate cyclase system and $G_{\alpha T}$ (transducin) for the phototransduction system. G-proteins exhibit considerable similarities in that they are composed of three subunits designated α, β and γ. The α subunit is believed to confer specificity of the G-protein with respect to both the receptor with which it interacts, and the effector system that the specific G-protein modulates. GTP hydrolysis is achieved by an intrinsic enzymatic activity of the G_{α} subunit. The GTPase activity has been attributed to a conserved region common to all G_{α} subunits which is homologous to the GTP binding site of elongation factor TU (Masters et al., 1986). The β and γ subunits, which exist predominantly as a dimer, were originally thought to constitute a common pool which can interact with all G_{α} subunits (Gilman, 1987). However, recent evidence suggests that a higher degree of selectivity exists between the interaction of G_{α} and the $\beta\gamma$ complex (Pronin & Gautam, 1992).

Several effector systems, in addition to adenylate cyclase and cGMP phosphodiesterase, have now been shown to be controlled *via* receptor activation of G-proteins. These include:- activation of phopholipase C (Smrcka et al., 1991; Taylor et al., 1991) activation of phospholipase A_2 and phospholipase D (reviewed by Cockcroft, 1992; and Cockcroft & Thomas, 1992); modulation of voltage-gated ion channels via pertussis sensitive G-proteins (Hescheler et al., 1987; Kleuss et al., 1991). G-proteins are also implicated in cellular differentiation. This activity was suggested by the localisation of $G_{\alpha o}$ with growth activating phosphoprotein (GAP 43) in PC12 cells (Strittmatter et al., 1990 & 1991), the altered expression of G-proteins during differentiation (Watkins et al., 1992) and by the modulation of differentiation in cells treated with antisense oligonucleotides to $G_{\alpha s}$ and $G_{\alpha i}$ (Watkins et al., 1992., Wang, H.Y. et al., 1992).

To date, at least 30 genes encoding G_{α} subunits have been identified although the specific

function and mode of action of many remain unclear. The development of techniques, such as antisense oligonucleotide (Wang H.Y. et al., 1992; Kleuss et al., 1991), should greatly assist in defining the roles of specific G-proteins in cellular activity.

1. 2. The GTPase cycle of G-protein activation and deactivation.

The early observations that GTP can stimulate adenylate cyclase activity (Rodbell et al., 1971) and that prolonged activation required continual addition of GTP or the addition of a non-hyrolysable analogue Gpp(NH)p (Spiegel & Aurbach, 1974) suggested that GTP hydrolysis acts as a temporal control mechanism. This was confirmed by Cassel & Selinger (1976), who demonstrated with eythrocytes that the release of ^{32}Pi from [γ-^{32}P] GTP occurred with high affinity under basal unstimulated conditions. In this system, the turnover of GTP was significantly enhanced by the addition of catecholineamines Cassel & Selinger (1976). Detailed examinations of the mechanisms involved in the regulation of cell transduction can be examined in reconstitution systems (reviewed by Cerione, 1991). This approach overcomes the difficulties associated with studies of biological preparations which invariably contain numerous G-proteins, receptors and effector molecules at varying concentrations.

Several investigators demonstrated that the addition of GTP analogues to reconstituted systems induced a dissociation of the $\alpha\beta\gamma$ heterotrimer, resulting in a free GTP bound α subunit (Northup et al., 1983; Fung, 1983., Codina et al., 1984); this effect was maximal in the presence of receptor agonist (Fung, 1983). It was suggested that receptor occupancy by agonists induce dissociation of the GDP bound to α subunit which facilitates GTP binding and that the dissociation of GDP is the rate limiting step (Ferguson et al., 1986). Brandt & Ross (1986) however, conducted elegant experiments in vesicles reconstituted with purified β-adrenergic receptors and $G_{\alpha s}$ which demonstrated similar rates of GTP binding and GDP dissociation. They proposed that receptor binding induced an 'open' conformation of the α subunit which can more readily exchange GTP for GDP. The dissociation of the GTP bound G-

protein/receptor complex occurs at a faster rate than GTP hydrolysis (Guy et al., 1990; Brant & Ross, 1986). This mechanism enables one receptor to activate more than one G-protein with a resulting increase in GTP turnover without increasing the rate of hydrolysis by the G-protein (Guy et al., 1990; Brant & Ross, 1986).

The process of receptor regulation of GTP binding and hydrolysis requires low concentrations of magnesium ions (Brant & Ross, 1986; Hilf & Jakobs, 1989). Although the role of magnesium is not entirely clear, it has been suggested that it chelates nucleotides and the resulting nucleotide-metal ion complex represents an active nucleotide triphosphate species. Receptor activation of G-proteins is also influenced by the relative stoichiometry of the α and $\beta\gamma$ subunits. It has been estimated that for rhodopsin catalysed fluorescence of transducin ($G_{\alpha T}$), which distinguishes between the GTP and GDP bound $G_{\alpha T}$, approximately half the total $G_{\alpha T}$ complexes could be activated at ratios of $\beta\gamma_T$ to $G_{\alpha T}$ ~1:150 and greater than 80% of $G_{\alpha T}$-GDP activated at ratios of $\beta\gamma_T$ to $G_{\alpha T}$ ~1:20 (Osawa et al., 1990). Reconstituted receptor, $G_{\alpha s}$ and adenylate cyclase systems, rather than being silent, have a basal intrinsic GTPase activity which is inhibited with the addition of $\beta\gamma$ subunits and gives an enhanced signal to noise ratio on receptor activation of the system (Cerione, 1991). This has also been observed with membranes preparations (Katada et al., 1984a)and $G_{\alpha s}$ in solution (Northup et al, 1983).

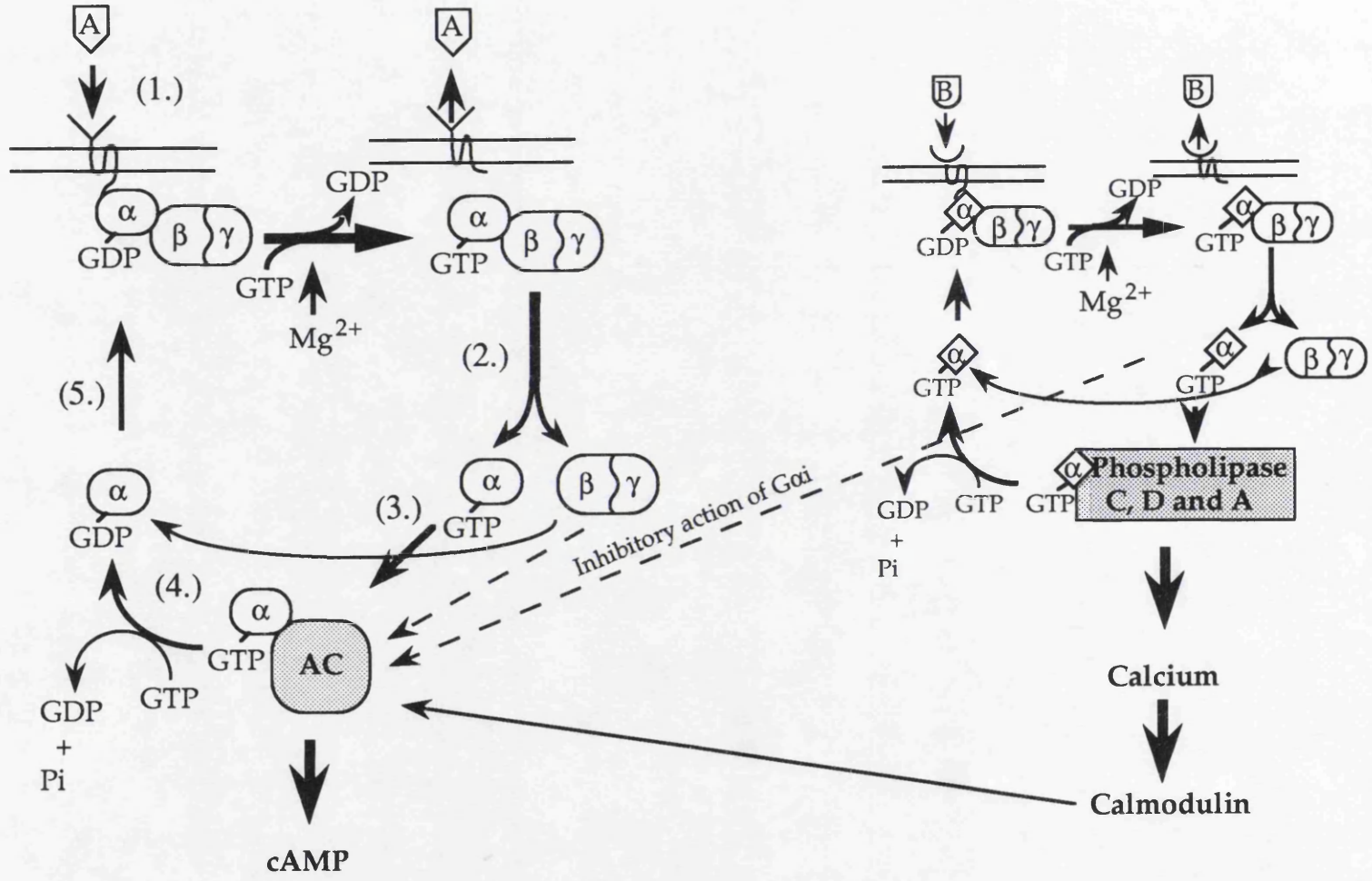
Gilman, (1987) proposed a model to describe a cyclic cascade of G-protein regulated signal transduction. The process is initiated by receptor activation which induces the activation and dissociation of the G-protein heterotrimer. The 'off' switch is the hydrolysis of GTP resulting in the reformation of the receptor G-protein complex. In biological systems this process is occurring at a basal intrinsic rate governed by the ratio of K_{diss} GDP/K_{cat} GTP. On receptor binding, the equilibrium is shifted in favour of the activation process. **Diagram I** illustrates the key points of the cascade of events believed to occur for the activation of the stimulatory $G_{\alpha s}$ and the inhibitory action of $G_{\alpha i}$ on adenylate cyclase activity. In addition, this diagram highlights the complexity of G-protein mediated signal transduction pathways. Recently is

has been demonstrated that G-proteins which operate *via* independent receptors, such as adenosine and β-adrenoceptors, have the capacity to cross-talk and cross-regulate (Hadcock et al., 1990). Furthermore, the recombinant forms of $G_{\alpha s}$ (large and small) have different rates of GTP hydrolysis due to differences in GDP dissociation rates, yet the K_{cat} (hyrolytic cycle per minute per molecule) were similar for both $G_{\alpha s}$ proteins (Graziano et al., 1989). Similarly, recombinant $G_{\alpha i}$ subtypes expressed different dissociation rates of GDP in comparison to $G_{\alpha o}$ although the K_{cat} were comparable (Linder et al., 1990). These observations underline the intricate nature of G-protein regulated signal transduction.

Diagram I: The regulation of adenylate cyclase activity by the GTPase cycle.

This diagram illustrates the molecular cascade which occurs on receptor activation and results in the activation of specific effector molecules which, in this case, is adenylate cyclase. The main events are shown in the left hand side of the diagram with each major stage annotated. The inclusion of a second receptor-linked pathway on the right hand side serves to illustrate the ability of different systems to interact. Although this diagram implies that this cascade occurs only on agonist binding to receptor, in fact this cycle is continuous with a significant basal activity of all stages such as GTP hydrolysis and adenylate cyclase activity. Agonist binding shifts the equilibrium to the right inducing an enhanced flow through the cycle.

(1) The unoccupied receptor is considered to be in a high affinity state and associated with the heterotrimer G-protein complex which is considered the dormant inactive configuration. Agonist binding induces an alteration in the conformation of the receptor and possibly the G_α subunit, which lowers the energy barrier for GDP dissociation and GTP binding. The GTP bound G-protein complex dissociates from the receptor with a concomitant shift in the receptor affinity from a high to a low affinity state. **(2)** The GTP bound G_α subunit dissociates from the $\beta\gamma$ dimer. At this stage the G_α is regarded as the active form and the free $\beta\gamma$ is available to interact with GDP-bound G_α subunits possibly from another pathway. Additionally, the $\beta\gamma$ dimer may interact directly with the effector molecule. **(3)** The active GTP bound free G_α subunit can interact with adenylate cyclase to stimulate cyclic AMP production, as is the case for $G_{\alpha s}$, or inhibit, as with $G_{\alpha i}$. **(4)** The activation of adenylate cyclase is terminated by the intrinsic hydrolysis of GTP to GDP. This induces dissociation of the GDP-bound G_α subunit and its reassociation with the $\beta\gamma$ dimer. **(5)** The GDP-bound heterotrimer can reassociate with the receptor, thereby completing the cyclic process.


A
(1.)
α
β
γ
GDP
GTP
Mg^{2+}
(2.)
(3.)
(4.)
(5.)
AC
cAMP
B
Inhibitory action of Gαi
Phospholipase C, D and A
Calcium
Calmodulin
Pi

1.3. Heterogeneity of the α subunits of G-proteins.

The application of cloning and sequencing techniques has identified multiple G-protein α subunits in the eucaryotic organism (reviewed by Birnbaumer et al, 1990; Simon et al., 1991). To date as many as thirty cDNA clones encoding α subunits have been reported, although the precise characterisations of receptor-effector interaction of many of the protein products awaits their isolation and purification. Of the α subunits that have been characterised, there is sufficient heterogeneity at their sequence, effector function and their capacity to act as substrates for ADP-ribosylation by bacterial toxins to allow G-proteins to be classified into one of four broad categories. These are: pertussis toxin sensitive, cholera toxin sensitive, pertussis toxin insensitive and the G12 class of α subunits. Some of the members of these categories have sufficient similarities to permit their being grouped into a separate subclass. As more information is acquired regarding the individual G-proteins, this classification system may yet prove to be inadequate and may have to be revised.

1.3.1 The $G_{\alpha o}$ subunits.

The $G_{\alpha o}$ protein (termed "o" for other since its function was unknown), originally isolated from bovine brain, has a molecular weight of 39 KDal (Sternweis & Robishaw, 1984). cDNA encoding two forms of mammalian $G_{\alpha o}$ and human chromosomal gene encoding $G_{\alpha o}$ have been isolated (Strathmann et al., 1990; Tsukamoto et al., 1991). The two protein products which have now been identified (Mullaney & Milligan, 1990; Asano et al., 1992) are substrates for pertussis toxin ADP-ribosylation which results in the inactivation of the protein. The proteins are thought to arise from a single gene by alternate splicing (Strathmann et al., 1990; Tsukamoto et al., 1991). $G_{\alpha o}$ is located mainly in neuronal and endocrine tissue, although a significant level of $G_{\alpha o}$ is located in the heart (Asano et al., 1988). Recently Asano et al. (1992), examined the distribution of the two forms of $G_{\alpha o}$, referred to as $G_{\alpha o}A$ and $G_{\alpha o}B$. $G_{\alpha o}A$ was located mainly in neural tissue whereas $G_{\alpha o}B$ is located in brain, pituitary gland,

adipose tissue, lung and testes.

The identification of the function of the $G_{\alpha o}$ protein has proved elusive. It has been suggested that $G_{\alpha o}$ can modulate the phosphatidylinositol via phospholipase C (Moriarty et al., 1990). There is however strong evidence which suggests that its prime activity is associated with the regulation of ion channels. These include four distinct types of potassium channels in cell free membrane patches of hippocampal pyramidal neurons (VanDongen et al., 1988); inhibition of voltage dependent calcium channels in neuroblastoma x glioma hybrids (Hescheler et al., 1987; Bergamaschi et al., 1992) and in rat pituitary GH_3 cells (Kleuss et al., 1991). In the latter study, Kleuss et al. (1991) demonstrated that both species of $G_{\alpha o}$ are capable of inhibiting calcium channel activity. Membrane depolarisation which induces changes in the affinity of muscarinic receptors is mediated by a pertussis sensitive G-protein, suggested to be $G_{\alpha o}$ (Cohen-Armon & Sokolovsky, 1991). Recently $G_{\alpha o}$ has been shown to be associated with growth activating polypeptide (GAP 43) in neuronal growth cone membranes (Strittmatter et al., 1990 &1991).

1.3.2. The $G_{\alpha i}$ subunits.

This subclass of G-proteins were first described by Hilderbrant et al, (1982) who observed that pertussis toxin treatment of liver preparations blocked the inhibitory regulation of adenylate cyclase; they defined this protein as the inhibitory G-protein, $G_{\alpha i}$. Subsequent purification and molecular cloning studies have shown that a $G_{\alpha i}$ family of proteins exist which is comprised of three highly related proteins: $G_{\alpha i}1$, $G_{\alpha i}2$ and $G_{\alpha i}3$ (Milligan & Klee, 1985; Jones & Reed, 1987; Itoh et al., 1988). These proteins have molecular weights of 41 KDal, 40 KDal and 39 KDal respectively. They exhibit a similar amino acid sequence, ($G_{\alpha i}1$ and $G_{\alpha i}3$ share 95% homology) and they are respectively 88% and 85% identical to $G_{\alpha i}2$ (Jones & Reed, 1987). $G_{\alpha i}1$ and $G_{\alpha i}2$ are present in all tissue but most abundant in brain (Asano et al., 1989); whereas $G_{\alpha i}3$ is undetectable in mammalian brain (Kanaho et al., 1989), although a recent

report identified low levels of $G_{\alpha i}3$ in brain myelin (Berti-Mattera et al., 1992).

Although the $G_{\alpha i}$ nomenclature implies that all three $G_{\alpha i}$ proteins are capable of an inhibitory effect on adenylate cyclase, $G_{\alpha i}2$ is the only member which has been demonstrated to exhibit this function (Simonds et al., 1989; McKenzie et al., 1990; Bushfield et al., 1990a). The mechanism by which $G_{\alpha i}$ achieves its inhibitory influence is still to be clearly defined. Katada et al. (1984a) suggested that the inhibition of adenylate cyclase involves the interaction of the free $\beta\gamma$ subunit with $G_{\alpha s}$; whereas other studies in $G_{\alpha i}2$ deficient cell lines suggest the inhibition is achieved by a direct action of $G_{\alpha i}$ on adenylate cyclase (Hildebrandt et al., 1982). Recently, Taussig et al. (1993b) reported that $G_{\alpha i}1$ is inhibitory to type V adenylate cyclase but only weakly inhibitory for type 1 adenylate cyclase. There is also evidence that members of the $G_{\alpha i}$ family can regulate the activity of phospholipase C (Bizzarri., 1990). Since $G_{\alpha o}$ is also a substrate for pertussis toxin, its involvement in this activity cannot be discounted.

The high degree of homology between the different $G_{\alpha i}$ species has hindered their assignment to specific cellular activities, although the development of more selective techniques is however beginning to delineate some of their roles. For instance, $G_{\alpha i}3$ has been implicated in controlling epithelial sodium channels by activating phospholipase A_2 (Cantiello et al., 1990), whilst $G_{\alpha i}2$ is reported to be involved in cellular differentiation (Watkins et al., 1992).

1.3.3 Transducin ($G_{\alpha T}$) subunits.

Transducin or $G_{\alpha T}$ is a 39 KDal protein which is sensitive to pertussis toxin and communicates the signal from light activated rhodopsin to cyclic GMP phosphodiesterase (reviewed by Liebman et al., 1987). The carboxy-terminal region of $G_{\alpha T}$ contains the binding site for rhodopsin (Hamm et al., 1988) which is also thought to correspond to the region on $G_{\alpha s}$ which

interacts with β-adrenergic receptors (Sullivan et al 1987). Two types of $G_{\alpha T}$ have been cloned: the $G_{\alpha T}$-r is composed of 350 amino acids and located in rod cells; $G_{\alpha T}$-c is 354 amino acids long and located in cone cells (Liebman et al., 1986). These $G_{\alpha T}$ members share 80% homology at their amino acid level and are closely related at the carboxy-teminal to $G_{\alpha i}$ proteins. Mechanistically, the $G_{\alpha T}$ protein, like all other G-proteins, exists as a trimer composed of $\alpha\beta\gamma$ subunits which on activation go through the GTPase cycle of activation and deactivation (Fung et al., 1983). Indeed, as discussed in relation to the GTPase cycle, the transducin-rhodopsin system has been intensely studied and has contributed greatly to the current understanding of the interactions involved in G-protein controlled signal transduction.

1.3.4. The $G_{\alpha s}$ subunits.

Two $G_{\alpha s}$ subunits with distinct molecular weights of 45 KDal ($G_{\alpha s}$S for small) and 52 KDal ($G_{\alpha s}$L for large) were originally purified from from rabbit liver (Northup et al., 1983). Both $G_{\alpha s}$ proteins are substrates for ADP-ribosylation by cholera toxin which results in their constitutive activation (Birnbaumer et al., 1990). Molecular cloning has identified four forms of $G_{\alpha s}$ cDNAs which arise from an alternative splicing reaction (Bray et al., 1986). The $G_{\alpha s}$ splice variants comprise of a pair of shorter and a pair of longer molecules which differ by a block of 14 amino acids encoded on exon 3. Each pair are formed by two polypeptides that differ by the presence and absence of a single serine residue at the insertion-deletion junction (Kozasa et al., 1988; Robishaw et al., 1986; Bray et al., 1986).

The stimulation of adenylate cyclase by $G_{\alpha s}$ has been unequivocally demonstrated by the ability to reconstitute hormonal, fluoroaluminate and GTP sensitivity of adenylate cyclase in *cyc*$^-$S49 cells which are deficient in $G_{\alpha s}$ (Codina et al., 1984), and by their ability to activate the purified catalytic unit of adenylate cyclase (Tang et al., et al., 1991). Patch clamp studies have also shown that $G_{\alpha s}$ can regulate the activity of cardiac and skeletal L-type calcium channels (Yatani et al., 1987). This activity can be achieved by three of the four

splice variants which are also equally functional in stimulating adenylate cyclase (Mattera et al., 1989). Additionally, $G_{\alpha s}$ has been shown to activate a novel calcium transporter in *Xenopus* oocytes (Murphy & McDermott, 1992). Thus, $G_{\alpha s}$ appears to be involved in the regulation of multiple effector systems.

Recently an additional member of the $G_{\alpha s}$ subclass has been described as predominantly localised in olfactory neurons and was therefore termed $G_{\alpha olf}$ (Jones & Reed, 1989). A detailed biochemical investigation of the three $G_{\alpha s}$ proteins, $G_{\alpha olf}$ $G_{\alpha s}L$ and $G_{\alpha s}S$, was conducted by expressing their respective cDNAs in *cyc⁻kin⁻* S49 cells which lack $G_{\alpha s}$ proteins (Jones et al., 1990). The data revealed that $G_{\alpha olf}$ is 88% homologous to $G_{\alpha s}$ at the amino acid sequence level, has a molecular weight of 47 KDal and is a substrate for cholera toxin. Functionally, $G_{\alpha olf}$ can couple to β-adrenergic receptors to stimulate adenylate cyclase, although the efficacy of the coupling is poor in comparison to the large and small $G_{\alpha s}$.

1.3.5. The $G_{\alpha q}$ subunits.

The involvement of G-proteins (termed $G_{\alpha p}$) in the signal transduction mechanisms mediating agonist stimulation of phosphoinositol hydrolysis has been suggested for some time. The evidence for the involvement of G-proteins in this process is based on the observations that phospholipase C stimulation is dependent on GTP; agonist stimulation of phospholipase C results in enhanced high affinity GTPase; and agonist stimulation of phospholipase C is enhanced by non-hydrolysable GTP analogues and fluoroaluminate. Additionally, while this activity can be inhibited in some instances by pertussis toxin, (for example in neutrophils), other tissues exhibited a refractory response to pertussis toxin (reviewed by Cockcroft & Thomas, 1992b). Recently, molecular cloning has identified a pertussis toxin insensitive class of G-proteins which are now believed to be coupled to phospholipase C and are termed $G_{\alpha q}$ (Smrcka et al., 1991; Wilkie et al., 1991; Taylor et al., 1991). The $G_{\alpha q}$ family comprise of at least five G-proteins, $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15}$ and $G_{\alpha 16}$ (Wilkie et al., 1991) which lack the

cysteine residue at their carboxy-terminal required for ADP-ribosylation. The $G_{\alpha q}$ and $G_{\alpha 11}$ share 88% homology at the amino acid sequence level while $G_{\alpha 14}$ and $G_{\alpha 15}$ are 81% and 58% identical to $G_{\alpha q}$ respectively (Wilkie et al., 1991).

The $G_{\alpha q}$ and $G_{\alpha 11}$ proteins have similar molecular weights of 42 KDal which, in combination with their high degree of homology and their insensitivity to pertussis toxin, hindered their assignment to specific receptors and phospholipase C subtypes (of which five have been identified). However, it has been shown that $G_{\alpha q}$ protein(s) isolated from liver can couple to brain purified phospholipase $\beta 1$ isotype, but not phospholipase $\gamma 1$ and $\sigma 1$ isotypes (Taylor et al., 1991). $G_{\alpha q}$ can also enhance the maximal activity and calcium dependence of phospholipase C isolated from bovine brain (Smrcka et al., 1991). The $G_{\alpha q}$ /$G_{\alpha 11}$ are expressed in a wide variety of tissue (Wilkie et al., 1991), whereas $G_{\alpha 14}$, $G_{\alpha 15}$ and $G_{\alpha 16}$ have a more restricted tissue distribution (Wilkie et al., 1991). Recently, Milligan (1993) reported that $G_{\alpha q}$ was heterogeneously distribution in regions of rat brain whereas the levels of $G_{\alpha 11}$ was more uniformly expressed throughout rat brain.

1.3.6. The $G_{\alpha z}$ (or $G_{\alpha x}$) subunit.

The $G_{\alpha z}$ protein was first cloned from rat brain (Matsuoka et al., 1988) and subsequently from human retina (Fong et al., 1988) and human brain (Matsuoka et al., 1990). The protein has a molecular weight of 40 KDal and is located primarily in neuronal tissue (Casey et al., 1990). $G_{\alpha z}$ has a similar amino acid sequence to $G_{\alpha i}$ but is divergent in the cysteine residue, which is the site for ADP-ribosylation, and at the GTP binding domain. Biochemical investigation of the recombinant $G_{\alpha z}$ demonstrated a 100 fold reduction in the GTPase activity of $G_{\alpha z}$ compared to $G_{\alpha o}$, $G_{\alpha i}$ and $G_{\alpha s}$ (Casey et al., 1990). The divergence of the GTP binding domain of $G_{\alpha z}$ may represent a structural modification which could potentially enhance the activation time of this G-protein. To date the function of $G_{\alpha z}$ is unknown however its interesting kinetic profile and its predominantly neuronal location have provided excellent

incentives for its continued investigation.

1.3.7. The $G_{\alpha 12}$ and $G_{\alpha 13}$ subunits.

Very little is known about the $G_{\alpha 12}$ and $G_{\alpha 13}$ proteins. The clones for these proteins were isolated alongside the $G_{\alpha q}$ clones (Strathmann et al., 1989). Their mRNA is ubiquitously expressed yet their function remains unknown.

1.4. The role of the $\beta\gamma$ subunit complex in signal transduction.

The early observation by Sternwies, (1986) that purified G_{α} subunits fail to associate with phospholipid vesicles unless $\beta\gamma$ subunits were present, led to the suggestion that the $\beta\gamma$ dimer may serve to anchor the G_{α} protein to the membrane. This proposal is supported by the findings of Milligan & Unson, (1989) who showed that agonist activation of G-proteins (resulting in dissociation of the $\alpha\beta\gamma$ complex) can result in the removal of the G_{α} subunit from the plasma membrane. It has also been shown, however, that a subset of G_{α} proteins namely $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha i}3$, and $G_{\alpha z}$ undergo co-translational N-myrystolyation (Mumby et al., 1990). This modification could assist membrane association of G-proteins (Mumby et al., 1990; Jones et al., 1990b). On the other hand, $G_{\alpha s}$ and mutants of $G_{\alpha i}2$ are not myristicated (Jones et al., 1990; Gallego et al., 1992) yet can associate with the plasma membrane. Thus, although $\beta\gamma$ dimers are required for the association of the heterotrimer with the receptor on the internal plane of the plasma membrane (Sternweis, 1986), the α subunit is also influential in this activity. It is now emerging that the $\beta\gamma$ dimer plays a more dominant role in various G-protein controlled signal transduction pathways: $\beta\gamma$ can interact directly to modulate the activity of phospholipase A_2 in phototransduction (Jelsema & Axelrod, 1987), phospholipase C-β2 in transfected COS-1 cells (Camps et al., 1992; Katz et al., 1992) and adenylate cyclase (Tang & Gilman, 1991; Taussig et al., 1993a). Considerable attention is now being focused on the $\beta\gamma$ dimers to clarify their roles in the mechanisms of signal transduction.

To date four β subunits have been identified (β1, β2, β3 and β4) which share more than 80% amino acid sequence homology (reviewed by Clapham & Neer, 1993). β1, β2 and β3 are ubiquitously expressed whereas β4 is abundant in brain and lung but expressed at low levels in other tissue (Simon et al., 1991). The literature commonly refers to two classes of β subunits, β_{35} and β_{36} due to their molecular weights of 35 KDal and 36 KDal respectfully. The β_{35} and β_{36}, which can be separated by electrophoresis, probably contain a mixture of the four β subunits. Similarly, the $G_{\alpha s}$ subtypes may contain products of the additional $G_{\alpha s}$ genes.

Six species of the γ subunit have now been identified however their functional and biochemical characteristics remains to be established. Three γ-protein isotypes have been described. $G_{\gamma 1}$ is located only in photoreceptors, $G_{\gamma 2}$ is differentially expressed in all tissue and $G_{\gamma 3}$ is present in brain and testis only (Simon et al., 1991). The carboxy terminal of both $G_{\gamma 1}$ and $G_{\gamma 2}$ contain a Cys-Ali-Ali-Xaa motif (Ali, aliphatic amino acid; Xaa, any amino acid) which is similar to the *ras* oncoprotein. The C-terminal of the *ras* oncoprotein and the G_{γ} protein undergoes a post-translational modification which involves the prenylation of the cystein residue (Spiegal et al., 1991; Fukada et al., 1990). This process is thought to be critical for membrane association but not for the assembly of, or the stability of the αβγ heterotrimer. The multiplicity of the γ and β subunits have the potential to greatly increase the divergence of G-protein receptor coupling. Indeed, it has recently been shown that β and γ subunits interact in a selective manner (Pronin & Gautam, 1992) which could generate heterogeneous pools of βγ dimers.

1.5. The adenylate cyclase system.

The hormonal regulation of intracellar cyclic AMP levels *via* G-protein control of adenylate cyclase activity is one of the most thoroughly investigated signal transduction systems. The enzyme is under the dual regulation of the stimulatory G-protein, $G_{\alpha s}$ and the inhibitory

action of $G_{\alpha i}$ (reviewed by Gilman 1987). Additionally, in some tissue adenylate cyclase activity was found to be regulated by intracellular free calcium levels (reviewed by Cooper & Brooker, 1993). Subsequent protein purification and amino acid sequencing has allowed the cloning of several forms of adenylate cyclase. These proteins have been classified into two groups, the calmodulin-sensitive and the calmodulin-insensitive adenylate cyclase.

To date six adenylate cyclase enzymes have been cloned and partially characterised. Type I (Tang et al., 1991) and type III (Bakalyar & Reed, 1990) are calmodulin sensitive. Type II (Feinstein et al., 1991), type IV (Gao & Gilman 1991), type V and type VI (Katsushika et al., 1992) are insensitive to calcium/calmodulin. Tissue distribution studies of the various mRNA has established that type I is restricted to neural tissue; type II and type IV are broadly distributed, although type II is most abundant in brain; type III is restricted to olfactory neurons; and that type V and type VI are widely expressed, although type VI mRNA is most abundant in brain (Xia et al., 1991 & 1993). Monoclonal antibodies have recently been produced and have been used to identify three antigens with molecular weights of 160 kDal, 150 kDal and 115 kDal (Mollner et al 1991). It has yet to be confirmed that these proteins are adenylate cyclase isotypes. These antibodies should prove to be useful tools in establishing the relative concentration of the adenylate cyclase subtypes in various tissue, the functional significance of differential expression and the mechanisms involved in their control.

1.6. The identification of receptors coupled to G-proteins.

It has been suggested that as many as 80% of the known hormones and neurotransmitters mediate their cellular activities through G-proteins *via* interactions with receptors (Birnbaumer et al., 1990). The assignment of a receptor population to the G-protein coupled group is primarily based on several observations; the GTP modulation of pharmacological ligand-binding characteristics, the alteration of high affinity GTPase activity and known G-protein controlled effectors by specific agonists, and, in some instances, on the ability of the

bacterial toxins, pertussis or cholera, to interfere with the agonist stimulation of effector molecules. It is clear that within a single cell there exist many different G-proteins and receptor populations, many of which contain numerous subtypes. Additionally, there is now evidence for cross-talk between different receptor mediated signaling pathways (Hadcock et al., 1990). Thus, the assignment of a specific G-protein to a particular receptor, which is based on current empirical evidence, will in most cases need to be reassessed as more specific receptor and G-protein probes are developed. **Table 1** gives examples of different receptor types, the G-protein to which they are coupled, and the effector system which they modulate. The reader is referred to mainly to reviews which provide detailed information on the variations in the coupling of receptors and G-proteins in different tissues.

The molecular basis for receptor interaction with G-proteins has been examined in relation to several receptor types, most notably the photoreceptor rhodopsin, the muscarinic and adrenergic receptor classes (reviewed by Bonner 1989 & 1992; Lefkowitz and Caron 1988). From the deduced amino acid sequence of many G-protein coupled receptors, the most striking feature is that each contains seven stretches of 20-28 hydrophobic amino acids which, based on the rhodopsin template, are likely to represent membrane spanning regions. Of these regions, the third cytoplasmic domain, which connects the fifth and sixth transmembrane domain, has been proposed as the receptor/G-protein coupling site and also confers selectivity of the receptors for specific G-proteins. This was demonstrated by introducing deletion mutations of the third cytoplasmic domain of the β_2-adrenoceptor which removed its ability to stimulate adenylate cyclase (Dixon et al 1987; Strader et al 1987). Further evidence was obtained from the observations that chimeric α_2-adrenoceptor (inhibitory to adenylate cyclase), containing part of the fifth and the complete sixth domains from the β_2-adrenoceptor, could stimulate the activity of adenylate cyclase (Kobilka et al 1988). Similar studies conducted on the muscarinic receptors subtypes also concluded that the third domain was responsible for G-protein interaction. More specifically, the first 16-21 amino acids of the third domain of the cloned m_2 and m_3 receptors (expressed in *Xenopus* oocytes) confer the

selectivity of these subtypes for the phosphoinositol and the adenylate cyclase pathways respectively (Bonner, 1992). Although these data strongly implicates the third cytoplasmic domain as being the main site for G-protein interaction, the efficiency of chimeric receptors in producing an effector response is generally less than the wild type molecules. Thus it is equally likely that the regions distal to the third cytoplasmic domain can also have a significant influence on the specificity and efficacy of receptor G-protein coupling.

TABLE 1. Examples of receptor modulated transduction system of neuronal tissue that are controlled by G-proteins.

Type of receptor	Effector system	G-protein	References
Muscarinic M1 type			
(M1, M3)	phosphoinositol	Gαq,	(1), (3)
Muscarinic M2 type			
(M2, M4)	K+ channels	Gαo,Gαi,	(1), (4)
	adenylate cyclase	Gαo, Gαi, Gαq	(1), (4),(6)
metabotropic glutamate			
mGluR1, mGluR5	phosphoinositol	Gαo?	(5)
	adenylate cyclase	Gαi?	(5), (2)
mGluR2, mGluR3, mGluR4	adenylate cyclase	Gαi?	(5), (2)
Somatostatin	adenylate cyclase	Gαi	(1)
	calcium channels	Gαo?	(1)
Opioid			
δ	adenylate cyclase,	Gαi2	(1), (7)
	K. channels	Gαi2	(1), (7)
κ	Ca. channels	Gαi, Gαo?	(1), (7)
μ	adenylate cyclase,		
	K. channels	Gαi, Gαo?	(1), (7)
Adrenergic α–type			
α1	phosphoinositol	Gαq	(6), (9), (10)
α2	adenylate cyclase	Gαi	(6), (9), (10)
	Ca, K channels	Gai? Gao?	(6), (9), (10)
Adrenergic β–type			
β1	adenylate cyclase	Gαs	(6), (9), (10)
β2	adenylate cyclase	Gαs	(6), (9), (10)
Dopamine			
D1	adenylate cyclase	Gαs	(1)
D2	potassium channels	Gαi/Gαo	(1)
	adenylate cyclase	Gαi	(1)
GABAB	Ca channels, K. channels	Gαi/Gαo	(1), (8)
	adenylate cyclase	Gαi/Gαo	(1)
Serotonin (5HT)			
S-1a	adenylate cyclase	Gαi/Gαo	(1)
	K channels	Gαi/Gαo	(1)
S-1c	phosphoinositol	Gαq	(1)
S-2	adenylate cyclase	Gαs	(1)
	Ca channels	Gαo	(1)
Adenosine			
A1	adenylate cyclase	Gαi	(1)
A2	adenylate cyclase	Gαs	(1)
VIP	adenylate cyclase	Gαs	(1)
Neuropeptide Y	adenylate cyclase	Gαs	(1)
	Ca, K channels	Gαo?	(1)

References:

(1) Birnbaumer et al., (1990), (2) Itano et al., (1991), (3) Mullaney et al., 1993, (4) Baumgold, (1992), (5) Shoepp& Conn, 1993, (6) Milligan,(1993a), (7) Childers, (1991), (8) Holz et al., (1989) . (9) Hadcock & Malbon, 1993, (10) Summers & McMartin (1993)

1.7. Disease induced alteration of G-protein mediated signal transduction.

There is now a growing body of evidence that indicates that alterations in G-proteins are associated with a variety of clinical conditions. Perhaps the earliest examples of targeting G-proteins was revealed by the action bacterial toxins, produced by the causative organisms of cholera (cholera toxin) and whooping cough (pertussis toxin). Pertussis toxin selectively blocks G-protein function while cholera toxin results in over-expression of the second messengers cAMP by constitutively activating $G_{\alpha s}$ proteins (Birnbaumer et al., 1990). A recent report identified sequences in the human cytomeglovirus, a herpesvirus, that were highly homologous to the seven membrane spanning peptides of the rhodopsin like G-protein coupled receptor (Chee et al., 1990). This raises the interesting possibility that viral transformation of the host cell may be achieved *via* a transduction system.

More reliable information on the plastic response of G-proteins to disease has been reported for other conditions. G-proteins have been implicated in cardiovascular disease, the first evidence of which was the observed reduction in the contractile response to β adrenergic agonists (Bristow et al., 1982). This response was attributed to down regulation of the β_1-adrenoceptor (Fowler et al., 1986; Bristow et al., 1986). Investigations of signal transduction components in diseased hearts reported alteration in the level of pertussis toxin binding and concomitant changes in the activities of adenylate cyclase (Feldman et al., 1988). Similarly, modifications of selective G_α subunits ($G_{\alpha i}2$ and $G_{\alpha i}3$) in the heart of spontaneously hypertensive rats has recently been reported (Anand-Strivastava, 1992).

The most closely-studied clinical disorder of G-proteins is probably Albright hereditary osteodystrophy, an uncommon genetic disorder that is characterised by skeletal defects. Affected members are classified as exhibiting either pseudohypoparathyroidism (PHP) or pseudopseudohypoparathyroidism (PPHP) depending on whether or not they are resistant to parathyroid hormone, thyroid hormone and gonadotrophins respectively. In both cases, it

has been demonstrated that $G_{\alpha s}$ mRNA levels are reduced by approximately 50% when compared with controls in all tissue examined (Levine et al., 1988). In chemically induced hypothyroidism of the rat, there is a decreased responsiveness to agents which stimulate adenylate cyclase, whereas the inhibitory response is enhanced. In this condition the effect is due to increased amounts of $G_{\alpha i}$ and $G\beta$ proteins (Milligan et al., 1987) although the mechanisms responsible for the altered G-protein levels are unclear. Regarding Albright hereditary osteodystrophy, examination of genomic DNA by polymerase chain reaction identified mutations of the $G_{\alpha s}$ gene from. The mutations in PHP patients consisted a single base substitution (Glycine to Cysteine) at the donor splice junction at exon 10, and a frame shift mutation created by a single base deletion within exon 10 in both PHP and PPHP (Weinstein et al., 1990). It was proposed that this frameshift mutation resulted in an abnormally truncated $G_{\alpha s}$, whereas the substitution mutation could alter the steady state level of the $G_{\alpha s}$ mRNA. While the genetic bases for full phenotypic expression are still unclear, this syndrome is a clear example of a genetic abnormality of a G-protein.

Mutations of $G_{\alpha s}$ isolated from thyroid (Lyons et al., 1990) and pituitary adenomas (Landis et al., 1989; Valler et al., 1987) have also been reported. These proteins contain a substitution of either of two conserved amino acids which result in the inhibition of GTPase activity, producing a constitutively active $G_{\alpha s}$ protein, termed *gsp* (Masters et al.,1989; Landis et al.,1989). Lyons et al. (1990) also described a mutation of the $G_{\alpha i}$ subunit (*gsp* 2) which, in a similar fashion to $G_{\alpha s}$, was subsequently shown to constitutively inhibit the activity of adenylate cyclase (Wong et al., 1991). These are further examples of genetic mutations of G-proteins that have, in this case, potential oncogenisity.

Alterations in G-protein mediated signal transduction have also been described in diabetes. This was first demonstrated by Gawler et al. (1987), where streptozocin induced type I diabetes in rat resulting in a marked reduction in the level of $G_{\alpha i}$ together with a functional loss in the inhibitory effect on adenylate cyclase and a marked reduction in basal adenylate

cyclase in hepatocytes. This effect was found to be partially reversed in diabetic animals which were administered sufficient insulin to normalise their blood glucose levels.

The disease conditions described above represent examples where alterations in G-protein levels with concomitant functional changes have been closely examined. It is likely that improved technologies will lead to the identification of additional pathological conditions where G-proteins may be implicated. Indeed, alterations in G-protein levels have been reported in obesity (Begin-Heick, 1990), bipolar affective disorder (Young et al., 1991), olivopontocerebellar atrophy (Kish et al.,1993) and it has been suggested that an altered equilibrium of the GTPase cycle occurs in brain ischemia (Takenaka et al., 1991).

1.8. Drug induced alteration of G-protein mediated signal transduction.

Investigation into the mechanisms of cell tolerance to long term opioid exposure has revealed complex changes in both receptor and G-protein subtypes. Opioid receptors have been classified into three subtypes, mu (μ), delta (δ)and kappa (κ) based on pharmacological and ligand binding studies all of which are coupled to G-proteins (reviewed by Childers, 1991). Studies performed on different preparations of cells with neural origin have shown that opioids can modulate a variety of cellular activities. These include the inhibition of adenylate cyclase activity (Attali & Vogel, 1989), and inhibition of calcium currents (Hescheler et al., 1987). Of these cellular responses, the adenylate cyclase system has received most attention and it has been proposed that modifications in this neurochemical pathway underlie the development of opioid tolerance.

In NG108 neuroblastoma cell line, the δ subclass has an inhibitory input on the adenylate cyclase pathway which is mediated through $G_{\alpha i}$ (McKenzie et al., 1988). In this cell line, long term exposure to the opiate receptor agonists altered basal and stimulated GTPase activity without altering the level of G-proteins $G_{\alpha o}$ and $G_{\alpha i}$ (Lang & Costa, 1989). Attali &

Vogel, (1989) showed that the $G_{\alpha i}1$ level was significantly reduced in rat spinal cord-dorsal root ganglion co-cultures following chronic long term opiate exposure. Nestler et al. (1989), on the other hand, showed that chronic morphine administration resulted in an increased level of both $G_{\alpha i}$ and $G_{\alpha o}$ of the dopamergic neurons of the ventral tegmental area such as the nucleus accumbens and in associated target neurons of the locus coeruleus. The complexity of the G-protein alterations following chronic opioid treatment is also illustrated by the selective changes in different G_{α} subunits which have been observed in cultured neurons derived from rat cortex (Eriksson et al., 1992). In this cell culture system *k* receptor agonist exposure increased $G_{\alpha s}$ mRNA and $G_{\alpha i}2$ mRNA; δ agonists had no effect on either $G_{\alpha s}$ mRNA, $G_{\alpha i}1$ mRNA or $G_{\alpha i}2$ mRNA, while μ agonists decreased the level of $G_{\alpha s}$ mRNA, $G_{\alpha i}1$ mRNA and $G_{\alpha i}2$ mRNA. Reductions in the pertussis toxin sensitive G-protein levels $G_{\alpha i}$ and $G_{\alpha o}$ following chronic cocaine administration in rats have also been observed (Nestler et al, 1990; Striplin and Kalivas, 1992). Thus, modulating the levels of selective G-proteins may underline the augmentation of motor stimulant effects and possible behavioral augmentation induced by these psychoactive drugs.

Lithium, which stabilises the mood fluctuations of manic depression, is believed to exert its therapeutic effect at the post-receptor G-protein level (Avissar et al., 1988). The biochemical basis of lithium action is unclear There is evidence which suggests that lithium can exert an inhibitory influence on adenylate cyclase action (Colin et al 1991), while inhibition of the phosphoinositol system has also been suggested (Berridge et al., 1982). Lithium has an inhibitory effect on the coupling of muscarinic receptors and β-adrenoceptors to G-proteins which is reversed by magnesium (Avissar et al., 1991). Since magnesium is required for G-protein activation, lithium therefore has the potential to modulate both the adenylate cyclase and the inositol pathways. It has been demonstrated that chronic lithium treatment of rat resulted in an increased expression in the cortex of type I and type II adenylate cyclase, decreased $G_{\alpha i}1$, whilst $G_{\alpha o}$ $G_{\alpha s}$ and Gβ remained unchanged (Colin et al., 1991).

The effect of exposure of adrenergic agonist on receptors, associated G-proteins and adenylate cyclase action, is probably the most extensively studied and best characterised system (reviewed by Hadcock & Malbon, 1993). The α_1-adrenergic receptors mediate the regulation of phospholipase C, β_1-adrenergic activation stimulates adenylate cyclase while α_2-adrenergic has an inhibitory input on adenylate cyclase (reviewed by Summers and McMartin 1993). Short term and long term exposure of a variety of cells to adrenergic agonists are believed to induce distinct cellular transduction adaptations.

Acute, short term agonist exposure results in a desensitisation of many adrenergic receptors where phosphorylation of the receptor plays a prominent role in this adaptation. The short term response appears to be largely independent of changes in the steady state levels of G-protein and receptor mRNA (Hausdorff et al., 1990). Chronic long-term agonist exposure, on the other hand, induced adaptive changes in the transduction pathways by altering the steady state level and expression of G-proteins and receptors. The persistent activation of β-adrenergic receptors induced an increase in $G_{\alpha i}1$ mRNA and protein expression, and a reduction in $G_{\alpha s}$ mRNA, although the $G_{\alpha s}$ protein levels were unaltered (Hadcock et al., 1990). The alterations were also achieved by the persistent activation of adenylate cyclase by forskolin (Hadcock et al., 1990).

Thus, the investigations described above have demonstrate that drug exposure can induce alterations in a variety of signalling pathways which, in some instances, involves adaptive changes at the G-protein level.

2. Neurochemical and pathological features associated with the neurodegenerative disorder of Alzheimer's disease.

2.1 Methodological considerations in human brain research.

The investigations of human neurological disorders, in general, adopt one of three approaches: (1) use of animal models in which specific neuronal damage has been induced; (2) use of fresh biopsy material and (3) use of postmortem tissue obtained at autopsy. Each of these strategies has restrictions (reviewed by Dodd et al., 1988). While animal models can allow for the control of genetic, environmental and dietary factors, and are a source of fresh tissue, it is difficult, if not impossible, to accurately induce the complex neuroanatomical and neuropathologic changes associated with disorders such as Alzheimer's disease. Thus, although animal models have generated valuable data concerning adaptive changes as a consequence of disruption of specific neural circuits, extrapolation of these data from animal to human conditions must be interpreted with caution. Fresh biopsy material is not widely available since, in general, neurosurgery is not performed in patients suffering from intractable neurological conditions. Where biopsy material is available the quantity of tissue is usually inadequate for a comprehensive functional neurochemical analysis. Thus, the majority of studies of human brain disorders are performed on postmortem tissue. Postmortem material is not the ideal tissue source for conducting functional biochemical analysis since the interval between death and the freezing of brain tissue, and the agonal status of the patients are but two factors which may influence the quality of the data generated for a particular parameter. However, valuable information has been obtained from studies conducted on human postmortem material particularly where the stability of various receptors and enzymes has been established.

2.1.1 The effect of the interval between death and brain freezing (postmortem interval) and agonal state on various enzymes and receptors.

Several studies have examined the effect of postmortem delay interval on various neurochemical aspects of brain function. Whitehouse et al. (1984) used rat as a model to examine neurotransmitter binding over a range of postmortem delay. The design of their experiment was such that the temperature changes in the rat postmortem closely resembled the cooling curve profile of human brains. In addition to following the cooling curve to $4^{o}C$ (mortuary storage temperature), rat heads were maintained at room temperature ($22^{o}C$) for extended postmortem delay intervals. Under both sets of conditions there was a significant reduction in agonist binding to muscarinic receptors (*N*-methylscolpolamine), although the extent of the decrease was more marked at $22^{o}C$. At $22^{o}C$ a statistically significant reduction (43%) in muscarinic ligand binding, compared to fresh tissue, was observed at a 6 hours postmortem interval; at $4^{o}C$ the ligand binding was stable until a 24 hours postmortem interval where a 23% reduction in binding achieved statistical significance. Kinetic analysis showed that B_{max} was reduced without significant changes in K_D, suggesting a loss of muscarinic ligand binding sites rather than an alteration in receptor affinity. Similarly, the binding of α_2-adrenergic agonist (*p*-aminoclonidine) was significantly reduced at $22^{o}C$ at 48 hours postmortem interval but not significantly reduced in brains cooled to $4^{o}C$. Scatchard analysis revealed a single binding site for *p*-aminoclonidine in fresh tissue , whereas tissue with a 48 hour postmortem delay interval displayed both high and low affinity binding sites. Reduced binding was also observed for dopamine agonist while benzodiazepine binding was increased due to an increase in K_D. None of the agonist binding profiles were effected by freezer storage times.

In a more recent study, Syapin et al. (1987) compared postmortem delay effect on the binding of various agonist and enzymatic activity of two strains of rat. With regards to receptor binding, their results in general agree with the findings reported by Whitehouse et al (1984):

a statistically significant reduction in muscarinic ligand binding (quinuclidinyl benzilate) occurred at 48 hours, a rapid reduction in opioid ligand binding (2-D-alanine-5-D-leucine enkephalin) was evident at 6 hours, while benzodiazepine binding ($GABA_A$ agonist) was unaffected by prolonged postmortem delay intervals. Ligand binding to muscarinic, opioid and benzodiazepine receptors was unaffected by postmortem interval of brains kept at 4^oC. They also observed that at 4^oC and 25^oC the Na^+, K^+-activated ATPase and low affinity Ca^{2+}dependent-ATPase activity were stable, while Mg^{2+} dependent, oubain sensitive-ATPase activity was significantly reduced at 24 and 48 hours but significantly elevated at 72 hours. The postmortem interval effect on enzymatic activities and receptor binding was similar for both rat strains, although an increase in diazepam binding was observed with brains from Sprague-Dawley rats which was not evident in the N/Nih strain. Postmortem interval would appear to have a selective effect on receptor binding which in some cases is influenced by the animal strain. The apparent stability of ATPase activity (with the exception of the Mg^{2+} dependent species) agreed with an earlier study by Nicol et al. (1981). The activities of adenylate cyclase activity and low affinity GTPase is stable at postmortem intervals up to 10 hours, however, a decrease in adenylate cyclase activity occurred in homogenates preparations but not in washed membranes (Nicol et al., 1981).

The selective vulnerability of proteins to postmortem interval may be due to alterations in the optimal membrane lipid environment specific to different membrane associated proteins. It has been shown that both mono and diacylglycerol kinase activities are reduced in brains stored at 4^oC and 23^oC for 24 hours in comparison to fresh tissue (Farooqui & Horroks, 1991). The decay profiles of both enzymes exhibited a sharp fall in activity when stored for up to 8 hours after death, thereafter a gradual decline in activity continued until 24 hours (Farooqui & Horroks, 1991). A further indication that membrane lipid metabolism is altered between death and autopsy is the reported increase in the basal rate of hydrolysis of phophatidylinositol in rat cortex which had been stored for 4 hours after death (Candy et al 1984). The effect of postmortem delay interval on membrane lipid composition, which may

subsequently alter the functional activities of membrane associated proteins, may also be compounded by the influence of the mode of death (agonal status) of the patient.

Agonal state can be divided into two broad groups; where death occurs relatively quickly such as cardiac failure, and prolonged death for example bronchopneumonia where death occurs after periods of prolonged, severe illness. A number of neurochemical differences have been identified between the two categories. Brains from patients who have suffered a protracted death have reduced activities of phosphofructokinase and glutamate decarboxylase, elevated tryptophan and reduced pH (Perry et al., 1982). Perry (In Dodd et al 1988) also observed that in the patients with a long terminal phase, a postmortem delay induced reduction in glutamate decarboxylase is more pronounced than in brains from the rapid death category. Premortem hypoxia has also been shown to influence the stability of selected mRNA (reviewed by Barton et al., 1993) and may therefore be a significant contributing factor the the degradation of selected mRNA species that is frequently encountered in postmortem studies (Ross et al., 1992). Recently it has been shown that the basal binding of the glutamate receptor subtype *N*-methyl-D-aspartate ligand MK-801 is significantly reduced in slow death cases (Piggott et al., 1992). Thus agonal status may influence the outcome of receptor binding studies.

Extensive neurochemical analysis of human brain disorders therefore necessitates the use of human postmortem material. The period between death and autopsy, together with possible differences in the mode of death of the cases examined, will impose restrictions on the type of analysis that can be performed to generate reliable data. These factors can be minimised by closely matching , where possible, the control and diseased groups. While this will not completely eliminate the potential variability encountered by these factors, by acknowledging their contribution the interpretation of data should be more secure.

2.1.2. Effect of aging on various enzymes and receptors.

Most of the current knowledge of Alzheimer's disease originates from histological and biochemical investigations conducted on postmortem material of diseased and age matched control samples. In addition to the potential variations introduced by postmortem and agonal status as described above, the aging process introduces another complicating factor in studies of age-related neurodegenerative disorders. There is evidence of deficits in pre- and postsynaptic neurochemical markers with aging (reviewed by Decker, 1987). Thus the aging effect may, in some instances, prevent accurate neurochemical analysis.

A dysfunction of the cholinergic processes, one of the most consistent neurochemical abnormalities associated with Alzheimer's disease, has stimulated numerous studies of the effect of aging on the status of the cholinergic neurons. Although the nature and severity of aging effect on the cholinergic processes remain in dispute, on balance the more recent studies suggest that these neurons are susceptible to aging induced alterations. Age-related decrements in choline acetlytransferase activity (Arujo et al., 1990), depolarisation induced acetyl choline release (Araujo 1990) and muscarinic receptor binding (Vannucchi & Goldman-Rakic, 1991) has been described. Zawia et al. (1992) recently reported that ibotenate lesion of the nucleus basilis induced reductions in cortical choline acetyltransferase and acetylcholinesterase activity. The magnitude of reduced acetyltransferase and acetylcholinesterase activity was significantly greater in aged compared to young rats. Zawia et al. (1992) suggested that the basal forebrain cholinergic system is more susceptible to neurotoxic damage.

An aging effect has also been described for a variety of biochemical activities in the brain. For example, alterations in aging brains compared to young brains have been reported for:- lipid composition in various regions of aging brains (Soderberg et al., 1990); changes in subcellular distribution of opioid receptors and G-proteins (Bem et al., 1991); brain protein

oxidation (Smith et al., 1991); mitochondrial function (Bowling et al., 1993); and eukaryotic initiation factor 2 activity (Kimball et al., 1992). The diversity of these age related changes may be of particular consequence to the central nervous system since neurons, unlike most other cells types, do not have the capacity to divide. Thus, in order for neurons to survive the aging effect, they must express a complex adaptive response to maintain their general house-keeping biochemical activities in addition to their highly specialised roles.

2.2 Neuropathological features of Alzheimer's disease.

Alzheimer's disease has, historically, been subdivided into two, categories; early onset which affects patients less than 50-65 years of age, and late onset which develops from approximately 65 years. Additionally there are other dementias affecting the population such as, vascular dementia and multi-infarct dementia. Confirmation of the clinical diagnosis of Alzheimer's type dementia is achieved only on neuropathological examination by the presence of the characteristic senile plaque, cerebrovascular amyloidosis and neurofibrillary tangles which were first described by Alzheimer in 1907 (reviewed by Henderson & Finch, 1989). Over the last decade there has been significant advancement in our knowledge of the neuroanatomical distribution and molecular composition of these lesions.

2.2.1 Amyloidogenesis in Alzheimer's disease.

The classical senile plaque is composed of a proteinous core of β amyloid surrounded by dystrophic neurites and glia cells . The term amyloid is used to describe tissue protein precipitates which have a predominant β sheet secondary structure that confers a high affinity for the histochemical dye congo red, although a variety of histological staining techniques are now employed to examine the density and morphological appearances of plaques (Khachaturian, 1985). In Alzheimer's disease, plaques are present in high concentrations in various neuroanatomical locations but occur predominantly in the cortical

and hippocampal regions (Braak & Braak, 1991).). Amyloid plaques are not, however, restricted to Alzheimer's diseased brains; they are present in normal aged brains although at a significantly lower density than diseased brains, and in aged Downs syndrome (trisomy 21) subjects (Masters et al., 1985). Plaques, visualised histologically, are roughly spherical in appearance and can vary in diameter from a few μm to 200μm. Modified histological staining methods, which employ more sensitive silver staining and immunological techniques (Ikeda et al., 1988), have identified an amorphous or diffuse, noncongophilic plaque (Yamaguchi et al., 1989). It has been suggested by some authors that the diffuse plaque are the earliest lesion of Alzheimer's disease and that they mature into the senile or neuritic plaque (Yamaguchi et al., 1989). Others believe, however, that the diffuse plaque is a harmless accompaniment of the aging process (Masliah et al., 1990a).

Significant progress has been made in unravelling the biochemical and molecular nature of the major amyloid protein associated with plaques which should aid attempts to understand the process of amyloid deposition in the brain and explain the neurological significance of this event. The major protein component of the amyloid fibril is a 4.2 KDal protein, now referred to as βA4 protein. It was first isolated from brain by Glenner & Wong, (1984). The βA4 protein was originally reported as being derived from an integral membrane amyloid precursor protein (APP) containing 695 amino acids (Kang et al., 1987). The APP protein consists of a large amino-terminal extracellular domain, followed by a membrane spanning region containing the βA4 fragment, and a short carboxy-terminal intracellular domain. Three other isoforms of APP have since been identified (APP 593, APP 751, APP 750) which contain an additional exon encoding a sequence homologous to the N-terminal Kunitz protease inhibitor domain (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988).

The precursor protein can undergo several post translational modifications such as N-and O-glycosylation (Weidemann et al., 1989), phosphorylation (Gandy et al., 1988) and proteolysis (Weidemann et al., 1989; Buxbaum et al., 1990). The physiological role of APP, and the

detailed mechanisms involved in the processing of APP are at present extremely active areas of Alzheimer's disease research. A variety of proposals have been put forward to account for a role of the APP molecule in neurodegeneration. Bauxbaum et al. (1990) suggested that abnormal phosphorylation of APP is central to its abnormal processing leading to βA4 deposition. Koo et al. (1990) demonstrated that APP undergoes fast axonal transport. APP protein fragments have also been reported to potentiate the neurotoxicity by nerve growth factors to cultures (Yanker et al., 1990), and increase the sensitivity of neurons to glutamate toxicity (Mattson et al., 1992). Taken together, the majority of evidence would now indicate that abnormal processing of APP, leading to the deposition of βA4 is deleterious to neurons.

Downs syndrome patients, in which there is trisomy of chromosome 21, develop pathological features which are similar to Alzheimer's disease . Thus it was proposed that abnormal expression of a gene on this chromosome is central to the pathology. This hypothesis was fuelled by the discovery that the APP gene was located on chromosome 21 (Goldgaber et al., 1987; Tanzi et al., 1987) and by the identification of a mutation on chromosome 21 which was close to the APP gene (St George Hyslop et al., 1987). However, the mutation identified on chromosome 21, and the APP gene were found not to be co-inherited (St George Hyslop et al., 1990) which eliminated the possibility that Alzheimer's disease is genetically homogeneous. Recent studies of two separate families with kindred of early onset Alzheimer's disease demonstrated mutations of the APP gene (Goate et al., 1991; Chartier-Harlin et al., 1991). Similar substitution mutations produce abnormal βA4 accumulation in transfected cells (Citron et al., 1992) which supports a genetic basis for amyloidogenesis in familial Alzheimer's disease. Tanzi & Hyman, (1991) also speculated that APP mutations could alter the stability of the mRNA and thus influence the normal translational control of the APP gene. Additional genetic loci have recently been implicated in Alzheimer's disease. A second FAD locus has recently been mapped to chromosome 14 (Schellenberg et al., 1992) while the gene for apolipoprotein E on chromosome 19 is suggested to be involved in late-onset Alzheimer's disease (Poirier et al., 1993).

2.2.2 Neurofibrillary tangles in Alzheimer's disease.

The term neurofibrillary tangles (NFT) originated from the microscopic appearance of a non-membrane mass of fibres detected histologically in the cell bodies of neurons. On electron microscopy, they consist largely of abnormal filaments with 10nm wide constrictions every 80nm that are twisted into paired helical structures referred to as paired helical filaments (PHF) (Wischik et al., 1988a). Straight filaments ~15nm in diameter and cytoskeletal filaments of normal appearance may also be associated with NFT. Although the NFT lesion is present at a higher density in Alzheimers disease, similar structures are also visualised in normal aging, Downs syndrome, lateral sclerosis of Guam and pugalistic dementia. Terry et al., (1987) also observed that in a substantial minority of Alzheimer's diseased brains (30%), tangles were absent in the hippocampus and neocortex. However, in samples where tangles are present, the severity of the dementia had a tendency to be greater than those patients lacking tangle formations (Terry et al., 1987).

The biochemical composition of NFT has proved difficult to determine due to the highly insoluble nature of the PHF. Most of the protocols for the isolation of PHF require strong protein denaturents such as SDS and urea and separation of the insoluble fraction by centrifugation (reviewed by Selkoe, 1989). Alternative approaches have subjected preparations to mild proteolytic digestion (Wischik et al., 1988). These techniques have invariably led to contamination of the PHF fractions and modification of the isolated PHF and therefore the true identity of the PHF and other proteins associated with NFT has been complex.

To date two proteins, tau and ubiquitin, are the only definite components of PHF. Six isoforms have been identified that are thought to arise by alternate splicing of a single pre-mRNA (Neve et al., 1986; Kosik et al., 1989). In Alzheimer's disease, tau is abnormally phosphorylated (Bancher et al., 1989) which may effect its immunoreactivity and solubility

(Hanger et al., 1991; Goedert et al., 1992). The abnormal phosphorylation of tau has been proposed to arise from altered kinase activity in Alzheimer's diseased brains (reviewed by Saitoh et al., 1991). A strong positive correlation between the level of tau mRNA and APP mRNA in normal aged brains has recently been reported (Oyama et al., 1992). It will be interesting to see whether such a relationship exists in Alzheimer's diseased brains and also if these parameters correlate with kinase levels. The significance of the abnormal phosphorylation of tau in Alzheimer's disease is still unclear; however, Brion (1990), has suggested that it could alter the normal polymerisation of tubulin and consequently increase the amount of tau not associated with microtubules. This in turn could precipitate PHF formation.

Ubiquitin is a small protein (8.5 KDal), highly conserved and abundant in all tissue. The association of ubiquitin with NFT has been based largely on immunological evidence. The significance of the presence of ubiquitin in NFT is unknown, although brain ubiquitin levels are elevated in Alzheimer's disease and correlate with the degree of neurofibrillary changes (Wang G.P. et al., 1991). Recently, Bancher et al. (1991) have shown that abnormal phosphorylation of tau precedes its incorporation into PHF and that ubiquitin association occurs secondary to this event. Based on these findings Bancher et al. (1991) suggested the presence of ubiquitin could be an attempt by cells to inhibit protein precipitation. The amyloid βA4 protein is also a minor component of NFT (Yamaguchi et al.,1990b).

Considerable attention has also been devoted to the putative role of aluminium in Alzheimer's pathology. This has been based largely on the identification of aluminium silicates at the core of neuritic plaques (Perl and Brody, 1980; Candy et al., 1986 but see Landsberg et al., 1992) which has led to the postulate that exogeonous aluminium intake potentiates the development of senile plaques. Additionally, microprobe studies have demonstrated elevated alumunium levels associated with NFT (Perl and Brody, 1980; see Good et al., 1993) although other studies failed to detect elevated levels of aluminium in

Alzheimer's diseased brains (Chafti, 1991). Recently, however, Mattson et al. (1993) concluded that in cultured neurons, aluminium cannot induce the characteristic features associated with the NFT of Alzheimer's disease. Thus, the putative relationship between aluminium and the development of the characteristic Alzheimer's disease pathology remains speculative.

2.2.3 Neuronal loss and reactive gliosis in Alzheimers disease.

Neuronal loss in selective brain regions is considered part of the normal aging process. The extent to which this phenomena occurs in Alzheimer's disease is, however, debatable. Vogels et al. (1990) performed a careful and thorough investigation of the neuron content in the Nucleus basilis of Meynert complex (NBMC). Vogels et al. (1990) considered some of the factors such as selective neuron loss and shrinkage which may have contributed to the discrepancies in the literature regarding the extent of neuron loss in Alzheimer's disease. They examined specific anatomical regions of the NBMC in serial section to create a 3-dimensional picture of neuron density in these areas. Both large and small neurons were counted. An overall neuronal decrease of 15.5% was found, although the density of small neurons were increased while the density of large neurons (probably cholinergic) were found to be decreased. Vogels et al. (1990) suggested that the inverse alterations in the density of small and large neurons in Alzheimer's NBMC was a result of neuronal shrinkage. Similar findings were reported by Iraizoz et al, (1990) who conducted a total cell count of the NBMC, although a higher neuronal loss compared to Vogel et al, (1990) was observed. In addition, Iraizoz et al, (1990) observed an increased nuclear area of remaining neurons suggesting that regenerative and degenerative responses co-exist in the NBMC in Alzheimer's disease.

Neuronal loss in the frontal and temporal cortex of Alzheimer's disease has also been confirmed (Terry et al., 1981; Hubbard & Anderson, 1985), and in layers II and III of the entorhinal cortex (Hyman et al., 1987). These investigations generally agree that in

Alzheimer's disease the larger pyramidal neurons are more markedly reduced than the smaller intrinsic neurons. The synaptic density of the cortical regions are also reduced (Davies et al., 1987). A minimal reduction of 25% to 35% in the synaptic content of the temporal and frontal cortices was noted (Davies et al., 1987), although the degree of synaptic loss was less marked when correcting synaptic content for cortical atrophy (temporal cortex 30%; frontal cortex 14%). The marked reduction in the cortical synaptic content particularly in layer III of the frontal cortex probably reflects the loss of cholinergic afferents from the basal forebrain in addition to intrinsic connections (Scheff et al., 1990). Scheff et al. (1990) also observed a negative correlation between synaptic density and synaptic size which may suggest a compensatory response. More recently, Masliah et al. (1990b & 1991a) employed synaptophysin antibodies to demonstrate a loss of 45% of presynaptic terminals in the frontal cortex by both immunohistochemical and Western blotting analysis. The synaptophysin antibody may prove a useful marker for synaptic content both *in situ* and *in vitro*.

The process of reactive gliosis which results in an increased astrocyte content of grey matter, occurs in response to brain trauma (Mathewson & Berry, 1985), and also as a result of aging (Beach et al., 1989). Marked gliosis in Alzheimer's disease has been noted by several authors (Schechter et al., 1981; Beach et al., 1989). The analysis of astrocyte density is usually performed by immunocytochemistry, employing antibodies against glial acidic fibrillary protein (GFAP). While some authors have suggested that in the visual cortex there is a relationship between the level of gliosis and the severity of the pathological features of Alzheimer's disease (neuron and synaptic loss, plaque and tangle accumulation) (Beach et al., 1989), others found no correlation between gliosis and the severity of pathology observed in the hippocampus (Vijayan et al., 1991).

Although gliosis is a well documented reaction which is observed in a wide range of conditions affecting the brain, the significance of this phenomenon is not yet fully understood.

There is evidence that the astrocytes and glia glutamate uptake mechanism can restrict the extracellular glutamate concentration to a sub-toxic concentration (Bouvier et al., 1992; Sugiyama et al., 1989; and see Hertz, 1989), while the ability of astrocytes and glia to produce growth factors may imply a trophic effect (Araujo & Cotman, 1992).

2.3. Neurochemical features of Alzheimer's disease.

For the last few decades neurochemical investigations of Alzheimer's disease have identified a variety of abnormalities associated with the disease (reviewed by Francis et al., 1992; Dewar & McCulloch, 1993). Many research groups have pursued a neurochemical line of investigation in the hope of identifying a primary neurotransmitter lesion similar to that of the dopamergic deficit in Parkinson's disease, and thus to enable the development of appropriate therapeutic strategies. While the literature describes modulations in the level of several neurotransmitters, receptors and the enzymes involved in the regulation of selected neurotransmitters, it is only relatively recently that attention has focussed on the functional aspects of neuromodulatory events involving signal transduction. As yet no single neuronal lesion has been identified unequivocally as the major factor in Alzheimer's disease, although there is strong evidence to implicate the cholinergic and glutamatergic systems in this condition (Palmer & Gershon, 1990).

2.3.1 Cholinergic neurotransmitters and receptors in Alzheimer's disease.

One of the earliest and most consistently reported neurochemical deficiencies in Alzheimer's disease is that of significant reductions in various cortical and subcortical regions in the activity of the acetylcholine synthesising enzyme, choline acetyltransferase (ChAT) (reviewed by Perry, 1986). The severest alterations are reported to occur in the neocortex and hippocampus (Perry, 1986). Losses in other cholinergic markers such as acetyl choline levels and acetylcholinesterase (AChE) activity have also been found in Alzheimer's disease

(Perry, 1986). The ChAT deficit reported by Perry (1986) was also found to exhibit a negative correlation with cognitive decline suggesting a causal relationship between the neurochemical abnormality and the severity of dementia.

More recent investigations have examined the regulatory events associated with acetyl choline turnover. Slotkin et al, (1990) examined the ChAT activity and synaptosomal choline uptake in rapid autopsy material and found the ratio of choline uptake to ChAT activity to be significantly elevated in the cortex of Alzheimer's disease. They interpreted the cortical changes as an upregulatory, compensatory response. Interestingly, the hippocampus displayed the reverse with a significant reduction in the ratio. Pascaul et al. (1991), on the other hand, demonstrated a significant reduction in the binding sites of a high affinity choline uptake marker ([3H] hemocholinium-3) in the frontal cortex and hippocampus of Alzheimer's disease postmortem samples. Pascual et al. (1991) did observe, however, that a significant proportion of the samples displayed normal values and that the overall reductions were not as marked as the ChAT deficit. Pascaul et al (1991) suggested that this observation may indicate a compensatory response by the remaining cholinergic neurons.

Numerous studies have examined the status of the cholinergic receptor populations in Alzheimer's disease, to establish whether or not regulation of receptors occurs in response to cholinergic afferent denervation. The findings of these investigations, however, have been contradictory. The muscarinic subclass of receptors, M1 have been shown to be preserved (Kellar et al., 1987; Rinne et al., 1989), while some investigators found a moderate decrease (Mash et al., 1985; Shimohama et al., 1986) and modest increases have also been observed (Danielsson et al., 1988; Probst et al., 1988). Additionally, the level of m1 mRNA was shown to be increased in Alzheimer's disease (Harrison et al., 1991). While this finding does not necessarily imply that the protein level is concomitantly increased, it does support the notion of an upregulation in the muscarinic receptors translation efficiency. However in conflict with the findings of Harrison et al. (1991), Wang et al. 1992 found a reduction in the m1 muscarinic

recptor message in Alzheimer's disease cortex. The different results between the two groups may be due to the particular method employed to isolate the mRNA.

In Alzheimer's disease, the M2 subclass was reported to be decreased in the frontal cortex (Araujo et al., 1988; Mash et al., 1985), decreased in the hippocampus (Rinne et al., 1989), preserved in the cortex (Kellar et al.,1987; Rinne et al., 1989) and increased in the cortex (Nordberg et al., 1992). In Nordberg (1992), found that the M2 receptors were preserved in multi infarct dementia. They suggested that misclassification of the patients may be a factor which contributes to the conflicting data regarding muscarinic receptors in Alzheimer's disease. Other factors such as ligand selectivity, lack of anatomical resolution, and the affinity status of receptors could also account for the discrepancies regarding muscarinic receptors in Alzheimer's disease.

The literature on the nicotinic receptors in Alzheimer's disease tends to be more consistent with most investigations reporting a decrease in receptor density (Kellar et al., 1987; Nordberg et al., 1992). Like most of the major classes of receptors, the nicotinic receptors are now known to be a family of proteins for which multiple genes encoding different subtypes have been isolated. It remains to be established if the subtypes are differentially altered as a consequence of Alzheimer's disease.

2.3.2 Glutamatergic neurotransmitters and receptors in Alzheimer's disease.

The excitatory amino acids (EAA) glutamate and aspartate have received considerable attention in studies of neurodegeneration and have been implicated in the pathogenesis of cerebral ischemia (reviewed by Meldrum & Garthwaite, 1991). They are also referred to as excitotoxins due to their excitatory transmitter action and their toxic effect on cells exposed to high concentrations for prolonged periods. There is evidence that glutamate is a major neurotransmitter of pyramidal cells of the cortex and hippocampus (Palmer et al., 1989).

Given that there is a significant loss of pyramidal cells in the cortex of Alzheimer's disease (Terry et al., 1981; Hubbard & Anderson, 1985; Davies et al., 1987), it is postulated that glutamatergic dysfunction is involved. Further studies have examined the glutamatergic binding sites in an attempt to substantiate this hypothesis (for reviews see Palmer & Gershon, 1990; Francis et al., 1993; Dewar & McCulloch 1993).

In Alzheimer's disease brain, the concentration of glutamate and aspartate has been difficult to assess. Glutamate is metabolised in neurons and glia as part of intermediary metabolism, which has led to difficulties in segregating the neurotransmitter and metabolic glutamate pools. Nonetheless, Hyman et al, (1987) examined the perforant pathway of the hippocampus (which is rich in glutamatergic terminals) and found a marked reduction in L-glutamate content. Evidence that neurotransmitter metabolism is altered in Alzheimer's disease was reported by Procter et al. (1988), who observed a reduction in ^{3}H aspartate uptake in rapid autopsy material. Reductions in the content of neurons which contain the glutamate synthesising enzyme, glutaminase, has been described in Alzheimer's disease (Kowell and Beal, 1991), yet the enzyme activity is reported unchanged (Procter et al., 1988). The ligand ^{3}H-aspartate was considered a marker for glutamatergic terminals and deficits in binding in cortical and hippocampal regions has been demonstrated (Chalmers et al.,1990). However, recent investigations has questioned the validity of ^{3}H-aspartate binding to assess glutamatergic binding sites (Browne et al., 1991; Greenamyre et al., 1990).

The glutamate receptors have been classified into two groups. The ionotropic receptors are ligand gated integral ion channels. Subclasses of the ionotropic class include N-methyl-D-aspartate (NMDA); a-amino-3-hydroxy-5-methyllisoxazole-4-propionate (AMPA); L-2-amino-4-phosphobutyrate (AP4); and Kainate (Sommer & Seeburg, 1992). The metabotropic glutamate receptors are coupled to G-proteins and modulate a variety of transduction pathways (Itano et al., 1991). Several subtypes have now been cloned (Schoepp & Conn, 1993).

The subclasses of the ionotropic receptors have been examined in various regions of Alzheimer's disease brain. The NMDA subtype exists as a receptor-channel complex and has an interesting characteristic in that its activity is allosterically modulated by glycine, zinc and polyamines. A derivative of phencyclidine, N-[1-(2-thienyl) cyclohexyl]-3-,4-piperidine (TCP) binds to the high affinity NMDA sites. The TCP binding sites were found to be significantly reduced in the frontal cortex (Ninomiya et al., 1990). However, the functional capacity of the remaining complexes has been shown to be preserved in Alzheimer's disease (Ninomiya et al 1990). In contrast, Procter et al, (1989) reported that the noncompetitive NMDA antagonist MK-801 was reduced in the frontal cortex, but only in the presence of glycine. Chalmers et al, (1990), on the other hand, found a small reduction in NMDA sensitive ^{3}H glutamate binding in the frontal cortex which bordered on statistical significance; AMPA binding sites were preserved whereas kainate binding was increased, a finding which may indicate a compensatory response in selective glutamate binding sites. Cowburn et al., (1989) reported that the kainate receptor was preserved in Alzheimer's disease cortex and hippocampus while quisqualate sensitive glutamate binding (Greenamyre et al., 1985), AMPA and glutamate metabotropic sites (Dewar et al., 1991) were shown to be decreased in the hippocampus. The discrepant findings of investigations of the iontropic subtypes may be the result of different ligands and binding conditions. Thus, although there exists some confusion regarding the individual glutamatergic receptor subtypes, the evidence, in general, suggests that the glutamatergic excitatory amino acid neurons and their receptors are compromised in Alzheimer's disease.

2.3.3 Monoaminergic neurotransmitters and receptors in Alzheimer's disease.

Investigations of the momaminergic system in Alzheimer's disease have identified alterations in a number of neurochemical systems. Reductions in noradrenaline (Palmer et al., 1987), dopamine β hydroxylase (Cross et al., 1981) (which synthesises noradrenaline) and the

noradrenaline metabolite 3-methoxy-4-hydroxyphenolglycol (Crow et al., 1984) have been observed in Alzheimer's disease. Investigations of adrenergic receptors have reported the α2 types to be preserved in the cortex (Cross et al., 1984; Shimohama et al., 1986); decreased in frontal cortex (Kalaria and Andorn., 1991; Meana et al., 1992) and preserved in the hippocampus (Meana et al., 1992). A recent autoradiographic study however, described reductions of approximately 50% in both frontal cortex and hippocampus (Pascaul et al., 1992). Data regarding the α1 receptors in Alzheimer's disease are more limited: Shimohama et al. (1986) found a decrease in the receptor density and a change in K_D in the hippocampus and cerebellum with no change in the frontal cortex. Cross et al. (1984), on the other hand, found no change in the hippocampus, while Kalaria, (1989) described a small, but significant, reduction (25%) in the prefrontal cortex. The discrepancies may be partly explained by group size, the different ligands and binding conditions employed, which may be influenced by the integrity of the receptor/G-protein coupling

The total number of β adrenoceptors in Alzheimer's disease were reported to be unchanged in the cortex and hippocampus (Cross et al.,1984). Analysis of the subtypes, however, have found a differential alteration. For instance, Shimohama et al. (1987) found a reduction in β1 in the hippocampus and an increase in the NBMC and cerebellum. Conversely, the β2 receptors were reduced in the NBMC, thalamus and cerebellum but increased in the hippocampus (Shimohama et al., 1987). Neither β1 or β2 were altered in the frontal cortex (Shimohama et al., 1987). Kalaria et al. (1989) also found that in the frontal cortex, the total β adrenoceptors were preserved, however the β1 subtype was decreased while the β2 type was increased. Kalaria et al. (1989) also reported that in the hippocampus the total β1 and β2 types were increased. Interestingly, a more recent report examined the laminar binding of a variety of ligands to a variety of receptors in the cingulate cortex of Alzheimer's disease samples (Vogt et al., 1991) . In this study the Alzheimer's disease samples were sub-divided on the basis of the severity of neuronal loss. Vogt et al. (1991) reported that β agonist binding was elevated only in groups with a marked loss in neurons. Thus, the elevated binding of β

agonists in Alzheimer's samples may, as suggested by Kalaria et al. (1989) and Vogt et al. (1991), indicate a compensatory change or alternatively reflect gliosis.

Alterations in the serotonergic systems, at various neurochemical levels, has been described in Alzheimer's disease. Serotonin (5-hydroxytryptamine) and its monoamine oxidase metabolite 5-hydroxyindoleacetic acid (5-HIAA) have been found to be decreased in the axon terminals in Alzheimer's disease (Gottfries , 1990). Various studies have found alterations in the serotonergic binding sites in Alzheimer's disease. Significant reduction of 5HT binding have been reported for the frontal and temporal cortex (Cross et al., 1984a; Sparks, 1989). Autoradiographic analysis identified specific laminar reductions in the 5HT binding sites (Cross et al., 1988). On the other hand Dewar et al, (1990) failed to observe a significant change in 5HT sites as assessed by autoradiography.

The dopaminergic system is possibly the least extensively studied monoaminergic system in Alzheimer's disease research. A reduction in the level of dopamine and its metabolite homovanillic acid (HVA) in subcortical regions, the amygdala, striatum but not in cortical regions, suggests that the nigrostriatal rather than the mesocortical pathway is affected (Cortes et al., 1988). In the striatum of Alzheimer's disease, the D2 s recepor subtypes were reported to be reduced, wheras the D1 receptor type was found to be preserved (Cross et al., 1984b).

2.3.4. Inhibitory neurotransmitters and neuropeptides in Alzhemer's disease.

The short intrinsic neurons of the cerebral cortex contain the inhibitory transmitter γ-aminobutyric acid (GABA) which often co-exists with neuropeptides such as somatostatin (SRIF) and neuropeptide Y (NPY) (Beal et al., 1986; Chan-Paly et al 1987; Davies et al., 1990). The neuronal content of both SRIF and NPY has been examined in Alzheimer's disease. While some studies report a decrease in SRIF in cortical regions (Davies et al 1990), others

have found no change (Whitford et al., 1988). Reduction in NPY in the cortex of Alzheimer's disease has beem reported (Beal et al., 1986; Davies et al., 1990). Davies et al. (1990) concluded, that by allowing for neuronal atrophy, there was a significant reduction of both SRIF and NPY containing neurons in addition to a reduced fibre length. These findings are supportive of previous reports that SRIF and NPY neurons are depleted in cortical areas in Alzheimer's disease (Kowell & Beal, 1988; Chan-Palay, 1987). Decrements in NPY and SRIF binding in the hippocampus and temporal cortex cortex has been observed (Martel et al., 1990; Beal et al., 1986). Recently, the high affinity SRIF binding site (SS1) and the low affinity site was examined although indirectly. The SS1 receptors were reduced in the frontal and temporal cortex whereas the non-SS1 (presumably the low affinity sites), were preserved in the frontal cortex but reduced in the temporal cortex (Krantic et al., 1992). Reductions in the binding sites of $GABA_B$ has also been described for Alzheimer's disease (Chu et al., 1987)

Sustance P immunoreactive neurons have been reported to be depleted in the cerebral cortex of Alzheimer's disease (Quigley & Kowall, 1991). Morphological changes, similar to those described for SRIF neurons, (distorted fibres and reduced perikarya) were also observed (Quigley & Kowall, 1991). An earlier report found small, non-significant reductions in Substance P levels in Alzheimer's disease samples (Sakurada et al., 1990), although the analysis was performed on tissue homogenates which may have masked localised alterations. A recent report of a protective action of substance P to β-amyloid induced toxicity of primary neuron cultures (Yanker et al., 1990) has raised the interesting possibility that substance P may play an active role in arresting the neurodegenerative processes associated with the pathological development in Alzheimer's disease and may be a potential therapeutic agent.

Cholecystokinin (CCK), vasoactive intestinal polypeptide (VIP) and corticotrophin-releasing hormone (CRH) are additional neuropeptides which have been examined in Alzheimer's disease. Perry et al. (1981) observed a marked loss in CCK immunoreactivity in

the enterhinal cortex, although modest reductions were reported for the frontal and temporal cortex (Mazurek & Beal, 1991) while unchanged CCK levels in the hippocampus and primary sensory cortices have also been reported (Mazurek and Beal, 1991). CRF is reported to be reduced in the cerebral cortex (Leake et al 1991) and VIP levels were reported to be unaltered in Alzheimer's disease (Ferrier et al., 1983).

Thus, categoric conclusions regarding the integrity of inhibitory and excitatory neurotransmitter systems, can only be achieved with the thorough assessment of their regulatory biochemical mechanisms together with the pharmacological status of their receptors.

2.3.5. Transmembrane signalling in Alzheimer's disease.

At the outset of this investigation, limited information was available on the integrity of signal transduction mechanisms in Alzheimer's disease, a factor which prompted this study. Probably the first study that addressed the effect of Alzheimer's disease on G-proteins, although indirectly, was conducted by Smith et al. (1987). They examined the effect of GTP analogues and Mg^{2+} on the binding of muscarinic ligands to membranes prepared from the parietal cortex of Alzheimer's disease and age matched control samples. They found a decrease in the ability of Gpp(NH)p to induce the formation of the low affinity state of the M1 type muscarinic receptor, as determined by carbachol inhibition of parenzipine binding. In the study by Smith et al. (1987) however, ligand binding and displacement was performed at one concentration of the relevant compound. A postmortem effect was also examined and while there were no significant changes in the binding characteristics of rat cortex left for 24 hours postmortem. Smith et al. (1987) did observe that the ability of Mg^{2+} to enhance carbachol binding was attenuated at 24 hours postmortem compared to fresh tissue. Nonetheless, since the postmortem periods were comparable for control and Alzheimer's disease groups, the data suggests that the coupling of M1 type receptors to G-proteins is

altered in Alzheimer's disease. Indeed, Smith et al. (1989) supported their findings by demonstrating a significant reduction in the pool of oxotremorine binding sites that can adopt the high affinity configuration in the presence of Gpp(NH)p.

Danielsson et al. (1988) examined the activity of adenylate cyclase, its responsiveness to various stimulants and the activity of guanylate cyclase activity in various regions of Alzheimer's disease samples. They reported a preservation of muscarinic binding sites and guanylate cyclase activity in the caudate, putamen, thalamus, hippocampus and temporal cortex. The basal adenylate cyclase was seen to be significantly elevated only in the hippocampus, while vasoactive intestinal polypepide (VIP) and forskolin stimulation was comparable for control and Alzheimer's disease samples. This study demonstrated that one of the central effector molecules of G-proteins, adenylate cyclase, can be assayed in postmortem tissue, and the enzyme is still responsive to receptor dependent (VIP) and receptor independent (forskolin) stimulation.

At the initiation of this project, the inositol phospholipid signaling system was also beginning to emerge as a neurochemical abnormality associated with Alzheimer's disease. Stokes & Hawthorne (1987), measured the content of free *myo*-inositol and and its products phosphoinositol (PI), phosphoinositol mono and di phosphate (PIP and PIP_2 respectively) in the temporal cortex and found a significant reduction in PIP levels in Alzheimer's disease. Non-significant reductions in the other lipids were also recorded. Stokes and Hawthorne (1987) also observed that data variation was considerable and these authors attributed this to the large postmortem delay. An alteration in this signalling system was supported by Young et al. (1988), who reported a reduction in [^{3}H]inositol binding in Alzheimer's disease cortex.

These studies, therefore, indicated that transmembrane signalling pathways are disturbed in Alzheimer's disease and that investigations of relevant parameters were possible. However,

large variations in the data can be expected. Significant progress has since been made regarding many different aspects of both the adenylate cyclase and the phosphoinositol pathways. These findings shall be examined in more detail in the discussion section of this thesis.

2.3.6 Drug treatment in Alzheimer's disease.

In contrast to Parkinsons disease, where dopamine deficiency is the primary cause of this neurological disorder, Alzheimer's disease appears to be characterised by a multiplicity of neurochemical and pathological abnormalities affecting many brain regions. While the development of therapeutic agents is a daunting task, (given the complex nature of the disease, and difficulties associated with the delivery of localised doses of agents which may poorly transverse the blood brain barrier), pharmacological intervention based on several strategies has been pursued (reviewed by Gottfries, 1992; Francis et al., 1992).

The cholinergic system has been the main target for therapeutic intervention. Replacement therapy to overcome the deficit has targeted different points in the regulatory mechanism for acetyl choline production: at the presynaptic level by supplying acetyl choline precursors; at the synaptic level by inhibiting the degradation of acetyl choline; and postsynapticaly by supplying analogues that can interact with the muscarinic (M1) and nicotinic receptors. Various compounds which meet the above criteria have been examined in clinical trials: for example, phosphatidylcholine; physostigmine and tetrahydroaminoacridine (Tacrine); arecoline and nicotine which function as acetylcholine precursors, inhibitors of acetylcholine degredation, and acetylcholine receptor agonists respectively. Combination drug treatment using acetylcholine precursors and inhibitors of acetylcholine degradation are reported to enhanced cognitive function of Alzheimer's disease patients (Eagger et al., 1991). There are, however, no reports of marked clinical improvements of Alzheimer's disease patients administered with cholinergic agents. Additionally, cholinergic agents can inflict severe

side effects (Adem, 1992).

The drug trials reported to date have failed to demonstrate major relief of the cognitive and non-cognitive symptoms associated with Alzheimer's disease. Perhaps new strategies are required given that aspects of signal transduction are also altered in the disease and that βA4 deposition is now thought to be a central, possibly instigative event in the neurodegenerative process. Indeed, a recent report has suggested that anti-inflammatory compounds can slow the onset of Alzheimer's disease (see Schnabel, 1993), although a detailed report has yet to be published.

2.4. Models of Alzheimer's disease.

One of the major limitations in Alzheimer's disease research is the necessity to use postmortem tissue which, in effect, analyses the end point of the disease. While this approach has established certain characteristic neurochemical and pathological abnormalities, this strategy gives little indication of the sequence of events that lead to such changes. It is necessary, therefore, to develop suitable models which allow controlled investigation of various parameters known to be altered in the disease to identify the causative agents and explain the temporal progression of this condition. Additionally, such models would be extremely valuable in assessing the effects of therapeutic intervention. Although there has recently been significant progress in the development of models representing the pathological changes associated with senile plaque and neurofibrillary tangle formation, this research is still in its infancy. The majority of studies have therefore concentrated on the neurochemical anomalies associated with Alzheimer's disease.

The two cholinergic pathway, originating from the Nucleus basilis of Meynert complex (NBMC) and the medial septal nucleus to innervate, primarily, the neocortex and hippocampus respectfully, have been the main focus of intervention to induce deficits

characteristic of Alzheimer's disease (reviewed by Smith, 1988; Francis et al., 1992). In rodents and primates, several different lesion techniques have been employed: electrolytic lesioning; neurotoxic lesioning; and surgical transsection of neuronal circuits. Although these techniques are not entirely selective for cholinergic neurons, there have been significant improvements in the selectivity and reproducibility of lesions induced by neurotoxins. The most commonly employed agents are the excitotoxins: N-methyl-D aspartate (NMDA), kainate, ibotenate quisqualate and quinolinic acid; ethylcholine mustard aziridinium (AF64A), a toxic analogue of choline which disrupts the high affinity choline uptake mechanism (Tamer et al., 1992); and colchicine, which disrupts cytoskeletal elements has been shown to induce cholinergic denervation (Ginn & Peterson, 1992). Excitotoxins produce relatively selective lesions that destroy neuronal dendrites and cell bodies but spare axons (reviewed by Meldrum & Garthwaite, 1991). The magnitude of the lesion induced cholinergic deficit, as assessed by changes in choline acetyltransferase (ChAT) and acetylcholineesterase (AChE) activity and loss of immunopositive neurons, can vary. Factors which influence the degree of cholinergic denervation include the choice of neurotoxin and concentration employed (Rugg et al., 1992; Beal et al., 1991), the post-treatment survival period (Tamer et al., 1992), and lesion placement (Wenk et al., 1984) Similarly, markedly different degrees of cholinergic denervation are achieved by partial compared to full transsection of the fimbria-fornix connections (Lapchak et al., 1991b).

Investigations, conducted on animals with cholinergic brain lesions have produced evidence to support a disruption in the abilities of animals to learn and recall certain tasks (Gray et al., 1990). Common techniques include the radial arm maze, and hole boards as well as place navigation in the water maze where rats learn the location of submerged platforms. Although similar impairment of cognitive function following excitotoxic lesioning of the medial septal area and the NBMC are reported (Arendt et al., 1989), different responses to the two lesion models have also been observed (Gray et al., 1990).

Although similar impairment of cognitive function following excitotoxic lesioning of the medial septal area and the NBMC are reported (Arendt et al., 1989), different responses to the two lesion models have also been observed (Gray et al., 1990).

Behavioural and neurochemical investigations of animals with induced cholinergic deficiency, has proved to be a useful system for examining the capacity of neuronal populations to compensate for selective denervation, and to examine the effects of various treatments on the recovery process. Lapchak et al, (1991b) demonstrated, for example, a compensatory increase in hippocampal acetylcholine synthesis 3 weeks after partial fimbrial transsection; and Tamer et al, (1992) also conclude that the cholinergic disruption of the hippocampus by AF64A is reversible.

Investigations of the integrity of receptor coupling to signal transduction systems, have been conducted, albeit to a limited extent. Two studies have shown that in rats lesioned at the NBMC, the stimulation of of phosphoinositol degredation by carbachol is comparable with control animals (Scarth et al., 1989; Raulli et al., 1989). Scarth et al, (1989) also demonstrated that neurotoxic lesion of the cortex caused a marked reduction on carbachol induced inositol-1-phosphate accumulation. Decreased $G_{\alpha o}$ mRNA in NBMC lesioned animals has been reported: the reduction was observed at 3 days post-lesion and returned to normal at 28 days (Wood & de Belleroche, 1990). Enhanced carbachol stimulation, of phosphoinositide hydrolysis following electrolytic lesion of the septum has been described (Connor & Harrell, 1989). A similar finding was reported following fimbrial transection (Smith et al., 1989), although this enhancement response was less marked in aged rats compared to young adult rats (Court et al., 1990).

Lesions of the entorhinal cortex, which destroys the glutamatergic projection to the hippocampus, (Geddes et al., 1992), have also been employed as paradigms for Alzheimer's disease. Geddes and colleagues (1985) have shown that lesion of this pathway results in cell

loss and a complex reorganisation of neuronal circuits. In Alzheimer's diseased brains the entorhinal cortex exhibits severe pathology (Hyman et al., 1987) which is likely to damage the connection between the entorhinal cortex and the dentate gyrus (Geddes et al., 1986). Lesions of the entrohinal cortex has also been shown to potentiate the increase in inositol phospholipid hydrolysis by excitatory amino acids (Nicoletti et al., 1987). Recently, Geddes et al. (1992) has shown that following partial lesion of the fimbria-fornix pathway in the rat, there is complex changes in the intrinsic circuitry and glutamate binding in selected areas. The relationship between these alterations and the changes observed in Alzheimer's disease is yet to be established, but future work should generate exciting information on the plastic response of the hippocampus to denervation.

Despite the fact that denervation models have provided valuable insight into the neurochemical and behavioral changes, the failure of such models to induce the pathological characteristics of β-amyloid deposition severely weakens the confidence with which these findings can be considered to reflect the Alzheimer's disease condition. Nonetheless, models of the pathological features are beginning to emerge. For example, transgenic mice have been developed which express APP protein fragments and neuropathological features associated with Alzheimer's disease (Kammesheidt et al., 1992). It has also been reported that the injection of abnormally phosphorylated tau into rat brain induces filament formation and amyloid deposition (Shin et al., 1993). Refinements of these models, to achieve the expression of selected APP fragments in specific brain areas, will greatly facilitate Alzheimer's disease research.

2.5 Aims of this thesis

At the onset of this study it was apparent that transmembrane signalling activities of the CNS, in most cases, involves G-proteins which are the first components in the intracellular signalling mechanism. It was also clear that a characteristic of Alzheimer's disease is the degeneration of certain neuronal populations, especially the cholinergic system. Thus, this investigation sought to establish whether the receptors are functionally coupled to G-proteins, and whether alterations in signal transduction down stream of the receptor had occurred as a consequence of the disease process. It is imperative that the status of this aspect of cellular signalling is determined if, in Alzheimer's disease, effective therapeutic intervention is to be realised.

EXPERIMENTAL PROCEDURES

3.1 Materials

The majority of reagents used were of the highest grade commercially available and purchased from the following companies:

Sigma Chemical Company Ltd, Poole, Dorset, UK:

Tris, ethylenedianinetetraacetate (EDTA), sodium deoxy cholate, trichloroecetic acid, glycerol, ammonium persulphate, N,N,N',N'-teramethylene-ethylenediamine (TEMED), tween-20, ortho-dianosidine, diaminobenzamidine, neutral chromatographic alumina, imadazole, sodium salt of cyclic 3'-5' adenosine monophosphphate (cAMP), sodium salt of adenosine triphosphate (ATP) sodium salt of guanosine triphosphate (GTP), creatine phosphate, creatine phospho kinase, aluminium chloride, sodium fluoride, acetyl coenzyme A, acetonitrile, kalignost (sodium tetra phenyl boron), bovine serum albumin, diethyl pyrocarbonate, 2-mercaptoethanol, charbacol, baclofen, commasie brilliant blue R, thimerosal, ouabain, bromophenol blue, trypsin, dithiothreitol, charcoal, folic acid, perchloric acid.

BDH Ltd., Glasgow, UK :

Sodium chloride, magnesium chloride, potassium cloride, triton X-100, hydrogen peroxide, folic acid, coppersulphate, sodium patassium tartrate.

Fisons Ltd., Loughborough, Leicester, UK:

Acrylamide, bisacrylamide, sodium hydroxide, sodium carbonate,

Genetic Research InstrumentationLtd., Dumow, Essex, UK:

Fugi RX X-ray film and Saran wrap.

Amersham International PLC, Buckinghamshire, UK:

^{125}I labelled anti rabbit IgG from donkey (5-20μCi/μg protein), 8-^{3}H cAMP (ammonium salt 1;1 in ethanol; 30Ci/mmol), 14C-acetyl coenzyme A (60Ci/mmol), γ-^{32}P ATP (ammonium salt in 5mM 2-mercaptoethanol; 3,000 Ci/mmol), γ-^{32}P GTP (ammonium salt in 5mM 2-mercaptoethanol; 3,000C/mmol).

Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K:

Dowex resin (AG50W-X4 200-400 mesh), mixed bed resin (AG501-X8 20-50 mesh) and polyprep columns.

BRL Ltd., Uxbridge, Middlesex, U.K:

Pre-stained molecular weight markers for Immunoblotting.

May and BakerLtd., Dagenham, Essex, U.K:

Ethanol, methanol, acetic acid, hydrochloric acid and isopropanol.

Anderman and Co. Ltd., Dumow, Essex., UK:

Nitrocellulose (Schleicher and Schuel, 0.2μM)

Packard Bell, Reading, Berkshire, UK:

Ecoscint (a toluene based scintillation fluid).

Scottish Antibody Production Unit, Carluke, Lanarkshire, UK:

Normal rabbit serum, normal sheep serum, anti-rabbit peroxidase linked sheep antisera, anti-mouse peroxidase linked rabbit antisera. Supplied free of charge.

3.2 Clinical details of subjects.

Human brains used in this study were obtained postmortem with ethical permission from Gartnavel Royal and the Southern General Hospitals, Glasgow. The control subjects had no known history of neuropathological or psychiatric illness. Alzheimer's diseased patients had a clinical diagnosis of dementia that was subsequently confirmed by histopathological quantification of senile plaques. The autopsy criteria for diagnosis was as described by Khachaturian, (1985): a minimal of three areas of the neocortex was examined; within a microscopic field of 1 mm square, patients between the age of 50-65 years must have a minimum of 8 senile plaques; in patients between 66-75 years, the number of plaques must exceed 10; any patient greater than 75 years, the senile plaques must exceed 15 per field. All samples were assayed for the activity of choline acetyltransferase (ChAT) since deficits in this enzyme is a basic hallmark of Alzheimer's disease (Perry , 1986). Patient information which is of relevance to this biochemical investigation are shown on **Tables 1a** and **1b.**

3.3 Dissection of human brains.

At autopsy, brains were removed and transported to the Wellcome neuroscience category 2 containment laboratory. The brain dissections were performed inside a flowhood after which all utensils and work surfaces were washed with chloros.

The brainstem was removed and remaining brain tissue sliced into 1cm thick coronal sections from the frontal to the occipital poles. Discrete anatomical brain regions were carefully dissected from the left and right hemispheres of alternate brain slices. The frontal cortex (Brodmann area) and hippocampus (dissected out at the level of the lateral geniculate body) used in this thesis werer from the left hemisphere. The tissue blocks were quickly frozen by immersion into isopentane at -40°C then placed into sealable plastic bags and covered with

dry ice. Tissue blocks were stored at -80 ^{o} C until required. The remaining brain material was fixed in formaldehyde and used for histopathological analysis. Blocks were marked on one side with indian ink to indicate the anterior face in relation to histological sections. Blood samples were routinely taken and screened for hepatitis and Creutzfeldt-Jakob syndrome. The stored brain material was only used when clearance of potential pathogens was confirmed.

3.4 Plaque quantification.

All histological analysis was carried out in the Neuropathalogical unit at the Southern General Hospital by Professor Graham. Tissue blocks, adjacent to the regions used for biochemical analysis were fixed in 10% formaline and used for histopathological examination. 28μM thick sections were stained by Kings amyloid silver stain to identify neuronal plaques (Chalmers et al., 1990). Plaque numbers were counted in superficial and deep cortical layers and in the subiculum of the hippocampus using the Quantimet 520 analyser. Plaque densities were expressed as number per mm^2 **(Tables 9 and 10).**

3.5 Dissection of grey and sub-cortical white matter from human brain tissue.

It was intended that the brain material should be used to investigate a variety of biochemical processes, but the stability of many had not been confirmed in postmortem tissue. Thus, every precaution was taken to minimise possible harmful physical manipulation of the tissue and it was decided not to routinely dissect grey from sub-cortical white matter since this would require thawing the material and also greatly reduce the amount of tissue available for analysis. However, one control cortex sample was dissected to give some indication of the contribution of grey and white matter to transduction events.

A tissue block from C23 temporal cortex was placed on a petri dish on a tray of ice and allowed

to thaw. The grey and subcortical white matter were carefully separated using a scalpel. The remaining tissue was considered to be a mixture of grey and white matter. The grey, subcortical white and grey/white mixture were powdered on liquid nitrogen and processed for membrane preparation as described in the section 3..

3.6 Assay of choline acetyltransferase activity.

Analysis of choline acetyl transferase activity was based on the method developed by Fonnum (1977). Tissue homogenates (5% weight per volume) were prepared in 10mM Tris HCl, pH 7.4, and 1mM EDTA as described in section 1.3. The homogenates were activated by the addition of an equal volume of 1% triton X-100 in 20mM EDTA and 10µl of activated homogenate was added to 25µl of substrate solution. The final concentration of the incubation mixture was; 0.2mM [14 C] acetyl CoA (approximately 125.000 dpm), 300mM NaCl, 50mM sodium phosphate buffer pH 7.4, 8mM choline bromide, 20mM EDTA (pH 7.4) and 0.1mM physostigmine. The reaction was initiated by the addition of activated tissue homogenate. The tubes were incubated at 37^{o}C. for 15 minutes, then placed on ice for 5 minutes. The reaction tubes were then carefully cut in half, placed in a scintillation vial and the contents washed out with 40mM chilled sodium phosphate buffer, pH 7.4. 2 mls of acetonitrile containing 10mgs of kalignost and 10mls of toluene scintillation mixture were added to the vial which were inverted to extract [^{14} C] labelled acetylcholine. The radioactivity was quantified by liquid scintillation analysis.

SAMPLE CALCULATION

The dpm values used in this calculation were the mean of assay triplicates measured in a control cortex sample tissue homogenate.

dpm values minus assay blank (890 dpm)

Total [^{14} C] acetyl choline per tube	22,7500 dpm
Sample	2255 dpm

Amount of acetyl choline per tube

Specific activity of [^{14} C] acetyl choline	=50μCi/μmole
Since 1μCi=2.22 x 10^6 dpm,	
then 22,7500 dpm/2.22 x 10^6	=0.102 μCi of [^{14} C] acetyl choline
Thus, 0.102 μCi	=2.04 nmole [^{14} C] acetyl choline per tube
Cold acetyl choline	=0.2mM=5 nmoles per tube

Therefore, a total of 7.2 nmoles of acetyl choline is added to each reaction tube.

Incubation time	=15 minutes and 45mg of protein assayed
Enzyme activity	=2255/227500 x 7.2
	=0.071nmoles/15minutes/45mg of protein
	=6.33 nmoles/hour/mg of protein.

Table2(a). Information available on the control subjects.

Patient	Age (Years)	PMD (Hours)	Storage time (Months)	Sex	Cause of death	Drug treatment
C8	80	23	37	F	Bronchopneumonia	Thyroxine
C14	88	11	37	F	Bronchopneumonia	Thyroxine
C15	87	12	37	F	Pulmonary thromboembolism Renal failure/Cervical pelvic abscess	Diamorphine
C16	76	17	36	M	Bronchopneumonia Pulmonary thromboembolism Brochial carcinoma	Metodopramide Diamorphine
C18	86	19	35	F	Sigmoid carcinoma	Diamorphine
C19A	84	7	33	F	Myocardial infarction	Diamorphine
C20	78	14	34	F	Myocardial infarction	None
C21	90	11	29	F	Carcinoma of the colon	None
C22	72	8	17	F	NK	NK
C23	74	4	19	M	Peritonitus	None
C24	96	3	16	F	NK	NK
C25	67	12	16	M	Myocardial infarction	None
C28	55	12	13	M	NK	NK
C33	92	2	10	F	Bronchopneumonia	Thioridazine
C34	83	6	9	F	NK	NK
C35	66	7	9	M	Myocardoal infarction	Diamorphine
C37	86	6	8	M	Bronchopneumonia	NK

Table2(b). Information available on the Alzheimer's disease subjects.

Patient	Age (Years)	PMD (Hours)	Storage time (Months)	Sex	Cause of death	Drug treatment
A17	89	4	39	F	Bronchopneumonia Pulmonary thromboembolism	Temazepam Morphine
A18	85	3	39	F	Bronchopneumonia	Morphine
A21	80	6	36	F	Conjestive cardiac failure	NK
A22	69	6	34	M	Bronchopneumonia	Nitrazepam Procyclidine Thioridazine Haloperidol Ophenadrine
A23	92	15	34	F	Bronchopneumonia	Bendrofluazide
A26	76	2	33	F	Pulmonary oedema/ Cardiac failure	None
A29	97	4	31	F	NK	NK
A30	85	4	30	F	NK	NK
A33	71	8	28	F	Bronchopneumonia	None
A35	NK	3	28	F	Bronchopneumonia	Thioridazine/ Orphrenadine/ Diamorphine.
A53	80	8	14	F	Cerebrovascular accident/ Pulmonary thromboemboli	Loperamide/ Thioridazine/ Temazepam.
A54	84	6	13	F	Respiratory failure Pneumonia	Morphine
A55	91	19	13	F	NK	NK
A56	88	16	13	F	NK	NK
A63	93	9	8	F	Bronchopneumonia	Thioridazine Temazapam Morphine
A65	76	11	6	F	Bronchopneumonia	Diamorphine

3.7 Human and rat brain tissue preparations.

All human tissue dissection and homogenate preparations were carried out in a category 2 containment facility. The entire blocks of frozen human tissue were powdered in a pre-chilled mortar in liquid nitrogen. Alternate control and Alzheimer's disease samples were powdered, transferred to a universal container and returned to liquid nitrogen. Aliquots of approximately 0.5g of powdered tissue were removed for processing and the remaining tissue stored at -80^{o}C. This provided a reservoir of homogeneous tissue powder thereby allowing repeat analysis if required. Tissue aliquots were homogenised by Polytron Homogeniser in 5 volumes of ice chilled buffer composed of 10mM Tris/HCl 1mM EDTA pH 7.4. 100µl of homogenate solution was removed and stored at -20^{o}C for subsequent analysis for choline acetyltransferase activity.

From the remaining tissue homogenate, crude membrane fractions were prepared by a modification of the method described by Milligan, (1993b). Small sample preparations, that required sample volumes less than 2 mls, were performed in a Beckman benchtop ultracentifuge. Homogenates were centrifuged at 1,000g K_{av} for 15 minutes at 4^{o}C to remove a nuclear, mitochondrial and cytoskeletal fraction. The supernatant was carefully removed and centrifuged at 4^{o}C for 20 minutes at 37,000g K_{av}. The crude membrane pellet was washed by resuspension in a chilled solution of 10mM Tris/HCl and 1mM EDTA pH 7.5 followed by recentrifugation at 37,000g for 20 minutes. The final pellet was resuspended in chilled 10mM Tris/HCl pH 7.4. The membrane preparations were aliquoted and stored in 1.5ml eppendorf tubes at -20^{o}C until required for biochemical analysis.

Eight control and eight Alzheimer's disease samples were prepared for the frontal cortex and hippocampus studies (see table 1 for a list of the samples designated to each region). The control and Alzheimer's disease samples were homogenised alternately and stored on ice until all samples were obtained. Similarly, individual centrifugation steps such as removal of the

supernatant, were carried out by alternating between control and Alzheimer's disease samples. Sample centrifugations were performed in a Bechman J21 rotor which has the capacity for eighteen tubes which allowed all samples to be processed at the same time thus eliminating batch variations.

3.8 Protein quantification.

Protein levels were assayed in duplicate by the method of Lowry et al. (1951) employing bovine serum albumin as a standard. Protein samples were diluted to 25µl with distilled H_2O with a minimal dilution of 1/4 required for accurate protein determination. At this dilution there was no interference of the sample buffer on the optical density value. 1ml of a fresh solution composed of 0.02% (weight/volume) sodium potassium tartrate, 0.02% (weight/volume) copper sulphate and 2% (weight/volume) sodium carbonate, was added to the diluted sample and the tubes vortexed. 100µl of Folins reagent (Sigma Ltd) diluted 1:1 was added to each tube, the tubes revortexed and left at room temperature for 20 minutes. Optical density values were then measured at 680nm.

3.9 Polyacrylamide gel electrophoresis.

3.9.1 Protein denaturation and alkylation.

For routine denaturation of samples, 100µg of membrane proteins were extracted in a solution of 0.1% (weight/Volume) sodium deoxy cholate in a total volume of 1ml and precipitated on ice with the addition of trichloroacetic acid to 5% (weight/volume). Samples were centrifuged briefly at 1000g $_{av}$. The pellets were neutralised by the addition of a small volume of 1M Tris base pH 10.8 (a 20µl volume of Tris base was found to be sufficient to neutralise 100µg of membrane preparation). Denaturation was achieved by adding an equal volume of buffer composed of bromophenol blue indicator, 5M urea, 5% dithiothreitol (DTT),

5% sodium dodecyl sulphate (SDS) and 0.1M Tris HCI pH 8.0.

Where required, protein alkylation was carried out in a 1.5 ml microfuge tube in a total volume of 50µl containing 100µg of protein in 10mM Tris, 1mM EDTA, pH 7.4, buffer containing 10mM DTT and 5% weight/volume SDS. Tubes were incubated at 90^{o}C for 15 minutes then placed on ice for 5 minutes. 10µl of 100mM N-ethyl malanide (NEM) was added and the tubes incubated at room temperature for 15 minutes followed by the addition of 20µl denaturing buffer. Such samples were ready for electrophoresis.

3.9.2 Denatured gel electrophoresis.

The discontinuous slab gel system of Laemmli(1970) was used. Resolving gels were composed of 12.5% acrylamide, 0.062% bisacrylamide, 0.38M Tris/HCl, pH 8.4, 0.1M SDS and 4.2% glycerol. Stacking gels contained 3% acrylamide, 0.08% bisacrylamide, 0.38M Tris/HCI, pH 6.8, and 0.1% SDS. In both the human and rat medial septal immunoblot studies (sections **4.4** and **4.6** respectively), control and diseased/lesioned samples were loaded onto alternate lanes of the gels. In addition, rat brain control preparations and prestained molecular weight markers were run with each immunoblot. For a single gel, the protein samples were run through the stacker at 20mA and through the resolver overnight at 8mA in a water chilled LKB electrophoresis apparatus.

In order to obtain reproducible resolution of the $G_{\alpha i}1$ and $G_{\alpha i}2$ subtypes, which are detected with the same antibody, the dye-front was ran off the gel and electrophoresis continued until the 18 KDal pre-stained marker protein approached the end of the gel. Analysis of fresh samples and samples left at room temperature confirmed that immunopositive, small molecular weight proteins did not migrate off the gel as a result of this modification.

3.9.3 Protein detection by immunoblotting (Western blotting).

Details of the primary antibodies and the dilutions employed for each are listed in **Table 3.** Proteins were transfered to nitrocellulose membrane by a modification of the method described by Towbin et al. (1979). After electrophoresis, the 3% acrylamide stacker was removed and the resolving gel incubated for 20 minutes in a chilled transfer buffer composed of 190mM glycine, 25mM Tris and 20% methanol. The gel was then placed on top of three sheets of Whatman number 1 filter paper which had been pre-soaked in transfer buffer. A sheet of the nitrocellulose paper was soaked in transfer buffer and placed on top of the resolving gel. Air bubbles were carefully removed by rolling a glass pipette over the nitrocellulose/resolving gel stack. Three sheets of wet Whatman number 1 filter paper was placed on top of the stack, enclosed in a plastic holder and placed in a LKB transfer tank. Proteins were transferred at 75V for 3 hours in transfer buffer which was chilled by a water cooling system.

After transfer the nitrocellulose films were carefully removed and the non-specific binding sites were blocked by incubating the nitrocellulose film with Tris/salt (0.5M NaCl and 20mM Tris/HCl pH 7.5) containing 3% dried milk for 3 hours at room temperature. Nitrocellulose sheets were then washed briefly in distilled/deionised H_2O, and incubated overnight at room temperature with a primary antibody prepared in a solution of Tris/salt containing 1% dried milk. Nitrocellulose sheets were then rinsed twice in Tris/salt containing 0.05% Tween 20 each for 10 minutes.

For the detection of $G_{\alpha o}$, $G_{\alpha i}1$ and $G_{\alpha i}2$, and glial fibrillary acid protein (GFAP), nitrocellulose sheets were incubated with peroxidase linked secondary antibody (supplied by the Scottish Antibody Production Unit) at a 1/200 dilution in 1% dried milk in Tris/salt at $37^{o}C$ for 3 hours at room temperature. Immunoreactive bands were visualised by the formation of a colour complex using 0.02% (weight/volume) ortho-dianosidine substrate in 10mM

Tris/HCl, pH 7.4, and the reaction catalysed by adding hydrogen peroxide (10ml of 30% per 50mls of substrate solution). Analysis of $G\alpha_s$ and Gβ proteins required a more sensitive technique and [^{125} I]-anti-rabbit Ig G (Amersham International) was used as a secondary antibody. Immunoreative complexes were visualised by autoradiography on FUJI RX X-ray film. Initial experiments were performed using normal rabbit serum as a primary antibody to confirm that the secondary antibodies did not crossreact with the proteins of interest. Some batches of secondary cross reacted with proteins of molecular weights approximately 60KDal.

Thimerosal was routinely added to antibody solutions to prevent bacterial contamination. The primary antibody solutions, and the peroxidase labelled secondary antibody solutions could be used for three separate immunoblot experiments without any significant effect on the specificity or the intensity of the immunocomplex. Fresh solutions were however employed in the human and rat semiquantitative studies.

3.9.4 Coomassie blue staining of acrylamide gels.

Gels were routinely stained, post transfer, by immersing the gels overnight at room temperature in a solution of 40% (volume/volume) methanol, 10% glacial acetic acid containing 0.1% (weight/volume) coomassie brilliant blue (R). The gels were destained by several washes in 40% methanol and 10% glacial acetic acid until protein bands were visible. Although most of the original protein loaded on to the gel had been transferred to nitrocellulose, there remained a sufficient quantity of the high molecular weight stained proteins to permit visual comparison of the amount of protein loaded for each sample.

3.10 Densitometric analysis of immunoblots.

Densitometric analysis of both stained blots and autoradiography were performed using a Quantimet 970 (Cambridge Instruments, Cambridge UK) computer assisted image analysis.

Images on the blots or autoradiography were illuminated in computer controlled intensity by light emitted from four 24 W lamps. The images were focused by a micro zoom lens connected to a video camera. Immunoreactive bands were analysed by spot densitometry and values expressed as optical density readings. To ensure reproducible readings, the image analyser was routinely calibrated using Kodak neutral density filter (0.D =0 1). Optical density values are the mean of two readings.

Densitometric analysis of glial fibrillary acid protein (GFAP) immunoblots were performed on a Joyce-Loebl Chromoscan 3 system. This system generated a scanning profile with six discernible peaks. The GFAP data reported in this thesis corresponds to the peak high values obtained by scanning densitometry. A comparison of both densitometric methods concluded that the data generated by spot densitometric analysis of G-proteins was comparable to the peak height measurements by scanning densitometery.

3.11 Antisera production.

All G-protein antisera were supplied by Dr G Milligan of the Department of Biochemistry, Glasgow University. The α and β subunits antibodies (CS1, SG2, OC1 and BN1) were raised in New Zealand white rabbits against the synthetic peptides (**Table 3**) coupled to keyhole-limpet haemocyanin by gluteraldehyde. The synthetic peptides corresponded to the various protein sequences: CS1 corresponded to the C-terminal decapeptide of $G_{\alpha s}$; SG2 corresponded to the C-terminal of transducin, which crossreacts with $G_{\alpha i}1$,$G_{\alpha i}2$ and $G_{\alpha i}3$ (Milligan & Unson, 1989); OC1 corresponded to the C-terminal of $G_{\alpha o}$ (Mullaney et al., 1988) and BN1 was raised against the sequence corresponding to amino acid 1-10 of β1 subunit (Carr et al., 1990). Anti GFAP antibody was purchased from DAKO limited and raised in rabbit against GFAP purified from bovine brain.

Table 3. Generation and characterisation of the antisera employed for the immunoblot analysis of G-protein subunits and for glial fibrillary acidic protein (GFAP).

Antibody	Antigen sequence	Protein detected	Molecular weight (KDal)	Working dilution
CS1	RMHLRQYELL	$G_{\alpha s}L$, $G_{\alpha s}L$	44, 42	1/200
SG2	KENLKDCGLF	$G_{\alpha i}1$, $G_{\alpha i}2$	40, 41	1/200
OC1	ANNLRGCGLY	$G_{\alpha o}$	39	1/10,000
BN1	MSELDQLRQE	G_{β}	36	1/200
Anti-GFAP	purified bovine brain GFAP		approx 55-30	1/2,000

3.12 Liquid scintillation analysis.

All isotopes were measured on a Packard 1900 CA TRI-CARB Liquid Scintillation Analyser. Quantification of [^{32}P] was calculated from the cpm values since the operational efficiency was greater than 90%. [^{14}C] choline and [^{3}H] cAMP cpm values were converted to dpm using appropriate quench curves. For quantitation of GTPase activity (section **3.13.**), 500µl of radioactive supernatant was mixed with 8mls of ecosinct and counted for 4 minutes. Quantification of the adenylate cyclase assay (section **3.14**)required 4mls of column eluate to be mixed with 16mls of ecoscint and counted for 4 minutes. For quantitation of [^{14}C] acetylcholine (section **3.5**) the contents of the reaction tube was washed out with 5mls of 50mM sodium phosphate buffer, then extracted from the aqueous phase by adding 2mls of acetonitrile containing 10mg of Kalignost followed by 10mls of toluene. The vials are left overnight then counted for 4 minutes.

3.13 Assay of GTPase activity.

The analysis of GTPase activity was based on the method developed by Cassel & Selinger, (1976) but incorporated modifications described by others (Hilf & Jakobs, 1989; Hoss et al., 1988). The GTPase assay was conducted in an ATP-regenerating system that inhibits both GTP hydrolysis by nonspecific nucleosidase and the transfer of phosphate from GTP to ATP.

All samples were assayed in triplicate and each assay included a rat brain membrane preparation as control. The assay medium contained final concentrations of, 5-10µg of membrane protein, 1mM ATP, 0.1mM 5'-adenyl-imidodiphosphate (AppNHp), 1mM oubain, 15mM creatine phosphate, 60U/ml creatine phosphokinase, 2mM dithiothreitol, 5mM $MgCl_2$, 100mM NaCl, 0.1mM EDTA, 20mM Tris HCl (pH 7.4) and GTP at either 0.2mM or 0.1mM concentrations and [γ^{32}P] GTP (60,000-80,000 cpm).

For a final assay volume of 100µl, 20µl of membrane suspensions in a buffer of 10mM Tris/HCl (pH 7.4), 0.5mM EDTA were pipetted into 1.5ml microcentrifuge tubes. Each tube contained the following solutions prechilled on ice; 50µl of reaction mixture, 10µl of deionised distilled H_2O or where applicable, 10µl of 10mM receptor agonist (carbachol or baclofen), and 10µl 1mM GTP. 20ml of sample buffer replaced membrane sample to determine endogenous free γ^{32}P [Pi] (assay blank) and the total radioactivity per tube of each assay.

The reaction was initiated by the addition of membrane suspension, incubation was for 15 minutes at 25°C and was terminated by the adding 900µl of chilled 5% (weight/volume) charcoal in 20mM phosphoric acid (pH 2.5). A fresh charcoal solution was prepared each day. 10g of charcoal was mixed in 200mls of 20mM phosphoric acid, allowed to sediment, then 100mls of 20mM phosphoric acid was removed and replaced with a fresh solution. The total amount of radioactivity per tube for each assay, was determined by adding 900ml of 20mM phosphoric acid (minus charcoal). Samples were kept on ice for 10 minutes then

centrifuged at 1000g $_{av}$ for 10 minutes in a Beckmans E microcentrifuge. A 500ml aliquot of the supernatant was removed for liquid scintillation analysis. For practical purposes, each assay was restricted to a maximum of 60 tubes. This assay size allowed 3 individual samples and a rat control to be assayed for non-specific, basal and stimulation of basal GTPase by two agonists. For the human (section **4.4**) and the medial septal lesion study (section **4.6**), samples were assayed over three days.

Appropriate modifications were employed in preliminary experiments to characterise the assay of fresh rat brain preparations. To assess the contribution of the nonspecific, low affinity GTPase to the effect of the agonists, a final concentration of 1mM agonist was added to reaction tubes containing 100mM GTP. No significant differences were observed with either carbachol or baclofen compared to non-stimulated samples. To exlude the possibility that significant hydrolysis of GTP occured on ice, the basal, stimulated and nonspecific activity was determined after a 15 minute incubation on ice. No significant activity was observed.

As described by Cassel & Selinger (1976), the high affinity GTPase activity can be calculated by subtracting the cpm values measured with 100μM GTP (low affinity GTPase) from that observed with 0.2μM GTP. The complete data obtained from the postmortem (section **4.3.3**) human (sections **4.4.3** and **4.4.6.**) and medial septal lesion (section **4.6.3**) GTPase analyses, are illustrated as the total, high affinity (basal) and low affinity (nonspecific) values.

SAMPLE CALCULATION

The cpm values used in this calculation are the mean of assay triplicates measured in 500ml aliquots of supernatant from a representative rat brain sample. 5μg of membrane protein was incubated for 15minutes.

cpm values minus blank (105 cpm)

Total	36,530
Nonspecific	299
Basal (high affinity)	2208
Carbachol	3139
Baclofen	3029

AMOUNT OF HOT GTP PER TUBE

Hot GTP per tube 2 x 36530 cpm = 73,060 cpm

Since 1μCi of [^{32}P] GTP~2.22x10^{6} cpm, then 73,060/2.22x10^{6} = 0.032μCi

1μCi of hot GTP=0.1nmole GTP, then 0.032 μCi=3.2pmole GTP is added.

AMOUNT OF COLD GTP PER TUBE

High affinity measurements	20 pmoles per tube
Low affinity	10 nmoles per tube
Basal activity	=[(2208-299)/36530] x(20+3.2)
	=1.212 pmoles per 15 minutes per 5μg of protein
	=16.16 pmoles per minute per mg of protein
Carbachol stimulated	[(3039-299)/36,530] x 23.2=1.74
	=23.2 pmoles per minute per mg of protein
	=23.2/16.1
	=144% of basal GTPase activity
Nonspecific activity	[299/36,530] x 10nmoles GTP
	=0.0818nmoles per 15minutes per 5μg
	=1.09nmoles per minute per mg of protein

3.14 Assay of adenylate cyclase activity.

3.14.1 Adenylate cyclase catalysed production of cAMP.

Adenylate cyclase activity was assayed by a modification of the method described by Salomon et al. (1974). The final reaction mixture was composed of 20µg of membrane preparations, 100mM Tris/HCl pH 7.5, 50mM NaCl, 5mM MgCl, 1mM cAMP, 200µM ATP containing 1µCi of α-[^{32}P] ATP, 20mM creatine phosphate and 100U/ml of phosphocreatine kinase. Where receptor-independent stimulation of adenylate cyclase was investigated, the reaction mixture included 10µM aluminium chloride and 5mM sodium fluoride. The reaction was initiated by the addition of 20µl of membrane preparation in 10mM Tris/HCl buffer and the tubes were incubated at 30^{o}C for 15 minutes. In assay blanks, 20µl of 10mM Tris/HCl replaced the membrane preparation. Termination of the reaction was achieved by adding 150µl of a solution containing 2% (weight/volume) SDS, 40mM ATP, 1.3mM cold cAMP and [^{3}H] labelled cAMP (approximately 30,000 cpm) which serves as an internal standard used to calculate the column recovery. Assay tubes were transferred to ice for 10 minutes then placed in boiling water bath for 10 minutes. At this stage the samples can be stored overnight at -20^{o}C.

3.14.2 Separation of cAMP from ATP.

The [^{32}P] cAMP formed was isolated on Dowex and aluminia columns. Dowex columns were preactivated with 1M HCl then rinsed with distilled H_2O. The aluminia columns were equilibrated with 0.1M imidazole pH 7.5. 600µl of distilled H_2O was added to reaction tubes and the total contents then loaded onto the dowex column which was washed with 2mls of distilled H_2O and the eluate discarded. A further 4mls of distilled H_2O was added and the eluate was allowed to run into the aluminia column. Aluminia columns were washed with 1ml of 0.1M imidazole and initial eluate discarded. The bound fraction was eluted with 4mls of

0.1M imidazole into vials containing 16mls of ecoscint and the radioactivity quantified by liquid scintillation.

SAMPLE CALCULATION

^{32}P assay blank	90cpm
Total 2,000,000cpm	
3H total	30,000 cpm
Incubation period	15 minutes
protein per tube	20µg
^{3}H sample	22,000cpm
^{32}P sample	1000cpm
Total ATP	20,000
Actual sample counts	1000-90=910
Column recovery	22,000/30,000=73%
Corrected sample counts	1240cpm
pmols cAMP	1240/2,000,000 X 20,000pmols =12.4pmoles per tube
pmols/minute/mg	(12.5/15) X (1000/20)=41.66.

3.15 Induction of postmortem interval in rat (the interval between death and freezing of brain tissue).

Male Sprague-Dawley rats weighing between 250-300g were used in this study. Rats were sacrificed by cervical dislocation and after the designated postmortem time period (zero hours for control animals) the animals were decapitated and the brains were rapidly removed, dissected and stored at $4^{o}C$. A period of 15 minutes, between the physical cervical dislocation and brain removal was typical for the control (zero hour) samples. The rats that were subjected to postmortem delay were therefore left at room temperature for 15 minutes after sacrifice, then stored at $4^{o}C$ for periods of 6, 12, 24 and 48 hours.

The brains were removed and processed as follows; the cranium was exposed by elongated laceration from the back of the head to the snout, muscle and skin tissue was removed and the cranium carefully opened, the dura was carefully cut and the brain eased out of the skull and placed onto a petri dish kept on ice. Superflous blood was washed away with saline and the cortex, cerebellum, hippocampus and subcortical regions dissected out. Specific brain regions were placed into universal containers (prechilled in liquid nitrogen) and dropped into liquid nitrogen. Samples were stored at $-80^{o}C$ until required. One control brain was severely damaged during removal and not included in the study. In total, five animals were prepared for controls and all other time points consisted of three animals.

3.16 Medial septal lesion of the rat.

Experiments were performed on male Sprague-Dawley rats (250-300g). Rats were housed in an environment of natural day/night cycles and kept at 21^{o}C. for a minimal period of 7 days prior to surgical intervention. Post surgery, animals were weighed and their food and water intake closely monitored. Any animals exhibiting signs of distress were examined by a veterinary surgeon and when necessary sacrificed. In this study a total of 24 animals underwent surgical intervention of which three animals required to be sacrificed because of animal welfare/ legislation requirements.

3.16.1 Stereotactic lesioning of rat medial septal nucleus.

Rats were placed in a perspex box into which anaethsetic gas was flowing composed of 70% nitro oxide, 30% oxygen and 2% halothane. Anaesthesia was maintained during surgical procedure by injection i.p. of chloral hydrate (400mg/Kg) and diazepam (2mg/Kg). The rats were placed in a stereotaxic frame and restrained using ear bars and an incisor bar. The scalp was incised, the underlying tissue retracted from the skull, and a burrhole was made anterior to the bregma. Buffered ibotenate or saline was injected into the medial septum in a total volume of 0.6ml at the rate of 0.1ml/minute. The stereotoxic co-ordinates used to determine the injection site were 0.8mm inferior to bregma at the midline, and lowered 5.8mm below the surface of the dura, Horsburgh et al. (1993).

At 21 days survival rats were sacrificed by decapitation, and the brains were rapidly removed as described in section 4.13. The brains were placed on to a petri dish, on ice and sliced transversely behind the injection site. The anterior portion of the brain was fixed in 10% formaldehyde and used for subsequent histological analysis to examine the medial septum. Subcortical tissue was carefully removed and the hippocampus was carefully dissected free from the cortical tissue, placed in a 1.5ml microfuge tube, rapidily frozen in

isopetone -40 ^{O}C. and stored at -80 ^{O}C.

3.16.2 Histological examination of medial septum post surgery.

For haemotoxylin and eosin and cresyl violet staining, brain tissue was fixed in formal saline then paraffin embedded. 10μm sections were dehydrated by incubating with xylene and rehydrated through a series of decreasing alcohol solutions. Formaldehyde fixed tissue was paraffin embedded, and 10μm microtome cut sections processed for cresyl violet and haemotoylin and eosin staining by the standard methods.

3.17 Immunocytochemistry of brain tissue.

The objective of this investigation was to examine by means of histological distribution to determine whether discrete alterations in the laminar pattern of G-protein localisation occurs as a consequence of neurodegeneration. Thus, the antibodies used in this study (**Table 3**) were employed in the immunocytochemical analysis of brain tissue. In addition, anti-GFAP antibody was employed since successful immunocytochemical analysis of this protein has been demonstrated with postmortem tissue and therefore served as a antibody control. The following is an outline of the techniques and modification employed in this study.

Microscope slides were coated with either gelatine or 1% weight/volume polylysine solution. 10μm thick sections were frozen on dry ice then fixed in formal saline for 30 minutes. In some experiments a variety of fixation solutions (e.g.acetone, gluteraldehyde) were employed. Sections were dehydrated by immersion in a series of 70,90 and 100% alcohol solutions. Endogenous peroxidase activity of the sections was blocked by incubating in methanol containing 0.3% volume/volume hydrogen peroxide. Sections were then dissected ready for immunocytochemistry. They were washed twice for ten minutes in a solution of Tris buffered saline containing 0.01% volume/volume Triton-X 100 (TTBS). Nonspecific binding sites were

blocked by incubating with normal goat serum at a 1/10 dilution for 1 hour at room temperature. The blocking antisera was poured off and the sections incubated overnight with anti-GFAP primary antisera diluted 1/400 with 1/10 dilution of normal goat serum. Sections were then incubated at 4 ^{o} C overnight. After this incubation, the sections were again washed twice with TTBS, then incubated for 1 hour at room temperature with horse radish peroxidase linked secondary antibody diluted 1/50 in Tris buffered saline before again being washed in TTBS for 2x10minutes. To enhance the immunocomplex, the sections were incubated for 1 hour at room temperature with peroxidase linked antiperoxidase antibody diluted 1/50 with Tris buffered saline. Immunopositive cells were visualised by incubating the sections with 0.5% diaminobenzamidine containing 0.01% hydrogen peroxide prepared in 50mM Tris/HCl, pH 7.4, and examined microscopically to ensure that the cells were sufficiently stained. The sections were counterstained by immersion in haemotoxylin solution for 5 minutes and placed in Scotts tap water until the nucleus was stained blue. The sections were then dehydrated, cleared in Histoclear solution and mounted.

While GFAP staining was successful in both human and rat tissue, no convincing staining was obtained with any of the G-protein antibodies. Since these antibodies were raised against specific peptide sequences, it was likely that the epitopes were inaccessible in fixed sections. To try and overcome this a variety of pretreatments were tried including incubation of sections with trypsin (1%), folic acid (10% volume/volume to neat solution) and SDS (0.1% weight/volume to 1%) and a range of fixation methods. However non of these methods were successful. Perhaps polyclonal antibodies raised against purified G-proteins are required since such a population of antibodies would react with various epitopes some of which may be exposed in tissue sections.

3.18 Statistical analysis of data.

Statistical analysis was performed using Macintosh Statswork software programme. Sample groups were compared by unpaired Student's t-test where sample distribution was normal. Groups were considered to by significantly different at significance levels of 5% and 1%. Correlation analysis was conducted by simple linear regression and significant correlations designated at 5% and 1% confidence level. Data generated in sections **4.3** and **4.5** were analysed by one way analysis of varience (ANOVA). Post hoc calculations of the statistical power of particular experimental designs were assessed from the following equation:

$$n = \frac{2\sigma^2}{\Delta^2}\left[\mathcal{E}_{(1-\alpha)} + \mathcal{E}_{\beta}\right]^2$$

where n= number of observations in the group

σ= standard deviation

Δ= difference between control and experimental group

with the values of $\mathcal{E}_{(1-\alpha)} + \mathcal{E}_{\beta}$ obtained from tables of the inverse normal function (with typically α=5% and β=80%)

RESULTS

4.1 Standardisation of the immunoblot technique.

4.1.1 Antisera characterisation.

Preliminary experiments were conducted to assess the specificity of the various antisera employed in this study, and to confirm the ability of these antisera to cross-react with both rat and human brain preparations. Since the pertusis toxin sensitive G-proteins $G_{\alpha i}1$, $G_{\alpha i}2$ and $G_{\alpha o}$ have similar molecular weights of 41, 40 and 39Kd respectfully (Birnbaumer et al., 1990), it was necessary to obtain electrophoretic separation of these proteins so that the specificity of the anti-$G_{\alpha o}$ and anti-$G_{\alpha i}$ antisera could be determined. The resolution of these proteins was enhanced by employing low amounts of bisacrylamide in the resolving gel and covalently modifying the proteins by alkylation. **Figure 1(A)** demonstrates the immunoblot profile of rat brain preparations analysed under these conditions. Antisera SG2 **(Table 3)** reacts with two polypeptides in the expected position of the two $G_{\alpha i}$ species $G_{\alpha i}1$ and $G_{\alpha i}2$. Similarly, the antisera OC1 detects one polypeptide species in the expected position of $G_{\alpha o}$. A solution containing both SG2 and OC1 antisera at the appropriate dilution (1/200 and 1/10,000 respectively) detects three immunoreactive bands corresponding to $G_{\alpha i}1$, $G_{\alpha i}2$ and $G_{\alpha o}$. The SG2 and OC1 antisera therefore cross-react with polypeptides as described by Milligan and Unson, (1987) and Mullaney et al. (1988) This experiment demonstrated the selectivity of the antisera and that $G_{\alpha o}$, $G_{\alpha i}1$, and $G_{\alpha i}2$ could be detected within the same immunoblot. Nevertheless, it was decided to analyse the $G_{\alpha o}$ and $G_{\alpha i}$ subtypes on separate immunoblots because it was probable that analysis of the human samples would produce variation in the relative intensities of the different proteins which could potentially restrict the resolution of the three protein bands.

Normal rabbit serum failed to detect any protein bands with these preparations. However, antisera occasionally cross-reacted with a faint protein band at approximately 60 KDal. The identity of this protein remained unknown but since it had a migration distance distinct from the Gα subunits, its presence was not considered to have any practical significance.

The anti G-protein antisera OC1, SG2, CS1 and BN1 cross-react with proteins of near identical molecular weight from membranes prepared from rat and human brains (**Figure 1(B)**). This suggests that these antisera can detect the appropriate G-protein subunits in both species. However, the anti $G_{\alpha s}$ antisera CS1 detects two species in human, $G_{\alpha s}L$ (large) and $G_{\alpha s}S$ (small), whereas in rat, the $G_{\alpha s}L$ predominates with $G_{\alpha s}S$ barely visible. Thus, the relative amounts of the $G_{\alpha s}$ subtypes varies between species. Antisera BN1, in addition to detecting Gβ, also reacts with an unknown protein at approximately 60 KDal which was detected in both human and rat. Subsequent analysis of grey and sub-cortical white matter demonstrated this protein predominates in white matter (see section **4.5.1**)

With fresh rat brain preparations, anti glial fibrillary acidic protein (GFAP) antibody cross-reacted with a predominant polypeptide of approximately 55 KDal and with less abundant lower molecular weight protein bands which were just visible. With postmortem human brains however, the same antibody cross-reacted with multiple polypeptides ranging in size from approximately 35-55 KDal as illustrated in **Figure 1(C)**. Densitometric analysis of the human samples using the Joyce Loebl Chromoscan 3 system (section **3.10**) detected six discernable peaks. Thus, the GFAP data reported in sections **4.4** and **4.5** are expressed as peak heights which corresponds to the polypeptides designated bands 1 to 6.

FIGURE 1. The immunoblot profile and selectivity of the anti G-protein antisera and anti glial fibrillary acidic protein (GFAP) antibody in human and rat membrane protein extracts. **(A)** 100μg of alkylated rat membrane extract incubated with SG2 at 1/200 dilution detects both $G_{\alpha i}1$ and $G_{\alpha i}2$; 50μg of membrane incubated with OC1 at 1/10,000 dilution detects $G_{\alpha o}$. A mixture of both SG2 (1/200) and OC1 (1/10,000) detects $G_{\alpha i}1$, $G_{\alpha i}2$ and $G_{\alpha o}$. **(B)** Both human and rat brain tissue react with anti-G-protein antisera; OC1 (1/10,000) reacts with $G_{\alpha o}$, SG2 (1/200 dilution) reacts with $G_{\alpha i}1$ and $G_{\alpha i}2$, CS1 (1/200) reacts with $G_{\alpha s}L$ and $G_{\alpha s}S$ and BN1 (1/200) reacts Gβ subunits. In rat $G_{\alpha s}L$ is the major $G_{\alpha s}$ subtype with $G_{\alpha s}S$ just visible. Antisera BN1 in addition to detecting Gβ proteins also crossreacts with a protein approximately 60 KDal referred to as BN1-A (artifact). **(C)** Immunoblots with anti GFAP antibody displays a multiple band appearance in humans that is distinct from protein pattern of rat brain. Where densitometric analysis was performed on GFAP immunoblots, the values were expressed as peak heights corresponding to the bands numbered 1 to 6.

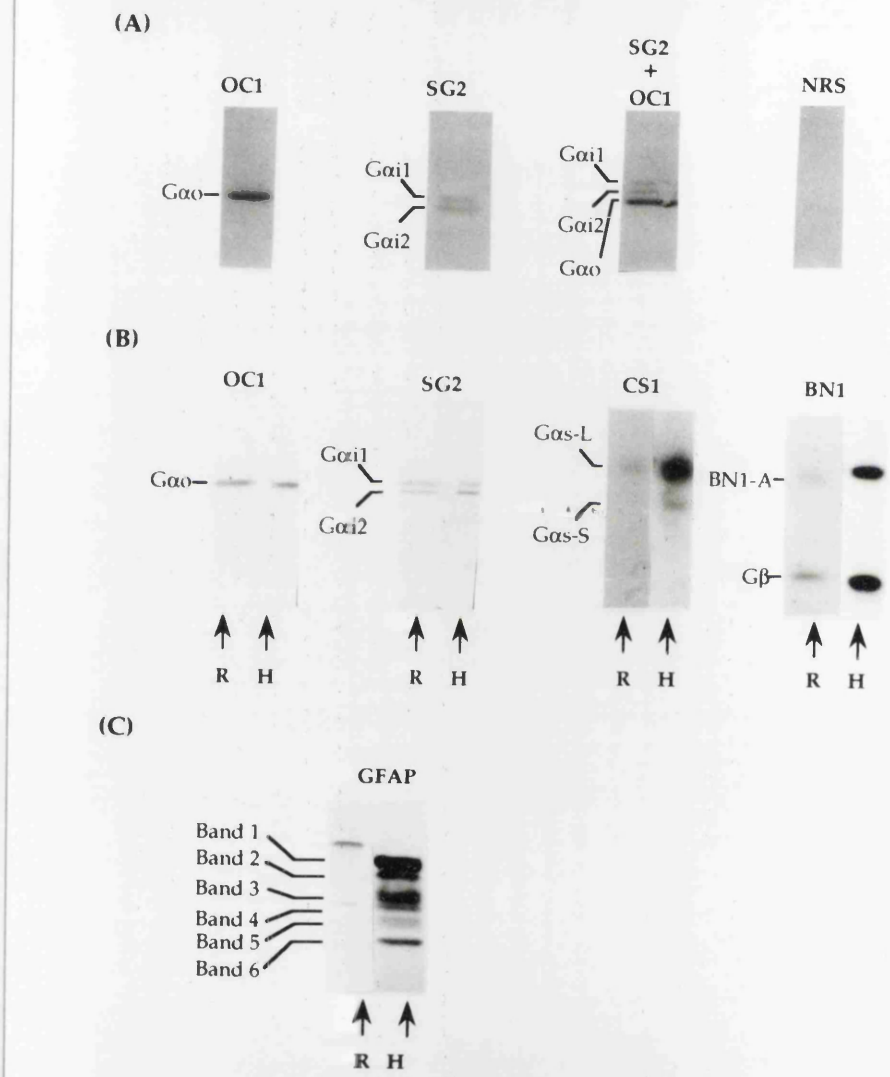

(A)
OC1
SG2
SG2
+
OC1
NRS
Gαo
Gαi1
Gαi2
Gαi1
Gαi2
Gαo
(B)
OC1
SG2
CS1
BN1
Gαo
Gαi1
Gαi2
Gαs-L
Gαs-S
BN1-A
Gβ
R H
R H
R H
R H
(C)
GFAP
Band 1
Band 2
Band 3
Band 4
Band 5
Band 6
R H

FIGURE 1.

4.1.2 Linearity of the immunoblot technique.

The intensity of immunoreactive complexes detected with anti $G_{\alpha o}$ and anti $G_{\alpha i}$ sera over a range of protein concentrations are illustrated in **Figure 2.** Densitometric measurements of the immunoblots are shown in **Figure 3.** The results demonstrate that while each antisera (OC1 and SG2), yields a linear relationship between the protein content and optical density value, the relationship is however saturable. Therefore, by loading the appropriate amount of membrane protein and routinely employing rat brain controls the intensity of the immunocomplex generated from the membranes being investigated invariably fell within the linear range of the technique. A similar experiment was conducted in this laboratory to establish the optimal amount of protein required for the detection of $G_{\alpha s}$ (Ross, 1992).

Antibody BN1 became available only in the latter stages of the study and the relationship between the intensity of the immunocomplex was not performed over the range of protein loaded onto the gel. However, 75μg of human and rat membrane protein gave 50% of the signal generated for 150μg of protein which would suggest that this amount of protein satisfies the sensitivity requirements of the immunoblot conditions for Gβ analysis.

FIGURE 2. Immunoblot profile of G_α subtypes, $G_{\alpha o}$, $G_{\alpha i}1$ and $G_{\alpha i}2$ obtained from polyacrylamide gels loaded with a range of rat brain extract (μg) and detected with the indicated antisera. **(A)** OC1 diluted 1/10,000, **(B)** SG2 diluted 1/200.

FIGURE 3. Densitometric analysis of immunoblots of the $G_{\alpha o}$, $G_{\alpha i}1$ and $G_{\alpha i}2$ subtypes over a range of rat brain protein (μg) loaded onto polyacrylamide gels. **(A)** OC1 at 1/10,000 dilution gave a linear signal to protein relationship for $G_{\alpha o}$ up to approximately 100μg. **(B)** SG2 at 1/200 dilution gave a linear signal to protein relationship for $G_{\alpha i}1$ and $G_{\alpha i}2$ up to approximately 150μg.

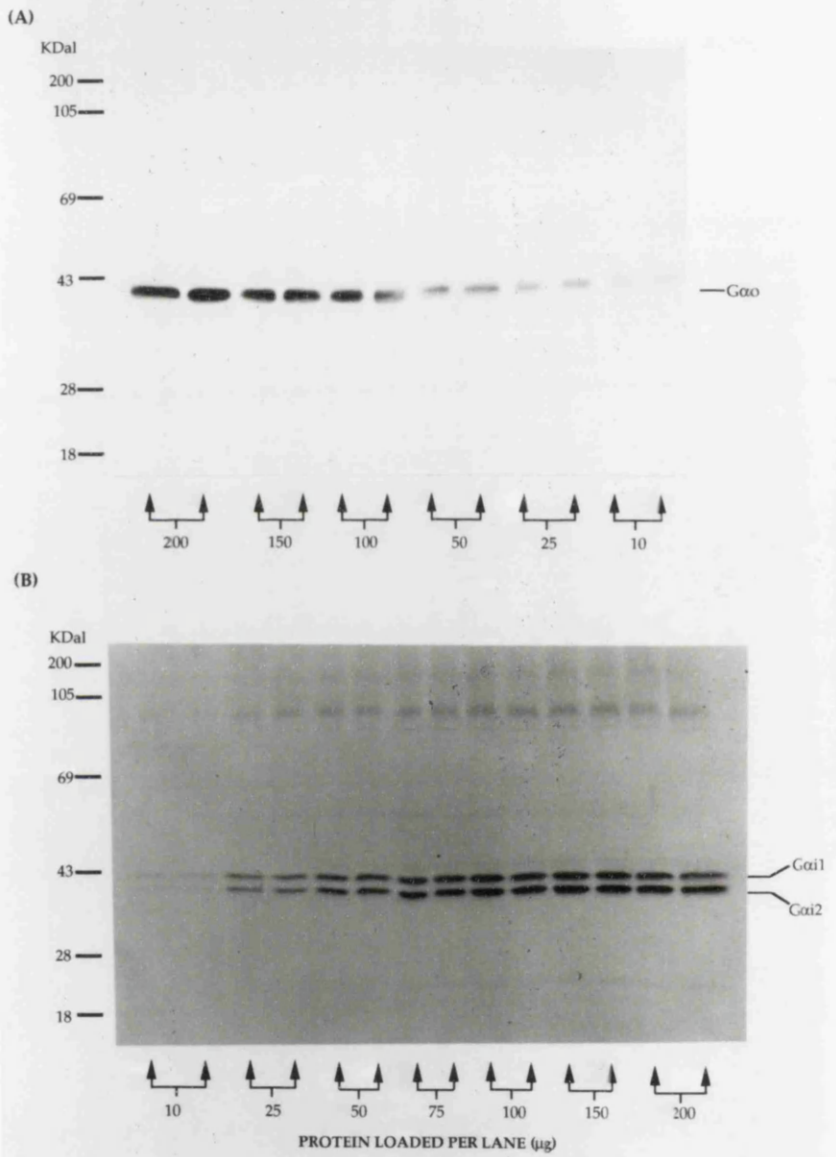
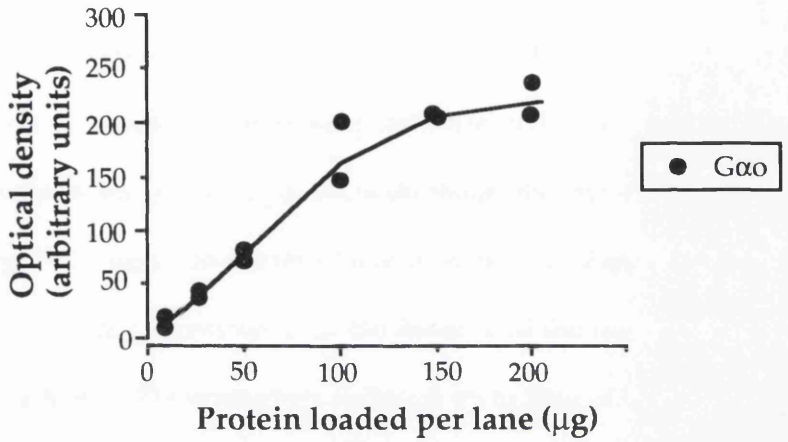

(A)
KDal
200
105
69
43
28
18
Gαo
200
150
100
50
25
10
(B)
KDal
200
105
69
43
28
18
Gαi1
Gαi2
10
25
50
75
100
150
200
PROTEIN LOADED PER LANE (μg)

FIGURE 2.

(A)


Optical density (arbitrary units)
300
250
200
150
100
50
0
0
50
100
150
200
Protein loaded per lane (μg)
Gαo

(B)

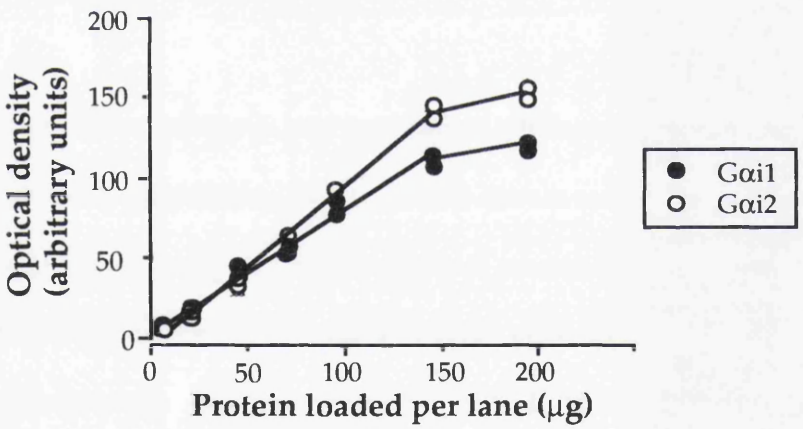

Optical density (arbitrary units)
200
150
100
50
0
0
50
100
150
200
Protein loaded per lane (μg)
Gαi1
Gαi2

FIGURE 3.

4.2 Characterisation of the GTPase assay.

4.2.1 Linearity of the GTPase assay.

Preliminary experiments were carried out to examine the relationship between the rate of GTP hydrolysis, the amount of membrane protein (μg) and the assay incubation period. **Figure 4 (A)** demonstrates that with 5μg of rat brain tissue, the rate of both high and low affinity GTPase is linear for up to 20 minutes incubation period. **Figure 4 (B)** shows the effect of increasing the protein concentration on the linearity of the reaction and illustrates that the high and low affinity GTPase reactions are linear up to 20μg of brain extract. Therefore, with 5-10μg of membrane protein extract the substrate concentration and the creatine phosphate ATP regenerating components are not limiting over 15 minutes.

4.2.2 Kinetic analysis of GTPase activity.

The kinetics of GTPase hydrolysis was analysed in whole rat brain and human membrane preparations from the frontal cortex of Alzheimer's disease sample 30 and control sample 35. **Figure 5** illustrates the effect of GTP concentration on percent hydrolysis of [γ^{32}P] GTP. There is a sharp fall in the hydrolysis of the total [γ^{32}P] GTP up to approximately 5μM GTP. Above this concentration the rate of hydrolysis is more gradual. This observation is consistent with the GTP hydrolysis profile described by Cassel and Selinger, (1976), and indicates the presence of two enzyme species, the high and low affinity (non-specific) GTPase, operating with differing enzyme kinetics.

By extrapolating Lineweaver Burk plot of the data as shown in **Figure 5**, the Vmax and Km values for each species (the high affinity and low affinity GTPase respectfully), can be estimated (**Figure 6**). It should be noted however, that there are only three points of GTP concentrations used to estimate the low affinity activity in the human samples, and in any

case the calculations are with impure enzymes. Thus, the values generated are only approximates. The calculated values are listed in **Table 4**. These values suggest that the kinetics for high and low affinity GTPase are comparable for rat, human control and Alzheimer's disease brain tissue. Although the Km values for the low affinity GTPase of the control and Alzheimer diseased samples are similar, the Vmax value obtained would appear to be higher for the Alzheimer's disease sample when compared with the human control sample. Similar observations were made in the human frontal cortex and hippocampus studies (sections **4.4.3** and **4.4.6**).

Table 4. Km and Vmax values of high and low affinity GTPase in membranes from rat brain and from the frontal cortex of Alzheimer's disease and age-matched control subjects.

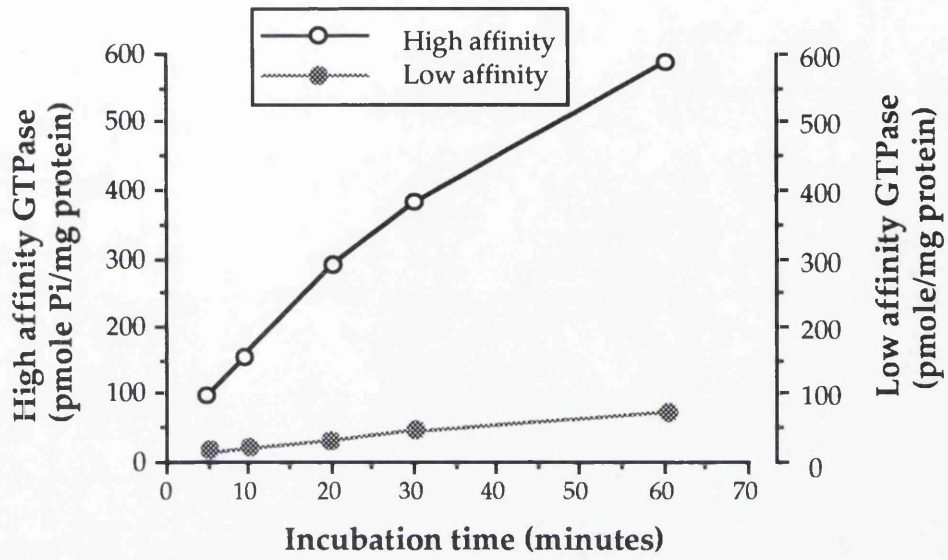
	LOW AFFINITY		HIGH AFFINITY	
	Km(mM)	Vmax (pmol/min/mg)	Km(mM)	Vmax (pmol/min/mg)
RAT	48.4	1092	0.467	39.1
Alzheimer 30	66.7	910	0.783	52.8
Control 35	52.9	789	0.756	46.3

FIGURE 4. The effect of assay incubation period and the amount of membrane protein extract from whole rat brain on the hydrolysis of GTP. **(A)** High and low affinity (nonspecific) GTPase activity measured in assays incubated for up to 60 minutes at 25^{o} C. **(B)** High and low affinity (nonspecific) GTPase measured within a range of 1-20μg of protein.

FIGURE 5. Analysis of GTP hydrolysis at concentration of 0.02-100μM GTP expressed as the percent hydrolysis of the total [γ^{32}P] GTP. **(A)** 10μg of membrane prepared the frontal cortex of sample C35; **(B)** 10μg of membrane prepared the frontal cortex Alzheimer's disease (AD) sample 30; **(C)** 5μg of whole rat brain sample.

FIGURE 6. Lineweaver-Burk plots of the high affinity GTPase activity **(A)**, and low affinity GTPase activity **(B)**, in human control (C35), Alzheimer's diseased (AD 30) and rat brain preparations. Data from these experiments quantifies the magnitude of GTP hydrolysis at a series of GTP concentrations from 0.02 to 1.02μM GTP **(A)**, and 5 to 100μM GTP **(B)**. Within these GTP concentrations, the Km and Vmax values were calculated and the values are shown in **Table 4.**

(A)


High affinity
Low affinity
High affinity GTPase
(pmole Pi/mg protein)
Low affinity GTPase
(pmole/mg protein)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
Incubation time (minutes)

(B)

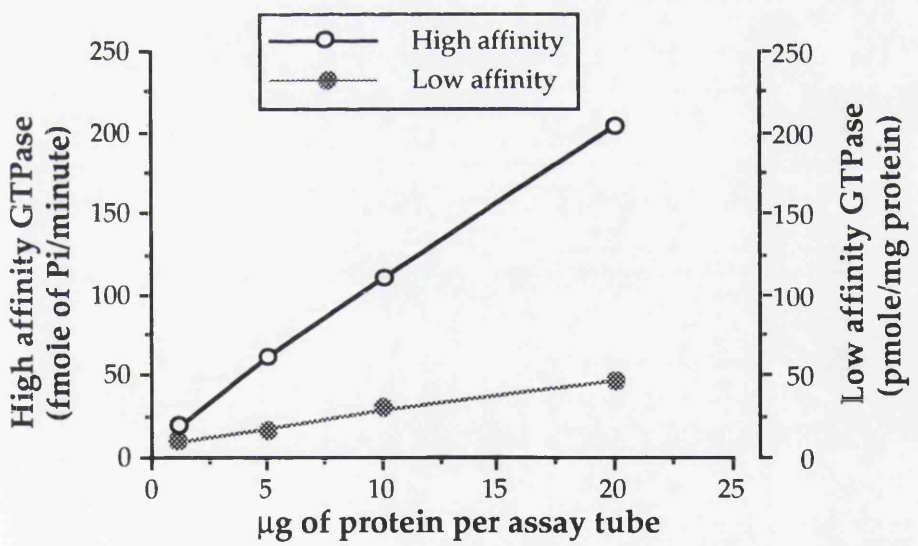
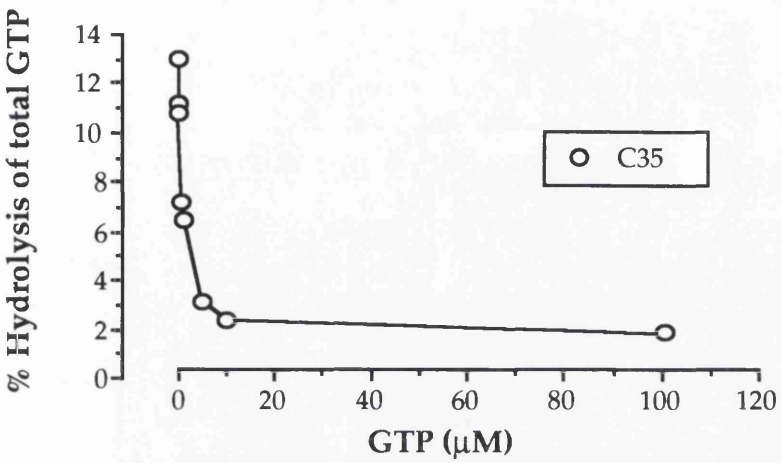
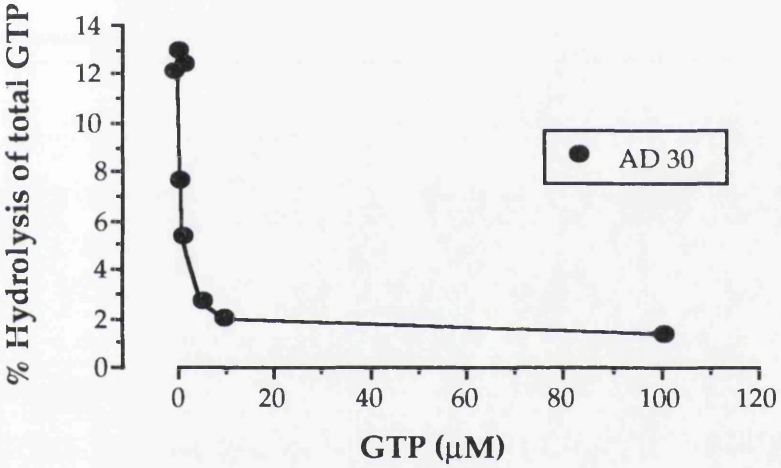

High affinity
Low affinity
High affinity GTPase
(fmole of Pi/minute)
Low affinity GTPase
(pmole/mg protein)
0
50
100
150
200
250
0
5
10
15
20
25
µg of protein per assay tube

FIGURE 4.

(A)


% Hydrolysis of total GTP
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
GTP (μM)
C35

(B)


% Hydrolysis of total GTP
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
GTP (μM)
AD 30

(C)

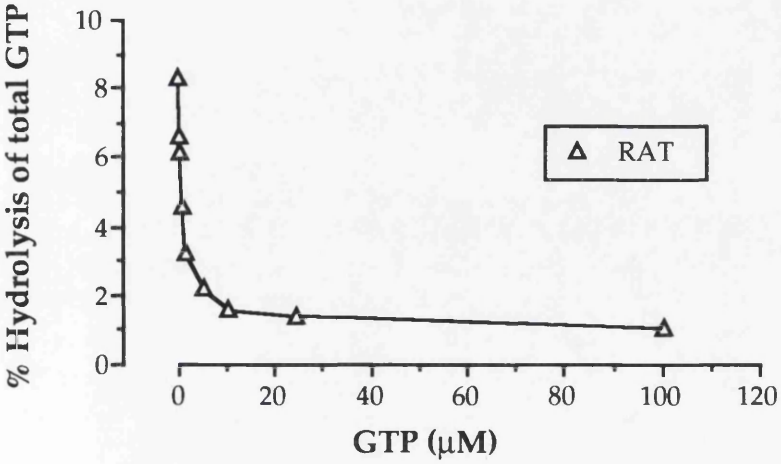
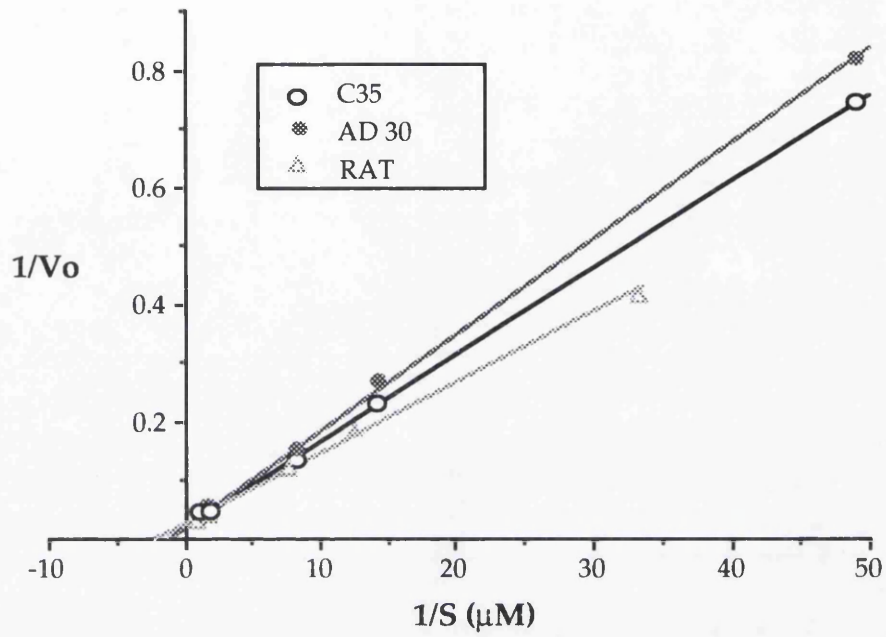

% Hydrolysis of total GTP
10
8
6
4
2
0
0
20
40
60
80
100
120
GTP (μM)
RAT

FIGURE 5.

(A)


C35
AD 30
RAT
1/Vo
0.8
0.6
0.4
0.2
-10
0
10
20
30
40
50
1/S (μM)

(B)

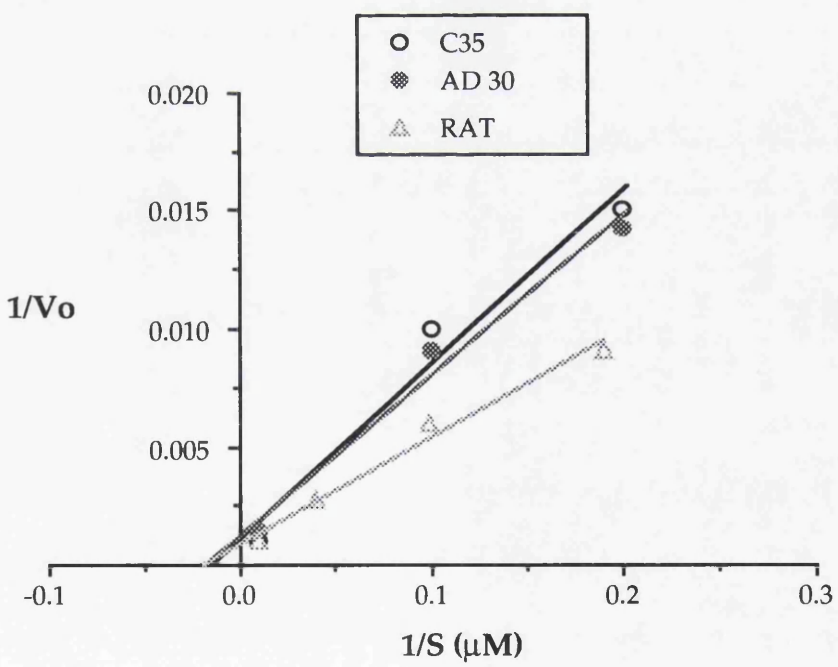

C35
AD 30
RAT
1/Vo
0.020
0.015
0.010
0.005
-0.1
0.0
0.1
0.2
0.3
1/S (μM)

FIGURE 6.

4.2.3 Agonist stimulation of GTPase.

The dose response stimulation of the basal high affinity GTPase activity with the muscarinic agonist carbachol, and the $GABA_B$ agonist baclofen was assayed in rat and human brain samples (**Figure 7**). The rat brain preparation gave stimulation of 130-150% of the basal activity which was achieved routinely. The human samples, on the other hand, failed to demonstrate the same magnitude of stimulation with either carbachol or baclofen. Alzheimer's diseased sample 30 demonstrated an increase of only approximately 125% of basal while a negligible degree of stimulation was observed for the control sample 35.

Given that a requirement of GTPase activity is the presence of low concentrations of Mg^{2+} (Gilman, 1987) and that nucleotides (present at high concentrations in the assay medium) have a chelating capacity (Bartfai, 1979), the possibility existed that the human samples required a specific divalent ion concentration for maximum stimulation. This was investigated by conducting the GTPase assay over several concentrations of the divalent chelator EDTA (**Figure 8**). Maximum stimulation with carbachol and baclofen was achieved for the rat and Alzheimer sample 30 at 0.1mM EDTA, whereas both agonists failed to stimulate the control human sample. While it remained possible that specific permutations of the selective ion concentration of the assay cocktail are required for stimulating human GTPase activity, this experiment gave no indication that the ionic environment of the assay was the source of the poor stimulation. Subsequent analysis suggested that the interval between the removal and freezing of brain had a significant influence on the activity (section **4.3.3**).

FIGURE 7. Effect of the muscarinic agonist carbachol and the $GABA_B$ agonist baclofen on rat and human brain high affinity GTPase activity. Human frontal cortex membranes (10μg) from control 35 **(A)**, Alzheimer's disease (AD) sample 30 **(B)**, and whole rat brain membrane (5μg) **(C)** were assayed in the presence of the agonists at the concentration indicated. The basal GTPase activities were assayed with 10mM Tris/HCl, pH7.4, replacing the agonists in the assay medium. The degree of stimulation is expressed as the percent of basal high affinity GTPase activity.

FIGURE 8. The effect of EDTA concentration on the activity of high and low affinity (nonspecific) GTPase measured in rat brain and human control and Alzheimer's disease frontal cortex preparations. The high affinity GTPase was assayed in the presence and absence of carbachol (1mM) and baclofen (1mM) and the data shown as absolute activity.

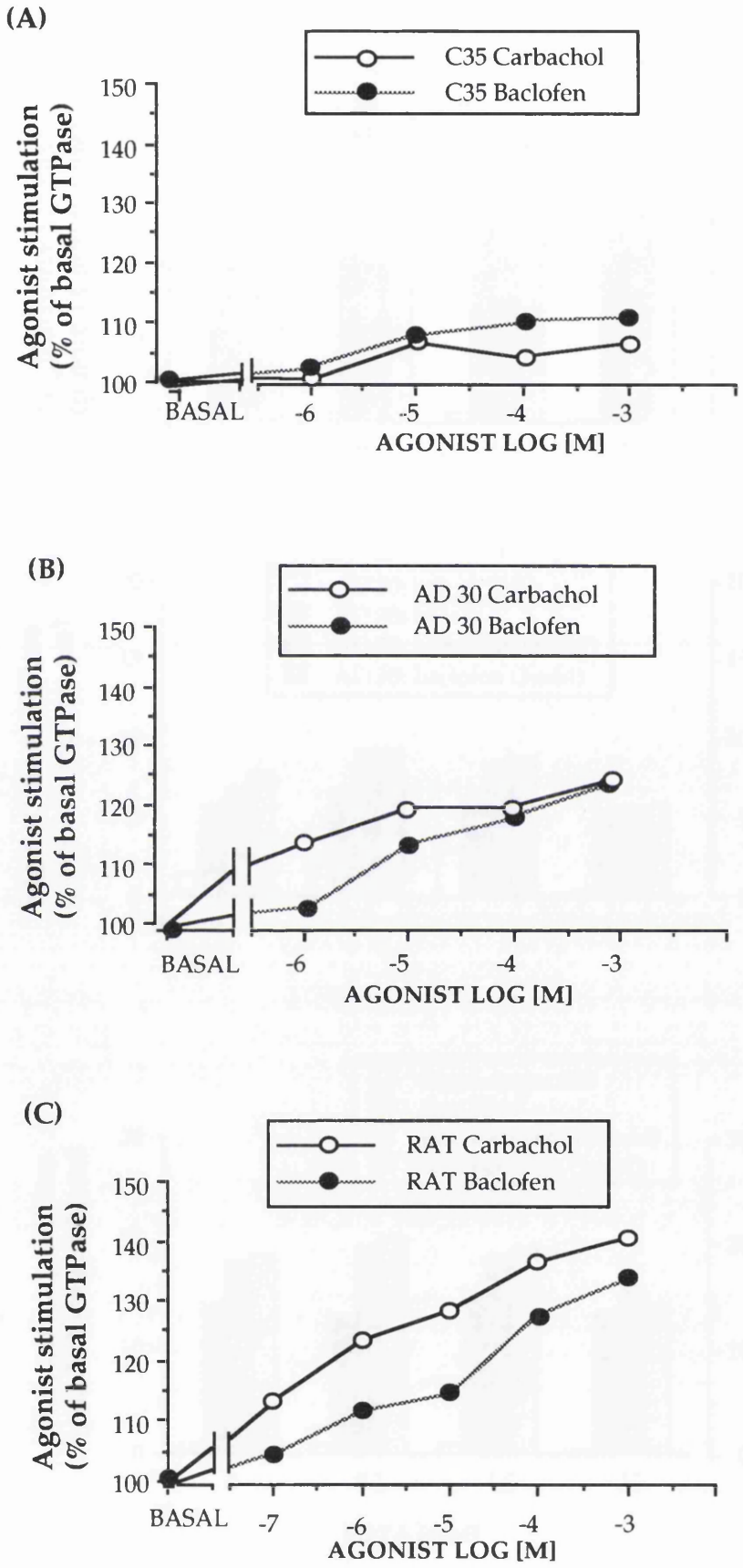

(A)
C35 Carbachol
C35 Baclofen
Agonist stimulation (% of basal GTPase)
150
140
130
120
110
100
BASAL
-6
-5
-4
-3
AGONIST LOG [M]
(B)
AD 30 Carbachol
AD 30 Baclofen
Agonist stimulation (% of basal GTPase)
150
140
130
120
110
100
BASAL
-6
-5
-4
-3
AGONIST LOG [M]
(C)
RAT Carbachol
RAT Baclofen
Agonist stimulation (% of basal GTPase)
150
140
130
120
110
100
BASAL
-7
-6
-5
-4
-3
AGONIST LOG [M]

FIGURE 7.

4.3 The influence of the interval between death and brain freezing on G-proteins.

This experiment examined the influence of the interval between death and freezing of brain on the stability of G-protein subunits and their high affinity GTPase activity. Rats were sacrificed and stored at $4^{o}C$ for different time periods. The brains were then dissected and frozen until required (section 3.5).

4.3.1 Immunoblot analysis of the G-protein subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, G_{β}.

The effect of the interval from death to the freezing of the brains (postmortem interval) on the stability of the proteins $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, and G_{β} was measured by the formation of an immunocomplex. **Figure 9** illustrates the immunoblot pattern obtained for **(A)** $G_{\alpha o}$; **(B)** $G_{\alpha i}1$ and $G_{\alpha i}2$; **(C)** G_{β}. None of the G-protein subunits analysed showed any visible sign of protein degradation which would have been suggested by either a decrease in the signal intensity of the specific protein(s), or by the appearance of low molecular weight immunopositive degradation products containing the epitope fragment. Densitometric analysis of the immunoblots are shown in **Figure 10.** As previously stated (section 4.1.1), the G_{β} antisera (BN1) became available at the latter stages of the project. By this time, the postmortem interval samples had been stored for a prolonged period. This is likely to have contributed to the large variation in signal intensity for each time period when compared to the $G_{\alpha o}$ and $G_{\alpha i}$ data. However, at 48 hours the G_{β} proteins were still visible. Analysis of the data by one-way analysis of variance (ANOVA) found that for each protein measured, there was no difference between the postmortem delay values when compared to the zero hour controls. The stability of $G_{\alpha s}$ over prolonged postmortem interval was confirmed in an earlier study in this laboratory (Ross, 1992).

This experiment demonstrated that G-proteins are not subjected to gross degradation during the postmortem intervals that are encountered with human brain material. Indeed, there was

no significant correlation between the postmortem interval and the intensity of G-protein levels observed in the study of Alzheimer's disease (section **4.4.8**)

FIGURE 9. The effect of the interval between the death and brain freezing on the rat cortex immunoblot profile of G-proteins subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, G_{β}. **(A)** $G_{\alpha o}$ of rat cortex (50μg) samples stored at 4°C for 0, 6, 12, 24, and 48 hours detected with OC1 at 1/10,000 dilution; **(B)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with SG2 (1/200); **(C)** G_{β} (50μg) incubated with BN1 (1/200).

FIGURE 10. Densitometric analysis of the G-protein immunoblots of rat cortex postmortem interval samples (**Figure 9**). **(A)** $G_{\alpha o}$ level presented as the mean±SEM where n=5 at zero hour and n=3 at all other points; **(B)** $G_{\alpha i}1$ and $G_{\alpha i}2$ level; **(C)** G_{β}. Statistical analysis by ANOVA failed to demonstrate a significant difference ($P>0.05$) between the zero hour samples when compared to the other postmortem delay periods in any of the subunits analysed.

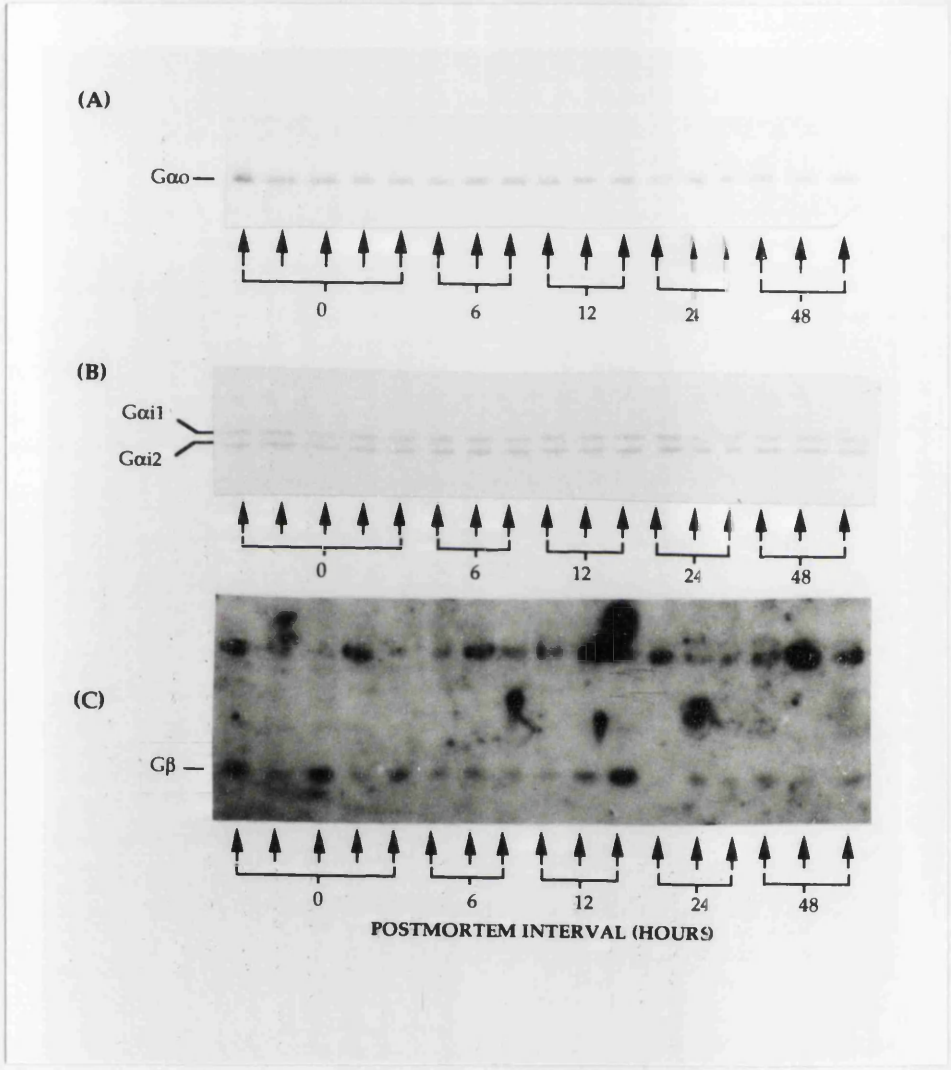
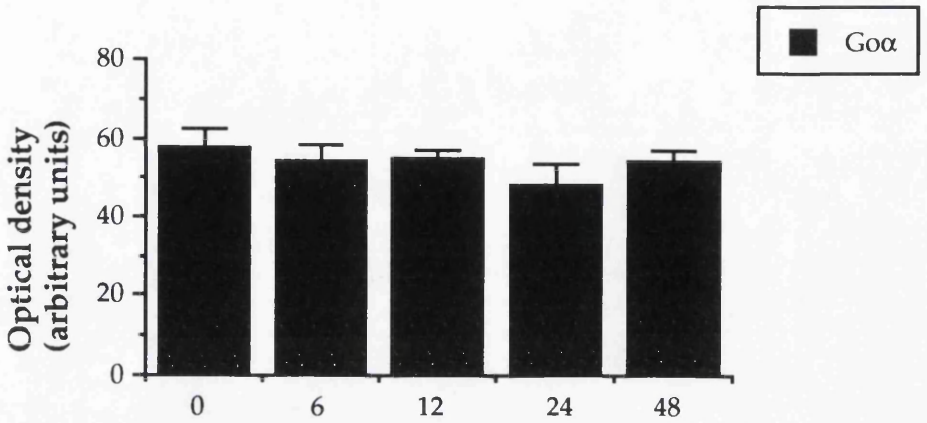
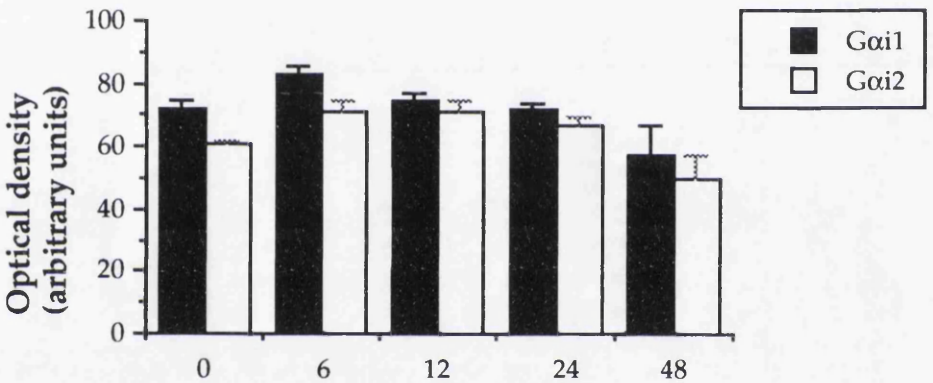

(A)
Gαo
0
6
12
24
48
(B)
Gαi1
Gαi2
0
6
12
24
48
(C)
Gβ
0
6
12
24
48
POSTMORTEM INTERVAL (HOURS)

FIGURE 9.

(A)



(B)



(C)

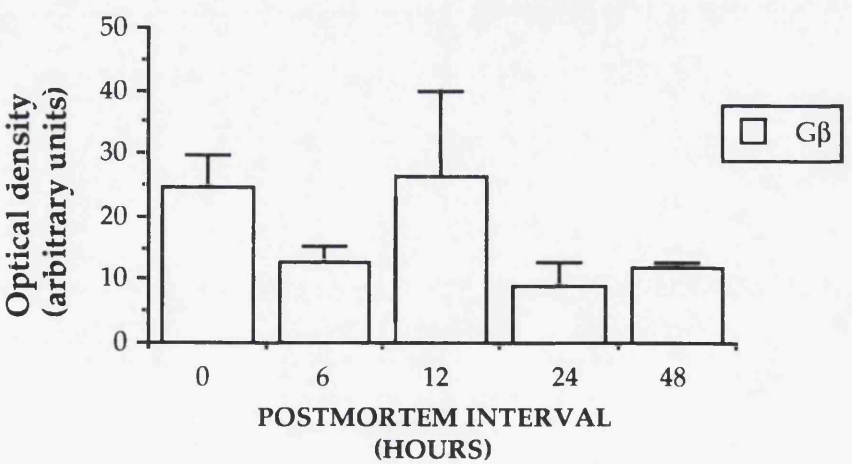


FIGURE 10

4.3.2 Analysis of choline acetyltransferase activity (ChAT).

The activity choline acetyltransferase was assayed in aliquots of the homogenate fraction of the rat cortex postmortem interval samples. **Figure 11** illustrates the stability choline acetyltransferase activity with increasing postmortem delay to brain freezing. There was a 14% reduction in choline acetyltransferase activity at 6 hours postmortem compared to the activity measured in the fresh, zero hour control samples. Statistical analysis by ANOVA demonstrated that the overall reduction observed did not achieve statistical significance. Thus, the activity of choline acetyltransferase was minimally affected by postmortem interval.

4.3.3 Analysis of basal and agonist stimulation of the high affinity GTPase activity.

This experiment demonstrated that the period between the removal and storage of brain tissue had a significant effect on the activity of the high affinity basal GTPase and on the stimulatory capacity of carbachol and baclofen on the high affinity GTPase (**Figure 12**). The low affinity (nonspecific GTPase) activity however, was stable over the range of postmortem intervals investigated and this is in agreement with the findings of Nicol et al. (1981). When compared to the fresh zero hour samples, the basal high affinity activity were reduced by 50% and 56% for the postmortem interval periods of 24 and 48 hours respectively. Statistical analysis by ANOVA demonstrated that these reductions were statistically significant ($P<0.05$).

This study also demonstrated that the stimulatory capacity of carbachol and baclofen of the high affinity GTPase activity was compromised in brains which had been left for prolonged periods before freezing. Carbachol stimulation was reduced from 147% of basal GTPase activity in fresh samples, to 124%, 116%, 112% and 108% at 6, 12, and 24 hours postmortem interval respectively. Analysis by ANOVA demonstrated that compared to the fresh

samples, these reductions achieved statistical significance at the 24 hour and the 48 hour time intervals ($P<0.05$). Baclofen stimulation was also reduced from 134% of basal GTPase activity in fresh samples to 128%, 125%, 113% and 102% at 6, 12, and 24 hours postmortem intervals. Analysis by ANOVA demonstrated that compared to the fresh samples, the reduction observed for the 48 hour time point achieved statistical significant ($P<0.05$). Thus, the integrity of the G-protein coupling to muscarinic and $GABA_B$ receptors were influenced by prolonged delays between death and freezing of brain tissue.

FIGURE 11. The effect of the period between death and brain freezing (postmortem interval) on the activity of choline acetyl transferase (ChAT) of rat cortex. Rats were sacrificed and the brains rapidly removed and frozen (zero hours) or stored at 4°C for 6 to 48 hours. The activity was measured in aliquots of the tissue homogenates from cortex dissected from the rat brains. The values shown are mean±SEM where n=5 for zero hour controls and n=3 for all other postmortem time points. At 6 hours postmortem interval, the activity was decreased by 14 percent compared to the zero hour sample. This reduction failed to reach statistical significance ($P>0.05$) when the data was analysed by ANOVA.

FIGURE 12. The effect of the period between death and freezing (postmortem interval) of rat brain on the activities of the high and low affinity GTPase and the agonist stimulation of high affinity GTPase of rat cortex. Rats were sacrificed and the brains rapidly removed and frozen (zero hours) or removed after the rats were stored at 4°C for 6-48 hours. **(A)** GTPase activity was assayed with 5µg of membrane preparation and the total (high and low affinity), basal (high affinity) and nonspecific (low affinity) GTP hydrolysis are shown as mean±SEM. At zero hours n=5 and n=3 at all other postmortem interval time points. Statistical analysis by ANOVA demonstrated a significant reduction in the total and basal GTPase levels at 24 and 48 hours compared to the zero hour samples (where * denotes $p<0.05$). **(B)** The high affinity GTPase assayed in the presence and absence of carbachol(1mM) and the presence and absence of baclofen (1mM). The effect of the agonists is expressed as the percent stimulation of basal high affinity activity. Comparison of the zero hour values to that obtained for the postmortem samples by ANOVA demonstrated a significant reduction in the magnitude of stimulation by carbachol at 24 hour and 48 hour time intervals; and in the stimulation achieved by baclofen at 48 hours (where * denotes $p<0.05$).

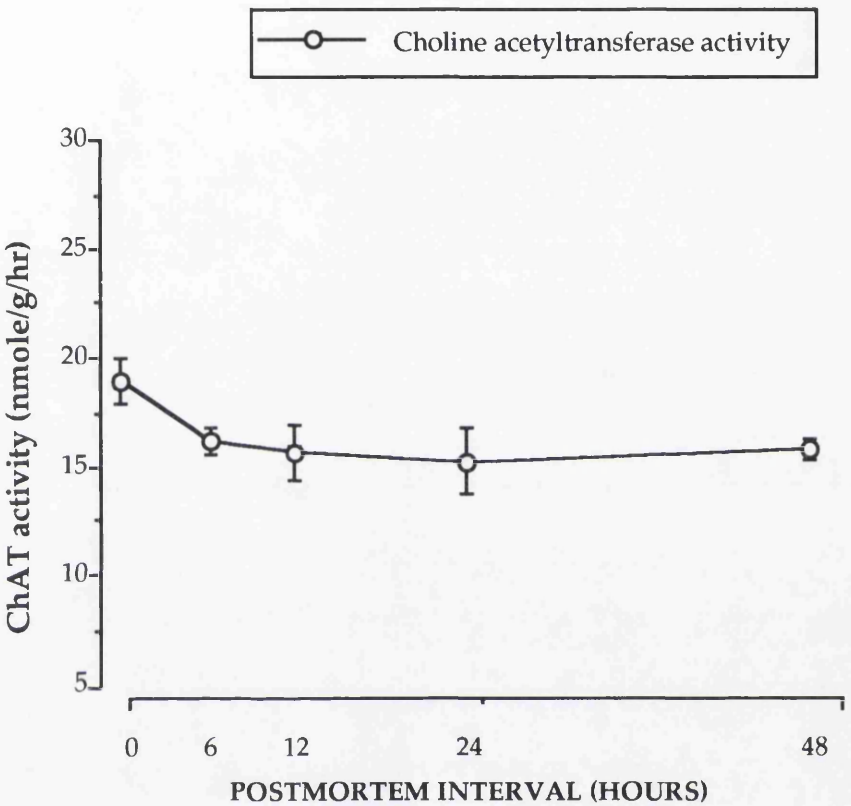
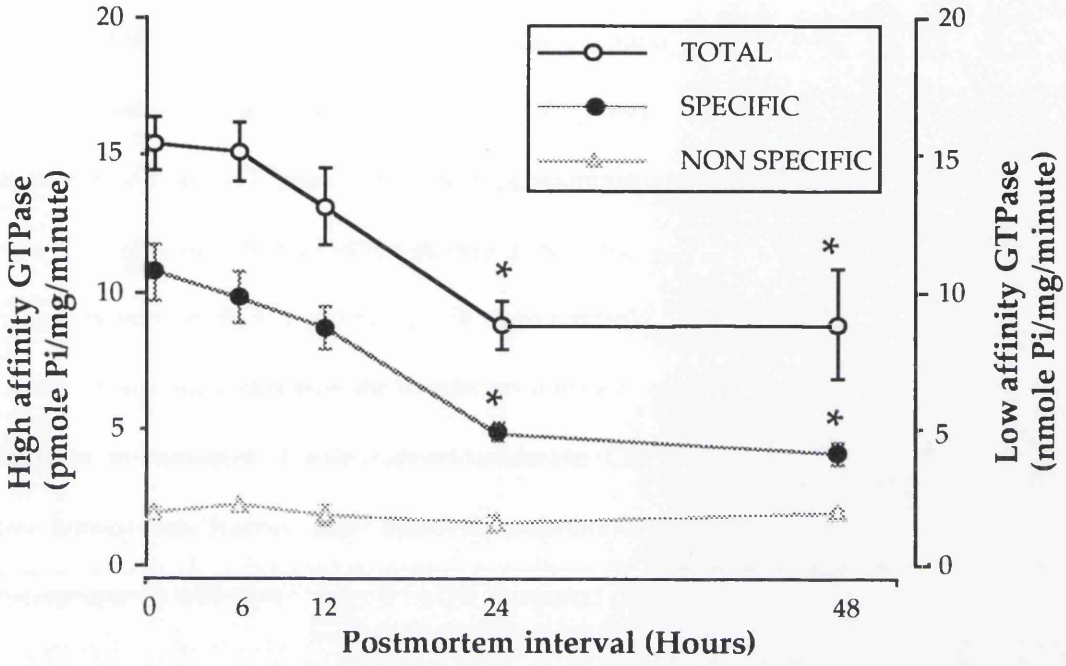

Choline acetyltransferase activity
ChAT activity (nmole/g/hr)
30
25
20
15
10
5
0
6
12
24
48
POSTMORTEM INTERVAL (HOURS)

FIGURE 11.

(A)


TOTAL
SPECIFIC
NON SPECIFIC
High affinity GTPase (pmole Pi/mg/minute)
Low affinity GTPase (nmole Pi/mg/minute)
Postmortem interval (Hours)
0
5
10
15
20
0
6
12
24
48
*

(B)

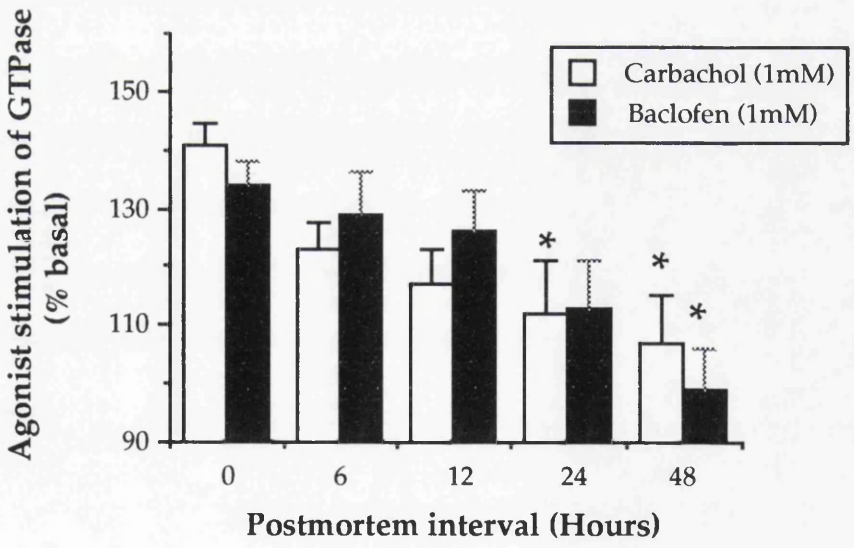

Carbachol (1mM)
Baclofen (1mM)
Agonist stimulation of GTPase (% basal)
Postmortem interval (Hours)
90
110
130
150
0
6
12
24
48
*

FIGURE 12.

4.4 G-proteins and related mechanisms in Alzheimer's disease.

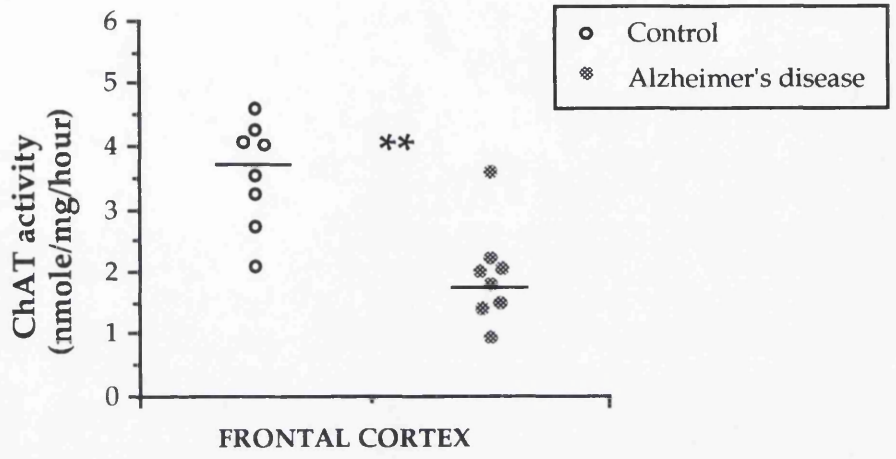
In order to gain the maximum information on the effect of Alzheimer's disease on G-protein mediated transduction event, crude membranes were prepared in sufficient quantities from each sample to permit the analysis of a variety of G-proteins and related activities. This study was performed on the frontal cortex and hippocampus regions since these regions have been shown to be severely effected by the disease at both the neurochemical and pathological level. In each region studied, a group size of eight controls and eight Alzheimer's disease samples were chosen since this was the maximum number of samples that can be analysed within the same immunoblot. Choline acetyltransferase (ChAT) activity was also measured in the same homogenate fraction from which the membranes were prepared. Therefore, the various measurements within each region can be compared directly.

4.4.1 Analysis of choline acetyltransferase activity in the frontal cortex and hippocampus.

Figure 13 illustrates the scatter distribution of ChAT activity measured in tissue homogenates prepared from the frontal cortex (A), and the hippocampus (B) of eight control and eight Alzheimer's disease samples. In both brain regions there is a significant reduction in the activity measured in the Alzheimer's disease group when compared to the control level. The frontal cortex activity was reduced by 47% and the hippocampus activity was reduced by 65% which is in agreement with the cholinergic deficit almost universally observed in Alzheimer's disease.

FIGURE 13. Choline acetyltransferase activity (ChAT) of eight Alzheimer's disease (AD) subjects and eight age matched control subjects measured in frontal cortex **(A)** and hippocampus **(B)** tissue homogenates. The activity measured in the frontal cortex was reduced by 47% in the Alzheimer's disease group compared to the control group sample mean. The hippocampus Alzheimer's disease values was reduced by 65% compared to the mean value of the control group. Statistical analysis by Students t-test demonstrated that the reductions observed in both regions were statistically significant (where ** denotes $P<0.01$.).

(A)


Control
Alzheimer's disease
ChAT activity
(nmole/mg/hour)
0
1
2
3
4
5
6
**
FRONTAL CORTEX

(B)

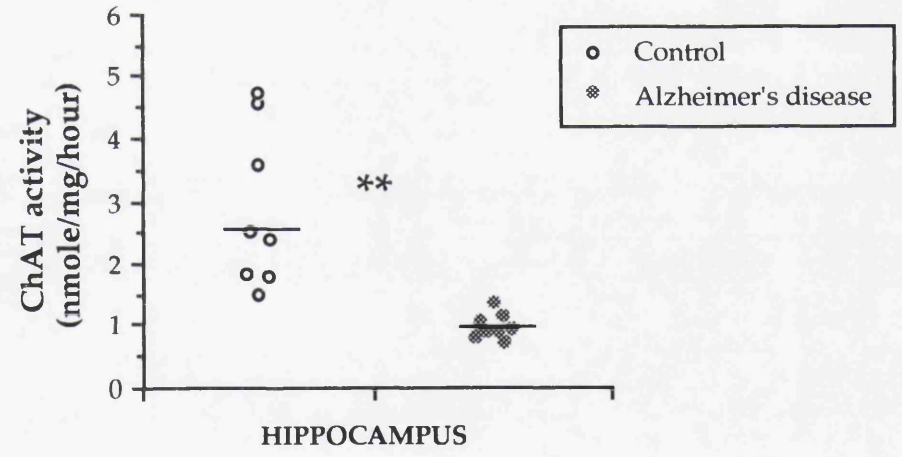

Control
Alzheimer's disease
ChAT activity
(nmole/mg/hour)
0
1
2
3
4
5
6
**
HIPPOCAMPUS

FIGURE 13.

4.4.2 Immunoblot analysis of G-proteins in the frontal cortex.

The immunoblot profile of the Gα subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ and the Gβ subunits, of eight control and eight Alzheimer's disease samples are shown in **Figure 14** and the results of the densitometric analysis of the immunoblots is shown as scatter plots in **Figure 15.** Since antisera SG2 is raised against an epitope which is identical for both $G_{\alpha i}1$ and $G_{\alpha i}2$ (section 3.9) then direct comparisons can be made between the level of each $G_{\alpha i}$ subtype. Similarly both $G_{\alpha s}S$ and $G_{\alpha s}L$ are detected with the same antisera (CS1) and these subtypes can be compared. **Figure 16.** illustrates the ratio of $G_{\alpha i}1$ to $G_{\alpha i}2$ and also $G_{\alpha s}S$ to $G_{\alpha s}L$.

Each G protein subunit(s) measured typically gave a coefficient of variance of between 25 to 40 percent. This variability is similar to that encountered in most studies of Alzheimer's disease. Statistical analysis by Student's t-test failed to demonstrate a significant difference in the levels of $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$ and $G_{\alpha s}S$ measured in Alzheimer's disease samples when compared to control samples. Similarly, the ratio of $G_{\alpha i}1$ to $G_{\alpha i}2$ was not significantly different between the two control and Alzheimer's disease groups. The difference between the ratio of $G_{\alpha s}L$ to $G_{\alpha s}S$ observed in the Alzheimer's samples compared to the control samples was found to be statistically significant when analysed by Student's t test ($P<0.05$).

The mean Gβ subunit level was reduced by 57 percent in the Alzheimer's samples when compare to the mean control values. This reduction achieved statistical significance when analysed by Student's t-test ($P<0.05$). Two factors should be considered which are relevant to the Gβ data; first, the analysis of the Gβ subunit was conducted on membrane samples which had been stored for 10 months (section 4.1.1); second, the Gβ immunoblot, **Figure 14 (A)**, also detected a major band at approximately 100 KDal which was not observed with fresh preparations. Taken together, these factors could indicate that the frontal cortex samples had been subjected to proteolysis during storage. Nonetheless, the sharp intense signal of the BN1-A and the Gβ proteins suggests that these proteins have remained undegraded.

FIGURE 14. Immunoblot analysis of G-protein subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ and G_{β} detected with eight Alzheimer's disease and eight control frontal cortex membrane preparations. **(A)** G_{β} (50μg) detected with BN1 at 1/200 dilution; **(B)** $G_{\alpha o}$ (50μg) detected with OC1 at 1/10,000 dilution; **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with SG2 at 1/200 dilution; **(D)** $G_{\alpha s}L$ and $G_{\alpha s}S$ (50μg) detected with CS1 at 1/200 dilution.

FIGURE 15. Scatter distribution presentation of the densitometric analysis of the G-protein subunit immunoblots (**Figure 14**) with the mean values indicated by the plain line (—). **(A)** G_{β} values. **(B)** $G_{\alpha o}$ values. **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$ are shown on the same graph since both $G_{\alpha i}$ subtypes are detected with the antisera SG2 and the values for each are therefore comparable. **(D)** $G_{\alpha s}L$ and $G_{\alpha s}S$ also shown on the same graph since both detected with antisera CS1. Statistical analysis by Student's t-test demonstrated that the reduction observed for the level of G_{β} in the Alzheimer's disease samples compared to control levels achieved statistical significant (where * denotes $P<0.05$).

FIGURE 16. Comparison of the ratio of $G_{\alpha i}1/G_{\alpha i}2$ and $G_{\alpha s}L/G_{\alpha s}S$ detected with the frontal cortex of eight Alzheimer's disease and eight control frontal cortex membrane preparations. Both $G_{\alpha i}1$ and $G_{\alpha i}2$ are detected within the same immunoblot by antisera (SG2), similarly $G_{\alpha s}L$ and $G_{\alpha s}S$ are both detected with antisera CS1. Therefore, the level of each subtype can be expressed as a ratio. Statistical analysis by Student's t-test demonstrated a significant difference between the ratio of $G_{\alpha s}L/G_{\alpha s}S$ measured in Alzheimer's disease samples compared to controls (where* denotes $P<0.05$). The difference between the ratio of $G_{\alpha i}1/G_{\alpha i}2$ observed for control and Alzheimer's samples failed to reach statistical significance.

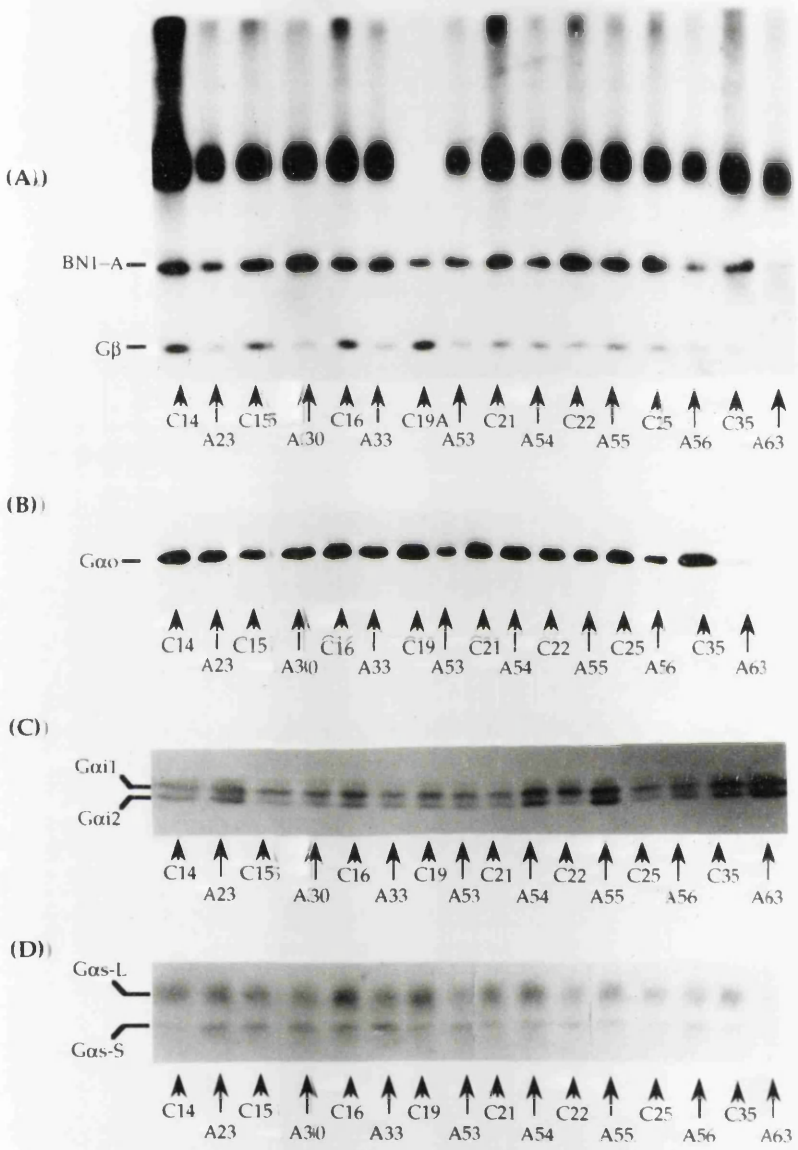

(A)
BN1-A
Gβ
C14
A23
C15
A30
C16
A33
C19A
A53
C21
A54
C22
A55
C25
A56
C35
A63
(B)
Gαo
C14
A23
C15
A30
C16
A33
C19
A53
C21
A54
C22
A55
C25
A56
C35
A63
(C)
Gαi1
Gαi2
C14
A23
C15
A30
C16
A33
C19
A53
C21
A54
C22
A55
C25
A56
C35
A63
(D)
Gαs-L
Gαs-S
C14
A23
C15
A30
C16
A33
C19
A53
C21
A54
C22
A55
C25
A56
C35
A63

FIGURE 14.

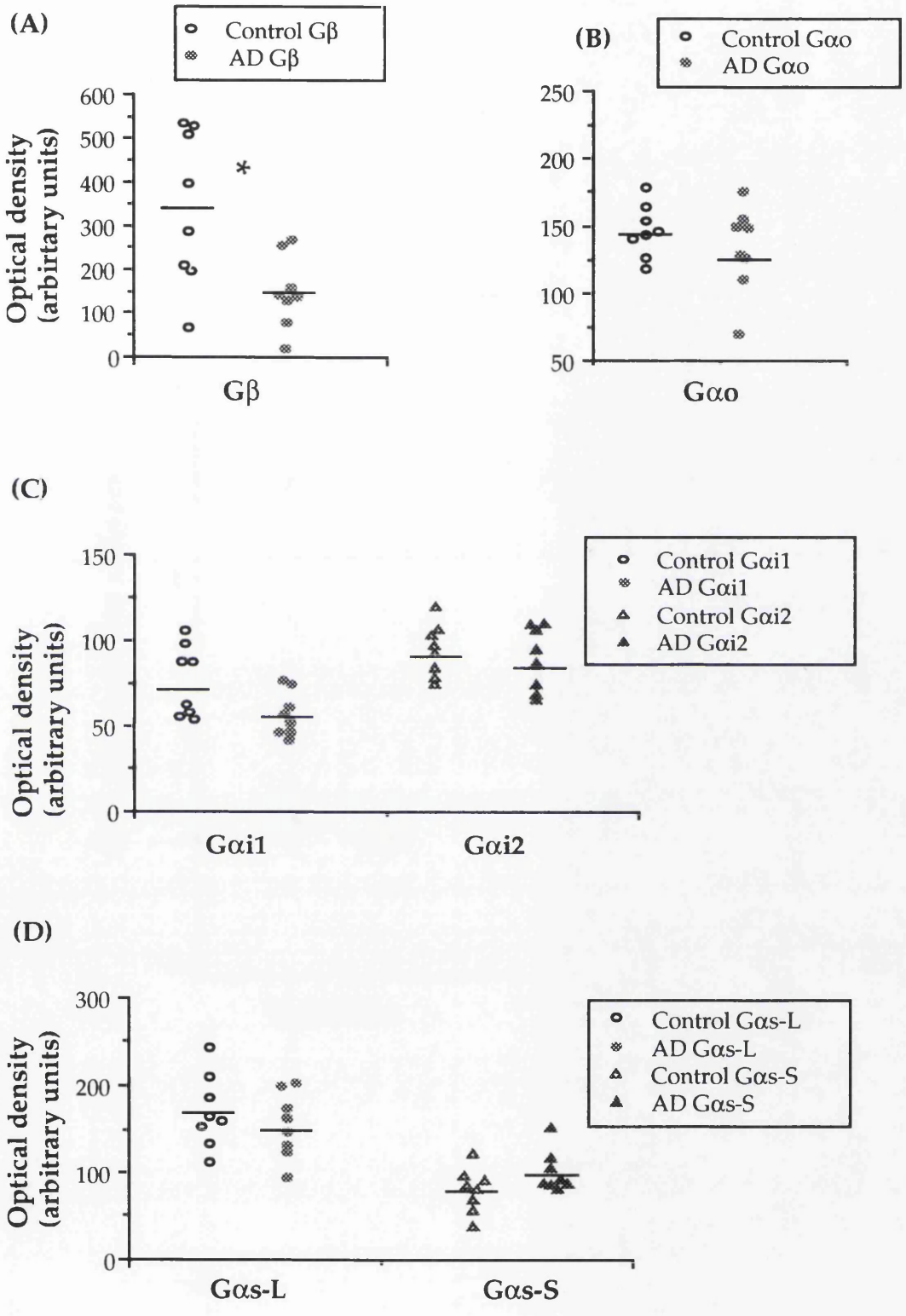

(A)
Control Gβ
AD Gβ
Optical density (arbitrary units)
600
500
400
300
200
100
0
*
Gβ
(B)
Control Gαo
AD Gαo
250
200
150
100
50
Gαo
(C)
Control Gαi1
AD Gαi1
Control Gαi2
AD Gαi2
Optical density (arbitrary units)
150
100
50
0
Gαi1
Gαi2
(D)
Control Gαs-L
AD Gαs-L
Control Gαs-S
AD Gαs-S
Optical density (arbitrary units)
300
200
100
0
Gαs-L
Gαs-S

FIGURE 15.

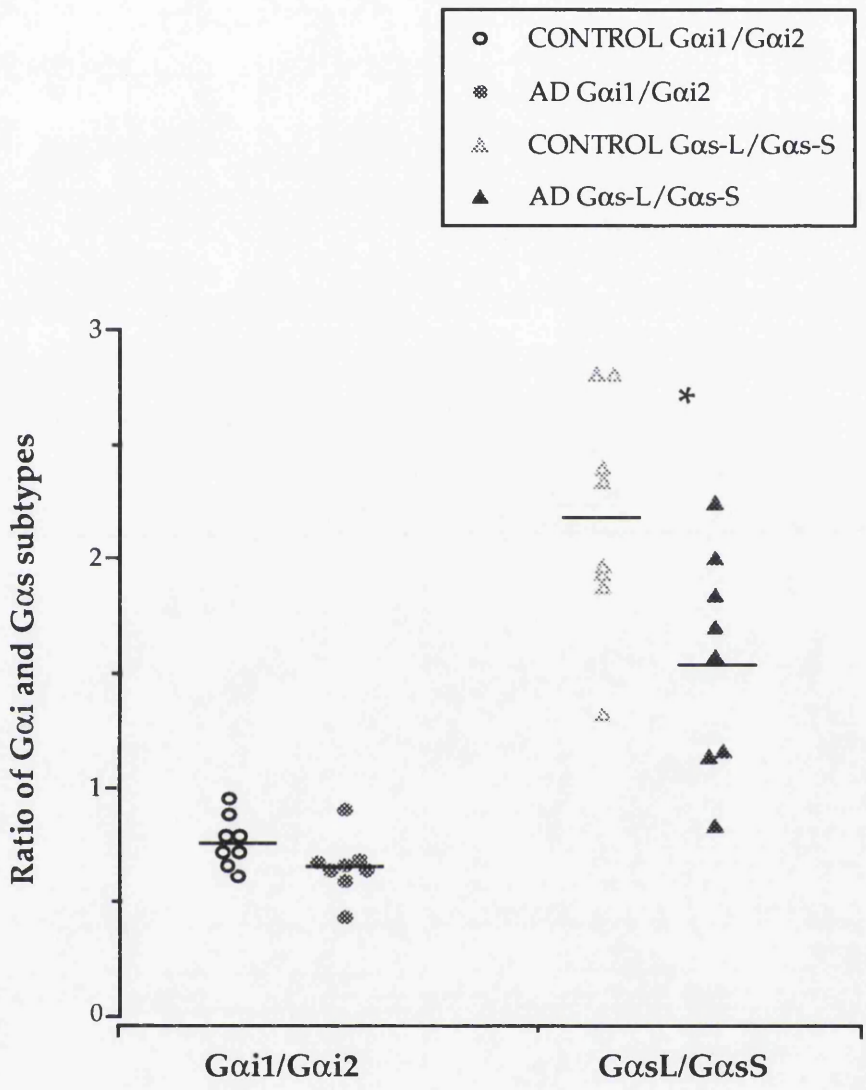

CONTROL Gαi1/Gαi2
AD Gαi1/Gαi2
CONTROL Gαs-L/Gαs-S
AD Gαs-L/Gαs-S
Ratio of Gαi and Gαs subtypes
3
2
1
0
*
Gαi1/Gαi2
GαsL/GαsS

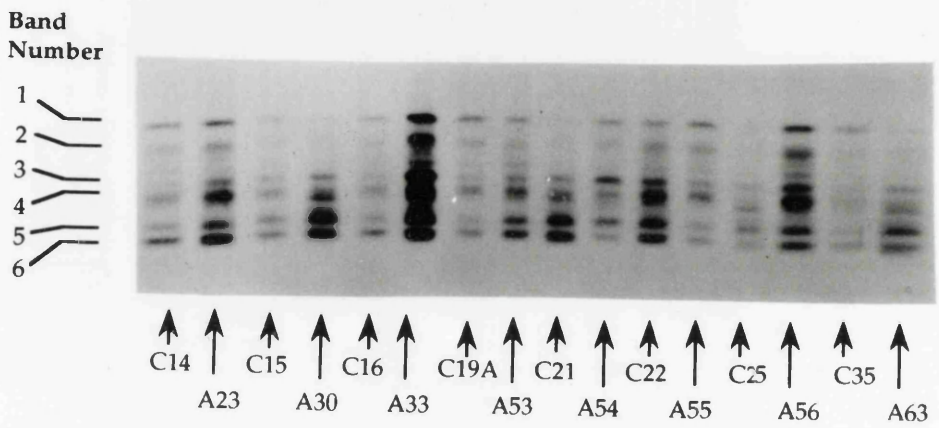
FIGURE 16.

4.4.3. Immunoblot analysis of glial fibrillary acidic protein in the the frontal cortex.

Glial fibrillary acidic protein (GFAP) was examined to access the contribution of both sub-cortical white matter and cortical astrocytes to the membrane preparations which were used for the investigation of signal transduction activities. The antibody used for this analysis detected multiple protein bands of between 55 and 30 KDal (section 4.1.1). Analysis by scanning densitometry routinely detected six peaks which are referred to as bands 1 to band 6. The immunoblot obtained with this antibody is shown in **Figure 17 (A)** and the mean peak hight values (±SEM) of the six protein bands plus the sum of the six bands are presented in **Figure 17 (B)**. While the mean values for each protein band and the sum of the peak heights were elevated in the Alzheimer's disease samples, the differences between the control and Alzheimer samples failed to reach statistical significance $P>0.05$ (Student's t-test).

FIGURE 17. Immunoblot analysis of glial fibrillary acidic protein (GFAP) of eight control and eight Alzheimer's frontal cortex membrane preparations. **(A)** The immunoblot detects multiple polypeptides of between 55 and 30 KDal. Scanning densitometry produced a profile where six peaks were clearly detected. These peaks were annotated bands 1 to band 6. **(B)** Histogram presentation of the mean peak heights ±SEM and of the sum of the six bands referred to as the total GFAP. The differences between the measurements for the control and Alzheimer's disease samples failed to achieve statistical significance as assessed by Student's t-test.

(A)


Band Number
1
2
3
4
5
6
C14
A23
C15
A30
C16
A33
C19A
A53
C21
A54
C22
A55
C25
A56
C35
A63

(B)

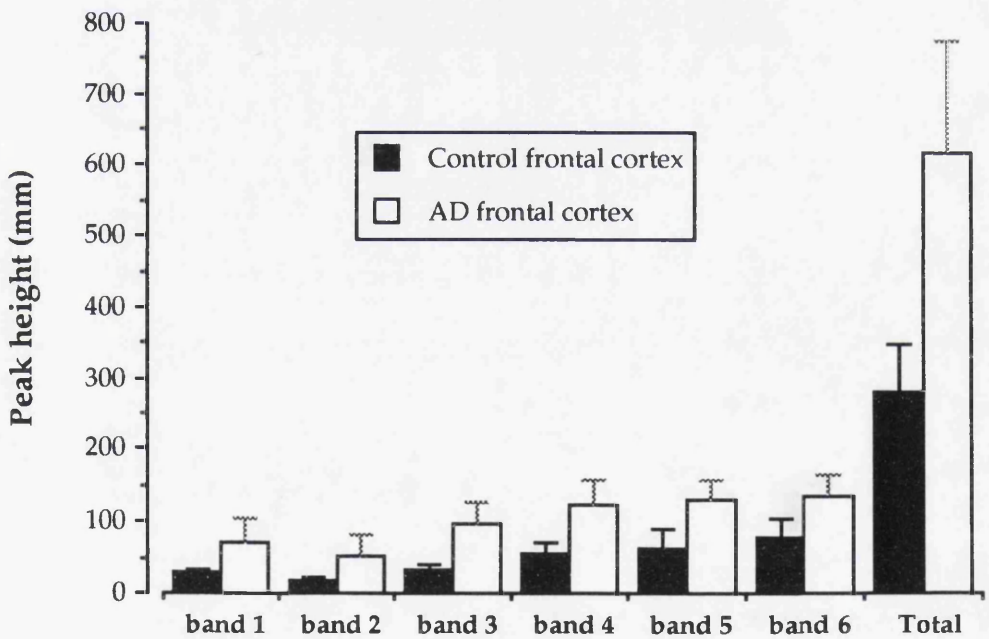

Peak height (mm)
800
700
600
500
400
300
200
100
0
Control frontal cortex
AD frontal cortex
band 1
band 2
band 3
band 4
band 5
band 6
Total

FIGURE 17.

4.4.4. Analysis of GTPase activity in the frontal cortex.

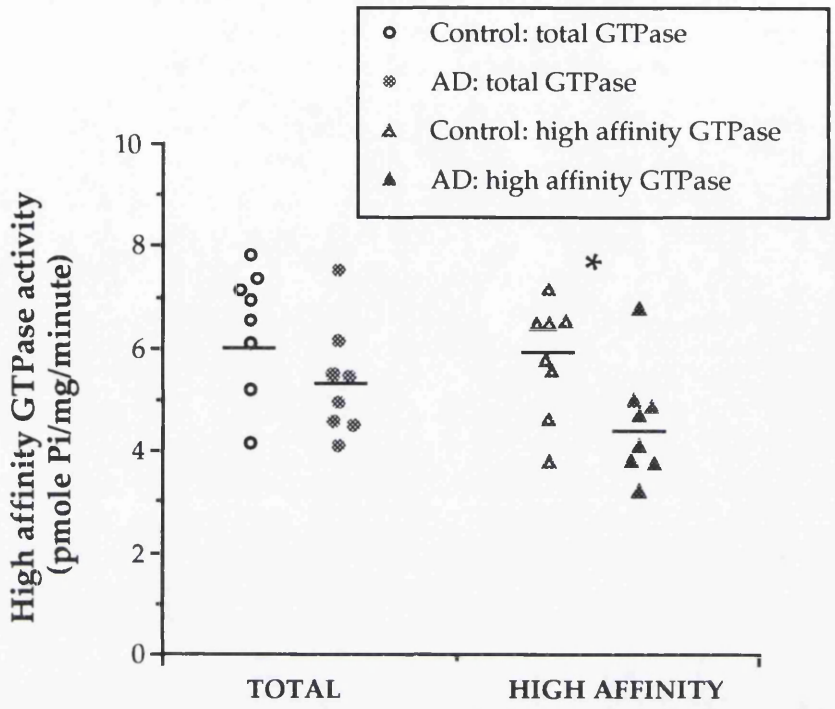
The GTPase assay was employed to analyse the basal intrinsic GTPase activity associated with G-proteins and to assess the integrity of G protein-receptor coupling. The basal high affinity GTPase, low affinity (nonspecific) GTPase and total (high and low affinity) GTPase activities were measured in membrane preparations from the frontal cortex of eight control and eight Alzheimer's disease samples (**Figure 19**). Statistical analysis by Student's t-test demonstrated the specific basal GTPase activity was significantly decreased (by 25 percent) in the Alzheimer's disease group when compared to the activity measured in the control samples ($P<0.05$). In contrast, the nonspecific, low affinity GTPase activity was significantly increased by 34% when compared to control values ($P<0.05$).

The effect of the muscarinic agonist carbachol, and the $GABA_B$ agonist baclofen on the high affinity GTPase activity was examined. **Figure 20** illustrated the degree of stimulation achieved with each agonist with the control and Alzheimer's disease samples. The data are expressed as a percent of basal GTPase activity. Analysis by Student's t-test found that there was no significant difference between the magnitude of stimulation (achieved by baclofen or carbachol) observed with the Alzheimer's disease samples when compared to the control samples. In the majority of the human samples, the magnitude of stimulation achieved with carbachol and with baclofen failed to reach the level of stimulation routinely achieved with fresh rat brain (130 to 150 percent of the basal activity). As reported in section 4.3.3, the length of the interval between death and the removal and freezing of the tissue may influence the responsiveness of G-proteins to the agonists carbachol and baclofen. However, there was no correlation between the postmortem interval and the degree of agonist stimulation of the basal GTPase activity (section **4.4.10**).

FIGURE 18. Scatter distribution of GTPase activity measured in membranes prepared from the frontal cortex of eight Alzheimer's disease and eight control samples. **(A)** illustrates the total rate of GTP hydrolysis assayed in the presence of 0.2μM cold GTP and the specific high affinity basal GTPase; **(B)** shows the low affinity (nonspecific) GTPase activity assayed in the presence of 100μM cold GTP. The specific high affinity activity shown in **(A)** is calculated from the difference between the total and nonspecific cpm values. The basal high affinity GTPase activity of the Alzheimer's disease group was significantly reduced when compared to the control sample values by Student's t-test (where * denotes $P<0.05$).

FIGURE 19. Agonist stimulation of high affinity GTPase activity of membranes prepared from the frontal cortex of eight Alzheimer's disease (AD) and eight age matched control samples. The activity of the high affinity GTPase was assayed in the presence and absence (basal) of carbachol (1mM) and in the presence and absence of baclofen (1mM) at 0.2μM cold GTP. The values are expressed as the percent of basal values indicated by the dashed line (---). Comparison of the mean values (——) by Student's t test demonstrated that for both carbachol and baclofen stimulation, there was no significant difference ($P>0.05$) between the control and Alzheimer's disease groups.

(A)


Control: total GTPase
AD: total GTPase
Control: high affinity GTPase
AD: high affinity GTPase
High affinity GTPase activity
(pmole Pi/mg/minute)
10
8
6
4
2
0
*
TOTAL
HIGH AFFINITY

(B)

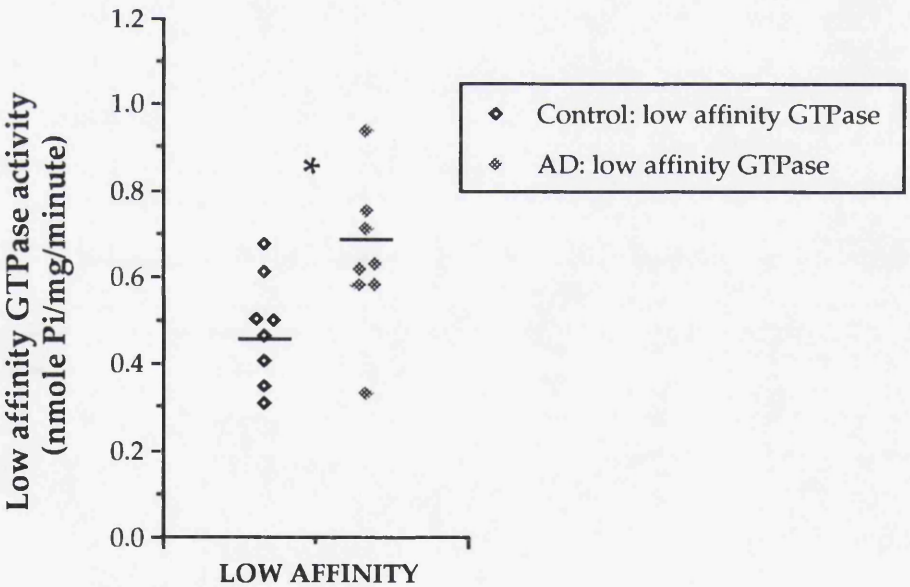

Control: low affinity GTPase
AD: low affinity GTPase
Low affinity GTPase activity
(nmole Pi/mg/minute)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
LOW AFFINITY

FIGURE 18.

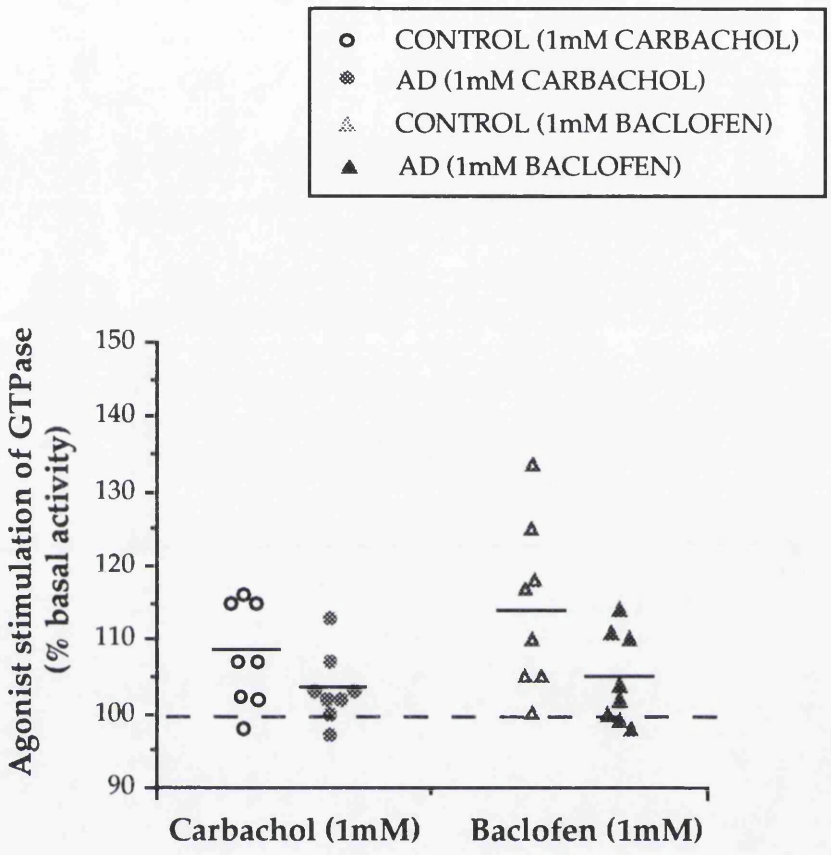

CONTROL (1mM CARBACHOL)
AD (1mM CARBACHOL)
CONTROL (1mM BACLOFEN)
AD (1mM BACLOFEN)
Agonist stimulation of GTPase
(% basal activity)
150
140
130
120
110
100
90
Carbachol (1mM)
Baclofen (1mM)

FIGURE 19.

4.4.5 Analysis of adenylate cyclase activity in the frontal cortex.

The adenylate cyclase activity of eight control and eight Alzheimer's disease frontal cortex membrane preparations measured in the presence (stimulated) and absence (basal) of fluoroaluminate is shown in **Figure 20.** The basal adenylate cyclase activity was significantly reduced in the Alzheimer's disease group by 44% ($p<0.01$ Student's t-test) with a similar reduction observed for the absolute activity assayed in the presence of fluoroaluminate. There was no significant difference in the magnitude of stimulation above the basal values by fluoroaluminate observed for Alzheimer's disease and control samples. Thus, the basal adenylate cyclase activity is reduced in Alzheimer's disease compared to age matched control levels, yet both groups have the same capacity for receptor independent stimulation as assessed by fluoroaluminate.

FIGURE 20. Adenylate cyclase activity of membranes prepared from the frontal cortex of eight Alzheimer's disease (AD) and eight age matched control samples. **(A)** Scatter distribution of the amount of cAMP formed in the presence (stimulated) and absence (basal) of fluoroaluminate (5mM sodium fluoride and 10μM aluminium chloride); **(B)** Stimulation above basal values of the samples expressed as percent of the basal adenylate cyclase activity. Statistical analysis by Student's t-test demonstrated a significant reduction in the basal values (where ** denotes $P<0.01$) of the Alzheimer's disease samples compared to activity in the control samples. There was no significant difference between the receptor independent stimulation by fluoroaluminate observed in the Alzheimer's disease when compared to the controls samples.

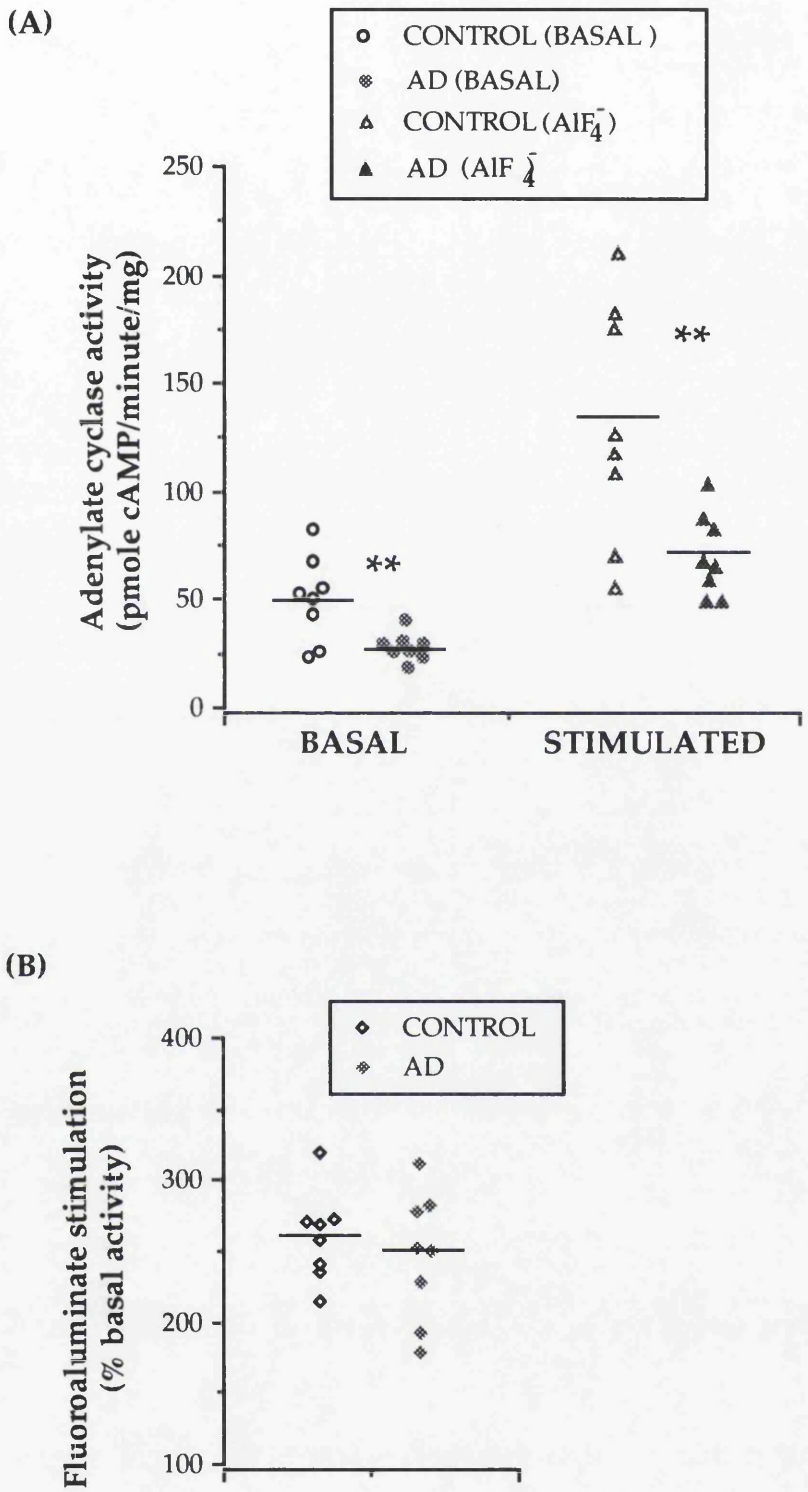

(A)
CONTROL (BASAL)
AD (BASAL)
CONTROL (AlF_4^-)
AD (AlF_4^-)
Adenylate cyclase activity
(pmole cAMP/minute/mg)
250
200
150
100
50
0
**
**
BASAL
STIMULATED
(B)
CONTROL
AD
Fluoroaluminate stimulation
(% basal activity)
400
300
200
100

FIGURE 20.

4.4.6 Immunoblot analysis of G-proteins in the hippocampus.

The immunoblot profile of the Gα subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ and the Gβ subunits, of eight control and eight Alzheimer's disease samples are shown in **Figure 21** and the results of the densitometric analysis of the immunoblots is shown as scatter plots in **Figure 22.** Since antisera SG2 is raised against an epitope which is identical for both $G_{\alpha i}1$ and $G_{\alpha i}2$ (section 3.9) then direct comparisons can be made between the level of each $G_{\alpha i}$ subtype. Similarly both $G_{\alpha s}S$ and $G_{\alpha s}L$ are detected with the same antisera (CS1) and these subtypes can be compared. **Figure 23** illustrates the ratio of $G_{\alpha i}1$ to $G_{\alpha i}2$ and also $G_{\alpha s}S$ to $G_{\alpha s}L$. Statistical analysis by Student's t-test failed to demonstrate a significant difference in the levels of $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ and Gβ measured in Alzheimer's disease samples when compared to control samples. Similarly, the ratio of $G_{\alpha i}1$ to $G_{\alpha i}2$ and $G_{\alpha s}L$ to $G_{\alpha s}S$ were not significantly different between the two control and Alzheimer's disease group ($P>0.05$).

4.4.7 Immunoblot analysis of glial fibrillary acidic protein in the hippocampus.

Glial fibrillary acidic protein (GFAP) was examined to access the contribution of both sub-cortical white matter and cortical astrocytes to the hippocampus membrane preparations which were used for the investigation of signal transduction activities. **Figure 24** shows the GFAP immunoblot **(A)**, and a histogram presentation of the mean peak height values±SEM of the individual protein bands 1 to 6 (section 5.1.1, **Figure 1**) and the sum of protein bands 1 to 6 (total) measured in Alzheimer's disease and control samples. Statistical comparisons of the levels in Alzheimer's disease and control samples found no significant difference ($P>0.05$) in the intensity of either the individual polypeptides bands or the sum of the anti-GFAP immunoreactive polypeptides.

FIGURE 21. Immunoblot analysis of G-protein subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ and G_{β} detected with eight Alzheimer's disease (AD) and eight control hippocampus membrane preparations. **(A)** G_{β} (50μg) detected with BN1 at 1/200 dilution; **(B)** $G_{\alpha o}$ (50μg) detected with OC1 at 1/10,000 dilution; **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with SG2 at 1/200 dilution; **(D)** $G_{\alpha s}L$, $G_{\alpha s}S$ (50μg) detected with CS1 at 1/200 dilution.

FIGURE 22. Scatter distribution plots of the densitometric analysis of the G-protein subunit immunoblots shown in **Figure 21** with the mean values (—). **(A)** G_{β} values; **(B)**, $G_{\alpha o}$; **(C)** $G_{\alpha i}1$, $G_{\alpha i}2$ are plotted on the same graph since both $G_{\alpha i}$ subtypes are detected with the antisera SG2 and the values for each are therefore comparable; **(D)** $G_{\alpha s}L$ and $G_{\alpha s}S$ also plotted on the same graph since both detected with antisera CS1. Statistical analysis by Student's t test found no significant difference ($P>0.05$) between the levels or ratio of any of the G-protein subunits detected in Alzheimer's disease and control hippocampus samples.

FIGURE 23. Comparison of the ratio of $G_{\alpha i}1/G_{\alpha i}2$ and $G_{\alpha s}L/G_{\alpha s}S$ detected with eight Alzheimer's disease and eight control hippocampus membrane preparations. Both $G_{\alpha i}1$ and $G_{\alpha i}2$ are detected within the same immunoblot by antisera (SG2), similarly $G_{\alpha s}L$ and $G_{\alpha s}S$ are both detected with antisera CS1, therefore the level of each subtype can be expressed as a ratio. Statistical comparison of the Alzheimer's disease and control values by Student's t test found no significant difference ($P>0.05$) in the ratio of $G_{\alpha s}L/G_{\alpha s}S$ or $G_{\alpha i}1/G_{\alpha i}2$.

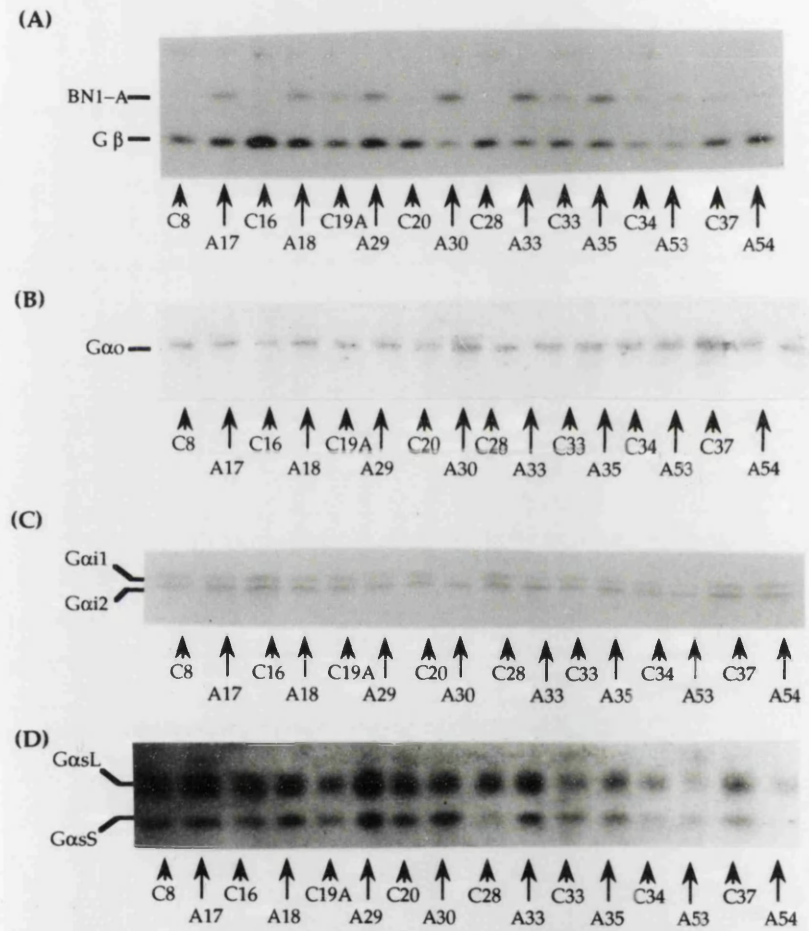

(A)
BN1–A
Gβ
C8
C16
C19A
C20
C28
C33
C34
C37
A17
A18
A29
A30
A33
A35
A53
A54
(B)
Gαo
C8
C16
C19A
C20
C28
C33
C34
C37
A17
A18
A29
A30
A33
A35
A53
A54
(C)
Gαi1
Gαi2
C8
C16
C19A
C20
C28
C33
C34
C37
A17
A18
A29
A30
A33
A35
A53
A54
(D)
GαsL
GαsS
C8
C16
C19A
C20
C28
C33
C34
C37
A17
A18
A29
A30
A33
A35
A53
A54

FIGURE 21.

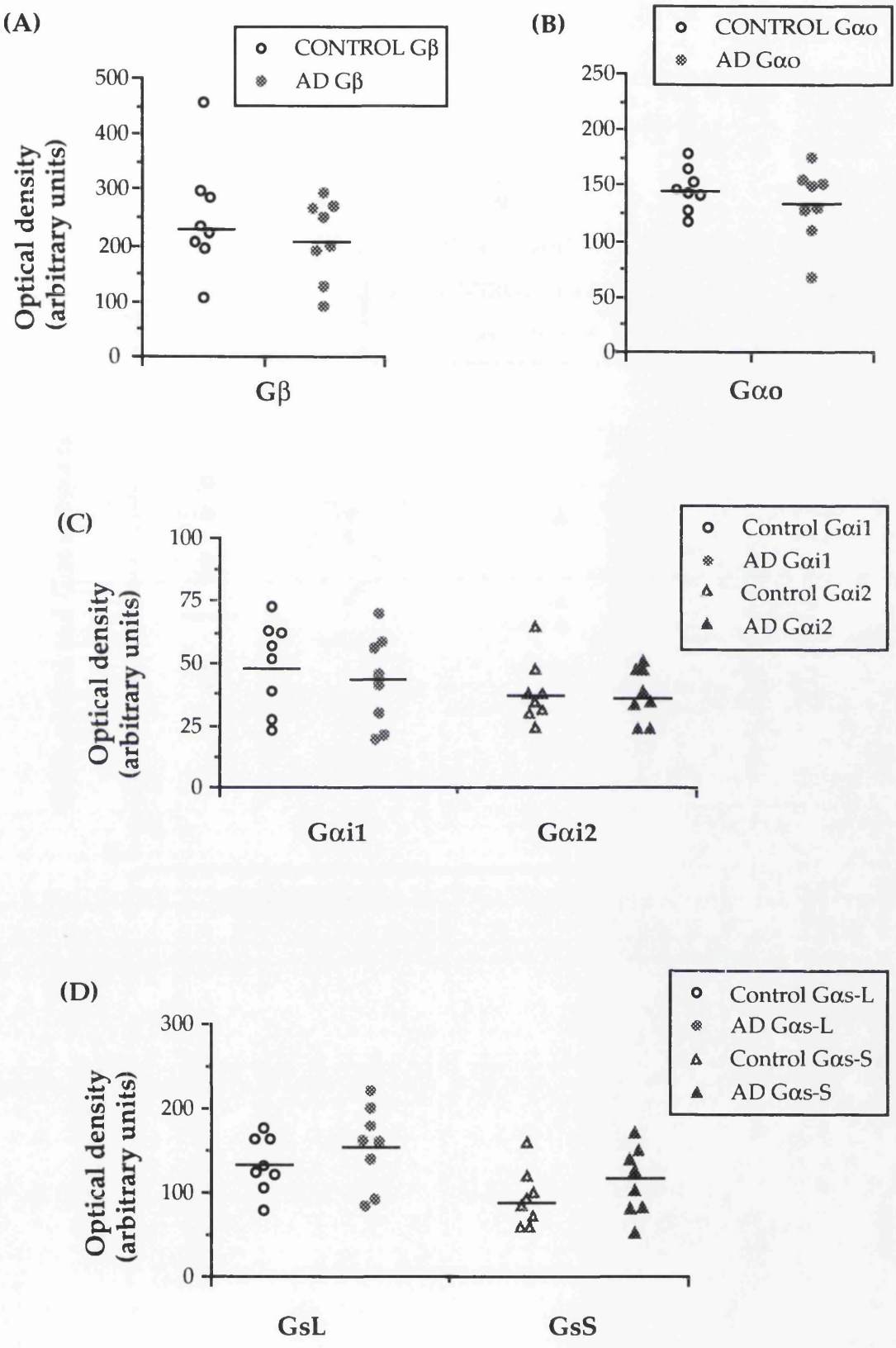

(A)
CONTROL Gβ
AD Gβ
Optical density (arbitrary units)
500
400
300
200
100
0
Gβ
(B)
CONTROL Gαo
AD Gαo
250
200
150
100
50
0
Gαo
(C)
Control Gαi1
AD Gαi1
Control Gαi2
AD Gαi2
Optical density (arbitrary units)
100
75
50
25
0
Gαi1
Gαi2
(D)
Control Gαs-L
AD Gαs-L
Control Gαs-S
AD Gαs-S
Optical density (arbitrary units)
300
200
100
0
GsL
GsS

FIGURE 22.

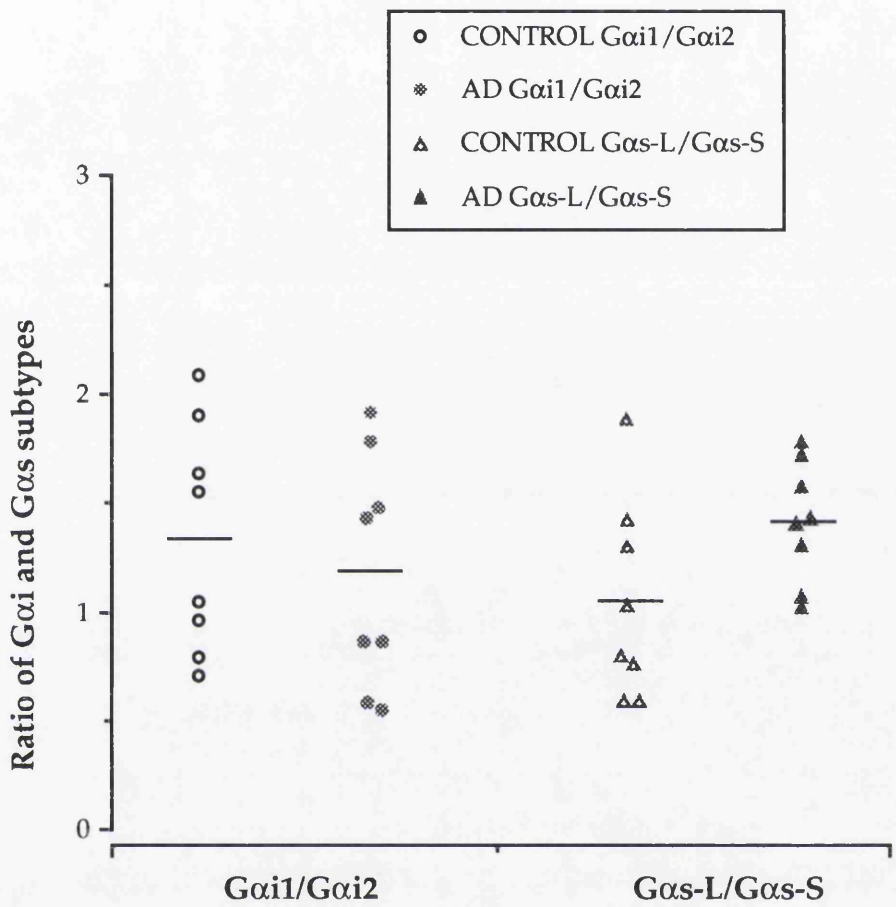
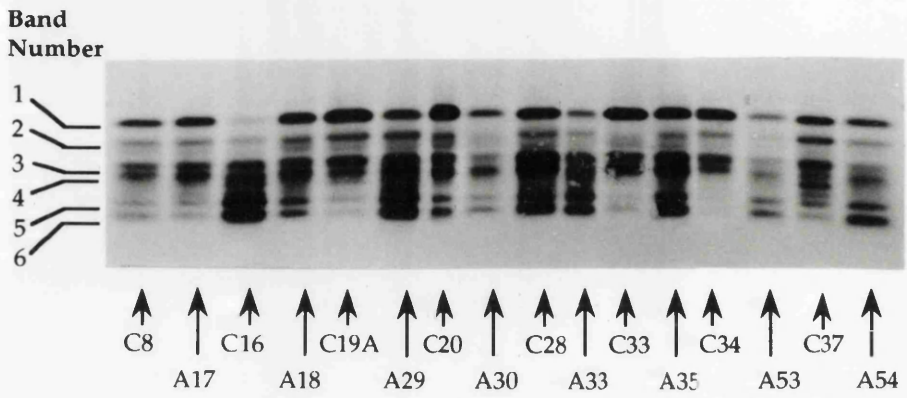

CONTROL Gαi1/Gαi2
AD Gαi1/Gαi2
CONTROL Gαs-L/Gαs-S
AD Gαs-L/Gαs-S
Ratio of Gαi and Gαs subtypes
3
2
1
0
Gαi1/Gαi2
Gαs-L/Gαs-S

FIGURE 23.

FIGURE 24. Immunoblot analysis of glial fibrillary acidic protein (GFAP) of eight control and eight Alzheimer's disease hippocampus membrane preparations. **(A)** The immunoblot detects multiple polypeptides of between 55 and 30 KDal. Scanning densitometry produced a profile where six clear peaks could be observed. These peaks were annotated bands 1 to band 6. **(B)** Histogram plot of the mean peak heights ±SEM and of the sum of the six bands, referred to as the total GFAP. Statistical comparison by Student's t-test of the GFAP polypeptides detected in Alzheimer's disease and control samples found no significant difference ($P>0.05$).

(A)


Band
Number
1
2
3
4
5
6
C8
A17
C16
A18
C19A
A29
C20
A30
C28
A33
C33
A35
C34
A53
C37
A54

(B)

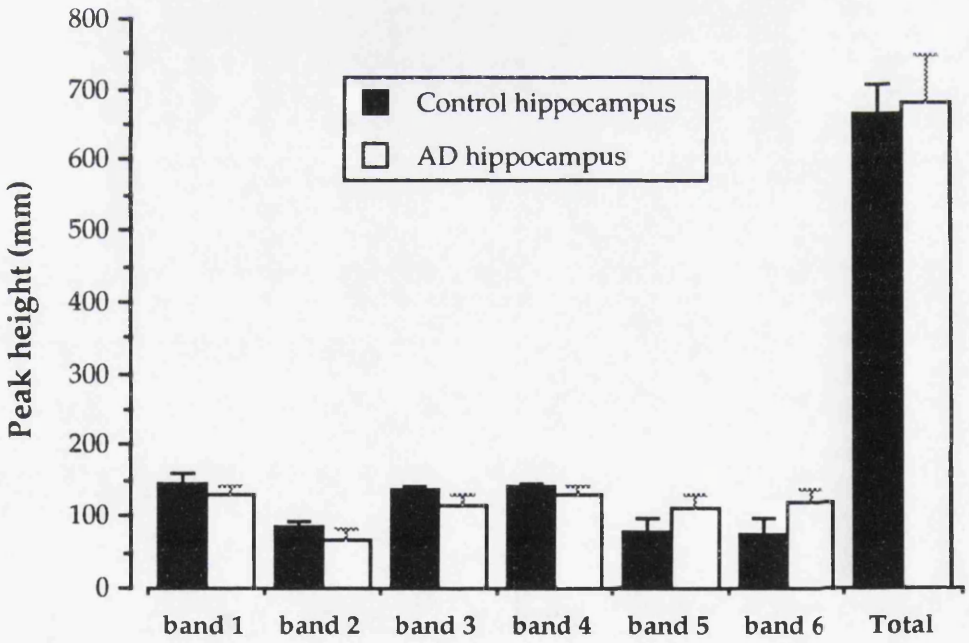

800
700
600
500
400
300
200
100
0
Peak height (mm)
Control hippocampus
AD hippocampus
band 1
band 2
band 3
band 4
band 5
band 6
Total

FIGURE 24

4.4.8 Analysis of GTPase activity in the hippocampus.

The basal high affinity GTPase, the low affinity GTPase (nonspecific) and total (high and low affinity) GTPase activity measured in the hippocampal membrane preparations from eight control and eight Alzheimer's disease samples (**Figure 25**). Statistical analysis by Student's t-test found no significant difference ($P>0.05$) between the total and specific basal GTPase activity measured with Alzheimer's disease samples when compared to the activity of the control samples. However, the mean specific basal activity of the Alzheimer's disease samples were reduced by 26 percent compared to the controls which failed to achieve statistical significance ($P=0.51$).

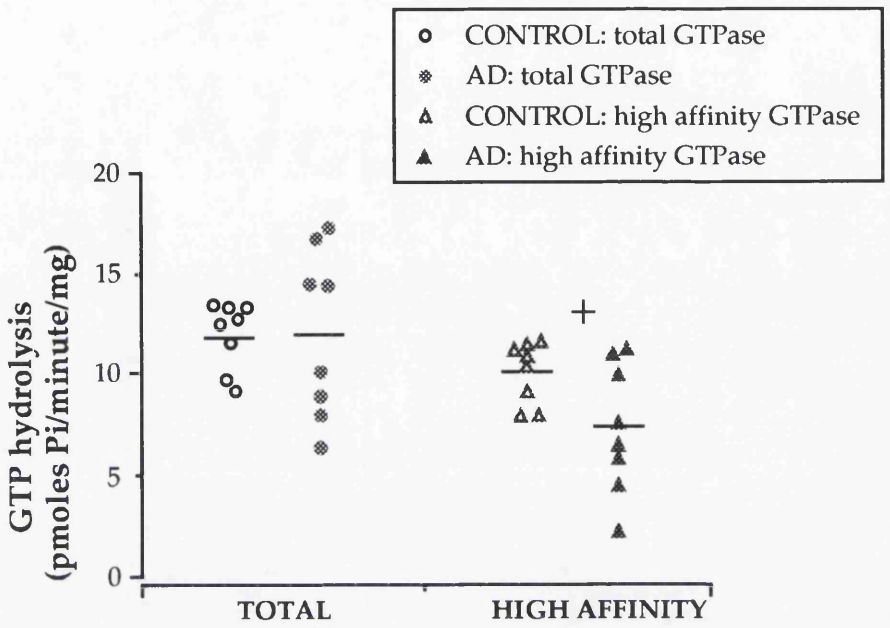
The mean nonspecific, low affinity GTPase values of the Alzheimer's disease group were increased by 55 percent from the control values. The variation about the mean of each group were comparable (38 and 37 percent coefficient of variance for Alzheimer's disease and control groups respectively) and Student's t test demonstrated a highly significant change ($p<0.01$). A similar increase in the Alzheimer's disease nonspecific GTPase was observed in the frontal cortex study, however the magnitude of the change (34 percent) was not as marked as that observed for the hippocampus study.

The effect of the muscarinic agonist carbachol, and the $GABA_B$ agonist baclofen on the high affinity GTPase activity was examined. **Figure 26** illustrated the degree of stimulation achieved with each agonist with the control and Alzheimer's disease samples. The data are expressed as a percent of basal GTPase activity. Analysis by Student's t test found that there was no significant difference ($P>0.05$) between the magnitude of stimulation (achieved by baclofen or carbachol) observed with the Alzheimer's disease samples when compared to the control samples.

FIGURE 25 Scatter distribution of GTPase activity measured in membranes prepared from the hippocampus of eight control and eight Alzheimer's disease samples. **(A)** illustrates the total rate of GTP hydrolysis assayed in the presence of 0.2μM cold GTP and the specific high affinity basal GTPase; **(B)** shows the low affinity (nonspecific) GTPase activity assayed in the presence of 100μM cold GTP. The specific high affinity activity shown in **(A)** is calculated from the difference between the total and nonspecific cpm values. Comparisons of the control and Alzheimer's disease values by Student's t-test found no significant change between the two groups. However, the basal specific activity was reduced by 27 percent which bordered of statistcal significance (where + denotes P=0.51). The nonspecific activity was significantly elevated in the Alzheimer's disease group (55 percent) when compared to the control group (where ** denotes P<0.01).

FIGURE 26. Agonist stimulation of high affinity GTPase activity of membranes prepared from the hippocampus of eight Alzheimer's disease (AD) and eight age matched control samples. The activity of the high affinity GTPase was assayed in the presence and absence (basal) of carbachol (1mM) and in the presence and absence of baclofen (1mM) at 0.2μM cold GTP. The values are expressed as the percent of basal values indicated by the dashed line (---). Comparison of the mean values (——) by Student's t-test demonstrated that for both carbachol and baclofen stimulation, there was no significant difference (P>0.05) between the control and Alzheimer's disease (AD) groups.

(A)


CONTROL: total GTPase
AD: total GTPase
CONTROL: high affinity GTPase
AD: high affinity GTPase
GTP hydrolysis
(pmoles Pi/minute/mg)
20
15
10
5
0
TOTAL
HIGH AFFINITY

(B)

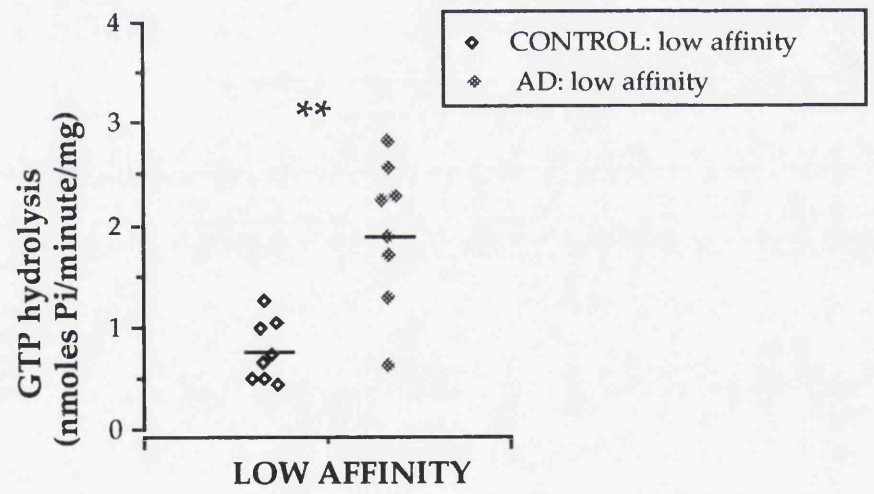

CONTROL: low affinity
AD: low affinity
GTP hydrolysis
(nmoles Pi/minute/mg)
4
3
2
1
0
**
LOW AFFINITY

FIGURE 25.

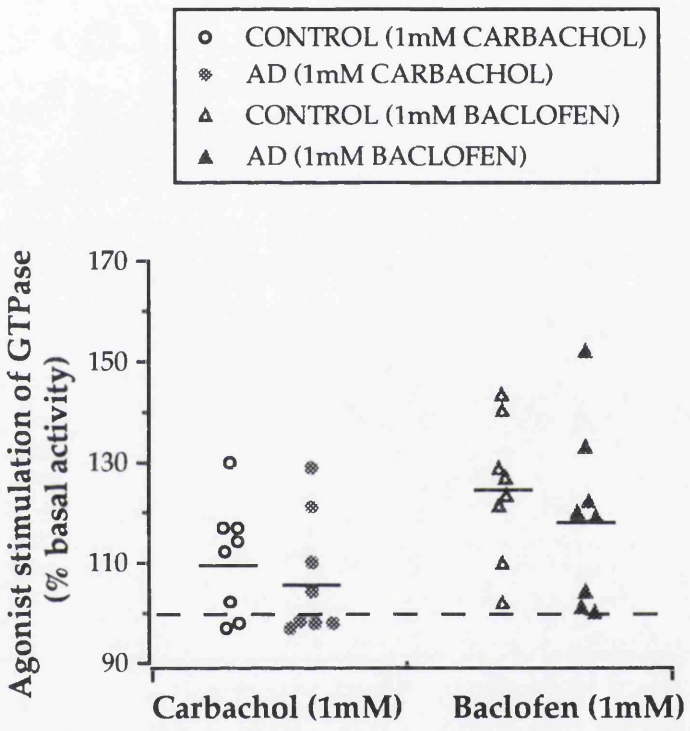

CONTROL (1mM CARBACHOL)
AD (1mM CARBACHOL)
CONTROL (1mM BACLOFEN)
AD (1mM BACLOFEN)
Agonist stimulation of GTPase
(% basal activity)
170
150
130
110
90
Carbachol (1mM)
Baclofen (1mM)

FIGURE 26.

4.4.9 Analysis of adenylate cyclase activity in the hippocampus.

The adenylate cyclase activity of eight control and eight Alzheimer's disease frontal cortex membrane preparations measured in the presence (stimulated) and absence (basal) of fluoroaluminate are shown in **Figure 27**. Statistical analysis by Student's t test found no significant difference ($P>0.05$) between the basal and stimulated activity of the Alzheimer's disease group when compared to the control group. Similarly, there was no significant difference ($P>0.05$) in the magnitude of stimulation achieved by fluoroaluminate when the data was expressed as a percent of the basal values.

4.4.10 Linear regression analysis of human data.

It has been established that specific components of signal transduction mechanisms have a biological relationship. For example, the activation of $G_{\alpha s}$ proteins leads to the stimulation of adenylate cyclase activity (Gilman, 1987). In this thesis, the individual parameters were all measured within the same membrane samples prepared from Alzheimer's disease and control tissue block. The data generated for the different parameters were analysed by linear regression. Additionally, factors such as the interval between death and brain freezing, age and period of freezer of the brains were examined. Several significant correlations were identified and are listed in **Table 5** as a hierarchy from most significant to least significant. It is acknowledged that by examining the different parameters within the same sample preparation the measurements are no longer statistically independent which weakens the statistical power of this analysis. Thus, although the significant correlations listed in **Table 5** are suggestive of biological relationships, the are not conclusive. **Figure 28** shows examples of significant correlations identified in the frontal cortex and hippocampus control samples.

FIGURE 27. Adenylate cyclase activity of membranes prepared from the hippocampus of eight Alzheimer's disease (AD) and eight age matched control samples. **(A)** Scatter distribution of the amount of cAMP formed in the presence (stimulated) and absence (basal) of fluoroaluminate (5mM sodium fluoride and 10μM aluminium chloride) with mean values; **(B)** Stimulation above basal values of the samples expressed as percent of the basal adenylate cyclase activity. Mean values are illustrated by a plain line (---). Statistical analysis by Student's t-test found no significant difference between Alzheimer's disease and control groups in either the basal or stimulated adenylate cyclase activity, or in the magnitude of stimulation achieved with fluoroaluminate.

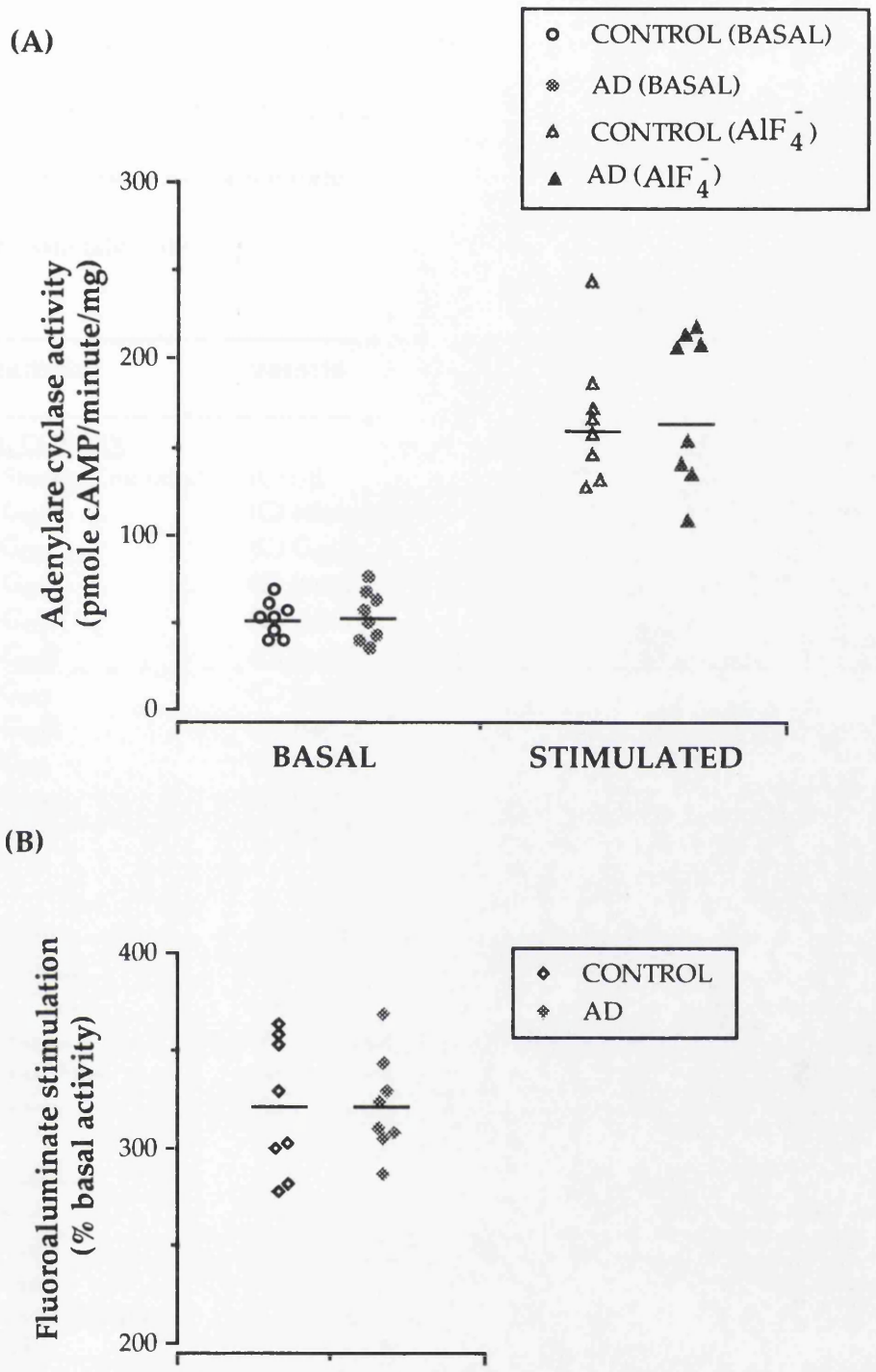

(A)
CONTROL (BASAL)
AD (BASAL)
CONTROL (AlF_4^-)
AD (AlF_4^-)
Adenylare cyclase activity
(pmole cAMP/minute/mg)
300
200
100
0
BASAL
STIMULATED
(B)
CONTROL
AD
Fluoroaluminate stimulation
(% basal activity)
400
300
200

FIGURE 27.

TABLE 5. Linear regression analysis of parameters measured in the frontal cortex **(A)** and hippocampus of control and Alzheimer's disease samples. Significant correlations are shown as Pearson's correlation (r). Abbreviations: Control samples (C); Alzheimer's disease samples (AD); adenylate cyclase (AC); fluoroaluminate stimulate AC expressed an percent of basal activity (percent); total adenylate cyclase activity assayed in the presence of fluoroaluminate (absolute).

Parameter	versus	r	P
FRONTAL CORTEX			
(C) Storage (months)	(C) Gβ	0.928	<0.002
(C) $G_{\alpha i}2$	(C) stimulated AC	0.883	<0.01
(C) $G_{\alpha o}$	(C) $G_{\alpha i}2$	0.835	<0.01
(C) $G_{\alpha i}1$	(C) basal GTPase	0.826	<0.02
(C) $G_{\alpha i}1$	(C) stimulated AC	0.809	<0.02
(C) $G_{\alpha i}2$	(C) basal GTP	0.792	<0.01
(C) $G_{\alpha o}$	(C) basal GTPase	0.790	<0.01
(C) $G_{\alpha i}2$	(C) baclofen stimulated GTPase	0.781	<0.05
(C) $G_{\alpha o}$	(C) basal AC	0.753	<0.05
(C) $G_{\alpha o}$	(C) $G_{\alpha i}1$	0.751	<0.05
(C) $G_{\alpha i}2$	(C) basal AC	0.739	<0.05
(AD) $G_{\alpha o}$	(AC) Gβ	0.856	<0.01
(AD) $G_{\alpha i}2$	(AC) stimulated AC (percent)	0.793	<0.02
(AD) ChAT	(AC) basal AC	0.775	<0.05
(AD) $G_{\alpha o}$	(AC) stimulated AC (percent)	0.765	<0.05
(AD) GTPase	(AC) stimulated AC (absolute)	0.749	<0.05
(AD) $G_{\alpha o}$	(AC) stimulated AC (absolute)	0.739	<0.05
HIPPOCAMPUS			
(C) $G_{\alpha o}$	(C) carbachol stimulated GTPase	0.939	<0.002
(C) $G_{\alpha o}$	(C) baclofen stimulated GTPase	0.873	<0.01
(C) $G_{\alpha i}1$	(C) basal GTPase	0.853	<0.01
(C) basal GTPase	(C) basal AC	0.846	<0.01
(C) Gβ	(C) carbachol stimulated GTPase	0.839	<0.01
(C) Gβ	(C) $G_{\alpha i}2$	0.795	<0.01
(C) $G_{\alpha i}2$	(C) basal GTPase	0.758	<0.05
(C) $G_{\alpha o}$	(C) Gβ	0.744	<0.05
(C) basal GTPase	(C) stimulated AC (absolute)	0.734	<0.05
(AD) Gβ	(AD) GTPase	0.900	<0.01
(AD) $G_{\alpha o}$	(AD) Gβ	0.842	<0.01
(AD) $G_{\alpha o}$	(AD) $G_{\alpha i}2$	0.794	<0.02
(AD) $G_{\alpha i}2$	(C) basal GTPase	0.731	<0.05

FIGURE 28. Examples of significant correlations determined by linear regression analysis between various parameters measured in frontal cortex and hippocampus preparations from control samples. The Pearson's correlation coefficients (r) are shown and the confidence intervals for P are denoted *<0.05, **<0.02, ***<0.01, ****<0.002. **(A), (B),** and **(C)** illustrate the correlations between GTPase and Gαi1, GTPase and Gαi1, and GTPase and Gαi2 measured in the frontal cortex of control subjects. The correlsponding measurements in Alzheimer's disease samples failed to achiecve statistical significance. **(D), (E),** and **(F)** illustrate the correlations between basal GTPase and Gαi1, basal GTPase and Gαi2, and basal GTPase and basal adenylate cyclase activity measured in the hippocampus of control subjects. The corresponding measurements recorded in Alzheimer's disease samples failed to achiecve statistical significance except for the relationship between basal GTPase and Gαi1 (r=0.731; P<0.05).

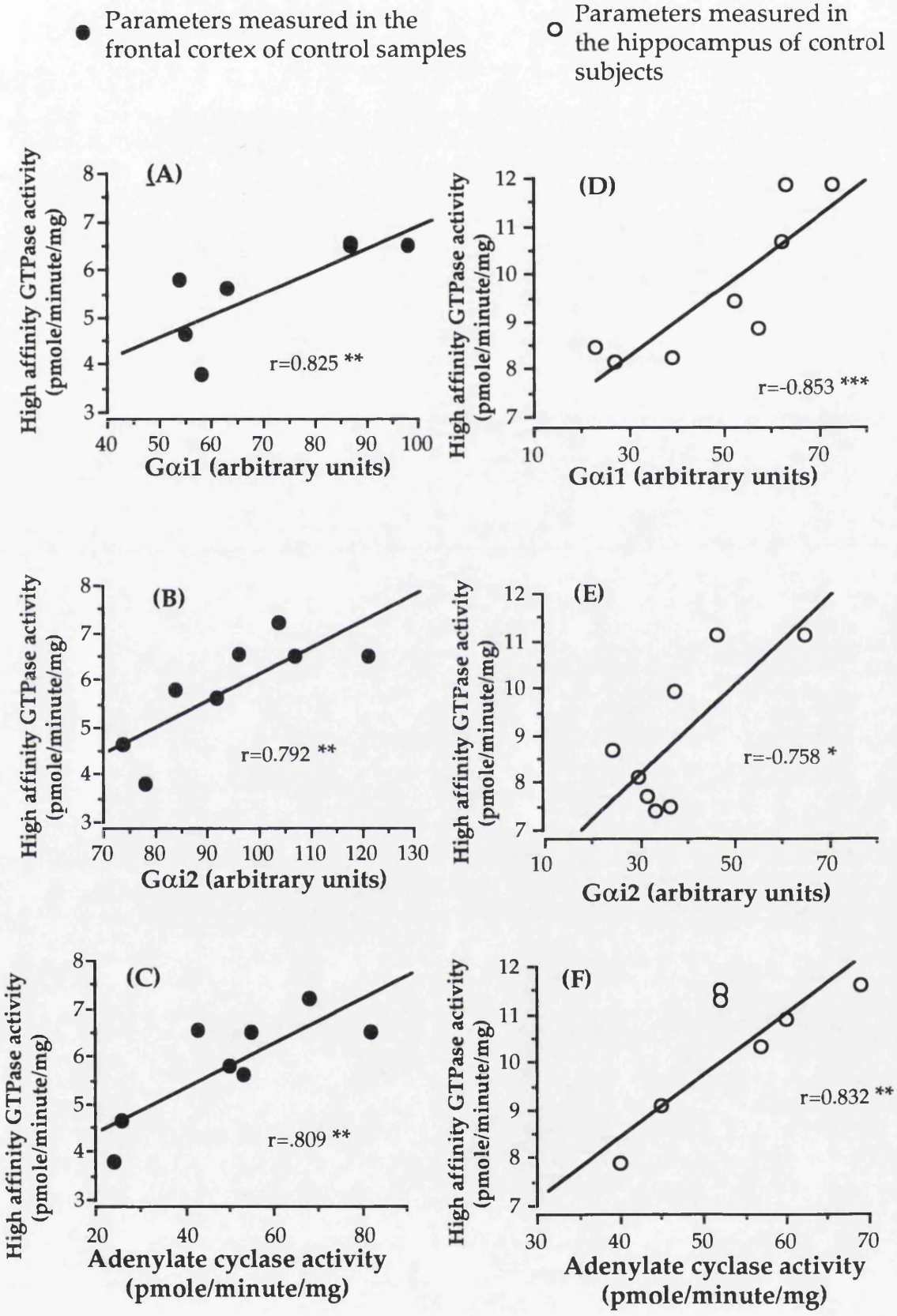

● Parameters measured in the frontal cortex of control samples
○ Parameters measured in the hippocampus of control subjects
(A)
High affinity GTPase activity (pmole/minute/mg)
r=0.825 **
Gαi1 (arbitrary units)
(D)
r=-0.853 ***
Gαi1 (arbitrary units)
(B)
r=0.792 **
Gαi2 (arbitrary units)
(E)
r=-0.758 *
Gαi2 (arbitrary units)
(C)
r=.809 **
Adenylate cyclase activity (pmole/minute/mg)
(F)
r=0.832 **
Adenylate cyclase activity (pmole/minute/mg)

FIGURE 28.

4.5 The distribution of G-proteins and adenylate cyclase activity in human brain

4.5.1 G-protein distribution in cortical grey and sub-cortical white matter.

The relative amounts of the G proteins subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, $G_{\alpha s}S$ G_{β} and glial fibrillary acidic protein (GFAP) in cortical grey, sub-cortical white and a mixture of grey and white matter were analysed by immunoblotting. The crude membranes were prepared from cortical grey, sub-cortical white and a mixture of grey and white matter dissected from a control human temporal cortex, sample number C23 (**section 3.5**). The immunoblot profile of the G-proteins and GFAP are shown in **Figure 29** with the corresponding densitometric values shown in **Figure 30**.

In grey matter, the level of $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$ and G_{β} were higher than that measured in sub cortical white matter. In white matter the level of $G_{\alpha s}S$ and the level of the BN1-A artifact protein (see section **4.1.1**) are more abundant compared to the levels observed in grey matter. In grey matter $G_{\alpha s}L$ is the more abundant $G_{\alpha s}$ subtype while in white matter $G_{\alpha s}S$ is more abundant than $G_{\alpha s}L$. A similar reversal in the predominance of the two $G_{\alpha i}$ subtypes was observed although the altered ratio in grey and white matter was not as marked as that observed for the $G_{\alpha s}$ proteins. Similarly, the intensity of the six polypeptide proteins detected with anti-GFAP antibody in grey matter gave an inverted profile compared to that observed with the sub-cortical white matter sample.

4.5.2 Enzymatic activity of grey and sub cortical white matter of human brain.

The following enzymes were assayed in cortical grey and sub-cortical white matter preparations: GTPase measured in the presence and absence of carbachol and baclofen; adenylate cyclase assayed in the presence and absence of fluoroaluminate and choline acetyltransferase. The results are shown as histogram plots in **Figure 31**. The basal GTPase,

basal adenylate cyclase. and choline acetyltransferase was markedly lower in sub-cortical white compared to grey matter. The nonspecific GTPase activity, and the percent stimulation of basal adenylate cyclase activity by fluoroaluminate were similar in grey and sub-cortical white matter. Both agonists carbachol and baclofen failed to stimulate the basal high affinity GTPase in any preparation.

FIGURE 29. Immunoblot profile of the distribution of G-protein subunits and glial fibrillary acid protein (GFAP) between sub-cortical white matter (W), a mixture of grey and white matter (M) and grey matter (G) membrane preparations from the temporal cortex of subject C23. **(A)** Gβ and BN1-A (artifact) (50μg) detected with antisera BN1 at 1/200 dilution; **(B)** $G_{\alpha o}$ (50μg) detected with OC1 at 1/10,000 dilution; **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with antisera SG2 at 1/200 dilution; **(D)** $G_{\alpha s}L$ and $G_{\alpha s}S$ (50μg) detected with antisera SG2 at 1/200 dilution; **(E)** GFAP detected with 25μg protein at 1/2,000 dilution.

FIGURE 30. Semi-quantitation by densitometric analysis of the G-protein subunit and GFAP polypeptides detected by immunoblot shown in **Figure 29.** **(A)** Gβ subunit; **(B)** $G_{\alpha o}$; **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$; **(D)** $G_{\alpha s}L$ and $G_{\alpha s}S$; **(E)** GFAP bands 1 to 6 (section **4.1.1**).

FIGURE 31. Distribution of enzymatic activity measured in sub-cortical white matter(W), a mixture of grey and white matter (M) and grey matter (G) membrane preparations from the temporal cortex of subject C23. **(A)** choline acetyltransferase activity; **(B)** low affinity (nonspecific) GTPase and high affinity basal GTPase assayed in the presence of carbachol and baclofen; **(C)** adenylate cyclase activity measured in the presence and absence of fluoroaluminate (AlF^-_4).

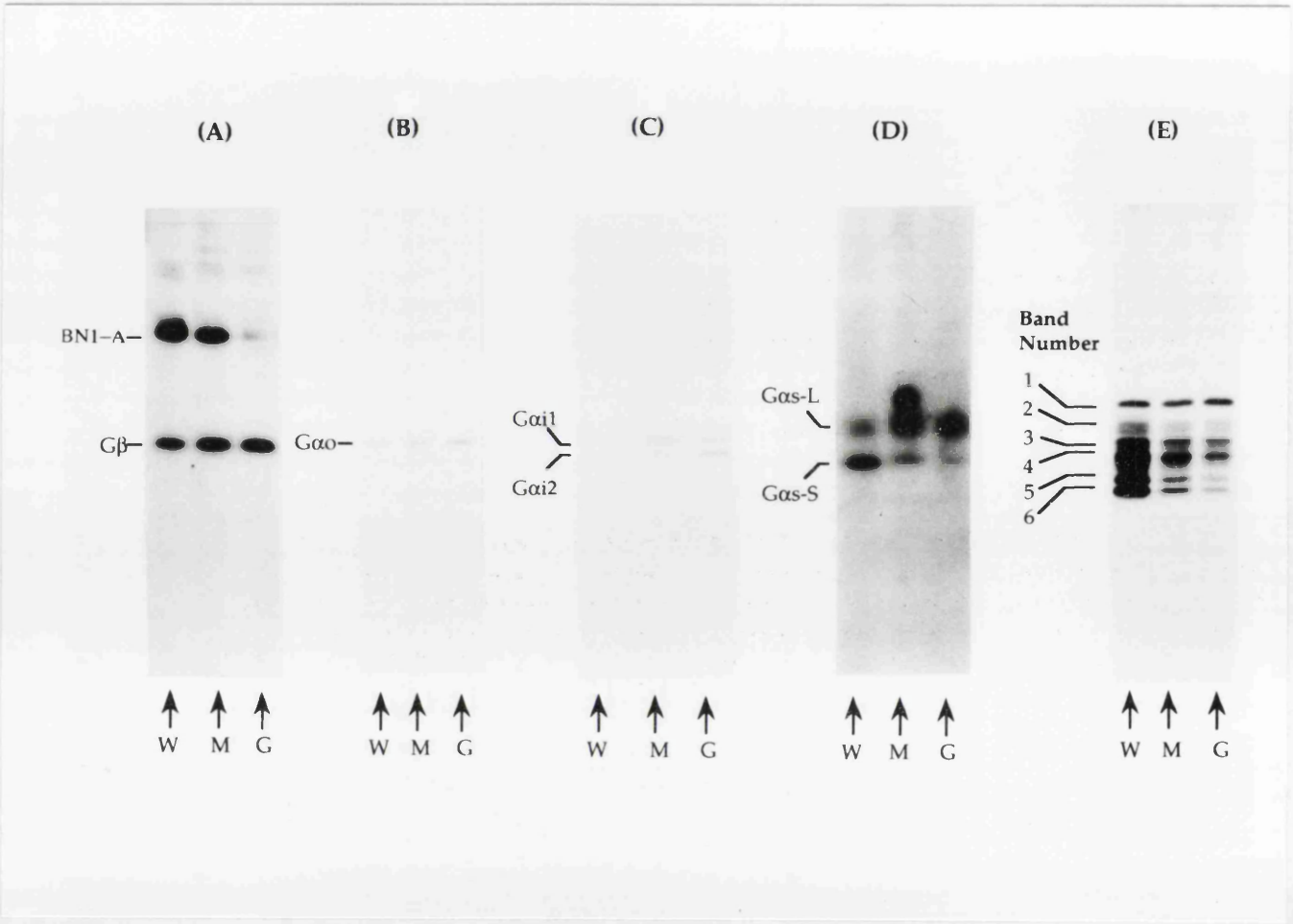

(A)
(B)
(C)
(D)
(E)
BN1–A–
Gβ–
Gαo–
Gαi1
Gαi2
Gαs-L
Gαs-S
Band Number
1
2
3
4
5
6
W M G
W M G
W M G
W M G
W M G

FIGURE 29

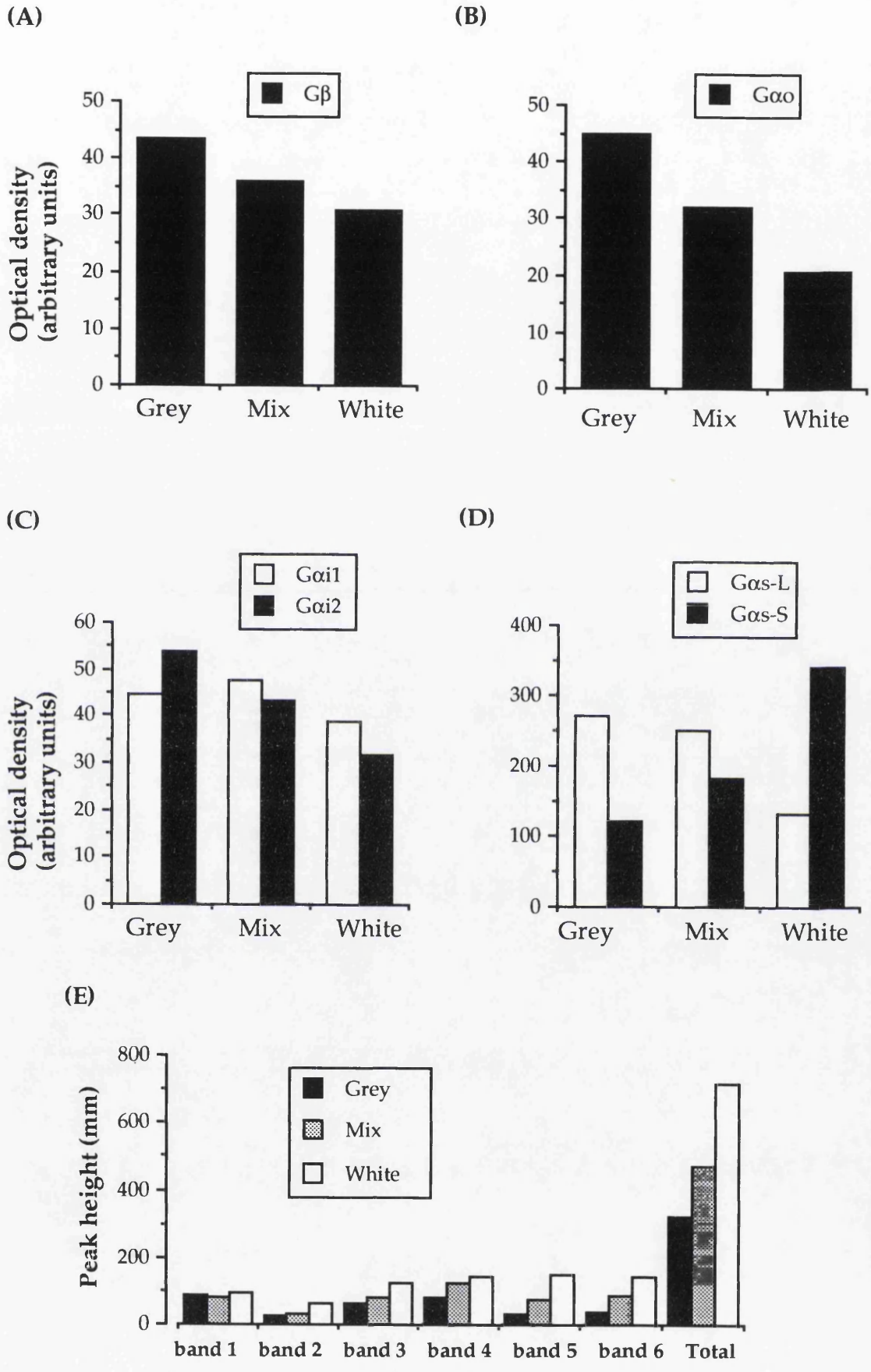

(A)
Gβ
Optical density (arbitrary units)
50
40
30
20
10
0
Grey
Mix
White
(B)
Gαo
50
40
30
20
10
0
Grey
Mix
White
(C)
Gαi1
Gαi2
Optical density (arbitrary units)
60
50
40
30
20
10
0
Grey
Mix
White
(D)
Gαs-L
Gαs-S
400
300
200
100
0
Grey
Mix
White
(E)
Peak height (mm)
800
600
400
200
0
Grey
Mix
White
band 1
band 2
band 3
band 4
band 5
band 6
Total

FIGURE 30.

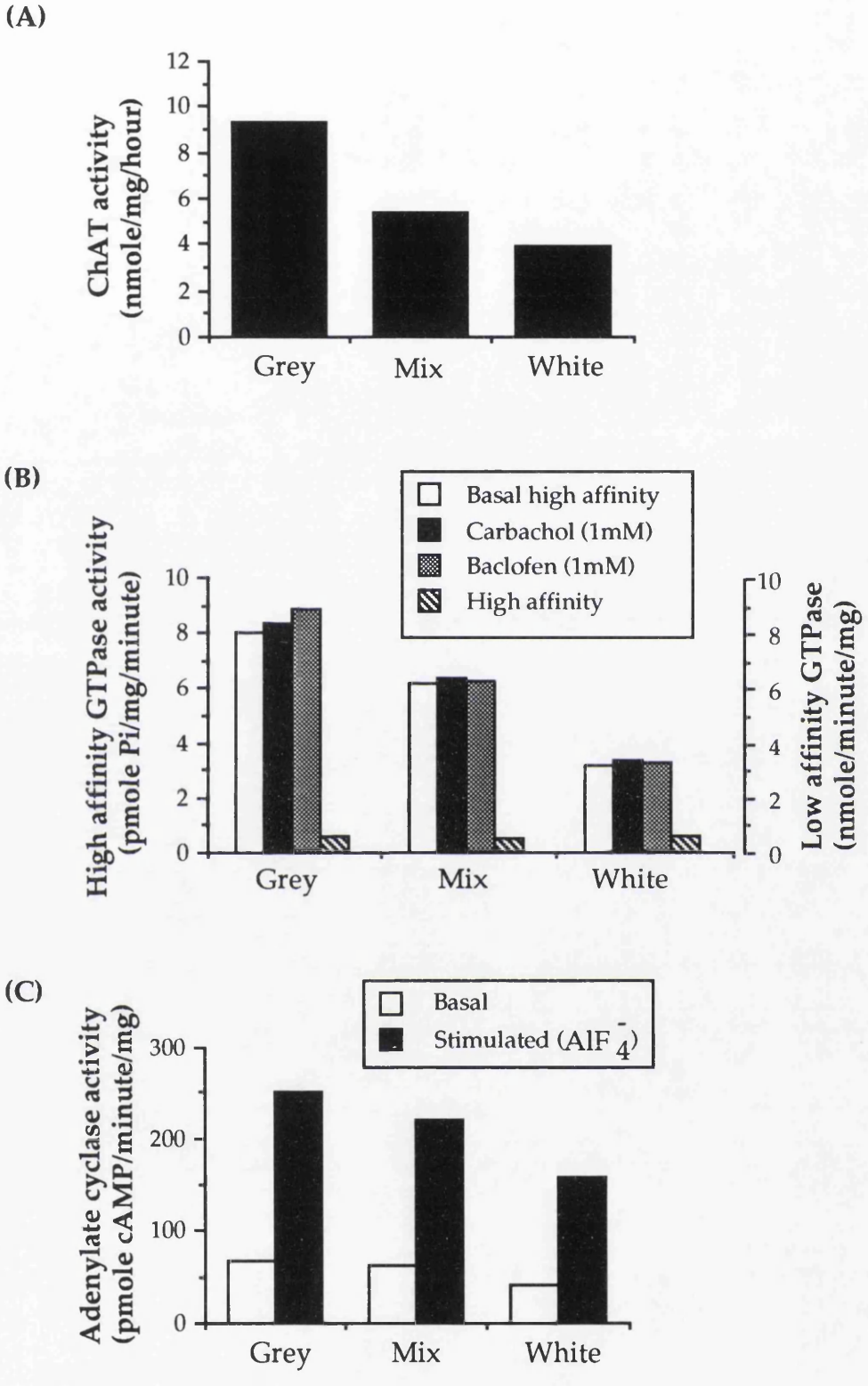

(A)
ChAT activity
(nmole/mg/hour)
12
10
8
6
4
2
0
Grey
Mix
White
(B)
Basal high affinity
Carbachol (1mM)
Baclofen (1mM)
High affinity
High affinity GTPase activity
(pmole Pi/mg/minute)
Low affinity GTPase
(nmole/minute/mg)
10
8
6
4
2
0
Grey
Mix
White
(C)
Basal
Stimulated (AlF 4 -)
Adenylate cyclase activity
(pmole cAMP/minute/mg)
300
200
100
0
Grey
Mix
White

FIGURE 31.

4.5.3 Regional distribution of G-protein subunits and related enzymatic activity in human brain.

In this study, membranes prepared from five brain regions, the frontal cortex, hippocampus, striatum, cerebellum and pons of three control brains (C14, C19A and C34) were analysed by immunoblotting and assayed adenylate cyclase activity. Due to freezer failure, aliquots which were reserved for GFAP and GTPase activity were considered to be no longer suitable for accurate and reliable biochemical analysis. The immunoblot profile of the Gα subunits are shown in **Figure 32.** The $G_{\alpha s}$ immunoblot also identified a third band in the striatum samples with a molecular weight between that for $G_{\alpha s}L$ and $G_{\alpha s}S$. This autoradiogram was over-exposed so that this polypeptide could be illustrated photographically (**Figure 32 B**). The data generated from densitometric analysis of the immunoblot is presented as mean±SEM in **Figure 33.** $G_{\alpha i}1$ and $G_{\alpha i}2$ are plotted on the same graph since both $G_{\alpha i}$ subtypes are detected with the antisera (SG2) and the values for each are therefore comparable. Similarly, $G_{\alpha s}L$ and $G_{\alpha s}S$ are also plotted on the same graph since both are detected with antisera CS1. The adenylate cyclase activity measured in each brain region is illustrated in **Figure 34.**

Statistical analysis by ANOVA demonstrated that the level of each G-proteins were heterogeneously distributed between the brain regions examined. There was no significant difference ($P>0.05$) between different brain regions in the basal adenylate cyclase activity, or the absolute stimulated activity (assayed in the presence of fluoroaluminate). The percent stimulation of adenylate cyclase was significantly different in selected brain region (for example cerebellum compared to hippocampus $P<0.025$). The rank order of abundance of the various G-proteins and adenylate cyclase activity measured in different brain regions are shown in **Table 6.**

The different parameters were examined for biological relationships by conducting linear regression analysis. Significant correlations are shown in **Table 7.**

FIGURE 32.. Immunoblot profile of membrane fractions prepared from the frontal cortex (F), hippocampus (H), striatum (ST), cerebellum (C) and pons (P) of the three control subjects C14, C19A and C34. **(A)** $G_{\alpha s}L$ and $G_{\alpha s}S$ (50μg) detected with antisera CS1 at 1/200 dilution; **(B)** the $G_{\alpha s}$ immunoblot was exposed for three times longer than the film shown in **(A)**. Note that in the striatum samples a third band is visible between $G_{\alpha s}L$ and $G_{\alpha s}S$.; **(C)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with SG2 at 1/200 dilution; **(D)** $G_{\alpha o}$ detected with antisera OC1 at 1/10,000 dilution.

FIGURE 33. Densitometric analysis of the immunoblots shown in **Figure 32.** The values are presented as the mean±SEM of three control samples C14, C19A and C34. Statistical analysis by ANOVA demonstrated that each G-protein subunit was heterogeneously distributed between the different brain regions. The abbreviations for the different regions are: frontal cortex (FCX); hippocampus (HIP); striatum (ST); cerebellum (CBL); pons (PONS).

FIGURE 34. Regional distribution of basal and stimulated adenylate cyclase activity. **(A)** adenylate cyclase activity measured in the presence and absence of 5mM sodiumfluoride and 10μM aluminium chloride; **(B)** Fluoroaluminate stimulation expressed as percent of the basal activity. The abbreviations for the different regions are: frontal cortex (FCX); hippocampus (HIP); striatum (ST); cerebellum (CBL); pons (PONS). Statistical analysis by ANOVA found no significant difference ($P>0.05$) between regions in activity measure in the presence or absence of fluoroaluminate. There was however significant differences ($P<0.05$) between regions in the magnitude of stimulation when expressed as a percent of the basal activity.

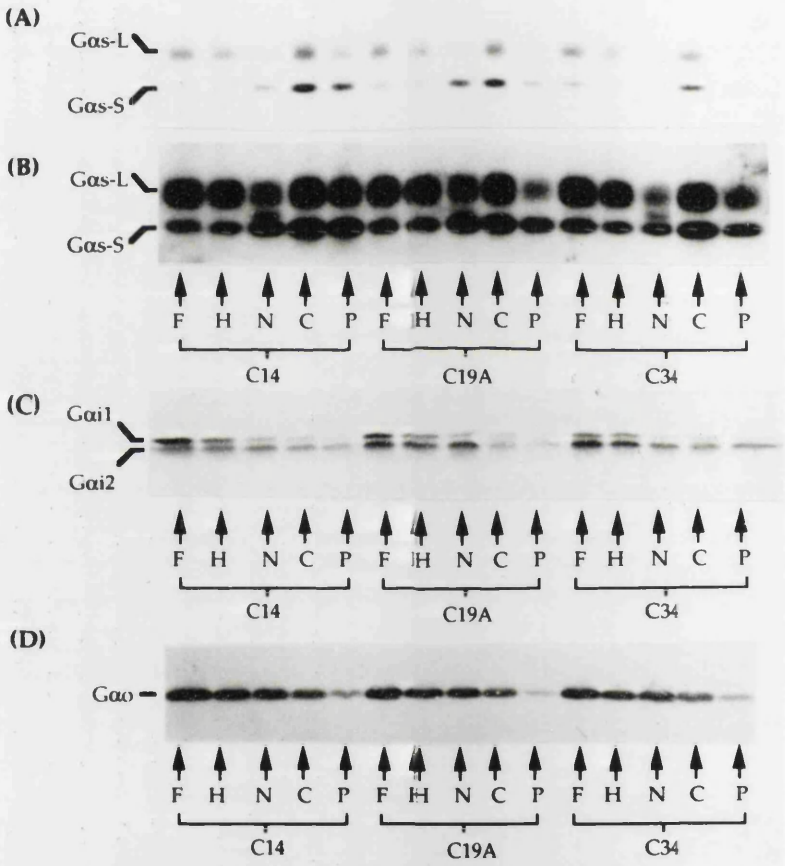

(A)
Gαs-L
Gαs-S
(B)
Gαs-L
Gαs-S
F H N C P F H N C P F H N C P
C14
C19A
C34
(C)
Gαi1
Gαi2
F H N C P F H N C P F H N C P
C14
C19A
C34
(D)
Gαo
F H N C P F H N C P F H N C P
C14
C19A
C34

FIGURE 32

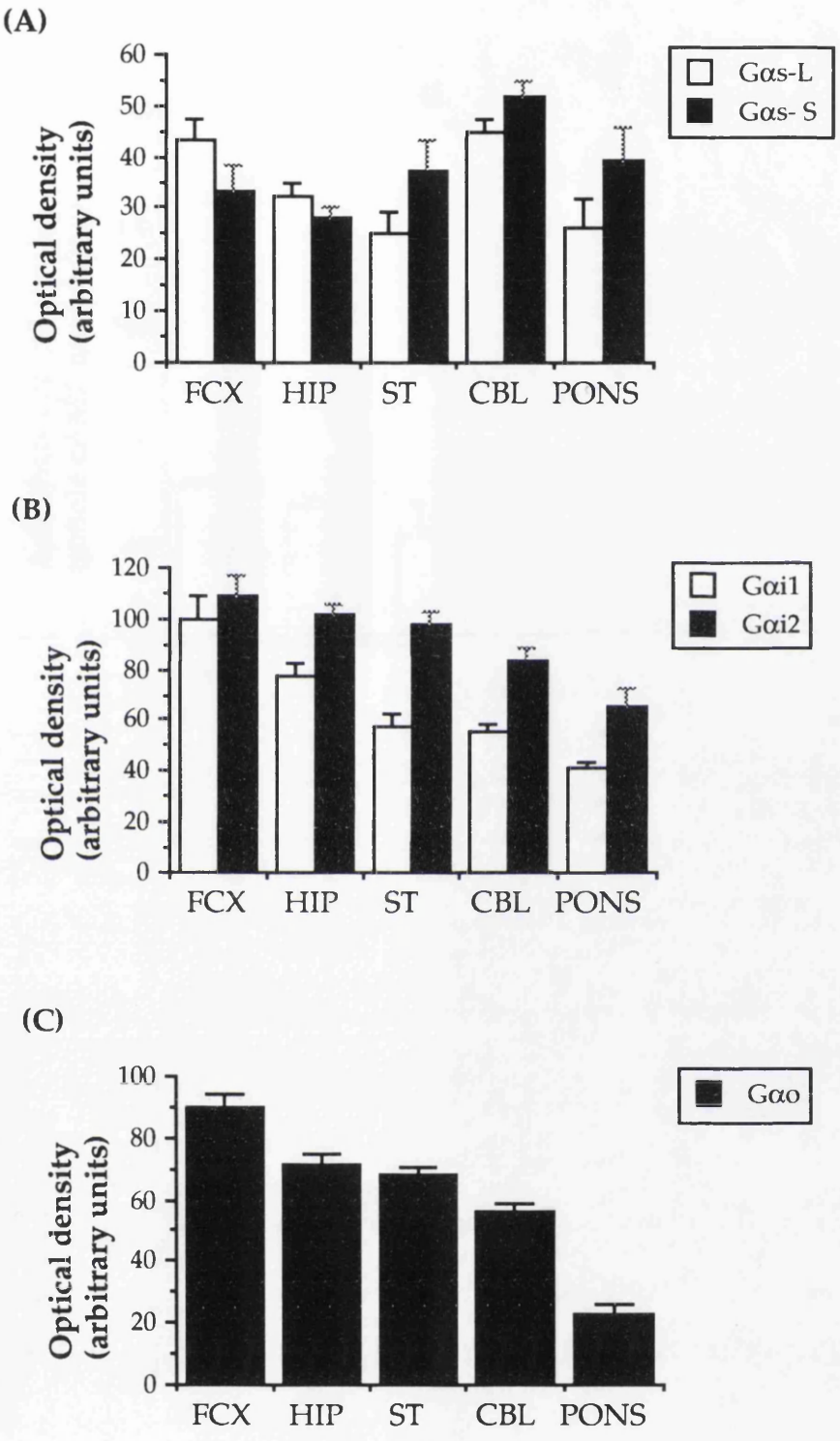

(A)
Optical density (arbitrary units)
60
50
40
30
20
10
0
FCX
HIP
ST
CBL
PONS
Gαs-L
Gαs- S
(B)
Optical density (arbitrary units)
120
100
80
60
40
20
0
FCX
HIP
ST
CBL
PONS
Gαi1
Gαi2
(C)
Optical density (arbitrary units)
100
80
60
40
20
0
FCX
HIP
ST
CBL
PONS
Gαo

FIGURE 33.

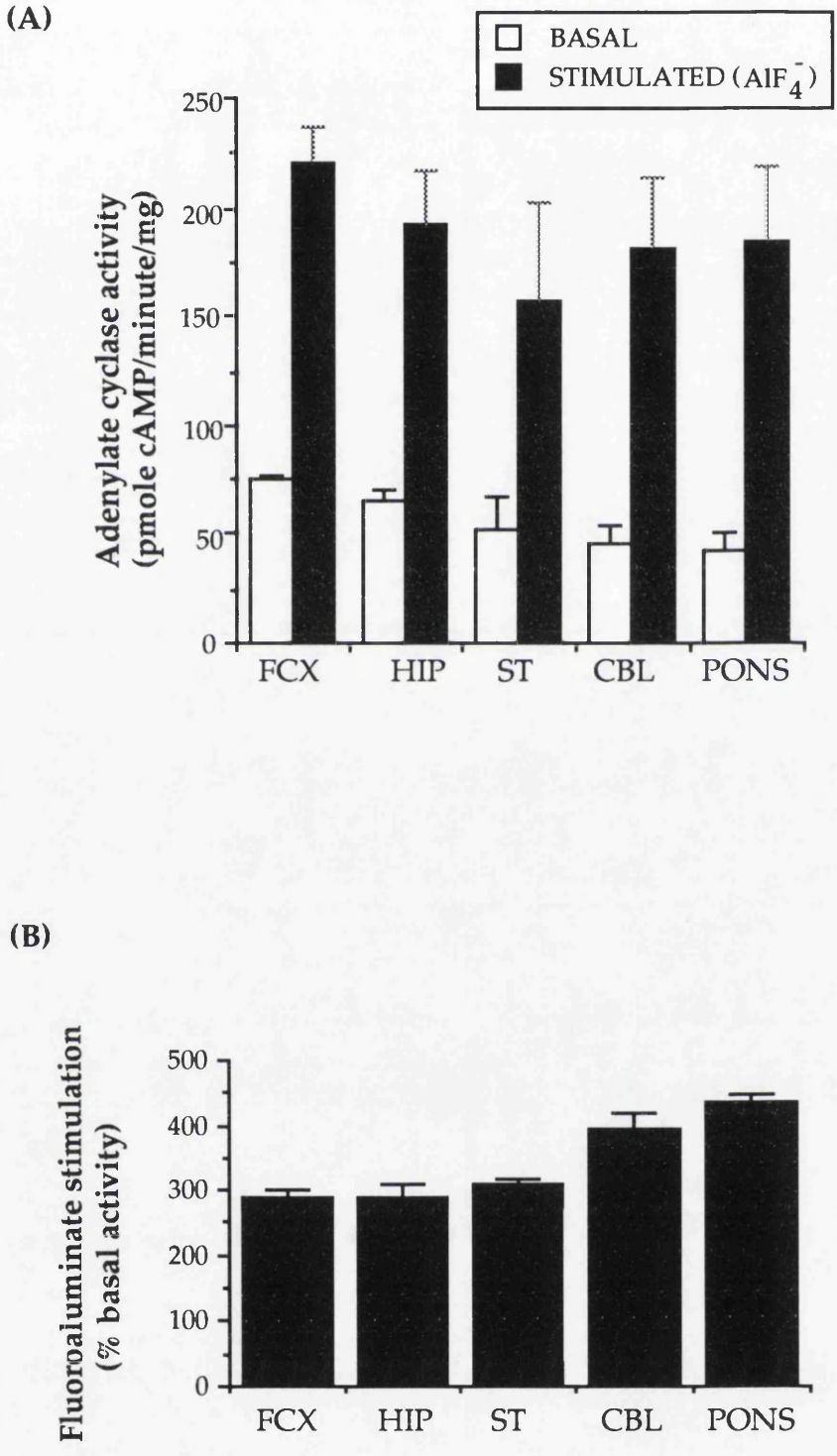

(A)
BASAL
STIMULATED (AlF_4^-)
Adenylate cyclase activity
(pmole cAMP/minute/mg)
250
200
150
100
50
0
FCX
HIP
ST
CBL
PONS
(B)
Fluoroaluminate stimulation
(% basal activity)
500
400
300
200
100
0
FCX
HIP
ST
CBL
PONS

FIGURE 34.

TABLE 6. The relative order of abundance of G-proteins and adenylate cyclase measured in five brain regions of three control human samples. The adenylate cyclase activity was expressed as basal (no fluoraluminate) absolute activity measure in the presence of fluoroaluminate (absolute), and the magnitude of stimulation by fluoroaluminate as a percent of the basal activity (percent)

Parameter	Relative order of abundance in different brain regions
$G_{\alpha s}L$	cerebellum ≥ frontal cortex ≥ hippocampus > striatum ≥ pons
$G_{\alpha s}S$	cerebellum ≥ pons ≥ neostriatum ≥ frontal cortex ≥ hippocampus
$G_{\alpha i}1$	frontal cortex ≥ hippocampus ≥ striatum ≥ cerebellum ≥ pons
$G_{\alpha i}2$	frontal cortex ≥ hippocampus ≥ striatum ≥ cerebellum ≥ pons
$G_{\alpha o}$	frontal cortex ≥ hippocampus ≥ striatum ≥ cerebellum ≥ pons
Basal AC	frontal cortex ≥ hippocampus ≥ striatum ≥ cerebellum ≥ pons
Stimulated AC (absolute)	frontal cortex ≥ hippocampus≥ pons ≥cerebellum ≥ striatum
Stimulated AC (% of basal)	pons ≥ cerebellum ≥ striatum ≥ hippocampus ≥ frontal cortex

TABLE 7. Linear regression analysis of the relative amounts of G-proteins and adenylate cyclase measured in various human brain regions. The relationship between G-protein levels and adenylate cyclase activity was performed using linear regression analysis and shown as Pearsons correlation (r). Abbreviations: Total $G_{\alpha s}$ refers to the sum of the optical density values obtained for $G_{\alpha s}L$ and $G_{\alpha s}S$; Total $G_{\alpha i}$ refers to the sum of the optical density values obtained for $G_{\alpha i}1$ and $G_{\alpha i}2$; Total PTX refers to the sum of the optical density values obtained for the pertussis toxin sensitive G-proteins $G_{\alpha i}1$, $G_{\alpha i}2$ and $G_{\alpha o}$. Basal adenylate cyclase refers to the activity recorded in the absence of fluoroaluminate; stimulated adenylate cyclase refers to the % of basal activity achieved by fluoroaluminate.

Parameter	versus	r	P
$G_{\alpha o}$	Total $G_{\alpha i}$	0.849	<0.002
$G_{\alpha o}$	$G_{\alpha i}1$	0.846	<0.002
$G_{\alpha o}$	$G_{\alpha i}2$	0.784	<0.002
$G_{\alpha o}$	GsL/GsS	0.673	<0.01
Basal adenylate cyclase	$G_{\alpha i}2$	0.791	<0.002
Basal adenylate cyclase	$G_{\alpha i}1$	0.765	<0.002
Basal adenylate cyclase	$G_{\alpha s}L/G_{\alpha s}S$	0.696	<0.01
Basal adenylate cyclase	$G_{\alpha o}$	0.599	<0.02
Basal adenylate cyclase	$G_{\alpha s}L$	0.517	<0.02
Stimulated adenylate cyclase	Total $G_{\alpha s}$/Total PTX	0.881	<0.002
Stimulated adenylate cyclase	Total PTX	-0.861	<0.002
Stimulated adenylate cyclase	$G_{\alpha o}$	-0.845	<0.002
Stimulated adenylate cyclase	Total $G_{\alpha i}$	-0.835	<0.002
Stimulated adenylate cyclase	$G_{\alpha i}2$	-0.786	<0.002
Stimulated adenylate cyclase	$G_{\alpha i}1$	-0.745	<0.002
Stimulated adenylate cyclase	$G_{\alpha i}S$	0.535	<0.05

4.6 The effect of medial septum lesion on signal transduction activities.

The hippocampus receives a major cholinergic and γ-aminobutyric acid (GABAergic) input from the the medial septal and diagonal band. Several studies have demonstrated in rat that following denervation of the septal/hippocampus pathway there is a complex neuronal structural and neurochemical reorganisation within the hippocampus. One of the hallmarks of Alzheimer's disease is a hippocampal deficit in the cholinergic marker enzyme choline acetyl transferase. Thus, animal models lesioned at the medial septum can provide valuable information on the plasticity of the hippocampus similar to that described in Alzheimer's disease. In this study, the effect of denervation of the cholinergic medial septum-hippocampus pathway in the rat was examined by lesioning the medial septum with the neurotoxin ibotenate. The group size composed of twelve lesioned and twelve sham lesioned (injected with saline) animals that served as controls. From these animals eight were chosen at random for biochemical analysis since this is the maximum number (total of 16) that can be examined within the same immunoblot.

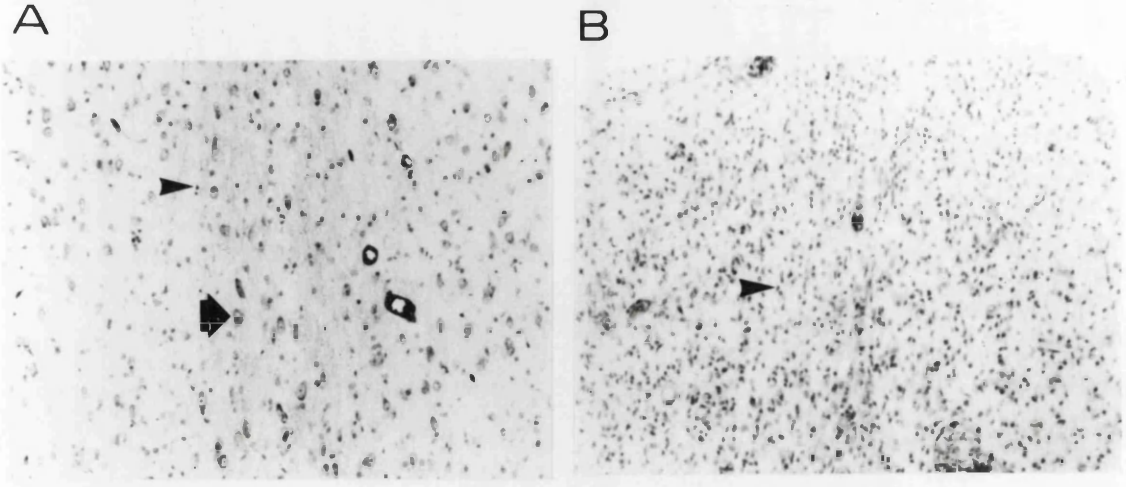
4.6.1 Histological and neurochemical assessment of medial septum lesion.

The location and size of the lesion was examined by microscopic analysis of cresyl-violet stained sections of the medial septum. **Figure 35 (A)** shows a representative example of the histology of a control **(A)** and lesioned **(B)** animal. The lesion was localised to the medial septal in all animals with minimal damage to the lateral septum and vertical diagonal band. In control animals (injected at the medial septum with phosphate buffered saline) there was minimal evidence of neuronal damage.

The activity of choline acetyltransferase (ChAT) was measured in hippocampal homogenates prepared from control and lesioned animals and is presented in **Figure 35 (c)**. The ChAT activity was significantly reduced by 24% ($P<0.01$) in the lesioned animals compared

to the control group (Student's t-test). Sample (L3) the recorded the lowest ChAT activity, and in this animal the lesion area appeared larger that the other samples of the lesion group. Thus, the magnitude of cholinergic deficite appears to correlate with the degree of neuronal damage in the medial septum.

FIGURE 35. Histological evaluation of ibotenate lesion in the medial septum. Coronal sections through the medial septum were stained by with cresyl violet. **(A)** Control animal (C5): cell bodies are visible (arrow) with minimal density of glial cells (arrow heads). **(B)** Lesioned animal (L6): neuronal cell bodies are absent and glial cells are present at a high density. Magnification = x130. **(C)** Choline acetyl transferase recorded with hippocampal preperations from control and lesioned animals (±SEM). The activity was significantly reduces by 24 percent ($P<0.01$) compared to the control animals (Student's t-test)


A
B

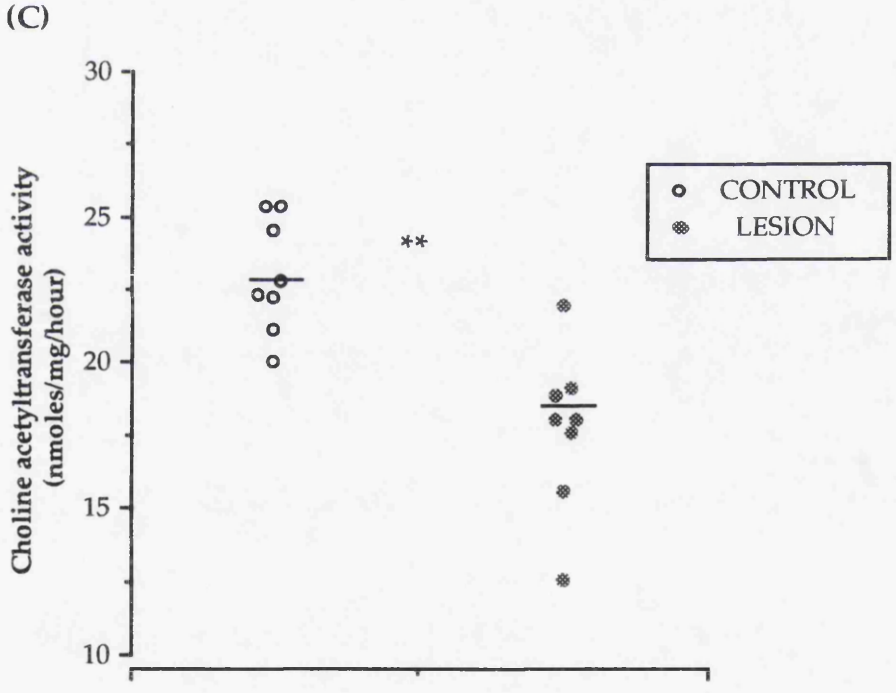

(C)
Choline acetyltransferase activity
(nmoles/mg/hour)
30
25
20
15
10
**
CONTROL
LESION

FIGURE 35

4.6.2 Immunoblot analysis of G-proteins in the hippocampus of rats lesioned at the medial septum.

The immunoblot profile of the Gα subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$ and the Gβ subunits of eight control (sham lesion) and eight lesioned animals are shown in **Figure 36** . The results of the densitometric analysis of the immunoblots are shown as scatter plots in **Figure 37**. Although the $G_{\alpha s}S$ signal could not be accurately analysed densitometrically, there was no obvious difference between the $G_{\alpha s}S$ levels of the control and lesioned groups. In this study there is clearly no significant difference in the level of any of the subunits measured in each group, which was confirmed by Student's t-test ($P>0.05$).

FIGURE 36. Immunoblot analysis of G-protein subunits $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$, ($G_{\alpha s}S$ was just visible) and Gβ detected with eight lesioned and eight control (sham lesioned) hippocampus membrane preparations. **(A)** Gβ (50μg) detected with BN1 at 1/200 dilution; **(B)** $G_{\alpha o}$ (50μg) detected with OC1 at 1/10,000 dilution; **(C)** $G_{\alpha s}L$, (50μg) detected with CS1 at 1/200 dilution; **(D)** $G_{\alpha i}1$ and $G_{\alpha i}2$ (100μg) detected with SG2 at 1/200 dilution.

FIGURE 37. Scatter distribution of the values obtained by densitometric analysis of the immunoblots shown in **Figure 36** with the mean values indicated by the plain line (—). **(A)** Gβ values; **(B)**, $G_{\alpha o}$; **(C)** $G_{\alpha i}1$, $G_{\alpha i}2$ are plotted on the same graph since both $G_{\alpha i}$ subtypes are detected with the antisera SG2 and the values for each are therefore comparable; **(D)** $G_{\alpha s}L$. Statistical analysis by Student's t test found no significant difference ($P>0.05$) between the levels of any of the G-protein subunits detected in lesioned and control (sham lesioned) hippocampus samples.

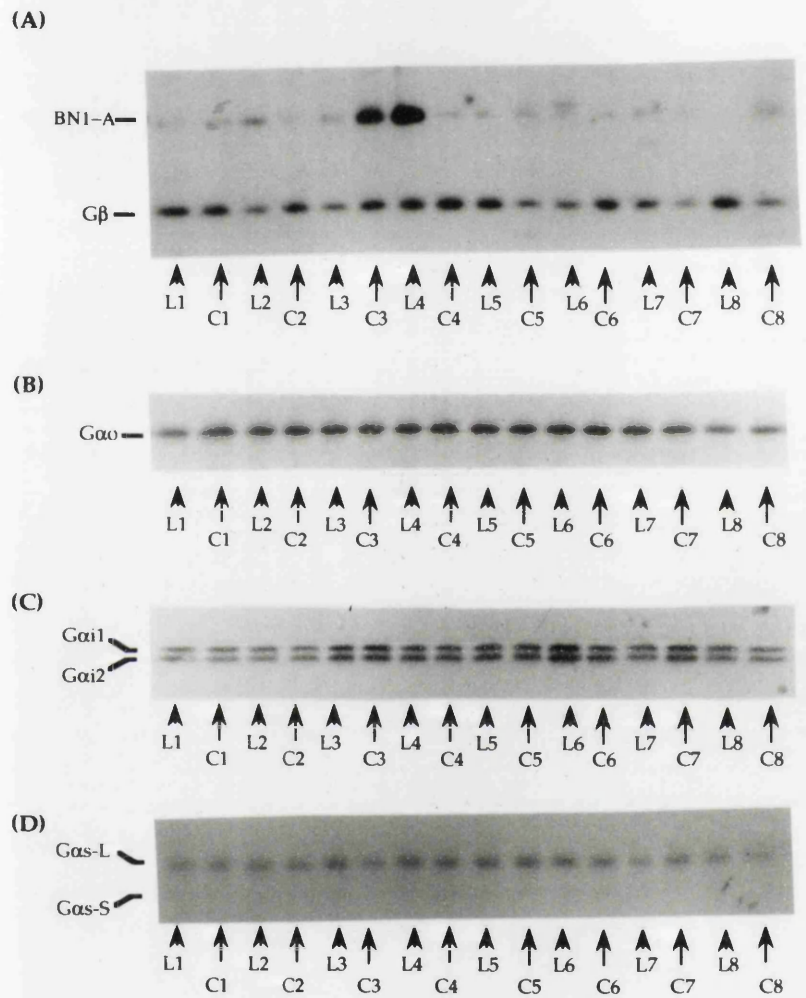

(A)
BN1-A
Gβ
L1 C1 L2 C2 L3 C3 L4 C4 L5 C5 L6 C6 L7 C7 L8 C8
(B)
Gαo
L1 C1 L2 C2 L3 C3 L4 C4 L5 C5 L6 C6 L7 C7 L8 C8
(C)
Gαi1
Gαi2
L1 C1 L2 C2 L3 C3 L4 C4 L5 C5 L6 C6 L7 C7 L8 C8
(D)
Gαs-L
Gαs-S
L1 C1 L2 C2 L3 C3 L4 C4 L5 C5 L6 C6 L7 C7 L8 C8

FIGURE 36.

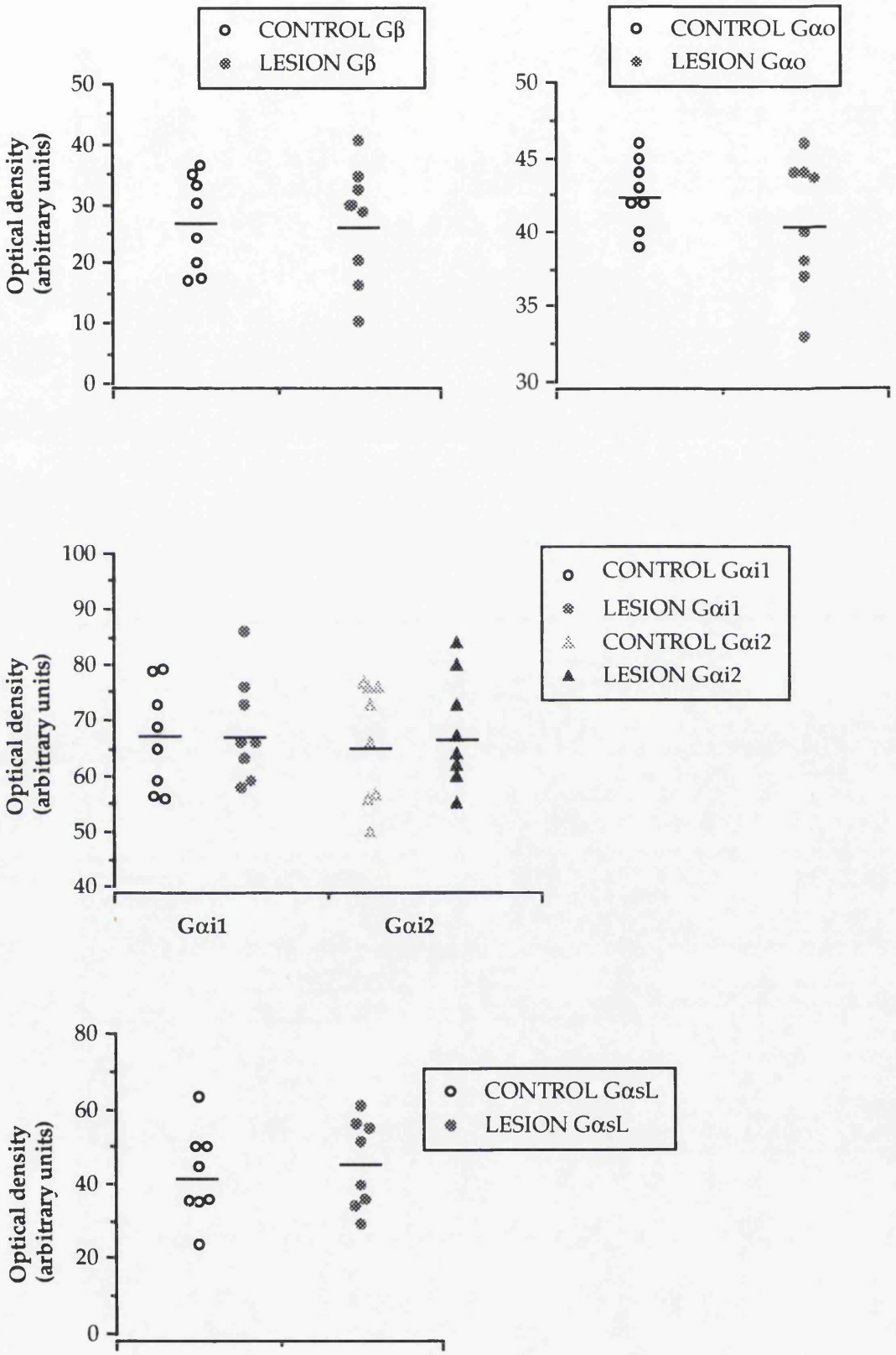

CONTROL Gβ
LESION Gβ
Optical density (arbitrary units)
50
40
30
20
10
0
CONTROL Gαo
LESION Gαo
50
45
40
35
30
CONTROL Gαi1
LESION Gαi1
CONTROL Gαi2
LESION Gαi2
100
90
80
70
60
50
40
Gαi1
Gαi2
CONTROL GαsL
LESION GαsL
80
60
40
20
0

FIGURE 37.

4.6.3 Analysis of GTPase activity in the hippocampus of rats lesioned at the medial septum.

The analysis GTPase activity was conducted in the same preparations used for the immunoblot analysis. Data for the total, basal and stimulated high affinity and the nonspecific GTPase was conducted in the same preparations used for the immunoblot analysis (**Figure 38**). The mean basal high affinity GTPase activity was increase by 20% in the lesioned group compared to controls. The difference however failed to reach statistical significance with Student's t-test ($P<0.05$). The low affinity GTPase was also increased but again failed to reach statistical significance. There was no significant difference between the degree of stimulation achieved with both carbachol and baclofen, although the mean stimulation was higher with both agonists in the lesioned group.

4.6.4 Analysis of adenylate cyclase activity in the hippocampus of rats lesioned at the medial septum.

The adenylate cyclase activity of the hippocampus membrane preparations from eight control (sham lesion) and eight lesioned were measured in the presence (stimulated) and absence (basal) of fluoroaluminate are shown in **Figure 37.** One sample shown on the scatter distribution illustration was found to exhibit abnormally low basal activity and was omitted from the statistical analysis. This sample was re-assayed and gave similar values. This sample showed no other abnormality and the cause of the low activity is unknown. Statistical analysis by Student's t test found no significant difference ($P>0.05$) between the basal and stimulated activity of the Alzheimer's disease group when compared to the control group. Similarly, there was no significant difference ($P>0.05$) in the magnitude of stimulation achieved by fluoroaluminate when the data was expressed as a percent of the basal values.

This study found that following lesioning of the medial septum, there is no gross alteration in the level or activity of G-proteins and related activity in the hippocampus.

FIGURE 38. Scatter distribution of the GTPase activity measured in membranes prepared from the hippocampus of eight lesioned and eight control (sham lesioned) samples. **(A)** illustrates the total rate of GTP hydrolysis assayed in the presence of 0.2μM cold GTP and the specific high affinity basal GTPase; **(B)** shows the nonspecific (low affinity) GTPase activity assayed in the presence of 100μM cold GTP. The specific high affinity activity shown in **(A)** is calculated from the difference between the total and nonspecific cpm values. Comparisons between the activities of the control and lesioned groups by Student's t test found no significant difference ($P>0.05$) The activity of the high affinity GTPase was assayed in the presence and absence (basal) of carbachol (1mM) and in the presence and absence of baclofen (1mM) at 0.2μM cold GTP **(C)**. The values are expressed as the percent of basal values. Comparison of the lesion and control values by Student's t test demonstrated that for both carbachol and baclofen stimulation, there was no significant difference ($P>0.05$) in the magnitude of stimulation.

FIGURE 39. Adenylate cyclase activity of membranes prepared from the hippocampus of eight lesioned and eight control (sham lesioned animals). **(A)** Scatter distribution of the amount of cAMP formed in the presence (stimulated) and absence (basal) of fluoroaluminate (5mM sodium fluoride and 10μM aluminium chloride) with mean values indicated by the plain line (—); **(B)** Stimulation above basal values of the samples expressed as percent of the basal adenylate cyclase activity. Mean values are illustrated by a plain line (--). Statistical analysis by Student's t test found no significant difference between the basal or stimulated adenylate cyclase activity, or in the magnitude of stimulation achieved with fluoroaluminate.

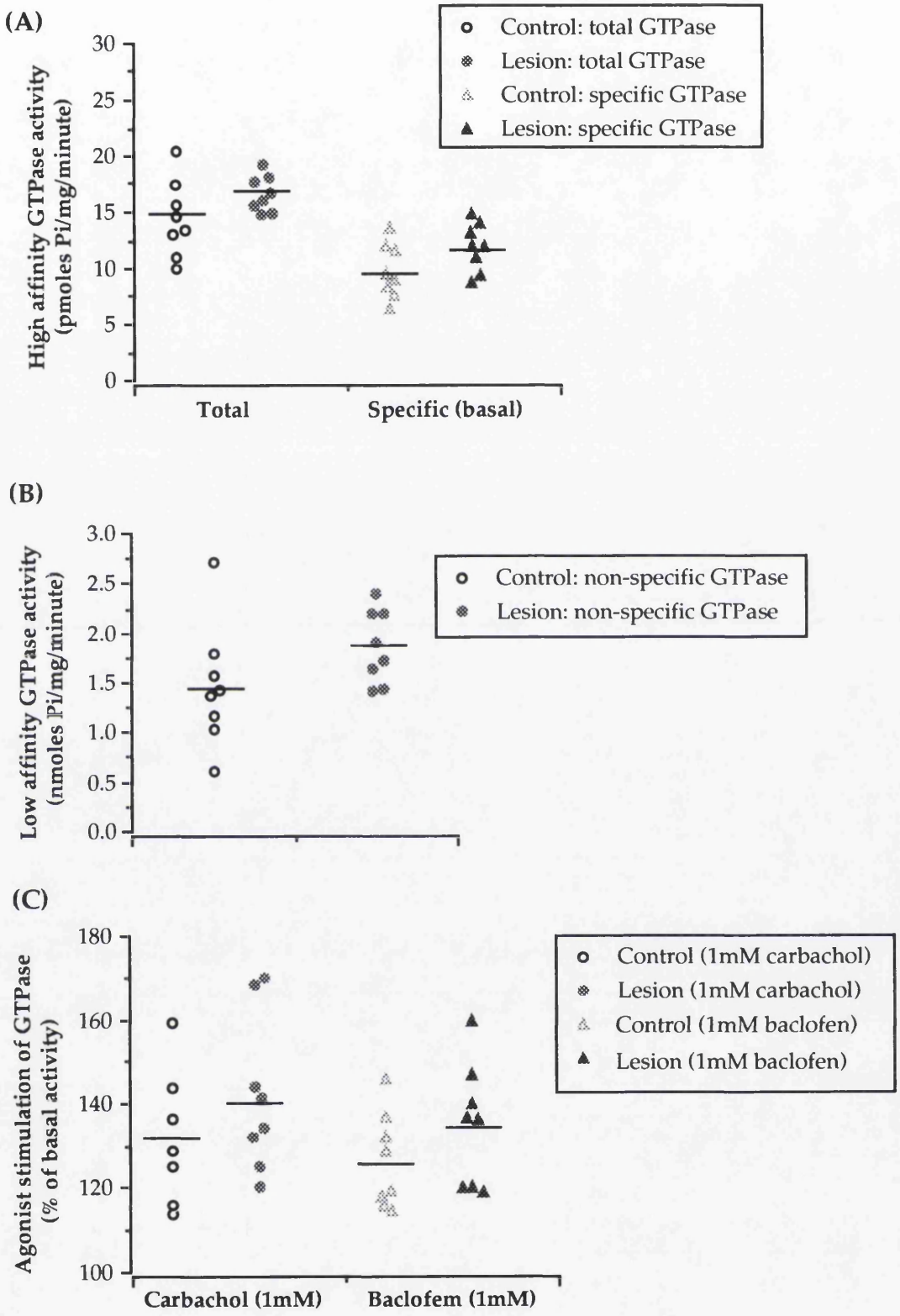

(A)
High affinity GTPase activity (pmoles Pi/mg/minute)
30
25
20
15
10
5
0
Total
Specific (basal)
Control: total GTPase
Lesion: total GTPase
Control: specific GTPase
Lesion: specific GTPase
(B)
Low affinity GTPase activity (nmoles Pi/mg/minute)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control: non-specific GTPase
Lesion: non-specific GTPase
(C)
Agonist stimulation of GTPase (% of basal activity)
180
160
140
120
100
Carbachol (1mM)
Baclofem (1mM)
Control (1mM carbachol)
Lesion (1mM carbachol)
Control (1mM baclofen)
Lesion (1mM baclofen)

FIGURE 38

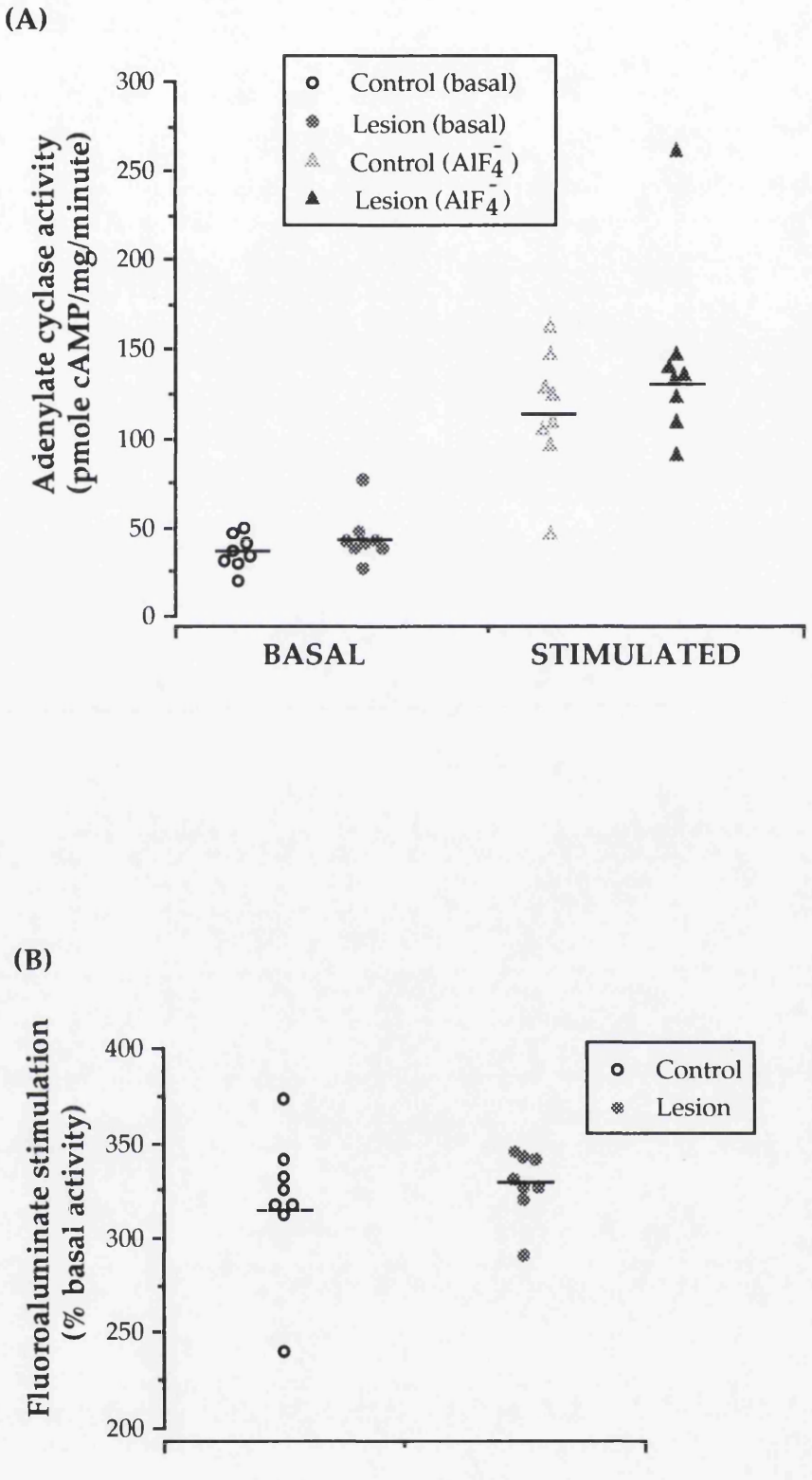

(A)
Adenylate cyclase activity
(pmole cAMP/mg/minute)
300
250
200
150
100
50
0
Control (basal)
Lesion (basal)
Control (AlF_4^-)
Lesion (AlF_4^-)
BASAL
STIMULATED
(B)
Fluoroaluminate stimulation
(% basal activity)
400
350
300
250
200
Control
Lesion

FIGURE 39.

4.7 Summary of results.

The findings of this thesis are summerised in the follwing tables. **Table 8** lists the results reported in section **4.3** which examined the effect of the interval between death and the freezing of brain tissue (postmortem interval) on G-protein levels and related activities. Values are expressed as mean±standard deviation (SD). Statistical analysis was conducted using one way analysis of varience (ANOVA) where * denotes $P<0.05$ and ** denotes ** $P<0.01$.

Tables 9 and **10** summerise the findings reported in section **4.4** which examined the effect of Alzheimer's disease on G-protein controlled signal transduction in the frontal cortex (**Table 9**) and hippocampus (**Table 10**). Values are expressed as the mean±SD. Statistical comparisons between control and Alzheimer's disease groups were conducted using Student's t-test where ** denotes $P<0.01$ and * denotes $P<0.05$.

Table 10. summerises the findings of section **4.6.** which examined the effect of medial septal lesion of the rat on G-protein controlled signal transduction in the hippocampus. Values are expressed as the mean±SD. Statistical comparisons between control (sham lesion) and lesioned animals were conducted using Student's t-test where ** denotes $P<0.01$ and * denotes $P<0.05$.

Table 8. The effect of the interval between death and freezing of brain tissue (postmortem interval=**T**) on G-protein levels and GTPase activity. Values are mean±SD wher n=5 at **T=0**, and n=3 at all other time points.

Parameter	**T=0 hours**	**T=6 hours**	**T=12 hours**	**T=24 hours**	**T=48 hours**
ChAT (nmole/mg/hr)					
P value	>0.05	>0.05	>0.05	>0.05	>0.05
$G_{\alpha o}$	57±11	54±7	55±3	48±10	54±5
P value	>0.05	>0.05	>0.05	>0.05	>0.05
$G_{\alpha i}1$	72±6	83±5	75±3	72±4	58±15
P value	>0.05	>0.05	>0.05	>0.05	>0.05
$G_{\alpha i}2$	60±3	71±6	70±7	67±4	50±13
P value	>0.05	>0.05	>0.05	>0.05	>0.05
G_{β}	24±11	13±4	26±22	9±7	12±2
P value	>0.05	>0.05	>0.05	>0.05	>0.05
Total GTPase	15.4±2.3	15.1±2	13.1±2.5	10±1.6	9.9±0.8
P value	>0.05	>0.05	>0.05	<0.05*	<0.05*
Basal GTPase	10.6±2.3	9.7±1.5	8.6±1.3	5.3±0.5	4.7±0.4
P value	>0.05	>0.05	>0.05	<0.05*	<0.05*
Low affinity GTPase	19.2±4	20.6±1.5	17.6±6	15.5±5	19.2±2.1
P value	>0.05	>0.05	>0.05	>0.05	>0.05
Carbachol stimulated (% of basal GTPase)	147±14	124±7	116±10	112±16	108±13
P value	>0.05	>0.05	>0.05	<0.05*	<0.05*
Baclofen stimulated (% of basal GTPase)	134±9	128±16	125±15	113±14	102±8
P value	>0.05	>0.05	>0.05	>0.05	<0.05*

Table 9. Summary of the results from the investigation of the effect of Alzheimer's disease on G-proteins in the frontal cortex. Values are mean ±SD where n=8 control and n=8 Alzheimer's disease samples. Statistical analysis by Student's t-test.

Parameter	Control	Alzheimers	P value
Age (Years)	79±9	85±7	0.15
Postmortem delay (Hours)	10±3	11±6	0.98
Tissue storage (Months)	27±11	19±10	0.17
Plaque density/mm^2	0.1±0.1	45.7±5.5	0.001*
ChAT (nmole/mg/hour)	3.62±0.84	1.92±0.79	0.001*
$G_{\alpha o}$	148±38	134±55	0.186
$G_{\alpha i}1$	75±20	58±14	0.064
$G_{\alpha i}2$	94±16	90±18	0.578
$G_{\alpha i}1/G_{\alpha i}2$	0.788±0.11	0.656±0.13	0.656
$G_{\alpha s}L$	170±42	154±37	0.442
$G_{\alpha s}S$	82±26	101±24	0.132
$G_{\alpha s}L/G_{\alpha s}S$	2.19±0.49	1.58±0.48	0.025*
Gβ	34±18	15±8	0.014*
GFAP band 1	30.1±14.8	71.8±88.5	0.209
GFAP band 2	18.2±9.9	54.2±75.7	0.204
GFAP band 3	32.1±28.8	97.8±85.9	0.059
GFAP band 4	55.6±43.5	124.6±87.0	0.065
GFAP band 5	64.1±71.1	132.0±72.3	0.077
GFAP band 6	79.5±66.7	133.7±87.1	0.184
GFAP total	279±193	615±453	0.075
Total GTPase	6.38±1.22	5.00±1.52	0.067
Basal GTPase	5.82±1.13	4.54±1.11	0.037*
Low affinity GTPase	0.477±0.12	0.64±0.17	0.046*
Carbachol stimulation (% of basal GTPase)	108±7	104±5	0.164
Baclofen stimulation (% of basal GTPase)	114±11	105±6	0.058
Basal adenylate cyclase	50.1±19.6	28.1±6.5	0.009*
Fluoroaluminate stimulated (% basal activity)	259±31.2	246±45.8	0.510
Fluoroaluminate stimulated (absolute activity)	132±53	70±20	0.009*

Table 10. Summary of the results from the investigation of the effect of Alzheimer's disease on G-proteins in the hippocampus. Values are mean ±SD where n=8 control and n=8 Alzheimer's disease samples. Statistical analysis by Student's t-test.

Parameter	Control	Alzheimers	P value
Age (Years)	81±5	83±7	0.59
Postmortem delay (Hours)	11±7	6±4	0.11
Tissue storage (Months)	22±13	28±10	0.39
ChAT (nmole/mg/hour)	2.9±1.1	1.0±0.6	0.001**
Plaque density/mm^2	0.2±0.5	7.9±7	0.001**
$G_{\alpha o}$	146±20	133±34	0.168
$G_{\alpha i}1$	49±18	43±18	0.472
$G_{\alpha i}2$	38±12	37±10	0.848
$G_{\alpha i}1/G_{\alpha i}2$	1.33±0.52	1.18±0.53	0.58
$G_{\alpha s}L$	133±32	154±47	0.305
$G_{\alpha s}S$	86±22	113±39	0.225
$G_{\alpha s}L/G_{\alpha s}S$	1.05±0.45	1.41±0.28	0.080
G_{β}	25±10.1	21±7.2	0.358
GFAP band 1	144±49	131±34	0.536
GFAP band 2	84±30	68±36	0.357
GFAP band 3	139±13	116±39	0.138
GFAP band 4	141±13	131±34	0.468
GFAP band 5	79±54	113±47	0.202
GFAP band 6	76±62	120±49	0.142
GFAP total	667±123	681±194	0.848
Total GTPase	11.9±1.7	12.0±4.1	0.957
Basal GTPase	10.0±1.56	7.4±3.2	0.051
Low affinity GTPase	0.77±0.29	1.92±0.72	0.001**
Carbachol stimulated (% of basal)	110±11.2	106±13.0	0.449
Baclofen stimulated (% of basal)	124±13.8	118±17.7	0.501
Basal adenylate cyclase	51.7±10.3	53.7±14.4	0.755
Flouroaluminate % basal	320±34	322±25.6	0.936

Table 11. Summary of the results from the investigation of the effect of partial denervation of the septal/hippocampal pathway on the level and related activities of G-proteins in rat hippocampus.

Parameter	Control	Lesion	P value
ChAT (nmole/mg/hour)	22.9±1.9	17.7±2.7	0.001
$G_{\alpha o}$	42.6±2.4	40.7±4.5	0.315
$G_{\alpha i}1$	66.8±9.5	68.3±9.4	0.776
$G_{\alpha i}2$	66.3±10.7	68.1±10.0	0.742
$G_{\alpha s}L$	42.2±12.1	45.1±12.3	0.646
G_{β}	26.7±7.9	26.8±10.1	0.991
Total GTPase	14.0±3.0	16.5±2.6	0.069
Basal GTPase	9.77±2.42	11.86±2.12	0.089
Low affinity GTPase	1.45±0.61	1.86±0.37	0.13
Carbacholstimulation (% of basal)	132±15	139±22	0.487
Baclofen stimulation (% of basal)	124±14	131±18	0.420
Basal adenylate cyclase	35.7±9.7	43.7±14.2	0.207
Fluoroaluminate stimulation (absolute)	113±38	132±46	0.227
Flouroaluminate stimulation (% basal)	319±37	328±17	0.438

DISCUSSION

The intensity with which the scientific community has pursued the aetiology of Alzheimer's disease has been driven by the devastating nature of this condition on the sufferer and their family. Furthermore, it has been estimated that in Europe as much as 10% of the aged population of 80-89 years may develop Alzheimer's disease (Rocca et al., 1991) which will impose a severe social and economical strain on the community. The research aims are to identify the significant event(s) particular to this disease so that appropriate therapeutic strategies can be adopted in the hope of either preventing the onset of Alzheimer's disease, or at least alleviate the impairment of cognitive processes. By achieving these aims the dependence of patients on nursing and community care should be reduced. However, effective therapeutic treatment of Alzheimer's disease cannot be realised if transduction mechanisms of cellular signalling are compromised. The present study was conducted to examine the integrity of the biochemical transmembrane signalling processes in Alzheimer's disease. Specifically, the integrity of guanine nucleotide binding proteins (G-proteins) was investigated. These proteins are a central control element of transmembrane signalling systems which operate via numerous receptors to modulate a multiple array of diverse cellular processes (for reviews see Gilman, 1987; Birnbaumer et al., 1990; Simon et al., 1991)

This thesis reports the findings of an investigation of G-protein controlled signal transduction activities in neurodegenerative conditions. The G-proteins were examined at various levels: 1) The coupling of G-proteins to selective receptors, specifically muscarinic and γ-amino-butyric acid B-type ($GABA_B$) receptors, were assessed by analysing the response of the basal high affinity GTPase when challenged with the agonists carbachol and baclofen. Additionally, the measurement of the basal GTPase in membranes assessed the collective activity of the various G-proteins associated with membranes in the resting, unstimulated state. 2) The relative abundance of five G-proteins α subunits $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha o}$, $G_{\alpha s}S$, $G_{\alpha s}L$ and Gβ subunit were compared by the semiquantatative immunoblot technique. 3) Effector

molecule activaty was assessed by analysing the basal and receptor independent stimulation of adenylate cyclase.

These analyses were performed on the frontal cortex and hippocampus of Alzheimer's disease and age matched control, samples. These are two regions which are associated with learning and memory and exhibit severe pathology in Alzheimer's disease as demonstrated by a high density of plaques and tangles and by deficits in choline acetyltransferase (ChAT) (for review see Rossor and Iverson, 1986; Pearson and Powell, 1989; Henderson & Finch, 1989). Additionally, the parameters reported in this thesis were measured (where appropriate) within the same membrane preparations to eliminate possible variations which may be encountered between different batch preparations. This approach allows comparisons to be made between different parameters to establish the existence of a biological relationship. However, the statistical power of such comparisons is compromised since the different measurements are no longer considered independent. Nonetheless, the advantages of this strategy were considered to outweigh the statistical limitations encountered by conducting multiple analyses within the same tissue preparations.

5.1 Effect of postmortem interval on G-protein mediated signal transduction.

Each of the G-proteins and their related activities were examined for the effect of the interval from death to freezing of the brain to temperatures where biological processes of interest are arrested (postmortem interval). This experiment demonstrated that the subunits $G\alpha i1$, $G\alpha i2$, $G\alpha o$ and $G\beta$ were not subjected to gross proteolysis during prolonged postmortem intervals. A separate study in the same laboratory also confirmed that both species of $G\alpha s$ ($G\alpha sS$ and $G\alpha sL$) were undegraded during prolonged postmortem intervals (Ross, 1992). Thus, the relative levels of G-protein subunits could be reliably analysed in human postmortem material which is frequently subjected to extended intervals between death and the freezing of brain material. The stability of the G-proteins was further supported by the lack of

correlation between the G-protein level and postmortem interval in the human studies (section **4.4.10**). These findings are in agreement with Young et al. (1991) who also concluded that postmortem interval did not affect the immunoreactivity of G-protein subunits. In this laboratory, Ross (1992) also concluded that adenylate cyclase is stable over prolonged postmortem intervals which is in agreement with a study conducted by Nicol et al. (1981).

Although G-protein subunits and adenylate cyclase activity were stable during prolonged postmortem intervals, the basal high affinity GTPase activity and the degree of agonist stimulation of the GTPase were compromised with extended postmortem intervals (section 5.3.3). The basal GTPase activity was significantly reduced at an interval of 24 hours compared to the activity of fresh material ($P<0.05$). The degree of stimulation achieved with carbachol was significantly decreased after a postmortem interval of 24 hours ($P<0.05$), while baclofen stimulation was significantly reduced after a postmortem interval of 48 hours ($P<0.05$). To my knowledge, there have been no investigations on the effect of postmortem interval on the high affinity GTPase. However, there is some evidence, albeit indirect, that G-protein/receptor coupling is affected by the interval between death and the storage of brain material. Smith et al. (1987) observed that the ability of Mg^{2+} (which is a requirement for G-protein/receptor interaction) to enhance carbachol binding was attenuated at 24 hours postmortem interval compared to fresh preparations. Reductions in the number of muscarinic receptor binding sites with extended postmortem have also been reported (Whitehouse et al., 1984; Syapin et al.,1987). Whitehouse et al. (1984) also observed that after a 48 hours postmortem interval, scatchard analysis of*p*-aminoclonidine binding to $\alpha 2$ adrenoceptors revealed two binding sites while only a single binding site was evident in fresh tissue. This could result from an alteration in the proportion in the high and low affinity state of the $\alpha 2$ adrenoceptor which is consistent with an alteration in GTPase activity.

The exact mechanism responsible for the reduced GTPase activity reported in this thesis is unclear. However, changes in membrane lipid composition (Farooqui & Horrocks, 1991) and

protein phosphorylation (Florez et al.,1991; Girault et al., 1989) during prolonged postmortem intervals has been shown to influence GTPase activity (Ben-Arie et al., 1988; Sauvage et al., 1991; Bushfield et al., 1990a). Although the GTPase appears to be compromised with increasing postmortem interval, its analysis was considered worthwhile since the control and the Alzheimer's disease samples were closely matched for postmortem delay and this enzyme gives an excellent insight into a central activity associated with G-protein function.

5.2. The partitioning of G-proteins and related activities between human grey and sub-cortical white matter

One of the major methodological considerations of this project was whether the tissue preparations should be made from grey matter dissected free of sub-cortical white matter or alternatively from the entire tissue block. For most analyses, the preparation of the entire tissue block was chosen since this allowed preparation of adequate material for multiple analyses. This approach also eliminated the introduction of a prolonged thawing of the tissue which would then have to be re-frozen in liquid nitrogen for subsequent preparation. Although the analysis of pure grey matter would reduce the variation associated with unequal contamination of sub-cortical white matter, it did not merit compromising the tissue yield or the introduction of a unnecessary, potentially disruptive, thaw/freeze cycle. Nevertheless, a pilot study was conducted to assess the partitioning between grey and sub-cortical white matter of the G-proteins subunits, GTPase and adenylate cyclase activity, and glial fibrillary acidic protein (GFAP). Since human brain tissue from selected regions (frontal cortex and hippocampus) were limited, the measurements were conducted in a temporal cortex of a control sample with minimal postmortem delay.

Of the G-protein subunits measured, $G\alpha_o$, $G\alpha_i1$, $G\alpha_i2$, and $G\beta$ levels were more abundant in grey matter than white matter, a finding which is in agreement with a quantitative study of primary cultures of astrocyte and neuronal cells (Brabet et al., 1988). Asano et al. (1987 &

1990) also described a high concentration of $G_{\alpha o}$ in grey matter while minimal amounts of $G_{\alpha o}$ was observed in white. The distribution of these proteins is consistent with their role in synaptic transmission.

The present study also showed that in sub-cortical white matter the ratio of $G_{\alpha s}L/G_{\alpha s}S$ was inverted compared to grey matter. Although Jones et al. (1990) reported that both $G_{\alpha s}$ species exhibited similar biochemical properties, others have suggested that the two $G_{\alpha s}$ species have different efficacy for adenylate cyclase in selected cell cultures (Walseth et al., 1989). A similar reversal in the ratio of the $G_{\alpha i}$ species was evident ($G_{\alpha i}2$ more abundant in grey and $G_{\alpha i}1$ predominating in white matter) although not as marked as that observed for the $G_{\alpha s}$ subtypes. The inverted G-proteins ratio in grey compared to sub-cortical white matter could reflect the different signal transduction requirements of neurons and glia cells. Indeed differential coupling of muscarinic and adrenergic receptors to the phosphoinositol and adenylate cyclase pathways have been described for neurons compared to glia cultures (Wilson et al., 1990; Atkinson & Minneman, 1991).

Higher activities of the enzymes choline acetyltransferase (ChAT), GTPase and adenylate cyclase in grey matter again reflects their role in neurotransmission. The high affinity GTPase activity would be expected to parallel the G-protein distribution, particularly $G_{\alpha o}$ since this is the most abundant protein in the CNS (Brabet et al., 1988; Asano et al., 1990). The low affinity nonspecific GTPase activity was marginally increased in white matter compared to grey. This may reflect axonal or astrocytic activity associated with cytoskeletal proteins polymerisation (Carlier and Pataloni, 1981; Shpetner and Vallee, 1992). As discussed previously (section 5.1), the lack of stimulation of the basal GTPase by carbachol and baclofen may be due to a contribution of the effects of the postmortem interval, aging (Villalobos-Molina et al., 1992; Yamagami 1992) and alterations in the lipid bilayer (Ben-Aire et al., 1988). The higher activity of adenylate cyclase in grey matter compared to sub-cortical white is in agreement with Ebersolt et al. (1981) and with the forskolin binding (as a

measure of activatable adenylate cyclase) study of Poat et al. (1988). The magnitude of stimulation by the receptor independent activator, fluoroaluminate, was comparable for grey and sub-cortical white matter (260% of basal activity). Thus, the difference in $G_{\alpha s}L/G_{\alpha s}S$ ratio does not alter the maximal stimulatory capacity of these proteins for adenylate cyclase. In fact the sum of the densitometric values for $G_{\alpha s}L$ and $G_{\alpha s}S$ in grey and in white matter were approximately equal. This suggests that fluoroaluminate action on $G_{\alpha s}L$ and $G_{\alpha s}S$ is addative.

The GFAP immunoblot detected multiple protein bands in grey and sub-cortical white matter. The presence of multiple GFAP bands has been described in rat brain and suggested to be the result of calcium dependent proteolysis (De Armond et al., 1983). A similar multiprotein pattern (which was proposed as a proteolytic event) has been described in rats that were protected from N-methyl-D-aspartate (NMDA) induced neurodegeneration by the antagonist MK801 (Haglid et al., 1991). In this thesis there was also a striking reversal in the GFAP protein pattern observed in grey compared to sub-cortical white matter. The lower molecular weight species being more abundant in the white matter fraction which also has higher total content of GFAP positive proteins (**Figure 29**). The different GFAP patterns may reflect different dynamics of microtubule assembly and GFAP turnover in the astrocyes present in grey compared to sub-cortical white matter.

The studies in this thesis demonstrated a higher density of G-proteins and their related enzymatic activity in grey matter compared to sub-cortical white matter. This is consistent with these proteins having a central role in neurotransmission and therefore localised within synaptic-rich grey matter. The presence of these components at a lower density in white matter is probably associated with glia cell activity although contribution of axonal content should not be overlooked.

5.3. Regional distribution of G-proteins and adenylate cyclase in the human brain.

Several studies of rat brain have been described which examined the relative distribution of various G-proteins such as $G_{\alpha o}$ (Worley et al., 1986a), $G_{\alpha i}$ (Asano et al., 1990) $G_{\alpha s}$ (Largent et al., 1988) and of adenylate cyclase (Poat et al., 1988; Gehlert, 1986; Xia et al., 1991). However similar studies, conducted on human brains, have been limited (Bergstrom et al., 1991; Mengod et al., 1988; De Keyser et al., 1988). Furthermore, there has been no systematic study which has examined G-proteins and adenylate cyclase activity in tandem. The study in this thesis was conducted to examine the relative abundance of five G-proteins ($G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha o}$, $G_{\alpha s}S$ and $G_{\alpha s}L$) and adenylate cyclase activity in discrete brain regions of control human brains. Additionally, since these parameters were analysed within the same membrane preparations, the protein levels and enzymatic activity could be compared to determine whether any putative relationships were evident.

The rank order for the abundance for $G_{\alpha o}$ in different regions of human brain (**Table 6**) paralleled that for $G_{\alpha i}1$ and $G_{\alpha i}2$. The order of abundance of $G_{\alpha o}$, $G_{\alpha i}1$ and $G_{\alpha i}2$ is similar to that described for rat brain (Asano et al., 1990). However, Asano et al. (1990) concluded that in all regions examined, $G_{\alpha i}2$ was markedly more concentrated than $G_{\alpha i}1$. The discrepancy between the findings of the study in this thesis compared to that of Asano et al. (1990) may be due to species differences. Indeed, the ratio of the $G_{\alpha s}$ species is dissimilar in rat brain when compared to human brain (Granneman & Bannon, 1991). Alternatively, the discrepancy between the $G_{\alpha i}$ ratio described in this thesis compared to the findings of Asano et al. (1990) may be due to different methodology. Asano et al. (1990) used seperate antisera for each $G_{\alpha i}$ analysed whereas this study employed an antisera (SG2) which would theoretically be equally reactive for the two $G_{\alpha i}$ species since it had been generated against a common epitope (Milligan & Unson, 1989).

The rank order for $G_{\alpha o}$ and both $G_{\alpha i}$ subtypes parallels the basal GTPase activity measured

in rat (Ghodsi-Hovsepian et al., 1990) and human brain (Franklin & Hoss, 1984). Given that $G_{\alpha o}$ and $G_{\alpha i}$ are thought to be the most abundant species of G-proteins in brain (Brabet et al., 1988; Asano et al., 1990), these proteins (particularly $G_{\alpha o}$) may be the major contributor to the basal activity of GTPase. Worley et al. (1986b) has demonstrated a similar distribution for protein kinase C (PKC) which may involve $G_{\alpha o}$ and $G_{\alpha i}$, although Gq has recently been implied as the prime controller of this effector *via* phospholipase C (Camps et al., 1992). Indeed, the regional distribution of Gq is similar to that reported for PKC (Worley et al., 1986; Milligan, 1993).

The two $G_{\alpha s}$, gave a rank order of abundance in different brain regions which was distinct from that observed for $G_{\alpha o}$ and $G_{\alpha i}$ proteins with the highest levels recorded in the cerebellum (**Figure 33**). In addition, there was a reversal in the ratio of $G_{\alpha s}L/G_{\alpha s}S$ in the striatum, cerebellum and pons when compared to the frontal cortex and hippocampus. Unlike $G_{\alpha o}$ and $G_{\alpha i}$, the distribution of $G_{\alpha s}$ protein in brain has been poorly documented. Analysis of the mRNA by *in situ* hybridisation for $G_{\alpha s}$ has been described by Largent et al. (1988) who found significant staining in the rat cortex, hippocampus and cerebellum whereas the striatum exhibited low levels. Mengod et al. (1988) reported the highest $G_{\alpha s}$ mRNA levels to be in the human cerebellar granular layers. Additionally, high $G_{\alpha s}$ mRNA levels were found in the hippocampus and cortex with lower levels in the brainstem. Although the $G_{\alpha s}$ mRNA levels does not necessarily parallel the protein levels (Walseth et al., 1989; Granneman et al., 1990), these studies would agree with the protein distribution pattern described here. Granneman and Bannon (1991), employed the polymerase chain reaction (PCR) to examine $G_{\alpha s}$ mRNA as a proportion of total $G_{\alpha s}$ mRNA in human brain and found that in the striatum $G_{\alpha s}S$ mRNA was ~35% of total $G_{\alpha s}$ mRNA whereas the cortex and hippocampus expressed only ~20% of $G_{\alpha s}S$ mRNA compared to total $G_{\alpha s}$ mRNA. However, one of the limitations of the PCR technique is that it is not quantitative since there may be different efficiencies of amplification for separate substrates. Cooper et al. (1990) has shown that $G_{\alpha s}$ in rat striatum is predominantly $G_{\alpha s}S$ whereas $G_{\alpha s}L$ is the major species present in the rat cerebellum. Since

the protein levels reported in this thesis do not parallel the relative proportion of $G_{\alpha s}L$ to $G_{\alpha s}S$ observed by Cooper et al. (1990), this would support the suggestion made by Granneman et al. (1990) and Copper et al. (1990) that these proteins maybe differentially transcribed and perhaps have distinct turnover rates.

An interesting observation of this present study was that the immunoblot of the $G_{\alpha s}$ subtypes with antisera CS1, (see **Table 3**), revealed a third protein band with a molecular weight that was intermediate to those for $G_{\alpha s}L$ and $G_{\alpha s}S$ (**Figure 32**). This protein was detected in the three striatum samples but not in any other samples. It could be speculated that the third protein band represents an additional $G_{\alpha s}$ protein subtype. Indeed, a third $G_{\alpha s}$ isolated originally from olfactory tissue (Jones & Reed, 1989) and referred to as $G_{\alpha olf}$ has a similar molecular weight to the protein detected with CS1. The $G_{\alpha olf}$ protein has a high degree of sequence homology with the other $G_{\alpha s}$ subtypes (Jones et al., 1990) and can be detected with the CS1 antibody (Milligan unpublished observation). The mRNA encoding $G_{\alpha olf}$ protein has been identified in the striatum and reported to be ten times the level of $G_{\alpha s}$ mRNA (Drinnan et al., 1991). A recent report has confirmed that striatum and substantia nigra contain significant levels of $G_{\alpha olf}$ protein (Herve et al., 1993). Taken together, these observations would suggest that the immunoreactive protein band detected in the striatum region with CS1 antibody is the $G_{\alpha olf}$ protein. Also, a novel adenylate cyclase mRNA has been identified in the rat striatum and termed AC_{ST} (Glatt & Snyder, 1993). While the mRNA for AC_{ST} is concentrated in the striatum, type I, II, and III adenylate cyclase mRNA were not detected (Glatt & Snyder, 1993). Additionally, the sensitivity of adenylate cyclase to stimulation by forskolin and inhibition by acetylcholine is different in rat cortex when compared to rat striatum (Dokas & Ting, 1993). Taken together, these findings could be interpreted to suggest that within the striatum $G_{\alpha olf}$ and the putative adenylate cyclase AC_{ST} may interact in a specific manner to meet the cAMP requirements of the neurons of the striatal areas. This may be reflected by atypical dopamine binding and G-protein coupling characteristics which has been reported to occur within this tissue (De Kyser 1989b, Sidhu et

al., 1991).

The regional distribution of adenylate cyclase was heterogeneous and exhibited a rank order which paralleled the $G_{\alpha o}$ and $G_{\alpha i}$ distribution; the highest activity was found in the frontal cortex followed by the hippocampus, striatum, cerebellum and then the pons (**Figure 34**). This pattern agrees, in general, with that described in rat brain for type 1 (Calmodulin sensitive) adenylate cyclase (Xia et al., 1991), although the highest staining was noted to occur in the cerebellum. A distribution pattern for human brain adenylate cyclase, comparable to the data described in this present study was reported by Bergstrom et al. (1991). However, Bergstrom et al. (1991) found the highest adenylate cyclase activity to be in the cerebellum. Bergstrom et al. (1991) adopted conditions which were optimal for examining receptor modulation by including 10μM GTP in their reaction solution. Thus, the difference in cerebellum activity, compared to the results reported in the present study, may be due to different methodological conditions.

The basal adenylate cyclase activity is thought to be controlled by the balance between the $G_{\alpha s}$ and $G_{\alpha s}$ activities (Katada et al, 1984a & 1984b; Gilman, 1987). In the present study however the basal activity of adenylate cyclase paralleled the regional distribution of $G_{\alpha i}$ proteins but not the $G_{\alpha s}$ proteins (**Figure 32**). This suggests that under resting unstimulated conditions, $G_{\alpha i}$ has a more potent effect on adenylate cyclase than the $G_{\alpha s}$ proteins. This was supported by linear regression analysis which revealed a strong correlation with the $G_{\alpha i}$ proteins measured in all samples versus the adenylate cyclase activity in the samples (**Table 6**). While the individual $G_{\alpha s}$ proteins failed to correlate with basal activity, a significant correlation with the ratio of $G_{\alpha s}L/G_{\alpha s}S$ with basal adenylate cyclase was observed. This suggests that in the resting state, the relative proportion of $G_{\alpha s}L$ to $G_{\alpha s}S$, rather than the level of the individual proteins, exert the stimulatory input on basal adenylate cyclase activity. A similar proposal was suggested by Granneman et al. (1990). Alternatively, the optimal $G_{\alpha s}L/G_{\alpha s}S$ ratio requirement of different regions may be governed by the proportion

of the calmodulin sensitive to calmodulin insensitive forms of adenylate cyclase (Cooper et al., 1990).

The receptor independent stimulation of adenylate cyclase by fluoroaluminate gave an inverted rank order compared to the basal activity of the enzyme. Gelhert (1986) observed a similar regional pattern of adenylate cyclase levels in rat when fluoroaluminate was used to enhance forskolin binding (as a marker for of activatable adenylate cyclase). It has been demonstrated that fluoroaluminate treatment is capable of activating $G_{\alpha i}$ (Inoue et al., 1990), Gq (Fisher et al., 1993) as well as $G_{\alpha s}$. Thus, the inverse relationship between the basal adenylate cyclase and the magnitude of stimulation of adenylate cyclase by fluoroaluminate may be due to activation of the $G_{\alpha i}$ proteins which dominate the activity of $G_{\alpha s}$. This proposal is supported by the strong negative correlation between $G_{\alpha i}$ protein levels and the magnitude of stimulation of adenylate cyclase by $G_{\alpha i}$ subtypes (section **4.5.3, Table 7**). This may be achieved by direct inhibition of the enzyme by $G_{\alpha i}$ or indirectly by contributing free $\beta\gamma$ complexes (Tang & Gilman, 1991; Taussig et al., 1993).

Linder et al. (1990) hypothesised that the supply of free $\beta\gamma$ complexes can inhibit adenylate cyclase activity by binding to and inactivating the free $G_{\alpha s}$ proteins. If this was the case, the $G_{\alpha o}$ protein, which is the most abundant G-protein in brain, would be able to influence the activity of adenylate cyclase by modulating the levels of free $\beta\gamma$ dimers. Indeed, there was a strong correlation between $G_{\alpha o}$ and the magnitude of stimulation of adenylate cyclase by fluoroaluminate ($r=0.841$; $P<0.002$).

In conclusion, this study has demonstrated a heterogeneous distribution of five G-proteins $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}S$ and $G_{\alpha s}L$ and adenylate cyclase in the the human brain. In general, the highest levels of these parameters were observed in cortex and hippocampus. This is in general agreement with the distribution of adrenergic (Joyce et al., 1991; ; De Vos et al., 1992)

and muscarinic receptors (Levey et al., 1991) and is consistent with their role in learning and memory (Xia et al., 1991). This study also suggests that adenylate cyclase regulation in the striatum is distinct from other brain regions. Furthermore, these results suggest that the basal and stimulatory adenylate cyclase response is controlled by the balance between the stimulatory and inhibitory G-proteins although $G_{\alpha o}$ is also implicated. Finally, these results may be suggestive of the cross-talk which exists between the different G-proteins and different signalling pathways.

5.4 Analysis of G-protein mediated signal transduction activities in Alzheimer's disease.

5.4.1 Analysis of G-protein levels

In an original study of a small sample size (n=6) the levels of G_α subunits were found to be robust in Alzheimer's disease brain regions compared to controls (M^{C}Laughlin et al., 1991). Considering the heterogeneous nature of Alzheimer's disease and the variability of biochemical measurements, increasing the sample size may help to detect subtle changes in the measurments in Alzheimer's disease compared to controls. Therefore, in the present study, the group size consisted of eight samples. Although, in this followup study G_α subumit levels remained unchanged, a significant difference was observed in the frontal cortex between the ratio of the $G_{\alpha s}$ subtypes $G_{\alpha s}S/G_{\alpha s}L$ in Alzheimer's disease compared to controls. The samples used in present study, were assayed for sodium/potassium dependent ATPase, which is a marker for plasma membrane (Begin-heick, 1990) and were similar for Alzheimer's disease and control samples (Ross, 1992). Thus, the membrane preparations of Alzheimer's disease and contol samples were of a similar purity. Additionaly, the GFAP immunoreactive proteins levels were analysed which served as a marker for astrocytes. Although the levels of the individual GFAP bands were elevated in the frontal cortex Alzheimer's disease samples, the difference between Alzheimer's disease and control measurements did not reach statistical significance. Thus, while the increased levels of GFAP protein bands may reflect the process of reactive gliosis which occurs in Alzheimer's disease (Duffy et al., 1980; Beach et al., 1989), the dilutional effect on the membrane preperations by astrocytes was not significant.

The investigation of this thesis strongly suggests that alteration in the G_α subunit level is not a central feature of Alzheimer's disease. Nonetheless, it is conceivable that subtle alteration, beyond the sensitivity of the immunological detection approach employed here, would have significant functional consequences. Indeed modest reductions of 10-15% in the

pertussis toxin (PTX) sensitive G-proteins ($G_{\alpha o}$ and $G_{\alpha i}$) have been shown to induce marked changes in membrane ion channel activity (Innes et al., 1991). However, PTX catalysed ADP-ribosylation of G-proteins is believed to prefer the heterotrimeric confirmation of G-proteins and may therefore reflect the proportion of the inactive G-protein complex (Katada et al., 1986) rather than the absolute amount of the G_{α} subunit. Indeed, following an ischaemic insult in rat brain there was a reduction in the level of the PTX labelled G-proteins, while immunoblot analysis found the level of $G_{\alpha i}$ and $G_{\alpha o}$ were preserved (Takenaka et al., 1991). These authors proposed that this disparity was due to a reduction in the free GDP bound G_{α} PTX sensitive proteins since addition of free $\beta\gamma$ complexes failed to restore the ADP ribosylation level to that achieved in the non-ischaemic animal.

Harrison et al. (1991) demonstrated, by *in situ* hybridisation, that $G_{\alpha s}$ mRNA in the hippocampus and temporal cortex were significantly increased in Alzheimer's disease. This suggests that an increased rate of transcription of the $G_{\alpha s}$ is required to maintain the $G_{\alpha s}$ protein levels. Alterations in $G_{\alpha s}$ mRNA levels to preserve to protein corresponding protein levels have been observed in the adipocytes of rats which were exposed to cold temperatures (Granneman et al., 1990). In another study, Ross et al. (1992) found no significant alteration in mRNA for $G_{\alpha s}$ in the frontal cortex of Alzheimer's brains while a low signal intensity for $G_{\alpha s}$ in the hippocampus prevented accurate densitometric analysis. However, Ross et al. (1992) observed a large variability in the integrity of the mRNA material isolated from different human brain samples. Ross et al. (1992) concluded that the small sample size employed in his study and large variability in the intensity of the $G_{\alpha s}$ mRNA which he encountered prevented a definitive conclusion being draw regarding the effect of Alzheimer's disease on the level $G_{\alpha s}$ mRNA.

Although the present study found no significant alteration in the level of G_{α} subunits in Alzheimer's disease, there was a significant reduction in the Gβ (35/36 KDal) subunit(s) measured in the frontal cortex of Alzheimer's disease compared to control samples. This

analysis was performed on membrane samples that were stored for 10 months at -80^{o}C and had been through 3 freeze/thaw cycles (section **4.4.2**). The Gβ immunoblot of the frontal cortex samples also identified a prominant high molecular weight protein (**Figure 16**). It was also found that within the frontal cortex control group, there was a strong correlation between the level of Gβ and freezer storage time ($r=0.928$, $p<0.001$). These observations may indicate that the samples have been subjected to a proteolytic process during the prolonged storage. Thus, the Gβ analysis was conducted on samples that did not meet the stringent criteria for sample handling, storage and preparation which was adopted throughout this project to ensure a high standard of biochemical analyses. However, the Gβ proteins (**Figure 16**) were detected as sharp and clearly visible bands which would indicate that the Gβ proteins were minimally affected by the prolonged storage.

There is now convincing evidence that the Gβ subunit may play an active role in the modulation of effector systems rather than serve a relatively passive role involving the aggregation and dissociation of the heterotrimer on receptor activation. It has been demonstrated that the Gβ, or more specifically the Gβγ complex, can directly activate the calcium/calmodulin insensitive adenylate cyclase activity (Federman et al., 1992; Tang & Gilman, 1991; Taussig et al., 1993); inhibit calcium sensitive adenylate cyclase activity (Tang et al., 1990; but see Mangels et al., 1992); directly activate phospholipase C (Camps et al., 1992) and modulate muscarinic receptors activity (Haga & Haga, 1992). The reductions in Gβ observed in this thesis could lead to a significant modulation in the activity of adenylate cyclase activity, a finding that is indeed observed in this study. It is also possible that the putative alteration in the βγ levels could affect the inositol phosphate signalling pathways. Alterations in the protein kinase C (PKC) translocation in Alzheimer's disease (Cole et al., 1988; Masliah et al., 1990c; Shimohama et al., 1990; but see Horsburgh et al., 1991) strongly indicates an alteration in the inositol signalling system. However, a recent report has shown that phospholipase C, which is modulated by Gβ (Camps et al., 1992) is not altered in Alzheimer's disease (Shimohama et al., 1992; but see Shimohama et al., 1991).

Alterations in G-protein levels have been described for psychiatric conditions and in neurodegenerative disorders other than Alzheimers disease. Young et al. (1992) reported a significant increase of both $G_{\alpha s}L$ and $G_{\alpha s}S$ in the prefrontal cortex (22%) and occipital cortex (80%) in patients with bipolar affective disorders. Since Young and co-workers, (1991) found increased $G_{\alpha s}$ in patients that had received lithium and those that had not, it is unlikely that lithium had induced these changes. A recent investigation of olivopontocerebellar atrophy disease reported increased levels of $G_{\alpha s}$ and reduced $G_{\alpha i}$ proteins in cerebellar cortex (Kish et al., 1993). Although the significance of these alterations, regarding neuronal function and the neurodegenerative process, are unclear, they demonstrate that alterations in G-protein levels can occur within selected brain regions in specific neurodegenerative disorders.

5.4.2. Analysis of GTPase activity

The high affinity GTPase activity was found to be significantly reduced (by 25%) in the frontal cortex of Alzheimer's disease when compared to control samples. A reduction in high affinity GTPase was also observed in the hippocampus of Alzheimer's disease samples (27%) which failed to reach statistical significance (reduced by 27% P=0.051). Post hoc power analysis would suggest that for the level of variability encountered for the basal GTPase activity, a group size of 18 samples would be required to to give a significant difference at the 5% level with 80% statistical power using a two-tailed Student's t-test.

These findings present evidence for a functional alteration in G-protein mediated signal transduction process in Alzheimer's disease. There are several reasons to explain the reduction in GTPase activity in Alzheimer's disease. First, there is evidence that $G_{\alpha i}$ can be phosphorylated (Bushfield et al., 1991) which has been shown to lower the intrinsic GTPase activity of human brain extracts (Sauvage et al., 1991). Recently, it was reported that

several G-proteins ($G_{\alpha s}S$, $G_{\alpha s}L$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha i}3$, $G_{\alpha o}$ and purified transducin) are phosphorylated at tyrosine residues by the product of the potential oncogene $pp60^{c\text{-}src}$ (Hausdorff et al., 1992). Functional analysis of $G_{\alpha s}S$ revealed that phosphorylation was inhibited by $\beta\gamma$ subunits in a dose dependent manner and that basal and agonist stimulated GTPγS binding was enhanced by phosphorylation (Hausdorff et al., 1992). Taken together, these observation suggest that the GTPase activity associated with G-proteins can be modulated by phosphorylation of the Gα subunits and this mechanism of controlling G-protein activity is sensitive to the level of free $\beta\gamma$ dimers. In Alzheimer's disease, aberrant protein phosphorylation of for example tau and neurofilaments (Grundke-Iqbal et al., 1986; Bancher et al., 1991; Zhang et al.,1989), and synapsin I (Parks et al., 1991) is well documented. This has been partly attributed to alterations in the action of various kinases such as protein kinase C (Cole et al., 1988; Masliah et al., 1990c; Shimohama et al., 1990; but see Horsburgh et al., 1991), casein kinase II (Iimoto et al., 1989), tyrosine kinase (Shapiro et al., 1991), and recently, mitogen activated protein (MAP) kinase (Drewes et al., 1992). These findings support the contention that abnormal phosphorylation activities in Alzheimer's disease may be involved in the modification of GTPase activity.

Second, the activity of GTPase has been shown to be influenced by the lipid content of the membrane (Ben-Arie et al., 1988). In Alzheimer's disease changes in membrane lipid composition has been reported (Soderberg et al.,1992; Sofic et al., 1991) and may therefore result in altered GTPase activity. Finally, alterations in GTPase activity has been shown to occur as a result of specific point mutations of $G_{\alpha s}$ and $G_{\alpha i}$ proteins isolated from endocrine tumours (Landis et al., 1989; Wong et al., 1991) and with remonbinant Gq (Conklin et al., 1992). Detailed analysis of the amino acid composition of G-proteins isolated from Alzheimer's disease brain is required to determine if point mutations are the cause of the reduced GTPase activity observed in the present study.

The nonspecific, low affinity GTPase activity was significantly increased in both the frontal

cortex and hippocampus in Alzheimer's disease. The GTPase assay was conducted under conditions that selectively enhanced the high affinity activity over the low affinity activity. This was achieved by including an ATP regenerating system and subtracting the hydrolysis of [γ-^{32}P] GTP measured in the presence of excess cold GTP. The inclusion of 100μM cold GTP was used to minimise the low affinity activity, and under these conditions the assay remains sensitive for the low affinity GTPase as demonstrated by the linear rate of hydrolysis against time and protein level (**Figure 4**). The source of the low affinity GTPase associated with crude membrane preparations is unclear. The presence of GFAP in these samples confirm that cytoskeletal proteins are co-sedimented by this method. It is tempting to speculate that the activity reflects the GTP hydrolysis associated with cytoskeleton since tubulin (Carlier & Pantaloni, 1981) and dynamin (Shpetner &Vallee, 1992) have higher GTP hydrolysis rates than G-proteins (K_{cat} 2-4 minutes). Indeed, Hoss et al., (1988) reported that purification of synaptic membranes gave an enhanced signal to noise ratio of the high to low affinity GTPase when compared to washed membranes. The increased low affinity GTPase activity in Alzheimer's disease may thus be due to an enhanced cytoskeletal associated GTPase action. This suggestion is supported by the recent demonstration that tau isolated from Alzheimer's disease brain is less effective in enhancing tubulin polymerisation than tau isolated from non-demented brain (Lu & Wood, 1993).

The GTPase activity measured in membranes reflects the cycling between the inactive and the active configurations of G-proteins and this cycle is dependent on the presence of receptors (see section **1.2**). Although the basal activity gives some insight into the integrity of G-protein coupling to receptors this method is unable to distinguish between the individual receptors and their G-proteins. This can be partly overcome by measuring the GTPase activity in response to selected agonists. Various studies have indicated that the coupling of specific receptors to G-proteins is altered in Alzheimer's disease. These receptors include the M1 muscarinic receptor (Smith et al., 1987; Flynn et al., 1991) and nicotinic receptors (Nordberg et al., 1992) although Pearce & Potter (1991) reported that the high affinity M1 receptor site

was preserved in Alzheimer's disease. Other receptors that have been reported as having an altered coupling with G-proteins in Alzheimer's disease are the dopamine D1 receptor (De Keyser et al., 1990) and the α2-adrenoceptor (Pascual et al., 1992). O'Neill et al. (1991a & 1991b) reported that the coupling of G-proteins to the α2 and 5HT receptors were unaltered in Alzheimer's disease.

In the present study, receptor/G-protein coupling was examined by monitoring the magnitude of agonist stimulation of basal GTPase activity. Carbamylcholine (carbachol) was used as it can interact with the M1 and M3 subclasses of muscarinic receptors to stimulate phospholipase C activity via a pertussis toxin insensitive G-protein (probably the Gq). Carbachol can also interact with M2 and M4 receptors to inhibit adenylate cyclase via $G_{\alpha i}$ proteins (Peralta et al., 1988; Fukamauchi et al., 1991). Baclofen, (a selective $GABA_B$ agonist) was also examined. $GABA_B$ receptors are thought to inhibit voltage dependent calcium channels *via* pertussis sensitive G-proteins possibly $G_{\alpha o}$ (Holz et al., 1989). Within the frontal cortex there was a tendency for the magnitude of stimulation by both agonists to be lower in the Alzheimer's disease group compared to the controls. The reductions in the magnitude of stimulation by both agonists however, failed to achieve statistical significance. It is also apparent from the present study that some of the hippocampus and frontal cortex preparations from both Alzheimer's disease and control samples gave a poor response to agonist stimulation. One the other hand, some patients gave stimulation in both the frontal cortex and the hippocampus (e.g. patient C19A). It is unlikely that the poor stimulation observed for some samples is due to methodolgical limitations since both the tissue preparation and GTPase analysis of the frontal cortex and hippocampus regions were conducted independent of each other. There are several reasons why some of the human samples gave a poor response to agonist stimulation of GTPase. In this thesis the effect of the interval between death and freezer storage of rat brain (postmortem interval) was demonstrated to reduce both the basal GTPase activity and the response of this enzyme to carbachol and baclofen (**Figure 12**). There was however no correlation between postmortem

interval and the basal and stimulated GTPase activity of the human brain samples. The muscarinic receptor and α1-adrenoceptors stimulation of GTPase stimulation has been shown to be reduced in aged compared to young rats (Villalobos-Molina et al., 1992; Yamagami et al., 1992). Agonal status has been shown to effect a variety of neurochemical activities (Perry et al., 1982; Piggot et al., 1992; Barton et al., 1993) and may therefore influence the stimulation of GTPase activity by receptor agonists.

Taken together, these data suggest that GTP hydrolysis by G-proteins and by other unidentified protein(s) is altered in Alzheimer's disease. The reduction in the high affinity GTPase in Alzheimer's disease frontal cortex and hippocampus may indicate an alteration in G-protein function. It was also noted that in the control samples the basal GTPase correlated with the levels of $G_{\alpha o}$, $G_{\alpha i}1$ and $G_{\alpha i}2$ measured in the frontal cortex, and with $G_{\alpha i}1$ and $G_{\alpha i}2$ in the hippocampus (**Table 5**). There was no correlation between the level of these proteins and activity of GTPase that was measured in the Alzheimer's disease samples, except in the hippocampus of Alzheimer's disease where the basal GTPase correlated with the levels of $G_{\alpha i}2$ ($r=0.73$, $P<0.05$). This suggests that in Alzheimer's disease there is a disruption of G-protein function which is independent of an alteration in the levels of G-proteins. The enhanced low affinity activity may be a consequence of altered cytoskeletal dynamics. It is possible that these changes are related since it has been proposed that the cytoskeletal protein tubulin serves as a GTP sink (Rasenick & Wang, 1988). The exchange of GTP with $G_{\alpha i}$ and $G_{\alpha s}$ has been shown to alter adenylate cyclase activity (Rasenick & Wang, 1988). Additionally, tubulin has been demonstrated to reduce muscarinic stimulation high affinity GTPase in rat striatum (Ravindra & Aronstam, 1990). This action may be achieved by the interaction of tubulin with the G-protein and/or muscarinic receptor (Ravindra & Aronstam, 1991). The alteration in GTP hydrolysis observed in the present study may reflect a disruption of the equilibrium between the free GTP bound G_{α} subunit and the receptor coupled G-protein heterotrimer. One consequence of this effect could be an alteration in the proportion of a given receptor which is in its high affinity state and which has been

described in Alzheimer's disease (Smith et al., 1988; Flynn et al., 1991; Nordberg et al., 1992; De Keyser et al., 1990; Pascual et al., 1992)

5.4.3. Analysis of adenylate cyclase activity in Alzheimer's disease.

In the present study, the basal activity of adenylate cyclase was significantly reduced by 44% in the frontal cortex of Alzheimer's disease compared to control samples. There was no significant difference in the hippocampal basal adenylate cyclase activity of Alzheimer's disease compared to controls. In both regions the percentage stimulation above basal levels by the receptor independent action of fluoroaluminate was comparable between the Alzheimer's disease and control groups.

The investigation of adenylate cyclase activity in Alzheimer's disease has now been conducted by several laboratories (Danielsson et al., 1988; Ohm et al., 1991; Bergstrom et al., 1991; Cowburn et al., 1992a; Cowburn et al., 1992b). The findings of these different studies have however been inconsistent. Danielsson et al. (1988), reported that the basal activity was significantly increased (46%) in the hippocampus of Alzheimer's disease brain but was similar to control samples in the parietal cortex. The degree of stimulation by vasoactive intestinal polypeptide (VIP) and forskolin was unchanged by the presence of Alzheimer's disease. Ohm et al. (1991) found a significant reduction in basal adenylate cyclase activity in the hippocampus although the percentage stimulation achieved with isoprenaline, Gpp(NH)p and forskolin were unchanged (although the absolute amount of cAMP generated in their presence was reduced). Cowburn et al. (1991) reported that the basal adenylate cyclase activity of the temporal cortex was preserved but somatostatin inhibition was reduced. In a subsequent study, Bergstrom et al. (1991) reported that in Alzheimer's disease that basal activity and somatostatin inhibition of adenylate cyclase was unchanged in the frontal cortex, hippocampus, caudate nucleus and cerebellum. Recently Cowburn et al. (1992a) reported that in five brain regions (frontal, occipital and temporal cortex; angular gyrus and

cerebellum) the basal activity of adenylate cyclase was preserved in Alzheimer's disease, although a 26% reduction was observed in the frontal cortex. The percentage stimulation achieved with fluoroaluminate and forskolin (1 and 100mM) was significantly reduced in the neocortex (Cowburn et al. 1992a). Finally, Cowburn et al. (1992b) examined the inhibitory arm of the adenylate cyclase system and under these conditions basal values were significantly reduced in the frontal and temporal cortex, the angular gyrus but was unchanged in the cerebellum. The inhibition achieved with fluoroaluminate was unchanged suggesting the $G_{\alpha i}$ activity is preserved. They also observed that while a biphasic response to Gpp(NH)p (10nM-1mM) was evident in the control samples, the increased adenylate cyclase activity at high concentrations of Gpp(NH)p was lost in the Alzheimer's disease samples.

These studies, together with the findings of this thesis, indicate that the adenylate cyclase system is altered in Alzheimer's disease. They are however inconsistent regarding the regional and mode of altered adenylate cyclase activities. The differences may be attributed, in part, to the particular sample preparation procedure and/or the assay system employed by the investigators. For instance, Ohm et al. (1991) homogenised their samples at 37^{o}C to remove endogenous cAMP and analysed the activity in the homogenates (resuspended pellet after 15,000g spin), yet Nicol et al. (1981) demonstrated that adenylate cyclase in whole tissue homogenates is labile. Cowburn et al. (1992a), on the other hand, employed similar conditions to that used in the present study, yet although Cowburn et al. (1992a) reported that the basal activity was not significantly altered (decreased by 22%), the degree of stimulation of adenylate cyclase was impaired. In the study by Cowburn et al. (1992a), the magnitude of stimulation of adenylate cyclase achieved by fluoroaluminate was markedly lower (29% stimulation above basal for control versus 9% for Alzheimer's disease) than that observed in the present study (260% above basal for control and Alzheimer's disease). Perhaps the discrepancy is due to methodological differences such as the inclusion of sodium ions or calcium chelators. Finally, Cowburn et al. (1992b) included calmodulin in their assay mixture when examining the inhibitory arm of the pathway. Their assay conditions did not include

exogenous calcium at stimulatory concentrations (10^{-7}-10^{-5}M). However, it has been recently demonstrated that reagents commonly used in the adenylate cyclase assay are a potential source of calcium contamination (Yovell et al., 1992). Thus, it is possible that the assay conditions employed by Cowburn et al. (1992) was influenced by the calcium/calmodulin sensitive adenylate cyclase subtypes. The assay conditions employed in the present study (repeated washing of membranes with buffer containing 1mM EDTA) reflects primarily the activity of the calmodulin insensitive adenylate cyclase under stimulatory conditions. However, as demonstrated by Caldwell et al. (1992), it is likely that particulate membrane preparations contain calmodulin (since addition of only 5nM calmodulin can induce stimulation) Therefore, the basal activity of adenylate cyclase reported in this thesis, while preferential for the calmodulin insensitive enzyme, probably reflects a contribution of the calmodulin sensitive types.

There are several possible explanations for the reduction in basal adenylate cyclase activity in the frontal cortex of Alzheimer's disease observed in this thesis. A reduction in the absolute amount of adenylate cyclase may have occurred. However, in situations where reductions in basal adenylate cyclase activity has been described, this does not appear to be due to changes in the enzyme level. Gawler et al. (1987), found reduced basal activity in diabetic animals although Bushfield et al. (1990), reported that the enzyme level was in fact increased. Recently Hershberger et al. (1992) examined adenylate cyclase activity under a variety of conditions in failing and non-failing hearts. The basal adenylate cyclase activity was decreased in pathologic tissue when assayed in the absence of GTP (termed "G-protein conditions") but unaltered when GTP was present. The altered adenylate cyclase activity in failing heart was attributed to an increase in a pertussis toxin sensitive $G_{\alpha i}$ protein (Hershberger et al., 1991). Similarly, changes observed in basal adenylate cyclase activity in the diabetic animal (Gawler et al., 1987) may be a result of an increased $G_{\alpha i}3$ level (Strassheim et al., 1990). Alterations in the functional activity of the inhibitory and stimulatory G-proteins may also explain the decreased basal adenylate cyclase activity

observed in diabetic animals (Bushfield et al., 1990). Thus, modifications in the levels of the adenylate cyclase molecule does not appear to be a common mechanism whereby cells regulate basal cAMP levels. Confirmation of the preservation of adenylate cyclase levels in Alzheimer's disease may be achieved by immunoblot analysis of the enzyme (Mollner et al., 1990).

There may be a reduced residual effect of $G_{\alpha s}$ on adenylate cyclase which is thought to exist under basal conditions (Birnbaumer et al., 1990) which may account for the reduced activity reported in this thesis. Indeed Dewar et al. (1991), observed a reduction in forskolin binding in the Alzheimer's disease frontal cortex and this ligand, under appropriate conditions, binds preferentially to the $G_{\alpha s}$/adenylate cyclase complex. This finding may relate to the altered ratio of $G_{\alpha s}L/G_{\alpha s}S$ observed in the frontal cortex (**Figure 16**). It has been shown that during development of mice brain there is a change in the ratio of $G_{\alpha s}L/G_{\alpha s}S$ proteins with a concomitant increase in basal adenylate cyclase Gpp(NH)p (Rius et al., 1991).

Normal control of basal adenylate cyclase activity which is regulated by the equilibrium of the stimulatory and inhibitory inputs, may be compromised causing an enhanced tonic inhibition of adenylate cyclase. This has been reported to occur in the diabetic induced rat (Strassheim et al., 1990). Although correlation analysis has a limited value when applied to small sample sizes and the large number of potential analyses, it is interesting that in the frontal cortex both $G_{\alpha i}$ proteins have a positive association with the basal adenylate cyclase activity which is not evident in the Alzheimer's disease group (**Figure 28**). This may reflect a breakdown in the normal inhibitory action of $G_{\alpha i}$ on adenylate cyclase which has been attributed to $G_{\alpha i}$ phosphorylation (Bushfield et al., 1992). It is also tempting to speculate that if the reduced basal GTPase reported in this thesis is due to a lowering of intrinsic activity associated with $G_{\alpha i}$, then this may prolong the inhibitory action without altering the protein level. Again, this may be inferred by the correlation of basal adenylate cyclase activity with basal GTPase in control frontal cortex which is not evident in the Alzheimer's

disease group. An increase of endogenous neurotransmitters, which inhibits adenylate cyclase, could also produce the reduction in the basal activity observed in the present study. Hershberger et al (1992) found that although basal activity was reduced in heart disease (assayed in the absence of GTP), the basal levels were preserved after treatment of preparations with adenosine deaminase to remove adenosine inhibition of adenylate cyclase. This proposal does not agree with the findings of Cowburn et al. (1992b) who reported that the inhibitory arm of adenylate cyclase is preserved in Alzheimer's disease. As mentioned above however, the assay conditions employed by Cowburn et al (1992b) may reflect primarily the calmodulin-sensitive forms of adenylate cyclase.

Finally, the reduced basal adenylate cyclase activity could be a consequence of the putative reduction in the Gβ protein reported in this thesis. This may result from the loss of stimulatory action described for the βγ complex (Tang & Gilman, 1991; Federman et al., 1992; Taussig et al., 1993) on type II and IV (calmodulin insensitive adenylate cyclase). However, the βγ complex has also been described to have an inhibitory effect on type I adenylate cyclase (Tang & Gilman, 1991; Taussig et al., 1993). Thus, to achieve a net reduction in the basal activity, the loss of stimulation by βγ is required to be significantly greater than the loss of inhibitory action on calmodulin-sensitive activity. Alternatively, this putative reduction in the β subunit could result in a higher abundance of the free α subunits of G-proteins. Since $G_{\alpha o}$ and the $G_{\alpha i}$ subtypes are thought to be more abundant in brain than $G_{\alpha s}$, (Asano et al., 1990) it is reasonable to suggest that these protein would be most influenced by the lower available β subunit level.

In conclusion, it can be hypothesised that the lower basal adenylate cyclase activity results from either a reduction in the enzyme level or a misbalance between the inhibitory and stimulatory constraints imposed on the enzyme under resting levels. It can be envisaged that an alteration in the normal localised concentration and metabolism of GTP, as indicated by the GTPase data, could influence the adenylate cyclase action. It is also emerging that

protein kinase C (PKC) can selectively activate the type II adenylate cyclase (Ca^{2+}/calmodulin-insensitive) (Yoshimura & Cooper, 1993). Given that the activity of PKC is considered to be altered in Alzheimer's disease, this may present another mechanism to explain the alterations in the adenylate cyclase pathway in Alzheimer's disease.

5.4.4. Significance of G-protein mediated signal transduction in Alzheimer's disease.

The main findings of this investigation were as follows: the levels of G-protein α subunits were preserved in Alzheimer's disease frontal cortex and hippocampus; the basal activity of adenylate cyclase was reduced in the frontal cortex but not in the hippocampus; in both regions, there was a decrease in the basal high affinity GTPase activity and a significant increase in the low affinity GTPase. In the preceding sections these observations are discussed in relation to the specific molecular interactions relative to signal transduction events. The following discussion will incorporate the significant developments that have been made regarding the role of the amyloid precursor protein (APP) in Alzheimer's disease. Since this thesis focussed on G-protein mediated transduction events, and did not examine the amyloidogenic aspect of Alzheimer's disease, the following proposals are acknowledged as being highly speculative. **Diagram II** is a schematic representation of the possible sequence of events leading to the formation of senile plaques and neurodegeneration in Alzheimer's disease. It also includes factors which are thought to influence this amyloidogenic process and interact with signal transduction mechanisms.

There is convincing evidence that senile plaque formation in Alzheimer's disease results from abnormal processing of the transmembrane amyloid precursor protein (APP) resulting in the aggregation and deposition of the βA4 protein. Several alternative proteolytic pathways for APP have been described which produce different degradation products: the normal secretory pathway involves a cleavage within the βA4 region and thus generates non-amyloidogenic amino and carboxy terminal products (Sisodia et al., 1990; Sisodia et al., 1990;); a secretory

pathway which produces soluble βA4 fragments (Haass et al.,1992a; Seubert et al., 1993); an endosomal-lysosomal pathway which results in the formation of C-terminal fragments some of which contain the βA4 protein and are therefore amyloidogenic (Cataldo & Nixon, 1990; Golde et al., 1992; Haass et al., 1992b). It remains to be established whether abnormal processing of APP is instigative of neurodegeneration in Alzheimer's disease or if this is a secondary response to an as yet unidentified event.

Several studies have suggested that denervated brain areas of Alzheimer's disease can mount an aberrant neuritic sprouting response which may indicate a defective regenerative process (Geddes et al., 1986; Hyman et al., 1987; Masliah et al., 1991b). Recently Masliah et al. (1992) reported the co-localisation of GAP-43 (growth activating phosphoprotein or B50 or F1), which is considered to be a marker for neuritic growth (Benowitz et al., 1989), and APP in neurites of AD frontal cortex. This suggests that APP is involved in the putative aberrent sprouting response.

Several investigations have provided evidence that carboxy terminal fragments of APP have neurotoxic properties. Yankner et al. (1989) reported that phaeochromocytoma cells (PC12) transfected with APP secreted a caboxy terminal peptide (βAPP-C104) which is neurotoxic to cultured hippocampal neurons. This effect may be mediated by a neuronal receptor for βAPP-C104 (Kozlowski et al., 1992). Yankner et al. (1990) subsequently demonstrated that NGF potentiated the neurotoxic effect of synthetic βA4 containing peptides. There is also evidence that βA4 can render neurons more susceptible to the neurotoxic action of excitatory amino acids (Mattson et al., 1992; Koh et al., 1990). Mattson et al. (1992) suggested βA4 acts by disrupting neuronal calcium homeostasis. The alteration in neuronal calcium influx has been proposed as an event which leads to altered phosphorylation activities and to neurofibrillary tangle formation (Mattson et al., 1991; Ueda et al., 1990).

There is now evidence that signal transduction mechanisms are involved in amyloidogenesis

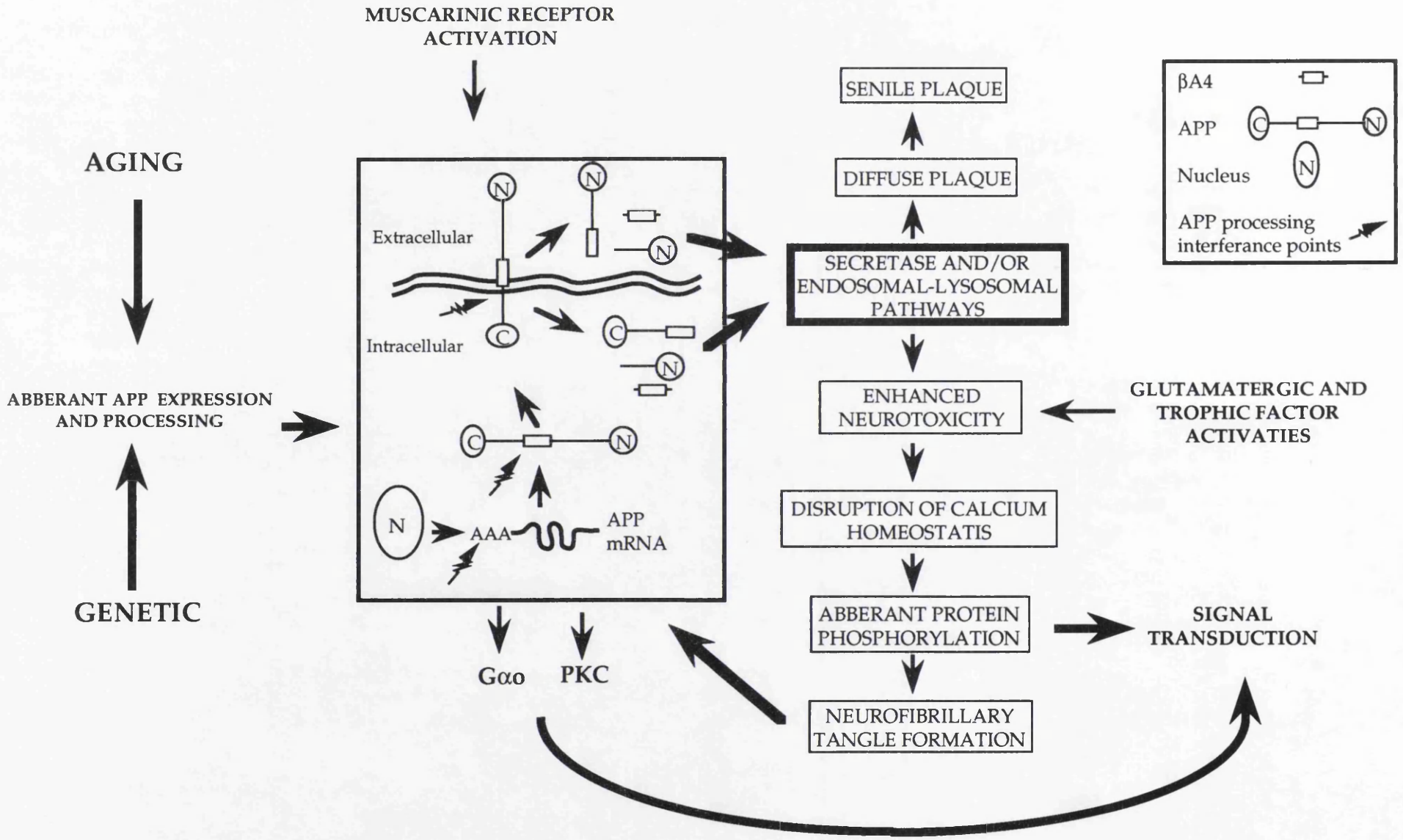
associated with Alzheimer's disease. It has been demonstrated that the activation of protein phosphorylation and dephosphorylation by agents which modulate protein kinase C and phophatases activity can regulate the processing of APP (Buxbaum et al., 1990). This activity can be achieved *in vitro*through the activation of muscarinic receptors (Bauxbaumet al., 1992) of the M1 and M3 subtypes (Nitsch et al 1992). Since the muscarinic receptors are coupled to G-proteins, then it likely that the stiochiometric relationship between G-protein subunits and receptors are altered in Alzheimer's disease.

Direct G-protein involvement in APP processing has also been demonstrated. Nishimoto et al. (1993) report that APP is coupled to $G_{\alpha o}$ through the cytoplasmic cytoplasmic domain outside the βA4 region . The association of $G_{\alpha o}$ with APP is inhibited by GTPγS treatment and is magnesium dependent. This suggests that the interaction between $G_{\alpha o}$ and APP proteins are similar to other G-protein receptor coupling mechanisms. Nishimoto et al. (1993) proposed that aberrant processing of APP may result in the over production of the $G_{\alpha o}$ coupling APP derivative and result in constitutive activation of the protein. If this were the case, one effect would be to occupy a significant proportion of the total cellular G-protein pool thereby reducing the basal GTPase activity, an effect observed in this thesis. It could also be speculated that occupancy of the $G_{\alpha o}$ by APP derivatives could induce downregulation of the G-protein β subunit to compensate for the shift in equilibrium between the active α subunits and the heterotrimeric complex. A knock-on effect could be a release of free G_{α} subunits such as $G_{\alpha i}$ and $G_{\alpha s}$. Since the $G_{\alpha i}$ are thought to be more abundant than $G_{\alpha s}$ (Asano et al., 1990), this could account for the reduced basal adenylate cyclase activity observed in the frontal cortex in the present study. However, in the present study the reduction in basal adenylate cyclase activity was not observed in the hippocampus. Since both regions exhibit marked amyloid pathology, this suggests that in Alzheimer's disease, amyloidogenesis has a different effect on the signalling mechanism in selected brain regions. GAP43 has also been shown to stimulate the binding of GTPγS to $G_{\alpha o}$ (Strittmatter et al., 1990) by a presently poorly defined mechanism which does not appear to require βγ subunits (Strittmatter et al.,

1991). Given that GAP 43 and APP are co-localised in Alzheimer's disease cortex and hippocampus (Masliah et al 1992), together with the ability of both proteins to interact with $G\alpha o$, it is tempting to speculate that APP derivatives and GAP43 have a functional association. GAP 43 and APP may also be intimately associated with the putative aberrant sprouting response.

In conclusion, the aberrant processing of APP is currently favoured as the key biochemical event involved in the neurodegeneration of Alzheimer's disease. Recent reports which examined various aspects of APP processing and neurotoxic properties support a muscarinic and glutamatergic involvement which may underlie an abnormal regenerative reaction. Signal transduction pathways may be effected as a consequence of changes such as alterations in phosphorylation and calcium regulation. Additionally, G-protein function may be directly altered by the action of APP and GAP43 on $G_{\alpha o}$ which is the most abundant G-protein in brain. Further investigations of the dynamics of G-proteins action, specifically $G_{\alpha o}$, in Alzheimer's disease and additional neurological disorders together with transfected cell lines may prove to be rewarding.

Diagram II. The significance of G-protein in Alzheimer's disease. This schematic diagram illustrates the mechanisms that are thought to be involved in the developement of Alzheimer associated pathology. A central event in plaque and tangle formation is the missprocessing of APP protein. This may be due to a combination of the aging process and genetic factors. This process could disrupt signal transduction at various levels. Recent evidense has suggested that $G_{\alpha o}$ is associated with APP fragments. It has also been demonstrated that PKC activity can influence APP processing. Thus, these components of signal transduction systems can be directly influenced by abberant APP processing. Signal transduction molecules may also be altered by the changes in cellular control of protein phosphorylation associated with Alzheimer's disease.


MUSCARINIC RECEPTOR ACTIVATION
AGING
ABBERANT APP EXPRESSION AND PROCESSING
GENETIC
Extracellular
Intracellular
AAA
APP mRNA
Gαo
PKC
SENILE PLAQUE
DIFFUSE PLAQUE
SECRETASE AND/OR ENDOSOMAL-LYSOSOMAL PATHWAYS
ENHANCED NEUROTOXICITY
GLUTAMATERGIC AND TROPHIC FACTOR ACTIVATIES
DISRUPTION OF CALCIUM HOMEOSTATIS
ABBERANT PROTEIN PHOSPHORYLATION
SIGNAL TRANSDUCTION
NEUROFIBRILLARY TANGLE FORMATION
βA4
APP
Nucleus
APP processing interferance points

5.5. G-protein activity in the hippocampus of the rat following lesion of the medial septal nucleus.

Animal models are frequently employed to examine various aspects of neurodegeneration as a consequence of the denervation of specific neuronal pathways. The advantage of such paradigms is that factors such as diet, age, the interval between death and freezing of the brain, agonal state and genetics can be controlled. However, species differences impose severe restrictions on the confidence with which observations from animal studies can be extrapolated to the particular human condition of interest. Here the medial septal-hippocampal pathway was lesioned in the rat and the effect of hippocampal G-protein mediated transduction mechanisms examined.

The medial septum (MS) lesion model was employed for several reasons. First, this model is relevant to the hypothesis that cognitive defects in Alzheimer's disease is correlated with the decreases in cholinergic function in the septohippocampal and basal forebrain cortical pathways (Perry et al., 1986). Second, the medial septal pathway has been reasonably well characterised: the cell bodies originating from the medial septal and diagonal band project through the fimbria fornix and terminate in a laminar pattern in the hippocampus innervating predominately areas CA2-CA4 (Geddes et al., 1992; Palacios et al 1991). The hippocampal afferents from the medial septum/diagonal band consist of a large population of cholinergic and GABAergic neurons (Freund & Antal, 1988). Third, lesions of the septal/hippocampal pathway has been shown to induce impairments in the ability of rats to learn and recall certain maze tasks (Nagahara & McGaugh, 1992). Fourth, there is evidence that lesions of this pathway can induce alterations in the inositol phosphate signalling pathway (Smith et al., 1989, Court et al., 1990; Connor & Harell, 1989). However, there has been no detailed investigations of the signalling mechanisms associated with these changes. To date, there has been minimal investigation of the adenylate cyclase system following specific neuronal lesions. Finally, the surgical expertise existed within the group to produce

reliable septal damage with highly reproducible reductions in choline acetyltransferase (ChAT) in the hippocampus (Horsburgh et al., 1993).

The present investigation established that in the although there was a significant reduction in the activity of choline acetyltransferase (23% $P<0.01$) in the hippocampus, there was no alteration in the level of $G_{\alpha o}$, $G_{\alpha i}1$, $G_{\alpha i}2$, $G_{\alpha s}L$ and $G\beta$. Longer exposure times of the $G_{\alpha s}$ autoradiogram allowed a visual inspection of $G_{\alpha s}S$ which also appeared to be comparable for the control and the lesioned groups. While the G-protein levels were unaltered in this lesion model, there is clearly a loss of the presynaptic input as indicated from the reduction in ChAT activity and by the loss of neuronal cell bodies in the septal area (**Figure 35**). It could be postulated that the apparently maintained G-protein levels following partial cholinergic denervation is indicative of an upregulation of G-protein levels in the remaining cholinergic terminals. Compensatory alterations at postsynaptic sites are also a possibility (Joyce et al., 1989). Although there is no direct G-protein evidence to support this hypothesis, compensatory changes in the hippocampus following cholinergic denervation have been reported. Following partial fimbria-fornix lesion the remaining intact cholinergic neurons appear to compensate for the reduced ChAT activity by upregulating their capacity to synthesize and store acetyl choline (Lapchak et al., 1991). This plasticity can be enhanced by administering nerve growth factor administration at the lesion site (Lapchak & Hefti, 1991).

The functional activity of the G-proteins as assessed by analysis of the GTPase activity was not significantly altered by medial septal lesion. Similarly the magnitude of stimulation achieved with both baclofen and carbachol was unaltered between the control and lesioned groups. Although there are no statistically significant changes in the GTPase data measured in either the presence and absence of agonists, the lesion group gave a slight increase in basal high affinity GTPase activity (20% above control values). The magnitude of baclofen stimulation of the GTPase activity was also non-significantly increased in the lesioned group compared to the controls. These observations may indicate a trend for an upregulatory

response in G-protein activity following cholinergic denervation. Post hoc power analysis would suggest that for the level of variability encountered for the basal GTPase activity, a group size of 17 samples would be required to to give a significant difference at the 5% level with 80% statistical power using a two-tailed Student's t-test. Using this criteria, it was also calculated that group sizes of 19 were required to observe a statistically significant change in the magnitude of agonist stimulation of GTPase by baclofen.

The adenylate cyclase activity was not significantly different in lesioned compared to control animals. Horsburgh et al. (1993) has used this same model to show that forskolin binding is increased in the polymorph layer of the dentate gyrus demonstrating a focal enhancement of $G_{\alpha s}$ coupled to adenylate cyclase. Horsburgh and her colleagues (1993) also concluded that phorbol ester binding to protein kinase C was unaltered in the hippocampus proper, but elevated in the entorhinal cortex. Given the highly localised changes in forskolin binding, and that the investigation reported in this thesis was conducted on whole hippocampal formations, perhaps subtle focal alterations in signal transduction mechanisms may occur beyond the spacial resolution of the approach which I employed.

The preserved levels of each G-protein related parameter may be interpreted to reflect a compensatory response. Indeed, lesion of the fimbria-fornix is reported to induce an upregulation of both the M1 and M2 receptors, which is partially reversed following reinnervation by transplantation (Joyce et al., 1989). It has also been reported that medial septal lesions by AF64A increased the levels of M2 receptors but not M1 receptors (Dawson & Wamsley, 1990). Supersensitivity responses have also been described for lesions of the nucleus basalis of Meynert complex (NBMC) (McKinney & Coyle, 1982). In contrast, preservation of receptors in the hippocampus have been reported following medial septal lesion (Overtstreet et al., 1980; Smith et al., 1989) and in the frontoparietal cortex following NBMC lesions (Raulli et al., 1989, Vige & Briley, 1989). However, Vige & Briley (1989) did observe an increase in the number of muscarinic receptors in the frontoparietal cortex following chronic

scopolamine treatment suggesting that antagonism is capable of inducing upregulation. Smith et al. (1989) and Court et al. (1990) also proposed a supersensitivity response to account for the enhanced phosphoinositol turnover in lesioned animals without a concomitant change in muscarinic receptor binding. A potential source of the the discrepancies regarding adaptive neurochemical changes may be the particular lesion system employed. Ibotenic acid lesions employed in this thesis produced a moderate reduction in ChAT and histological examination of the septal area displayed highly localised cell loss. Transsection and electrolytic lesions are regarded however, as being less specific both anatomically and pharmacologically.

Denervation of the septal input to the hippocampus has been shown to induce a complex process of sympathetic ingrowth (HSI) of noradrenergic fibres that invade the dentate gyrus and the CA3 region (Crutcher et al., 1981). This response may influence signal transduction mechanisms. Ayyagari & Harell (1992) recently showed that HSI can result in alteration in the affinity of phorbol ester binding to protein kinase C in the dorsal hippocampus and increase in the B*max* in the ventral hippocampus. The anatomically circumscribed changes may account for an earlier observation by the group (Connor & Harell, 1989) that while cholinergic stimulation of phosphoinositide hydrolysis is enhanced in lesioned animals, the noradrenergic response is not increased. Thus, moderate increases in GTPase activities reported in this thesis, could be a reflection of the HSI phenomena.

In conclusion, partial denervation of the septal hippocampal pathway in the rat does not induce gross alterations in the level or related activity of G-proteins in the hippocampus. These findings are consistant with the results of the investigation of G-proteins in Alzheimer's disease hippocampus. However, while the GTPase activity was found to be reduced in Alzheimer's disease hippocampus compared to control samples, a moderate increase in basal GTPase activity was observed in lesioned animals. This may be interpreted to reflect a compensatory reaction in response to the cholinergic deficiency in animals which is lost in Alzheimer's disease.

5.6. Conclusion

This thesis reports the findings of an investigation of the effect of the neurodegenerative disorder of Alzheimer's disease on G-protein controlled signal transduction mechanisms. The study has demonstrated that while there are no gross alteration in G-protein levels, alterations in the GTPase and adenylate cyclase activities indicate that there is a tendency for the functional activities to be compromised in Alzheimer's disease. These alterations could be in responce to the processes involved in amyloid deposition which is characteristic of Alzheimer's disease. These modest changes, particularly the GTPase reductions could have a significant impact on the responsiveness of patients to drugs targeted at specific receptors.

This study has also shown that while the biochemical analysis of postmortem tissue has its limitations, some parameters of the transduction process can be investigated to generate reliable data. Investigation of transduction components in different regions has shown a heterogeneous pattern of distribution and suggests that common mechanisms for controlling adenylate cyclase activity are employed by various regions. An apparent exception however is the striatum and this may reflect the role of specific G-proteins expressed in this region.

Finally, although there are no animal models for Alzheimer's disease, rats lesioned at the medial septal support the hypothesis that major alterations in G-protein levels are not employed as a compensatory mechanism to cholinergic denervation. However subtle changes in GTPase activity may indicate an upregulatory response in the fuctional activity of G-proteins.

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