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A STUDY OF G PROTEIN COUPLED SIGNAL TRANSDUCTION MECHANISMS IN ALZHEIMER'S DISEASE.

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ACKNOWLEDGEMENTS

I would like to thank my supervisors, Prof. James McCulloch and Prof. John Knowler, for their advice, encouragement and patience while carrying out this work.

I am also indebted to Prof. Murray Harper for his help and advice, and use of facilities in the Wellcome Surgical Institute, to Dr. Kasumoto Kuramaji for carrying out middle cerebral artery occlusions, to Prof. David Graham, Department of Neuropathology, Institute of Neurological Sciences, for neuropathological data, and to Dr. Graeme Milligan, Institute of Biochemistry, University of Glasgow, for providing anti-G protein antisera.

Special thanks go to my friend and colleague Mark McLaughlin with whom I have worked over the past three years. The analysis of G protein levels in human brain was a collaborative venture between us, with Mark executing the end stages of several of the experiments concerning Goα and Giα proteins.

I would also like to thank Dr. Anuj Bhatt, Dr. Suzanne Griffiths, Dr. Linda Jones, Colin Loney and Dr. Ian Mullaney, of the Institute of Biochemistry, University of Glasgow, and my colleagues at the Wellcome Surgical Institute, Sue Browne, Deborah Dawson, Dr. Deborah Dewar, Dr. Karen Horsburgh, Fiona Inglis, Kenneth MacKay, Dr. Mhairi Macrae, for their help and encouragement.

Last, but by no means least, I would like to express my gratitude to all the technical and secretarial staff of the Wellcome Surgical Institute, especially to Margaret Roberts, without whose expertise and diligence the assays of choline acetyl transferase would not have proved possible.

ABBREVIATIONS

ATP, adenosine triphosphate; **ADP**, adenosine diphosphate; **ATPase**, adenosine triphosphatase; **AMPA**, 2-amino-3-hydroxy-5-methyllisoxazole-4-propionic acid; **ANOVA**, analysis of variance; **acetyl CoA**, acetyl coenzyme A; **A.Ch.**, acetyl choline; **APP**, amyloid precursor protein; **AHO**, Albright's heriditary osteodystropy.

ChAT, choline acetyl transferase; **CV**, coefficient of variance; **cAMP**, cyclic adenosine-3',5'-monophosphate; **CRF**, corticotrophin releasing factor; **CNS**, central nervous system.

DAG, diacylglycerol; **DAT**, dementia of the Alzheimer type; **DEPC**, diethylpyrocarbonate.

EDTA, ethylenediaminetetraacetate.

IgG, Immunoglobulin G; **IP_3**, inositol triphosphate.

Gαs,i,o,p,z,olf,q or 11, G protein α subunits; **GsH**, the heavy form of Gsα; **GsL**, the light form of Gsα; **Gi1, Gi2 and Gi3**, Giα subtypes. Note - in the context of this text GsH, GsL, Gi1, Gi2 and Gi3 refer specifically to the G protein alpha subunits and not to the trimeric structure. **Gβ**, G protein β subunit; **Gi**, the inhibitory G protein of adenylate cyclase; **Gs**, the stimulatory G protein of adenylate cyclase; **Gp**, the stimulatory G protein of phospholipase C; **Ga**, the stimulatory G protein of phospholipase A_2; **Gd**, the stimulatory G protein of phospholipase D; **Gk**, the stimulatory G protein of atrial potassium ion channels; **GTP**, guanosine triphosphate; **GDP**, guanosine diphosphate; **GTPase**, guanosine triphosphatase; **GppNHp**, guanyl-imido-diphosphate; **GAPDH**, glyceraldehyde-phosphate dehydrogenase.

HRP, horse radish peroxidase.

MCA, middle cerebral artery; **MOPS**, 3-[*N*-morpholino] propane sulphonic acid.

NMDA, *N*-Methyl-D-Aspartate; **NADH**, reduced nicotinamide adenine dinucleotide; **Na/K ATPase**, sodium potassium dependent ATPase.

PEI, polyethyleneimine; **PHF**, paired helical filaments; **PLA_2**, phospholipase A_2; **PLC**, phospholipase C; **PLD**, phospholipase D; **PMD**, post mortem delay; **PHF**, paired helical filaments; **PHP**, pseudo-hypoparathyroidism; **PPHP**, pseudo-pseudo-hypoparathyroidism.

SEM, standard error of the mean; **SD**, standard deviation; **SDS**, sodium dodecyl sulphate; **SSC**, standard saline citrate; **SSPE**, standard saline phosphate.

TEMED, N,N,N',N'-tetramethylene-ethylenediamine; **TCA**, trichloroacetic acid.

U, unit of enzyme activity equal to 1μmol of product formed per minute.

SUMMARY.

The specific objective of the work described in this thesis was to study aspects of signal transduction in the post mortem brains of persons who had suffered from dementia of the Alzheimer type (DAT). There already exists much data describing the state of many neurotransmitter systems in the disease, but little information was available regarding the events that take place subsequent to receptor activation. Such knowledge is important in order to assess the potential for neurotransmitter replacement therapies in the treatment of Alzheimer's disease, as well giving further insight into the neurodegenerative mechanism of this disease.

The levels of the guanine nucleotide binding protein (G protein) α subunits, GsH, GsL, Gi1, Gi2 and Gsα, were measured by western blotting utilising highly specific anti-G protein antisera. Similarly, the messenger RNA (mRNA) encoding the G protein subunits Goα, Giα and Gβ, as well as 28S ribosomal RNA (28S mRNA), were analysed by northern blotting utilising radiolabelled oligonucleotide probes. In addition, the activities of the enzymes adenylate cyclase, sodium potassium dependent ATPase and choline acetyl transferase were assayed using standard methods.

(i) Effect of post mortem delay on different components of signal transduction in rat brain.

Since multiple parameters (e.g. levels of G proteins and their mRNAs, adenylate cyclase activity, etc.) were to be measured in post mortem human tissue, there was a concern that the delay between death and freezing of the tissue (the post mortem delay) would influence the reliability of any measurements made. This possibility was investigated by experiments in which rats were sacrificed and left at room temperature for 24 hours, or at 4°C for up to 72 hours. The subsequent analysis of crude membranes prepared from the brains of these animals brains showed that no significant

degradation of G protein α subunits, or loss of activity of adenylate cyclase or sodium-potassium dependent ATPase, occurred over these time periods. In addition G protein encoding mRNA appeared to be relatively stable for up to 72 hours at 4°C, although there was a time dependent increase in 28S rRNA breakdown.

(ii) Measurement of G protein levels, and enzyme activities in DAT diseased post mortem human brain.

The mean activity of choline acetyl transferase (ChAT), assayed in the hippocampus of eight persons who had suffered from DAT, was significantly reduced to 34% of that observed in eight age-matched control subjects. Similarly, the mean activity of ChAT in DAT diseased frontal cortex was 54% of that measured in control subjects.

When adenylate cyclase activity was assayed in the same subjects, both the basal and fluoroaluminate stimulated activities were significantly reduced in DAT diseased frontal cortex, being 56% and 53% of the mean values observed in control subjects. In contrast, no statistically significant change was observed in the hippocampus.

A second membrane-bound enzyme, sodium potassium-dependent ATPase, was assayed in the same preparations. No statistically significant change in the mean activity of this enzyme was detected between DAT diseased and control subjects, in either the frontal cortex or hippocampus.

The western blot analysis of G protein levels also revealed no statistically significant difference, in either brain region, in the levels of GsH, GsL, Gi1, Gi2 or Goα, between DAT and control subjects. However the mean ratio of GsH to GsL was found to be significantly reduced in DAT diseased frontal cortex, to a value of 72% of that observed in controls.

Thus while some components of the signal transduction process are preserved in DAT, other key components are significantly altered by the disease process.

None of the parameters measured showed any significant linear correlation with either age or post mortem delay. However the activity of sodium potassium-dependent ATPase was significantly correlated with the length of time the tissue had been stored at -80°C, in both the frontal cortex and hippocampus ($r = 0.746$ and 0.707 respectively).

(iii) Distribution of G protein α subunits and adenylate cyclase in human brain.

The levels of the G protein α subunits, and the activity of adenylate cyclase, were measured in five brain regions obtained from three control subjects. Adenylate cyclase, Gi1, Gi2 and Goα were similarly distributed throughout the brain, each possessing their highest levels in the frontal cortex, and lowest in the pons. However the distributions of GsH, and especially GsL, were dissimilar to that of the other α subunits and adenylate cyclase. In addition, an extra "Gsα-like" immunoreactive band, which migrated slightly more slowly than GsL, was observed in the western blot analysis of human neostriatum. It is suggested that this species may be the G protein α subunit, Golfα, which is normally associated with olfactory neurons.

Similar experiments demonstrated that GsH, Gi1, Gi2, Goα and adenylate cyclase were more strongly expressed in cortical grey matter than white, with the opposite being true for GsL.

(iv) Analysis of G protein encoding mRNA in post mortem human brain.

The levels of the mRNA encoding the G protein subunits Gsα, $G\beta_1$ and $G\beta_2$ were measured, by northern blotting, in the frontal cortex and hippocampus of six DAT diseased subjects, and in six age-matched control subjects. There was no significant difference, in either brain region, between the control and DAT diseased groups, for any of the G protein mRNAs measured.

The degree of intersubject variability was very high, giving an average coefficient of variation, or CV, (SD as percentage of mean) of 65%, with the highest CV being 92%, observed in the measurement of $G\beta_1$ mRNA levels in the frontal cortex. This compares with an average CV of approximately 30%, observed in the measurement of G protein levels, or enzyme activities.

The extent of generalised RNA degradation was assessed by detecting the breakdown products of 28S rRNA. RNA degradation was present in tissue samples from every human subject studied. The extent of 28S rRNA degradation in each subject was found to be related to the levels of G protein mRNA detected. In addition, the degree of RNA degradation in human subjects was found to be very variable and unaffected by the presence of DAT. RNA degradation (and G protein mRNA levels) were not significantly correlated with subject age, post mortem delay or storage time.

The effect that periods of ischemia, and freeze/thawing of tissue, had upon RNA integrity was investigated in rat brain. A single cycle of freeze/ thawing greatly increased the rate of RNA degradation, while three hours of permanent focal forebrain ischemia had little effect upon RNA integrity.

The variable extent of RNA degradation means that great care must be taken to ensure the validity of RNA analyses undertaken in human postmortem brain, in order to avoid drawing incorrect conclusions from such studies.

I. INTRODUCTION

1. Introductory comments.

The evolution of complex multicellular organisms has necessitated the development of mechanisms which allow intercellular communication to take place. This is vital for the co-ordination of body functions, as well as in mediating a response to external stimuli. Cellular communication is of particular importance in the central nervous system which, being comprised of fairly simple cell types, relies for its function on the interactions which take place between individual cells. The study of these mechanisms has revealed that what is an apparently simple process, namely the release of a transmitter substance by one cell eliciting a response in another, is associated with an array of complex molecular events. The major components of such processes, for example neurotransmitter receptors, G proteins, and protein kinases, will be described in the following section.

The ubiquitous and important nature of these systems makes it likely that they are involved in a variety of disease states. Indeed, there is much current interest in the role that such processes play in ailments such as diabetes, heart disease and affective disorders of the brain (described in Section 3). One disease in which dysfunction of intercellular communication has clearly taken place, is dementia of the Alzheimer type (DAT), a severe neurological disorder prevalent in later life. The role which different neurotransmitters play in the disease, as well as the receptor proteins to which they bind, has been extensively studied over the last two decades. It has become apparent however that, in many cases, the binding of transmitter to a receptor is only the first in a series of events which cause a response to occur in the responding cell. These components, distal to the activation of receptors, have received relatively little attention in DAT which, given their demonstrated involvement in other neurological disorders, is a situation which requires to be remedied.

Tissue samples from the human subjects used in this study have already been used in a variety of other investigations. For example, that of Dewar *et al.* (1990) has indicated that alterations in the levels of G proteins or adenylate cyclase may have occurred in DAT, while Chalmers *et al.* (1990) and Dewar *et al.* (1991) have observed changes in the levels of a number of different receptor proteins. This thesis builds on these results by using biochemical techniques to measure both the levels and functioning of a variety of different moieties involved in the signal transduction process.

2. G protein coupled signal transduction.

2.1 Overview.

The elucidation of the processes by which the release of a transmitter substance (also referred to as hormones or agonists) by one cell causes a response in another is central to the understanding of how the central nervous system (CNS) functions at a molecular level. Typically this relies on the binding of the transmitter to a receptor molecule on the extracellular surface of the cell membrane. This binding event, or signal, must be communicated, or transduced, across the plasma membrane allowing the cell to respond in some way to the stimuli - hence signal transduction. In some cases the neurotransmitter binds directly to an ion channel causing it to open or close (ionotropic receptors), examples of this being the $GABA_A$ receptor (Sieghart, 1989), as well as several types of glutamate receptor (Farooqui & Horrocks, 1991). However in other cases the occupied receptor does not, in itself, cause any intracellular events to occur, but instead activates a second class of proteins termed heterotrimeric guanine nucleotide binding proteins (G proteins). These in turn modulate the activity of a range of enzymes and ion channels, the purpose of which are to cause some form of change within the cell. This multistep process possesses the potential for more varied, and finer, control than is possible for ionotropic receptors.

G proteins are part of a super family of guanine nucleotide binding proteins, which includes the initiation and elongation factors of protein synthesis, and the small GTP binding proteins such as the *ras*-related family of proto-oncogene products (Bourne *et al.*, 1991). In view of their ability to bind guanine nucleotides, it is not surprising that the main feature of G protein coupled signal transduction systems is their requirement for GTP (Casey & Gilman, 1988). The first indications that such systems existed came from Rodbell *et al.* (1971) who demonstrated that the stimulation of adenylate cyclase by glucagon in liver was dependent upon GTP, a

requirement which was subsequently found to be due to the presence of the adenylate cyclase stimulatory G protein, or Gs (Northup *et al.*, 1980). A large number of other G proteins have subsequently been identified having functions such as the inhibition of adenylate cyclase (Hildebrandt *et al.*, 1983), the regulation of phospholipid metabolism (Birnbaumer *et al.*, 1990) and the gating of ion channels (Sternweis & Pang, 1990). In addition the activity of G proteins can be modulated by a wide variety of receptors such as muscarinic, adrenergic and serotonergic (Birnbaumer *et al.*, 1990).

2.2 Mechanism of G protein action.

Despite being able to associate with a wide range of receptors and effectors, all G proteins possess a similar heterotrimeric structure composed of α, β and γ subunits (Lochrie & Simon, 1988). The α subunits, of which many forms exist, confer receptor and effector specificity upon the G protein, whilst the β and γ subunits are required for the expression of G protein activity (Kanaho *et al.*, 1984 ; Florio & Sternweis, 1989), and are thought to be interchangeable between different α subunits in a non discriminating manner (Birnbaumer *et al.*, 1990). As well as their similarities in structure, each G protein appears to have a similar mechanism of action. Fundamental to this process is the ability of the α subunit to bind GTP and subsequently hydrolyse it by means of an intrinsic GTPase activity (Pedersen & Ross, 1982). In its inactive state the α subunit is bound to GDP and complexed to the β and γ subunits (Figure 1). However when this trimeric structure interacts with an occupied receptor protein, GDP is exchanged for GTP, and the receptor-α-$\beta\gamma$ complex dissociates to give receptor, $\beta\gamma$ subunits and free GTP-bound α subunit (Taylor, 1990). The α subunit can then interact with specific effector proteins in the membrane, such as adenylate cyclase, and modulate their activity. Finally the GTPase activity of the α subunit hydrolyses the bound GTP to GDP, causing the α subunit to reassociate with the $\beta\gamma$ subunits, thereby returning the G protein

to its inactive state. It is not certain at what stage the α subunit actually becomes competent to modulate effector activity, since Codina *et al.* (1984) have demonstrated that, under certain conditions, a GTP-bound αβγ structure can be isolated. It is therefore possible that dissociation of the α subunits from the β and γ subunits is not mandatory for G proteins to exert control over effectors; a feature which may allow a more rapid response to the binding of transmitter molecules. The mechanism of G protein action therefore takes the form of a cycle, which is activated by the presence of transmitter bound receptors, and "switched off" by the hydrolysis of GTP. This GTPase activity can easily be measured by virtue of the α-subunits possessing a higher affinity for GTP (low Km GTPase) than the other GTPases which are present in the cell (high Km GTPases) (Cassel & Selinger, 1976). As might be expected, low Km GTPase activity is enhanced in the presence of certain agonists, a feature which has been useful in determining which receptors are coupled to G proteins (Cassel & Selinger, 1976; Franklin & Hoss, 1984). However low Km GTPase activity is also measurable in the absence of agonists, which indicates that there is a continuous basal rate of activation of G proteins. This feature may be important in determining the activity of effector proteins in the absence of external stimuli. The formation of active α subunits can also be stimulated by the addition of non-hydrolysable analogues of GTP, such as guanyl-imido-diphosphate (GppNHp), which bind to the α subunit and permanently activate it (Schramm & Rodbell, 1975). A similar action is also performed by fluoroaluminate (AlF_{3-5}^-), which also binds to and activates the α subunit (Bigay *et al.*, 1987). However this requires that the α subunit is in its GDP-bound form, leading to the proposal that fluoroaluminate acts as a high affinity phosphate analogue, which binds next to the β phosphate of GDP mimicking a γ phosphate group, hence converting the α subunit to its active conformation (Bigay *et al.*, 1987). One further feature of G protein coupled signal transduction is that the agonist bound receptor protein can exist in

two different conformations, each of them possessing a different affinity for agonists (Iyenger *et al.*, 1980; Rojas *et al.*, 1985; Koo *et al.*, 1983). The high affinity state is thought to be indicative of a receptor G protein complex, with the low affinity state representing free receptors (Taylor, 1990; Birnbaumer *et al.*, 1990). The addition of non-hydrolysable GTP analogues causes the conversion of high affinity to low affinity agonist binding, since it causes the formation of activated α subunits, which then dissociate from the receptor (Figure 1).

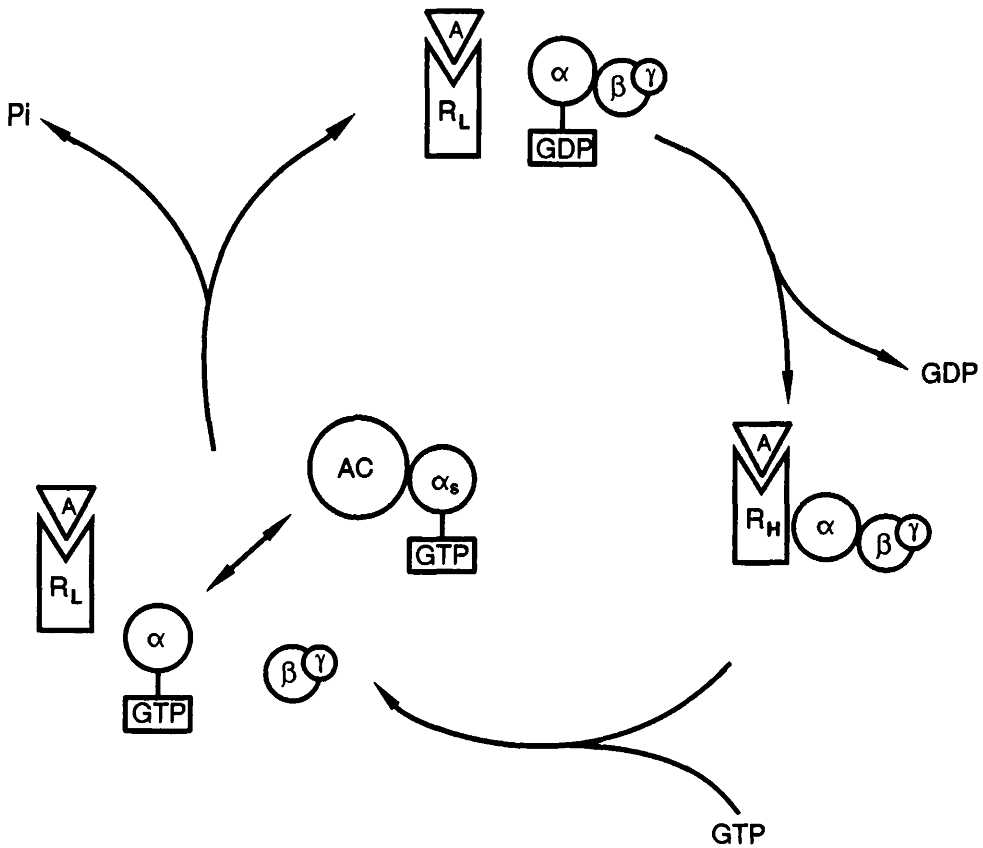


Figure 1.

The cyclical nature of G protein function.

A = Agonist, R_L and R_H = Receptor, α, β and γ = G protein subunits, AC = Adenylate Cyclase.

When an agonist bound receptor protein interacts with an inactive trimeric G protein, the G protein α subunit loses its bound GDP, and the receptor changes from its low (R_L) to its high affinity state (R_H). The α subunit then binds GTP causing the dissociation of the receptor/G protein complex into free α subunit, βγ subunits and receptor in its low affinity state. The α subunit can now interact with, and modulate the activity of, effector proteins. In this case Gsα is shown activating adenylate cyclase. The cycle is terminated by the α subunit hydrolysing its bound GTP, causing reassociation of the α and βγ subunits and returning the G protein to its inactive form.

2.3 Classification of G proteins.

As mentioned in Section 2.1, there exists a wide range of different enzymes and ion channels, the activity of which can be modulated by G proteins. In addition, many different α subunits have been identified by the use of molecular cloning and protein purification. In many cases the specific function of the identified subunits is unclear. Conversely, many of the effector systems are still "seeking" a controlling G protein. It is therefore possible to classify G proteins in two ways; by characterised protein species and by the effector systems which are thought to be G protein controlled.

2.3.1 Physically identified G protein subunits.

The highly homologous nature of G protein subunits has meant that their purification to homogeneity by conventional means has proved difficult. However the use of molecular cloning has allowed the identification of numerous G protein encoding mRNAs and genes. The amino acid sequence deduced from these clones has allowed the preparation of highly specific anti-peptide antisera against individual G protein subunits, the use of which has proved invaluable in the process of elucidating the functions of these proteins. Such antisera will be used in this study to measure the levels of several G protein subunits. It is therefore important to examine the large variety of G proteins which have been identified in the CNS, before attempting to describe the specific functions of these proteins.

β and γ subunits.

The use of molecular cloning has identified two species of β subunit mRNAs termed β_1 and β_2 present in human and bovine brain (Fong *et al.*, 1986; Fong *et al.*, 1987). These correspond to the two forms of β subunit protein which can be identified by SDS-PAGE (36kDa and 35kDa respectively; Sternweis *et al.*, 1981; Birnbaumer *et al.*, 1990). In addition, a

third form of β subunit mRNA has recently been cloned from bovine retina (Levine *et al.*, 1990b), although a corresponding protein has not, as yet, been identified.

The G protein γ subunits have not been fully characterised. They are much smaller than the β or α subunits (approximately 8kD), and under normal circumstances are found tightly bound to the β subunit (Tamir *et al.*, 1991). At least six forms of γ subunits are known to exist, with three being present in bovine brain (Tamir *et al.*, 1991). These can associate in any combination with the different β subunits (Birnbaumer *et al.*, 1990), and while different tissues possess differing amounts of the β and γ subtypes (Birnbaumer *et al.*, 1990), no functional role is known for this heterogeneity.

α subunits.

The α subunits are a rapidly growing family of homologous proteins, possessing molecular weights ranging from 37 to 52 kDa, the amino acid sequences of which differ in the regions of the protein which confer receptor and effector specificity (Lochrie & Simon, 1988). A feature of the α subunits which has proved useful in the elucidation of their function, is their susceptibility to ADP-ribosylation by bacterial toxins isolated from *Bordetella pertussis* (Pertussis toxin or PTX) and *Vibrio cholera* (Cholera toxin or CTX), which cause a change in the activity of the proteins that they modify (Cassel & Pfeuffer, 1978; Heyworth *et al.*, 1984). Each toxin only modifies those proteins containing a specific amino acid sequence, which fortuitously are not present within every α subunit. Hence discovering whether or not a process is susceptible to disruption by these toxins has helped to identify which G proteins are involved in each task. In addition, the use of radiolabelled substrate for the ADP-ribosylation reaction, allows partial quantification of the levels of cholera and pertussis toxin substrates, although the availability of highly specific anti-G protein antisera has reduced the need to use the toxins for this purpose.

The major α subunits which are known to exist in the CNS are as follows.

Gsα. Bray *et al.* (1986) have isolated four cDNA clones from human brain which correspond to Gsα. These are all derived from the same gene and arise from alternative splicing events (Kozasa *et al.*, 1988 and Figure 2). The four mRNAs fall into two distinct groups - two species which encode the heavy form of Gsα, GsH, and those that encode the light form, GsL. GsH differs from GsL by containing a sequence of 14 or 15 additional amino acid residues, which corresponds to exon 3 of the Gsα gene (Kozasa *et al.*, 1988). Within each group, the proteins differ by a single amino acid residue, also caused by an alternative splicing reaction at the 5′ end of exon 4 of the Gsα gene. This additional residue is a serine and may have some functional significance since it represents a potential phosphorylation site. All forms Gsα can be ADP ribosylated by cholera toxin, but not by pertussis toxin (Jones *et al.*, 1990).

Giα. The use of cDNA cloning has revealed the presence of three homologous mRNAs, known as Gi1, Gi2 and Gi3, which, unlike Gsα subtypes, are all derived from separate genes (Itoh *et al.*, 1988). While Gi1 and Gi2 are prevalent species in brain, Gi3 is only found in very low concentrations in discrete brain regions such as the substantia nigra and hippocampus (Cortes *et al.*, 1988). Newton & Klee (1990) have recently provided evidence for the existence of subspecies of Gi2 termed Gi2-A and Gi2-B. It is not known whether these derive from a difference in primary sequence or to post-translational modifications, but there are indications that they possess differences in function (Newton & Klee, 1990). In contrast to Gsα, Giα molecules can be ADP ribosylated by pertussis toxin but not by cholera toxin (Linder *et al.*, 1990).

Goα. Two Goα cDNA clones, thought to derive from alternative splicing of one gene product, have been identified in mouse brain (Strathmann *et al.*, 1990). The deduced amino acid sequence of Goα is

similar to that of Giα (Lochrie & Simon, 1988), and can also be ADP ribosylated by pertussis toxin (Linder *et al.*, 1990). Goα is found in large amounts within the CNS, where it comprises approximately 1% of total membrane protein (Gierschik *et al.*, 1986). It has also been identified in other tissues such as heart and retina (Angus *et al.*, 1986; VanDongen *et al.*, 1988), but is thought to derive from the neuronal elements within these tissues. Various protein purification strategies have shown the existence of four Goα-like proteins in bovine brain, termed Go-1,2,3 and 4 (Inanobe *et al.*, 1990). Although these subspecies may all be due to differing post translational modifications, peptide mapping has suggested that two forms of Goα, each possessing a different amino acid sequence, exist in brain. Whether these correspond to the two cDNA clones which have been isolated is, at the moment, unclear. It also possible that other species of Goα await isolation, since the northern blot analysis of RNA purified from brain reveals the existence of several RNA species containing Goα like sequences, although this may be due to differences within the 3' untranslated regions of these mRNA species rather than within the coding region (Price *et al.*, 1990).

Golfα. This α subunit was cloned from a cDNA library derived from olfactory neurons (Jones & Reed, 1989), in which it is predominantly found. It is highly homologous to Gsα and can also be ADP-ribosylated by cholera toxin (Jones *et al.*, 1990).

Gzα. This α subunit, also known as Gxα, was originally cloned from bovine retina, but is also expressed in adrenal medulla and brain (Fong *et al.*, 1988; Casey *et al.*, 1990). It cannot be modified by either pertussis toxin or cholera toxin (Casey *et al.*, 1990).

Gqα. This species, and the highly homologous α_{11}, were cloned from mouse brain (Strathmann *et al.*, 1989; Strathmann & Simon, 1990), and are expressed in many different tissues. Like Gzα, neither protein can be ADP- ribosylated by pertussis toxin (Blank *et al.*, 1991).

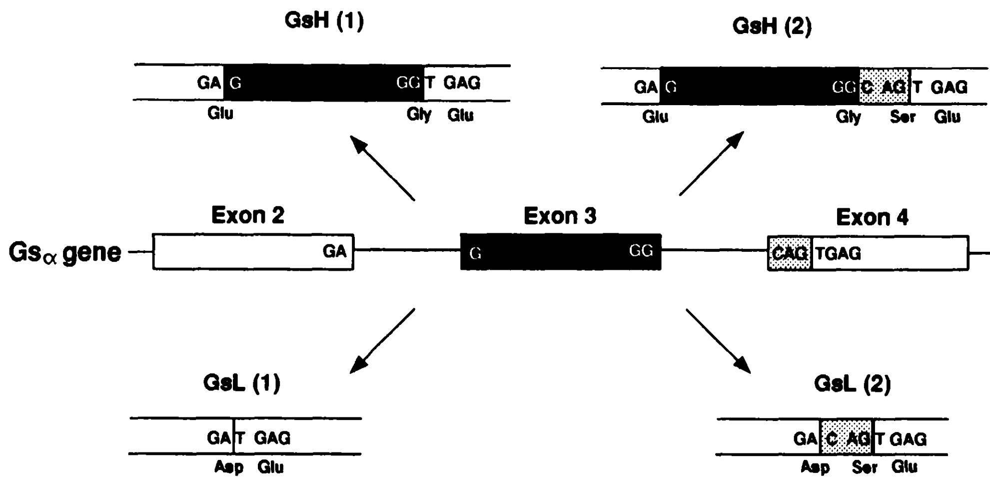


Figure 2.

The four species of Gsα mRNA produced by alternative splicing reactions.

Exons 2, 3 and 4 of the Gsα gene are shown along with the nucleotide sequence adjacent to the splice points. The inclusion of the sequence encoded by exon 3 gives rise to GsH encoding mRNA, while its exclusion produces GsL mRNA. Furthermore, GsH (2) and GsL (2) mRNA, contain an additional codon (shaded) encoding a serine residue.

2.3.2 G protein coupled effector systems.

Adenylate cyclase.

The best characterised of the G protein controlled effector enzymes is adenylate cyclase. This is a membrane bound enzyme which catalyses the hydrolysis and cyclisation of ATP to form cyclic adenosine monophosphate (cAMP) (Rall *et al.*, 1957; Rall & Sutherland, 1961). The cAMP formed then acts as a "second messenger" (the transmitter substance being the "first messenger") by diffusing into the cell's cytoplasm and activating a protein kinase, known either as protein kinase A or cAMP-dependent protein kinase. This in turn phosphorylates and alters the activity of a variety of cellular components including other enzymes (Martin, 1987), ion channels (Levitan, 1988), and cytoskeletal proteins (Nestler & Greengard, 1989). It has been known for many years that some transmitters can stimulate the activity of adenylate cyclase, for example adrenaline and dopamine, and that this can be altered by the action of cholera toxin (Cassel & Pfeufer, 1978). Since the only widely distributed cholera toxin substrate is Gsα, it was concluded that this protein was responsible for the coupling of these receptors to the stimulation of adenylate cyclase. This has subsequently been proved by reconstituting purified adenylate cyclase with purified Gsα (May *et al.*, 1985). More recently both GsH and GsL have been shown to be able to stimulate adenylate cyclase (Jones *et al.*, 1990).

There exists only one other α subunit which is known to activate adenylate cyclase, this being Golfα. This G protein has been proposed to couple olfactory receptors to adenylate cyclase (Jones & Reed, 1989), although it is also able to couple β-adrenergic receptors to the enzyme (Jones *et al.*, 1990). With this one exception, the hormonal stimulation of adenylate cyclase is thought to be mediated exclusively by Gsα.

Gsα was named because of its stimulatory effects on adenylate cyclase. Similarly, Giα subunits were thought to be involved in inhibitory processes. However, the exact mechanism by which receptors can cause the

inhibition of the enzyme is unclear. Firstly, it is not known with which G protein inhibitory receptors interact. This is likely to be one of the Giα proteins, since the stimulation of low Km GTPase activity by opiate receptors (which inhibit adenylate cyclase), is blocked by anti-Giα antisera in NG-108 cells (McKenzie *et al.*, 1988). Furthermore, the inhibition of adenylate cyclase by α-adrenergic agonists in platelets can be inhibited by anti-Gi2 antisera (Simonds *et al.*, 1989), while the inhibition caused by non-hydrolysable GTP analogues in hepatocytes, can be blocked by the phosphorylation of Gi2 (Houslay, 1991). While these results indicate that Gi2 mediates the inhibition of adenylate cyclase, Katada *et al.* (1986) have demonstrated that purified Gi1 is able to inhibit the enzyme which, along with other less direct evidence (Wang *et al.*, 1990), suggests that the Gi1 is also able to fulfil this role. These results are not necessarily contradictory, since it is possible that different receptors may mediate the inhibition of adenylate cyclase via different G proteins.

The mechanism by which G proteins cause the inhibition of adenylate cyclase is also unclear. One possibility is that the enzyme is inhibited directly by the α subunit of an inhibitory G protein, in an analogous manner to its stimulation by Gsα. This view is supported by the work of Katada *et al.* (1986) who, as mentioned above, observed that Gi1 can directly inhibit adenylate cyclase. However similar experiments by others have failed to repeat this observation (Linder *et al.*, 1989; Newton & Klee, 1990). Alternatively, the fact that βγ subunits can also cause inhibition of the enzyme (Hildebrandt & Kohnken, 1990), has lead to the suggestion that βγ subunits released from an activated inhibitory G protein can combine with free Gsα molecules thereby preventing them from stimulating the enzyme. This implies that the activation of any G protein other than Gs could cause the inhibition of adenylate cyclase, since βγ subunits are thought to be interchangeable between α subunits. However, titration of Gsα by βγ subunits cannot be the sole mechanism by which Gi mediates inhibition of

adenylate cyclase, since non-hydrolysable GTP analogues and inhibitory agonists can reduce the activity of adenylate cyclase in cells which totally lack any functional Gsα (Katada *et al.*, 1984a). As a compromise between the two possibilities, Hildebrandt & Kohnken (1990) have proposed that inhibition can occur by both mechanisms, with the release of βγ subunits mediating "tonic" inhibition i.e. counteracting the effect of free Gsα molecules released under basal conditions (see Section 2.2), while actual transmitter-mediated inhibition is due to the direct effect of a Giα molecule upon adenylate cyclase.

Thus the term Gi is somewhat ambiguous. It can mean either a G protein which is capable of inhibiting adenylate cyclase, or the highly homologous family of cloned Giα proteins which, may, or may not, be the actual mediators of the agonist dependent inhibition of adenylate cyclase. In this text the terms Giα, Gi1, Gi2 and Gi3, refer to the actual protein species, while the term Gi is used to mean a G protein through which receptors can cause the inhibition of adenylate cyclase.

Phospholipases.

The membrane-bound enzyme, inositol phopholipid-specific phospholipase C, serves to catalyse the breakdown of inositol phospholipids in the cell membrane to form inositol triphosphate (IP_3) and diacylglycerol (DAG) (Fisher & Agranoff, 1987). The activity of the enzyme can be stimulated by a number of transmitter substances in a GTP dependent manner, indicating that it is a G protein coupled process (Kikuchi *et al.*, 1986). The IP_3 that is produced by phospholipase C diffuses into the cytoplasm, where it binds to specific receptors causing the release of calcium ions from intracellular stores (Berridge, 1990). This causes an increase in intracellular free calcium ion concentration, leading to a variety of effects including the activation of calcium/calmodulin dependent protein kinases, other phospholipases (Kennedy, 1989) and proteases (Melloni & Pontremoli,

1989), as well as causing various cytoskeletal rearrangements (Forscher, 1989). The other product formed, DAG, acts in unison with calcium to activate protein kinase C (Huang, 1989), an enzyme of particular interest due it postulated role in the pathology of Alzheimers disease (Masliah *et al.*, 1991a, and references therein).

The identity of the G protein which stimulates phospholipase C, termed Gp, remains unclear. In some cases the stimulation of the enzyme can be inhibited by pertussis toxin, while in others it has no effect (Moriarty *et al.*, 1989). This difference probably arises from the existence of isoenzymes of phospholipase C (Crooke & Bennet, 1989), which can presumably interact with different G proteins. In HL-60 cells the stimulation of phospholipase C is abolished by pertussis toxin treatment, but this can be overcome by the addition of purified Goα and Giα (Kikuchi *et al.*, 1986). In addition, Moriarty *et al.* (1990) have demonstrated that the addition of purified Goα but not Gi1, Gi2 or Gi3, to *Xenopus* oocytes causes the opening of a calcium sensitive chloride channel, an effect which is accredited to the stimulation of phospholipase C. Although the limited tissue distribution of Goα makes it likely that other G protein species can activate phospholipase C, it seems that one role of Goα may be to modulate inositol phospholipid metabolism in brain.

The identity of the G protein which mediates the pertussis toxin-insensitive stimulation of phospholipase C has only recently become clearer. The two cloned α subunits which are the best candidates for Gp, are Gzα and Gqα (and α11), both of which are insensitive to pertussis toxin. The experiments of Shenker *et al.* (1991) have demonstrated that the stimulation of low Km GTPase activity in platelets by the thromboxane A_2 receptor (a receptor which is coupled to the stimulation of phospholipase C), can be abolished by the addition of antibodies against Gqα but not Gzα. This indicated that, in platelets at least, Gqα was associated with the modulation of phospholipase C activity. Confirmation of this has come from Smrcka *et*

al. (1991), who have shown that both Gqα and α11, purified from bovine brain, are able to stimulate phospholipase C, while Goα, Gi1 and Gi2 are not. Although it is as yet unknown with which receptors Gqα can interact, it is likely that this relatively unstudied G protein will prove to be important in neural function. Thus it appears that a range of different G proteins may serve to stimulate the activity of phospholipase C, each possibly interacting with different receptors and phospholipase C subtypes.

Although most is known about the G protein coupled control of phospholipase C, the activity of other phospholipases may also be modulated in this way. For example, phospholipase A_2, which catalyses the breakdown of phospholipids to form arachidonic acid, appears in some circumstances to be stimulated by transmitters via a pertussis toxin sensitive G protein, termed Ga (Burch *et al.*, 1986). In addition, the activity of phospholipase D in hepatocytes is also thought to be coupled to receptors by a G protein referred to as Gd (Bocckino *et al.*, 1987), although other mechanisms involving protein phosphorylation or calcium ion concentration are also known to be involved (reviewed in Billah & Anthes, 1990). Phospholipase D may constitute an important part of the cell's signal transducing repertoire, since it catalyses the breakdown of phosphatidyl choline to form phosphatidic acid. This can be further degraded to DAG by the action of phosphatidic acid phosphohydrolase (Billah & Anthes, 1990). Since phosphatidyl choline is a much more abundant lipid than phosphatidyl inositol (Houslay & Stanley, 1984), it represents a much larger potential source of DAG, which as explained above, is required for the activation of protein kinase C. As it also appears that protein kinase C can in turn stimulate phospholipase D activity in astrocytoma cells (Martinson *et al.*, 1989), there potentially exists a positive feedback mechanism between the two enzymes that could result in the persistent activation of protein kinase C. This may be of particular importance in the CNS, since protein kinase C is thought to play an important role in the formation and

maintenance of long term potentiation (Malenka *et al.*, 1989b), a process by which repeated stimulation of certain neurons gives rise to a lasting increase in their excitability. It is thought to be a mechanism by which information is stored in the brain i.e. memory (Barnes *et al.*, 1990).

Ion channels.

The conductance of many types of ion channels can be altered by the action of kinases activated by the mechanisms described above (Levitan, 1988). However, the use of the patch clamp technique for monitoring ion channel activity has shown that G proteins can directly interact with, and modulate the conductance of, several types of ion channel. This direct effect was initially investigated in heart in which a G protein, termed Gk and subsequently shown to be Gi3 (Codina *et al.*, 1988), was able to control the opening of potassium channels (Codina *et al.*, 1987). In addition, a Giα protein can activate ATP sensitive-potassium channels in rat RINm5F cells (Ribalet *et al.*, 1989). Other G proteins are also involved in the regulation of ion channels. For example, Gsα can interact with sodium channels in guinea pig heart (Schubert *et al.*, 1989), and dihydropyridine-sensitive calcium channels in guinea pig heart (Imoto *et al.*, 1988) and rabbit muscle (Mattera *et al.*, 1989). Although this facet of Gsα action has not been demonstrated in the CNS, it seems likely that it does occur. Thus Gsα may mediate a rapid response to a neurotransmitters via its direct effect on ion channels, which may then be consolidated by the phosphorylation of other ion channel proteins by protein kinase A, acting in response to increased cAMP levels. There exists two demonstrations of G protein control of neural ion channels. Firstly, Goα has been shown to enhance the conductance of four types of potassium channels in rat hippocampus (VanDongen *et al.*, 1988). Secondly, the same protein can inhibit the opening of calcium ion channels in dorsal root ganglion cells (Ewald *et al.*, 1988). Both these actions would be expected to have an inhibitory effect on the cell, which is somewhat at odds with the

postulated role of Goα as an activator of phospholipase C; an action expected to increase the intracellular concentration of calcium ions. It is therefore likely that Goα does not mediate both effects within one cell, and raises the possibility that the different subtypes of Goα are functionally distinct.

2.4 G protein coupled receptors.

The preceding sections have concentrated mainly upon the relationship between different G protein and effector proteins. It has also been important to determine which receptors couple with which G protein linked pathways. This has been accomplished by monitoring the effect that receptor specific agonists have on the activity of the various effector proteins, reconstituting purified receptors and G proteins in phospholipid vesicles, and the use of anti-G protein antisera to block the actions of certain G proteins. A large number of G protein coupled receptors are known to exist in brain and some of the most important are listed in Table 1.

Table 1.

Examples of G protein linked neurotransmitter receptors.

The table illustrates a range of neurotransmitters, and their receptors, that modulate the activity of G protein coupled effector enzymes.

Abbreviations - AC = adenylate cyclase, PLC = phospholipase C, PLA_2 = phospholipase A_2 , PLD = phospholipase D, ↑ or ↓ indicates stimulation or inhibition respectively, CRF = corticotrophin releasing factor.

References are -

(1) Bonner (1989)

(2) Peralta *et al.*, (1988)

(3) Egan *et al.* (1986)

(4) Birn baumer *et al.* (1990)

(5) Cotecchia *et al.* (1990)

(6) Burch *et al.* (1986)

(7) Bocckino *et al.* (1987)

(8) Bowery (1989)

(9) Childers (1991)

(10) Hoyer and Schoeffter (1991)

Transmitter	Receptor type	Effector	Reference
Acetyl Choline	M1	↑ PLC	(1)
		↑ AC (?)	(2)
		↓ K channel	(1)
	M2	↓ AC	(1)
		↑ K channel	(3)
Adenosine	A1	↓ AC	(4)
	A2	↑ AC	(4)
Adrenaline (Noradrenaline)	β_1	↑ AC	(4)
		↑ Ca channel	(4)
	β_2	↑ AC	(4)
	α_1	↑ PLC	(5)
		↑ PLA_2	(6)
		↑ PLD	(7)
	α_2	↓ AC	(5)
Cholecystokinin		↑ AC	(4)
		↓ PLC	(4)
CRF		↑ AC	(4)
Dopamine	D1	↑ AC	(4)
	D2	↓ AC	(4)
GABA	$GABA_B$	↓ AC	(8)
		↑ K channel	(8)
Histamine	H1	↑ PLC	(4)
	H2	↑ AC	(4)
Opioid	μ, κ, δ	↓ AC	(9)
		↑ PLC	(9)
Serotonin (5-HT)	$5\text{-}HT_{1A}$	↓ AC	(10)
	$5\text{-}HT_{1B}$	↓ AC	(10)
	$5\text{-}HT_{1C}$	↑ PLC	(10)
	$5\text{-}HT_{1D}$	↓ AC	(10)
	$5\text{-}HT_2$	↑ PLC	(10)
	$5\text{-}HT_3$	↑ K channel	(10)
	$5\text{-}HT_4$	↑ AC	(10)
Somatostatin		↓ AC	(4)
		↑ K channel	(4)

2.5 Summary.

It is evident that in some cases one neurotransmitter can bind to several different receptors, which in turn interact with different G proteins, which may themselves have more than one function. Additionally, the activity of G proteins may be dependent on the membrane potential of the cell (Cohen-Armon & Sokolovsky, 1991), thus offering the possibility that ionotropic receptors can also effect G protein coupled signal transduction. Furthermore, the functional activity of several receptors and G proteins can be altered by phosphorylation, and "cross talk" can occur between different effector systems e.g. protein kinase C, activated by the action of phospholipase C, can effect the control and activity of adenylate cyclase (Lo, 1988). Although all of these possibilities are unlikely to occur in every cell, it is apparent that a large variety of subtle interactions can exist between the various mechanisms responsible for transducing signals across the cell membrane. Thus, it is likely that even small alterations in the functioning of any of these components may have far reaching consequences for the assimilation and processing of intercellular messages, especially within the CNS.

3. Changes in G protein coupled signal transduction associated with disease.

During the last decade it has become increasingly apparent that alterations in the mechanisms of signal transduction are associated with a wide range of diseases. This has already been mentioned with respect to the effect that some bacterial toxins have upon G proteins; however many more examples are known. In some cases the disruption of a signal transducing cascade is the primary cause of the ailment, while in others they may represent an adaptive response to the disease state. Probably the best studied example of the former is Albright's hereditary osteodystrophy (AHO). This is a rare genetic disorder characterised by numerous physical abnormalities including skeletal defects, short stature and obesity. Two forms of the disease exist in which the sufferers are either resistant to hormones which stimulate adenylate cyclase, such as parathyroid hormone, or show a normal response to these substances. These subclasses are termed pseudo-hypoparathyroidism (PHP) and pseudo-pseudo-hypoparathyroidism (PPHP) respectively (Weinstein *et al.*, 1990). The majority, though not all, of those with the disease (both PHP and PPHP) demonstrate an approximately 50% deficiency in the levels of Gsα throughout the body (Levine *et al.*, 1986; Levine *et al.*, 1988), with both GsH and GsL being affected (Carter *et al.*, 1987). The implication of this is that while a deficiency in Gsα contributes to the disease, other elements in the signal transduction cascade must also be altered in some way for the disease to develop. The use of molecular biological techniques has revealed that the Gsα deficiency can be caused by a variety of genetic defects. For example, in one patient Weinstein *et al.* (1990) observed a heterozygous splice junction mutation, which would presumably lead to abnormal RNA splicing and hence decreased levels of functional Gsα. In another patient however, the same authors found a heterozygous frameshift mutation within the coding region

of the Gsα gene. In addition, a heterozygous mutation within the initiation codon of Gsα has been reported by Patten *et al.* (1990). Thus AHO is associated with a variety of dominant heterozygous abnormalities within the Gsα gene.

As well as disease caused by too little functional Gsα being present, too much Gs activity would also appear to be detrimental. In some human pituitary tumours, the cancerous cells are characterised by high levels of intracellular cAMP (Vallar *et al.*, 1987). This is due to the presence of a variety of point mutations within the Gsα gene, which prevents Gsα from hydrolysing GTP (Landis *et al.*, 1989). The molecule therefore becomes constitutively active, leading to a high basal activity of adenylate cyclase. This bypasses the cell's normal requirement for trophic hormones and leads to uncontrolled cell division. Thus, Gsα can be thought of as a proto-oncogene product, which is a potential source of tumours occurring in other tissues.

There are also a number of diseases where dysfunction of signal transduction is not the primary cause, but may contribute to the expression of the disease state. A well studied example of these, are disorders affecting the thyroid gland. In contrast to PHP in which parathyroid hormones are present but have reduced effect, these illnesses are characterised by either elevated (hyperthyroidism) or decreased (hypothyroidism) serum levels of thyroid hormones. One of the hallmarks of thyroid disorders is a change in the potency of those hormones which modulate the activity of adenylate cyclase. Thus hypothyroid rats display a decreased responsiveness to stimulatory hormones (Malbon & Gill, 1979), while hyperthyroidism is accompanied by a marked increase is this response (Guarnieri *et al.*, 1980). It might be expected from what is known about pseudo-hypoparathyroidism, that hypothyroidism will be associated with decreased Gsα levels, however this does not appear to occur (Levine *et al.*, 1990a). Rather the ailment is accompanied by the increased expression of Giα and Goα (Orford *et al.*,

1991; Levine *et al.*, 1990a) and of G protein β subunits (Rapiejko *et al.*, 1989). This would presumably lead to a greater release of βγ subunits from Gi under basal conditions, which would then compete with adenylate cyclase for free Gsα molecules, thereby reducing the response to stimulatory hormones. In contrast, the increased responsiveness to stimulatory hormones observed in hyperthyroidism is associated with decreased expression of Giα (Ros *et al.*, 1988), accompanied by slightly increased levels of Gsα as assessed by cholera toxin mediated ADP-ribosylation (Rapiejko & Malbon, 1987). Thus, at least one function of thyroid hormones is to regulate the effect that other hormones have on the cell, a feature which is mediated via their control of G protein levels. When dysfunction of the thyroid gland occurs, these effects are amplified, possibly contributing to the clinical symptoms associated with the disease. It is worthwhile noting at this point that other hormones can also alter the levels of G proteins. For example, the administration of glucocorticoid or sex steroid hormones results in increased levels of Gsα and decreased levels of Giα in rats (Saelo *et al.*, 1989; Riemer *et al.*, 1988). It would appear therefore, that the actions of a number of different hormones are mediated, in part, by affecting the functional characteristics of the adenylate cyclase transduction system. Furthermore, any disease which causes a change in the levels of such hormones, may produce physical symptoms via its effect upon this transduction system.

Considerable attention has also been given to the association between human heart disease and G protein function. A reduction in basal, GppNHp-stimulated and adrenergic receptor-stimulated adenylate cyclase activity has been reported to occur in failing heart. This is associated with increased levels of Giα and unaltered levels of Gsα (Bohm *et al.*, 1989; Bohm *et al.*, 1990), a situation which is reminiscent of that observed in hypothyroidism. In contrast however, no change in the levels of β subunits have been observed in heart disease (Bohm *et al.*, 1990). The decreased response of adenylate cyclase to adrenergic agonists may be accounted for

by a reduction in the numbers of β-adrenergic receptors present in failing heart (Bohm *et al.*, 1990). However, it is difficult to explain the reduced stimulatory effect of GppNHp in heart disease, if one believes that inhibition of adenylate cyclase is mediated solely by βγ subunits. In addition, the reduction in stimulation can be removed by inactivating Gi with pertussis toxin (Feldman, 1991). The most logical explanation therefore is that Giα can have a direct inhibitory effect upon cardiac adenylate cyclase. Aside from the interest in this system for the testing of theoretical models of G protein function, the alterations observed may be of considerable physiological relevance, given that control over cardiac muscle contraction is mediated via the adenylate cyclase transduction system (Drummond *et al.*, 1979).

The study of animal models of diabetes has also demonstrated changes in the G proteins which control adenylate cyclase. This disorder is due to reduced levels of, or reduced effect of, insulin. The disease is associated with a wide range of metabolic aberrations, some of which are simply due to the primary loss of insulin effect upon glucose transport (Taylor & Agius, 1988). However the disease is also accompanied by alterations in the response of tissues to many other hormones. The development of an animal model of the disease, in which diabetes is induced in rats by the administration of streptozotocin, has proved invaluable in investigating the molecular basis for these "secondary" effects of diabetes. Using this model Gawler *et al.* (1987) have demonstrated a loss of Giα in hepatocytes of diabetic rats. In addition, the remaining Gi possesses decreased functional capacity (Bushfield *et al.*, 1990b), an effect which appears to be mediated by the phosphorylation of Gi2. This effect is even more apparent in rat adipocytes, in which diabetes does not produce any change in the levels of Giα, but does reduce the ability of this G protein to inhibit adenylate cyclase (Strassheim *et al.*, 1990). As well as providing a greater understanding of the disease, these studies have also been important

in emphasising the need to consider the functioning, as well as the levels of, the components involved in signal transduction.

The study of affective disorders (mania and depression) has indicated that alterations in interneuronal communication occur in these extremely common illnesses. Various early studies of the disease suggested that depression is caused by a deficiency of monoamine neurotransmitters, especially noradrenaline, while mania is caused by an excess of these transmitters (reviewed in Malenka *et al.*, 1989a). However this hypothesis fails to explain many aspects of the diseases and their treatment. A more satisfactory molecular basis for the affective disorders has been proposed by Wachtel (1990), in which an imbalance occurs between the adenylate cyclase and phosphatidylinositol-specific phospholipase C signal transduction systems. Thus in depression, there is hypofunction of the adenylate cyclase system with a relative dominance of the inositol phospholipid system, while the opposite in proposed to occur in mania. This hypothesis offers a rational as to how various treatments for depression may operate. For example, a common therapy is the administration of lithium which has been shown to attenuate phosphoinositide turnover by inhibiting the resynthesis of inositol phospholipids. Lithium also inhibits the coupling of β-adrenergic and muscarinic receptors to G proteins (Avissar *et al.*, 1991), although the length of time that lithium takes to exert a clinical effect suggests that its major role is in depressing phosphoinositide metabolism (Malenka *et al.*, 1989a). Additionally, two other treatments, tri-cyclic antidepressants and electro-convulsive treatment appear to increase the production of cAMP in rat brain, by enhancing the coupling between Gsα and adenylate cyclase (Ozawa & Rasenick, 1989; Ozawa & Rasenick, 1991). The molecular mechanism of this effect is unknown, but may be due to antidepressant treatment causing alterations in the "complexes" which are proposed to exist between Gs, Gi and the cytoskeleton. The suggestion that such interactions actually take place, derives from the apparent exchange of GTP

between Gsα and Giα, and between G proteins and the cytoskeleton (Hatta *et al.*, 1986; Rasenick & Wang, 1988; Wang *et al.*, 1990). After anti-depressant treatment, the exchange of nucleotides between Gsα and Giα appears to be increased (Ozawa & Rasenick, 1991). These studies, deriving from the investigation of disease, indicate that the proteins species involved in signal transduction may not simply move randomly in a "sea" of lipids, but may be assembled into functional units, held together by the cytoskeleton. There have been limited investigations of signal transduction mechanisms in the post mortem brains of manic-depressive human subjects, although Young *et al.* (1991a) have very recently demonstrated an increase in the levels of Gsα post mortem, within the cortex of persons who had suffered from manic depression. It will be interesting to discover if the drug treatments given to such patients work in a similar way as that observed in animal models.

Finally, the functioning of signal transduction mechanisms is also changed by the chronic administration of certain drugs. For example, the treatment of NG-108 cells (a neural-glial hybrid cell line) with ethanol for 48 hours causes a reduction in their response to agonists which stimulate adenylate cyclase. This was found to be due to a reduction in the levels of Gsα in the treated cells (Mochly-Rosen *et al.*, 1988). Ethanol also uncouples receptors from the stimulation of phospholipase C in NG-108 cells, an effect which is once again attributed to its modulation of G protein activity (Simonsson *et al.*, 1991). The chronic administration of opiates also leads to alterations in G protein levels, with a decrease in Giα being reported in rat spinal cord neurons, resulting in the cells becoming desensitised to opiates (Attali & Vogel, 1989). Curiously, Nestler *et al.* (1989) have reported a small increase in the levels of Giα in rat locus coeruleus in response to opiate treatment. Despite this, desensitisation still takes place, suggesting that a decrease in receptor-Gi coupling has occurred.

Thus a growing number of disease states, or adaptive responses to external agents, are either caused by, or are manifested through, alterations

in the functioning of signal transduction systems. In addition, the study of these mechanisms in disease provides an ideal opportunity to test current ideas of how such systems operate. As will be made clear in the next section, the investigation of signal transduction in dementia of the Alzheimer type has concentrated largely on neurotransmitters and their receptors, with little attention given to other components. Given the growing awareness that events distal to receptor activation are frequently affected by disease processes, it is imperative that such studies be carried out. Only by doing so can therapeutic strategies, which attempt to manipulate signal transduction, be designed with any confidence.

One general conclusion that can be drawn from the above examples, is the changes in the levels or activity of G proteins largely occur in response to chronic conditions. They can therefore be considered to be a means by which cells can respond to long term alterations in their environment. This makes them an ideal candidate for study in non-acute disorders such as dementia of the Alzheimer type.

4. The major features of Dementia of the Alzheimer Type.

4.1 Overview.

The combination of an increasing birth rate and decreasing mortality have led to a steady rise in the numbers of elderly persons. It is estimated that over the next ten years there will be a 25% increase in the numbers of individuals aged over 65 (Phelps, 1990). This has resulted in a heightened prevalence of the diseases of later life, among which are several neurological disorders collectively known as dementias. These are characterised by a loss of cognitive functions, impairment of memory, and a decreased ability to learn (Katzman, 1986). The most common form of dementia is Alzheimer's disease, first reported in 1907 by Alois Alzheimer (Alzheimer, 1907), in which the sufferers ability to reason is severely reduced. Alzheimer's disease was initially thought to be a pre-senile dementia since the case described by Alzheimer was of a 51 year old woman. However a very similar disease occurs in later life, the incidence of which increases with age (Evans *et al.*, 1989). While the severity of the illness appears to be greater in the presenile compared to senile form (Mann *et al.*, 1985), the only major difference between the two is age of onset. It is usual therefore to refer to any person whose brain displayed the pathological features of Alzheimer's disease, as having suffered from Dementia of the Alzheimer Type (DAT).

The disease has typically been investigated by one of two distinct strategies. The first has attempted to discover the cause of the disorder, with initial studies using mainly epidemiological approaches. Most recently, the application of powerful molecular genetics techniques has resulted in significant advances in our understanding of the disease's aetiology. A general conclusion of these studies has been that there is no single cause of DAT, but a variety of different risks factors exists including serious head trauma (Heyman *et al.*, 1986), myocardial infarction (Aronson *et al.*, 1990), aluminium intoxication (Crapper-McLachlan & Van Berkum, 1986), and a variety of possible genetic abnormalities (Weitkamp *et al.*,1983; Schellenberg

et al., 1991; Pericak-Vance *et al.*, 1991; Goate *et al.*, 1991; Chartier-Harlin, 1991). It is possible that the relative contributions of each factor will result in small differences in the pathological changes which take place within the DAT diseased brain. However, at autopsy, the brains of all DAT subjects possess a number of distinct pathological features. Macroscopically the brain shows significant atrophy, particularly of the frontal and temporal lobes, associated with narrowing of sulci, widening of gyri and ventricular enlargement (Perry, 1986b). There also exists in the literature, a large number of studies describing a range of neuropathological and neurochemical changes associated with DAT. The second research strategy employed has therefore been to characterise these changes and discover the mechanisms by which they occur. This review will be limited to (i) those neuropathological changes which allow DAT to be definitively diagnosed post mortem, an important consideration since it is not possible at present to unambiguously diagnose DAT before death, and (ii) neurochemical evidence that alterations in signal transduction systems may have occurred.

4.2 Neuropathological changes.

The classical features of DAT are characterised neuropathologically, these being the presence of neuritic plaques and neurofibrillary tangles within the association cortex and hippocampus. In addition, the disease exhibits loss of cells within particular populations of neurons, although the severity of loss in each area may differ between subjects. In general, it is the larger neurons that are lost, thus the large pyramidal neurons of the association cortex (especially in the frontal and temporal cortices) are severely affected, while smaller cells are relatively spared (Terry *et al.*, 1981). In addition, the pyramidal cells of the hippocampus (Hyman *et al.*, 1984), and cells in various subcortical nuclei, including the cholinergic nucleus basalis of Meynert (Whitehouse *et al.*, 1982), the adrenergic locus coeruleus and the serotonergic dorsal raphe (Mann *et al.*, 1984), are also affected by the

disease. The death and dysfunction of these neurons is undoubtedly the physical basis for the cognitive decline observed in DAT. In contrast, other regions such as the brain stem, cerebellum and neostriatum are relatively unaffected by the disease (Pearson & Powell, 1989). The underlying cause of the selective vulnerability of certain cellular populations is unclear, but it is noticeable that they are within, or have connections with, the association cortex, suggesting that the disease is cortical in origin (Pearson & Powell, 1989). Quite why larger neurons are most effected is also unknown, but may be due to differences in the biochemical makeup of these cells, with the presence or absence of certain neurotransmitter receptors being a prime candidate (Phelps, 1990).

The quantification of neuritic plaques, which are present in large numbers within DAT diseased cortex, is commonly used to confirm the clinical diagnosis of DAT (Khachaturian, 1985). Plaques are most frequently composed of a core of amyloid protein surrounded by degenerating neuronal terminals, as well as microglia and astrocytes, although other forms do exist (reviewed in Probst *et al.*, 1991). Most recently, several groups have identified a new form of plaque, known as amorphous or diffuse plaques, which are composed of non compacted deposits of amyloid protein (Joachim *et al.*, 1989). These are present in large numbers within DAT diseased brain, and most interestingly are found in areas which are thought to be relatively unaffected by the disease, such as the neostriatum and cerebellum, in addition to the cortex and hippocampus (Tagliavini *et al.*, 1988; Yamaguchi *et al.*, 1988; Joachim *et al.*, 1989). Furthermore, it has been suggested that diffuse plaques are precursors of neuritic plaques (Motte & Williams, 1989). It is therefore of interest to determine what differences exists between, for example the cortex and cerebellum, such that diffuse plaques do not develop into neuritic plaques throughout the brain.

The composition of amyloid has been extensively studied, and is now known to be composed of a small 4kD protein known as β-amyloid (Masters

et al., 1985). Although amyloid may merely serve to occupy the space left by dead neurons, there are indications that β-amyloid has neurotoxic effects (Yankner *et al.*, 1989). The use of protein sequencing and molecular cloning, has revealed that β-amyloid is the proteolytic degradation product of a larger protein termed the amyloid precursor protein (APP) (Goldgaber *et al.*, 1987). The normal function of APP is unclear, but it is thought to exist in both membrane bound and secreted forms (Palmert *et al.*, 1989). Molecular cloning has revealed the presence of three major species of APP, two forms of which contain a domain which is homologous to that of Kunitz serine protease inhibitors, with the other lacking this sequence (Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988). The mRNAs are expressed in many tissues (Tanzi *et al.*, 1988; Ponte *et al.*, 1988), which agrees with the finding that APP is a typical housekeeping gene (Salbaum *et al.*, 1988). The expression of APP mRNA is reported to be increased in DAT, with many, though not all, reports showing an increase in the mRNA containing the protease inhibitor domain (König *et al.*, 1991 and references therein). This has led to speculation that altered expression of the different forms may alter the susceptibility of APP to proteolytic degradation, possibly increasing the amount of β-amyloid that is formed. This is supported by the observation that deposits of β-amyloid are formed in the brains of transgenic mice, in which APP mRNA containing the protease inhibitor domain was overexpressed (Quon *et al.*, 1991).

An alternative explanation is that the proteolytic processing of APP is altered in DAT. In normal brain, APP is largely cleaved within the β-amyloid region, thereby excluding the formation of the peptide (Tagawa *et al.*, 1991). Thus in DAT an alternative pathway must occur which cleaves APP on either side of the β-amyloid region. Indeed, Chartier-Harlin *et al.* (1991) have reported the presence of a mutated APP gene in some persons suffering from a form of DAT in which genetic factors are clearly involved (familial DAT). Such a defect may increase β-amyloid formation by making

APP a better substrate for the alternative pathway of processing. The recent purification and characterisation of proteases which can cleave APP (Tagawa *et al.*, 1991), makes it likely that the sequence of events which lead to the formation of β-amyloid may soon be elucidated. The release of trophic factors by glia in response to neuronal death may also contribute to increased amyloid deposition, since nerve and epidermal growth factors cause the increased secretion of APP from PC12 cells (Refolo *et al.*, 1989). In addition, it has been suggested that an alteration in the phosphorylation of APP in DAT may alter its proteolytic processing (Buxbaum *et al.*, 1990).

The other main pathological feature observed in DAT diseased brain is neurofibrillary tangles (NFT). These are large, non-membrane bound filaments which occur in the neuronal cell body and often extend into the dendrites (Probst *et al.*, 1991). They are mainly found in the brain regions which experience the greatest cell loss (Bondareff *et al.*, 1989), leading to the conclusion that NFT occur within degenerating neurons. For this reason there has been great interest in how these structures are formed, since an understanding of this process may indicate a general mechanism for the cellular dysfunction that occurs in the disease.

NFT consist mainly of pairs of filaments twisted around each other (paired helical filaments or PHF), but straight filaments and vesicular materials are also found (Yagishita *et al.*, 1981; Wisniewski *et al.*, 1984). Although PHF were originally thought to be a form of normal neurofilaments, electron microscopy has demonstrated that PHF are not composed of filamentous structures (Wisniewski *et al.*, 1984), but of a chain of small globular units (Wischik & Crowther, 1986). Such a configuration is not typical of the other proteins which make up the normal neuronal cytoskeleton. Despite this, immunohistochemical studies using antibodies raised against various cytoskeletal elements, including the 150kD and 200kD neurofilament proteins (Anderton *et al.*, 1982; Sternberger *et al.*, 1985), MAP2 (Yen *et al.*, 1987) and vimentin (Yen *et al.*, 1984), have demonstrated that PHF

share common epitopes with these proteins, leading to the suggestion that PHF are composed of modified cytoskeletal proteins. Indeed the major, if not the only, component of PHF is thought to be a modified form of the microtubule accessory protein, tau (Goedert *et al.*, 1991). The normal role of tau is to promote tubulin polymerisation (Weingarten *et al.*, 1975), but in DAT it is found to be abnormally phosphorylated, as well as possessing an altered cellular localisation (Kowall & Kosik, 1987; Flament *et al.*, 1990). It has been proposed that the phosphorylation of tau, by as yet unidentified kinases, promotes its polymerisation into PHF (Mori & Ihara, 1991). The same authors have also suggested that this occurs as a consequence of changes in the functioning of one, or more, signal transduction systems. Thus, it is possible that aberrant kinase activity, possibly mediated by altered signal transduction mechanisms, may play a role in the formation of neurofibrillary tangles, as well as in amyloidogenesis.

4.3 Changes in the levels of neurotransmitters and receptors.

The measurement of the levels of neurotransmitters and their receptors, as well as the activity of enzymes involved in transmitter synthesis or breakdown, has revealed a wide variety of neurotransmitter abnormalities in DAT. In general these changes are thought to be due to the loss or dysfunction of specific neuronal types, although in the case of some neurotransmitter receptors, compensatory alterations in expression may occur. There follows a description of the effects that DAT has on the major neurotransmitter systems in the brain.

One of the most dramatic neurochemical changes that takes place in DAT diseased cortex is a reduction in the activity of choline acetyl transferase, the enzyme which catalyses the formation of acetyl choline (Procter *et al.*, 1988). In addition, levels of the M2 subtype of muscarinic receptors, which are thought to be located presynaptically, are decreased in DAT diseased cortex, although the postsynaptic M1 receptors are largely

unchanged (Mash *et al.*, 1985). Since the major cholinergic projection into the cortex is from the nucleus basalis of Meynert (nbM), it was postulated that degeneration of this subcortical nuclei was the primary event in DAT, which in turn caused the dysfunction of the neurons in the cortex (Perry, 1986a). This hypothesis was particularly appealing since Parkinson's disease appears to be caused by the death of neurons in the substantia nigra, which forms part of the brains dopaminergic system (Sourkes, 1989). It was therefore thought that DAT may be the cholinergic analogy of Parkinson's disease. However, marked cortical pathology can occur without severe cell loss from the nbM (Pearson *et al.*, 1983b). In addition, degeneration of neurons in the nbM has been observed to occur after cortical damage in human subjects, and in primates (Pearson *et al.*, 1983a). It is probable therefore that the primary damage in DAT occurs in the cortex, which causes damage to the projecting fibres and subsequent death of the neuronal cell body with the nbM. This seems likely since cell loss also occurs within other subcortical nuclei such as the locus coeruleus.

Although muscarinic receptors, being G protein coupled, are of most interest in the context of this study, the ionotropic nicotinic receptor has also been studied with conflicting results - Shimohama *et al.* (1986a) have reported no change in the levels of nicotinic receptors in DAT diseased cortex or hippocampus, while Flynn & Mash (1986) have demonstrated a 65% decrease within the cortex.

Although, the cholinergic deficit is probably the most characteristic and robust neurochemical change which occurs in DAT, alterations also occur in a wide range of other neurotransmitter systems. For example, reductions in the levels of adrenalin have been reported to occur in post mortem DAT diseased brain (Adolfsson *et al.*, 1979), a not unexpected observation given the damage that occurs to the locus coeruleus in the disease. Shimohama and co-workers have demonstrated changes in the levels of adrenergic receptors in DAT, these being decreases in α_1 receptors

in the hippocampus, α_2 receptors in the nucleus basalis and β_1 receptors in the hippocampus and frontal cortex, but an increase in the levels of β_2 receptors in the hippocampus and frontal cortex (Shimohama *et al.*, 1986b; Shimohama *et al.*, 1987).

In a similar way, the cellular loss found in the dorsal raphe indicates an involvement of the serotonergic neurons in the disease. This appears to be so, since Bowen *et al* (1983) have demonstrated a decrease in serotonin uptake in brain biopsies, while Cross *et al.* (1986) have reported large decreases in the levels of 5-HT_2 , but not 5-HT_1 , receptors in the cortex of DAT diseased subjects.

The lack of any substantial cell loss in the neostriatum or substantia nigra in DAT, would suggest that the brain's dopaminergic system is relatively unaffected by the disease process. Cortical dopamine levels are largely unaltered in the disease, although low dopamine concentrations have been recorded in the neostriatum (Arai *et al.*, 1984), as well as reductions in the levels of D_1 and D_2 receptors (Cross *et al.*, 1984b).

The levels of the peptide transmitter somatostatin are greatly reduced in the cortex and hippocampus, a change which correlates with impaired cognitive function (Tamminga *et al.*, 1987). In addition, the number of somatostatin receptors are significantly reduced within these two regions (Beal *et al.*, 1985). The levels of another peptide transmitter, corticotrophin releasing factor, are also decreased within DAT diseased cortex. However, this is accompanied by an increase in the levels of the receptor for this peptide, indicating that a compensatory response may have taken place in the postsynaptic neurons (De Souza *et al.*, 1986). This observation is of some importance, since it indicates that despite widespread neuronal damage, some cells retain their capacity to respond to changes which occur in their extracellular environment.

Finally, the mainly ionotropic amino acid receptors will be considered, due to their postulated role in the pathological mechanism that

takes place in DAT. There is considerable interest in the receptors of glutamate, since it putatively represents the major excitatory neurotransmitter of the large cortical pyramidal neurons which are preferentially lost in DAT (Fonnum *et al.*, 1981). This, and the observation that there is a reduction in cortical glutamatergic terminals in DAT (Procter *et al.*, 1986), has led some to suggest that the these neurons are damaged by a glutamate mediated excitotoxic mechanism. Glutamate acts via at least four receptor types, these being *N*-Methyl-D-Aspartate (NMDA) receptors, kainate receptors, and 2-amino-3-hydroxy-5-methyllisoxazole-4-propionic acid (AMPA) sensitive quisqualate receptors, all of which are thought to be ionotropic, and AMPA-insensitive quisqualate receptors, which are linked to the modulation of phospholipase C activity, presumably via Gp (Farooqui & Horrocks, 1991). The measurement of the levels of these receptors in DAT diseased brain has given rather inconclusive results. Chalmers *et al.* (1990) have reported an increase in the levels of cortical kainate receptors, a small decrease in NMDA receptors, and no change in the levels of AMPA sensitive quisqualate receptors. Greenamyre *et al.* (1987), and Jansen *et al.* (1990), have both demonstrated a large loss of NMDA receptors in the hippocampus, although Geddes *et al.* (1986) did not detect any change. The role of glutamatergic receptors in DAT therefore remains unclear. Most recently, and most relevantly to this study, a decrease in the numbers of hippocampal AMPA-insensitive quisqualate receptors has been observed (Dewar *et al.*, 1991).

The receptors of the inhibitory amino acid transmitter gamma aminobutyric acid ($GABA_A$ and $GABA_B$) are also found to be decreased in DAT diseased cortex (Chu *et al.*, 1987). In addition, Lowe *et al.* (1988) found a decrease in the cortical concentration of GABA determined post mortem in DAT diseased subjects.

5. Summary and objectives.

Recent advances in study of the molecular mechanisms responsible for the reception and processing of intercellular messages, have demonstrated the existence of a variety of complex interactions between the various components of such systems. This most likely derives from these mechanisms being involved in a large variety of different functions throughout the body. However, as described in Section 3, the potential for dysfunction of these processes is similarly great. With regard to dementia of the Alzheimer type, a large number of studies have demonstrated widespread changes in the levels of neurotransmitters and their respective receptors in DAT diseased brain, the most pronounced being to the cholinergic, serotonergic and somatostatinergic systems. However, little attention has been paid to the other components of signal transduction, such as G proteins and the various effector enzymes and ion channels. This situation is reminiscent of the investigation of affective disorders, which was initially confined to the determination of neurotransmitter levels. However the recent shift of emphasis onto the other components of the signal transduction cascade, has proved fruitful in unravelling the mechanisms associated with these illnesses. While the presence of widespread neuropathological features in DAT makes it somewhat different to that of affective disorders, it remains likely that the investigation of signal transduction mechanisms in DAT will prove to be similarly beneficial. Such knowledge is of particular importance to the design therapeutic strategies for the disease. For example, the observation that muscarinic M1 receptors appear to be relatively unaffected by the disease, has prompted attempts to increase the stimulation of this receptor type, in the hope that this will ease the cognitive decline of sufferers. Various approaches have been used including the administration of drugs that raise the levels of acetyl choline in the brain (Dysken, 1987), or by the administration of muscarinic agonists (Read *et al.*, 1990). Unfortunately, this form of treatment, known as

cholinergic replacement therapy, has met with little or no success. While this may be due to increasing the stimulation of these receptors in a global manner, it could also be caused by dysfunction of the signal transduction mechanisms which are required for the activation of M1 receptors to have any effect. For this reason, and the possibility that other neuropathological features such as NFT, may be in part due to changes having occurred in aspects of signal transduction, it is important to examine the functioning of these mechanisms in DAT diseased brain.

The specific objectives of this study will firstly be to examine the feasibility of examining the components of signal transduction in human post mortem brain tissue. There is a concern that during the inevitable delay between death and freezing of the brain tissue, proteins or RNA may be degraded by either chemical or enzymatic means, thereby reducing the reliability of any measurements performed in such tissue. Hence the effect that this delay has upon the integrity of G proteins, G protein encoding mRNA, and the activity of several enzymes, will initially be studied in rat brain. Should the results of these experiments indicate that measurement of these moieties in post mortem brain is worthwhile, then the same standard biochemical techniques will be used to examine the effects that dementia of the Alzheimer type has on the same components in human brain.

II. METHODS

1. Materials.

1.1 Reagents.

All reagents used were of the highest grade commercially available and are listed according to source.

Tris, ethylenediaminetetraacetate (EDTA), sodium deoxycholate, trichloroacetic acid, glycerol, ammonium persulphate, N,N,N',N'-tetramethylene-ethylenediamine (TEMED), tween-20, *o*-dianosidine, neutral chromatographic alumina, imazadole, sodium salt of cyclic 3'-5' adenosine monophosphate (cAMP), sodium salt of adenosine triphosphate (ATP), creatine phosphate, creatine phospho kinase (from rabbit muscle), aluminium chloride, sodium fluoride, acetyl coenzyme A, acetonitrile, kalignost (sodium tetra phenyl boron), bovine serum albumin, diethyl pyrocarbonate, sodium N-lauryl sarcosine, phosphostigimine (eserine), 2-mercaptoethanol, 8-hydroxyquinoline, polyethyleneimine (PEI) cellulose, 3-[*N*-morpholino] propane sulphonic acid (MOPS), ficoll 400, polyvinyl pyrollidone, salmon testes DNA, methylene blue, comassie brilliant blue R, thimerosal, ouabain and bromophenol blue were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K.. Dithiothreitol, phosphoenoylpyruvate, reduced nicotinamide adenine dinucleotide (NADH), pyruvate kinase (from rabbit muscle), lactate dehydrogenase (from hog muscle) and T4 polynucleotide kinase were purchased from Boehringer Mannheim Ltd., Lewes, Sussex, U.K.. Urea, glycine, hydrogen peroxide (30% v/v), magnesium chloride, potassium chloride, triton X-100, sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium citrate, sodium acetate, chloroform, isoamylalcohol, sodium pyrophosphate, copper sulphate and sodium potassium tartrate were purchased from BDH Ltd., Glasgow, Lanarkshire, U.K.. Acrylamide, bis acrylamide, sodium hydroxide, sodium carbonate and formaldehyde were purchased from Fisons Ltd., Loughborough, Leicester, U.K.. Sodium dodecyl sulphate (SDS) and phenol were purchased

from IBI Ltd., Cambridge, U.K.. Methanol, hydrochloric, acetic acid, isopropanol, ethanol were purchased from May and Baker Ltd., Dagenham, Essex, U.K.. Agarose, prestained protein molecular weight standards for western blotting and RNA molecular size markers for northern blotting were purchased from BRL Ltd., Uxbridge, Middlesex, U.K.. Fuji RX X-ray film and Saran wrap were purchased from Genetic Research Instrumentation Ltd., Dumow, Essex, U.K.. Dowex resin (AG50W-X4 200-400 mesh), mixed bed resin (AG501-X8 20-50 mesh) and poly prep columns were purchased from Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.. Nitrocellulose (Schleicher and Schuel, 0.2 μM) was purchased from Anderman and Co. Ltd., Surrey, U.K.. Guanidinium thiocyanate and formamide were purchased from Fluka Ltd., Bristol, U.K.. Ecoscint and POPOP (a toluene based scintillation fluid) were purchased from Packard Bell, Reading, Berkshire, U.K.. Hybond N, hybond N+, biotinylated anti-rabbit immunoglobulin G (IgG) antisera from donkey, streptavidin-linked horse radish peroxidase (HRP), ^{125}I labelled anti-rabbit IgG from donkey (5-20 μCi/μg protein), 8-^{3}H cAMP (ammonium salt 1:1 in ethanol; 30Ci/mmol), ^{14}C-acetyl coenzyme A (60Ci/mmol), γ-^{32}P ATP (ammonium salt in 5mM 2-mercaptoethanol; 3000 Ci/mmol), α-^{32}P ATP (ammonium salt in 5mM 2-mercaptoethanol; >400 Ci/mmol) were purchased from Amersham International PLC, Amersham, Buckinghamshire, U.K.. HRP linked anti-rabbit IgG was supplied free of charge from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K..

1.2 Apparatus.

Vertical polyacrylamide gel and protein blotting apparatus were purchased from LKB Ltd., U.K.. Horizontal agarose gel apparatus was purchased from BRL Ltd., Uxbridge, Middlesex, U.K.. Intensifying screens were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K.. Radioactive samples were counted in a Packard Bell Tri Carb 1900CA liquid scintillation counter.

2. Brain tissue used.

Brains were obtained post mortem from nineteen subjects who had no neurological disorders (age 78 ± 3 years, (mean ± SE), delay between death and freezing the tissue 14 ± 3 hours), and ten subjects with a clinical diagnosis of dementia of the Alzheimer type (DAT), (age 84 ± 2 years, delay between death and freezing the tissue 7 ± 1 hours), as described in Table 2. The diagnosis of DAT was confirmed histopathologically after death according to the criteria of Khachaturian (1985). At autopsy, brains were cut into 1cm thick slices, dissected into different regions, frozen in isopentane (-40°C) and stored at -80°C. The freezer used for storage has a CO_2 backup system as well as a high temperature alarm which is monitored continuously. Tissue blocks adjacent to those used in this study were fixed in 10% formalin, sectioned and used to determine regional densities of neuritic (senile) plaques. The DAT group had plaque densities of 42.5 ± 4.7 plaques/mm^2 in the frontal cortex, and 7.2 ± 1.4 plaques/mm^2 in the hippocampus, whilst the control group had plaque densities of 2.0 ± 1.4 plaques/mm^2 in the frontal cortex, and 0.5 ± 0.4 plaques/mm^2 in the hippocampus (Table 2). All manipulations involving undenatured human tissue were performed in a level 2 containment facility at the Wellcome Surgical Institute.

The rats used in the study were adult male Wistar rats which had a body weight of between 300 and 350g except in the case of experiments involving middle cerebral artery occlusion where male Sprague Dawley rats were used.

Table 2.

The human subjects used in this study.

The subjects are listed according to their code number this being Cxx for controls or Axx for DAT subjects. PMD refers to the post mortem delay of the subject i.e. the time between death and freezing of the tissue. The drug treatment category lists any medication which was being administered to the subject before their death. Only drugs which are known to have effects on the CNS are listed. NK indicates that the cause of death and/or drug treatment were not known. The neuritic plaque density in plaques/ mm^2 of tissue blocks adjacent to those used in this study was derived as described in "Methods". The value given is the mean of the left and right hemisphere densities. The table also shows (as indicated by •) which patients were used in each of the major studies presented herein, these being:

(a) Measurement of G protein levels, adenylate cyclase etc. in the left frontal cortex.

(b) Measurement of G protein levels, adenylate cyclase etc. in the left hippocampus.

(c) Measurement of G protein mRNA levels in the right frontal cortex.

(d) Measurement of G protein mRNA levels in the right hippocampus.

Control Subjects

Subject	Age (years)	PMD (hours)	Sex	Cause of death	Drug treatment	Plaque density		Study			
						FCX	HIP	a	b	c	d
C8	80	23	F	Bronchopneumonia	Thyroxine	4	1		•		
C14	88	11	F	Bronchopneumonia	Thyroxine	0	0	•		•	•
				Kyphoscirrhosis							
C15	87	12	F	Pulmonary thromboembolism	Diamorphine	0	0	•			
				Renal failure							
				Cervical pelvic abscess							
C16	76	17	M	Pulmonary thromboembolism	Metodopramide	0	0	•	•	•	
				Bronchopneumonia	Diamorphine						
				Bronchial carcinoma							
C18	86	19	F	Sigmoid carcinoma	Diamorphine	1	0			•	
C19A	84	7	F	Myocardial infarction	Diamorphine	0	0	•	•	•	
C20	78	14	F	Myocardial infarction	None	0	0		•		•
C21	90	11	F	Carcinoma of colon	None	0	0	•			
C22	72	8	F	NK	NK	1	0	•			
C23	74	4	M	Peritonitis	None	0	0			•	
C24	96	3	F	NK	NK	8	5				•
C25	67	12	M	Myocardial infarction	None	0	0	•			
C28	55	12	M	NK	NK	0	0		•		•
C33	92	2	F	Bronchopneumonia	Thioridazine	NK	NK		•		•
C34	83	6	F	NK	NK	NK	NK		•	•	•
C35	66	7	M	NK	NK	NK	NK	•			
C37	86	6	M	Bronchopneumonia	NK	NK	NK		•		

Table 2 (continued) DAT subjects.

Subject	Age (years)	PMD (hours)	Sex	Cause of death	Drug treatment	Plaque density		Study			
						FCX	HIP	a	b	c	d
A17	89	4	F	Bronchopneumonia Pulmonary thromboembolism	Temazepam Morphine	56	5		•		
A18	85	3	F	Bronchopneumonia	Morphine	41	4		•		
A21	80	6	F	Congestive cardiac faliure	NK	27	11			•	
A22	69	6	M	Bronchopneumonia	Nitrazepam Procyclidine Thioridazine Haloperidol Ophenadrine	15	7			•	
A23	92	15	F	Bronchopneumonia	None	15	10	•		•	
A26	76	2	F	Pulmonary oedema Cardiac failure	None	39	12			•	
A29	97	4	F	NK	NK	80	3		•		
A30	85	4	F	NK	NK	45	2	•	•	•	•
A33	71	8	F	Bronchopneumonia	None	60	22	•	•	•	•
A35	NK	3	F	NK	NK	68	5		•		•
A53	80	8	F	NK	NK	45	8	•	•		•
A54	84	6	F	NK	NK	48	14	•	•		
A55	91	19	F	NK	NK	54	0	•			
A56	88	16	F	NK	NK	53	11	•			•
A63	93	9	F	NK	NK	NK	NK	•			
A65	76	11	F	NK	NK	NK	NK			•	•

3. Preparation of crude membranes.

Tissue blocks were ground in liquid nitrogen before a portion was homogenised in 6 volumes of 10mM Tris/HCl, pH7.5, 1mM EDTA for 30 seconds in a polytron homogeniser at full speed. This and all further manipulations were carried out at 4°C. If required, a small aliquot was removed for the assay of choline acetyl transferase and stored at -80°C. The remainder of the homogenate was centrifuged at $1000g_{av}$ for 15 minutes in a Beckmann TL-100 tabletop ultracentrifuge using a TL-100 rotor, or a Beckmann J2-21M/E ultracentrifuge using a JA21 rotor. The supernatant was removed and spun at $40,000g_{av}$ for 15 minutes. The pellet was resuspended in 10mM Tris/HCl, pH 7.5, using a needle and syringe, before being spun at $40,000g_{av}$ for 15 minutes. This pellet was then resuspended in 10mM Tris/HCl , pH 7.5, at an approximate concentration of 5mg protein/ ml. The preparation was then stored at -20°C until required.

4. Western blotting.

4.1 Antisera used.

The antisera used were provided by Dr. Graeme Milligan, Department of Biochemistry, Glasgow University. The antisera were raised against synthetic peptides corresponding to specific amino acid sequences predicted from cDNA sequences encoding the α subunit of Gs, Gi and Go (Mullaney *et al.,* 1988; Milligan & Unson, 1989). Briefly the peptides were linked to keyhole lympet haemocyanin and injected into New Zealand white rabbits. Blood was removed from the rabbits and sera separated by centrifugation. In brain tissue antisera OC1 detects Goα, SG2 detects two forms of Giα, Gi1 and Gi2, and CS1 detects two forms of Gsα, GsH and GsL. Details of the sequences to which the antisera were raised are given in Table 3.

Antisera	Peptide Used	G protein sequence	Recognises	Reference
OC1	ANNLRGCGLY	Goα 345-354	Goα	(1)
SG2	KENLKDCGLF	Transducin α 341-350[1]	Gi1, Gi2	(1)
CS1	RMHLRQYELL	Gsα 385-394	GsH, GsL	(2)

Table 3.

The anti-G protein antisera utilised in western blotting.

The peptide sequence against which the antisera used were raised (using one letter abbreviations), the position in the G protein that this corresponds to, and the subunits that the antisera recognises in brain are shown. References are: (1) Mullaney *et al.* (1988), (2) Milligan and Unson (1989).

[1] - Transducin is a G protein, also known as Gt, which couples photoreceptors to guanylate cyclase. It is only present in the retina.

4.2 Preparation of membrane samples for electrophoresis.

Crude membranes were prepared for the electrophoretic analysis of proteins by precipitating with tri-chloroacetic acid as follows. An appropriate quantity of protein was made up to 1ml with distilled water in a micro centrifuge tube, and sodium deoxycholate was added to a concentration of 1%. After vortex-mixing the samples, ice cold tri-chloroacetic acid was added to a concentration of 10% (w/v) to precipitate the proteins. The tubes were then left on ice for 30 minutes before the protein was pelleted by spinning briefly in a microcentrifuge. The supernatant was poured off and the tubes drained. The protein pellet was neutralised by the addition of 10 µl of 1M Tris, and then resuspended in denaturing buffer composed of 5% dithiothreitol, 5% sodium dodecyl sulphate (SDS), 5M urea and 0.1M Tris/HCl pH 8.0. A small volume of denaturing buffer containing 0.1% bromophenol blue was then added to each sample.

4.3 Polyacrylamide gel electrophoresis.

The denatured proteins were separated by discontinuous slab gel electrophoresis (Laemmli, 1970). A resolving gel, consisting of 12.5% acrylamide, 0.062% bisacrylamide, 0.38M Tris/HCl, pH 8.8, 0.1% SDS and 4.2% glycerol, was poured between a former composed of 2 glass plates 1.5mm apart, to give a gel approximately 13cm in length. Polymerisation was initiated by the addition of TEMED and ammonium persulphate to final concentrations of 0.05% (v/v) and 0.04% respectively. While the gel was polymerising, the top surface of gel was covered with 0.1% SDS. After the resolving gel had polymerised, the 0.1% SDS was removed and a stacking gel, consisting of 3% acrylamide, 0.08% bisacrylamide, 0.38M Tris/HCl, pH6.8, and 0.1% SDS, was poured above the resolving gel, such that there was approximately 1.5cm of stacking gel between the bottom of each well and the resolving gel. Polymerisation was initiated in the same manner as

for the resolving gel. After the stacking gel had polymerised, the electrophoresis apparatus was assembled and the denatured protein samples loaded onto the gel. When control and DAT subjects were being compared the two groups were loaded alternately onto the gel. Molecular weight markers were applied to at least one lane per gel. The markers used were prestained which allowed them to act as an indicator of protein transfer after blotting. However the bands produced by these markers were rather diffuse, and consequently only approximate molecular weights could be determined using them. The gels were run overnight at a constant current of 14mA per gel using an electrophoresis buffer composed of 190 mM glycine, 25 mM Tris and 0.1% SDS. Electrophoresis was stopped when the bromophenol blue dye front reached the bottom of the gel.

4.4 Protein blotting.

After electrophoresis, the resolving gel was separated from the stacking gel and incubated in transfer buffer composed of 190mM glycine, 25mM Tris and 20% methanol, for 15 minutes. The gel was removed from the buffer and a sheet of nitrocellulose was placed on top of it. After any air bubbles had been removed, the gel and nitrocellulose were sandwiched between sheets of wet Whatman 3MM paper, and immersed in transfer buffer in the blotting apparatus. Proteins were then transferred to the nitrocellulose at 70V for 3 hours. The extent of transfer and equity of protein loading on the gel, were then checked by staining the gel in a solution containing 0.05% comassie brilliant blue R, 50% methanol and 10% acetic acid, and destaining the gel in the same solution minus the comassie brilliant blue R.

4.5 Detection of G protein bands.

After transfer the nitrocellulose blot was incubated in TS buffer (0.5M sodium chloride, 20mM Tris/HCl, pH7.5) containing 3% fat free dried milk

for 3 hours at room temperature to block non-specific binding sites. All incubations involving nitrocellulose sheets were carried out on an orbital shaker set at approximately 1 revolution per second. The nitrocellulose sheets were then rinsed twice with TS buffer and incubated overnight at room temperature in primary antisera diluted in TS buffer containing 1% dried milk and 0.01% thimerosal. The latter substance served to inhibit microbial growth. Antisera CS1 was normally used at a dilution of 1/200, SG2 at 1/200, and OC1 at 1/10000. The blots were then washed for 10 minutes at room temperature in TS buffer containing 0.05% Tween 20, and then for 10 minutes at room temperature in TS buffer. Those blots which had been incubated with antisera OC1 or SG2 were then incubated for 3 hours at room temperature with horse radish peroxidase linked anti-rabbit IgG, used at a dilution of 1/200 in TS buffer containing 1% dried milk. The blots were then washed for 10 minutes at room temperature in TS buffer containing 0.05% Tween 20, and then for 10 minutes at room temperature in TS buffer. Immunoreactive bands were visualised by incubating with a buffer containing 10mM Tris/HCl, pH 7.5, 0.015% hydrogen peroxide and 0.02% *o*-dianosidine. The colour reaction was terminated by rinsing the blot twice in distilled water. In a few experiments antisera CS1 was processed in the same manner as SG2 and OC1, however it emerged that more sensitive techniques were required for successful blotting with this antisera. In one experiment, after incubation with CS1 and subsequent washing, the blot was incubated for 2 hours at room temperature with biotinylated anti-rabbit IgG used at a dilution of 1/400 in TS buffer containing 1% dried milk. The blot was then washed for 3x5 minutes at room temperature in TS buffer containing 0.05% Tween 20, and then for 3x5 minutes at room temperature in TS buffer. After this the blot was incubated at room temperature with streptavidin linked horse radish peroxidase used at a dilution of 1/3000 in TS buffer containing 1% dried milk. The blot was then washed for 3x5 minutes at room temperature in TS buffer containing 0.05% Tween 20, and

then for 3x5 minutes at room temperature in TS buffer. Immunoreactive bands were then visualised by incubating with a buffer containing 10mM Tris/HCl, pH 7.5, 0.015% hydrogen peroxide and 0.06% *o*-dianosidine. The colour reaction was terminated by rinsing the blot twice in distilled water. For the majority of the experiments involving CS1, after incubation with the primary antisera and subsequent washing, the blot was incubated for 2 hours at room temperature with ^{125}I- labelled anti rabbit IgG, used at a concentration of 2-4μCi per 50 ml of TS buffer containing 1% dried milk. The blot was then washed for 2 x 10 minutes in TS buffer containing 0.05% Tween 20, before being dried, wrapped in Saran wrap, and exposed to Fuji RX X-ray film with "lightning fast" intensifying screens at -80°C for between 2 and 5 days.

The blots were analysed using a Quantimet Image Analyser 970 (Cambridge Instruments) by taking a number of spot densitometric readings along the width of the band which were then averaged to give the final measurement.

5. Assay of adenylate cyclase activity.

The activity of adenylate cyclase was essentially assayed as described in Salomon *et al.* (1974) with some variations.

5.1 Preparation of chromatography columns.

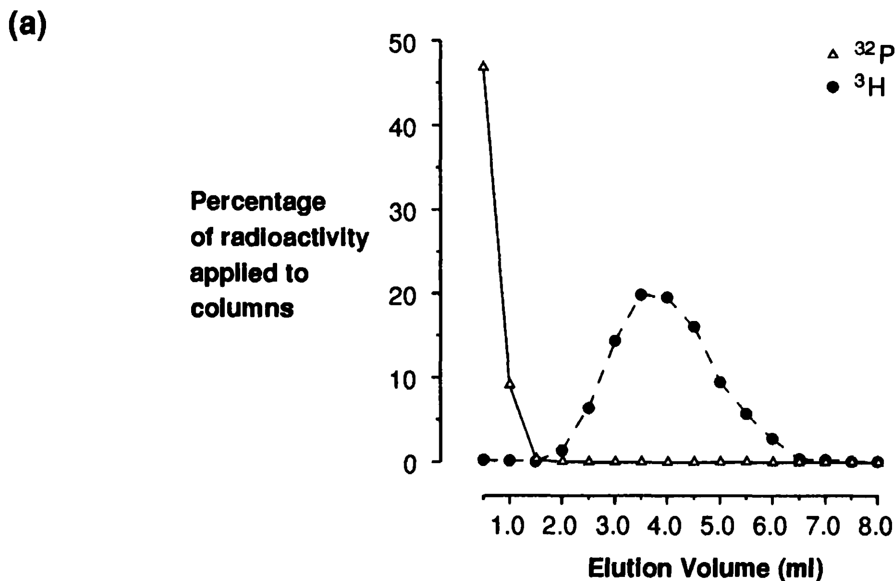
An equal volume of water and Dowex AG50W-X4 200-400 mesh resin was mixed for 10 minutes, the resin allowed to settle out and the water removed. This was repeated until the water which had been mixed with the resin remained clear. A further volume of water was added to the resin and, whilst stirring rapidly, 2ml of the mixture was pipetted into a Biorad polyprep column to give a bed volume of 1ml. The columns were then washed once with 4ml of 1M NaOH, then with 4ml of 1M HCl, and finally the column was stoppered and a further 4ml of 1M HCl added.

The alumina columns were prepared by mixing an equal volume of neutral chromatographic alumina and 0.1M imidazole/HCl, pH 7.3. While stirring rapidly, 2 ml of the mixture was pipetted into a Biorad polyprep column to give a bed volume of 1ml. The column was washed once with 4 ml of 0.1M imidazole/HCl, pH 7.3, before the column was stoppered and a further 4 ml of 0.1M imidazole/HCl, pH 7.3, added.

Since the columns were to be used to separate ATP from cAMP, it was necessary to determine the elution profiles of the columns. A tube was prepared which was processed as per an assay blank (See Section 5.2) except that no ^{3}H labelled cAMP was added. Another tube was prepared in a similar way except that no ^{32}P labelled ATP was added. The contents of each tube were mixed with 0.6ml of water and applied to separate Dowex resin columns. The first 0.6 ml of eluate was collected in a scintillation vial. A series of additions of 0.5ml of water was then applied to each column and the eluate collected. After scintillation counting, the elution profiles of the columns were plotted (Figure 3). From this it was decided to wash the Dowex resin columns with 2 ml of water to elute most of the ATP, then with

4 ml of water to elute the cAMP. The elution profiles of the alumina columns were derived by using the Dowex columns as determined above, and letting the 4ml water wash drip through onto, and through, the alumina columns. After this 0.5 ml volumes of 0.1M imidazole/HCl, pH 7.3, were applied to the alumina columns, and the eluate collected and the radioactivity in each fraction determined by scintillation counting. The elution profiles for these columns (Figure 3) showed that most of the cAMP was eluted after 1-5ml of 0.1M imidazole/HCl, pH 7.3, had been applied to the column. It was therefore decided to first wash the alumina columns with 1ml of 0.1M imidazole/HCl, pH 7.3, and then elute the cAMP with 4ml of 0.1M imidazole/HCl, pH 7.3.

(a)



(b)

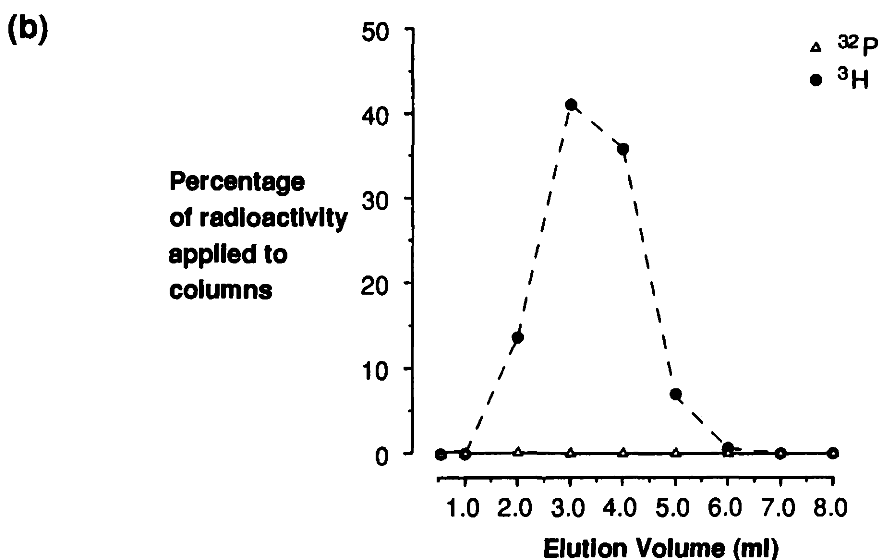


Figure 3.

Elution profiles of Dowex resin and alumina columns.

Both ^{32}P labelled ATP and ^{3}H labelled cAMP were applied to Dowex resin or alumina columns as described in the text. Dowex resin columns (a) were washed with 0.5ml volumes of water, while alumina columns (b) were washed with 0.5ml volumes of 0.1M Imadazole/HCl, pH 7.3. The eluate was collected and the amount of ^{32}P or ^{3}H it contained determined by liquid scintillation counting. The results are expressed as the percentage of the quantity of radioactivity originally applied to the columns.

5.2 Formation of ^{32}P labelled cAMP.

Aliquots of membrane protein preparations were diluted to 1mg protein/ml with 10mM Tris/HCl, pH 7.5, and kept on ice before use. Adenylate cyclase activity was assayed by incubating 20µg of membrane protein in 100µl of a buffer containing 100mM Tris/HCl, pH 7.5, 50mM sodium chloride, 5mM magnesium chloride, 1mM cAMP, 200µM ATP containing 1µCi of α-^{32}P ATP, 20mM creatine phosphate and 100U/ml of creatine phosphokinase. The incubations were carried out at 30°C for 15 minutes, the linearity of the reaction having been confirmed. In some cases adenylate cyclase was assayed in the presence of 10µM aluminium chloride and 5mM sodium fluoride. Two tubes per assay were incubated with no membrane protein added to act as assay blanks. The reaction was terminated by the addition of 150µl of stop solution containing 2% SDS, 40mM ATP, 1.3mM cAMP and approximately 30,000 cpm of ^{3}H labelled cAMP, before placing the tube in an ice bath. This was followed by boiling for 10 minutes. If required the assay tubes were frozen to -20°C and stored overnight. All preparations were assayed in duplicate.

5.3 Purification of ^{32}P labelled cAMP.

^{32}P labelled cAMP was purified from the much more abundant ^{32}P labelled ATP according to the method of Salomon (1974). The Dowex resin columns were prepared for use by draining them of the 4ml of 1M HCl that they had been stored with, washing with 10ml of water and finally with 4ml of water. The alumina columns were simply drained of the 4ml of 0.1M imidazole/HCl, pH 7.3, that they had been stored with. To each assay tube was added 0.6ml of water and the entire contents of each tube applied to Dowex resin columns. After this had run into the column, the column was washed with 2ml of water and the eluate discarded. The Dowex resin columns were then placed above the alumina columns, and 4ml of water applied to the Dowex resin columns. The eluate from the Dowex resin

columns was allowed to drip through the alumina columns, the eluate from the alumina columns being discarded. The alumina columns were then washed with 1ml of 0.1M imidazole/HCl, pH 7.3, and the eluate discarded. After this 4ml of 0.1M imidazole/HCl, pH 7.3, was added to the columns, the eluate being collected in scintillation vials filled with 16 ml of Ecoscint. The vials were shaken and counted, for four minutes per vial, in a liquid scintillation counter set to count ^{3}H and ^{32}P. The ^{3}H counts were used to monitor the recovery of cAMP from each column, this being used to correct the ^{32}P counts to give the total amount of ^{32}P labelled cAMP produced in the reaction. Column recoveries of cAMP were in the order of 70 to 80%. The activity of adenylate cyclase in pmols cAMP formed/mg protein/minute was calculated by the following expression :

$$\begin{array}{l}(^{32}P\text{ recovered} \\ \text{- assay blank) (cpm)}\end{array} \times \frac{^{3}H\text{ cAMP added (cpm)*}}{^{3}H\text{ recovered (cpm)}} \times \frac{20000\text{ pmols **}}{^{32}P\text{ added (cpm)***}}$$

$$\times \frac{1000}{\text{Protein }(\mu g)} \times \frac{1}{\text{Time (minutes)}}$$

* the amount of ^{3}H cAMP solution added after the incubation to monitor recovery.
** the 20000 pmols refers to the amount of ATP in each assay tube.
*** the total amount of ^{32}P labelled ATP added to each tube.

After each experiment the alumina columns were washed with 4ml of 0.1M imidazole/HCl, pH 7.3, stoppered and a further 4ml of 0.1M imidazole/HCl, pH 7.3 added. The Dowex resin columns were recycled by washing them with 4ml of 1M NaOH, then with 4ml of 1M HCl. Finally the columns were stoppered and a further 4ml of 1M HCl added.

6. Assaying of sodium potassium-dependent ATPase activity.

Sodium potassium-dependent ATPase activity was assayed according to the method of Esmann (1988) with minor variations. The basis of the assay is the coupling of the hydrolysis of ATP to form ADP plus Pi, to the oxidation of NADH, an event which can be followed spectrophotometrically. The membrane preparations were diluted to 1mg protein/ml with 10mM Tris/HCl, pH 7.5, and sodium deoxycholate was added to a final concentration of 0.2% and the mixture put on ice for 15 minutes. 1ml of a buffer containing 30mM histidine, 3mM ATP, 20mM potassium chloride, 130mM sodium chloride, 1mM phosphenolpyruvate, 0.2mM NADH, 10U/ml pyruvate kinase and 30U/ml lactate dehydrogenase was pre-incubated in a cuvette for 90 seconds at 37°C. The reaction was then started by addition of 20μg of membrane protein. After 15 minutes at 37°C, the A_{340nm} of the incubation mix was measured to determine the amount of NADH oxidised and hence the amount of Pi produced. The linearity of the reaction had been confirmed previously. The non-specific ATPase activity of each sample was measured in the presence of 1mM ouabain. Three cuvettes containing no membrane protein were processed per assay to act as blanks. All preparations were assayed in triplicate. The activity of sodium potassium-dependent ATPase in nmols Pi formed/mg protein/min was calculated as follows :

$$(A_{340nm}\text{ assay blank} - A_{340nm}\text{ after incubation}) \times \frac{1}{0.00622^{*}} \times \frac{1}{\text{Time (mins)}} \times \frac{1000}{\text{Protein }(\mu g)}$$

*the value of ε(NADH) - ε(NAD) in μM/cm or nmols/ml/cm. (The path length used was 1cm and the assay volume was 1ml).

7. Assay of choline acetyl transferase activity.

Choline acetyl transferase activity was assayed according to the method of Fonnum (1975) with minor modifications. Tissue samples were homogenised in 6 or 10 volumes of ice cold 10mM Tris/HCl, pH 7.5, 1mM EDTA and diluted 1:1 with 1% Triton X-100 in 20mM EDTA. The homogenates were kept on ice until ready to use. 10μl of each homogenate (containing 30-50μg of protein) was incubated for 20 minutes at 37°C in a buffer containing 300mM sodium chloride, 50mM sodium phosphate buffer, pH 7.4, 8mM choline bromide, 20mM EDTA, 0.1mM physostigimine and 0.2 mM acetyl CoA containing approximately 125,000 dpm of ^{14}C labelled acetyl CoA. Three tubes which contained no protein were processed in each assay to act as blanks. All samples were assayed in triplicate. The microtube was then opened by cutting the top off and both pieces were put into a scintillation vial containing 5ml of 50mM sodium phosphate buffer, pH 7.4. The vial was then shaken for 30 seconds and 2ml of acetonitrile containing 10mg of kalignost added. Finally 10ml of a toluene based scintillation mixture (POPOP) was added to each vial. The vial was then shaken lightly for one minute and the two layers left to separate for 10 minutes. This procedure extracted the ^{14}C acetyl choline formed during the incubation into the organic phase (which contains fluor), while leaving the acetyl CoA in the aqueous phase. The radioactivity in the organic phase was then determined by liquid scintillation counting. Choline acetyl transferase activity in nmols acetyl choline formed/mg protein/hour, was calculated using the following expression -

$$(^{14}C \text{ of sample} - \text{assay blanks})\ (\text{cpm}) \times \frac{(5 \text{ nmols} + \text{contribution from } ^{14}C \text{ ACoA})^{*}}{^{14}C \text{ added (cpm)}} \times \frac{60 \text{ mins}}{\text{Time (mins)}} \times \frac{1000}{\text{Protein } (\mu g)}$$

* the amount of acetyl CoA added to each assay tube

8. Assaying of protein concentrations.

Proteins were assayed according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

9. Purification of total RNA.

All apparatus and solutions used in the purification and analysis of RNA were treated to remove any RNase contamination as described in Sambrook *et al.* (1989) : glassware was oven baked at 200°C for 16 hours, or washed in chloroform before being autoclaved at 15lb/inch^2 for 20 minutes; plasticware was autoclaved for 20 minutes at 15lb/inch^2; aqueous solutions were treated, where appropriate with diethyl pyrocarbonate (DEPC) by adding DEPC to a concentration of 0.1%, shaking vigorously for 30 seconds, leaving the solution overnight and then autoclaving for 20 minutes at 15lb/inch^2; certain articles of apparatus e.g electrophoresis kits, were steeped in 3% hydrogen peroxide for 1 hour before being rinsed with DEPC treated water. In addition the water used to resuspend RNA was filtered through a sterile 0.2µm filter before being DEPC treated.

Total RNA was prepared, with minor variations, according to the method of Chomczynski & Sacchi (1987). Tissue which had been previously ground in liquid nitrogen was homogenised in 10 volumes of GIT buffer (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sodium sarcosine, 0.1M 2-mercaptoethanol; this solution was filtered through a 0.8µm filter before use) using a teflon-glass homogeniser. The homogenate was then transferred to a 15 or 30ml glass centrifuge tube (Corex). To this was added sequentially; 0.1 volumes (relative to the homogenate volume) of 2M sodium acetate, pH 4.0, 1 volume of water saturated phenol containing approximately 0.1% 8-hydroxyquinoline, and 0.2 volumes of a 49:1 chloroform, isoamyl alcohol mixture. The top of the centrifuge tube was sealed with para film, and the mixture shaken vigorously for 2 minutes

before being left on ice for 15 minutes. It was then centrifuged at $3000g_{av}$ for 30 minutes at 4°C in a Mistral 5L centrifuge, or in some experiments at $10,000g_{av}$ for 20 minutes at 4°C in a Sorvall superspeed centrifuge. The upper aqueous phase was removed and mixed with an equal volume of cold (-20°C) isopropanol. This was then placed at -20°C overnight to precipitate RNA. The RNA was sedimented by centrifugation at $3000g_{av}$ for 30 minutes at 4°C, or at $10,000g_{av}$ for 15 minutes at 4°C, and the resulting pellet was resuspended in 3ml of GIT buffer and reprecipitated using isopropanol at -20°C for 1 hour. After centrifugation, the pellet was washed once with cold 70% ethanol before being dried in a vacuum dessicator. The RNA was then resuspended in DEPC treated water, and the RNA concentration estimated by measuring the OD_{260} of the solution in triplicate (1OD unit = 40μg RNA/ ml; Sambrook *et al.*, 1989). The purity of the RNA was assessed by measuring the OD_{260}/OD_{280} ratio of the solution. This purification procedure usually gave a ratio of above 1.90 and in all cases above 1.85. Pure RNA should give a ratio of 2.0 (Sambrook *et al.*, 1989).

10. Northern blotting.

10.1 Generation of oligonucleotide probes.

Oligonucleotides were synthesised using an Applied Biosystems DNA synthesiser, and are described fully in Table 4. Those which hybridise to G protein mRNA were designed by Prof. J.T. Knowler, Department of Biological Sciences, Glasgow Polytechnic. In summary, oligonucleotides were made with unique complementary to all four forms of rat Gsα mRNA, the two large forms of human Gsα mRNA, human $G\beta_1$ and $G\beta_2$ mRNA, rat Gi1 mRNA, rat Gi2 mRNA, human Gi1 mRNA, human Gi2 mRNA and human 28S rRNA. Oligonucleotides were 5' end labelled with γ-^{32}P labelled ATP by incubating 100ng of oligonucleotide in a buffer (final volume 50μl) containing 10mM magnesium chloride, 100mM Tris/HCl, pH 7.6, 20mM 2-mercaptoethanol, 100μCi γ-^{32}P ATP and 10 units T4 polynucleotide kinase.

Incorporation of ^{32}P into the oligonucleotide was monitored by thin layer chromatography on PEI cellulose plates using 0.5M potassium phosphate buffer, pH 3.5, as a running buffer. The plate was exposed to X-ray film to determine the position of the oligonucleotide, ATP and Pi. The plate was then cut up and Cherenekov counted to calculate the percentage incorporation of ^{32}P into the oligonucleotide. This was typically between 50% and 70%.

Table 4.

The oligonucleotides utilised in northern blotting.

The sequences of the oligonucleotides are shown in the 5′ to 3′ direction. The amino acid sequence numbers represent the amino acids encoded by the mRNA to which the oligonucleotides are complimentary.

* = the sequence given refers to the nucleotide number. RM = number of mismatches between oligonucleotide sequence and sequence in rat. HM = number of mismatches between oligonucleotide sequence and sequence in human. NK = not known.

Oligo	Sequence	Amino acid sequence	RM	HM	References
Gs	CTCATCCTCCCACAGAGCCTGGCATGCTCATA	Rat Gsα 147-157	0	1	Jones and Reed (1987)
Gs-52	ATCGCTGTTGCTCCTTGCAGCCTGCGGGTCCTC	Rat Gsα 75-85	0	0	Bray *et al.* (1986)
Rat Gi1	GCTGTCCTTCCACAGTCTCTTTATGACGCCGGC	Rat Gi1α 124-135	0	7	Jones and Reed (1987)
Rat Gi2	ATGGTCAGCCCAGAGCCTCCGGATGACGCCCGA	Rat Gi2α 125-135	0	1	Jones and Reed (1987)
Human Gi1	ACTATCTTTCCACAATCTCTTTATAACTCCAGC	Human Gi1α 124-135	7	0	Bray *et al.* (1987)
Human Gi2	ATGGTCAGCCCAGAGCCTCCGGATGACGCCGGA	Human Gi2α 125-135	1	0	Didsbury *et al.* (1987)
Human Gβ	TGGACCTTGTTGGTGGTGTAGCTGTCCCA	Human Gβ 82-91	NK	0	Fong *et al.* (1986)
					Fong *et al.* (1987)
28S rRNA	AACGATCAGAGTAGTGGTATTTCACC	Human 28S 4010-4035*	1	0	Barbu and Dautry (1990)

10.2 Electrophoresis and blotting of RNA.

The deionised formamide used in the following procedures was produced by mixing formamide with mixed bed resin (AG501-X8 (20-50 mesh)) at 10ml formamide per gram of resin for 30 minutes. The formamide was then filtered to remove the resin, and stored at -20°C.

RNA (usually 20μg) was denatured at 65°C for 5 minutes in a buffer (final volume 25μl) containing 2M formaldehyde, 50% deionised formamide (v/v) and 1xMOPS (1xMOPS = 20mM MOPS, 5mM sodium acetate, 1mM EDTA pH 8.0). To each tube was added 5μl of loading buffer containing 50% glycerol and 0.4% bromophenol blue. This was then applied to a 1% agarose gel, of dimensions 1.2 cm deep and 14cm long, containing 2.2M formaldehyde and 1xMOPS buffer. The gel was run overnight at a constant voltage of 24V, using 1xMOPS as an electrophoresis buffer. In some cases RNA size markers were run in one lane per gel. After electrophoresis, RNA was transferred onto nylon membranes by capillary blotting according to Sambrook *et al.* (1989). The gel was inverted and placed on a piece of 3MM paper (Whatmann), the ends of which had been dipped into a reservoir of 20xSSC (1xSSC = 150mM sodium chloride, 15mM sodium citrate pH 7.0). A piece of Hybond N or Hybond N+ was placed on top of the gel, and covered with two pieces of 3MM paper. On top of these was placed a 5cm pile of paper napkins. The whole sandwich was compressed with a 1kg weight, and transfer allowed to continue for 6 hours, with frequent changing of the paper napkins. After transfer, the RNA was fixed to the nylon membrane by exposing to short wavelength UV light for 1 minute (Hybond N), or placing the blot onto 3 pieces of 3MM paper soaked in 0.05M sodium hydroxide, leaving for 5 minutes and rinsing in 2xSSC (Hybond N+).

10.3 Detection of specific RNA species.

For the detection of Gsα, Gi1, Gi2 or Gβ mRNA the blots were prehybridised at 42°C for 4-6 hours in hybridisation solution (5xSSPE, (1xSSPE = 150mM sodium chloride, 10mM sodium dihydrogen phosphate, 1mM EDTA, pH 7.4), 30% deionised formamide (v/v), 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% denatured

salmon testes DNA and 0.1% SDS) and then hybridised overnight in hybridisation buffer containing 3-5x10^6 cpm/ml of the appropriate labelled oligonucleotide. After hybridisation, the blots were rinsed twice in 2xSSPE, 0.1% SDS at 42°C and then washed in 0.5xSSC, 0.1% SDS at 60°C for 15 minutes. The 28S rRNA oligonucleotide was used in a similar way except that prehybridisation and hybridisation took place in a solution containing 4xSSPE, 0.1% sodium pyrophophate, 0.2% SDS and 0.05% denatured salmon testes DNA. The blot was rinsed twice in 2xSSPE, 0.1% SDS, 0.1% sodium pyrophosphate, and then washed in the same solution for 30 minutes at 37°C. This oligonucleotide was washed to a lower stringency than the G protein oligonucleotides due to its lower GC content and smaller size.

The location of bound oligonucleotide was visualised by exposure to Fuji RX X-ray film using "lightning plus" intensifying screens at -80°C. Blots which had been probed to show G protein mRNAs were exposed for 1 to 3 days, while those probed to show 28S rRNA were exposed for 5 to 20 minutes.

Densitometric analysis of the autoradiograms was carried out using a Quantimet Image analyser 970 (Cambridge Instruments). Spot densitometric readings were taken along the length of the band. The optical density reported is the highest OD within the length of the band. This is analogous to using peak heights on a scanning densitometer.

After hybridisation and autoradiography, total RNA was stained on the blots with methylene blue to show the position of 28S and 18S rRNA. The blot first immersed in 5% (v/v) acetic acid for 15 minutes, and then stained in a solution containing 500mM sodium acetate, pH 5.5, and 0.04% methylene blue, for 3 minutes. The blot was then washed in water until the background was destained.

The use of nylon membranes allowed the oligonucleotide probes to be stripped from the blot and the blot rehybridised to another probe. This was accomplished by immersing the blot in a solution of boiling 0.1% SDS and allowing it to cool to room temperature. Autoradiography was then used to check that the probe had been removed.

11. Mid cerebral artery occlusion with 30 minutes of hypotension.

The following procedures were carried out by Dr. Kasumoto Kuramaji at the Wellcome Surgical Institute.

11.1 Preparation of animal.

The experiments were carried out on male Sprague-Dawley rats of body weight 250-300g. Anaesthesia was induced by placing the rat in a perspex box into which was fed 5% halothane in 30% O_2 and 70% N_2O. The animal was moved to an operating table where anaesthesia was maintained with 2% halothane in 30% O_2 and 70% N_2O delivered via a funnel placed over the animal's nose. The trachea was exposed and a tracheotomy tube inserted. Respiration was maintained using a Palmert small animal positive pressure respirator pump delivering 0.5% halothane, 30% O_2, and 70% N_2O via the tracheotomy. Peripheral body temperature was recorded continuously by a rectal thermometer. Two femoral arteries and one femoral vein were cannulated with polyethylene catheters (internal diameter 0.58mm, external diameter 0.96mm). The arterial lines were used to obtain continuous, direct arterial blood pressure measurements using a Gould blood pressure transducer. They also served to withdraw arterial blood to induce haemorrhagic hypotension or perform intermittent arterial blood gas analysis using a Corning 168 pH/blood gas analyser. The venous line was used to deliver blood to return the animals blood pressure to normal after the period of hypotension.

11.2 Middle cerebral artery occlusion.

The occlusion of the middle cerebral artery was carried out exactly as described in Tamura *et al.* (1981). Briefly the left mid cerebral artery (MCA) was exposed by a small cranioectomy using a saline cooled dental drill. The skull was opened close to the foramen ovale. After the dura was opened with a fine needle, the middle cerebral artery was occluded by

thermocoagulating and severing it between its cortical branch to the rhinal cortex and the lateral striate arteries. The cranioectomy was then covered by a small piece of gel foam. Sham operated animals were treated identically except that no MCA occlusion was performed.

11.3 Hypotension.

The blood pressure of the animal was lowered to a target value of 55mm Hg for 15 minutes after MCA occlusion, and lasted for a period of 30 minutes. This was performed as described in Osborne (1987). By continuous reference to the blood pressure trace (set on average) the animals blood pressure was lowered to the target value by withdrawing blood from the arterial line into a heparinised syringe. After transferring the syringe to the venous line and attaching another syringe to the arterial line, the blood pressure was maintained by withdrawal and administration of blood via the arterial and venous lines respectively. At the end of the thirty minute period, the blood pressure was restored to normal by slow reperfusion of blood through the venous line. Three hours after MCA occlusion the animal was killed by decapitation and the brain carefully removed. The region of the cortex fed by the middle cerebral artery (as described in Yamori *et al.* (1976)) was dissected from the left and right hemispheres, frozen in liquid nitrogen and stored at -80°C. The length of time between decapitation and freezing of the brain tissue was no more than 10 minutes.

12. Statistical methods.

The mean values of a variable measured in two groups were compared using a two tailed, unpaired Students test using pooled variance as described in Milton & Tsokos (1983). Each dataset was checked for any evidence of a non-normal distribution using a goodness of fit test (Milton & Tsokos, 1983). These two tests were usually carried out with the aid of the statistical package "Statworks" running on an Apple Macintosh microcomputer. In addition the assumption that the variances of both populations were equal was checked using a "F-test" (Milton & Tsokos, 1983) to compare sample standard deviations. The statistical significance of the result of a the test was either determined automatically by the Statworks program, or derived using Neave's (1983) tables. One way analysis of variance was accomplished manually as described in (Milton & Tsokos, 1983).

The correlation between two variables was investigated using linear regressional analysis as described in Milton & Tsokos (1983). The statistical significance of the calculated regression coefficient was determined using Neave's (1983) tables.

The percentage difference between the mean value of two data sets, each possessing any given value of standard deviation, which was required to give a statistically significant result using a Students t-test was calculated as follows. A computer program was written that calculated the value of the t-statistic using the two datasets. If this was not statistically significant the mean value of one of the groups was changed by a small amount. The standard deviation of the data was also altered, such that the standard deviation as a proportion of the mean (the coefficient of variation) remained constant. The t-statistic was then recalculated. The program repeated this procedure until the value of the t-statistic became statistically significant, whereupon the percentage difference between the two means was reported. In this way the effect of different degrees of intersubject variability upon the likelihood of obtaining a statistically significant difference between two groups of data could be investigated.

III. RESULTS

1. Characterisation of the procedures used to analyse human brain tissue.

The analysis of transduction systems in human brain required the use of various biochemical techniques. These procedures have previously been applied mainly in the investigation of animal and cell line systems. It was necessary therefore to check that the assays that were to be used would function correctly when used to analyse human brain tissue.

1.1 Western Blotting.

The measurement of the relative amounts of the α subunits of Gs, Gi and Go was accomplished using western blotting utilising specific antisera. The antisera used were CS1 which detects Gsα, OC1 which detects Goα, SG2 which detects Giα (see "Methods").

The procedure that was employed to quantitate the relative amount of G proteins present on the western blots, was to measure the optical density of the G protein bands. For this to be successful it was necessary to ensure that a linear relationship exists between the amount of G protein present on the blot, and the optical density of the detected band. This was accomplished by loading an increasing quantity of membrane protein onto a gel, measuring the optical density of the resulting band(s), and determining over what range the two values are directly proportional to each other. Membranes prepared from rat brain were used to test antisera OC1 and SG2 due to the large amount of protein required for this procedure and the relative scarcity of the human tissue. Figure 4 shows a western blot of rat brain membrane proteins incubated with antisera OC1, the resulting immunocomplex being detected with a horse radish peroxidase (HRP) linked secondary antibody. The antisera detected one band, corresponding to Goα, which possessed an approximate molecular weight of 40kD. The optical density is directly proportional to the quantity of protein which was

loaded onto the gel, within the range 0 to 150μg of protein. In all subsequent blots 50μg of protein was used. The procedure was also carried out for antisera SG2 (Figure 4), which detects two bands corresponding to two forms of Giα, Gi1 and Gi2, having approximate molecular weights of 39 and 37kDa respectively. This antisera also gave a linear response up to 150μg of protein loaded for both Gi1 and Gi2; 50 or 100μg of protein was used in subsequent experiments. Antisera SG2 also detected higher molecular weight proteins, which did not correspond to any known G protein species. However these were not present on all blots.

The use of antisera CS1, which detects Gsα, proved to be more problematical. The detection method used for OC1 and SG2, namely a secondary anti-rabbit immunoglobulin antisera which had been linked to HRP, was too insensitive to detect Gsα protein in brain. When the antisera was used at a dilution of 1/200, even loading 400μg of membrane protein onto the gel proved to be inadequate (data not shown). A more sensitive method was tried which employed a biotinylated secondary antibody with subsequent application of streptavidin-linked HRP. This procedure allowed the detection of bands which were the size of Gsα, but had the disadvantage that many other bands were also visible on the blot (data not shown). Another detection system was tried which used a ^{125}I labelled anti-rabbit immunoglobulin antibody. When used with CS1 as the primary antibody, two major bands were visible on the resulting autoradiogram corresponding to the two forms of Gsα detected by CS1, GsH and GsL, possessing approximate molecular weights of 45 and 42 kDa respectively (Figure 5). The antisera gave a linear response up to 100μg of protein loaded onto the gel for both GsH and GsL; 50μg protein was used in subsequent experiments. A further three faint bands were also visible at molecular weights which do not correspond to any known variants of Gsα. They were not detected when the blot was incubated with secondary antibody alone, and are therefore not due to the detection system employed (data not

shown). In addition these bands were present on some blots but not on others. However even when present, the higher molecular weight bands were barely discernible at the protein loadings which were routinely used.

To confirm that the same bands were being detected in both rat and human brain, membrane proteins prepared from both species were analysed by western blotting using the antisera OC1, SG2 and CS1 (Figure 6). The resulting blots show bands of the same molecular weight in rat and human brain, the only difference being that the band corresponding to GsH is wider in membrane proteins prepared from human brain, than from those prepared from rat brain. In addition the relative abundance of GsL compared to GsH is very low in membranes prepared from whole rat brain.

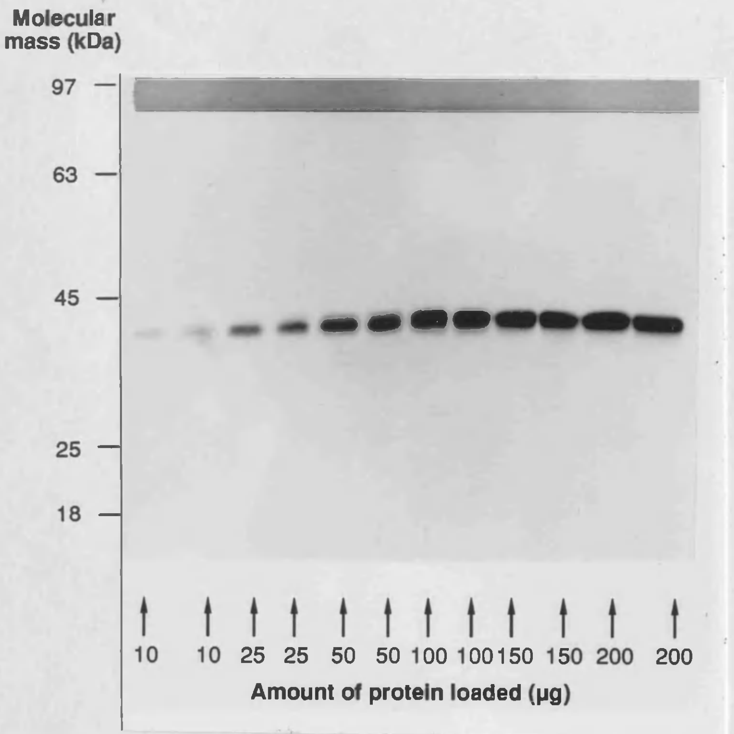
Figure 4.

Relationship between the amount of protein loaded onto gels and the intensity of the Goα band detected with antisera OC1, and the Gi1 and Gi2 bands detected with antisera SG2.

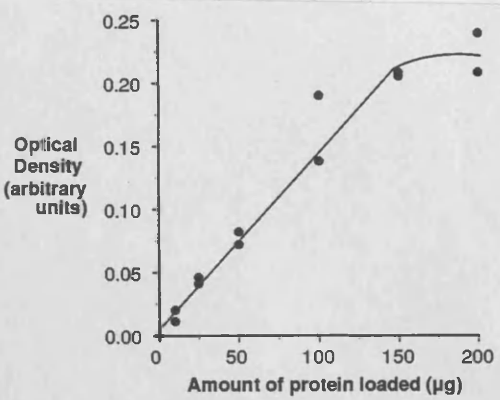
Crude membrane protein extracts were prepared from whole rat brain and analysed by western blotting as described in "Methods". A polyacrylamide gel was run overnight with each gel lane having been loaded with between 10 and 200μg of protein. After transfer of the proteins onto a nitrocellulose membrane, the blot was incubated with the antisera OC1, and then with HRP linked anti-rabbit IgG antisera. After development, a single band was visible on the blot (a), which was then analysed by densitometry (b) to show the relationship between the amount of protein loaded and the optical density of the resulting Goα band. A second polyacrylamide gel was loaded with between 25 and 250μg of protein per lane, run overnight and the proteins transferred onto a nitrocellulose membrane. The blot was incubated with the antisera SG2, and then with HRP linked anti-rabbit IgG antisera. After development, the blot (c) shows two bands which were then analysed by densitometry (d), to show the relationship between the amount of protein loaded and the optical density of the resulting Giα bands.

Molecular weight markers, run along with the membrane preparations, are shown alongside each blot.

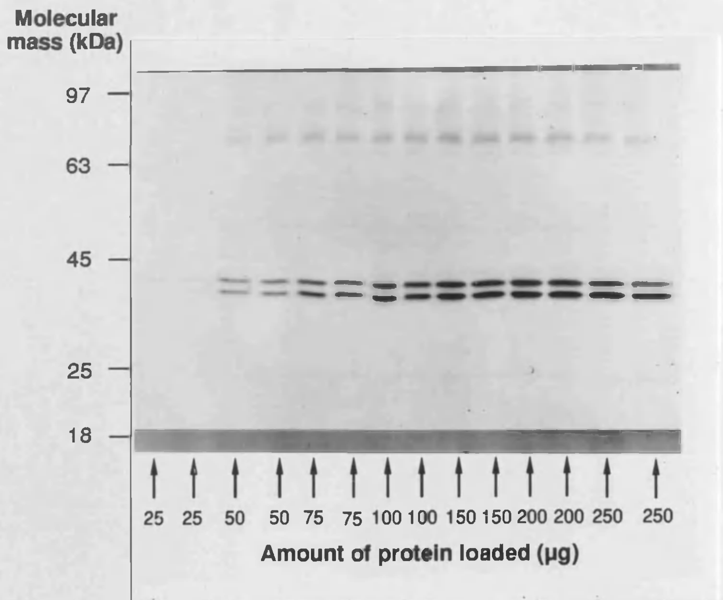
(a)



(b)



(c)



(d)

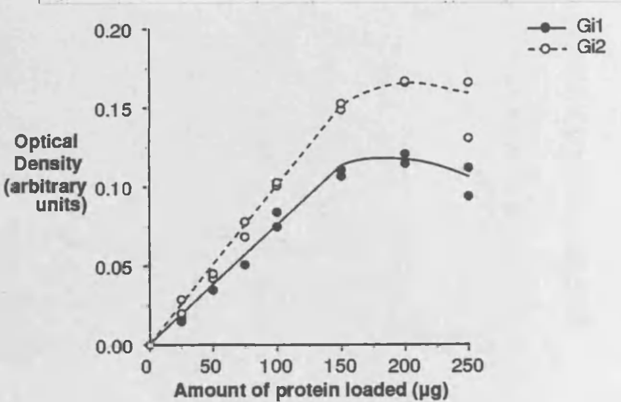


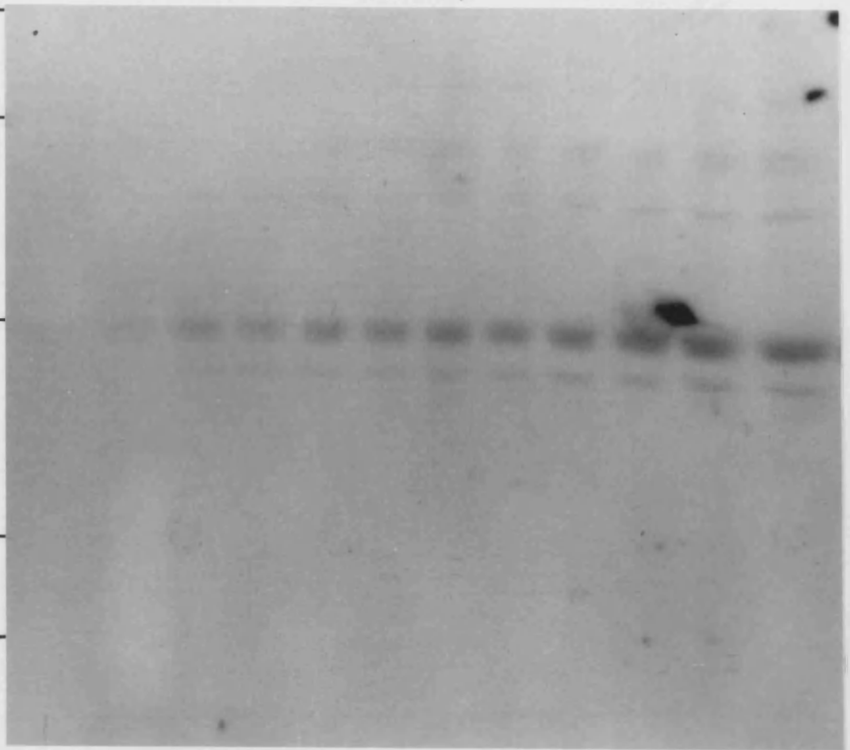
Figure 5.

Relationship between the amount of protein loaded onto the gel and the intensity of the GsH and GsL bands detected with antisera CS1.

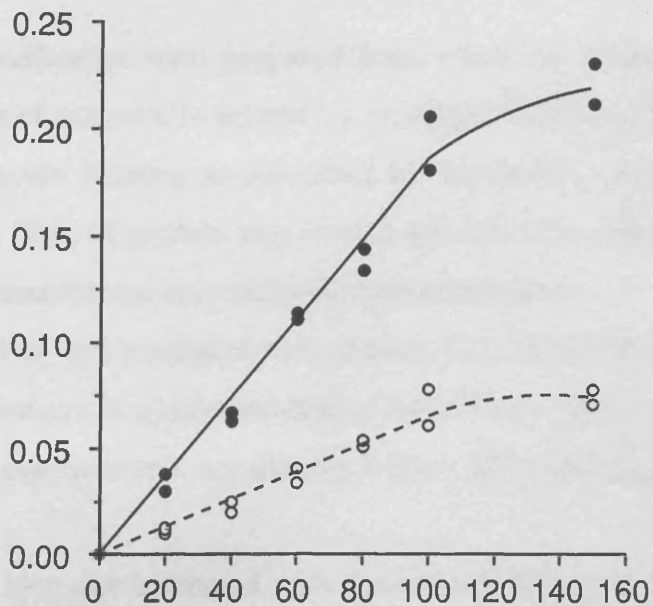
Crude membrane protein extracts were prepared from the right frontal cortex of subject C8 and analysed by western blotting as described in "Methods". The amount of protein loaded onto the polyacrylamide gel ranged from 20 to 150μg. The gel was run overnight and the proteins transferred onto a nitrocellulose membrane. The blot was incubated with antisera CS1, and then with ^{125}I linked anti-rabbit IgG antisera at a concentration of 3μCi/50ml. The immunocomplex was detected by exposure to X-ray film for 2 days. The resulting autoradiogram (a) shows two bands of the correct molecular weight, which were then analysed by densitometry (b) to show the relationship between the amount of protein loaded and the optical density of the Gsα bands.

Molecular weight markers, run along with the membrane preparations, are shown alongside the autoradiogram.

(a)



(b)



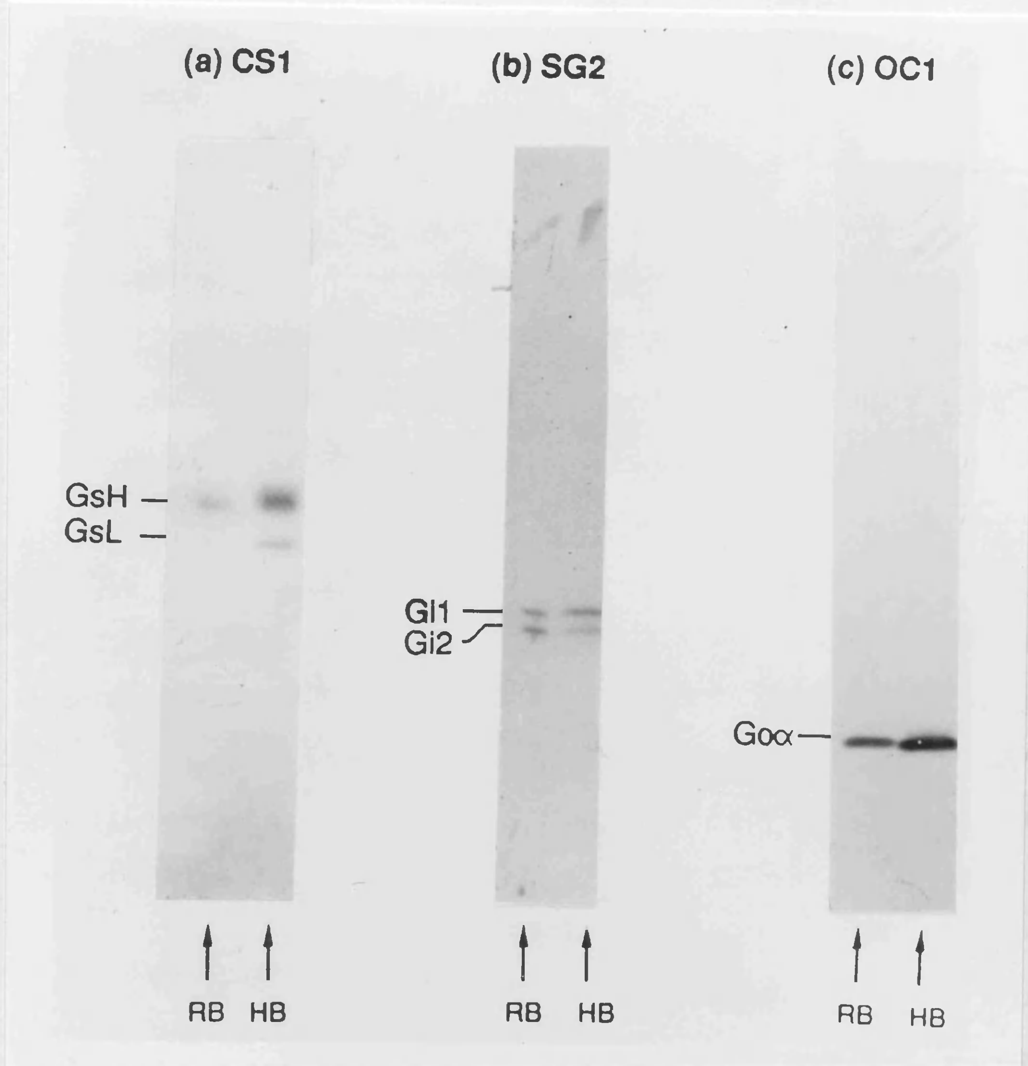


Figure 6.

The detection of Gsα, Giα, and Goα proteins in human and rat brain.

Crude membranes were prepared from whole rat brain and from the left frontal cortex of subject C14 (a) and (c), or subject C20 (b). These were then analysed by western blotting as described in "Methods". For both rat and human samples, 50μg of protein was loaded per lane, the gels run overnight and the proteins transferred onto nitrocellulose membranes.

(a). One blot was incubated with antisera CS1, and then with ^{125}I linked anti-rabbit IgG antisera at a concentration of 3μCi/50ml. The immunocomplex was detected by exposure to X-ray film for 3 days. RB = rat brain, HB = human brain.

(b). This blot was incubated with the antisera SG2, and then with HRP linked anti-rabbit IgG antisera.

(c). The blot was incubated with the antisera OC1, and then with HRP linked anti-rabbit IgG antisera.

1.2 Adenylate Cyclase assay.

Adenylate cyclase activity was assayed by monitoring the formation of ^{32}P labelled cAMP using α-^{32}P labelled ATP as a substrate, and then separating the cAMP from the ATP using chromatography. The enzyme was assayed both under basal conditions, and stimulated in the presence of sodium fluoride and aluminium chloride (fluoroaluminate). To determine for how long the enzyme maintained a constant rate of reaction, and hence the number of samples that could be processed in one batch, a time course experiment was carried out using human membranes as the source of adenylate cyclase (Figure 7). It was evident that for both stimulated and basal activities that the initial rate of reaction was maintained for 20 minutes. In all subsequent assays the reaction was allowed to proceed for between 15 and 17 minutes. Given that up to four assay tubes may be processed per minute, this allowed up to 68 tubes to be processed in one batch. This number was sufficient to allow all assays to be carried out in a single batch, thereby removing the possibility of inter-batch variation. Another experiment was performed to confirm that the amount of cAMP produced in the assay was directly proportional to the quantity of adenylate cyclase present. Figure 7 shows this to be true for both basal and stimulated activities up to at least 60μg of protein assayed. In addition the extent of stimulation by fluoroaluminate is independent of the amount of protein added to the assay (Figure 7).

The degree of stimulation of adenylate cyclase, when it is incubated in the presence of fluoroaluminate, is mainly dependent on the sodium fluoride concentration. Only small amounts of aluminium are required for stimulation to occur (Inoue *et al.* 1990), and in all assays performed the aluminium concentration was kept constant at 10μM. In both rat and human brain sodium fluoride caused an increase in the activity of adenylate cyclase (Figure 8), with maximal activity occurring at 5mM sodium fluoride after which stimulation decreased. This response to sodium fluoride is the same as that reported elsewhere (Inoue *et al.*, 1990). A sodium fluoride concentration of 5mM was used in all subsequent assays.

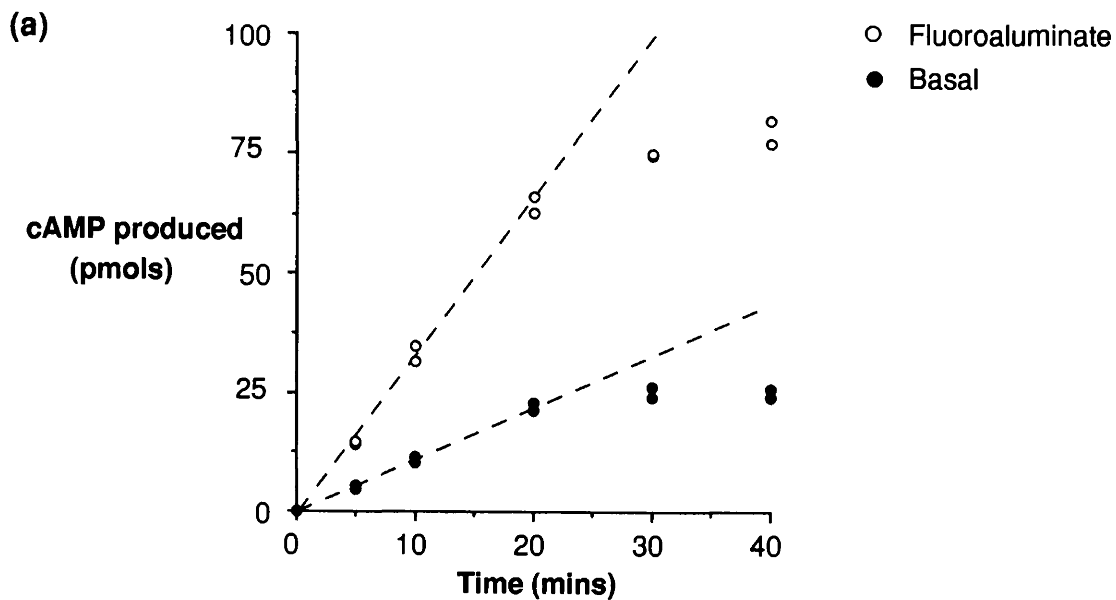
Figure 7.

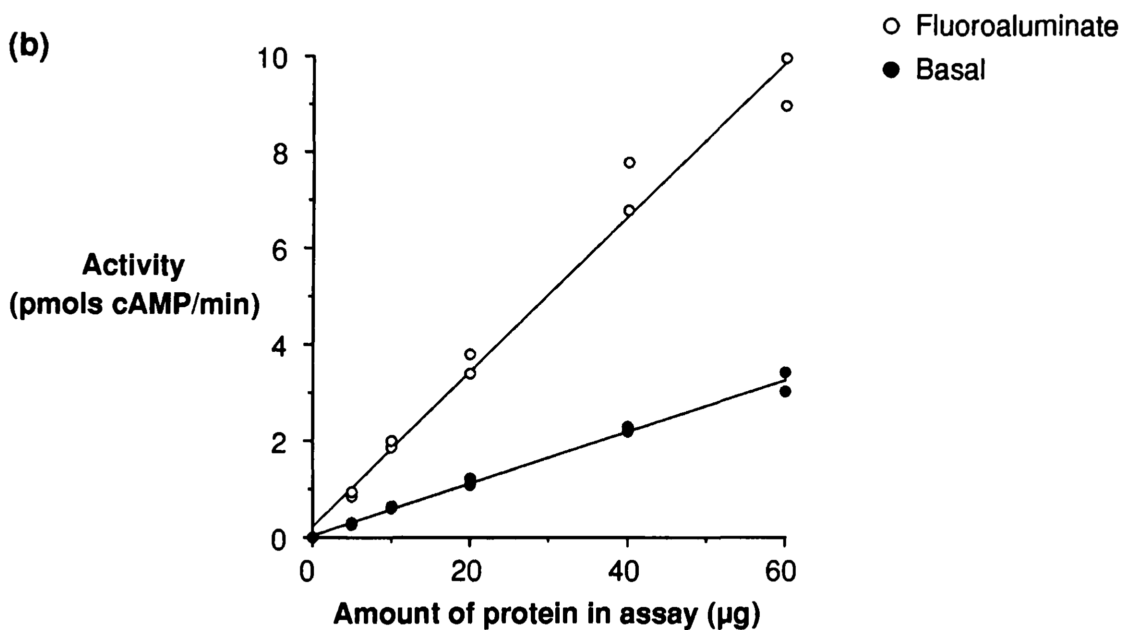
The effect of time and protein concentration on adenylate cyclase activity in human brain.

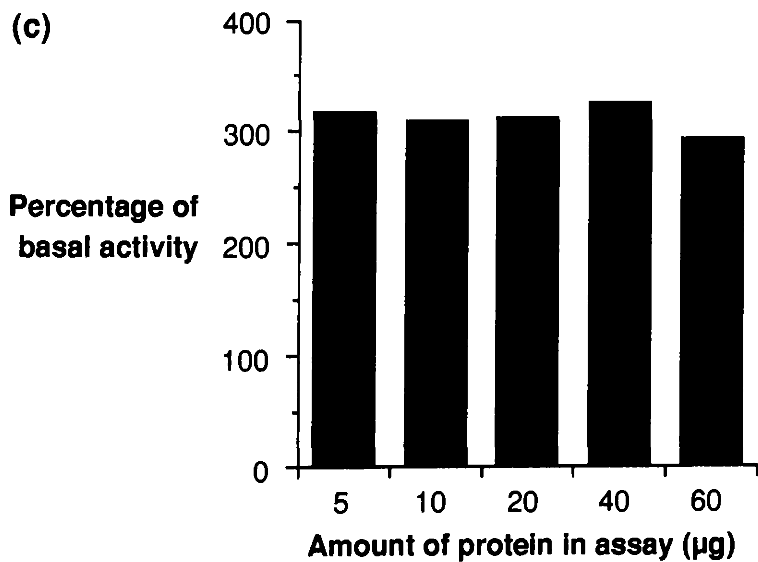
Crude membranes were prepared from the left frontal cortex of subject C14 and assayed for adenylate cyclase activity under the conditions described in "Methods".

(a). Reactions were started by the addition of 20μg of membrane protein and terminated after 5, 10, 20, 30 or 40 minutes at 30°C. The enzyme was assayed under basal conditions and in the presence of 10μM aluminium chloride and 5mM sodium fluoride (fluoroaluminate). The amount of cAMP formed during this period was calculated and and plotted against time, for both basal and fluoroaluminate stimulated activity. The dotted line demonstrates that the reaction rate is constant for the first 20 minutes of the assay.

(b) and (c). Reactions were started by the addition of 5, 10, 20, 40 or 60μg of membrane protein and terminated after 15 minutes at 30°C. The rate of cAMP formation is shown plotted versus protein (b). For both basal and fluoroaluminate stimulated activities a "best fit" line calculated by linear regressional analysis is shown. This give a regression coefficient of 0.998 for basal activity and 0.987 for stimulated activity. Stimulated adenylate cyclase activity expressed as a percentage of basal activity is also shown (c).


(a)
Fluoroaluminate
Basal
cAMP produced
(pmols)
100
75
50
25
0
0
10
20
30
40
Time (mins)


(b)
Fluoroaluminate
Basal
Activity
(pmols cAMP/min)
10
8
6
4
2
0
0
20
40
60
Amount of protein in assay (µg)


(c)
Percentage of
basal activity
400
300
200
100
0
5
10
20
40
60
Amount of protein in assay (µg)

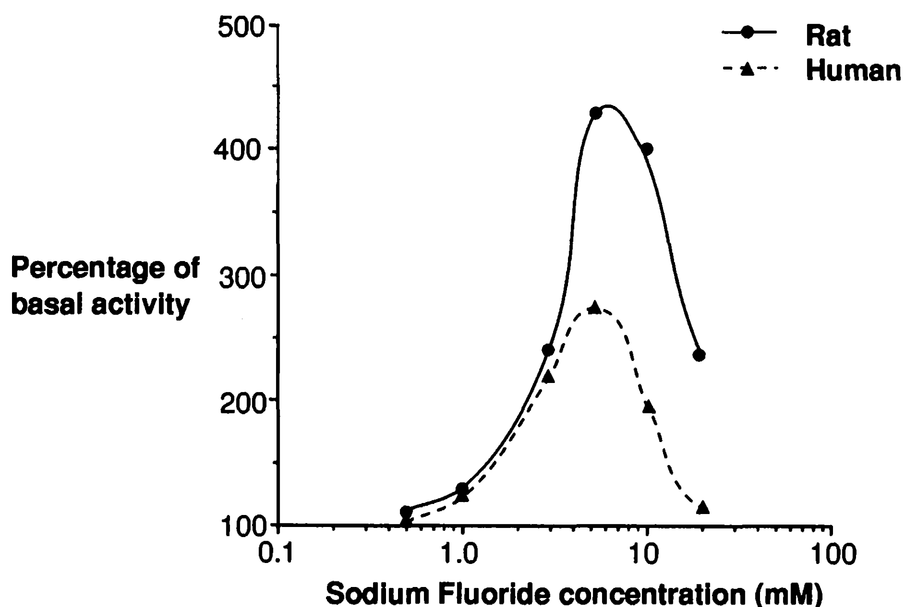


Figure 8.

The effect of sodium fluoride concentration on adenylate cyclase activity in human and rat brain.

Membrane preparations were prepared from whole rat brain and from the left frontal cortex of human subject C14, and assayed for adenylate cyclase activity as described in "Methods". Reactions were carried out at 30°C for 15 minutes. Each preparation was assayed in the presence of 10μM aluminium chloride along with a range of sodium fluoride concentrations. The enzyme activity is expressed as a percentage of the basal activity which was determined in the same experiment. Basal activity was 61 pmols cAMP/min/mg protein for human brain, and 52 pmols cAMP/min/ mg protein for rat brain.

1.3 Sodium potassium-dependent ATPase.

The hydrolysis of ATP by sodium potassium dependent ATPase was assayed by coupling the formation of ADP by the enzyme to the oxidation of NADH, an event which can be followed spectrophotometrically. The assay as described in Esmann (1988) calls for continual monitoring of the A_{340nm} of the assay mix during the reaction. However this method of working is somewhat impractical when large numbers of samples are to be assayed. The feasibility of measuring the A_{340nm} of the assay mix after a fixed time interval was therefore considered. For this to be possible the initial rate of reaction must be maintained for a reasonable length of time. Figure 9 shows the time course of the reaction for various concentrations of membrane protein present in the assay. The reaction proceeds at a constant rate until approximately 200 pmols of Pi have been formed after which Pi accumulation appears to stop. When lower amounts of protein are used there is no change in the reaction rate over 30 minutes. The reaction stops when 200 nmols of Pi have been formed due to the supply of NADH being exhausted. At this point the A_{340nm} of the assay mix is approximately 0.1. Based on the data illustrated in Figure 9, assays were routinely performed using 20µg of membrane protein over a 15 minute time course. However they were repeated using half the quantity of protein if the A_{340nm} of the incubation mix had fallen below 0.2.

As well as sodium potassium-dependent ATPase there are other ATPases present in the membrane preparations. Since ouabain is a specific inhibitor of sodium potassium-dependent ATPase, the contribution of other non-specific ATPases can be measured by assaying ATPase activity in the presence of ouabain. The effect of ouabain concentration on ATPase activity in rat and human brain is shown in Figure 9. Both species show a similar sensitivity to ouabain, with maximal inhibition occurring between 0.1 and 1mM. The latter concentration was routinely used to measure non-specific ATPase activity.

Figure 9.

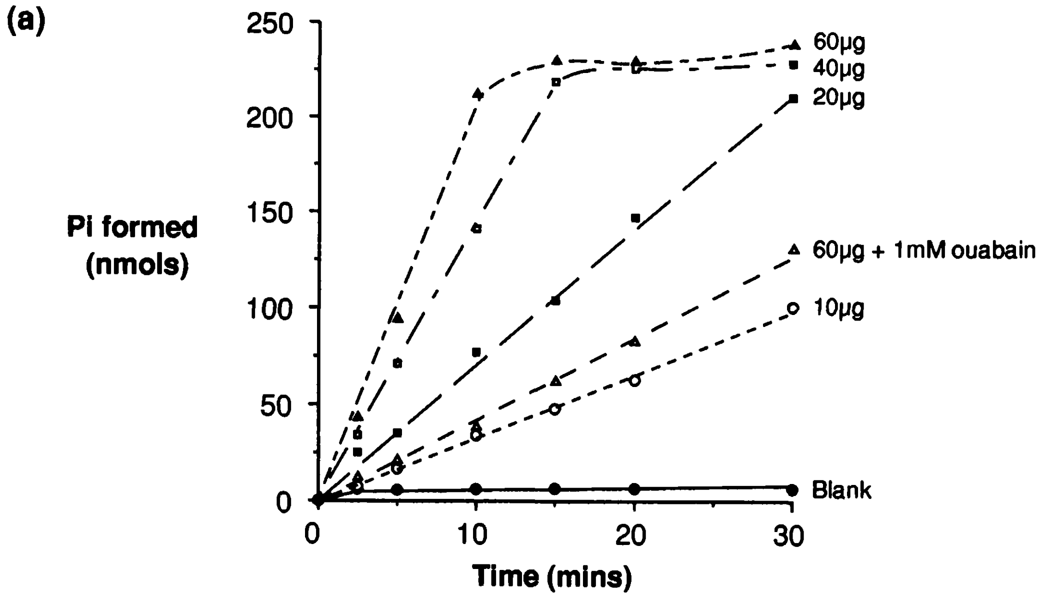
The effect of time, protein concentration and ouabain concentration on sodium potassium dependent ATPase activity in brain.

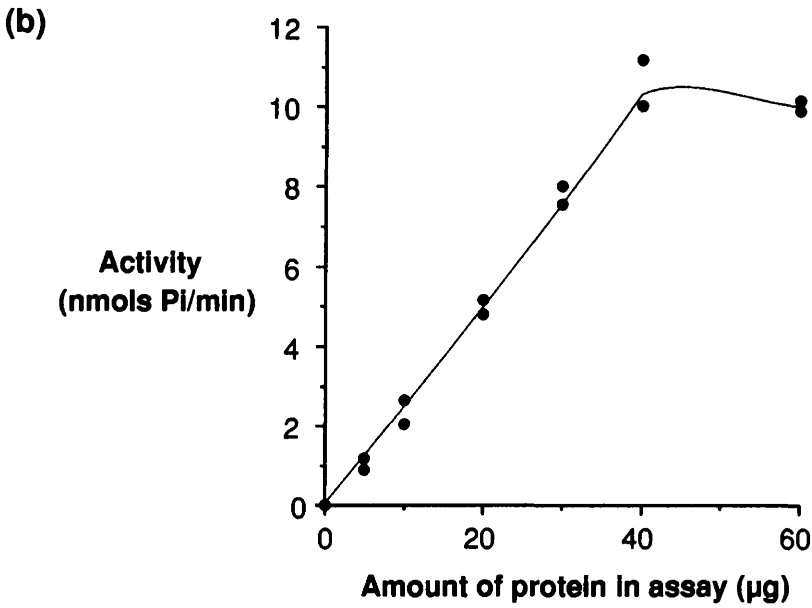
A crude membrane preparation was prepared from the left frontal cortex of subject C19A and assayed for sodium potassium dependent ATPase activity under the conditions described in "Methods".

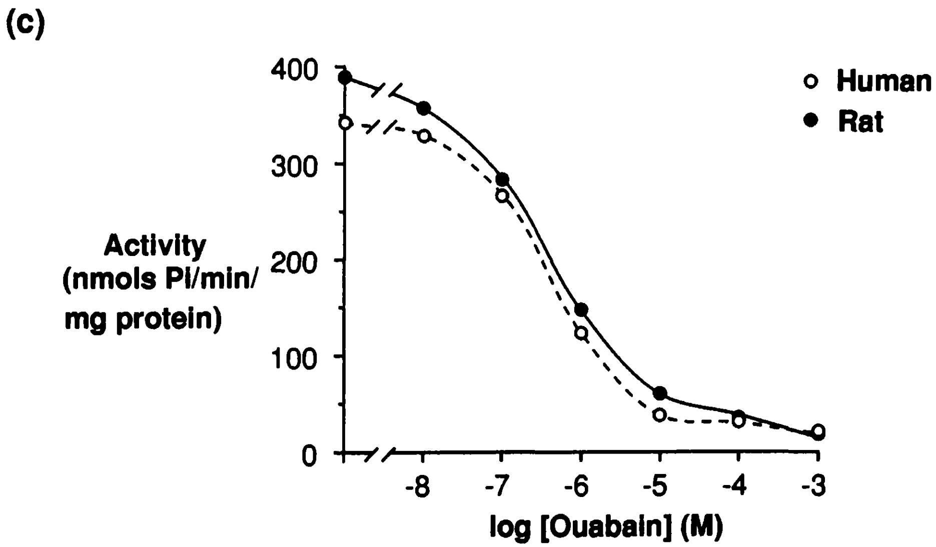
(a) Reactions were started by adding 10, 20, 40 or 60µg of protein to a cuvette containing assay mix which had been preincubated at 37°C for 90 seconds. In one case the assay mix contained 1mM ouabain and in another no protein was added to act as a blank. After 2½ minutes the cuvette was removed from the water bath, the A_{340nm} of the assay mix read, and the cuvette returned to the water bath. This was repeated after 5, 10, 15, 20 and 30 minutes. The change in A_{340nm} was then converted to nmols Pi and plotted against time.

(b) Reactions were started by the addition of 5, 10, 20, 30, 40 or 60µg of membrane protein to a cuvette containing assay mix. Each protein concentration was assayed in duplicate. After 15 minutes at 37°C the A_{340nm} of the assay mix was measured, allowing the enzyme activity to be calculated and plotted against the amount of protein used. The activity shown is minus non specific ATPase activity, which was measured at each protein concentration used by including 1mM ouabain in the assay.

(c) Total ATPase activity was determined in human and rat brain in the presence of a range of ouabain concentrations. The assay was carried out at 37°C for 15 minutes. The values plotted are the mean of duplicate determinations.


(a)
Pi formed
(nmols)
250
200
150
100
50
0
0
10
20
30
Time (mins)
60µg
40µg
20µg
60µg + 1mM ouabain
10µg
Blank


(b)
Activity
(nmols Pi/min)
12
10
8
6
4
2
0
0
20
40
60
Amount of protein in assay (µg)


(c)
Activity
(nmols Pi/min/
mg protein)
400
300
200
100
0
-8
-7
-6
-5
-4
-3
log [Ouabain] (M)
Human
Rat

1.4 Northern Blotting.

For some of the G protein mRNAs assayed in this study it was only possible to prepare oligonucleotides complimentary to one of the species studied - see Table 4. It was therefore necessary to be satisfied that the probes could be used to detect the relevant RNA species in both rat and human, and that cross reaction between different G protein mRNAs was not a problem. Each oligonucleotide probe was tested on RNA purified from the brain of a human subject and from fresh rat brain. The results of these hybridisations are shown in Figure 10. In all cases band smearing is observed in the human RNA, indicating that it is at least partially degraded.

The oligonucleotide which was used to detect mRNA encoding Gβ hybridises to two species of mRNA in human brain. These have an approximate molecular size of 3.0 kilobases (kb) and 1.4 kb (Figure 10). The molecular sizes of each of these RNA species are similar to those reported by others (Fong *et al.,* 1986; Fong *et al.,* 1987). The oligonucleotide hybridises to only one species in rat brain RNA. This has a molecular size slightly greater than that of the smaller human component (approximately 1.5 kb). It is assumed that this species was Gβ2, as the oligonucleotide hybridises to a second band the same size as human Gβ1 when the blot is washed at low stringency. This may indicate that rat Gβ1 is more divergent from its human counterpart than Gβ2 is, but it should be remembered that only a small region of the mRNA is being hybridised and that the variation may be confined to this area. A possible disadvantage of using the one oligonucleotide to detect both forms of Gβ stems from the fact that Gβ1 mRNA can also be detected at a similar molecular weight to Gβ2. It is therefore possible that some of the hybridisation attributed to Gβ2 is actually to Gβ1. However the abundance of the small form of Gβ1 mRNA in brain is low compared to the large form (Fong *et al.,* 1987), and so this is unlikely to be a major problem. In any case this is only true for human RNA since washing rat RNA at high stringency abolishes the hybridisation of the probe

to Gβ1 mRNA.

The oligonucleotide which should hybridise to all four species of Gsα mRNA detects only one major species in rat (Figure 10). However the band is rather elongated and has a molecular size ranging from 2.1 to 1.6 kb. This size and shape of Gs band has been observed by others (Harris on *et al.*, 1991a; Bray *et al.*, 1986). When this oligonucleotide is hybridised to human RNA, four bands are visible. Two bands, of size 2.0 and 1.6kb, were within the range of sizes observed for rat. The doublet appearance of human Gsα mRNA has been observed before (Harris on *et al.*, 1991a). The two species are too different in size to be the two major subgroups of Gsα mRNA reported by Bray *et al.* (1986). The third band detected with this probe has an approximate size of 1.2 kb. However when human RNA is hybridised to the Gs-52 oligonucleotide, which should only hybridise to the large forms of Gsα mRNA, this species is not detected. Instead a band of size 0.6 kb is visible. It is likely therefore that this band and the 1.2 kb band detected with the Gs oligonucleotide are degradation products. However the Gs-52 oligonucleotide does hybridise to the 2.0 kb and 1.6 kb species detected by the Gs oligonucleotide. This confirms that the two bands are not derived from the large and small Gsα mRNA subgroups. For the purpose of estimating the amount of Gsα mRNA in human brain, the optical densities of these two bands were measured and the values averaged. An additional faint higher molecular size species (approximately 4.5kb) is also observed in rat and human RNA. This may be the aberrant form of Gsα mRNA produced using an alternative promoter that was reported by Ishikawa *et al.* (1990).

Giα mRNA was detected in rat brain RNA using oligonucleotides complimentary to Gi1 and Gi2 mRNA. The Gi1 oligonucleotide hybridises to a single species of approximate size 3.8 kb while the Gi2 oligonucleotide hybridises to a species of size 2.5 kb (Figure 10). Both these values are very similar to those reported by others (Nukada *et al*, 1986, Jones & Reed 1987).

However due to the low abundance of Giα mRNA, and the number of mismatches between both types of rat Giα oligonucleotides and the region to which they hybridise to in human RNA (especially of Gi1 - see Table 4), separate oligonucleotides complimentary to the human Giα mRNA species were made. The human Gi1 oligonucleotide hybridises very weakly to a species which is of a similar size to Gi1 mRNA in rat. The oligonucleotide also appears to hybridise to both 28S and 18S ribosomal RNA. Other human subjects had similarly low, or undetectable, amounts of Gi1 mRNA (data not shown). Due to this, the measurement of Gi1 mRNA in human brain was not pursued. A similar situation occurred with Gi2 mRNA in human brain. Despite there being one mismatch between the human Gi2 oligonucleotide and that of rat Gi2 mRNA, this oligonucleotide actually gave a stronger hybridisation signal for Gi2 mRNA in rat brain than in human brain. Again other human subjects contained a low abundance of Gi2 mRNA in RNA purified from brain, and no further attempts were made to measure Gi2 mRNA.

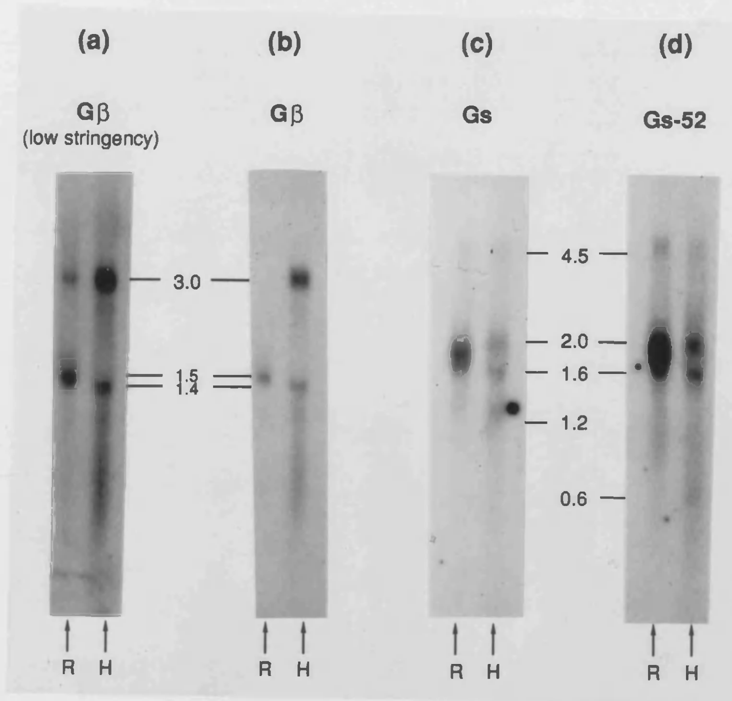
The final oligonucleotide used was made complimentary to human 28S rRNA (Figure 10). This was intended to act as a control RNA species that would allow a normalisation of the amount G protein mRNA between different total RNA preparations. 28S rRNA was chosen in preference to the other common control RNA species, such as actin or GAPDH, since the hybridisation signal stays constant in a wide variety of circumstances (Leeuw *et al.*, 1989). The oligonucleotide hybridised more strongly to human 28S rRNA than to rat 28S rRNA. However there were a number of distinct lower molecular weight species detectable in human RNA not detectable in rat; these are most probably degradation products.

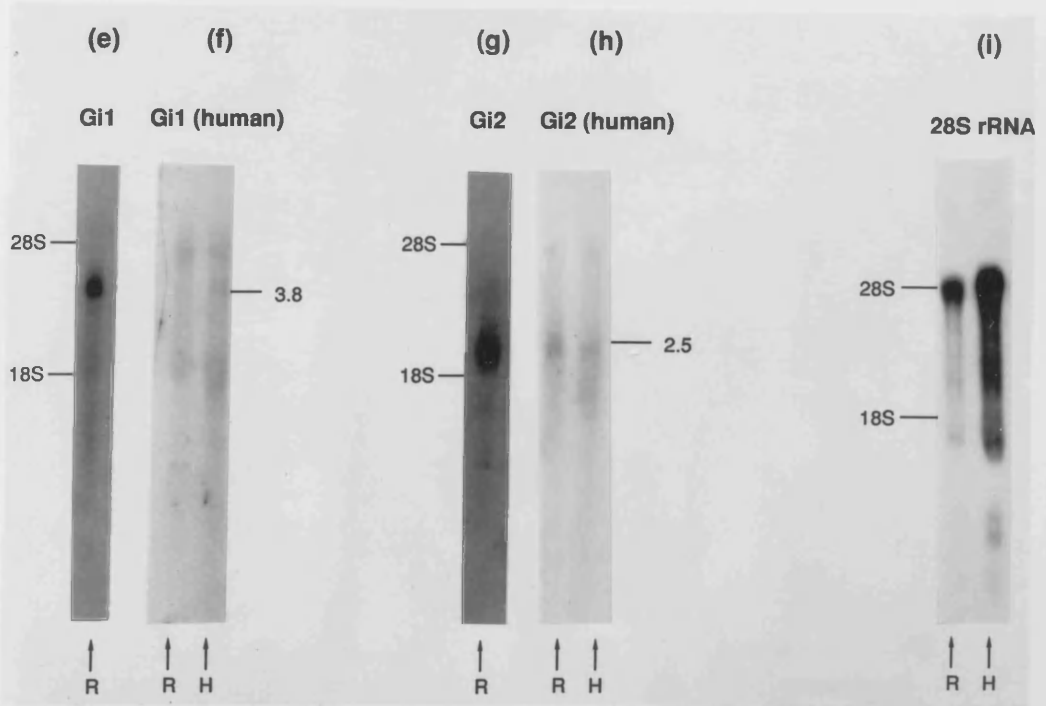
The suitability of using northern blots to quantitate G protein mRNA was previously confirmed by Dr. S. Griffiths, Department of Biochemistry, University of Glasgow. She demonstrated that the optical density of the G protein mRNA bands on the resulting autoradiogram is directly proportional to the amount of RNA loaded onto the gel, up to 40μg (S. Griffiths; personal communication).

Figure 10.

Use of northern blotting to detect the mRNAs encoding Gβ, Gsα, Gi1, Gi2, and 28S rRNA in human and rat brain.

RNA was purified from whole rat brain and from the right frontal cortex of human subject C34. The RNA was analysed by northern blotting as described in "Methods". An agarose gel was loaded with 20μg of denatured rat or human RNA per lane, and run overnight. After transfer of the RNA to Hybond N, the blot was cut into strips and hybridised to labelled oligonucleotides that were complimentary to the mRNAs encoding (a and b) $G\beta_1$ and $G\beta_2$ mRNA (oligo Gβ), (c) all four forms of Gsα (oligo Gs), (d) the two large forms of Gsα (oligo Gs-52), (e) rat Gi1 (oligo Gi1), (f) human Gi1 (oligo hGi1), (g) rat Gi2 (oligo Gi2), (h) human Gi2 (oligo hGi2) and (i) 28S rRNA. The rat Gi oligonucleotides were hybridised to rat RNA only. The strip which had been hybridised to the Gβ oligonucleotide was first washed at low stringency (2xSSPE/0.1% SDS at 42°C for 15 minutes) and exposed to X-ray film for 1 day (a). The blot was then washed to high stringency (0.5xSSC/0.1% SDS at 60°C for 15 minutes) and exposed to X-ray film for 2 days (b). The strips that had been hybridised to oligo Gs, oligo Gs-52 and the Gi oligos were washed to a stringency of 0.5xSSC/ 0.1%SDS at 60°C for 15 minutes and exposed to X-ray film for 2 or 3 days. The strip which had been hybridised to the 28S rRNA oligonucleotide was washed to a stringency of 2xSSPE/0.1% SDS/0.1% sodium pyrophosphate at 37°C for 30 minutes and exposed to X-ray film for 10 minutes. The approximate molecular size (in kb) of each RNA species visible on the autoradiogram is shown. These were estimated by comparing the bands migration to RNAs of known size (4.4, 2.4 and 1.4 kb).


(a)
Gβ
(low stringency)
(b)
Gβ
(c)
Gs
(d)
Gs-52
3.0
1.5
1.4
4.5
2.0
1.6
1.2
0.6
R H
R H
R H
R H


(e)
Gi1
(f)
Gi1 (human)
(g)
Gi2
(h)
Gi2 (human)
(i)
28S rRNA
28S
18S
3.8
28S
18S
2.5
28S
18S
R
R H
R
R H
R H

2. The feasibility of studying signal transduction systems in human post mortem brain.

The study that was envisaged required the use of post mortem tissue. The time between death and freezing of the tissue was typically in the order of 12 hours, this time interval being termed the post mortem delay or PMD. The following series of experiments attempted to discover the stability post mortem of G proteins, their mRNAs, adenylate cyclase or sodium potassium-dependent ATPase, in rat brain. No attempt was made to accurately model the cooling of the human brain post mortem; the experiments were designed merely to indicate the feasibility or otherwise of studying the forementioned substances in human post mortem brain tissue.

2.1 The effect of post mortem delay on G protein stability in rat brain.

Adult male Wistar rats were killed by cervical dislocation and stored at 4°C for up to 72 hours, or at room temperature for 24 hours. After each time interval the brain was removed and ground in liquid nitrogen to yield a homogeneous powder, a proportion of which was used to prepare crude membranes. These preparations were then analysed by western blotting utilising antibodies to Gsα (Figure 11), Giα (Figure 12) and Goα (Figure 13). Since the abundance of GsL is very low in membrane preparations made from whole rat brain, no information about this protein was obtained.

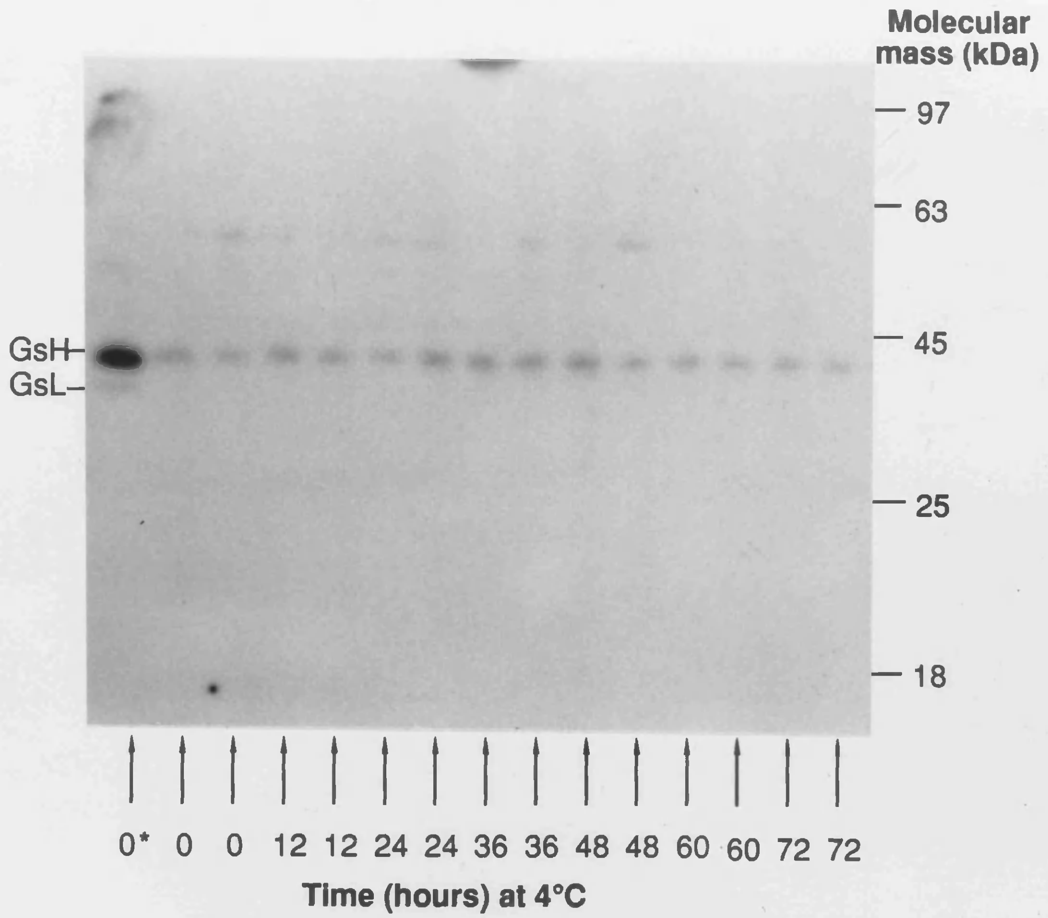
In no case was a change in the molecular weight of any of the G protein bands observed nor were there any smaller size species apparent (there was a small amount of smearing of the Giα bands in the experiment conducted at room temperature, however this was also present in the zero time point preparations). In addition, there was no significant change (SL = 0.05) in the optical density of any of the G protein bands with increasing time post mortem as tested by a one way ANOVA for the experiment conducted at 4°C, or an unpaired Students t-test for that conducted at room temperature (data not shown). Thus these proteins appear to be very stable post mortem in rat brain, at least in respect to their mobility on polyacrylamide gels.

Figure 11.

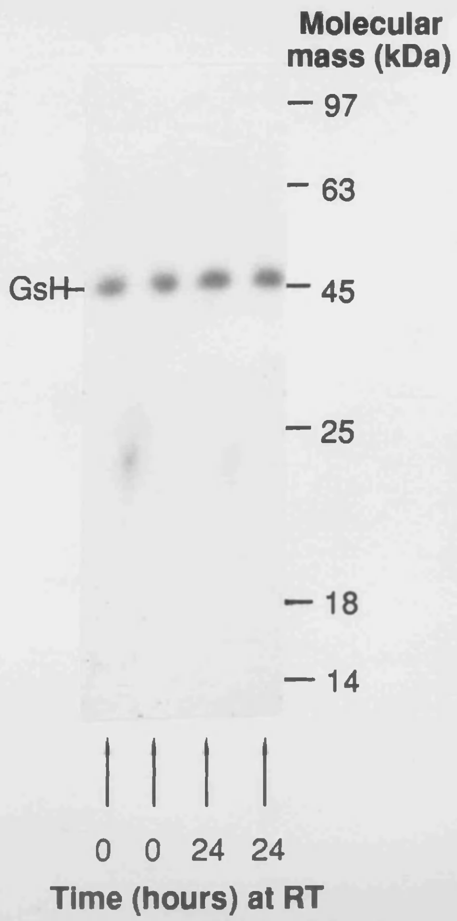
The effect of post mortem delay on the integrity of Gsα.

Fourteen male Wistar rats were sacrificed and kept at 4°C for up to 72 hours before the brains were removed and stored at -80°C. In another experiment the animals were kept at room temperature (approximately 20°C) for twenty four hours after death. Separate zero time point animals were used for the experiments conducted at room temperature (RT) and 4°C. The brains were ground in liquid nitrogen and a portion used to prepare crude membranes as described in "Methods". 50μg of membrane protein was loaded per lane onto a polyacrylamide gel (except for the lane marked with * in which 150μg of protein was loaded) and the gel run overnight. After transferring the proteins onto a nitrocellulose membrane, antisera CS1 was used to show the position of the Gsα bands. The immunocomplex was detected by use of ^{125}I labelled anti-rabbit IgG antibody used at 4μCi/50ml. The autoradiogram was exposed for 3 days.

Molecular weight markers, run along with the membrane preparations, are shown alongside the autoradiogram.



Molecular mass (kDa)
97
63
45
25
18
GsH
GsL
0* 0 0 12 12 24 24 36 36 48 48 60 60 72 72
Time (hours) at 4°C



Molecular mass (kDa)
97
63
45
25
18
14
GsH
0 0 24 24
Time (hours) at RT

Figure 12.

The effect of post mortem delay on the integrity of Giα.

Membrane preparations were made from the brains of rats which had been kept at 4°C and at room temperature (RT) after death, as described in the legend to the previous figure (Figure 11). 100μg of membrane protein was loaded per lane onto a polyacrylamide gel and the gel run overnight. After transfer of the proteins onto nitrocellulose, the blot was incubated with antisera SG2 and then with horse radish peroxidase linked anti-rabbit IgG antisera.

Molecular weight markers, run along with the membrane preparations, are shown alongside each blot.

Molecular mass (kDa)

— 97
— 63
— 45
Gi1
Gi2
— 25
— 18

0 0 0 12 12 24 24 36 36 48 48 60 60 72 72

Time (hours) at 4°C

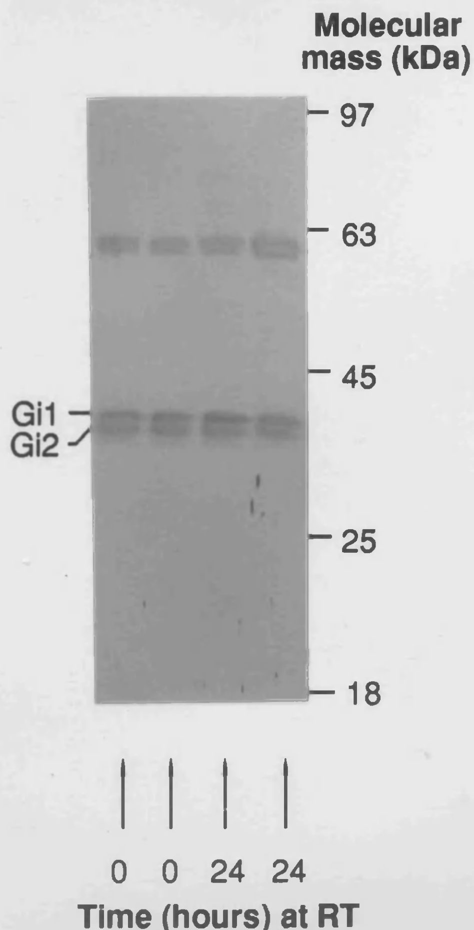


Figure 13.

The effect of post mortem delay on the integrity of Goα.

Membrane preparations were made from the brains of rats which had been kept at 4°C and at room temperature (RT) after death, as described in the legend to Figure 11. 50μg of membrane protein was loaded per lane onto a polyacrylamide gel and the gel run overnight. The proteins were then transferred onto a nitrocellulose membrane. Antisera OC1 was used to show the position of the Goα band, the immunocomplex being detected with horse radish peroxidase linked anti-rabbit IgG antisera.

Molecular weight markers, run along with the membrane preparations, are shown alongside each blot.

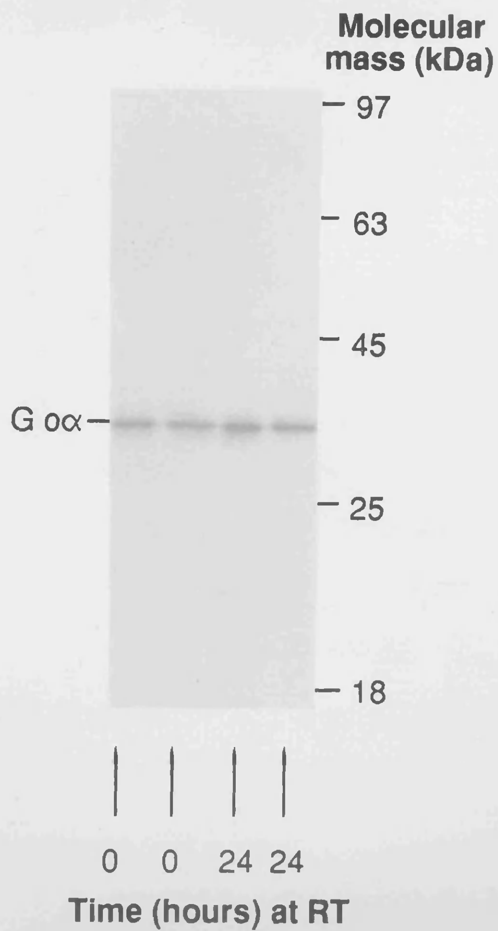
* - 150μg of protein was used in this lane.

Molecular mass (kDa)
97
63
45
25
18

G oα

0 0 12 12 24 24 36 36 48 48 60 60 72 72 0*

Time (hours) at 4°C



2.2 The effect of post mortem delay on adenylate cyclase activity in rat brain.

A subset of the membrane preparations which were used to assess the effect of post mortem on G protein integrity were used to examine its effect on adenylate cyclase activity. There was no significant change in either basal or fluoroaluminate stimulated activities during up to 48 hours at 4°C (Figure 14). There was a decrease in the order of 25% in both basal and fluoroaluminate stimulated activities after 24 hours at room temperature when compared with fresh tissue (Figure 14). This decrease approached, but did not reach, statistical significance. There was no change in fluoroaluminate stimulated adenylate cyclase activity when expressed as a proportion of basal activity. Since fluoroaluminate stimulates adenylate cyclase via its effect on G proteins, this parameter provides a measure of the ability of adenylate cyclase and G proteins to interact. It would therefore appear that adenylate cyclase is a fairly stable enzyme in rat brain, and is therefore a suitable candidate for study in post mortem human brain. In addition G proteins are still able to interact with adenylate cyclase in rat brain for at least 24 hours after death.

2.3 The effect of post mortem delay on sodium potassium-dependent ATPase activity in rat brain.

The effect of post mortem delay on the enzyme sodium potassium-dependent ATPase was examined using the same rat brain membrane preparations as those described for G proteins and adenylate cyclase (Sections 2.1 and 2.2). When compared to fresh brain tissue their was no statistically significant change observed in the activity of this enzyme in intact sacrificed rats which had been stored 48 hours at 4°C or 24 hours at room temperature, when compared to fresh tissue (Figure 14). This enzyme is therefore an ideal candidate for study in human post mortem tissue.

Figure 14.

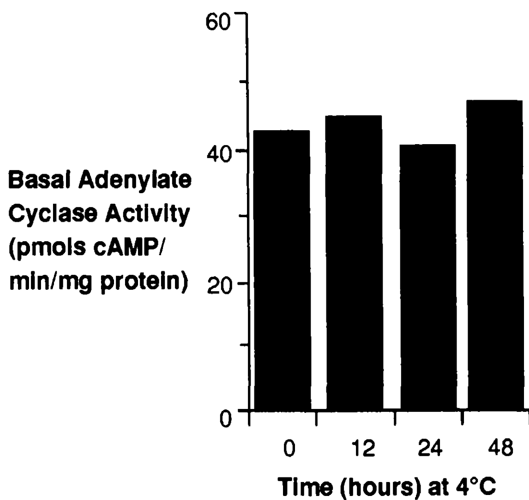
The effect of post mortem delay on the activity of adenylate cyclase and sodium potassium dependent ATPase.

Fourteen male Wistar rats were sacrificed and kept at 4°C for up to 72 hours before the brains were removed and stored at -80°C. In another experiment the animals were kept at room temperature (approximately 20°C) for twenty four hours. Separate zero time point animals were used for the experiments conducted at room temperature (RT) and 4°C. The brains were ground in liquid nitrogen and a portion used to prepare crude membranes as described in "Methods". (Note that other aliquots were used to analyse G proteins - see Figures 11 to 13).

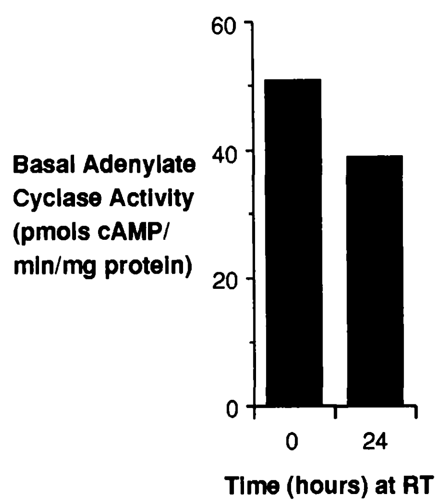
Adenylate cyclase activity was assayed (as described in "Methods") at 30°C for 15 minutes using 20μg of membrane protein per tube, for both the animals which had been kept at 4°C, (a) and (c), and for those that had been kept at RT, (b) and (d). Both basal activity, (a) and (b), and fluoroaluminate stimulated activity, (c) and (d), were measured. In the latter case the concentrations of aluminium chloride and sodium fluoride were 10μM and 5mM respectively. The activities shown are the mean of duplicate analyses of two animals.

Sodium dependent ATPase activity was also assayed in these membrane preparations, at 37°C for 15 minutes using 20μg of membrane protein per tube as described in "Methods". This is shown for the animals which had been stored at 4°C (e) and room temperature (f). Non-specific ATPase activity was determined in the presence of 1mM ouabain.

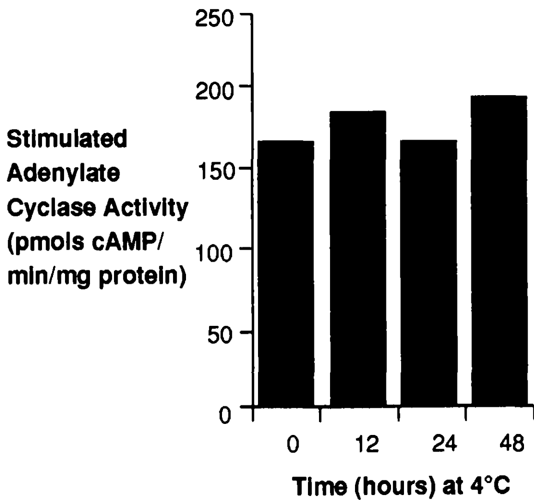
(a)



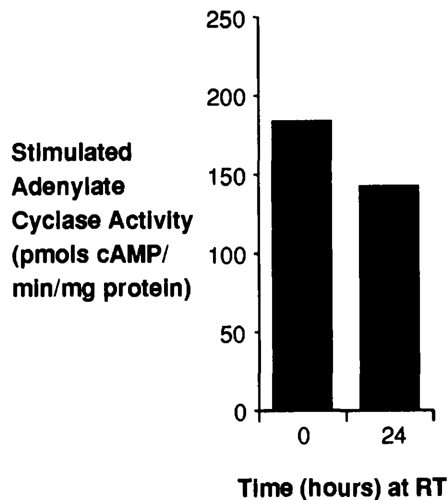
(b)



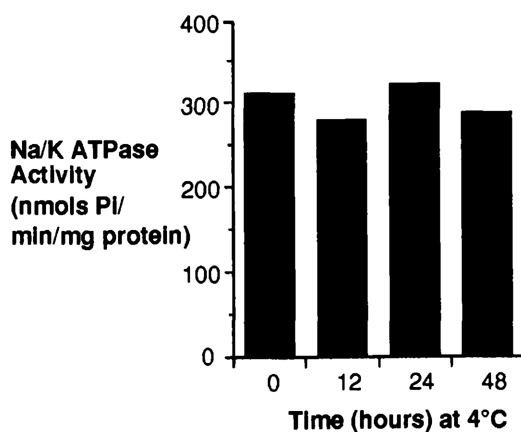
(c)



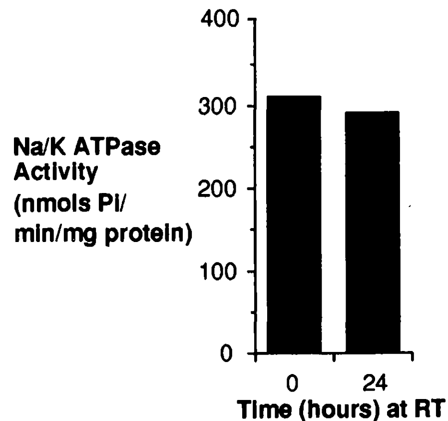
(d)



(e)



(f)



2.4 The effect of post mortem delay on RNA stability in rat brain.

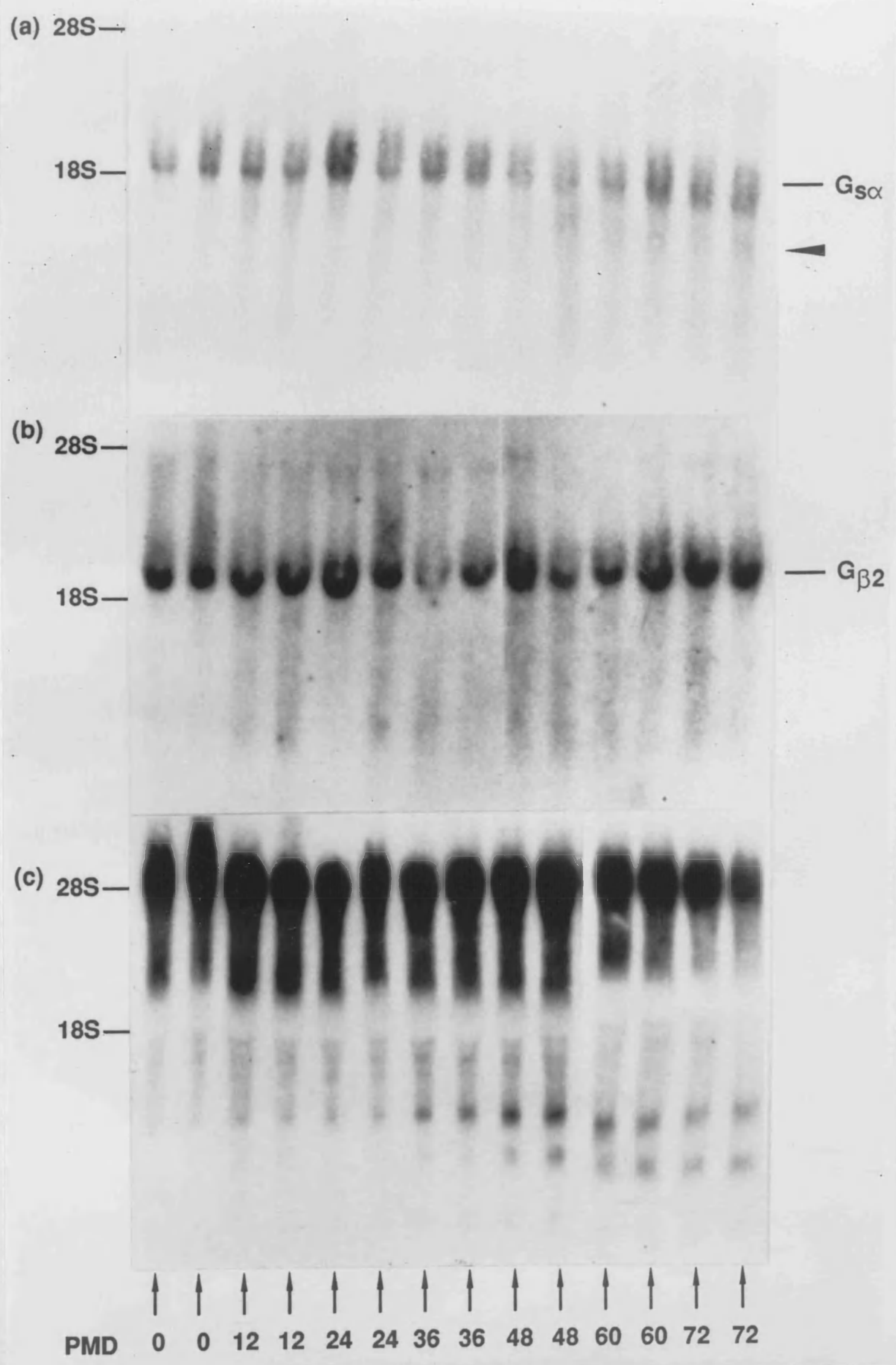
The stability of RNA post mortem was studied in rat brain using the same animals as were used to study protein stability. RNA was purified from an aliquot of brain powder derived from each animal which had been stored at 4°C for up to 72 hours. The RNA was subsequently analysed by northern blotting using ^{32}P labelled oligonucleotides complimentary to Gsα and Gβ mRNA, and to 28S rRNA (Figure 15). Each G protein mRNA showed little degradation after 72 hours post mortem, as witnessed by the small amount of band smearing and constant molecular size, relative to the zero time point animals. Of the band smearing that was apparent in the later time point preparations, it appears to be more marked for Gsα than Gβ mRNA. Degradation of Gsα was most apparent after 48 hours where a weakly hybridising second band is visible. This band may be analogous to the 1.2 kb band detected in human Gsα mRNA (Figure 10). In the case of 28S rRNA there was a gradual time dependent increase of low molecular weight 28S rRNA degradation products, of which there are three major species that hybridise to the oligonucleotide.

It appeared at this stage that G protein mRNA could be reliably measured in post mortem brain, and analysis of mRNA was conducted in parallel with those of G proteins (Section 3). During the course of these investigations however doubts emerged on the reliability of the measurement of G protein mRNA in human post mortem brain, and for this reason the data together with a further investigation of RNA degradation is presented in Section 5.

Figure 15.

The effect of post mortem delay on the integrity of Gsα and Gβ mRNA, and 28S rRNA.

Fourteen male Wistar rats were sacrificed and kept at 4°C for up to 72 hours before the brains were removed and stored at -80°C. The brains were ground in liquid nitrogen and a portion used to purify RNA. The RNA was then analysed by northern blotting as described in "Methods". Three agarose gels were loaded with 20μg of RNA per lane and run overnight. The RNA was then transferred to Hybond N membranes. The blots were hybridised to a labelled oligonucleotide probe complimentary to (a) Gsα mRNA, (b) Gβ mRNA and (c) 28S rRNA. After washing, blots (a) and (b) were exposed to X-ray film for 2 days, while blot (c) was exposed for 25 minutes. The arrow head on blot (a) indicates the position of the second band found at the longer time points using the Gsα probe. The positions of 18S and 28S rRNA are shown to indicate molecular size. The ribosomal bands were visualised by staining the entire blots with methylene blue stain after the autoradiograms had been exposed.


(a) 28S
18S
Gsα
(b) 28S
18S
Gβ2
(c) 28S
18S
PMD 0 0 12 12 24 24 36 36 48 48 60 60 72 72

3. Analysis of G protein linked coupled transduction in demented and control subjects.

3.1 Comparison of choline acetyl transferase activity in control and DAT subjects.

A group of eleven subjects with a neuropathologically confirmed clinical diagnosis of DAT was compared to a group of fifteen age matched control subjects. From these, sixteen subjects (eight from each group) were chosen to study the effects of DAT on choline acetyl transferase (ChAT), G protein levels, adenylate cyclase and sodium potassium-dependent ATPase in the left frontal cortex. For experimental reasons a further different sixteen were employed to study the same parameters in the left hippocampus. Tissue from each subject was homogenised and a small aliquot was removed for the determination of ChAT activity. The remainder of the homogenate was used to make membrane preparations.

There was a large statistically significant decrease observed in the activity of ChAT in the frontal cortex and hippocampus of DAT subjects compared with that of controls (two-tailed Students t-test; $P<0.002$; Figure 16). The mean ChAT activity in the frontal cortex of DAT subjects was 54% of that found in controls, while that observed in the hippocampus was 34% of that measured in controls.

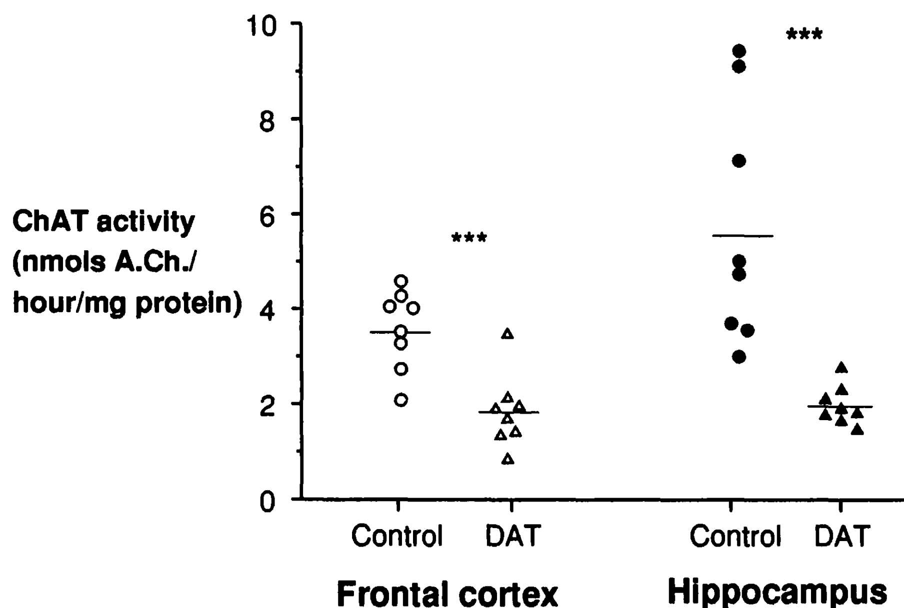


Figure 16.

The activity of choline acetyl transferase activity in the frontal cortex and hippocampus of control and DAT subjects.

The left frontal cortex and left hippocampus from DAT and control subjects were powdered in liquid nitrogen and homogenised in six volumes of 10mM Tris/HCl, pH 7.5, 1mM EDTA. A small aliquot was removed and assayed for choline acetyl transferase (ChAT) activity as described in "Methods". The assay was carried out at 37°C for 20 minutes using 30μg of protein. Eight control and eight DAT subjects were analysed for both the hippocampus and frontal cortex, although different sets of patients were used for each region. All assays were performed in triplicate. The distribution of ChAT activity within each group is shown for both regions. Bars indicate the mean enzyme activity of each group. *** indicates a statistically significant difference between control and DAT subjects (two tailed Students t-test; $P<0.002$). The ChAT activities measured in each subject are listed in Appendix A1.

3.2 comparison of G protein alpha subunit levels in control and DAT subjects.

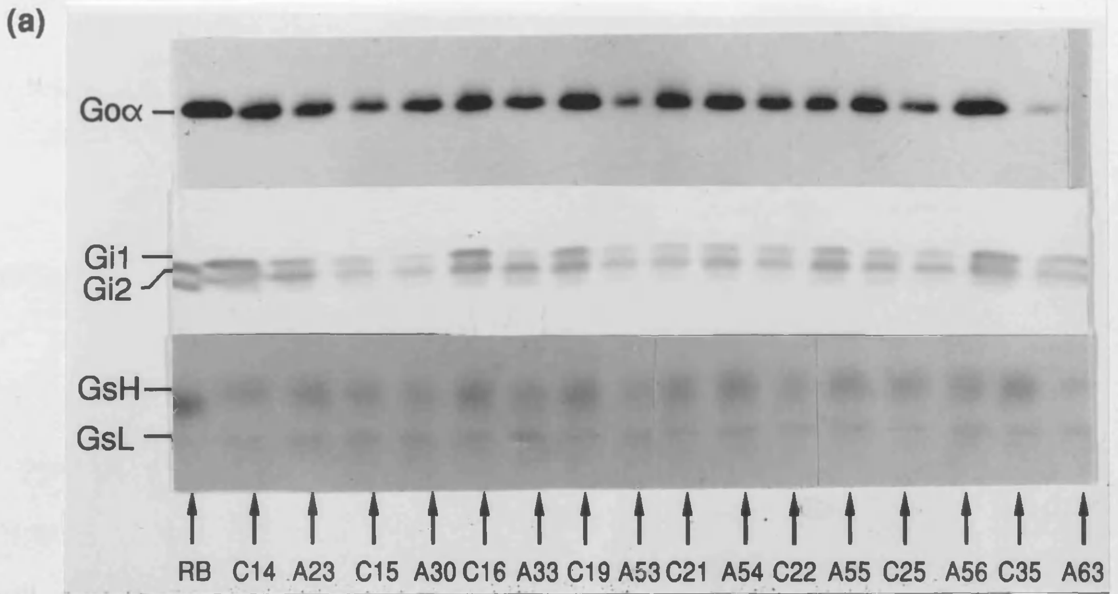
The levels of the G protein subunits GsH, GsL, Gi1, Gi2 and Goα were compared in the left frontal cortex and left hippocampus of DAT and control subjects, using membrane preparations which had been prepared from homogenates in which ChAT activity had been assayed (section 3.1). The levels of the various G protein alpha subunits were measured by western blotting using the antisera CS1, SG2 and OC1 (Figures 17 and 18).

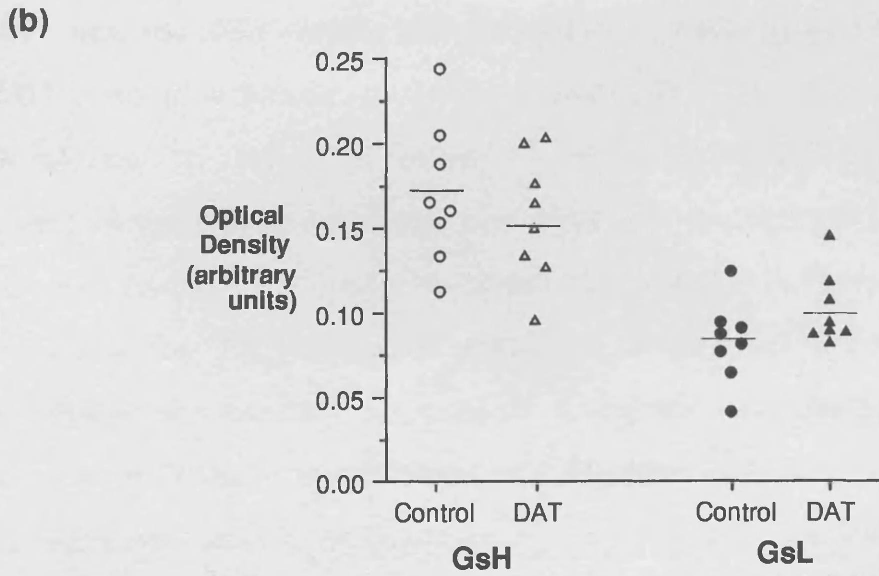
No statistically significant difference was observed between control and DAT groups, as assessed using a two-tailed Students t-test (significance level = 0.05), in either region for any of the alpha subunits which were analysed. However a statistically significant decrease (28%) was observed ($P<0.05$) in the mean ratio of GsH to GsL found in the frontal cortex, but not the hippocampus, of DAT subjects compared with controls (Figure 19). This can occur since the ratio is determined for individual subjects, whilst G protein levels represent the overall value for each group of subjects. It would appear therefore that a small increase in the relative levels of GsL compared to GsH has taken place in DAT frontal cortex, although some caution should be exercised in the interpretation of this finding as a degree of overlap exists between the ratios obtained in the two groups.

Figure 17.

The levels of the alpha subunits of the G proteins Gs, Gi and Go in the frontal cortex of control and DAT subjects.

Crude membrane proteins were prepared from the left frontal cortex of eight control and eight DAT subjects, and analysed by western blotting as described in "Methods". A polyacrylamide gel was loaded with 100μg of protein per lane (to be used with antisera SG2), while another two gels were loaded with 50μg of protein per lane (to be used with antisera OC1 or CS1), and run overnight. After transfer of the proteins to nitrocellulose, one blot was incubated with antisera CS1, one with OC1 and the other with SG2. The blots were then incubated with either HRP linked anti-rabbit IgG antisera, or ^{125}I labelled anti rabbit IgG antisera at 3μCi/50ml as appropriate. The latter was then exposed to X-ray film for 3 days. The parts of the blots or autoradiogram showing the G protein bands are shown in (a). The patient number is shown beneath each lane (C = Control, A = DAT, RB = rat brain). Subsequent densitometric analysis of these bands was used to determine the relative levels of each G proteins in the control and DAT groups. The distribution of band intensities in each group is shown for (b) Gsα, and (c) Giα and Goα. Bars indicate the mean optical density in each group. The band intensity measured for each G protein in each subject is listed in Appendix A1.


(a)
Goα
Gi1
Gi2
GsH
GsL
RB C14 A23 C15 A30 C16 A33 C19 A53 C21 A54 C22 A55 C25 A56 C35 A63


(b)
Optical Density (arbitrary units)
0.25
0.20
0.15
0.10
0.05
0.00
Control
DAT
Control
DAT
GsH
GsL

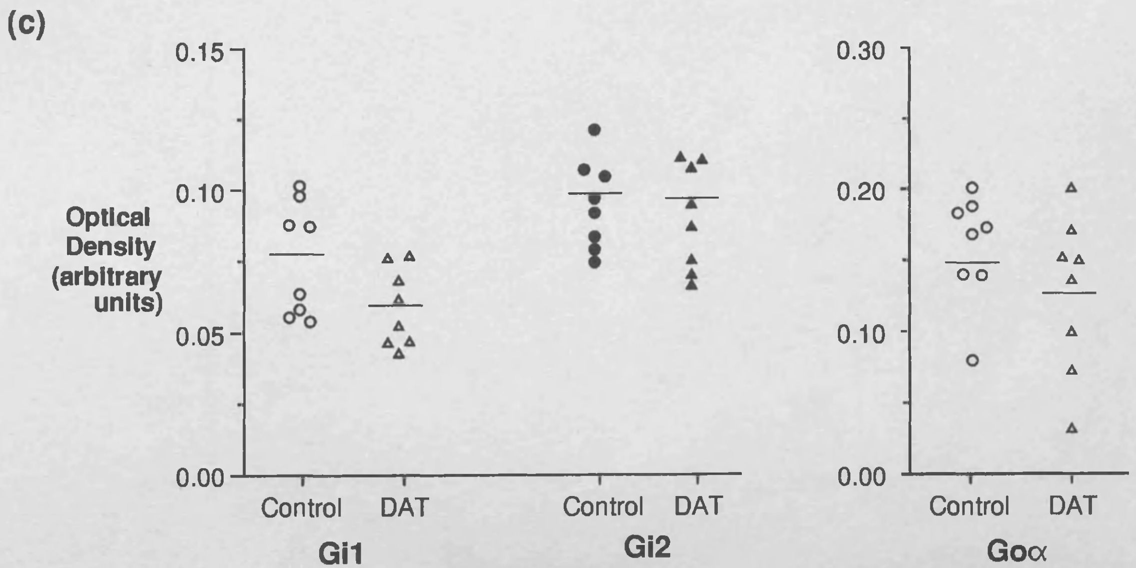
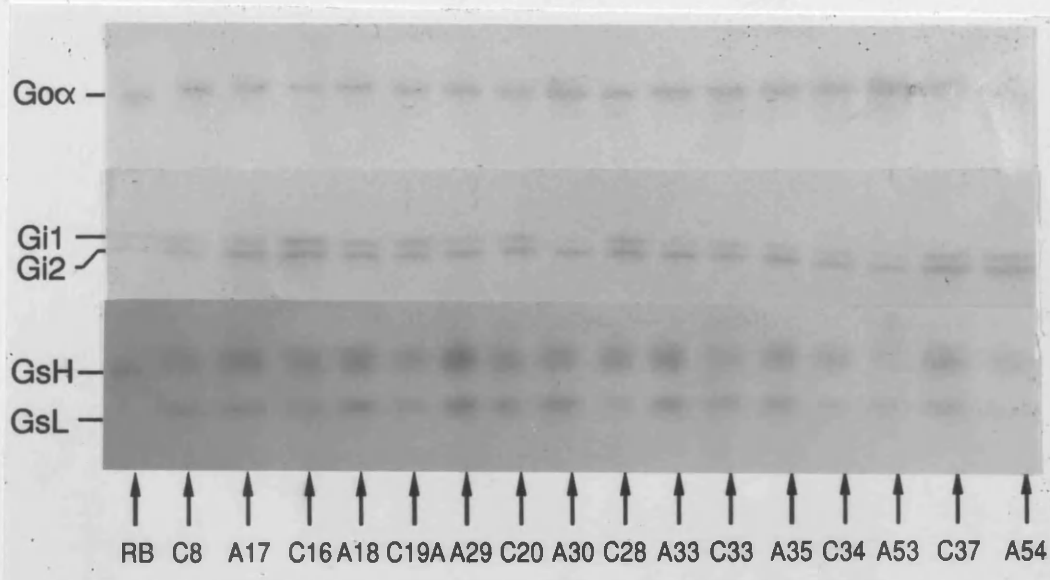

(c)
Optical Density (arbitrary units)
0.15
0.10
0.05
0.00
Control
DAT
Control
DAT
Gi1
Gi2
0.30
0.20
0.10
0.00
Control
DAT
Goα

Figure 18.

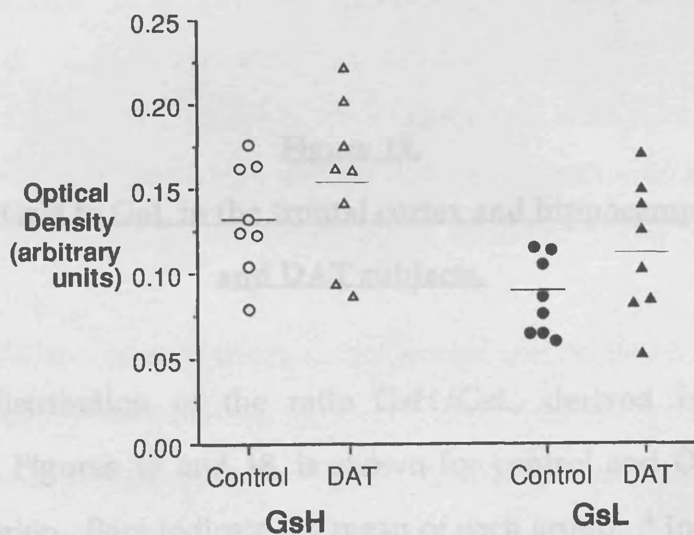
The levels of the alpha subunits of the G proteins Gs, Gi and Go in the hippocampus of control and DAT subjects.

Crude membrane proteins were prepared from the left hippocampus of eight control and eight DAT subjects, and analysed by western blotting as described in "Methods". Three polyacrylamide gels were prepared and run overnight. One gel was loaded with 100μg of protein per lane (to be used with antisera SG2), while another two gels were loaded with 50μg of protein per lane (to be used with antisera OC1 or CS1). After transfer of the proteins to nitrocellulose, one blot was incubated with antisera CS1, one with OC1 and the other with SG2. The blots were then incubated with either HRP linked anti-rabbit IgG antisera, or ^{125}I labelled anti-rabbit IgG antisera at 3μCi/50ml as appropriate. The latter was then exposed to X-ray film for 2 days. The parts of the blots or autoradiogram showing the G protein bands are shown in (a). The patient number is shown beneath each lane (C = Control, A = DAT, RB = rat brain). Subsequent densitometric analysis of these bands was used to determine the relative level of the G proteins in each the control and DAT groups. The distribution of band intensities in each group is shown for (b) Gsα, and (c) Giα and Goα. Bars indicate the mean optical density in each group. The band intensity measured for each G protein in each subject is listed in Appendix A1.

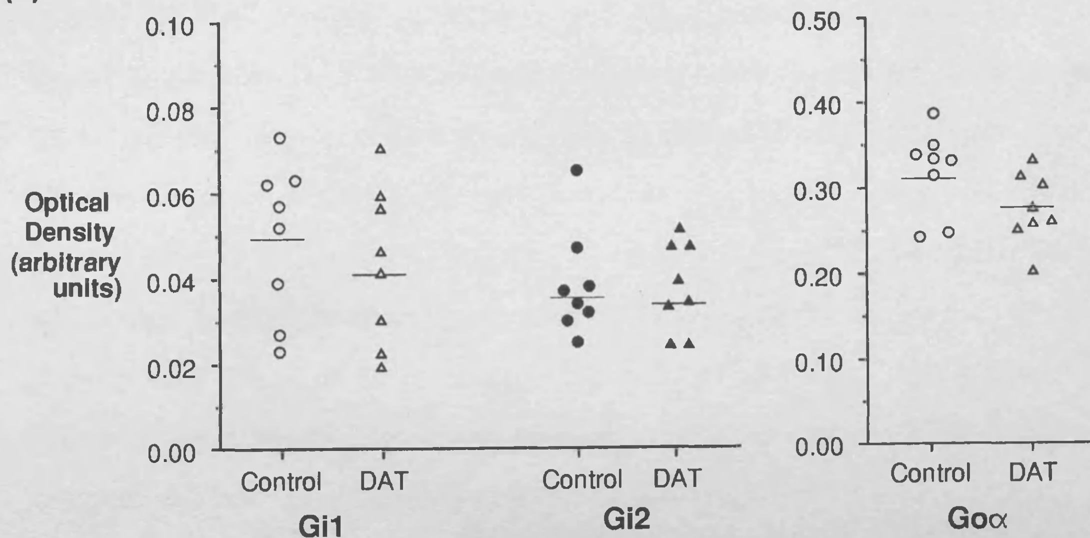
(a)



(b)



(c)



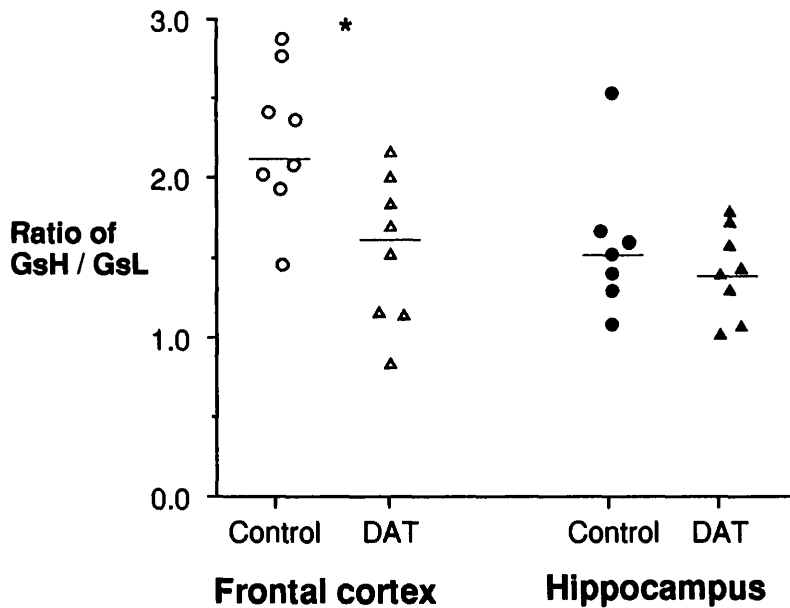


Figure 19.

The ratio of GsH to GsL in the frontal cortex and hippocampus of control and DAT subjects.

The distribution of the ratio GsH/GsL, derived from the data presented on Figures 17 and 18, is shown for control and DAT groups in each brain region. Bars indicate the mean of each group. * indicates $P<0.05$ when the data was analysed using a two-tailed Students t-test.

3.3 The activity of adenylate cyclase in control and DAT subjects.

The activity of adenylate cyclase was assayed in crude membranes prepared from control and DAT left frontal cortex and hippocampus. The membrane preparations used were those in which the levels of G protein alpha subunits had previously been measured (section 3.2). The activity of adenylate cyclase was assayed under basal conditions and while stimulated by fluoroaluminate.

Both the basal and stimulated enzyme activities were found to be significantly decreased in the frontal cortex of DAT subjects (Figure 20), as assessed by a two-tailed Students t-test ($p<0.01$). The mean basal activity measured in DAT subjects was 56% of that determined in control subjects, while fluoroaluminate stimulated activity in DAT subjects was 53% of that found in controls. In contrast, no significant difference in adenylate cyclase activity was found between the control and DAT groups in the hippocampus. When fluoroaluminate stimulated adenylate cyclase activity was expressed as a percentage of basal activity in each subject, no significant difference was found between the control and DAT groups in either region (Figure 20).

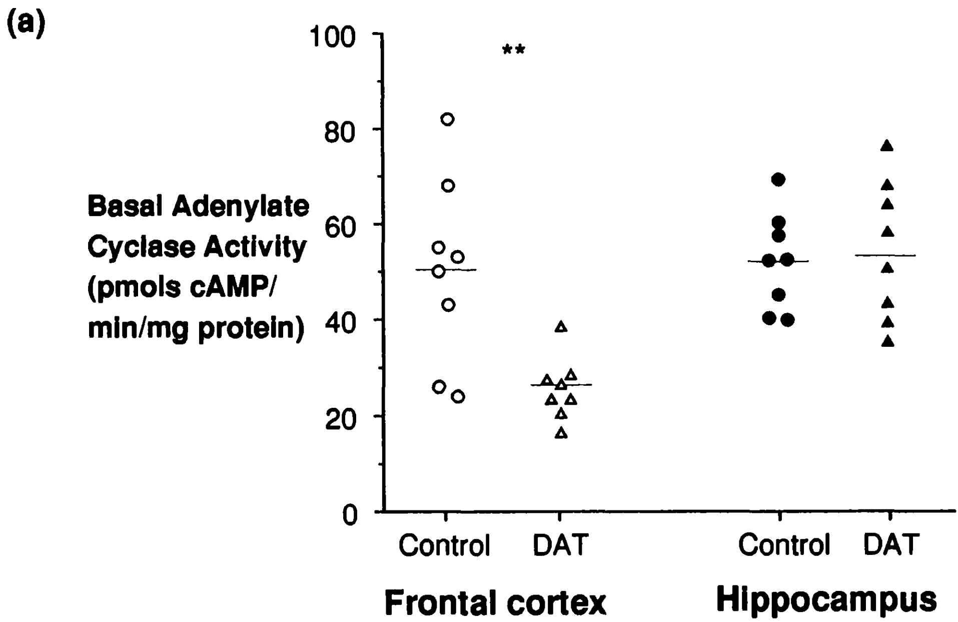
Within the control group in the frontal cortex, two subjects (C15 and C22) gave values more akin to the DAT group. However these subjects had normal ChAT activities and normal neuritic plaque densities, and are therefore not incorrectly classified DAT cases.

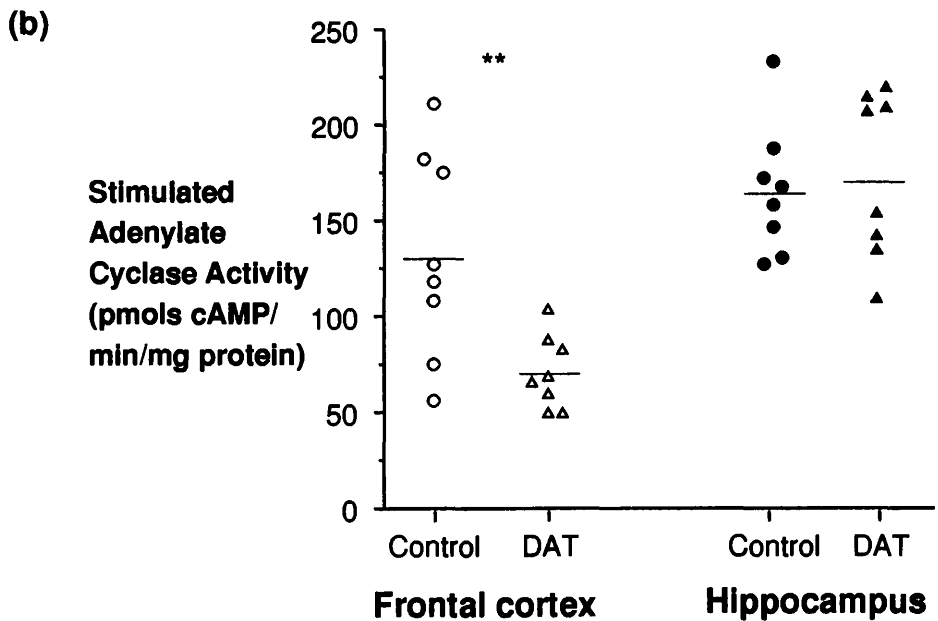
An attempt was made to find a correlation between adenylate cyclase activity in DAT frontal cortex and two other measures which had been found to alter in DAT frontal cortex, namely neuritic plaque density and ChAT activity. There was no statistically significant linear correlation found between plaque density and both basal or stimulated adenylate cyclase activity (Figure 21; $r = 0.152$ and 0.091 respectively). However there was a significant linear correlation observed between basal adenylate cyclase activity and ChAT activity (Figure 21; $r=0.770$; $p<0.05$). The correlation between stimulated adenylate cyclase activity and ChAT activity approached, but did not reach, statistical significance ($r = 0.675$).

Figure 20.

The activity of adenylate cyclase in the frontal cortex and hippocampus of control and DAT subjects.

Crude membrane preparations were prepared from the left frontal cortex and left hippocampus of subjects who had suffered from DAT, and from control subjects as described in "Methods". Eight control and eight DAT subjects were analysed for both the hippocampus and frontal cortex, although for experimental reasons different sets of patients were used for each region. The preparations were assayed for adenylate cyclase activity as described in "Methods". Assays were carried out in duplicate at 30°C for 16 minutes, using 20μg of protein per tube. Both the basal (a) and fluoroaluminate stimulated (b) activities were measured. The latter was determined by including 5mM sodium fluoride and 10μM aluminium chloride in the assay. Fluoroaluminate stimulated activity is also shown expressed as a percentage of basal activity (c). Bars indicate the mean activity of each group. ** indicates a statistically significant difference between control and DAT subjects (Students t-test; $P<0.01$). The adenylate cyclase activities measured in each subject are listed in Appendix A1.


(a)
Basal Adenylate
Cyclase Activity
(pmols cAMP/
min/mg protein)
100
80
60
40
20
0
**
Control
DAT
Control
DAT
Frontal cortex
Hippocampus


(b)
Stimulated
Adenylate
Cyclase Activity
(pmols cAMP/
min/mg protein)
250
200
150
100
50
0
**
Control
DAT
Control
DAT
Frontal cortex
Hippocampus

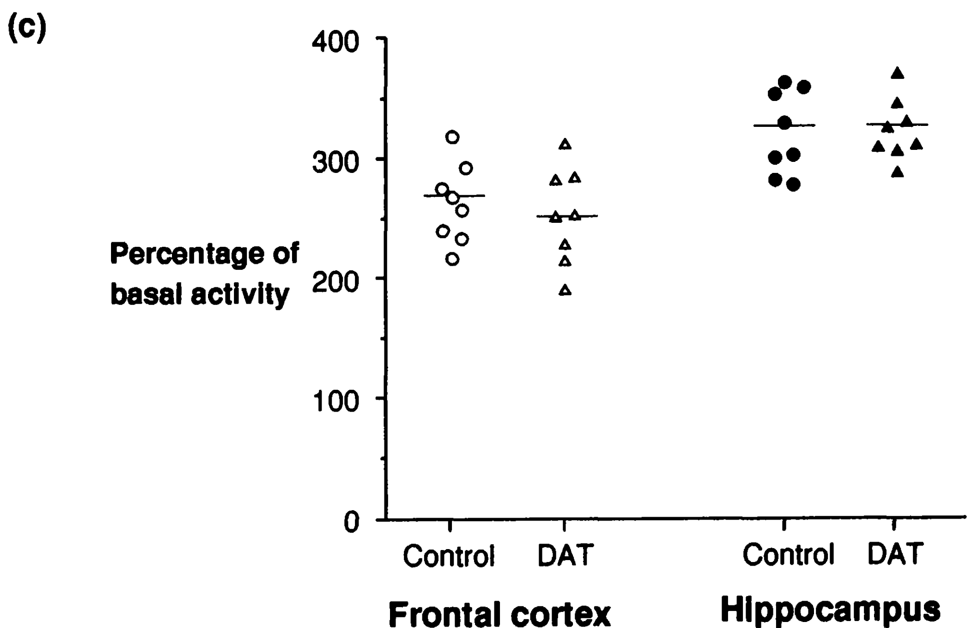
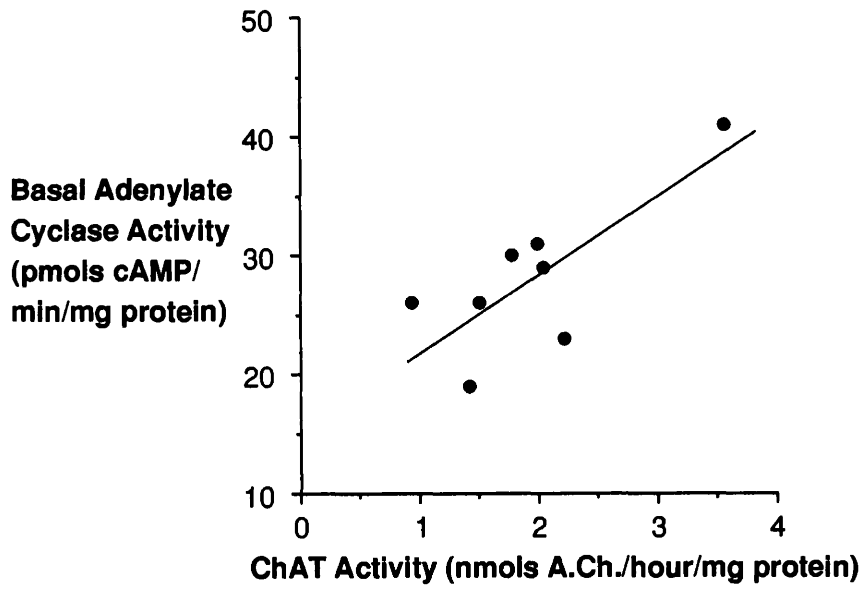

(c)
Percentage of
basal activity
400
300
200
100
0
Control
DAT
Control
DAT
Frontal cortex
Hippocampus

Figure 21.

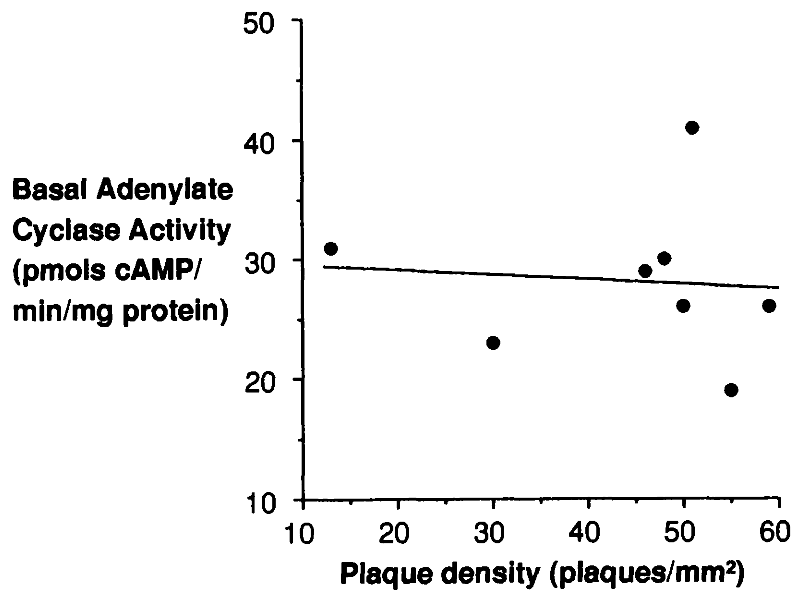
A comparison of basal adenylate cyclase activity in DAT frontal cortex, with choline acetyl transferase activity and neuritic plaque density.

Basal adenylate cyclase, assayed in the left frontal cortex of eight DAT subjects, is shown plotted against (a) the activity of choline acetyl transferase (ChAT), or (b) density of neuritic plaques. In each case linear regressional analysis was used to determine the "best fit" line. When adenylate cyclase activity is compared to ChAT activity a statistically significant linear correlation is present ($P<0.05$, $r=0.770$). In contrast no statistically significant linear correlation exists between basal adenylate cyclase activity and neuritic plaque density ($r=0.091$).

(a)



(b)



3.4 The activity of sodium potassium-dependent ATPase in control and DAT subjects.

In the preceding sections a change in adenylate cyclase activity was described in DAT subjects which was not accompanied by any changes in the level of G protein alpha subunits. Another membrane bound enzyme, sodium potassium dependent ATPase, was assayed to act as a control against differences in membrane purity etc. having occurred between the two groups. Using the same membrane preparations that were assayed for adenylate cyclase activity (section 3.3), the activity of sodium potassium dependent ATPase was determined in the frontal cortex and hippocampus of control and DAT subjects. Although the mean activity of this enzyme was decreased by 20% in DAT frontal cortex compared to controls, this did not reach statistical significance. There was also no statistically significant difference in mean hippocampal activity between DAT and control groups (Figure 22).

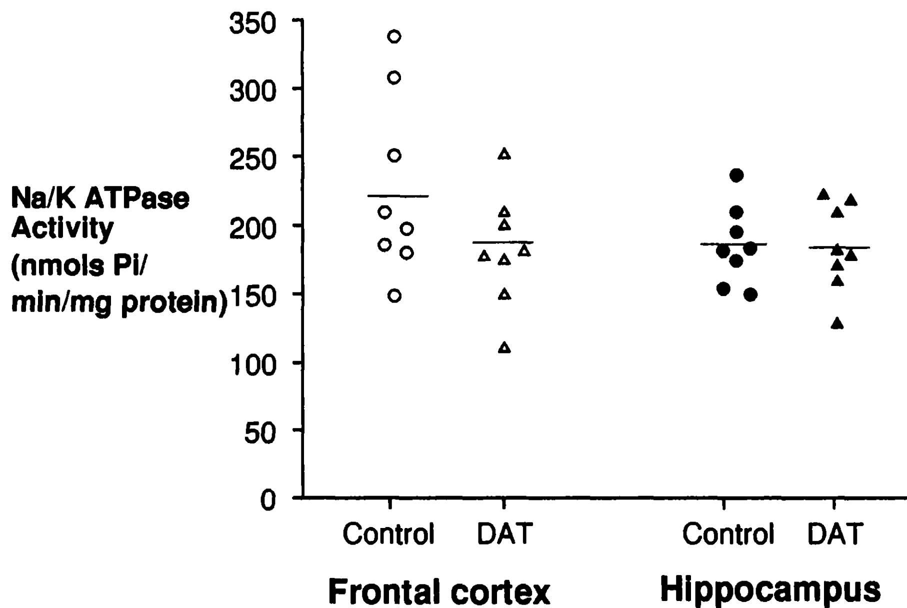


Figure 22.

The activity of sodium potassium dependent ATPase in the frontal cortex and hippocampus of control and DAT subjects.

Crude membrane preparations were prepared from the left frontal cortex and left hippocampus of subjects who had suffered from DAT, and from control subjects as described in "Methods". The preparations were assayed for sodium potassium dependent ATPase activity. Assays were carried out in triplicate at 37°C for 15 minutes, using 20μg of protein per tube. Eight control and eight DAT subjects were analysed for both the frontal cortex and hippocampus, although different sets of patients were used for each region. The distribution of the enzyme activity for each region is shown above. Bars indicate the mean activity of each group. The activity of sodium potassium dependent ATPase measured in each subject is listed in Appendix A1.

4. The distribution of the proteins measured in this study in five regions of the human brain.

In section three little evidence was found that the levels of five G protein alpha subunits were altered in DAT. There were indications though that the proteins exhibited an uneven distribution within different regions of the the normal and diseased brain. In this section these indications are pursued.

4.1 Distribution of G proteins in five regions of the human brain.

The left frontal cortex, left hippocampus, right neostriatum (caudate/ putamen), left cerebellum and pons of three control subjects (C14, C19A and C34) were used to make crude membrane preparations. These were analysed by western blotting to determine the relative amounts of each G protein α subunit in the different brain regions. The distribution of each subunit was broadly similar in each subject, the results being averaged to arrive at a final value. While it was possible to use a single antiserum to compare the relative amounts of individual G protein in different brain regions, it was not possible to compare the amounts of different G protein i.e. Gsα, Giα and Goα. This is because the different antisera are expected to vary in their affinities for their respective antigens.

Figure 23 shows the western blot analysis of Gsα in each subject. This shows the normal pattern of bands except in the neostriatum, where an extra band possessing an approximate molecular weight 43 kD is present. It is of low abundance relative to the Gsα bands but is clearly visible at longer exposures in C14 and C34. The band is just visible in C19A in which it is partially obscured by the GsL band. This 43 kD band is not an artefact of the secondary antibody since this did show any bands in either frontal cortex or neostriatum (Figure 23). It is therefore possible that this band represents a "Gsα-like" protein which is only present in the neostriatum (out of the five regions analysed). Of the levels of the known Gsα proteins, the cerebellum

had the highest concentration of GsL within the five regions examined, followed (in descending order) by the pons, neostriatum, frontal cortex and hippocampus (Figure 23). The concentration of GsH was also highest in the cerebellum, but in this case was followed by the frontal cortex and hippocampus. The neostriatum and pons contained the lowest amounts of GsH. It is also clear from Figure 23 that in the frontal cortex and hippocampus GsH is the major species, while the reverse is true for the other three regions examined.

The relative concentrations of the two Giαs were also examined (Figure 24). Of all the α subunits measured, Gi2 has the least variable distribution within the regions analysed. It was highest in the frontal cortex followed by the hippocampus, neostriatum, cerebellum and pons. The distribution of Gi1 followed the same order as for Gi2, but was more variable between the different regions. The concentration of Gi2 was greater than that of Gi1 in all the regions examined (except in C14 frontal cortex), this being most pronounced in the neostriatum, cerebellum and pons.

Finally Goα was found to be distributed among regions in a similar manner to Gi1 and Gi2 (Figure 25), with the pons containing a very low concentration of Goα.

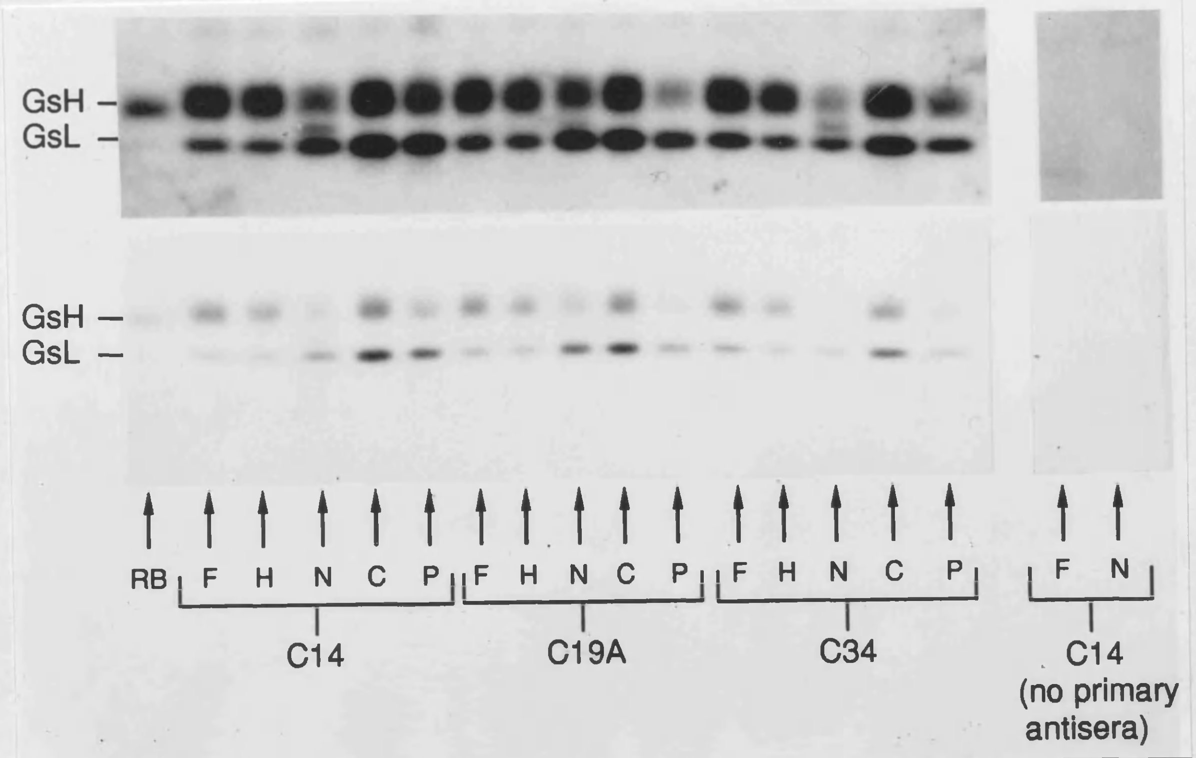
Figure 23.

The distribution of Gsα in human brain.

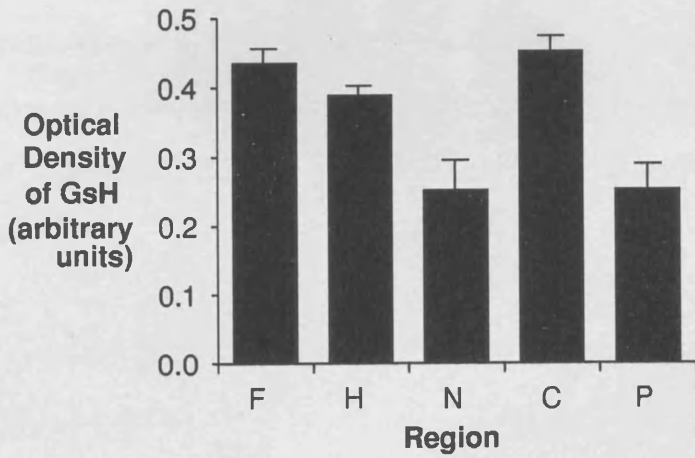
Crude membranes were prepared from the frontal cortex, hippocampus, neostriatum, cerebellum and pons of subjects C14, C19A and C34, and analysed by western blotting as described in "Methods". A polyacrylamide gel was loaded with 50μg of protein per lane and run overnight. The proteins were then transferred onto a nitrocellulose membrane. The blot was incubated with antisera CS1, and then with ^{125}I anti-rabbit IgG antisera at a concentration of 4μCi/50ml. A strip of nitrocellulose to which had been transferred samples of C14 frontal cortex and neostriatum was incubated in the secondary antisera alone. The blot was then exposed to X-ray film for 1 or 3 days (a). The longer exposure is shown to illustrate more clearly the position of the third band visible between GsH and GsL in human neostriatum. However the autoradiogram which had been exposed for 1 day was analysed densitometrically. Regions are indicated thus F = Frontal cortex, H = Hippocampus, N = Neostriatum, C = Cerebellum, P = Pons. RB = rat brain. Only the portion of the blot containing the G protein bands is shown.

The levels of GsH and GsL in each region are given in (b) and (c) respectively. The values shown are the mean of the three subjects; error bars indicate the SEM.

(a)



(b)



(c)

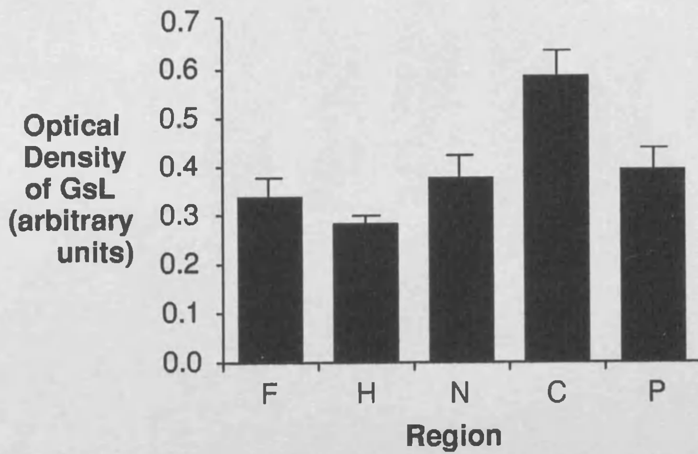


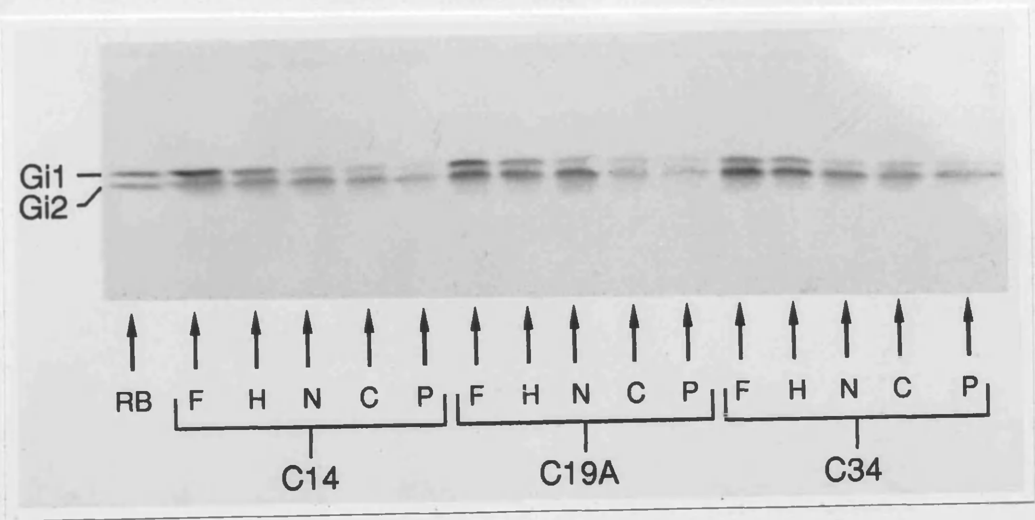
Figure 24.

The distribution of Giα in human brain.

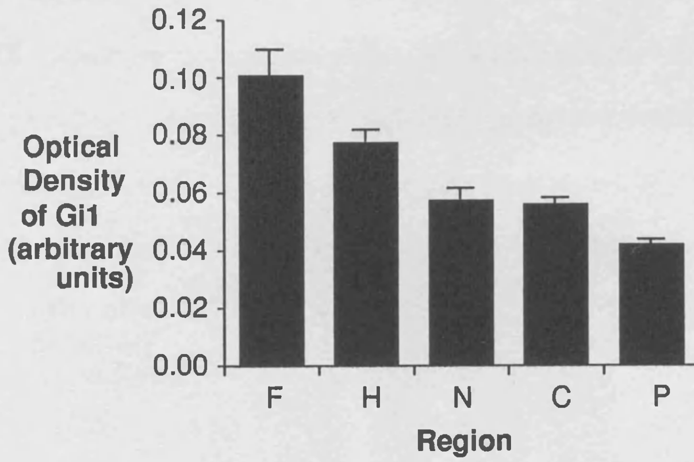
Crude membranes were prepared from the frontal cortex, hippocampus, neostriatum, cerebellum and pons of subjects C14, C19A and C34, and analysed by western blotting as described in "Methods". Each sample was loaded onto a polyacrylamide gel (100μg protein per lane) and the gel run overnight. After transfer of the proteins onto a nitrocellulose membrane, the blot was incubated with antisera SG2, and then with horse-radish peroxidase linked anti-rabbit IgG antisera. The resulting blot is shown in (a) with regions indicated thus, F = Frontal cortex, H = Hippocampus, N = Neostriatum, C = Cerebellum, P = Pons. RB = rat brain. Only the portion of the blot that contained the G protein bands is shown.

The blot was then analysed densitometrically. The levels of Gi1 and Gi2 in each region are given in (b) and (c) respectively. The values shown are the mean of the three subjects; error bars indicate the SEM.

(a)



(b)



(c)

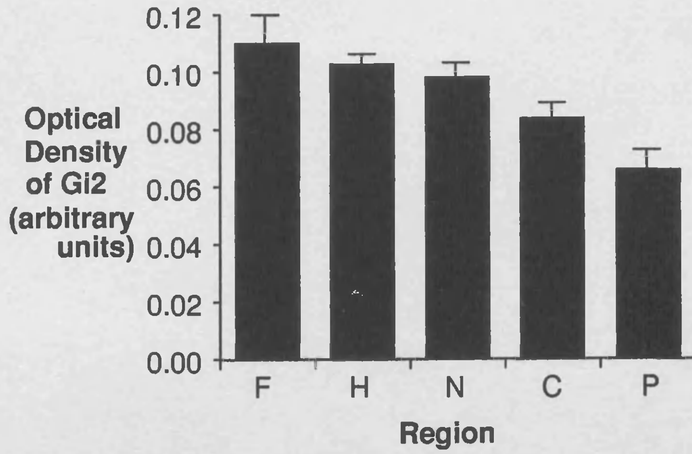


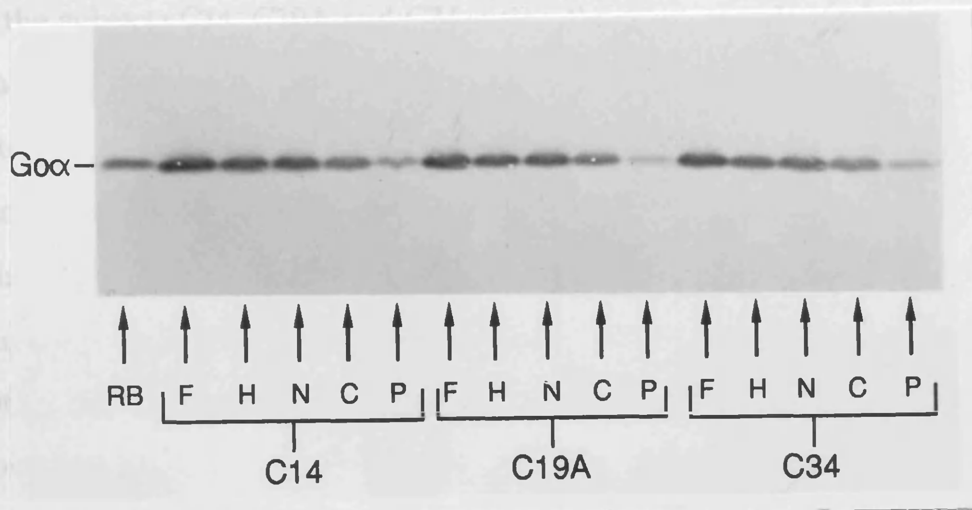
Figure 25.

The distribution of Goα in human brain.

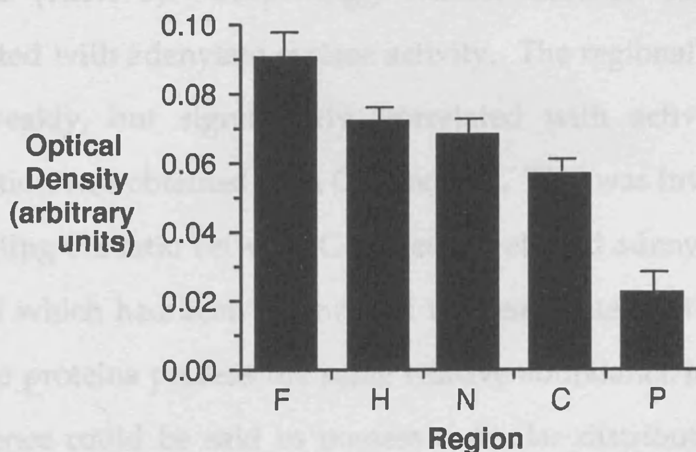
Crude membranes were prepared from the frontal cortex, hippocampus, neostriatum, cerebellum and pons of subjects C14, C19A and C34, and analysed by western blotting as described in "Methods". A polyacrylamide gel was loaded with 50μg of protein per lane and run overnight. The proteins were then transferred onto a nitrocellulose membrane and the blot incubated with antisera OC1, and then with horse radish peroxidase linked anti-rabbit IgG antisera. The resulting blot is shown in (a) with regions indicated thus, F = Frontal cortex, H = Hippocampus, N = Neostriatum, C= Cerebellum, P = Pons. RB = Rat Brain. Only the portion containing the Goα band is shown.

The blot was then analysed densitometrically. The level of Goα in each region is given in (b). The values shown are the mean of the three subjects; error bars indicate the SEM.

(a)



(b)



4.2 Distribution of adenylate cyclase in human brain.

The activity of adenylate cyclase was determined in five brain regions of the subjects C14, C19A and C34, using the same membrane preparations that were used to analyse the G proteins (section 4.1). Once again the activities observed in each region were similar in each subject except in the neostriatum which gave basal activities of 37.9, 83.4 and 33.2 pmols cAMP/min/mg for C14, C19A and C34 respectively. The average value for basal adenylate cyclase activity in the neostriatum should therefore be viewed with some caution. Of the five regions basal activity was highest in the frontal cortex followed by the hippocampus, neostriatum, cerebellum and pons (Figure 26). The relationship between the distribution of adenylate cyclase and each G protein was investigated using linear regressional analysis (Table 5). Surprisingly neither GsH or GsL was significantly correlated with adenylate cyclase activity. The regional distribution of $G_o\alpha$ was weakly, but significantly, correlated with activity while the best correlation was obtained with Gi1 and Gi2. This was investigated further by calculating the ratio between G protein levels and adenylate cyclase activity, both of which had been normalised to their values in the frontal cortex. If the two proteins possess the same relative abundance in each brain region, and hence could be said to possess a similar distribution, the normalised ratio between them should be unity. By using this approach, the distribution of adenylate cyclase activity in human brain appears to be most closely matched by that of Gi1 (Figure 27), although the analysis of more regions in a larger number of subjects would be needed to confirm this.

When adenylate cyclase was stimulated by fluoroaluminate the variation in activity between different regions was smaller than that observed for basal activity, since the extent of stimulation observed in the cerebellum and pons was greater than that of the other three regions (Figure 26). Since fluoroaluminate acts via its effect on G proteins, the level of these proteins were compared with the observed stimulation of adenylate cyclase

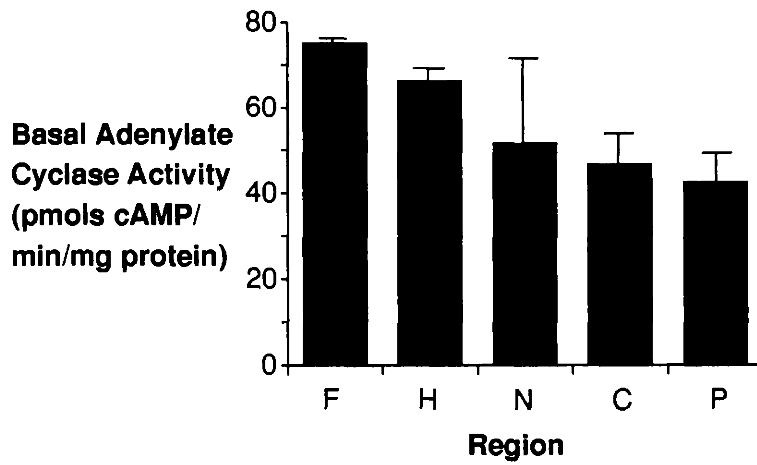
in each preparation. Adenylate cyclase stimulation was not significantly correlated with GsH levels (Table 5), and only weakly correlated that of GsL. However the ratio GsH/GsL was more strongly (negatively) correlated with adenylate cyclase stimulation, as were the levels of Gi1, Gi2 and Goα. The strongest correlation however was that observed between stimulation and the ratios Gsα/Giα, and Gsα/Goα.

Figure 26.

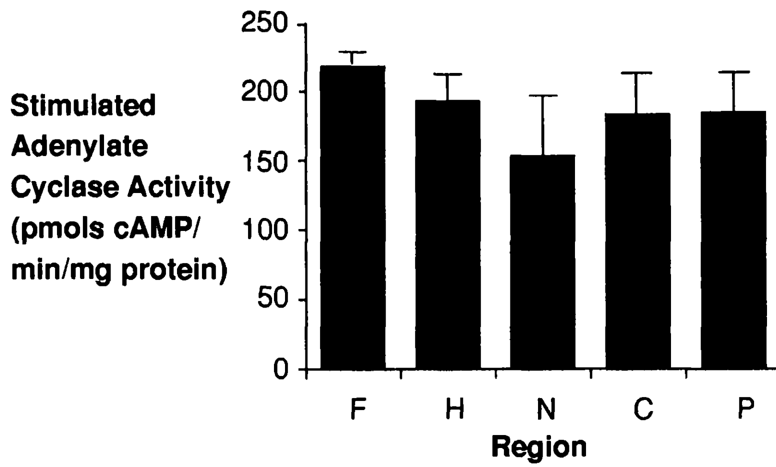
The distribution of adenylate cyclase activity in human brain.

Crude membranes were prepared from the frontal cortex, hippocampus, neostriatum, cerebellum and pons of subjects C14, C19A and C34, and assayed for adenylate cyclase activity as described in "Methods". Adenylate cyclase was assayed at 30°C for 16 minutes using 20µg of membrane protein per tube. Each sample was assayed in duplicate for both basal activity (a) and fluoroaluminate stimulated activity (b). The latter was carried out in the presence of 10µM aluminium chloride and 5mM sodium fluoride. Fluoroaluminate stimulated activity is also shown expressed as a percentage of basal activity for each region (c). Each region is indicated thus, F = Frontal cortex, H = Hippocampus, N = Neostriatum, C = Cerebellum, P = Pons. The values shown are the mean of the three subjects; error bars indicate the SEM.

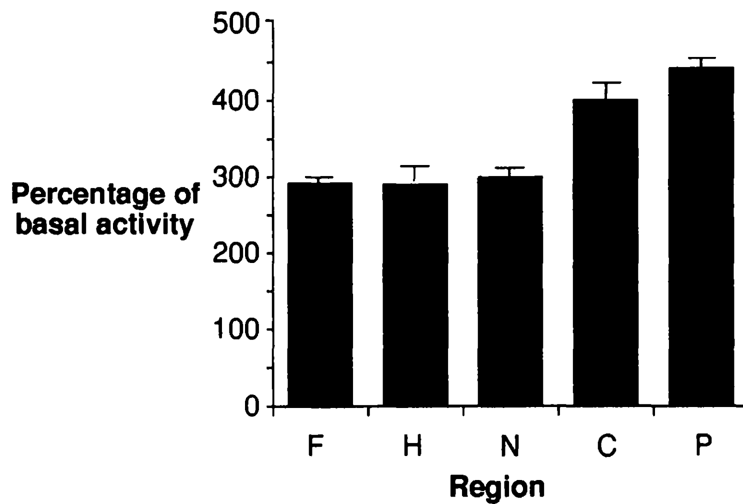
(a)



(b)



(c)



G protein level or ratio	Versus	Correlation coefficient
GsH	Basal activity	0.503
GsL	" "	-0.138
Gi1	" "	0.710 **
Gi2	" "	0.787 **
Go	" "	0.581 *
GsH	Stimulation	-0.174
GsL	"	0.525 *
GsH/GsL	"	-0.616 *
Gi1	"	-0.746 **
Gi2	"	-0.789 **
Go	"	-0.748 **
Total Gsα/Total Giα	"	0.804 **
Total Gsα/Total Goα	"	0.889 **

Table 5.

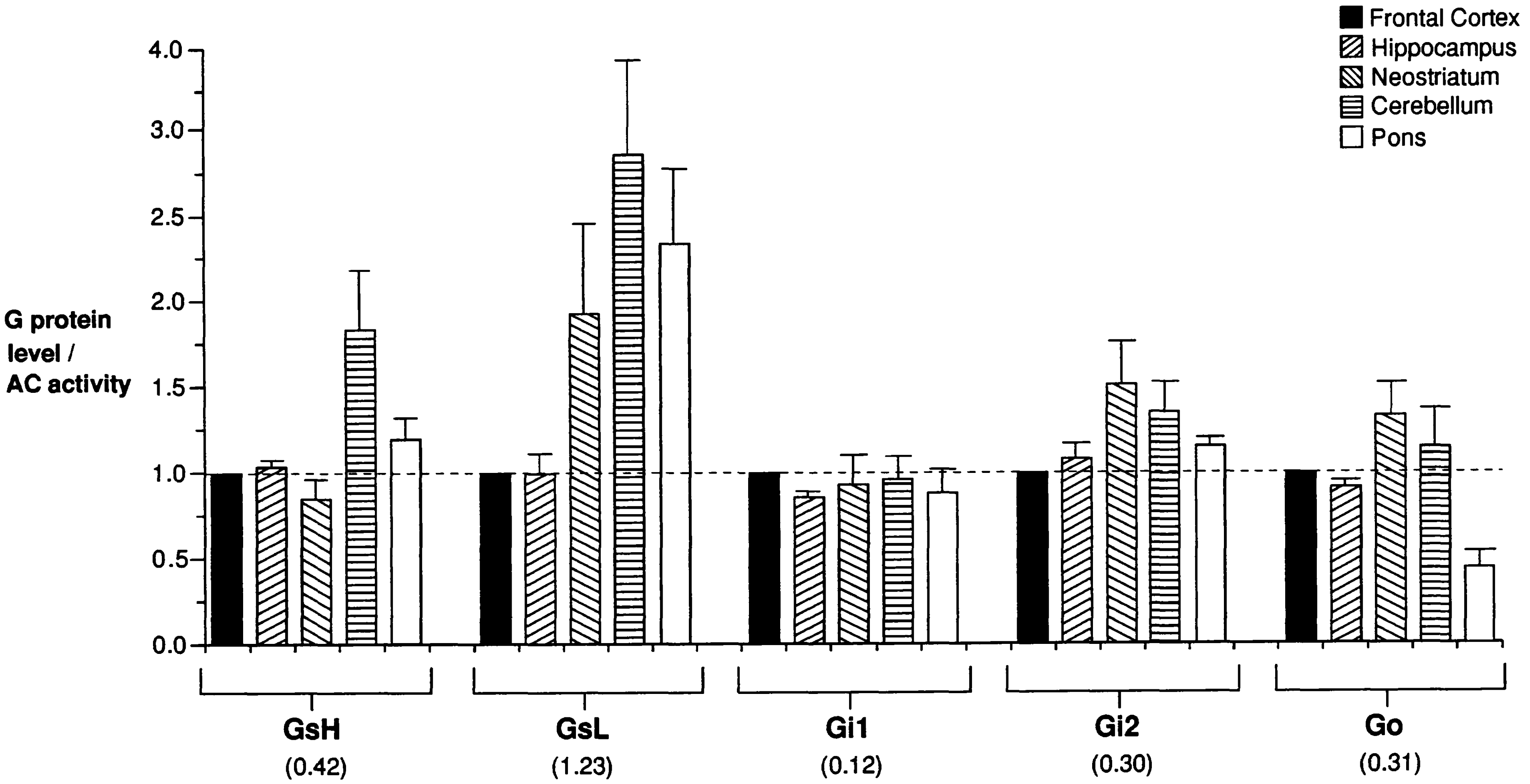
The relationship between G protein levels and basal adenylate cyclase activity, or its stimulation by fluoroaluminate.

The basal activity of adenylate cyclase, or its stimulation by fluoroaluminate relative to basal activity, in the frontal cortex, hippocampus, neostriatum, cerebellum or pons of subjects C14, C19A and C34 (Figure 26) was related to the levels of Gsα, Giα, Goα, or the ratio of Gsα to Goα, or Giα (Figures 23 to 25). This was accomplished by calculating Pearsons correlation coefficient to evaluate the strength of any linear relationship. Total Gs is the total of the optical densities of the bands corresponding to GsH and GsL on the western blot. Similarly total Gi is the sum of Gi1 and Gi2. The statistical significance of the observed regression coefficient is indicated thus, * = $P<0.05$, ** = $P<0.01$.

Figure 27.

Comparison of the distribution of basal adenylate cyclase activity in human brain to that of G protein alpha subunits.

The activity of adenylate cyclase and the levels of each G protein in each subject/region combination (Figures 23 to 26), were normalised to their value measured in the frontal cortex of each subject. The normalised G protein levels were then divided by the normalised adenylate cyclase activities, and are shown as the mean ratio of the three subjects, with bars showing the SEM. If the G protein possesses the same distribution as that of adenylate cyclase, the ratio between the two normalised values should be equal to unity in each region (shown by the dotted line). The numbers in brackets beneath are the average deviations from a ratio of one for each G protein. The ratio for the frontal cortex was not used for this calculation since this will always be equal to unity.


Frontal Cortex
Hippocampus
Neostriatum
Cerebellum
Pons
G protein
level /
AC activity
4.0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GsH
(0.42)
GsL
(1.23)
Gi1
(0.12)
Gi2
(0.30)
Go
(0.31)

4.4 Distribution of sodium potassium-dependent ATPase in the human brain.

The activity of sodium potassium-dependent ATPase was determined in the same preparations used in Sections 4.1 and 4.2. As before all subjects gave similar results. The highest activity was observed in the cerebellum, followed by a similar activity in the frontal cortex and pons, the lowest activities being observed in the hippocampus and neostriatum (Figure 28). The activity of the enzyme was not significantly correlated with any other measurement performed with the exception of GsL ($r=0.601$; $P<0.05$). Thus the heterogeneous distribution throughout the brain of adenylate cyclase etc. is not due to differences in the purity of crude membranes prepared from different regions.

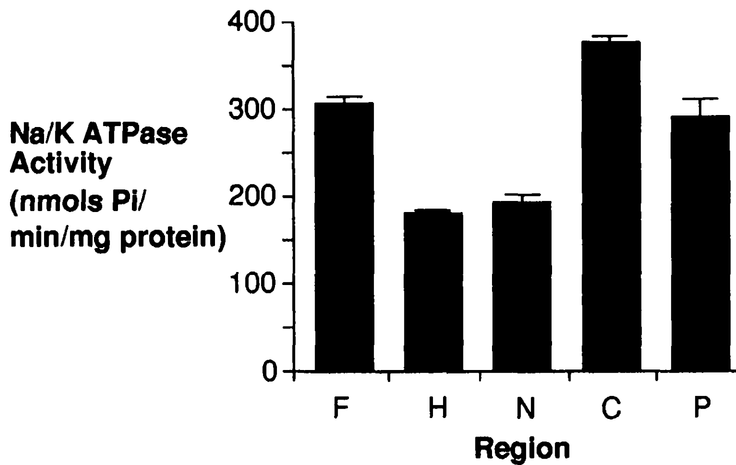


Figure 28.

The distribution of sodium potassium dependent ATPase activity in human brain.

Crude membranes were prepared from the frontal cortex, hippocampus, neostriatum, cerebellum and pons of subjects C14, C19A and C34, and assayed for sodium potassium dependent ATPase activity as described in methods "Methods". The enzyme was was assayed at 37°C for 15 minutes using 20μg of membrane protein per tube. Each sample was assayed in triplicate. Non specific ATPase activity was measured in the presence of 1mM ouabain. Each region is indicated thus, F = Frontal cortex, H = Hippocampus, N = Neostriatum, C = Cerebellum, P = Pons. The values shown are the mean of the three subjects; error bars indicate the SEM.

4.4 Distribution of G proteins, adenylate cyclase and sodium potassium-dependent ATPase in grey and white matter.

The right temporal cortex of subject C20 was dissected into grey matter, white matter and a mixture of both. Crude membrane preparations were made from all three and the levels of GsH, GsL, Gi1, Gi2 and Goα, and the activities of adenylate cyclase and sodium potassium-dependent ATPase measured. There was a marked difference in every parameter measured between grey and white matter (Table 6). In every case, except for Gi1, the mixture of grey and white gave a value intermediate to that of substantially grey and substantially white matter. Except for GsL the proteins were more abundant in grey matter than in white matter. However fluoroaluminate stimulated adenylate cyclase activity, expressed relative to basal activity, was approximately the same in grey and white matter.

Parameter	Grey Matter	Grey/White Matter	White Matter
GsH	0.207	0.186 (90%)	0.122 (59%)
GsL	0.058	0.111 (191%)	0.303 (522%)
GsH/GsL	3.57	1.68 (47%)	0.40 (11%)
Gi1	0.027	0.033 (122%)	0.019 (70%)
Gi2	0.035	0.022 (63%)	0.018 (51%)
Goα	0.245	0.206 (84%)	0.156 (64%)
Basal adenylate cyclase activity	68	62 (90%)	41 (60%)
Fluoroaluminate stimulated adenylate cyclase activity -			
(i) Absolute	251	221 (88%)	159 (67%)
(ii) Percent of basal	369	356 (96%)	389 (105%)
Na/K dependent ATPase activity	349	202 (58%)	102 (29%)

Table 6.

Levels of G proteins, adenylate cyclase and sodium potassium-dependent ATPase in temporal cortical grey and white matter.

The temporal cortex of subject C20 was thawed on ice and dissected into grey matter, white matter, and a mixture of grey and white matter. These were used to prepare crude membranes which were then analysed by western blotting to determine the levels of Gsα, Giα, and Goα, as well as being assayed for adenylate cyclase activity and sodium potassium-dependent ATPase activity as described in "Methods". Adenylate cyclase was assayed in duplicate at 30°C for 15 minutes, while sodium potassium dependent ATPase was assayed in triplicate 37°C for 15 minutes. G protein levels are given in optical density units, adenylate cyclase activity in pmols cAMP/min/mg protein and sodium potassium-dependent ATPase activity in pmols Pi/min/mg protein. The numbers in brackets indicate the percentage of that measured in grey matter.

5. RNA stability in human post mortem brain.

The results presented in Section 2.1 appeared to show that G protein mRNA levels could be reliably estimated in post mortem brain. As a result, experimental analyses of the effect of DAT on G protein levels were conducted in parallel with analyses of the level of G protein mRNAs. As will be seen however, the early indications that the mRNAs could be accurately measured in post mortem human brain proved to be unfounded, and data was generated that cast some doubt on other determination of mRNAs in post mortem brain that have been published by other laboratories. Because of this the investigation of human brain mRNA, together with supporting experiments with rat brain, are presented as a separate Section of the results.

5.1 Analysis of RNA prepared from human tissue.

RNA was prepared from human right frontal cortex and right hippocampus, using six control subjects and six DAT subjects in each case. To ensure that the purification procedure was not introducing any degradation, RNA was prepared in parallel from fresh rat brain. The RNA was then analysed by northern blotting using oligonucleotide probes complimentary to Gsα and Gβ mRNA, and to 28S rRNA. Autoradiograms derived from the analysis of the frontal cortex are shown in Figure 29, and that from the hippocampus is reproduced in Figure 31. The hybridisation pattern observed for each probe was similar in every human subject, and allowed the detection of the mRNAs encoding Gsα, Gβ1 and Gβ2. The hybridisation pattern for each probe is described fully in Section 1.4. The frontal cortex blot which had been hybridised to the probe complimentary to Gsα mRNA (Figure 29), was reprobed with Gs-52 oligonucleotide which is complimentary to the large forms of Gsα (Figure 30). This did not hybridise to the lower molecular size (1.2kb) species observed using the probe complimentary to all four forms of Gsα mRNA, in any of the subjects

analysed. This confirmed the view taken earlier (Section 1.4) that this band should not be included in any attempted quantification of Gsα mRNA. A visual inspection of the autoradiograms did not reveal any obvious difference in the intensity of the G protein mRNA bands in control and DAT subjects, however an attempt was made to quantify this using densitometry. Due to the smearing of the G protein mRNA bands and the large variability in signal intensity between subjects, densitometry proved difficult. However the values that were obtained are illustrated in Figure 32. There was no statistically significant difference in the relative amounts of Gβ1 or Gβ2 mRNA in the frontal cortex and hippocampus between control and DAT subjects, and no change in the relative amount of Gsα mRNA in the frontal cortex. (No numerical information was obtained about Gsα mRNA in the hippocampus since the hybridisation signal was too weak to allow densitometric analysis.) What is apparent is that the variability within each group is large (average standard deviation = 65% of mean). This high degree of variability is considered further in Section 5.3.

There was a significant degree of correlation observed between the amounts of each mRNA species measured in each subject. For example, Gsα versus Gβ2 mRNA in the frontal cortex gives a correlation coefficient of 0.90 (Figure 33). This suggested either an error in gel loading or that another factor, such as RNA degradation, is acting equally on each mRNA species.

The northern blots showed a considerable degree of band smearing (especially that of Gsα mRNA) in the human RNA, which was not present in the rat brain controls. This indicates that the RNA was at least partially degraded. When the blots were re-hybridised with the 28S rRNA probe, a number of bands are visible in addition to the 28S rRNA band itself (Figures 29 and 31). In many cases the intensity of the 28S rRNA band itself is low, and is accompanied by a relatively higher hybridisation to the lower molecular size species. This indicates that the larger species are being broken down to form the smaller ones. The pattern of bands was similar to

that seen in the longer time points of the experiment used to assess the effect of delaying the freezing of the brain in rat (Section 2.3). They are therefore assumed to be degradation products of 28S rRNA. It is apparent that the extent of 28S rRNA breakdown differs between human subjects. In the two brain regions examined there was a marked correlation between the extent of 28S rRNA degradation, and the intensity of the G protein mRNA bands. Thus, where a tissue exhibited extensive degradation of 28S rRNA, in most cases the hybridisation signal of G protein mRNAs were also relatively weak and smeared. It therefore became clear that the degradation of 28S rRNA degradation was an excellent indicator of overall RNA degradation that could be used in further experiments designed to investigate RNA degradation. It should be noted that the 28S rRNA oligonucleotide hybridises less strongly to rat RNA than to human RNA, making it difficult to compare 28S rRNA degradation in rat and human RNA on the same blot by this method. However, the two can be compared on ethidium bromide-stained gels, or on the blots themselves using the RNA shadow method (Herrera & Shaw, 1989).

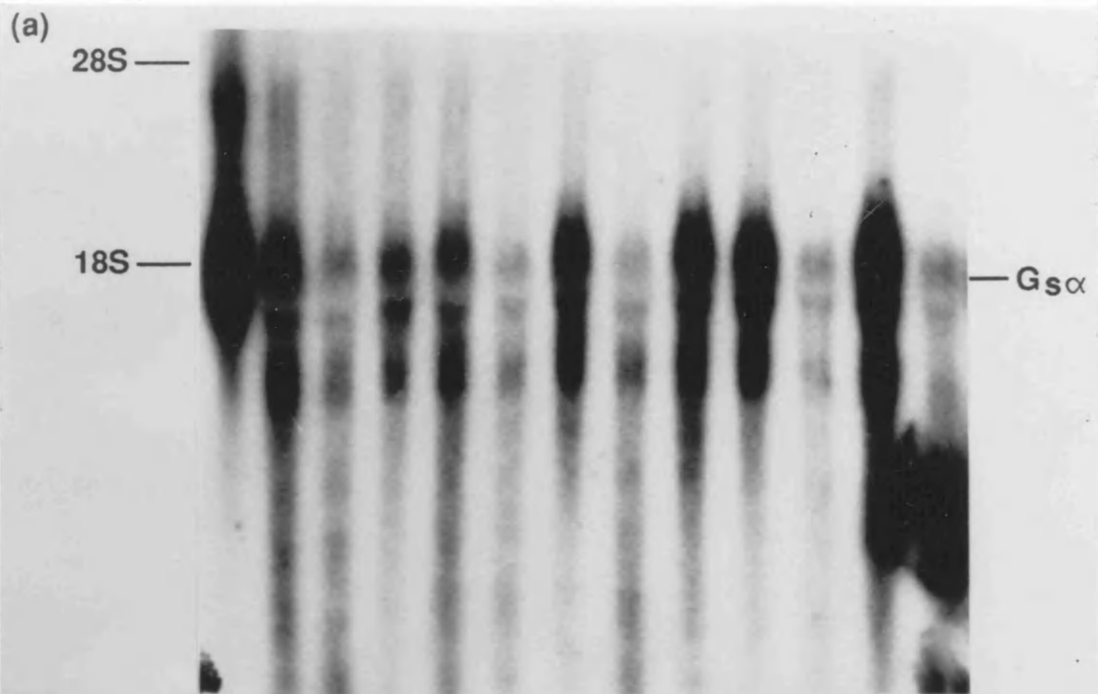
There was no evidence for increased RNA degradation in diseased brain compared to normal tissue. Neither was there a strong correlation between the degree of degradation and the time between death and freezing of the tissue. The effects of this variable, along with age and length of storage time, are considered further in subsequent sections.

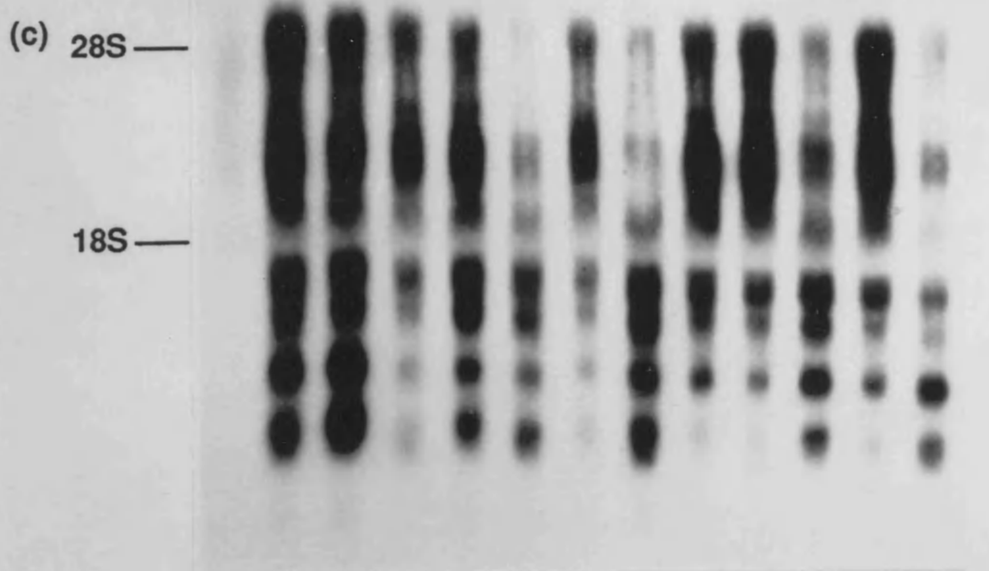
Figure 29.

Analysis of RNA purified from the frontal cortex of control subjects and from those who had suffered from DAT.

RNA was purified from the right frontal cortex of six control subjects and six DAT subjects as described in "Methods". In parallel to these preparations, RNA was purified from fresh rat brain. The RNA was then analysed by northern blotting as described in "Methods". Two agarose gels were loaded with 20μg of denatured RNA per lane and run overnight. The RNA was then transferred to Hybond N membrane. One blot was hybridised to a labelled oligonucleotide complimentary to Gsα mRNA (a) and the other to an oligonucleotide complimentary to Gβ mRNA (b). After washing, the blots were exposed to X-ray film for 3 days. Blot (b) was then stripped and rehybridised to an oligonucleotide complimentary to 28S rRNA (c). This was then exposed to X-ray film for 20 minutes.

The subject number is shown beneath each lane (C = Control, A = DAT, RB = Rat Brain). PMD indicates the post mortem delay of each subject in hours i.e. the time between death and freezing of the tissue. Each blot was stained with methylene blue stain to show the positions of 28S and 18S rRNA, which acted as molecular size markers.





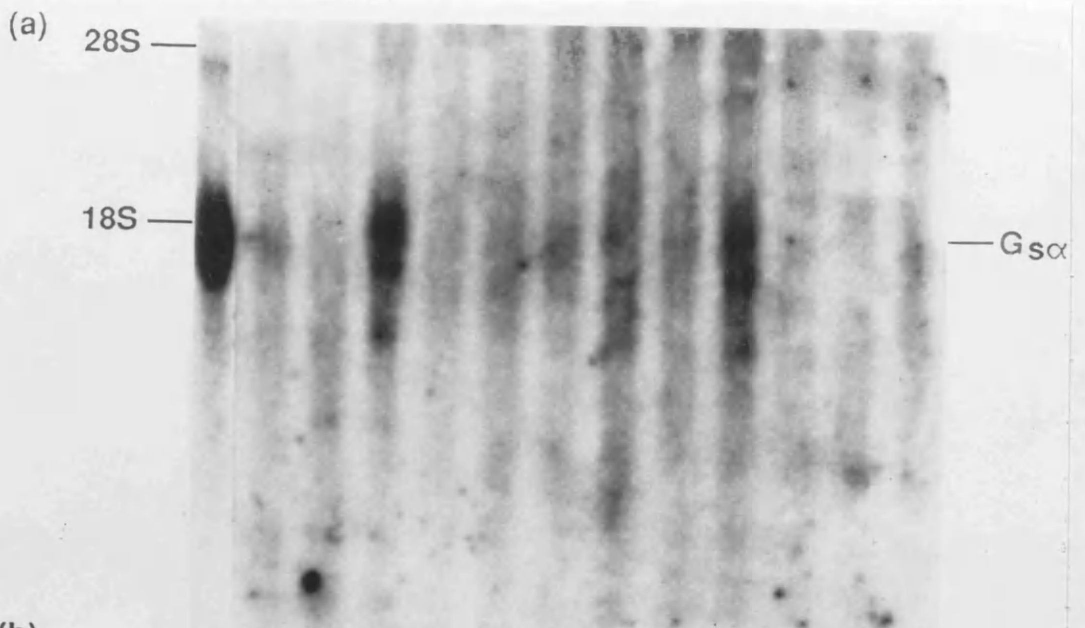
	Rat	C14	A21	C16	A22	C18	A23	C19	A26	C23	A30	C34	A33
PMD	0	11	6	17	6	19	15	7	2	4	4	6	8

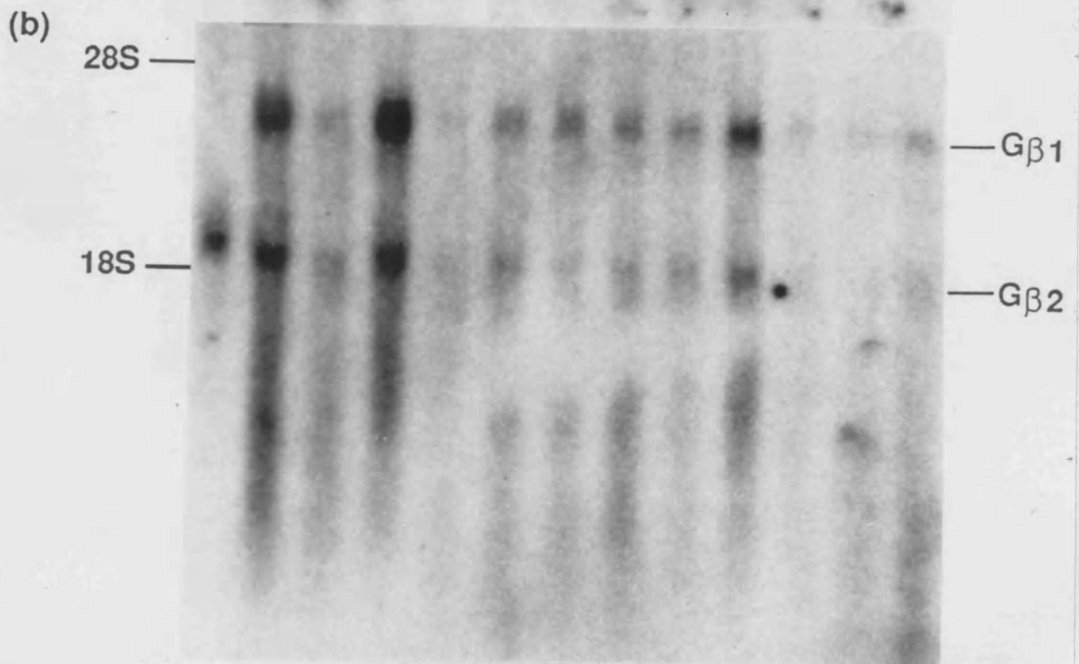
Figure 31.

Analysis of RNA purified from the hippocampus of control subjects and from those who had suffered from DAT.

RNA was purified from the right hippocampus of six control subjects and six DAT subjects as described in "Methods". In parallel to these preparations RNA was purified from fresh rat brain. The RNA was then analysed by northern blotting as described in "Methods". Two agarose gels were loaded with 20μg of denatured RNA per lane and run overnight. The RNA was then transferred to Hybond N+ membrane. One blot was hybridised to a labelled oligonucleotide complimentary to Gsα mRNA (a) and the other to an oligonucleotide complimentary to Gβ mRNA (b). After washing the blots were exposed to X-ray film for 4 days. Blot (a) was then stripped and rehybridised to an oligonucleotide complimentary to 28S rRNA (c). This was then exposed to X-ray film for 20 minutes.

The subject number is shown beneath each lane (C = Control, A = DAT, RB = Rat Brain). PMD indicates the post mortem delay of each subject in hours i.e. the time between death and freezing of the tissue. Each blot was stained with methylene blue stain to show the positions of 28S and 18S rRNA, which acted as molecular size markers.


(a)
28S
18S
Gsα


(b)
28S
18S
Gβ1
Gβ2

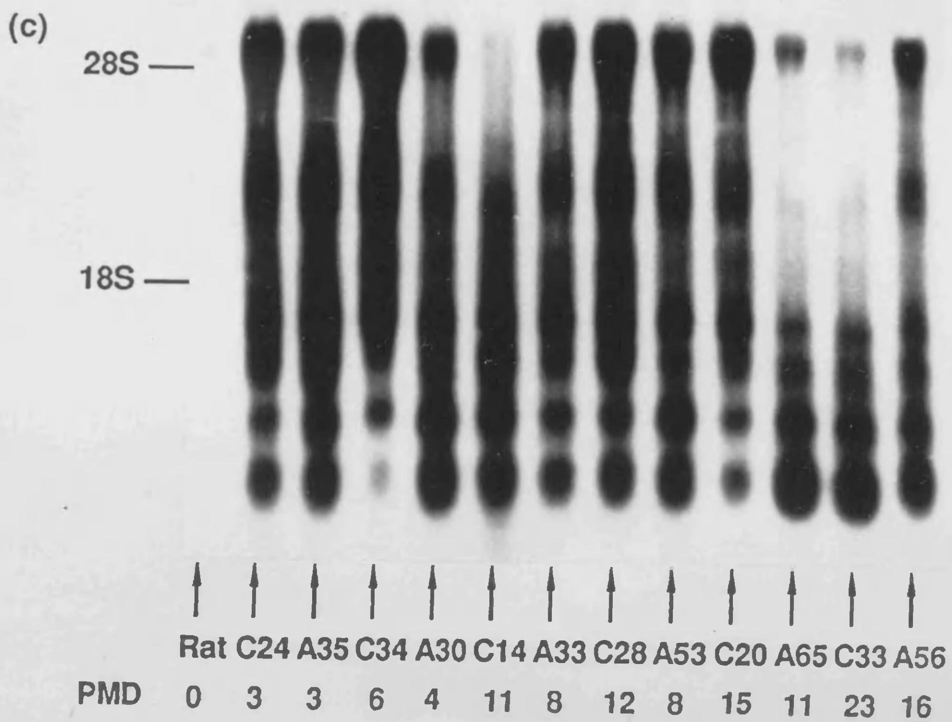
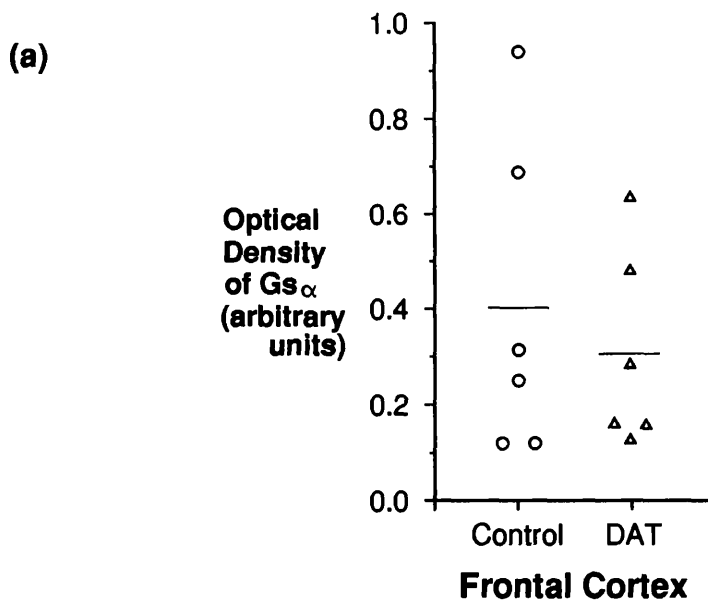
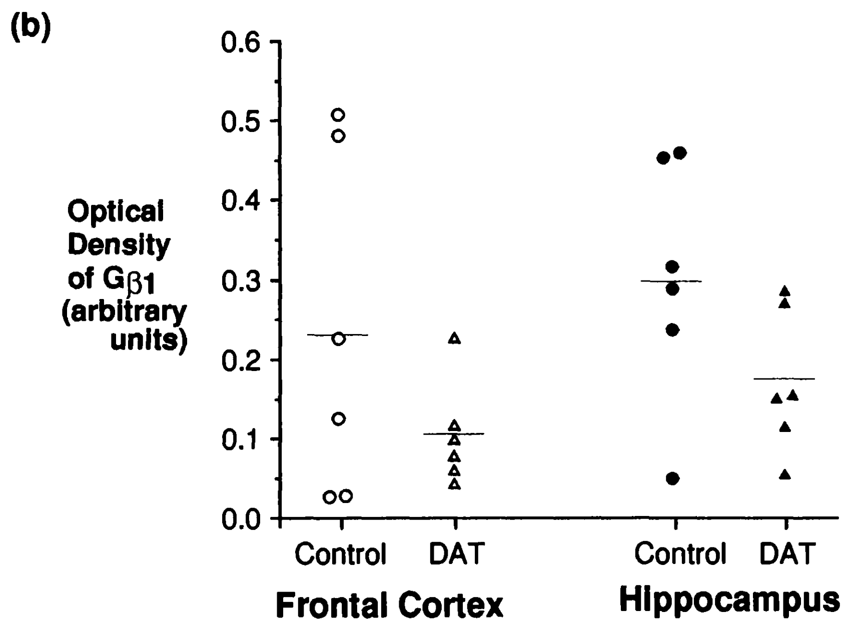

(c)
28S
18S
Rat C24 A35 C34 A30 C14 A33 C28 A53 C20 A65 C33 A56
PMD 0 3 3 6 4 11 8 12 8 15 11 23 16

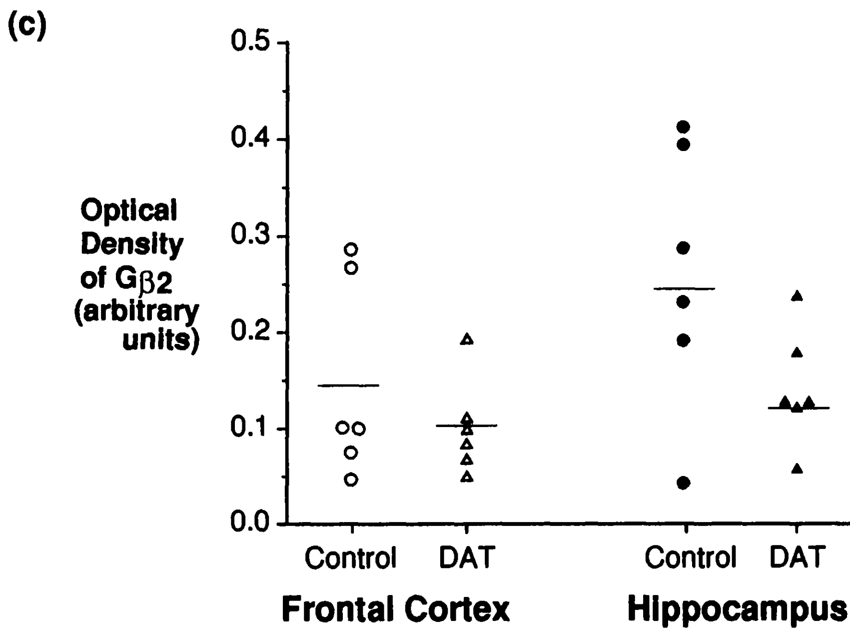
Figure 32.

Densitometric analysis of northern blots in Figures 29 and 31.

The northern blots showing the analysis of RNA prepared from human frontal cortex and hippocampus (Figures 29 and 31) were analysed by densitometry. The distribution of optical densities in each group are shown for (a) Gsα, (b) Gβ1 and (c) Gβ2 mRNA. No analysis is shown for Gsα mRNA in the hippocampus since the hybridisation signal was too weak to allow this. Bars indicate the mean optical density in each group.


(a)
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density of Gsα (arbitrary units)
Control
DAT
Frontal Cortex


(b)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density of Gβ1 (arbitrary units)
Control
DAT
Control
DAT
Frontal Cortex
Hippocampus


(c)
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density of Gβ2 (arbitrary units)
Control
DAT
Control
DAT
Frontal Cortex
Hippocampus

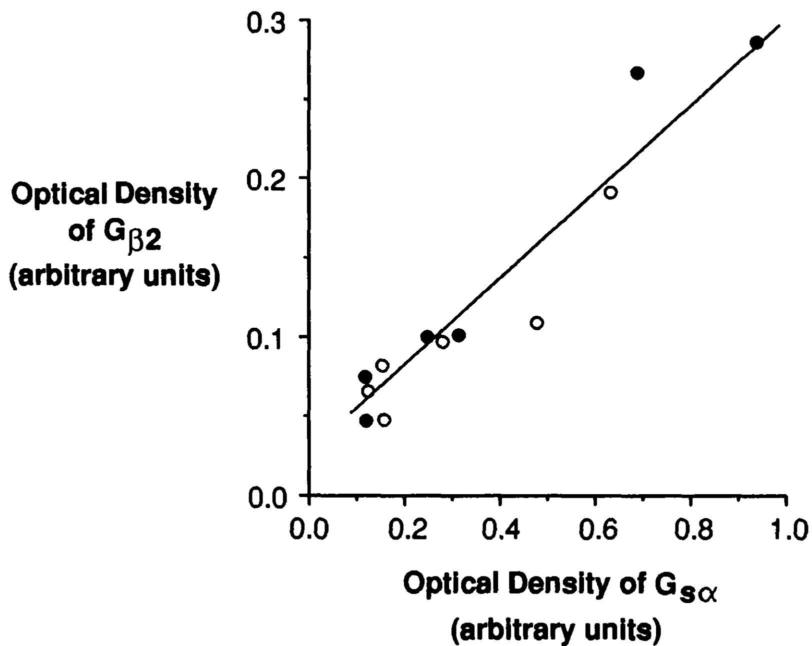


Figure 33.

The relationship between the amounts of Gsα and Gβ2 mRNAs detected in each subject by northern blotting.

The northern blots in Figure 29 (human frontal cortex) were analysed densitometrically to give the relative amounts of each RNA species (Figure 32). The graph opposite shows the relationship between the optical density of the Gsα bands and that of Gβ2. Filled circles indicate control subjects while open circles indicate DAT subjects. The line drawn through the points was determined by linear regression and has a correlation coefficient of 0.90.

5.2 The degradation of 28S rRNA in three regions of the human brain.

To explore the possibility that RNA degradation could differ in various brain regions, RNA was purified from the frontal cortex, hippocampus and cerebellum of three human subjects, all of whom had been used in the investigation of G protein mRNA (Figures 29 and 31). In two cases both hemispheres were examined. The subsequent analysis of 28S rRNA (Figure 34) shows that the extent of degradation is broadly similar in all the regions examined within each subject, .

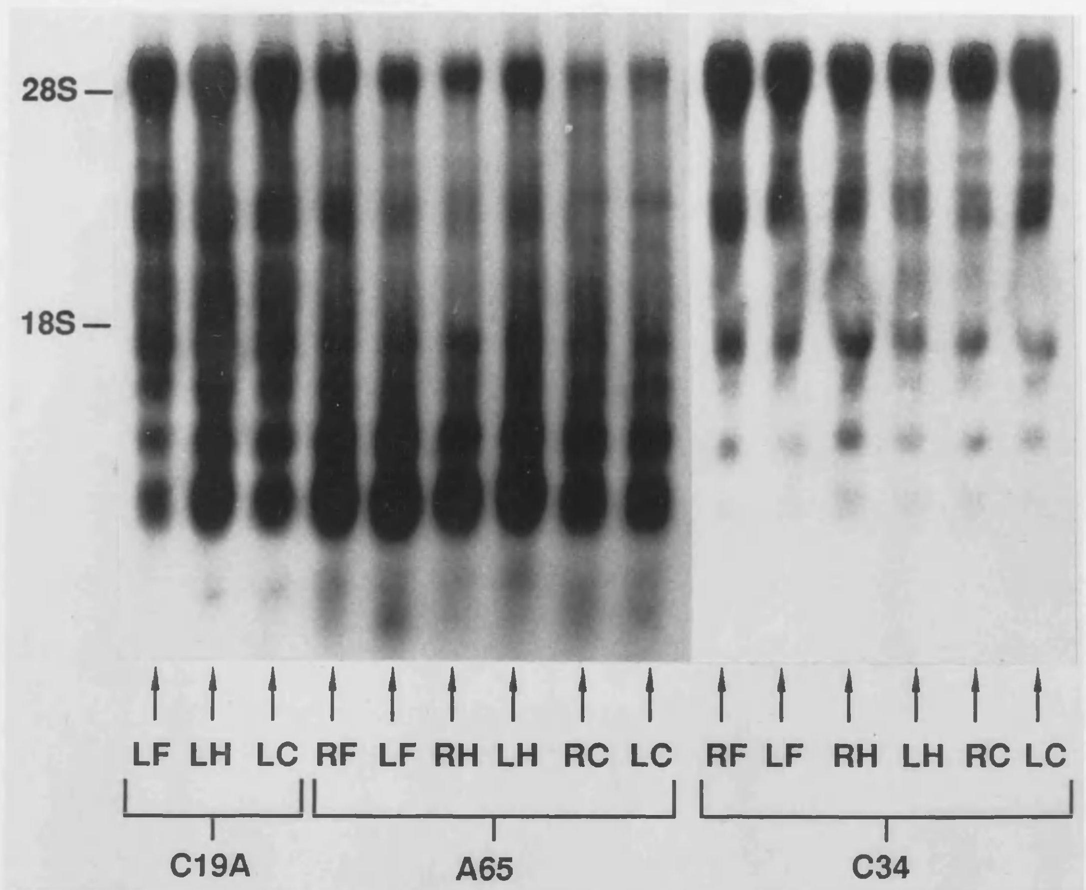


Figure 34.

The extent of 28S rRNA degradation in different regions of three human subjects.

RNA was purified from the frontal cortex, hippocampus and cerebellum of subjects C19A, C34 and A65 as described in "Methods". In the latter two cases, both right and left hemispheres were used. The RNA was then analysed by northern blotting as described in "Methods". 20µg of RNA per lane was loaded onto two agarose gels and the gels ran overnight. After transfer of the RNA onto Hybond N+ membranes, the blots were hybridised to a labelled oligonucleotide complimentary to 28S rRNA. After washing, the blots were exposed to X-ray film for 20 minutes. The positions of 18S and 28S rRNA were visualised by methylene blue staining of the blot, and serve to indicate molecular size. F = Frontal cortex, H = Hippocampus, C = Cerebellum. These are preceded by L or R to indicate left or right hemisphere.

5.3 The inter subject variability of the parameters measured in human brain.

In view of the data in Section 5.1 indicating that RNA degradation could be substantial in human post mortem brain, it was decided to look further at some of the parameters that could be linked with macromolecular instability. The variability of RNA levels assayed was therefore compared with that of the other parameters measured and related to patient age, post mortem delay and the length of time for which the tissue had been stored.

The standard deviation of the data presented in the preceding Sections was calculated for all of the parameters measured in each of the regions examined. For each of the datasets the standard deviation in the control and DAT groups was compared using an F-test (see "Methods"). In no case was there any evidence to suggest that the variability of the data was greater in one group than the other (data not shown; these tests had been done previously to ensure the validity of using t-tests with pooled variance). To allow comparison of variance between the various measures employed all standard deviations were converted to coefficients of variation (CV) i.e. SD expressed as a percentage of the mean. The various datasets were arranged into groups and the average CV calculated for each category as shown in Table 7. For all of the categories which involved the measurement of protein levels or enzyme activities, the CV was in the order of 30%. The least variable measure was that of stimulated adenylate cyclase expressed as a percentage of basal activity which gave an average CV of 11%. The highest inter subject variability was observed in the measurement of G protein mRNA, which had a high average CV of 65%. This included the highest individual CV recorded in the entire study, this being 92% observed in the measurement of Gβ1 mRNA in the right frontal cortex of control subjects. For comparison to the human studies, the CV was calculated for the basal adenylate cyclase activities measured in rat brain in the study used to assess the effect that post mortem delay (at 4°C) had on this enzyme

(Section 2.5). This study detected no change in activity over the period studied, and therefore the variance of this data can be used to estimate the extent of variability that is achievable using the techniques employed, this being a CV of 9%.

The effect of the various levels of variance in the data on the statistical methods employed in this study were examined using a form of *post hoc* power analysis. This allowed the smallest difference that could be detected between two groups, using an unpaired, two-tailed Students t-test, to be calculated. The results of this are listed in Table 7, and show that, for measures on proteins, a difference of 20-30% between mean values would be required to give statistically significant difference between control and DAT groups using a significance level of 0.05. In contrast the studies on RNA would require a difference of 70% between the groups for significance level of 0.05, and 107% for a significance level of 0.01.

The effect, upon the parameters measured, of post mortem delay, subject age, and the time the tissue had been kept in storage prior to use, was examined using linear regressional analysis. There was no significant linear correlation between post mortem delay or subject age, and any parameter measured in human tissue (Table 8). The length of time that the tissue had been stored at -80°C showed no significant correlation with any measurement except with that of sodium potassium dependent ATPase activity in the frontal cortex and hippocampus ($r = 0.746$ and 0.707 respectively; $p<0.01$). Note that the correlation (illustrated in Figure 35) is positive i.e the longer the tissue has been stored the higher the activity of the enzyme.

Parameter	Sample size	Average CV (%)	Required difference (%)	
			P<0.05	P<0.01
G protein mRNA	6	65	71	107
G proteins (left hemisphere)	8	30	31	42
Basal adenylate cyclase	8	27	28	40
Stimulated adenylate cyclase -				
Absolute	8	29	30	42
Proportion of basal	8	11	12	18
Na/K dependent ATPase	8	22	23	33
Choline acetyl transferase	8	32	33	46
Adenylate cyclase activity in rat	8	9	10	14

Table 7.

The inter-subject variability of the parameters measured in this study.

The standard deviation of each of the parameters measured in human tissue was expressed as a percentage of the mean value (the coefficient of variation or CV). The various CVs were arranged into the groupings given in the table and averaged to give a measure of the variability of that parameter. The sample size used for each study is also shown. The sample size and average inter-subject variability were used to calculate the smallest difference in the mean values of two groups e.g. control and DAT, that was required to give a statistically significant result using an unpaired, two tailed, Students t-test, assuming that the variability in the data is equal in both groups. The change required to a statistically significant difference is shown for significance levels of 0.05 and 0.01. The "adenylate cyclase in rat brain" category uses the variability of the values derived from the study to assess the effect of post mortem delay, at 4°C, on basal adenylate cyclase activity (Section 2.2).

Table 8.

Correlation of various parameters with the age, post mortem delay and length of tissue storage time of each subject.

The levels of G protein alpha subunits and the activities of several enzymes measured in the left frontal cortex (F. Cortex) and left hippocampus (Hipp.), together with the levels of G protein mRNAs measured in the right frontal cortex and right hippocampus, were related, using linear regressional analysis, to the post mortem delay, age, or length of time in storage. The correlation coefficient for each analysis is shown. Where there was no significant difference between control and DAT subjects for the parameter being examined, both groups were analysed together. Where a difference had been detected, the two groups were analysed separately the result of the correlation analysis being given as control, DAT. A statistically significant correlation of $P<0.01$ or $P<0.001$ is indicated by ** or *** respectively. The number of subjects used for the mRNA correlations was 12, while the protein studies employed 16 subjects, except in the case of correlations with subject age in the hippocampus where the size was 15, as the age of subject A35 was unknown. ND = not done.

Parameter	Post mortem delay		Age		Time in storage	
	F. Cortex	Hipp.	F. Cortex	Hipp.	F. Cortex	Hipp.
GsH	0.307	-0.430	-0.331	0.052	-0.078	0.053
GsL	0.036	-0.003	-0.124	-0.245	0.099	0.147
Gi1	0.322	-0.036	-0.007	-0.350	0.325	-0.490
Gi2	-0.052	0.237	0.318	-0.106	-0.240	0.393
Goα	-0.384	0.485	-0.355	-0.254	-0.084	0.439
Gsα mRNA	0.305	ND	-0.026	ND	-0.416	ND
Gβ1 mRNA	-0.308	0.206	-0.116	0.091	-0.461	0.157
Gβ2 mRNA	-0.334	0.099	-0.124	0.022	-0.421	0.165
Adenylate cyclase activity -						
Basal	-0.028, 0.535	-0.245	0.264, 0.560	0.247	0.356, -0.112	0.150
Stimulated (absolute)	-0.323, 0.560	-0.128	-0.253, 0.387	0.194	-0.273, 0.154	0.284
Stimulated (relative to basal)	0.333, 0.237	0.255	0.292, -0.703	-0.032	0.368, 0.523	0.341
Na/K dependent ATPase	0.019	0.053	-0.272	0.055	0.746***	0.707**
Choline acetyl transferase	-0.398, 0.380	-0.626, 0.083	-0.059, 0.470	0.338, 0.057	0.090, -0.501	-0.501, -0.135

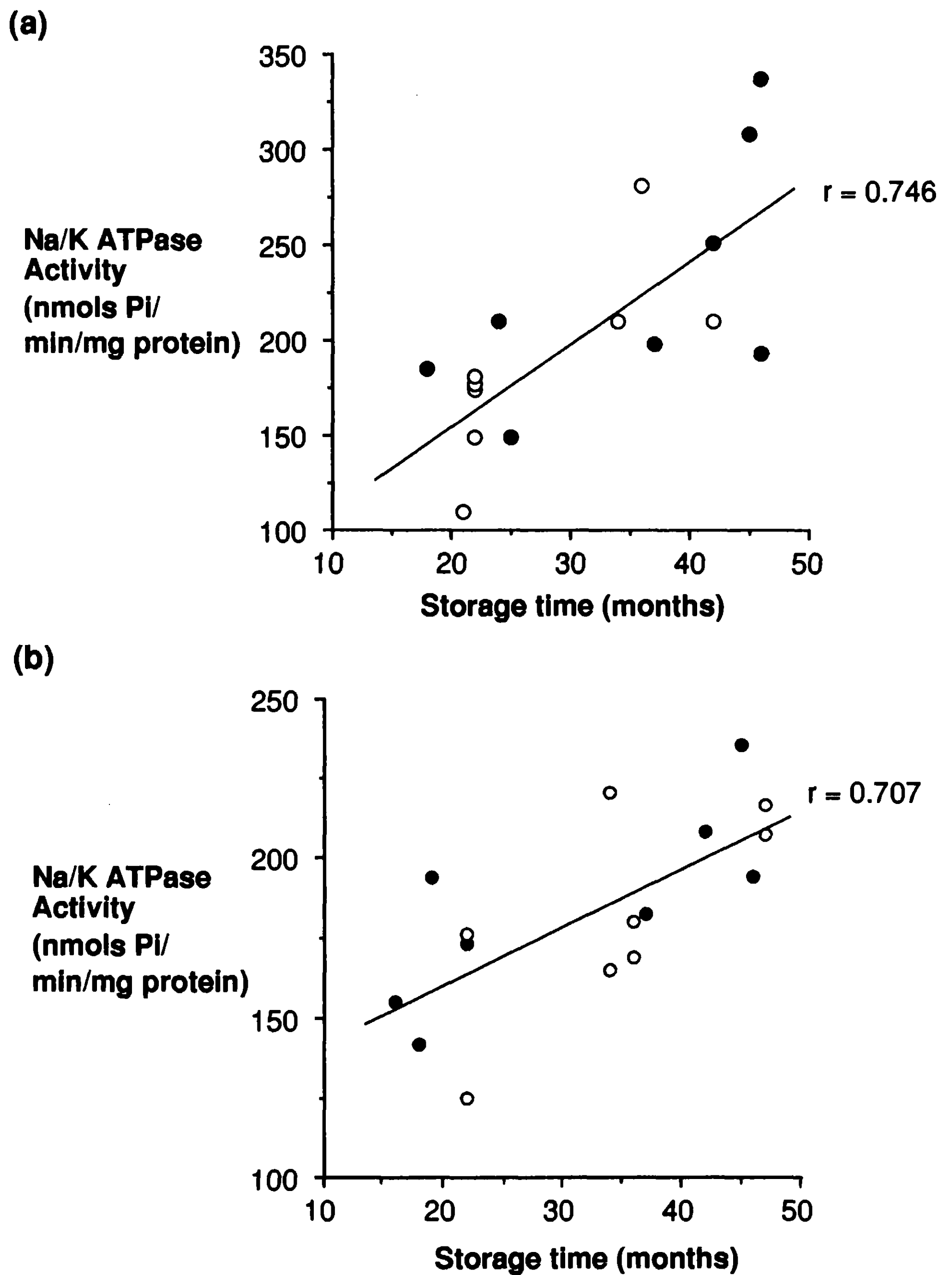


Figure 35.

Correlation between sodium potassium dependent ATPase activity in frontal cortex and hippocampus, and the length of tissue storage time.

The activities of sodium potassium dependent ATPase in the brains of eight control and eight DAT subjects (section 3.5), were compared to the length of time each brain had been stored at -80°C. This was accomplished using linear regressional analysis. The relationship between the two variables is shown for (a) the frontal cortex and (b) hippocampus. Filled circles indicate control subjects, and open circles represent DAT subjects.

5.4 Possible sources of RNA degradation modelled in rat brain.

The previous Sections have shown that, of all the parameters studied in human tissue, the measurement of mRNA levels show the largest inter subject variation. There were also indications that this variability might be due to different amounts of RNA degradation in each subject. These analyses failed however to relate the RNA degradation to subject age, post mortem delay, time of tissue storage or disease state. An attempt was therefore made to find conditions that reproduced this degradation in rat brain.

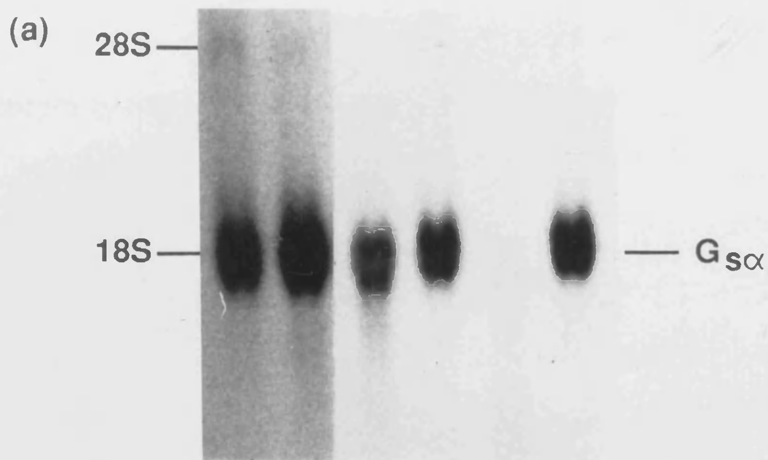
5.4.1 The effect of freeze/ thawing of rat brain on RNA integrity.

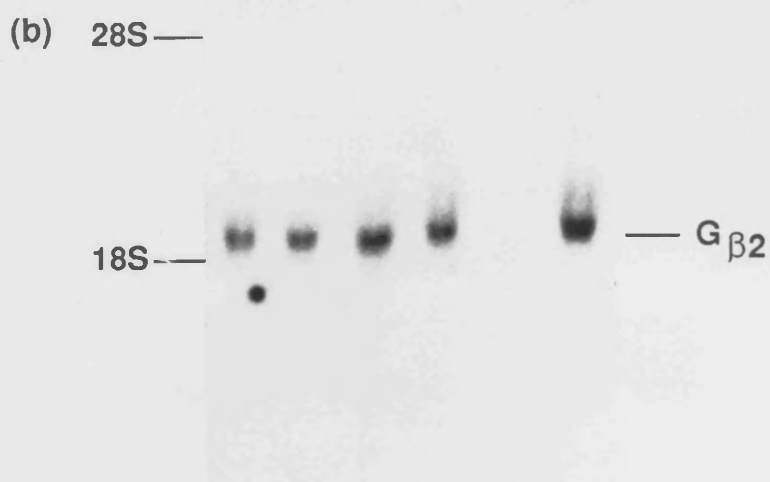
The brains of sacrificed adult male Wistar rats were rapidly removed, the cerebellum dissected away and the brain cut in half down the mid line. One hemisphere was left at 20°C for 15, 30 or 60 minutes, while the other was frozen to -80°C then left at 20°C for the same length of time. Figure 36 shows the result of the northern blot analysis of RNA purified from each brain hemisphere. After fifteen minutes there was no detectable difference between control and freeze/thawed hemispheres, while at thirty minutes a small amount of degradation was apparent in the freeze/thawed tissue. However, after one hour there was no G protein mRNA detectable in the freeze/thawed tissue, this being accompanied by pronounced 28S rRNA degradation.

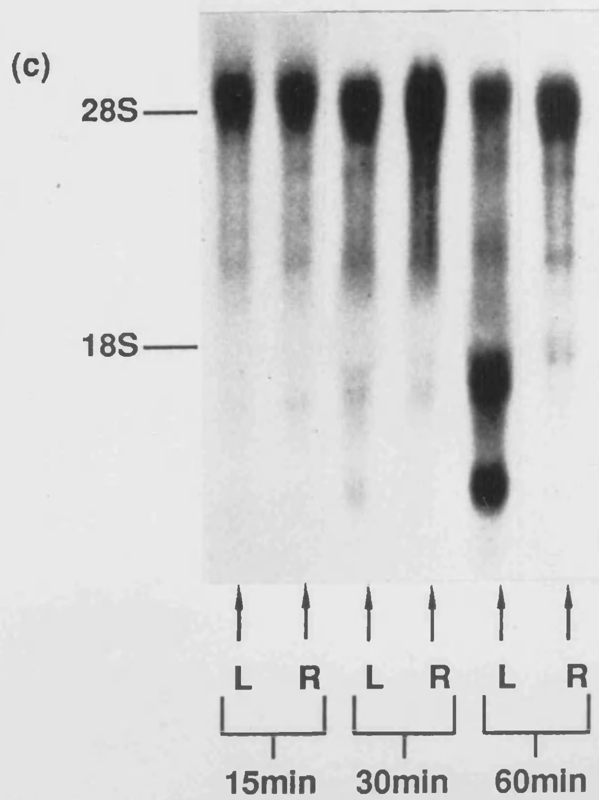
Figure 36.

The effect of freeze/thawing of tissue on the integrity of RNA in rat brain.

Male Wistar rats were sacrificed, and their brains removed and cut into two hemispheres. The right hemisphere was placed at 20°C for 15, 30 or 60 minutes as indicated. The left hemisphere was frozen to -80°C and then left at 20°C for the same length of time, before RNA was purified as described in "Methods". L or R indicates the hemisphere used. The RNA was then analysed by northern blotting as described in "Methods". An agarose gel was loaded with 20μg of RNA per lane and ran overnight. The RNA was then transferred onto a Hybond N+ membrane and hybridised to labelled oligonucleotide probes complimentary to (a) Gsα mRNA, (b) Gβ mRNA and (c) 28S rRNA. After washing, the blots were exposed to X-ray film for 2 days ((a) and (b)), or 10 minutes (c). The position of 18S and 28S rRNA was visualised by methylene blue staining of the blot, and serve to indicate molecular size.


(a)
28S
18S
$G_{s\alpha}$


(b)
28S
18S
$G_{\beta 2}$


(c)
28S
18S
L R L R L R
15min 30min 60min

5.4.2 The effect of focal forebrain ischemia on RNA integrity in rat brain.

The human subjects from which the brains in the present study were derived, died from various causes but most common was pneumonia. In pneumonia, breathing difficulties may lead to tissue hypoxia well before death. Indeed it is possible that such an effect occurs, to varying degrees, in any persons who did not die suddenly. One effect of ante mortem hypoxia may be to cause increased RNase activity leading to greater RNA degradation (see "Discussion", Section 1.2.4). An attempt was therefore made to mimic this in rats by inducing focal forebrain ischemia.

The left middle cerebral arteries of five male Wistar rats were occluded as described in "Methods". Two sham operated animals were also produced. Blood was withdrawn from the animals to reduce the blood pressure to between 50 and 55 mm Hg for a period of thirty minutes. This period of hypotension has been shown to increase the volume of damaged tissue caused by mid cerebral artery occlusion (Osborne 1987). The blood pressure of the animals was monitored throughout the experiment and is shown in Figure 37. The animals were sacrificed 3 hours after middle cerebral artery occlusion, the brains removed, and the right and left cortices dissected and stored at -80°C. RNA was subsequently purified from the tissue and analysed by northern blotting, using oligonucleotide probes complimentary to Gβ mRNA and 28S rRNA (Figure 38). Unfortunately no information about Gsα mRNA was obtained since this oligonucleotide would no longer incorporate ^{32}P. The Gβ oligonucleotide also exhibited very low incorporation of ^{32}P (11%) and gave very high background hybridisation. The reason for the failure of the G protein oligonucleotides is unclear since they were stored under the same conditions as the 28S rRNA oligonucleotide. Despite this the Gβ bands are still visible on the autoradiogram. There was no band smearing apparent in any of the RNA preparations, whether ischemic (left cortex) or control (right cortex). In addition the Gβ signal intensity is broadly similar in most preparations. The

variability in signal intensity that did occur was mimicked by that of 28S rRNA hybridisation, indicating that this was due to errors in gel loading rather than to differences in RNA degradation in each preparation. Some 28S rRNA degradation products were visible in each sample, however they were not consistently greater in abundance within the ischemic cortex than that observed within the control cortex, or in the sham operated animals.

Figure 37.

Time course of blood pressures during the three hour survival period following middle cerebral artery occlusion.

The blood pressures of the seven animals used to assess the effect of middle cerbral artery occlusion on RNA integrity (as described in the legend to Figure 38) are shown at several time points during the experiment. The values shown are the mean of seven animals; bars indicate the SEM.

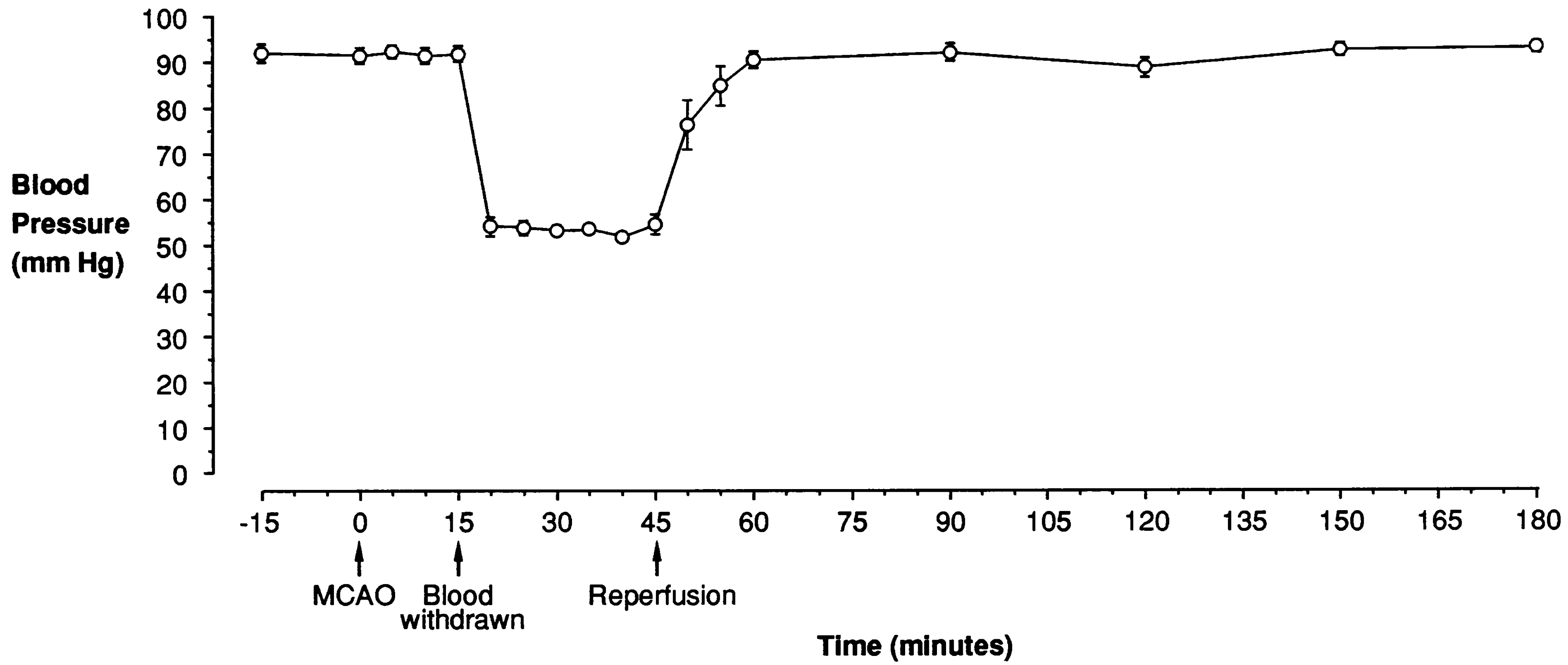
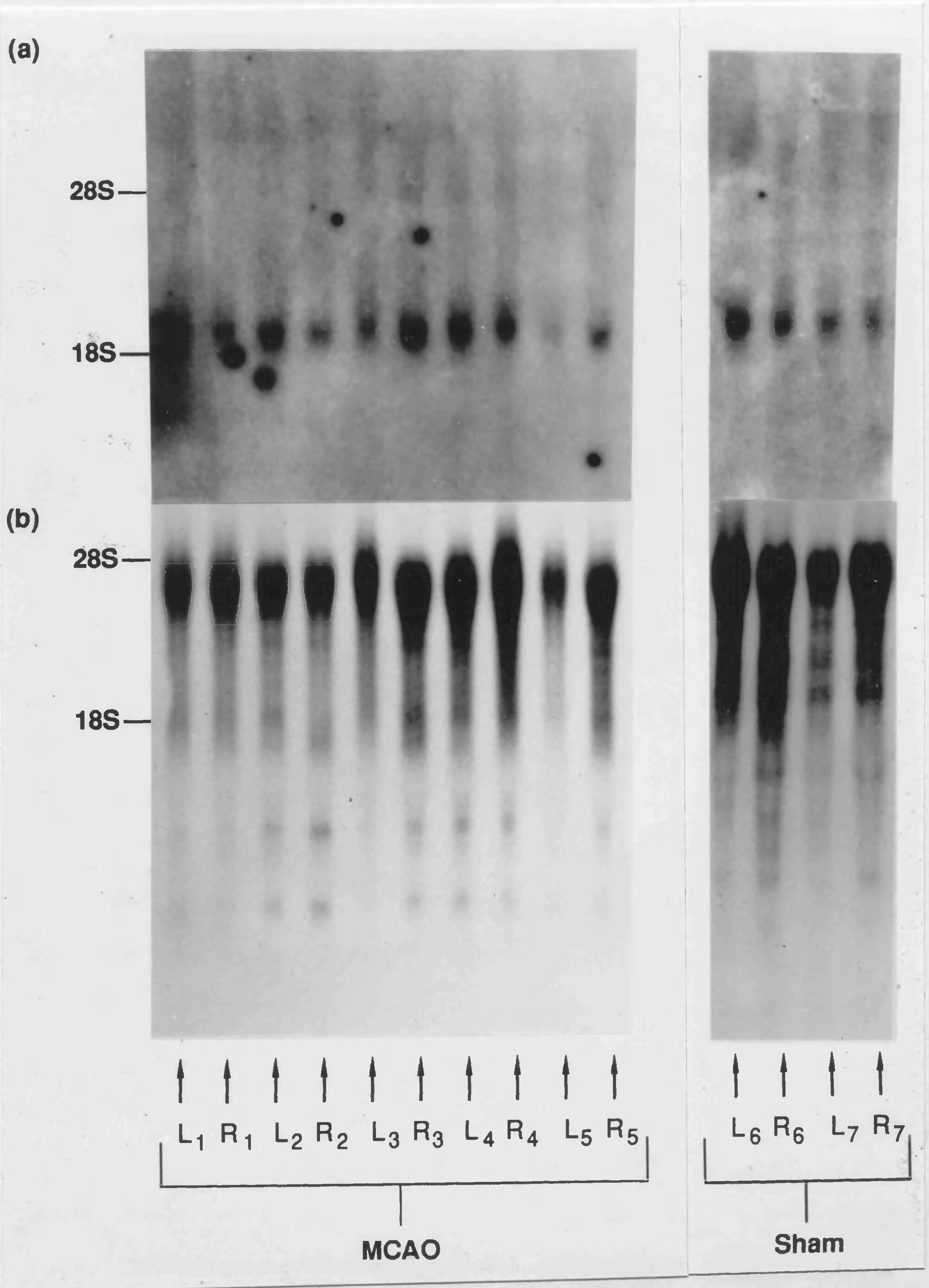

Blood
Pressure
(mm Hg)
100
90
80
70
60
50
40
30
20
10
0
-15
0
15
30
45
60
75
90
105
120
135
150
165
180
MCAO
Blood
withdrawn
Reperfusion
Time (minutes)

Figure 38.

The effect of three hours of focal forebrain ischemia on RNA integrity in rat brain.

Seven male Wistar rats were anaesthetised using halothane. Five of the animals were then subjected to left middle cerebral artery occlusion as described in "Methods". The other two rats, that acted as sham animals, were treated identically except that no occlusion was performed. Fifteen minutes after occlusion, the animals blood pressure was reduced to between 50 and 55 mm Hg by the removal of approximately 3 ml of blood. Hypotension was maintained for 30 minutes before blood was reinjected to bring the blood pressure back to normal (Figure 37). The animals were sacrificed 3 hours after occlusion, the brains removed and the left and right cortices carefully dissected. These were then frozen in liquid nitrogen and stored at -80°C. RNA was then purified from the tissue and analysed by northern blotting as described in "Methods". The agarose gel was loaded with 20μg of RNA per lane and run overnight. After transfer of the RNA to Hybond N+ membrane, the blot was hybridised to a labelled oligonucleotide complimentary to Gβ mRNA (a), washed and exposed to X-ray film for 3 days. The blot was then stripped and hybridised to a labelled oligonucleotide complimentary to 28S rRNA (b). This was exposed to X-ray film for 15 minutes. The positions of 18S and 28S rRNA were visualised by methylene blue staining of the blot, and serve to indicate molecular size. MCAO = mid cerebral artery occlusion, L or R indicates left (ischemic) or right (control) cortex, the subscript indicating which animal the RNA was prepared from.



(a)
28S
18S
(b)
28S
18S
L1 R1 L2 R2 L3 R3 L4 R4 L5 R5
MCAO
L6 R6 L7 R7
Sham

IV. DISCUSSION

1. The feasibility of biochemical analyses of human post mortem brain.

The breakdown of interneuronal communication in DAT which is central to the symptomology of the disease, is characterised by a variety of neurochemical alterations in the brain of the sufferer, not least of which are changes in aspects of cellular signal transduction. While some neurotransmitter systems appear to be severely effected by the disease, others remain relatively unscathed. The study of such systems has been mainly confined to what are essentially extracellular aspects of signal transduction, namely neurotransmitters and their respective receptors. However the events which occur subsequent to receptor activation are also of importance, possibly more so, since a variety of different receptors exert their effects through the same transduction mechanisms. It is possible therefore that neurotransmitters which appear to be relatively unaffected in DAT, may have impaired function due to damage having occurred to other parts of the signal transduction cascade. Alternatively the loss of neurotransmitters such as acetyl choline could be counteracted by a heightened response of post receptor elements. To address these possibilities, a systematic analysis is required of all the components which serve to carry signals across the cell membrane. The primary purpose of this study therefore was to examine the events that occur subsequent to receptor activation in DAT using a variety of established biochemical techniques. These included western and northern blotting to measure the levels of G proteins and their mRNAs, as well as enzyme assays to assess the functional integrity of some aspects of signal transduction in DAT. However, the use of human post mortem tissue presents potential difficulties, since the actual value of any parameter measured can be considered to be a combination of the effects of DAT, and that of other variables such as subject age, tissue storage time and the length of the delay between death and freezing of the

tissue (the post mortem delay). It is necessary to know the contribution of such factors in order to assess the feasibility of any study which is to use post mortem tissue. In essence one must ensure that any changes which occur due to the presence of DAT, are not exceeded, modified or negated by the action of other processes. Secondly such information allows groups of human subjects to be matched in such a way as to minimise the impact of variables other than that under investigation. Their importance can be assessed either by using animal models, or by attempting to correlate the variability observed between human subjects with a variety of factors such as post mortem delay, tissue storage time etc. A combination of both approaches was used in this investigation.

The determination of the average variance between human subjects for each technique used, allowed two general observations to be made. Firstly the inter subject variability of the parameters measured in human subjects was in all cases higher than that observed using rat brain. In addition, the variation in measurements of the levels or activities of proteins in human brain, was less than that observed in the analysis of mRNA. Due to the apparent difference between protein and RNA, the factors influencing the intersubject variability for each will be discussed separately.

1.1 Measurement of G protein levels and enzyme activities in human post mortem brain.

1.1.1 Statistical considerations.

Measurements of protein levels, or activities, typically gave a coefficient of variance (sd as percentage of mean) of approximately 30% compared to around 10% for measurements involving rats (Table 7). The effect that this level of variance has upon the likelihood of obtaining a significant change using Students t-test was determined, it being found that on average, using a sample size of eight and a significance level of 0.05, a 31% difference in the mean of two groups of subjects could be detected, as

opposed to 11% when rat brain was used. Thus, in the case of human brain, quite large neurochemical changes in DAT may not result in a statistically significant difference being detected. The possible causes of the variability observed therefore merit closer examination.

1.1.2 Methodological considerations.

Although it might be expected that studies of human brain would possess greater variation between subjects due to for example, genetic or environmental effects, it is also possible that variables such as post mortem delay contribute to the measurements performed in human brain. This of course assumes that there are no methodological differences between how the rat and human studies were performed, an assumption which is true except for one aspect, that of tissue sampling. In a complex tissue, such as the brain, it is important to ensure that exactly the same areas of tissue are used in each case. The rat studies involved the use of whole brains, thereby removing any problems of associated with sampling. The protocols which determines how the human brains are dissected into different regions are followed carefully, but since the dissections are carried out by a number of different persons, it is possible that small variations occur in the area of brain which is dissected.

One factor which may have a significant effect is the proportion of grey and white matter contained within each tissue block, since the levels of all the moieties measured in this study varied substantially between the two (Table 6). However, the variety of measurements carried out in the same preparations offers some safeguard against the possibility that differences in tissue sampling between groups of subjects are wrongly attributed to being due to the presence of DAT. Some investigators avoid this problem entirely by dissecting out grey matter from each block, a procedure which is most usefully carried out before the brain is frozen, but can be done afterwards. However, since in this study, grey matter was not dissected out before freezing of the tissue, it would have required that the tissue was thawed to

allow dissection. Since at some stage RNA was to be purified from the same tissue blocks, there was a concern (later confirmed) that thawing the brain could effect RNA integrity.

The level of intersubject variability encountered in this study is not atypical when compared with that observed by others (for example, Cross *et al.*, 1984a; D'Amato *et al.*, 1987; Shapiro *et al.*, 1991). In particular it is not noticeably higher than that observed in those studies in which grey matter was dissected out (Kalaria & Andorn, 1990), or those using *in situ* techniques which allow the same region of brain to be "sampled" in each subject (Dewar *et al.*, 1990). In addition, if substantive differences were occurring in the relative amounts of grey and white matter used for each preparation, it might be expected that measurements in the cortex would possess greater variance than those in the hippocampus. There was however no indication that this had occurred. It seems likely therefore that other factors are acting on the tissue prior to its use.

1.1.3 The effect of post mortem delay.

One variable which might be expected to have a major effect on post mortem tissue, is the delay which occurs between death and freezing of the tissue. In the present study this ranged from two to twenty three hours. During this period, enzymatic degradation and/or protein denaturation may have an influence on parameters subsequently measured in the tissue. There exists in the literature many studies which assess the stability post mortem of a wide range of cellular constituents. A general conclusion that may be drawn from these investigations is that the stability of different cellular components is very variable. For example, the levels of some neurotransmitters change quickly after death (Sloviter & Conner, 1977; Spokes & Koch, 1978), while most enzymes are relatively unaffected by any delay in tissue freezing (Syapin *et al.*, 1987; Spokes & Koch, 1978; Fahn & Côté, 1976). Similarly, the stability of neurotransmitter receptors post mortem is very variable (Syapin *et al.*, 1987; Whitehouse *et al.*, 1984; Benwell

& Balfour, 1985). It is therefore important to discover the stability post mortem of any moiety that is to be investigated.

The effect that post mortem delay has on brain, can be readily assessed using rats which have been stored for increasing lengths of time post mortem before being analysed. This offers an advantage over the alternative approach of using non-diseased human brains with a range of post mortem delays, since its effect can be studied in isolation in rat, whilst human subjects are likely to exhibit other sources of variation in addition to post mortem delay. However, differences have been found in the stability of benzodiazipine receptors between strains of rats, and even between males and females of the same strain (Syapin *et al.*, 1987). It would be prudent therefore to use the results of studies on the effect of post mortem delay in rats, merely as an indicator to the feasibility of studying a parameter in human tissue. Experiments were carried out to discover what effect delaying freezing of the brain after death has upon G proteins, G protein mRNA, adenylate cyclase and sodium potassium-dependent ATPase. Some investigators attempt to accurately model the cooling of the human brain which, due to its larger mass, cools more slowly than rat brain. However the simpler experiments presented here, which were carried out at either 4°C or room temperature, proved to be a useful indicator of the effect that post mortem delay had on the same parameter in human brain.

The mobility of G proteins on polyacrylamide gels using membrane extracts prepared from rats which had been stored at 4°C for up to 72 hours, or at room temperature for 24 hours, was not significantly different from those prepared from fresh tissue (Figures 11 to 13). There was also no significant correlation between G protein levels and post mortem delay in the human subjects studied. Both these findings suggest that these proteins are relatively stable. This conclusion is supported by measurements of the *in vivo* half life of G$o\alpha$ measured in cell cultures, which varied from 28 hours in undifferentiated N1E-115 neuroblastoma cells to 154 hours in cerebellar granule cells (Brabet *et al.*, 1991). The apparent slow turnover of G proteins

suggests that their levels will not change rapidly in response to stimuli, but rather represent a mechanism by which adaptations to long term changes in the cells environment may occur.

The influence that post mortem delay has on the activity of sodium potassium-dependent ATPase was also studied in rat brain. The results of this study were similar to those of Syapin *et al.* (1987) and Fahn & Côté (1976), in that the enzyme was found to be stable post mortem under the conditions examined (Figure 14). In addition no significant correlation was found between human sodium potassium-dependent ATPase activity and post mortem delay. Adenylate cyclase proved to be slightly less stable, in that while its activity did not change in rat brain when the animals had been stored at 4°C, a small decrease was observed in animals which had been stored at room temperature for 24 hours (Figure 14). These results are in agreement with those of Nicol *et al.* (1981), who showed that adenylate cyclase activity was stable for at least 10 hours post mortem at room temperature in bovine striatum. As has previously been observed by Ohm *et al.*, (1991), there was no correlation between post mortem delay and activity in human brain. The stability of the enzyme in brain is in contrast to that in heart, in which a rapid decrease in enzyme activity occurs after death (Vatner *et al.* 1990). It is unclear whether this is a consequence of the enzyme's cellular environment differing between brain and heart, or to different subtypes of the enzyme being present in the two tissues. The latter possibility seems most likely since cardiac adenylate cyclase has recently been shown to be inhibited by a rise in intracellular calcium ion concentration (Cooper, 1991), in contrast to neural adenylate cyclase which is stimulated (Harrison *et al.*, 1989). The degree of stimulation of adenylate cyclase by fluoroaluminate also remained unaltered with increasing post mortem delay. This would indicate that G proteins are still functionally active post mortem, at least with regard to their ability to interact with adenylate cyclase.

The stability of choline acetyl transferase (ChAT) post mortem was

not investigated in rat brain, but has been shown to be stable by Spokes & Koch (1978). This was confirmed by the failure to find any significant correlation between ChAT activity and post mortem delay in human brain. Thus, it is evident that the proteins examined in this study may be reliably assayed in human brains possessing post mortem delays of up to at least twenty four hours. In addition, it is evident that the importance usually given to the matching of post mortem delays between different groups of subjects, perhaps at the expense of other factors, is not always justified, although it will continue to be a wise precaution.

1.1.4 The effect of subject age.

The failure to find that the post mortem delay had a major effect on any of the measured proteins studied led to other variables being considered, such as subject age. This was not significantly correlated with any measurement (Table 8), although caution should be exercised in the interpretation of this finding since the range of ages used was small. Indeed, the use of a larger age range by Young *et al.* (1991b), has demonstrated a statistically significant negative correlation between the levels of Giα and age in human frontal cortex, and a positive correlation between GsH levels and age. The apparent increase in the relative levels of stimulatory compared with inhibitory G proteins, may indicate the occurrence of age related changes in the activity of different neurotransmitter systems, or even that the levels of adenylate cyclase are reduced with age.

1.1.5 The effect of storage at -80°C.

The length of time which the brains had been stored at -80°C did not correlate with any measurement except with sodium potassium-dependent ATPase activity, where a positive correlation with storage time was observed (Figure 35). Since this occurred in both the frontal cortex and hippocampus, it is reasonable to assume that this is a real effect. It is unlikely that a single event such as freezer failure could account for this

observation since there is a continuous range of enzyme activities i.e. the activities do not fall into two distinct groups. The most likely explanation therefore is that long term storage damages a factor which has an inhibitory effect on the enzyme, whether this be another protein, or a domain within the enzyme itself. This phenomenon should have had little effect on the comparison of sodium potassium-dependent ATPase activities in control and DAT subjects, since no significant difference in the mean length of storage time existed between the groups of subjects used. However it does demonstrate that long term storage of brain tissue at -80°C can have a gradual effect on the integrity of some neural components. The time taken to assemble a sufficient number of brains which will allow useful analyses to be carried out, frequently means that there is a wide variation in the storage time of different subjects. Unfortunately this variable is usually given a low priority when attempting to match groups of subjects, and given these results and those of others (Burke *et al.*, 1991), this may be rather unwise.

Other factors such as the persons sex,

were also considered, but neither had any obvious effect. Thus, the source of the variability observed between human subjects is most likely due to a variety of factors which, by themselves, are not large enough to be readily detectable. Fortunately, the variability encountered was not of such a magnitude that it precluded a useful study of transduction system proteins in human brain.

1.2 The analysis of RNA in human post mortem brain.

1.2.1 A possible explanation for the high intersubject variability encountered.

The intersubject variability in the levels of G protein mRNAs, as measured by northern blotting, was much higher than that observed for that for the G proteins themselves when measured using western blotting (Table 7). It is unlikely that the methodology used to measure RNA levels has an intrinsically greater scope for error, since multiple RNA preparations using rat brain did not normally give high levels of variability. A clue as to the source of this phenomenon was given by the observation that when the human RNA preparations were analysed by northern blotting, varying degrees of band smearing of each mRNA species was evident. This indicated that some RNA degradation had taken place, and it therefore seemed likely that different amounts of degradation in each subject could account for the variability in G protein mRNA levels.

1.2.2 Assessment of the degree of RNA degradation.

It was difficult to assess the extent of degradation from band smearing alone, not least because the smaller degradation products may have run off the end of the gel. However, when 28S rRNA degrades it gives clearly definable products since the secondary structure and normal association with protein leaves some parts of the molecule more vulnerable to RNase action than others. The extent of breakdown of 28S rRNA can be easily determined by visual inspection and provided a more rigorous assessment of degradation than band smearing *per se*. In most cases, the greater the degree of 28S rRNA degradation, the smaller the amount of $Gs\alpha$ or $G\beta$ mRNA that was detected (Figures 29 and 31). This suggests that the amount of G protein mRNA detected in each subject is dependent, at least in part, on the extent of RNA degradation which has taken place in the tissue. The use of densitometric analysis of band smearing to provide an

assessment of degradation has been used by Clark *et al.* (1989). A similar scheme to assess 28S rRNA degradation was not considered to be useful, as the intensity of each band depends not only on the extent of degradation but on the actual amount of RNA loaded onto the gel. Although the latter should be the same for each sample, small variations in the actual quantity loaded does occur. Indeed, it was to control for such variations that first prompted the use of the 28S rRNA probe. Furthermore, the similarity which was observed between 28S rRNA and G protein degradation may not be true for all mRNAs. Alternatively, if degradation is effecting most RNA species to the same extent, it would imply that normalising the levels of each RNA species under investigation to another control species would be advantageous. Indeed it was found that there was a high degree of correlation between the levels of Gsα and Gβ mRNA in each subject (Figure 33). The problem associated with this is the choosing of a suitable control species. Common choices such as GAPDH or actin are known themselves to vary in their levels of expression (Leeuw *et al.*, 1989). One possible answer to this problem has been to use the hybridisation of polyT DNA to polyA$^+$ RNA, thus detecting most mRNA species (Harrison *et al.*, 1991a). This is somewhat problematical for use with northern blotting, but can be advantageously utilised as a control for use with *in situ* hybridisation, or slot blotting. Whether the level of hybridisation of polyA$^+$ RNA provides a good indicator of overall RNA integrity is unclear, since it has not been demonstrated that regions of polyA mRNA are degraded to a similar extent to the rest of the RNA molecule.

1.2.3 Statistical considerations.

The coefficient of variance (SD as percentage of mean) for the measurement of Gsα and Gβ mRNA levels ranged from 50% for Gβ2 mRNA in DAT hippocampus, to 93% for Gβ1 in control frontal cortex, with an average CV of 65%. This level of variability would only allow large

differences between means, in the order of 80%, to be detected using Students t-test (Table7). Similarly, to detect, for example, a 40% difference between the DAT and control groups would require the use of over 40 patients in each group. Thus, although there was no significant difference detected between the control and DAT groups, there is a strong possibility of a false negative conclusion. Alterations in response to the neurodegenerative process, are thus likely to be dwarfed by the interpatient variability as a consequence of RNA degradation. It is unlikely that variable degradation of RNA is peculiar to this study, or to G protein mRNA (and 28S rRNA); published northern blots from human postmortem brain almost invariably displays some evidence of degradation in a diverse range of RNA species (e.g. Johnson *et al.*, 1986; Clark *et al.*, 1989; Iacopino & Christakos, 1990; Crapper-McLauchlin *et al.*, 1988; Goedert, 1987). In addition the ability of RNA extracted from the human brain to be translated *in vitro* is very variable (Perret *et al.*, 1988), again indicating varying degrees of degradation in different subjects. Harrison *et al.* (1991a) have also investigated Gsα mRNA in control and DAT diseased human brain. They found a similar level of intersubject variability to that reported here, but the use of large sample sizes allowed the detection of a large (300%) increase in Gsα mRNA expression in DAT diseased hippocampus compared to controls. The frontal cortex was not analysed, although no change was detected in the temporal cortex. Due to the low hybridisation signal of Gsα mRNA in the hippocampus, no quantitative data was obtained about its levels in control and DAT diseased subjects. However there was no subjective difference apparent between the control and DAT groups. The reason for the contrasting findings of the two studies may relate to the greater spatial resolution afforded by *in situ* hybridisation, although Harrison *et al.* (1991a) did detect alterations in Gsα mRNA expression throughout the hippocampus. As explained above, it not impossible that had larger sample sizes been used in this study, a change in Gs mRNA levels in DAT would

have been detected.

As well as the risk of false negative conclusions being drawn, the large intersubject variability also heightens the likelihood of false positive conclusions. For example, in this study although there were no statistically significant changes in any of the mRNAs measured, in all cases the mean levels were decreased in the DAT group relative to controls. This was most likely due to the presence of a greater number of samples in the control group possessing modest amounts of RNA degradation. Such an effect was most clearly seen in the hippocampus, where of the two subjects which had the least RNA degradation, both were controls. Had this bias been any greater, and had no attempt been made to assess RNA degradation, it is possible that a statistically significant, but artefactual, difference would have been detected between the two groups.

1.2.4 The possible causes of RNA degradation in post mortem human brain.

The presence of varying degrees of RNA degradation in different subjects makes it especially important to determine what factors influence this RNA stability in the human CNS in order to identify criteria for matching groups of patients. There was no correlation between the extent of RNA degradation (or the amount of G protein mRNA detected), with either the diagnosis of DAT nor its histopathological severity. RNA degradation was not influenced by the age of the subjects or tissue storage time. Burke *et al.* (1991) also have examined the effect of storage time on RNA levels. These authors found a significant negative correlation between this variable and both actin and APP751 mRNAs, but not with the other mRNA species they examined. It is possible therefore that different RNA species possess different susceptibilities to long term freezer storage and, as suggested by Burke *et al.* (1991), that this variable may not possess an equal importance in each subjects.

There was also no correlation between post mortem delay and G protein mRNA degradation in human brain. This result confirmed the experiment to assess the effects of PMD in rat brain which showed that even after 72 hours at 4°C, only small amounts of degradation of Gsα mRNA were observed, and practically none of Gβ mRNA (Figure 15). Although there was a time dependent increase in 28S rRNA breakdown, the amount was small compared to the total amount of 28S rRNA and was much less than that observed in human brain. Most human subjects are not placed at 4°C immediately after death, so greater opportunity for degradation in human tissue cannot be excluded. Nevertheless, the results of both the human and rat studies suggest that the delay in freezing the tissue after death is not the major influence on RNA stability (see also Johnson *et al.*, 1986 and Kobayashi *et al.*, 1990). In contrast, Burke *et al.* (1991) found the levels of actin and APP751 mRNA were negatively correlated with post mortem delay, although other mRNA species were not.

It has been suggested that mechanical damage to human tissue during dissection may increase RNA degradation (Johnson *et al.*, 1986). This seems unlikely since in the experiment which assessed the effect of freeze/ thawing on RNA stability, the control brains, which were removed from the skull and dissected, exhibited no detectable increase in RNA degradation. Only the freeze/thawing of the tissue greatly accelerated the rate of RNA degradation (Figure 36). It is highly unlikely that accidental thawing during storage could account for the RNA degradation observed in the human RNA for the following reasons. Firstly, the tissue was stored at -80°C in alarmed freezers with automatic solid CO_2 backup. The experiments described here suggest that a complete thaw lasting at least 30 minutes is needed before significant degradation occurs. Secondly, there was no correlation between storage time and degradation. Thirdly, the RNA preparation method employed ensured that the tissue was ground up in liquid nitrogen and thawed into a buffer that instantly denatures RNase

(Chomczynski & Sacchi, 1987). These precautions were taken because it has been reported that RNA extracted from human brain is especially vulnerable to degradation during RNA purification (Ilaria *et al.*, 1985). It is highly unlikely that the methodology used herein was responsible for the RNA degradation observed, since separate RNA preparations made from the same tissue block displayed similar amounts of RNA degradation. For example, the extent of 28S rRNA degradation observed in subjects C19A, C34 and A65 in the experiment which compared degradation in different brain regions, was the same as that observed in previous preparations (e.g. compare Figures 29 and 31, to Figure 34).

The enhanced rate of RNA degradation observed after freeze/ thawing the tissue may provide a clue to the actual cause of the degradation observed in the human RNA. Rapid freezing of the tissue will cause damage to the membrane structures within the cell, most notably lysosomes. These organelles contain many hydrolytic enzymes, among which is a type of RNase (Koenig *et al.*, 1964), known as acid RNase due its pH optima (Franzoni & Argiz, 1978). Under normal circumstances, acid RNase is confined within lysosomes and will have no effect on RNA present in the cytoplasm, but if the lysosomes are damaged, RNase can leak out. Although physiological pH is not the ideal condition for acid RNase activity, a large release of the enzyme may be enough to cause considerable RNA breakdown. The absence of lysosomal damage would provide an explanation as to why there is little RNA degradation observed over long post mortem intervals in rat brain. The non-lysosomal, cytoplasmic RNases which are responsible for the normal turnover of RNA may be relatively inactive after death due to cooling of the tissue, and because they are complexed with an inhibitor protein (Brockdorff and Knowler, 1987) . An explanation for the variable degree of RNA degradation in the brains used in the present study may reside in events immediately prior to death, such as the degree of ischemia or anoxia of the brain tissue caused by respiratory

or circulatory malfunction (i.e. the "agonal state"). The degree of ischemia before death influences a variety of cellular components, most notably reductions in the activities of phosphofructokinase and glutamate decarboxylase (Perry *et al.*, 1982). Animal models have shown that ischemia causes both tissue acidosis, due to lactate accumulation (Nemeto & Frinak, 1981), and membrane damage caused by the action of phospholipases (Edgar *et al.*, 1982) and lipid peroxidation by free radicals (Kogure *et al.*, 1985). These events may lead to leakage of RNase from the lysosomes, (Maschoff *et al.*, 1989), as well as providing more acidic conditions in which the enzyme can act. Indeed oxygen free radicals have been shown to cause the release of lysosomal enzymes in canine heart (Kalra *et al.*, 1989). A recent study demonstrated that tissue acidity was significantly increased in those individuals who died agonally as opposed to those who died suddenly (Yates *et al.*, 1990), suggesting that ischemia or hypoxia was occurring in the former case. Unfortunately, although the cause of death of many subjects in this study was known, their agonal state was not adequately assessed. However, a study by Harrison *et al.* (1991b) found a relationship between the amount of M1 muscarinic receptor RNA (but not total polyA$^+$ mRNA) detected by *in situ* hybridisation and the agonal state of the subject. In addition, relatively undegraded RNA has been purified from subjects who had been oxygenated for several days prior to death, thus minimising the risk of antemortem hypoxia (Roses *et al.*, 1987).

It is known that the hippocampus is especially vulnerable to ischemic damage (Pulsinelli, 1985) and in view of the above observations it was expected that there would be increased RNA degradation observed in this region. However, when the extent of 28S rRNA breakdown was examined in the frontal cortex, hippocampus and cerebellum of three subjects, there was little difference observed between each region; although there were marked differences in degradation between the three patients (Figure 34). In an effort to gain greater understanding of the effects of ischemia/hypoxia on

RNA stability in human brain, an attempt was made to model these events in rat brain. Permanent focal forebrain ischemia was induced in the rats by permanently occluding the left middle cerebral artery (Tamura *et al.*, 1981). A series of preliminary experiments showed that dissection of the ischemic area only yielded 40 to 60mg of tissue. To increase the area of ischemic damage, the animals were made hypotensive for thirty minutes after occlusion (Osborne, 1987). The restoration of normal blood pressure after the period of hypotension also facilitates some recirculation to take place in the tissue adjacent to the most severely ischemic areas. Having a degree of reflow is important, since during this period processes which cause additional tissue damage can occur (Halsey *et al.*, 1991). For example, during ischemia the rise in intracellular calcium levels increases the activity of phospholipase A_2 leading to the production of arachidonic acid (Chan *et al.*, 1985). Its subsequent metabolism to form leukotrienes, prostoglandins or thromboxanes, is thought to cause the production of superoxide radicles (Kuehl & Egan, 1980). This however requires the presence of molecular oxygen (Wolfe, 1989), and therefore during ischemia arachidonic acid accumulates. During reflow, the renewed availability of oxygen allows the rapid metabolism of accumulated arachidonic acid to take place, giving rise to a burst of free radicles which may lead to further lipid peroxidation and membrane damage (Chan *et al.*, 1985).

The analysis of RNA purified from the ischemic areas did not show any marked increase in 28S rRNA degradation compared to controls (Figure 38). In addition, Gβ mRNA did not appear to be significantly degraded in the ischemic regions, although methodological problems hindered the assessment of mRNA integrity. The failure to find increased degradation after an ischemic insult may indicate that, despite much circumstantial evidence, antemortem ischemia is not the major cause of the degradation observed in the human subjects. Alternatively, it is possible that permanent focal forebrain ischemia does not provide an accurate model of what takes

place during an agonal death. This is likely to involve short periods of partial ischemia or anoxia, followed by reflow, rather than one period of severe ischemia as provided by the model. Thus, a number of relatively small ischemic or anoxic events over a period of hours, may have a more pronounced effect on RNA integrity than one severe event. A useful development of the experiments already carried out, may therefore be to produce a number of short periods of global ischemia in the rat by reversibly occluding the carotid artery. In addition, the brains of elderly persons, as used in this study, may be particularly vulnerable to RNA degradation. A study carried out in the rat has shown that there is an age dependent increase in acid RNase activity, which may be due to to increased levels of the enzyme or to weakened lysosomes (Alberghina & Stella, 1988). Furthermore Goto *et al.* (1969) showed that the proportion of acid RNase activity recoverable in the "particulate" as opposed to the "cytosolic" cell fraction in mice liver was greater in aged compared to younger animals, indicating a decrease in lysosomal integrity with increasing age. It is possible therefore that the RNA present in the brain of elderly individuals is particularly vulnerable to degradation. Although no correlation exists between age and degradation was found, the range of ages used was small. It would be interesting therefore to compare the effects of ischemia, or post mortem delay, on RNA integrity in aged rats to that in younger animals. Alternatively the integrity of RNA extracted from younger human subjects may help to clarify this issue.

It seems likely that no single cause of RNA degradation observed in human brain exists, the phenomena being due to a combination of factors which may vary in their importance in each subjects, or for different RNA species. However the most important finding is that degradation occurs and its extent is variable. The small groups sizes frequently encountered in human studies make it possible that variable RNA degradation might give rise to incorrect conclusions. An assessment of the RNA degradation that

has occurred in each subject is therefore highly advantageous, especially when using in situ hybridisation where unlike northern blotting, there is no indication of RNA integrity. Since RNA degradation was found to be similar in different regions of the brain, it may be possible to use a small part of the brain for the assessment of RNA degradation whilst the rest is used for *in situ* hybridisation studies. The finding that the measurement of G protein mRNA in post mortem human brain is unreliable, may lead one to question data on these species that was not accompanied by an assessment of stability.

In many ways it was unfortunate that the measurement of G protein mRNA levels proved so problematical, especially since there are reports of changes in the levels of G proteins mRNAs, independent of the actual protein levels, and vice versa (Granneman & Bannon, 1989; Klinz & Costa, 1990). Fortunately the high degree of stability possessed by G proteins themselves allowed them to be reliably measured in human post mortem brain.

2. The distribution of G proteins and adenylate cyclase in human brain.

2.1 Distribution of Giα and Goα.

It became evident from preliminary studies of G protein levels that these proteins were heterogeneously distributed throughout the brain. This presented an opportunity to discover in which regions G protein were most abundant, and to compare this to that of other signal transduction components. It was hoped that such information would facilitate the interpretation of data obtained in the analysis of DAT diseased brains. Of all the G proteins measured Gi2 had the least variable distribution throughout the five regions. However it possessed a similar regional distribution trend to that of Gi1 and Go with all three having their highest levels in the frontal cortex, hippocampus and neostriatum and their lowest levels in the cerebellum and pons (Figures 24 and 25). These findings are in broad agreement with that of Asano *et al.* (1990) who measured the levels of Gi1, Gi2 and Go in rat brain. These authors reported however that Gi1 was substantially more abundant than Gi2 in all the regions they examined, while the present study suggests that both species have approximately the same levels of expression in human brain. The distribution of the mRNAs encoding Giα and Goα have also been examined (Brann *et al.*, 1987); both species having a similar level of expression in rat cortex, hippocampus and cerebellum. Curiously, these authors could detect no Gi1 or Gi2 mRNA in the neostriatum, although both proteins are present in human and rat neostriatum (Cooper *et al.*, 1990). This suggests that Giα levels in brain may be partly determined by post transcriptional mechanisms. The low levels of Goα and Giα that were found in the pons suggested that they were mainly expressed in grey matter, this being confirmed by the observation that cortical white matter contained low levels of these G proteins. Such a distribution is to be expected given the role of G proteins in signal transduction.

2.2 The third Gsα-like immunoreactive species in human neostriatum.

The measurement of Gsα levels in the human brain produced a number of interesting results, including the detection of a third immunopositive band in the neostriatum, in addition to those of GsH and GsL (Figure23). While this may represent a breakdown product of GsH, the stability post mortem of this protein makes this unlikely. It is more plausible that the band represents a "Gsα-like" protein, the expression of which is limited to the neostriatum. One such species is Golfα which shares extensive amino acid homology with Gsα (88%). Although the sequence of human Golfα is not known, within the decapeptide sequence recognised by antisera CS1, an arginine residue in rat Gsα is replaced by a lysine residue in Golfα. Despite this, antisera CS1 is still able to detect Golfα in rat (G. Milligan, personal communication). If a similar difference exists between the two species in humans, it is unlikely that the antisera will have the same affinity for Golfα and Gsα. Thus the abundance of the two proteins cannot be directly compared by western blotting using this antisera. The third band detected by CS1 is also of the correct molecular weight to be Golfα, as this protein contains two additional amino acids compared to GsL, and migrates just above GsL on denaturing polyacrylamide gels (Jones *et al.*, 1990). As described in Section 2.3.1 of the Introduction, Golfα was originally cloned from olfactory sensory neurons. However Drinnan *et al.* (1991) have reported the presence of Golfα encoding mRNA throughout the basal ganglia in rat brain. These results may therefore suggest that human neostriatum contains, in addition to GsL and GsH, a third stimulatory G protein, Golfα. However Drinnan *et al.* (1991) failed to detect a third immunoreactive band in rat neostriatum and, since they also observed low levels of total Gsα mRNA compared to high levels of Golfα mRNA in this region, they concluded that what appeared, on western blots to be GsL, was in fact Golfα. This would imply that the major stimulatory G protein in the neostriatum is not Gsα but Golfα. However these authors assumed that the

low levels of Gsα mRNA present in the neostriatum implied low levels of Gsα protein. This may not be correct, since the levels of GsL mRNA in this region represents only a small proportion of the total Gsα mRNA present (16%) (Granneman & Bannon, 1991). Thus the degree of hybridisation of a probe complimentary to all forms of Gsα, will be mainly determined by the amount of GsH mRNA present, the abundance of GsL mRNA having only a minor effect. Hence the low levels of total Gsα mRNA detected by Drinnan *et al.* (1991) in rat neostriatum, may derive from the fact that this region contains a low abundance of GsH. Alternatively the possibility that Giα mRNA is translated with greater efficiency in the neostriatum i.e. normal protein levels associated with low mRNA levels, may also apply to Gsα, explaining the relatively low abundance of this mRNA species compared with that of Golfα mRNA. Furthermore, Jones *et al.* (1990) observed that S49 cyc⁻ cells could express Golfα less well than either GsL or GsH, possibly suggesting that Golfα mRNA is translated with lesser efficiency than that of Gsα. The failure of Drinnan *et al.* (1991) to find three immunopositive bands in rat neostriatum may simply be due to the difficulty in resolving GsL from Golfα on polyacrylamide gels (see subject C19A on Figure 23), and to the apparent low abundance of this protein although, as stated above, this may be due to antisera CS1 possessing a lower affinity for Golfα than for Gsα. Further experiments using Golfα specific antisera will be needed to confirm the presence of this G protein in human neostriatum, and to conclusively differentiate it from GsL. It is interesting however to speculate as to the role of Golfα in the neostriatum. It has been suggested that Golfα may couple D1 receptors to adenylate cyclase in the neostriatum (Drinnan *et al.*, 1991). However Schiffmann *et al.* (1990) have reported that the mRNA encoding a putative G protein coupled receptor is present abundantly and exclusively in rat basal ganglia, perhaps indicating an association between this receptor and Golfα.

2.3 Distribution of Gsα.

The distribution of Gsα throughout the brain, especially that of GsL, was somewhat different to that of Gi1, Gi2 and Goα : the levels of both types of Gsα were highest in the cerebellum, while GsL exhibited an almost mirror image of Giα and Goα expression (Figure 23). In addition GsL appeared in general to be more highly expressed in those structures containing significant amounts of white matter. This was supported by the observation that GsL was present in relatively high amounts in cortical white matter, and in low quantities in grey matter (Table 6).

2.4 Distribution and stimulation of adenylate cyclase.

In an attempt to discover the reasons for the rather odd regional distribution of GsL, adenylate cyclase was assayed in the same membrane preparations (Figure 26). The basal enzyme activity exhibited significant correlations with the levels of Gi1 and Gi2, and to a lesser extent Goα, it having its highest activity in the frontal cortex and hippocampus with lower activities in the cerebellum and pons. Of the two Giα molecules, Gi1 most closely followed the distribution of adenylate cyclase (Figure 27), which offers some, albeit weak, evidence that Gi1 rather than Gi2 represents the predominant Gi activity in brain. In contrast, neither the levels of GsH or GsL were significantly correlated with adenylate cyclase activity (Table 5). This may suggest that Gsα is associating with other membrane components in addition to adenylate cyclase, such as the calcium and sodium ion channels that it interacts with in heart and muscle (see "Introduction", Section 2.3.2). However the high levels of GsL observed in white matter, makes it unlikely that it is concerned mainly with the modulation of ion channel activity. Another possible explanation lies in the existence in brain of at least two subtypes of adenylate cyclase, one of which can be stimulated by calcium (in association with calmodulin) while the other cannot (Mollner & Pfeuffer, 1988). Cooper *et al.* (1990) have suggested that GsL is

preferentially associated with the calcium-insensitive form of the enzyme. Moreover, the distribution of the mRNA which encodes calcium sensitive-adenylate cyclase has recently been determined in rat brain (Xia *et al.*, 1991); high levels of this mRNA were found in the cerebellum, high to moderate amounts in the cortex and hippocampus and none in the neostriatum and pons. Since this distribution is similar to that of GsH in human brain, it may suggest that an association exists between GsH and the calcium-sensitive form of adenylate cyclase. Confirmation of this awaits the co-expression of the different forms of adenylate cyclase and Gsα in cell cultures. If it is correct however, it would imply that white matter contained very little calcium- sensitive adenylate cyclase, a not unexpected situation given that this form of the enzyme has been implicated in the processes of learning and memory (Xia *et al.*, 1991).

The distribution of adenylate cyclase in brain has also been investigated using the binding of ^{3}H-forskolin to tissue sections. When forskolin is used at concentrations in excess of 5-10μM, it causes the activation of adenylate cyclase activity (Seamon & Daly, 1981). This effect does not require the presence of Gs, and it has been suggested that forskolin interacts directly with adenylate cyclase (Seamon & Daly, 1981). However, at lower concentrations (50nM) forskolin does not stimulate the enzyme, but can potentiate the actions of stimulatory agonists (Seamon *et al.*, 1981). This has lead to the proposal that forskolin is binding to a Gsα/adenylate cyclase dimer. The binding of forskolin to this so called high affinity site is therefore dependent on the presence of both Gsα and adenylate cyclase. However, since Gsα is present in much higher concentrations than adenylate cyclase (Alousi *et al.*, 1991), the level of forskolin binding should be mainly determined by the abundance of adenylate cyclase. High affinity forskolin binding to rat brain is highest in the neostriatum and the molecular layer of the cerebellum, with lower levels occurring in the cortex and hippocampus (Poat *et al.*, 1988), findings which do not concur with the activity of

adenylate cyclase found in these regions of human brain. In the cerebellum this discrepancy is most probably due to the use of the whole cerebellum to measure adenylate cyclase activity rather than just cerebellar grey matter. It is unclear however as to why the neostriatum possesses such high levels of forskolin binding. While it is possible that differences exist in the levels of adenylate cyclase found in rat and human neostriatum, forskolin is also known to interact with moieties other than adenylate cyclase, such as ion channels and the glucose transporter (reviewed in Laurenza *et al.*, 1989). It is possible therefore that forskolin binding in the neostriatum is not all attributable to it binding to adenylate cyclase. Furthermore, Poat *et al.* (1988) have shown that differences in certain aspects of high affinity forskolin binding exists between the neostriatum and other brain regions. On the other hand, the affinity of the binding site for forskolin is the same in rat neostriatum, cerebellum and hippocampus (Poat *et al.*, 1988), suggesting that the nature of the binding site is similar in each brain region. It is interesting to speculate that the postulated presence of Golfα in the neostriatum enhances forskolin binding, although the mechanism of such an action is unclear since, if anything, Golfα appears to be less efficiently coupled to adenylate cyclase than GsH or GsL (Jones *et al.*, 1990). Indeed, it seems unlikely that increased coupling between Gsα and adenylate cyclase could explain the high levels of forskolin binding in this region, since the ability of fluoroaluminate to stimulate the enzyme was similar in the hippocampus, cortex and neostriatum. Nevertheless, the stimulation of adenylate cyclase by fluoroaluminate was noticeably higher in the cerebellum and pons compared to other brain areas (Figure 26). There exists some, largely circumstantial, evidence to suggest that GsL is more efficaciously coupled to adenylate cyclase than GsH (Walseth *et al.*, 1989). In contrast, recent studies of GsH and GsL expressed in S49 cyc$^-$ cells, indicate that GsH is the slightly more potent of the pair (Jones *et al.*, 1990). The existence of a small but significant correlation between the degree of stimulation of adenylate

cyclase in human brain, and the levels of GsL, or the ratio of GsL to GsH (Table 5), may indicate that GsL is better able to stimulate adenylate cyclase than GsH. However, despite cortical grey and white matter possessing the largest difference in the ratio of GsH to GsL (3.6 and 0.4 respectively), stimulation was similar in both, making it unlikely that the relative amounts of GsH and GsL determines the stimulatory ability of fluoroaluminate (Table 6). In cells which do not contain functional Gsα (Hildebrandt *et al.*, 1983), or in situations where Gs is inaccessible (Inoue *et al.*, 1990), fluoroaluminate can inhibit adenylate cyclase. Since fluoroaluminate is able to activate Gi as well as Gs (Katada *et al.*, 1984b), it is probable that the final degree of stimulation of adenylate cyclase is a combination of the influences of stimulatory and inhibitory G proteins. Indeed a strong correlation was found between the ratio of Giα to Gsα and stimulation, although a similar correlation was also found between the ratio of Goα to Gsα and stimulation (Table 5). In both cases, fluoroaluminate stimulation may be being reduced by the release of $\beta\gamma$ subunits, which have been shown to decrease fluoroaluminate stimulated activity in brain (Premont & Iyengar, 1989). Thus the stimulation of adenylate cyclase by fluoroaluminate may represent a simple means to assess the relative amounts of functional stimulatory and inhibitory G proteins present in different preparations.

3. Signal transduction in Dementia of the Alzheimer Type.

3.1 Choline acetyl transferase.

The subjects which were designated as having suffered from dementia of the Alzheimer type had both a clinical and neuropathologically confirmed diagnosis of the disease. The diagnosis was also confirmed by measuring the activity of choline acetyl transferase (ChAT) in each subject. This was found to be substantially reduced in the frontal cortex and hippocampus of DAT diseased subjects compared to that of controls, confirming that changes in neuronal communication had taken place in the DAT diseased brains which were used (Figure 16). A similar reduction in ChAT activity has been reported by a number of groups; for example Procter *et al.* (1988) observed a ChAT activity equal to 50% of controls in DAT diseased frontal cortex, and 44% in the hippocampus. Although the decrease in ChAT activity is a useful confirmation of the diagnosis of DAT, it is not possible to use it as an index of the severity of the disease, since no correlation has been observed between ChAT activity and the overall cognitive function of patients, using either cortical biopsies (Neary *et al.*, 1986), or in subjects whose cognitive abilities were tested before death (Perry, 1986a). However, the reduction in ChAT activity represents a severe, and accepted, neurochemical alteration in DAT with which other neurochemical measures may be usefully compared. In this respect, it offers an advantage over other markers of the disease, such as plaque density, since ChAT activity can be determined in the same preparations which are used to investigate other moieties.

3.2 Sodium potassium-dependent ATPase.

The use of biochemical techniques to investigate brain disorders usually necessitates the analysis of relatively large areas of tissue containing a heterogeneous population of cells. This leads to problems in interpreting the data obtained, since a significant change in the parameter being assayed

may be due either to an up or down regulation of the moiety being measured, or to the loss of a particular cell type. It is therefore useful to measure other cellular components in parallel to the one of interest. The assay of several different G proteins in the same preparation, allowed each one to act as a control for the others. However, although adenylate cyclase activity was determined in the same preparations as those used to determine G protein levels, it was felt that the activity of another enzyme should also be measured. The ATP-dependent sodium potassium pump, or sodium potassium-dependent ATPase, which is responsible for the active transport of Na^{2+} and K^{+} ions, was chosen for this purpose as it located on the cell membrane and will be present in the same preparations as those used to measure the signal transduction components. The enzyme is important in its own right as it maintains the gradients of sodium and potassium ions necessary for nerve impulse conduction (Albers *et al.*, 1989). It is also estimated that it is responsible for 50% of the energy expenditure of the brain (Astrup *et al.*, 1981). In addition, the activity of sodium potassium-dependent ATPase can be stimulated by adrenergic or serotonergic agonists in synaptosomes (Albers *et al.*, 1989). Although the mechanism of such effects is unclear, it may involve phosphorylation of the enzyme by protein kinase C (Collins *et al.*, 1987; Hootman *et al.*, 1987), possibly acting via a G protein coupled mechanism (Danilenko *et al.*, 1991). Thus it appears that signal transduction mechanisms can modulate the activity of sodium potassium-dependent ATPase, which may in turn alter synaptic transmission by hyperpolarising the cell. The enzyme is also frequently used to control against differences in membrane purity, and therefore enzyme activity, that may arise during the preparation of membranes (Begin-Heick, 1990). It also offers the advantage of being similarly distributed between grey and white matter to G proteins and adenylate cyclase (Table 6). This offers a safeguard against the possibility that differences in the relative amounts of grey and white matter within each

tissue block, may bias the resulting data in favour of the control or DAT diseased groups (see Section 1.1.2).

Previous investigations of sodium potassium-dependent ATPase levels in DAT have used the binding of ^{3}H-ouabain to the enzyme to measure its abundance in human brain. Such studies have given conflicting results with Harik *et al.* (1989) reporting a decrease in ^{3}H-ouabain binding to DAT diseased frontal cortex using *in situ* autoradiography, while Colvin *et al.* (1991) using ^{3}H-ouabain binding in tissue homogenates found no change. The discrepancy between the two studies may arise from the use of different techniques to measure ouabain binding; autoradiography estimates the amount of binding per volume of tissue, while homogenate binding is usually expressed per weight of protein. Factors such as cell loss or atrophy may effect the former measure, while the latter might be expected to be less influenced by such changes. The measurement of the enzyme's activity in membrane preparations would therefore be expected to give similar results to that obtained using ouabain binding to tissue homogenates. In the present study, this is indeed the case since no significant difference in the activity of sodium potassium-dependent ATPase was detected between DAT and control subjects, in the hippocampus or frontal cortex (Figure 22). These results indicate that this important enzyme is largely unaltered in the disease, both in terms of activity, and possibly enzyme levels. Alternatively, if the results of Harik *et al.* (1989) do indeed mean that the abundance of the enzyme is lower in DAT diseased frontal cortex, the fact that its activity is unaltered may suggest that there exists a compensatory stimulation of the enzyme in DAT, possibly mediated by protein phosphorylation. It should be noted that the assay of ^{3}H-ouabain binding and enzyme activity are not completely comparable measures. Of the α and β subunits which comprise sodium potassium-dependent ATPase, ouabain is thought to bind to the α subunit. There are three forms of α subunit present in brain, each differing in their affinity for ouabain. The low ($<$100nM) concentrations of ouabain

used in binding studies will only bind to α2 and α3 subtypes, which have a Kd for ouabain in the order of 15nM (Colvin *et al.*, 1991), compared to the α1 subtype which possesses a Kd of 50μM (Marks & Seeds, 1978). Under the assay conditions used herein, it was observed that a ouabain concentration of 100nM could only inhibit approximately 25% of enzyme activity. This suggests that the activity measured is mainly due to sodium potassium-dependent ATPase containing the α1 subunit. Studies using cell cultures have suggested that α1 is mainly present in astrocytes, and α2 and α3 in neurons (Markwell *et al.*, 1991). However, immunohistochemical investigations have demonstrated that there is no exclusively neuronal or glial forms of sodium potassium-dependent ATPase (McGrail *et al.*, 1991), thus the enzymes activity cannot be assigned to any one cell type. Since G proteins are also thought to exist in both neurons and glial cells (Brabet *et al.*, 1988), sodium potassium-dependent ATPase represents a useful negative control with which the levels of other membrane proteins may be compared. The failure to find a difference between the control and DAT groups also indicates that the membrane purity of the preparations, or the amounts of grey and white matter in the tissue from which they were prepared, did not differ markedly between the two groups of patients.

3.3 G protein levels.

The first signal transduction components to be investigated in DAT diseased brains were the α subunits of G proteins. This gave a similar result to that obtained with sodium potassium-dependent ATPase, namely that there was no significant change in the levels of Goα, Gi1, Gi2, GsH and GsL in the frontal cortex and hippocampus of DAT subjects compared to controls (Figures 17 and 18). This suggests that G protein levels are relatively unaffected by the disease process. However a small, but statistically significant increase in the relative amount of GsL compared to GsH was observed in the frontal cortex of DAT subjects relative to controls. As

discussed in Section 2.4, the functional significance of having two forms of Gsα is unclear, but an altered ratio of GsL to GsH, may indicate alterations in the stimulation of adenylate cyclase activity, or in the sensitivity of adenylate cyclase to intracellular calcium concentration; a possibility which is considered in more detail later.

Since this study was completed many other G protein α subunits have been identified, the best characterised being Gzα and Gqα. Although both α subunits are present in brain, the possibility that Gqα can act to stimulate phospholipase C activity (see "Introduction", Section 2.3.2), and may represent a G protein through which muscarinic receptors can modulate inositol phospholipid metabolism, makes it of particular relevance to DAT. It cannot be ruled out that changes in the levels of Gqα or other newly discovered α subunits occur in DAT, and it would of interest to measure the levels of these α subunits as, and when, antisera become available.

Although the results presented here suggest a maintenance of α subunit levels in DAT, it is possible that small cell-specific changes are occurring, which are masked by the use of areas of brain containing a heterogeneous population of cells. For example, a decline in G protein levels due to neuronal loss (Davies *et al.*, 1987) may be compensated by the proliferation of glial cells that occurs in the disease (Beach *et al.*, 1989). Both cell types are known to contain G proteins (Brabet *et al.*, 1988). The sub-cellular localisation of G proteins may also contribute to their robustness in the disease, since it appears that synaptic areas may be more severely affected than other parts of the cell (Hamos *et al.*, 1989). In particular the use of western blotting has demonstrated the partial loss of synaptophysin, a synaptic marker protein, in DAT diseased frontal cortex (Masliah *et al.*, 1991b). Therefore, since Goα has been localised to areas of the cell outwith synaptic densities (Gabrion *et al.*, 1989), it may explain why no significant change in the levels of this protein was observed. Whether this is true for all

G proteins is unclear, since ultrastructural studies of their intracellular localisation in neurons are unavailable. Clearly, an immunohistochemical study is required to resolve such questions. Unfortunately, the anti-G protein antisera which were available did not give immunopositive staining when used upon tissue sections, despite possessing excellent immunoreactivity against denatured proteins on western blots. This is most likely due to the antisera having been raised against small peptides; successful attempts to examine G protein expression by immunocytochemistry have used antisera raised against whole proteins.

3.4 G protein-receptor coupling.

Whilst G protein α subunit levels appear to be maintained in DAT, there may exist some functional impairment of these proteins in the disease. For example the ability of somatostatin to inhibit adenylate cyclase is decreased in DAT diseased temporal cortex, but the number of somatostatin receptors are unchanged (Cowburn *et al*, 1991). A problem associated with such studies however is their inability to determine at what point in the transduction system a change has taken place. It is therefore advantageous to use techniques which examine specific events in the transduction cascade such as effector-G protein or receptor-G protein coupling. The latter event has been investigated by several groups, who have measured the ability of GTP analogues that cause the conversion of high affinity agonist binding sites (which are thought to represent receptor-G protein complexes), to low affinity sites (see "Introduction", Section 2.2). When applied to human brain, this and similar techniques, have demonstrated a substantial decrease in the relative amounts of high affinity agonist binding to M1 and D1 receptors (Flynn *et al.*, 1991; De Keyser *et al.*, 1990), in the frontal cortex of DAT diseased subjects compared to controls. This may be due to a specific alteration of these receptors, possibly by phosphorylation, inhibiting their ability to interact with G proteins. Alternatively, a more generalised change

in G protein-receptor coupling may have occurred in DAT diseased frontal cortex. This seems likely since the effect is observed with different receptors linked to several types of G proteins (D1 to Gs, and M1 to Gq, Go or possibly Gs). It should be noted that high affinity binding to somatostatin receptors was found to be unchanged in DAT (Bergström *et al.*, 1991). However since somatostatin causes the inhibition of adenylate cyclase (Schettini *et al.*, 1989), it is possible that Gi is not affected in the same manner as other G proteins. A possible explanation for reduced receptor-G protein coupling in DAT, may lie in the altered membrane composition that is reported to occur in the disease (Söderberg *et al.*, 1991). Alternatively, the interaction of G proteins with the cell's cytoskeleton may play a role. Tubulin, itself a GTP binding protein, can interact with G proteins (specifically Gi and Gs) and alter their activity (Rasenick & Wang, 1988; Wang *et al.*, 1990, and "Introduction", Section 3). There is strong evidence to suggest that there is a disruption of the cell's cytoskeleton in DAT, for example, the accumulation of neurofibrillary tangles, and the abnormal intracellular localisation and phosphorylation of the microtubule accessory protein tau (see "Introduction", Section 4). It is conceivable that such events may disrupt G protein function, leading to a decrease in their ability to interact with receptors. Indeed, work done in this laboratory has demonstrated a significant decrease in low Km GTPase activity in DAT diseased frontal cortex, suggesting that either not all of the α subunits measured by western blotting are functional, or there is an overall change in G protein activity. A reduction in GTPase activity, or in receptor-G protein coupling, could also be due to a decrease in the levels of β or γ G protein subunits, since their presence is required for both GTPase activity and the interaction of G proteins with neurotransmitter receptors (Kanaho *et al.*, 1984; Florio & Sternweis, 1985; Florio & Sternweis, 1989). Although no significant change was found in the levels of Gβ mRNA, as discussed above, only large changes in mRNA levels would have been detectable. Thus the northern blot study

cannot rule out that a small change in β subunit expression has occurred. Indeed, preliminary data obtained in this laboratory suggests that a decrease in the levels of β subunits has taken place in DAT diseased frontal cortex. Even a small change in β subunit expression, independent of that of α subunits, would have far reaching consequences for many aspects of signal transduction. Clearly this observation may be of great importance and experiments are underway to confirm or refute the preliminary data. Whatever the explanation for the apparent change of receptor-G protein coupling in DAT, it is unclear as to what the *in vivo* consequences of such events may be. However, a reduced ability of M1 receptors to interact with G proteins may account for the failure of cholinergic replacement strategies to significantly improve memory or cognition in DAT patients.

3.5 G protein-effector coupling.

The discussion so far has concentrated on the functioning of G proteins in DAT with respect to their ability to interact with neurotransmitter receptors. To perform any useful function however, G proteins must also be able to couple to their various effector systems. The enzyme adenylate cyclase was chosen to examine the effect that DAT has on such interactions, as it has been well characterised with regard to its interactions with G proteins. As explained in Section 2.4, the ability of G proteins to interact with adenylate cyclase can be estimated by activating the enzyme with fluoroaluminate. The use of fluoroaluminate to stimulate adenylate cyclase activity offers an advantage over using agonists, in that it can be used to specifically assess G protein-effector coupling. The ability of agonists to stimulate the enzyme can however be additionally modified by changes in receptor number and/or receptor-G protein coupling.

Both basal and stimulated activities of adenylate cylase, assayed in membrane preparations in which G protein levels had been previously determined, were found to be substantially decreased in the DAT diseased

frontal cortex, while no significant change was detected in the hippocampus (Figure 20). In contrast, the ratio of stimulated to basal adenylate cyclase activity was not significantly different between the DAT and control subjects, in either of the brain regions examined. This indicated that the presence of DAT has not adversely altered the ability of G proteins and adenylate cyclase to interact. It is therefore unlikely that any increase in the functional activity of inhibitory G proteins is responsible for the decrease in cortical adenylate cyclase activity observed, and that the disease has therefore affected adenylate cyclase itself. Decreased adenylate cyclase activity in DAT diseased frontal cortex may lead to a lowering of intracellular levels of cAMP, and hence decrease the *in vivo* activity of protein kinase A. Alterations in the phosphorylation of various cellular components have been reported to occur in DAT. For example, as well as changes in the phosphorylation of tau, the ability of synapsin 1 (a protein kinase A substrate) and GAP-43 to be phosphorylated *in vitro* is altered in DAT (Parks *et al.*, 1991; Florez *et al.*, 1991), and it has been suggested that phosphorylation of amyloid precursor protein by protein kinase C affects its metabolism (Buxbaum *et al.*, 1990). Furthermore, a lack of phosphorylation by protein kinase A may allow increased phosphorylation at competing sites by other kinases. Despite this, since no change was observed in the activity of hippocampal adenylate cyclase in DAT subjects, it would seem unlikely that the alterations observed in the frontal cortex play a fundamental role in the pathogenesis of the disease. They are therefore likely to be a consequence, rather than the cause of, other events.

3.6 Other studies of adenylate cyclase in DAT.

There exists in the literature several other investigations of adenylate cyclase in DAT diseased brain, giving mostly contradictory results. The results of these studies and that of the present investigation are summarised in Table 9. The study by Danielsson *et al.* (1988) claimed to have detected a

significant increase in basal adenylate cyclase activity in DAT hippocampus. However, calculation of the t-statistic using the data given in the paper does not give a statistically significant change using a two-tailed test ($P = 0.068$). This discrepancy may be due to a misprint or to the manner in which the t-test was carried out. Even if the original data did give a significant change it is likely that it only just reached statistical significance. The actual enzyme activities recorded by Danielsson *et al.* (1988) are much lower than those quoted elsewhere, this being due to the enzyme being assayed in a homogenate which had been pelleted at 1650g, rather than a high speed membrane fraction. Given the non-standard methodology used for the Danielsson study, in addition to the questionable statistical analysis, it is not felt that the conclusions reached by these authors are reliable. The investigation by Ohm *et al.* (1991) detected a significant reduction in adenylate cyclase activity in the hippocampus of DAT diseased subjects. This data is in contrast to those of this study and that of Bergström *et al.* (1991). There are no obvious methodological reasons for the difference in findings of the studies, albeit that Ohm *et al.* (1991) did include the step of homogenising their tissue samples at 37°C in a buffer containing magnesium ions. Although any adverse effects of this treatment should be felt equally by control and DAT diseased groups, it is conceivable that different levels of proteases, or in the localisation of proteases in DAT diseased brain may account for a lower adenylate cyclase activity observed in this group of subjects. Bergström and collaborators also found no change in adenylate cyclase activity between control and DAT groups in the temporal cortex (Cowburn *et al.*, 1991), cerebellum, caudate nucleus or frontal cortex (Bergström *et al.*, 1991), this latter finding being in contrast with that of this study. However the data presented herein is in agreement with Dewar *et al.* (1990) who found a significant decrease in high affinity ^{3}H-forskolin binding in the frontal cortex of DAT diseased subjects, but found no change in the hippocampus. In addition, there existed a significant correlation between

the activities of ChAT and adenylate cyclase in DAT diseased frontal cortex, further suggesting that the disease process is affecting adenylate cyclase in the frontal cortex of DAT diseased subjects. No correlation however was found between adenylate cyclase activity and the density of neuritic plaques in the frontal cortex. However plaques were counted in tissue adjacent to those used for the assaying of adenylate cyclase, and it is possible that local neuropathology differs between the two areas of brain. Even small differences would be enough to affect the strength of correlation due to the relatively low number of subjects used for the analysis. It should be noted though that the investigation of high affinity forskolin binding by Dewar *et al.* (1990), used subjects from the same population of patients as that used in the present investigation. It is possible therefore that there are pharmacological or aetiological reasons for the different findings reported by the various laboratories.

Region	Study	Control	DAT	Significance
Frontal Cortex	(1)	50.1 ± 6.9	28.1 ± 2.3	P<0.01
	(2)	85.6 ± 15.5	72.5 ± 10.9	NS
Temporal Cortex	(3)	60.3 ± 11.8	47.4 ± 6.0	NS
Parietal Cortex	(4)	14.5 ± 1.4	16.4 ± 3.1	NS
Hippocampus	(1)	52.1 ± 3.6	54.1 ± 5.2	NS
	(2)	42.7 ± 4.9	33.2 ± 4.6	NS
	(4)	9.8 ± 1.0	14.3 ± 2.1	P<0.05 [1]
	(5)	42.0 ± 3.0	22.3 ± 2.9	P<0.01
Caudate nucleus	(2)	59.7 ± 5.9	57.5 ± 5.7	NS
Cerebellum	(2)	82.7 ± 10.3	65.1 ± 7.9	NS

Table 9.

Comparision of basal adenylate cyclase activities in several studies of the enzyme in human post mortem brain.

The values quoted are derived from the following studies, and are quoted as mean basal activity (pmols cAMP/min/mg protein) ± SEM.

(1) present study.

(2) Bergström *et al.*, 1991.

(3) Cowburn *et al.*, 1991.

(4) Danielsson *et al.*, 1988

(5) Ohm *et al.*, 1991.

In each case mean activity in control and DAT diseased subjects were compared using an unpaired, two-tailed, Students t-test, this being reported as a significance level (NS = not significant).

[1] - the authors quote a signicance of P<0.05, however the data given in this paper does not give a statistically significant change.

3.7 Possible mechanisms leading to the loss of adenylate cyclase activity in DAT.

The obvious questions that must be addressed are by what mechanism is cortical adenylate cyclase activity decreased in DAT, and why this does not happen in the hippocampus. Since the activity of ChAT is decreased by a large amount in both the hippocampus and frontal cortex, it would suggest that the reason for the robustness of hippocampal adenylate cyclase in DAT, does not lie in this region being less severely effected by the disease than the frontal cortex. However, differences may exist in the localisation, nature, or control of the enzyme in the two regions. It is unclear whether the reduction in activity observed in the frontal cortex, is due to a decrease in the levels of the enzyme, or to a change in its functional capacity. The experiments of Wadman *et al.* (1991) have indicated that phosphorylation of adenylate cyclase may reduce the activity of the enzyme, while those of Yoshimasa *et al.* (1987) strongly suggest that the enzyme can be phosphorylated by protein kinase C. While phosphorylation of adenylate cyclase may take place in DAT diseased frontal cortex, it is not known whether such a modification would be maintained over the post mortem delays encountered in the human tissue. Furthermore, as mentioned previously, Dewar *et al.* (1990) detected a 50% decrease in high affinity forskolin binding in DAT frontal cortex, suggesting a decrease in the actual levels of adenylate cyclase rather than a change in its activity. It is possible that adenylate cyclase, in the frontal cortex, is localised to parts of the cell, such as synapses, which are thought to be preferentially lost in DAT. However since the levels of Gs and Gi are unaltered (which would be expected to be colocalise with adenylate cyclase), it is perhaps more likely that there is a specific downregulation of the enzyme in the disease. This may simply be due to aberrant transcription of the adenylate cyclase gene in DAT. Alternatively a prolonged stimulation of the enzyme may have caused a compensatory decrease in its levels. If the enhanced stimulation

was caused by mechanisms which were only active *in vivo*, or ceased shortly after death, the result would be a decreased activity when the enzyme was subsequently assayed *in vitro*. It is unlikely that increased stimulation by neurotransmitters would result in a downregulation of adenylate cyclase, since in other systems the mechanisms which compensate for such events either involve the phosphorylation and inactivation of specific receptors (Sibley *et al.*, 1984), or decreased ability of Gs to stimulate adenylate cyclase (Hernandez-Sotomayor *et al.*, 1991). However it cannot be ruled out that the loss of specific receptors which cause the inhibition of adenylate cyclase e.g. somatostatin, results in a downregulation of the enzyme, especially since the stimulation of inhibitory opiate receptors in rat, results in increased adenylate cyclase activity (Duman *et al.*, 1988). A potentially reversible increase in adenylate cyclase activity has been observed in frog erythrocytes after treatment with phorbol ester, a substance which activates protein kinase C (Yoshimasa *et al.*, 1987). This effect is thought to be due to phosphorylation of adenylate cyclase. However in duck erythroctes, phorbol esters have the opposite effect, although this may not involve the direct modification of adenylate cyclase itself (Sibley *et al.*, 1984). Increased adenylate cyclase activity can also arise from the removal of the inhibitory influence of Gi (Heyworth *et al.*, 1984). In rat hepatocytes Giα is phosphorylated, probably by protein kinase C, leading to a stabilisation of the G protein trimer, and hence a loss of functional Gi (Bushfield *et al.*, 1990a). Since the addition of phosphatase inhibitors results in a rapid increase in Gi phosphorylation (Bushfield *et al.*, 1991), the dephosphorylation of Gi in hepatocytes appears to be a continuous process, a feature which would preclude its study in post mortem tissue. Of particular interest is the inactivation of rat adipocyte Gi2, which occurs in experimentally induced diabetes (Strassheim *et al.*, 1990, and "Introduction" Section 3). This is accompanied by a large reduction in basal adenylate cyclase activity but without any change occurring in the levels of Gi1, Gi2 or

Gsα. The loss of Gi function appears to be limited to its tonic inhibitory effect upon adenylate cyclase, rather than on receptor mediated inhibition. Although reduced adenylate cyclase activity in diabetic rat adipocytes could be mediated by a number of mechanisms, it is possible that the deactivation of Gi2 leads to an increase in basal adenylate cyclase activity and subsequent downregulation of the enzyme. Since the reduced activity of adenylate cyclase in DAT frontal cortex is also accompanied by a maintenance of G protein α subunit levels, it is tempting to speculate that a mechanism similar to that in adipocytes occurs in brain. Although it is not known as to whether neural Giα can be phosphorylated, Sauvage *et al.* (1991) have recently shown that a protein kinase C-like enzyme is able to phosphorylate and inactivate a G protein, thought to be Gi, which had been purified from human brain. Hence if, as postulated by Masliah and collaborators (Van Huynh *et al.*, 1989; Masliah *et al.*, 1990; Masliah *et al.*, 1991a), protein kinase C activity is altered in DAT, there exists the possibility that it catalyses the phosphorylation and inactivation of Gi in DAT diseased brain. Such an event would presumably lead to increased basal adenylate cyclase activity *in vivo*, and may subsequently elicit a compensatory reduction in the levels of the enzyme. Furthermore, the heterogeneous distribution of the various protein kinase C isotypes offers an explanation as to why such events take place in the frontal cortex, but not in the hippocampus (Nishizuka, 1988). It is possible that a change in protein kinase C activity could be mediated via a rise in intracellular calcium concentration, since calcium can stimulate the formation of diacylglycerol (which is also required for its activation), via its effects on phospholipase C (Kennedy, 1989), or on phospholipase D (Billah *et al.*, 1989).

Another possibility concerns the two subtypes of adenylate cyclase that exist in brain, namely the calcium-sensitive and insensitive enzymes. In cells which possess the calcium-sensitive form, a prolonged increase in intracellular calcium concentration, stimulating the cells adenylate cyclase

activity, may lead to a down regulation of both types of adenylate cyclase, or of the calcium-sensitive enzyme in particular. The latter possibility is supported by the small increase in the levels of GsL compared to GsH detected in the frontal cortex of DAT subjects, since as described in Section 2.4, GsL may be coexpressed with the calcium-insensitive form of the enzyme. In addition the possibility that a decrease in G protein β subunit levels has occurred in DAT diseased frontal cortex also supports this hypothesis, since the stimulation of adenylate cyclase by calcium can be inhibited by G protein βγ subunits (Katada *et al.*, 1987). Thus, a decrease in β subunit levels may potentiate the stimulation of adenylate cyclase by calcium. There is much circumstantial evidence suggesting that intracellular calcium may be elevated in DAT diseased brain. For example, the application of excitatory amino acid neurotransmitters to rat cortex, (which leads to an increase in calcium levels), causes similar patterns of neuronal death to that observed in DAT (Maragos *et al.*, 1987). In addition, increasing the intracellular calcium concentration of cultured rat hippocampal neurons, causes the formation of structures strongly resembling neurofibrillary tangles (Mattson, 1991). The use of primary cell cultures has also indicated that β-amyloid can interfere with calcium homeostatis (Mattson *et al.*, 1992). Furthermore, if the human hippocampus contains lower amounts of calcium sensitive adenylate cyclase, it could offer an explanation as to why adenylate cyclase activity is unaltered in the hippocampus. However, as described in Section 2.4, mRNA encoding this form of the enzyme possesses a similar abundance in rat hippocampus as that found in the cortex. The development of antisera to adenylate cyclase should allow the distribution of the two forms to be mapped in brain, as well as allowing any alterations that have occurred in DAT to be determined.

The possible mechanisms leading to down regulation of adenylate cyclase are summarised in Figure 39, which demonstrates that a decreased adenylate cyclase activity in DAT diseased brain is not incompatible with a

rise in intracellular calcium concentration having occurred, possibly due to the excitotoxic effects of glutamate.

3.8 Closing comments.

The effect that DAT has on cortical adenylate cyclase activity could have important consequences for potential therapeutic strategies which act through this enzyme. For example, the failure of cholinergic replacement therapies may lead to attempts to increase the levels of other transmitters which are reduced in DAT, for example somatostatin. Although such a strategy would apparently worsen a deficit in adenylate cyclase as discussed above, it is not certain whether decreased activity occurs *in vivo*. More generally, the data presented here demonstrate that significant alterations in signal transduction can occur in the DAT. It is therefore necessary to develop the investigation of the disease to include other effector systems such as phospholipase C. This particular enzyme is one of the major routes of cholinergic signal transduction, and has recently been immunohistochemically localised to neuritic plaques (Shimohama *et al.*, 1991). It would be of interest therefore to determine its activity in the brains of control and DAT subjects, especially since the receptor for the second messenger it produces, IP_3, has been found to be lowered in DAT diseased brains (Young *et al.*, 1988). Similarly, another phospholipid metabolising enzyme phospholipase A_2, may possess increased activity in the disease as an increased abundance of its metabolites are detected in DAT diseased frontal cortex (Blusztajn *et al.*, 1990). Thus, much work needs to be done to gain a full understanding of the mechanisms leading to the breakdown of intercellular communication in DAT. Once completed such studies will allow a more realistic assessment of whether any strategies designed to treat the disease once cognitive dysfunction has occurred, will ever prove successful. This will also provide insights into the pathological mechanisms involved in the disease process.

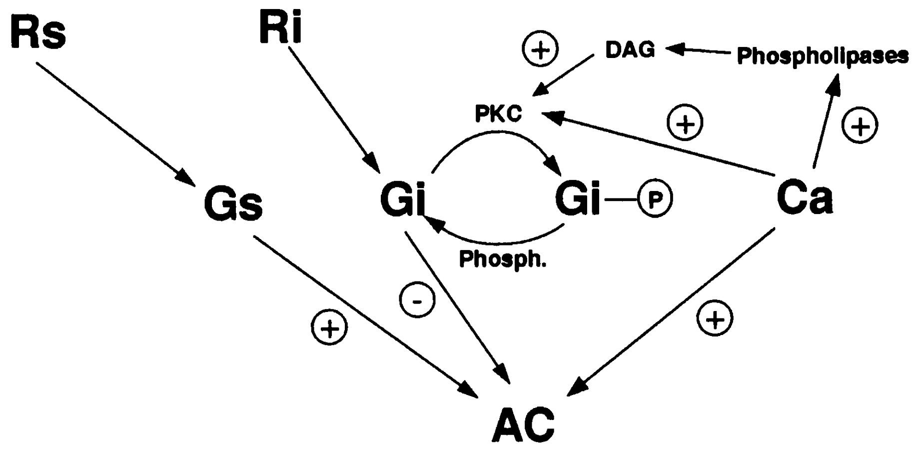
Figure 39.

Downregulation of adenylate cyclase in DAT.

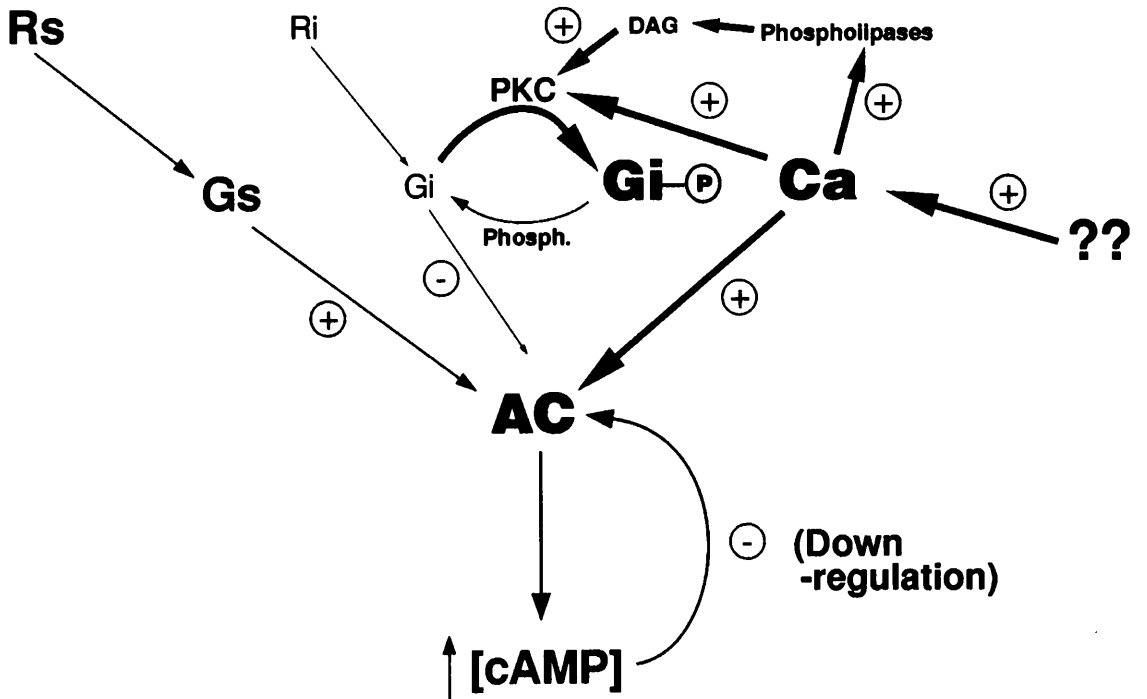
(a) Under normal circumstances adenylate cyclase activity is controlled by stimulatory receptors (Rs) acting via Gs, and inhibitory receptors (Ri) acting via Gi. Although it is not certain, it is likely that Gi in brain can be inactivated in brain by phosphorylation (Gi-P) by protein kinase C (PKC) (activated by diacylglycerol (DAG) and Ca^{2+}), an effect which is reversed by phosphatases (phosph.). In addition, adenylate cyclase can also be stimulated by a rise in intracellular calcium ion concentration.

(b) In DAT (line thickness and size of characters indicates the relative effect of each feature) a decline in inhibitory G protein activity, whether due to loss of inhibitory receptors e.g. somatostatin, or increased phosphorylation of Gi by protein kinase C, may lead to a rise in basal cAMP levels. Similarly, elevated intracellular calcium concentration would also be exspected to raise cAMP levels. These events may in turn lead to a compensatory downregulation of adenylate cyclase itself. Note though that, with the exception of the loss of inhibitory receptors, the other changes would only be apparent either, *in vivo* (rise in calcium concentration), or would be reversed soon after death (phosphorylation of Gi). Thus the net result would be decreased adenylate cyclase activity when the enzyme was assayed *in vitro.*

(a)



(b)



APPENDIX A1

The values of the parameters measured in each human subject used in the analysis of the frontal cortex and hippocampus ("Results", Section 3), are listed in Table 10.

Table 10.

G proteins levels and enzyme activities of each human subject.

The values of the parameters measured in each human subject, as described in Section 3 of "Results", are shown. GsH, GsL, Gi1, Gi2 and Go refer to the G protein α subunits, and are quoted in optical density units. Bas. AC and Stim. AC refer to basal and fluoroaluminate stimulated adenylate cyclase activity respectively, and are quoted in pmols cAMP/ min/mg protein. ATPase refers to sodium potassium-dependent ATPase activity and is quoted in nmols Pi/min/mg protein. ChAT refers to the activity of choline acetyl transferase, and is given as nmols acetyl choline/ hour/mg protein.

FRONTAL CORTEX

Subject	GsH	GsL	Gi1	Gi2	Go	Bas. AC	Stim. AC	ATPase	ChAT
C14	0.112	0.040	0.101	0.104	0.162	68	182	337	3.5
C15	0.133	0.099	0.055	0.074	0.069	26	70	180	4.5
C16	0.243	0.124	0.097	0.121	0.177	55	175	308	3.2
C19A	0.187	0.094	0.087	0.107	0.190	82	211	251	4.2
C21	0.153	0.081	0.053	0.083	0.128	50	108	198	2.0
C22	0.165	0.070	0.058	0.078	0.129	24	556	149	4.0
C25	0.160	0.057	0.063	0.091	0.157	53	127	210	2.7
C35	0.210	0.087	0.087	0.096	0.172	43	118	185	4.0
A23	0.175	0.086	0.076	0.109	0.124	31	87	210	1.9
A30	0.123	0.106	0.042	0.065	0.140	30	68	210	1.7
A33	0.132	0.153	0.046	0.107	0.138	19	59	281	1.4
A53	0.148	0.093	0.045	0.069	0.061	26	49	181	1.5
A54	0.199	0.088	0.060	0.094	0.189	29	82	174	2.0
A55	0.163	0.087	0.075	0.110	0.159	41	103	177	3.5
A56	0.202	0.117	0.051	0.086	0.088	26	65	149	0.9
A63	0.094	0.080	0.067	0.744	0.020	23	49	110	2.2

HIPPOCAMPUS

Subject	GsH	GsL	Gi1	Gi2	Go	Bas. AC	Stim. AC	ATPase	ChAT
C8	0.079	0.060	0.039	0.037	0.334	52	187	194	1.5
C16	0.104	0.174	0.063	0.065	0.387	39	131	235	1.8
C19A	0.132	0.221	0.027	0.034	0.350	69	244	208	2.5
C20	0.162	0.160	0.023	0.032	0.332	52	158	182	3.5
C28	0.163	0.201	0.062	0.038	0.339	45	127	172	2.3
C33	0.124	0.161	0.057	0.030	0.243	60	167	193	4.5
C34	0.122	0.085	0.052	0.025	0.248	57	172	142	4.7
C37	0.176	0.093	0.073	0.047	0.315	40	146	155	1.7
A17	0.140	0.081	0.041	0.047	0.275	76.	218	207	1.0
A18	0.174	0.125	0.030	0.051	0.331	39	134	216	1.1
A29	0.221	0.170	0.019	0.034	0.302	50	153	165	0.9
A30	0.160	0.149	0.021	0.024	0.250	43	141	220	0.7
A33	0.201	0.140	0.056	0.039	0.257	57	213	168	0.8
A35	0.161	0.102	0.059	0.033	0.260	63	206	180	0.8
A53	0.085	0.084	0.046	0.024	0.201	35	108	125	0.9
A54	0.093	0.052	0.070	0.047	0.312	67	208	176	1.3

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