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Susan Ann Valley
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ABSTRACT

Alteration of the Adenylate Cyclase System
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Creutzfeldt-Jakob Disease

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Creutzfeldt-Jakob (CJD) disease is an infectious, progressive neurological disorder which results in human dementia. Synaptic membranes from various brain regions of guinea pigs and hamsters infected with Creutzfeldt-Jakob disease show increased guanyl nucleotide mediated activation of adenylate cyclase which appears to be due to a greater coupling of stimulatory subunits (Ns) and not to a decreased coupling of inhibitory subunits (Ni) or to a change in the catalytic subunit of adenylate cyclase. In addition to the increase in Ns coupling, CJD infected membranes appear to be more fluidized than normal membranes especially in the cerebral cortex. Experiments with selected neurotransmitters and neuropeptides indicate that a sub-population of cyclases (dopamine specific) are involved in the CJD effect. It is possible that these effects are due to the direct action of the CJD infectious agent, or a pathological product resulting from that agent, upon synaptic membrane adenylate cyclase.

TABLE OF CONTENTS

INTRODUCTION

Stimulation or inhibition of adenylate cyclase (AC) by hormones or neurotransmitters is a complex process (see Appendix B) involving transduction of several different stimuli into one common intracellular signal thereby permitting summation of these signals. Such a process involves a large number of complex interactions which take place in the plane of the membrane. Several protein components and surrounding lipids of the membrane are responsible for changes in signal transduction.

Adenylate cyclase has been shown to be important in memory; alteration of the intracellular regulation of this enzyme results in decreased learning ability (18). Since a decline in cognitive and memory function are among the characteristic clinical findings in human victims of Alzheimer's disease and in the transmissible spongiform encephalopathy Creutzfeldt-Jakob disease (CJD), it is possible that alteration of adenylate cyclase components may be responsible for the decline in memory function of patients with these diseases. Whether this alteration is the result of a transmissible agent (especially in the case of CJD) or agent triggered changes in the membrane composition remains to be elucidated.

Alterations in neurotransmitters have also been shown in dementing illnesses including Alzheimer's disease and spongiform encephalopathies (including CJD and scrapie, see Appendix A). Changes in choline acetyltransferase are well documented in Alzheimer's disease and have been shown to occur in scrapie (62). Serotonin also has been shown to be altered in Alzheimer's disease (5) and scrapie (82). Since Alzheimer's disease and scrapie appear to share dysfunctions in their neurotransmitters, elucidation of the role of specific transmitters in Alzheimer's disease and CJD is important to

determine if these are specific defects or if they are part of a global effect of a related dementing process.

Creutzfeldt-Jakob Disease is known to produce severe membranes alterations. Thus via direct disruption of integral receptors and/or associated second messesger systems, changes in the adenylate cyclase system may be manifest. Preliminary studies investigating the functional capacity of membrane adenylate cyclase in CJD showed an increased interaction of adenylate cyclase regulatory subunits in CJD, resulting in an increased cyclase activity in basal ganglia and hippocampal membranes from guinea pigs infected with CJD as compared to normal membranes (79). The integrity of the adenylate cyclase system in CJD therefore appeared to be intact despite pathological changes at the synaptic and membrane level. We attempted to further characterize the alterations in adenylate cyclase from neuronal tissue in Creutzfeldt-Jakob disease.

APPENDIX A

Epidemiological studies indicate that between 2 and 4 million persons in the United States are currently afflicted with a nonreversible dementia (5). The National Center for Health Statistics estimates that by the year 2000 there will be 6.7 million people 85 years and older with the fastest growing segment of the population those age 75 years and older (44). The rapid growth of the elderly population at risk for dementing disorders predicts a significant burden on our society unless ways are discovered to prevent and treat this "silent epidemic" of the next century. The most common nonreversible dementia is neuronal degeneration of the Alzheimer type occuring in more than one half the cases that come to autopsy.

A small subset of human dementias, originally classified with degenerative dementing illnesses of the Alzheimer type are caused by infectious unconventional agents. Similarities exist between Alzheimer's disease (AD) and the slow virus group of dementing illnesses including Creutzfeldt-Jakob Disease (CJD). Both show a similar average age of onset: AD: 67years with a range of 40-89 years, CJD: 60 years with a range of 35-84 years. Familial cases constitute 10-15% of both AD and CJD cases with an apparent autosomal dominant inheritance. In an analysis of 73 families with CJD and 52 families with AD, four families were found in which one member had pathologically confirmed CJD and at least two members with AD.(61).

Creutzfeldt-Jakob Disease has an incidence of 1-2 per million per year worldwide. Kuru, a disease limited to a region of New Guinea has been shown to be transmitted by ritual cannabilism (22) and has been eradicated with the termination of this practice. A naturally occuring transmissible disease of sheep (Scrapie) is also caused by a similar unconventional

agent. All of these diseases share common pathological (spongiform) changes in the CNS, have been shown to be transmissible and lack an immune response to the infection. This class of infectious agents is still not well characterized, but notably these "viral" agents are small and resistant to a variety of physical and chemical treatments.

In 1959 William Hadlow noticed histological similarities between scrapie and the then newly described kuru. Kuru was subsequently transmitted to nonhuman primates by Gajdusek et al. (22). Histologic observations by Klatzo, Gajdusek and Zigas further documented the common pathological features in kuru and Creutzfeldt-Jakob disease. Subsequent transmission of CJD to apes by Gibbs et al. (23) and to guinea pigs by Manuelidis (51) further solidified the relationships between these diseases.

CJD is a progressive neurological disorder characterized by dementia, spongiform changes in the CNS and relatively rapid decline to death (1-2 year clinical phase). In humans, symptoms occur insidiously and their onset is difficult to date. Initially the patient exhibits vague symptoms such as: depression, anxiety, irritability followed by degrees of confusion, memory defects and progressive dementia accompanied by signs of upper and lower motor neuron disturbance and myoclonus. In terminal stages the patient becomes mute, rigid and unresponsive followed by coma and death. The clinical phase ranges between 1-120 months with the mean course about 10 months. EEG shows a characteristic pattern of periodic bursts of sharp waves on a diffuse slow backround (42).

The natural mode of transmission is still unknown. The incubation among familial cases is postulated to be from 10-40 years. In iatrogenic transmissions (via corneal transplant (19) and depth electrodes (3), incubation time ranged from 18-30 months. There is evidence against maternal transmission in experimental disease in rodents and primates (1,54). Viremia of both guinea pig and man has been demonstrated by

transmission of CJD from buffy coat preparations (52,53). Further studies indicated that viral replication may take place in lymphocyte populations (45). In animals, infectivity appears in the spleen several weeks before high levels are apparent in the brain.

About 65% of cases show some evidence of cortical atrophy in CJD, mostly in the frontal lobes. The most pronounced microscopic features are spongiform changes, neuronal degeneration and astrocytic changes without significant inflammatory infiltrates. The lesions are almost exclusively in the gray matter although a Japanese varient has been described with lesions in the white matter (65). The majority of lesions occur in the cerebral cortex although there is great variation between patients, and other regions have been noted to be involved including basal ganglia, thalamus, cerebellum, dentate nucleus, brainstem nuclei. Some of these variants may be due to different (variant) isolates of the infectious agent. Isolation of a variety of strains of the scrapie agent, including those producing amyloid-like plaques, has been accomplished using inbred mice with different alleles of the "scrapie incubation" gene ("SINC") that contols incubation time (16,93).

Spongiform changes consist of small, well delineated vacuoles scattered throughout the neuropil. They are most often seen in dendrites and in the neuropil. The degree of vacuolization is variable in different patients. Increased numbers of astrocytes may be present. Inoculated animals show essentially the same general histologic changes as seen in human material. Electron microscopy demonstrates a decrease in the number and organization of organelles in the perikarya of neurons and astrocytes (43). Occasional vacuolar membranous structures may be found in the perikarya. Neuronal processes exhibit swellings with clearing of the matrix These changes occur in both dendrites and axons as well as in astrocytes. Large vacuoles seen under light microscopy appear to be formed by progressive coalescing of

adjacent swollen processes. Both neurons and astrocytes show increased numbers of lysosomal structures.

It has been proposed (50), that some cases of Alzheimers disease may also be caused by an unconventional agent. However, thus far Alzheimers disease has not been shown to be transmissible and there are no animal models directly derived from this disease. Because the transmissible encephalopathies, especially CJD share some similarities in clinical picture, pathology and epidemiology, experimental CJD models are useful for the study dementias of unknown etiology. With such models the course of these degenerating dementias can be followed from beginning to end. The identification of specific disease mechanisms may shed light on potential common pathway in all progressive dementias.

To date the CJD agent has not been identified. The agent has been found in high titers in synaptosome enriched membranes and is thought to be bound and/or sequestered within the membranes at late stages of the disease (56). Membrane disrubting detergents (Sarkosyl, NP-40, Triton X-100) partially solubilize infectivity from synaptosomal membranes (60). In both CJD and scrapie but not in normal brain two components have been identified in the infectious fractions. One is a protein component described as a proteinase-K-resistant 27 to 30 kd gel band known as "protease resistant" protein PrP27-30, or prion protein (76). In CJD a 35 kd glycoprotein band (not digested with proteases) that cross reacts with anti-scrapie PrP 27-30 antibodies have also been identified (60). Although not visible in fractions from normal brain, this 35 kd protein (or prion protein) has been identified in normal homogenates (69). The cDNA of this protein has been sequenced and corresponds to a normal genomic DNA sequence that is transcribed as mRNA equally in both normal and scrapie brain (9). This protein, when isolated with SDS, shows no significant infectivity (57). Two theories exist concerning the role of this protein in infectivity. One group considers this protein itself is the infectious agent while

two other groups postulate that this protein may represent a marker or pathological product that co-sediments with infectivity in many cases of CJD and scrapie. Studies of humans have shown about 1/3 of cases fail to detect this protein (7).

The second component found in CJD and scrapie infectious fractions has been termed SAF (scrapie-associated fibrils)(63). SAF fibrils have a similar structure to βamyloid of Alzheimers disease but they are constituted by distinct peptides. SAF have not been found in uninfected brain tissue (64). Although these structures are present in purified infectious fractions, there have been reports of infectious scrapie fractions with no SAF present (77), although this may be a difference in terminology. SAF and prion protein appear to be constituted by the proteolytic cleavage products of Gp 34 (90). To date, no agent, non host molecules of any sort have been detected in CJD or scrapie .

APPENDIX B

CNS neurons often have more than a thousand synaptic inputs, many of these involve different neurotransmitters. Signals presented at various sites and times are integrated by converting them into the common intracellular languge of cyclic nucleotides or calcium ions which are the most common second messengers. Thus, integration of certain extracellular signals can be achieved by changing intracellular levels of cyclic nucleotides which serve as their second messengers. While cyclic nucleotide systems occur in both neurons and glial cell types, the specific activity of these enzymes is generally higher in neuronal cell or in neuronally derived cell lines (101).

The composition and interactions of the adenylate cyclase components within the plasma membrane of each cell type varies and is subject to regulation of gene expression. Under ordinary conditions it appears that receptor molecules and nucleotide binding proteins are in excess over catalytic units of the adenylate cyclase system. Occupancy of a receptor with an agonist increases the probability of a coupling of the agonist occupied receptor to the adenylate cyclase catalytic unit. This reaction involves the mechanism described below. Receptors are either stimulatory or inhibitory and are specific for the hormone. The number of each receptor varies with cell type, cell age, developmental status as well as hormonal and drug-induced variations. Some of the hormones/transmitters influencing the adenylate cyclase system include the following:

Stimulatory	*Inhibitory*
Glucagon	Opioids
Vasopressin	Acetycholine
Epinephrine (β)	Epinephrine (α_2)
Dopamine (D_2)	Dopamine (D_1)
LH,FSH,TSH	Adenosine
VIP,ACT	

Adenylate cyclase (AC) is a plasma membrane bound enzyme system which functions as a "second messenger" to catalyze the synthesis of cyclic AMP from ATP on the cytoplasmic side of the membrane (Figure 11). In the CNS, second messenger systems are integral components of cellular functions which include phosphorylation of proteins important for neurotransmitter effects, and cellular differentiation. The synthesized cyclic AMP functions as an intracellular mediator by virtue of its intracellular concentration, which can change 5-fold upon hormonal or other external membrane stimulation. The normal intracellular concentration of cAMP ($\leq 1 \mu M$) is in dynamic equilibrium and is influenced by two components: a)stimulation of AC (which will increase the intracellular concentration) and b)phosphodiesterases which hydrolyze cAMP to 5'-AMP thereby decreasing the concentration of cAMP intracellularly.

Receptors on the surface of the cell membrane activated by hormones or other extracellular molecules do not activate AC directly. Integral membrane proteins which binds GTP on its cytoplasmic surface, couple the two together. These proteins, called N proteins (or G proteins) were first suggested by the requirement of GTP for hormonal activation of cyclase (81). Addition of a hormone or other appropriate extracellular binding molecule to the receptor of an adenylate cyclase system results in three consequences: a)stimulation of the GTP-mediated activation of the system, b)stimulation of the GTPase activity of Ns (s indicates a stimulatory N protein, i indicates an inhibitory N protein, see below) and c)stimulation of guanine

nucleotide release and/or exchange. The hormone-receptor complex accelerates the activation of GTP-Ns by increasing the affinity of Ns for Mg. By increasing the affinity for Mg, the complex becomes fully saturated by Mg at physiologic concentration of Mg, instead of requiring the higher concentration needed without hormonal stimulation. Inhibitory hormones will not change the affinity of Ni-GTP for Mg, as this complex has a high affinity before interaction with the hormone.

The hormone receptor complex has sufficient affinity for the GDP-N complex that it drives their interaction and promotes dissociation of GDP. This allows a subunit of N, α, to bind GTP and become active. When GTP binds, the hormone receptor complex dissociates. The GTP-N is then free to become activated by its increased affinity for Mg (in the case of Ns) followed by dissociate into sub-units (Figure 12).

N proteins in the membrane are activated upon binding of guanine nucleotides. GTP or non hydrolyzable analogues of GTP such as Gpp[NH]p and GTPγS are used <u>in vitro</u> to assess N protein activation of cAMP in membrane preparations. Magnesium concentration also influences adenylate cyclase activity (see below).

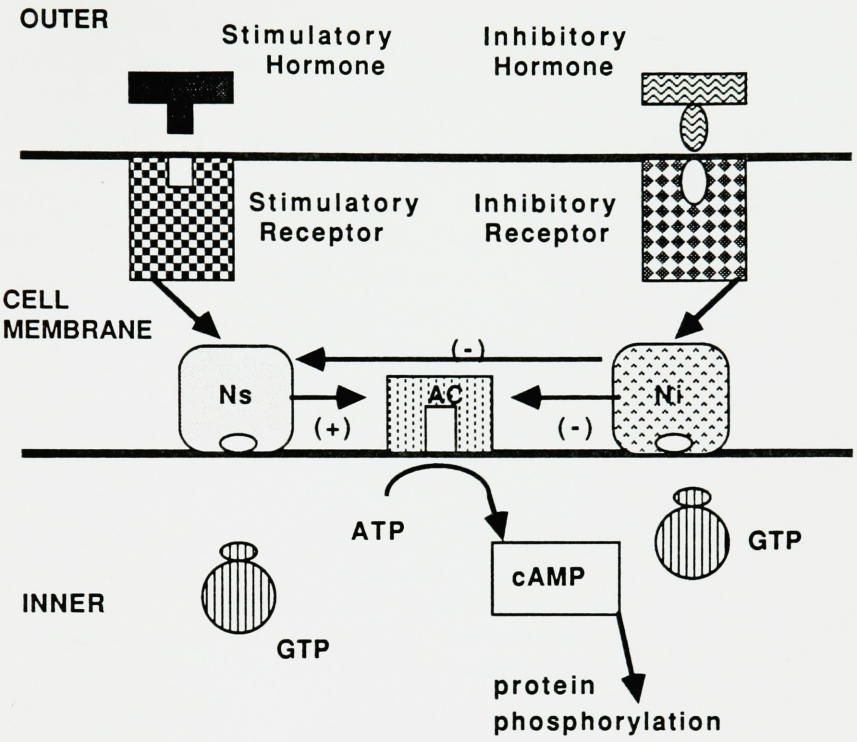

FIGURE 11: Theoretical model for adenylate cyclase system (41). AC represents the catalytic unit of adenylate cyclase. Stimulation via stimulatory hormones or other membrane targeting molecules enhances adenylate cyclase activity by binding via receptor to Ns (+) along with GTP and increases intracellular levels of cAMP. Cyclic AMP then activates specific kinases causing phosphorylation of protein(s). Inhibitory hormones act through Ni to either directly inhibit the catalytic unit (-) or to decrease Ns activation of the catalytic unit (-).

There are many classes of N proteins. A stimulatory N protein (Ns) activates cyclase and increases intracellular cAMP,

an inhibitory N protein (Ni) has a direct effect on cyclase decreasing the amount of cAMP produced, and also feeds back upon Ns decreasing the stimulatory action of that N protein (Figure 11). A third N protein (Nt) stimulates retinal cGMP phosphodiesterase. N proteins also stimulate phosphoinositide hydrolysis, and by this mechanism regulate intracellular calcium (11) which is important in neuronal function. N proteins have also been shown to regulate ion channels (32,73). The elucidation of specific N proteins with stimulatory or inhibitory functions was made possible by assays that purified N proteins (68) and by the study of cells that lack specific N proteins (i.e. cyc^- varients of S49 lymphoma cells that lack stimulatory N proteins) (36). In addition, the capacity of certain bacterial toxins to ADP-ribosylate specific N proteins was discovered for cholera toxin (8,24) and pertussis toxin (38,39) which proved useful in further characterization of N proteins. Much of the basic understanding of the AC system has come from studies on purified cyclase components. Although there is good evidence that these findings hold true in more complex systems (i.e. tissue membranes) the fact that animal tissues contain many membrane components other than the adenylate cyclase system, additional components in vivo may influence the adenylate cyclase system.

N proteins are characterized by shared actions (25). First both GTP and an appropriate hormone for the specific receptor are required to initiate a response. Second, the response can be provoked independently of the receptor by inclusion of nonhydrolyzable analogues of GTP (Gpp[NH]p or GTPγS) in in vitro studies. Third, there is negative feedback regulation between the binding of a hormone to a N-linked membrane receptor and the binding of guanine nucleotide to a N protein. Fourth, cholera toxin and/or pertussis toxin have characteristic effects on the functions of known N proteins.

The three well characterized N proteins (Ns,Ni,Nt) are close structural homologs (68). All appear to have a α-β-γ subunit structure. The α subunits contain a single high-affinity

binding site for guanine nucleotides. In addition, the α subunits are specific substrates for bacterial toxin catalyzed ADP-ribosylation. For example cholera toxin activates Nsα increasing intracellular cAMP and pertussis toxin acts on Niα to yield a decrease in intracellular cAMP. The α subunits of N proteins differ in molecular weight [Ns = 45 or 52kd, Ni = 41kd, Nt = 39kd]. β subunits of all three N proteins however are very similiar [35kd]. These polypeptides are functionally interchangable and their amino acid compositions and peptide maps are indistinguishable (91). The third subunit γ is also similar for all three N proteins [5-10kd]. Not much is known about the γ subunit which is tightly associated with β. β and γ dissociate together as a complex from Nα in the presence of activating hormones and like the β subunit are functionally interchangable (Figure 12).

Stimulation of the catalytic unit of adenylate cyclase by Ns requires the interaction of this catalytic unit along with the activated guanine nucleotide-Ns subunit to form guanine nucleotide-Nsα. The rate of activation of the coupling is dependent upon the concentration of Mg ion. Activation of cyclase is comparitively slow at physiologic concentrations of Mg (0.5mM) and is accelerated (4-5 fold) when the Mg concentration is increased (>10 fold). Rates of activation vary according to the guanine nucleotide used. Nonhydrolyzable Gpp[NH]p in vitro shows a slower activation than GTP. Studies on cyclase activation have shown Gpp[NH]p to take twice as long as GTP to reach maximal cAMP production (4).

Studies on the activation of biochemically purified Ns in the presence of non-hydrolyzable guanine nucloetides show the following: guanine nucleotide first interacts reversibly with N, then the guanine nucleotide-Nαβγ complex isomerizes in a Mg dependent manner to give a complex in which the guanine nucleotide does not dissociate. Finally the complex undergoes a temperature-dependent subunit dissociation reaction to give a Nα-G complex and a Nβγ complex (12). All these reactions are

reversible. Subunits reassociate on cooling and the guanine nucleotide dissociates on removal of Mg (Figure 12).

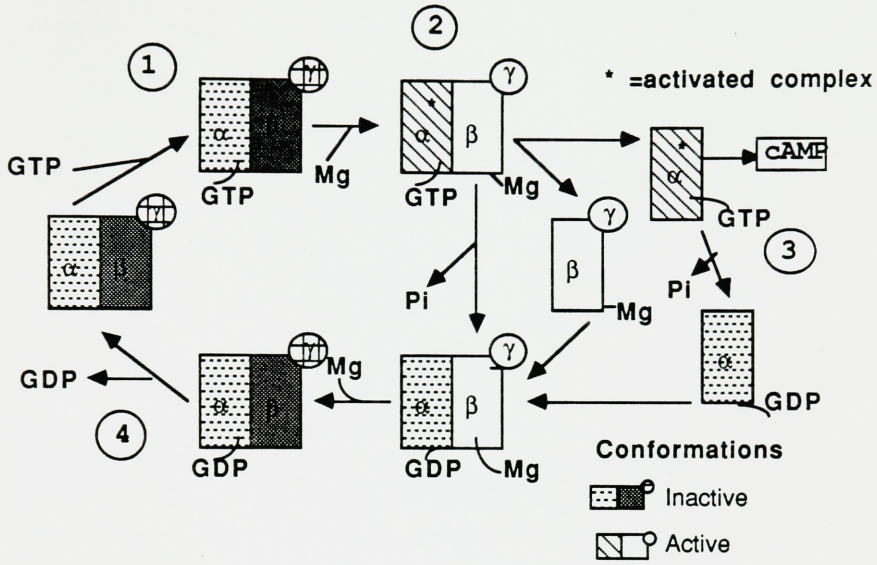

FIGURE 12. Regulatory cycle of N proteins (Ni or Ns) as it may occur under the influence of GTP and Mg (44). The cycle is divided into four steps: 1)association of GTP 2)activation of N and subsequent dissociation of N into $N\alpha^*$ and $N\beta\gamma$. 3)hydrolysis of GTP and deactivation of N 4)dissociation of GDP. The undissociated $\alpha^*\text{-}GTP_{\beta\gamma}\text{-}Mg$ and the dissociated α^*-GTP are represented as active GTPases as it is not known which form of the N protein hydrolyzes the GTP. This cycle can represent Ns and/or Ni. Mg is present and drives the activation in steps 1 and 2.

The apparent Km of Ns for guanine nucleotides is in the order of 10^{-6}M. Since the intracellular concentration of GTP is approximately 10^{-4}M, guanine nucleotide is in excess and Ns is always saturated. Under physiologic conditions, and in the absence of hormonal stimulation, Ns does not fully stimulate the AC system because Ns itself catalyzes GTP (GTPase) and

therefore metabolizes its stimulatory form. Furthermore, the Km for Mg is 5-10mM which is >10 fold higher than the intracellular concentration of 0.5mM. Therefore under (non-hormonal) stimulated physiologic conditions, the concentration of Mg is too low to more than slowly activate Ns.

Magnesium shifts the equilibrium of the reaction to favor activation of the N protein in the presence of GTP with consequent stimulation by Ns (Figure 12). The rate of dissociation of Gpp[NH]p from Nα is slow. However βγ increases Gpp[NH]p or GDP rate of dissociation by about three fold (29). Therefore high concentrations of Mg enhance subunit dissociation to yield an extremely stable endproduct Nα-Gpp[NH]p-Mg.

In vitro regulators of activity include non-hydrolyzable GTP analogs which are modified by substituting imidodiphosphate (Gpp[NH]p) for the pyrophosphate of GTP, or by substituting thiophosphate (GTPγS) for the phosphate of GTP. These analogs are useful because they retain their ability to interact with GTP-specific sites(and compete with GTP for these sites). They also are relatively resistant to hydrolysis. The latter feature depends on the modified phosphate contained in these analogs. Such GTP analogs are quantitatively more effective than GTP in stimulating cyclase activity. Although GTP without hormone generally is not very effective in stimulating adenylate cyclase (see above), nonhydrolyzable analogs are often quite effective. Also, analogs plus hormone generally give higher rates of cyclase activity than hormone plus GTP. Nonhydrolyzable analogs lead to a qualitatively different state of "persistent" activation causing an apparent increase in agonist potency for enzyme acitvation. In summary nonhydrolyzable analogs allow for stimulation of the adenylate cyclase system without the use of hormones. Since the hormone-receptor complex alters the adenylate cyclase system, use of these analogs allows investigation of the intrinsic adenylate cyclase stimulatory system.

Ni like Ns is a GTPase. The two differ in their affinity for Mg. While the affinity of Ns for Mg is low, that of Ni is high. Therefore at physiological conditions, Ni is saturated not only by guanine nucleotides but also by Mg. Ni can act on Ns (Figure 11). The inhibition of adenylate cyclase, via inhibition of the Nsα-catalytic unit complex arises from a local increase of $\beta\gamma$ complex (from stimulation of Ni by GTP and Mg, and by the concomitant subunit dissociation reaction) (4). In addition to inhibition via this indirect mechanism there is evidence for a direct effect of Niα on the cAMP catalytic unit. This is illustrated in studies showing that Ni is active in reducing AC activity in Ns-deficient systems such as in the cyc$^-$ varient of the S49 lymphoma cells (30,36). These two mechanisms of inhibition are non-competitive. The direct mode of inhibition is not due to displacement of Nsα from a common site on the catalytic unit.

Under conditions with no hormonal influence,these two systems (Ns, Ni) continuously cycle giving rise to steady state levels of active Ns and Ni as well as basal rates of GTP hydrolysis. Therefore whenever GTP (or GTP analog) effect on AC is measured, the activity measured is a balance of both the amount of activated Ns (stimulating AC) and activated Ni (inhibiting Ns and AC) (4).

Several other chemicals are useful for investigating specific features of the adenylate cyclase system. Forskolin, a diterpine, activates adenylate cyclase systems. Although its exact mode of action is unknown, studies indicate that it functions by interacting with the catalytic unit of adenylate cyclase causing an increase in cAMP. In addition, the expression of its effect is dependent on, and/or affected by, additional factors and/or compounds including Ns (10). <u>In vitro</u> forskolin stimulates the complete adenylate cyclase system many more times than other stimulators such as Gpp[NH]p or NaFl (3 fold the stimulation by Gpp[NH]p alone (89) and thus can be used to determine maximal cyclase

response of a system. In addition hormones acting through Ni are better seen in a forskolin-stimulated assay than in an unaffected system (4,89).

Mangenese activates the catalytic unit of the AC system. Under known assay conditions it reflects activity of the catalytic moiety of adenylate cyclase independent of the guanine nucleotide-Ns complex. Activity stimulated by Mn^{2+} can therefore be used to evaluate the relative quantity of intrinsic AC activity in different membrane preparations.

The lateral mobility of the AC components within the membrane is necessary for the formation of complexes that lead to activation of the enzyme and production of cAMP. Lateral mobility may be influenced by integral membrane proteins kept in place by both submembranous structures (spectrin) and cytoskeletal proteins. The mobility of adenylate cyclase components is also restricted by membrane lipids. Increases in membrane fluidity augment enzyme activity whereas activity is reduced when membranes become more rigid.

One membrane fluidizer is ethanol. Ethanol exerts its physiological effects by interacting with the lipid components of cellular membranes. Numerous studies have demonstrated that ethanol and other alcohols disrupt or fluidize the lipid structure of membranes. Ethanol appears to increase the rate of activation of cyclase by guanine nucleotides and concomitantly increase the affinity of the system for magnesium. The effect of ethanol can vary in different regions of the brain (98); thus it has been suggested that ethanol may have selective effects on different receptor-coupled adenylate cyclase systems.

Colchicine and vinblastin, antimicrotubule drugs, have also been reported to potentiate hormone-stimulated cAMP production in a variety of cell systems. Studies have shown these compounds release a 42kd GTP-binding protein from

synaptic plasma membranes along with loss of G-unit activity (80). Loss of these adenylate cyclase components after colchicine treatment suggests tubulin can modify the coupling process within the adenylate cyclase system.

Amphotericin B is a polyene that binds sterols. Cholesterol is a major lipid component of the plasma membrane that interacts with phospholipids in the membrane to suppress non-specific leakage of polar substances across the bilayer and modulates bilayer fluidity. Elevation of cholesterol concentrations in plasma membranes has been shown to lead to an inhibition in adenylate cyclase activity (102).

Amphotericin B by binding cholesterol results in increased adenylate cyclase activity. Interestingly, scrapie infected animals treated with amphotericin B extended their incubation times (i.e. decrease the agent replication) even after intra-cerebral inoculation of the agent (75). Because of this effect in scrapie, and its role in influencing adenylate cyclase, the possibility exists that amphotericin B may be shown to reverse the CJD alterations in cyclase.

MATERIALS AND METHODS

Animals:

Tissue was obtained from a serially passaged models of CJD. Guinea pigs were Hartley strain from Charles River Laboratory and hamsters were LVG strain also from Charles River. Both species were inoculated intracerebrally as weanlings with 10% homogenate of infected brain tissue in normal saline. Inoculations were made into the right side of the cortex. Animals were sacrificed when showing typical signs of CJD. For guinea pigs this was approximately 20 weeks while for hamsters it was closer to 18 weeks.

Animals were sacrificed and brains removed rapidly. The brains were disected on ice into regions: basal ganglia, cortex (occipital-parietal and frontal), hippocampus and cerebellum. Each region was placed in a cold Dounce homogenizer with a 10 fold volume of Buffer A*, and homongenized 15 strokes with a loose fitting (B) size pestle. The homogenate was then poured into a sealed polypropelene tube and centrifuged in a Sorval centrifuge HB-4 rotor at 1500 rpm (365g)for 15 minutes. The supernatant was poured off into another tube and spun again at 9500 rpm (14,600g) for 20 minutes. Supernatants from this last spin were discarded and the pellet vortexed up in 0.5 to 1ml of the buffer and frozen in liquid nitrogen. Small samples (20µl) were taken before freezing for protein assay. These frozen pellets were composed of synaptosome-enriched membrane fractions. Normal control animals at approximately the same age as the CJD infected animals were prepared in the same way.

*Buffer A: KCl 800mM, NaCl 150mM, Spermine 1.5mM, Spermidine 5mM, Tris Acetate 150mM, EDTA 2mM, EGTA 0.5mM, 2-Mercaptoethanol 15mM, Sucrose 0.11M, PMSF 0.5mM.

Adenylate Cyclase Assay:

The AC assay used was according to the method of Salomon (86) with the following buffers:.Membrane Buffer: Hepes (GIBCO) 25mM, Dithiothreitol (DTT) (Sigma) 1mM, PMSF (Sigma) 0.75mM, $MgCl_2$ 0.1-1mM. Pre-Incubation Buffer: Hepes (GIBCO) 25mM, NaCl 60mM, DTT (Sigma) 1mM, EGTA 0.5mM, $MgCl_2$ 1-5mM. Incubation Buffer: ATP (Sigma).1mM, IBMX (Sigma) 0.5mM, NaCl 60mM, cAMP (Sigma) 0.05mM, EGTA (Sigma) 0.5mM, Hepes (GIBCO) 25mM, DTT (Sigma) 1mM, Creatine Phosphokinase (Sigma) 0.14mg/ml, Creatine Kinase (Sigma) 0.5mg/ml, Myo-Kinase (Sigma) 15U/ml, Adenosine Deaminase (Sigma) 1U/ml, BSA 0.25mg/ml, $MgCl_2$ 1-5mM, $\alpha^{32}P$ ATP 500,000 cpm/tube.(New England Nuclear) Stop Solution: SDS 2%, ATP 45mM.

Guanine Nucleotides: Gpp[NH]p (Boeringer Mannheim) , and GTP (Sigma) were added at similar dilutions (10^{-9}-10^{-4}M). Neurotransmitters tested included Dopamine(DA) (Sigma) 10^{-6}-10^{-8}M in 0.1% ascorbic acid, Serotonin(5HT) (Sigma) 10^{-6}-10^{-8}in 0.1% ascorbic acid, D-Ala2-Met5-Enkephalinamide (Sigma) 10^{-5}M, Vasoactive Intestinal Peptide(VIP) (gift from Dr. B. Bunney) $5x10^{-7}$M. The fluidizers Forskolin (Sigma) 10^{-2}M, Ethanol 500mM(3%), Colchicine (Sigma) 10^{-7}M, Amphotericin B (Sigma) 10^{-5}M were also tested. (All additive are at their final concentrations.)

Procedure: Tubes were prepared in triplicate (12x75 mM borosilicate glass) and placed on ice. Pre-incubation components were added to a total final volume of 65λ/tube as follows: 25µl of Pre-Incubation Buffer , 10µl of guanine nucleotides (GTP or Gpp[NH]p) , 10µl of fluidizers (Etoh, Amp B, Colchicine), neurotransmitters (dopamine, 5HT etc.), or forskolin, and adjusted with distilled water up to the final

volume. Guanine nucleotides and buffer were used in virtually all tubes, with the other components added in separate experiments.

Membranes were thawed and the pellet was washed in approximately lml of Membrane Buffer, then centrifuged at 11,000rpm in an Eppendorf refrigerated centrifuge. The pellet was resuspended in Membrane Buffer to a final concentration of 5-20 μg of protein/tube. Protein determinations were completed on samples of membranes taken before freezing. Membranes (25μl) were added to the tubes containing pre-incubation buffer. Tubes were pre-incubated at 30°C for 10 minutes. After 10 minutes, 10μl of Incubation Buffer was added and the reaction continued at 30°C for another 10 minutes (5 minutes of incubation was used if inhibitory reactions were to be enhanced). The reaction was terminated with 100μl of Stop Solution. 50μl of ^{3}H cAMP (10,000 cpm/tube) were added to each tube followed by 1ml of water. These preparations were then chromatographed on columns.

Chromatography columns for assay of cAMP were prepared according to the method of Salomon (86). Dowex AG 50W-X4 (200-400 mesh) columns were washed with 20ml of de-ionized water prior to each use. Alumina columns were washed with 10ml of 0.1M Imidazole buffer before each use. Reaction tubes were inverted over the columns and allowed to drain. This was followed by washes with water (two 1.25ml washes that were allowed to drain and one 5ml wash after the Dowex columns were stacked on top of the Alumina). The Dowex columns were removed, scintillation vials were placed under the Alumina columns and each column was washed with 5.5ml of 0.1M Imidazole buffer. Sixteen milliliters of scintillation fluid (Optifluor) was added to each vial and they were counted on a combined ^{3}H and ^{32}P channel for 5 minutes/vial. The tritium window was set at 1-400 and the ^{32}P at 415-1000. The Dowex columns were recharged with 2ml of 1N HCl before storing.

All results were calculated and corrected for protein. Protein values were based on analysis of the membrane suspensions after they were prepared for the assay. Results are expressed in cAMP pM/mg protein/minute of reaction.

RESULTS

Initial experiments showed an increase in coupling of stimulatory subunits (Ns) in CJD membranes (79). Using Gpp[NH]p to study the Ns dependent activation of adenylate cyclase, guinea pig basal ganglia membranes activated cyclase 66%(±17,S.D.) greater than normal membranes. In subsequent experiments with different samples, synaptosomal membranes from CJD guinea pig basal ganglia similarly gave a greater fold Gpp[NH]p stimulated cyclase than did membranes from normal animals. Synaptosomal membranes from CJD and control guinea pig basal ganglia were incubated with Gpp[NH]p under stimulatory conditions (high Magnesium) with no pre-incubation (Figure 1).

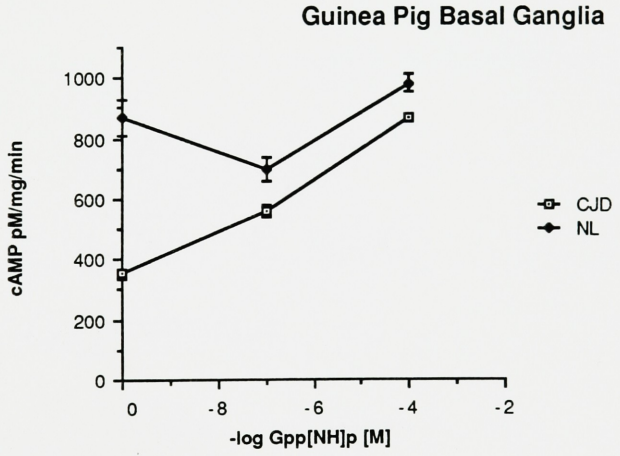

FIGURE 1: CJD and normal membranes were incubated for 10 minutes at 30°C in Incubation buffer (see methods) containing 4mM $MgCl_2$, Gpp[NH]p and 1mM EGTA. No pre-incubation was used. Remainder of protocol was as per methods.

Stimulation of CJD membranes with Gpp[NH]p shows a greater activation in cyclase (cAMP) as compared to uninfected membranes. CJD tissues gave a Gpp[NH]p stimulation of 2.5 fold. Basal activity was 354pM of cAMP/mg protein/minute. (Basal activity refers to the amount of cAMP generated from membranes in a tube without addition of guanine nucleotides, hormones or other modifiers of activity. The fold stimulation was determined at Gpp[NH]p concentrations of 10^{-4}M.) The cAMP/mg protein/minute increased steadily to a maximal value of 872. In contrast normal tissues yielded only a 1.1 fold stimulation over basal levels with Gpp[NH]p. Unlike the CJD membranes, the normal gave a higher basal activity of 868pM/mg/min. and did not steadily increase with rising Gpp[NH]p concentration but instead decreased to 697pM/mg/min at a Gpp[NH]p concentration of 10^{-7}M before again rising to a peak of 984pM/mg/min. (Figure 2)

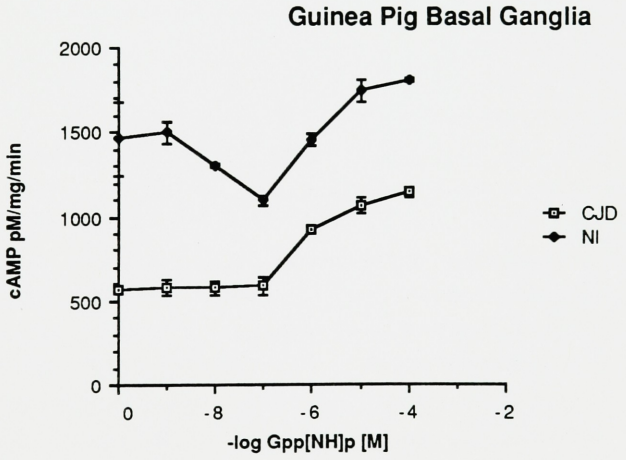

FIGURE 2: Guinea pig basal ganglia membranes were incubated as above in Incubation buffer containing 9mM $MgCl_2$ and 1mM EGTA, with Gpp[NH]p ranging from 10^{-9} to 10^{-4}M.

In studies utilizing a more extensive range of Gpp[NH]p concentrations, CJD membranes gave a stimulation of 2.0 fold. The basal activity of 565pM/mg/min. remained stable up to a Gpp[NH]p concentration of 10^{-7}M. Activity then steadily increased to a maximum of 1146pM/mg/min. Normal membranes, with a stimulation of 1.4 fold (compared to basal [no Gpp[NH]p]), showed the same curve of activity as seen in figure 1. Basal activity of 1463pM/mg/min remained unchanged at the Gpp[NH]p concentration of 10^{-9}M then decreased to a low of 1100pM/mg/min at a Gpp[NH]p concentration of 10^{-7}M, before steadily rising to a maximum of 1810pM/mg/min.

Figures 1 and 2 depict three pertinent differences between the CJD and normal membranes: a) normal membranes show a dip in activity at low Gpp[NH]p concentrations while CJD activity remains stable; b) CJD shows a lower total amount of cAMP/mg protein produced throughout the curve, and c) CJD continues to show a higher fold stimulation compared to basal (no Gpp[NH]p) than does normal. The higher fold stimulation of CJD membranes supports the earlier evidence suggesting that coupling of the stimulatory subunit (Ns) is greater in CJD than in normal membranes; the portion of decreased activity in the normal curve may suggest that normal tissues contain an inhibitory component of their cyclase system that is lacking in CJD. This inhibitory activity appears to be specific for Gpp[NH]p concentrations between 10^{-9} and 10^{-7}M. This presumed lack of Ni in CJD was investigated with inhibitory hormones (see pg.23-26). Variations in the amount of intrinsic Ns-GTP present in the membranes however could limit the maximal amount of activity and might account for the differences seen between CJD and normal membranes in the presence of Gpp[NH]p (Figure 2). Additionally, the in vivo state of the receptor influenced by hormone, might also lead to normal and CJD differences in the assay.

In order to investigate another potential variability we used manganese and forskolin to study maximal amounts of stimulatable cyclase. Mangenese and forskolin are substances which maximally stimulate cyclase (see Appendix B). Comparison of maximal values between CJD and normal membranes allowed us to determine if the amount of stimulatable cyclase was the same in both normal and CJD membranes, i.e. if steady state in vivo factors such as hormones were responsible for the above differences.

Initial experiments with manganese showed stimulation was equal in CJD and normal membranes (79). Repeat experiments on cortex and basal ganglia in hamster and guinea pig as well as hamster cerebellum and guinea pig cortex also showed that in all of the above tissues, the fold stimulation by Mn was equal in CJD and normal membranes. This finding indicates that even with the membrane damage inflicted by the CJD agent, the cAMP catalytic moiety of adenylate cyclase at the very least remains intact and functional since stimulation of the cyclase system by mangenese is a measure of the catalytic activity of the system without the influence of Ns components or hormones.

Forskolin stimulates cyclase by primarily acting directly on the catalytic unit (as does Mn) but it also is thought to act through other poorly described mechanisms, one of which is through Ns. Experiments showed that in all of the above tissues (except guinea pig basal ganglia), forskolin stimulation (fold cAMP) was equal in CJD and normal membranes (data not shown). Guinea pig basal ganglia was different in that CJD membranes had a higher fold stimulation with forskolin than did normal membranes (1.35 ± 0.10). Thus in general there appears to be no difference in the catalytic moiety in CJD and normal membranes, although in guinea pig basal ganglia there is increased coupling of forskolin on Ns (leading to the increased fold stimulation). This forskolin change may be due to its other poorly understood effects on Ns.

Since normal membranes showed a higher basal cyclase activity than those of CJD (Figures 1 and 2), the initial (steady state) activities of membranes were investigated by varying conditions of pre-incubation. (Steady state refers to the proposed state of membranes in vivo in a non-stimulated state.) Pre-incubation of membranes (under standard Gpp[NH]p concentrations) before addition of substrate should allow the formation of a new steady state in vitro, independant of the in vivo steady state. Changes in activity with varying time of pre-incubation yielded information on the initial state of the CJD and normal membranes in vivo.

Normal membranes without additions of guanine nucleotides, show decreasing activity with increasing time of pre-incubation (with the exception of the zero time point which could be an inaccurate point.). CJD tissues in contrast show a more stable but lower level of pre-incubation profile. With increasing time of pre-incubation, the activation in normal membranes decreases until it equals the CJD membranes at 20 minutes. The actual values of the basal cyclase after pre-incubation are very close in CJD and normal (i.e. CJD = 4798 pM cAMP/mg protein/minute, normal = 4703 pM/mg/min).

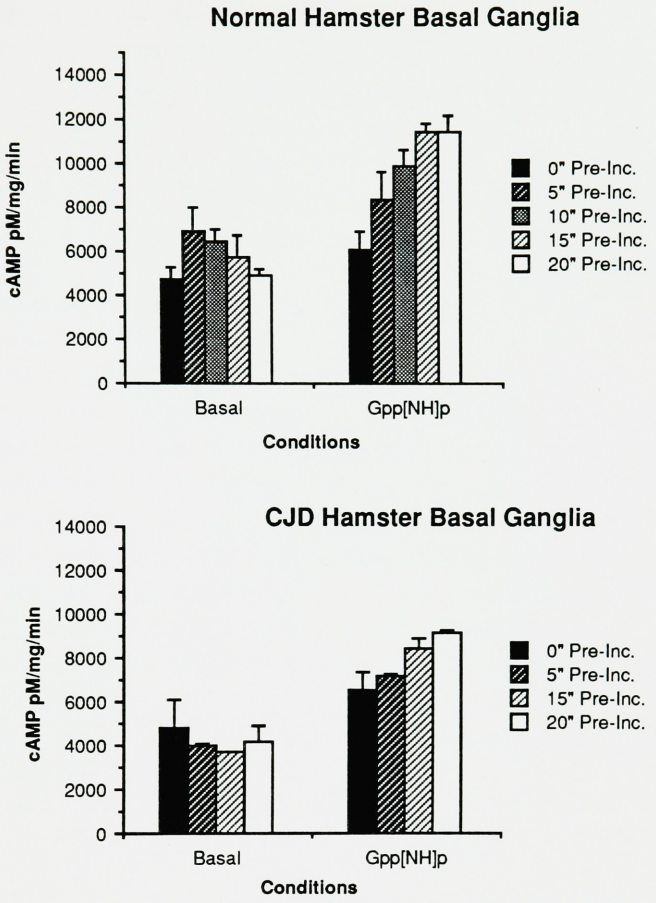

FIGURE 3: Hamster basal ganglia membranes (CJD and control) were pre-incubated in Pre-Incubation buffer (see methods) containing 5mM $MgCl_2$,and 10^{-4}M Gpp[NH]p for 0,5,10,15 and 20 minutes. This was followed by addition of Incubation buffer for an additional of 10 minutes at 30°C. (The CJD 10 minute point was not done under standard conditions.)

The overall impression from these experiments is that with the same amount of cAMP catalytic subunit (and therefore

the same quantity of stimulatable cyclase), normal tissues appear to have more GTP bound Ns in their steady state than do CJD membranes. A proposed mechanism would be for normal membranes to have a greater amount of GTP-Ns (activated complex, see Figure 12 in Appendix B) in the steady state than in CJD. With increasing time of pre-incubation the GTP will hydrolyze, and more of the inactive GDP-Ns form will appear. Thus after 20 minutes of pre-incubation both CJD and normal membranes will have the same amount of active (GTP-Ns) and inactive (GDP-Ns) forms.

While pre-incubation under basal conditions (no additives) gives some indication of the steady state of the membranes, pre-incubation with Gpp[NH]p gives additional information and helps further characterize the steady states. Pre-incubation of CJD with Gpp[NH]p showed an increase in stimulation over time. The Gpp[NH]p fold stimulation versus time of pre-incubation showed that CJD has a more rapid rise of Gpp[NH]p fold stimulation than normal. Furthermore in CJD there is a plateau after 10 minutes of pre-incubation, while normal membranes show a steady but slower rate of increase (Figure 6). This difference is due to the increased GTP-Ns in normal versus CJD membrane preparations.

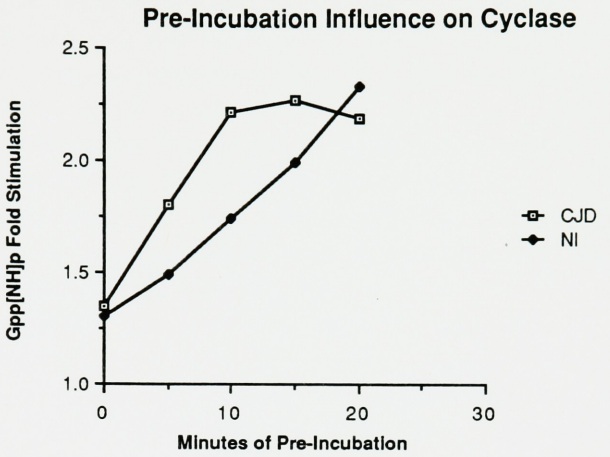

FIGURE 4: Plot of Gpp[NH]p fold stimulations verses pre-incubation. Data is taken from the previous pre-incubation experiment on hamster basal ganglia (see Figure 5). Since cAMP is initially higher in the normal at short pre-incubation times there is a shift to the right in the normal curve.

Thus in summary there appears to be more activated Ns (Ns-GTP complex) in membranes prepared from normal animals than in those prepared from CJD infected animals. This yields a higher basal level of activity without pre-incubation (Figure 1,2). In normal membranes the observed fall off in pre-incubation activity appears to be secondary to hydrolysis of GTP seen with increasing time of pre-incubation and more inactive (Ns-GDP) and reflects the initial in vivo differences in CJD and normal memranes. Further support for this comes from Gpp[NH]p experiments where an increase in normal membrane activity after a lag is a result of the conversion of Ns-GDP to an unbound state during pre-incubation. Because of the relative lack of basal activity in CJD, membranes appear initially to be a predominately inactive steady state (Ns-GDP) and therefore yield a rapid rise in cAMP with Gpp[NH]p.

Addition of Gpp[NH]p may not only fill available guanine nucleotide sites but perhaps enhance the affinity of Mg which is also an integral component for Ns activation (see Appendix B). Therefore in the steady state, CJD may be deficient in two important stimulatory components viz. GTP and Mg. However CJD membranes appear to retain their capacity to maximally stimulate cyclase when these components are added.

Since the maximal fold difference in Gpp[NH]p stimulated cyclase occured at 10 minutes (Figure 4), we used 10 minutes for our standard pre-incubation time in further experiments. Pre-incubation of 10 minutes results in approximately equal basal levels indicating that both membranes are in a similar activation state at this time. A parallel Gpp[NH]p dose response, with a 10 minute pre-incubation, now show basal levels of cAMP are equal in CJD and normal membranes (Figure 5).

In addition the normal membranes have lost their inhibitory dip and demonstrate a steady rise of activity as the concentration of Gpp[NH]p increases, most likely due to rendering normal membrane Ns equivalent to that in CJD. Notably even with equalization of basal levels, Gpp[NH]p fold stimulation remains higher in CJD (by 48%) than in normal membranes.

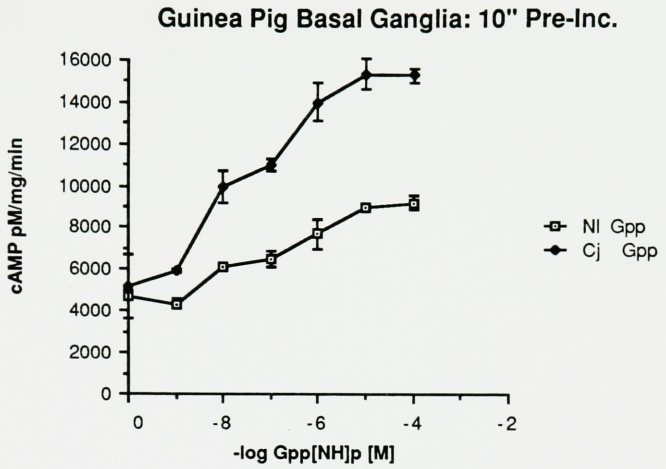

FIGURE 5: Guinea Pig basal ganglia membranes were pre-incubated in Pre-Incubation Buffer with 5mM $MgCl_2$ and Gpp[NH]p for 10 minutes at 30°C. Incubation Buffer was added and incubation continued for 10 minutes more at the same temperature. The reaction was terminated and continued as per methods. Bars indicate standard deviation.

Pathological changes in CJD infected brain vary from region to region, and regional involvement varies with species and agent isolate. We used one isolate to characterize adenylate cyclase changes in two species. In hamsters cortical tissues show vacuolization at 14 weeks of incubation, and there are only minimal basal ganglia changes at terminal disease in hamsters. The cerebellum also shows little evidence of vacuolar change. Guinea pigs show considerably more vacuolar changes in basal ganglia than hamsters when these species are inoculated with the same agent isolate. Since there is pathologic regional variation, Gpp[NH]p fold stimulation was studied in different regions of brain.

As described above the Gpp[NH]p fold stimulation of adenylate cyclase is higher in CJD than in normal membranes.

In guinea pig basal ganglia and in hamster cortex membranes this trend was clear and appears to reflect the predominant region that is involved in each species. For example there is a clear increase in CJD fold stimulation over that of normal in 5 out of 6 guinea pig basal ganglia experiments and in 4 out of 5 hamster cortex experiments, and these are statistically significant.

	Gpp[NH]p Fold Stimulation			
Exp	*CJD*	*Nl*	*%CJD>Nl*	
1	2.5	1.1	56%	
2	2.0	1.4	30%	Guinea Pig
3	2.8	1.8	36%	Basal Ganglia
4	2.5	2.1	16%	
5	3.7	2.7	28%	
6	3.1	1.6	49%	
	2.7 ± 0.56	1.7 ±0.52	36%	p=0.003

Exp.	*CJD*	*Nl*	*%CJD>Nl*	
1	3.3	2.5	25%	
2	2.4	2.2	9%	Hamster
3	5.8	4.5	23%	Cortex
4	3.8	2.6	32%	
5	4.9	3.4	31%	
	4.0 ± 1.28	3.0 ± 0.90	24%	p=0.012

TABLE 1: Summary of experiments investigating Gpp[NH]p stimulated adenylate cyclase. Experiments include those with and without pre-incubation. Results are expressed as fold stimulation and percentage increase in CJD versus normal in paired experiments. The negative value in experiment 2 in hamster cortex is likely due to inclusion of 2 brains from animals not showing clinical signs (occassional animals receive an inadaquate inoculum). If this experiment is omitted the p value is 0.004.

Hamster basal ganglia and guinea pig cortex gave inconsistant results (not shown) with Gpp[NH]p fold stimulation larger in CJD than normal in half the experiments and approximately equal in the other half. Since a smaller number of experiments were done on these regions, the findings are not as convincing as the two above. However they may relate to the predominant regional targets in these species. Cerebellar membranes showed no difference in Gpp[NH]p fold stimulation between CJD and normal. This is consistant with the fact that the cerebellum does not appear to be microscopically affected in these animal models of CJD. Notably, cerebellum contains ≥20 fold less infectivity than cortex at end stges of disease (59).

Since CJD disrupts neuronal membranes, alteration in membrane fluidity and/or cytoskeletal structure was considered as a possible explanation for the general increase in adenylate cyclase stimulation seen in CJD as compared to normal synaptosomal membranes.

Ethanol can interact with the lipid components of cellular membranes and "fluidize" these components. Its effects on membrane cyclase activity are well characterized (31,48,94,100). Since interactions between various components of the adenylate cyclase system take place within the lipid milieu of the plasma membrane, ethanol may be expected to affect these interactions by perturbing membrane lipids. Likewise, CJD may similarly influence interactions between adenylate cyclase components by disrupting the integral structure or fluidity of membranes. Incubating membranes with ethanol was used to determine if CJD membranes were comparably fluidized: If there is preexisting fluidization of membranes in CJD, one would expect to see an increase in cAMP with ethanol in normal membranes but little or no effect with ethanol in CJD, i.e. the membranes are maximally fluidized in CJD. Membrane fluidity was evaluated by examining the effect of ethanol on Gpp[NH]p dose response curves in parallel in CJD and normal membranes.

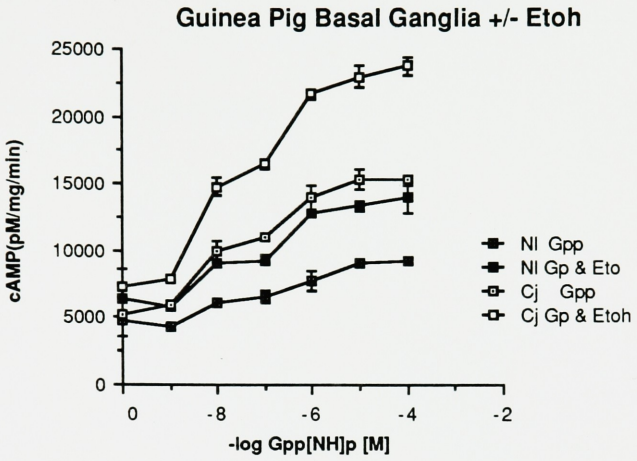

FIGURE 6: Guinea pig basal ganglia membranes were pre-incubated in Pre-Incubation buffer containing 5mM $MgCl_2$, Gpp[NH]p 10^{-9} to 10^{-4}M, and ethanol 500mM (3%). Membranes were pre-incubated 10 minutes at 30°C, Incubation buffer was added and membranes incubated for 10 additional minutes at the same temperature. Reaction was terminated and assay completed as per methods.

Ethanol increased the slope of the Gpp[NH]p dose response curve as well as the amount of cAMP (pM/mg/min) generated in both CJD and normal membranes. The Gpp[NH]p stimulation of cyclase in normal membranes by was 1.9 fold but increased to 2.2 fold upon addition of ethanol. In CJD Gpp[NH]p stimulation was 3 fold (greater than normal as previously). With addition of ethanol, although the total activity increased the fold stimulation was the same. In contrast the fold stimulation with ethanol was increased in normal membranes. The effect of ethanol on membranes in a true basal state (i.e. no activity) should be negligible. The fact that ethanol does affect the basal activity indicates that the membranes had varing amounts of Ns-GTP available for the

increasd coupling by ethanol. Data from other similar experiments showed the same effect; that is Gpp[NH]p + ethanol gave a higher fold stimulation than Gpp[NH]p alone in normal membranes, while it had much less effect on the fold stimulation in CJD membranes.

The difference in Fold Change
(Gpp[NH]p+Etoh / Gpp[NH]p)

Exp.	*Normal*	*CJD*	
1	1.15	1.00	Guinea Pig
2	1.13	0.96	Basal Ganglia
3	0.96	0.92	
1	1.12	0.81	Hamster
2	1.30	1.18	Cortex
Average :	1.13 ± 0.12	0.97 ± 0.13	p = 0.023

TABLE 2: Membranes were incubated in the presence of Gpp[NH]p ± ethanol (500mM). Values are expressed as the difference in the fold stimulation between Gpp[NH]p + ethanol and Gpp[NH]p alone (basal). The p value of all 5 experiments = 0.023 which is significant.

The data shown above reflects the fold differences between Gpp[NH]p + ethanol and Gpp[NH]p alone. Similar results are seen in plots of the cAMP generated (Figure 7). Since ethanol does not increase the fold stimulation of Gpp[NH]p in CJD to the same extent as it does in normal membranes, the CJD agent and ethanol may be altering the membranes in a similar fashion.

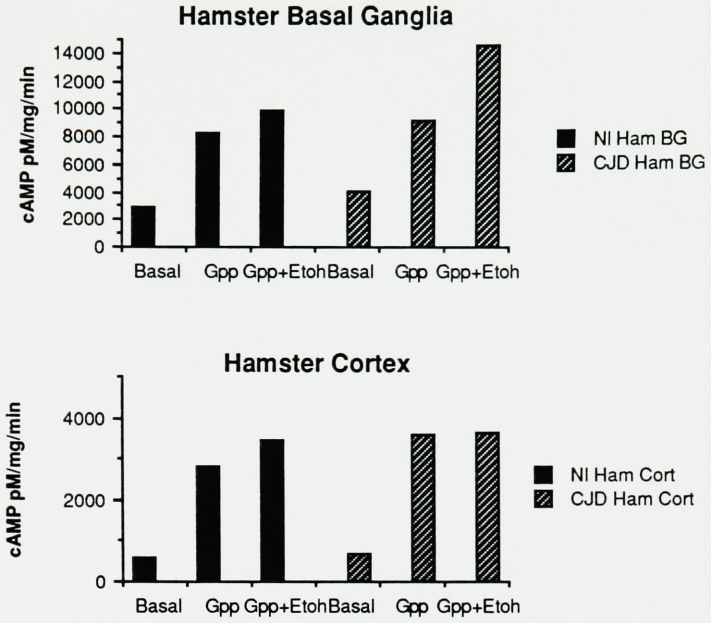

FIGURE 7: Membranes were pre-incubated in Pre-Incubation Buffer containing 5mM $MgCl_2$, Gpp[NH]p 10^{-4}M, ± ethanol 500mM (3%). Membranes were pre-incubated 10 minutes at 30°C, Incubation Buffer was added and membranes were incubated for 10 additional minutes at the same temperature. The reaction was terminated and the assay completed as per methods.

Notably the cortex of hamster CJD infected membranes was more fluidized then basal ganglia. Hamster cortex shows stimulation with ethanol in normal membranes but not in CJD, as would be expected if the CJD membranes were already fluidized. This suggests that fluidization is a major effect of CJD (disease or agent) on hamster cortical tissue. In hamster basal ganglia the lack of fluidization effects of ethanol were not surprising as this tissue is less involved by disease.

We also investigated other mechanisms that might lead to the cyclase stimulation in CJD. Treatment of brain synaptic membranes with colchicine or vinblastine and Gpp[NH]p results in augmentation of adenylate cyclase activation (80) due to disruption of tubulin within the plasma membrane. This leads to increased coupling of the N protein and increased AC activity (80). Membranes were incubated with colchicine in an effort to investigate this mechanism in CJD.

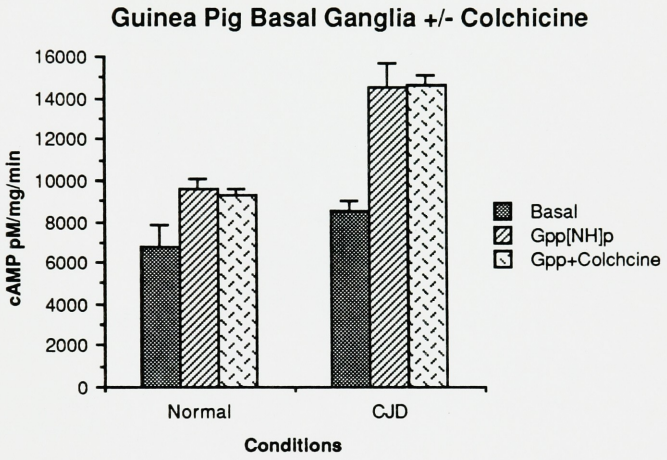

FIGURE 8: Guinea pig basal ganglia membranes were pre-incubated in Pre-Incubation buffer containing 5mM $MgCl_2$, 1mM EGTA, Gpp[NH]p 10^{-5}M and colchicine 10^{-6}M for 10 minutes at 30°C. Incubation buffer was added and reaction was incubated for an additional 10 minutes at the same temperature. Assay proceeded as per methods.

Colchicine appeared to have no effect on adenylate cyclase with fold stimulations of Gpp[NH]p + colchicine equal to that of Gpp[NH]p alone. Both CJD and normal membranes incubated as above but without Gpp[NH]p (basal activity) gave a 20% inhibition upon the addition of colchicine. It is possible that colchicine altered the coupling of N proteins in these

membranes but in such a way as to inhibit rather than stimulate adenylate cyclase activity, possibly by enhancing Ni subunit dissociation over Ns. Addition of Gpp[NH]p may overshadow this effect and give the overall result of no change of cyclase stimulation with colchicine. Regardless of the mechanism, both CJD and normal membranes respond to colchicine in the same way. This suggests that disruption of tubulin is not a factor in the mechanism of the observed CJD alterations in adenylate cyclase activity.

Amphotericin B not only affects membrane fluidity by binding cholesterol, but has also been shown to lengthen the incubation period of scrapie in experimental animals (74). If CJD affected membranes by binding cholesterol (as does amphotericin B) one would expect to see an increase in Gpp[NH]p stimulated cyclase in normal membranes while visualizing a smaller effect in the CJD membranes that had already been fluidized. Preliminary experiments on normal hamster basal ganglia showed no effect of amphotericin B on the Gpp[NH]p dose response curves. Therefore the adenylate cyclase system in this system does not appear to be affected by alterations of cholesterol in the membrane.

Previous studies in scrapie infected animals have shown decreased levels of serotonin (5-HT) in whole brain homogenates of mice and in peripheral blood of hamsters (82). Behavioral aspects have been ascribed to 5-HT supersensitivity, secondary to possible destruction of the 5-HT nerve terminals (26). Earlier experiments with CJD guinea pig basal ganglia were incubated with 10^{-6}M Gpp[NH]p over a range of 5-HT concentrations ranging 10^{-9} to 10^{-5}M (79)..

In the present experiments guinea pig basal ganglia, cortex and hamster cortex were incubated with Gpp[NH]p at a range of concentrations and 5-HT ($5x10^{-5}$M) in an effort to observe a supersensitivity response to the hormone in CJD membranes. In all experiments CJD failed to show an increased response to 5-HT over that of normal membranes. Stimulation

of cyclase by transmitters was enhanced by keeping the Mg at a relatively high concentration (5mM) thereby promoting Ns saturation with the ion (Appendix B) for a constant baseline to observe Ni effects. In addition to altering the Mg concentration, the use of forskolin, especially in conjunction with low Mg concentrations was used enhance visualization of inhibitory neurotransmitter effects (4). None of our experiments showed an increased sensitivity to 5-HT. The failure to elicit a response to 5HT is possibly due to a low number of 5-HT receptors in the membrane preparations of these brain regions. Depletion of 5-HT in regions of CJD brain would explain a part of the supersensitivity noted in other studies as well as the behavioral characteristics seen in these animals: increases in irritability, aggressiveness, activity and sensitivity. Since other studies examined whole brain preparations instead of specific brain regions, it is possible our experiments contained too few 5-HT sensitive regions.

Dopamine (DA) both stimulates and inhibits the formation of cAMP in the striatum (97). Stimulation of D1 receptor sites enhances activity of adenylate cyclase, while stimulation of D2 receptors does not (40). Some D2 receptor populations when stimulated cause inhibition of cyclase activity and reduction of intracellular cAMP (15,20). Cells may contain D1 or D2 exclusively, or may contain both receptors that function independently and have opposite effects on intracellular cAMP levels (41). Dopaminergic neurons have modulatory influence on the structures they innervate rather than any specific intrinsic function (47).

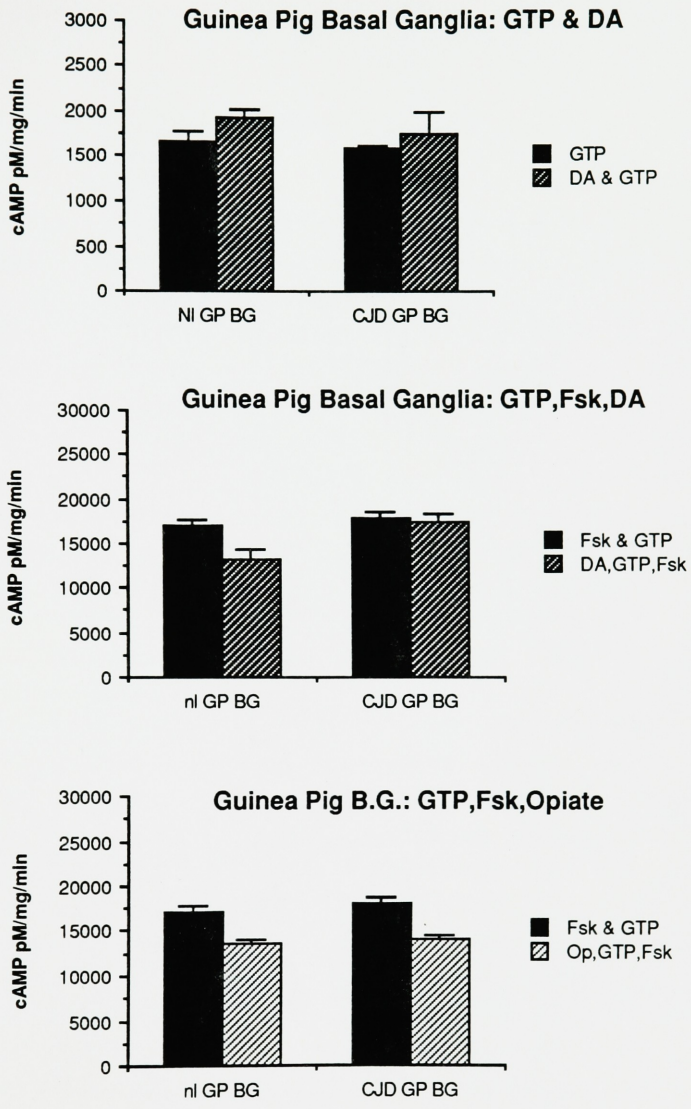

FIGURE 9: Membranes from guinea pig basal ganglia were pre-incubated for 10 minutes at 30°C in Pre-Incubation buffer that contained 1mM $MgCl_2$ (to enhance

inhibitory conditions), GTP 10^{-4}M, dopamine 10^{-4}M.or opiate(D-Ala^2-Met^5-Enkephalinamide) at 10^{-5}M. Forskolin was added to half of the tubes in a concentration of 10^{-5}M to further enhance the inhibitory effect of the hormones. Incubation buffer was added and incubation continued for 10 minutes at the same temperature. Error bars reflect S.D..

Guinea pig basal ganglia membranes were incubated with dopamine under inhibitory and stimulatory conditions to determine the response of the membranes to the dual effects of dopamine. In addition, membranes were also incubated with an opiate under inhibitory conditions to test the inhibitory capacity of the membranes.

Addition of dopamine with GTP enhanced stimulation over that of GTP alone: CJD=29% and normal=14%. When forskolin was added to enhance the inhibitory response, normal membranes gave a 22% inhibition with the addition of dopamine while CJD membranes had virtually no change in activity. Opiate added with GTP and forskolin gave an inhibitory response in both CJD and normal: CJD=23% and normal=21% inhibition.

This data suggests that the D2 (inhibitory) receptors are affected in CJD. The fact that opiate elicited the same inhibitory response in both CJD and normal membranes indicates that the cyclase inhibitory mechanism is intact in CJD and it contrary to our initial interpretation (see pg. 10). Stimulation of both CJD and normal membranes by dopamine indicates that the D1 receptor is intact. Normal membranes have an inhibitory response to dopamine when enhanced by forskolin, therefore dopamine is capable of inhibiting cyclase in this membrane system. The decrease in inhibitory dopamine action in CJD (and therefore unregulated stimulatory dopamine component) may be responsible for some of the movement disorders described in human victims of this disease

Other transmitters looked at included VIP, PIA, and PGE_1. These transmitters were examined under stimulatory conditions (5mM $MgCl_2$, Gpp[NH]p) and inhibitory conditions

(1mM $MgCl_2$, forskolin and GTP). These transmitters gave no consistent effect on CJD or normal membranes. Since CJD appears to affect the dopamine pathways, it is quite possible that other transmitter pathways are also affected. Possibly optimal conditions were not met for these transmitters resulting in the lack of effect.

Membranes from regions of hamster and guinea pig were assayed at various time points during disease incubation to determine when the effects of CJD on adenylate cyclase become evident. The time points chosen included pre-clinical (animals showing no clinical symptoms of the disease) as well as terminal (animals showing clinical symptoms) times. The time points chosen were: Hamster: 8,12,16, and 18.5(terminal) weeks, Guinea Pig: 12,15 and 22(terminal) weeks.

All membranes were frozen in liquid nitrogen and assayed at the same time. Membranes were pre-incubated for 10 minutes at 30°C in Pre-incubation buffer containing 5mM $MgCl_2$, Gpp[NH]p 10^{-9} to 10^{-4}M. Forskolin 10^{-5}M and ethanol 500mM (3%) were added as required. Incubation Buffer was added and reaction was incubated for 10 additional minutes at the same temperature.

Guinea pig basal ganglia and hamster cortex were the two tissues that consistently showed a greater stimulation of Gpp[NH]p in CJD membranes as described previously. Thus these regions were investigated in the time course experiment. Normal stimulation was 2.1. The percent increase of CJD/normal Gpp[NH]p stimulation at 12wk was 12%, at 15wk was 16% and at 22wk was 16%. Hamster cortex also showed a more pronounced progression in the degree of stimulation of the CJD membranes. Normal was 4.5 fold and percent increase of CJD/normal Gpp[NH]p stimulation at 8 wk was 10%, at 12wk was 15% and 18.5wk (terminal) was 22%.

Forskolin was used not only to maximally stimulate cyclase but also to enhance the detectability of inhibitory components (Ni) of the system in these time course

experiments. Since the stimulatory effect of Gpp[NH]p was not consistently seen in many brain regions , forskolin was used to effectively lower the "base line" and enhance the observed stimulatory effect of Gpp[NH]p.

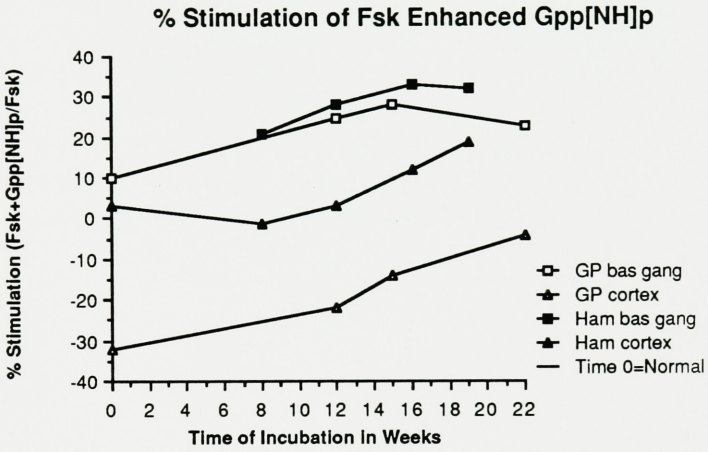

FIGURE 10: Percent stimulation of Gpp[NH]p enhanced by forskolin (Fsk+Gpp[NH]p/Fsk). 0 time indicates normal membranes with other points at the indicated weeks of disease incubation. Normal hamster basal ganglia (day 0) was not pre-incubated due to an oversight.

Through the incubation of the disease, Gpp[NH]p appears to have an increasing stimulatory effect on forskolin modified cyclase activity. Since the amount of catalytic unit is equal in all regions by virtue of equal stimulation of Mn, the difference in stimulation is likely due to the increased coupling of the Ns subunit in combination with the forskolin effect on the catalytic unit. The increase in coupling appears to progress as incubation time increases. Infectivity studies of hamster

cortical tissue using the side of the brain contralateral to the one initially inoculated with infectious material show a rapid increase in infectivity from 9.2 weeks to 15 weeks. After this 1.7 log rise, infectivity plateaus and remains relatively constant until the terminal stages at 20 weeks (Manuelidis L., unpublished data). Earlier time points are needed to clearly define the trend before the 8th week of incubation, but an increase is stimulation is clearly evident until a plateau of stimulation occurs at about 15-16 weeks in the basal ganglia of both species. Interestingly the cortical tissues of both species appear to continue to increase in stimulation up to the terminal stages. The slight fall off in very terminal guinea pig basal ganglia may be due to extreme destructive spongiform changes at this time.

CONCLUSIONS

We found that 1) adenylate cyclase is reproducubly stimulated by non-hydrolyzable guanine nucleotides in CJD significantly more than it is in control uninfected brain membranes. Greater Gpp[NH]p cyclase stimulation in CJD as compared with normal membranes was found in several brain regions in guinea pig and hamster, although the magnitude of the response varied from region to region. It appears that this is due to a greater coupling of stimulatory subunits (Ns), and not to a decreased coupling of inhibitory subunits (Ni). 2) There was no evidence for any change in the functional capacity of the cAMP catalytic unit in CJD membranes. 3) CJD membranes give a greater fold cyclase stimulation as compared to normal membranes under a variety of conditions. Although there appears to be more activated Ns (GTP-Ns complex) in membranes prepared from normal animals than in those prepared from CJD infected animals, equalization of this effect by pre-incubating membranes still yielded significant increases in Gpp[NH]p stimulation in CJD. 4) CJD membranes appeared to be more fluidized than normal membranes. Colchicine, tubulin and amphotericin B had no further effects which rules out involvement of filamentous proteins and cholesterol in the CJD effect. 5) Experiments with selected neurotransmitters and neuropeptides, indicated that only a sub-population of cyclases (dopamine receptors) are involved in the CJD effect. Therefore, although the magnitude of the fold stimulation increase in CJD is often modest, albeit significant, it probably represents a large magnitude change in a sub-population of cyclase complexes. It is remarkable that many components of the cyclase system remained completely functional in CJD since the infectious agent targets membranes and leads to severe neuronal membrane damage.

The molecular mechanism of this effect remains to be completely elucidated. However, it appears that in many brain regions, there is an increase in membrane fluidity that allows greater coupling of stimulatory subunits (Ns). This apparent change in fluidity can account for the effect in hamster and guinea pig cortex. In guinea pig basal ganglia (more so than in hamster), there appears to be some additional molecular process of unknown nature that may account for the majority of the CJD effect observed. The basis of this fluidity change is unknown. We were unable to document any indirect effect of the cytoskeletal system on the membrane fluidity even though morphologic studies suggest cytoskeletal system disruption in spongiform encephalopathies (43).

Enhancement of Ns coupling as depicted through increased fold stimulation of forskolin modified Gpp[NH]p appears to begin at least as early as 8 weeks in hamsters and 12 weeks in guinea pigs but more extensive time course studies are needed to assess the reproducibility or significance of the CJD changes. Such changes if reproduced early in the disease could be an important marker of infection when clinical signs are not apparent.

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STUDENT RESEARCH PROGRAM
Thesis Requirement

HISTORY OF STUDENT RESEARCH AT YALE

There is a long tradition at Yale University School of Medicine supporting the concept of medical student research. The first evidence that the thesis or dissertation was considered a requirement for the degree of Doctor of Medicine is in a statement in the catalog from 1839, which in part reads, ". . . the candidate must present a dissertation on some subject connected with the medical sciences." This requirement remains in effect to the present time, and is enthusiastically endorsed by the faculty as an important component of the "Yale System" of general medical education.

In a survey of Yale Medical School graduates performed 26 years ago, (with 1,034 of 1,206 graduates responding), 40% of graduates held academic appointments and 61% had done research since graduation. The experience in research during medical school was cited by graduates as a critical factor in their choice of careers.

GENERAL ASPECTS OF CURRENT MEDICAL STUDENT RESEARCH PROGRAM

All students at Yale University School of Medicine engage in research, with the exception of those students who have a Ph.D. degree before matriculation. A wide choice of subjects for research is permitted. Students may choose laboratory projects, or in many cases, investigate clinical, epidemiologic, economic or historical topics. A review of the literature or review of patients' charts may serve as a source of data for research. One basic requirement applies to all projects; there must be a hypothesis that can be supported or rejected by the data that are generated. Data must be subjected to statistical analysis, and results should either confirm or reject the original hypothesis.

A close working relationship between student and research mentor is a major goal of the program and is strongly encouraged. If laboratory research is performed, it is the responsibility of the faculty advisor to provide all necessary equipment and supplies. If the project is concerned with clinical or epidemiological investigation, the same commitment to guidance and support is expected. Weekly conferences between student and advisor are encouraged during the course of the research. The research must be designed and specifically performed by the student with the advice of the faculty mentor. Students may not work jointly on the research project.

The research is presented in written form during the fourth year and must fulfil the following minimal requirements:

a. A critical review and citation of the work of previous investigators.
b. A valid research design.
c. Evidence of the mastery of appropriate methodolgy.
d. Analysis of research data.
e. Conclusions that are supported by the data.
f. Summary (in abstract form).
g. A literate presentation and correct bibliography.

Before the written report is presented to the Office of Student Affairs, it must be approved by the student's advisor and a committee in the department where the work was done.

An Awards Committee critiques and ranks all student theses submitted for honors by various departments. The highest ranked papers are presented in a student program chaired by the Dean of the School and widely attended by students and faculty. Twelve prizes are awarded at graduation for outstanding student research.

The creative discipline required to carry out a project and prepare a thesis enables each student to become a physician-scholar whether the ultimate objective is clinical practice, research, teaching, or administration. Yale hopes to produce physicians who can evaluate data quickly and critically as they must do throughout their professional careers.

SPECIFIC ASPECTS OF MEDICAL STUDENT RESEARCH PROGRAM

Many students begin research work during the summer following their first year. In recent years the vast majority of first year medical students have remained in New Haven to work with various faculty members on a wide variety of projects. Stipends are available to support this summer research. Many medical students continue their research work afternoons, evenings, and weekends during the second year of medical school. The Curriculum Committee has affirmed the desirability of spending an additional six week block during the third year if students so wish. Additional time is also available in mid-fourth year for completion of research work. Thus a total of four to seven months is currently available for research by each Yale student during the four years at medical school.

The diagram below indicates the major blocks of free time in which a student may complete a thesis.

	Semester I			Semester II				Summer
Year I	BASIC SCIENCES							T
Year II	BASIC SCIENCES			NBI				T or 1
Year III	2	3	4	5	6	7	8	E/T
Year IV	E/T	E/T	E/T	E	E	E		

NBI = Part I National Boards
1 thru 8 = Required six week clerkship rotations
E/T = Six week blocks available for elective and/or thesis time

In the fourth year, it is important that a project started at this late date be one with reasonable expectation of completion and acceptance. Some projects can be done at odd times, evenings and weekends. The schedule shown above applies only to those projects that require solid blocks of time.

A reminder: We recommend that the actual time devoted to data collection (laboratory or other) be accomplished in a six-to eight-week period or its equivalent in days. Additional time is needed for planning and literature review, for evaluation of data, and final write-up.

One year medical student reseach fellowships

The School also encourages a few students to consider a fifth year of medical school and devote it exclusively to research. This Student Research Fellowship is facilitated by charging no tuition for the extra year and by the provision of a limited number of stipends that can be paid to students. These stipends will be available on a competitve basis and students in any year are eligible to apply.

Getting started

A list of faculty members and their areas of research is available in the Office of Student Affairs. After deciding upon the area of general interest, the students should check with members of the faculty in both the basic and clinical sciences who are working in that field. After appropriate discussions, the student should choose the faculty member with whom he/she wishes to work. Although it is not necessary that the idea for the thesis originate with the student, it is necessary that the work done be his/her own. The student can work as a member of a group project only if the student's contribution is carried out on an independent basis. Dissertations written jointly by two or more students are not acceptable. There is no required length for the thesis. In planning a project it should be clear that the prime goal is to learn the scientific method and not necessarily to publish a paper. The faculty member should make every effort to orient the student to a practical problem that can be fulfilled within the available time.

Need for a Hypothesis

A review of the literature or a review of patients' charts may be the source of data used for a thesis. However, the basic requirement still applies. There must be a hypothesis which can be supported or rejected on the basis of data gleaned from the patients' records and/or literature. These data should be subjected to statistical analysis, and the results should either cofirm or reject the original hypothesis. As with any other thesis, a review of the literature and a section dealing with the interpretation of the data and a discussion of its importance should be included. A chart or literature review can not be a simple narrative describing the information obtained from these sources.

Research Involving human subjects

All research involving human subjects must be approved by the Human Investigation Committee of the School of Medicine. Applications and guidelines are available in the Office of the Chairman of the Committee.

Literature review and Protocol Formulation

When the area of investigation has been approved by the preceptor and Departmental Thesis Committee the student is expected to explore the literature and formulate a protocol. This step provides an unusual opportunity for tutorial instruction in experimental design. Faculty members who assume responsibility as preceptors should take it seriously and provide the amount of guidance in design of the investigation that is necessary.

Joint Projects Not Acceptable

Dissertations written jointly by two or more students are not acceptable. This does not mean that they may not work on related problems but each student should have the experience of carrying out an investigation from beginning to end on his/her own initiative.

Research done outside of Yale Medical School

A student may wish to undertake his/her research project under the supervision of a qualified investigator who is not a member of the faculty of Yale University School of Medicine. The approval of an outside preceptor will be granted by the Dean's office upon receipt of a statement by a senior faculty member acting as sponsor, in whose area the research work will be done. The statement should indicate the sponsor has approved the preceptor and the facilities available for the research project. The same regulations concerning the dates of submission and review by the appropriate departmental committee will apply to the thesis done outside of the medical school.

Ph.D. Thesis in lieu of Medical School Thesis

For students enrolled in the combined M.D./Ph.D. program, the doctoral thesis submitted to and approved by the Graduate School will serve in lieu of the thesis requirement of the Medical School. For students in the M.D./M.P.H. program, the medical school requirement applies.

Typing and Binding Costs

The cost of typing and binding the dissertation is the responsibility of the student. Departmental or research funds should not be used for this purpose.

Statistical Analysis

When the results of an investigation lend themselves to statistical analysis the student should be encouraged to seek the aid of a biometrist for assistance in statistical methodology. It is hoped that this will enable the student to learn the value and limitations of statistical analysis as an aid in interpreting the results of an investigation.

Typing and Assembly Instructions

The thesis should be typed, double spaced, on a good 20 pound weight paper with a 1-1/2 inch left hand margin. All other margins may be one inch. Single spacing may be used within (double spacing between) block quotations, footnotes and bibliography. Corrasable bound is not acceptable. You will need an original and at least two copies (one copy for the department chairman's office and one copy for your advisor). The copies should be reproduced on a good copying machine. The original or best copy must be bound with a hard cover, any color. The other copies may be bound with soft covers.

The Office of Student Affairs has lists of typists, copying and binding services. A thesis which contains strikeovers, messy erasures, and careless spacing and centering, or in which the fundamentals of punctuation and spelling are not observed, may not be accepted.

The title, the student's full name and the year of the degree must be imprinted on the cover, and the student's last name and abbreviated title on the spine.

If charts, graphs, maps, tables or computer printouts that are larger than the standard page size are to be used, they should be folded carefully into the manuscript, with the fold at least 1/2 inch from the right hand edge of the page.

Each page in the thesis should be numbered except the title page (sample attached), abstract, acknowledgements and table of contents. The number should be placed either at the top center (approximately 4 lines from the top of the page) or at the top right hand corner (approximately 4 line from the right edge of the paper).

The abstract should be headed as follows:

ABSTRACT

FULL TITLE OF DISSERTATION (as on title page)

Full Legal Name (including middle name)

Year (of graduation)

Legends are placed below the illustrative material. A legend may appear, however, on a facing legend page when both illustration and the legend cannot be accommodated on one page.

To mount photographs, use Duco or equivalent cement, rubber cement or "permanent" glue. Regular glue, picture corners and adhesive cellophane are not acceptable.

Title Page Format

(Title of Dissertation)

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

by

(Legal name of author)

(Year of degree)

APPROVAL PROCESS

The Thesis Subcommittee of the Curriculum Committee:

A Thesis Subcommittee of the Curriculum Committee has been formed and meets regularly to recommend policy to the Curriculum Committee for all aspects of the Thesis requirement. Specifically, rules and regulations will be set and deadlines established. The committee will serve as a reviewing body to determine prizes and guidelines for the awarding of prizes.

Office of Student Research

The Chairman of the Thesis Subcommittee will also establish an office dedicated to student research where lists of preceptors will be kept up to date; where advice can be given to students searching for research projects; where information about past student projects can be kept; and where sources of funds can be filed and organized in such a way that timely applications can be made. For the present this office will be in the Office of Student Affairs under the direction of Dr. John Forrest.

Departmental Thesis Committees

Each department chairman will appoint a Departmental Thesis Committee of three senior faculty members who will be responsible for approving completed dissertations done within that department.

Outline of Proposal must be Approved

Each student will prepare an outline of his/her proposed research project. This outline will include a title, a brief outline of the project (including an hypothesis), the data that will be obtained to support the hypothesis and the resources available. In addition, it will include an estimate of the time necessary to accomplish the project. This resume will be signed by the student and approved by his/her advisor and submitted to the Departmental Thesis Committee six to eight weeks before the proposed starting date of the research project. The Departmental Thesis Committee will return the document to the student with their approval and/or reason for rejection. The review by the Departmental Thesis Committee will determine if the general scope of the work and resources available are consonant with the traditional Yale thesis requirement. The Committeee is not being asked for advance approval of the project.

Each student must have prepared this resume, obtained approval from his/her faculty advisor and submitted it to the Departmental Thesis Committee for review no later than May 1 of the third year. In the case of those who are spending more than four years in Medical School, the deadline will be May 1 preceding the final year.

Thesis approval and Deadlines

Early in the fourth year, at the time of the interview with the Dean of Students concerning postgraduate training, each student will submit a progress report describing the status of the research project and the anticipated date of completion. This brief document will be signed by the advisor and the student.

A draft of the thesis project must be submitted to the advisor by February 2 of the year of graduation.

Before a thesis is presented to the Office of Student Affairs, it must be approved by the student's advisor and a thesis committee in the department where the work has been done. It is not sufficient that the thesis be approved only by the student's advisor.

If changes are suggested by the departmental committee, these will be explained to the student and his/her advisor, and revisions made if necessary.

If the thesis is to be considered for an award or presentation of Student Research Day, it must be submitted to the Departmental Thesis Committee by February 16 and to the Office of Student Affairs by March 16. Departmental Thesis Committees nominate student theses for prizes with recommendation of the student's advisor.

When approved, the thesis should be typed in the format stated previously and presented to the Office of Student Affairs no later than March 16, accompanied by a letter stating that it has been approved by the Department. Extensions will be granted only on approval of the advisor and the Departmental Thesis Committee Chairman. Under no circumstances will any thesis be received by a department after April 1, with a bound, approved thesis to the Office of Student Affairs by May 1. Students missing these later deadlines will not graduate in May of that year.

The dates set in the fourth year are not arbitrary ones. Granting of the M.D. degree by the University is approved by the Board of Permanent Officers of the Medical School and the Yale Corporation. Your name cannot be presented to the Board of Permanent Officers until all of the requirements for the degree have been fulfilled; your name cannot be presented to the Yale Corporation until it has been approved by the Board of Permanent Officers. These two bodies do not meet frequently and the timing of their meeting with the date of Commencement necessitates our setting real deadlines.

YALE UNIVERSITY SCHOOL OF MEDICINE

OFFICE OF STUDENT AFFAIRS
L205 SHM

MEMORANDUM

February 1, 1988

To: Class of 1988

From; Betsy Winters

Attached is a release form to allow photocopying of your thesis for educational purposes in future years. If you would like to have this permission included with your thesis in the Medical Library, please return the form to me by May 21. This is entirely optional.

NO

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Permission for photocopying or microfilming of "_____

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(Title of thesis)

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Signature of Author

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Date

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Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE