Effect of AF64A on Rat Brain Septo-Hippocampal and Striatal Cholinergic Systems: A Neurochemical and Molecular Analysis

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LOYOLA UNIVERSITY CHICAGO

EFFECT OF AF64A ON RAT BRAIN SEPTO-HIPPOCAMPAL AND STRIATAL CHOLINERGIC SYSTEMS: A NEUROCHEMICAL AND MOLECULAR ANALYSIS.

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

BY QING IVY FAN

CHICAGO, ILLINOIS JANUARY, 1997
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This work is dedicated to my parents, Yangshi Fan and Meiyuan Shen, for their support.
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<tr>
<td>α</td>
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<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>µCi</td>
<td>microcurie</td>
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<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>acetyl CoA</td>
<td>acetyl Coenzyme A</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AF64A</td>
<td>ethylcholine mustard aziridinium</td>
</tr>
<tr>
<td>araC</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>BChE</td>
<td>butyrylcholinesterase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3'5'-monophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CDF/LIF</td>
<td>cholinergic differentiation factor/leukemia inhibitory factor</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary ribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
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<tr>
<td>DBB</td>
<td>diagonal band of Broca</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>dibutyryl cAMP</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>FF</td>
<td>fimbria fornix</td>
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<tr>
<td>FUdR</td>
<td>5'-fluoro-2'-deoxyuridine</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>H3.3</td>
<td>histone 3.3</td>
</tr>
<tr>
<td>HACHT</td>
<td>high affinity choline transport</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HC-3</td>
<td>hemicholinium-3</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HLDB</td>
<td>horizontal limb of the diagonal band of Broca</td>
</tr>
<tr>
<td>ic</td>
<td>intracerebral</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IR</td>
<td>immuno-reactive</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LACHT</td>
<td>low affinity choline transport</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>mAChR</td>
<td>muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini-Mental State Examination</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>medial septum</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic receptor</td>
</tr>
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</table>
NaCl  sodium chloride
NBM  nucleus basalis of Meynert
NFT  neurofibrillary tangles
ng  nanogram
NGF  nerve growth factor
NH2  nitrogen mustard
Oligo(dT)  oligodeoxythymidylic acid
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffer saline
pmol  picomole
poly(A)+  polyadenylated mRNA
QA  quinolinic acid
RNA  ribonucleic acid
rpm  rotations per minute
RT-PCR  reverse transcription - polymerase chain reaction
SDS  sodium dodecyl sulfate
SP  senile plaques
Taq  *Thermus aquaticus* DNA polymerase
TBE  tris-boric acid-EDTA buffer
trkA  high affinity receptor of nerve growth factor
U units

VACHT vesicular acetylcholine transport

w/v weight/volume
ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that affects a large proportion of old people. Extensive medical evidence has closely connected AD with a dysfunction of cholinergic systems in the human brain. Ethylcholine mustard aziridinium (AF64A) is a synthetic selective cholinergic neurotoxin, which has been used to produce an animal model of AD. A single icv injection of AF64A causes a long-lasting deficit of cholinergic function. One of the hypotheses underlying the mechanism of AF64A's effects is that it induces this effect by damaging DNA molecules of cholinergic neurons, which subsequently induces a decrease in gene transcription and translation, \textit{in vivo}. The purpose of this dissertation was to test this hypothesis by determining the effect of AF64A on the level of mRNA and protein of choline acetyltransferase (ChAT), a specific cholinergic marker, in the septo-hippocampal and striatal cholinergic systems.

In the septo-hippocampal cholinergic pathway, during the first two days after AF64A icv infusion, ChAT activity was reduced in the hippocampus but the levels of the 67 kD ChAT protein did not change in the same brain region. Concomitantly, AF64A caused a significant increase in ChAT mRNA levels in the septum, which was
followed by an increase in the amount of 67 kD ChAT protein amount and ChAT activity in the septum. Seven days after the administration of AF64A, ChAT mRNA levels in the septum were reduced below control level and stayed at this low level for at least four weeks. Meanwhile, ChAT activity in the hippocampus continued to remain low for at least four weeks, and this effect was matched by a decrease in the amount of 67 kD ChAT protein.

In the striatal cholinergic system, icv infusion of AF64A did not cause significant changes in ChAT activity, ChAT mRNA expression, or 67 kD ChAT protein expression over the entire time course observed.

These data indicate that the long-lasting hypofunction of the septo-hippocampal cholinergic pathway caused by AF64A icv infusion may, in part, be associated with its effect on gene expression of cholinergic neurons.
INTRODUCTION

Clinically, a variety of neurological disorders are associated with the loss of specific populations of neurons. Alzheimer's Disease (AD), Huntington's Disease, and Parkinson's Disease (PD) present unique constellations of behavioral and neurological abnormalities which result from the degeneration of cholinergic neurons in specific regions of the brain. An animal model that is capable of mimicking the cholinergic hypofunction of these diseases would help researchers to find a way to treat them, especially, Alzheimer's Disease.

AD is a progressive neurodegenerative disease that is characterized by the presence of senile plaques (SP) and neurofibrillary tangles (NFT) in the brain. Large populations of people over the age of 65 years old suffer from mild to severe dementia, which costs their relatives a considerable amount of money and requires extensive personal attention to these individuals. Substantial medical evidence has closely connected AD with a dysfunction of cholinergic systems in the human brain. This implies that an efficient treatment of the symptoms of AD might be found by using a proper animal model that replicates the cholinergic hypofunction of AD.

Ethylcholine mustard aziridinium ion (AF64A) is a synthetic neurotoxin, which selectively targets cholinergic neurons. A single dose intracerebroventricular administration of AF64A causes a long lasting deficit of the cholinergic function of central
nervous system in experimental animals. The ability of AF64A to selectively damage cholinergic neurons makes it an ideal agent with which to produce an animal model of AD.

Choline acetyltransferase (ChAT) is the enzyme that catalyzes the biosynthesis of the cholinergic neurotransmitter, acetylcholine (ACh). In AD patients, reduction of ChAT activity is closely related to their symptoms of decreased learning and memory ability. ChAT therefore is a specific cholinergic marker which has been used as an index of cholinergic function.

This study focuses on the mechanism of the effect of AF64A, especially on the possibility that AF64A affects DNA molecules in cholinergic neurons, in vivo. Since AF64A belongs structurally to the family of mustard agents, and causes a long lasting effect following single dose administration, the effects of AF64A might occur at the gene level, or be due to a decrease in gene transcription, and/or translation.
A. Cholinergic System:

The central cholinergic system constitutes a group of neurons that produce acetylcholine (ACh) as a neurotransmitter in their nerve terminals. Several distinct groups of cholinergic neurons in rat brain have been mapped out by the use of appropriate tracing techniques and specific markers for cholinergic neurons.

A. 1. Cholinergic System Markers:

A. 1. 1. Acetylcholine (ACh):

Acetylcholine, the specific cholinergic system neurotransmitter, is synthesized in a one-step reaction by choline acetyltransferase from its precursors choline and acetyl coenzyme A (acetyl CoA), packed in vesicles within the nerve terminals, and released into the synaptic cleft where it exerts its effect. Intracellular levels of ACh are maintained in a dynamic steady state between its synthesis and release.

A. 1. 2. Choline acetyltransferase (ChAT):

This enzyme is a specific marker for the functional status of cholinergic neurons, and is responsible for the synthesis of ACh from choline and acetyl CoA. There are at least
two active sites on this enzyme. One is the choline binding site; the other is the acetyl CoA binding site.

A. 1. 3. Acetylcholinesterase (AChE):

AChE is responsible for terminating ACh function by degrading it to choline and acetate in the synaptic cleft. This enzyme is not a strict cholinergic marker, since it also has been found to be expressed in some non-cholinergic neurons (Bernard et al., 1995).

A. 1. 4. High affinity choline transport (HACHT):

Most of the reported results indicate that HACHT exists primarily in cholinergic nerve terminal (Guyenet et al., 1973; Yamamura and Synder, 1973; Kuhar and Murrin, 1978; Jope, 1979). The HACHT system is responsible for taking up choline from the extracellular space into nerve terminals to be used for ACh synthesis. This step is the rate-determining step of the biosynthesis of ACh (Yamamura and Synder, 1973; Kuhar and Murrin, 1978; Murrin, 1980; Salvaterra and Vaughn, 1989). The transport of choline through this HACHT is a sodium dependent process. In brain tissue homogenates, HACHT was localized to the synaptosomal fraction with a $K_m$ of 1-5 µM for choline (Guyenet et al., 1973; Yamamura and Synder, 1973).

A. 1. 5. Low affinity choline transport (LACHT):

This choline transport system is not a strict cholinergic marker, has less specific function, and is present in cholinergic and non-cholinergic cells which deliver choline for cell membrane anabolism. In rat brain tissue, it is located in the cell body with a $K_m$ of 40-
90 µM for choline. The transport of choline through LACHT is much less dependent on
sodium than is HACHT (Yamamura and Synder, 1973).

A. 1. 6. Vesicular acetylcholine transport (VACHT):

This transport system is located within the cholinergic nerve terminal, on the
membrane of the synaptic vesicles. Most of the synaptic ACh appears to be packaged
inside synaptic vesicles that do not contain ChAT and are impermeable to ACh. This
transport system is responsible for transporting synthesized ACh from the cytoplasm into
vesicles (Anderson et al., 1983). The ACh in the vesicles can then be released into the
synaptic cleft; although the free cytosolic ACh also can be released into the synaptic cleft
(Isräel et al., 1984).

A. 2. Distribution of Cholinergic Neurons and Their Projections in Rat Brain:

In situ hybridization histochemistry studies, using labeled rat ChAT cRNA as
probes, have shown that a detectable cellular signal can be found in the striatum, basal
forebrain, pontomesencephalic tegmentum, various cranial nerve nuclei and spinal cord;
but not in the cerebral cortex (Oh et al., 1992; and Butcher et al., 1993) or hippocampus
(Oh et al., 1992; Butcher et al., 1993; Lauterborn et al., 1993). These studies suggest that
cholinergic neuronal cell bodies are located at the above mentioned areas except for the
cortex and hippocampus. However, a weak signal of ChAT mRNA was detected in the
hippocampus and cortex when reverse transcription polymerase chain reaction (RT-PCR)
methodology was used (Cavicchioli et al., 1991). This small amount of ChAT mRNA
observed in the hippocampus and cortex indicates that there might be a few intrinsic
cholinergic neurons located in these two areas which contribute to this small amount of ChAT (Tajima et al., 1991).

The localization of central cholinergic neurons has been illustrated very well in the following schematic figure by Butcher and Woolf (1986) (Figure 1). One type of cholinergic neuron, the interneurons, are arrayed entirely within the neural structure in which they are found. Cholinergic interneurons are located mainly in the striatum. Another type of cholinergic neuron is the projection neuron. The cell bodies of most projection cholinergic neurons are located in the basal forebrain and pontomesencephalic regions. Neurons in the basal forebrain project to the entire nonstriatal telencephalon while the pontomesencephalotegmental neurons project to the thalamus, other diencephalic loci, the pontine, the medullary and cranial nerve nuclei.

The focus of this dissertation is on the septo-hippocampal cholinergic pathway (projection neuron type) and the cholinergic neurons which are located in the striatal region (interneurons); both well-defined cholinergic systems.

A. 3. The Septo-Hippocampal and Striatal Cholinergic System:

The cholinergic neurons of the septum and diagonal band of Broca (DBB) project to the hippocampus through the fimbria-fornix (FF), form a part of the basal forebrain cholinergic neuronal system (Mesulam et al., 1983). In this cholinergic system, ChAT proteins are synthesized in the cell body and transported down to nerve terminal in the hippocampal pathway is one of the most affected systems in brain of Alzheimer's disease hippocampus, where ChAT is responsible for synthesis of ACh. The HACHT system
Figure 1. Schematic representation of the major cholinergic systems in the mammalian brain (Butcher and Woolf, 1986). (ms, medial septal nucleus; bas, nuclei basalis; td, diagonal band nuclei; si, substantia innominata; poma, magnocellular preoptic field; tpp, pendunculopontine tegmental nuclei; dltn, laterodorsal tegmental nuclei; ICj, Islands of Calleja complex).
primarily exists in the hippocampus where cholinergic nerve terminal located. The septohippocampal pathway is one of the most affected system in brain of Alzheimer’s disease patients, and has been used in regeneration studies and employing neurotrophic factors, such as nerve growth factor (NGF) (Hefti and Knusel, 1990).

The striatum contains the highest level of ACh in the central nervous system. Striatal cholinergic neurons are principally multipolar with aspiny or sparsely spined dendrites (Woolf and Butcher, 1981). Cholinergic neurons located in the striatum are interneurons, as has been demonstrated by fluorescent tracer experiments (Woolf and Butcher, 1981). Both the cell body and nerve terminal of cholinergic neurons exist within the striatum. Cholinergic neurons typically constitute a small proportion of the total number of cells in the striatal regions in which they are found, and account for 1-2% of the striatal neuronal population (Woolf and Butcher, 1981; Phelps et al., 1985).

A. 4. Axonal Transport of Cholinergic Enzymes:

Most studies dealing with cholinergic enzyme transport have relied on detection of enzyme activity for measuring transported enzymes. If the enzymes were synthesized and transported as nonenzymatically active precursors they would not have been detected by these studies (Salvaterra and Vaughn, 1989). Using histochemistry methodology, Kása et al. (1973) located ChAT and AChE in cholinergic neuronal axons. It is believed that the axonal transport of ChAT is unidirectional, as slow as 4 mm/day, and most of the transported ChAT is not associated with intra-axonal organelles (Tuček, 1975). In contrast to ChAT, the axonal transport of AChE is bi-directional, and associated with vesicles and
neurotubules, which indicates that AChE is bound to some components of the membrane inside the axon (Brimijoin and Wiermaa, 1978). The orthograde transport of AChE predominates during this bi-directional flow. The transport of AChE along the axons is about 400 mm/day, which is much faster than that of ChAT (Tuček, 1975; and Brimijoin and Wiermaa, 1978). These data suggest that AChE and ChAT are transported by different mechanisms and at different velocities inside the cholinergic neuronal axon.

A. 6. Effect of Nerve Growth Factor (NGF) on the Cholinergic System:

NGF is a polypeptide trophic factor which interacts with two distinctive types of receptors, trk A and p75. NGF is synthesized and released from the target sites of cholinergic neurons. After binding with its high affinity receptor (trk A) at the axon of the cholinergic neuron, NGF is internalized and retrogradely transported to the cholinergic neuronal cell body (Schwab et al., 1979; Korsching and Thoenen, 1983; Seiler, and Schwab, 1984). NGF is necessary for the survival and maintenance of central cholinergic neurons. It has been reported that NGF infusion can increase ChAT activity in normal animals and restore cholinergic function in animals with cholinergic deficits (Hefti et al., 1989).

Following chemical damage of cholinergic neurons via intracerebral injection of the excitotoxin quinolinic acid (QA) into the striatum, there was a decrease in the number of cells expressing trk A and ChAT, while the mRNA for trk A and ChAT in the surviving cells was down-regulated. NGF treatment prevented the decrease in the number
of the cells which express the above two markers in treated animals and increased their expression in control animals. However, NGF failed to prevent the down regulation of glutamic acid decarboxylase (GAD) induced by QA (Venero et al., 1994) in the striatum, which indicates that NGF is a specific cholinergic trophic factor.

B. Choline Acetyltransferase (ChAT)

The specific cholinergic functional enzyme, ChAT, was first discovered by Nachmansohn and Machado in extracts of rabbit brain (1943). This enzyme protein apparently is synthesized in the cell body of the cholinergic neuron and conveyed to synaptic sites of action in the slowly transported pool of proteins (Frizell et al., 1970; Kása et al., 1973; Tuček, 1975).

B. 1. ChAT Protein:

B. 1. 1. Half-life of ChAT protein:

In cell culture, the half-life of human ChAT protein is longer than 24 hours (Hersh, 1992). In in vivo experiments conducted on rat brain cortex, double labeling with L-[1-14C]leucine and L-[4,5-3H] leucine has been used to estimate the half-life of ChAT protein under steady-state conditions. The measured half-life of ChAT is 5.2 days (Wenthold and Mahler, 1975).

B. 1. 2. Isoform and molecular weight of ChAT protein:

ChAT protein has a very low expression in mammalian brain, which has been estimated at about 0.0001% of the brain protein (Eckenstein and Thoenen, 1982). This
makes it very difficult to obtain purified homogeneous ChAT protein, using the classical affinity column method. Usually, a purification factor of more than 1,000,000 is necessary to obtain the pure enzyme (Berrard et al., 1989). Based on the solubilizing characterization of this enzyme, multiple forms of ChAT protein existing in mouse and rat brain have been suggested (Benishin and Carroll, 1981; 1983); they have been identified as cytosolic soluble and membrane-bound ChAT forms. The cytosolic soluble form ChAT protein exists in supernatant when brain tissue is homogenized in phosphate buffer, while the membrane-bound ChAT protein is only released into supernatant when detergent (Triton) is present. An extra membrane-anchoring domain was expected to exist in membrane-bound ChAT protein. However, subsequently ChAT protein purified by immuno-affinity chromatography from rat and human tissue showed a single molecular weight peptide; epitope maps produced from both soluble and membrane-bound ChAT were identical (Bruce and Hersh, 1987). These results did not support the previous assumption that isoforms of ChAT proteins exist. Most researchers now believe that ChAT is a single-subunit globular protein (Salvaterra and Vaughn, 1989).

Several research groups have made efforts to purify and to characterize the ChAT protein from rat brain tissue. Rat cortex ChAT protein appears to have a molecular weight of 60-65 kD under non-denaturing conditions (Wenthold and Mahler, 1975). Most investigators suggest that the purified ChAT protein from rat brain has a molecular weight of about 67 kD (Dietz and Salvaterra, 1980; Schmidt and Rylett, 1993). Although multiple protein bands around this molecular weight have also been reported, the tryptic peptide
maps have indicated that they contain a nearly identical primary structure (Dietz and Salvaterra, 1980). There is no evidence suggesting the existence of low molecular weight ChAT protein in rat brain, although a low molecular weight ChAT protein has been reported in bovine brain with a molecular weight of 34 kD (Malthe-Sørenssen et al., 1978). In rat, purified ChAT protein was isolated from whole brain tissue, thus, there is no report on any possible differences of ChAT proteins among different brain regions.

B. 1. 3. Antibodies against ChAT protein:

Most antibodies raised against the ChAT protein bind to an epitope that is located at, or near the enzyme active site (Houser et al., 1982; Levey et al., 1982; Eder-Colli et al., 1989). Thus, these antibodies can be used to inhibit ChAT activity in a very specific manner. There are, however, some antibodies which bind to an epitope in ChAT protein which is different from the active sites (Eckenstein and Thoenen, 1982).

Antibody for ChAT protein raised from one species is capable of cross-reacting with other species, indicating a certain degree of immunological conservation of the protein structure of ChAT in mammals (Salvaterra and Vaughn, 1989).

B. 1. 4. Post-translation modification of ChAT protein:

ChAT protein can undergo post-translational modification by phosphorylation (Bruce and Hersh, 1989; Schmidt and Rylett, 1993), but a change in ChAT activity after this phosphorylation has not yet been established. Usually, the cytosolic fraction of the ChAT is phosphorylated under basal, unstimulated conditions. However, the
phosphorylation of ChAT does not appear to regulate cholinergic neurotransmission by a direct action on the catalytic activity of the enzyme (Schmidt and Rylett, 1993).

B. 2. ChAT mRNA:

ChAT cDNA was first cloned from *Drosophila melanogaster* head (Itoh et al., 1986). In mammals, a cDNA clone encoding the complete sequence of an active ChAT was first isolated from porcine brain (Berrard et al., 1987), and subsequently from rat brain (Brice et al., 1989; and Ishii et al., 1990), mouse spinal cord (Ishii et al., 1990) and human spinal cord (Oda et al., 1992). Comparison of these cDNA sequences revealed a high degree of amino acid sequence similarity among mammalian ChAT, but not with *Drosophila* ChAT (Brice et al., 1989; Wu and Hersh, 1994). Based on the amino acid sequence derived from the cDNA, there is no membrane-anchoring domain to be found in ChAT protein (Habert et al., 1992). These data also contradict the assumption for the existence of two isoforms of ChAT protein based on their solubilizing characterization. Accordingly, the membrane-bound form and membrane-free form of ChAT might exist due to differential post-translational processing, rather than due to difference in their primary structure.

The size of ChAT mRNA in adult rats is about 4 Kb (Ibanez et al., 1991; Nawa et al., 1991) and is expressed at a very low level in rat brain (Ibanez et al., 1991). Multiple transcripts were observed in rat spinal cord (Kengaku et al., 1992). However, all of the transcripts encoded the same ChAT protein; they were only different in their non-coding 5'-ends.
B. 3. ChAT Gene:

In all species examined so far ChAT is encoded by a single gene (Wu and Hersh, 1994). In humans, the ChAT gene has been mapped to chromosome 10 (Cohen-Haguenauer et al., 1990). In rats, on the other hand, the location of the ChAT gene is still not clear. The gene encoding ChAT protein in rat was first reported to include one 5' noncoding exon and 14 exons accounting for the entire coding sequence, which distributes over at least 64 Kb. The largest intron is 14 Kb that is located immediately after the noncoding exon (Hahn et al., 1992). The gene encoding VAChT has recently been localized within the first intron of the gene encoding ChAT and is in the same transcriptional orientation (Bejanin et al., 1994; Erickson et al., 1994; Berrard et al., 1995), which indicates these two specific cholinergic markers might be under control of the same regulators.

However, three 5' noncoding exons in rat gene have been demonstrated lately (Misawa et al., 1992; Kengaku et al., 1993), and there are three different promoter regions. It is therefore conceivable that five different types of ChAT mRNA are formed by a combination of differential alternative splicing of noncoding exons, and that multiple promoters play a role in this step. In the ChAT gene promoter region, there is a TATA-like element and numerous potential binding sites for transcription factors which include: AP-1 (Hahn et al., 1992), Sp1 (Hahn et al., 1992; Inoue et al., 1993), octamer-binding factor (Kitamoto and Salvaterra, 1995), CTF/NF-1 (Hahn et al., 1992), and the nuclear oncoprotein Myb (Hahn et al., 1992).
The active sites of ChAT protein have also been located in the rat ChAT gene. Exon 3 contains the amino acid sequence of EELDLPKLPVPPLQ, which was suggested to be the choline interaction site (Finocchiaro et al., 1991); and exons 8 and 9 code for the histidine residues that might participate in the catalytic reaction (Berrard et al., 1987; Brice et al., 1989).

There are three promoters for ChAT gene which are responsible for transcription of five different ChAT mRNA types. There is no knowledge on the identity of ChAT mRNA expressed in the septum and striatum. If ChAT mRNAs are different in the septum from striatum, this might indicate that different promoters are response for the regulation of ChAT gene expression in these two brain regions. This would indicate that ChAT gene expression in the septum and striatum might be under different regulation.

B. 4. Regulation of ChAT Expression:

B. 4. 1. Physiological conditions:

ChAT activity changes during different developmental stages (Large et al., 1986). Levels of ChAT mRNA undergo the same developmental stage-dependent changes as ChAT activity (Cavicchioli et al., 1991), although the changes in mRNA precede those of the activity (Barber et al., 1989).

In aged rats, there is a decrease in ChAT activity in cortex, hippocampus and striatum, but there is no difference in mRNA levels in the striatum and basal forebrain (Alberch, et al., 1991; Ogawa et al., 1994). Age-related impairments in ACh-mediated neuronal systems are caused primarily by disorders of post-transcriptional events.
Further studies conducted in *Drosophila*, using a wild type and two mutant type (temperature sensitive) flies, indicated that neurotransmission and the rate of neurotransmitter biosynthetic enzyme gene transcription are coupled for the cholinergic system (Tajima and Salvaterra, 1992). At a permissive temperature (18°C), the mutant flies attempted to compensate for production of more thermolabile form of enzyme (defective enzyme), by producing and/or maintaining higher levels of ChAT mRNA when compared to the wild type, although the mutant type flies have less ChAT activity. This indicates a negative feedback response for the regulation of ChAT gene expression in this condition. The wild type flies, on the other hand, increased their ChAT mRNA levels when they were transferred from a permissive temperature to a restrictive temperature (32°C), to adapt to the increase in synaptic activity which might be temperature dependent. These data suggested a positive feedback mechanism by which higher ChAT mRNA levels reflect a higher demand for ACh, to sustain cholinergic synaptic transmission at the higher temperature. This study suggests that ChAT gene transcription is a very important step in the regulation of cholinergic function; and that this step is regulated by a balance of both positive and negative feedback mediated through cholinergic transmission.

B. 4. 2. Axotomy and axonal transport blockade:

Injury in an axon can affect, in central cholinergic neurons, expression of ChAT, galanin, and trk A mRNA. The direction of changes in ChAT mRNA expression has, however, been reported to differ. In the peripheral system, axotomy caused a decrease in ChAT mRNA expression in motoneurons (Piehl et al., 1995). However, in the central
nervous system, one week after fimbria fornix transection (axotomy of septal-hippocampal pathway), neurons in the horizontal limb of the diagonal band of Broca (HLDB) showed an increase in ChAT mRNA without any change in ChAT protein levels, followed by a steady decrease in ChAT mRNA and ChAT protein content for at least 8 weeks (Weiser et al., 1994). Different from what occurs following axotomy, tetrodotoxin-induced blockade of neuronal activity did not change ChAT mRNA levels significantly, although there was a significant increase in mRNA of galanin, which coexists with acetylcholine in cholinergic neurons (Agoston et al., 1994).

B. 4. 3. Nerve growth factor (NGF):

It has been widely reported that NGF plays a prominent role in regulating the function of forebrain cholinergic neurons (Hefti, 1986; Large et al., 1986; Dreyfus, 1989; Williams and Oostveen, 1992). It is believed that NGF produces cholinergic neuronal hypertrophy through induction of gene expression of the NGF receptor within the basal forebrain (Higgins, 1989). Icv administration of NGF stimulates ChAT activity in the forebrain of neonatal and adult rats (Gnahn et al., 1983; Hefti et al., 1984). NGF also up-regulates ChAT mRNA expression both in neonatal and adult rats (Cavicchioli et al., 1991; Lorenzi et al., 1992). This phenomenon of NGF up-regulating ChAT activity, paralleled with an increase in ChAT mRNA in neonatal rats, indicates that during the developmental stage, transcriptional regulation is very important.
B. 4. 4. Other factors:

B. 4. 4. 1. Second messenger systems:

Various second messenger systems have been implicated as influencing the properties of cholinergic cells in general, and ChAT activity in particular.

a). Chemicals which relate to cAMP have been used to induce ChAT expression in cell culture. These include: cAMP (Hersh, 1992); cAMP derivatives, such as dibutyryl cAMP (dbcAMP) (Brenneman and Warren, 1983); and phosphodiesterase inhibitors (Brenneman and Warren, 1983). These agents can increase ChAT activity in immature primary cell cultures as well as the cultured neuroblastoma cells. The increase in ChAT activity can be blocked by cycloheximide and actinomycin D (Brenneman and Warren, 1983; and Kirshner et al., 1986), indicating that the increase of the ChAT activity involves ChAT protein synthesis and might be regulated at the transcription level.

b). In addition to cyclic nucleotide modulation of ChAT, the effects of protein kinase inhibitors K-252 and staurosporine on ChAT activity in rat spinal cord cultures have also been reported (Glicksman et al., 1993). It is believed that protein kinase A inhibits expression of the ChAT gene at the transcriptional level, by controlling the activity of an enhancer element.

B. 4. 4. 2. Some endogenous factors:

a). Retinoic acid (RA), an endogenous derivative of vitamin A, plays a role in the development of the nervous system. In sympathetic neuron cell cultures, RA increases the specific activity of ChAT; concomitantly, RA reduces the specific activities of two
catecholamine synthetic enzymes, tyrosine hydroxylase (TH) and dopamine-hydroxylase (DBH), and the level of norepinephrine (NE) (Berrard et al., 1993).

b). Cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) and interleukin 6 (IL-6): In cultured rat sympathetic neurons, 1 nM CDF/LIF induces mRNA for ChAT, somatostatin (SOM), substance P, and vasoactive intestinal polypeptide (VIP); lowering the mRNA of TH, and of neuropeptide Y (NPY) (Nawa et al., 1991). CDF/LIF increases both ChAT mRNA and immunoreactivity (Oh et al., 1994). However, IL-6 induces 6-fold increases in ChAT mRNA without any change in ChAT immunoreactivity, and without any effect on cell survival.

c). Hormones: Both thyroid hormones, T3 and T4 have been reported to increase ChAT activity in cultured neurons and in vivo, as well as to restore the decreased ChAT activity caused by hypothyroidism (Hayashi and Patel, 1987; Hashimoto et al., 1994; Juarez de Ku et al., 1994); the precise mechanism is unknown. Glucocorticoids have also been reported to have different effects on the regulation of ChAT activity in several brain regions (Torres et al., 1991), which might be through an activation of AP-1 by glucocorticoids (Schmitt et al., 1995)

B. 4. 4. 3. Antimitotic agents:

Cytosine arabinoside (araC) and 5'-fluoro-2'-deoxyuridine (FUdR), cause an increase in ChAT activity without changing the level of glutamic acid decarboxylase activity in embryo septal cultures. However, when embryo striatal cultures were used, incubation with araC or FUdR had no effect on ChAT activity. It is believed that the
different microenvironment of these two brain regions contributed to these different responses (Hayes et al., 1991).

C. Alzheimer's Disease and Cholinergic Hypofunction:

Alzheimer's Disease (AD), is a progressive neurodegenerative disease, which may affect up to 15% of the population over the age of 65 years (Whitehouse et al., 1982), and 50% of the population over the age of 85 years (Fisher et al., 1993). AD is characterized by the presence of senile plaques and neurofibrillary tangles in the brain cortex (Dournaud et al., 1995) and hippocampus (Ball, 1977; Ransmayr, 1992). In patients who suffer from AD, multiple central neuronal system deficits have been observed. However, a chronic deficiency in central cholinergic function has been consistently found in these AD patients. The most severely affected pathways are the nucleus basalis of Meynert (NBM)-cortical pathway (White et al., 1977; and Whitehouse et al., 1981), and the septal-hippocampal (Perry et al., 1977; Ransmayr, 1992) pathway.

C. 1. Neurochemical and Histological Studies:

The cholinergic hypothesis of AD emerged from the findings of a reduced activity of the enzyme ChAT in postmortem AD brain tissue (Bowen et al., 1976; Davies and Maloney, 1976; Perry et al., 1977). Reduced activity of this enzyme has been consistently shown in all subsequent studies (DeKosky et al., 1985), and extended to include losses of a number of other cholinergic markers including AChE (DeKosky et al., 1985; Tune et al., 1985), HACHT (Pascual et al., 1991), and the content of ACh (Davis et al., 1985).
It has been hypothesized that the decrease in ChAT activity in AD patients might be due to mutation of ChAT protein or the loss of a part of the particular isoform of this enzyme. However, purified ChAT protein is identical in brains obtained from normal people and AD patients. This rules out the idea of selective loss of one particular isoform or the mutation of this functional enzyme to explain the reduced levels of ChAT activity observed in AD (Bruce, 1985). The degeneration of cholinergic neurons in the basal forebrain has been reported widely (Whitehouse et al., 1982; Hedreen et al., 1984; Pearson and Powell, 1987). The expression of ChAT mRNA might be down-regulated in the surviving cholinergic neurons in the NBM of patients with AD, raising the possibility of functional restoration by stimulating ChAT synthesis (Strada et al., 1992)

C. 2. Correlation of Cholinergic Function and Mental Status in AD Patients:

In AD patients, it is believed that cholinergic hypofunction is closely related to their symptoms of decrease/loss in learning and memory abilities. Comparing cholinergic enzyme activity from biopsy samples and the level of cognitive function as measured by the Mini-Mental State Examination (MMSE) from AD patients; a significant correlation between the decrease in ChAT activity and mental status before death has been reported (Perry et al., 1978; Wilcock et al., 1982; DeKosky et al., 1992; Bierer et al., 1995). Moreover, ChAT activity in the cortex autopsied from AD patients was found to be significantly lower when compared with control cortices (DeKosky et al., 1992), indicating continuous decline of ChAT activity throughout the course of the disease.
C. 3. Clinical Effects of Cholinergic Agents on AD Patients:

Since cholinergic hypofunction has been consistently found in AD patients and correlated with their symptoms, replacement of lost cholinergic function has, therefore, been thought of as having a potential therapeutic benefit for AD patients. This can be accomplished by enhancing endogenous levels of ACh through the inhibition of its degradation by AChE, or by directly mimicking its actions at postsynaptic muscarinic receptors (mAChR); which could be performed by using AChE inhibitors or mAChR agonists, respectively (Winblad et al., 1992).

Tacrine (tetrahydroaminoacridine), a mixed, reversible inhibitor of cholinesterase, has been shown to significantly improve several measures of cognitive performance in probable AD patients although not all patients responded to this agent (Minthon et al., 1994; Pacheco et al., 1995; Woo and Lantz, 1995). A similar effect with other AChE inhibitors, physostigmine (Cummings et al, 1993) and velnacrine maleate (Jackson et al., 1995); and cholinergic agonists, such as arecoline (Soncrant et al., 1993), also have been reported.

Based on animal research showing that NGF treatment benefits central cholinergic neurons, an intracranial infusion of NGF to an AD patient has been tried. The result showed that there is an improvement in memory, without severe side effects. This study suggests that further clinical trials of NGF infusion in AD are warranted (Seiger et al., 1993).
D. Effect of Ethylcholine Mustard Aziridinium (AF64A):

Using the selective cholinergic neurotoxin AF64A, a rodent model of human brain disorders has been developed, in which a central cholinergic hypofunction has been induced (Mantione et al, 1981; Fisher et al., 1982; Fisher and Hanin, 1986; Hanin, 1990, 1994). This animal model exhibits behavioral, anatomical, and neurochemical deficits analogous to those observed in AD. AF64A-induced cholinergic hypofunction is expressed by both loss of some cholinergic neurons and impairment in the functioning of the spared neurons (Pittel et al., 1992). The effects of AF64A on the cholinergic system have been explored both in neurochemical studies and in animal behavioral tests.

D. 1. Structure and Selectivity of AF64A:

AF64A is a structural analogue of choline, except that two of the methyl groups of choline have been converted to the aziridinium moiety in AF64A, and the third methyl group has been elongated to an ethyl group (Figure 2).

Usually, only a single dose intraventricular injection is required to produce a long-lasting cholinergic deficit in animals (Mantione et al., 1981). There have been some arguments in the literature as to whether AF64A is really a selective cholinergic neurotoxin, since some researchers have shown non-specific effects of AF64A (Levy et al., 1984; Jarrard et al, 1984; Eva et al., 1987; Allen et al., 1988; Gomez et al., 1993). However, this depends on how much AF64A is used and where it is administered (Baskey et al., 1989; Kása et al., 1986; Hanin, 1990). Since AF64A is structurally similar to choline, it can be recognized by the choline transport system. When low doses of AF64A
are used, AF64A is only recognized and taken up by the HACHT into cholinergic neurons and would thus appear to be a cholinergic selective toxin, since HACHT only exists in cholinergic neurons (Guyenet et al., 1973; Suszkiw and Pilar, 1976; Kuhar and Murrin, 1978; Jope, 1979). However, when a high dose of AF64A is used, it can be taken up by the HACHT as well as the LACHT system into cells and thus cause an abundance of damage without cell selectivity, since LACHT exists in all types of cells and is responsible for uptake of choline for the synthesis of phosphatidylcholine which is one component of the plasma membrane (Pittel et al., 1987; Uney and Marchbanks, 1987; Hanin, 1990).

D. 2. Neurochemical Studies on the Effect of AF64A:

D. 2. 1. Effect of AF64A on ACh level and release:

In vivo studies have shown that AF64A treatment can cause a decrease in ACh content in rodent animal brain tissue (hippocampus, cortex, and striatum) which happens as early as 1 day after its intracerebral (ic) or intracerebroventricular (icv) injection (Mantione et al., 1983; Sandberg et al., 1984b; Potter et al., 1986; Leventer et al., 1985, 1987; Blaker and Goodwin, 1987; Tateishi, 1987; Mouton et al., 1989; Murai et al., 1994). In synaptosomes which were obtained from AF64A-treated rats, there was a decrease in their ability to synthesize and release ACh (Leventer et al., 1985, 1987; Potter et al., 1986; Mouton et al., 1988; Pittel et al., 1992). In vivo microdialysis experiments have shown that AF64A can induce a dose-dependent decrease in extracellular ACh release (Meana et al., 1992; Tajima et al., 1993).
Figure 2. Structural similarity of AF64A with choline.
D. 2. 2. Effect of AF64A on the HAChT system:

Icv administration of AF64A caused a significant decrease in HAChT activity in the hippocampus and cortex in mice and rats (Mantione et al., 1981, 1983; Leventer et al., 1987; Chrobak et al., 1989; Morley and Garner, 1990; El Tamer et al., 1992). Pretreatment with excess choline, or with the specific HAChT inhibitor, hemicholinium-3 (HC-3), was able to prevent the effect of AF64A on HAChT and to block other effects of AF64A, which indicates that the toxic effects of AF64A are dependent upon its uptake via the HAChT site (Davies et al., 1986; Chrobak et al., 1989, Brake and Pappas, 1994). Similar results have also been obtained in cell culture studies (Santiago et al., 1995), in which 10 times higher concentrations of choline chloride than AF64A delayed or diminished the neurotoxic effect of AF64A. These data indicated there is a competition between choline and AF64A for the HAChT (Davies et al., 1986; and Santiago et al., 1995).

In vitro, the effect of AF64A on choline transport was also studied in hippocampal, striatal, and cerebrocortical synaptosomes. Synaptosomes prepared from these three brain regions were equally sensitive to AF64A, and HAChT was more sensitive to the toxin than the LAChT (Pittel et al., 1987). Choline and HC-3 protected the choline transport against attack by AF64A (Curti and Marchbanks, 1984).

It also has been observed that AF64A and hemicholinium-3 were much less potent in neuronal cultures than in synaptosomes (Kelley et al., 1988).
D. 2. 3. Effect of AF64A on cholinergic enzymes:

*In vivo:*

Following icv administration of AF64A, ChAT and AChE in the hippocampus were markedly decreased in a dose-dependent manner, but were unchanged in the neocortex and striatum (Vickroy et al., 1985; Leventer et al., 1987; Chrobak et al., 1988; El Tamer et al., 1992; Gaspar et al., 1992; Ricci et al., 1992). The activity of ChAT started to decline in the hippocampus within 24 h (Hörtnagl et al., 1991), and the reduction of ChAT activity reached its maximum within 4-7 days and persisted for as long as several months (Leventer et al., 1987; Hörtnagl et al., 1991; El Tamer et al., 1992;). This cholinergic marker (ChAT) was most affected in the ventral part of the hippocampus (Hörtnagl et al., 1991).

Although icv infusion of AF64A has no effect on the activity of cholinergic enzymes the striatum, direct injection of AF64A in rat striatum caused a decrease in ChAT activity (Sandberg et al., 1984 a, b, c). Meanwhile intracerebral (ic) injection of AF64A into the NBM or cortex also caused a decrease in ChAT activity in the cortex.

*Cell culture and in vitro:*

Cell culture studies showed that ChAT activity decreased rapidly, long before cell lysis occurs (Sandberg et al., 1985). Further *in vitro* studies showed that there was a concentration- and time-dependent inhibition of the activity of partially purified ChAT by AF64A. This effect could be prevented by co-incubation of the enzyme and AF64A with choline but not with acetyl-coenzyme A. Similar phenomena have also been observed in
tissue homogenate studies (Mantione et al., 1984). These data suggest that AF64A might act as an irreversible active site directed inhibitor of ChAT (Sandberg et al. 1985), which binds at the choline binding site of the ChAT protein.

D. 3. Histological Studies on the Effect of AF64A:

D. 3. 1. Axonal transport blockade:

AChE and ChAT are normally transported from the cell body, where they are synthesized, by different transport systems down the cholinergic neuronal axon (Tuček, 1975). In 1985, Kása and Hanin reported an interesting phenomenon. Following icv injection of a high dose of AF64A (5 nmol/lateral ventricle), a heavy accumulation of AChE along the axons of the septo-hippocampal pathway was observed. This observation was interpreted as indicating that AF64A blocked axonal transport of this enzyme.

There is no direct evidence supporting the concept that the transport of ChAT protein is also affected by AF64A treatment. However, some neurochemical studies provide clues that there might be a blockade of ChAT transport as well. These clues are based on results from AF64A/NGF experiments by Willson and Hanin (1995). In these experiments, ChAT activity significantly increased in the septum following AF/NGF treatment. This increase in ChAT activity was even higher than that observed in the vehicle/NGF treated rats. In the hippocampus, on the other hand, ChAT activity in AF/NGF treated rats was significantly lower than that in vehicle/NGF treated rats. These data indicated the possibility of some kind of blockade of the transport of ChAT from
septum to hippocampus following AF64A infusion, an accumulation of ChAT in the cell bodies of the septum, and a progressive reduction of enzyme content in the nerve terminal.

D. 3. 2. Neuronal degeneration:

The loss of ChAT immunoreactive (IR) positive neurons following AF64A administration has been reported by several groups of investigators. Following direct injections in the hippocampus (Tonnaer et al., 1986; Messer et al., 1991), or icv infusion of AF64A (Lorens et al., 1991; Chrobak et al., 1988), immunocytochemistry studies showed ChAT-IR profiles revealing shrinkage and disappearance of cholinergic neurons in the medial septum (ms) and DBB. These data implied a retrograde degeneration of cholinergic neurons following injection of AF64A administration. Meanwhile, intracortical AF64A infusions caused a significant atrophy, but not degeneration, of NBM cholinergic cell bodies (Mouton and Arendash, 1990). This retrograde degeneration starting at the nerve terminal also has been observed in primary neuron cell cultures under low concentrations (<25µM) of AF64A treatment, which could be prevented by the presence of high concentrations (500µM) of choline (Amir et al., 1988), indicating that retrograde cellular atrophy might be associated with inhibition of presynaptic HACHT on cholinergic neurons (Mouton and Arendash, 1990).

D. 4. Effect of AF64A on Animal Behavior:

AF64A treated rats exhibited a severe deficit in their working memory (Blaker and Goodwin, 1987; Chrobak et al., 1987, 1988; Masuda et al., 1992; Murai et al., 1994) when they were subjected to T maze or radial arm maze tasks. In open-field tests, the
cholinergic lesion caused by AF64A infusion induced a dose-dependent increase in activity (Sandberg et al., 1984c; Bailey et al. 1986; Gaspar et al., 1992; Lamberty et al., 1992). Animals also showed a significant reduction in their spatial learning ability when the Morris water maze or hole-board exploration were conducted (Gaspar et al., 1992; Lamberty et al., 1992; Nakamura et al., 1992; Opello et al., 1993).

The septo-hippocampal and the NBM-cortical cholinergic pathways are the most affected systems in AD patients; both pathways are very important for the acquisition of learning and memory in animals (Bartus et al., 1982). AF64A-induced cholinergic hypofunction in these two pathways has been studied using behavioral tests. The cholinergic deficits were confined to the hippocampus, which produced significant learning and memory impairments in situations where intermediate or long term memory formation is required (Walsh et al., 1985; Blaker and Goodwin, 1987; Chrobak, 1987; Opello et al., 1993) Furthermore, following intracortical AF64A infusion, animals exhibited deficits in their active avoidance ability (Mouton et al., 1989; Nakamura et al., 1992).

D. 5. Effect of AF64A on Cholinergic Receptors:

It has been well defined that there are two types of cholinergic receptors (AChR), nicotinic (nAChR) and muscarinic (mAChR) respectively. In mammalian brain, type one mAChR (M1 receptor) are located at the postsynaptic plate, while most type two mAChR (M2 receptor) are autoreceptors which are located presynaptically. In rats, M1 receptors were either unaffected, or showed super-sensitivity following AF64A administration.
(Vickroy et al., 1985; Moroi-Fetters et al., 1990; Ricci et al., 1992), whereas M2 sites were reduced after injection of the neurotoxin (Ricci et al., 1992). Some investigators have also reported that adaptive changes in nAChR and mAChR occur in AF64A treated rats which are similar to those reported in AD patients (Potter and Nitta, 1993).

D. 6. Effect of AF64A on Genes and Gene Expression:

The suggestion that AF64A may be causing a long-term cholinergic hypofunction due to damage of DNA molecules originated from the observation of its unique chemical structure (Futscher et al., 1992). Besides choline, AF64A is also structurally similar to the reactive intermediate of the antitumor agent mechlorethamine (nitrogen mustard, HN2) (Figure 3), which is known as a chemotherapeutic agent that exerts its effect by acting at DNA molecules (Lawley and Brookes, 1965). Both AF64A and HN2 contain a highly reactive aziridinium moiety which can serve to crosslink DNA molecules and subsequently cause the cytotoxicity.

To test this hypothesis, in vitro experiments were conducted, which showed that AF64A was capable of producing extensive dose-dependent N-7 guanine alkylations in DNA fragments in vitro, although no sequence specificity of this AF64A attack could be discerned (Futscher et al., 1992). Moreover, in vitro studies also showed that AF64A could inhibit cDNA transcription without selectivity, since all DNA molecules subjected to AF64A in these in vitro studies, such as, N-myc, AChE, and butyrylcholinesterase (BChE) were affected (Futscher et al., 1992; Lev-Lehman et al., 1994; Hanin et al.,
Figure 3. Structural similarity of AF64A with nitrogen mustard.
1995). Furthermore, G, C-rich genes appear to be more sensitive to AF64A than A, T-rich genes (Futscher et al., 1992; Lev-Lehman et al., 1994).

In cell culture studies, AF64A induced-cytotoxicity was accompanied by DNA lesions (Futscher et al., 1992) which supported the hypothesis that AF64A alters the structure and function of cellular DNA, which might help to explain the observed long-term cholinergic deficits in vivo. Further studies conducted in cultured neuroblastoma cells found that AF64A caused a transient decrease of steady state levels of both N-myc mRNA and ChAT mRNA, which also indicated that AF64A can affect gene expression in cell culture systems and cause DNA damage without gene selectivity (Santiago, 1995).

In vivo, icv administration of AF64A (2 nmol/lateral ventricle) caused a transient change in the steady state levels of AChE mRNA in the septo-hippocampal pathway (Lev-Lehman et al., 1994). The decrease in AChE mRNA in the septum was explained as an effect of AF64A at the gene level of cholinergic neurons.

AF64A also caused changes in mRNA levels of secretogranin II (increases in the hypothalamus, amygdaloid nuclei and the reticular thalamic nuclei), chromogranin B (increase in the hippocampus, temporal cortex, substantia nigra compacta,) and a growth factor VGF (increase in magnocellular neurons). The changes in mRNA expression of these peptides are however considered as a consequence of cholinergic deficits caused by AF64A treatment, rather than due to a direct effect of AF64A (Mahata et al., 1993).
D. 7. Treatment and Prevention of the Effect of AF64A:

AF64A treated animals have been used to mimic the cholinergic hypofunction aspect of AD patients and to screen for therapeutic agents which have the potential for treatment in AD (Hanin, 1996). For example, AF102B and AF150(s), new muscarinic agonists, highly selective for M1 receptors, were tested for their ability to reverse cognitive impairments in a step-through passive avoidance task and in an 8-arm radial maze (Fisher et al., 1989, 1991; Brandeis et al., 1995). These compounds may prove useful for treatment of cholinergic deficiencies and cognitive impairments which have been reported in Alzheimer's disease. However, AF102B also exhibited low toxicity in this animal experiment, which should be a warning in future studies. Similar studies have been also conducted using AChE inhibitors, such as physostigmine (Yamazaki et al., 1991), and tetrahydroaminoacridine (THA) (Murai et al., 1994), and cognition enhancers (Hanin, 1996).

In the interest of reversing or preventing the presynaptic cholinergic damage caused by AF64A, some additional treatments have also been attempted, with some success:

a) Transplantation of fetal cholinergic neurons into the hippocampus can attenuate both the behavioral and neurobiological alterations induced by AF64A (Ikegami et al., 1989, 1990, 1991; Walsh and Opello, 1992; Emerich et al., 1992).

b) Application of some potential treatment chemicals with antioxidant properties, such as tolcapone (Khromova et al., 1995), and Vitamin E (Johnson et al., 1988;
Maneesub et al., 1993; Wortwein et al., 1994) has also been attempted. It has been demonstrated that pretreatment with these antioxidants can attenuate the effect of AF64A presynaptically, which would also include the attenuation of the decrease in ACh content and ChAT activity.

c) NGF is the first neurotrophic factor which was identified as a cholinergic trophic factor. NGF up-regulates cholinergic function in the intact animal. Persistent treatment (14-28 days) with NGF following AF64A administration could conceivably restore the ChAT activity in the hippocampus (Willson and Hanin, 1995).

This success of the chemical treatment by using NGF and Vitamin E, as well as cholinergic tissue transplantation, on the AF64A induced animal model, may provide clues for the treatment of AD.
CHAPTER III
MATERIALS AND METHODS

A. Animals

Adult, Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, PA) weighing between 175-250 grams were used in this study. After shipping, rats were housed in our animal room 10-14 days before they were subjected to the surgery. The rats were housed 2-3 per cage, in a room on a 12 hour light-dark cycle. They had access to food and water ad libitum.

B. Preparation of AF64A Solutions

AF64A was prepared as previously described by Fisher et al. in 1982. Briefly, an aqueous solution of acetyl ethylcholine mustard HCl was adjusted to pH 11.5 with NaOH and stirred at room temperature for 20 min, after which the pH was brought to 7.3 with HCl and the solution stirred at room temperature for another 60 min. This solution was prepared freshly prior to each experiment and kept on ice during the experiment. The solution was used within 6 hours after its preparation.
C. Stereotaxic Surgery and Intraventricular Infusion

Animals were anesthetized with equitensin* (3 ml/kg body weight) and positioned in a Kopf small animal stereotaxic frame. Iodine (1%) was applied to the skin surface prior to incision. Two needles (26 gauge) were passed through parallel drilled holes in the skull and positioned bilaterally in the ventricles, at the following stereotaxic coordinates from bregma: posterior 0.8 mm, lateral ± 1.5 mm and ventral 3.8 mm. AF64A (1.0 nmol/1.5 µl) or an equal volume of vehicle (distilled water prepared according to the same procedure as the AF64A preparation), were infused bilaterally at a flow rate of 0.5 µl/min. for 3 minutes. The needles were left in place for 3 minutes after completion of infusion, then they were slowly pulled out. Postoperatively, chloromycetin ointment (1%) was used locally and wounds were closed with autoclips, and the rats received ampicillin (50 mg/kg) to protect them from subsequent infection.

* Equitensin (100 ml): pentobarbital sodium (0.972 g), 4.25 g chloral hydrate, 2.125 g magnesium sulfate-7H2O, 39.99 ml propylene glycol, 10.0 ml ethanol, and distilled water added to bring the final volume up to 100 ml.

D. Tissue Preparation

In vivo study:

At predetermined time points (1, 2, 4, 7, and 28 days after AF64A infusion) the rats were decapitated, and septum, hippocampus and striatum were dissected from
each rat brain, frozen on dry ice immediately, and stored at -70°C until they were used for assay.

*In vitro* study procedures:

a) Striatal tissues from four untreated rats were pooled together and homogenized in 12 ml sodium phosphate buffer (75 mM sodium phosphate, pH 7.4). The above preparations were aliquoted into equal amounts (50 µl/tube) and stored at -70°C until used for further tests.

b) Purified bovine ChAT protein (Sigma C3388) was dissolved in sodium phosphate buffer (1 unit per 50 µl), then aliquoted into equal amounts and stored at -20°C until used for further tests.

c) Striatal tissue homogenates or purified ChAT proteins prepared as described above were incubated with various concentrations of AF64A (0.5 µM - 0.5 mM for purified ChAT protein, and 5 µM - 5 mM for tissue homogenates) at 37°C for 30 minutes, then placed on ice. The ChAT activity assay was conducted immediately after the AF64A incubation. The remaining samples (~30 µl for each sample) had 4 x protein loading buffer* added (10 µl for each) and stored at -20°C for western blotting assays.

*4 x protein loading buffer (100 ml):* Glycerol (40%), 8% SDS, 0.260 M Tris, 1% bromophenol blue, and pH = 6.8.
E. Total Protein Measurement

Tissue proteins were measured spectrophotometrically, using the procedure described by Lowry et al. (1951). Bovine serum albumin was used as standard.

F. ChAT Activity Assay

This assay was performed as described by Fonnum (1975). Briefly, tissue homogenates (10 µl) were added to 10 µl of buffer substrate mixture which contained: sodium phosphate (pH 7.4), 75 mM; sodium chloride 600 mM; MgCl₂, 40 mM; eserine, 2.0 mM; bovine serum albumin (BSA), 0.05%; choline-iodide, 10 mM; and [³H]-acetyl-CoA, 0.87 mM (18.6 µCi/mmol). After 30 min incubation at 37°C, the tubes were placed on ice and 150 µl of sodium tetraphenylboron (TPB) solution (75 mg TPB per ml 3-heptanone) was added to each tube. After vortexing and centrifugation, 100 µl of the top organic layer was taken to measure the amount of [³H] ACh extracted from the buffer, using liquid scintillation spectrometry. Blank controls consisted of cold phosphate buffer instead of tissue homogenates.

G. Isolation of Total RNA

The isolation of total RNA was performed according to the protocol which is described by Chomczynski (1987). TRI REAGENT™ (mainly containing guanidine thiocyanate and phenol) was used. Briefly, tissue samples were homogenized in TRI REAGENT™ (50-100 mg/ml), and stored at room temperature for 5 minutes. Chloroform...
was added as 0.2 ml per ml TRI REAGENT™ originally used, vortexed for 15 seconds, and stored at room temperature for 3 minutes. The samples were next centrifuged at 12,000 g for 15 minutes at 4°C. The upper, aqueous phase was transferred to a fresh tube, and a second phenol/chloroform extraction was conducted. After the upper aqueous phase was transferred again to a fresh tube, isopropanol 0.05 ml/ml TRI REAGENT™ was added, and the samples then were stored at room temperature for 5 minutes. They were next centrifuged at 12,000 g for 10 minutes at 4°C to remove possible DNA contamination. The liquid phase was then transferred into a fresh tube, isopropanol 0.45 ml/ml TRI REAGENT™ was added, and the samples then were mixed, and stored at -20°C for 1-2 hours. Finally, the samples were again centrifuged at 12,000 g for 10 minutes, at 4°C. RNA was precipitated on the side and bottom of the tube. The RNA pellets were washed with cold 75% ethanol and centrifuged at 12,000 g for 5 minutes at 4°C. After air-drying, the RNA pellets were next resuspended in DEPC-treated double distilled H₂O, and stored in a -70°C freezer. The concentration of the final preparation of total RNA was detected spectrophotometrically at 260 nm.

H. Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

H. 1. Reverse Transcription (RT):

This procedure was performed according to the protocol for reverse transcription from GIBCO BRL, with minor modifications. Briefly, a 20 µl reaction volume was used for each sample. To make first strand cDNA from 2-3 µg total
RNA, 1 µl Oligo (dT)_{12-18} (500 µg/ml) and sterile distilled water were added to bring the volume to 12 µl. The mixture was heated up to 70°C for 10 min. and then rapidly chilled on ice. The contents were collected by brief centrifugation. Then, to each sample, 4 µl 5X First Strand Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl\textsubscript{2}, pH 8.3), 2 µl 0.1 M DTT, 1 µl Mixed dNTP (10 mM) and 1 µl (200 units) of SuperScript RT were added. The contents were mixed gently, and the tubes were placed at room temperature for 10 min., then incubated at 42°C for 50 min. The reaction was stopped by heating the tubes at 95°C for 5 min. After chilling on ice the contents were brought down to the bottom of the tube by brief centrifugation. This RT-product was stored at -20°C until used.

H. 2. PCR Amplification of cDNA:

The oligonucleotides used for the PCR reaction were as follows:

Histone 3.3 5': CCACTGAACCTTCTGATTCGC 3'
Histone 3.3 3': TTCTGTAAGTCTCGATCGTGCG 5'
ChAT 5': GGCTTACTACAGGCTTTACCAGAG 3'
ChAT 3': GTAGTGGGAGACGTAGAGGTCAAA 5'

The expected PCR products were 465 bp for ChAT and 214 bp for Histone 3.3.

The reaction was performed in a total volume of 50 µl for each sample. RT-product (5 µl) was added to the mixture which contained 10 µl PCR Optimizer\textsuperscript{TM} buffer A [Invitrogen: 300 mM Tris-HCl, pH 8.5, 75 mM (NH\textsubscript{4})SO\textsubscript{4} and 7.5 mM
MgCl₂], 3 µl dNTP mix (2.5 mM each of dATP, dGTP, dCTP, and dTTP), 20 pmol primer for each, 2 µCi (3000 Ci/mmol) α-³²P dCTP, 1 unit of Taq polymerase, and PCR water* were used to bring the final volume to 50 µl. The polymerase amplification was carried out for 32 cycles (95°C, 30 sec; 58°C, 1 min.; 72°C, 2 min.) followed by a final extension cycle (72°C, 10 min.).

**PCR water**: It is highly purified double distilled water which was obtained from Invitrogen with PCR Buffer.

I. Labeling of pGEM Markers

The pGEM marker DNA (DNA molecular weight markers) purchased from Promega was radiolabeled by using the following reaction. pGEM marker DNA (1 µl), 1 µl T4 Polynucleotide Kinase (10 units), 2 µl 10 X Kinase buffer (700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT), and 1 µl γ-³²P ATP (10 µCi, 5000 Ci/mmol) were combined in a microcentrifuge tube and brought to a final volume of 20 µl with distilled water. The mixture was incubated at 37°C for 30 min, and the kinase reaction was inactivated at 65°C for 10 min. The free incorporated nucleotide was eliminated by using Ultrafree-MC Filters (MILLIPORE). The end-labeled DNA marker was stored at 4°C until used.
J. Restriction Enzyme Analysis

An aliquot of radiolabeled DNA (ChAT PCR product) obtained as described above, was used for the following restriction enzyme digestion. DNA was added to 10 units of, respectively, \textit{Nco I} in REact 3 Buffer* (50 mM Tris-HCl, pH 8.0, 10 mM MgCl\textsubscript{2}, 0.1 M NaCl); \textit{Pvu II} in REact 6 Buffer* (50 mM Tris-HCl, pH 7.4, 6 mM MgCl\textsubscript{2}, 50 mM KCl, 50 mM NaCl); and \textit{Rsa I} in REact 1 Buffer* (50 mM Tris-HCl, pH 8.0, 10 mM MgCl\textsubscript{2}), and incubated at 37°C for 60 min. After these restriction enzyme reactions, samples were extracted with an equal volume of phenol:chloroform and then the aqueous phase was recovered by centrifugation at 4,000 x g for 15 min.

*Reaction buffer from GIBCO BRL.

K. Detection of PCR Products

To the samples of PCR product, 6 x DNA loading buffer* was added the samples were subjected to electrophoresis on 4% polyacrylamide gel (PAGE) in 1 x TBE buffer at 100 volts until the BPB (bromophenol blue) dye had reached the bottom of the gel. After fixing in 10% acetic acid, and drying with an air drier, the gels were quantitated on a Betagen Betascope 603 analyzer. The ratio of the counts between the investigated gene (ChAT) and the control gene (Histone 3.3) was calculated for each sample. The gels were also exposed under Fuji film.

* 6 x DNA loading buffer: Bromophenol blue (0.25%), 0.25% xylene cyanol FF, and 15% Ficoll (Type 400) in water. Stored at room temperature until used.
L. Isolation Of Total Protein

Total proteins were obtained from the same samples which were used to isolate total RNA. The isolation of total protein was performed according to the protocol which is described by MOLECULAR RESEARCH CENTER, INC., and TRI REAGENT™ was used. Briefly, after the aqueous phase (containing RNA) was removed from the homogenate, 0.3 ml of 100% ethanol per one milliliter of TRI REAGENT used for the initial homogenation was added to the rest of solution. The samples were mixed by inversion and stored at room temperature for 3 min. The samples were next centrifuged at 2,000 g for 5 min. at 4°C, and the supernatants were used for further protein isolation. Isopropanol as 1.5 ml per 1 ml of TRI REAGENT was added, and samples were mixed and stored at room temperature for 10 min., then centrifuged at 12,000 g for 10 min. at 4°C. The supernatants were discarded, and the pellets were washed 3 times with a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. This washing solution as 2 ml per 1 ml TRI REAGENT was added, and the pellets were stored in the washing solution for 20 min at room temperature each time, then centrifuged at 7,500 g for 5 min. at 4°C. Next, ethanol (100%) as 2 ml per 1 ml TRI REAGENT was added, samples were stored at room temperature for another 20 min., and centrifuged at 7,500 g for 5 min. at 4°C. After air drying, the protein pellets were resuspended in 1% SDS, incubated overnight at 50°C, then the samples were centrifuged at 12,000 g for 10 min. at 4°C. The supernatants were collected and stored at -20°C for subsequent Western-blot analysis.
**M. Western Blotting Assay**

**M. 1. Electrophoretic Blotting:**

Total proteins (30 µg) which were stored in protein loading buffer were heated up to 95°C for 5 minutes, then subjected to electrophoresis in the 7.5% SDS-PAGE gel at a consistent voltage of 100 volt in the 1x protein electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and pH = 8.3). After the bromphenol blue dye has been run out off the bottom of the gel, the gel was taken out. Next the gels were firmly and evenly pressed against nitrocellulose paper (NCP) in an electrophoretic destaining chamber, with the nitrocellulose paper facing the cathode. The proteins were then electrophoretically transferred to NCP (BIO-RAD, 0.45 mm pore size in roll form) in the 1 X transfer buffer (25 mM Tris, pH = 8.3, 192 mM glycine and 20% methanol) at 100 volts for 2 hours or at 30 volt overnight, in a cold environment. The NCPs were saved for further analysis.

**M. 2. Immunological Detection of Proteins on Nitrocellulose Paper:**

The NCPs obtained as in M.1. were soaked in a blocking solution (10% wt/vol. nonfat dry milk, 1% calf serum, 0.02% sodium azide, 0.05% NP-40 in PBS) for 2 hours at room temperature. After rinsing with PBS, the NCP were incubated overnight at 4°C, with the primary antibody (Anti-ChAT, mouse host, Boehringer Mannheim, #1464272) in a 1:10 dilution in PBS, 5% BSA, 1% calf serum, 0.05% NP-40 and 0.02% sodium azide. After rinsing with PBS, 15 min. x 1, and 5 min. x 2, the NCPs were incubated with the secondary antibody (Anti-mouse IgG1, goat host, Boehringer Mannheim, #100831) which was linked with horseradish peroxidase, in a 1:500 dilution in PBS, 5% nonfat dry milk,
and 0.05% NP-40 for 2 hours at room temperature. Then, the NCPs were washed thoroughly with PBS 20 min. x 1, and 5 min. x 4.

The signal was detected by using ECL (enhanced chemiluminescence) Western blotting detection reagents (Amersham #RPN2209). The NCPs were incubated in the detecting reagent for 1 min. at room temperature, and immediately exposed to Hyper-film (Amersham) for various time periods.

The results were analyzed using an NIH Image 1.57 System. Gels were quantitated with respect to uncalibrated optical densities (O.D) where gray scale values were measured in the range of 1 to 256. 1 was equivalent to 100% transmittance and 256 was equivalent to 0% transmittance. The O.D from an area adjacent to the protein band was read as background. Standard curves showed good linearization (correlation factor = 0.998).

N. Chemicals and Enzymes

Acetyethylcholine mustard HCl was obtained from UCB (Belgium). TRI REAGENT™ was purchased from Molecular Research Center, INC. (MRC). [3H]-acetyl coenzyme A was purchased from DuPont; α-[32P]-dCTP and γ-[32P]-dATP were purchased from Amersham. All other chemicals were purchased from Sigma unless stated otherwise. Enzymes for RT-PCR and restriction enzyme analysis were purchased from GIBCO BRL. Antibodies were purchased from Boehringer Mannheim. PCR buffer and PCR water were purchased from Invitrogen.
O. Statistics:

The data are represented as group means and standard errors of the means (S.E.M.). The data were analyzed by two way analyses of variance (ANOVA). Group means were compared by Newman-Keuls' test. Correlation analysis was conducted by using GB-STAT.
CHAPTER IV

RESULTS

A. General Observations

AF64A treated rats were more aggressive compared to control animals.

When tissues were dissected, a softer septum was observed in AF64A treated rats within one week after treatment. Twenty-eight days after the icv infusion of AF64A, the septum from treated rats showed distinct shrinkage compared to control animals at the same time point. At all time points, increased cerebral spinal fluid was observed in dissected brain which confirmed findings which had been reported before. No significant differences in hippocampal and striatal tissue between the control and AF64A treated animals were observed over all the time points.

These markers have been used to serve as the first index of whether AF64A treatment was successful.

B. ChAT Activity Studies

B. 1. Septo-Hippocampal Pathway:

The effect of single icv administration of AF64A (1 nmol/lateral ventricle) on ChAT activity in the hippocampus was examined (Figure 4). Statistical analysis
indicated that a significant AF64A treatment x time course interaction occurred $(F(4,32) = 3.42, P = 0.02)$. There was a significant decrease in ChAT activity following the AF64A treatment $(F(1,8) = 155.35, P < 0.001)$ and over the time course observed $(F(4,32) = 4.94, P < 0.01)$ in the hippocampus. The decrease in ChAT activity was observed as early as one day after AF64A infusion, (82.82 % of control; $P < 0.05$) in this study. The most severe decrease in ChAT activity (61.39 % of control; $P < 0.01$) in the hippocampus was observed at 7 days after AF64A infusion, and the extent of the decrease lasted for at least as long as 28 days (62 % of control; $P < 0.01$), the last time point observed in this study.

In the septum (Figure 5), on the other hand, the activity of ChAT increased following AF64A administration. Statistical analysis indicated that a significant AF64A treatment x time course interaction occurred $(F(4,20) = 11.3, P < 0.0001)$. A significant alteration occurred in ChAT activity following AF64A treatment $(F(1,5) = 96.84, P < 0.001)$ and over the time course observed $(F(4,20) = 12.06, P < 0.0001)$. The increase in ChAT activity in the septum was transient. This increase was observed starting at day 2 after treatment (143.73% of control; $P < 0.01$), and remained proximal level until day 7 (164.36 % of control; $P < 0.01$). By 28 days after AF64A administration, ChAT activity in the septum did not show significant difference from control.
B. 2. Striatum:

In the striatum (Figure 6), statistical analysis indicated that there was no significant AF64A treatment x time course interaction ($F(4,32) = 0.81, P = 0.53$). There was no significant alteration in ChAT activity following the AF64A treatment and over the time course observed.

The alterations of ChAT activity in these different brain regions have been used to serve as an index of whether AF64A treatment was successful.

C. Optimization of RT-PCR Working Conditions

C.1. Selection of the Primer for PCR & Restriction Enzyme Analysis of ChAT cDNA PCR Product.

Over the past few years, many researchers have attempted to quantitate ChAT mRNA using various methods. Due to the low level of expression of ChAT mRNA in rat brain tissue, Northern blot analysis and RNase protection analysis are not sensitive enough for the detection of ChAT mRNA levels. Therefore, a more sensitive method, semi-quantitative RT-PCR, was used for the purpose of measuring ChAT mRNA levels in this study.

The primers for amplifying ChAT cDNA were designed based on the cDNA sequence which was published by Brice et al. (1989). A set of forward (sense, 1500-1523) and reverse (antisense, 1941-1964) PCR primers were chosen by using a computer program, Oligo 4.0. The ideal annealing temperature for this pair of primers
Figure 4: Effect of AF64A on ChAT activity in the hippocampus. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Enzyme assay was performed in duplicate on tissue homogenates according to the procedure described in the Methods chapter. The data were obtained from 4 - 9 animals per group. The activity of ChAT in control group: mean ± SEM (30.5 ± 0.76 nmol/mg protein/hr). Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Neuman-Keuls test). * P < 0.05; ** P < 0.01.
Figure 5: Effect of AF64A on ChAT activity in the septum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Enzyme assay was performed in duplicate on tissue homogenates according to the procedure described in the Methods chapter. The data were obtained from 3 - 6 animals per group. The activity of ChAT in control group: mean ± SEM (42 ± 1.7 nmol/mg protein/hr). Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Neuman-Keuls test). ** P < 0.01.
Figure 6: Effect of AF64A on ChAT activity in the striatum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Enzyme assay was performed in duplicate on tissue homogenates according to the procedure described in the Methods chapter. The data were obtained from 8 animals per group. The activity of ChAT in control group: mean ± SEM (120.3 ± 3.03 nmol/mg protein/hr). Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Neuman-Keuls test).
is 58°C, and the expected size of the PCR product is 465 bp. Restriction enzyme analysis of this 465 bp PCR product was conducted with three enzymes, which were chosen based on the ChAT cDNA sequence by using computer program DNASIS, and produced unique cuts within the DNA to yield different sizes of DNA fragments: Nco I, 366 & 99 bp; Pvu II, 293 & 172 bp; and Rsa I, 259 & 206 bp (Figure 7). These sizes of the DNA fragments were expected according to the sequence of ChAT cDNA (Brice et al., 1989).

C. 2. Optimization of PCR Buffer Conditions:

In this semi-quantitative RT-PCR study, a housekeeping gene, Histone 3.3, was chosen as an internal control for both the reverse transcription and amplification. As stated in my overview hypothesis, AF64A might cause non-specific gene damage, and thus the histone gene of cholinergic neurons could also be damaged by AF64A. However, in this in vivo system study, histone 3.3 still can be used as a very good internal standard to normalize the variation of RT-PCR analysis from sample to sample for the following reasons: It has been known that, in the central nervous system, the ratio between neurons and astrocytes is one to ten. Meanwhile, cholinergic neurons are not the only neuron type in any brain region. In the striatum, only 1-2 % of neurons belong to the cholinergic system (Woolf and Butcher, 1981; Bolam et al., 1984; and Phelps et al., 1985). Similar or even smaller populations of cholinergic neurons exist in the septum. Therefore, less than 1 % of all the cells in the septum or striatum are cholinergic neurons, and only those histone genes which exist in these less than 1 % of
**A**

ChAT cDNA Sequence

Upstream primer

1501 GCTTACTACA GGCTTTACCA GAGACTGGTG CCCACCTATG AGAGCGCATC 51
1551 CATCCGCCGC TTCCAGGAAAG GTCGGGTGGA CAACATCAGA TCAGCCACTC 101
1601 CAGAGGCTCT TTGGTTTTGTG CAAGCCATGA CTGACCACAA GGCTGCCATG 151
1651 CCGGCTTCTG AGAAACTG CATGCTGCTGCA GAGCCCATGC AGGCCCAGAC 201
1701 TGAGTACACA GTGATGCCCA TAAACCGGAT GGCATTGAC AACCATCTTC 251
1751 TGGCACTGAG GGAGCTGGCC CGAGACCTGT GCAAAGAGCC ACCTGAGATG 301
1801 TTCATGGATG AAACATACCT GATGAGCAAC CGTGTTGTC TCTCCACCAG 351
1851 CCAGGTGCCT ACAACCATGG AGATGTCTG CTGTATCGCA CCGTGCTGCC 401
1901 CCAATGGCTA TGGAGCCTGC TACAACCCCC AGCCCGAGGC CATCACCTTC 451
1951 TGCATCTCCA GTTT

**B**

PCR Product Sequence

\[ \text{G} \quad 1 \]

Downstream primer

RT blank control
PCR blank control
ChAT 465 bp
Nco I 365/89 bp
Pvu II 283/172 bp
Rsa I 259/206 bp

**Image Details**

- **A**: ChAT cDNA Sequence with Upstream and Downstream primers.
- **B**: PCR Product Sequence with enzyme restriction sites.

**Genetic Analysis**

- The ChAT cDNA sequence is presented with restriction enzyme sites indicated (Pvu II, Rsa I, Nco I).
- The PCR product sequence is shown with the expected bands for the given restriction enzymes.

**Quality Assurance**

- The text is presented in a clear and organized manner, facilitating easy reading and understanding.
- The use of specific enzymes and the expected band sizes support the genetic analysis presented.

**Relevance**

- The document appears to be a part of a broader scientific study, possibly focusing on genetic analysis and sequence variations within the ChAT gene.
- The presence of enzyme restriction sites suggests the study's emphasis on genetic modification or genetic mapping.
Figure 7: Setup of primers for ChAT cDNA amplification and restriction enzyme analysis of the ChAT PCR product. Panel A. Sequence of ChAT cDNA PCR product. A set of primers for amplification of ChAT cDNA was designed based on the ChAT cDNA sequence published by Brice et al. (1989). A 465 bp PCR product was expected. Three restriction enzymes were chosen to verify the correctness of this 465 bp PCR product. Panel B. Autoradiograph of the restriction enzyme analysis of the 465 bp ChAT cDNA. The PCR product was incubated with the following enzymes which produced unique cuts within the DNA to yield different size DNA fragments: Nco I (366&99 bp); Pvu II (293&172 bp); and Rsa I (259&206 bp). Lane 1: DNA marker; Lane 2: PCR blank control; Lane 3: RT-PCR blank control. Lane 4: Uncut 465 bp ChAT cDNA; Lane 5: Nco I; Lane 6: Pvu II; Lane 7: Rsa I.
cells have a chance to be affected by AF64A. Most of the housekeeping gene histone 3.3, which exists in the remaining more than 99% of cells will thus not be directly affected by AF64A. So, a possible alteration of the expression of the histone gene within the small population of cholinergic neurons would be neutralized by the large pool of the expression of histone gene in the whole brain region.

According to the primer design, the expected size of PCR product from H3.3 was 214 bp (Santiago, 1995).

Utilizing an optimized buffer kit from Invitrogen (Invitrogen PCR Optimizer™), the efficiency of amplification of both H3.3 and ChAT cDNA were tested in different buffer solutions which varied in pH and MgCl₂ concentrations (Table 1). There were clearly two products amplified in buffers A-F, H, and J. Only H3.3 was amplified in buffer G, K, N, and P. There was no PCR product found in buffers I, L, M and O (Figure 8).

C. 3. RT-PCR Blank Control.

To confirm the specificity of the RT-PCR products, a series of control experiments was conducted (Figure 9): 1) PCR blank control was used to exclude the possibility of contamination happening during the amplification process. In this control, RT-products were replaced by PCR water in the PCR reaction. 2) RT blank control was used to exclude the possibility of contamination happening during the reverse transcription process. In this control, during the reverse transcription reaction, total RNA was replaced by PCR water, and this RT product was subsequently
subjected to the PCR reaction. 3) Genomic DNA control was used to show, in the event that there was genomic DNA contamination in the total RNA samples, what would be observed as the final result of the RT-PCR. In this control, genomic DNA 100 ng was added in the PCR reaction tube instead of the RT-product. 4) Total RNA control was used to exclude the possibility of genomic DNA contamination. In this control, total RNA was subjected directly to the PCR reaction. Since Taq polymerase does not recognize RNA as template and amplify it, any positive signal must come from contaminating DNA. If the RT blank control and PCR blank control were negative, then this signal may be due to contamination from the total RNA sample itself.

Total RNA control was conducted here to confirm that the method of total RNA isolation has efficiently eliminated all genomic DNA from the samples.

PCR blank control and RT blank control were used for each of the following experiments.

C. 4. Optimization of the Number of PCR Cycles and the Concentration of Two Pairs of Primers.

Buffer A, B, E, and F were further subjected to an optimization analysis for the number of PCR cycles. Buffer A (pH = 8.5; MgCl₂, 1.5 mM) consistently provided good amplification of both H3.3 and ChAT cDNA PCR products (Figure 10A).

The range of linearity with regard to the number of amplification cycles was determined to be 32 cycles since the H3.3 almost reached a plateau at 35 cycles, while
ChAT started to show a very clear band at 30 cycles and was still within the linear range for up to 45 cycles (Figure 10B).

Since the PCR product of ChAT cDNA is relatively small, an attempt to increase this product was made by increasing the concentration of the primer for ChAT cDNA. However, there was a severe competition between H3.3 and ChAT cDNA primer (Figure 11). After adjustment, the final concentration of both pairs of primers was fixed at 20 pmol of each primer for each sample.

D. Time Course Study of ChAT mRNA Steady State Level:

D. 1. Formula for the Calculation of Steady State Levels of ChAT mRNA.

The steady state levels of ChAT mRNA were quantified using RT PCR amplification.

ChAT mRNA expression was calculated as follows:

1) First, from each individual sample, the counts of PCR products for both ChAT cDNA and H3.3 cDNA were obtained from the reading of the Betagen Scope. Then the ratios of the counts of ChAT over histone for each individual sample were calculated from control and AF64A treated rats ($R_C$ and $R_A$), respectively.

\[
R_C = \frac{\text{Counts of ChAT (control)}}{\text{Counts of histone (control)}}
\]

\[
R_A = \frac{\text{Counts of ChAT (AF64A-treated)}}{\text{Counts of histone (AF64A-treated)}}
\]
## TABLE 1

Invitrogen PCR Optimizer™ Buffers

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<th>[MgCl₂] (mM)</th>
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<th>pH 9.5</th>
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<td>E</td>
<td>I</td>
<td>M</td>
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<td>B</td>
<td>F</td>
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<td>G</td>
<td>K</td>
<td>O</td>
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<td>D</td>
<td>H</td>
<td>L</td>
<td>P</td>
</tr>
</tbody>
</table>
Figure 8: Autoradiograph of the effect of different PCR OptimizerTM (Invitrogen) buffers on amplification of ChAT and H3.3 cDNA signal. Total RNA from striatal tissue was reverse transcribed, and the polymerase amplification was carried out for 35 cycles as described in the Methods chapter. Predicted PCR products for ChAT and H3.3 were 465 and 214 bp, respectively.
Figure 9: Autoradiograph of RT--PCR control experiment. Lane 1: DNA molecular weight marker; Lane 2: PCR blank control; Lane 3: RT blank control; Lane 4: Genomic DNA control; Lane 5: Total RNA control; and Lane 6: RT-PCR product. The upper band is the ChAT cDNA PCR product; the lower band is the histone cDNA PCR product.
A

Number of PCR Cycles

ChAT=465 bp
H3.3=214 bp

B

Betagen Counts (cpm)

[Histone 3.3] = 4 μM
[ChAT] = 4 μM

Number of Cycles
Figure 10: Reverse transcription-polymerase chain reaction (RT-PCR) analysis of ChAT and H3.3 signals with increasing amplification cycles. Total RNA (3 µg) from striatal tissue was subjected to RT-PCR analysis using ChAT and H3.3 primers. Aliquots of the reaction were removed at 20, 25, 30, 35, 40, and 45 cycles. The expected PCR products were 465 bp for ChAT and 214 bp for H3.3. Panel A: Autoradiograph of the RT-PCR reactions. Panel B: Densitometric results after the gel was scanned in a Betagen analyzer.
Primer:  [ChAT] = 6 µM  
[His3.3] = 4 µM

Figure 11: Autoradiograph of the competition effect of these two pairs of primer. The PCR products of ChAT cDNA were increased along with the cycles, while the PCR products of histone 3.3 cDNA were decreased.
2). The average of $R_C$ ($\overline{R_C}$) for each time point was calculated as:

$$\overline{R_C} = \frac{\sum R_C}{N}$$

(where $N$ = the number of rats for control group at each time point)

3). The percentage of the $R$ ($R\%$) from each individual sample (both control and AF64A treated animals) compared to this $\overline{R_C}$ value was calculated as:

$$R_C\% \ (or \ R_A\%) = \left[ \frac{R_C \ (or \ R_A)}{\overline{R_C}} \right] \times 100$$

4). The mean ± SEM of this $R_C\%$ or $R_A\%$ was used to represent the expression of ChAT mRNA in the final results.

D. 2. Septo-Hippocampal Pathway:

Total RNA isolated from rat septal tissue was first reverse transcribed to cDNA. Then, the cDNA was amplified using two pairs of primer for ChAT and H3.3 respectively, and the steady state level of ChAT mRNA was calculated as described above. Statistical analysis indicated that a significant AF64A treatment x time course interaction occurred ($F(4,20) = 6.7, P = 0.001$) in the septum. There was a significant alteration in ChAT mRNA over the time course observed ($F(4,20) = 4.4, P = 0.01$), although there was no significance following AF64A treatment ($F(1,5) = 0.3199, P = 0.6$). However, comparing AF64A and vehicle-injected rats at each time point using Newman-Keuls test, AF64A treatment appeared to significantly affect ChAT mRNA levels in the septum (Figure 12). A significant increase (up to 167% of control; $P < 0.05$) in ChAT mRNA levels was observed as early as 1-2 days after the administration of AF64A; this increase was followed by a significant decrease (down to 42.5% of
control; $P < 0.05$) at 7 days after AF64A treatment. This reduction of ChAT mRNA levels in the septum was still observed at day 28 (37.6% of control; $P < 0.05$).


In the striatum (Figure 13), statistical analysis indicated that there was no significant AF64A treatment x time course interaction ($F(4,28) = 0.63, P = .0.65$). There was no significant alteration in ChAT mRNA levels following AF64A treatment ($F(1,7) = 1.27, P = 0.3$) and over the time course observed ($F(4,28) = 0.38, P = 0.82$).

E. Time Course Study of the Amount of ChAT Protein:

E. 1. Normalization of the Conditions of Western Blot Analysis:

In this study, a monoclonal antibody which binds ChAT protein to an antigenic determinant different from the active site was used to perform Western blot analysis (Eckenstein and Thoenen, 1982). Even if ChAT protein had been alkylated by AF64A at its active site and inactivated (Sandberg et al, 1985), it would still be recognized by this antibody.

To test the ability of this antibody to recognize ChAT protein in its denatured condition, commercially available (Sigma) purified bovine ChAT protein, was first used to conduct a Western blot analysis. This antibody recognized two protein bands from purified bovine ChAT protein at molecular weights of 68 kD and 66 kD, respectively (Figure 14 A). This result confirmed what has been reported. The density of the protein bands showed very good dose-dependent changes (Figure 14 B), with a
Day 7

Day 28
Figure 12: Effect of AF64A on ChAT mRNA expression in the septum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). RT-PCR was performed in duplicate on septal tissue total RNA according to the procedure described in the Methods chapter. The data were obtained from 6 animals per group. Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Newman-Keuls' test.). * P < 0.05. Panel A: Autoradiographs of the RT-PCR reactions. Each autoradiograph represents the result from one time point. Starting from the left, the first three lanes are: DNA molecular weight marker (1); PCR blank control (2); and RT-PCR blank control (3); respectively. For the remaining lanes, each lane represents a result from an individual animal. There are six animals in each group. Panel B: Densitometric results after the PAGE gels were scanned in a Betagen analyzer.
Day 1

Day 2

Day 4
Day 7

ChAT=465bp
H3.3=214bp

Day 28

ChAT=465bp
H3.3=214bp
Figure 13: Effect of AF64A on ChAT mRNA expression in the striatum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). RT-PCR was performed in duplicate on striatal tissue total RNA according to the procedure described in the Methods chapter. The data were obtained from 8 animals per group. Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Newman-Keuls' test). Panel A: Autoradiographs of the RT-PCR reactions. Each autoradiograph represents the result from one time point. Starting from the left, the first three lanes are: DNA molecular weight marker (1); PCR blank control (2); and RT-PCR blank control (3); respectively. For the remaining lanes, each lane represents a result from an individual animal. There are eight animals in each group. Panel B: Densitometric results after the PAGE gels were scanned in a Betagen analyzer.
correlation factor of 0.98. This commercially available purified bovine ChAT protein was therefore used as a positive control for Western blot analysis in all of the following experiments.

Next, the total protein isolated from rat brain tissue was subjected to the same analysis. Unexpectedly, two bands were observed from the rat brain tissue protein samples, at MW = 67 kD, and MW = 45 kD, respectively. Both bands were recognized by the same monoclonal antibody. When the linearization experiment was conducted, the densities of both protein bands also showed dose-dependent changes (Figure 15).

The protein amount was read from the protein band and calculated for each individual sample as:

\[
\text{O.D. of protein} = \text{area of band} \times (\overline{\text{O.D. of band}} - \overline{\text{O.D. of background}}).
\]

\[
\overline{\text{O.D.}} = \text{mean of optical density}.
\]

The calculation of the expression of protein is similar to step 2-4 of the calculation of expression of ChAT mRNA (page 59 &-67). However, reading of O.D. was using for protein instead of using the R for mRNA.

E. 2. Septo-Hippocampal Pathway:

In the septum (Figure 16), a single icv injection of AF64A induced a transient increase in the expression of the 67 kD protein \( (F(1,5) = 7.46, P < 0.05) \). Statistical analysis indicated that there was no significant AF64A treatment x time course interaction \( (F(4,20) = 1.56, P = 0.22) \). A significant increase in the amount of 67 kD
protein was first observed at day 2 (143.4 % of control, \( P < 0.05 \)), which peaked at day 4 (+150%, \( P<0.05 \)) and was reduced to control levels at day 28. Meanwhile, there was a more extensive and long-lasting increase in the expression of the 45 kD protein. Statistical analysis indicated that there was a significant AF64A treatment x time course interaction occurred (\( F(4,20) = 19.45, P < 0.0001 \)). There was a significant alteration in the amount of the 45 kD ChAT protein following the AF64A treatment (\( F(1,5) = 302.66, P < 0.0001 \)) and over the time course (\( F(4,20) = 23.95, P < 0.0001 \)) in the septum. The increase in the 45 kD protein was observed at the first time point (day 1, 639.9 % of control, \( P < 0.01 \)) and peaked at day 2 (+1407%, \( P < 0.01 \)); the level of the 45 kD protein remained at a high level for at least for 28 days (190.7 % of control, \( P < 0.5 \)) after the infusion of AF64A. When the septal proteins from day two were tested, two commercially available ChAT proteins, human placenta and bovine brain, respectively, were used as positive controls. In addition to the high molecular weight protein band, a faint band at molecular weight of 45 kD was also observed in both of these purified ChAT proteins.

In the hippocampus (Figure 17), unlike the results from the ChAT activity assay, there was no immediate decrease in the amount of the 67 kD ChAT protein. Statistical analysis indicated that there was a significant AF64A treatment x time course interaction (\( F(4,32) = 7.86, P =0.002 \)). There was a significant alteration in the amount of the 67 kD protein following AF64A treatment (\( F(1,8) = 24.41, P = 0.001 \)) and over the time course observed (\( F(4,32) = 7.11, P = 0.0003 \)). The decrease of this
protein was first observed at day 4 (down to 53.5% of control, \( P < 0.01 \)) following treatment with AF64A. This change appears to be long-lasting; the 67 kD expression in the hippocampus was still low at the last time point measured, on day 28 (56.5% of control, \( P < 0.01 \)). There also was an alteration in the amount of the 45 kD protein in the hippocampus. Statistical analysis indicated that there was a significant AF64A treatment x time course interaction (\( F(4,32) = 2.91, P = 0.037 \)). There was a significant alteration in the amount of the 45 kD protein following the AF64A treatment (\( F(1,8) = 24.66, P = 0.001 \)) and over the time course observed (\( F(4,32) = 4.42, P = 0.006 \)). This increase in the 45 kD protein lasted from day 2 to day 7 following AF64A administration (180% of control, \( P < 0.05 \)).

E.3. Striatum

In the striatum (Figure 18), a single icv injection of AF64A did not significantly affect the expression of the 67 kD protein which corresponded to the results of ChAT activity analysis. Statistical analysis indicated that there was no significant AF64A treatment x time course interaction (\( F(4,24) = 0.67, P = 0.62 \)). There was no significant alteration in the amount of the 67 kD protein following AF64A treatment (\( F(1,6) = 3, P = 0.13 \)) and over the time course observed (\( F(4,24) = 0.92, P = 0.47 \)). However, there was a significant change in the amount of the 45 KD protein. Statistical analysis indicated that there was a significant AF64A treatment x time course interaction (\( F(4,24) = 6.69, P = 0.0009 \)). There was a significant alteration in the amount of the 45 kD protein following AF64A treatment (\( F(1,6) = 75.75, P = \))
A

Loading Amounts of ChAT Protein (unit \(10^{-2}\)):

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<th>4.0</th>
<th>3.5</th>
<th>3.0</th>
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MW

68.000

66.000

B

OD (Densitometry)

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Figure 14: Correlation of Western blot analysis for the measurement of ChAT protein amounts. Panel A: Autoradiograph of Western blot analysis. Different amounts of purified bovine ChAT protein, commercially available, were loaded on the 7.5 % SDS-PAGE gel. The expected molecular weights of this protein were 68 & 66 kD. Panel B: Densitometric results after the autoradiograph was scanned in an NIH Image 1.57 System. The density from both bands was counted.
Brain Tissue from AF64A Treated Rats

A1

Increase in the Protein Loading Amount

MW
67,000
45,000

Purified Bovine ChAT Protein

B1

<table>
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</table>

Protein Loading Amount (ug)

- MW = 67 kD
- MW = 45 kD
Brain Tissue from Control Rats

A2
Increase in the Protein Loading Amount

Purified Bovine ChAT Protein

MW
67,000
45,000

B2

O.D.

MW = 67 kD
MW = 45 kD

Protein Loading Amount (μg)
Figure 15: Linearization of tissue ChAT protein. Panel A: Autoradiograph of the Western blot analysis. Different amounts of total protein from septum were loaded on the 7.5 % SDS-PAGE gel. Two proteins with different molecular weight (67 & 45 kD, respectively) were detected. A1. Septal tissue harvested from AF64A treated animals. A2. Septal tissue harvested from control animals. Panel B: Densitometric results after the autoradiograph was scanned in an NIH Image 1.57 System. The density from both bands was counted.
**A1**

Control | AF64A
---|---
MW | MW
68,000 | 68,000
66,000 | 66,000
45,000 | 45,000

Purified Bovine ChAT Protein

Day 1

**A2**

Control | AF64A | Control | AF64A
---|---|---|---
MW | MW | MW | MW
68,000 | 68,000 | 68,000 | 68,000
66,000 | 66,000 | 66,000 | 66,000
45,000 | 45,000 | 45,000 | 45,000

Day 2

Human; Bovine Purified ChAT Protein

**A3**

Control | AF64A
---|---
MW | MW
68,000 | 68,000
66,000 | 66,000
45,000 | 45,000

Purified Bovine ChAT Protein

Day 4
Purified Bovine ChAT Protein

Day 7

Purified Human ChAT

Day 28
Figure 16: Effect of AF64A on ChAT protein expression in the septum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Western blot analysis was performed by using total protein isolated from septal tissue according to the procedure described in the Methods chapter. The data were obtained from 5-6 animals per group. Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Newman-Keuls' test). * P < 0.05; ** P < 0.01. Panel A: Autoradiographs of Western blot analysis. Each autoradiograph represents the result from one time point. In panels A1, A3, A4 and A5, starting from the left, the first lane was loaded with purified bovine ChAT protein as positive control. For the remaining lanes, each lane represents a result from an individual animal. There were 5-6 animals in each group. Panel B: Densitometric results after the films were scanned in an NIH Image 1.57 System. B1 shows the data for the 67 kD protein. B2 shows the data for the 45 kD protein (common log scale for the Y-axis).
**A1**

Purified Bovine ChAT Protein

Day 1

**A2**

Purified Bovine ChAT Protein

Day 2

**A3**

Purified Bovine ChAT Protein

Day 4
B1

MW = 67 kD

![Bar graph showing ChAT protein expression (% of control) over time (days) for Control and AF64A groups. The graph compares MW = 67 kD protein expression over time.](image)

B2

MW = 45 kD

![Bar graph showing ChAT protein expression (% of control) over time (days) for Control and AF64A groups. The graph compares MW = 45 kD protein expression over time.](image)
**Figure 17:** Effect of AF64A on ChAT protein expression in the hippocampus. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Western blot analysis was performed by using total protein isolated from hippocampal tissue according to the procedure described in the Methods chapter. The data were obtained from 7 animals per group. Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Newman-Keuls' test) * $P < 0.05$; ** $P < 0.01$. **Panel A:** Autoradiographs of Western blot analysis. Each autoradiograph represents the result from one time point. Starting from the left, the first lane is purified bovine ChAT protein. For the remaining lanes, each lane represents a result from an individual animal. There are 7 animals in each group. **Panel B:** Densitometric results after the films were scanned in an NIH Image 1.57 System. B1 shows the data for the 67 kD protein. B2 shows the data for the 45 kD protein (common log scale for the Y-axis).
A1
MW
Control  AF64A
68,000  68,000
66,000  66,000
45,000  45,000

Purified Bovine ChAT Protein  Day 1

A2
MW
Control  AF64A
68,000  68,000
66,000  66,000
45,000  45,000

Purified Bovine ChAT Protein  Day 2

A3
MW
Control  AF64A
67,000  67,000
45,000  45,000

Day 4
MW
68,000
66,000
45,000

Control
AF64A

Purified ChAT Bovine Protein  Day 7

MW
68,000
66,000
45,000

Control
AF64A

Purified Bovine Protein  Day 28
Figure 18: Effect of AF64A on ChAT protein expression in the striatum. Animals were decapitated at different time points after icv administration of AF64A (1 nmol/side, bilaterally). Western blot analysis was performed by using total protein isolated from striatal tissue according to the procedure described in the Methods chapter. The data were obtained from 4 - 7 animals per group. Statistical significance was calculated from a comparison of the AF64A and vehicle-injected rats, at the same time point (Newman-Keuls' test) * P < 0.05; ** P < 0.01. Panel A: Autoradiographs of Western blot of analysis. Each autoradiograph represents the result from one time point. Starting from the left, the first lane is purified bovine ChAT protein. For the remaining lanes, each lane represents a result from an individual animal. There are 4 - 7 animals in each group. Panel B: Densitometric results after the films were scanned in an NIH Image 1.57 System. B1 shows the data for the 67 kD protein. B2 shows the data for the 45 kD protein (common log scale for the Y-axis).
0.0001) and over the time course observed (F(4,24) = 8.95, P = 0.0001) in the striatum. The increase of the amount of the 45 kD protein peaked at day 2 (558.4 % of control, P < 0.01) after the treatment with AF64A; this increase was observed at all the time points, with statistical significance (Figure 18).

Generally, changes in the expression of 67 kD protein were paralleled by changes in ChAT activity in all brain regions which have been observed. However, there was no relationship between changes in the amount of 45 kD protein and ChAT activity.

F. In Vitro Studies of ChAT Protein:

Since the amount of the low molecular weight protein (MW, 45 kD) increased significantly after AF64A treatment in all brain regions which were studied, there was a possibility that AF64A might bind to ChAT protein and break it down to smaller molecules. The following in vitro experiments were conducted to test this hypothesis.

F. 1. Effect of AF64A on Purified Bovine ChAT Protein.

Co-incubation of AF64A (0.5 µM - 0.5 mM) with purified bovine ChAT protein (37°C, 30 minutes) caused a significant decrease in ChAT activity in a dose dependent manner (K_i = 0.1 mM), without significant change in ChAT protein amounts at MW 68 & 66 kD. There were also no other new bands appearing following AF64A treatment (Figure 19).
F. 2. Effect of AF64A on ChAT Protein in Tissue Homogenates.

Co-incubation of AF64A (5 µM - 5 mM) with tissue homogenate (from rat striatum) caused a significant decrease in ChAT activity in a dose dependent manner ($K_i = 1$ mM) without significant changes in ChAT protein amounts at either molecular weight of 67 kD or 45 kD (Figure 20).

These data suggest that AF64A reduces ChAT activity by masking the active site of the enzyme rather than by breaking this protein down to smaller molecules.

F. 3. The 45 kD Protein is an Unstable Protein.

During the above experiments, protein was stored in a 1% SDS solution and in the presence of the proteinase inhibitor PSMF. However, following repeated freezing, thawing, and heating up to 95°C several times, the 45 kD protein disappeared from the Western blot. Meanwhile, no significant changes in the 67 kD protein were observed. This result suggests that the 45 kD protein is less stable than the 67 kD protein (Figure 21).
**A**

- **ChAT Activity (% Control)**
- **ChAT Protein**
- **ChAT Activity**

![Graph showing ChAT Activity and ChAT Protein](image)

**AF64A Concentration (M)**

- Control
- Increase in the Concentration of AF64A

**B**

- **MW**
  - 68,000
  - 66,000
  - 45,000

- **Control**
- **Increase in the Concentration of AF64A**
Figure 19: Effect of AF64A on purified ChAT protein. Different concentrations of AF64A (0.5 µM - 0.5 mM) were incubated with purified ChAT protein for 30 minutes at 37°C. Then the protein samples were subjected to the ChAT activity assay and Western blot analysis.
A ChAT Activity

Concentration of AF64A (M)

ChAT Activity (% Control)

ChAT Protein MW = 67 kD
ChAT Protein MW = 45 kD

B

Increase in the concentration of AF64A

MW

68,000
66,000
45,000

Purified Bovine ChAT Protein
Control Tissue Sample
Figure 20: Effect of AF64A on ChAT protein of tissue homogenate. Different concentrations of AF64A (5 µM - 5 mM) were incubated with tissue homogenate (striatal tissue from untreated rats) for 30 minutes at 37°C. Then the protein samples were subjected to the ChAT activity assay and Western blot analysis.
Striatal ChAT Protein:

A

Control AF64A

MW

68,000 66,000 45,000

Purified Bovine ChAT Protein Day 2

B

Control AF64A

MW

68,000 66,000 45,000

Purified Bovine ChAT Protein Day 2
Figure 21. Autoradiographs for Western blot analysis. The protein samples used for both experiments are identical; they are the proteins which were isolated from day 2 striatum. A. Protein loading samples were prepared freshly before loading for electrophoresis. Both molecular bands were detected. B. Protein samples which had been frozen, thawed and heated to 95°C for 5 min. a couple of times before loading. The low molecular weight bands have disappeared in this autoradiograph.
CHAPTER V

DISCUSSION

The actions of AF64A have been widely studied, and several possible targets of AF64A action have been reported, which include the HACkT (Curti and Marchbanks, 1982; Uney and Marchbanks, 1987), ChAT protein (Sandberg et al., 1985), and axonal transport (Kása and Hanin, 1985) of the cholinergic neuron (Figure 22).

Since AF64A is structurally a mustard, it is possible that AF64A directly alkylates the molecules of the HACkT and ChAT proteins, as well as those proteins that are involved in the axonal transport mechanisms of cholinergic neurons (Calabresi and Chabner, 1990). The alkylation of these molecules would affect their function, which could appear as a decrease in the activity of ChAT and HACkT, and blockade of axonal transport, respectively. According to previous studies, a single icv dose of AF64A administration causes long term (months) damage in the central cholinergic system of animals (Fisher et al., 1986; Leventer et al., 1987; Hörtagl et al., 1991; El Tamer et al., 1992). If this long-lasting deficit of cholinergic function was caused by a direct effect of AF64A on any or all of those molecules, then AF64A would have to be in its active form for a very long time period, since all of these molecules have a relatively rapid turnover time. For example, the half life of ChAT protein is only several days (Hersh, 1992;
Figure 22. Schematic representation of the septo-hippocampal cholinergic pathway and possible sites (*) of action of AF64A.
and Mahler, 1975). Any ChAT protein damaged directly by AF64A would not stay in the tissue too long, since it would be rapidly replaced by newly synthesized molecules. Therefore, to induce a long term cholinergic deficiency by directly acting on these molecules, the active form of AF64A would have to be present in tissues for the duration of the study; and constantly alkylate those newly synthesized molecules to induce their functional deficit. However, AF64A has a very short half life (Pilar et al., 1987; Goldstein et al., 1988) at physiological conditions, which is about 3-6 hours in CSF (pH = 7.4, and 37°C). A chemical with such a short half life (hours) appears to have a long lasting (months) toxicity in vivo, which is difficult to explain by AF64A only acting on the ChAT protein or HACHT themselves. There are several possibilities which could cause a long-term decrease in these parameters in the hippocampus. These include: 1) A loss of cholinergic innervation in the hippocampus; and/or 2) AF64A damage of DNA molecules of cholinergic neurons in the septo-hippocampal pathway. The damage of DNA molecules in cholinergic neurons could subsequently cause a decrease in the transcription of mRNA and translation of protein, which would finally appear as a functional reduction in the cholinergic neurons. This dissertation has focused on the second possibility.

The possibility of AF64A targeting DNA molecules was considered since AF64A is structurally similar to other nitrogen mustard (HN2) compounds (Figure 3). HN2 is a member of a family of alkylating agents which are used for the chemotherapy of neoplastic diseases (Lawley and Brookes, 1965; Calabresi and Chabner, 1990). The chemotherapeutic and cytotoxic effects of HN2 are directly related to the alkylation of
DNA. The 7 nitrogen atom of guanine is particularly susceptible to the formation of a covalent bond with both monofunctional and bifunctional alkylation of nitrogen mustards. However, this does not exclude the possibility that other sites in the DNA may also be attacked; thus, other atoms in the purine and pyrimidine bases of DNA may also be alkylated to a lesser degree by HN2, such as the phosphate atoms of the DNA chains and the proteins associated with DNA (Lawley and Brookes, 1965; Calabresi and Chabner, 1990).

Up to now, in vitro and cell culture experiments, which had been conducted to test the effects of AF64A at the gene level, are consistent with this hypothesis. In vitro (Futscher et al., 1992; Lev-Lehman et al., 1994; Hanin et al., 1995) and cell culture studies (Futscher et al., 1992; Santiago et al., 1992; Santiago, 1995) strongly support the hypothesis of AF64A acting on DNA molecules. However, whether AF64A acts in vivo by a similar mechanism has not yet been demonstrated. In a study employing cell culture, the neuroblastoma (LA-N-2) cell line was used by Santiago (1995); AF64A can be readily taken up directly into the cell body where the nucleus is located. In the in vitro studies AF64A was directly incubated with naked DNA molecules. Thus, in both preparations studied so far, AF64A has had an excellent chance to react with DNA molecules, and thus to cause DNA damage.

However, there are differences between in vitro, and in vivo studies. As shown in Figure 22, in the septo-hippocampal cholinergic pathway cholinergic neuron cell bodies are located in the septum, and project long axons into the hippocampus (Butcher and Woolf, 1986). A HACHT system primarily exists at the nerve terminal (Guyenet et al.,
1973; Suszkiw and Pilar, 1976; Kuhar and Murrin, 1978; Jope, 1979); which means that, in the case of the septo-hippocampal pathway, the HACHT system exists mainly in the hippocampus. It is known that uptake of AF64A into cholinergic neuron occurs via the HACHT system when low doses of AF64A are used. Thus, in vivo, if the effect of AF64A is exerted at the cell body, after delivery into the lateral ventricles, AF64A would have to be taken up by the HACHT in the hippocampus and transported in a retrograde manner into the cell body of these cholinergic neurons, which are in the septum.

Only after AF64A has arrived in the cell body where the nucleus is located, would it be possible for it to act directly on the DNA molecules of the cholinergic neurons. In this case, a decrease in the steady state level of ChAT mRNA would be expected to appear, as has been observed in the cell culture studies (Santiago, 1995), since the damage of DNA molecules by AF64A would affect the gene transcription rate, resulting subsequently in a decrease in ChAT mRNA levels.

Based on all of the above information, this project was developed to test the HYPOTHESIS that: in vivo, AF64A induces a long-lasting cholinergic hypofunction by damaging the DNA molecules of cholinergic neurons, which subsequently induces a decrease in gene transcription and translation.

A. Response of the Septo-Hippocampal Cholinergic Pathway to icv AF64A Infusion

The response of the septo-hippocampal cholinergic pathway to the icv AF64A infusion was variable, in different parts of the pathway, and at different time points.
One day after the treatment, there was an immediate decrease in ChAT activity in the hippocampus (Figure 4) without any change in ChAT protein amount in the same brain region (Figure 17, A1). Concomitantly, a significant increase in ChAT mRNA levels was observed in the septum (Figure 12, A1), which was followed by an increase in the 67 kD ChAT protein content (Figure 16, A2), as well as in ChAT activity (Figure 5) in the septum, by the next day. ChAT mRNA levels reduced below control level 7 days after the administration of AF64A and stayed at this low level for at least four weeks in the septum (Figure 12, B). Meanwhile, ChAT activity continually remained low in the hippocampus, and this was matched with the decrease in the amount of the 67 kD ChAT protein starting from 4 days after the treatment (Figure 17, A3-A5).

The long term effects of AF64A, which include the long lasting decrease in ChAT mRNA level in the septum and decrease in ChAT protein and ChAT activity in the hippocampus, agree with my hypothesis as stated above. However, the increase in ChAT mRNA in the septum at this early stage was not expected.

A. 1. Elevation of ChAT mRNA and Protein in the Septum at an Early Stage.

The steady state level of mRNA is balanced by its synthesis and degradation. Therefore, this elevation in steady state expression of ChAT mRNA in the early stage of the experiment could be due to: 1) an increase in the transcription of the ChAT gene; 2) a decrease in the degradation of ChAT mRNA; or 3) both.

The best way to test the first possibility was to determine the transcription rate of ChAT gene by using a nuclear run-on analysis. However, due to relatively low
transcription levels of ChAT gene in brain tissue (Cavicchioli, 1991), there have been no reports on the measurement of the transcription rate of this particular gene. So, the first possibility could not be tested directly, although, it is very possible that the increase in ChAT mRNA levels in the septum may be due to a up-regulation of transcription of the ChAT gene.

The studies conducted on primary culture of rat sympathetic neurons indicate that neuronal activity determines the level of mRNA by regulating the transcription rate of the ChAT gene (Brice et al., 1989). When these sympathetic neurons were cultured in a muscle-conditioned medium, the expression of ChAT mRNA was up-regulated.

More information about the regulation of ChAT mRNA expression has been obtained from studies on a simple in vivo system, Drosophila, using wild type and temperature-sensitive mutant flies. It appears that both a positive and negative feedback mechanism contribute to the regulation of the expression of ChAT mRNA in Drosophila (Tijima and Salvaterra, 1992). When Drosophila were placed at higher temperature (restrictive temperature 30°C), a higher level of ChAT mRNA was detected in wild type flies compared to flies which were placed under permissive temperature (18°C). This reflects a higher demand for ACh to sustain cholinergic synaptic transmission at the higher temperature.

All these studies have provided clues that a demand for neuronal activity could be a regulator of ChAT gene expression. When higher neuronal activity is required,
target cells may conceivably generate more trophic factors, or some unidentified signal system in the cholinergic neuron may be triggered, resulting in an up-regulation of the function of the whole system.

The exact mechanism for the regulation of the expression of ChAT mRNA in rat brain has not yet been clarified. However, there are some factors which could enhance cholinergic function by up-regulating gene expression of cholinergic neurons, such as NGF (Cavicchioli et al., 1991), which is derived from the target cells of cholinergic neurons. This trophic polypeptide is necessary for the normal development and survival of certain populations of neurons in the central nervous system (CNS) and peripheral nervous system (PNS), especially cholinergic neurons.

It has been reported that injury in the axon of cholinergic neurons could cause an increase in ChAT mRNA levels. This phenomenon has been observed both in the PNS and the CNS (Ibanez et al., 1991; Weiser et al., 1994). In the peripheral system, an increase in ChAT mRNA expression following axonal injury has been explained as being due to an up-regulation of the ChAT gene. It is believed that an increase of ChAT mRNA expression is necessary for regeneration of motor neurons (Ibanez et al., 1991). There is a hypothesis to explain the above phenomenon which occurs in PNS: 1) Axonal injury or denervation of motor neurons (initial insult) will reduce ACh release; 2) Decrease in cholinergic function will trigger muscle cells to synthesize and release more NGF; and 3) NGF will promote motor neuron sprouting and up-regulation of ChAT expression (compensatory response). However, in the CNS, a similar compensatory effect is
incomplete. Damage caused by the initial insult might exceed the compensatory effect, and thus result in loss of neurons. In this case, exogenous NGF delivery might help support survival of cholinergic neurons (Stewart and Appel, 1988).

In the present in vivo studies, icv AF64A infusion caused an initial functional cholinergic decrease in nerve terminals, which appeared as a decrease in ACh, and in the activity of ChAT and HACHT in the hippocampus. This disequilibrium, in the form of a lower cholinergic neuronal activity with the relative high target demand, might trigger a compensatory mechanism as described above.

However, this is still an assumption. If a more sensitive method, which could be used to test gene transcription rate, was available, a positive experimental result which confirmed the up-regulation of the transcription of the ChAT gene, would be convincing.

The second possibility which could conceivably cause an increase in ChAT mRNA levels in the septum is one in which AF64A treatment affects the stability of ChAT mRNA. AF64A might cause a decrease in the degradation of ChAT mRNA, which would cause an elevation of ChAT mRNA levels in the cholinergic neuron. To eliminate this possibility, the stability of ChAT mRNA has to be tested following AF64A treatment. Actinomycin D, an RNA transcription inhibitor, could be used in this experiment. Administration of actinomycin would exclude the effect of gene transcription. Following the infusion of actinomycin D with AF64A or vehicle, comparing ChAT mRNA levels in the septum between these two groups could give a clue whether AF64A treatment alters the stability of ChAT mRNA.
Thus, the following sequence of events was proposed to explain the mechanism of action of AF64A on ChAT mRNA expression in the septo-hippocampal cholinergic neuron in vivo in the early stage: 1) An rapid decrease in ChAT activity (Figure 4) in the hippocampus following AF64A infusion is observed. A concurrent decrease in the cholinergic neurotransmitter, ACh, the product of ChAT, had also been reported earlier (Leventer et al., 1986). 2) The reduction of neuronal activity in the nerve terminal triggers certain signal systems which activate the function of the cell body of the cholinergic neuron by a feedback regulation. 3) The cell body, trying to compensate for the functional reduction occurring at the nerve terminal over-expresses some genes, such as the functional enzyme ChAT; this first appears as an increase in the steady state level of ChAT mRNA, followed by an increase in the amount of ChAT protein and ChAT activity in the septum.

A. 2. Long-Lasting Reduction of ChAT mRNA in the Septum.

The decrease in ChAT mRNA level in the septum, which happens at a later stage following administration of AF64A (7 days to 4 weeks), occurs in parallel with a long term decrease in protein amount and activity of ChAT in the hippocampus. This alteration is in response to the long term deficit of cholinergic function in the septo-hippocampal pathway. The long-lasting decrease in ChAT mRNA in the septum might be due to a decrease in gene transcription which results from the direct effect of AF64A at the gene level, similar to what has been inferred in the cell culture and in vitro studies.
Mustard agents cause DNA damage without gene selectivity. Besides the ChAT gene, AF64A could also damage other genes. The effect of AF64A on other genes which are necessary to keep the neurons alive, such as the housekeeping genes, might affect the viability of these affected cholinergic neurons. The final result could be the cell death which appears as a loss of cholinergic neurons in the septum.

Immunocytochemical studies have been conducted to explore whether there is a loss of cholinergic neurons following AF64A treatment. There were significant decreases in the number of ChAT immunoreactive (ChAT-IR) neurons in the septum, 1-3 months after icv AF64A infusion (Chrobak et al., 1988; Lorens et al., 1991). Double-labeling experiments, using the retrograde fluorescent tracer, FluoroGold, combined with immunocytochemistry for ChAT, confirmed that there was death of cholinergic neurons in the septum following icv AF64A infusion (Lorens, S. A., personal communication). Administering AF64A directly into the hippocampus (Messer et al., 1991; Tonnaer et al., 1986) or cortex (Mouton and Arendash, 1990) also induced similar morphological changes in cholinergic cell bodies in the septum.

However, despite the direct effect of AF64A on DNA molecules causing the cell death that has been described above, there is another possibility that might explain the observed loss of cholinergic neurons in the septum. AF64A may have an effect only at cholinergic nerve terminals, which would initiate a retrograde degeneration of the cholinergic neurons.
As early as 1986, it has been reported that icv infusion of AF64A (5 nmol/lateral ventricle) caused a specific degeneration of cholinergic axons in the septo-hippocampal pathway, without influencing neuronal perikarya and non-cholinergic fibers (Kása et al., 1986). A similar phenomenon was reported in other cholinergic pathways (Morley et al. 1991; and Stephens et al., 1986). Furthermore, primary cell culture studies showed that there was a cholinergic cell loss following incubation with AF64A, and the surviving cholinergic neurons appeared with intact somata but damaged processes (Amir et al., 1988). All these data indicate that a retrograde degeneration is induced by AF64A, and that it starts at the nerve terminal. This retrograde degeneration might occur because of an impairment of trophic factor function, or due to an inadequacy of trophic effects to keep pace with the damage of cholinergic nerve terminals and axons by AF64A (Stewart and Appel, 1988). The massive loss of cholinergic neurons in the septum caused by retrograde degeneration might also result in a decrease in ChAT mRNA levels in this brain region.

There are, therefore, two possibilities that now may be responsible for the decrease in the ChAT mRNA expression and loss of cholinergic neurons in the septum. They are: 1) AF64A causes extensive gene damage in cholinergic neurons when it arrives at the cell bodies in the septum by retrograde transport from hippocampus, which finally affects the viability of the cell and causes cell death; or 2) AF64A acts only at cholinergic nerve terminals and/or axons, which initiates a retrograde degeneration of the cholinergic neuron mimicking the effect of axotomy.
Both of these effects would result in a loss of ChAT-IR neurons and a decrease in expression of ChAT mRNA in the septum.

Axotomy caused retrograde degeneration in septo-hippocampal cholinergic neurons has been reported (Hefti, 1986). The injury occurring at these axons induced a transient increase, followed by a long lasting decrease in ChAT mRNA expression in the septum, which is similar to what was observed in this AF64A study. According to the in situ hybridization study, axotomy induced up-regulation of ChAT mRNA, which peaked at 7 days after the surgery, and returned to control levels in about two weeks (Weiser et al., 1994). Unlike the response to the axotomy, a rapid drop in ChAT mRNA within one week was observed in the septum (Figure 12) in AF64A studies. The time period of changes in ChAT mRNA levels does not coincide in these two different studies.

Furthermore, electron microscopic examination combined with Fluoro-Gold retrograde tracing showed that axotomy of the septo-hippocampal projection neurons significantly attenuates the expression of transmitter-synthesizing enzymes and causes cell shrinkage. Nevertheless, a majority (>70%) of the cells survived for extended periods of time (10 weeks) (Naumann et al., 1992). However, a significant loss of ChAT-IR neurons (36-51%) has been observed as early as 4 weeks following icv AF64A infusion (Lorens et al., 1991).

Therefore, the long lasting decrease in cholinergic function and loss of ChAT-IR neurons might be due to the combination of both possibilities (direct effect of
AF64A on gene level and retrograde degeneration), but, the rapid drop in ChAT mRNA is more likely due to a direct effect of AF64A on DNA molecules of cholinergic neurons. Possibly, a double tracer immunocytochemistry, and an in situ hybridization parallel study concerning ChAT in the septum at earlier time-points, would help to clarify the problem, since we have observed a decrease in ChAT mRNA in the septum as early as 7 days after AF64A administration.

A. 3. Alterations of ChAT Protein in the Septo-Hippocampal Pathway.

As a protein, ChAT, the key enzyme in acetylcholine (ACh) synthesis, has been studied for years. It has been suggested that this enzyme is a single peptide protein, encoded by a single gene with a predicted molecular weight of about 72 kD in rat brain tissue (Brice et al., 1989), although the purified ChAT protein measures at about 67 kD in SDS-PAGE gel analysis (Dietz, 1980). In the current study, the monoclonal anti-ChAT antibody recognized two proteins with molecular weight of about 67 kD and 45 kD, respectively (Figure 15).

In the septum, the alteration of the expression of the 67 kD protein was closely related to the change in ChAT activity (Figure 5, and Figure 16, B1). However, there was no close relationship between the expression of the 45 kD protein and ChAT activity (Figure 5, and Figure 16, B2); the extensive increase in the expression of the 45 kD protein following AF64A administration was not accompanied by an increase in ChAT activity.
The anti-ChAT antibody used in this study recognizes ChAT protein in an epitope different from the active sites of this enzyme. Thus, even if the active sites of ChAT protein were masked by AF64A and lost their function (Sandberg, 1985), the ChAT protein could still be tested by this antibody. In the hippocampus, the decrease in 67 kD protein expression appeared several days later than the decrease in ChAT activity, implying that, in the very early stage, the decrease in ChAT activity in the hippocampus might be due to a direct alkylating effect of AF64A on ChAT protein. So, in the first two days, although normal amounts of ChAT protein existed in this brain region (Figure 17), the decrease in cholinergic function was still observed (Figure 4). To eliminate the gap between ChAT protein content and enzyme activity, the system needs several days to replace these alkylated ChAT proteins since the normal half life of ChAT protein is 5.6 days (Wenthold and Mahler, 1975). Therefore, the parallel decrease in both ChAT protein and ChAT activity later on in the hippocampus indicated that the long lasting decrease in ChAT activity in the hippocampus was due to a decrease in the amount of ChAT protein (Figure 4; and Figure 17, B1).

A. 4. Overall summary of the Response of the Septo-Hippocampal Cholinergic Pathway to AF64A icv Infusion.

As shown in Figure 23, after icv administration, AF64A diffuses into the hippocampus which is adjacent to the lateral ventricle, where it is taken up by HACht (which primarily exists in cholinergic nerve terminals) into cholinergic neurons and is accumulated inside the neuron. AF64A first causes local damage at the nerve
Figure 23: Summary of the response of the septo-hippocampal cholinergic pathway to the icv infusion of AF64A. (Based on figure 4, 5, 12, 16 and 17)
terminal, which includes direct binding with ChAT proteins, resulting in a reduction in neuronal activity in the hippocampus. This acute insult to cholinergic nerve terminals triggers a compensatory reaction by the cholinergic neuron, which results in a transient increase in the expression of ChAT mRNA and ChAT protein in the septum. At a later stage, due to an inhibitory effect by AF64A on gene expression of cholinergic neurons, the expression in both parameters (ChAT mRNA and ChAT protein) is reduced. Meanwhile, axonal transport is also affected by AF64A (Kásá and Hanin, 1985). This induces some accumulation of synthesized ChAT in the cholinergic cell body, which helps to maintain ChAT protein amount and activity at normal levels in the septum. However, in the interim, there is a severe shortage of ChAT protein in the hippocampus, which appears as a long lasting significant decrease in ChAT activity at this site. The alteration in the amounts of the 67 kDa ChAT protein in the hippocampus was significant correlated with the change in ChAT mRNA levels in the septum (r = 0.37, P < 0.01).

Changes in levels of the 45 kDa protein after AF64A administration did not agree with changes in ChAT activity both in the septum and in the hippocampus. More about this protein will be discussed later.

B. Response of the Striatal Cholinergic Region to icv AF64A Infusion

The striatum is another brain region abundant cholinergic neurons, which also is anatomically adjacent to the lateral ventricle. Cholinergic neurons in the striatum are interneurons; their cell bodies, axons and nerve terminals are all contained within
the striatum (Butcher and Woolf, 1986). This anatomical characteristic of cholinergic neurons in the striatum is different from that of cells in the septo-hippocampal pathway. Therefore, the effect of AF64A on the striatum was also tested in these studies.

There was no significant change in ChAT activity in the striatum following AF64A treatment (Figure 6), which raises several possibilities: 1) The cholinergic neurons in the striatum might be resistant to AF64A action; 2) Similar changes to those seen in the septo-hippocampal pathway might occur in the striatum, however, the time scale for these effects is different from that in the septo-hippocampal pathway. Striatal cholinergic interneurons might recover faster from AF64A damage compared to the septo-hippocampal cholinergic pathway. 3) In view of the previous findings showing that ChAT activity is altered in different directions at the cell body and the nerve terminal in the septo-hippocampal cholinergic pathway following AF64A treatment (Figure 4, and Figure 5), the possibility cannot be excluded that similar changes also happened in the striatum. If opposite changes in the cell body and nerve terminal also happened in the striatum, the changes of ChAT activity in cell body and nerve terminal might compensate for each other. Thus, a significant change in ChAT activity would not be observed. In this case, ChAT activity from the striatal tissue homogenates is not the best marker to measure the effect of AF64A on striatal cholinergic system.
With this in mind, the expression of ChAT mRNA in the striatum was measured, with the hope of observing a change in expression of ChAT mRNA in the striatum, similar to what was observed from the septo-hippocampal pathway. However, in this study I did not observe any significant overall changes in ChAT mRNA in the striatum, although there were large variations in measured levels of this parameter within groups (Figure 13), therefore, the last possibility is eliminated.

In view of the considerations listed above, there are only two possibilities available to explain the lack of any observed response in cholinergic neurons in the striatum to AF64A treatment.

The first is that the cholinergic neuron in the striatum is relatively less sensitive to AF64A compared to the septo-hippocampal pathway. The differential sensitivity of different cholinergic pathways in the brain to AF64A has been reported before for other cholinergic pathways (Morley et al., 1991; and Smith et al., 1989), and it might relate to the density of the HACht in the nerve terminals on different cholinergic neurons (Gomez et al., 1993). However, the HACht activity in the striatum is three times higher than that in the hippocampus (our own, unpublished observations). Furthermore, it has been shown that synapsomes prepared from both hippocampus and striatum are equally sensitive to AF64A (Pittel et al., 1987). These studies therefore do not support the hypothesis that the lesser sensitivity of striatum to AF64A treatment results in a lesser change of ChAT mRNA and ChAT activity in that brain region.
It would appear, therefore, that the present studies may have missed the time points in which one should observe change in ChAT mRNA and ChAT activity in the striatum.

In primary neuron cell culture studies, AF64A induced a similar degree of inhibition in ACh synthesis both in co-cultures of septum-hippocampus and cultures of striatum. However, ACh-synthesis recovered more rapidly in the striatal cultures then in septal-hippocampal co-cultures after AF64A washout (Meyer et al., 1993). This indicates that there is a special processing mechanism in striatal cholinergic neurons which helps them to recover rapidly from AF64A induced damage.

Further *in vivo* studies on ChAT protein amount in this brain region make this assumption more plausible. Although no significant change was found in the 67 kD protein (Figure 18, B1), there was a tremendous increase in the amount of 45 kD protein following AF64A treatment (Figure 18, B2). The extent and duration of time of the increase of this low molecular weight protein was compatible to that which was observed in the septum (Figure 16, B2).

This phenomenon indicates that the striatal cholinergic system does indeed respond to AF64A treatment. However, its response is different from that of the septo-hippocampal cholinergic pathway. Perhaps, a rapid recovery mechanism of striatal cholinergic neurons plays an important role during the process.
C. Response of ChAT Protein to AF64A Incubation

A monoclonal anti-ChAT antibody (Boehringer Mannheim, Clone 1.B3.9B.3) was used in Western blotting analyses. Besides the expected 67 kD ChAT protein, a 45 kD protein also was recognized by this antibody. Linearization experiments showed a good correlation between the density of the protein bands and the amount of protein loaded (Figure 15), suggesting that this protein band was not an artificial phenomenon. The low molecular weight protein also has been observed in purified ChAT proteins from bovine brain and human placenta (Figure 16, A2 A4), although the signal is very faint. These data suggest that this 45 kD protein is very closely related to ChAT protein, the 67 kD protein which has been reported before.

Following AF64A icv infusion, there was a significant increase in the expression of the 45 kD protein in all the brain regions which were studied (Figure 16, 17, 18). The most extensive and long lasting increase in this protein was found in the septum and striatum, the brain regions where cholinergic neuron cell bodies exist.

This 45 kD protein could be one of the following: 1) an isoform of ChAT protein; 2) a small molecule resulting from the breakdown of ChAT protein which was induced by AF64A; 3) another protein sharing the same epitope with ChAT protein which is recognized by the anti-ChAT antibody; or 4) ChAT protein which is translated from an truncated ChAT mRNA induced by AF64A damage on the ChAT gene.

There have been no earlier reports on the low molecular weight ChAT protein in rat brain. Therefore, I first considered this 45 kD protein as a degradation product of the
67 kD ChAT protein. To test whether AF64A could directly break down the 67 kD ChAT protein to this low molecular weight protein, purified bovine ChAT protein (there is no purified rat ChAT protein available) was incubated with different concentrations of AF64A. Although AF64A caused a significant reduction in ChAT activity in a dose-dependent manner, no significant changes were observed in the protein amount, nor in the molecular weights (Figure 19). At this point, I considered that the breakdown of ChAT protein by AF64A might need some additional factors which exist in tissue. So, in the next experiment, tissue homogenates were incubated with varied concentrations of AF64A. As was observed in the purified enzyme study, there were no significant changes in either molecular weight protein, although AF64A did cause a decrease in ChAT activity in the tissue homogenates in a dose-dependent manner (Figure 20).

These *in vitro* studies suggested that AF64A reduces ChAT activity by masking the active site of enzyme rather than by breaking this protein down to smaller molecules, and that the 45 kD protein is not likely resulting from the breakdown of the 67 kD protein.

Since this low molecular weight protein has also been observed in brain tissue from control animals and purified ChAT protein (bovine and human), the data do not support my last assumption, which means that the 45 kD protein most probably is not translated from a truncated ChAT mRNA induced by AF64A treatment.

The protein is normally expressed in rat brain tissue, and the administration of AF64A causes its over-expression. This protein could therefore be an isoform of the
67 kD ChAT protein, or, another protein, which shares the same protein sequence with the 67 kD protein.

In the early 1980s, possible multiple isoforms of ChAT protein had been reported based on the solubilizing characterization of the enzymes; these were named cytosolic ChAT and membrane bound ChAT, respectively (Benishin and Carroll, 1981; 1983). It was assumed that these two enzymes should be a little bit different in their structure, since the latter one needs a lipophilic area to allow its binding with the membrane. However, ChAT mRNA studies indicate that both of these enzymes have an identical amino acid sequence (Habert et al., 1992).

A low molecular weight ChAT protein has been reported in bovine brain as 34 kD, however, no further information about this protein is available. No low molecular weight ChAT protein has been reported in other mammalian species.

Due to the low expression of this 45 kD protein in normal rat brain, and its unstable character (Figure 21), this protein is not easily noticed during the purification of ChAT protein from normal animals. Protein sequencing or/and protein footprint analysis could help to demonstrate whether this protein is an isoform of ChAT protein and whether it shares its most amino acid sequence with that 67 kD protein. However, purification of this protein would be a challenge due to the low expression and the instability of this protein.

There is another possibility to explain this 45 kD protein. This protein might not be related to the ChAT protein; however, it could possibly share certain amino
acid sequence with the ChAT protein. This could cause an immuno-crossing recognition by the anti-ChAT antibody which I used for the Western blot analysis.

When comparing ChAT protein to other cholinergic related proteins, it has been reported that ChAT protein (*Drosophila*) shares a limited sequence homology with AChE (*Torpedo*) and with the alpha subunit of nAChR (rat) (Mori et al., 1987; Salveterra, 1987). This alpha subunit of nAChR expressed in rat brain tissue has 474 amino acids and a predicted molecular weight of 54.8 kD (Boulter et al., 1985), although Western blot analysis on rat brain tissue showed that this alpha subunit protein has a molecular weight of about 40 kD (Hawrot et al., 1986; and Reuss et al., 1992). However, the *Drosophila* ChAT protein only shares a 60% sequence homology with rat ChAT protein. The protein sequence homology between ChAT and the alpha subunit of nAChR has not been reported. Western blot analysis using the antibody against to this alpha-subunit of nAChR would help to solve this puzzle.
CHAPTER VI

SUMMARY

1. AF64A icv administration causes immediate inhibition of ChAT activity in the nerve terminal (hippocampus) and triggers a compensatory response in the septo-hippocampal pathway.

2. Initially, AF64A causes an inhibition of hippocampal ChAT activity by directly alkylating the active site of the ChAT protein.

3. The increase in septal ChAT mRNA, 67 kD ChAT protein and ChAT activity is transient.

4. The long-lasting decrease in ChAT activity in the hippocampus is due to a decrease in the amount of the 67 kD ChAT protein in this brain region. These phenomena are associated with the long-lasting decrease ChAT mRNA level in the septum.

5. AF64A did not cause significant changes in ChAT activity and ChAT mRNA expression in the striatum over the time course observed.

6. AF64A caused a significant increase in the 45 kD putative ChAT protein in all tested brain regions.

7. This study cannot exclude the possibility that disruption of cholinergic axonal transport induced by AF64A also contributes to the long-lasting cholinergic hypofunction.
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This dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

10/23/96
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