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**Hemicholinium-3 (HC3) Attenuates the Cholinotoxic
Effects Induced by Ethylcholine Mustard Aziridinium
(AF64A) in the Developing Rat**

by

Wayne Gerard Brake

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of**

Master of Science

**Department of Psychology
Carleton University
Ottawa, Ontario
September 9, 1993**

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**"Hemicholinium-3 (HC3) Attenuates the Cholinotoxic Effects
Induced by Ethylcholine Mustard Aziridinium (AF64A)
in the Developing Rat"**

submitted by Wayne Brake, B.Sc.
in partial fulfillment of the requirements for
the degree of Master of Science

Thesis Supervisor, Bruce A. Pappas, Ph.D.

Chair, Department of Psychology
Bill Jones, Ph.D.

Carleton University
September 23, 1993

Abstract

AF64A has been reported to induce hypofunction of the cholinergic system both *in vitro* and *in vivo*. Thus, this compound has a potential to be used for an animal model of cholinergic dysfunction. Yet, the results of some investigations have shown that the AF64A-induced toxicity may not be specific to the cholinergic system. This study was designed to test the effects of AF64A on the development of the cholinergic system and to examine its specificity for ACh by pre-treatment with the high-affinity choline uptake blocker; hemicholinium-3 (HC3). On post-natal day (PND) 3-4 rats were either injected bilaterally with 20 µg/µl HC-3 followed by 0.1 nmol/µl AF64A or injected with saline followed by AF64A (0.5 µl/ventricle i.c.v.). Pups infused with HC-3 plus vehicle or saline plus vehicle served as controls. At PND 60 all groups were tested on the Morris water maze (MWM) and then the elevated-plus maze. They were then subjected to biochemical and histological analysis. There was a significant difference between groups on the MWM behavioural task. AF64A impaired performance in the MWM, while HC3 blocked this effect. There were no differences between groups on performance in the elevated-plus maze. The level of choline acetyltransferase (ChAT) activity did not measurably differ between groups for any brain region investigated. Animals infused with saline-AF64A showed a marked degeneration of the frontal cortex and dilation of the ventricles as well as a larger dentate gyrus. Notably, none of these effects occurred in rats pre-treated with

hemicholinium. Thus, HC3 blocked behavioural and gross neuropathological effects of AF64A. It can be concluded from these results that; (1) AF64A exerts its effects on infant brain via high-affinity choline uptake mechanisms, (2) at the dose used here, AF64A selectively lesions the cholinergic system in the developing rat brain, (3) when administered to the neonate, AF64A causes severe impairments in spatial memory at adulthood and alters the development of structures in the brain. The marked cortical degeneration following neonatal cholinergic hypofunction may be a contributing factor to the behavioural alterations seen in adulthood.

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**Hemicholinium-3 (HC3) Attenuates the Cholinotoxic Effects
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Introduction

Recently there has been an increase of investigations into the synthesis and evaluation of agents which are toxic to specific neurotransmitter systems in the brain. These neurotoxins may be used to model neurotransmitter deficits which occur in neuropsychiatric diseases. They may also be used in animal research to investigate new therapeutic approaches which can later be applied in clinical treatment of these diseases. In addition, specific neurotoxins may serve investigators in the study of basic mechanisms pertaining to synthesis, metabolism, and development of various neurotransmitter systems and their involvement in behaviour (Hanin, Fisher, Hortnagl et al., 1987). To date there has been only moderate success in characterizing a neurotoxin specific for the cholinergic system.

It is well established that, in mammalian brain, the presynaptic cholinergic apparatus and cholinergic receptors develop early in pre- and postnatal life (eg. Schlumpf, Palacios, Cortes, & Lichtensteiger, 1991; Coyle & Yamamura, 1976). The topographical distribution and density of these markers further change notably in relation to developmental stage. Consequently, it follows that drug sensitivity of individual regions in the immature brain can vary from the adult condition. Examination of the effects of early neurochemical depletion are important-especially in the case of the cholinergic system whose development may be quite significant for brain function later in life. However, the importance of this system in development has remained largely unexplored due to the lack of a specific and potent cholinotoxin with long-lasting effects.

The cholinergic system is highly resistant to depletion. That is, lesions to cholinergic fibres have resulted in only temporary reduction of this transmitter. It appears that undamaged cholinergic cells are capable of efficiently sprouting new terminals to functionally compensate for loss of damaged cholinergic cells. As a result, investigators have had difficulty studying long-term, developmental depletions of this system.

Characterization of ontogenic cholinergic dysfunction is important because a dysfunction of cholinergic cells in early development has been implicated in disorders such as Down's Syndrome (DS). A specific, long-lasting cholinotoxin would prove invaluable to the study of cholinergic development. Recently, the toxin AF64A has been suggested to be cholinotopic. Initial studies have shown that AF64A depletes brain acetylcholine longer than

previous cholinotoxins (eg. hemicholinium-3 and styrylpyridine). Yet, the specificity of AF64A for cholinergic cells has been debated. Use of this agent in studies of cholinergic ontogeny has significant potential if the cholin specificity of this compound were clarified. The following study was designed to determine the cholin specificity of AF64A as well as to investigate the effects of early cholinergic dysfunction on development.

The Cholinergic System¹

Anatomy of Cholinergic Fibres:

The cholinergic system is comprised of neurons in the central nervous system (CNS) and peripheral nervous system (PNS) which secrete the neurotransmitter acetylcholine (ACh). The identification of acetylcholine-releasing neurons in the PNS has been documented for some time. It is known that ACh is released at the autonomic ganglia, at parasympathetic postganglionic synapses, and at the neuromuscular junction.

The location of cholinergic neurons in the CNS has only been identified in recent years. There are two types of ACh-releasing fibres in the CNS. A system of cholinergic neurons may be morphologically located entirely within one neural structure. Such neurons are referred to as local circuit cells. These neurons are mostly small interneurons found in the cortex as well as neurons in

¹ Unless otherwise indicated, this discussion is referenced from Cooper, Bloom, and Roth (1991); Kandel, Schwartz, and Jessel (1991); and Carlson (1986).

the nucleus accumbens, the caudate-putamen, the olfactory tubercle, and the amacrine cells of the retina.

The other cholinergic neurons in the CNS are projection neurons. These are cells which project to a different structure from where the cell body is found. There are two main groups of projection neurons. The pontomesencephalotegmental cholinergic complex is comprised of pedunculopontine and laterodorsal tegmental nuclei which project to diencephalic nuclei such as the thalamus. This system also contains descending projections to the medullary and pontine reticular formations as well as vestibular, deep cerebellar, and cranial nuclei. It has been proposed that the centrally projecting cholinergic fibres of the pontine area could be an anatomical substrate of the ascending reticular activating system (Shute & Lewis, 1967). This group of neurons is also likely to be involved in the control of cardiovascular function, ingestion, motor activity, temperature regulation, and respiration (Woolf & Butcher, 1986).

The other group of cholinergic projection neurons, the basal forebrain cholinergic complex, is comprised of cells in the nucleus basalis, the medial septal nucleus, the diagonal band, the substantia innominata, and the magnocellular preoptic field. These nuclei send cholinergic projections to the olfactory bulb, the hippocampus, the amygdaloid body, and all areas of the cerebral cortex.

Morphological characteristics of cholinergic neurons in septum, NBM, and striatum differ from one another. Morphometric analysis of individual cholinergic neurons revealed that total neurite length of cholinergic neurons of striatum and nucleus basalis is

greater and there are more branching points compared to septal cholinergic neurons (Hefti, Hartikka, Junard, & Knusel, 1990).

The basal forebrain cholinergic complex has been functionally implicated in learning and memory (see Dekker, Connor, & Thal, 1991 for review). Specifically, the nucleus basalis magnocellularis (NBM) in rodents, referred to as nucleus basalis of Meynert (nbM) in primates, and the medial septum are thought to be important. The importance of nbM has gained recent attention since there is up to a 79% decrease of cholinergic neurons in this structure in Alzheimer's patients

The NBM in rats consists of large multipolar cells located in the ventromedial region of the globus pallidus. NBM projection neurons account for 70-80% of cholinergic innervation to the cortex. The projection from the NBM to the neocortex is predominantly ipsilateral. These projections are also somewhat topographical with the anterior areas of the nucleus projecting more to the frontal and temporal cortex while the posterior areas project more to the parietal-occipital regions of the neocortex. The medial septal nucleus projects largely to the hippocampus via the fimbria fornix. The basal forebrain complex receives afferents from the amygdala, hypothalamus, and various brain stem nuclei.

Cholinergic Receptors:

There are two classes of acetylcholine receptors; nictotinic and muscarinic (Table 1). The majority of CNS cholinergic receptors are muscarinic. All muscarinic ACh receptors are coupled to G

proteins and either directly gate ion channels or activate an assortment of second messenger systems. The activation of muscarinic receptors can lead to either hyperpolarisation or depolarization of the postsynaptic cell. This is achieved through the opening or closing of potassium, calcium, or chlorine ion channels. There have been up to five subtypes of muscarinic receptors identified and cloned. These subtypes are referred to as M_1 to M_5 respectively. The muscarinic subtypes have been characterized through pharmacological manipulations. For example, M_1 receptors exhibit a high affinity for the antagonist pirenzepine, M_2 for the antagonist AFDX-116, and 4-DAMP possesses the highest affinity for M_3 . The antagonists QNB and atropine block all muscarinic receptors equally with no selective affinity for the subtypes. It is thought that some of the muscarinic subtypes may have functional specificity. For example, the M_1 receptor in cortex is likely to be associated with cognitive function while M_2 is thought to be involved in the mediation of memory function (Shannon et al., 1990).

The nicotinic receptors have been identified morphologically from research with monoclonal antibodies and cDNAs. These receptors are receptor-channel configurations which, upon binding to ACh, allow ions to pass through a central pore. The nicotinic receptor contains four different subunits in torpedo electric fish labeled alpha (α), beta (β), gamma (γ), and delta (δ). All nicotinic receptors contain a total of five subunits comprised of some combination of these four. To date four different nicotinic receptor subtypes have been identified based on the sequence of subunits and

pharmacological manipulation. Cloning studies have demonstrated that the α and β subunits are also expressed in humans.

Acetylcholine Metabolism and Release:

The neurophysiological activity of acetylcholine can be characterized by the metabolic reactions which synthesize and break down this transmitter as well as the other enzymes and substances involved in these reactions (figure 1). ACh, along with coenzyme A (CoA), is synthesized from a reaction between acetyl coenzyme A (acetyl CoA) and choline according to the following reaction:

Acetyl CoA is synthesized primarily in mitochondria and is probably synthesized from acetate through acetate thiokinase. Once synthesized, acetyl CoA is thought to be transported out of the mitochondria by the carrier citrate where it participates in ACh synthesis in the cytoplasm. The other component of ACh, choline, cannot be synthesized in the CNS *de novo* and is the rate-limiting substance in ACh synthesis. As a result, 35-50% of choline is recycled back into the cell via high- and low-affinity choline uptake mechanisms. Other sources of choline come from dietary products such as eggs, liver, and lecithin. In lecithin choline is part of a larger molecule which is synthesized into choline.

Figure 1. Steps involved in the synthesis, release, and degradation of acetylcholine (ACh). Acetyl coenzyme A (Ac CoA) and choline react with the assistance of the enzyme *choline acetyltransferase* (ChAT) to produce ACh. ACh is packaged in synaptic vesicles or stored in cytoplasm until its release in to the synapse. The enzyme *acetylcholine esterase* (AChE) cleaves ACh to produce choline and acetate. Choline is recycled by being re-uptaken by the cell through *high affinity choline uptake* (HACU) or *low affinity choline uptake* (LACU). *Ethylcholine mustard aziridinium* (AF64A) is believed to enter the cell via HACU and *hemicholinium-3* (HC-3) is a HACU antagonist.

The catabolic enzyme responsible for ACh synthesis is choline acetyltransferase (ChAT). The highest activities of this enzyme are found in the caudate nucleus, retina, corneal epithelium, and central spinal roots. Within the cell, ChAT is found in the cytoplasm of the nerve terminal. To date, a specific and potent inhibitor of ChAT has not been identified. Styrylpyridine derivatives can inhibit ChAT but are moderately insoluble and vary in the degree of anticholinesterase action.

Once synthesized, ACh is stored both in cytoplasm and in synaptic vesicles. These vesicles contain adenosine triphosphate (ATP) in addition to ACh. Uptake of ACh by synaptic vesicles is thought to occur in the same manner as catecholamines. Catecholamines are taken up by the vesicles as a result of ion trapping and by the formation of ATP complexes with internal proteins. Biochemical measurements of vesicle content revealed that a single cholinergic vesicle contains about 2000 molecules of ACh.

An action potential of the cholinergic cell will stimulate release of acetylcholine from cytoplasmic and vesicular sources. Before cholinergic vesicles are able to release their contents into the synapse via exocytosis, they must migrate to the active zone at the synaptic membrane. This process is called mobilization. Both mobilization and exocytotic release of ACh are mediated, in part, by calcium ions. In the resting state, vesicles are anchored to one another by actin. An action potential arriving at the terminal activates Ca^{2+} channels. The calcium influx into the cell then dissolves some of the actin filaments causing mobilization of ACh vesicles. Calcium further assists in the fusion of vesicles with the

plasma membrane and subsequently transmitter is exocytosed into the synaptic junction.

Upon its release into the synapse, ACh is quickly hydrolyzed by cholinesterases according to the following reaction:

There are two classes of cholinesterase; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE is referred to as true cholinesterase whereas BChE is called pseudo cholinesterase. These two compounds are similar, yet they are encoded by different genes. It is suggested that BChE is more abundant in lower life forms while AChE is more abundant in higher, more evolved species. With few exceptions, in mammals AChE is found in brain and BChE predominates extraneural organs. AChE is one of the most efficient enzymes known. It is capable of hydrolyzing 5000 molecules of ACh per molecule of enzyme per second due to its turnover rate of 150 microseconds.

As mentioned previously, choline is recycled back into the cholinergic terminal to be reused in ACh synthesis. There are two transport mechanisms which re-uptake choline. The low-affinity choline uptake (LACU) system is not sodium-dependent and does not utilize energy. LACU exists in all cells and occurs at the same rate as simple membrane diffusion. High-affinity choline uptake (HACU), also known as high-affinity choline transport (HACHT), is mediated by a sodium-dependent transport carrier which uses energy. This carrier protein is found predominantly on, if not

restricted to, the terminals of cholinergic neurons. The regional distribution of HACU correlates well with other cholinergic markers such as ACh levels and ChAT activity. In rat brain membrane preparations, HACU has the following distribution: striatum >> hippocampus > cortex >> cerebellum (Kuher et al., 1973; Yamamura & Snyder, 1973; Yamamura et al., 1974). The rate-limiting step in ACh synthesis is HACU (Kuhar & Murrin, 1978) which is closely coupled to the synthetic enzyme ChAT. The rate of HACU, $V_{\max}$ and $K_{\text{transport}}$, is the same as the rate of ACh synthesis, $V_{\max}$ and $K_{\text{acetylation}}$ (Barker & Mittag, 1975). Further, the rate of ACh synthesis changes with neuronal activity levels. As demand for releasable ACh changes, HACU velocity increases or decreases to support this demand. As a result, HACU regulates ACh synthesis by regulating choline availability. The regulation of HACU appears to be due to changes in the number of carrier sites, as indicated by [^3H]HC3 binding, rather than due to increased activity of existing carrier sites (Happe & Murrin, 1993).

Cholinotoxins

In light of the manner in which ACh is metabolized and re-uptaken, researchers searched for a chemical analog of choline to act as a specific cholinergic neurotoxic agent. This choline analog would have to selectively target the HACU system because of structural similarities to choline but concomitantly be cytotoxic at the site of accumulation. Fisher and Hanin (1980) discovered a neurotoxin which appears to fulfill these requirements. They

proposed that nitrogen mustard analogs of choline might be useful as specific cholinotoxins within the brain. The most potent of these is the ethylcholine mustard aziridinium ion (AF64A) which irreversibly binds to HACU carriers (Rylett & Colhoun, 1980). AF64A is structurally similar to choline but contains a cytotoxic aziridinium moiety and is, therefore, a good candidate for a specific cholinotoxin. It has been theorized that AF64A is specifically transported into the nerve terminal through the HACU carrier. Once inside the terminal, the agent could inhibit specific enzymes for choline and engage a cascade of intracellular events which are toxic to the cholinergic neuron (Sandberg, Schnaar, McKinney, et al., 1985). However, it should be noted that if AF64A were to actually cause cell death it would have to be transported retrogradely along the axon to the cell body. That is, a cholinergic neuron will not die from damage to its axon terminal alone.

AF64A Lesions:

In vitro experiments on AF64A have demonstrated that it produces an irreversible inhibition of HACU (Rylett & Colhoun, 1980). Further, AF64A is many times more potent on HACU than on LACU, muscarinic acetylcholine receptor (mAChR), ChAT, or AChE activity or serotonin uptake *in vitro* (Rylett & Colhoun, 1982; Mantione, Zigmond, Fisher, & Hanin, 1983).

AF64A has been evaluated *in vivo* to examine its putative specific cholinotoxic effects. Overall, intracerebroventricular (i.c.v.) injections of AF64A result in a reduction of ACh, ChAT, and

HACU in the hippocampus and cortex in rats (eg. Fisher & Hanin, 1986; Hanin, Fisher, Hortnagl, et al., 1987). The effects of AF64A appear to be dose dependent, appear in 2-3 days, and persist up to 12 months.

Cognitive deficits induced by AF64A seem to mimic those observed in AD patients. It appears that "working memory" rather than the "reference memory" is impaired in the 8-arm radial arm maze (Walsh et al., 1984). Specifically, Jarrard, Kant, Meyerhoff, and Levy (1984) found that after AF64A i.c.v. injection, "working memory" was impaired on both 'place' and 'cue' tasks while "reference memory" performance was impaired only on the 'place' task. Similar results have been observed on the one-trial passive-avoidance test (Brandeis et al., 1986). In addition, spatial learning memory is disrupted by AF64A in the Morris water maze (Armstrong & Pappas, 1991). AF64A-induced lesions in animals have not, however, mimicked the histological characteristics observed in AD patients. That is, AF64A administration does not produce neuritic plaques or neurofibrillary tangles in brains of rats (Fisher & Hanin, 1986). Furthermore, the specificity of AF64A as a cholinotoxin is yet to be delineated. Studies have produced results which contradict the findings that AF64A affects only the cholinergic system.

Many investigators have observed specific effects such as a reduction in cholinergic markers upon AF64A administration without disturbance to other neurotransmitter levels (eg. Walsh et al., 1984; Brandeis et al., 1986). Yet, others have shown non-specific effects such as damage to serotonin (5-HT), dopamine (DA),

norepinephrine (NE), and gamma-aminobutyric acid (GABA) levels as well as varying signs of cell necrosis and ventricular enlargement (Asante, Cross, Peakin, Johnson, & Slater, 1983; Jarrard et al., 1984; McGurk & Butcher, 1985; Hanin et al., 1987; Armstrong & Pappas, 1991). Ventricular enlargement and cell necrosis are indicators of non-specific neuronal damage in electrolytic lesion studies.

Fisher and Hanin (1986) proposed that nonspecific damage to tissue could be a result of factors other than nonspecific effects of AF64A. One explanation is that destruction of a specific neuronal population could cause secondary morphological changes such as ventricular dilation. Fisher and Hanin (1986) suggested that ventricular enlargement observed after AF64A infusion may be misinterpreted as a nonspecific lesion. This would be reminiscent of electrolytic lesions, when it is in fact collateral damage caused by cholinergic cell loss. Hanin et al. (1987) suggested that because the cholinergic system interacts with other transmitter systems *in vivo*, other systems may be disrupted secondary to, and as a result of, cholinergic hypofunction. It is also possible that such ventricular enlargement may be caused by hydrocephalus.

Hemicholinium-3:

Another agent that is believed to be specific for the cholinergic system is an antagonist which is the hemicholinium analog of choline (viz. hemicholinium-3). Long and Schueler (1954) first reported the synthesis of alkyl bisquaternary ammonium salts containing two choline-like moieties produced by hemiacetal formation, thus hemicholiniums. The most potent derivative, 2,2'-(4,4'-

biphenylene)-bis(2-hydroxy-4,4-dimethylmorpholinium bromide), was called hemicholinium-3.

Initially, hemicholinium-3 (HC3) was evaluated as an AChE inhibitor. Although it did not inhibit AChE, HC3 was found to produce a toxicity consisting of a delayed and progressive respiratory failure (Long & Schueler, 1954). HC3 was later investigated as an antagonist which may be used in animal models of AD. Smart (1981) showed that HC3, *in vitro*, produced significant and irreversible inhibition of choline uptake. Moreover, he found that the site of action was also the site of HACU. However, *in vivo* studies demonstrated that, although HACU may be inhibited, the cholinergic system as a whole was negligibly affected (Hanin et al., 1987). Hanin et al. (1987) found that HACU had been virtually restored to control levels within 2 days and ACh levels had doubled within 2 hours and returned to normal levels 2 days following HC3 injection. It is now known that HC3 is a potent inhibitor of HACU ($K_i = 25\text{-}100\text{ nM}$) and a much weaker inhibitor of LACU ($K_i = 50\text{ }\mu\text{M}$) (Happe & Murrin, 1993).

AF64A is presumably taken into the cell via the HACU system where it exerts its cytotoxic effects (Hanin et al., 1987). If the HACU system is impeded (ie. with HC3) it follows that AF64A could no longer enter the cell to exert its effects. As a result, AF64A-induced cholinotoxicity could be prevented. Under this assumption, the cholin specificity of AF64A could be evaluated via preinfusion with HC3.

Effects of HC3 on AF64A:

Pre-injection with HC3 followed by AF64A infusion could result in one of three observations. One would be that cholinergic hypofunction is observed after infusion with HC3 followed by AF64A. This would suggest that AF64A does not exert its cytotoxic effect through the HACU system and, thus, is probably not specific for cholinergic neurons. Another possibility is that HC3 would prevent AF64A-induced cholinergic hypofunction and other neurotransmitter systems are not affected either. In this case, AF64A would appear to be cholinergic specific. It could thus be concluded that the reduction in other transmitter systems observed in previous studies were due to secondary disturbances caused by cholinergic cell loss. Finally, if HC3 prevented cholinergic hypofunction but other neurotransmitters were affected, AF64A may be specific for cholinergic neurons but toxic to other systems as well. That is, AF64A would be exerting its toxicity via the HACU system in cholinergic cells, but additionally exerting its toxicity for other transmitter systems via some yet to be discovered mode of action.

Chrobak, Spates, Stockman, and Walsh (1989) conducted a study to investigate the effects of i.c.v. pretreatment of HC3 (2 µg/1 µl/ ventricle) on AF64A (1 nmol/3 µl/ ventricle) induced hypofunction. The results were in support of the hypothesis that AF64A is a specific toxin for the cholinergic system. Chrobak et al. (1989) found that pretreatment with HC3 prevented working memory impairment on the standard radial arm maze task and the

delayed non-match to sample variation of this task. Furthermore, HACU was found to be significantly reduced in animals infused with AF64A alone in reference to those pretreated with HC3 before AF64A injection.

This experiment (Chroback et al., 1989) does not unequivocally demonstrate the cholinergic specificity of AF64A. Chroback et al. (1989) revealed that cell necrosis and ventricular dilation had occurred in both AF64A alone and HC3 followed by AF64A treatment conditions. The authors suggested that this histological aberration may be due to impurities in the AF64A medium or too high a concentration of AF64A near the tip of the injection cannula. However, this conclusion is questionable in the absence of data on non-cholinergic transmitter levels.

Another study (Potter, Tedford, Kindel, & Hanin, 1989) evaluated the effects of i.c.v. pre injection of HC3 on AF64A-induced cholinergic hypofunction with regards to non-cholinergic transmitter levels. Potter et al. (1989) pre-administered HC3 (20 µg/1 µl/ ventricle) into rats before AF64A (2 nmol/ 1 µl/ ventricle) administration. This study investigated ACh, NA, 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) levels. It was observed that pretreatment with HC-3 significantly attenuated the AF64A-induced reductions in ACh, NA, 5-HT, and 5-HIAA.

While these results seem compelling, it should be noted that this study used a smaller dose of AF64A than was used by Chroback et al. (1989). Potter et al. (1989) infused a total of 2 nmol into the rat while Chroback et al. (1989) injected a total of 6 nmol of AF64A. It has been shown that the histopathological effects of

AF64A are dose dependent (Fisher & Hanin, 1986; Fisher et al., 1987; Armstrong & Pappas, 1991). Moreover, Potter et al. (1989) did not examine for tissue necrosis or behavioural change. While this study demonstrated that HC3 attenuated the reduction of non-cholinergic as well as cholinergic transmitter levels at a low dose of AF64A, it did not show that this low dose causes behavioural effects which may be reversed with HC3. Additionally, the absence of histopathological data in this study fails to illuminate the issue of whether the aberrant ventricular dilation observed by Chrobak et al. (1989) was due to effects on non-cholinergic neurons. Consequently, it would be prudent to evaluate behavioural, histopathological, and cholinergic neurotransmitter data in the same study using a dose of AF64A which is known not to cause non-specific damage.

Development

Cholinergic Development:

Ontogeny of central cholinergic neurons has been investigated *in vivo* and *in vitro* using cholinergic markers such as ChAT activity and ACh concentration. Autoradiographic techniques for HACU (using [^3H]3-quinuclidinyl benzilate (QNB) and [^3H]HC3) as well as for muscarinic receptors (using [^3H]N-methyl scopolamine) have also been used.

The concentration of acetylcholine in fetal brain has been measured in whole brain tissue extracts using an enzymatic radiometric assay (Coyle and Yamamura, 1976). ACh levels were found to be high in relation to other cholinergic markers. ACh was at 22% of adult levels at 15 days of gestation. ACh increased to 38% of adult levels at two days prior to parturition. Interestingly, ACh concentration decreased at birth to 27% of adult levels. This apparent decrease may be due to the different method used on fetal brain and neonate brain. Coyle and Yamamura (1976) evaluated fetal brain ACh levels after decapitation but measured neonatal brain ACh after sacrificing the animal with focused microwave irradiation. Upon birth, whole brain ACh concentration increased linearly until four weeks of age when adult levels were attained.

Regional distribution of acetylcholine concentration is heterogeneous in the developing brain. The highest concentration of ACh in adult rat is in the striatum and the lowest is in the parietal

cortex and cerebellum. At parturition, the level of ACh in the medulla-pons is the same as adult levels. At the same time, the combined midbrain-hypothalamus is 25% of adult levels while the parietal cortex is 50% of adult levels. The concentration of ACh in the striatum is 28% of adult levels up until one week after birth at which point it increases linearly to adult levels by four weeks of age.

Coyle and Yamamura (1976) revealed that specific activity of ChAT increases from 1-8% of adult levels between 15 days of gestation and PND 7 in whole brain. ChAT activity then increases linearly to 83% of adult levels by four weeks of age. ChAT activity in adult brain exhibits regional heterogeneity; the specific activity of the striatum has a 3-fold increase over the medulla-pons which has a 20-fold increase over the cerebellum.

The regional distribution of specific ChAT activity in the fetal brain is quite different. Prenatally, the rhombencephalon (which includes the cerebellar anlage) possesses a several-fold higher activity than the more cephalad mesencephalon and telencephalon. At birth, the medulla-pons maintains the highest ChAT activity as it is between 3 and 10-fold higher than all other brain regions. At three weeks of age, there is a transient overexpression of ChAT in the midbrain + brainstem and hippocampus (Zahalka, Seiler, Lappi, et al., 1993). Whereas medulla-pons achieves adult ChAT activity levels by four weeks of age, all other brain regions have only attained 70-80% of adult levels by this time (Coyle & Yamamura, 1976).

Zahalka et al. (1993) found that the marked increase in ChAT activity from late gestation through young adulthood was in parallel with the expansion of total membrane protein. A relative expansion

of membrane surface is commensurate with axonal outgrowth, synaptogenesis, and dendritic arborization. Therefore, it is not surprising that ChAT develops in parallel to membrane protein because this enzyme is a marker for cholinergic nerve terminals.

The general interpretation of ChAT development is that this measure corresponds to synaptic proliferation. Thus, ChAT activity includes a transient period of excess expression which chronologically parallels cell hyperinnervation prior to the pruning of excess synaptic terminals.

Development of the HACU carrier protein, in whole brain and several brain regions occurs relatively late. Major increases occur only after the first postnatal week (Sorimachi & Katoaka, 1975; Coyle & Yamamura, 1976; Shelton et al., 1979; Yamada et al., 1986; Happe & Murrin, 1992). The ontogenesis of HACU correlates to the timing of increased efficiency in synthesis of choline into ACh. ACh synthesis reaches adult efficiency at PND 17 (Shelton et al, 1979; Kotas & Prince, 1987). While development of HACU closely corresponds to the development of other presynaptic cholinergic markers, several studies indicate that HACU develops a few days before ChAT activity in certain brain regions (Sorimachi & Katoaka, 1975; Shelton et al., 1979).

Zahalka et al. (1993) found that HACU sites in rat brain, as measured by [³H]HC3 binding, were extremely high during late gestation and the first days of life. The high levels of HACU sites was particularly notable in the midbrain-brainstem area and hippocampus. These levels declined to the same low levels as other cholinergic markers by postnatal day (PND) 10 before resuming the

increases associated with bulk synaptogenesis. Conversely, the density of HACU sites in cortex and striatum transiently rose to a minor peak at PND 10. This corresponds to a transient period of cholinergic hyperactivity in the regions.

Muscarinic cholinergic receptors first appear in the rat CNS by 13-14 days of gestation. These receptors are only present in spinal cord at this stage of development. As cells begin to appear in the brain stem, such as the trigeminal nucleus and pontine gray between 13 and 17 days of gestation, muscarinic receptors develop shortly after cell birth. The increase in muscarinic receptors is prominent in motor nuclei of the hypoglossal and trigeminal nerve between days 16 and 18 of gestation.

In rat neocortex, muscarinic binding sites are diffusely distributed across the late fetal cortex including the cortical plate (Schlumpf, Palacios, Cortes, & Lichtensteiger, 1991). By the end of the first and beginning of the second week of life, muscarinic receptor density increases in cortical layers II/III and V/VI (Miyoshi, Kito, Shimizu, & Matsubayashi, 1987). This pattern is reminiscent of the transient increase of AChE activity in layers III/IV and VI at the beginning of the first postnatal week (Robertson, 1987).

Naeff, Schlumpf, and Lichtensteiger (1992) used [³H]nicotine to evaluate the development of nicotinic cholinergic receptors in rats. They found that receptor sites begin to appear in the spinal cord and caudal medulla oblongata at gestational day (GD)12. Nicotinic receptors develop in the cerebellar anlage and caudal caudate - putamen by 16 days of gestation. It appears that the density of receptors at lower brainstem sites is reduced during late gestation to

early postnatal life. At the same time, nicotinic receptor density increases in forebrain, especially in the neocortex.

The first nicotinic receptor sites in neocortex are observed at GD 20 in the subplate layer and become progressively more dense up to early postnatal life. By the second week of life, this deep band of nicotinic receptors disappears. This is interesting because the subplate is thought to play an important role in cortical development. That is, this zone is a site of early synapse formation (Kristt & Molliver, 1976; Kostovic & Rakic, 1990) and a waiting zone for ascending projections to cortex which appear to establish synaptic connections with subplate neurons migrating to the cortical plate (Friauf, McConnell & Shatz, 1990; Kostovic & Rakic, 1990). Also by the second week of life, a high density of receptors are seen in cortical layers III/IV which is reflective of adult distributions. Finally, the pattern of nicotinic receptors in rat brain is similar to that of adult by 27 days of age.

In summary, it appears that cholinergic tone is very high during late gestation and the first days of postnatal life. Acetylcholine, high-affinity choline uptake sites, and cholinergic receptors show transient overexpression during this stage of development. It has been shown that stimulation of cholinergic receptors by exogenous cholinergic agonists during this ontogenic stage results in premature termination of cell replication (Navarro, Seidler, Eylers, et al., 1989; McFarland, Seidler, & Slotkin, 1991).

Considering that cholinergic stimulation is important in both target cell differentiation (Spitzer, 1991) and in fetal and early neonatal behaviours (Schlumpf et al., 1991), the cholinergic system,

particularly HACU, may play a role in coordinating the timing of membrane development, synaptogenesis, and macromolecule synthesis (McFarland et al., 1991; Zahalka et al., 1993).

It is also conceivable, during this heightened expression of cholinergic markers, that non-cholinergic cells are transiently expressing these binding sites. Cholinergic markers have been observed in areas that do not possess either the cells or the cholinergic characteristics at adulthood (eg. Gould and Butcher, 1987). The expression of cholinergic markers by other cells could mediate target cell development and may play a role in the physiological needs of the fetus and neonate.

Whether developmental overexpression of cholinergic markers, such as HACU sites, are a result of very high cholinergic tone, despite few cholinergic nerve terminals, or a result of transient expression by non-cholinergic cells, this period of ontogeny is important. In either case, ontogenic elevations in cholinergic input or transient cholinergic phenotypic expression may be linked to important developmental cellular and physiological events.

ACh Development and Human Pathology:

A disease in which the cholinergic system is hypothesized to play an aetiological role is Alzheimer's Disease (AD). A pattern of nerve cell loss is common in Alzheimer's Disease. Studies have demonstrated (see Terry & Davies, 1983 for review) that populations of nerve cells have been degenerated in the frontal and temporal cortices, the medial septum, diagonal band of Broca, and

the nucleus basalis of Meynert (nbM). Incidentally, the nbM consists of the cell bodies of a cholinergic pathway whose projections terminate in the neocortex and hippocampus. Moreover, it has been shown that presynaptic cholinergic markers are significantly reduced in the cortex and hippocampus of those afflicted with AD (Francis, Palmer, Sims, et al., 1985). Such markers include choline acetyltransferase, and acetylcholinesterase activities, as well as sodium dependent high affinity choline uptake, acetylcholine synthesis, and muscarinic receptor binding.

The decrease in specific nerve systems and cholinergic markers correlates with an increase of morphological alterations in AD brains. This increase concomitantly parallels an increase in dementia scores of AD patients. The morphological alterations are speculated to be a result of nerve terminal degeneration (Fisher & Hanin, 1986). Additionally, pharmacological studies have consistently shown that the cholinergic system has a primary function in learning and working memory in both animals and humans (Francis, Palmer, Sims, et al., 1985; Bartus, Dean, Beer, & Lippa, 1982; Davis & Yamamura, 1978). The cholinergic regions of the brain are believed to be involved in mnemonic functions, and specifically working memory. Consequently, it has been hypothesized that AD is a result of cholinergic hypofunction in specific brain areas (Francis, Palmer, Sims, et al., 1985; Terry & Davies, 1983; Davies, 1979). There is evidence that other neuron systems are effected in AD such as serotonergic, noradrenergic, and somatostatin-like immunoreactive neurons. Yet, these appear to be affected to a lesser extent and/or at a latter stage of the disease than

the cholinergic hypofunction (Rossor & Iversen, 1986; Whitehouse & Unerstall, 1988).

While it is unlikely that pathology (viz. cholinergic cell loss) incurred early in development could cause AD in the latter stages of life, it is feasible that cholinergic ontogeny may play a role in this disease. A paradigm of Alzheimer's is that perinatal cholinergic damage may be of insufficient magnitude to alter cognitive functions until the age related decline of ACh markers in the brain augments this loss. Consequently, there is a potential for the effects of ontogenic cholinergic reduction to combine with age-related cholinergic decline resulting in a presenile dementia such as AD. It should be noted at this point that AF64A lesioned neonates are in no way considered to be representative of an animal model of Alzheimer's disease. This discussion is simply to reflect that there are similar patterns seen in this model and AD.

Effects of Perinatal ACh Lesions:

The importance of a specific cholinotoxin is reflected by its potential use in investigating the role of the cholinergic system in cognitive and brain development. Previous studies on cholinergic development using electrolytic lesions illustrate the need for a specific toxin.

Hohmann, Brooks, and Coyle (1988) investigated the effects of cholinergic hypofunction on subsequent brain development. They studied cortical development in mice with electrolytic lesions of the basal forebrain cholinergic neurons. The lesion reduced ChAT activity by 90% at PND's 4/5 (2/3 days post lesion). However, this

reduction was transient. ChAT activity was reduced only by 40% at PND 14 and was restored to control levels by PND 30. Similarly, transient reductions were found in NE and 5-HT in response to the basal forebrain lesion. Hohmann et al. (1988) also observed morphological disruptions due to cholinergic loss. There was a delay in expression of pyramidal cells in layer V and a more lasting disruption of the laminar boundaries which characterize the cortex.

Interpretation of the Hohmann et al. (1988) results is complicated by the fact that electrolytic lesion of the basal forebrain could damage many different cortical afferents as well as the cholinergic cell bodies. In this case, a cholinergic toxin would help clarify the issue of the effects of cholinergic hypofunction in neonates on subsequent brain ontogeny.

Neuronal turnover and metabolism of ACh is extremely rapid and much more rapid than any of the other transmitters (Fisher, Hanin, & Hortnagl, 1987). Consequently, the cholinergic system is difficult to block pharmacologically. Those agents which have been discovered to date (eg. pilocarpine, arecoline, oxotremorine, and hemicholinium-3) have been found to have a limited period of action. In fact, cholinergic function is commonly restored within hours after insult to the cholinergic system with these agents. While these agents may provide some insight into the mechanisms underlying the cholinergic system, they are of limited use in studying the ontogeny of the cholinergic system. A long-term specific cholinergic neurotoxin is thus paramount to investigating cholinergic function in brain development (Fisher & Hanin, 1980).

Armstrong and Pappas (1991) studied rat behavioural and cortical development in response to cholinergic insult via AF64A. They administered AF64A (i.c.v.) at 0.1, 0.5, and 2.0 nmol/ventricle dosages at PND 2. Consistent with the observations after electrolytic lesions of the NBM by Hohmann et al. (1988), they found that ChAT activity exhibited a transient reduction. There was a 42% decrease at PND 16 (14 days post-lesion) returning to control levels at PND 58 for all three drug doses. Although Armstrong and Pappas (1991) did not evaluate the levels of non-cholinergic transmitters over the course of development, they found a non-significant, dose-dependent reduction in DA at PND 58.

Similarly, Speiser, Amitzi-Sonder, Gitter, and Cohen (1988) observed that ChAT activity was reduced by approximately 14% at PNDs 14-18 (14 days post lesion) upon i.c.v. administration of AF64A (1 nmol/ ventricle). Speiser et al. (1988) found that neonatal AF64A treated animals displayed hyperactivity and reduced performance in the passive avoidance task as adults. In accordance with this, Armstrong and Pappas (1991) demonstrated that infusion with AF64A into neonates later attenuated spatial learning in the Morris water maze and caused hyperactivity in the elevated-plus maze at adulthood. However, these authors did not attribute the effects to cholinergic dysfunction. They argued that because the behavioural deficits only occurred in the 2.0 nmol dose of AF64A even though lower doses of AF64A resulted in comparable cholinergic decreases, the deficits could not be attributed to the cholinergic decreases.

Conversely, Gáspár, Heeringa, Markel et al. (1992) found that i.c.v. injection of 0.25, 0.5, 1.0, or 2.0 nmol doses of AF64A at PND 8 produced long-term depletions of ChAT and AChE. The decreases in ChAT and AChE were dose dependent with the more severe depletions occurring at the higher doses. In the same experiment, it was found that AF64A treated animals displayed abnormal novelty-induced behaviour and there was a dose-dependent impairment of spatial learning in the hole-board test.

There may be a number of reasons why Gáspár et al. (1992) found permanent ChAT reductions as opposed to transient reductions. In this experiment, the reductions in ChAT were found to be significant only in the hippocampus and only at 1.0 and 2.0 nmol doses. It is well established that, at such high doses, AF64A may act as a non-specific toxin. Furthermore, AF64A was administered at PND 8 in this study. This could be a significant variable because high-affinity choline uptake mechanisms only appear around PND 3-4 and are well established at PND 8. Perhaps the transient reductions in cholinergic markers observed in other studies (ie. Speiser et al., 1988 ; Armstrong & Pappas, 1991) were a result of the immaturity of the cholinergic system at the time of lesion.

Obviously, the effects of specific neonatal cholinergic dysfunction on cortical and behavioural development is yet to be clarified. AF64A, if found to be unequivocally a cholinergic toxin, would be an ideal agent for studies of cholinergic ontogeny. Furthermore, it may be concluded that AF64A may provide an animal model of Alzheimer's disease insofar as it mimics the

cholinergic loss and cognitive deficits found in AD patients. Yet, the mode of action of AF64A remains a contentious issue. In order for AF64A induced cholinergic hypofunction to be a functional model for evaluating treatment for AD or cholinergic development, the cholin specificity of this neurotoxin must be determined.

Overview

The cholinergic system within the CNS is implicated in many brain functions such as respiration, ingestion, motor activity, cardiovascular function, arousal, and cognitive functions such as learning and memory. This system is known to be compromised in Alzheimer's Disease which is characterized by severe memory loss.

To date, little is known about the consequences of long term, developmental depletion of the cholinergic system. This is largely due to the fact that cholinergic neurons cannot be effectively destroyed in experimental conditions. If brain acetylcholine could be permanently compromised in the neonate, then this model could be used to characterize the importance of cholinergic cells in development. This model could also be used to study the utility of new therapeutic approaches to disorders which result from cholinergic hypofunction.

AF64A is a promising neurotoxin which is shown to potently reduce cholinergic markers for an extended period of time and produce long-term cognitive impairments. Yet, this agent has not been shown, unequivocally, to be specific for cholinergic neurons.

AF64A could be used to study the ontogenic importance of the cholinergic system by administering it early in development. Yet, this agent would have to concomitantly be shown to only be acting on cholinergic cells.

This study proposed to investigate the importance of acetylcholine-releasing neurons in the CNS in development. Specifically, the purpose was to characterize behavioural, neurochemical, and histological changes which may result from cholinergic hypofunction sustained early in development. This study also proposed to use AF64A as a cholinotoxin while investigating the cholin specificity of this agent at the same time.

In the following study, neonatal rats were infused with AF64A and evaluated upon development (viz. PND 60) on behavioural, histological and neurochemical parameters. The specificity of AF64A was examined by pre-administering HC3 prior to AF64A infusion in some of the animals. Animals underwent behavioural and histopathological evaluation as well as an assessment of cholinergic markers. Specifically, animals were evaluated for ChAT upon development. Animals were also assessed via the Morris water maze task and elevated-plus maze and then assessed by histological examination.

An additional study was conducted to investigate the behavioural effects of various doses of HC3. One of the pioneering studies on this compound (Long & Schueler, 1954) found HC3 infusion to cause a progressive respiratory failure in adult rats. Although investigations since then have not reported such effects of HC3, pilot research in this study revealed severe respiratory failure,

often becoming fatal, in neonates infused with this drug. Furthermore, these animals appeared to have impaired spatial memory as adults. For this reason, an additional dose response study was conducted to measure the mortality rates as well as memory performance, via Morris water maze, of neonates injected with HC3.

It was hypothesized that i.c.v. injection of AF64A into neonates will produce a transient reduction in ChAT activity. ChAT was expected to return to control levels by the time the animal reached adulthood. Nonetheless, adult animals were hypothesized to sustain prolonged cognitive impairment on behavioural tasks and display histological impairment such as ventricular dilation as a result of neonatal cholinergic lesions. These results would be attributed to cholinergic and collateral non-cholinergic denervation sustained during early development.

Furthermore, preinfusion with HC3 was expected to prevent the AF64A-induced reduction in cholinergic markers. This would demonstrate that AF64A is cholinergic specific. It would also suggest that AF64A-induced non-cholinergic transmitter reduction is a secondary effect of cholinergic hypofunction and not due to non-specific effects of AF64A. In addition, adult animals who were pre-injected with HC3 prior to AF64A as neonates, were expected to display a marked improvement in behavioural measures in relation to animals receiving AF64A alone. Finally, preinfusion with HC3 was hypothesized to attenuate the histological pathology induced by AF64A.

Methods

Animals:

The litters of 30 pregnant female Long-Evans hooded rats, bred in our colony, were used in this study. Each litter was culled and cross fostered to approximately eight male pups and housed with dams in polypropylene cages (45 x 23 x 15 cm) until weaning. Upon weaning, rats were dually housed in polypropylene cages as described above. Rats were subjected to reverse light-dark cycle (lights off at 08:00 h, on at 20:00 h) with testing occurring during the dark cycle. Pups were subjected to one of four drug treatments. 16 pups were pre-injected with HC-3 followed by AF64A (H-A group), 16 received HC-3 followed by vehicle control (H-V group), another 16 were injected with physiological saline followed by an injection of AF64A (S-A group), and a final 16 animals were injected with physiological saline followed by a vehicle control injection (S-V group). For the HC3 dose response study, an additional 12 animals were administered one half and 12 animals given one quarter the amount of HC3 used in the primary investigation

The animals from each drug treatment were evaluated at approximately PND 60 on the Morris water maze task and elevated-plus maze. After behavioural testing, eight animals from each group were assayed for ChAT activity while the remaining eight underwent histopathological examination.

Drug Synthesis:

AF64A was synthesized from acetylcholine mustard hydrochloride (acetyl-AF64, Research Biochemicals) as described by Jackson and Hirst (1972). It has been shown that the purity of AF64A prepared by this method is 95% or greater (Fisher, Mantione, Abraham, & Hanin 1982). On the day of injection, acetyl-AF64 was dissolved in distilled water (2.0 mg/ml). The pH was adjusted to 11.5 with a small amount of 10 N NaOH (1.0 μ l/ml) and maintained between 11.5 and 11.8 for 30 min by addition of 1 N NaOH (approximately 10 μ l/ml). The pH was then be reduced to 7.4 with 6 N HCl (1.5 μ l/ml). Distilled water was added to bring the final concentration of AF64A to 0.1nmol/ μ l. The AF64A solution was left at room temperature for at least 60 min and was injected within 6 h of preparation. For preparation of vehicle control, 2 ml of distilled water was adjusted to pH 11.5 with 10 N NaOH and processed exactly as the AF64A preparation and adjusted to a final pH of 7.4 prior to injection. Hemicholinium-3 (Sigma; 20, 10, 5 μ g/ μ l) was dissolved in distilled water and pH was adjusted to 7.

Surgery:

Pups underwent stereotaxic surgery on post-natal day (PND) 2-3. Pups were placed in a stereotaxic apparatus which has been modified for neonatal surgery as described by Lithgow and Barr (1982). After initial anesthetization with a mixture of halothane and

oxygen (4% halothane/ 1.5% oxygen), animals were maintained on this mixture (1.5% halothane/ 1.5% oxygen) for the duration of surgery. Each pup received an incision along the midline to expose bregma.

32 animals (groups Sal-AF64A and Sal-Veh) received a bilateral pre-injection with physiological saline (0.5 μ l/ventricle) and 32 animals (groups HC3-Veh and HC3-AF64A) were given a bilateral pre-injection of HC3 (20 μ g/ μ l : 0.5 μ l/ventricle). After a period of 10 min, each animal was given a second injection. The second injection consisted of either AF64A (0.1 nmol/ μ l : 0.5 μ l/ventricle) for groups HC3-AF64A and Sal-AF64A or vehicle control (0.5 μ l/ventricle) for groups HC3-Veh and Sal-Veh. An additional 24 animals were pre-injected with saline and then administered 10 μ g/ μ l or 5 μ g/ μ l HC3 for the HC3 dose response study.

All injections were administered at the following coordinates: 0.5 mm anterior from bregma, +/- 1.5 mm lateral from midline, and 3.0 mm ventral from dura. Coordinates were established by Armstrong and Pappas (1991) and confirmed by pilot research. The injection needle remained in place for a minimum of 2 min following each injection to allow for the drug to diffuse into the cerebral spinal fluid (CSF) of the lateral ventricles. After completion of surgery, animals were removed from the stereotaxic apparatus and the scalp incision was occluded with tissue adhesive (Vetbond™). Animals were placed on a heating pad until their body temperature returned to normal and they had recovered from the anesthetic, at which point they were returned to their respective dams.

Behavioural Testing:

Cognitive evaluation was carried out using the Morris water maze (MWM) task to evaluate spatial learning and the elevated-plus maze to monitor spontaneous behaviour. All behavioural tests were recorded via an overhead video camera mounted on the ceiling of the testing room.

Morris water maze:

Spatial memory was evaluated on all 16 animals from each treatment group as well as 12 rats from 5µg/µl HC3 group at approximately PND 60 using the MWM. The water maze consisted of a large cylindrical tank (150 cm in diameter, 45 cm deep) filled to a depth of 30 cm with opaque water (ie. water containing powdered milk). The water was maintained at room temperature. A transparent Plexiglas platform was placed 1 cm below the surface of the water in the middle of one quadrant in the tank. The platform was visible to the animal. The tank was placed in the centre of the testing room which contained extramaze clues such as posters on the walls, shelves, etc. During spatial navigation animals were allowed 4 trials on each day for 5 consecutive days. At the onset of testing each day, a random sequence of 4 starting locations (90° apart) were assigned. All rats will followed the same sequence for that day. For each trial, the animal was placed in the water facing the wall at the designated start location. The rat was given 120 s to find the platform, climb

onto it and rest for 30 s. The latency to reach the platform was recorded. If the animal did not reach the platform by the allotted time, it was lifted out of the water and placed on the platform for that test interval. At the next test interval, each rat was removed from the platform and placed at the next start location.

On the sixth day of testing, the platform was removed from the tank and the rat was assessed on the spatial probe trial. Each animal was allotted 60 s to swim about the platformless tank. The distance that the rat swam within the quadrant previously containing the platform (ie. the training quadrant) was measured versus the distance the rat swam in total.

On the seventh day of testing, a black platform was placed at the surface of the water. Each rat was then given four cued navigation trials. Animals were allotted 60 s to locate the visible platform. As in the spatial navigation trials, a random sequence of the four starting positions were assigned for all animals. The latency to reach the platform was recorded and used to evaluate each rats swimming ability.

Elevated-plus maze:

Spontaneous activity was measured from 16 rats per group (total of 64) by means of an elevated-plus maze following MWM testing. This maze contains two open arms (50 x 10 cm), and two enclosed arms (50 x 10 x 40 cm) with an open roof. The maze was designed for the like arms to face opposite each other. The entire maze is elevated to a height of 50 cm from the floor of the testing

room. Each rat was exposed to the maze for 5 min and the number of open/closed arm entries were recorded. Each rat was evaluated according to the total number of arm entries and the ratio of open to total arm entries. In this testing paradigm, normal rats are expected to show aversion to the open arms.

Neurochemistry:

All 32 animals who underwent neurochemical evaluation were sacrificed by decapitation immediately following behavioural testing. The brains were immediately removed and dissected over a saline-rinsed ice cold plate into frontal cortex, hippocampus, and caudate sections. One half of the samples were taken from the right half of the brain and the other half were taken from the left side. An assay for ChAT activity was conducted on these animals.

ChAT activity was measured following the method described by Fonnum (1975). In brief, tissue was weighed and homogenized in a buffer containing 2.9 g/l EDTA, 8.0 mM choline bromide, and 0.10 mM physostigmine. Buffer pH was adjusted to 7.4. Following homogenization, tissue was held on ice for 20 min and then rehomogenized. 20 μ l tissue extracts were obtained in duplicate for the assay while the remaining tissue was used for protein analysis. A denatured tissue blank was obtained by boiling 1.0 ml of unused frontal cortex in water for 15 min to denature the enzyme. The tissue blank was used as a reference for all tissue values.

20 μ l of 650 μ M [3 H]acetyl CoA was added to each 20 μ l tissue extract to initiate the reaction. Reactions were carried out for 15 min

at 37°C. The reaction was terminated by adding 500 µl of 'stop' solution. 'Stop' solution consisted of 10 mg sodium tetraphenyl boron, 5.0 ml (10 nM) sodium phosphate buffer, and 2.0 ml acetonitrile. The pH of the stop solution was maintained at 7.4. The resulting solution was quantitatively transferred to a scintillation vile containing 10 ml of toluene based scintillation cocktail. The scintillation vial was rocked exactly 6 times to perform phase extraction. This process extracts newly synthesized [³H]ACh into the toluene phase and allows unreacted [³H]acetyl CoA to remain in the aqueous phase. 20 µl of 650 µM [³H]acetyl CoA was added to Aquasol scintillation cocktail for a baseline radioactivity count. Final enzyme activity was expressed in nmol/ hr/ mg protein.

Histology:

Upon completion of behavioural testing, 8 rats from each drug treatment group were perfused with 10% formalin after receiving a lethal dose of pentobarbital (100 ml/kg) intraperitoneally. The brains were stored in 10% formalin for later sectioning on a cryostat at 40 µm in the coronal plane. Twenty-four hours prior to freezing the brains for sectioning, they were stored in a formalin solution containing 30% sucrose. Approximately every fourth section was selected and mounted on a slide. Each section was stained with standard Cresyl Violet for illumination of nissl substance.

Sections were dehydrated in 70%, 95%, 100%, 100% alcohol and then defatted in xylene. The sections were then rinsed in 100%

and 70% alcohol, followed by distilled water and stained with 0.5% Cresyl Violet (pH 3.9). Once stained, the sections were quickly rinsed in distilled water and then dipped in an acetic acid differentiator and rinsed in distilled water again. Subsequently, they were dehydrated in 70%, 95%, 100%, and 100% alcohol before being immersed in xylene and cover-slipped with Permount.

Following the procedure of Diamond et al. (1972; 1975), tissue was quantified using commercially available software (Imaging Research™, St. Catharines, Ont.). An image of each section was captured, digitized and subsequently projected onto a monitor. Measurements were obtained manually or by an automated subroutine to estimate extent of morphological damage resulting from each drug treatment.

Five sections obtained from each of the total of 40 animals were included in the morphological analysis. These sections included the section at the most rostral position showing the decussation of the corpus callosum (section CC), a section at the decussation of the anterior commissure (section AC), and a section remotely caudal to the appearance of the posterior commissure (section PC) so that the ventral hippocampus was included. Another section was obtained at the area of the prefrontal cortex to include any structural damage incurred more rostrally. Finally a section was taken just prior to section PC so that only the dorsal hippocampus was exposed.

Corresponding to Diamond et al.'s (1972; 1975) cortical areas B, C, and D, thickness measurements were taken from dorsal, lateral, and ventral cortex for both hemispheres. Anatomically, these areas represent the frontal cortex, forelimb area of the cortex,

and the parietal cortex, respectively, at sections AC and CC. At section PC, these areas represent the medial occipital cortex, lateral occipital/parietal cortex and temporal cortex, respectively.

Additional measurements were obtained from section PC to include the area of the granule cell layer in the dentate gyrus, the area of the CA3 cell field, and the area of the most lateral cell field of the hippocampus consisting of CA1 and CA2.

Results

Surgery:

The fatality rates following surgery are reported in Table 2 for each drug treatment administered. For the AF64A injected animals, the survival rate (96%) was the same or better than that which has been reported in the literature. The mortality rate for the HC3 treated animals was very high at both the 20 μ g/ μ l and 10 μ g/ μ l doses (mortality rate = 82% and 100% respectively). At the 5 μ g/ μ l dose, HC3 does not produce the respiratory failure and subsequent death which higher doses produce (mortality rate = 0%). As a result, the HC3 dose response study was conducted using only the 20 μ g/ μ l and 5 μ g/ μ l doses, as all the animals treated with 10 μ g/ μ l of HC3 died.

Behaviour:

Morris water maze. All animals displayed normal swimming ability in this task. A repeated measures analysis of variance (ANOVA), using five blocks of four daily trials as the within-subject factor and the four drug groups as between-subject factor, was carried out for place navigation (invisible platform) trials (appendix A.1). Figure 2 shows that there was an interaction between drug treatment and test day . Post hoc analysis revealed that, on test day 1, the saline-vehicle control group performed better than the HC3-vehicle group ($p < 0.05$, Tukey HSD) as well as the saline-AF64A group ($p < 0.01$, Tukey HSD). On day 2, the control group reached the hidden platform faster than all other groups ($p < 0.01$, Tukey HSD). On the third day of navigation trials, both the HC3-AF64A and saline-vehicle control group performed better than the other two groups ($p < 0.01$, Tukey HSD). The AF64A-saline group displayed impaired performance versus other groups on days 4 and 5 of testing ($p < 0.05$, Tukey HSD).

A separate repeated-measures ANOVA was carried out to assess the cued navigation (visible platform) trials for the Morris water maze (appendix A.2). As can be seen in figure 3, there was no significant interaction between test trial and drug treatment group, nor was a main group or trial effect observed. This demonstrates equal swimming performance between all groups when spatial memory is not necessary in order to find the platform.

Figure 2. Mean latencies (sec) to reach hidden platform across navigation test days (4 daily trials) on Morris water maze task.

There was a significant difference between groups for each test day ($F_{(12,228)} = 1.93$; $p < 0.05$). The Sal-Veh group performed better than the other groups on days 1 and 2. The HC3-AF64A group and Sal-Veh control group performed better than the Sal-AF64A group on days 3 and 4. (* $p < 0.05$, ** $p < 0.01$, Tukey HSD).

Figure 3. Performance (over four trials) on the cued navigation trial of the Morris water maze. There were no differences apparent on this test, as all treatment groups displayed equal swimming ability.

A one-way ANOVA was performed to compare groups on the total distance swam during the spatial probe trial (appendix A.3). There was no difference between groups in total distance swam in the pool during a 60 sec exposure to the platformless pool (figure 4). The ratio of total distance swam in the pool versus the distance swam in the training quadrant (previously containing the platform) was analysed via a one-way ANOVA (appendix A.3). There were no observable differences between groups on this measure. Figure 5 reveals that all groups performed slightly better than chance (chance = 0.25) at this task.

For the HC3 dose response study, a repeated-measures ANOVA was carried out on the three drug dose groups with five blocks of four daily test trials being the within-subject factor (appendix A.4). While no interaction was found between dose and test day, a main group effect was observed ($F_{(2,39)} = 9.159$; $p < 0.001$). Figure 6 illustrates that the 20 µg/µl dose group needed much more time to find the hidden platform than did animals who received 5 or 0 µg/µl doses of HC3 ($p < 0.01$, Tukey HSD)

Elevated-plus maze. A one way ANOVA was performed to assess the spontaneous behaviour (viz. total entries of maze arms) of rats in each drug treatment group (appendix A.5). There were no measurable differences between animals receiving different drug treatments in the amount of spontaneous behaviour they exhibited (figure 7). A separate ANOVA was carried out to investigate the ratio of open to total arm entries (appendix A.5). Figure 8 shows that animals in each group performed equally on this measure with each group displaying a normal aversion to the more vulnerable

Figure 4. Total swim distances (cm) for each drug treatment group during the spatial probe trial of the Morris water maze. There was no difference between groups during a one minute exposure to the platformless pool.

Figure 5. Ratio of distance swam for each treatment group during the spatial probe trial of the Morris water maze. The ratio is expressed in terms of total distance swam in the training quadrant (previously containing the platform) over total distance traveled in the pool (chance = 0.25). There were no differences between drug treatment groups.

Figure 6. Mean latencies (sec) to reach hidden platform across navigation test days (4 daily trials) on Morris water maze for the HC3 dose response study. While there was no significant interaction between group and test days, there was a group effect ($F_{(2,39)} = 9.159$; $p < 0.001$) showing that the $5\mu\text{g}/\mu\text{l}$ dose and control groups performed better than the $20\mu\text{g}/\mu\text{l}$ dose group ($p < 0.01$, Tukey HSD).

Figure 7. Total arm entries for each group during a five minute exposure to the elevated-plus maze. All groups made the same number of entries.

Figure 8. Ratio of open to total arm entries for each group during a five minute exposure to the elevated-plus maze. There were no apparent differences between treatment groups. Animals in all groups exhibited the normal avoidance of open arms.

open field arms.

Neurochemistry:

As shown in appendix A.6, three separate ANOVAs were conducted for choline acetyltransferase activity in the brain at adulthood. In the frontal cortex, no differences were found between groups in the level of enzyme activity (figure 9). There was, however, a non-significantly higher level of activity for animals who received saline-AF64A infusions at this brain site. There were no group differences in the level of ChAT activity in the hippocampus or caudate nucleus (figures 10 & 11 respectively). All drug treatment groups displayed normal levels of enzyme activity for these brain areas.

Histology:

The brains of animals who received saline-AF64A neonatally were globally damaged whereas all other drug treatment combinations did not produce any global pathology. As can be seen from the examples in figures 12 and 13, 6 of the 7 animals examined from the saline-AF64A group had virtually hollowed out frontal cortices with holes leading from the prefrontal cortex down to the lateral ventricles.

A more specific analysis was carried out on these brains. Appendix A.7 shows the ANOVA summary tables for the analyses performed on the thickness of cortex at area B (corresponding to

Figure 9. Choline acetyltransferase (ChAT) activity (nmol/hr/mg of protein) in the frontal cortex for each drug treatment group. There were no group differences in the level of ChAT activity.

Figure 10. Choline acetyltransferase (ChAT) activity (nmol/hr/mg of protein) in the hippocampus of each treatment group. There were no apparent differences between groups in the level of activity.

Figure 11. Levels of choline acetyltransferase (ChAT) activity (nmol/hr/mg of protein) in the caudate nucleus for each treatment group. No differences between groups were found.

Figure 12. Photographs of the adult brain of a rat treated with AF64A neonatally and one pre-treated with HC3 before AF64A administration. HC3 prevented the morphological damage indicated by the severe malformation of frontal and prefrontal cortex.

Figure 13. Example of a typical saline-AF64A and HC3-AF64A treated animal. Morphological damage is observed at the pre-frontal cortex (PFC), at the crossing of the corpus callosum (CC), and at the appearance of the anterior (AC) and posterior (PC) commissures of the saline-AF64A rat. HC3 pretreatment blocked this effect of the AF64A. Note the developmental abnormality of the corpus callosum and hippocampus of the animal treated with saline-AF64A.

saline-AF64A

HC3-AF64A

PFC

CC

AC

PC

dorsal cortex) at sections CC, AC, and PC. Figure 14 illustrates the mean thickness measurements (taken from both hemispheres) of cortex between each group at each section. At section CC, representing the frontal cortex, there was a difference between groups ($F_{(3,24)} = 11.769$; $p < 0.001$). The saline-AF64A group had a shallower frontal cortex than all other treatment groups ($p < 0.01$, Tukey HSD). There were no differences between groups at the more caudal portion of the frontal cortex (section AC) or the medial occipital cortex (section PC).

Three separate ANOVAs were conducted on the thickness measurements from area C, corresponding to lateral cortex, at sections CC, AC, and PC (appendix A.8). Figure 15 shows that there were differences between groups at section CC ($F_{(3,24)} = 3.270$; $p < 0.05$). Post hoc analysis revealed that the thickness of the forelimb area of the cortex was reduced in saline-AF64A treated animals compared to saline-vehicle controls ($p < 0.05$, Tukey HSD). The thickness of the more caudal forelimb area of the cortex (section AC) as well as the lateral occipital/parietal cortex (section PC) did not differ between groups.

For area C (ie. ventral cortex), a separate ANOVA was carried out for each of the three sections; CC, AC, PC (appendix A.9). As can be seen in figure 16, there were no differences between groups in the mean thickness of parietal cortex (sections CC and AC) or temporal cortex (section PC) taken from both hemispheres.

The area of the corpus callosum was measured from each hemisphere at sections CC and AC. The mean of the two

Figure 14. Cortical thickness measurements (mm) for area B (Diamond et al., 1972; 1975: corresponding to the dorsal cerebral cortex) taken from three separate coronal sections (CC, AC, PC). The Sal-AF64A group had a thinner cortex than other groups at section CC ($F_{(3,24)} = 11.769$). Measurements represent a mean of both hemispheres (* $p < 0.01$, Tukey HSD).

Figure 15. Cortical thickness measurements (mm) for area C (Diamond et al., 1972; 1975: corresponding to the lateral cerebral cortex) taken from three separate coronal sections (CC, AC, PC). There was a difference between groups at section CC ($F_{(3,24)} = 3.270$; $p < 0.05$). The Sal-AF64A group (indicated by X) had a thinner cortex than the Sal-Veh group (indicated by O) ($p < 0.05$, Tukey HSD). Measurements represent a mean of both hemispheres.

Figure 16. Cortical thickness measurements (mm) for area D (Diamond et al., 1972; 1975: corresponding to the ventral cerebral cortex) taken from three separate coronal sections (CC, AC, PC). There were no measurable differences between drug treatment groups. Measurements represent a mean of both hemispheres.

hemispheres for each animal was then analysed by a separate ANOVA for each section (appendix A.10). At the more rostral area of the brain (viz. section CC) there was a significant difference between groups ($F_{(3,24)} = 17.829$; $p < 0.0001$). Figure 17 (top graph) shows that animals injected with saline-AF64A had a smaller area of fibres decussating the hemispheres at the corpus callosum than all other groups ($p < 0.01$, Tukey HSD). However, at the more caudal section AC these differences were no longer apparent (figure 17, bottom graph).

The amount of ventricular dilation was calculated by averaging the area from the lateral ventricles of both hemispheres at sections CC and AC. Appendix A.11 shows the ANOVA summary tables for the separate analyses for sections CC and AC. There was a significant difference in ventricular size between treatment groups at section CC ($F_{(3,24)} = 6.691$; $p < 0.01$) but not at section AC (figure 18). At section CC, the saline-AF64A treated animals exhibited greater ventricular enlargement than all other groups ($p < 0.01$, Tukey HSD), whereas at section AC there was the same trend but the differences were not significant.

The mean area of the caudate nucleus, taken from both hemispheres, was analyzed by a one-way ANOVA (appendix A.12). There were no significant differences between drug treatment groups although there was a trend for the saline-AF64A group to be smaller (figure 19).

The hippocampus was evaluated by dividing it up into four measureable parts. Area measurements of the dorsal horn as it occurs just rostral to section PC were taken along with measurements

Figure 17. Area measurements (mm²) taken from the corpus callosum at sections CC and AC respectively. There was a significant difference in the size of the corpus callosum at section CC ($F_{(3,24)} = 17.829$; $p < 0.0001$). At this site, the Sal-AF64A group had a smaller corpus callosum than all other groups (* $p < 0.01$, Tukey HSD). There were no differences between groups at section AC. Measurements represent a mean of both hemispheres.

SECTION CC:

SECTION AC :

Figure 18. Area measurements (mm²) of the lateral ventricles at sections CC and AC respectively. The ventricles were significantly different at section CC ($F_{(3,24)} = 6.691$; $p < 0.001$). The Sal-AF64A group displayed more ventricular dilation than all other groups ($*p < 0.01$, Tukey HSD). The ventricles were non-significantly larger for this group at section AC. Measurements represent the mean area for both hemispheres

Figure 19. Area measurement (mm²) of the caudate nucleus. There was no notable difference in caudate size between groups. Measurements represent a mean area of the caudate from the left and right hemisphere at section CC.

of the area of the dentate gyrus, area of the CA3 cell field, and the lateral area of the hippocampus including the CA1 and CA2 cell fields.

Appendix A.13 summarizes the separate ANOVAs conducted on area measurements of the dentate gyrus and dorsal hippocampus respectively. There was a difference in area of the dentate gyrus as a result of drug treatment ($F_{(3,23)} = 6.652$; $p < 0.01$). Figure 20, top graph, illustrates that the saline-AF64A group (indicated by O) had a larger dentate gyrus than either the HC3-AF64A group ($p < 0.05$) or the HC3-vehicle group ($p < 0.01$, Tukey HSD). Figure 20 (bottom graph) illustrates that drug treatment had no effect on size of the dorsal hippocampus.

The combined area of the CA1 and CA2 cell fields were analysed using a one-way ANOVA (appendix A.14). There was no effect of drug on the development of the CA1 and CA2 cell field area (figure 21). An ANOVA performed on the area measurements of the CA3 cell field revealed that there was no difference in size of this structure between groups (appendix A.14, figure 21).

The thickness of the entorhinal cortex was also measured to determine if there was any effect of drug treatment. A one-way ANOVA revealed that there were no significant group effects (appendix A.12; figure 22).

Resolution test chart showing patterns of vertical and horizontal lines with numerical values: 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.6, 6.3, 7.1, 8.0.

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figure 20. Area measurement (mm²) of the dorsal horn and the dentate gyrus of the hippocampus respectively. There was no significant difference between groups for dorsal hippocampus area. There was a difference in the size of the dentate gyrus ($F_{(3,23)} = 6.652$; $p < 0.01$). The Sal-AF64A group (denoted by O) showed a larger dentate area than either the HC3-AF64A ($p < 0.05$, Tukey HSD) or HC3-Veh group ($p < 0.01$, Tukey HSD). Measurements represent the mean area from both hemispheres.

DENTATE GYRUS:

DORSAL HIPPOCAMPUS:

Figure 21. Area measurements (mm²) of the combined CA1 and CA2 cell fields as well as the CA3 cell field of the hippocampus respectively. There were no differences between groups for either of these measures. Measurements represent an average of hippocampal area from both hemispheres at section PC.

CA1 AND CA2 :

CA3:

Figure 22. Cortical thickness measurement (mm) of the entorhinal cortex at area PC. There was no measurable difference between groups at this area. Measurements represent a mean cortical thickness from both hemispheres.

Discussion

Neonatal i.c.v. administration of AF64A (0.1nmol/ventricle; 0.5µl/ventricle) produced long term cognitive impairments and permanent histopathological alterations in the brain. The levels of the cholinergic marker, choline acetyltransferase (ChAT), were equal to control levels at the time of sexual maturity (60 days of age). The pre-administration of hemicholinium-3 (20µl/µg) 10 min prior to AF64A injection attenuated the cognitive impairments and prevented the histopathology observed later in development. Animals which received the HC3 pre-administration did not differ from control animals on any histological measure.

The behavioural impairments induced by AF64A in this study are inconsistent with previous findings. Armstrong and Pappas (1991) found deficits in MWM performance only at AF64A doses as high as 2.0 nmol. Similarly, Gáspár et al., (1992) found impairments of spatial memory on the hole-board test but did not use a dose of AF64A below 0.25 nmol. Considering that higher doses of AF64A may produce non-specific lesions, it was questionable as to whether the behavioural impairments observed in developmental studies using AF64A could be attributed to cholinergic loss only. This study demonstrated that AF64A produced spatial memory impairments at a low dose and that HC3 attenuated these impairments. This is evidence that the behavioural deficits seen in this and previous developmental studies may be attributable to specific cholinergic loss sustained early in life.

The animals treated with 0.1 nmol of AF64A in this study did not exhibit hyperactivity in the elevated-plus maze. Previous findings (viz. Speiser et al., 1988 ; Armstrong and Pappas, 1991 ; Gáspár et al., 1992) have shown that at higher doses of AF64A (i.e. 0.25 - 2.0 nmol) animals display hyperactivity. Gáspár et al. (1992) found that animals were hyperactive only at AF64A doses of 0.5 nmol or higher. Perhaps hyperactivity in these developmental studies was not a result of cholinergic lesions but may be attributed to the non-specific effects of AF64A seen at higher doses.

Although the level of choline acetyltransferase (ChAT) was not affected at adulthood in this study, previous studies (e.g., Speiser et al., 1988 ; Armstrong and Pappas, 1991) have shown that ChAT activity is transiently reduced upon AF64A administration and is restored by adulthood. This is evidence that cholinergic depletions in early development will only temporarily affect this cholinergic marker. Gáspár et al. (1992) did, however, find that ChAT activity was permanently reduced in the hippocampus upon AF64A administration. This is interesting because Gáspár et al. (1992) infused AF64A at PND 8 whereas this study and others who found only transient reductions in ChAT administered AF64A at PND 2-3.

These data are interesting in light of how the cholinergic system develops over the first two weeks after birth. It appears that cholinergic tone is very high during late gestation and the first few days of life. ACh, HACU, and AChR's show a transient overexpression during this period of development. This overexpression decreases to normal levels by PND 10 when the distribution and density of these markers is more similar to that of

adult levels. However, a week later in development there is a transient overexpression in the level of ChAT activity in the brainstem and hippocampus. Perhaps a cholinergic lesion sustained at PND 8 is interrupting processes which are more sensitive to hippocampal ChAT development whereas at PND 2-3 ChAT activity is not well established let alone overexpressed. Thus, a cholinergic lesion sustained at PND 2-3 may only temporarily affect ChAT because this system is not yet as sensitive to cholinergic depletion.

The morphological alterations seen in animals treated with AF64A were contained to areas surrounding the lateral ventricles and prefrontal cortex. Indeed, this is evidenced by the very large ventricles found in the AF64A rats. It was found that the prefrontal cortex and the rostral portion of the frontal cortex was most severely affected in these animals. There were also developmental abnormalities at the rostral corpus callosum and dentate gyrus of the hippocampus in animals infused with AF64A.

The alterations in frontal cortex is interesting considering that, at the period of development when the cholinergic insult was sustained in these animals (i.e. PND 2-3), there is an overexpression of cholinergic markers at this site. There is an overexpression of AChE and muscarinic AChR's which occurs in the neocortex during the first postnatal week. Further, there is an expression of nicotinic receptors in the subplate layer of the neocortex during the first week of postnatal life which disappears by the second week. The subplate is thought to play a role in development as it is a site of early synapse formation and a waiting zone for projections migrating to the cortical plate.

As mentioned previously, cholinergic tone is very high during the first postnatal week. This heightened expression of cholinergic markers appears to be very important in brain development. For example, it appears that AChR stimulation by exogenous ACh will prematurely terminate cell replication during this stage of development (Navarro et al., 1991). Cholinergic stimulation is important in target cell differentiation (Spitzer, 1991) and early neonatal behaviours (Schlumpf et al., 1991). The cholinergic system may play a role in coordinating the timing of membrane development, synaptogenesis, and macromolecule synthesis. This role seems to have a critical period in late prenatal and early postnatal development. It stands to reason that an insult to the cholinergic system during the first week of postnatal life will severely compromise the development of cholinergic mechanisms and possibly other transmitter systems as well.

It has been suggested that the increased expression of cholinergic markers during this period may be due to transient expression of these markers by non-cholinergic cells. If this is the case, then cholinergic lesions at this stage of development may be affecting the ontogeny of other non-cholinergic systems. This a possibility as non-cholinergic reductions have been observed as a result of cholinergic insult sustained during this time (e.g. Armstrong & Pappas, 1991).

Nonetheless, the transient overexpression of cholinergic markers in early development, whether it be a result of high cholinergic tone or expression of these markers by other cells, seems to occur in the neocortex and hippocampus. A lesion to the

cholinergic system may interrupt a trophic role which this system may play at this stage of ontogeny. The histological alterations seen in this study would be evidence of such a phenomenon.

Abnormalities in the frontal cortex, corpus callosum and hippocampus seen here may be the result of the loss of trophic stimulation for cells in these areas at a critical stage in their development.

The long-term alterations in brain morphology and impairment of spatial memory which resulted from neonatal AF64A injection appear to be a result of the specific effects of this agent on the cholinergic terminals in the brain. HC3 is an inhibitor/antagonist of the high-affinity choline uptake (HACU) carrier which is present only on the terminals of ACh neurons. HACU has been indicated to be the mechanism which transports AF64A into the cell (Rylett & Colhoun, 1980) where AF64A may exert its cytotoxic effects. Seeing that the toxic effects of AF64A have been prevented by HC3, these toxic effects must be mediated via the cholinergic cells as HC3 should not prevent AF64A damage to other cells.

There is an alternative explanation for the ventricular enlargement seen in the brains of AF64A-injected animals. Perhaps these animals are experiencing hydrocephalus as a result of infusion with AF64A. The morphological characteristics of these brains seem reflective of animals suffering severe hydrocephalus. This may come about as a result of meningeal infection or hemorrhage of CSF into the subarachnoid space. Yet, considering that no other group experienced ventricular dilation, it would be unlikely that this condition would come about as a result of rupture of the ventricular

wall via the injection needle or infection due to surgery. Perhaps AF64A creates an environment where there is an increased opportunity for hydrocephalic infection.

It may be possible that AF64A is entering non-cholinergic cells via low-affinity choline uptake (LACU) which exists on all cells. Therefore, AF64A would be acting non-specifically. However, HC3 is a very weak inhibitor of low-affinity choline uptake (Happe and Murin, 1993) and it is unlikely that it could prevent the action of AF64A to the extent as was seen in this study if it were the LACU sites that AF64A were actually acting on. Furthermore, it has been demonstrated that AF64A is a much higher inhibitor of HACU than LACU (Rylett & Colhoun, 1982 ; Mantione et al., 1983).

AF64A is shown to reduce non-cholinergic markers as well as produce excitotoxin-like morphological damage in several studies (e.g. Asante et al., 1983; Jarrard et al., 1984; & Hanin et al., 1987). Yet, it has been proposed that these effects are secondary to, and as a result of, long-term cholinergic reduction. Because the cholinergic system has not been specifically compromised for long periods of time, the consequences of severe cholinergic loss remains largely unexplored. The damage incurred as a result of AF64A administration which have been reminiscent of non-specific lesions may be an eventuality of severe and permanent cholinergic loss. Such a premise is supported by several lines of evidence.

Cholinergic cells, specifically ACh, are known to play an important role in the development of other transmitter systems in addition to being important in maintaining synaptogenesis in the

PNS. The withdrawal of cholinergic input from PNS sites, such as the neuromuscular junction, causes atrophy of postsynaptic assemblies (i.e. receptors and functional synapses).

This premise allows for a reinterpretation of one aspect of Alzheimer's disease. Human patients suffering from Alzheimer's disease exhibit a notable deterioration of cholinergic markers which correlates with a deterioration of cognitive abilities. Furthermore, the non-cholinergic systems which also degenerate as the disease progresses, appear to deteriorate secondary to, and to a lesser extent than, the cholinergic system. Regardless of what the aetiology of AD may be, the non-cholinergic systems in the AD brain may be degenerating as a result of cholinergic hypofunction.

The effects of HC3, when administered early in development, seem to be very toxic at doses higher than 5 µg/µl. Animals receiving 10 or 20 µg/µl doses of HC3 at PND 2-3 exhibited a delayed (1 hr post-surgery) and progressive respiratory failure. For most of these animals, this effect was fatal. Those animals surviving the toxin were impaired on the Morris water maze. Previous studies using HC3 have been conducted on adult animals only. These studies did not report this effect of HC3 after acute injection except for Long and Schueler (1954). Yet, it should be noted that the doses of HC3 used in this study were extremely high.

Obviously, the neonate is more sensitive to higher concentrations of HC3. Those animals which received the 5µg/µl dose did not exhibit respiratory failure and were not impaired on the behavioural task. It may be that the impairment of spatial memory was a result of the respiratory failure in the neonate and not directly

a result of the HC3 itself. It is well established that periods of anoxia sustained early in development impairs spatial memory on the Morris water maze task (e.g. Boksa et al. 1993).

Interestingly, those animals who received both AF64A and HC3 performed better on the MWM than those receiving HC3 alone. Perhaps the actions of both these agents counter each other. That is, both HC3 and AF64A bind to the HACU sites in the brain. Perhaps when both are introduced, their competitive binding does not allow either agent enough binding sites to for its toxic effects to be exerted in any measurable manner. It may be argued that HC3 and AF64A are interacting with each other directly to alter their configuration and subsequently reduce their toxic potential. However, there is no theoretical reason, based on molecular configuration, that these two compounds would interact (Abram Fisher, personal communication Nov. 1992).

The manner in which HC3 produces respiratory failure is unknown. It is possible that this compound is acting on cholinergic sites in the brainstem responsible for respiration which are expressing a high cholinergic tone during this stage of development. This would explain why this effect is not seen when HC3 is administered to the adult at the same doses use here.

In summary, AF64A acts specifically on the cholinergic terminals at a dose of 0.1nmol. When administered neonatally, this agent produces long term deficits in spatial memory and alters brain structures although it does not permanently reduce ChAT activity. The alteration in brain morphology corresponds to the areas where there is a heightened cholinergic tone during the first postnatal week

(viz. cortex and hippocampus). The cholinergic system may play a trophic role in the development of these areas and lesions to this system result in permanent structural abnormalities. Furthermore, the behavioural impairments may be a result of the developmental, histological abnormalities seen in AF64A-injected brains.

HC3 is able to attenuate the effects of AF64A on behavioural measures and prevents its effects of morphological abnormalities seen in the brain. HC3 is, in itself, toxic when applied to the neonate at doses higher than 5µg/µl. The delayed respiratory failure incurred as a result of this anoxia may be responsible for the behavioural impairment exhibited by HC3 injected animals.

Future studies on this topic should explore the levels of cholinergic markers other than ChAT as well as the level of other non-cholinergic markers to determine which systems are specifically affected as a result of cholinergic dysfunction in early postnatal life. If the cholinergic system is indeed playing a trophic role for non-cholinergic cells in development, it would be interesting to see exactly what is affected by cholinergic loss. It would also be interesting to produce specific cholinergic lesions within a range of early development (e.g. 1 week prenatally to 2 weeks postnatally) to determine if there is a critical period for cholinergic tone where depletions of this system result in developmental abnormalities like those seen here. This would also demonstrate whether such a putative critical period corresponds to the period of overexpression by cholinergic markers in certain brain areas.

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Table 1. Characterization of cholinergic receptor subtypes.

Receptor	Nicotinic	Muscarinic
Mode of Action	Na ²⁺ ion channel coupling	c-AMP
Agonists	nicotine (low dose)	oxotremorine
Antagonists	mecamylamine nicotine (high dose)	scopolamine atropine
CNS Location	cerebral cortex interpenduncular n. medial habenula substantia nigra -pars compacta thalamus -ventrobasal complex -med. lat. geniculate neostriatum hippocampus	all areas
PNS Location	autonomic ganglion motor end plates	autonomic effectors (ie. smooth muscles & glands)

Table 2. Fatality rates of neonates in each drug treatment group.

Group	Total pups injected	Fatalities	Percentage
saline-Vehicle	20	0	0%
saline-AF64A	43	7	6.14%
HC3-AF64A	37	6	16.22%
HC3-vehicle (20 μ g/ μ l)	114	94	82.44%
HC3-vehicle (10 μ g/ μ l)	12	12	100%
HC3-vehicle (5 μ g/ μ l)	12	0	0%

APPENDIX A.1

Repeated measures ANOVA summary table for
Morris water maze latencies during navigation test days.

	SS	df	MS	F-test	p
GROUP	55416.51	3	18472.17	10.85	0.000
ERROR	97067.54	57	1702.94		
DAY	184814.47	4	46203.62	164.91	0.000
GROUP BY DAY	6491.58	12	540.97	1.93	0.032
ERROR	63881.20	228	280.18		

APPENDIX A.2

Repeated measures ANOVA summary table for latencies during
cued navigation trials in the Morris water maze.

	SS	df	MS	F-test	p
TRIAL	1187.621	3	395.87	1.012	0.3943
ERROR	21905.206	56	391.16		
GROUP	191.988	3	63.996	0.163	0.9209
GROUP BY TRIAL	188.169	9	20.908	0.053	1.0000
ERROR	65810.088	168	391.727		

APPENDIX A.3

ANOVA summary tables for Morris water maze
spatial probe trial (for total distance swam and
distance ratio respectively).

TOTAL DISTANCE SWAM:

	SS	df	MS	F-test	p
GROUP	31962.18	3	10654.06	0.379	0.768
ERROR	1574006.67	56	28107.26		

QUADRANT/TOTAL DISTANCE RATIO:

	SS	df	MS	F-test	p
GROUP	0.028	3	0.009	1.516	0.220
ERROR	0.345	56	0.006		

APPENDIX A.4

Repeated measures ANOVA summary table
for Morris water maze latencies for the
Hemicholinium-3 dose response study.

	SS	df	MS	F-test	p
GROUP	19830.751	2	9915.376	9.159	0.0005
ERROR	42218.563	39	1082.527		
DAY	112960.862	4	28240.216	97.800	0.0000
GROUP BY DAY	3775.421	8	471.928	1.634	0.1191
ERROR	45045.759	156	288.755		

APPENDIX A.5

ANOVA summary tables for the elevated-plus maze
(total entries and open/closed ratios respectively).

TOTAL ARM ENTRIES:

	SS	df	MS	F-test	p
GROUP	9.933	3	3.311	1.676	0.1827
ERROR	110.667	56	1.976		

RATIO OF OPEN/CLOSED ARM ENTRIES:

	SS	df	MS	F-test	p
GROUP	0.043	3	0.014	0.549	0.651
ERROR	1.475	56	0.026		

APPENDIX A.6

ANOVA summary tables for choline acetyltransferase
activity in each brain region

FRONTAL CORTEX:

	SS	df	MS	F-test	p
GROUP	20725.717	3	6908.57	2.868	0.058
ERROR	57809.181	24	2408.72		

HIPPOCAMPUS:

	SS	df	MS	F-test	p
GROUP	15910.082	3	5303.36	0.298	0.827
ERROR	409805.705	23	17817.64		

CAUDATE NUCLEUS:

	SS	df	MS	F-test	p
GROUP	167992.406	3	55997.47	1.927	0.1510
ERROR	726312.561	25	29052.50		

APPENDIX A.7

ANOVA summary tables for thickness of
dorsal cortex at sections CC, AC, and PC respectively.

SECTION CC:

	SS	df	MS	F-test	p
GROUP	1.837	3	0.612	11.769	0.0001
ERROR	1.248	24	0.052		

SECTION AC:

	SS	df	MS	F-test	p
GROUP	0.605	3	0.202	0.219	0.8821
ERROR	22.063	24	0.919		

SECTION PC:

	SS	df	MS	F-test	p
GROUP	0.157	3	0.052	1.852	0.1647
ERROR	0.679	24	0.028		

APPENDIX A.8

ANOVA summary tables for thickness of
lateral cortex for sections CC, AC, and PC respectively.

SECTION CC:

	SS	df	MS	F-test	p
GROUP	0.353	3	0.118	3.270	0.0386
ERROR	0.863	24	0.036		

SECTION AC:

	SS	df	MS	F-test	p
GROUP	0.046	3	0.015	1.257	0.3115
ERROR	0.295	24	0.012		

SECTION PC:

	SS	df	MS	F-test	p
GROUP	0.154	3	0.051	1.077	0.3777
ERROR	1.143	24	0.048		

APPENDIX A.9

ANOVA summary tables for thickness of
ventral cortex at sections CC, AC, and PC respectively.

SECTION CC:

	SS	df	MS	F-test	p
GROUP	0.196	3	0.065	1.855	0.1642
ERROR	0.843	24	0.035		

SECTION AC:

	SS	df	MS	F-test	p
GROUP	0.128	3	0.043	0.101	0.9586
ERROR	10.142	24	0.423		

SECTION PC:

	SS	df	MS	F-test	p
GROUP	0.339	3	0.113	1.260	0.3161
ERROR	1.705	19	0.090		

APPENDIX A.10

ANOVA summary tables for area of the
corpus callosum at sections CC and PC respectively.

SECTION CC:

	SS	df	MS	F-test	p
GROUP	18.620	3	6.207	17.829	0.0000
ERROR	8.355	24	0.348		

SECTION AC:

	SS	df	MS	F-test	p
GROUP	0.605	3	0.202	0.219	0.8821
ERROR	22.063	24	0.919		

APPENDIX A.11

ANOVA summary tables for area of the lateral ventricles at sections CC and AC respectively.

SECTION CC:

	SS	df	MS	F-test	p
GROUP	514.082	3	171.361	6.691	0.0019
ERROR	614.662	24	25.611		

SECTION AC:

	SS	df	MS	F-test	p
GROUP	310.634	3	103.545	2.087	0.1285
ERROR	1190.746	24	49.614		

APPENDIX A.12

ANOVA summary tables
for area measurment of caudate nucleus
and thickness of entorhinal cortex respectively.

CAUDATE NUCLEUS:

	SS	df	MS	F-test	p
GROUP	17.037	3	5.679	1.618	0.2115
ERROR	84.263	24	3.511		

ENTORHINAL CORTEX:

	SS	df	MS	F-test	p
GROUP	0.360	3	0.120	1.348	0.2845
ERROR	1.955	22	0.089		

APPENDIX A.13

ANOVA summary tables for
area measurements of dentate gyrus and
dorsal horn of hippocampus respectively.

DENTATE GYRUS:

	SS	df	MS	F-test	p
GROUP	1.614	3	0.538	6.652	0.0021
ERROR	1.861	23	0.081		

DORSAL HIPPOCAMPUS:

	SS	df	MS	F-test	p
GROUP	3.043	3	1.014	1.928	0.1546
ERROR	11.577	22	0.526		

APPENDIX A.14

ANOVA summary tables for
area measurements taken from the combined CA1 & CA2
and from the CA3 cell fields of the hippocampus.

CA1 AND CA2:

	SS	df	MS	F-test	p
GROUP	10.241	3	0.043	0.101	0.0760
ERROR	28.619	24	1.301		

CA3:

	SS	df	MS	F-test	p
GROUP	1.006	3	0.335	2.624	0.3735
ERROR	6.761	22	0.307		

END

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