To the Graduate Council:

I am submitting herewith a dissertation written by Brian J. Hurlbut entitled "Basal Forebrain Infusion of Hemicholinium in Rats: Maze Learning Deficits and Neuropathology." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Psychology.

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We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

BASAL FOREBRAIN INFUSION OF HEMICHOLINIUM IN RATS: MAZE LEARNING DEFICITS AND NEUROPATHOLOGY

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Brian J. Hurlbut

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DEDICATION

To Frances Pena Hurlbut, my wife and true love, I dedicate this study, for without her support it would not exist. A boy's dream has become reality. Patience, love, and wisdom have been her gifts all the years of our marriage. Truly, I am blessed.

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ABSTRACT

Ten adult male Sprague-Dawley rats were infused with hemicholinium (HC-3) using miniosmotic pumps over a 14 day period through bilateral, chronically implanted cannuli in the nucleus basalis magnocellularis (nbm). Ten matched controls were infused in the same fashion with saline.

HC-3 rats receiving implants demonstrated a signficant deficit in maze-learning ability compared with individual and group performances before receiving the implants. In saline rats there was no significant difference in maze-learning ability before and after receiving implants. The HC-3 group receiving implants demonstrated a significant deficit in maze-learning ability compared with the saline control group. Serial sections through nbm from control and HC-3 rats indicated that all cannuli were located wtihin infusion range of nbm. In HC-3 subjects, cholinergic cell bodies were destroyed with concurrent degeneration of terminal fields in cortex. Except for cannula insertion damage, the cholinergic neurotransmitter system appeared unharmed in controls. Stains for neuritic plaques and neurofibrillary tangles were negative in both groups. The memory deficit in experimental subjects supported by the demonstrated destruction of nbm cholinergic neurons suggests that HC-3 may be useful in the development of an animal model for Alzheimer's Disease.

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CHAPTER 1

A REVIEW OF ALZHEIMER'S DISEASE

Introduction

Alzheimer's Disease: History and Statistics

Aging is a universal experience. In man, the physical changes and deterioration of bodily function are apparent to all and have been the subject of investigation since the beginning of written history. It has long been observed that a percentage of those growing old become absent minded and confused. In fact, cognitive impairment of some kind and to some degree may be expected in the aged. "Senile dementia" is the medically accepted term to describe the cognitively defective state observed in aged humans, "aged" usually meaning past 65 years of age. However, senile dementia is a term of general description that, until fairly recently, encompassed a host of symptoms and an unknown number of causes.

The French psychiatrist Esquirol, in 1838, was the first to use the term "demence senile" (Torack, 1978). At first used as a clinically descriptive term, it was not until the 1890s that a study by Kraepelin associated senile dementia with specific neuroanatomical correlates (cited in Torack, 1978). Kraepelin's study indicated that "insanity" among the aged was one of the few psychiatric syndromes accompanied by gross brain changes. Half of the brains studied had cerebral infarcts which were attributed to arteriosclerosis. The other

half had cortical atrophy but no significant arteriosclerosis (cited in Torack, 1978). In 1899, Alois Alzheimer, a German neurologist, postulated a vascular disease involving the arterioles that caused dementia in patients without cerebral infarcts. Pathological changes associated with this nonarteriosclerotic senile dementia included "senile plaques" and "neurofibrillary tangles."

In 1907, Alzheimer reported a case of "pre-senile dementia" in a 55 year old woman (cited in Torack, 1978). All of the behavioral symptoms and pathology associated with noninfarct senile dementia were present in this first reported case of presenile dementia. Subsequently, noninfarct senile dementia was referred to as Alzheimer's Disease (AD), presenile dementia, or senile dementia of the Alzheimer's type. Presenile dementia (i.e., AD) was believed by many to arise from a separate etiology from senile dementia, although no reliable data existed to support this conclusion. Until the mid-1960s, the predominant opinion was that arteriosclerosis or an analogous affliction caused a narrowing of cerebral blood vessels and resulted in a gradual loss of blood supply to the brain (Schneck, Reisberg, & Ferris, 1982). This slow strangulation was thought to cause cerebral atrophy and as a result, senile dementia. A study by Corsellis and Evans in 1965 of demented and nondemented elderly patients demonstrated that arteriosclerotic changes were approximately the same in both groups (cited in Schneck et al., 1982). This was later confirmed by other investigators and it was also found that 50% of the demented patients in later studies demonstrated the pathology associated with Alzheimer's

Disease (Tomlinson, Blessed, & Roth, 1968; Tomlinson, Blessed & Roth, 1970). Another 18% of the dementia cases were due to both AD and cerebrovascular changes. A more recent study by Jellinger reported approximately the same findings (Jellinger, 1976). These studies and others have shown a clear distinction in the pathology associated with infarct dependent senile dementia and that of AD. Hachinsky, Lassen, and Marshall (1974) suggested a strict distinction in diagnosis between senile dementia resulting from infarct dependent cerebral softening, and dementia resulting from causes reflected in the histological changes as reported by Tomlinson et al. In the Diagnostic and Statistical Manual of Mental Disorders, 3rd edition (DSM-III), a distinction is made between two types of AD. The first is termed primary degenerative dementia--senile onset. The second is, primary degenerative dementia--presenile onset. These two categories are each further divided into the subtypes delirium, delusions, depression, and uncomplicated, depending on the presence or absence of these symptoms. Major differences in symptomology between senile dementia and presenile dementia have not been established, and many published authorities make no distinction between the DSM-III classifications except as an indicator of age of onset (Katzman, 1976; Schneck et al., 1982). There is also no pathological distinction between AD and senile dementia where the dementia is not caused by a known agent (Rathmann & Conner, 1984; Adams & Victor, 1981; Richardson & Adams, 1980).

It is stated in the DSM-III that 2 to 4% of the population under 65 years of age may have AD, and that the incidence over age 65 is higher. Katzman (1976) and Terry (1976) estimated that the prevalence of severe dementia in the population of Northern Europe at 4 to 5%, and the prevalence of mild to moderate dementia at 11 to 12%. Several studies have indicated that 50 to 60% of patients diagnosed as "demented" demonstrate a neural pathology indicative of AD (National Center for Health Statistics, 1975; Schneck et al., 1982; Tomlinson et al., 1968; Tomlinson et al., 1970; Heston & Mastri, 1982; Jellinger, 1976; Report of the Panel on Inflammatory, Demyelinating and Degenerative Diseases to the National Advisory Neurological and Communicative Disorders and State Council, 1979). Heston and Mastri (1982) have studied the frequency of AD and other causes of dementia by age group. In their survey it was found that the percentage of demented patients with AD ranged from 47% for those under 50 years of age, to almost 100% for patients over 80 years. Roth (1978) and Tomlinson et al. (1970) have reported a higher rate of AD in women. However, these data were based on hospitalized patients. Schneck et al. (1982) have criticized this conclusion since more women than men are hospitalized and this may have biased the data. Other studies have reported equal prevalence between men and women (Hagnell, 1970; Nielson, 1962). Schneck et al. (1982) noted that the reported incidence of AD varied greatly according to various studies, ranging from a low of .38% to a high of over 18% in studies by Akesson (1969) and Nielson (1962), for individuals over 65 years of age. However, a majority of

researchers in the available American literature indicate a range from 2% (DSM-III reported low) to about 8% incidence in the population over 65 years of age (Goldsmith, 1984; Rathmann & Conner, 1984; Johns, Greenwald, Mohs, & Davis, 1983; Heston & Mastri, 1982; Schneck et al., 1982; Whitehouse et al., 1982). This equates to 500 thousand to over 2 million American citizens suffering from AD (Bartus, Dean, Beer, & Lippa, 1982; Miller & Cohen, 1981). AD inevitably leads to death through complications such as pneumonia, and may account for 100 to over 200 thousand deaths annually in the United States. This makes it the fourth leading cause of death (Schneck et al., 1982).

It has been estimated that nursing home costs in the U.S. for AD patients may be 3 billion dollars annually, and that AD is responsible for 26 billion dollars in lost productivity each year (Goldsmith, 1984; Coyle, Price, & DeLong, 1983). In 1983, the United States Congress appropriated $25 million for research on AD. In 1984, appropriations totaled about $40 million, reflecting an increased concern by citizens and elected representatives (Goldsmith, 1984).

Clinical Features

There are over 50 causes of dementia. Approximately 20% of all dementias can be reversed with appropriate treatment (Rathmann & Conner, 1984). AD may account for over 50% of all dementias. At the present time, there is no cure for AD, and no treatment that is reliably effective in reducing the severity of symptoms or slowing the progress of the disease. In some limited experimental studies, some

drug treatments have temporarily alleviated some symptoms for relatively short periods under specific conditions (see Treatments, below).

AD is characterized by progressive, irreversible loss of all cognitive capabilities, including memory, attention, logic, emotional stability, and language (Steel & Feldman, 1979; Rathmann & Conner, 1984). The onset of AD is difficult to pinpoint due to the insidious nature of the disease. According to different authorities various stages of deterioration have been delineated. A "Global Deterioration Scale" for AD and age related cognitive diseases has been developed by Reisberg, Ferris, DeLeon, and Crook (1982). This scale lists seven phases, ranging from normal (1) to late dementia (7). Schneck et al. (1982) reported the widespread use of a three phase scale. In Phase I, the "Forgetfulness Phase," the individual may be aware of increased forgetfulness of where things were placed, or appointments, or names, or seldom used words. Some investigators believe the progress of AD demonstrates the tendency to retain past significant memories but to forget recent events (Adams & Victor, 1981). However, studies demonstrate a progressive loss of past memory in addition to loss of short term memory capability (Rathmann & Conner, 1984). Phase I may be characterized by increased general anxiety or depression. Decrements in cognitive abilities may be quantifiable using psychological tests; however, intellectual impairment is usually not severe enough to interfere with employment or daily function.

In Phase II, the "Confusional Phase," the individual displays a clear deficit in cognitive ability. Typically, the deficit is most

pronounced in short term memory capability (Schneck et al., 1982). Confusion and disorientation are common. Concentration may be affected. Speech is still possible but may be somewhat halting as the patient searches for appropriate words. Denial of the affliction may replace or accompany anxiety and/or depression.

Phase III, the "Dementia Phase," is associated with obvious dementia. The individual may confuse or not remember the identity of well known individuals. Concentration and memory are quite poor and get progressively worse. Cognitive abulia, or loss of the will to think and plan, eventually develops. Anxiety, depression, and/or denial may become acute. In late stages psychotic symptoms of delusion, paranoia, and hallucination may occur. Central nervous system control begins to fail. Facial weakness, gait disturbances, incontinence, hemiparesis, extrapyramidal disorders, and focal or general seizures may occur (Sulkava, 1982; Liston, 1979). An increase may develop in lip puckering, grasping and sucking reflex, snout reflex, palmomental reflex (flexation of fingers upon stroking of the palm), and corneomandibular reflex (Rathmann & Conner, 1984).

Life expectancy is significantly reduced depending on the age of onset of the disease (Kay, Norris, & Post, 1956; Beamish & Roth, 1964; Liston, 1979; Peck, Wolloch, & Rodstein, 1973). Some AD patients deteriorate rapidly within one or two years, while others may survive 10 to 15 years (Rathmann & Conner, 1984; Wurtman, 1985). Liston (1979) found an average survival of seven years. Terry and Davis (1983) found the average remaining life span of those with

severe dementia to be less than three years, based on a study of the entire population of elderly individuals on a group of islands near the Scandinavian coast. A possible explanation for the wide variation in survival time may be gleaned from the conclusion of Breitner and Folstein, reported by Wurtman (1985). They suggested that two types of AD may exist: a relatively mild, nonfamilial form generally affecting very old people, and a genetically transmitted variant that accounts for about three-quarters of all cases. A review of available literature indicates some, but still limited support for this conclusion.

Pathology

Neuropathology of the Alzheimer patient, especially in Phase III of the disease, is very distinctive. Most studies of neuropathology are based on the examination of brains from deceased individuals. However, computed tomography (CT), positron emission tomography (PET), single proton emission computed tomography (SPECT), samples collected during diagnostic neurosurgery, and other advanced procedures have allowed in vivo examination of AD. Generally, the neurological changes that accompany AD may be divided into three interrelated categories: gross anatomical changes, microscopic anatomical changes, and changes in neurochemistry.

Gross Anatomy

It has been demonstrated that the mean brain weight of normal adults begins to decrease after the age of 50 years (deKaban &

Sadowsky, 1978). Histologic studies show a continuing loss of cortical neurons, including hippocampal cells, and purkinje cells (Brody, 1955, 1970; Coyle et al., 1983; Corsellis & Evans, 1965). However, a study by Tomlinson et al. (1968) indicated no significant difference in brain weight between AD patients and nondemented controls. Numerous investigators have demonstrated significant cortical atrophy in AD brains, although not all AD brains show gross degenerative changes (Schneck et al., 1982; Richardson & Adams, 1980; Tomlinson et al., 1968). Atrophy may be prominent in parietal, temporal, and frontal cortex. Generally, primary motor and sensory areas of the precentral and postcentral gyri and occipital area tend to be preserved. In vivo CT scans have demonstrated enlargement of the 3rd and lateral ventricles, and this enlargement has been correlated with degree of cognitive dysfunction (Damasio, 1983; DeLeon, Ferris, & George, 1978; Merskey, Ball, & Blume, 1980). SPECT scans show a marked decrease in blood flow in occipital-temporal and parietal regions in AD patients (Goldsmith, 1984).

Microscopic Anatomy

As noted, in normal aging adults there is a general loss of cortical neurons. Although research results are mixed, a preponderance of evidence indicates that cortical cell loss in AD patients is greater than in age matched, nondemented controls (Shefer, 1973; Ball, 1977; Coyle et al., 1983). Frontal and temporal locations are most affected with losses also reported in hippocampus and subiculum. In AD brains,

Whitehouse et al. and other researchers have demonstrated over a 75% loss of acetylcholine rich cells in the Nucleus Basalis of Meynert (nbm), located in the substantia innominata (Whitehouse et al., 1982; Price et al., 1982). Scheibel and Scheibel (1975) have reported shrinkage of the dendritic tree in AD patients. Changes include loss of dendritic spines, and the irregular swelling of the soma and initial segment of apical and major dendritic bifurcations. Hirano, Malamud, and Elizan (1966) have reported the presence of a small eosinophilic rod-shaped structure (Hirano Body) within the neurons of AD brains. There is evidence to suggest that they may be actin aggregates, or that they are formed from ribosomes; however, Tomlinson (1979) regards them as nonspecific to AD since they are observed in other diseases. Mandybur, Nagpaul, and Pappas (1977) described a congophilic angiopathy (i.e., blood vessel disease or deterioration characterized by pronounced staining with congo red) occasionally noted in the brain of AD victims. The significance of the congophilic angiopathy, Hirano Body, and dendritic tree changes are unknown. However, the loss of cholinergic neurons in nbm and the related effect on cortical neurons has been studied in depth, with subsequent elucidation of an important neurochemical aspect of AD (see Neurochemical Changes, below). In addition to the above, the brains of AD individuals typically demonstrate three microscopic anatomical changes. These pathological changes are in the form of neurofibrillary tangles, neuritic (senile) plaques, and granulovacuolar bodies (GB).

Neurofibrillary tangles are found in the cerebral cortex and especially in the hippocampus (Schneck et al., 1982). Ball (1977) demonstrated that the posterior half of the hippocampus is significantly more affected with neurofibrillary tangles than the anterior half. The number of neurofibrillary tangles has been correlated with degree of dementia by a number of investigators (Farmer, Peck, & Terry, 1976; Blessed et al., 1968; Wilcock & Esiri, 1982). However, tangles are not unique to AD patients. In a study by Matsuyama and Nakamura (1978), 50% of brains from normal, nondemented individuals between 50 and 59 years of age had neurofibrillary tangles. Tangles were found in 80% of brains from patients 60 to 69 years old, and in 100% of the brains from control patients over 70 years old. Neurofibrillary tangles have not been found to occur in animals other than man in the natural state, although aluminum induced tangles of a different ultrastructure have been demonstrated in cat and rabbit brains (Schneck et al., 1982; Torack, 1978). It should be noted that the dementia observed in about 55% of all Parkinson's Disease victims, and virtually all Downs Syndrome individuals who live to age 35, very closely resembles AD in both clinical features and pathology, including the presence of neurofibrillary tangles (Goldsmith, 1984; Whitehouse et al., 1982).

The ultrastructure of the neurofibrillary tangle has been examined with the use of the electron microscope. Terry and Davies (1983) described the tangle as composed of twisted linear structures with a half period of 80 nm and a maximum width of 24 nm. Structurally the twisted elements appear as paired helical filaments, and are found in

the neuronal cell body, synaptic terminals, and in neuritic plaques. A number of studies indicate that neurofibrillary tangles are chemically related to normal neurofilaments and neurotubules and may be the result of a chemical alteration of these subunits (Schneck et al., 1982). Neurofibrillary tangles are associated with other diseases including Parkinson dementia, adult Downs Syndrome, and dementia pugulistica.

Neuritic or senile plaques are primarily found in cerebral cortex, more commonly in temporal rather than frontal lobes; however, they have been found in amygdala, basal ganglia, white matter, brain stem, and cerebellum (Schneck et al., 1982). Neuritic plaques consist of a central core of amyloid surrounded by degenerated cells and neurites (Gershan & Herman, 1982; Terry, Gonatas, & Weiss, 1964). The plaques may contain axonal and dendritic processes, within which are masses of paired helical filaments, degenerating mitochondria, and lamellar lysosomes (Schneck et al., 1982). Terry (1980) has hypothesized that neuritic plaques result from degenerated nerve endings, and, in fact, neuritic plaques are generally localized to areas containing axons and terminals, rather than the soma. Plaques undergo an orderly change in appearance as they mature. Beginning as aggregates of neurites, they develop into spherical masses 50 to 200 microns in diameter, and expand to encompass degenerating nerve terminals and glial cells. Amyloid accumulates in a central core, surrounded by degenerating axons, glia, and macrophages. Finally, all debris is cleared away and the amyloid deposit remains (Wurtman, 1985). It should be noted that Struble, Cork, Whitehouse, and Price (1982)

found immature plaques to contain acetylcholine rich axons which disappear as the plaques condense with age.

Several studies have correlated degree of intellectual deterioration with the number of neuritic plaques per area of brain (Farmer et al., 1976; Tomlinson et al., 1970; Blessed et al., 1968; Wilcock, Esiri, Bowen, & Smith, 1982). Tomlinson et al. (1968) reported neuritic plaques to be more common than neurofibrillary tangles in the non-demented elderly. Matsuyama and Nakamura (1978) reported the opposite. Neuritic plaques are also characteristic of Kuru and Creutzfeld-Jakob diseases (CJD) which also cause dementia. Clinically, Cruetzfeld-Jakob disease may be indistinguishable from AD. Stellate amyloid plaques are found in about 10% of brains with CJD, and about 70% of brains with Kuru (Beck, Daniel, & Gajudusek, 1969), although appearance and topography of CJD and Kuru plaques differ from AD plaques. The nature of the protein in the amyloid of the plaque can vary, apparently depending on precursor proteins available in a particular disease state (Wurtman, 1985). In addition to the amyloid containing neuritic plaque found in AD brains, amyloid may also be found as deposits in and adjacent to cerebral blood vessels. Abnormal proteins of the neurofibrillary tangle may also stain like amyloid. Glenner found that 92% of 350 AD brains contained cerebrovascular amyloid (cited in Wurtman, 1985). Wurtman has suggested a pathogenic process that may explain why brains may display both arteriosclerotic disease and AD pathology, thus leading to the confusion of diagnosis between dementia caused by AD or by multiple

small strokes.[1] In addition to accumulating abnormal proteins, it has been reported that AD brains synthesize subnormal quantities of proteins in general (Wurtman, 1985).

In 1911, Simchowicz described the degeneration of hippocampal pyramidal cells in severe cases of senile dementia (cited in Woodard, 1962). This specific type of neuronal degeneration has become known as "granulovacuolar degeneration." The presence of this type of degeneration is not, by itself, indicative of AD. Ball (1977) found granulovacuolar bodies (GB) in the brains of normal elderly individuals. AD brains, however, demonstrated 2 to 100 times the number of granulovacuolar bodies as found in aged matched controls. Woodard (1962) has suggested that GB found in conjunction with the presence in the brain of neurofibrillary tangles and senile plaques, especially when the GB are focused in hippocampus, establishes a strong diagnostic for AD. Ball (1977) found GB in the hypothalamus of AD brains, with the posterior portion more severely affected than the anterior half. Ball (1977) also noted GB to be concentrated in approximately the same areas as were neurofibrillary tangles, and suggested a common underlying cytotoxic mechanism in AD that forms both lesions.

The morphology of granulovacuolar degeneration has been described by a number of researchers (Ball, 1962, 1977; Woodard, 1962).

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[1] Amyloid collects in the middle, muscular layer of brain blood vessels and advances outward. Sometimes it entirely replaces the vascular wall, possibly weakening it and resulting in hemorrhage. Thus, brains from advanced AD diagnosed patients may show pathology typical of AD and also multiple small infarcts from the deposition of both types of amyloid.

Typically, a GB occurs in clusters of intracytoplasmic vacuoles with diameters up to 5 microns. Each vacuole contains a central granule between five tenths and one and one-half microns in diameter. Woodard (1962) stated that in advanced cases, there is substantial neuron loss and gliosis. Involved neurons may show other signs of degeneration, including nuclear eccentricity, coagulation, and nuclear lysis.

Neurochemical Changes

Biochemical analysis has been conducted on the brains of deceased AD patients to determine abnormalities in quantities or other features of neurotransmitters. Norepinephrine, dopamine, gamma-aminobutyric acid (GABA), serotonin, and acetylcholine are among those which have been examined. There is evidence to support loss of noradrenergic, serotonergic, GABAergic, and cholinergic neurons in association with AD (Reisberg et al., 1983; Reisine, Yamamura, Bird, Spokes, & Enna, 1978; Bowen et al., 1983; Sims et al., 1983). However, it has not been established whether the deficits in individual neurotransmitter systems are associated with the immediate cause of AD or are the result of a central debilitating factor that, in the course of the disease, eventually, perhaps indirectly, affects various individual systems.

Studies of noradrenergic activity in AD patients have relied on the measurement of markers including tyrosine hydroxylase, dopamine-beta-hydroxylase (DHB), 3-methoxy-4-hydroxyphenylglycol (MHPG), monoamine oxidase B, and beta-adrenergic receptor binding (Bowen, Smith, White, & Davison, 1976; Carlsson, Adolfsson, & Aquilonius,

1980; Bowen et al., 1979). Adolfsson, Gottfries, Orlandez, Wilberg, and Winblad (1979) reported a general loss of norepinephrine in several regions of cerebral cortex and specifically putamen and gyrus frontalis. Adolfsson et al. (1979) also found an increase in both platelet and brain monoamine oxidase (MAO), with the effect of decreasing available norepinephrine by increasing the rate of breakdown. Loss of producing cells would naturally result in decreased available brain norepinephrine. However, no correlation was found between a neocortical marker of noradrenergic terminals and changes or loss of cell bodies in the brain stem. Also, cerebrospinal fluid enzyme activity of MAO, MHPG, and cortical measurements of DBH do not correlate with mental test scores or degree of dementia (Mann, 1981).

In a study by Bondareff, Mountjoy, and Roth (1982), a group of AD patients exhibited an 80% loss of noradrenergic neurons in the locus ceruleus. Another AD group demonstrated a 20% loss. An earlier study by Perry et al. (1981) reported losses in two separate AD groups of 67% and 17% of neurons in locus ceruleus compared to normal controls. However, measurement of cortical DBH was not found to correlate with locus coeruleus cell loss, and no significant relationship was found between cell loss and reduced levels of acetyltransferase. Also, at least one study has shown no relationship between cell loss and mental status scores (Tomlinson, Irving, & Blessed, 1981).

Unequivocal data regarding deficits in the serotonergic system are also not available. It is difficult to study serotonin (5-HT) containing neurons in humans because of the ephemeral nature of enzymatic

markers, specifically tryptophan hydroxylase, in postmortem tissue (McGeer & McGeer, 1981). Nonenzymatic markers, including [^{3}H](5-HT) uptake, [^{3}H] imipramine binding, and 5-hydroxyindo-lactate, have been utilized to study the loss of terminals (Bowen et al., 1983). Postsynaptic 5-HT receptors have also been studied using [^{3}H]5-HT and [^{3}H] lysergic acid diethylamide (LSD) binding. Bowen et al. (1983) found a significant reduction in the uptake of [^{3}H]5-HT, and binding of [^{3}H]5-HT. In addition, [^{3}H] imipramine and indolamines were reduced in AD brains. However, when examined by age group, a reduction in [^{3}H]5-HT binding was significant only for younger patients (a linear relationship by age). A problem with the use of [^{3}H] imipramine is that it probably binds to several postsynaptic receptors (U'Prichard, Greenberg, & Sheehan, 1978). In studies using drugs that diminish serotonergic activity (e.g., periactin, methysergide), no symptoms characteristic of AD were reported (Greenwald & Davis, 1983).

Parkinson's disease (PD) involves the selective loss of dopamine producing cells of the nigrostriatal neurotransmitter system. Nearly 55% of Parkinson patients develop cognitive deficits very much like those found in AD (Whitehouse, Hedreen, White, & Price, 1983). An examination of PD brains where the patient developed dementia demonstrates the senile plaques and neurofibrillary tangles characteristic of AD. Various studies have also shown a loss of neurons in nucleus basalis of Meynert, and the diagonal band of those PD patients suffering from AD. In patients with AD, studies have included measurement

of dopamine, homovanillic acid (the major metabolite of dopamine), and receptor binding of dopamine. Generally, concentration of dopamine has been found to be reduced in thalamus, pons, caudate, and hypothalamus, but remained normal in hippocampus and frontal cortex (Adolfsson et al., 1983; Carlsson et al., 1980; Mann et al., 1981). Receptor binding sites were found to be reduced in caudate, but not reduced in frontal cortex, putamen, or hippocampus (Reisine et al., 1978). Greenwald and Davis (1983) have criticized conclusions by Reisine and others because of a lack of rigorous attention to appropriate diagnosis in those individual cases included in the studies. Use of L-dopa on AD patients has generated conflicting results. Drachman and Stahl (1975) found improvement, Kristensen, Olsen, and Thilgaard (1977) found no effect, and Van Woert, Yahr, Henenger, Ratney, and Bowers (1970) found a worsening of symptoms. Use of bromocriptine, a dopamine postsynaptic agonist, failed to improve symptoms (Phvapradit, Philips, Lees, & Sterm, 1978).

Greenwald and Davis (1983) report that most studies of free GABA in hippocampus of AD patients show no difference in concentration than that measured in control brains. However, decreases in glutamic acid decarboxylase, the enzyme required for synthesis of GABA from glutamic acid, is reduced in midbrain, hippocampus, and cortex, depending on the advancement of the disease (Greenwald & Davis, 1983).

The most striking and significant apparent neurochemical change in the AD brain is that of acetylcholine levels. Essentially, within the

cholinergic neuron, choline acetyltransferase (CAT) catalyzes the acetylation of choline with acetyl coenzyme A, producing acetylcholine (ACh). Acetylcholinesterase (AChE) hydrolyzes ACh back to an inactive substrate. AChE has been used as a postmortem marker for ACh. However, in the brain, the presence of AChE in noncholinergic neurons reduces the effectiveness of using AChE as a marker for the specific study of the cholinergic neurotransmitter system. The presence of CAT has proven a much more reliable indicator of acetylcholine, especially in the presence of AChE.

Early biochemical assays of postmortem AD brains showed a marked reduction in CAT. Pope, Hess, and Lewin (1964) first reported below normal levels of CAT in AD brains. Spillan et al. (1977), and Davies and Maloney (1976) confirmed this finding and the latter also reported reduced levels of AChE. Later studies all demonstrated a reduction of cortical CAT by 50% or more in AD brains (White et al., 1977; Reisine et al., 1978; Davies, 1979; Rossor et al., 1980). Bowen et al. (1982) reported a reduction of CAT in AD patients within one year of onset of symptoms based on diagnostic craniotomies. Research by numerous investigators has resulted in conflicting data regarding CAT activity in the cortex, caudate nucleus, putamen, and hippocampus of elderly normal humans (Bartus et al., 1982).

Reduction in CAT levels has been found to be related to the degree of histopathological change (Bowen et al., 1976; Perry et al., 1978). It has also been correlated to the mental test scores of AD patients (Perry, 1978), and the severity of dementia (Wilcock, Esiri,

Bowen, & Smith, 1982; Blessed et al., 1968). Numerous studies have indicated no large or reliable decreases in CAT activity as a function of aging in normal individuals (Bowen et al., 1976; Carlsson et al., 1980; Davies & Verth, 1978; McGeer & McGeer, 1975; McGeer & McGeer, 1976; Spillane et al., 1977; White et al., 1977). In AD, CAT activity is consistently depressed in hippocampus and cortex, the same area associated with neurofibrillary tangles, neuritic plaques, and GB (Bartus et al., 1982; Johns, Greenwald, Mohs, & Davis, 1983). CAT activity has also been quantitatively correlated with plaque number (Perry et al., 1978). Various studies have shown AD brains are not significantly different from normal, age matched control brains in their ability to bind muscarinic antagonists in the cortex, indicating that postsynaptic muscarinic cholinergic receptors are not affected in AD (Antuona, Sorbi, Bracco, Fusco, & Amaducci, 1980; Davies & Verth, 1978; Perry et al., 1978; Perry et al., 1981; Reisine et al., 1978; White et al., 1977). However, Mash, Flynn, and Potter (1985) have demonstrated that two subtypes of muscaranic receptors may exist. Type M1 receptors are located in postsynaptic cells and facilitate cellular excitation. Type M2 receptors function in cholinergic nerve terminals and apparently regulate the release of ACh. Cerebral cortex samples from AD patients exhibited a decrease in the number of M2 receptors but no change in M1 receptors. This is significant because it suggests that AD may be confined to presynaptic structures, and that ACh levels may change due to a loss of M2 receptors.

An in vivo study by Davis and Mohs (1982) of cerebral spinal fluid from 14 AD patients indicated a correlation between a deficit in ACh and cognitive impairment. There was no correlation between mental test scores and other neurotransmitter metabolities measured.

Diagnosis

A preliminary or first step in the diagnosis of AD is the exclusion of other causes of dementia. Drug intoxication, heavy metal or other toxic poisoning, fluid and electrolyte imbalance, frontal or temporal lobe tumors, subdural hematomas, endocrine-metabolic disorders, hydrocephalus, vitamin B-12 deficiency, and depression are some of the more likely causes of dementia, although AD is the most common (Rathmann, 1984). A thorough history of the patient and, very importantly, family members is essential. A much increased incidence of AD has been found in families where earlier generations have had AD (see Theories of Cause below). An individual history is necessary to describe the age at onset of symptoms plus the order, pace, and degree of cognitive change. Approximately 15% of dementias are due to multiple infarcts. Onset of brain infarcts and symptoms is usually abrupt and most often occurs between 40 and 60 years of age, with many remissions and spells of increased debilitation (Gershon & Herman, 1982). The onset and progression are clearly different from the clinical features of AD, although certain features, age of onset and dementia, are suggestive of AD.

Other forms of dementia may be even more difficult to distinguish from AD, and require an extremely thorough historical workup for early elucidation. Late life depression, for example, may appear as a pseudodementia, involving selective memory loss, changes in sleep patterns, general apathy and mood change. Distinctive differences between late life depression or other dementias and AD are probable attempts to conceal cognitive deficits by an AD patient, and a lack of response or worsening of condition as a result of antidepressant therapy and drug treatment for the AD patient. Within the last five years, advances have been made in clinical testing for AD. Reisberg et al. (1982) have developed an aid for physicians in the diagnosis of AD. The "Global Deterioration Scale" correlates specific clinical characteristics with a phase or stage of cognitive decline. Together with available mental test batteries, the likelihood of correct and early diagnosis is enhanced.

Fortunately, physical and neurological examinations, together with radiologic studies and laboratory analysis, have proved valuable in eliminating other possible causes of dementia (Steel & Feldman, 1979; Adams & Victor, 1981; Gershon & Herman, 1982; Rathmann & Conner, 1984). Unfortunately, many of the physical abnormalities associated with AD are not apparent until more advanced stages of the disease. Physical symptomology may include enlargement of the third and lateral ventricles, and decreases in cerebral blood circulation. Damasio (1983) correctly predicted AD as the basis of dementia in 84% of patients in one group, and 77% in a second group of elderly patients with

unspecified dementia, using CAT scans to determine the ratio between ventricular and brain area measurements. These changes may also be seen using pneumoencephalography (Adams & Victor, 1981). Other indicators are available. Approximately one-quarter of all AD patients may have abnormal cisternograms indicating a decreased flow in cerebral spinal fluid (Steel & Feldman, 1979). Cerebral spinal fluid examination may also indicate elevated protein levels (Adams & Victor, 1981).

An additional diagnostic tool is the electroencephalogram (EEG). As early as 1931, Berger showed an abnormal EEG pattern was correlated with AD (cited in Johannesson, Hagberg, Gustafson, & Inguar, 1979). In this early study, a relationship was demonstrated between AD and slowed EEG waves. Many studies have since found a correlation between the degree of dementia and the quantity of the low frequency EEG activity (Johannesson et al., 1979). A number of studies have also demonstrated a diffuse increase in theta and delta waves associated with AD (Adams & Victor, 1981; Johannesson et al., 1979). There is evidence that the EEG of AD dementia patients is distinguishable from EEG's of patients with dementia from other causes, including cerebrovascular dementia (Johannesson et al., 1979). Diagnosis utilizing a detailed history, EEG, laboratory, and physical examinations can greatly enhance correct diagnosis.

A second category of direct measurement of cortical and subcortical electrical activity may lend increased specificity to the diagnosis of dementia and AD. The event-related evoked potential (ERP) can be

recorded from the human scalp by using signal averaging techniques and standard EEG recording equipment. In response to sensory stimulation, a sequence of waves reflecting gross electrical potential amplitude shifts reliably develop in brain activity within a narrowly defined period of time. These waves are subdivided into endogenous or exogenous components (Donchin, 1975). Exogenous components appear within a few milliseconds of stimulus onset and extend to 200 milliseconds, depending on stimulus modality, and indicate the movement and processing of stimulus data as it ascends sensory pathways (Squires, Chippendale, Wrege, Goodin, & Starr, 1980). Endogenous components are less dependent on stimulus modality and relate more closely to the physical and cognitive state of the subject, and the context of stimulus presentation. Endogenous components typically occur after 150 milliseconds (Goodin, Squires, & Starr, 1978). Evoked potentials and ERP's are now used as objective tests for definition and location of brain stem lesion appearing in infants and retarded individuals, and as a diagnostic tool for neurologic abnormalities like multiple sclerosis (Epstein & Andriola, 1983; Starr, 1978). The most prominent of endogenous ERP is the P3 or P300 evoked potential. The P3, characteristically occurring as the third positive amplitude potential shift after stimulus onset, usually has a latency of 250 to 450 milliseconds (Goodin et al., 1978). Numerous studies have linked the P300 with perception and cognition in normal subjects (Tueting, 1978; Donchin, 1978, for review).

Many studies have correlated a change in P300 amplitude and latency with dementia (Goodin et al., 1978; Squires et al., 1980). Squires et al. (1980) reported that 80% of demented individuals were correctly diagnosed using this methodology. It was also noted that functional disorders like depression were distinctly different in terms of ERP from organically based dementias (e.g., AD), or disorders of sensory or motor systems. Advancements in techniques for P300 testing may eventually allow highly specific diagnosis of the cause of dementia. A review of the literature indicates a lack of standardization in P300 test methodologies and P300 tests for AD candidates. The high cost of current diagnostic tools may foster development of noninvasive, relatively low cost ERP or P300 test capability.

Theories of Cause

The cause of AD is unknown; however, various hypotheses have been suggested to explain the observed characteristics of the disease and its possible origin. Generally, these theories may be grouped into three categories: infectious agent, toxic substances, and genetic. Evidence exists to support, and detract from, proposals in each of the categories.

Infectious Agent

There is evidence suggesting that AD may result from an infectious viral or other agent. For example, AD shares several clinical and pathological features with Creutzfeldt-Jakob Disease (CJD). CJD

is a transmissible subacute spongiform encephalopathy, sometimes clinically indistinguishable from AD. Intracellular vacuolic and stellate amyloid plaques are also characteristic of CJD, although the plaques display a different appearance and topography from AD plaques. In addition, families have been reported with some members suffering from CJD and other members from AD.

Attempts to induce AD in nonhuman primates by some investigators have proved unsuccessful (Goudsmith et al., 1980). However, Gibbs and Gajdusek (1978) reported transference of a "chronic, progressive neurologic illness" to chimpanzees after their brains were infected with tissue from the brains of deceased AD patients. A subviral structure termed a "prion" is currently under investigation as a probable agent in the transmission of CJD, Kuru, and Scrappie. Kuru is a progressive and fatal disease of the nervous system seen in inhabitants of the eastern highlands of New Guinea. Scrapie is a disease that causes nervous system deterioration in sheep. Both diseases are termed "slow virus" infections because an incubation period of at least nine months is required after initial innoculation with infected material. A prion is a protein particle containing neither DNA nor RNA. It has been suggested that this protein unit may also be an infectious agent in AD (Wurtman, 1985).

Toxic Substance

Klatzo, Wisniewski, and Streicher (1965) injected an aluminum solution in a rabbit brain and noted the formation of neurofibrillary

tangles. Terry, Gonatas, and Weiss (1964) repeated this study and found that the morphology of the tangles differed from that of AD patients. In 1973, Crapper, Kirshan, and Dalton discovered aluminum concentrations in AD brains to be 10 to 30 times normal levels. Crapper et al. (1973) also found that aluminum injected into cat brain caused memory defects and abnormal behavior after neural degeneration and neurofibrillary formation. Aluminum toxicity has been blamed for "dialysis dementia," due to corresponding high serum and brain concentrations of aluminum, although symptoms and rate of progression are different than those found in AD patients. Aluminum is concentrated in nuclear chromatin in AD patients, and in rabbits and cats that have been brain injected with aluminum. Wisniewski, Sturman, and Shek (1980) have demonstrated that 550 mg of Al/g of tissue is necessary to produce only a 20% decrease in CAT, less than half of the decrease noted in AD patients. AD brains and animals with aluminum induced tangles have only 5 mg of Al/g. Also, reduction in CAT activity does not occur in patients with dialysis dementia (Perry, 1978). Shore and Wyatt (1983) state that no antialuminum treatments or reduced aluminum diets have proven effective in affecting the progress of AD. Toxic accumulation of aluminum is still under investigation by many researchers. There does, indeed, seem to be some link between AD and abnormal concentrations of aluminum and other metals in the brain. However, it is possible that brain accumulations are a result of the degenerative process, rather than the root cause of the degeneration.

Genetic

Models or ideas categorized as "genetic" hypothesize an aberrant gene or genes as the root cause of AD. A genetic component underlying AD has been indicated in a number of studies (Schneck et al., 1982; Heston, Mastri, Anderson, & White, 1981; Breitner & Folstein, 1983). Case studies that examine the incidence of AD in close relatives, siblings, and children of AD patients consistently report an increased risk. For example, in studies by Breitner and Folstein (1984) and Heston et al. (1981), the incidence under certain conditions suggests an autosomal dominant condition for transference of the disease. Heston et al. (1981) found that the time of disease onset in an affected parent was a critical determinant in the risk factor for children. Onset after age 70 indicated a risk factor for children little different from that of the general population. However, when dementia occurred before age 70, and the AD victim's parents also suffered from senile dementia, the incidence among children approached 50%. When onset was before age 70, but the husband's parents were unaffected, the risk was about 18%. In addition to autosomal dominance as indicated by Heston et al. (1981), and Breitner and Folstein (1983), Larsson, Sjogren, and Jacobson (1963) have suggested a polygenetic model of inheritance. Wurtman (1985) has suggested the possibility of a familial genetic component that allows the expression of a nonfamilial, at-large gene for AD. Some researchers have proposed a nonfamilial gene for a mild form of AD and a genetically transmitted gene for severe forms. The concept of a genetically based cause is supported by obervations

in other diseases. Virtually all Down's Syndrome patients surviving to adulthood, for example, develop the pathology associated with AD (Heston, 1981). This suggests a genetic factor carried by the extra chromosome of the Down's victim. Also, in Huntington's disease, the progressive dementia and motor abnormalities characteristically become apparent only after the age of 40. This is comparable to AD in that onset is delayed over a period that is decades in length.

The possibility exists that a time locked genetic code for AD is activated by biochemical changes resulting from normal aging, or exogenous factors associated with advanced maturity. This hypothesis may account for both the increased incidence in some families and the similarity to pathology found in other dementing diseases. A time code defect in the immune system, it may be conjectured, may pave the way for invasion and proliferation of an infectious agent. Various encephalopathic diseases are known to lie dormant for years or require exceedingly long periods of incubation. Thus, actual infection could occur at any time, including before birth, or result from a secondary infection made possible by an immune system malfunctioning against a primary infection. Another possibility is suggested by the work of Nandy (1972). Aging mice were found to have brain reactive antibodies. These antibodies, which increase with age, may account for the progressive loss of a specific type of neuron. Tkach and Hokama (1970) reported increased brain antibodies in AD patients. A high level of specific antibodies may trigger the genetic code for onset or susceptibility to AD.

Treatment

Research into the pathology of AD has resulted in data that indicate a dramatic change in the cholinergic transmitter system of AD patients. Decreased levels of ACh, CAT, and AChE, correlated with the physical anomalies of neurofibrillary tangles, granulovacuolar bodies, and neuritic plaques, together with corresponding decreases in mental test scores as these degenerative changes increase, have led researchers to focus attention on treatment strategies that would alter available supplies of ACh in cortex. Adjunct treatments seek to alleviate cognitive symptoms, such as depression and anxiety, that develop as a consequence of AD. Unfortunately, no treatment has been found to be consistently effective in reducing symptomology or in slowing the course of the disease. In some cases, temporary, significant improvement has been demonstrated in individual cases, although improvement has always been transitory and specific to certain symptoms.

Physostigmine

Choline acetyltransferase, the enzyme that catalyzes the conversion of choline to acetylcholine, is very markedly reduced in AD patients. This loss suggested to some researchers that a reduction in available ACh may exist in cortex afflicted with AD. Alleviation of this condition, it has been reasoned, may improve cognitive function. Studies with scopolamine, an ACh muscarinic receptor blocker, can induce memory deficit (Carlton & Vogel, 1965; Hamilton & Grossman, 1969). Reversal of this effect, and enhancement of memory through

the application of physostigmine, a cholinesterase inhibitor, has been demonstrated (Bartus et al., 1978; Davis et al., 1978). Physostigmine has been used to treat AD according to various methodologies of application. Sullivan, Shedlack, Corkin, and Growdon (1982) found that infusion of physostigmine in a double blind experiment did not alter the mental tests of the experimental (AD) group; however, various individuals within the group did show improvement on specific tests in the battery of tests utilized. Other studies using physostigmine administered via infusion or oral dose demonstrated the same effect, namely some improvement in specific abilities by some individuals although, overall, a statistically nonsignificant and temporary effect. Physostigmine in combination with other drugs has also not proven to be reliably effective.

Choline

Attempts to change the chemical balance of ACh precurors in cortex have included the use of choline sources to increase extracellular choline. Lecithin in various forms, via different methods of administration, and in combination with other chemicals, has been tried. Although transient improvements were noted in many cases for specific individuals, overall results were generally negative (Mohs et al., 1981). It has been suggested that the loss of muscarinic receptors coupled with the decline in CAT activity may explain the ineffectiveness of choline therapy (Enna & Strong, 1981). Logically, if the structural integrity of the neuron has been compromised, an increase in precursors could not be utilized if uptake, synthesis or other capability is nonfunctional or reduced.

Vasopressin

Arginine vasopressin (AVP), a peptide synthesized by nuclei in the hypothalamus, has been implicated in animal studies to have an effect on quality of REM sleep, pain sensitivity, synchronization of biorhythms, fluid and electrolyte balance, and, importantly in this instance, memory. AVP has been experimentally demonstrated to affect avoidance task learning in rats (Telegdy & Kovacs, 1979). Desamino-9-D-arginine-vasopressin (Desmopressin), a synthetic analog of AVP, was tested on patients with primary degenerative dementia (PDD). Used in conjunction with tetrahydroaminoacridine (THA), it was found to improve serial learning temporarily in a subgroup of PDD patients, and in a second study to facilitate "access to semantic memory" (Chase, Durson, & Fedio, 1982). It should be noted that the clinical syndrome of PDD is a general descriptive term and includes the subgroup of AD patients. In this study, no distinction was made between AD patients and patients with other types of dementia.

In another study by Chase et al. (1982), lysine-8-vasopressin administered via nose spray was found to have no effect on AD patients. Tinklenberg, Pigache, Pfefferbaum, and Berger (1982) tested three PDD patients (including two with alcohol induced amnesia) after intranasal administration of desglycenamide-9-arginine-8 vasopressin (Organon 5667). No significant memory related improvement was noted. In a second study, two AD and four alcoholic patients were again given vasopressin. Aside from a suggested effect on mood and attention, no significant improvements were noted.

Acetylcholinesterase Inhibitors

Diisopropylphosphyluoridate (DFP) and THA, both potent AChE inhibitors, have been tested. THA has a longer half life than DFP and seems to have fewer side effects. Greenwald and Davis (1983) reported that THA tested on AD patients in three separate studies was shown to increase the capability to learn new information. In a pilot study by Summers et al. (1981) THA produced a "general global improvement" in patients with AD.

Vasodilators

Numerous chemicals have been tested for the treatment of cognitive deficits in the elderly. Most studies were not specific to AD, and included a multitude of probable causes for the observed dementia. There is evidence that a number of vasodilators may be somewhat effective according to data collected in some studies; however, positive results were most often indicated in studies without effective controls. In any case, very little data are available to suggest that cognitive improvement follows administration in AD patients. Funkenstein, Hicks, Dysken, and Davis (1981) provide a review of vasodilators and possible effects on dementia in the aged. Chemicals reviewed include:

Papervine (Pavabid)

Isoxsuprine (Vasodilan)

Cyclandelati (Cyclospasmol)

Sehydroergotoxin (Hydregine)

Nicotinic Acid

Carbon Dioxide

Tocopherol (Vitamin E)

Metabolic Enhancers

Piracetam, alone and in combination with other drugs such as choline, has been the most widely studied metabolic enhancer relative to aging and AD specifically. Piracetam is currently in widespread use in Europe for the treatment of AD in the elderly. The rationale for the use of piracetam in the treatment of AD is based on the sensitivity of the cholinergic system to anoxia or impaired metabolic function, and on studies of reduced glucose metabolism in the aging rat and aging human (Ferris, Crook, & Bartus, 1982). It has been suggested that piracetam increases the synthesis of ATP from ADP thus increasing the supply of available energy for metabolic production of ACh. Controlled trials have indicated some weak stimulant-like effect on attention and memory retrieval (Ferris et al., 1982). Ferris et al. (1982) reported stronger beneficial effect for some patients when piracetam was used in conjunction with choline. Currently, three analogues of piracetam (aneracetam, pramiracetam, and oxiracetam) are under study by various investigators.

Naloxone

Naloxone, a synthetic opioid antagonist, has been tested in limited studies by Reisberg, London, Ferris, and Anand (1983), with some clinical improvement noted in some subjects. Naloxone may function by blocking the effect of endogenous opiate receptor-activating agonists, thereby increasing effective GABAergic function.

Arecoline

Arecoline, a muscarinic agonist, although effective in augmenting memory in normal and aged humans, was found ineffective on AD patients (Christie, Shering, Ferguson, & Glen, 1981).

CHAPTER 2

THE CHOLINERGIC NEUROTRANSMITTER SYSTEM

Anatomy and Projections

Introduction

The most distinctive pathologic feature of AD is the progressive destruction of the cholinergic pathway originating at the level of the basal forebrain and projecting to cortex. It is the destruction of this pathway and the resulting loss of ACh available to cortex that is probably the basis of memory and perhaps other cognitive deficits. The cause of destruction is unknown. It is unknown whether damage to individual neurons begins at the soma, along the axon, within the dendritic tree, or at the very termination of the cell in the synaptic buttons. There are no data regarding the way the neuron is damaged, or the cellular mechanism(s) that are the first to malfunction. In fact, there is no definitive information regarding the specifics of atrophy once a neuron sustains mortal damage and begins its decline. Two things are essential to the investigation and collection of data that will eventually serve to answer the unknowns of AD. The first is a comprehensive schematic of the cholinergic transmitter system, and the second is a thorough understanding of the ACh producing mechanism.

In 1896, A. von Kolliker published "Handbuch der Gewerbelehre des Menschen." In Volume 11 "Mnb: History, Anatomy, Comparative

Phylogeny," he described the topological relationships and microscopic anatomy of a nucleus of cells in human first discovered by Theodor Meynert in 1872 (cited in Gorry, 1963). Meynert called it Ganglion Ansae lenticularis. Working with Carmin and Weigert stains, Kolliker described a collection of very large cell bodies, multipolar, 20-30 microns (later observations indicate cell diameter may approach 45 microns) in size, with deep though irregular staining of nissl substance. He also described a profuse network of fibers among the cells. The type of stains used did not allow the observation of axons and so the projection of this "Meynert's basal ganglion," as it was termed, remained unknown. Later investigation of the nucleus was conducted by Brissaud and Forel in 1907, and Beccari in 1911 (cited in Gorry, 1963). Beccari, using a Golgi preparation, noted columinous multipolar nerve cells of the basal ganglia with long dendritics and little branching. He emphasized that the basal ganglia were composed of two types of cells, the very large Meynert type, and other small cells. The Golgi stains allowed observation of only the beginning of axons.

Kodoma (1929; cited in Gorry, 1963) was the first researcher to provide evidence for a projection from the nucleus basalis of Meynert (nbm) to the neocortex. Kodoma noted several human cases that exhibited cell loss in nbm associated with lesions of the temporal cortex. Similar lesions of the frontal and parietal lobe did not cause the same reduction in volume. It was observed that shrinkage and approximation of nbm cells were accompanied by a loss of fibers from the inferior thalamic peduncle. Experiments with dogs and cats involving

temporalectomy and hemidecortication confirmed the specific cell loss noted in humans.

Gorry (1963) reported the existence of only three investigatory efforts into the nature of nbm between 1929 and 1963. Grunthal (1932; cited in Gorry, 1963) undertook a comparative anatomical study, primarily cytoarchitectonic, in which was included the nbm. Grunthal made the mistaken assumption that the basal ganglion of lower mammals corresponds to the external pallidus in primates, and thus, nbm dwindles to rudimentary significance (Gorry, 1963). In 1942, Brockhaus rectified this error and described the basal ganglia as part of a basal nuclear complex, consisting of three nuclear formations (cited in Gorry, 1963). From Gorry (1963) they are described as

> (1) The nuclear formation of Broca's diagonal band, extending farthest medially and dorsally, and rising dorsally into the Septum; (2) the tubercular nuclear formation, lying in the Tuberculum olfactorium; and (3) the actual Nucleus Basalis, situated lateral and dorsal to the tubercular group. It is always in topographic relation to the Globus pallidus, although the extent of this relation varies with the species.

Von Buttlar-Brentano (1952; cited in Gorry, 1963) studied nbm in the brains of nine schizophrenics and ten senile, but otherwise normal, humans. She found changes in nbm in the senile brains and a reduction in size of the large cells of the basal ganglia in the schizophrenic brains. In 1963, Gorry undertook a comprehensive comparative study of nbm in mammals. In man, nbm is approximately 1.0 mm wide by about 0.18 mm thick and extends from its most rostral presence, where the external capsule connects to the superior frontal gyrus, caudally to the level of the mammillary bodies. Based on his

comparative study, Gorry concluded that the nbm follows a phylogenetic gradation: Marsupialia-Insectivora-Chiroptera-Lagomorpha-Rodentia-Ungulata-Carnivora-Primate. Differentiation undergoes a gradual increase as the phylogenetic scale is ascended. Nuclear differentiation correlates with total brain and especially neocortical development. Gorry pointed out that if nbm does project to the temporal lobe as proposed by Kodoma, the correlation between neocortical development and nbm differentiation would support this concept. He did emphasize, however, that although phylogenetic development was consistent through the different orders, there was also a stepwise development in the species of each order. Thus, although the overall development of primates may be more extensive than carnivora, a primitive primate may demonstrate more rudimentary development of the nbm than an advanced carnivore.

Nucleus Basalis of Meynert: Afferent Projections and Methodology

Shute and Lewis, in a classic series of experiments, investigated the anatomy and afferent projection of cholinergic neurons (Shute & Lewis, 1967; Lewis & Shute, 1967). They hypothesized that cholinergic neurons contain acetylcholinesterase in the soma, axon, and nerve terminals. By lesioning at serial levels of the brain and staining histochemically for AChE, they proposed to map brain cholinergic pathways. Horseradish peroxidase, radiological markers, and other innovations allowing determination of direction of axonal projection did not exist at that time. Lewis and Shute relied upon differential accu-

mulation of AChE on either side of a lesion to determine the direction of a fiber track (Shute & Lewis, 1967, 1961). The side on which AChE accumulated was considered proximate to the soma, the side on which AChE diminished was considered distal. Thus, not only a map of cholinergic pathways could be produced, but also a directional schematic.

From their studies, Shute and Lewis concluded that two major ascending cholinergic pathways exist in the brain. These tracts, termed the dorsal and ventral tegmental pathways, originate in the reticular and tegmental nuclei and terminate in the forebrain. The dorsal tegmental was supposed to originate primarily in the nucleus cuneiformis and innervate tectal and thalamic areas. The ventral pathway was thought to arise in the pars compacta of the substantia nigra and project to the hypothalamus and areas of the basal forebrain. It is now known that AChE is contained in axons and terminals of neurons that are noncholinergic (Fibiger, 1982). The dopamine neurotransmitter system contains significant levels of AChE, and it is now known that the ventral tegmental pathway identified by Shute and Lewis corresponds to this system (Fibiger, 1982). Hoover and Jacobowitz (1979) have confirmed that the cholinergic dorsal pathway of Shute and Lewis does exist, as do a number of other cholinergic pathways first proposed by Shute and Lewis. From the work of Shute and Lewis and later investigators, it has been suggested that high AChE activity may be a necessary but not a sufficient criterion for identification of central cholinergic pathways (Fibiger, 1982).

More recently developed methodologies provide a wider margin of evidence in the investigation of cholinergic pathways. Recently created procedures include the placement of electrolytic lesions in an area suspected of containing cholinergic cell bodies or axons and then subsequently measuring in vitro for CAT activity, choline uptake, or ACh levels in apparent terminal fields (Kuhar, Sethy, Roth, & Aghajanian, 1973; McGeer & McGeer, 1979). Subsequent development of chemicals that cause dysfunction or destroy cell bodies only eliminate a serious problem with damage to fibers of passage or important noncholinergic cell bodies caused by even the most discrete of electrolytic lesions. Within the last decade, additional methodological advances permitted the elucidation of heretofore unknown cholinergic pathways. These advances include: (1) pretreating the subject brain with irreversible AChE inhibitors a few hours before processing for AChE histochemistry, (2) purification of CAT and subsequent production of CAT antibodies for use in immunohistochemistry, and (3) the autoradiographic identification of muscarinic and nicotinic receptor sites using labeled ligands.

Fibiger (1982) has suggested that AChE staining is a necessary but not a sufficient criterion for identification of a cholinergic pathway, since AChE is also found in noncholinergic cells. This is also based on the observation that known cholinergic perikarya consistently contain high levels of newly synthesized AChE 4 to 8 hours after treatment with diisopropylphosphyluoridate (DFP), an irreversible AChE inhibitor. Whereas 4 to 8 hours is sufficient time for the

synthesis of AChE in the soma, it is not sufficient time for it to be transported to axon terminals. This procedural criterion applied to all brain areas that stain for AChE eliminates many areas from consideration. According to this reasoning, the cholinergic neurons in the forebrain are located only in the basal forebrain, olfactary tubercle, nucleus accumbens, and striatum (Lehmann & Fibiger, 1979). It should be noted that this procedure only allows identification of cholinergic positive perikarya. This technique must be combined with some other procedure to examine axonal projections specifically of cholinergic neurons. Specific localization of cholinergic neurons in the forebrain of the rat utilizing the latest histochemical techniques is a significant development, and provides correlative evidence for conclusions drawn as a result of data collected by the older methods described above.

Nucleus Basalis Magnocellularis: Anatomy and Afferent Projection

Investigation of cholinergic activity in the cortex has been extensive, and includes studies across numerous species. An often repeated experiment is the undercutting of cortex and the subsequent measurement of cholinergic markers in the denervated tissue. This procedure has generally resulted in large decreases in the presence and/or activity of AChE and CAT (Lehmann, Nagy, Atmadja, & Fibiger, 1980; Fibiger, 1982; Hebb, McGeer, McGeer, & Perkins, 1964). These studies clearly indicate that although some cholinergic neurons may exist in cortex, most cholinergic activity is dependent on

afferent projections into cortex. Additional evidence exists that suggests the total absence of cholinergic perikarya in cortex (Fibiger, 1982). Lehmann et al. (1980) found that kainic acid injected into cortex resulting in massive cell death did not affect cortical CAT levels. Lehmann also found that no AChE remains in cortex at the end of 4 to 8 hours after treatment with DFP. Kumura et al. (1980, 1981) found numerous choline-receptive cells in cortex utilizing immunohistochemical techniques, but no cholinergic cell bodies.

Mesulam and Van Hoesen (1976), using retrograde axonal transport of horseradish peroxidase (HRP), and other studies by Kievet and Kuypers (1975) and Divac (1975), confirmed the findings of Kodoma, and later Shute and Lewis, that cells of the neocortex are innervated by cholinergic perikarya located in the nbm. Studies by Mesulam and Van Hoesen (1976) and a more recent investigation by Lehmann et al. (1980), using a combined HRP/AChE histochemical procedure, identified a region, termed nucleus basalis magnocellularis (nbm), in the rat that is the homologue of the more clearly defined nucleus basalis of Meynert (of the substantia innominata) in the primate. Magnocellularis cells are so named because of their large size, 30-45 microns, equalling cell diameters observed in primates.

Gorry's (1963) comparative anatomical study across species included the order rodentia, species rattis norvegicus, which may be similar to the variants used in current laboratory research. Figure 1 is a schematic of the location and extent of nbm through a series of rat brain coronal sections. It should be noted that cholinergic projections

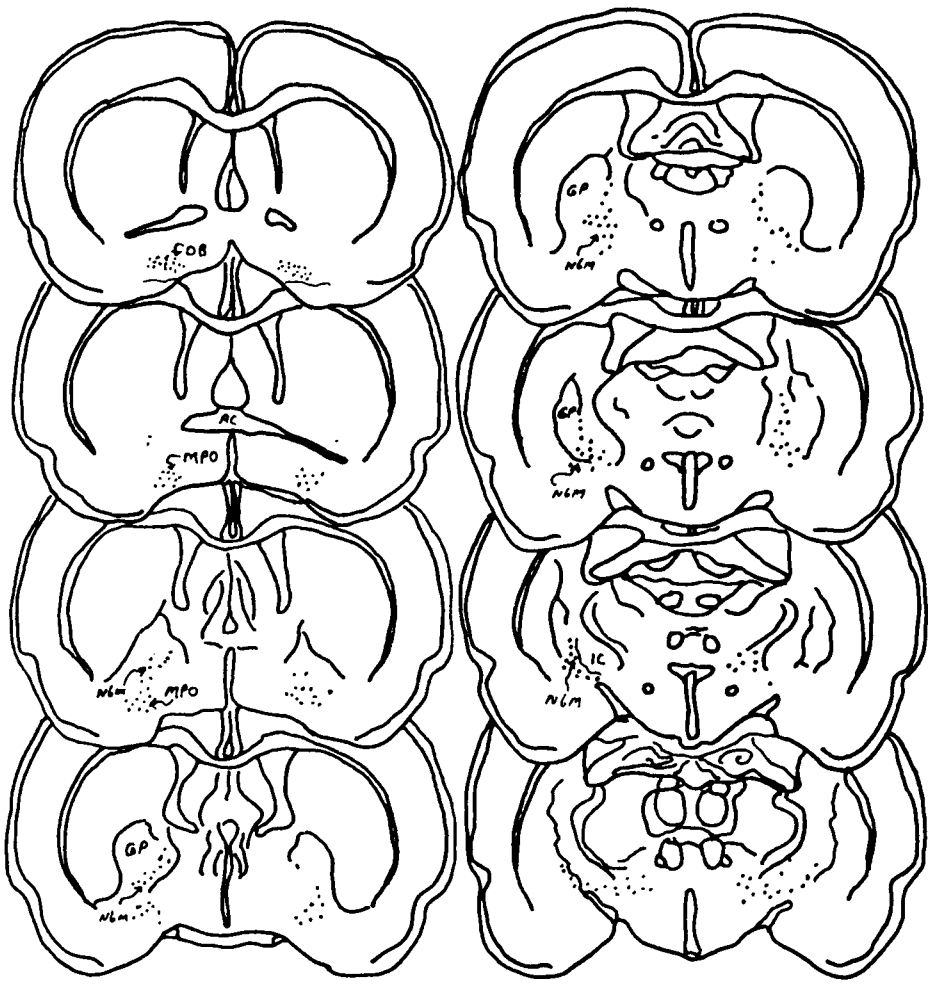


Figure 1. Location in the rat of nucleus basalis magnocellalaris (nbm), diagonal band (db), and medial preoptic area (mpo).

to neocortex from the basal forebrain are not confined to nbm. Magnocellularis type cholinergic neurons also exist in the horizontal limb of the diagonal band (Kimura, McGeer, Peng, & McGeer, 1981; Price & Powell, 1970; Shute & Lewis, 1967). Other cholinergic afferent fibers to neocortex arise in globus pallidus, entopeduncular nucleus, and the lateral preoptic area (Divac, 1975; Kievet & Kuypers, 1975; Fibiger, 1982). Figure 1 also indicates the distribution of these neurons. Although cholinergic neurons may be divided into separate nuclei that appear to maintain some uniformity of grouping in a serial rostral to caudal manner, caution should be used in assuming unanimity of projection, function, and even anatomy. These subdivisions are historically reported and a substantial body of evidence supports the groupings, at least in terms of general projection. It has been demonstrated that (1) magnocellular neurons of the horizontal limb of the diagonal band project to the olfactory bulb, and entorhinal cortex (Wenk, Bigl, & Meyer, 1980; Fibiger, 1982; Bigl, Woolf, & Butcher, 1982); (2) the vertical limb of the diagonal band and the medial septum innervate the hippocampus and medial limbic cortex (Lewis & Shute, 1967; Lynch, Rose, & Gall, 1978; Mesulam, Van Hoesen, & Rosene, 1977; Wenk et al., 1980); (3) preoptic, medial septal nucleus, and horizontal limb neurons project to amygdala (Bigl et al., 1982; Emson, Paxinos, LeGol, La Salle, Ben-Ari, & Silver, 1979); and (4) neurons of the nucleus basalis magnocellularis primarily, and possibly neurons of the preoptic nucleus and horizontal limb of the diagonal band, may, secondarily, project to the neocortex (Emson

et al., 1979; Kelly & Moore, 1978; Lehmann et al., 1980; Mesulam et al., 1976; Wenk et al., 1980; Fibiger, 1982). The nbm may also innervate the amygdala to some extent (Fibiger, 1982). Figure 2 schematically demonstrates this cholinergic afferent system.

Price and Powell (1970), and Millhouse (1969) have gathered experimental evidence that there is considerable overlap in these areas, with the possibility of collaterals of individual perikarya innervating widely divergent regions. The same studies indicate that there is evidence that collaterals travel to the contralateral nucleus, and that axons may bifurcate into ascending and descending branches.

Fibiger (1982) stated that although a well developed body of experimental evidence indicates the origin of cholinergic neurons and the general area of afferent projection, little is known about specific localities of innervations (i.e., cortex layer, postsynaptic innervation) and functions. He suggested the development of a toxin selective to cholinergic neurons. As has been noted above, cholinergic neurons have an extensive rostro-caudal distribution without clearly defined boundaries, are often interspersed with noncholinergic neurons, may send collaterals to diverse regions of the forebrain, and tend to be located in the course of the fiber-rich medial forebrain bundle. These factors will present substantial interpretive difficulties for studies that employ either electrical stimulation or electolytic lesioning techniques. The presence of noncholinergic cell bodies interspersed with nbm and in adjacent areas limits the usefulness of excitotoxic chemicals like ibotenic or kainic acid. Behavioral measurement studies that seek to

Figure 2. Cholinergic neurotransmitter system of the rat.

OB: olfactory tubercle
DBL: horizontal limb of diagonal band
DBV: vertical limb of diagonal band
AMB: amygdala
MPO: medial preoptic area
nbm: nucleus basalis magnocellularis
Hp: hippocampus
TL: thalamus
VTA: ventral tegmental area
IPN: interpenduncular nucleus

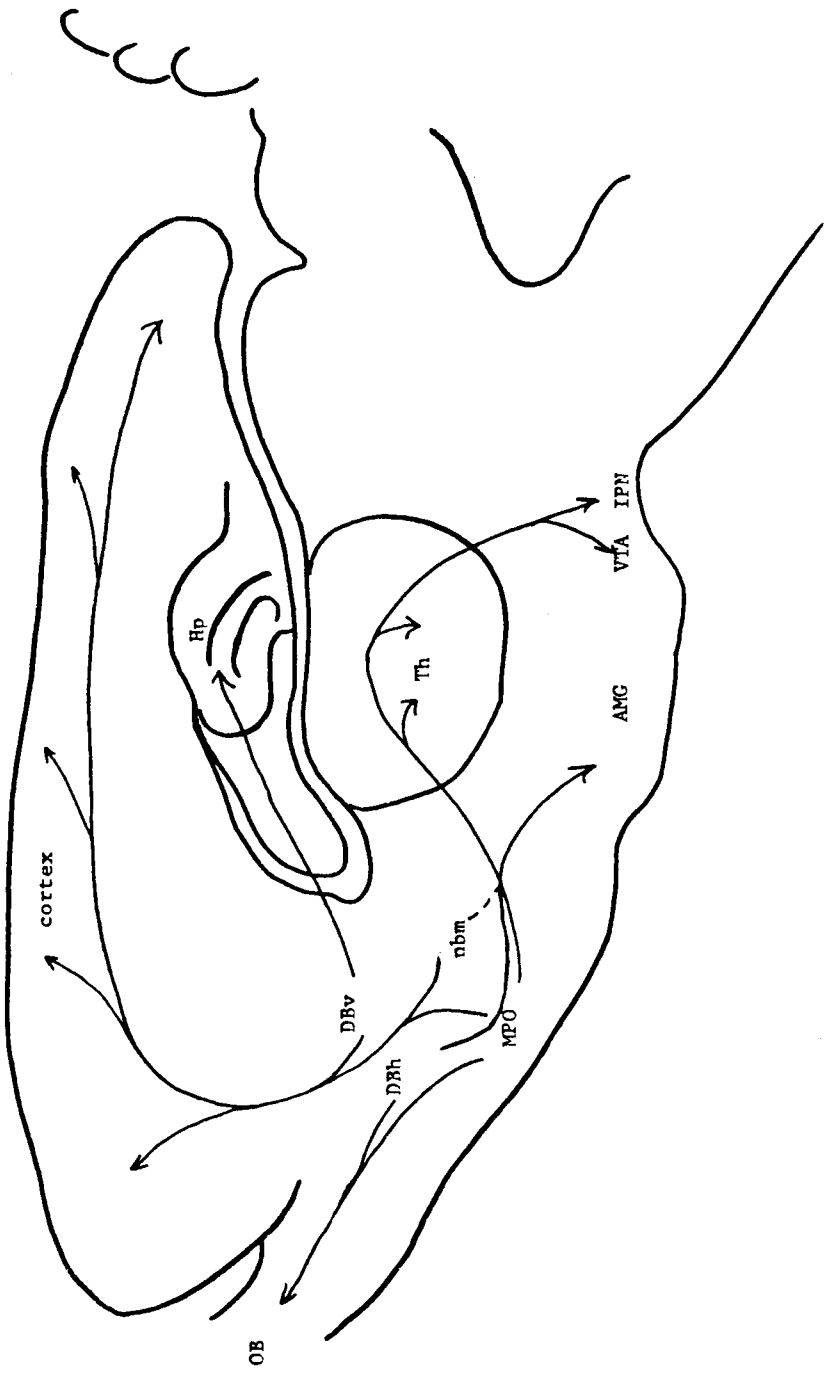


Figure 2.

examine cognitive deficits resulting from destruction of nbm may be compromised using these techniques. Damage to important fibers of passage or nearby perikarya in globus pallidus may result in damage to neural networks supporting or controlling fixed action patterns, or other behavior and thus preclude accurate measurement of behavior or motor activity. A selective toxin for cholinergic neurons would be of great utility, but the unequivocal action of such a chemical has not been demonstrated.

The Cholinergic Neuron

A great deal of investigative effort has been devoted to the elucidation of cholinergic pathways. Over the past 20 years, no less effort has been expended toward understanding the metabolism and function of the cholinergic neuron. Much has been learned, but much more is yet to be understood. Choline was discovered in 1862 by Strecker (Ansell & Spanner, 1979). It was a known constituent of lecithin and sphingomyelin, two phospholipid membrane components. During the 1930s and 1940s there was considerable effort expended in studying the biosynthesis and catabolism of choline as a constituent of acetylcholine. After the development of chromatographic methods of analysis, Kennedy and Weiss (1956) investigated the incorporation of choline into the phospholipid, phosphatidylcholine. Generally, up to this time, the study of the metabolism of acetylcholine was conducted by pharmacologists and the investigation of choline-containing lipids was done, separately, by biochemists. In the 1960s and 1970s,

interest developed in a possible link between the metabolism of choline lipids and the cholinergic system of the brain. It has been reported that choline is not synthesized in the brain from unmethylated precursors such as serine or aminoethanol (Ansell and Spanner, 1967, 1971, 1972, 1976). However, controversial research by Kewitz, Pleul, Dross, & Schwartzkopff (1975) has suggested that some small amount may be manufactured from ethanolamine or dimethylaminoethanol.

Because the brain lacks the metabolic machinery to synthesize substantial amounts of choline from its precursors, the major supply of choline must ultimately come from the liver via plasma and through the blood brain barrier (BBB) (Bremer & Greenberg, 1961; Lymann et al., 1967). The kidney is also actively involved in choline blood concentration control. The chief means of removal of choline from plasma (approximately 85%) is due to uptake by various tissues of the body, with a 20-fold difference between the most active (adrenals) and the least active (CNS) (Freeman & Jenden, 1976). Three minutes after injection of choline into the bloodstream, 69% is present as free choline in tissues (Gardiner, 1974), and most of this is converted to phosphorylcholine, phospholipids and betaine (Rossiter & Strickland, 1970).

The transport of choline and phospholipids across the BBB is a bidirectional balanced system. The net flux of free choline is out of the brain, thus requiring a continuous degradation of brain choline lipids (Jenden, 1979). Table 1 lists the concentration of choline sources in the brain. It should be noted that the concentration of

Table 1

Choline Compounds in Rat Brain

Compound	Concentration (μmoles/g fresh tissue)	
Choline	0.024	cerebrum
Acetylcholine	0.070	
Phosphocholine	0.38	whole brain
Glycero-3-phosphocholine	0.40	
CDP-choline	0.05	
Phosphatidylcholine	14.7	
Choline plasmalogen	0.6	
Sphingomyelin	3.7	

Source: Ansell, G. B. and Spanner, S., Sources of choline for acetylcholine synthesis, Nutrition and the Brain, vol. 5, Raven Press, N.Y., 1979.

free choline is very small. This is a result of the metabolically highly reactive nature of choline. Free choline in the brain is very rapidly phosphorylated or acetylated (Ansell & Spanner, 1979). Choline half life in plasma has been demonstrated to be less than 1 minute (Freeman & Jenden, 1976). There are, in theory, three routes through which choline is made available in the brain; by de novo synthesis (probably very small amounts), by direct entry of free choline from the blood-stream (again, very small amounts), and degradation of phospholipids or simpler compounds (largest source).

Although net flow is out of the brain, it has been repeatedly demonstrated that unbound or free choline can enter the brain from plasma (Ansell & Spanner, 1975). Free choline enters the brain by diffusion and by a carrier mediated process (Dross & Kewitz, 1972; Freeman & Jenden, 1976). However, data suggest that this is not a major source of brain choline. It has also been shown that choline may enter the brain in a lipid bound form (Illingsworth & Portman, 1972; Freeman & Jenden, 1976). Phospholipids are probably the major source, ultimately, of choline used for brain ACh synthesis and other metabolic functions (Dross, 1975; Dross & Kewitz, 1972; Illingsworth & Portman, 1972). Choline containing phospholipids can be synthesized in the brain or outside of the brain and transported to the brain. Phosphorylcholine is formed as a result of the action of choline kinase, forming the Kennedy pathway.

Lecithin (phosphatidylcholine) can also be formed via acetylation of lysolecithin by acetylcoenzyme A. Ansell and Spanner (1979) have

suggested three possible sites for lipid bound choline release: (1) free choline could be directly released from phosphatidylcholine and lysophosphatidylcholine in the blood as these pass through the brain, with diffusion or mediated carrier transport across the BBB (as indicated this contributes a relatively small amount); (2) A pool of phosphatidylcholine in extracellular brain could release free choline via the Kennedy or other pathways; and (3) choline could be released from its phospholipid-bound form as it enters the neuron and crosses the cell membrane, or by internal neuronal processes.

There is considerable evidence for the existence of an extracellular pool of phosphatidylcholine (Ansell & Spanner, 1979; Horrocks, Toews, Thompsin, & Chin, 1976; Miller, Benjamins, & Morrell, 1977; Illingsworth & Portman, 1972). Studies indicate a continual release of choline from this pool, with some choline taken up by neurons, some reincorporated into the pool, some acetylated, and some lost from the brain. From Table 1 notice that the highest concentration of source choline is that of phosphatidylcholine. Thus, both uptake of free choline from the blood and release of choline from a bound form in an extracellular pool contribute to the availability of free choline for intraneuronal use. However, the relative contribution from either source may vary across species. Ansell and Spanner (1979), Freeman and Jenden (1976), Porcellati (1974), and Rossiter and Strickland (1970) have described possible mechanisms for the release of choline from phosphatidylcholine and lysophosphatidylcholine. One process, the base exchange reaction, is not energy dependent and

thus does not require the presence of ATP or CTP. Evidence also exists which suggests that a significant amount of choline is available from intraneuronal processes (Jope and Jenden, 1981). Ansell and Spanner (1975) have shown that choline is stored as phospholipid within the neuron. This intraneuronal phosphorylated choline later undergoes catabolization to release choline for the synthesis of ACh. After ACh is released into the synaptic cleft, it is hydrolyzed by AChE and the released choline is taken-up into the neuron.

Approximately 50% of the choline required for synthesis of acetylcholine is reclaimed from the synaptic cleft (Tucek, 1982). Thus, of the remaining required 50%, the probable sources are (1) free choline crossing the blood brain barrier which is either immediately transported into the neuron and acetylated or phosphorylated for storage, or incorporated into the extracellular pool of phospholipid; (2) choline transported across the BBB as a phospholipid where it augments the extracellular pool; or (3) choline released from the extracellular phospholipid pool and transported into the neuron.

Yamamura and Snyder (1973) and Haga and Noda (1973) demonstrated the existence of a specialized system in the synaptosomal membrane for the uptake of extraneuronal choline. This high-affinity, sodium-dependent, energy consuming uptake system (HAS) is almost exclusively localized to nerve terminals in the cholinergic neuron and is specifically associated with providing choline for the synthesis of acetylcholine (Suszkiw & Pilar, 1976). The HAS may be kinetically, although is probably not directly, coupled to the rate of ACh

synthesis (Kuhar & Murrin, 1978; Suszkiw & Pilar, 1975; Wheeler, 1979; Jope & Jenden, 1979).

Jope and Jenden (1981) have proposed a model for the transport of choline and regulation of ACh synthesis in the cholinergic nerve ending:

> The intracellular Ch equilibrates with the specific activity of Ch in the medium. Intracellular Ch and ACh are equilibrated by ChAT by mass action and labile bound and stable bound ACh in turn equilibrate. When ACh is released the cytoplasmic concentration of ACh is reduced. This drives ACh synthesis by mass action which utilizes intracellular Ch. Utilization of intracellular Ch reduces its concentration and results in increased net Ch transport and acetylation in the nerve ending. It is implicit in this model that AcCoA [acetyl coenzyme A] becomes available at a sufficient rate for the ACh concentration to be maintained. The production of AcCoA in the cytoplasm could determine the kinetics of the entire system and itself be subject to regulatory control. (pp. 509-510)

This model accounts for most observed phenomena in the resting state. However, prolonged stimulation of the cholinergic neuron may require further explanation. It is not clear whether the HAS is responsible for the formation of all releasable ACh (Kuhar & Murrin, 1978; Kewitz et al., 1975; Guyenet, Lefrusne, Beaujounan, & Glowinski, 1975; Blusztajn & Wurtman, 1983; Yamamura & Snyder, 1973; Ansell & Spanner, 1975; Tucek, 1982). It has been suggested that somatic synthesis of ACh followed by axonal transport may provide a reserve mechanism for supplying additional ACh at nerve terminals under various conditions (Jenden, Weiler, & Gundersen, 1982; Suszkiw & Pilar, 1975).

A second system also transports choline across the cell membrane. This is the low-affinity, calcium-dependent, chiefly energy-independent choline transport system (LAS) described by Diamond and Milfay (1972). Choline transported by the LAS is primarily incorporated into the phospholipid pool and was found by Diamond and Milfay to be present in mitochondrial, microsomal, synaptosomal and synaptic vesicle fractions of the rat brain. It has been demonstrated in brain cell cultures that choline transported by the LAS can be utilized for ACh synthesis (Caroll & Goldberg, 1975; Suszkiw & Pilar, 1976). Thus, the HAT is apparently primarily localized to synaptic terminals and is essentially tied to the uptake of choline for ACh synthesis; and the LAS exists in the soma, axon, and terminal area and is probably chiefly tied to the uptake of choline for renewal of the phospholipid pool. Suszkiw and Pilar (1976) and Pilar, Jenden, and Campbell (1973) have shown that ACh is also synthesized in the soma and utilizes choline garnered by the LAS. Koike, Eisenstadt, and Schwartz (1972) and others have shown transport of ACh down the axon to the terminal area, with evidence for both slow and fast transport.

Acetyl Coenzyme A

Cholinergic neurons synthesize, store, and release ACh. The synthesis and metabolism of ACh has been studied extensively, notably by Birks and MacIntosh (1961). ACh is catalyzed by the enzyme choline acetyltransferase and follows the reaction:

$$\text{choline} + \text{acetyl coenzyme A} \overset{\text{CAT}}{\rightleftharpoons} \text{acetylcholine} + \text{CoA}$$

In theory there are a number of possible sources for acetyl coenzyme A; through glycolysis and the pyruvate system from glucose; from acetate through acetate thiokinase; or from citrate either by reversal of the condensing enzyme citrate synthetase, or by citrate lysase, the citrate cleavage enzyme. In some in vitro preparations (e.g., brain slices and preparations of nerve endings) glucose or citrate has proven to be the best source for ACh synthesis with little contribution from acetate. In lobster axons, the electric organ of Torpedo, corneal epithelium, and other tissue, acetate appears to be the best substrate (Cooper, Bloom, & Roth, 1982). The exact source of acetyl CoA in vivo is not known and remains a controversial topic. It is known that acetyl CoA is manufactured primarily in mitochondria, although the transport mechanism out of mitochondria to the site of ACh synthesis is open to conjecture. The problem lies in the necessity of acetyl CoA leaving the mitochondria by passing through both an inner and outer membrane. The permeability of the outer membrane is high, but the inner membrane has been demonstrated to be largely impermeable to large and charged molecules similar to acetyl CoA. Tucek (1982) reports that acetyl groups are only allowed to leave mitochondria after they have been converted to a "carrier" compound such as citrate, acetylcarnitine, acetate, glutamate, or acetylated amino acid. After passing the inner membrane, the carrier compound may be converted back to acetyl CoA in the outer membrane. Although no carrier compound has yet been isolated, acetylcarnitine has been demonstrated to be a source of acetyl groups for ACh synthesis at about

two-thirds the efficiency of glucose. The utilization of acetate, citrate, acetylaspartate, or glutamate is very low (Tucek, 1982). It has been suggested that acetyl CoA may be converted to a number of different acetyl groups and leave the mitochondria in the form of these other compounds (Tucek, 1978). It has, in fact, been shown that the acetyl groups of ACh in brain appear to be different from those in other organs.

Choline Acetyltransferase

Choline acetyltransferase, first identified by Nachmansohn and Machado in 1943, is manufactured in the soma and axonally transported to the synapse where it catalyzes the synthesis of ACh (MacIntosh, 1981). Fonnum and Walburg (1973) estimated that hypoglossal nucleus perikarya contain 2% of total neuronal CAT, the main axon 42%, and preterminal axons and nerve endings 56%. Primary axonal transport is 1-6 mm/day in mammals (i.e., "slow" axonal transport), unidirectional, and includes all manufactured enzyme (Fonnum & Walburg, 1973; Tucek, 1982). There is evidence that some portion of CAT is carried by fast axonal transport (Davies, Whittaker, & Zimmerman, 1977). Cell fractionation studies indicate that most CAT in nerve endings is free in the cytosol, although some portion may adhere to the outside of vesicles (Fonnum, 1972; MacIntosh, 1981).

Although purification of CAT is difficult, a homogeneous preparation has been produced. CAT is a globular protein, with an estimated radius of 3.39 nanometers, a high positive surface charge, and a molecular weight of 67,000-68,000 (Rossier, 1976; Malthe-Sorenssen,

1976). Three molecular forms have been identified in rat, ox, mouse, and monkey, while two forms have been found in the cat, and one form in the guinea pig, pigeon, and rabbit (Tucek, 1982). However, it is unclear whether these forms result from true isoenzymes coded by different genes, whether they are artifacts caused by treatment of tissue for analysis, or whether posttranslational processing introduces specific modification. According to Tucek (1982), available data suggest the last possibility as most likely. Although CAT is a weak immunogen, antibodies have been successfully produced and further investigative studies are underway using this technique to identify sources and pathways.

Acetylcholinesterase

After release into the synaptic cleft, ACh is reduced to its constituents for re-uptake and re-utilization in the cholinergic neuron. Two classes of cholinesterase that will hydrolyze ACh are acetycholinesterase ("true" or specific cholinesterase), and butyrylcholinesterase ("pseudo" or nonspecific cholinesterase). Acetylcholinesterase is the primary enzyme found in synaptic clefts that catalyzes the hydrolysis of ACh:

$$\text{AChE} + \text{ACh} \rightleftharpoons \text{AChE}\cdot\text{ACh} \rightarrow \text{AChE}\cdot\text{Acetyl} \xrightarrow{H_2O}$$
$$\text{AChE} + \text{Acetic Acid} + \text{Choline}$$

AChE is generally localized to neuronal tissue, although it is a constituent of blood erythrocytes and it is found together with pseudocholinesterase in some neuronal tissue and organs (e.g., autonomic

ganglia, liver, and lung). AChE apparently has several forms that are catalytically identical glycoproteins that differ in molecular weight and physical properties (MacIntosh, 1981). One form, which is probably the precursor of the other forms, is a monomer and has the smallest molecular weight, about 65,000 daltons. Some forms are membrane bound and others exist free in the synaptic cleft. One variant is composed of three tetrameric globular catalytic units attached to a collagen-like tail. The tail has a composition similar to that of basement membrane and is believed to play a structural role in the localization of the enzyme (Cooper, 1982; MacIntosh, 1981). In neurons, AChE is manufactured in the soma and axonally transported to the nerve terminals. In the synapse AChE is in some fashion externalized with a portion remaining attached to the neural membrane and part secreted into the extracellular space (Skau & Brimijohn, 1978). At synapses most AChE remains membrane-bound with its active sites facing outward enabling the enzyme to hydrolyze newly released ACh (MacIntosh, 1981). As indicated above, the collagen tail may be important in this localization and orientation. AChE is recognized as one of the most efficient enzymes, with a turnover rate of 150 ms. This is equivalent to hydrolyzing 5,000 molecules of ACh per second per molecule of AChE.

Hemicholinium

Long and Schueler (1954) investigated a series of bisquaternary ammonium compounds based on a a'-dibromo-4:4-bisacetophenone. Schueler (1955) suggested the hemiacetal formulation of one particular

dibromo compound that had been treated with dimethylaminoethanol. This new compound he described as "hemicholinium," usually referred to as HC-3. Long and Schueler (1954) found that when HC-3 was injected into animals it caused paralysis of respiration that could be reversed by injecting choline or anticholinesterases. MacIntosh et al. (1956) found that low concentrations of HC-3 inhibited the synthesis of ACh in the perfused superior cervical ganglion of the cat, which could be reversed by adding choline. Gardiner (1961) proposed that HC-3 inhibited the acetylation of choline by competing with choline for passage through the cellular membrane. This has been confirmed by others (Diamond & Kennedy, 1969; Slater, 1973; Yamamura & Snyder, 1973). It has also been determined that HC-3 does not enter the neuron (Slater, 1973; Yamamura & Snyder, 1973). HC-3 has been found not to inhibit the activity of CAT (Diamond & Milfay, 1972). However, there is evidence to support the conclusion that choline kinase is inhibited (Ansell & Spanner, 1975).

The latter finding is important relative to the uptake of choline into phosphorylcholine and phosphatidylcholine. Gomez, Domino, and Sellinger (1970a, 1970b) found that HC-3 stimulates the formation of phosphatidylcholine in dog caudate nucleus. This would appear to be a contradiction since the primary pathway for formation of phosphatidylcholine in brains is the Kennedy pathway (choline→phosphorylcholine→CDP-choline→phosphatidylcholine). However, as noted above, choline may be incorporated into phosphatidylcholine by a base exchange reaction. Since choline kinase may be inhibited, a larger pool

of choline would be available for a base exchange. This supposition would seem to be supported by the research of Ansell and Spanner (1975) wherein labeled choline, [Me-^{14}C] choline, was injected into six regions of the brain. Labeled choline uptake for synthesis of phosphorylcholine and phosphatidylcholine was then measured. It was found that phosphorylcholine synthesis was inhibited by HC-3 in cerebellum and midbrain, but the synthesis of phosphatidylcholine was stimulated. In cortex, HC-3 inhibited phosphorylcholine synthesis, but synthesis of phosphatidylcholine was not stimulated. They concluded that different areas of the brain may utilize different synthesis techniques. Cerebellum and midbrain may employ both the Kennedy cycle and base exchange (stimulated by HC-3 through the inhibition of choline kinase). In cortex only the Kennedy pathway may be available for use, thus accounting for the lack of effect due to HC-3 inhibition of choline kinase.

Studies by Domer and Schueler (1960), Bhatnagar and MacIntosh (1967), Bull and Hemsworth (1965), and Giarman and Pepeu (1962) have demonstrated the inability of quaternary ammonium compounds to pass the BBB. HC-3 is no exception. HC-3 injected into cortex, ventricles, caudate nucleus and other areas has been conclusively demonstrated to reduce ACh in those areas invaded by the HC-3 (Gardiner, 1961; Ansell & Spanner, 1975; Hebb et al., 1964; Slater, 1968). Schueler (1960) has determined in agreement with MacIntosh, Birks, and Sastry (1958), that HC-3 affects only presynaptic processes at low concentrations. HC-3 blocks both the LAS and the HAS, but

the sensitivity of the HAS to HC-3 is much greater, perhaps 50 times greater (Kewitz et al., 1975).

CHAPTER 3

THE EFFECTS OF CHRONIC INFUSION OF HEMICHOLINIUM

Introduction

No cure or reliably effective treatment exists for AD. Prevailing law and morality strictly limit the testing of experimental drugs or the use of invasive research methods on humans. Various authorities have proclaimed the need for an animal model for Alzheimer's Disease to permit assessment of various drugs in halting the progress of AD, on the restoration of cognitive function, and for the investigation of AD etiology (Report of the Panel on Inflammatory, Demyelinating and Degenerative Diseases of the National Advisory Neurological and Communication Disorders and State Council, 1979; Report of the Secretary for the Task Force on Alzheimer's Disease, 1984). No animal model currently exists.

As described above, numerous studies have strongly tied AD to the progressive deterioration of the cholinergic neurotransmitter transport system. This link is based on observed correlation between the degree of deterioration of the system, as indicated by microscopic anatomical and biochemical changes, and degree of cognitive deficit. Previous research has demonstrated a vital role for ACh in memory and learning (Pope et al., 1964; Spillan et al., 1977; Davies & Maloney, 1976; Davies, 1979; Reisine et al., 1978; Rossor et al., 1980). In addition, various studies have demonstrated that lesions in the nbm of

the rat cause deficits in passive avoidance learning or maze learning and concomitant destruction of the cholinergic system (Salamone, Beart, Alpert, & Iversen, 1984; Friedman, Lerer, & Kuster, 1983; LoConte, Bartolini, Casamenti, Marcocini-Pepeu, & Pepeu, 1982; Flicker, Bean, Watkins, Fisher, & Bartus, 1983). Unfortunately, these lesion studies have not been sufficient for the creation of an animal model. The problem lies in the nature of the lesion and its effect on the measurement of cognitive deficit.

The magnocellularis cells of the rat are embedded in a matrix of fibers of passage and noncholinergic perikarya. The individual cells that collectively comprise a "nucleus" are not, in fact, an anatomically well defined mass. Rather, they are dispersed rostrally and caudally over a rather large area. Lesions caused by electrolytic means would necessarily destroy other noncholinergic cell bodies and axons. Since most measurements of cognitive ability rely on behavioral indices, wholesale destruction via electrolytic lesion may introduce behavioral deficits, and possibly cognitive abnormalities unrelated to nbm function. For example, the globus pallidus, located immediately adjacent to nbm, is intimately involved in the generation and regulation of species specific fixed action patterns. Electrical stimulation or destruction of loci controlling fixed action patterns may result in modified behavior including changes in response to sensory stimulation and reinforcement, and the endogenously generated emission of behavior (Caggiula, 1970; Lynch, Rose, & Gall, 1970, Komisaruk & Old, 1968; Towle & Jennings, 1969; Huston, 1971). Destruction of nbm using electricity

cannot be sufficiently restricted so as not to involve the globus pallidus or other adjacent structures, or noncholinergic perikarya and fibers of passage. Some researchers have used ibotenic acid or kainic acid in an effort to limit damage to only cell bodies. Although fiber of passage destruction may be reduced, noncholinergic cell bodies in the lesioned area are not spared by these chemicals. There exists a need for a chemical or some methodology whereby only the nbm is inhibited or destroyed. This would permit reliance on the validity of behavioral measurements designed to assess cognitive ability insofar as the tests employed are valid and reliable. It should also reduce problems in the interpretation of anatomical and biochemical changes that may result upon the inhibition or destruction of the cholinergic neurotransmitter system. The use of noncholinergic specific lesions could cause changes in other neurotransmitter or regulatory systems that might obscure nbm related changes.

Montione, Fisher, and Hanin (1981) injected ethylcholine mustard aziridinium ion (AF64A) into the cerebral ventricles of mice and observed a loss in the functional, sodium-dependent, high-affinity choline transport sites and reduction in total brain ACh. Sandberg et al. (1982) reported an in vitro study wherein it was concluded AF64A enters the cell via the HAS and inhibits the enzymes involved in choline metabolism thereby reducing phosphatidylcholine concentrations and possibly disrupting the cell membrane. AF64A was injected into the nbm of rat by Antuono, Sorbi, Bracco, Fusco, and Amaducci (1984). A deficit in passive avoidance learning was observed, as was

a significant reduction in frontal cortex CAT activity. AF64A may prove to be the cholinergic specific chemical required for the development of an animal model for AD. However, certain questions may be raised regarding this compound. First, AF64A is new and has undergone only limited testing. There has been insufficient testing against other neurotransmitters, enzymes, etc., to demonstrate conclusively its specificity to choline containing neurons. Second, the HAS of the cholinergic neuron is localized to axon terminals (Suszkiw & Pilar, 1976). AF64A supposedly gains entry into the neuron through the high affinity transport system. The question might then be raised as to why application of AF64A directly to nbm should have any effect since transport via HAS is not available at the soma, unless, of course, AF64A is not specific to the HAS of the cholinergic neuron. Indeed, in a study by McGurk and Butcher (1985), it was demonstrated that administration of AF64A at effective dose levels was not specific to cholinergic neurons at all. Injection of AF64A into various brain sites, including nbm, resulted in the destruction of all cell bodies and fibers of passage at high doses, or no significant cell damage (including cholinergic neurons) at lower doses. Finally, when AF64A was injected into the lateral ventricle of rats "animals developed neurological disturbances (such as hypokinesis) and various ataxic syndromes with variable frequency, and typically lost 10 to 20 percent of their original weight" (Montione et al., 1981). This occurred within three days of treatment and suggests an effect not limited to the cholinergic

system. Thus, specificity of AF64A to the cholinergic neuron has not been conclusively demonstrated, and may be seriously questioned.

There is an additional consideration relative to the use of AF64A in the development of an animal model for AD. AD is a slowly progressive disease. If AF64A is immediately cytotoxic, even if specific to cholinergic neurons, this precludes using the chemical to investigate various stages of the disease. It is likely that the microscopic changes (i.e., neurofibrillary tangles, vacuoles, and plaques) characteristic of AD require considerable time to develop, and that changes in the cholinergic neurotransmitter system induce alterations in other neurotransmitter systems or neuromechanisms. A chemical that causes immediate wholesale destruction would not leave neurons behind that might be expected to develop the characteristic neuroanatomical anomalies. Also, testing drugs that may be efficacious for maintenance of the function of the cholinergic or other systems during the course of the disease would be difficult at best. Immediate cytotoxicity would remove the cholinergic system leaving no substrate for the action of beneficial drugs.

Hypothesis

It is hypothesized that direct infusion of HC-3 into the area of the nbm of the rat over a 2-week period will result in the inhibition or destruction of cholinergic neuron function by preventing the uptake of choline, the depletion of endogenous supplies of choline, and the subsequent failure of intraneuronal synthesis of ACh. Comparison of

HC-3 infused brains with brains from saline infused control subjects should demonstrate differences in the physical status of cholinergic neurons and markers for ACh. Further, it is hypothesized that inhibition of cholinergic neuron function will result in a memory based behavioral deficit, measurable by testing maze learning performance and comparison with the maze behavior of saline infused control subjects. Measurable differences should also be indicated when the performance of HC-3 infused subjects is compared to the performance of the same group before surgery.

It is proposed that inhibition or destruction of cholinergic neurons will result in a reduction of AChE in nbm perikarya, observable by appropriate staining for AChE in brain slices. If cholinergic nbm cells are destroyed as a result of the inhibitory or other effect of HC-3, cell body degeneration should be observable with the application of an appropriate nissl stain, and axonal and terminal field destruction should be evident through the application of a degenerative silver stain. If neurofibrillary tangles and neuritic plaques form as a result of neuron degeneration induced by HC-3 or some other effect, these structures may be observable with appropriate stains (e.g., Hicks stain or thioflavin-S stain).

Method

Subjects

Twenty adult male Sprague-Dawley rats, 10 control subjects and 10 experimental subjects, were used in this study. The free feeding

weight of each subject was between 450 g and 600 g at the beginning of the study. Experimental and control rats were individually housed in a single room at the University Animal Facility under constant temperature (20°C ± 1.7°) and humidity (50% ± 10%). The diurnal cycle was set at 12 hours, and water was continuously available.

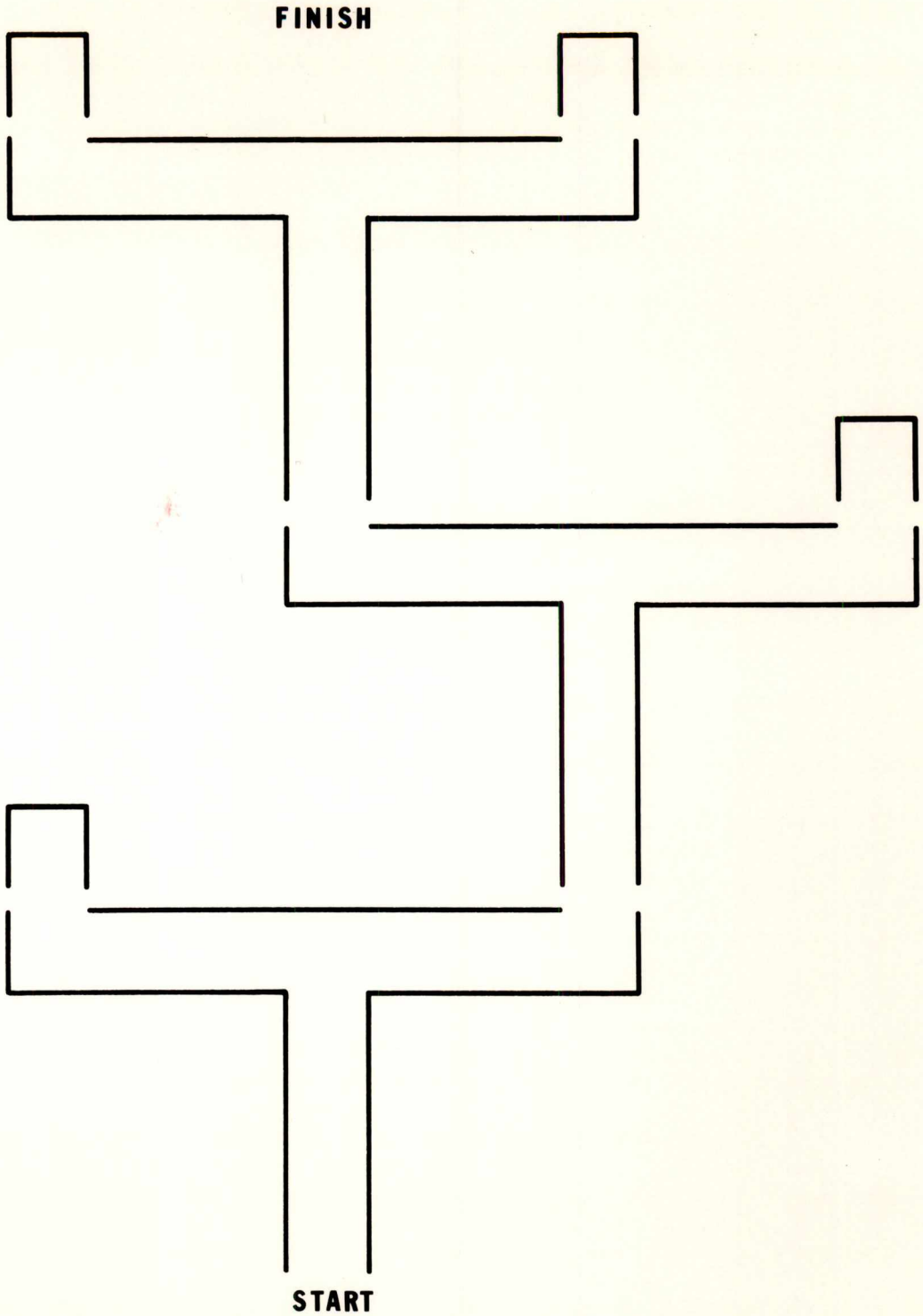
Apparatus

Three "T" mazes were combined to form a composite multiple "T" maze with three choice points in order to test short term spatial memory. Each "T" of the maze consisted of a transverse arm 105 cm in length and a perpendicular leg 46 cm long. Internal height and width of the arm and leg was 12.8 cm, respectively. A scale drawing of the maze is shown in Figure 3.

Procedure

Subjects. Prior to the start of the experiment, each subject was gradually reduced to approximately 80% of its free feeding weight and maintained at that weight on a diet of Purina #5001 rat chow. Each animal underwent a gentling procedure at the beginning of the project consisting of 2-4 minutes per day for 5 days of holding and stroking. Each rat gained familiarity with the maze and was fed in it for 10 minutes each day for three consecutive days. This occurred after reduction to 80% of free feeding weight. During this familiarization process free access was allowed to one 5 gram food pellet in the reward

Figure 3. A three "T" maze. A combined three "T" maze as diagrammed was used to test each subject for memory based cognitive deficit. Each "T" and reward box could be removed and repositioned to create eight different mazes of equal difficulty.


FINISH
START

box. The configuration of the maze was not changed over this 3-day period.

Maze configuration. The "T's" were combined by attaching in a perpendicular direction the open end of the leg of one "T" to either the left or right upper extent of the transverse arm of another "T." The reward box, with 12.8 x 12.8 x 12.8 cm internal dimensions, was attached to the left or right upper extent of the last "T." Thus, a subject placed at the end of the leg distal to the transverse arm of the first "T" must correctly choose a right or left turn upon reaching the transverse arm to gain entry to the distal leg of the next "T," and so on, until the reward box is entered. Empty boxes of the same dimensions as the reward box were placed at the opposite end of the transverse arm from the site of attachment of the leg of the next "T." The three "T"'s, with reward and dummy goal boxes, could be recombined into eight different combinations providing eight different maze configurations of the same difficulty. The eight possible combinations are illustsrated in Appendix E. The configuration of the maze was changed for each experimental trial, thus requiring subjects to utilize short term memory to learn the correct path to the reward box.

Maze test procedure. After gentling and familiarization with the maze but before surgery, each experimental and control subject was tested in the maze. Each subject was placed at the open end of the leg of the first "T." When the hind legs of the animal crossed the threshold of the maze a stopwatch was started. At the intersection of

each transverse arm a record was made regarding the direction first chosen by the subject. When a subject's front legs crossed a line placed 10.26 cm down an arm from the intersection, that choice of direction was recorded as correct or incorrect depending on the maze configuration. When a subject's nose crossed the threshold of the reward box, the time on the stopwatch was recorded. Each subject was allowed 10-15 seconds access to food once a food pellet of #5001 Purina rat chow was grasped. If a subject did not begin eating within 1 minute of crossing the threshold of the reward box, it was removed from the reward box and started at the beginning of the maze for the second of 10 consecutive runs.

If a subject did not eat in the reward box at the end of run two, it was removed from the maze, returned to its home box, and not retested for 24 hours. All previous runs for that trial were discarded. If a subject ate, it was returned to the start of the same maze configuration within 3 minutes of its feeding period. This procedure was repeated until a subject ran the same maze configuration and received food reinforcement 10 consecutive times. At the conclusion of the 10th run, the subject was returned to its home cage. Ten consecutive runs composed one trial. Each rat received a new trial, with a new maze configuration for each trial, every 3 to 6 days, until three complete trials were recorded. If a subject "froze" in some part of the maze, after 2 minutes it was prompted to move by a light nudge at the dorsal base of the tail with a ruler. If the rat continued to remain immobile, it was removed from the maze after a total elapsed time of eight

minutes. All previous runs for that trial were discarded and the subject not tested again for at least 24 hours.

Surgical preparation and testing. Following initial baseline testing in the maze, each subject was anesthetized with an interperitoneal injection of sodium pentobarbital (45 mg/kg) and positioned in a Kopf stereotaxic instrument. A sagittal incision was made in the scalp and two holes drilled through the skull for placement of 30 gauge cannuli to terminate in the region of the nbm. Coordinates were derived empirically in pilot studies on adult males to preclude error as a result of variability in bregma and lambda from the continued elongation of the skull in immature subjects. Final coordinates correspond to Konig and Klippel: A: 5660 m; V: -1.7 mm; L: 2.8 mm.

Each cannula was chronically cemented in place with cranioplast, and subcutaneously attached via silastic tubing to an Alzet model 2002 miniosmotic pump inserted beneath the skin of the flank approximately 5 cm posterior to the cranium and 3 cm medial and ventral to the spinal column. Each pump, tube, and cannula was previously filled with 214 µl of 0.9% saline solution for control animals, or 214 µl of HC-3, 95% concentration (Sigma No. H-1632), and 0.9% saline vehicle for experimental animals. The incision was closed with chromic gut, one suture anterior to the cranioplast and either one or two sutures posterior to the cranioplast. Over the next 14 days, 0.385 µg/hr of HC-3 was delivered to each nbm in experimental animals. Control subjects received saline vehicle only for 14 days. Twenty-four to 30 hours after surgery each subject was tested in the maze according to

the above methodology. A second and third complete trial of 10 runs was obtained by the end of the 14-day infusion period. For each subject, a maze configuration was selected at random at the beginning of each test session from those configurations not previously utilized. The maze configuration used in each trial for each subject is recorded in Appendix D. Additional trials were obtained from those subjects allowed to survive past the infusion period, although these trials were not averaged with the three trials obtained during the 14 days of HC-3 infusion.

Five control and 5 HC-3 subjects were sacrificed within 16 days of surgery. Five control and 5 experimental subjects were allowed to survive from 27 to 106 days after surgery, for the purpose of several histological studies. One experimental and 1 control subject were sacrificed at approximately 27 days, 43 days and 106 days postsurgery. Two experimental and 2 saline subjects were sacrificed at approximately 70 days postsurgery.

Histological preparation. After anesthesia with sodium pentobarbital (45 mg/kg) each animal was perfused through application of the technique described by Wolf (1971), except that the descending aorta was clamped with mosquito forceps. Perfusion was performed utilizing 100 ml of 0.9% saline followed immediately with 100 ml of 10% phosphate buffered formalin. The animal was then decapitated, the brain removed, again according to the technique of Wolf (1971), and placed in 10% phosphate buffered formalin. After sectioning the brain on a rotating or sliding microtome, 40 micron thick serial sections of nbm

were obtained from the level of the anterior commissure through the level of the mammillary bodies. These sections were divided into serial groups of five without disturbing the rostral to caudal order. Serial sections within each group were then treated with the following stains, (1) thiocholine method of Koelle for AChE, (2) thionine for confirmation of infusion site and cell body destruction, (3) the cupric silver method of de Olmos for axon and terminal field degeneration, (4) thioflavin-S for plaque and neurofibrillary tangle formation, and (5) a modified Hicks stain, also for plaques and neurofibrillary tangles. The protocol for each of these stains is listed in Appendix A.

Statistical analysis. For the statistical analysis, the average number of errors over 10 runs for all 3 trials in the presurgery condition was computed for each control and each experimental subject. The average number of errors over 10 runs for all 3 trials for each subject in the postsurgery condition was also computed. The standard "t" test was used to generate probabilities relative to the null hypothesis for each of the following conditions:

1. All HC-3 presurgery subjects vs. all HC-3 postsurgery subjects.
2. All saline presurgery subjects vs. all saline postsurgery subjects.
3. All saline postsurgery subjects vs. all HC-3 postsurgery subjects.
4. All saline presurgery subjects plus all HC-3 presurgery subjects (baseline) vs. all HC-3 postsurgery subjects.

5. Baseline vs. all saline postsurgery subjects.

Individual means for each subject are listed in Appendix B.

The same statistical analysis was used to examine the same groups relative to the total time spent in the maze. An analysis of variance was also performed for errors and also for time in the maze. Results of this analysis are reported in Appendix C.

Results

Within 24 hours after surgery neither saline nor HC-3 rats displayed observable reduced motor or sensory capabilities. Both experimental and control subjects demonstrated good appetites, maintained self-grooming behavior, and engaged in exploratory behavior in the maze. It was noted that HC-3 postsurgery subjects appeared more active in the maze than in the presurgery condition, and more active than postsurgery saline rats. However, no objective measurement of this observation was attempted. Three HC-3 rats and three saline rats were retained over 43 days from the date of surgery. All 3 HC-3 subjects but no saline subjects began a gradual decline in health, evident by weight loss and apparent muscular weakness, beginning approximately in the 6th week after surgery. The longest surviving rat (106 days) was subjectively estimated to be the most debilitated. No experimental subject died as a result of infusion of HC-3.

Maze Behavior

Table 2 lists the "t" value and probability for each group statistically analyzed relative to error. Table 3 lists the "t" value and

Table 2

Statistical Comparison of the Average Number of Errors in Maze Made by HC-3 Experimental, Saline Control, and Baseline Groups

Groups	1st group M	2nd group M	"t"	Probability	d.f.
Presurgery HC-3 vs. postsurgery HC-3	.91	1.76	10.62	<.001	18
Baseline vs. postsurgery HC-3	.895	1.76	6.24	<.001	28
Postsurgery saline vs. postsurgery HC-3	1.05	1.76	8.49	<.001	18
Postsurgery saline vs. presurgery saline	1.05	.88	1.52	>.10	18
Baseline vs. postsurgery saline	.895	1.05	0.71	>.10	28

Table 3

Statistical Comparison of the Average Total Time in Maze Made by HC-3 Experimental, Saline Control, and Baseline Groups

Groups	1st group M	2nd Group M	"t"	Probability	d.f.
Presurgery HC-3 vs. postsurgery HC-3	72.3	150.2	8.22	<.001	18
Baseline vs. postsurgery HC-3	74.6	150.2	9.09	<.001	28
Postsurgery saline vs. postsurgery HC-3	94.3	150.2	4.68	<.001	18
Postsurgery saline vs. presurgery saline	94.3	76.9	1.23	>.10	18
Baseline vs. postsurgery saline	74.6	94.3	1.92	>.10	28

Figure 4. Performance in maze relative to error. Average errors per run. The "baseline" group includes all experimental and all control subjects before surgery (N = 20). The HC-3 group includes all subjects infused with Hemicholinium after surgical implantation of cannuli and pumps (N = 10). The saline group includes all subjects infused with saline after surgical implantation of cannuli and pumps (N = 10).

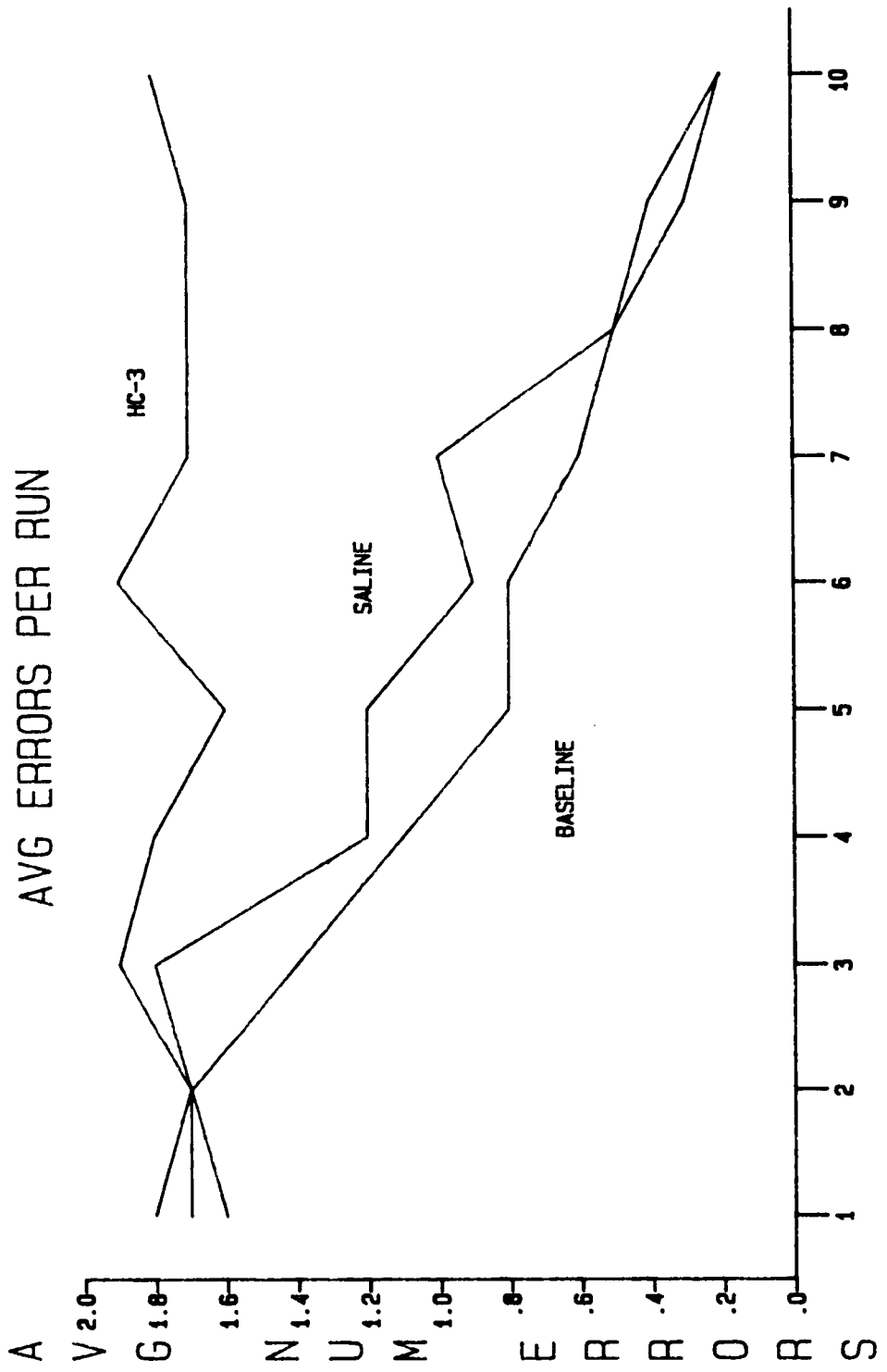

AVG ERRORS PER RUN
A
V 2.0
G 1.8
1.6
N 1.4
U 1.2
M 1.0
.8
E
R .6
R .4
O .2
R .0
S
HC-3
SALINE
BASELINE
1
2
3
4
5
6
7
8
9
10
RUN NUMBER

Figure 4.

probability for total time in the maze. Figure 4 illustrates the learning curve for HC-3 postsurgery subjects, saline postsurgery controls, and a combined baseline of presurgery HC-3 and presurgery saline subjects. Figure 5 illustrates the same groups relative to total time in the maze. Note that the HC-3 postsurgery group was unable to reduce the number of errors made in the maze, demonstrating a significantly different average in the presurgery condition from the postsurgery saline group, and from the baseline group. Postsurgery HC-3 rats also required significantly more time to reach the reward box despite appearing generally more active in the maze. There was no significant difference in average errors or average time in the maze between saline postsurgery and baseline groups, or postsurgery saline and presurgery saline groups.

Maze configurations that were utilized in this study for each subject are listed in Appendix D. All possible maze configurations are diagramed in Appendix E.

Histology

All cannuli were located within effective infusion range of nbm in both control and experimental subjects. Figure 6 illustrates the area affected by infusion as demonstrated by damage to ACh perikarya and total area of gliosis. Figure 7 is an example of a saline infused nbm stained for AChE. Except for incidental damage caused by cannula insertion, all saline subjects displayed no damage to nbm perikarya. Figure 8 is an example of a HC-3 infused brain stained for AChE. Note the virtual absence of cholinergic magnocellularis cells. All

Figure 5. Performance in maze relative to time. Average time per run. The "baseline" group includes all experimental and all control subjects before surgery (N = 20). The HC-3 group includes all subjects infused with Hemicholinium after surgical implantation of cannuli and pumps (N = 10). The saline group includes all subjects infused with saline after surgical implantation of cannuli and pumps (N = 10).

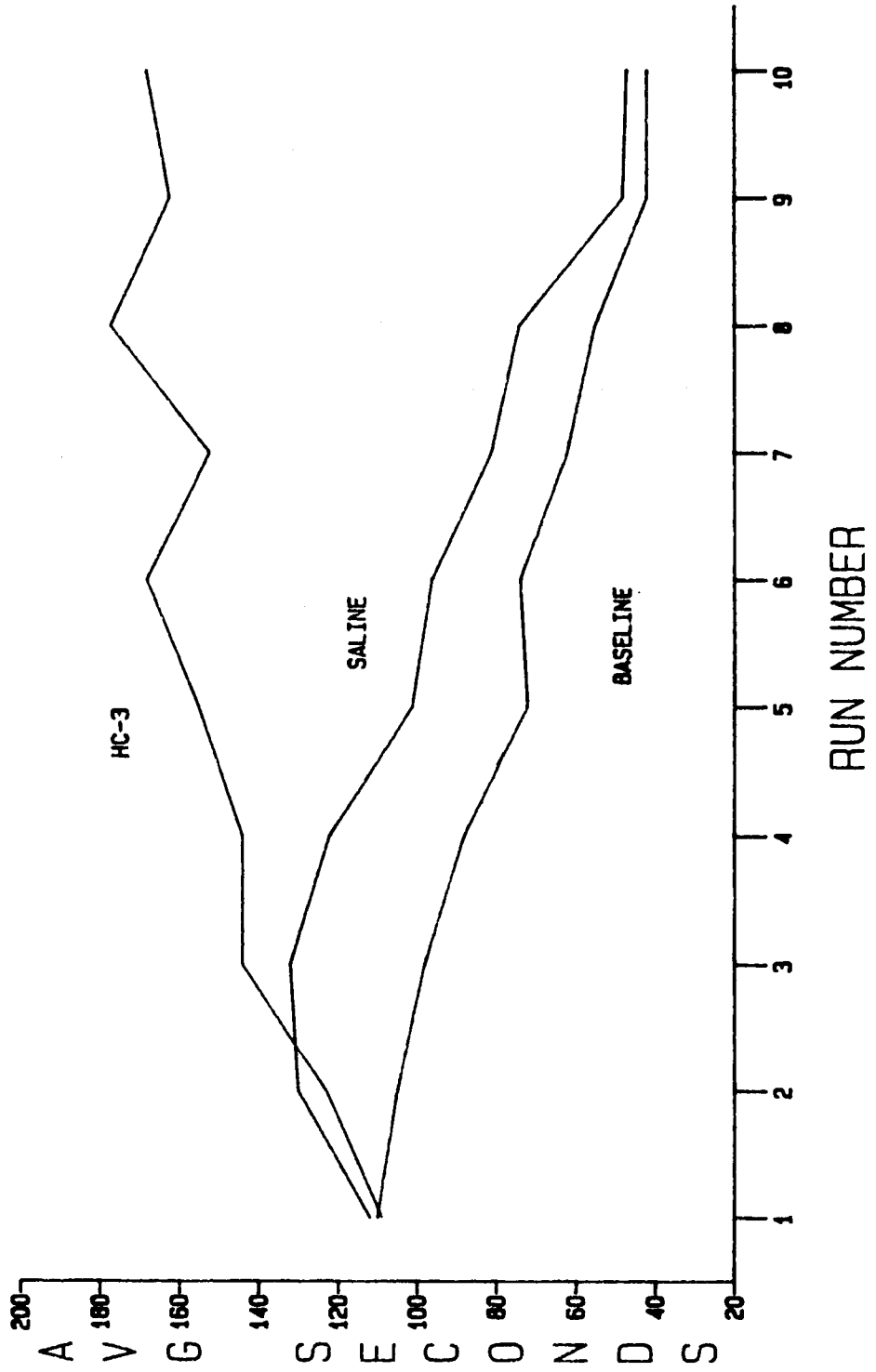

AVG TIME PER RUN
HC-3
SALINE
BASELINE
AVG SECONDS
RUN NUMBER
200
180
160
140
120
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10

Figure 5.

Figure 6. Extent of HC-3 infusion. Extent of the HC-3 infusion in all experimental subjects is indicated for the right and left hemispheres. The extent of infusion was determined by cholinergic neuron destruction and general gliosis in all brain sections from the same rostral/caudal level. The target area for cannuli placement was the midpoint of the rostral/caudal extent of nbm.

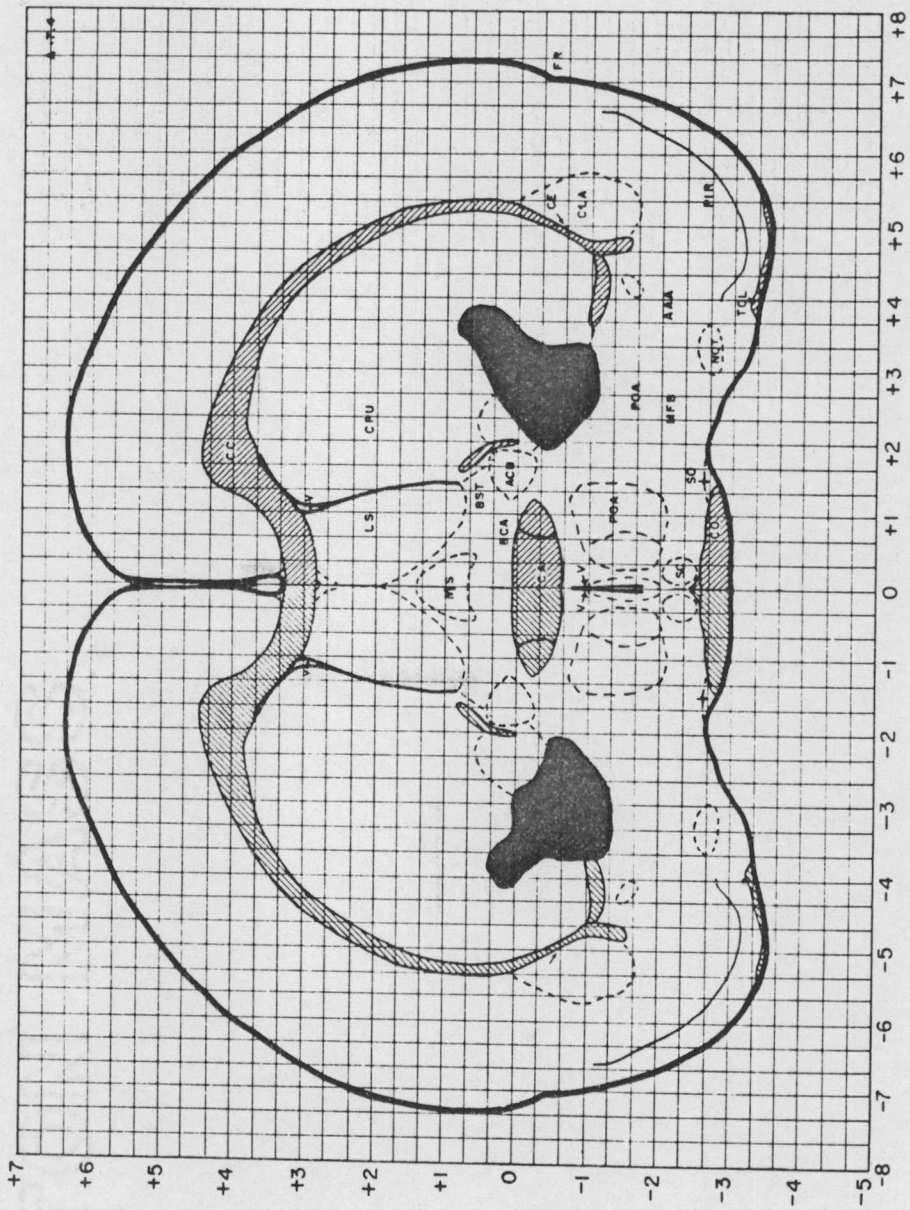

FR
CLA
CE
RIR
ATA
TDL
NCT
PGA
BFB
SO
CPU
LS
BST
ACB
ECA
MTS
POA
SCI
CC
+8
+7
+6
+5
+4
+3
+2
+1
0
-1
-2
-3
-4
-5
-6
-7
-8

Figure 6.

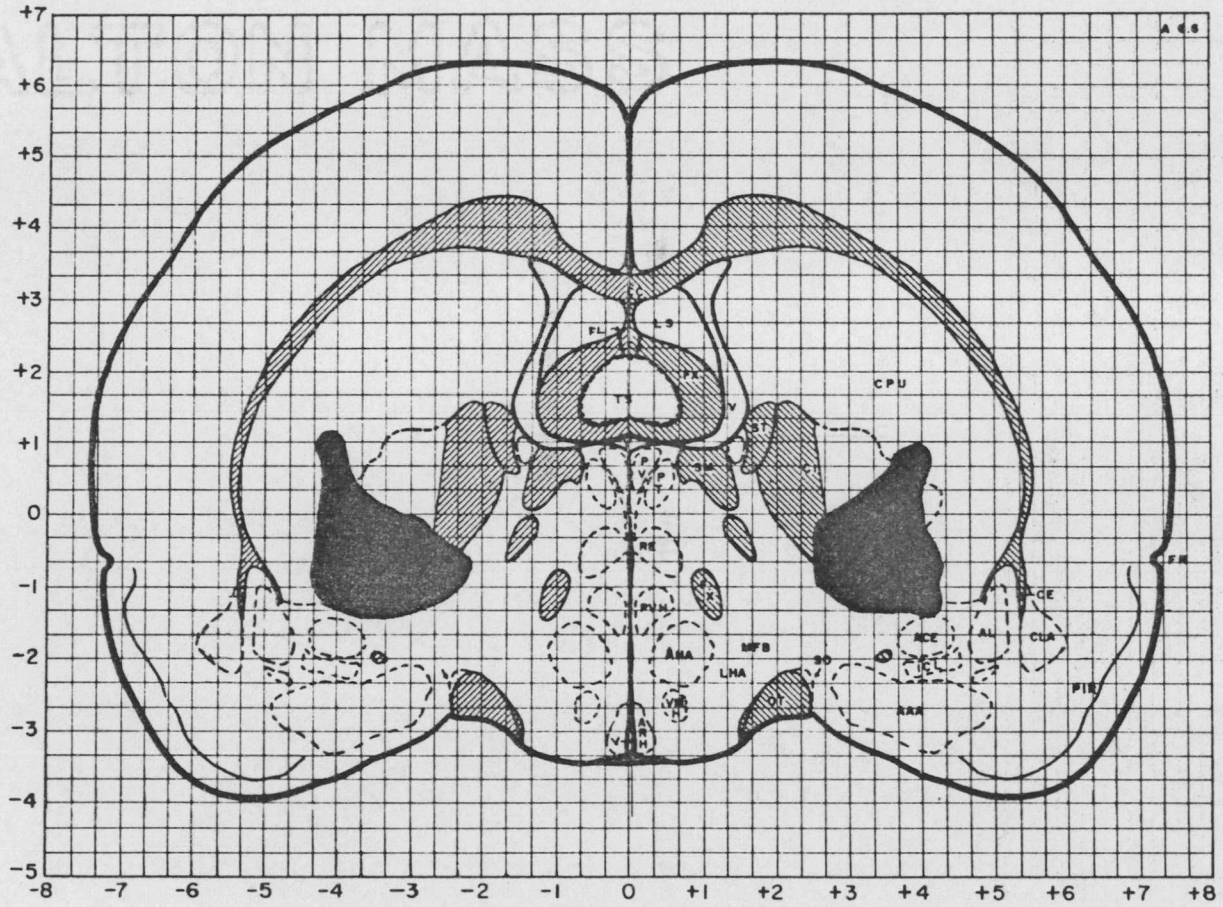


Figure 6 (continued)

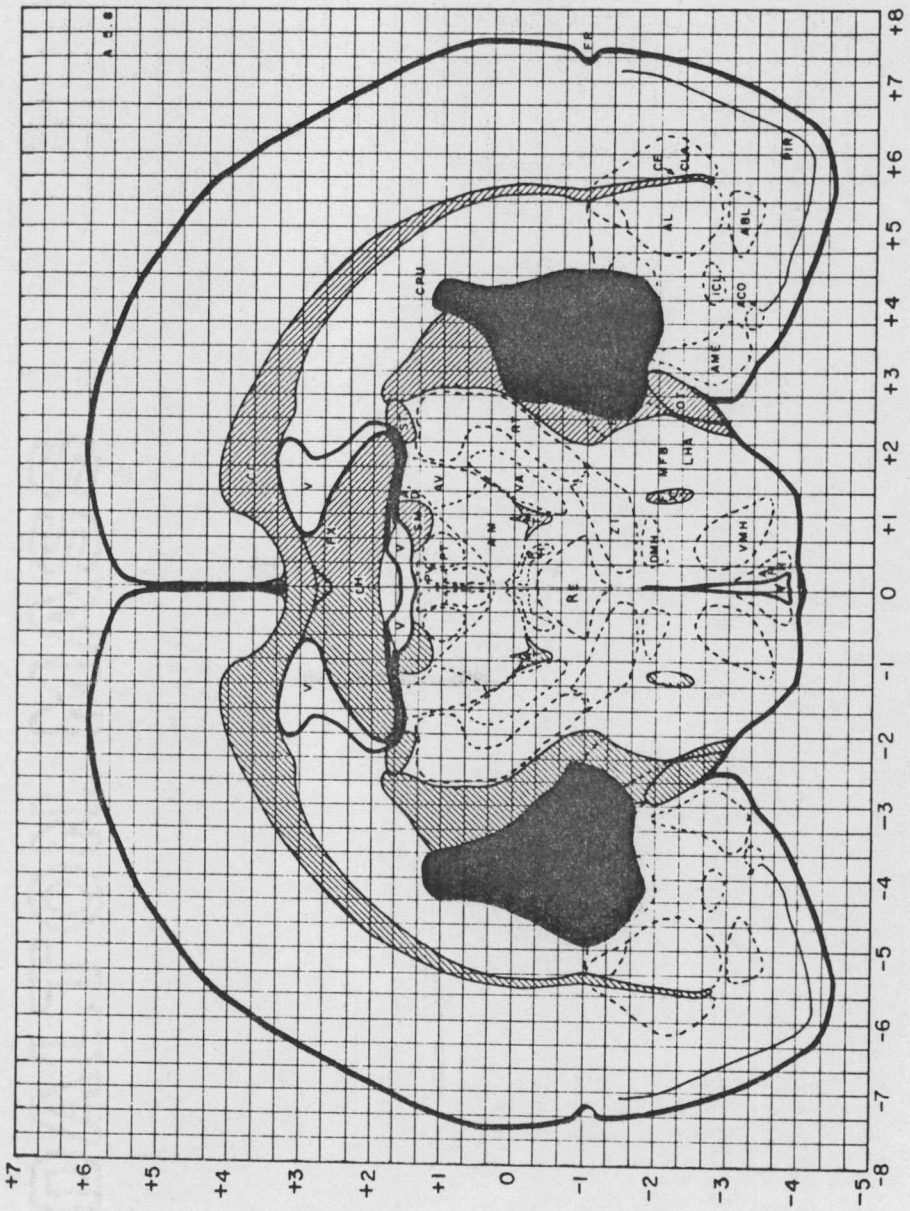


Figure 6 (continued)



Figure 6 (continued)

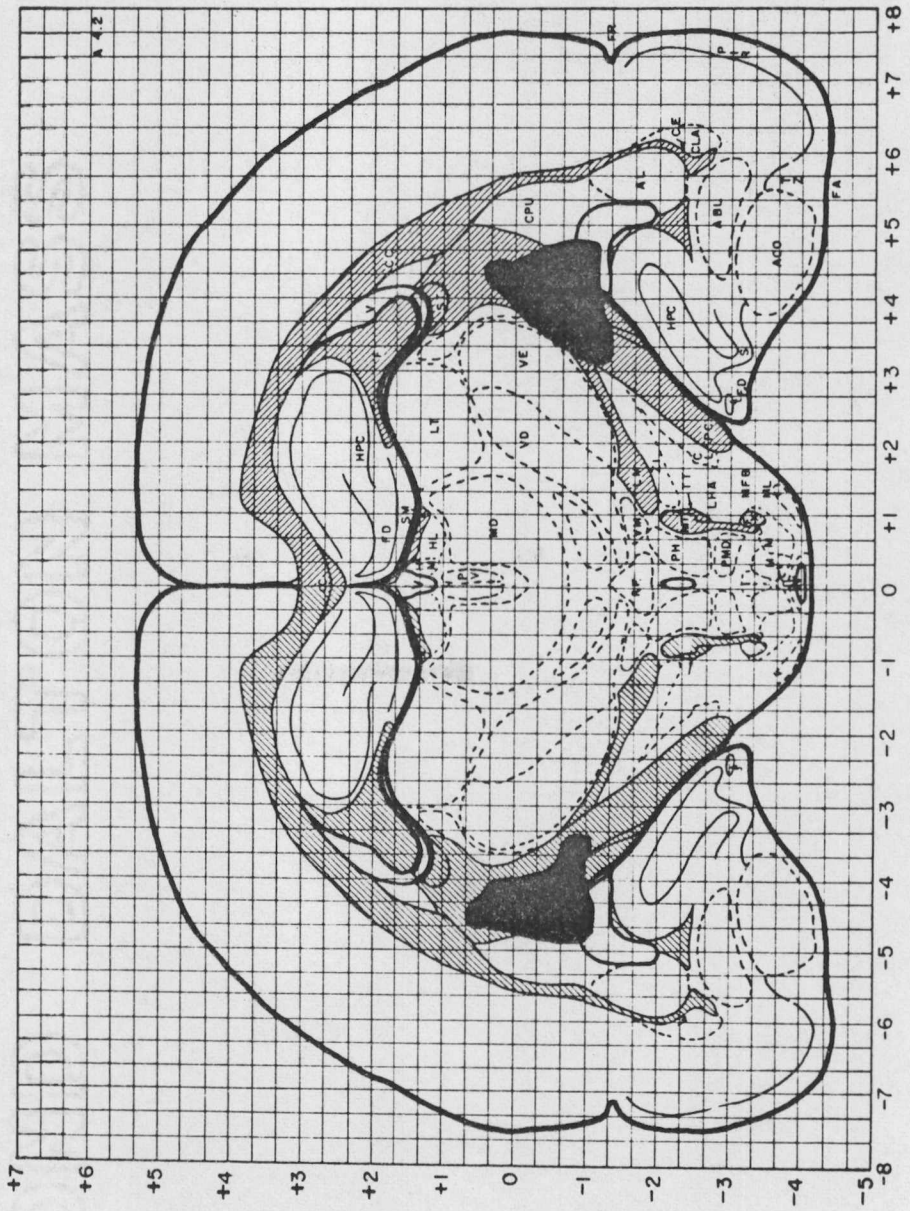


Figure 6 (continued)

Figure 7. An example of a saline infused brain stained for AChE. It is matched to Figure 12 for cannula placement, section level through nbm, and survival past surgery. Except for incidental damage caused by cannula insertion, all saline subjects showed no damage to nbm cholinergic neurons. Magnification: 80X.

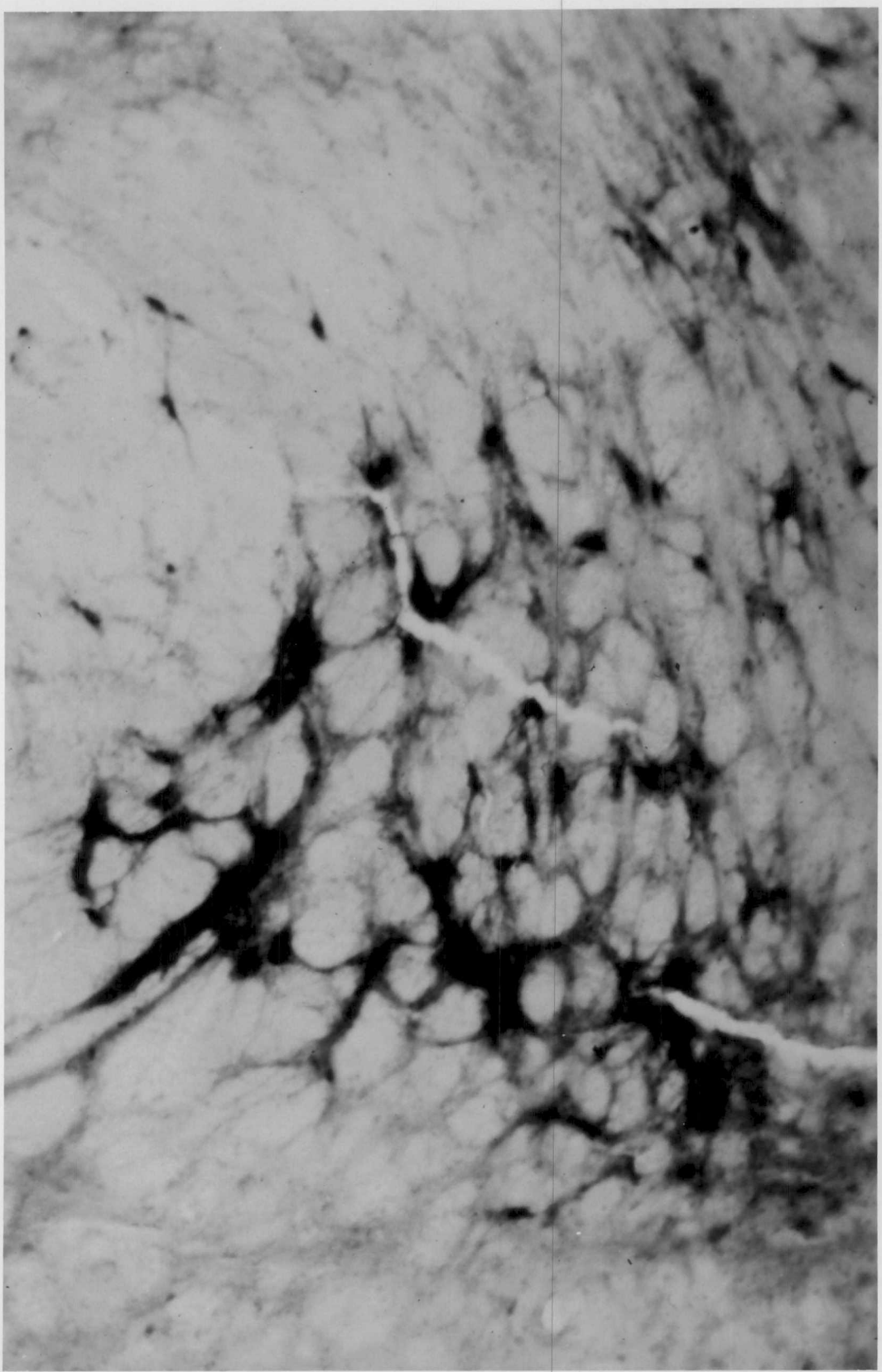
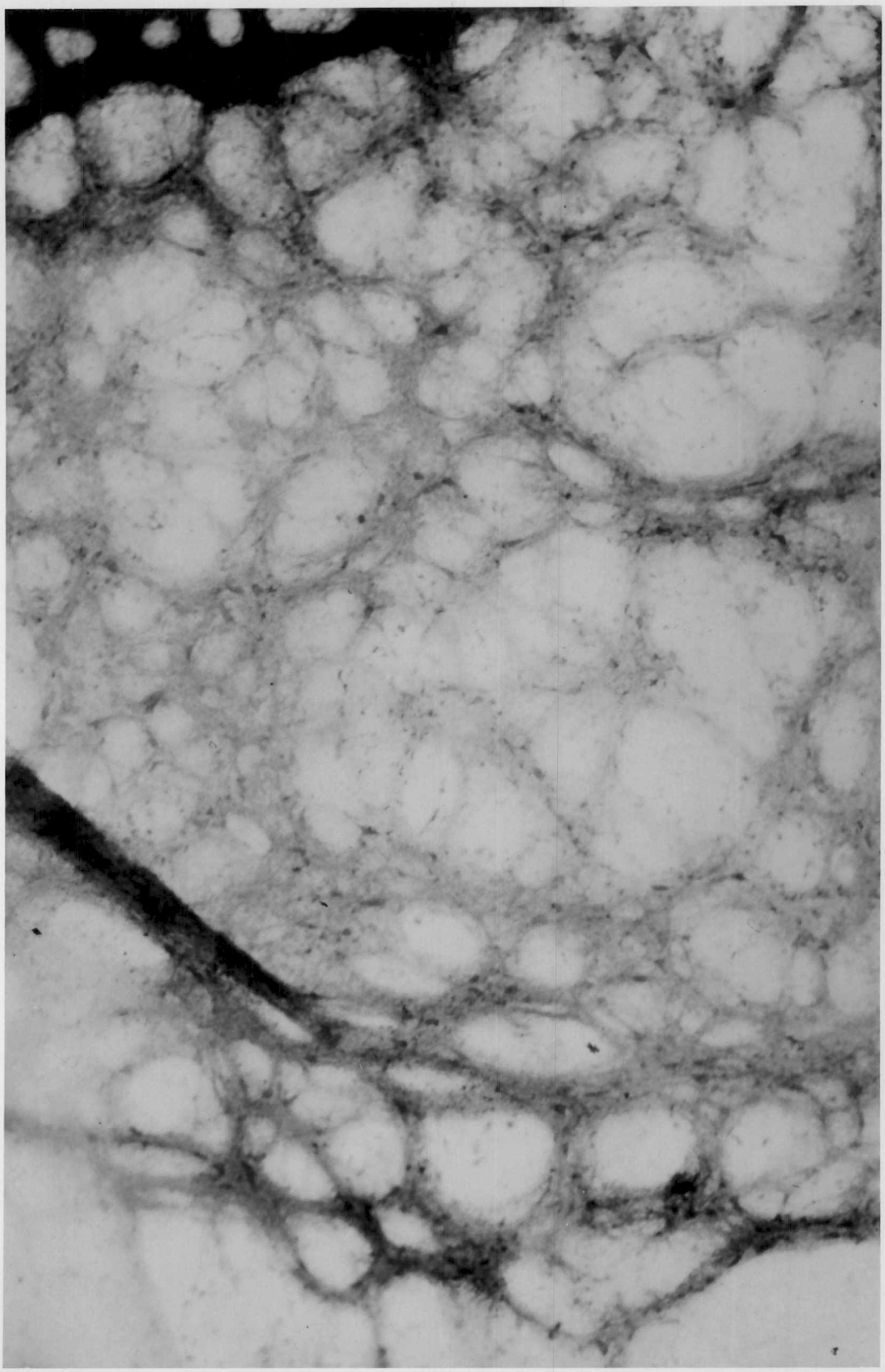


Figure 8. An example of a HC-3 infused brain stained for AChE. It is matched to Figure 11 for cannula placement, section level through nbm, and survival past surgery. All brains infused with HC-3 demonstrated nearly complete destruction of nbm cholinergic neurons. Mangification: 80X.



brains infused with HC-3 over 14 days demonstrated nearly complete destruction of nbm cholinergic neurons. In pilot studies to determine appropriate dosage and cannula placement, some brains were infused for periods less than 14 days but at the same HC-3 delivery rate (.385 µg/hr) as that used in the experiment proper. Figure 9 is a section displaying nbm from a brain that received HC-3 with appropriate cannula placement but with an infusion period of approximately 48 hours. Magnocellularis cells are still present, some partially damaged, with cellular debris suggesting either immediately prior or ongoing destruction of cholinergic neurons.

Figures 10 and 11 are sections from a saline infused brain and a HC-3 infused brain stained for axon and terminal field degeneration. These sections are matched for location of cannula, portion of cortex examined, and survival time past surgery. There was no apparent terminal field or axonal damage in the saline brain nor was there damage, other than that due to cannula insertion, in all other saline brains. The HC-3 infused brain, however, had sustained substantial degeneration, as did all HC-3 brains examined.

Although amyloid may not be produced in the rat CNS, the possibility of amyloid plaques perhaps resulting from the artificially induced destruction of nbm over time could not be ignored. Neurofibrillary tangles and neuritic plaques, distinctive of AD brains, may be the time related degeneration of neurofilaments of neurons. It was for this reason that saline and HC-3 subjects were maintained for varying lengths of time past the infusion period. Specific stains for amyloid plaques and for neurofibrillary tangles were negative.

Figure 9. Nbm stained with Koelle cholinesterase stain from a pilot study rat. Subject was infused with HC-3 for approximately 48 hours. Magnocellularis cells are still present, some partially damaged, with cellular debris suggesting either immediately prior or ongoing destruction of cholinergic neurons. Magnification: 250X.

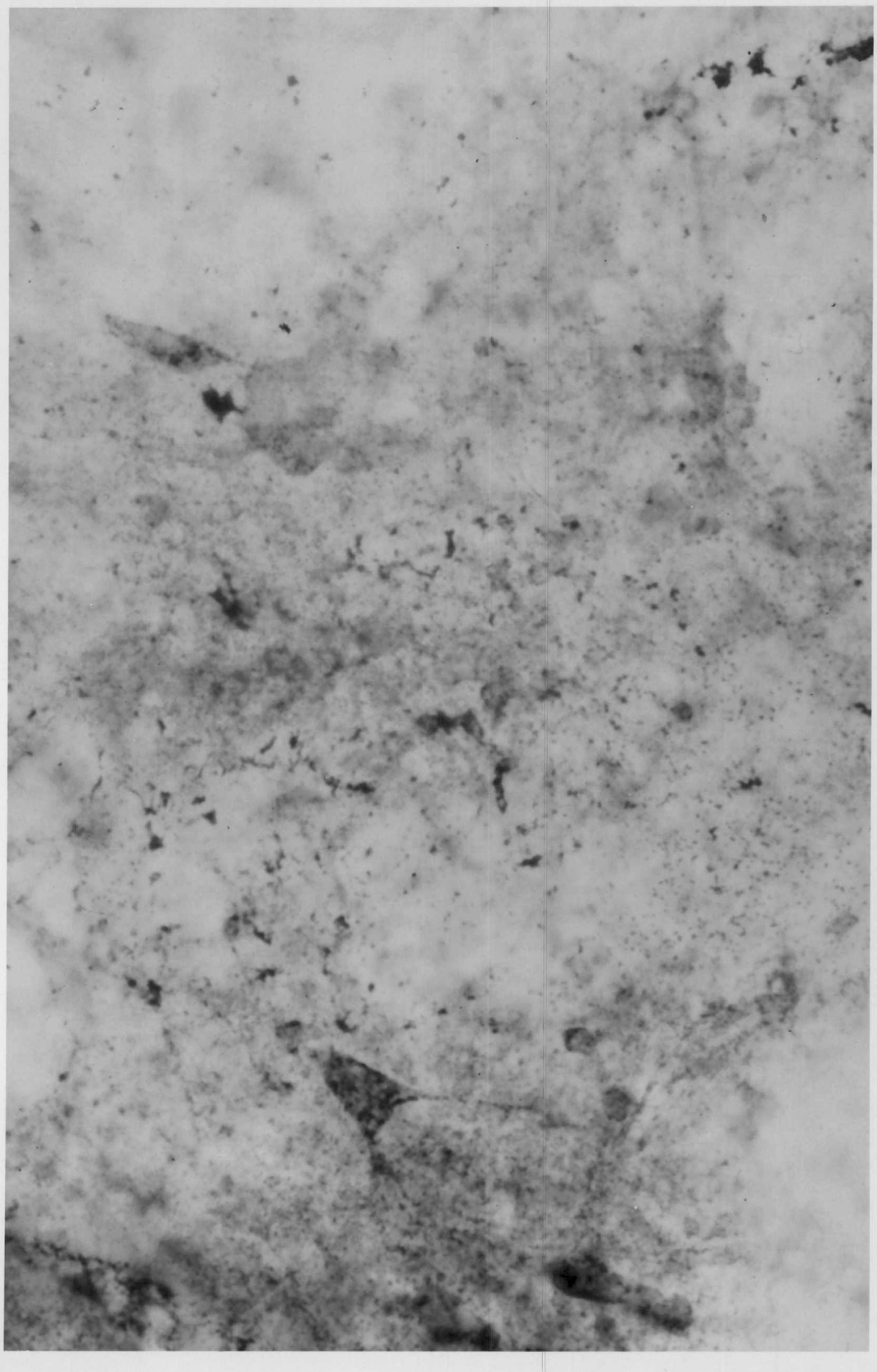


Figure 10. An example of cortex taken from a saline infused brain stained for axon and terminal field degeneration. It is matched to Figure 13 for cannula placement, portion of cortex examined, and survival past surgery. There was no apparent terminal field damage in all saline brains. Magnification: 80X.

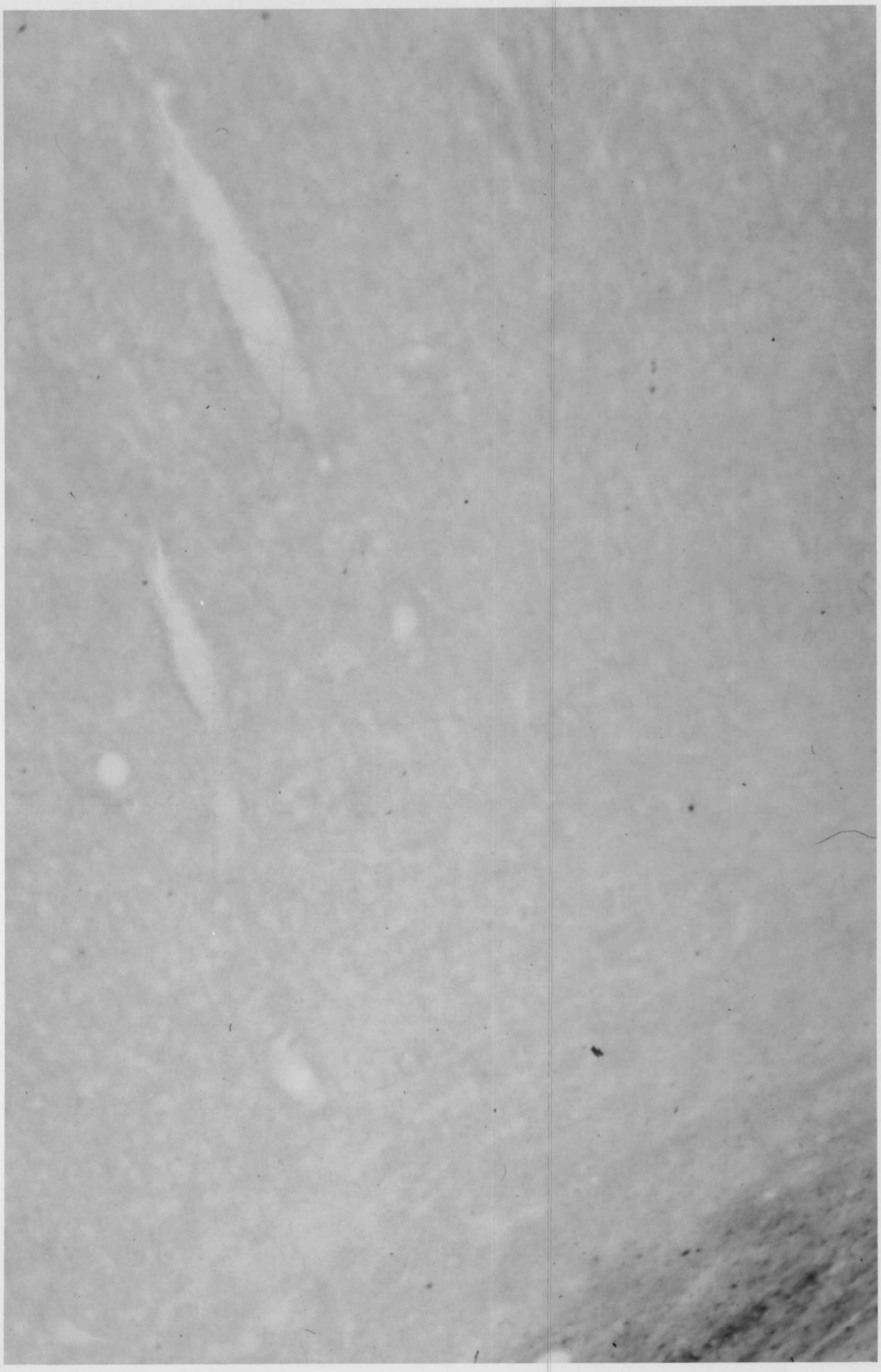
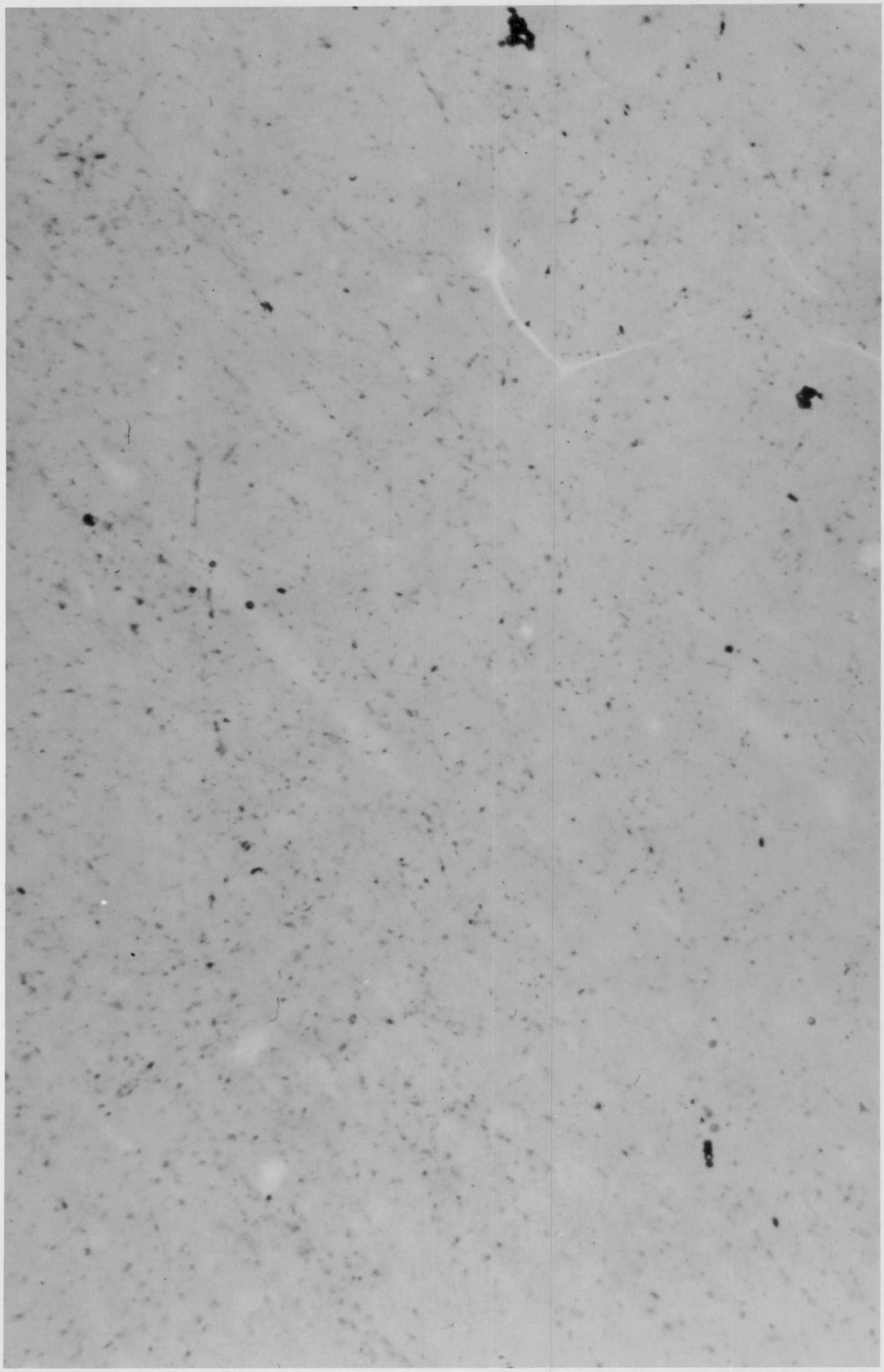


Figure 11. An example of cortex taken from a HC-3 infused brain. It is matched to Figure 14 for cannula placement, portion of cortex examined, and survival past surgery. There was substantial terminal field destruction in all HC-3 brains. Magnification: 80X.



Discussion

Rats that received a chronic infusion of HC-3 into nbm lost the ability to learn new mazes. This cognitive deficit was measurable within 24 hours from the start of infusion, based on the observation that HC-3 subjects did not reduce the errors made in the maze during the first trial of ten runs. Controls that received saline infusion demonstrated no loss in maze learning ability. Histological examination of saline infused brains displayed little damage to nbm cholinergic neurons and no terminal field destruction in cortex. HC-3 infused brains demonstrated almost complete destruction of nbm cholinergic neurons concurrent with axonal and terminal field degeneration in cortex. This is consistent with research demonstrating memory based cognitive deficits as a result of the destruction of nbm in rat, and nbM in human (Bartus, 1982; Flicker, 1983; Friedman, 1983; Glick, 973; Lehman, 1980; Salomone, 1984). Other research indicates a role for cortex and frontal cortex in spatial learning (Kolb, 1983; Becker, 1980).

As noted previously, HC-3 is not immediately cytotoxic and affects cholinergic neuron function apparently only indirectly by competing with choline for receptor sites on the cell membrane without entering the cell. The exact cause of cholinergic cell death observed in this experiment is unknown. It may be conjectured that cell destruction not caused by cannula insertion may be the result of cellular atrophy produced by prolonged blockage of choline uptake, and thus the prevention of a vital cell function. It was noted that cholinergic

neurons located well within the infusion area were totally destroyed and that in areas more distal to the site of infusion, only a percentage of cholinergic neurons was destroyed. The boundary line denoting the end of the infusion area was defined by the presence of only healthy cells and no gliosis. Further research is required to eludicate the mechanism of cell death as a result of prolonged infusion of HC-3.

Other than impaired memory acquisition, no behavior deficits in experimental subjects were observed. However, it was noted that HC-3 subjects displayed a greater degree of activity in the maze in the postsurgical condition, despite the fact that postsurgically HC-3 infused rats required significantly more time to reach the reward box. The observed activity of these subjects in the maze was that of energetic exploratory behavior with frequent reversal of direction resulting in a continuous reinvestigation of areas explored only seconds before. Although caution is mandatory in anthropomorphic interpretations, over each single trial of 10 runs, HC-3 infused subjects seemed to display confusion and indecision, especially at left/right choice points. In fact, as indicated in Figure 5, p. 84, the average time per run actually increased with additional experience in a particular maze despite the pace of motor activity remaining high. This investigation was designed to measure only one type of cognitive deficit, that of spatial memory acquisition. The appropriateness and utility of an animal model is dependent on the similarity of the method used to cause debilitation in the animal compared to the actual process of destruction in the disease. It is also dependent on similarity of effect on behavior and

cognition. Additional measurement of behavior and cognitive capabilities affected by prolonged infusion of HC-3 is required.

An additional observation requires further investigation. HC-3, but not saline, infused rats maintained over 6 weeks from the date of surgery began an apparent decline in health. In pilot studies to determine appropriate HC-3 dose levels, high levels of HC-3 (>2.25 µg/hr) caused piloerection, moderate to severe muscular weakness, and loss of appetite that was immediately obvious after recovery from surgery. This condition lasted until that animal was sacrificed. It would seem unlikely that HC-3, per se, was the cause of debilitation in the HC-3 postsurgery animals given the time span after infusion and before symptoms began. It may be that destruction of the cholinergic neurotransmitter system has an eventual dilatory effect on the function of other CNS systems. It is noteworthy that AD patients displaying severe cognitive deficits also experience a marked physical decline. It is much too early to draw parallels indicative of cause between the HC-3 induced destruction of nbm in the rat and the destruction of the cholinergic neurotransmitter system in humans resulting from AD. However, investigation of this possibility should be vigorously pursued.

Data from pilot studies wherein subjects received HC-3 for less than 14 days, and demonstrated only partial nbm degradation, together with reduced cholinergic cell destruction in the periphery of the infusion area in experimental animals, suggest that controlled partial destruction of nbm may be achieved. This may require a reduction in

HC-3 concentration because cell death began to occur within 48 hours of infusion at the dose level used in this experiment. Additional research to examine this possibility and to look for a correlation between degree of destruction and level of cognitive deficit might prove to be the next investigative step. Also, the neuroanatomical abnormalities commonly observed in AD brains were not developed in rat brain with the procedure utilized in this investigation. Neurofibrillary tangles and neuritic plaques may develop as the result of the very slow degeneration of cholinergic neurons. Thus, infusion of HC-3 over several months at very low concentration may be required to promote these phenomena, assuming rat brain has the capability of generating these anomalies.

Conclusions

In this investigation three direct and one indirect observations were made to indicate an effect of HC-3 on the cholinergic neurotransport system. These were: (1) destruction of nbm cholinergic perikarya as indicated by a loss of AChE staining magnocellularis cells, (2) destruction of magnocellularis cell bodies indicated by nissl stain, (3) degeneration of axons and terminal fields in cortex projected from nbm, demonstrated by silver degenerative stain, and (4) (the indirect indicator) a significant difference in maze learning ability in HC-3 experimental subjects relative to unoperated and saline infused controls. Although these indicators demonstrate an effect, it is acknowledged that this single experiment is certainly not nor was it intended to be

sufficient to prove that HC-3 is the most appropriate chemical for producing an animal model of AD. Several variables were not addressed in this investigation and these raise relevant questions that preclude such an assumption. For example, this study in no way examined the possible effect of HC-3 on noncholinergic neural mechanisms or elements. Although HC-3 has been demonstrated in earlier research to affect cholinergic neurons, there is little evidence regarding its effect on other types of cells. Also, AF64A has been criticized for its immediate cytotoxicity. In this study, cholinergic neuron degeneration was noted within 48 hours from the onset of HC-3 infusion. This could be a toxic effect that may be related to HC-3 dose level (either too high in concentration or too fast in delivery rate). If dose level is not the salient factor, then HC-3 may be limited in its applicability as is AF64A.

In summary, the infusion of HC-3 into nbm of rats caused the destruction of the cholinergic neurotransmitter system and a concomitant memory-based cognitive deficit. This suggests that HC-3 may be a tool for use in the development of an animal model for AD. Further research is required to investigate this possibility, including direct measurements of ACh, CAT, other neurochemicals, and additional indices of cognitive deficit using other behavioral tasks.

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APPENDIXES

APPENDIX A

Thionine, pH 4.5
(Nissl Stain)

Preparation

Fixation: Buffered formalin
Technic: Frozen sections 15-40 μ

Procedure

1. EtOH; 95%: 10 min
2. EtOH, Formalin; 95%: 5 min
3. EtOH; 95%: 3 min
4. Chloroform, ether, absolute EtOH: 10 min
5. EtOH; 95%: 3 min
6. HCl, EtOH; 10%, 95%: 5 min
7. EtOH; 70%: 3 min
8. Distilled H_2O; Rinse: 2 min
9. Thionine stain (longer as stain ages): 3 min

Go back to step 8

10. EtOH; 70%: 3 min
11. EtOH; 95%: 3 min
12. Differentiate in 95% EtOH, acetic acid (watch closely; check every 2-3 min)
13. EtOH; 95%: 3 min; (dehydrogenation)
14. EtOH; 100%: 3 min
15. EtOH; 10%: 3 min
16. EtOH, Xylene; 100%, 1000%: 3 min
17. Xylene; 100%: 3 min
18. Xylene; 100%: 3 min
19. Coverslip

Solutions

1. 37% formaldehyde: 40 ml; 37% EtOH: 460 ml
2. 95% EtOH: 25 ml; ether: 25 ml; chloroform: 20 ml
3. HCl: 50 ml; 95 EtOH: 450 ml
4. Thionine, pH 4.5
   12.5 ml 1% acqueous thionine solution
   56.5 ml 0.2 M acetic acid
   42.5 ml Na Acetate 0.2 M
   138.5 ml H_2O
5. Acetic Acid, EtOH
   Glacial acetic acid
   500 ml 95% EtOH
   Replenish after 60-80 slides

Notes:
1. To restain, go back to Step 5 of procedure.
2. Make thionine fresh once a week.

Modified Hicks
(neurofibrillary tangle and plaque stain)

Preparation

Fixation: buffered formalin
Technic: Frozen sections 15-40 μ

Procedure

1. After sectioning collect in 10% formalin.
2. Rinse in distilled H_2O to remove formalin.
3. Place the sections to be stained (no more than 4 sections at a time) in ammonia water: 5 min; agitate gently.
4. Rinse sections in two changes of distilled water: 1 min each.
5. Place the sections into silver staining solution: 40 min
6. 1% citric acid: 3 min.
7. 0.5% acetic acid: 3 min.
8. Physical developer: 90 sec to 3 min; monitor with dissecting scope with underlighting.
9. 0.5% acetic acid: 2 min.
10. Water.
11. Mount in acetate buffer: Triton X mounting solution.
12. Dehydrate, clear and coverslip.

Solutions

1. Ammonia Water:
   Ammonium Hydroxide: 2 ml
   Distilled H_2O--make just before use: 100 ml

2. 1% Silver Nitrate:
   Silver Nitrate ($AgNO_3$): 0.6 gm
   Distilled H_2O: 60 ml

3. 1% Potassium Carbonate: Potassium Carbonate (K_2CO_3)
   Anhydrous: 0.5 gm
   Distilled H_2O: 50 ml

   K_2CO_3 MUST be pure (reagent grade) and anhydrous

4. Pyridine-Silver-Carbonate Staining Solution:
   1% $AgNO_3$: 60 ml
   Pyridine (Baker Reagent Grade 1-3348): 2.7 ml
   1% K_2CO_3: 45 ml

   Add the pyridine to the silver nitrate and stir; then add the potassium carbonate to the silver-pyridine mixture and stir well. It is important to add the solutions together in the order listed so no precipitate will form.

5. 1% Citric Acid:
   Citric acid: 1 gm
   Distilled H_2O: 100 ml

6. 0.5% Acetic Acid:
   Acetic acid: 1 ml
   Distilled H_2O: 200 ml

7. Physical Developer:

   Solution A: 5% Sodium carbonate: 25 gm
   Distilled H_2O: 500 ml

   Solution B: Dissolve in the following order:
   Ammonium nitrate: 1.0 gm
   Silver nitrate: 1.0 gm
   Tungstosilicic acid anhydrous, fw 2874
   (various with degree of hydration): 5.0 gm
   Distilled H_2O: 500 ml

   Solution C: Prepare exactly as Solution B and then add:
   3.5 ml of 37% formaldehyde (Commercial Strength)

   The physical developer is prepared by mixing the following amounts of A, B, and C, in the order given, with vigorous stirring:

   A--50 ml
   B--45 ml
   C-- 5 ml

   If the solution is anything but clear, discard and remix, slowly adding B and then C.

8. Mounting Solution:

   Stock Acetate Buffer, pH 4.6
   0.2 M sodium acetate: 500 ml; 13.6 gm to 500 ml H_2O
   0.2 M acetic acid: 500 ml; 6 ml to 500 ml H_2O

   Acetate Buffer Mounting Solution:
   Stock Acetate Buffer: 25 ml
   Distilled H_2O: 1000 ml
   Triton X: 4 drops

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Thioflavine-S
(Renders amyloid fluorescent under UV light)

Preparation

Fixation: buffered formalin
Technic: frozen sections 15-40 μ

Procedure

1. Mount sections and allow to air dry.
2. 95% Alcohol: 5 min
3. H_2O; wash to remove alcohol
4. Thioflavine-S; stain: 10 min
5. 70% alcohol; differentiate: 5-10 dips
6. H_2O; wash: 7 times 1 mm each
7. Dehydrate, clear, and coverslip with DPX mountant.

NOTE: May use Apthy's gum, 50% Karo Syrup, or glycerin to coverslip after Step 6.

Solutions

Thioflavine-S: 10 g dissolved in 1000 ml distilled H_2O

Cupric Silver Method of DeOlmos, 2.5%
(silver degeneration stain)

Preparation

Fixation: buffered formalin
Technic: Frozen sections 15-40 μ

Procedure

1. Distilled water: 3 min; try to make transfer time very short (see text)
2. Distilled water: 3 min
3. Distilled water: 3 min
4. 2.5% cupric-silver nitrate solution: 3-4 days
5. Pure acetone: 3-5 min; Start with 4 min. Pour off acetone and pour in diamine. Diamine must be prepared while sections are in acetone.
6. Diamine:
   7-15 min (cat)
   7 min (rat)
7. Reducer: until reddish highlights begin to diminish; use lesion are as a guide
8. Distilled water: usually 1½-2 min; timing not critical--hold point; timing determined by examination
9. 0.5% K ferricyanide: 3-7 min; color should be golden yellow
10. Distilled water x 2: 3-5 min

Solutions

1. 2.5% silver nitrate solution (make up no more than you will use that day). Add solutions in following order:

	25 gm Ag / 1000 ml
2.5% silver nitrate	
0.5% cupric nitrate	15 ml
1% cadmium nitrate	18 ml
pyridine	40 ml
95% ethanol	80 ml

2. Ammoniacal silver (Diamine):

   20% $AgNO_3$: 5 ml
   0.36% NaOH: 2.55 ml
   28% Conc. NH_4OH: 1.15 ml

3. Reducer (not more than 1-2 days old):

    10% commercial formalin: 12 ml
    1% citric acid: 7 ml
    95% ethanol: 100 ml
    Distilled water: Up to 1000 ml

4. 0.5% potassium ferricyanide (diluted 1:4 when used):

    K ferricyanide: 20 gm
    Borax: 2.5 gm
    Distilled water: 1000 ml

Acetylcholinesterase Stain

Preparation

1. Neutral buffered formalin, or formalin-sucrose ammonia of Pearson, or fix used for horseradish peroxidase (HRP)
2. Cryostat or on frozen stage 25-50 μ
3. If freeze sectioning, collection sections in trays containing saturated sodium sulfate 50 gm/1 over a bed of ice ca. 4°C

Procedure

1. Rinse: 7 times

2. Incubation: transfer slides or sections to incubation medium prewarmed to 37°C and incubate for times to be varied for optimal staining at areas of interest. Typically, these times may be 10 min to 3 hours; e.g. 90, 120, 150, 180 min

3. Distilled H_2O; Rinse: 7 times 1 min each

4. 1.25% sodium: 1-2 min (reducer)

5. Distilled H_2O; rinse: 7 times; 1 min each

6. 1% silver nitrate: 1 min 1% (silver intensification)

7. Distilled H_2O; rinse: 2 times, 1-2 min

8. 1% sodium; 1% sodium thiosulfate: 1 min

9. Distilled H_2O; rinse: rinse briefly in distilled H_2O twice

10. Acetate buffer: storage

11. Mount out of: acetate buffer-alcohol onto gelatinized slides, air dry, counterstain [see Thionine pH 4.5]

Solutions

1. Incubation medium:

   Stock solution:

   $CuSo_4$: 16 mM; 4 gm/L
   Glycine: 80 mM; 6 gm/L
   Acetate buffer: .2 M; pH 5
   30 ml Acetic acid: .2 M; 12 ml/l
   70 ml Na Acetate: .2 M; 27.2 gm/l

Iso-OMPA: 2×10^{-4} M made from a 2×10^{-3} M and solution; 68.4 mg/100 l

Final solution: mix A & B 1:1

A. Acetylthiocholine iodide: 0.226 gm/100 ml of acetate buffer
B. stock glycine: 25 ml
   stock $CuSO_4$: 25 ml
   acetate buffer: .2 M; 50 ml
   stock Ios-OMPA: 1 ml

3. Reducer:

   1% sodium sulfite: Na_2SO_3 10 gm/1 H_2O

5. Silver Intensifier:

   1% $AgNO_3$; 5 gm/500 ml H_2O

7. Fixative:

   1% sodium thiosulfate: 10 gm/1 H_2O

8. Acetate buffer alcohol:

   10 ml 0.2 M acetic acid
   10 ml 0.2 M Na acetate
   100 ml 95% EtOH
   130 ml dist. H_2O

APPENDIX B

Table B-1

Presurgery HC-3 Subjects: Average Errors Over Three Trials

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
8	1.33	2	1.67	.33	.67	1.33	.67	.33	1	0
9	1.67	2.33	.67	.33	,67	.33	.33	1	1	0
13	1	1.33	1.33	1.33	1	1.33	0	.33	0	0
15	1.67	1.67	1.67	1	0	1	.33	.33	.33	.33
17	1.33	1.33	1.67	1.67	1.67	1.67	.33	0	0	.33
18	1.67	2	1.33	1	1	.33	0	.33	0	.33
25	1.67	2	1.67	1.33	1.33	.67	1	0	.67	0
26	1.67	2	1.33	1.33	.33	.1	1	1.33	.33	.67
34	1.33	2.33	2.33	2	1.33	1.33	1	1.33	.67	0
41	1.33	1.33	.33	.67	0	0	.33	0	0	0
Total	14.67	18.32	14	10.99	8	8.96	4.99	4.98	4	1.66
Avg.	1.5	1.8	1.4	1.1	.8	.9	.5	.5	.4	.2

Table B-2

Presurgery HC-3 Subjects: Average Time Over Three Trials (Seconds)

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
8	45	67	50	39	33	67	35	47	53	28
9	93	87	42	31	41	29	34	37	32	18
13	76	82	82	112	79	100	42	37	44	53
15	86	62	82	52	43	58	37	34	60	68
17	80	101	203	155	121	90	91	46	31	32
18	68	74	82	62	42	44	28	33	27	38
25	127	99	127	72	78	56	67	53	50	41
26	117	109	86	80	78	146	80	104	34	107
34	120	139	170	169	112	166	163	166	96	74
41	184	112	57	90	18	18	15	25	15	14
Total	992	932	981	862	645	774	592	582	442	444
Avg.	99	93	98	86	65	77	59	58	44	44

Table B-3

Postsurgery HC-3 Subjects: Average Errors Over Three Trials

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
8	1.33	1.33	1.67	2.67	1.33	2.67	1.67	2	2	1.33
9	2.33	2.33	2	2	2.33	2	1.67	1.67	1.67	2
13	1.67	1.67	2.33	1.33	1	2.33	1.33	2.33	2	2.33
15	1.67	.67	.67	.67	.67	.33	.67	1.33	.67	1.67
17	2	2.67	2.67	2.33	2.33	2	2.67	2	2.33	2.67
18	2.67	2	1.67	2	2	.67	2	1	1.33	1.33
25	2.33	2	2	2	2.33	2	2.67	1.67	2	2
26	.33	1.67	2.33	2.33	2.33	2.33	1.33	1.33	1.67	1.33
34	2	1.67	2	1.33	1.67	1.67	2	2.33	2.33	2.33
Total	18.33	17.01	19.34	17.66	16.32	19	16.68	16.99	17.0	17.99
Avg.	1.83	1.70	1.93	1.77	1.63	1.9	1.67	1.70	1.70	1.80

Table B-4

Postsurgery HC-3 Subjects: Average Time Over Three Trials (Seconds)

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
8	48	78	64	91	87	90	76	144	124	155
9	209	123	256	210	212	232	203	238	222	205
13	65	143	175	106	122	160	124	216	130	183
15	136	146	104	155	251	236	173	224	171	205
17	68	109	115	110	122	221	247	241	175	234
18	89	75	54	102	131	104	113	130	142	158
25	168	203	147	184	226	242	248	224	205	110
26	147	208	309	278	235	221	177	151	178	226
34	102	104	133	156	132	145	133	162	233	173
41	58	40	82	46	32	30	27	40	41	35
Total	1090	1229	1439	1438	1550	1681	1521	1770	1621	1684
Avg.	109	123	144	144	155	168	152	177	162	168

Table B-5

Presurgery Saline Subjects: Average Errors Over Three Trials

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
12	2.33	.33	.67	.67	0	.33	.67	0	0	.33
19	2.33	1.67	1.67	1.33	.67	.33	.33	.33	.67	.33
21	1.33	2	1	1.67	.67	1	1	1	.67	.33
22	1.67	2	1	.67	1.33	.33	.33	.33	.33	0
23	1.67	2	1.67	.67	.67	.67	.67	.33	.33	0
24	2	1.67	1.33	1.33	1	1	.33	.33	.67	0
27	1.33	1.67	2	1.33	1.33	1.33	.67	.33	.67	0
30	2	1.67	1.67	.67	.33	.33	.67	1.33	0	0
36	1.33	1.33	2	1	1.33	.67	.33	1	.33	0
39	1	1.67	1.33	.33	.67	.67	.67	0	0	0
Total	16.99	16.01	14.34	9.67	8.0	6.66	5.67	4.98	3.67	.99
Avg.	1.7	1.6	1.4	1.0	.8	.7	.6	.5	.4	.1

Table B-6

Presurgery Saline Subjects: Average Time Over Three Trials (Seconds)

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
12	52	22	9	22	20	24	28	14	22	28
19	64	71	113	72	29	24	25	27	27	24
21	119	88	62	79	55	66	39	51	44	32
22	113	114	64	123	158	86	74	59	35	49
23	80	90	77	68	50	46	56	39	32	27
24	59	74	77	81	118	81	79	49	54	50
27	112	112	147	101	99	62	59	32	43	42
30	229	222	144	149	98	105	118	115	72	76
36	74	114	154	116	96	101	83	70	49	42
39	308	268	124	78	66	112	79	50	25	24
Total	1210	1175	971	889	789	707	640	506	403	394
Avg.	121	118	97	89	79	71	64	51	40	39

Table B-7

Postsurgery Saline Subjects: Average Errors Over Three Trials

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
12	1.33	.67	2	1	.33	.67	.33	0	0	0
19	1.33	2	2.33	1	1.67	.67	1	1	0	0
21	2.67	1	2	1.33	1.33	1	1.67	.67	0	0
22	1.67	1.67	1.67	2	.33	1.33	1.67	.33	0	0
23	1.67	2	2	1	1	1	1.33	.33	0	1
24	2.33	2	1	1	.67	1.67	.67	.67	1	.67
27	1.33	2.33	1.33	1.33	2	1.67	.67	.67	1	0
30	1.67	1.67	1.33	.67	1	.67	.33	.33	0	0
36	1.67	1.67	2.33	1	1.33	1	1.33	.67	.33	.67
39	1.67	1.67	2	2	2	.67	1	.33	.67	0
Total	17.34	16.68	17.99	12.33	11.66	9.36	9.67	5	3	2.34
Avg.	1.73	1.67	1.8	1.23	1.17	.94	.97	.5	.3	.23

Table B-8

Postsurgery Saline Subjects: Average Time Over Three Trials (Seconds)

	Trial									
Rat #	1	2	3	4	5	6	7	8	9	10
12	70	57	119	100	70	58	52	25	25	25
19	42	77	77	50	61	66	74	71	37	29
21	87	79	114	95	59	71	80	64	35	31
22	82	154	141	120	60	97	134	92	25	43
23	65	158	114	135	94	81	95	94	41	53
24	31	65	49	41	53	114	37	42	47	40
27	216	234	157	128	178	98	79	95	97	84
30	231	193	207	159	159	141	110	86	71	72
36	67	98	136	124	119	139	82	115	48	52
39	232	188	201	266	157	151	65	60	49	40
Total	1123	1303	1315	1218	1010	958	808	744	475	469
Avg.	112	130	132	122	101	96	81	74	48	47

APPENDIX C

Table C-1

Analysis of Variance for Errors and Time in Maze (Treatment: Saline or HC-3; Admin.: Preinfusion or Postinfusion

Test	SS	MS	DF	P<
	Errors			
Main effect of treatment vs. admin. (pre)	9.8	9.8	1	0.6352
Main effect of treatment vs. admin. (post)	2121.8	2121.8	1	0.0002
Contrast preinfusion saline vs. postinfusion saline	174.05	-	1	.1181
Contrast preinfusion saline vs. preinfusion HC-3	9.8	-	1	.7062
Contrast preinfusion HC-3 vs. postinfusion saline	101.25	-	1	.2299
Contrast preinfusion saline vs. postinfusion HC-3	3511.25	-	1	.0001
Contrast preinfusion HC-3 vs. postinfusion HC-3	3150.05	-	1	.0001
Contrast postinfusion saline vs. postinfusion HC-3	2121.8	-	1	.0001
	Time			
Main effect of treatment vs. admin. (pre)	74176.2	74176.2	1	.7694
Main effect of treatment vs. admin. (post)	13826182.05	13826182.05	1	.0148
Contrast preinfusion saline vs. postinfusion saline	1453144.05	-	1	.3099
Contrast preinfusion saline vs. preinfusion HC-3	74176.2	-	1	.8173
Contrast preinfusion HC-3 vs. postinfusion saline	2183944.09	-	1	.2149
Contrast preinfusion saline vs. postinfusion HC-3	24244020.0	-	1	.0002
Contrast preinfusion HC-3 vs. postinfusion HC-3	27000232.2	-	1	.0001
Contrast postinfusion saline vs. postinfusion HC-3	13826182.05	-	1	.0031

APPENDIX D

Table D-1

Maze Configurations Used for Each Subject

	Configuration		
Rat #	Trial 1	Trial 2	Trial 3
HC-3			
8	LLL	RLL	RLR
9	LLR	RRL	RLR
13	RLL	RLR	LLL
15	RLL	LLR	RRR
17	RRR	LRR	RRL
18	RLR	LRR	RRL
25	RLL	RLR	LRL
26	LRR	LLL	LRL
34	LLL	RLR	RLL
41	LRL	LRR	RLR
Saline			
12	LRR	RRL	RLL
19	RLR	LLL	RLL
21	RLR	LLL	RLL
22	RLR	LRL	RLL
23	RLR	RLL	LLL
24	RLR	RLL	LRR
27	LRR	LRL	RRL
30	LRR	LRL	RRL
36	LRL	RLL	LLL
39	LRR	RLR	RLL

L = left turn; R = right turn.

APPENDIX E

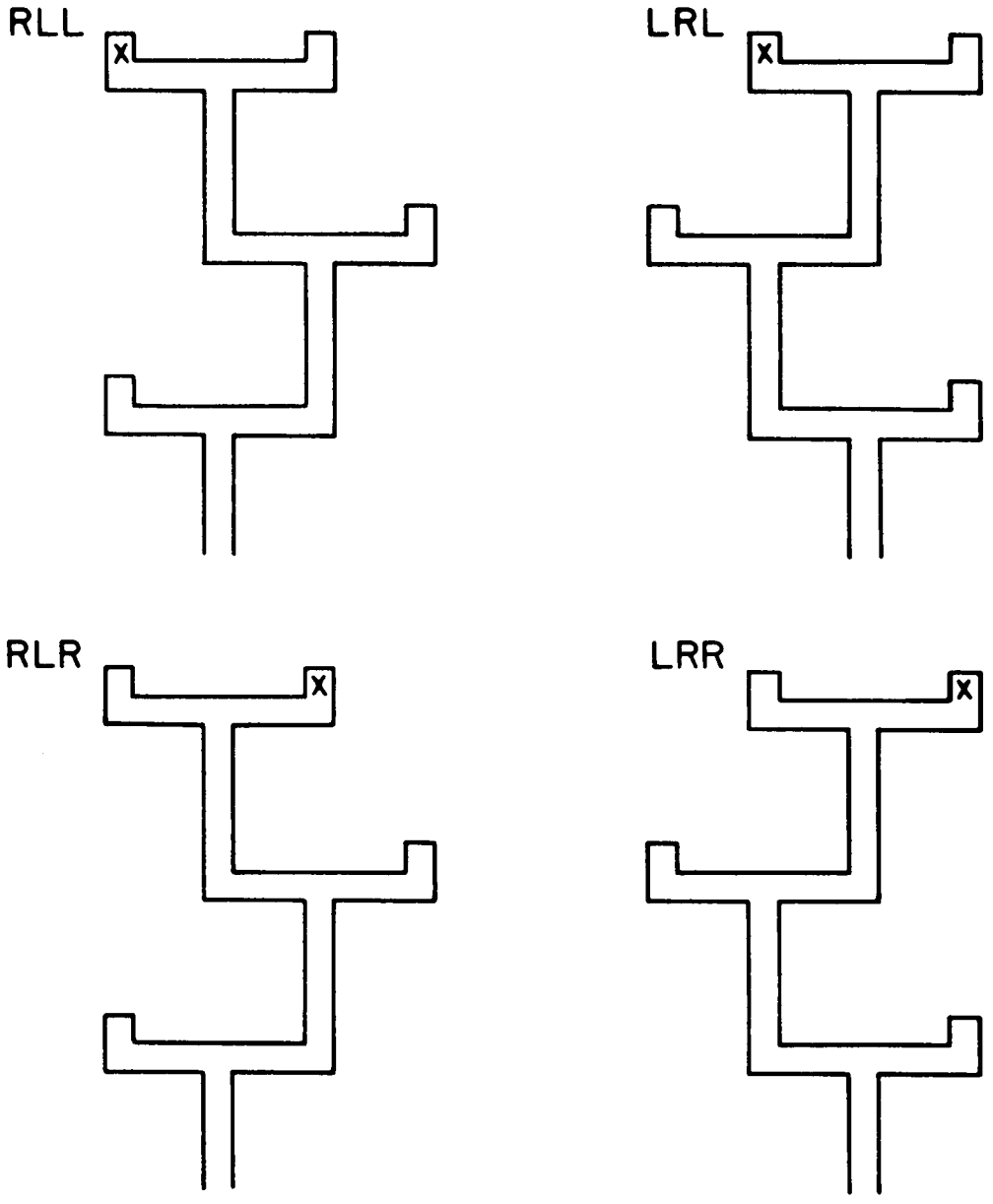


Figure E-1. All maze configurations ("x" indicates reward box).

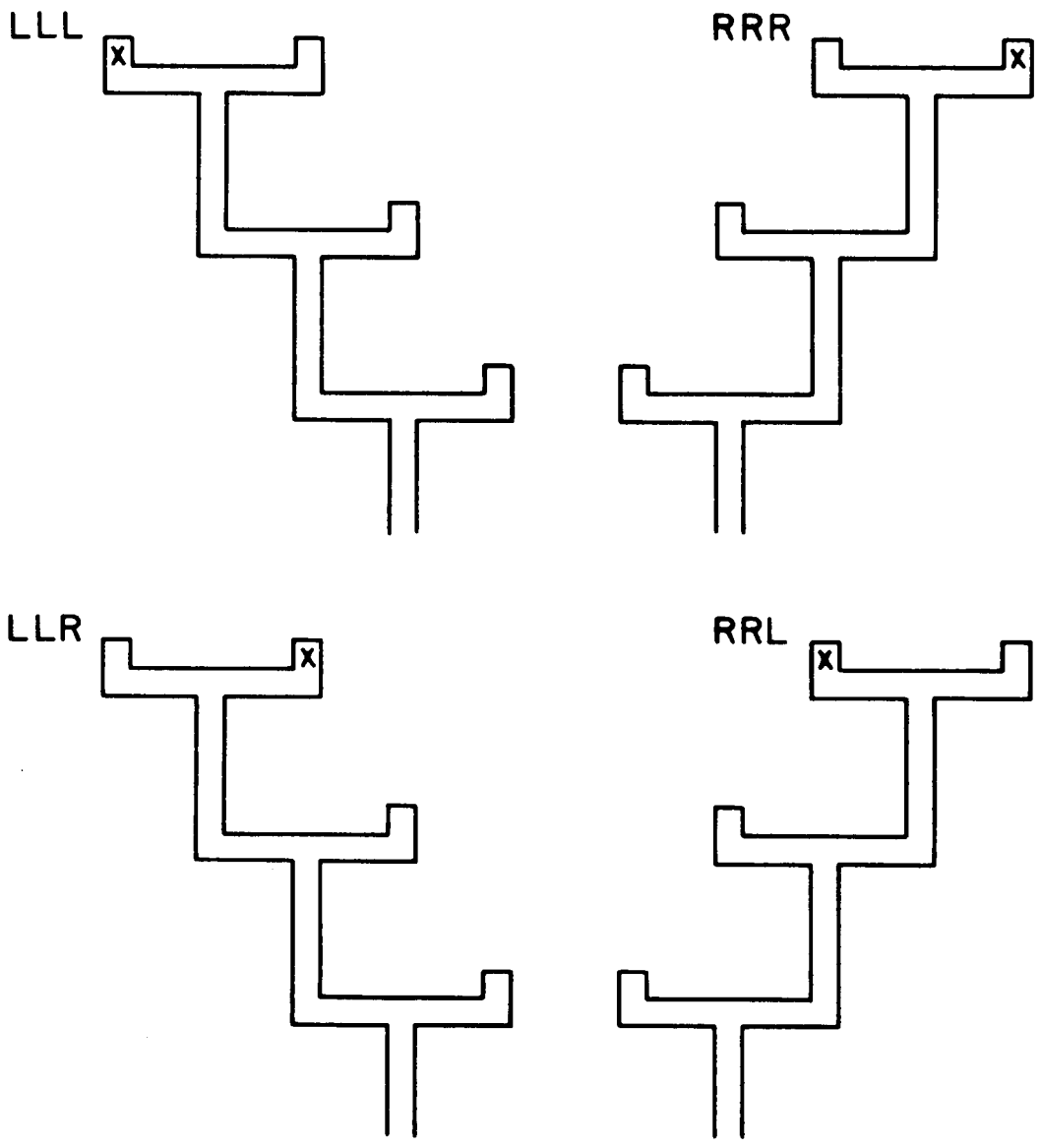


Figure E-1 (continued)

VITA

Brian James Hurlbut was born in Grand Rapids, Michigan, November 27, 1948. He attended elementary school in Grand Rapids until he moved with his parents to Lake Worth, Florida, in 1961. He graduated from Lake Worth High School in 1966, and immediately entered Florida State University.

In 1968, Jim Hurlbut entered the Army Security Agency and served his country in Vietnam and various locations within the United States. He was honorably discharged in 1972.

In 1972, he reentered Florida State University and, in 1974, received a Bachelor of Arts degree in Psychology. In June of 1980 he received a Master of Science in Planning from the University of Tennessee, Knoxville.

Mr. Hurlbut's work experience includes over two years with the Oak Ridge National Laboratory working in the Urban and Regional Studies Division on the Building's Energy Use staff, and two years with the Tennessee Valley Authority as an energy planner. In 1982, Mr. Hurlbut and decided to attend the University of Tennessee, Knoxville, and study in experimental psychology. He graduated in August of 1986 with a Ph.D.