FUNCTIONAL RELATIONSHIP BETWEEN FOREBRAIN CHOLINERGIC PROJECTIONS AND SOMATOSTATIN NEURONS IN THE RAT

by

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(ABSTRACT)

The two neuron types that initially degenerate with Alzheimer's Disease are the cholinergic projections from the septum to the hippocampus and from the substantia innominata to the cortex, and the somatostatinergic neurons in the hippocampus and cortex. The functional relationship between these two types of neurons was investigated using folic acid, a neuro-excitant, and cysteamine, a somatostatin depleter.

Folic acid causes a neuron to fire at a much higher rate than normal (Spector, 1971). Folic acid was injected into either the septum or the substantia innominata, and the long-term effect of the resulting acute hyperactivity of the cholinergic neurons on somatostatin neurons was measured as somatostatin-like immunoreactivity in the hippocampus and cortex. Glutamic acid decarboxylase activity, a marker for gamma-amino butyric acid (GABA) neurons, was also measured because it has been shown to decrease in the cortex after injection of folic acid into the substantia innominata. The

administration of folic acid to the cholinergic neurons did not have a significant long-term effect on somatostatin-like immunoreactivity nor glutamic acid decarboxylase activity; therefore, a hyperactivity of the cholinergic neurons did not result in degeneration of GABAergic nor somatostatinergic neurons.

Cysteamine causes a short-term depletion of somatostatin. Cysteamine was injected subcutaneously and the effect of an acute decrease of brain somatostatin on the cholinergic neurons was studied by measuring high affinity choline uptake, an indicator of cholinergic activity. Administration of cysteamine had no measured effect on high affinity choline uptake in the hippocampus or frontal cortex; therefore, a depletion of somatostatin did not effect cholinergic activity. The assay for high affinity choline uptake was tested by injection of pentobarbital, a drug known to decrease high affinity choline uptake. We detected a decrease in high affinity choline uptake after pentobarbital administration, indicating that if cysteamine were decreasing high affinity choline uptake, the assay would have detected it.

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Introduction

Alzheimer's Disease causes marked degeneration of certain neurons of the forebrain. Two neuron types that are initially affected are the cholinergic projections from the septum to the hippocampus and from the substantia innominata to the cortex, and the somatostatin neurons within the projection target regions. There are several pieces of evidence that suggest a functional relationship between these two types of neurons in the healthy condition. Because both are invariably damaged in Alzheimer's Disease, examination of their relationship to one another may yield insights to the disease. This thesis further examined the relationship between the cholinergic projections and the somatostatin neurons in two ways.

The first follows a hypothesis that there is an initial hyperactivity of the cholinergic neurons whereby they fire at a much higher rate than normal before they die out (Wurtman, 1985; Blusztajn, 1986) If this is true, this hyperactivity may affect the somatostatinergic neurons. This first part studies the effect of an induced hyperactivity on the somatostatin neurons. The cholinergic neurons were made hyperactive using the neuroexcitant folic acid and the effect

on the somatostatin neurons was measured.

Hyperactivity may adversely effect a post-synaptic neuron by overstimulating it. Recently a mechanism has been described for kainic acid excitotoxicity whereby kainic acid opens receptor-gated channels, leading to an influx of sodium ions and an efflux of potassium ions. Accompanying these ion fluxes is the passive influx of chloride ions, with water following the osmotic gradient. It is the influx of water that causes the neuron to swell and eventually lyse (Mayer, 1987; Rothman, 1987). The hyperactivity of the cholinergic neurons may act similarly on a somatostatin neuron. Acetylcholine administration to cortical cell cultures is known to stimulate them and cause the release of somatostatin (Robbins, 1982). Overstimulation may also cause the excessive influx of sodium ions accompanied by the passive influx of chloride and water leading to swelling and cell death.

There is already indirect evidence for this hypothesis. The effects of folic acid injection into the substantia innominata have been reported to cause degeneration of neurons distal to the substantia innominata injection. The specific type of neuron depleted was reported to be GABAergic, as measured by glutamic acid decarboxylase activity (McGeer, 1983). Somatostatin is co-localized within a subset of cortical and hippocampal GABAergic neurons (Hendry, 1984b;

Kosak, 1988: Somogyi, 1984). In the first part of this study, folic acid was injected into the area of the cell bodies of the cholinergic projection neurons, and the distal effects of the resulting hyperactivity were measured in the GABA neurons as glutamic acid decarboxylase activity and in the somatostatin neurons as somatostatin-like immunoreactivity. Althogh this injection would effect the cholinergic projections, the folic acid would also effect any other neuron in that region.

The second method to investigate the relationship between the cholinergic projection neurons and the somatostatin neurons was the converse of the first, in that the somatostatin neurons were manipulated and the effect on the cholinergic neurons was measured. The somatostatin neurons were manipulated with cysteamine. Subcutaneous injection of cysteamine results in a reversible depletion of somatostatin-like immunoreactivity (Sagar, 1982; Beal, 1984; Cook, 1989; Patel, 1985; Widmann, 1988; Cameron,1986). Cysteamine was administered to rats and its effect on cholinergic projection neurons was examined by measuring high affinity choline uptake, a biochemical indicator of cholinergic activity. High affinity choline uptake takes place in the neuron terminals and therefore was measured in the terminal regions within the cortex and hippocampus.

The review of the literature describes Alzheimer's Disease and details the evidence of loss of neurons that occurs with the disease. It then describes the three neuron types that were investigated in this study, the cholinergic neuron, the somatostatinergic neuron, and the GABAergic neuron. The review then presents background on the neurotoxins folic acid and cysteamine, used in this study, and the experiments that provide some indication that there is a relationship between the cholinergic projection neurons and the somatostatinergic neurons that initially degenerate with Alzheimer's Disease. The experimental design is described, as well as the experimental methodology used to study this question. The results are given followed by a discussion of how these results relate to results already reported.

Review of Literature

Alzheimer's Disease

Alzheimer's disease and senile dementia of the Alzheimer type (SDAT) are defined clinically as progressive mental deterioration, with a loss of memory and cognitive function, and a resultant inability to carry out normal daily activities (Plum, 1986). The earliest description of Alzheimer's disease was simply a presenile dementia with onset before age sixty-five. Elderly persons with the same type of symptoms were considered to be suffering from senility (Wurtman, 1985). In 1907, Alois Alzheimer first described the post-mortem changes seen with the disease in a 51-year-old demented female, where he noted marked neuronal loss and high incidence of abnormal neuron characteristics (see below) (Alzheimer, 1907). More recently, it has been found that some of the brains of the elderly suffering from dementia share the same characteristics as the presenile dementia brains at autopsy, and these have now been collectively termed senile dementia of the Alzheimer type (SDAT), and loosely Alzheimer's disease.

For the cases with later onset, the damage is less severe and the disease takes longer to run its course (Rossor, 1981). Generally, it is about three to ten years between the time the symptoms first become apparent to when the patient becomes bedridden. The early stages of the disease are a time of cognitive dysfunction only, with the main difficulty being recall. No physical disabilities form until the later stages. (Plum, 1986) The cause of death is normally some complication that arises from becoming bedridden, such as bronchopneumonia.

There are an estimated 1.5 to 2.0 million Americans afflicted with SDAT today, with 650,000 cases of the most severe state. Some surveys suggest that between four and ten percent of the over-sixty-five population are presently affected with some form of SDAT (Kwentus, 1986), and the disease is responsible for more than 100,000 deaths per year. Cost for the disease in the United States for nursing home care alone is over ten billion dollars a year. If the frequency of SDAT in our population continues as it is, and our overall population continues to age, the incidence of this disease will triple by the year 2050. The fiscal impact of SDAT by that time will be enormous (Wurtman,1985).

There are three characteristic markers in SDAT: amyloid plaques, neurofibrillary tangles, and neuronal loss. Although any of these may be found with normal aging, none occurs to

the extent that they do with SDAT (Tomlinson, 1970). When the progressive process of SDAT begins, the normal changes seen in aging spread to include severe neuronal degeneration, especially in the hippocampus, and the development of plaques and neurofibrillary tangles, particularly in the cortex.

The amyliod plaques, also called neuritic plaques, have a core containing extracellular amyloid fibers. It is unclear as to the normal function of the amyloid protein nor what triggers its deposit. The core is surrounded by abnormal neurites, some of which have now been found to be cholinergic nerve endings (Struble, 1982; Armstrong, 1986; Tago, 1987). The number of plaques is particularly increased in the cortex over that of the normal aged brain.

The second marker, neurofibrillary tangles, is very much increased in the hippocampus, and their occurance in the neocortex occurs almost exclusively in SDAT. They are more prominent in patients with the earlier onset than those exhibiting symptoms of SDAT later in life (Terry, 1983). They occupy the cell body of medium and large neurons. Electron microscopy reveals that the abnormal fibers in these tangles are composed of a pair of twisted filaments, sometimes called paired helical filaments (PHF). The PHF have also been found in the neurites contained in the amyloid plaques.

The third characteristic of an SDAT brain, and the focus of this thesis work, is neuronal loss. Neuronal loss can be measured by loss of weight of the brain. The degree of brain atrophy is extremely variable, but generally the weight loss in an Alzheimer brain is about nine to ten percent of a normal age matched brain (Mountjoy, 1984). One study looking at cortical neuronal counts found a significantly lower number of neurons in the cortex in patients with SDAT than in age-matched controls. Also, there was a trend toward a negative correlation between neuronal number and plaque number, and a high negative correlation between neuronal number and neurofibrillary tangle estimates (Mountjoy, 1983). Another study found decreased granular cells in the medial septal nuclei (Gertz, 1987).

The two types of neurons that show a large decrease in number in SDAT are cholinergic and somatostatinergic. The earliest evidence for a deficit in cholinergic neurons came in 1964 when Pope reported that acetylcholinesterase levels were lower than normal in the cortex of patients with SDAT upon autopsy. Levels of choline acetyltransferase (ChAT), the enzyme which synthesizes acetylcholine, are also decreased in the Alzheimer brain in specific brain regions (Sims, 1983). One prominent area is the nucleus basalis of Meynert, which shows losses of greater than 75 percent (Whitehouse, 1982;

Rossor, 1982). These losses also correlate with neurofibrillary tangle estimates (Mountjoy, 1984).

Decreases in acetylcholine and in the rate of its synthesis have been demonstrated in SDAT (Davies, 1976). Histopathologically, the characteristics of Alzheimer's Disease are particularly pronounced in areas of high concentration of cholinergic neurons, such as cortical and hippocampal areas and in the nuclei of the basal forebrain (Coyle, 1983). Also decreased is the activity of choline acetyltransferase (ChAT) (Bowen, 1976), which decreases greater than fifty percent in the substantia innominata (Nagai, 1983). Widespread decreases in ChAT are also found in the cingulate gyrus, frontal cortex, and hippocampus (Davies, 1979; Rossor, 1982b), including a 97% decrease in the hippocampus in one patient (Henke, 1983). There is now a general consensus that this neuron type is very much involved in SDAT.

After the establishment of the participation of cholinergic neurons in SDAT, other neurotransmitters were investigated. Decreases in somatostatin are consistently reported in post-mortem studies of SDAT. There is a decrease in cortical somatostatin-like immunoreactivity, defined as the amount of substance that reacts to antibody raised against somatostatin (Davies, 1980), and a decrease in temporal cortex

somatostatin in patients diagnosed with SDAT (Dawburn, 1986). A 47% decrease in somatostatin-like immunoreactivity correlates with decreases in cholinergic deficits in the temporal cortex (Rossor, 1980). Other regions with decreases in somatostatin-like immunoreactivity are the orbital cortex, hippocampus, and putamen of Alzheimer patients (Arai, 1984). Decreases in somatostatin are also observed in association with neuritic plaques (Morrison, 1985; Roberts, 1985). Also correlating with decreases in somatostatin are decreases in somatostatin receptor number in both the hippocampus and cortical regions (Beal, 1985). The role of somatostatin in SDAT is a matter of recent speculation. GABA neurons may also play a role, but reports of this decrease are variable.

Although these two types of neurons are both decreased in SDAT, their relationship to one another in both the healthy and diseased states has yet to be thoroughly investigated. The cholinergic neurons that degenerate in SDAT project from the septum to the hippocampus and from the substantia innominata to the cortex. The somatostatin neurons are neurons within the two target regions (see figure 1). Because they are both invariably damaged in Alzheimer's Disease, examination of their relationship may yield insight into this disease.

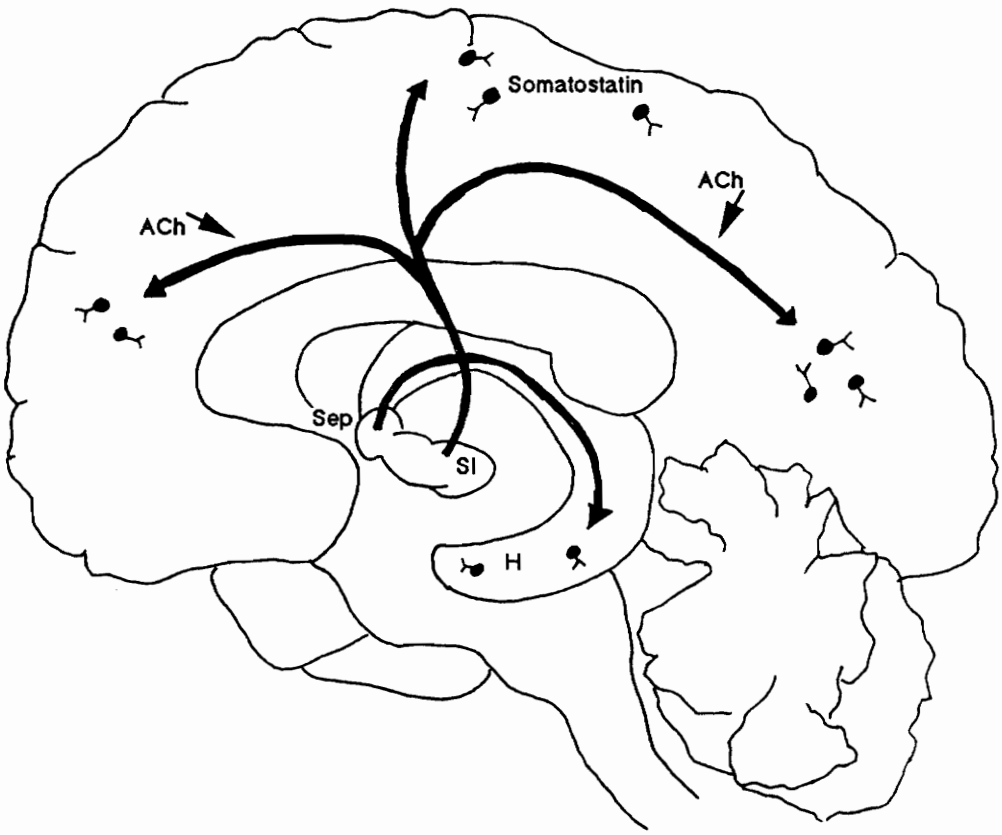

Figure 1. Neurons lost in Alzheimer's Disease. The primary neurons that are lost in the early stages of Alzheimer's Disease are cholinergic (ACh) and somatostatinergic. The cholinergic neurons are projection neurons from the septum (sep) to the hippocampus (H) and from the substantia innominata (SI) to the cortex. The somatostatin neurons are colocalized with GABA.

Acetylcholine

Acetylcholine is perhaps the most widely distributed neurotransmitter in the human body. It was as early as 1921 that acetylcholine was reported as having chemical transmission properties (Loewi, 1921). The enzyme responsible for the synthesis of acetylcholine is choline acetyltransferase (ChAT). The reaction it catalyzes is:

Choline + acetyl-CoA -> ACh + CoA

ChAT, like acetylcholine, is distributed through the length of the cholinergic neuron and is concentrated at the neuron terminal (see figure 2).

Unlike ChAT, the most prominent enzyme responsible for catalysis of acetylcholine, acetylcholinesterase (AChE), is not exclusive to cholinergic neurons. It is also made in the cell soma and transported to the neuron terminal. Only when it is found in high concentrations is AChE reliable as a marker for cholinergic neurons. However, it is used in studies as a cholinergic marker because it is much more histochemically detectable than ChAT.

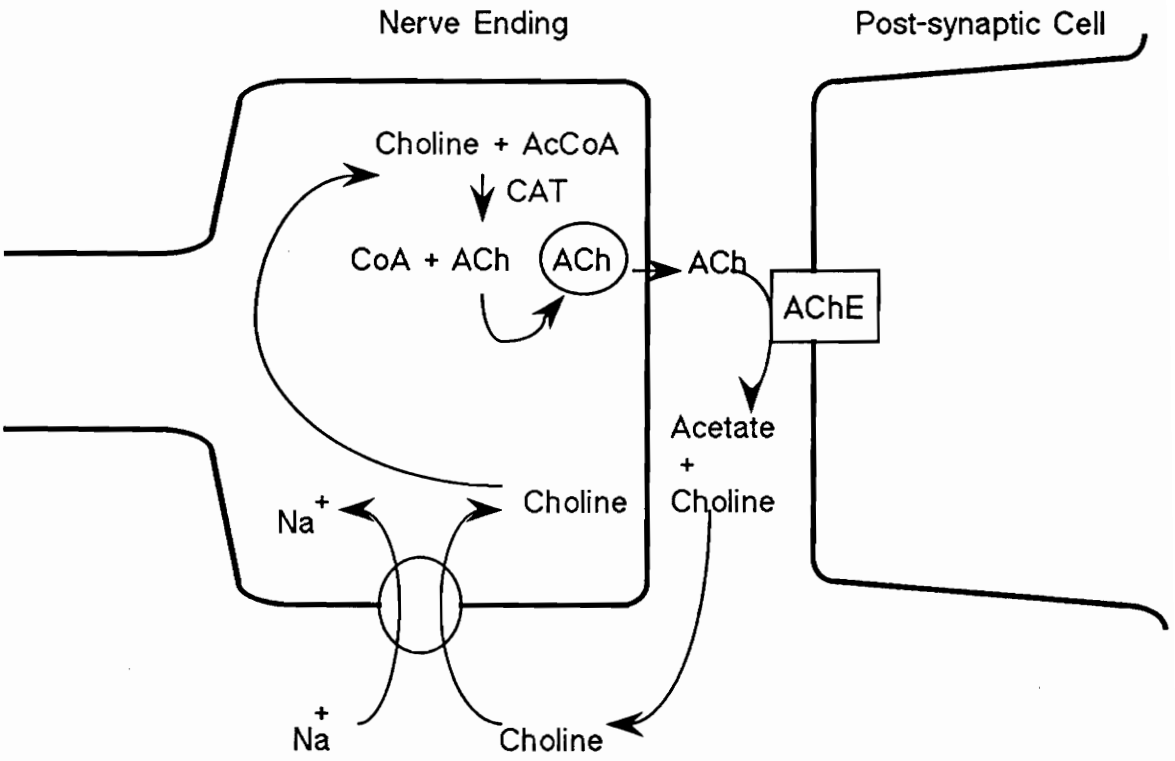

Figure 2. Metabolism of acetylcholine. Choline acetyltranferase (CAT) is made in the soma of the cholinergic neuron and transported down the axon where it functions to produce acetylcholine (ACh). After release, the ACh is broken down in the synapse by acetylcholinesterase (AChE) to acetate and choline. The choline enters an extracellular pool and is pumped back into the neurons by the sodium-dependent high affinity choline uptake. The activity of the pump is dependent on the activity of the cholinergic neuron.

The source of the substrates for ACh synthesis has been studied. There is little doubt that the source of the AcCoA is from the Embden-Meyerhof pathway (Blass, 1978). It is produced in the inner matrix of the mitochondria, and it is probably transported from there to the cytosol by the citrate shuttle for production of acetylcholine.

Neurons do not synthesize their own choline under normal conditions, and so rely entirely on the choline found free in the extracellular space. In conditions where there is not enough choline to keep up with the rate of acetylcholine production, choline can be obtained by phospholipid degradation. This could happen if the cholinergic neuron were firing at an increased rate. (Wurtman, 1985; Blusztajn, 1986) There are two systems to bring choline into the cell from the extracellular pool. One is the sodium-independent system. It was discovered first and is not exclusive to cholinergic neurons. Nor does it transport choline fast enough to meet the needs of the cholinergic neuron. Later studies revealed a second, more efficient transport system that was sodium-dependent (Yamamura, 1973). This system has been called the sodium-dependent, high affinity choline uptake system. After lesion of cholinergic neurons in the septal region, there is a sixty percent decrease in choline uptake in hippocampal

synaptosomes, suggesting that it is the cholinergic nerve endings that are responsible for choline uptake (Kuhar, 1973).

Choline uptake can be manipulated by many agents. When this occurs, it is almost always the high affinity choline uptake system that is affected (Simon, 1976). High affinity choline uptake can be manipulated by altering impulse-flow (Simon, 1975), administration of pentobarbital (Simon, 1976; Atweh, 1975; Richter, 1982b), pentylenetetrazol (Simon, 1976) and convulsants (Richter, 1982b). Three hemicholinium derivatives, hemicholinium-3 and -15, and terphenylhemicholinium-3, all inhibit the high affinity choline uptake system (Barker, 1975; Guyenet, 1973). Although the activity of high affinity choline uptake can be manipulated <u>in vivo</u>, once the system is homogenized into a synaptosomal preparation the activity of high affinity choline uptake no longer changes. High affinity choline uptake is a regulatory step in the synthesis of acetylcholine (Simon, 1976), and therefore is used as a biochemical assay of cholinergic activity.

<u>Somatostatin</u>

Although somatostatin, as well as most neuropeptides, has only in the past twenty years been recognized as a neurotransmitter within the central nervous system, its association with a number of neurological disorders continues to grow rapidly. Increased levels of somatostatin in the CSF have been observed in several inflammatory diseases such as spinal cord disease, nerve root compression, cerebral tumor, meningitis, and metabolic encephalopathy (Patel, 1977). Decreased levels of somatostatin in CSF have been found with senile dementia (Oram, 1981), Parkinson's disease (Dupont, 1982), and Alzheimer's disease (Wood, 1982; Gomez, 1986). Decreased levels of somatostatin have also been found in cases of depression (Black, 1986; Rubinow, 1986). Somatostatin has also been implicated in schizophrenia, but reports vary as to whether levels increase or decrease in the CSF (Rubinow, 1988).

The occurrence of somatostatin disturbances in such a wide variety of diseases has led to a thorough study of the neuropeptide. The peptide's full-length mRNA sequence has been discovered in both rat and human. In human, the RNA was derived from a human pancreatic somatostatinoma, and used to prepare a cDNA library. The mRNA includes the preprosomatostatin coding region, 105 nucleotides from the 5' untranslated region, and the complete 150-nucleotide

3'untranslated region (Shen, 1979). The most prominent form of the peptide is somatostatin-14. The numbering begins with +1 at the first amino acid of somatostatin-14. It is estimated that the signal peptide extends from position -102 to -79, the propeptide from -78 to -1, and somatostatin-28 from -14 to 14 (Shen, 1979).

The sequence of the preprosomatostatin was also analyzed in rat medullary thyroid carcinoma (Goodman, 1981). Although there are some differences in the propeptide, they are quite conservative and do not effect the somatostatin-28 or somatostatin-14 portion of the propeptide. The gene spans 1.2 kilobases and contains a single intron of 630 bases. The Goldberg-Hogness promoter (TATA box) is located 31 bases upstream from the transcriptional initiation site (Montimony, 1984).

In murine hypothalamus extracts, processing of the propeptide to yield the two predominant peptide species involves a minimum of four cleavage sites within the propeptide and produces at least seven different somatostatin species (Benoit, 1982). Not all of the seven fragments are thought to be biologically active. One of the primary enzymes responsible for the processing is somatostatin-28 convertase. It converts somatostatin-28 to somatostatin-14 and somatostatin-$28_{(1-12)}$ (Gluschankof, 1985). Because of this, it

is thought that the main role of somatostatin-28 is intermediary (for metabolism of somatostatin, see figure 3).

Somatostatin-28 was analyzed separately from somatostatin-$28_{(1-12)}$, and it was found that somatostatin-28 is located predominately in the perinuclear region of the neuron, while somatostatin-$28_{(1-12)}$ is found in the axons and dendrites (Lewis, 1986).

Somatostatin is found throughout the central nervous system and indeed throughout the human body. It was originally discovered in the pancreas as an endocrine hormone. In the central nervous system, somatostatin was originally isolated from the hypothalamus and is known to inhibit the release of growth hormone (Brazeau, 1973). After this discovery, many distribution studies were done to find how extensive the somatostatin distribution is.

In immunohistochemical studies, high concentrations of somatostatin have been found in the cerebral cortex (Beal, 1987; Lewis, 1986; Hendry, 1984a; Mizukawa, 1987; Yamashita, 1989), the nucleus accumbens and striatum (Beal, 1987), the amygdala and the bed nucleus of the stria terminalis (Beal, 1987; McDonald, 1987), the substantia innominata (Beal, 1987), and the hippocampus (Bakst, 1985; Morrison, 1982; Feldman, 1982; Scarfman, 1988). The distribution is largely consistent throughout the species of mammals.

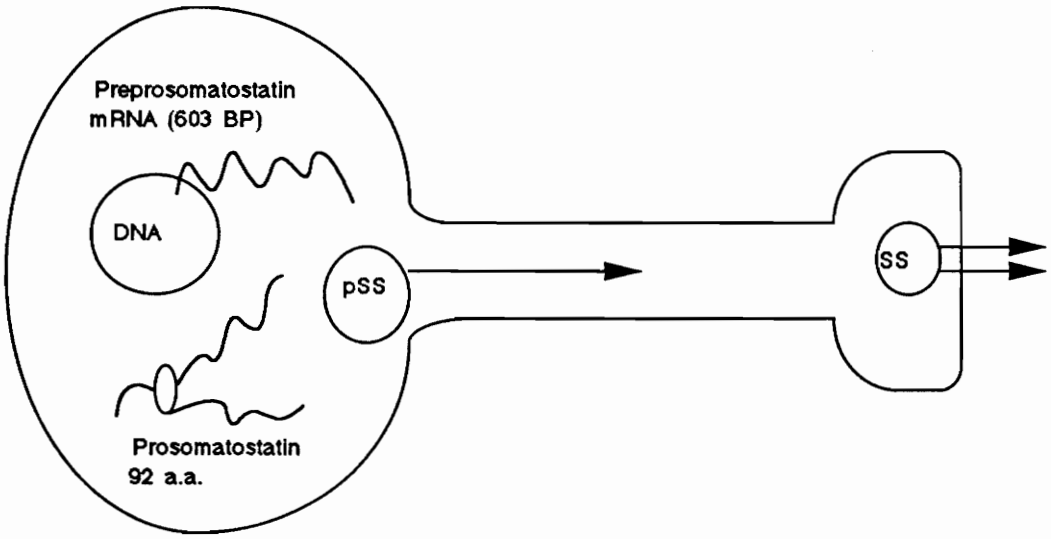

Figure 3. Metabolism of somatostatin. The preprosomatostatin mRNA is transcribed from nuclear DNA. It is translated to the prosomatostatin peptide which contains a signal peptide for placement into a synaptic vesicle. The propeptide and somatostatin-28 convertase travel down the axon together in the vesicle, and the prosomatostatin is transformed to somatostain-14 and somatostatin-28 (1-12). After release, the somatostatin peptide is degraded by a variety of peptidases. There is no reuptake system, and the peptide cannot be reused.

Prosomatostatin mRNA was studied using in situ hybridization in the anterior olfactory nucleus, hypothalamus, amygdala, and all regions of the cerebral cortex. This distribution correlated with the distribution of somatostatin immunoreactive regions (Fitzpatrick-McElligott, 1988). The same was found in the striatum (Weiss, 1989).

Somatostatin binding sites are also characterized by use of a radioiodinated analog of somatostatin and correlate with somatostatin-like immunoreactivity (Beal, 1986). Somatostatin receptors are abundant in the hippocampus (Palacios, 1986).

There are two types of somatostatin receptors, identified by use of cyclic somatostatin analogs (Tran, 1985). The two types have distinct topological localization (Reubi, 1987), but so far no functional distinction has been made between the two.

Most neuropeptides have been found coexisting with other neurotransmitters, and somatostatin is no exception. One of the most common colocalizations is somatostatin with neuropeptide Y. These compounds are located together in the cortex (Hendry, 1984a; Vincent, 1982) where up to 41% of somatostatin neurons also stain for neuropeptide Y in immunohistochemical studies (Hendry, 1984a). In the peripheral sympathetic nervous system, somatostatin is colocalized with norepinephrine (Hokfelt, 1977). So far there

has not been a demonstration of the coexistence of acetylcholine and somatostatin. Somatostatin has been found to be located in the same neuron as acetylcholinesterase in neurons cultured from rat cerebrum, and this suggests that the somatostatin neuron may be cholinoceptive (Delfs, 1984).

Somatostatin is also colocalized within GABAergic neurons, and this is a fairly widespread phenomenon in the central nervous system. In the cerebral cortex, neurons were stained immunohistochemically for glutamic acid decarboxylase, a marker enzyme for GABA neurons, and for somatostatin. Greater than ninety percent of the somatostatin immunoreactive cells were also glutamic acid decarboxylase positive in the cortex of cat and monkey (Hendry, 1984b). A similar study was done in rat hippocampus. Approximately ninety percent of somatostatin-like immunoreactive neurons were also glutamic acid decarboxylase-like positive, whereas only fourteen percent of glutamic acid decarboxylase staining cells were somatostatin positive (Kosaka, 1988). This is also true in the cat visual cortex and hippocampus with similar results (Somogyi, 1984). The somatostatinergic neurons are then a subset of GABAergic neurons in the hippocampus and cortex.

GABA

GABA, or gamma-amino butyric acid is found throughout the central nervous system, and as mentioned previously, has often been found to coexist with other neurotransmitters. Its chemistry is interesting, in that every time a molecule of GABA is destroyed metabolically, a molecule of precursor is formed to take its place (See figure 4). The enzyme responsible for breaking down GABA is GABA α-oxo-glutarate transaminase. It forms succinic semialdehyde, but the reaction can only take place if α-ketoglutarate is available to accept the amine group. The amination of α-ketoglutarate produces glutamate. A decarboxylation reaction by glutamic acid decarboxylase transforms the glutamate to GABA. This closed loop is called the GABA shunt. The α-ketoglutarate originates from the Krebs cycle, and thus originally from glucose. The shunt can also be fed by pyruvate. Once released, the GABA can return to the neuron that has released it, or it can be picked up by glial cells, thus entering the glutamine loop. GABA-transaminase is also present in these cells, thus converting the GABA to succinic semialdehyde and an α-ketoglutarate to glutamate. The glutamate cannot be converted to GABA by the glial cells however, because they lack glutamic acid decarboxylase. This makes the glutamic

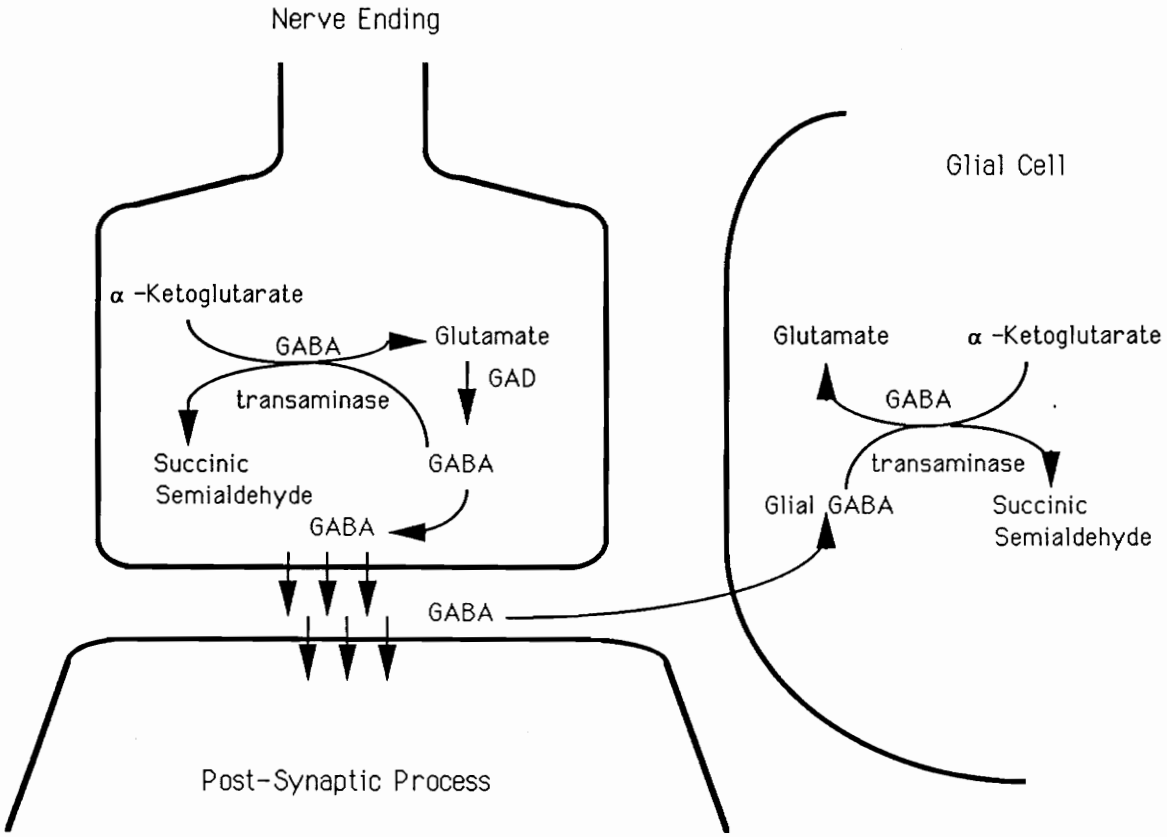

Figure 4. Metabolism of γ-amino butyric acid (GABA). Schematic diagram showing the GABA shunt and the glutamine loop. GABA is synthesized from glutamate which is exclusive to the GABA neuron. GABA is metabolized by GABA-transaminase which is found in both the GABA neuron and neighboring glial cells. Adapted from Siegel, Albers, Agranoff, and Katzman. Basic Neurochemistry, p 235, 1981.

acid decarboxylase a better marker enzyme than GABA transaminase because the glutamic acid decarboxylase is exclusive to GABA neurons. GABA transaminase is sometimes used as a marker enzyme because it is easier to assay. Instead of the glutamate being converted to GABA, it is converted to glutamine by glutamine synthetase, and the glutamine then readily crosses the cell membranes to the GABA neuron to feed back into the GABA shunt.

Neurotoxins

Cysteamine

Cysteamine, also known as 2-mercaptoethylamine, has long been known to produce perforating duodenal ulcers in rat (Seyle, 1973). In the course of studying the ulcerogenic effects of cysteamine, it was found that somatostatin administration prevents the formation of ulcers (Schwedes, 1977), and oral administration of cysteamine produces a decrease in somatostatin in the stomach and duodenal mucosa of the gastrointestinal tract and in the hypothalamus (Szabo, 1981). In isolated islet cells, cysteamine depletes somatostatin by fifty percent. Cysteamine also decreases glucose-induced insulin release (Ostenson, 1985). In monolayer cultures of neonatal rat islet cells, the decrease

in somatostatin and insulin is also observed (Patel, 1985). One study of isolated pancreatic islets did not find the increase in insulin *in vivo*, but still noted the decrease in somatostatin (Hashimoto, 1987).

Somatostatin is depleted in the retina, cerebral cortex (38%), and cervical spinal cord (65%) of rats following subcutaneous injection of cysteamine (Sagar, 1982). Another study looking at the depletion of somatostatin after local injection of cysteamine into the striatum showed the histological damage was seen only from the needle tract. The maximum depletion of somatostatin in the striatum was 50%. (Beal, 1984) The depletion is evident after two hours, with maximum depletion occurring at four hours post injection. The effects are reversed by one week (Sagar, 1982; Cook, 1989).

Associated with the decrease in somatostatin levels is a 1.7 fold increase in maximum binding to cerebrocortical receptors five minutes post injection. A slower increase in the affinity is also observed, with the disassociation constant being almost 2-fold lower than the control at thirty minutes post-injection. Incubation of synaptosomes *in vitro* with cysteamine shows the same depletion of somatostatin (Srikant, 1984). This suggests that a decrease in somatostatin will affect the somatostatin receptive cell, and so the effect of cysteamine is on the releasable pool of

somatostatin, which can effect the functionality of the somatostatin neuron. A similar observation was made in the gastrointestinal tract, where there was a parallel increase of somatostatin binding sites with the decrease in somatostatin (Rossowski, 1987).

Other neuropeptides have been measured after the administration of cysteamine, and the effects are selective for somatostatin. In the periventricular nucleus and median eminence, there are decreases in somatostatin of up to 80%, while there is no change in luteinizing hormone releasing hormone, vasopressin, enkephalin, vasoactive intestinal peptide, or cholecystokinin (Palkovits, 1982).

One neurohormone that does show a decrease in immunoreactivity after cysteamine administration is prolactin in the anterior pituitary. Prolactin, like somatostatin, has a disulfide bond in its secondary structure. In a study protecting the disulfide bond with reduced glutathione, prolactin was not effected by cysteamine <u>in vivo</u>. This suggests that cysteamine works through a thiol-related mechanism (Sagar, 1985). Further evidence that the disulfide bond is important in the cysteamine-induced depletion of somatostatin is that somatostatin-$28_{(15-28)}$, a disulfide bond containing somatostatin peptide, is depleted with cysteamine administration, but not the non-disulfide bond containing

peptide, somatostatin-$28_{(1-14)}$ (Patel, 1985).

In cases where the somatostatin and neuropeptide Y are colocalized in the same neuron, there are decreases of up to fifty percent for somatostatin following cysteamine injection, but there was no change in neuropeptide Y levels. This suggests that treatment with cysteamine does not affect the vesicles of somatostatin/neuropeptide Y neurons (Widmann, 1988). In another study, the effect of cysteamine on [^{35}S]-cysteine incorporation into somatostatin was looked at. The depletion of somatostatin was observed with a decrease in [^{35}S]-cysteine levels. The authors concluded that cysteamine inhibits the synthesis of somatostatin, however they had no control for the degradation of somatostatin (Cameron, 1986).

Cysteamine has been used in studies of epilepsy. Somatostatin causes seizures after intracerebroventricular injection. Intraperitoneal administration of cysteamine leads to profound suppression of kindled seizures (Higuchi, 1983). Cysteamine also induces myoclonic seizures for the first hour after cysteamine injection in hippocampal kindled rats (Cottrell, 1986). The effects of cysteamine on kindled seizures and myoclonic seizures is more marked in the frontal cortex than in the amygdala (Takazawa, 1988).

There have been some behavioral changes observed following treatment with cysteamine (Bakhit, 1986). One study

showed a decrease in locomotor activity at high doses and head and neck tremor with increased defecation at medium and high doses (Hartoutunian, 1987).

Folic Acid

Folate compounds, a different kind of neurotoxin, folate compounds, cause epileptiform activity when applied to brain tissue, thus working as an excitant (Spector, 1971). Folates occur normally in the body, and are responsible for carrying single carbon units in various synthesis pathways. When applied to neurons in higher than physiological doses, folates cause neurons to fire at highly increased rates, much like kainic acid; however, unlike kainic acid, folates do not cause neuronal death. Folic acid may cause this increased firing rate by acting at kainic acid receptors (Olney, 1981) Folic acid produces epileptic activity to varying degrees within different regions of the hippocampus. The area showing the highest activity after perfusion of a brain slice with folic acid is the pyramidal cells of the CA3 region of the hippocampus. This suggests that folic acid does not have a general effect, but acts in a specific manner, perhaps on neurons with kainic acid receptors (Clifford, 1983).

Cholinergic-GABAergic-Somatostatinergic Interrelationships

As stated earlier, the two main types of neurons that degenerate in Alzheimer's Disease are the cholinergic septo-hippocampal and substantia innominata-cortical projection pathways and the somatostatin interneurons found in the target regions. Of the two cholinergic pathways, the septo-hippocampal is better characterized both histochemically and neurophysiologically than the substantia innominata-cortical pathway. While both the septo-hippocampal and substantia innominata-cortical cholinergic projections are the major afferents to their respective target regions, in the hippocampus, the cholinergic projections are the only cholinergic input to that region, whereas in the cortex, the substantia innominata afferents account for only seventy percent of the total cholinergic activity with the source of the remaining thirty percent unknown (McGeer, 1982). So while they both degenerate in Alzheimer's Disease, there are some differences between the two systems.

Although all the cholinergic innervation to the hippocampus is from the septum, all the innervation to the hippocampus from the septum is not cholinergic. The reported percentage of total innervation from the septum to hippocampus that is varies depending on the method of staining used and

the region of the hippocampal formation injected. In one study, after hippocampal injection of horseradish peroxidase, a retrograde tracer, less than fifty percent of the neurons in the septum containing horseradish peroxidase injection product also stained for acetylcholinesterase (Baisden, 1984). In a study utilizing horse radish peroxidase and choline acetyltransferase staining, various regions of the hippocampal formation were injected. The proportion of retrogradely labeled cells that were also positively stained for choline acetyltransferase ranged from 23 to 74 percent, depending on the region of the hippocampus injected (Amaral, 1985). One type of study done to characterize the cholinergic septal-hippocampal pathway is to transect the fimbria-fornix, the pathway that the axons run along between the septum and hippocampus. When this is done, retrograde cholinergic neurons in the septum die. However, only fifty percent of the cells that die are cholinergic, with the other half suggested to be GABAergic and substance P (Gage, 1986).

In histochemical studies, the septo-hippocampal projections were shown to synapse onto somatostatin neurons in the hippocampus. In an immunocytochemical study using the anterograde transport of Phaseolus vulgaris leucoagglutinin (PHA-L), coupled with staining for PHA-L in the fibers and cellular presence of somatostatin, it was found that the

projecting fibers did in fact synapse onto somatostatin neurons (Yamano, 1989). However the study could not distinguish what type of projection neuron was stained with the PHA-L, and it therefore could not be concluded unequivocally that cholinergic neurons synapse onto somatostatin neurons.

In another study, the hilar region of the hippocampus was stained for choline acetyltransferase and glutamic acid decarboxylase, or choline acetyltransferase and somatostatin and examined under electron microscope. Both GABAergic and somatostatinergic non-pyramidal neurons in the hilar region receive a cholinergic input. The cholinergic inputs to the GABAergic and somatostatinergic neurons were classified on a morphological basis with electron microscopy to be the afferents from the septum (Leranth, 1987). There is then histochemical evidence of cholinergic input to somatostatin neurons (see figure 5).

There is some evidence that the two cholinergic projection neurons and the somatostatin neurons may be functionally related as well. Administration of acetylcholine to cultured cerebral cortical neurons causes the release of somatostatin into the cell media, suggesting that somatostatinergic neurons are able to receive innervation from cholinergic neurons (Robbins, 1982). Conversely, somatostatin

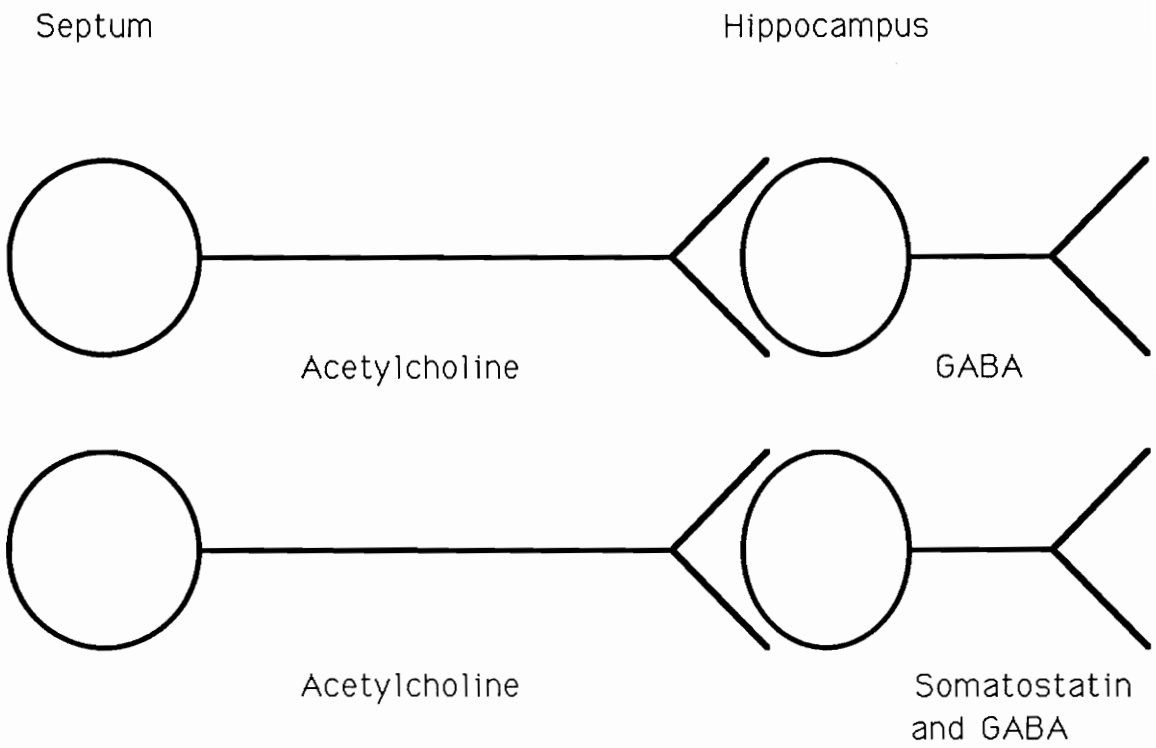

Figure 5. Model of septo-hippocampal cholinergic projections. The cholinergic neurons project from the septum to the hippocampus where they synapse on both GABAergic and somatostatinergic neurons. The somatostatin is colocalized with GABA. A similar model is assumed for the substantia innominta-cortical system.

stimulates the release of acetylcholine from hippocampal synaptosomes (Nemeth, 1979). In addition, when somatostatin is injected intracerebroventricularly, acetylcholine turnover rate increases in the hippocampus and cortex (Malthe-Sorenssen, 1978). These two facts suggest that cholinergic neurons are able to be innervated by somatostatin. However, somatostatin has been injected into the septum with no modification of hippocampal acetylcholine turnover (Wood, 1981).

Following electrolytic lesion to the septum, choline acetyltransferase activity is decreased by almost 90% two weeks later. Somatostatin-like immunoreactivity was measured in the hippocampus and found to be unaltered (Pierotti, 1986). In a similar experiment, ibotenic acid was used to lesion the nucleus basalis. Cortical choline acetyltransferase was decreased by over sixty percent, but levels of somatostatin, as well as noradrenaline, dopamine, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid, were unchanged (Fine, 1987).

Folic acid has been used to study the substantia innominata-cortical projection. The effect of folic acid-induced hyperactivity is found distal to the site of injection. Most of the damage seen at the site of injection can be attributed to the physical disruption caused by the needle. Folic acid was injected into the substantia

innominata and glutamic acid decarboxylase and choline acetyltransferase activities were measured in several regions throughout the brain. Injection of folic acid produced sustained seizure-like activity. After 200 nmol injection of folic acid into the substantia innominata, glutamic acid decarboxylase activities were down to twenty percent of control in the pyriform cortex and amygdala, fifty-eight percent of control in the thalamus, and seventy-four percent of control in the entorhinal cortex. The control was the side contralateral to the site of injection. There were modest decreases in choline acetyltransferase activities in the same regions (McGeer, 1983). In a follow-up study, folic acid was again injected into the substantia innominata and binding studies of various brain regions were done. Binding of [^{3}H]quinuclidinyl benzylate, a marker for cholinoceptive neurons, was significantly decreased from controls in the frontal cortex, pyriform cortex, substantia innominata, and amygdala. There was no effect on benzodiazepine binding, specific for GABA receptors, indicating that the loss was specific (Wong, 1983).

Materials and Methods

Experimental Design

The purpose of this thesis was to examine the functional relationship between the cholinergic septal-hippocampal and substantia innominata-cortical projections and the somatostatin neurons that lie within the projection regions. This was done in two ways. First, the cholinergic neuron activity was increased and somatostatin-like immunoreactivity was measured to see if hyperactivity of the cholinergic neurons results in degeneration of the somatostatin neurons, similar to the degeneration reported for GABAergic neurons (McGeer, 1983). Second, somatostatin levels were decreased and cholinergic activity measured to see if somatostatin levels affected cholinergic activity.

The rationale for increasing the activity of the cholinergic neurons was as follows: First, both the forebrain cholinergic projections and the somatostatin interneurons degenerate during the initial stages of Alzheimer's Disease. Cortical and hippocampal GABAergic interneurons contain a subset of cell which also contain somatostatin, and cholinergic septo-hippocampal projection neurons synapse upon

these somatostatin neurons. Previous work has shown that a hyperactivity of the cholinergic substantia innominata-cortical cholinergic projections results in a degeneration of GABA neurons as measured by glutamic acid decarboxylase activity (McGeer,1983). Thus, substantia innominata hyperactivity may decrease somatostatin through a degeneration of the GABAergic subset containing somatostatin.

The cholinergic projections were made hyperactive by injection of folic acid into the region of the cell bodies. The rapid firing of the cholinergic neurons after the injection of folic acid was expected to lead to the degeneration of the GABA and somatostatinergic neurons onto which the projections synapse. A first set of injections was made into the septum. Because both sides of the septum fall on the midline, spread of injected folic acid has a bilateral influence. For this reason the control group had to be a separate group of rats. These rats were injected with physiological saline. After injection into the septum, two weeks were allowed to pass. This time period allowed for any degenerated hippocampal or cortical neurons to be cleared away. The rats were killed and regions of the hippocampus were assayed for somatostatin-like immunoreactivity and glutamic acid decarboxylase activity.

A second set of injections of folic acid was made into the substantia innominata. Because the contralateral sides of the substantia innominata are not in close proximity, injection could be made to one side and the contralateral side used as the control. This method has been performed previously whereby the injected side was decreased from the control (McGeer, 1983). From that it was assumed that the injection of folic acid into the substantia innominata would not spread to the contralateral side. Again, two weeks were allowed to pass before killing the rats to ensure that any cellular debris would be cleared. The cortex was dissected and choline acetyltransferase and glutamic acid decarboxylase activities were measured.

The second approach to establishing a relationship between the two types of neurons that degenerate in Alzheimer's Disease was a converse to the first. Instead of looking for an effect of cholinergic neurons on somatostatin levels, an effect of decreased somatostatin on cholinergic activity was investigated. The rationale for looking at the effect of a decreased level of somatostatin was that somatostatin stimulates the release of acetylcholine from hippocampal synaptosomes (Nemeth, 1979) In addition, when somatostatin is injected intracerebroventricularly, acetylcholine turnover rate increases in the hippocampus and

cortex (Malthe-Sorenssen, 1978). These two facts suggest a possible innervation of cholinergic neurons by somatostatin.

Somatostatin was decreased with the subcutaneous injection of cysteamine. Cysteamine temporarily decreases somatostatin levels (Sagar, 1982; Beal, 1984; Cook, 1989; Patel, 1985; Widmann, 1988; Cameron, 1986) After four hours, the rats were killed. Four hours post-injection is the time of maximum somatostatin depletion (Sagar, 1982; Cook, 1989). Both the cortex and the hippocampus were dissected and the tissues were immediately homogenized. The samples were assayed for high affinity choline uptake, a biochemical indicator of cholinergic activity. The system for the uptake is found in the neuron terminals in the cortex and hippocampus. Because cysteamine was systemically injected, a separate group of rats had to be used as the control group. The control rats were injected subcutaneously with saline.

Materials

The [^{125}I]-somatostatin and rabbit antiserum to somatostatin used in the somatostatin RIA were purchased from Amersham Corporation, Arlington Heights, Illinois. Protosol and [^{3}H]-choline, both used in the high affinity choline uptake assay; [^{3}H]-acetyl coenzyme A, used in the choline

acetyltransferase assay; and [^{3}H]-glutamate, used for the glutamic acid decarboxylase assay, were purchased from New England Nuclear, Boston, Massachusetts. The Biosafe II scintillation cocktail, used in both the high affinity choline uptake assay and the glutamic acid decarboxylase assay, was purchased from Research Products International Corporation, Mount Prospect, Illinois. The anion exchange resin, AG 1-X8, analytical grade, 100-200 mesh, acetate form used in the glutamic acid decarboxylase assay was obtained from BioRad Laboratories, Richmond, California. The following were from Fisher Scientific Company, Fair Lawn, New Jersey: Bis-MSB, cupric sulfate, EDTA, scintanalyzed PPO, sodium carbonate, sodium tartrate, sodium chloride, and acetonitrile. All other reagents were obtained from Sigma Chemical Company, St. Louis, Missouri.

Methods

Folic Acid Injection

Male Sprague-Dawley rats, weighing between 250 and 270 grams, were anesthetized with sodium pentobarbital at 45-60mg/kg body weight. After loss of tail pinch reflex, the

rats were placed in a Kopf small animal stereotaxic instrument. The head was stabilized with ear bars and an incisor bar. The head was shaved and a cut made into the tissue overlaying the skull to reveal the bregma suture (see figure 6). The needle for injection was then lined up with bregma. The needle was stainless steel and was hooked by polyethylene tube to a Hamilton syringe. The syringe was loaded onto a syringe pump. A hole was drilled into the skull using a cable drill at the point of needle insertion. For injection into the septum, the sagittal sinus was avoided. The coordinates for the septum were AP -8.5 mm and L 0 from bregma, and -4.7 mm ventral to the dura. Instead of bringing the needle directly down to its coordinates, the needle was moved 1mm lateral from the sinus and then down 2 mm. This placed the needle tip ventral to the sinus, and the needle was then brought back to the midline, thereby pushing the sinus aside instead of puncturing it. The needle was then lowered the rest of the 4.7 mm. For injection into the substantia innominata, the coordinates were AP -1.0 mm, L 2.9 mm from bregma and down 7.3 mm from the dura.

The folic acid was dissolved in water to 200nmol/ul, and was brought to a pH between 6.8 and 7.6 with NaOH. The folic acid was injected using a syringe pump at a rate of 2μl/3 minutes. The total volume injected was 1 μl. After

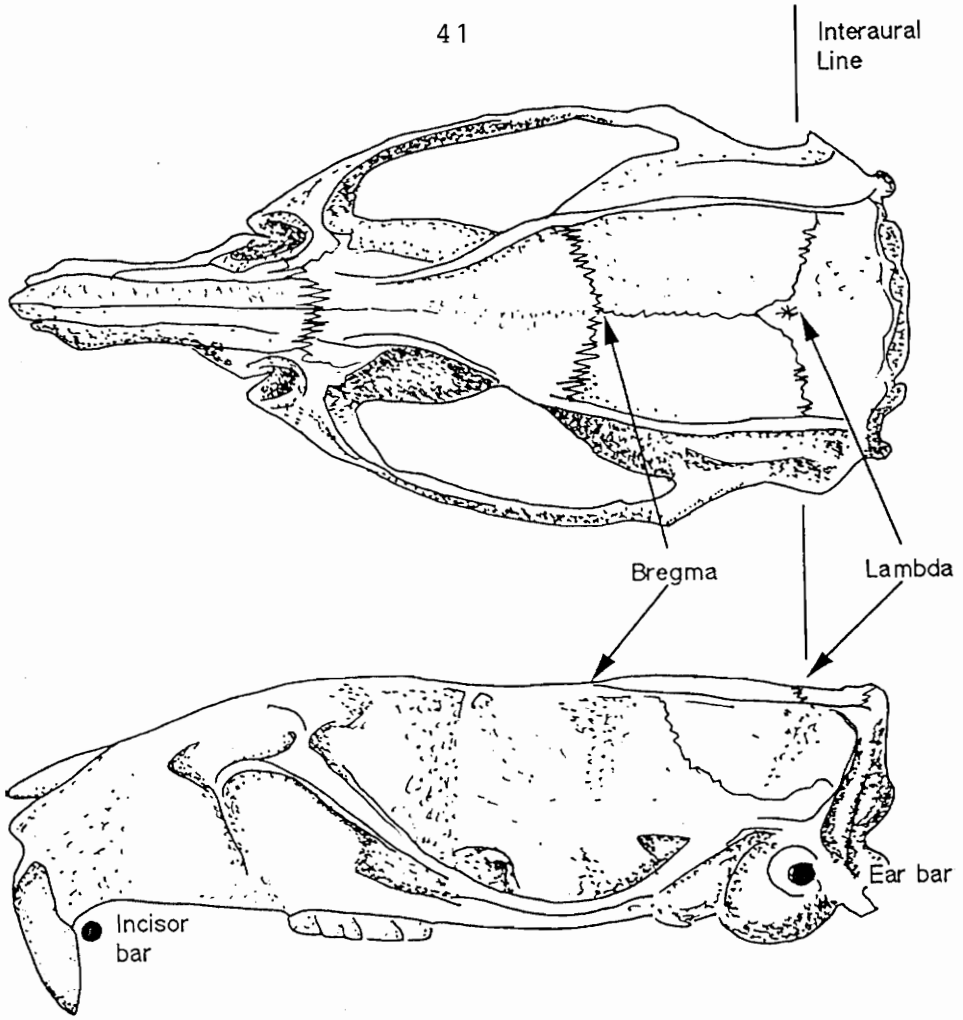

Figure 6. Dorsal and lateral views of rat skull. Adapted from Paxinos and Watson , The Rat Brain in Stereotaxic Coordinates, 1982.

injection, one minute was passed before pulling the needle out to avoid pulling the folic acid up the needle tract.

The skin was closed using staples and the rats were kept on low doses of pentobarbital for two hours to reduce the severity of the seizures. The doses averaged 26 mg pentobarbital/ kg body weight. The animals were awake for these two hours and continued to the reaction to the folic acid described by McGeer (1983). The seizures continued for six to eight hours post-injection and by twelve hours the rat no longer experienced these effects. The rats were kept for two weeks on a twelve hour light dark cycle with ad lib food and water intake.

After two weeks, the rats were killed in one of two ways. For tissue samples to be used in enzyme assays, rats were killed by decapitation. The brains were dissected on ice and the tissue immediately frozen and stored at -70°C. For tissue sample to be used for radioimmunoassays, the rats were killed by head focused microwave irradiation in a Cober Electronics Model S6F Industrial Microwave Generator. Such microwave irradiation denatures proteins enough to insure that none of the metabolic enzymes are functional but leaves the primary structure of proteins intact so that somatostatin can be detected by radioimmunoassay (Ikarashi, 1986).

Cysteamine Injection

Cysteamine was injected subcutaneously into male Sprague-Dawley rats. The rats weighed between 250 and 375 grams and were kept on a twelve hour light/dark cycle with ad lib food and water intake. The dose was 300 mg cysteamine/kg body weight. The cysteamine was dissolved in 0.9% saline. The pH was adjusted to 7.2-7.4 using 1N NaOH. The volume injected was approximately 0.2ml. The control rats were injected with 0.2ml 0.9% saline. The rats were killed by decapitation four hours post-injection. The brains were dissected on ice and the tissue immediately homogenized for high affinity choline uptake assay.

Assay for Choline Acetyltransferase

This assay was adapted from Fonum (Fonum, 1974). Tissue was homogenized in approximately 20 volumes of 10mM EDTA in a Radnoti glass tissue homogenizer on ice. (eg, for 10mg of tissue, homogenize in 200 ul of 10mM EDTA) 100ul of homogenate was transferred to a test tube on ice. 300ul of 0.67% Triton X-100 was added and the mixture was gently

shaken. The mixture stood for at least ten minutes.

The substrate media containing 120mM sodium phosphate, 48mM EDTA, 722mM sodium chloride, 18mM choline bromide, .2mM physostigmine, 0.4mM acetyl coenzyme A, and 16.2 uCi [^{3}H] acetyl coenzyme A was warmed to 37°C. All incubations took place in an American Sci Products BT-23 water bath. 4ul of Triton homogenate was transferred to a microtube and warmed to 37°C. 4μl substrate media was added to the microtube and incubated at 37°C for 15 minutes.

At the end of 15 minutes, the microtube was dried off and placed in a scintillation vial. The reaction was stopped by flushing the microtube with 1.5 ml cold 10mM sodium phosphate, pH7.4. The contents of the microtube were kept in the vial, and 0.6ml acetonitrile-TPB (300ml acetonitrile + 1.5g tetraphenylboron) was immediately added followed by 3.0ml toluene scintillation mixture (600ml toluene + 3g PPO + 120 mg bis MSB). This mixture was shaken moderately and then the phases were allowed to separate for at least ten minutes. The ^{3}H channel was counted for two minutes in a Beckman LS 5800 B counter.

The principle behind the choline acetyltransferase assay is illustrated in figure 7. After flushing the microtube with the aqueous buffer, the organic acetonitrile and toluene mixtures are added. Both the substrates and products are

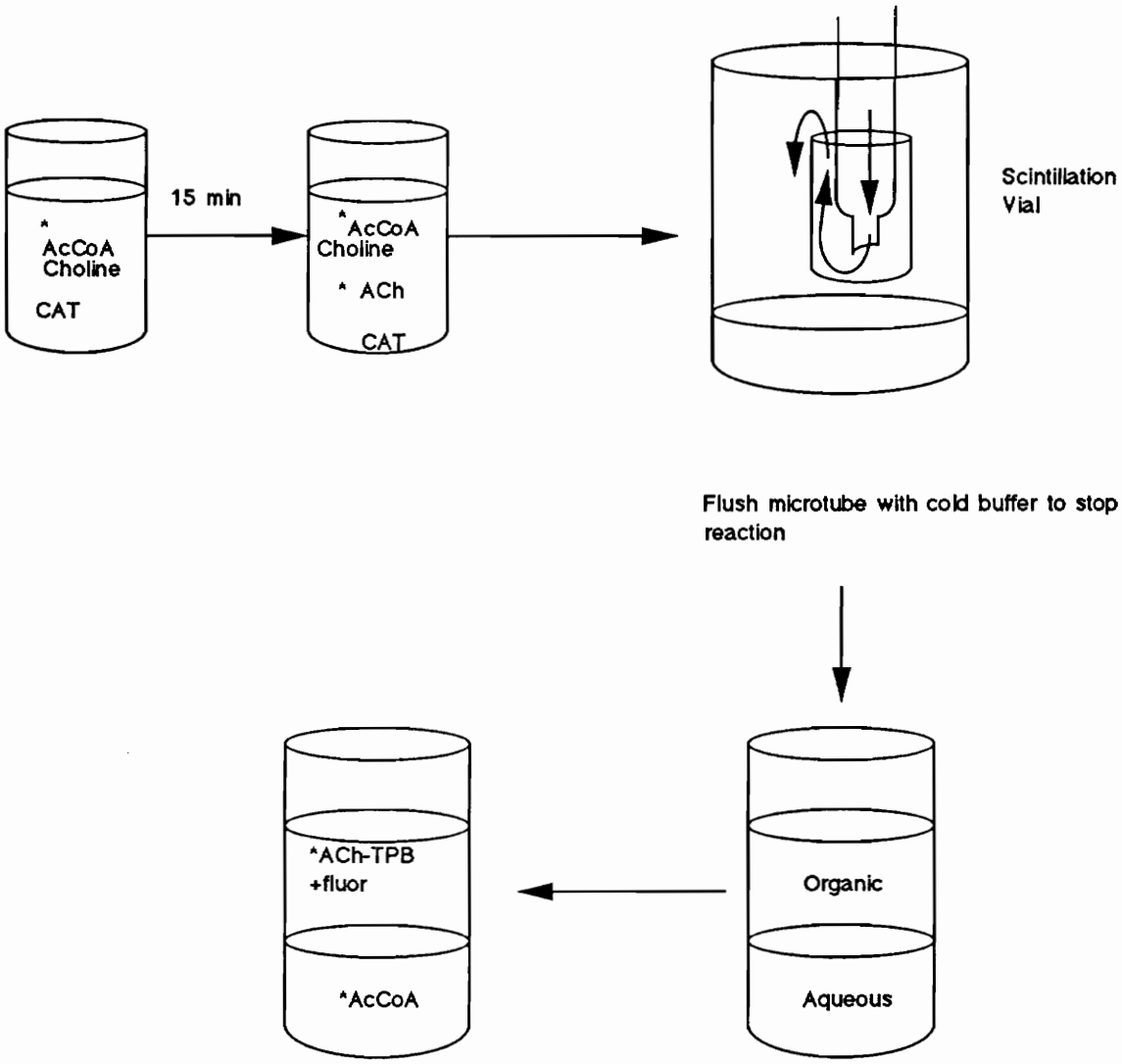

Figure 7. Choline acetyltransferase assay. The [3-H]-acetyl coenzyme A (*AcCoA), choline and choline acetyltransferase (CAT) are incubated for 15 minutes. The reaction is stopped by flushing with cold buffer solution. The labelled reaction product, [3-H]-acetylcholine (*ACh) is separated from the unreacted *AcCoA by partitioning of *ACh-TPB complex into the organic phase of the scintillation vial.

still present in the vial. Normally the newly formed acetylcholine would partition into the aqueous layer with the substrates. However, in this case the acetylcholine forms a complex with the TPB and then partitions into the organic phase. The fluors are only present in the organic phase, and so only the labelled acetylcholine is detected. Because the unreacted labelled acetyl coenzyme A is partitioned into the aqueous phase, it is not detected.

Assay for Glutamic Acid Decarboxylase

This assay was adapted from Hamel (1982). The tissue was homogenized in twenty volumes 10mM EDTA in a Radnoti glass tissue homogenizer. 25 μl of this homogenate was added to 25 μl of 100mM potassium phosphate, pH 7.2m, 0.2 mM pyridoxal-5 phosphate (PLP), 0.2mM EDTA, 10 mM β-mercaptoethanol, and 0.25% Triton X-100. The mixture was spun at 8.4K x g for ten minutes in an Eppendorf Centrifuge 5415. The supernatant was then assayed for glutamic acid decarboxylase activity.

The assay reaction mixture contained 10 μl of 0.5M potassium phosphate, pH 7.2, containing 1mM PLP, 10μl [^{3}H]-glutamic acid (10 μl contains 10 μCi), and 20 μl of tissue supernatant. The tissue extract was added to the mixture

last. The reaction mixture was deoxygenated under a nitrogen stream and incubated at 37°C for 45 minutes in a American Sci Products BT-23 water bath.

The reaction is stopped with 500 μl of copper-phosphate suspension. The suspension contains 27 g/l cupric chloride, 64.5 g/l trisodium phosphate, and 28.6 g/l water containing 50 ml 1M HCl of sodium borate. This suspension must be made up fresh every day. The tubes were then placed on ice for ten minutes. The excess copper-phosphate suspension was pelletted in a microcentrifuge at 12.1K x g for 5 minutes in an Eppendorf Centrifuge 5415. The entire supernatant containing both the substrates and products was pipetted onto a 2.5 x 0.5 cm column of Bio Rad AG 1-X8 anionic resin, 100-200 mesh, acetate form. Unreacted glutamic acid stuck to the column, but not the newly formed GABA. The column was rinsed with 1.0 ml water, and both effluents were collected into a scintillation vial. This was mixed with 15 ml aqueous scintillation fluid and counted in a Beckman LS 5800 B counter (see figure 8).

Somatostatin Radioimmunoassay

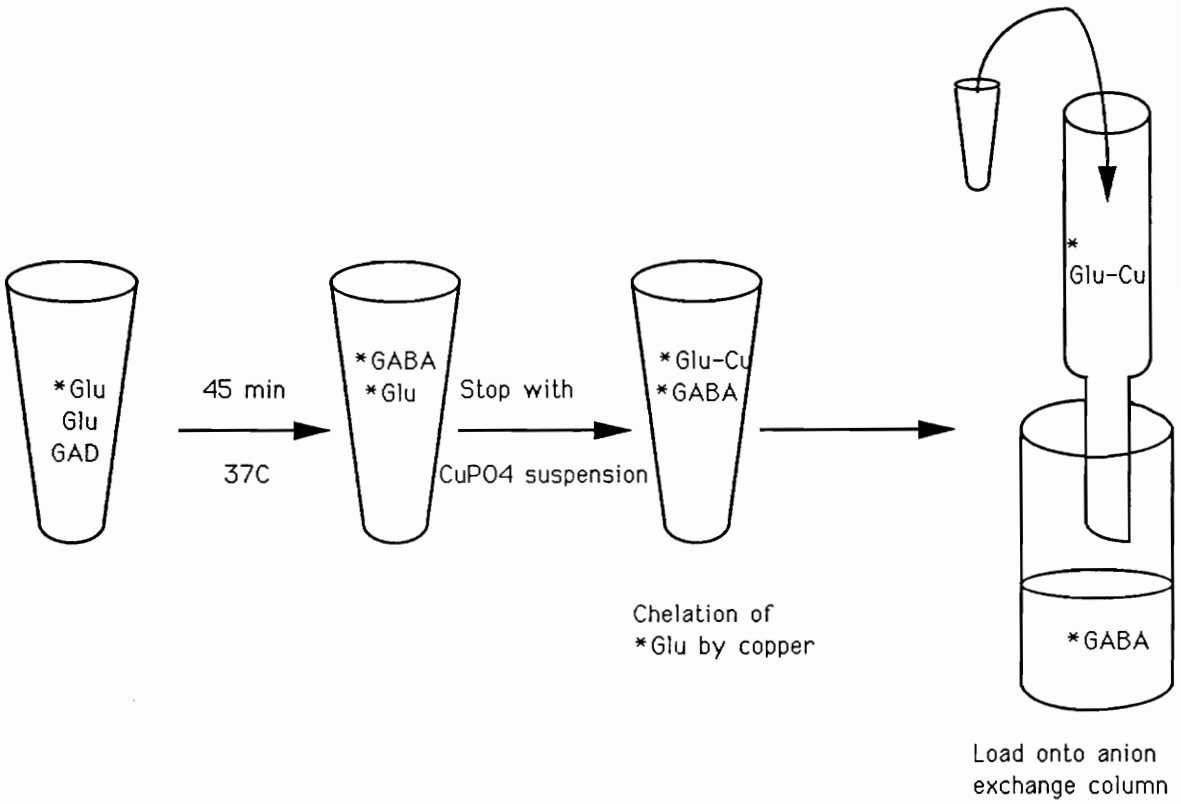

Figure 8. Assay for glutamic acid decarboxylase. The glutamate (glu) is incubated with tissue glutamic acid decarboxylase (GAD) to form γ-amino butyric acid (GABA). This reaction is stopped with a copper phosphate suspension. The copper chelates with the glutamate, but not the GABA, as copper is ineffective in chelating γ-amino acids. The copper helps the glutamate stick to the anion exchange column, while the GABA is washed into the scintillation vial.

The tissue was homogenized in 20 volumes of 50mM sodium phosphate, pH 7.2, 0.3% BSA, and 10 mM EDTA in a Radnoti glass tissue homogenizer. The homogenate was spun at 1000 x g for 15 minutes and the supernatant was used in the assay. Previous somatostatin RIAs have boiled the tissue in 2N acetic acid. This was unnecessary for this case because the degradative enzymes had already been denatured by microwave.

Each assay tube contained .005 μCi [^{125}I]-somatostatin, rabbit antiserum to somatostatin, and either standard quantities of somatostatin or tissue sample. Each tube was brought up to 600 μl with the same buffer that the tissue was homogenized in. These tubes were covered and allowed to incubate overnight at 4°C.

The charcoal suspension was made by adding 1.0g acid washed, activated charcoal and 0.1g dextran, MW = 60,000-70,000, to 50 ml assay buffer. The suspension was added to the tubes and incubated at room temperature for 15 minutes. The samples were then centrifuged at 1500 x g in a IEC Centra-7R refrigerated centrifuge for fifteen minutes and the supernatant was counted on a Beckman Gamma 5000.

Assay for High Affinity Choline Uptake

This assay was adapted from Richter (1982). Tissue was homogenized in 20 volumes of 0.32M sucrose in a Wheaton teflon homogenizer. The homogenate was centrifuged at 1000 x g for 10 minutes in an Eppendorf Centrifuge 5415. The pellet was discarded and the supernatant was centrifuged at 17000 x g for 15 minutes in a Sorval RC5C centrifuge using the SM24 rotor. The supernatant was discarded and the synaptosome-containing pellet (P2) was resuspended and rehomogenized in the original volume of 0.32M sucrose and assayed.

In a microtube, 0.400ml was added of either Krebs-Ringer phosphate media containing 126mM NaCl, 4.75mM KCl, 1.27mM $CaCl_2$, 15.8mM Na_2HPO_4, 1.42mM $MgCl_2$, and 2 mg/ml dextrose, or sodium free Krebs-Ringer phosphate media containing 4.75mM KCl, 1.27mM $CaCl_2$, 1.42mM $MgCl_2$, 252mM sucrose, 15.8mM tris phosphate, and 2 mg/ml dextrose. Both buffers were pH 7.4. The sodium-free tube served as the blank. To each tube was added 50 μl of choline solution containing 0.2 μCi [^{3}H]-choline and 0.5 μM choline.

The tubes were transferred to a American Sci Products BT-23 water bath at 30°C and 50 μl of the synaptosomal tissue homogenate was added. The samples were incubated for four minutes in the water bath. The reaction was stopped by transfer to an ice bath. The tubes were centrifuged at 6000 x g for 15 minutes in an Eppendorf Centrifuge 5415. The media

was aspirated off and discarded. The remaining pellet was washed with 1 ml 0.9% saline solution. The pellets were then digested overnight with 100μl Protosol. The Protosol mixture was transferred to a scintillation vial and the microtube washed twice with 1 ml Biosafe II scintillation cocktail. The volume in the vial was brought to a total of 5 mls. This was counted in a Beckman LS 5800 B counter (see figure 9).

Statistical Analysis

Comparison of the means for the somatostatin-like immunoreactivity and glutamic acid decarboxylase activity in the hippocampus following injection of folic acid into the septum was done using a student t-test. The values were reported as the mean $\pm$ standard error of the mean (S.E.M.).

Comparison of the pairs for choline acetyltransferase activity and glutamic acid decarboxylase activity in the frontal cortex following injection of folic acid into the substantia innominata was done using a paired student t-test. The values were reported as the mean $\pm$ S.E.M.

Comparison of the means for high affinity choline uptake activity after adminstration of cysteamine and pentobarbital was done using a student t-test. The values were reported as the mean $\pm$ S.E.M.

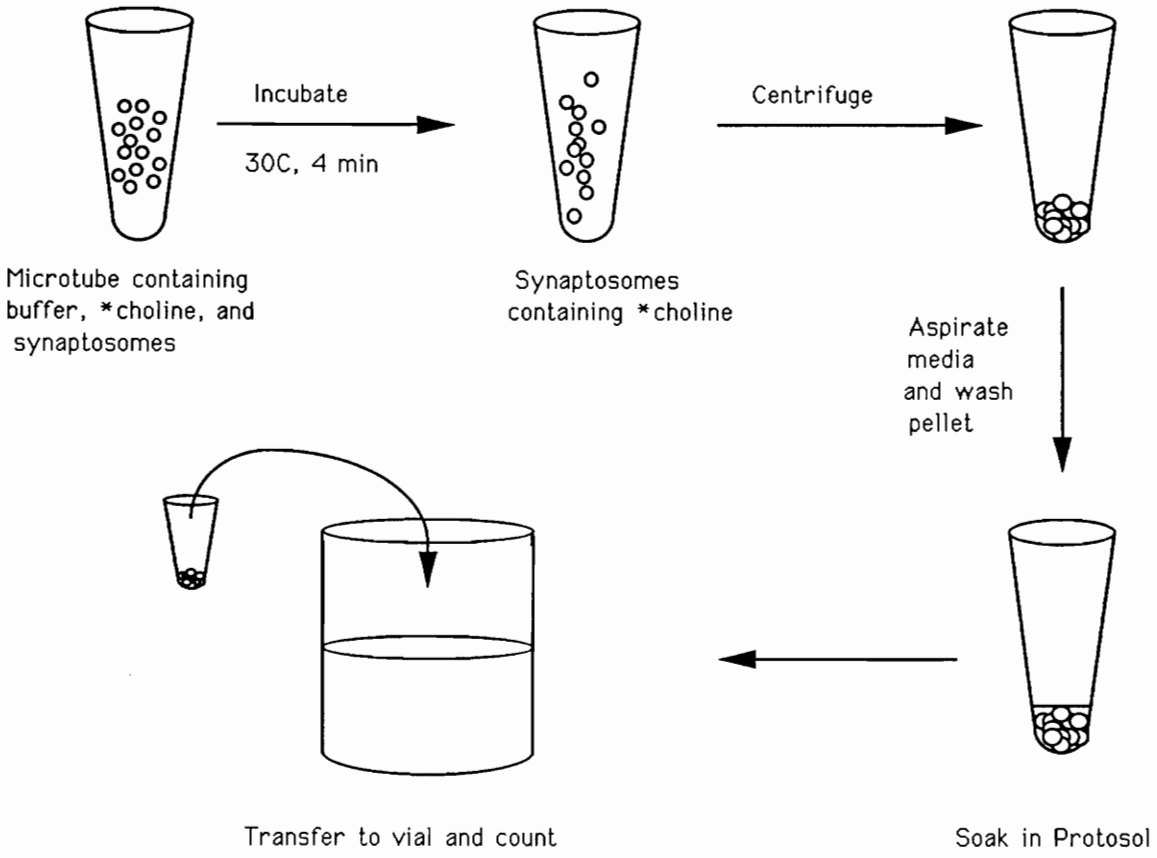

Figure 9. Assay for high affinity choline uptake. The synaptosomal preparation containing high affinity choline uptake (HACU) system is incubated with [3-H]-choline (*choline). The *choline is taken into the synaptosomes. The synaptosomes are pelleted and rinsed with 0.9% saline. They are then soaked in protosol to solubilize the vesicles so that after transfer into a scintillation vial , they can be counted.

Results

Effects of Altered Cholinergic Neuron Activity

Folic Acid Injection into the Septum

Folic acid injection into the septum caused seizures in the rat. The animals had tremors and walked continuously in a tight circle. This continued for six to eight hours and was controlled with low doses of pentobarbital for the first two hours to prevent terminal seizures. The animal was awake and still experiencing the seizure activity during the time of low dosage of pentobarbital.

The somatostatin level for each hippocampal region is in figure 10. None of the differences between the control groups and folic acid treated groups were significant. The largest decrease was seen in the dorsal hippocampus and was fifteen percent of control. The decreases in the other regions were less than five percent. Thus a hyperactivity of the septal cholinergic projections had no long-term effect on somatostatin level in the hippocampus.

The GAD activity for each hippocampal region is shown in figure 11. The p-values for the difference between the means of the control and folic acid treated groups for the dorsal medial hippocampus and the dorsal hippocampus were p

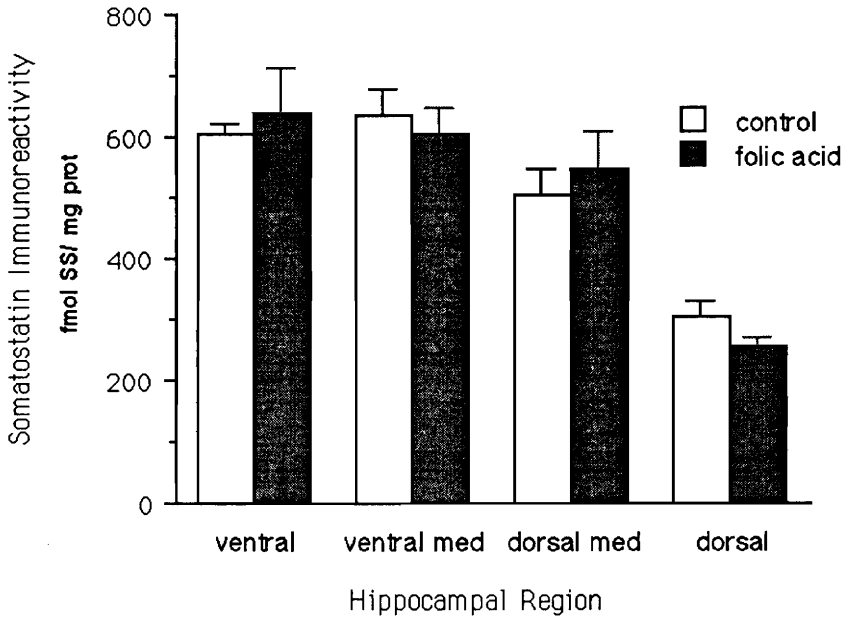

Figure 10. Somatostatin-like immunoreactivity in hippocampal regions after folic acid injection into the septum. The values are shown as the mean ± S.E.M. Each control had n = 7, and each treated group had n = 8.

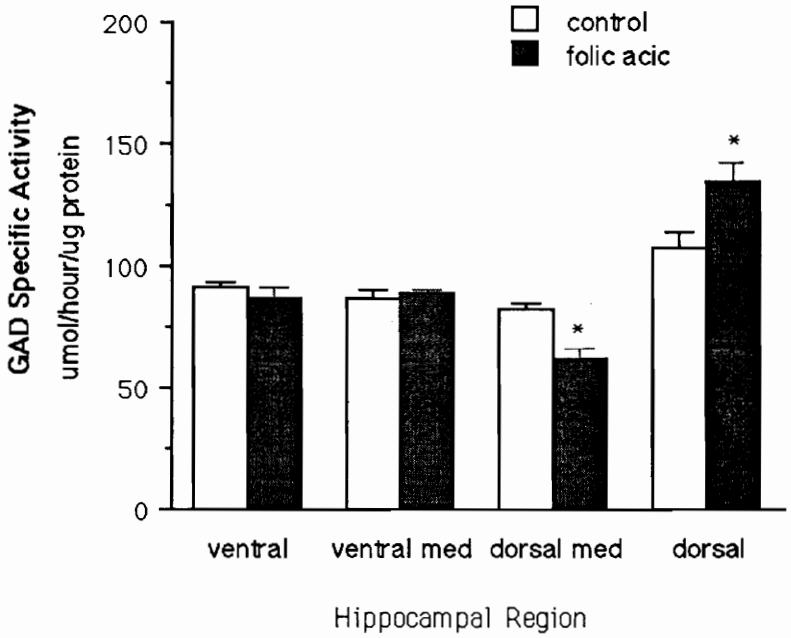

Figure 11. Glutamic acid decarboxylase activity in hippocampal regions after injection of folic acid into the septum. The values are shown as the mean ± S.E.M. (*$p < 0.05$)

= 0.01 and p = 0.022 respectively. The decrease in the dorsal medial hippocampus was twenty-five percent. This was not a pronounced decrease, as was seen previously (McGeer, 1983) The dorsal hippocampus had an increase of twenty-five percent.

Folic Acid Injection into the Substantia Innominata

Because the results following injection of folic acid into the septum did not follow McGeer's report, an attempt to confirm the report in the substantia innominata was done. Following the injection, the rats experienced seizures and tremors as described for the septal injection, but this time there was less walking in circles and the rats exhibited barrel-rolling behavior. The rats were kept on low doses of pentobarbital as for the septal injection.

The choline acetyltransferase activity on the folic acid treated side is presented in figure 12 as percent of control, for various brain regions. There was no significant change in choline acetyltransferase activity on the injected side. The choline acetyltransferase activity on the folic acid treated side was 97% of control in the frontal cortex, 119% of control in the hippocampus, and 118% of control in the parietal cortex. This suggests there was no degeneration of the cholinergic projections following injection of folic acid.

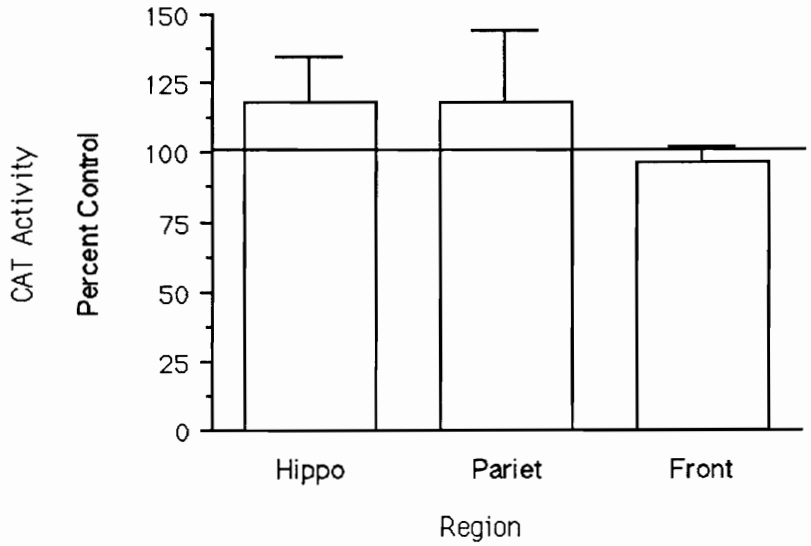

Figure 12. Choline acetyltransferase activity after folic acid injection into the substantia innominata. The mean ± S.E.M. percent of control for choline acetyltransferase activity is shown. In the frontal cortex, n = 7; in the hippocampus and parietal cortex, n = 6.

Glutamic acid decarboxylase activity on the folic acid treated side is presented in figure 13 as percent of control, for various brain regions. Frontal cortex 2 refers to the repeated experiment of injection of folic acid into the substantia innominata and measurement of glutamic acid decarboxylase activity in the frontal cortex. No significant differences in glutamic acid decarboxylase activity were observed between control and folic acid treated groups. The largest change in glutamic acid decarboxylase activity following injection of folic acid was only eight percent. This suggests that there was no degeneration of GABAergic neurons following injection of folic acid. This is in contrast to the results reported by McGeer (see discussion below).

Effect of Somatostatin Depletion on Cholinergic Activity

Cysteamine Administration

Cysteamine was administered to deplete somatostatin levels and measure the resultant effect on the cholinergic activity. After administration of cysteamine, the rats exhibited minor twitching in the facial area and increased defecation for the four hours before they were killed. The high affinity choline uptake activity in the hippocampus and

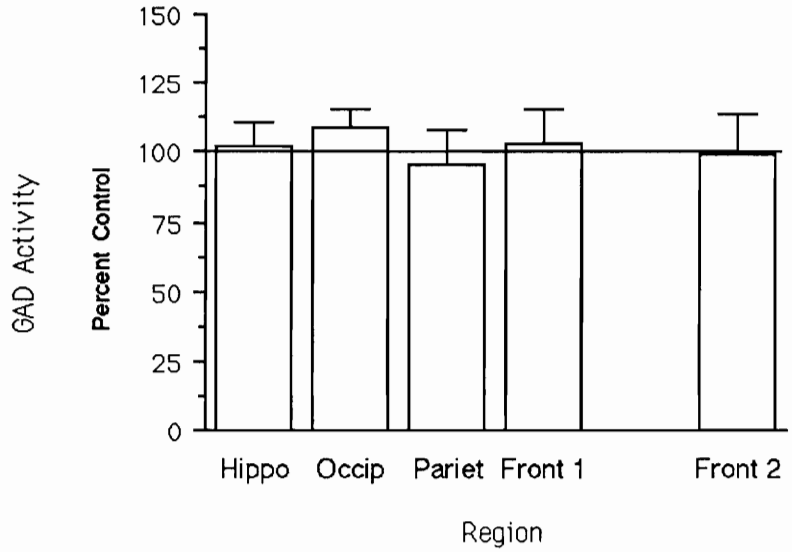

Figure 13. Glutamic acid decarboxylase activity as percent of control after folic acid injection into the substantia innominata. The mean ± S.E.M. percent of control of glutamic acid decarboxylase activity is shown. The experiment was done a second time, measuring only the frontal cortex. For the hippocampus and parietal cortex, n = 6; for the occipital cortex and frontal cortex 1, n = 7; and for frontal cortex 2, n = 8. Front 2 is from a second injection into the substantia innominata.

frontal cortex is given in figure 14. Hippocampal and frontal cortical high affinity choline uptake activity was increased only four percent and decreased eight percent, respectively, following administration of cysteamine. This indicates that decreased somatostatin levels had no effect on cholinergic neuron activity.

Pentobarbital Administration

High affinity choline uptake was measured in the hippocampus and frontal cortex after administration of pentobarbital. There was a significant decrease in high affinity choline uptake in the hippocampus following pentobarbital administration, where activity was decreased by fifty percent, and a trend toward a decrease in the frontal cortex, where the decrease was 32.5 percent (see figure 15), confirming that pentobarbital does decrease cholinergic activity and indicating that the assay is able to detect decreases in high affinity choline uptake activity.

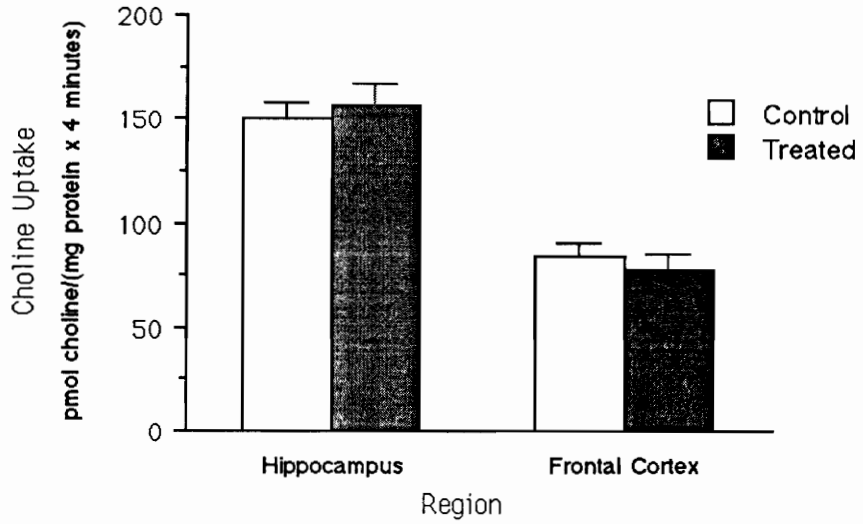

Figure 14. Effect of cysteamine on high affinity choline uptake. The activity of high affinity choline uptake is shown for the hippocampus and frontal cortex. In the hippocampus, n = 7 for the control group and n = 6 for the cysteamine group. In the frontal cortex, n = 10 for both groups.

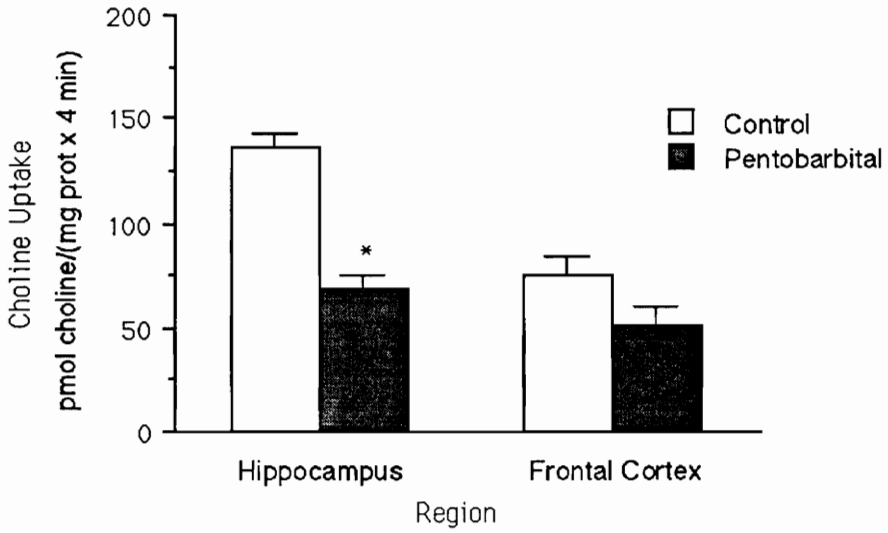

Figure 15. Effect of pentobarbital on high affinity choline uptake. The activity of high affinity choline uptake is shown for the hippocampus and frontal cortex. All groups had n = 6.

Discussion

In Alzheimer's Disease, there are two neuron types that degenerate: cholinergic and somatostatinergic. This project studied the functional relationship between the two neuron types. The first half of the project studied the long-term effect of an acute hyperactivity of cholinergic neurons on somatostatin neurons. The excitant folic acid was used to induce the increased cholinergic activity (McGeer, 1983; Wong, 1983). In their work, McGeer and colleagues injected 200 nmol folic acid into the substantia innominata and found a degeneration of cortical GABAergic neurons as indicated by a decrease in cortical GAD activity (McGeer, 1983). Somatostatin was measured in this project because it is colocalized with GABA in a subset of GABAergic neurons (Hendry, 1984b; Kosaka, 1988; Somogyi, 1984).

Although McGeer's work was done in the substantia innominata and cortex, our first injection was done in the septum because the septo-hippocampal cholinergic projection system is much better characterized, both histochemically and neurophysiologically. It was in the hippocampus that the cholinergic projection neurons were found to synapse onto the somatostatin neurons (Yamano, 1989; Leranth, 1987). Also,

while the septo-hippocampal projection is the only cholinergic input to the hippocampus, the substantia innominata afferents account for only seventy percent of the total cortical cholinergic activity, with the source of the remaining thirty percent unknown (McGeer, 1982). This first injection did not yield any significant differences in GAD activity nor somatostatin-like immunoreactivity between the control and treated groups in the hippocampus. This was thought to have been due to possible differences in the two cholinergic projection systems.

Because the injection of folic acid into the septum did not produce the results McGeer saw after injection into the substantia innominata (McGeer, 1983), a second experiment was done with the injection site in the substantia innominata. We measured cortical GAD and CAT as McGeer had done and compared our results to his. Again, there were no significant differences between the control and the treated groups for either enzyme activity. The experiment was repeated, measuring only GAD in the frontal cortex again with no decrease after injection of folic acid.

The differences between the methods used in this work and the methods used by McGeer are few. Their study used Wistar rats compared to Sprague-Dawley in this experiment. The Sprague-Dawley stock was started in 1925 by a female of Wistar

origin and a male of unknown origin (Baker, 1979). Although the stock was originally half Wistar, the time period that Sprague-Dawley was separated from Wistar would allow for some drift between the two outbred stocks. Genetic drift refers to random gene frequency changes from generation to generation. Because they are random, the differences between two finite populations would increase (Pirchner, 1983). For this reason, further studies could involve doing the experiment in the Wistar rat.

In any case, we did not observe the results which had been previously reported (McGeer, 1983). Nevertheless, the behavioral effects of our injections were similar to those reported by McGeer. Injection of folic acid into both the septum and substantia innominata resulted in seizures for six to eight hours, similar to the `seizure-like activity' reported by McGeer (McGeer, 1983). Post-injection behavioral activity reported by other investigators, such as hyperkinesis with running and jumping (Spector, 1972), `status' limbic seizures (Olney, 1981), and convulsive actions (Ruck, 1980) were also observed. These similarities suggest that our folic acid injections were analogous to those done previously. From our results, it can be concluded that hyperactivity of the cholinergic projections does not cause degeneration of the GABAergic neurons in the cortex and hippocampus, or the

somatostatin neurons in the hippocampus. This suggests that if there is a hyperactivity of the cholinergic neurons during the early stages of Alzheimer's Disease, this hyperactivity does not cause degeneration of the somatostatin neurons that are in the target regions.

One of the ambiguities of these experiments lies in the use of folic acid. Folic acid has a widespread effect as a cerebral excitant (Spector, 1972). Folate derivatives have been shown to interact with receptors for another neuroexcitant, kainic acid (Ruck, 1980). Folate derivatives and kainic acid both have convulsive properties (Ruck, 1980; Olney, 1981), but whereas kainic acid produces lesions both locally and distal to the site of injections, folic acid only produces the distant lesions (Olney, 1981). Kainic acid receptors are not well classified, and exist on a variety of neuron types. Because the effects of folic acid have not been fully elucidated and tend to be general, it is possible that folic acid not only caused the cholinergic neurons to be hyperactive, but other neurons types within the projection as well. If this were the case, a hyperactivity of a second type of neuron may have interfered with the stimulatory cholinergic activity, thus cancelling the overall effect on the post-synaptic neurons. Also, even if the folic acid injection had caused a distal degeneration of GABA and somatostatin neurons,

it would be impossible to ascertain from our experiments whether this was due to a hyperactivity of the cholinergic neurons or a hyperactivity of another neuron type within the projection system.

The second part of the study was the converse of the first and studied the effect of short-term depletion of somatostatin upon cholinergic neurons. Somatostatin stimulates the release of acetylcholine from hippocampal synaptosomes (Nemeth, 1979). In addition, when somatostatin is injected intracerebroventricularly, acetylcholine turnover rate increases in the hippocampus and cortex (Malthe-Sorenssen, 1978). This suggests that somatostatin can stimulate cholinergic neurons. In our study, there was no change in high affinity choline uptake in the frontal cortex and hippocampus after administration of cysteamine. This suggests that an acute depletion of somatostatin does not have an effect on cholinergic activity. A long-term depletion of the somatostatin neurons would be needed to investigate if the death of the cholinergic neurons in Alzheimer's Disease is caused by a loss of input by the somatostatin neurons. Although somatostatin-like immunoreactivity was not measured after administration of cysteamine due to technical problems, the rats did exhibit the reported signs of cysteamine administration, namely facial twitching and increased

defecation (Hartoutunian, 1987). In addition, brain somatostatin depletion by cysteamine has been consistently shown in several independent studies, with the dose used here producing a maximal effect at four hours (Sagar, 1982; Beal, 1984; Cook, 1989; Patel, 1985; Widmann, 1988; Cameron, 1986).

To test the efficacy of the assay for high affinity choline uptake, pentobarbital, known to decrease cholinergic activity was administered to rats and the assay done. There was significant decrease in high affinity choline uptake in both the hippocampus and cortex, demonstrating the ability of the assay to detect changes.

Although an acute depletion of somatostatin did not have an effect on cholinergic activity, it is still possible that the somatostatin neurons exert a functional influence on the cholinergic neurons. If the influence of the somatostatin neurons is phasic rather than tonic, a decrease in somatostatin activity may not have a large effect on the average activity of the cholinergic neurons. Tonic activity can be defined as neuronal activity that is continual. Depleting somatostatin would significantly decrease the tonic influence of the somatostatin neuron by stopping its constant signalling of the cholinergic neuron. Phasic neuronal activity can be described as occasional activity. Depletion of somatostatin in this case would have a limited effect on

cholinergic activity because the average activity of the receptive cholinergic neuron is not directly controlled by the somatostatinergic input. To test for phasic effects, one would need to selectively excite the somatostatin neurons or administer a somatostatin agonist, thus boosting its influence from an intermittent one to a continual one. Whether the effect were inhibitory or excitatory, an increase in somatostatin message to the receptor neuron would cause a change in the activity of that neuron.

Further investigations could go in many directions. One possibility in examining the effect that the cholinergic projections may have on somatostatin neurons is with the use of cholinergic agonists. By administering cholinergic agonists to the area of the somatostatin neuron, and measuring the change in somatostatin levels, one could ascertain whether acetylcholine affects somatostatin neurons. This approach would prove a more specific excitation of cholinergic neurons compared with the experiments reported above. To look at dynamic changes in somatostatin levels, one must look at somatostatin peptide levels, somatostatin propeptide levels, and somatostatin messenger RNA levels (Schwartz, 1986). This experimental design could not differentiate between a direct influence of acetylcholine on somatostatin neurons, or an indirect influence whereby acetylcholine effects a pre-

synaptic neuron which in turn affects the somatostatin neurons. The converse can also be done, where somatostatin agonists are administered to the neuron terminal regions of the cholinergic neurons and cholinergic activity measured. Neither of these would determine the etiology of Alzheimer's Disease, but would illuminate the functional relationship between the neurons involved.

Conclusions

In the first set of experiments, the lack of effect by folic acid on somatostatin shows that a hyperactivity of the cholinergic neurons does not produce degeneration of GABAergic neurons in the cortex and hippocampus, nor does it result in degeneration of somatostatinergic neurons in the hippocampus.

The second group of experiments shows that a depletion of somatostatin had no influence on the activity of the cholinergic neurons.

Summary

Hyperactivity of septal cholinergic neurons had no effect on glutamic acid decarboxylase activity nor somatostatin-like immunoreactivity in the hippocampus two weeks after injection of folic acid. Hyperactivity of the substantia innominata

cholinergic projections had no effect on glutamic acid decarboxylase activity nor choline acetyltransferase activity in the cortex two weeks after injection of folic acid. Cysteamine-induced depletion of somatostatin in the brain had no effect on high affinity choline uptake in the hippocampus at the time of presumed maximal somatostatin depletion.

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