INTERACTIONS OF ACETYLCHOLINE AND PHOSPHOLIPID METABOLISM IN RAT BRAIN

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Dedicated to Madeline.

INTERACTIONS OF ACETYLCHOLINE AND PHOSPHOLIPID METABOLISM IN RAT BRAIN

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ABSTRACT

The theme of this thesis has centered on the interactions between acetylcholine and phospholipid metabolism, specifically, on changes in phospholipid metabolism and mass of cortical terminal fields after removal of cholinergic innervation. Phospholipid and phospholipid metabolite levels in frontoparietal cortex are decreased following excitotoxic lesion of the basal forebrain cholinergic nucleus basalis magnocellularis (NBM). Histological verification of NBM lesions was shown by choline acetyltransferase immunocytochemistry and Nissl staining. NBM lesions were biochemically verified by the measurement of choline acetyltransferase activity and evoked acetylcholine release in cortical slices. Significant decreases in lesion-side choline acetyltransferase activity and evoked acetylcholine release was observed at 7 days following the lesion.

Cortical phospholipid levels, choline acetyltransferase activity, and evoked acetylcholine release recovered to control values after the lesion-induced deficit. The time course of recovery differed for these measures. Cortical phospholipid levels and evoked acetylcholine release returned to control values at 15 days following the lesion, while choline acetyltransferase activity returned to control levels at 3 months following the lesion.

Cholinergic deafferentation of the cortex by surgical sectioning of cortical fiber projections also resulted in decreased cortical phospholipids, while excitotoxic lesions of non-cholinergic projections to cortex did not change cortical phospholipid levels. Cholinergic deafferentation of the hippocampus did not alter phospholipid levels in this structure, indicating that the effects of removing cholinergic input are tissue specific.

Radiolabelling and enzyme assay experiments with cortical tissues indicate that choline phosphorylation and phospholipid biosynthesis is decreased following

the biosynthetic chain. Cortical muscarinic receptor-mediated inositol lipid hydrolysis increases in response to agonist in the cortex following NBM lesions. This may reflect compensatory increases in phospholipid turnover in cortex in response to lowered endogenous input following NBM lesions.

The decrease and recovery of cortical phospholipids is comparable with the response of other metabolic indices following deafferentation. Further study of the mechanisms underlying recovery processes following experimental deafferentation may be useful for the development of strategies aimed at reversing the effects of naturally occurring cholinergic hypofunction.

Thesis Supervisor: Dr. Richard J. Wurtman, Professor of Neuropharmacology

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Introduction

My hypothesis is that the partial removal of the cholinergic input into the frontal cortex leads to alterations in phospholipid metabolism in the terminal cortical fields by decreased phospholipid synthesis and increased phospholipid turnover. Such alterations reflect changes in cortical tissue (post-synaptic, relative to the NBM) and are not simply due to degeneration of the axonal arbors of the NBM. Choline, a membrane synthetic precursor and metabolite may contribute to maintaining compensatory acetylcholine release from remaining cholinergic terminals.

In order to test the hypothesis that neurotransmitter-specific inputs can influence terminal field phospholipid metabolism, I have utilized a chemical lesion method which destroys the cell bodies of the cholinergic basal forebrain. Subsequently, cortical tissues are analyzed for indices of acetylcholine and phospholipid metabolism. I have examined tissue levels of phospholipids and water-soluble phospholipid metabolites in cortex following a unilateral lesion of the nucleus basalis. Unilateral nucleus basalis lesions were verified by the measurement of choline acetyltransferase activity, comparing lesioned side frontal cortex with control side frontal cortex. The method for generating NBM lesions was effective: significant decreases occurred in ChAT activity in frontal cortex on the lesion side relative to control side. I noted significant decreases in frontocortical phospholipid and water-soluble phospholipid metabolites levels on the lesion side relative to the control side. These changes in phospholipids and their metabolites are specific to

the lesion of NBM, as shown by sham lesions. This eliminates the possibility of transneuronal cortical degeneration from the cannula placement. Increases in inositol phospholipid degradation in NBM lesion-side cortex were observed by measuring the activities of the receptor-coupled phospholipid degradative enzyme phospholipase C (PL-C) on lesioned vs. control sides. These results show that there is an increase in carbachol stimulated phospholipase C activity measured by inositol phosphate accumulation in lesion side cortex, indicating that muscarinic receptor-effector coupling increases following lesion of the NBM.

To determine whether lesion of the NBM alters phospholipid synthesis in frontal cortex, I have compared the activity of choline kinase and acetylcholine biosynthetic enzymes, in NBM lesion- and control-side cortical tissue. I have measured the levels of endogenous and radiolabelled biosynthetic intermediates and found decreased choline kinase activity and decreases in phosphatidylcholine formation.

I have compared the changes in phospholipid metabolism to other experimental systems of cholinergic hypofunction and pharmacologic treatments with choline and aminopyridines which alter phospholipid levels. These compounds have been shown to protect membrane phosphatides in other experimental systems under conditions of sustained acetylcholine release (Ulus et al, 1989, Buyukuysal et al, 1991). Choline is the biochemical precursor for both acetylcholine and phosphatidylcholine, whereas aminopyridines are potassium channel blockers. The potentiation of neurotransmitter release by aminopyridines appears to be mediated

by pre-synaptic potentiation of action potentials by the blockage of delayed potassium conductance, causing a subsequent increase in terminal Ca^{++} levels (Molgo et al., 1977). The mechanism of aminopyridine protection of phospholipid under depolarizing conditions has not been determined. It has been suggested that aminopyridines may increase the coupling between acetylcholine release and choline uptake (Buyukuysal and Wurtman, 1990). The specificity of action of aminopyridines on acetylcholine release may be improved by co-administration with choline.

My aims are to further elucidate brain acetylcholine and phospholipid metabolic pathway interactions. The effects of decreased cholinergic input on terminal field phospholipid metabolism were examined. This topic is of interest as changes in terminal field phospholipid metabolism could reflect compensatory events for maintaining acetylcholine release. I have compared the effects of manipulating neurotransmitter-specific systems with natural phenomena such as aging and neuropathological conditions, where neurotransmitter systems are compromised. Pharmacological treatments which augment neurotransmitter release and protect membrane phospholipids may be useful for treating cholinergic hypofunction.

Experiment Outline

The hypotheses tested and the experiments are as follows:

Hypothesis 1) Frontal cortex levels of phospholipids and water-soluble phospholipid metabolites are altered following lesions of the NBM.

a) Examine phospholipid levels in frontal cortex following lesion of nucleus basalis.

b) Examine water soluble phospholipid metabolites in frontal cortex following lesion of the nucleus basalis.

Hypothesis 2) Frontocortical phospholipid depletions following cholinergic deafferentation reflect alterations in the cortex, not terminal degeneration of axons.

c) examine the time course of phospholipid and ChAT depletion following cholinergic lesions.

d) determine whether the magnitude of phospholipid and ChAT depletion is linearly related.

e) examine the effects of cholinergic deafferentation in other brain tissues (i.e. the hippocampus)..

f) examine the effects removing non-cholinergic large axonal projections.

Hypothesis 3) Phospholipid biosynthetic enzyme activities decrease in frontal cortex following lesions of the NBM.

g) Examine the following phospholipid and transmitter enzyme synthetic activities

in frontal cortex following lesion of the NBM.

i) choline acetyltransferase

ii) choline kinase

iii) isotopic labelling with choline

Hypothesis 4) The activities of receptor-coupled phospholipid degradative enzymes increase following lesions of the NBM.

h) Examine muscarinic receptor activity as indexed by inositol phospholipid hydrolysis in frontal cortex following lesion of the NBM (inositol lipid PL-C activity).

i) Utilize isotopic phospholipid labelling in frontal cortex to determine whether nucleus basalis lesion results in altered phospholipid biosynthesis and/or turnover.

Hypothesis 5) The changes observed in frontocortical phospholipids following nBM lesions do not reflect local injury to cortex caused by cannula placement.

j) Examine the effects of sham lesion of the NBM on phospholipid levels and phospholipid metabolites in frontal cortex as a control for cannula placement.

Hypothesis 6) Acetylcholine release from cholinergic terminals in cortex decrease following NBM lesions, but acetylcholine release is not directly related to ChAT levels.

k) Examine endogenous acetylcholine release per unit terminal (as assayed by

ChAT) for compensatory increases following NBM lesions at a range of time points following the lesion and plot cortical acetylcholine release vs. ChAT.

l) Examine the time course and recovery of acetylcholine release and ChAT activity following NBM lesions.

Hypothesis 7) Lesion-induced depletion of frontocortical phospholipids are specific to cholinergic deafferention.

m) Examine the effects of non-cholinergic thalamic lesion (putative glutaminergic) on frontocortical phospholipid levels.

Choline Metabolism

All mammalian cells utilize choline for the biosynthesis of phosphatidylcholine. Cells which release the neurotransmitter acetylcholine have an additional requirement for choline in the biosynthesis of this compound via acetylation. There is evidence for interactions between these two pathways which both utilize choline (Wurtman, 1992).

Choline Sources and Transport

There are three sources of choline in the brain. The metabolism of brain choline is shown in figure 1.

Figure 1

Biochemical Pathways of Choline Metabolism

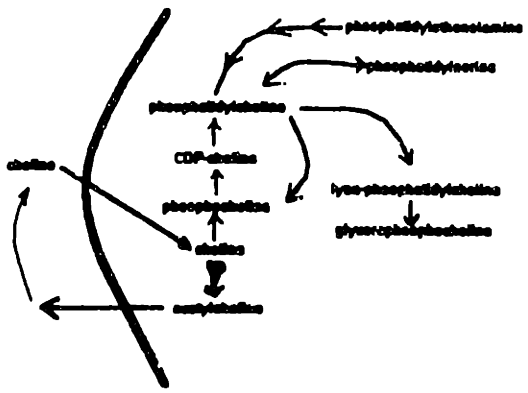


Fig. 1) Extracellular choline enters cells by low- or high-affinity carriers located on the cell surface. Phosphorylation of choline by choline kinase shunts choline into phosphatidylcholine biosynthesis, while acetylation by choline acetyltransferase shunts choline into neurotransmitter biosynthesis.

Choline of dietary origin is present in plasma (10 uM) and crosses the blood-brain barrier from circulation through an amine-specific facilitated carrier located on capillary endothelial cells (Pardridge et al., 1979). This carrier is not saturated under normal physiological conditions (K_m = 0.31 mM, V_{max} = 12.6 nM/min/g brain tissue), is bi-directional, and will not move choline against a concentration gradient. The transport of choline into the brain varies with age, choline enters the brain at a greater rate in neonatal animals (Braun et al., 1980). This increase in transport is due to selective alterations of the barrier as arginine, adenine also increase their rate of passage, in contrast to glucose transport, which shows no difference as compared to adults. These results show that the blood-brain barrier

is not "leaky" in immature animals. The increases in choline passage into the brain coincides with rapid growth of the brain and may constitute an adaptive response. Choline transport is reduced in aged animals (Mooradian, 1988), which may contribute to cholinergic hypofunction that occurs in aging. This reduction in choline transport may occur in response to decreased choline demand in aged brain and may also account for the difference between young and aged subjects in the efficacy of choline administration for improving memory performance.

The brain choline arteriovenous difference is negative in rats and humans (-2uM), indicating that there is net efflux of choline from the brain (Choi et al., 1975, Spanner et al, 1975). This indicates that there is a net production of choline in the brain. In contrast, there is no arteriovenous difference in choline containing phospholipids (Spanner et al., 1976). The arteriovenous difference is difficult to explain as the concentration of choline in cerebrospinal fluid is app. 2.5 uM. Choline carriers in choroid plexus will transport choline against a concentration gradient in the blood-CSF barrier (Ehrlich and Wright, 1982). The physiological role of the arteriovenous difference may be related to the regulation of organic bases in the brain. The negative arteriovenous choline difference can be reversed by small increases in plasma choline, implying that under these conditions, a net uptake of choline into brain occurs (Klein et al., 1990) This provides evidence for the dynamic regulation of brain choline levels by nutritional choline. Endogenous amines such as carnitine and spermidine probably do not inhibit choline flux a across the blood-brain barrier due to their low concentrations in plasma (Pardridge

et al., 1979).

The BBB choline carrier is blocked by diverse agents including deanol, hemicholinium-3, and lithium (Pardridge et al, 1979). These drugs block choline influx in addition to blocking choline efflux. Choline containing compound did not differ in human patients receiving lithium treatment relative to control patients as measured by in vivo proton magnetic resonance spectroscopy (Stoll et al., 1992). This conclusion should be viewed with caution as this study did not distinguish individual choline containing compounds and compared bipolar depressive patients with healthy control subjects. The efficacy of drugs which alter choline flux across the blood-brain barrier remains undetermined.

Choline containing phospholipids and lyso-phospholipids in plasma have been proposed as a potential source of brain choline (Ansell and Spanner, 1975; Jope and Jenden, 1979), however this is unlikely as these compounds are tightly bound to albumin in circulation. Although albumin may gain entry into the brain via cell-mediated endocytosis, such transport occurs at a much smaller rate as compared to facilitated choline transport. Additionally, the permeability of the blood-brain barrier to free phosphatidylcholine and lyso-phosphatidylcholine is very low, so it appears that choline lipids are not a source of brain choline (Pardridge et al., 1979).

The hydrolysis of choline containing molecules is the other major source of brain choline. Acetylcholine is hydrolyzed by acetylcholinesterase, an enzyme linked to the basal membrane in extracellular space of the synaptic junction

(Taylor, 1991), yielding choline and acetate. The choline from this reaction may then be reclaimed by neurons by carrier-mediated mechanisms (see below). The hydrolysis of phosphatidylcholine by receptor-mediated phospholipase D yields choline and phosphatidic acid (Taki and Kanfer, 1979; Pelech and Vance, 1989). This choline may be utilized for acetylcholine synthesis (Blusztajn and Wurtman, 1983).

Choline is also indirectly synthesized in the brain via the step-wise methylation of phosphatidylethanolamine to phosphatidylcholine, utilizing S-adenosylmethionine (SAM). Phosphatidylcholine synthesized by this pathway is then subject hydrolysis which can liberate choline by phospholipase D activity (Blusztajn and Wurtman, 1983). Kinetic estimates of the methyltransferase pathway may account for up to 10% of the efflux of brain choline.

Cellular Transport of Choline

Brain cells take up choline by two kinetically distinct carrier-mediated transport systems (Yamamura and Snyder, 1973). A low affinity carrier (k_m = 30-300 uM) is found in all cells, while a high affinity carrier (k_m = 0.1-10 uM) which requires sodium binding and is restricted in distribution in adult tissues to cholinergic nerve endings (Kuhar and Murrin, 1978) and photoreceptor cells of the retina (Pu and Masland, 1984). The high affinity transport of choline is measured in the synaptosomal preparation in order to distinguish this carrier from the more abundant low affinity carrier found on neuronal cell bodies and glial cells. High

affinity transport has also been described in several cell lines (Blusztajn and Wurtman, 1983).

Conversion of the high-affinity transporter to the low-affinity transport by a calcium dependent, ATP-induced mechanism has been reported (Chatterjee and Bhatnagar, 1990). Preincubation under conditions of decreased ATP increased the number of high-affinity sites, while treatment with AMP, ADP or other nucleotide triphosphates had no effect on the affinity state of the choline uptake sites. The number of total choline carriers was reduced in the presence of GTP. These authors suggest that the two choline carriers may be the same molecule which differs in affinity state by a post-translational modification(s) or association with other cellular components. This is of interest as ATP is co-released with acetylcholine (Richardson and Brown, 1987) and may contribute to regulation of choline uptake in an activity dependent manner. Arachidonic acid, a product of neurotransmitter receptor-mediated phospholipid hydrolysis, activates the sodium dependent high affinity uptake (SDHACU) system (Saltarelli et al., 1990).

In vivo treatment with nicotine and haloperidol increases high-affinity choline transport (subsequently measured in synaptosomes), while atropine and apomorphine have no effect on choline uptake (Happe and Murrin, 1992). These drugs appear to act indirectly as in vitro treatment of synaptosomes with these agents do not effect high-affinity choline uptake.

High-affinity choline uptake is increased in synaptosomes following both in vivo and in vitro electrical stimulation (Antonelli et al., 1981; Happe and Murrin,

1992) and in vitro potassium stimulation (Murrin and Kuhar, 1976) indicating that depolarization is a key regulatory step. The changes in high-affinity choline uptake described above following in vivo drug treatments and stimulation all appear to occur by alterations in the number of active sites at the cell surface as measured by radiolabelled hemicholinium-3, which selectively binds ot the carrier.

High-affinity choline uptake in synaptosomes prepared from striatum, hippocampus and cortex is increased following chronic intracerebral nerve growth factor treatment (Williams and Rylett, 1990).

The primary structure of the mammalian high affinity choline transporter has recently been characterized by molecular cloning (Mayser et al, 1992). It is a member of a family of sodium dependent neurotransmitter transporters. Transport proteins of this family share a common structure of 12 putative membrane spanning regions and 30-65% homology of primary amino sequence. This homology was used to isolate the choline transporter by polymerase chain reaction with degenerate oligonucleotide primers based on the sequence of the GABA transporter.

It is interesting to note that high affinity choline uptake is widely distributed throughout the brain during developmental periods of rapid growth (Kotas and Prince, 1987).

High-affinity choline transport can be selectively blocked by hemicholinium-3 (HC-3, IC50 = 2-80 nmol). This compound can also be used to map the relative distribution of high-affinity choline transporters in the brain at a higher level of

detail than biochemical assays (Happe and Murrin, 1993), showing that the transporter is located presynaptically and that the density of high-affinity choline uptake correlates well with other measures of cholinergic activity such as choline acetyltransferase activity and acetylcholine release. The relative distributions of HC-3 is striatum>>hippocampus>cerebral cortex>>cerebellum. HC-3 binding is approximately 35 times greater in striatum as compared to cerebral cortex. HC-3 binding to the high-affinity choline transporter may be blocked by choline (IC50=40 uM) and other choline derivatives.

Phospholipid biosynthesis

Phosphatidylcholine biosynthesis occurs by three pathways (Fig.1). The majority of brain choline is utilized for phosphatidylcholine biosynthesis via the Kennedy pathway (de novo synthesis). The majority of phospholipid synthesis occurs in the endoplasmic reticulum of the cell bodies, although nerve terminals have the enzymatic capacity to synthesize phospholipids (Abdel-Latif et al., 1973). Newly synthesized phospholipid is transported distally to dendrites and axons. Phosphatidylcholine can also be synthesized by the stepwise methylation of phosphatidylethanolamine and by the substitution of ethanolamine or serine polar head groups with choline, catalyzed by phospholipid base-exchange enzymes. The enzymes for methylation and base-exchange pathways have different distribution than enzymes for de novo synthesis, which have the highest activities in the cell body. The methylation and base-exchange pathways may contribute to

phospholipid remodelling in terminals. Phospholipid modelling reactions also alter the length and the degree of saturation of the fatty acid chains (Tacconi and Wurtman, 1985). The ratio of phospholipids and other lipid components in the membrane appears to be strictly regulated by the cell in order to maintain constant membrane fluidity, which may account for the multiplicity of pathways for membrane remodelling.

The absolute mass of cellular phospholipid relative to protein or DNA can be altered by conditions of cellular growth and regeneration, precursor availability and cellular activity. Electrically stimulated brain slices can sustain their release of acetylcholine at the expense of their membrane phospholipid contents (Maire and Wurtman, 1985; Ulus et al; 1989; Buyukuysal et al. 1991; Farber et al., 1993). This phenomenon does not occur by other means of cellular depolarization. Potassium depolarization does not alter phospholipid levels. Aminopyridines, which are depolarizing agents, actually spare electrical evoked membrane depletion (Buyukuysal and Wurtman, 1990; Buyukuysal et al., 1991).

The concentration of free choline in brain as compared to other choline containing compounds is low. Most brain choline is incorporated into phosphatidylcholine by way of de novo synthesis. The ratio of phosphatidylcholine to choline in brain is greater than 1000:1 (Jope and Jenden, 1975; Ansell and Spanner, 1977).

Choline enters the de novo synthesis pathway by phosphorylation by choline kinase (CK, E.C. 2.7.1.32). ATP serves as the phosphate donor and magnesium

is required for the reaction. Reports of Km values for choline range considerably (0.7-310 uM) depending on tissue preparation, subcellular localization, and assay conditions (Spanner and Ansell, 1979; Reinhardt and Wecker, 1983). This enzyme has been suggested as a key regulatory step in phospholipid synthesis (Bishop and Bell, 1988). Choline kinase activity is localized in the cytoplasm (Burt and Brody, 1975; Spanner and Ansell, 1979), although a membrane bound fraction has been reported (Reinhardt and Wecker, 1983). The cellular distribution of choline kinase in brain varies, where activity is lower in cytosolic fractions of lysed synaptosomal preparations as compared to whole brain cytosol. This indicates that lower activity is present in nerve terminals than is present in cell bodies (Spanner and Ansell, 1979). The gene for human choline kinase has recently been cloned, yielding a deduced sequence of 456 amino acids and a molecular mass of 52,065 (Hosaka et al., 1992).

The regulation of choline kinase is influenced by several factors. ADP and adenosine inhibit choline kinase (Burt and Brody, 1975; Wecker and Reinhardt, 1988) in rat brain homogenates. The activity of choline kinase in excised rat superior cervical ganglia is decreased following pretreatment with low extracellular choline and potassium depolarization (Ando et al., 1987). Blockage of choline transport with hemicholinium-3 also decreased choline kinase activity (Spanner and Ansell, 1979, Ando et al., 1987). Postganglionic axotomy has been shown to result in transient increases in ganglionic choline kinase activity, which presumably contributes to axon regeneration (Gilad and Gilad, 1982, Ando et al,

1987).

Choline administration has been shown to modulate levels of phosphocholine in rat striatum (Millington and Wurtman, 1982), indicating that precursor availability can influence synthesis in both neurotransmitter and phospholipid pathways. Treatments in cell culture associated with growth and transformation increase choline kinase activity. Mitogenic growth factors increase choline kinase activity in 3T3 fibroblasts in culture (Warden and Friedkin, 1985). Increased choline kinase activity and phospholipid turnover is also seen in 3T3 and C3H10T fibroblast cells transfected with the Harvey-ras oncogene (Macara, 1988; Teegarden et al., 1990). Phosphocholine is also generated by the breakdown of glycerophosphocholine by glycerophosphocholine cholinephosphodiesterase (E.C. 3.1.4.38).

Phosphocholine is transferred to a cytidine carrier by cytidine triphosphate: phosphocholine cytidyltransferase (CT, E.C. 2.7.7.15) (Kennedy and Weiss, 1956), forming CDP-choline. Most experimental evidence indicates that the CT reaction is the rate limiting step for this pathway in most tissues (Vance, 1991). There is additional evidence that the CK reaction may be an activity dependent regulatory step in cholinergic tissues (Ando et al, 1987, Bell and Bishop, 1988). CT activity is modulated by conditions of enhanced cell growth and repair. Colony-stimulating factor 1 increases CT mRNA levels and activity in murine macrophage cells (Tessner et al., 1991). CT activity increases in response to phospholipase C treatment (Sleight and Kent, 1983; Slack et al., 1991; Watkins and Kent, 1991)

and to direct treatments of fatty acids and diacylglycerol (Cornell and Vance, 1987; Kolesnick and Hemer, 1990). This step appears to be subject to end product inhibition by phosphatidylcholine (Jamil et al, 1990) and is also inhibited by sphingosine (Sohal and Cornell, 1990) and cAMP analogues (Pelech et al., 1981). This negative regulation may occur by phosphorylation of the CT enzyme by cAMP dependent kinase (Sanghera and Vance, 1989) and it is noteworthy that in vitro with drugs which promote dephosphorylation increase the activity of this enzyme (Watkins and Kent, 1991).

CDP-choline:1,2 diacylglycerol choline phosphotransferase catalyzes the final reaction, forming phosphatidylcholine (E.C. 2.7.8.2). The choline phosphotransferase enzyme does not appear to be saturated under physiological conditions and the in vivo formation of phosphatidylcholine appears to be limited by the availability of the substrates CDP-choline and diacylglycerol (Lim et al., 1985). Phosphatidylcholine may also be synthesized by step-wise methylation of phosphatidylethanolamine catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT, enzymes form 1 and 2). This pathway accounts for the indirect synthesis of brain choline (Blusztajn et al., 1979). There are least two methyltransferase enzymes in this pathway which differ in kinetic properties and location. The first methyltransferase is located on the cytoplasmic face of the synaptosomal membrane, whereas the second enzyme appears to be on the extracellular side of the membrane. Nerve terminals have the highest PEMT activity based on subcellular fractionation (Blusztajn and Wurtman, 1983).

Serine, choline, and ethanolamine bases may be directly incorporated into phospholipid by calcium-dependent base exchange (Goracci et al., 1973). Base-exchange activity is enriched in neurons with the highest activity in microsomal fractions and is present in nerve terminals (Holbrook and Wurtman, 1987). The redundancy of pathways for phospholipid biosynthesis is striking and other membrane remodelling reactions such as reacylation and alterations of the fatty acid chains take place (Bluzstajn and Wurtman, 1983). The physiological role of the methyltransferase and base-exchange pathways is poorly understood. They may regulate the ratio of phospholipid species in the membrane bilayer, as small alterations of this ratio can lead to significant changes in membrane fluidity, which can in turn influence the function of ion channels and other integral membrane proteins.

Phospholipid Catabolism

The breakdown of brain phospholipids is stimulated by receptor-mediated stimulation of phospholipases which cleave phospholipid molecules at specific bonds. These receptors bind to neurotransmitters, hormones, and growth factors and mediate signal transduction and membrane remodelling (Abdel-Latif et al., 1974; Billah and Anthes, 1990). The time course of activation of phospholipases ranges from seconds to hours. There is substantial crosstalk between these systems (Joseph et al., 1990; Liscovitch, 1992). Receptor binding can activate multiple phospholipase activities (Conklin et al., 1988; Sandmann et al., 1991).

The receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by phospholipase C (PL-C) is the best characterized system of lipid catabolism (Asaoka et al., 1992). PIP_2 is a quantitatively minor phospholipid. This reaction yields the second messengers diacylglycerol and inositol triphosphate, which mobilize activate protein kinase C and mobilize intracellular and extracellular calcium. Protein kinases phosphorylate proteins which may modulate cellular functions including ion channel properties, secretion, and cellular differentiation. There are multiple subtypes of guanine-nucleotide protein (G protein) coupled receptors (Gilman, 1987; Bonner et al, 1987). Neurotransmitter agonists which stimulate receptor-mediated PL-C activity (Berridge et al., 1982) also stimulate phospholipase D and A_2 activity (Sandmann and Wurtman, 1991; Conklin et al., 1988). The receptor-mediated hydrolysis of inositol lipids is useful as a general model of lipid breakdown because of the stability of the reaction product in the presence of lithium.

Cortical slices increase radiolabelled phosphate incorporation into phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol following neurotransmitter stimulation (Abdel-Latif et al., 1974). This reflects compensatory remodelling in response to phospholipid hydrolysis as de novo synthesis as indexed by labelled glycerol and glucose incorporation does not increase. Receptors coupled to phospholipases and subsequent phospholipid catabolism are subject to homologous regulation by agonists (Lee and Wolfe, 1989) and heterologous regulation, where transmitter-specific deafferentation of one system

can affect the response of another neurotransmitter system (Morrow et al., 1983). The regulation of receptor function appears to occur by the degree of feedback phosphorylation of receptors by activated protein kinases, leading to alterations in receptor sensitivity (El-Fakahany et al., 1988).

Abundant structural phospholipids such as phosphatidylcholine are also hydrolyzed by phospholipases, these reactions occur over a greater duration than the hydrolysis of PIP_2, yielding a sustained production of diacylglycerol (Liscovitch, 1992). Phosphatidylcholine is also cleaved by phospholipase D, yielding phosphatidic acid and choline. Phosphatidic acid may converted to diacylglycerol by phosphatic acid phosphohydrolase (Billah and Anthes, 1990).

Acetylcholine Biosynthesis and Release

The availability of a neurotransmitter's precursor has been shown to influence its biosynthesis and release. This has been demonstrated for several neurotransmitters including acetylcholine (Haubrich et al., 1975, Cohen and Wurtman, 1976, Ulus et al., 1989). Choline acetyltransferase (ChAT, E.C. 2.3.1.6) catalyzes the synthesis of acetylcholine from choline and acetyl-CoA. Both precursors are found in sub-saturating concentrations in the brain, the K_m of this enzyme is 0.4-1.0 mM for choline and 7-46 uM for acetyl-CoA (Singh and McGeer, 1979; Tucek, 1985). Increasing the availability of either precursor increases acetylcholine synthesis (Cohen and Wurtman, 1976, Jope, 1979).

Subcellular fractionation reveals that ChAT levels are highest in nerve

terminals, proximal to the site of acetylcholine release (Tucek, 1967). Most ChAT is located in the cytoplasm, acetylcholine synthesized in the cytoplasm is concentrated in vesicles by an energy-dependent carrier (Koenigsberger and Parsons, 1980).

The release of acetylcholine occurs spontaneously by non-quantal (Dunant et al., 1980) and calcium-independent quantal mechanisms (Dolezal et al., 1988). Activity-evoked acetylcholine release is calcium dependent and may occur over long periods of electrical stimulation in the absence of exogenous choline without significant changes in tissue acetylcholine levels in brain slices prepared from young and aged rats (Collier et al., 1972; Ulus et al, 1989; Buyukuysal et al., 1991; Holmes et al. 1990). Prolonged release of acetylcholine in vitro occurs at the expense of membrane phospholipids. This depletion is prevented by the administration of choline (Maire and Wurtman, 1985; Ulus et al., 1989; Buyukuysal et al., 1991; Farber et al., 1993). Choline administration enhances the release of acetylcholine in vitro under depolarizing conditions (Trommer et al., 1982; Ulus et al., 1989) and in vivo (Koshimura et al., 1990; Farber et al., 1993). Acetylcholine release varies in vivo; it peaks during awake, behaving states and correlates with activity and behavioral activation (Mizuno et al., 1991; Nillson et al., 1990).

Acetylcholine release is decreased in aged rat brain in vitro (Pedata et al. 1983; Holmes et al., 1990) and in vivo (Wu et al., 1988). The mechanism of this decrease is not known, however it does not appear to be due to decreases in

tissue ChAT or choline uptake (Bartus et al., 1983). Several neurotransmitters modulate the release of brain acetylcholine. Inhibition of acetylcholine release is mediated by muscarinic M2 receptors and serotonergic 5HT-1a receptors located on presynaptic terminals (Hadhazy and Szerb, 1977; Raiteri et al. 1989). Dopamine increases acetylcholine release in cortex, but is inhibitory in the striatum (Day and Fibiger, 1993; Scatton, 1982). Glutamate agonists increase acetylcholine release from striatal and cortical slices (Lehmann and Scatton, 1982). Glutamate-induced acetylcholine release is augmented by the co-administration of choline (Ulus et al., 1992). Aminopyridines increase acetylcholine release from striatal slices prepared from young and aged rats and simultaneously protect against electrical stimulation-induced membrane depletion (Buyukuysal and Wurtman, 1990; Holmes et al., 1990; Buyukuysal et al., 1991). These agents are potassium channel blockers. They may augment acetylcholine release by increasing the depolarizing phase of the action potential, thereby increasing calcium influx in nerve terminals.

Evidence for Interactions between Phospholipid and Acetylcholine Metabolism.

Previous work in this laboratory and others have shown that choline liberated from phosphatidylcholine can be utilized for acetylcholine synthesis (Blusztajn et al.,1983; Ulus et al., 1989; Yavin et al., 1989). Thus, membrane phospholipids in cholinergically innervated tissues may serve as a reservoir of choline. There is also evidence for a reciprocal regulation of the enzymes responsible for the synthesis

of phospholipids and acetylcholine: choline acetyltransferase activity increases under conditions of sustained release of acetylcholine and low concentration of extracellular choline, while choline kinase activity decreases under these conditions (Ando et al., 1987). Choline is preferentially shunted to phospholipid synthesis when extracellular choline concentrations are low in cholinergic sympathetic neurons (Suidan and Tolkovsky, 1993). This occurs under conditions of neuronal outgrowth. Regulation of phospholipid metabolism in these preparations occur at the level of synthesis as opposed to turnover.

It has been shown that muscarinic receptors are coupled to phospholipases, which cause the breakdown of phospholipids (Sandmann et al, 1991, Vance, 1991).

The most striking neurotransmitter deficit in Alzheimer's disease is the loss of cholinergic cells of the basal forebrain and the subsequent loss of cholinergic activity in cerebral cortex. Previous studies in our lab and others have shown that disturbances in membrane phospholipid metabolism occur in pathological conditions characterized by disrupted cholinergic neurotransmission. Phospholipid and phospholipid metabolite levels are abnormal in the brains of Alzheimer's disease (AD) patients (Barany et al., 1985, Blusztajn et al, 1990, Nitsch et al, 1992; Kienzl et al., 1993). Phospholipid levels are decreased by 12-15% in AD brain cortex relative to brains of aged matched controls (Nitsch et al., 1992; Kienzl et al., 1993). Phospholipid metabolite levels are altered in AD brain relative to age matched controls. Choline levels are lower (Nitsch et al., 1992) and

glycerophosphate membrane catabolites are increased (Barany et al., 1985, Miatto et al., 1986, Pettegrew et al, 1988, Nitsch et al, 1992). Lipase and lysophospholipase activities are elevated in AD brain (Farooqui et al., 1990).

Cholinergic deafferentation of cortex also occurs in the neurodegenerative disorder of dominantly inherited olivo-pontocerebellar atrophy (OPCA). Phospholipid metabolism is altered in the post-mortem samples of cerebral cortex from OPCA patients that show ChAT depletions (Nitsch et al., 1993). These individuals are exhibit moderate to severe mental retardation.

Although these observations are consistent with the hypothesis that ascending cholinergic projections may influence terminal field phospholipid metabolism, there is no strong evidence for a causative relationship in AD. This body of evidence suggests that cholinergic projections may regulate terminal region phospholipid metabolism. A greater understanding of such regulatory processes may be useful for modelling compensatory phenomena following transmitter-specific lesions and complex neurodegenerative disorders.

Anatomy of Brain Cholinergic Systems

The cerebral cortex receives major ascending inputs from neurotransmitter specific subcortical nuclei. These inputs are have been termed "modulatory" and have been implicated in processes such as learning and memory, and arousal.

These projections are compromised in the aging process and in neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's

disease (PD). The primary cholinergic projection to the cerebral cortex arises from the basal forebrain NBM. There is extensive morphological and biochemical evidence that the NBM decreases in both cell size and cell number (presumably decreasing terminal arbors) in normal mammalian aging (Buszaki et al., 1988; Altavista et al., 1990). The temporal coding of NBM output to cerebral cortex may also change during aging as shown by decreases in the conduction velocity of this projection (Aston-Jones et al. 1982).

The anatomy of cholinergic innervation of the frontal cortex in rats allows for the partial removal of cholinergic arbors in this structure by lesioning distant cell bodies in the basal forebrain. This approach minimizes local damage to the cortex. The neocortex of the rat has two types of cholinergic input: intrinsic cells and extrinsic projections. Intrinsic ChAT positive cells, which consist of both pyramidal and non-pyramidal cell type are observed in neocortex. These cells also co-localize with vasoactive intestinal peptide immunoreactivity (Nishimura et al, 1988, Houser et al, 1983). The intrinsic ChAT positive terminals comprise between 30-40 % of the cortical cholinergic input (Eckenstein et al, 1988). Extrinsic cholinergic projections (see figure 2) are predominantly from the nucleus basalis (Ch4, Mesulam et al., 1983) and there is a minor projection to medial frontal cortex from the midbrain cholinergic nuclei Ch.5 and Ch.6 which are associated with the reticular activating system (Vincent et al, 1983). These cholinergic midbrain cells also contain substance P (Eckenstein et al., 1988).

Figure 2

Anatomy of Cholinergic Cells and

their Projections in the Rat Brain

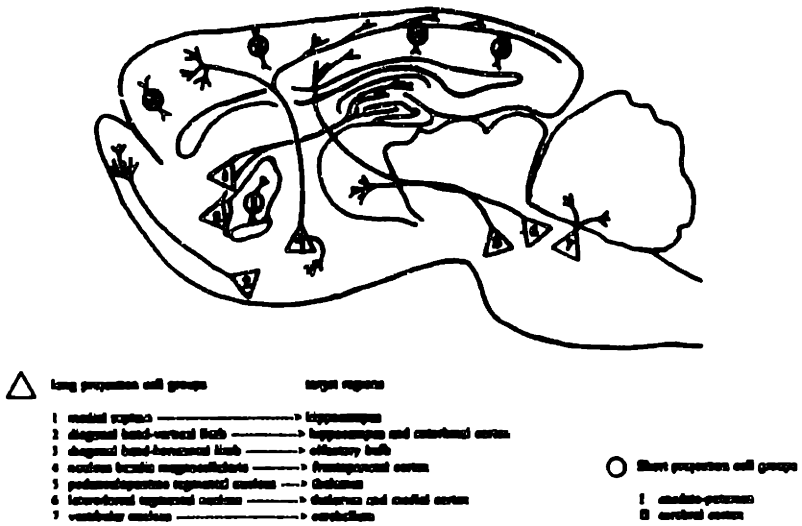


Fig. 2) Cholinergic cells are found as short projection interneurons in the striatum and as long projection cells of the septohippocampal projection. The cholinergic innervation of the rat cortex consists of both intrinsic interneurons and extrinsic projections from the basal forebrain nucleus basalis and the midbrain cholinergic nuclei.

Approximately 90% of the neurons in NBM which project to the cerebral cortex are ChAT positive. Almost all cells in NBM positive for HRP-WGA (injected in neocortex) are also AChase positive (ibid). NBM projections to the thalamus are

equally distributed between cholinergic and GABAergic terminals (Steriade and Buszaki, 1990). The cholinergic projection of the NBM is topographically organized and unilateral in the rat, this observation is supported by both anatomical and biochemical evidence (Eckenstein et al, 1988, Wenk et al, 1980). The laminar distribution of ChAT positive cortical terminals is typically strongest in layers I-III and layer V. There is less of a laminar appearance and greater ChAT immunoreactivity in layer V in frontal cortex, which appears to be the major target area for the NBM projection (Eckenstein et al, 1988). Numerous retrograde labelling studies indicate that individual cells of the NBM have restricted terminal arbors in cortex ranging from 1.0 - 1.5 mm sq. (Bigl et al., 1982; Lamour et al., 1982; Price and Stern, 1983). This restricted arborization is in contrast to other ascending systems such as the noradrenergic locus coeruleus, where single cells branch to innervate multiple targets (i.e. thalamus and cortex) and may terminate over a range of 10 mm sq. in cortex. These studies also show the topographical organization of NBM projections. Anterior, medially located cells of the NBM project to frontal areas of cortex (Lamour et al., 1982). This organization displaces earlier speculations of the "diffuse" projections of the basal forebrain. ChAT positive terminals form symmetrical synapses with small to medium dendrites and terminate on both apical and basal dendrites of pyramidal cells (Houser et al., 1985).

The hippocampus receives four cholinergic inputs. These projections are in discreet fiber bundles in contrast to the more diffuse frontal radial projection of

the nucleus basalis. The medial septal nucleus and nucleus of the diagonal band of Broca send cholinergic projections to the hippocampus via three dorsal pathways which travel through the fimbria, the dorsal fornix and the cingulate cortex (Meibach et al., 1977; Gage et al., 1983). The dorsal pathways account for approximately 85% of the cholinergic input to the hippocampus. The remaining cholinergic input projects from the medial septum and diagonal band of Broca by a ventral pathway via the ansa lenticularis (Gage et al., 1984). The septohippocampal projections contain a large proportion of non-cholinergic cells (GABA, galanin, enkephalin) in contrast to the basalocortical projection (Amaral and Kurz, 1985; Lamour et al., 1987). The cholinergic projections to the hippocampus exhibit a gradient pattern of termination: anterior and dorsal hippocampus are most heavily innervated (Amaral and Kurz, 1985). Individual cell projections have a limited terminal area in the hippocampus, based on combined retrograde tracing and ChAT immunohistochemistry (Amaral and Kurz, 1985). The majority of septohippocampal projections are unilateral. However, approximately 15% of these projections are contralateral and many of the contralateral cells have bilateral terminations (Amaral and Kurz, 1985; Peterson, 1988). Ultrastructural studies indicate that hippocampal pyramidal cells receive the greatest number of cholinergic synapses, which appear to be axodendritic (Kuhar, 1975).

The striatum has the densest cholinergic innervation in the forebrain as indexed by tissue levels of acetylcholine, ChAT activity, muscarinic receptor levels, high-affinity choline uptake, and acetylcholinesterase levels (Graybiel and Ragsdale,

1983). By comparison, high-affinity choline uptake sites are nearly forty times greater in striatum than cortex (Sandberg and Coyle, 1983). Cholinergic cells in the striatum are intrinsic medium sized cells comprising 1 % or less of the total cell population of the striatum (Wainer et al., 1984). It does not appear that the striatum receives a significant cholinergic input from the long projection cell groups of the basal forebrain or other brain regions (Wainer et al., 1984; see Graybiel and Ragsdale, 1983 for thorough review). The interneuronal organization of cholinergic cells in the striatum is not amenable to lesion techniques. The neurotoxin AF64A which has been proposed to be specific to cholinergic cells is also toxic to non-cholinergic cells (Fisher et. al. 1982; Levy et al., 1984).

The cholinergic cells of the striatum are thought to receive excitatory inputs from the cortex (Wood et al., 1979; Ulus et al., 1992). The transmitter of this input is probably glutaminergic/aspartergic based on lesion studies and antagonist and agonist actions in the striatum (Druce et al., 1982; Spencer, 1976; Ulus et al, 1992). The distribution of acetylcholinesterase in the striatum is not uniform; regions poor in acetylcholinesterases are referred to as striosomes, and correspond to the borders and distribution of glutamic acid decarboxylase and certain neuropeptides (Graybeil and Ragsdale, 1983).

Cholinergic pharmacology

There are pharmacological agents used in the study of cholinergic systems. These drugs are categorized by their effects on receptor subtypes,

acetylcholinesterase activity and choline uptake (see above). Receptor classes are divided as nicotinic and muscarinic based on agonist response. Nicotinic receptors are pentameric ligand-gated ion channels, which allow cation flux when in the open state. These receptors are noted for their fast activation and cause depolarization when open. Muscarinic receptors are linked to heterotrimeric guanine nucleotide binding proteins (G proteins), their activation modulates the activity of enzymes which regulate cellular second messenger production (Bonner et al., 1987). The duration of effects following muscarinic receptor activation ranges from hundreds of milliseconds to minutes, due to their coupling to enzymatic cascades (Liscovitch, 1992). Cholinergic transmission in the cortex, hippocampus, and striatum occurs by activation of both nicotinic and muscarinic receptors. It appears that nicotinic receptors are more restricted in their distribution in these tissues as compared to muscarinic receptors (Bonner et al., 1987; Wada et al., 1988). Nicotinic activation occurs preferentially in subcortical areas (McNamara et al., 1990).

Muscarinic receptors have recently been cloned and their primary sequence has been used to determine a model of seven membrane spanning domains based on hydrophobicity plots Bonner et al., 1987). All muscarinic receptors interact with G proteins which modulate the activity of enzymes which catalyze the productions of intracellular second messengers (Richards, 1991).

Biochemical and anatomical studies show that m1 and m3 receptors are positively coupled to phospholipase-C activation and cellular depolarization

through pertussis toxin-sensitive and -insensitive pathways and tend to be post-synaptically distributed in cortex and hippocampus. m2 and m4 receptors are negatively coupled to adenylate cyclase activity (mediating cellular hyperpolarization) and appear to be pre-synaptic on cholinergic projection neurons (Bonner et al., 1987; Ashkenazi et al., 1989; Mash and Potter, 1986; Beigon et al., 1989). An alternative mechanism for muscarinic activation of phospholipase C has recently been proposed involving a calcium-dependent tyrosine kinase mediated phosphorylation of the lipase (Gusovsky et al., 1993).

Carbachol and oxotremorine act as non-specific muscarinic agonists. Carbachol binds with similar affinities to m1-4 receptor subtypes based on dissociation constants and activates second messenger formation at similar concentrations (Richards, 1991; Sandmann et al., 1991). Improvements on muscarinic receptor subtype specific agonists have recently been reported (Fisher et al., 1993).

There are specific and non-specific muscarinic antagonists: atropine acts at all muscarinic receptor subtypes, while pirenzepine is specific to m1 receptors and AF-DX 116 is specific to m2 receptors (Richards, 1991).

Muscarinic receptors are subject to homologous and heterologous regulation (Lee and Wolfe, 1989; Lee and Fraser, 1993). Homologous regulation occurs following prolonged exposure to atropine which results in down-regulation of the number of receptors (Lee and Wolfe, 1989). Heterologous desensitization of muscarinic receptors occurs in response to cross-talk by chronic exposure of the beta-adrenergic agonist isoproterenol (Lee and Fraser, 1993). Heterologous

upregulation occurs in response to transmitter-specific brain lesions (Morrow et al., 1983). Both types of regulation may be mediated by several mechanisms. Phosphorylation of a domain of the third intracellular loop signals internalization of the receptor (Lee and Fraser, 1993). G proteins are subject to down-regulation be agonist exposure (Dell'Acqua et al., 1993). Intracellular increases in calcium induce muscarinic receptor desensitization that does not involve receptor sequestration or changes in G protein levels (Dell'Acqua et al., 1993). The agonist induced down-regulation of muscarinic receptors can be blocked by potassium channel blockers in brain slices (Shaw et al., 1989).

Acetylcholine evokes both inhibitory and excitatory responses in brain, based on the relative distribution of receptor subtypes and the intrinsic circuitry. Iontophoretic application of acetylcholine to cerebral cortex evokes a fast hyperpolarization, which is sometimes followed by a slow depolarization (Krnjevic et al., 1971). The inhibitory action of acetylcholine is mediated by the activation of GABAergic inhibitory interneurons (McCormick and Prince, 1985), while the slow depolarization is mediated by the reduction of a voltage-gated potassium current (Cole and Nicoll, 1984). The excitatory action of acetylcholine may also be due to the blockage of calcium-activated potassium channel mediated afterhyperpolarization that occurs following bursts of pyramidal cell firing (Cole and Nicoll, 1984; McCormick and Prince, 1985). Vidal and Changuex showed that in vitro frontal cortex, nicotine administration had an excitatory action on all responsive cells (14% of total cells), while muscarine decreased the excitatory

post-synaptic potential in all cells (100% of total cells).

Acetylcholine administration to the hippocampus is more uniformiy excitatory. this may be due to the direct activation of hippocampal pyramidal cells in contrast to inhibitory interneurons (Kuhar, 1974; Lamour et al., 1990). Striatal in vitro preparations show inhibitory responses to muscarinic agonists and slow depolarizations following physostigmine (Dodt and Misgeld, 1986; Akaike et al., 1988). Both of these studies utilized non-specific agonists, so pre-synaptic vs. post-synaptic effects could not be distinguished.

The unit activity recording in the NBM shows that activity is tonic and correlates with behavioral activity and desynchronized cortical EEG, which is most commonly seen in awake, behaving animals (Buzsaki et al., 1988b; Metherate et al., 1992). Direct stimulation of the NBM leads to striking changes in cortical activity as indexed by EEG, where a shift occurs from high voltage rhythmic activity to increases in low voltage desynchronized activity (Belardetti et al., 1977). Cholinergic hypofunction caused by NBM lesions, atropine administration, Alzheimer's disease or aging all cause a pronounced shift to rhythmic high-voltage slow activity EEG (Buzsaki et al., 1988a; Riekkenen et al., 1991; Metherate et al., 1992). These changes appear to mediated by alterations of the firing of layer 5 pyramidal cells in cortex. Parallel changes in single units in this layer can be evoked by physostigmine and are blocked by atropine (Metherate et al., 1992). The function role of NBM stimulation and endogenous acetylcholine release in cortex promotes the facilitation of cortical responsiveness to thalamic sensory

inputs (Metherate and Ashe, 1992; Hars et al., 1993).

The electrical activity of hippocampus and its response to acetylcholine is quite different, as compared to cerebral cortex. The hippocampus exhibits a pronounced rhythmical activity in awake, behaving animals (theta rhythm) and this rhythm can be reset by stimulation of the cholinergic septal nucleus (Lehman and Miller, 1974). Single unit recordings of septal hippocampal neurons indicate 45% of these cells exhibit endogenous rhythmic bursting activity. This is in contrast to nucleus basalis cells which never exhibit rhythmic bursting activity (Lamour et al., 1990). Septal nucleus lesions or atropine administration decrease the incidence of the theta rhythm and shift the pattern of electrical activity to increased non-rhythmicity and lower frequency (Vinogradova et al., 1980; Colom and Bland, 1991; Valjakka et al., 1991). Unit recording of septal nucleus cells and correlation analysis of terminal field activity indicate that these cells are theta-related, fire spontaneously, and drive the theta rhythm of the hippocampus (Colom and Bland, 1991).

Unit recording studies in striatal cholinergic cells indicate a slow, arrhythmic tonic pattern of firing (Wilson et al, 1990). Cholinergic striatal cells have been proposed to inhibit transmission in the basal ganglia (Akaike et al., 1988). As these cells are interneurons, they are much more difficult to study as compared to the long projection cholinergic systems.

Effects of Cholinergic Brain Lesions

Lesion studies of cholinergic and non cholinergic ascending systems are being

pursued in order to determine whether specific transmitter systems influence terminal region phospholipid metabolism.

There are several points worth noting with the utilization of the lesion technique of neurotransmitter specific systems.

1) Following brain lesions in young animals, compensatory events may occur. These include a partial reversal of the loss of choline acetyltransferase in cortex several months after a lesion of the nucleus basalis (Wenk and Olton, 1984). This is in contrast to aging and human neurodegeneration, where changes in phospholipid and neurotransmitter metabolism appear to be progressive. Indeed, these neurochemical deficits may be due to disruptions in uncharacterized compensatory processes in normal brain.

2) The lesion technique allows for transmitter-specific hypotheses to be tested. In other conditions where transmitter systems are compromised (i.e. normal aging and AD), there are a large number of interactions between these systems that make it difficult to test specific hypotheses.

3) The lesion technique allows for temporal control in the examination of the effects of compromising a specific transmitter system. This may be useful for determining whether neurochemical changes seen after a lesion reflect compensatory events.

4) The degree of a transmitter system lesion can be controlled, allowing for the examination of the effects of partial lesions and full lesions (i.e. transection of the cholinergic septo-hippocampal tract). This may also be important for elucidating

compensatory processes following brain lesions.

5) The conditions for tissue collection are under much greater control in animal lesion studies. The post-mortem time for human autopsy sample collection is generally in the range of 4-24 hours.

6) The brain lesioned animals do not appear to have major sensory, motor, or nutritional deficits.

7) The interpretation of drug effects may be more relevant in transmitter specific lesioned animals than in normal animals.

Excitotoxic agents such as ibotenic acid are employed to selectively destroy cell bodies without damaging adjacent fibers of passage. The mechanism of toxicity of excitotoxins is based on their potent glutamate receptor agonist properties, which causes disruptions in calcium homeostasis and cell death. This technique thus improves the anatomical specificity of the lesion over methods such as electrolytic lesion. The tissue damage from ibotenic acid is more localized and controlled as compared to other excitotoxins such as kainic acid. Kainic acid induces widespread seizures which may cause damage independent of the projections of the lesioned area. The metabolism of ibotenic acid involves a decarboxylase reaction yielding muscimol, a GABA agonist. This reaction may limit the spread of ibotenic acid induced cytotoxicity.

Ibotenic acid injections into the NBM result in gliotic scarring and cholinergic cell death as indexed by the loss of ChAT immuno-positive cells at the site of the

injection (Holmes et al., 1990; Unger and Schmidt, 1992). Decreases in cholinergic indices in the cortex are also observed. The most robust measure is a decrease in cortical ChAT activity and acetylcholinesterase staining (McKinney and Coyle, 1982; Wenk and Olton, 1984; Eckenstein et al., 1985; Unger and Schmidt, 1992). The magnitude of decrease in cortical ChAT activity following ibotenic acid injections in NBM usually range from 25-50% due to the distribution of the nucleus, the number of injections, and the amount of ibotenic acid injected. A 50-60% decrease in ChAT activity in the cortex is interpreted as a result of the complete destruction of the NBM, as there are cholinergic interneurons and midbrain cholinergic cortical projections. Cholinergic basal forebrain neurons in aged rats appear to be more vulnerable to excitotoxic damage as compared to young rats (Zawia et al., 1992). The recovery of ChAT activity following NBM lesions is controversial. The ChAT activity in cortex following excitotoxic NBM lesions recovers within 3-6 months (Wenk and Olton, 1984; Casamenti et al., 1988; T.Holmes, data below). A lack of recovery in ChAT activity has been reported by several groups (Bartus et al., 1985; El-Defrawy et al., 1985). Careful examination of these results indicates that recovery does not occur following larger lesions which may reflect complete destruction of the NBM. Recovery following partial lesions could reflect: 1) sprouting from surviving cells of the NBM of the same side or from contralateral NBM cells, 2) local compensatory sprouting of ChAT containing terminals into denervated areas 3) upregulation of ChAT activity in surviving terminals with no morphological changes. Recent improvements in

immunohistochemical staining may be used to determine the mechanism of recovery (Delacalle and Holmes, unpublished observations). Previous methods for ChAT immunohistochemistry do not yield sufficient resolution to examine terminal arborization. The laminar pattern of cortical acetylcholinesterase staining recovers 12 weeks after cortical knife cut sections of cholinergic pathways. This recovery appears to be mediated by collateral sprouting by remaining acetylcholinesterase positive axons. Axonal growth occurs past the gliotic scar of the knife cut (Farris et al., 1993).

In addition to decreases in cortical ChAT activity and AChase staining in cortex, NBM lesions result in decreases in SDHACU (Pedata et al., 1982; Bartus et al., 1985, El-Defrawy et al., 1985). The timecourse of the SDHACU decrease does not follow the same timecourse as ChAT depletion. The peak decrease in SDHACU occurs at 4 days following the lesion and rapidly recovers within twenty days (Pedata et al., 1982; Casamenti et al., 1988). This rapid recovery is noteworthy as choline availability is believed to be the rate limiting factor for acetylcholine biosynthesis and release.

Acetylcholine release evoked by high potassium measured in vitro and in vivo in cortex is decreased following NBM lesion (Gardiner et al., 1987; Herrera-Marschitz et al., 1990). Compensatory recovery of potassium evoked acetylcholine release in cortex occurs at 3 months following NBM lesion (Gardiner et al., 1987). Treatment with nerve growth factor partially reduces this deficit (Dekker et al., 1991). A potential problem with the interpretation of all of these studies is the use

of potassium depolarization for inducing acetylcholine release. Potassium evoked release is probably a better model for releasing tissue stores of acetylcholine. This treatment does not evoke sustained release and results in tissue depletion of acetylcholine (See fig. 25).

Muscarinic receptor binding and muscarinic agonist supersensitivity in cortex following NBM lesion has been examined. Small reductions were observed one to two weeks after lesioning in total muscarinic binding sites, muscarinic low-affinity binding sites, and muscarinic m1 receptors which are thought to be predominantly post-synaptic (McKinney and Coyle, 1982; Watson et al., 1985; de Belleroche et al., 1985; Atack et al., 1989). Larger reductions of m2 receptors have been observed three days following NBM lesions, this subtype of receptor is thought to be located primarily on pre-synaptic terminals (Mash et al., 1985). Receptor densities measured at longer post-lesion intervals yield conflicting results, some groups find persistent depressions in muscarinic binding in cortex at 13 weeks post-lesion (Atack et al., 1989), while long-term cholinergic denervation of the hippocampus results in increases in m1 and m2 receptors at 40-60 days post-lesion (Joyce et al., 1989).

Receptor-effector coupling in cortex, as measured by second messenger generation in response to receptor agonist has been examined following NBM lesions, yielding controversial results. Several groups find no change in carbachol responsiveness (Raulli et al., 1989; Scarth et al., 1989; Shoham et al., 1990) while the results of this study (see below) and one other group find increases in

carbachol responsiveness in cortex following NBM lesions (Reed and de Belleroche, 1988). Colchicine injections into the NBM result in increased inositol lipid hydrolysis in response to carbachol in cortex at three months after the injection (Mundy et al., 1991). This study also reported a long-term (14 months) increased responsiveness to quisqualate- and noradrenaline-stimulated hydrolysis of inositol lipids. The relatively late responses observed in this study may be due to the mechanism of colchicine, which destabilizes microtubules and disrupts axonal transport. Age-related effects may also contribute to changes in response. A previous report by this group reported a recovery in cortical ChAT activity in cortex, but a persistent reduction in ChAT immuno-positive neurons in the NBM at 12 weeks following this procedure (Mundy and Tilson, 1990). There is lack of strong evidence for cell death in the basal forebrain following colchicine injections. ChAT immunoreactivity is not a reliable measure for cellular viability. Other cholinergic systems exhibit increased agonist-induced hydrolysis of inositol lipids following de-afferentation, as shown in the hippocampus following septal lesions (Connor and Harrell, 1989). No changes in basal inositol lipid hydrolysis was observed in this study.

Regional increases in stimulated inositol lipid turnover have been observed in vivo in the cortex of NBM lesioned rats using a novel radiolabelling strategy (Nariai et al, 1991). Arachidonic acid is preferentially incorporated into phosphatidylinositol under these conditions and can be visualized by autoradiographic methods. Increases in arachidonate incorporation in lesioned side

frontoparietal cortex occurs in layers I, IV, and V following agonist stimulation, no differences were observed in regions which do not receive NBM innervation. The cortical layer specificity of increased lesion/control label incorporation correlates with the regional density of NBM innervation. While these results suggest that muscarinic receptor responsivity and lipid turnover can be increased in the cortex in vivo following NBM lesions, there were no differences in arachidonate incorporation between lesion and control side cortex in the absence of pharmacologic stimulation.

Additional evidence for increased receptor responsivity in cortex following NBM lesions comes from electrophysiological and pharmacological studies. Cortical neurons are more readily depolarized by the iontophoretic application of acetylcholine, carbachol, and nicotine at two weeks following NBM lesions (Lamour et al., 1982). Cellular responsivity also showed striking intralaminar changes, where excitatory responses to acetylcholine were recorded in layers II-III and IV following NBM lesion, such areas were non-responsive in control cortex. Cortical neurons did not change their responsivity to glutamate in this study. Supersensitivity to carbachol induction of the inward leak current and the calcium-dependent potassium current in hippocampal pyramidal cells increases after cholinergic deafferentation by fimbria-fornix lesions (Benson et al., 1989).

Behavioral responsivity to cholinergic drugs is increased after nBM lesions (LoConte et al., 1982). Peripheral administration and intraventricular infusion of cholinergic agonists such as carbachol and nicotine induce seizures. Seizure

severity and the doses required for seizure induction are much lower in NBM lesioned rats (Ksir and Benson, 1983; Mastropaulo and Crawley, 1988).

Transient decreases in cortical energy metabolism following NBM lesions have been observed utilizing the 2-deoxyglucose method (London et al, 1984, DeMicheli et al., 1993). The reduction in regional cerebral glucose utilization occurs earlier than indices such as the reduction in ChAT activity and recovers within two weeks after the lesion (DeMicheli et al., 1993). It is of interest that the time course of reduction and recovery of cerebral glucose utilization parallels another metabolic perturbation: that for high affinity choline uptake (Pedata et al., 1982; Casamenti et al., 1988).

Stimulation of the NBM results in increased cortical blood flow, this effect is blocked by atropine and by nitric oxide synthase inhibitors (Adachi et al., 1992). It is not clear whether alterations in regional cerebral blood flow occur by direct control by NBM projections to blood vessels or by indirect control by cortical activation (Scremin et al., 1991).

Several studies suggest that abnormalities in lipid metabolism occur following NBM lesions. In vivo cholinergic stimulation increases arachidonate incorporation into inositol lipids (Nariai et al., 1991). Increases in mono- and diacylglycerol lipase activities were observed in synaptosomal and plasma membrane fractions of cerebral cortex, midbrain, and hippocampus prepared ten days after unilateral NBM lesions in rats (Farooqui et al., 1991). These increases ranged from three to five fold, slightly lower increases were also observed at six months after the

lesion. ChAT depletions were significant in cortex, but not in hippocampus or midbrain regions after the lesion. The authors conclude the changes observed in midbrain and hippocampus may reflect transneuronal effects of cholinergic denervation of cortex or non-specific effects of the lesion. Both of these studies emphasize catabolic metabolism of lipids.

Morpholology

Alterations in cortical morphology have been observed following NBM lesions in neonatal and adult experimental preparations. Cortical layer V pyramidal cells show striking decreases in cell size and arborization one week after NBM lesions in neonatal mice (Hohmann et al., 1991). Although signs of recovery of neurochemical indices and pyramidal cell morphology occur as early as 14 days after neonatal NBM lesions, there are disruptions of cortical layer cytoarchitecture, alterations in both afferent and efferent connections, and impairments in behavior that persist into adulthood (Hohmann et al., 1988; Hohmann et al., 1991, Sengstock et al., 1992). Cortical morphological changes have also been observed in adult animals following NBM lesions (Inoue et al., 1992). Degenerated dendrites were observed as early as three days and were detected up to three weeks later in the cortex following ibotenate lesions of the NBM. Degenerated axons were observed consistently at three days, then declined at seven days, and were not observed at three weeks following the lesion. The number of degenerated dendrites was greater than degenerated axons at all time points examined. No significant changes in lesion side cortical cell numbers were observed at any of

these time points. The authors conclude that the ultrastructural changes observed in cortex following NBM lesions reflect transneuronal degeneration of cortex. Long-term alterations in cortical morphology have been observed following NBM lesions in adult rats. Decreases in the number of cortical neurons (20-27%) and shrinkage of cortical cells were observed at 14 months after NBM lesion (Arendesh et al., 1987; Terry et al., 1988). Cortical cell loss and shrinkage of cortical layers were observed 4 months after NBM lesions in aged twenty one month old rats (Socci et al., 1992). Cytoskeletal aberrations resembling neurofibrillary tangles and cell loss in frontoparietal cortex have been reported after NBM lesions (Panzarino et al, 1986; Arendash et al., 1987) and abnormal levels of phosphorylated neurofilaments are seen following transection of the cholinergic septal-hippocampal pathway (Koliatsos et al., 1989). Several groups have attempted and failed to replicate the finding of Arendash's group of the long-term induction of neurofibrillary tangle-like structures in cortex following NBM lesions in rats, but all groups do find consistent cell loss in cortex after lesioning (Terry et al., 1988; Pepue et al., 1990).

Materials and Methods

Rats receiving surgery (300-350 g) were anesthetized with equithesin (0.3 ml/kg body weight) and placed in a stereotaxic frame. The skull was exposed and a stereotaxically placed burr hole was drilled in NBM lesioned and sham lesioned rats. NMB lesioned rats received unilateral injections of ibotenic acid (Sigma

Chemical Co, I-0382), 10 mg/ml in phosphate buffered saline, 0.5 ul injected over four minutes) via a stereotaxically placed glass cannula using the following coordinates relative to bregma: AP = -0.8, ML = -2.8, and DV = -7.3 based on the atlas of Paxinos and Watson. The cannula was left in place for fifteen minutes to allow diffusion of the toxin, then slowly withdrawn to prevent the toxin from diffusing up the cannula tract. The cannula was withdrawn too quickly in one animal, which immediately suffered a seizure and died, presumably due to the action of the toxin. The procedures for cannula placement and withdrawal were the same for sham-lesion animals except for the cannula depth which was DV = -6.3 in order to avoid mechanical damage to the NBM.

Similar surgical procedures were used for animals receiving unilateral lesions of the ventrobasal nucleus of the thalamus, except these animals received two 5 ul injections at the following coordinates: AP = -2.3,-3.3, ML = -2.8,-3.2, DV = -6.3,-7.0 based on the atlas of Paxinos and Watson.

Rats receiving fimbria-fornix/cortical transections were anesthetized and placed in a stereotaxic frame as above. A thin slit was stereotaxically drilled from the midline to five millimeters to the left of the midline at AP = -0.2 relative to bregma. A stereotaxic surgical knife was lowered to DV = -5.0 relative to the brain surface at the midline, then moved laterally to ML = 5.0. This procedure was repeated three times to insure complete transection of the fimbria and the supracallosal cortical pathway.

The incision was closed over the skull with discontinuous sutures and the

animals were placed on a heating blanket for one hour, then transferred to their home cages for recovery in the vivarium.

The stereotaxic coordinates used for the NBM lesion were histologically verified in several animals following a pressure injection of 5 ul of Chicago Sky Blue dye into the NBM. The NBM injected animals were sacrificed one hour after injection. The stereotaxic coordinates for the ventrobasal thalamic ibotenate lesion were verified in several animals. These animals were sacrificed seven days after the lesion. All histology animals were sacrificed by trans-cardial perfusion with buffered 4% paraformaldehyde. A freezing microtome was used to cut 30 um coronal sections. Sections from NBM lesioned rats were processed for Nissl stain and choline acetyltransferase immunohistochemistry coupled to a DAB/peroxidase reaction. Sections from ventrobasal thalamus lesioned rats were processed for Nissl stain and the Fink-Heimer method of silver impregnation for the determination of axonal degeneration. Drawings of the sections were made using either a projection system or camera lucida and compared to the atlas of Paxinos and Watson.

Animals used for biochemical studies were sacrificed by decapitation at various times after lesioning. Frontoparietal cortical sections from lesioned and unlesioned sides were rapidly dissected and snap frozen in dry ice. Care was taken to match corresponding cortical sections for biochemical assays such that left and right sections represented the same cortical areas. Tissue samples were transferred from dry ice to a -70 degree freezer for storage.

Similar procedures were used for the collection of hippocampal tissues. Anterior hippocampus were collected for ChAT determination, the remainder of the hippocampus was used for other tissue assays.

Choline acetyltransferase (ChAT) activity was measured using a modification of the method of Fonnum. Dissected tissue was stored at -70 degrees C. Tissues were homogenized in 500 ul of buffer (EDTA:10mM; Triton-X:0.5%; pH=7.4) using a glass-teflon homogenizer. Aliquots of homogenate were collected for protein determination and for the assay. Triplicate aliquots of 5ul of homogenate and reaction blanks were incubated with 10 ul/tube of assay buffer (mM: NaCl=300; $NaPO_4$=50; EDTA=20; choline chloride=0.8; acetyl coenzyme A=0.2; eserine salicylate=0.1; pH=7.4) containing 0.05 uCi/tube of ^{3}H-acetyl coenzyme A for 30 minutes in a shaking water bath heated to 37 degrees C. The reaction was stopped by the addition of 200 ul of ice-cold 10 mM phosphate buffer to each tube. ^{3}H-acetylcholine was separated from the excess label by liquid-liquid extraction with the addition of 1 ml of 1.5% tetraphenylboron in heptanone. Fractions of the heptanone phase were counted using liquid scintillation spectrophotometry. All samples were converted to blank subtracted, quench corrected to D.P.M., and normalized to protein.

Tissue sections adjacent to those used for ChAT activity were used for the extraction of phospholipid and water soluble phospholipid metabolites. These sections placed frozen in 2 ml of ice-cold methanol, then homogenized using a polytron. This procedure was used to rapidly deactivate enzymatic activity.

Aliquots of the homogenate were collected for protein determination (see below), the remainder of the homogenate was used for Folch's extraction method of phospholipid and water soluble metabolites with chloroform:methanol:water (4:4:2 mls). The extraction tubes were centrifuged at 3000 r.p.m. and were left to stand overnight at in a cold room. Fractions were then collected for phospholipids from the organic phase and water-soluble metabolites from the aqueous phase and dried using a Savant Speed Vac.

Total phospholipid fractions were collects in quadruplicate for most experiments. Individual phospholipids were separated by thin layer chromatography on silica gel G plates using a solvent system of chloroform: ethanol: triethylamine: water (30:34;30;8 mls) by the method of Touchstone. Phospholipid standards were spotted in lanes for verification (Sigma Chemical Co.). The plates were then air-dried and sprayed with hexatriene in petroleum ether. Individual phospholipids were visualized under UV light and were marked according to their co-migration with standards, scraped off into tubes. Individual phospholipids and total phospholipids were hydrolyzed in perchloric acid, yielding inorganic phosphate, which was determined by the colorimetric method of Svanborg and Svennerholm. All phospholipid determinations were normalized to tissue protein.

Water soluble phospholipid metabolites including choline and glycerophosphocholine were separated by normal phase solvent gradient high pressure liquid chromatography using the method of Liscovitch. The elution times

for water soluble metabolites were determined by collecting fractions following the injection of radiolabelled standards (Fig 3). Individual metabolites were collected using a fraction collector, pooled and dried using a Savant Speed Vac.

Figure 3

Elution Profile of Water-Soluble Choline Metabolites

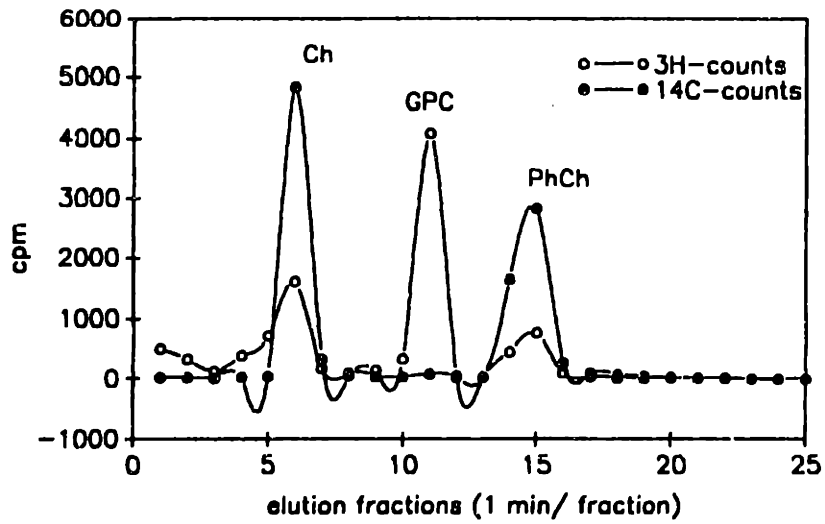


Fig. 3) Water-soluble choline metabolites were separated by normal phase high performance liquid chromatography using a gradient solvent system. The elution times of choline (5-7 min), glycerophosphocholine (10-12 min), and phosphocholine (13-17 min) was determined by counting 1 minute elution fractions of known radiolabelled standards.

Choline was directly quantified using a BAS 1000 reverse phase high pressure

liquid chromatography system coupled to an electrochemical detection system, sample values were normalized to known standard values determined during each run (Fig 4).

Figure 4

Electrochemichemical Detection of Acetylcholine and Choline

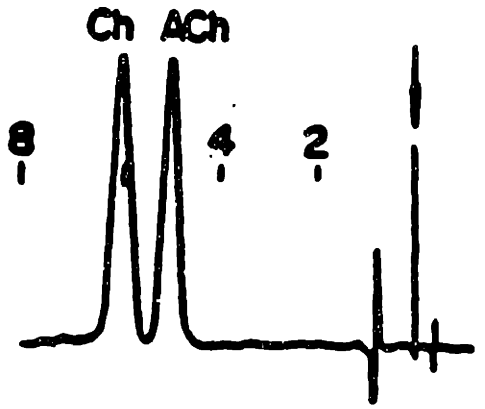


Fig. 4) Choline and acetylcholine were separated and quantified by reverse phase high performance liquid chromatography coupled to electrochemical detection.

This system utilized a BAS covalently linked choline oxidase/acetylcholinesterase column reactor system (Bioanalytical Systems). Choline values were normalized to tissue protein content.

Glycerophosphocholine was hydrolyzed to choline by hydrochloric acid. After

pH neutralization with phosphate buffer, samples were assayed for choline content as above. Recovery standards were run at all chromatography steps.

Tissue protein was assayed by one of two methods: the method of Lowry, or by the bicinchoninic acid/copper sulfate reaction using a commercially available kit (Pierce). Most tissue protein assays were done in triplicate or quadruplicate. Tissue total protein and individual proteins were compared to tissue wet weight. Individual tissue proteins were separated by gel electrophoresis using the Bio-Rad acrylamide tris/tricine mini-gel system. Gels were then stained with Coomassie Blue and densiometric determination was made with a Pharmacia LKB Ultroscan gel scanner system.

Tissue DNA as a normalization factor was assayed in some experiments (Labarca and Paigen, 1979). Tissue DNA fractions were collected in duplicate.

Choline kinase activity in brain tissue was determined using a modification of a radioenzymatic method used by Spanner and Ansell. Frozen tissue sections were transferred from dry ice and homogenized in 500 ul of ice-cold buffer (8.3 % sucrose, 10 mM HEPES, pH=7.2) using a glass/teflon homogenizer. Aliquots were collected for protein determination, the remainder was stored frozen at -70 degrees C. These samples were thawed, and diluted to uniform protein values of 10 ug/sample with buffer (8.6% sucrose in PBS, pH=7.2). Samples and boiled tissue blanks were incubated in duplicate for 10 minutes in buffer containing ^{14}C-choline (1 uCi), $MgCl_2$ (3.3 M), phosphocholine (0.05 M), ATP (2.22%), and either 10 uM or 80 uM choline. The reaction was stopped by the addition of 0.5 ml of ice-

cold methanol:water (1:1), the reaction product ^{14}C-phosphocholine was separated from the remaining choline label with Amberlite CG50 cation exchange column chromatography and was quantified by liquid scintillation spectrometry. The parameters for protein, incubation time, and choline concentrations were determined by criteria of reaction linearity and kinetic parameters determined in earlier experiments. The column separation procedure was verified using radioactive standards of ^{14}C-choline and ^{14}C-phosphocholine. Folch extractions were carried out in preliminary experiments, showing no detectable conversion of the label to phospholipid using these assay conditions.

Fractions of the homogenate samples used for the determination of choline kinase activity were utilized for the determination of serine base exchange activity using the method of Holbrook. Base exchange buffer (BXB) containing HEPES (50 mM), $CaCl_2$ (1 mM), L-serine (100 uM), ^{3}H-serine (5 uCi/ml) was prepared fresh, 150 ul of BXB buffer plus 50 ul of brain homogenate samples were incubated for 20 minutes in at shaking water bath (37 degrees C). The reaction was stopped by the addition of 3 ml of chloroform:methanol (2:1) and vortexed, 1 ml of water was added to this for Folch extraction of the labelled phosphatidylserine reaction product. The aqueous phase was discarded and the organic phase was washed twice with 0.75 % KCl in 50% methanol to remove any remaining serine label. Organic fractions were then dried and resuspended in chloroform:methanol (1:1) for liquid scintillation counting. Parallel fractions of homogenate were used for protein determination, labelled phospholipid values

were normalized to homogenate protein.

Receptor mediated hydrolysis of inositol lipid was examined using the method of Berridge. Rat brains were chilled in ice-cold oxygenated Kreb's buffer (calcium and eserine free) and frontoparietal cortices were rapidly dissected. Tissue sections were separated for ChAT determination. Tissue microprisms were cross-chopped at 350 um by 350 um with a McIlwain tissue chopper, then gently separated with a fine hair brush. The microprisms were washed to remove tissue debris and equilibrated for 1 hour in Kreb's buffer in a shaking water bath heated to 37 degrees C. The equilibration medium was changed with fresh oxygenated medium every 10 minutes. A stream of O_2/CO_2 (95:5) was run over the buffer surface to improve oxygenation. Microprisms were labelled for 1 hour in 3 ml of Kreb's buffer containing 0.5 uCi of myo-^{3}H-inositol (New England Nuclear; NET-114) in the shaking water bath. Labelled microprisms were washed with Kreb's buffer containing 10 mM LiCl and incubated for 2-60 minutes in the presence of carbachol (0-1000 uM) and other agonist in the shaking water bath. The reaction was stopped by transferring the tubes to an ice-water bath. The incubation medium was removed and labelled lipid and water-soluble metabolites were separated using a Folch extraction. Inositol phosphates were separated using Dowex anion exchange columns (Bio-Rad, formate form, 200-400 mesh) and quantified by liquid scintillation counting. Fractions were also counted from the organic phase for the determination of labelled inositol lipids. Inositol phosphate metabolites were normalized to total tissue label.

Neurotransmitter release from brain slices utilized a superfusion system for maintaining tissue viability. Rat brains were rapidly removed and placed in ice-cold oxygenated Kreb's buffer (mM: NaCl=120; KCl=3.5; $CaCl_2$=1.3; $MgSO_4$=1.2; NaH_2PO_4=1.2; $NaHCO_3$=25; glucose=10; eserine salicylate=0.02; bubbled with a mixture of 95% O_2 and 5% CO_2 for oxygenation and the maintenance of pH=7.4). The brain was allowed to chill for 30 seconds, brain regions of interest (striatum or cortex) were rapidly dissected and returned to ice-cold Kreb's buffer. 300 uM thick sections were cut using a McIlwain tissue chopper, the slices were then submerged in ice-cold Kreb's buffer and gently separated with sable hair brushes. The slices were washed several times to remove debris and transferred to chambers in a water bath held at 37 degrees and superfused with Kreb's buffer at a rate of 0.6 ml/minute for 45-60 minutes for equilibration.

All superfusion samples were collected on ice. Basal superfusate samples were collected in all experiments. Drugs were added to the incoming superfusion medium in some experiments and the superfusion medium was collected. Electrical field stimulation was administered by a Grass stimulator coupled to Ag/$AgCl_2$ electrodes in the superfusion chambers (1 ms pulses at 15 Hz with an amplitude of 15 V for one hour). In later experiments examining electrical field stimulation of cortical slices, a current control device which controlled the output of the Grass stimulation and bubble release chambers designed by Steven Farber were utilized. The amplitude of the current in these experiments was maintained at 125 mA, all other parameters remained constant.

The effects of potassium depolarization-induced release of acetylcholine from striatal slices and subsequent effects on tissue acetylcholine, choline, and phospholipid was measured. High potassium Kreb's buffer contained 50 mM KCl, NaCl was decreased accordingly to maintain physiological osmolarity. Rapid control of the potassium-augmented Kreb's medium and normal Kreb's medium entering the superfusion chambers was achieved with a valve assembly placed immediately before to chamber.

The superfusates from cortical release experiments were filtered with Millipore 0.2 uM syringe filters.

Acetylcholine and choline in striatal superfusates were separated with silicic acid columns and measured using a radioenzymatic method (Goldberg and McCaman, 1973). These compounds were measured in cortical superfusates using the BAS reverse phase HPLC-ED system described above. Superfusate values were normalized to tissue protein or DNA.

Amino acids released by brain slices into the superfusion medium were measured in some experiments on an ESA HPLC system utilizing an o-phthalaldehyde derivitization procedure (Alvarez-Coque et al., 1989). Amino acid uptake into cortical synaptosomes was determined used a modification of the method of Levi and Raiteri.

Slices were removed from the chambers after superfusion and kept on ice until homogenization. Striatal slices were homogenized in ice-cold water containing 0.02 mM eserine salicylate. Cortical slices were homogenized in ice-cold buffer

(mM: sucrose=230; $ZnSO_4$= 1; HEPES=20; pH=7.4). Homogenate fractions were then assayed for protein, DNA, phospholipid, and water-soluble choline metabolites. Cortical synaptosomes were suspended in a bicarbonate-buffered oxygenated physiological medium containing ^{3}H-glutamate (2.5 uM or 500 uM) and incubated for 5 minutes at 37 degrees. The assay was stopped by vacuum filtration. The filters were washed with medium several times and counted by liquid scintillation spectrophotometry. Subtraction boiled tissue blanks were run in parallel to account for non-specific binding to the filters and synaptosomes. All values were normalized to synaptosomal protein.

^{14}C-choline incorporation and metabolism in cortical slices was examined using a modification of a method developed by Farber and Savci. Cortical slices were prepared (see above; neurotransmitter release from slices) and superfused for 1 hours with oxygenated Kreb's buffer in chambers in a 37 degree water bath. Slices were transferred to glass tubes (5-6 slices/tube) and labelled for 1 hour in 1 ml of oxygenated Kreb's containing 5 uCi of ^{14}C-choline in a gently shaking water bath heated to 37 degrees. The labelled slices were washed with 35 ml/tube of fresh oxygenated Kreb's buffer. In some experiments, the labelled slices were immediately homogenized, in other experiments, the labelled slices were transferred to chambers for Kreb's basal superfusion or electrical field stimulation (see above, neurotransmitter release).

All labelled slices were homogenized in 1 ml of ice-cold water containing 20 uM eserine, aliquots were collected for protein. The remainder of the homogenate

was used for a Folch extraction (chloroform:methanol:water, 2:2:1 ml). Fractions of the aqueous phase and the organic phase were collected and dried.

Dried aqueous fractions were resuspended in 150 ul of water and centrifuged at 10,000 g for five minutes, 100 ul of this was injected on a normal phase HPLC described above for the separation of labelled acetylcholine, choline, glycerophosphocholine, and phosphocholine. The labelled eluates were verified by radiolabelled standards and were quantified by an on line Berthold counter. The output was analyzed using an computer program for peak integration. The values were quench corrected to D.P.M. values and normalized to tissue protein.

Dried organic fractions were resuspended in 1:1 chloroform:methanol and applied to silica G thin layer chromatography plates along with phospholipid standards for phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. The phospholipids were separated using the solvent system described above (phospholipid separation). The plates were dried and exposed for autoradiography with Kodak X OMAT AR5 film for 12-24 hours. The autoradiographs revealed the position of labelled phospholipids which were scraped off the plate and assayed by liquid scintillation counting. The values were quench corrected to D.P.M. values and normalized to tissue protein.

Statistics were determined using the Statgraph program (Jandel Scientific). Student's paired t test, Newman-Keuls paired test, and analysis of linear regression were used.

Results and Discussion

Histological identification of ibotenic acid lesions

A variety of histological methods were employed to verify the stereotaxic coordinates for ibotenic acid lesion injections. The verification of the target nuclei and transmitter specificity was important as ibotenic acid selectively destroys cell bodies and spares fibers of passage. Methods for qualitatively examining the effects of ibotenic acid lesions on axonal degeneration and regional activation of terminal areas were also used. Fiber transection experiments did not require histology as the extent of the section was clearly visible during microdissection.

The stereotaxic coordinates used for unilateral ibotenic acid injections into the nBM in rats were verified by several histological methods. Rats weighing 300 g received unilateral pressure injections of Chicago Sky Blue using coordinates which matched to reported position of the nBM in the stereotaxic atlas of Paxinos and Watson. Comparisons were made been the injected and the non-injected sides of coronal sections.

ChAT immunohistopositive cells in the expected region of the nBM correspond to the contralateral lesion (Fig 5,6). The cholinergic projection of the nucleus basalis is unilateral in the rat (Eckenstein et al., 1988), which allows for the comparison of lesion side tissues with control tissues from the non-lesioned side.

Figure 5

The Nucleus Basalis and

Adjacent Structures in the Rat

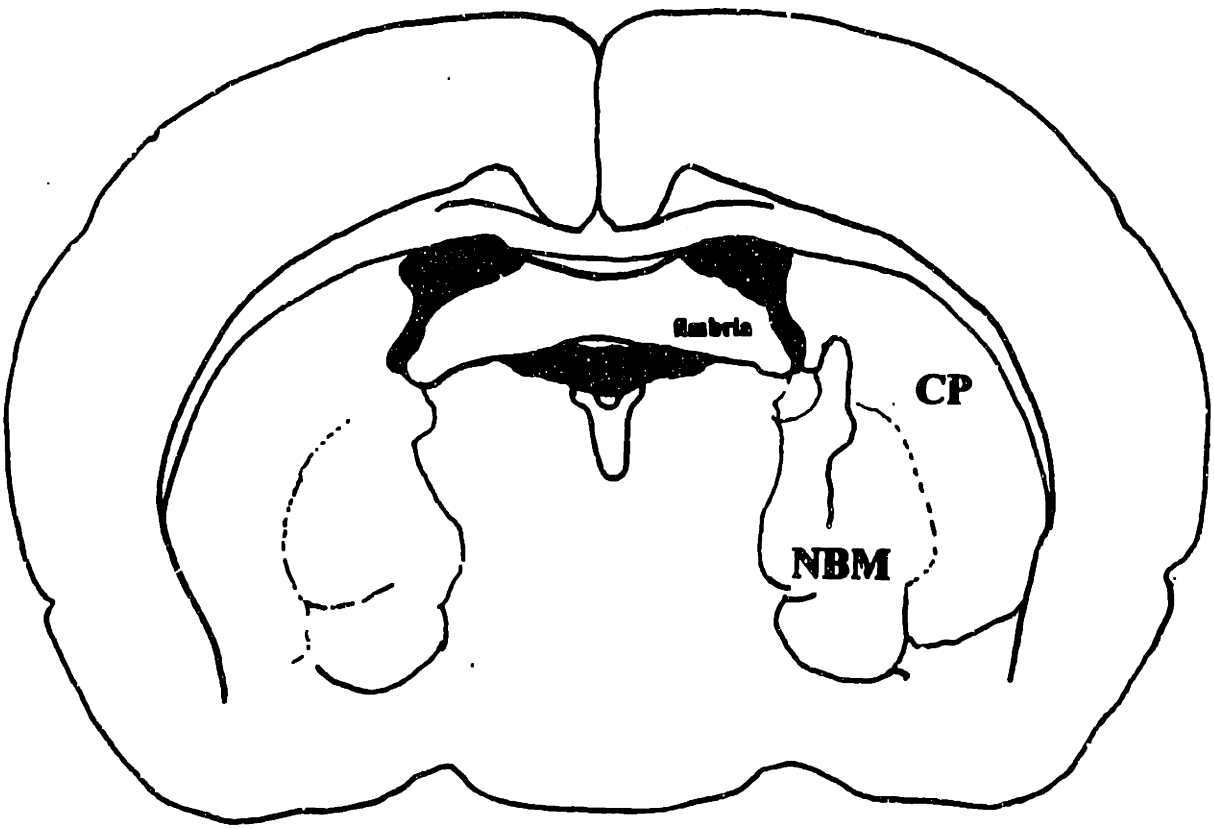


Fig. 5) The basal forebrain nucleus basalis and its relation to the globus pallidus and caudate-putamen are shown above (Adapted from Paxinos and Watson, The rat brain in stereotaxic coordinates).

Figure 6

Choline Acetyltransferase Immunohistochemical

Verification of Injection Coordinates for

the Nucleus Basalis

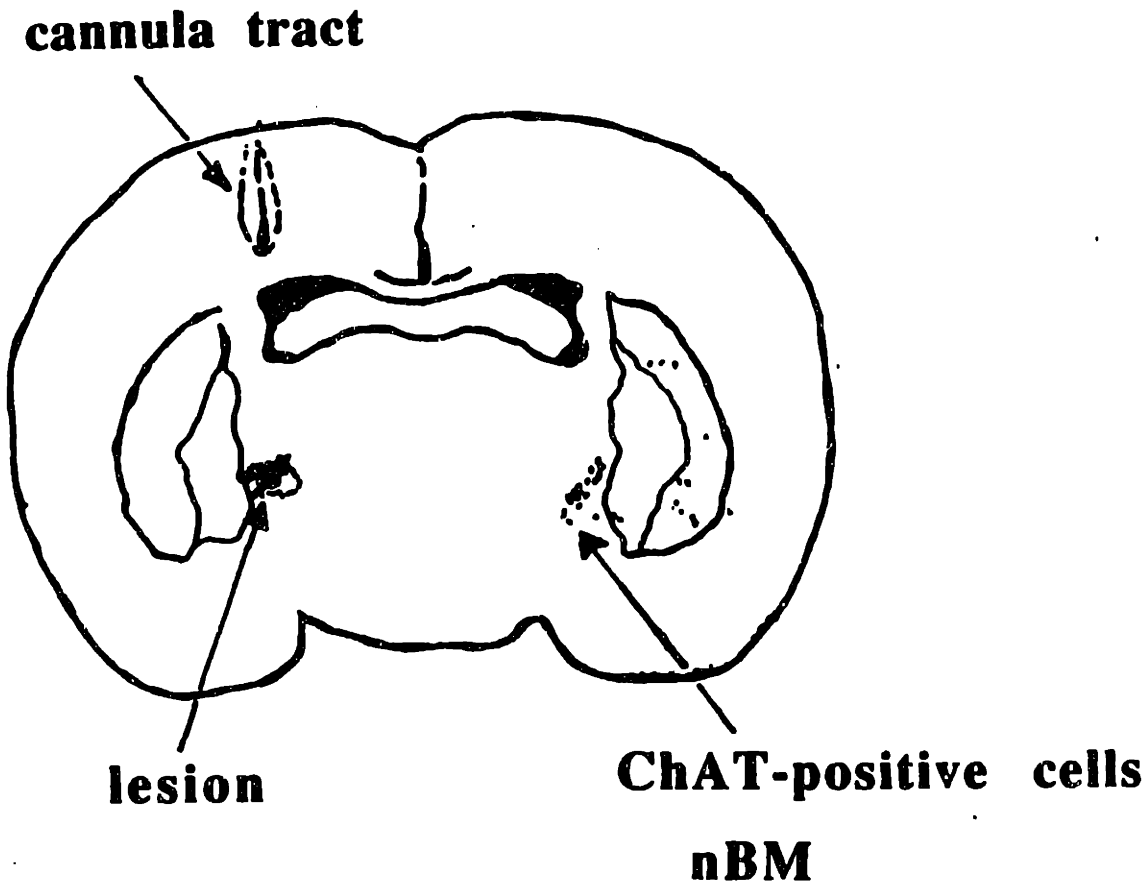


Fig. 6) Cholinergic cells were visualized by choline acetyltransferase immunohistochemistry (ChATi). ChATi-positive cells of the nucleus basalis correspond to the contralateral lesion created using the injection coordinates used for ibotenic acid infusion.

Close examination of the ChAT immunostained sections revealed staining of cell bodies in the striatum and the basal forebrain, but do not show staining of

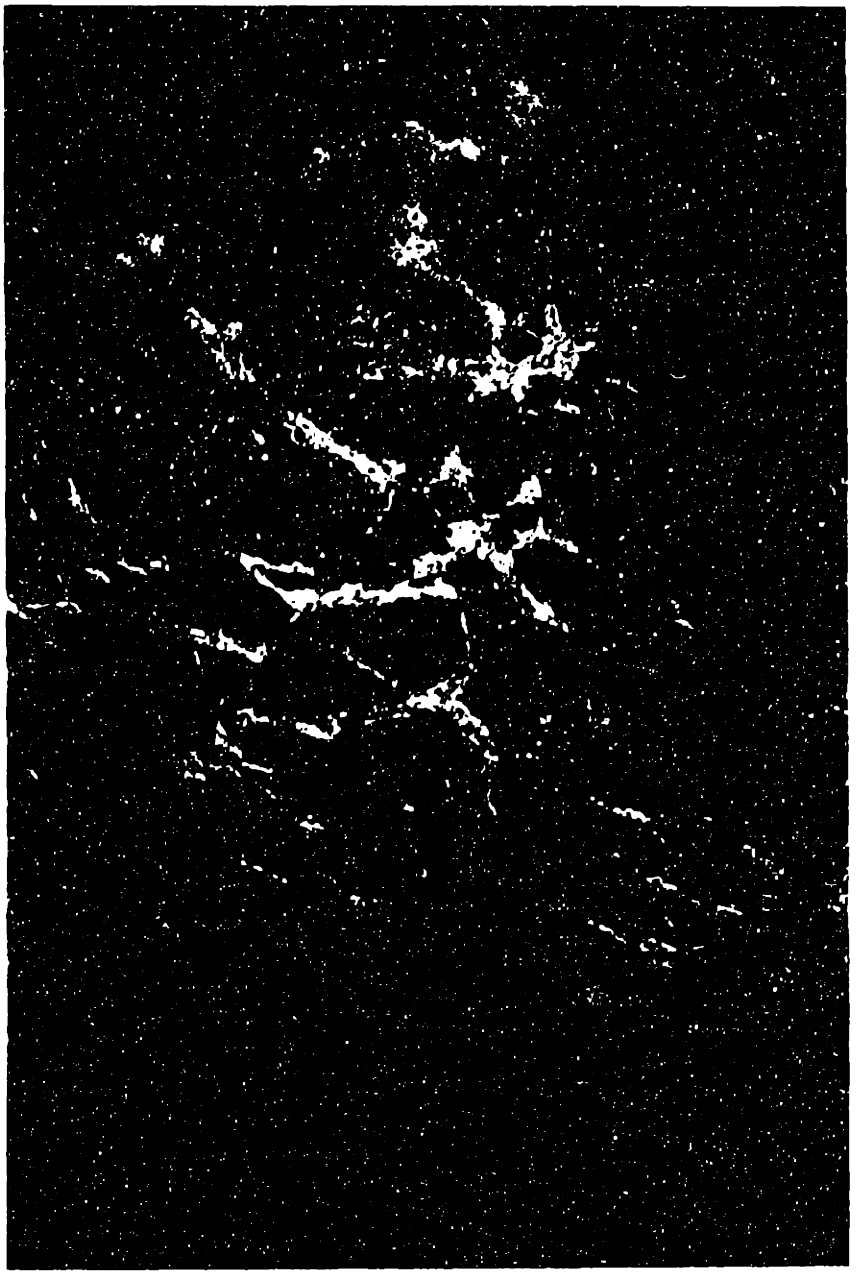
axonal projections to cortical regions. Thus, the resolution of this method was adequate for examining cell bodies in the basal forebrain, but not terminal projections.

Adjacent sections were processed for Nissl staining to determine the position of cell bodies and show correspondence of the position of the lesion with the region identified as the nBM in the atlas of Paxinos and Watson. There was no evidence of gliosis in the region of the injection because of the short interval between the injection and the perfusion.

Figure 7

Ibotenic acid lesion-side and control-side nucleus basalis as visualized by choline immunohistochemistry

Fig. 7) Photomicrograph on page 74 depicts the ChATi cells of the control NBM, the lesion-side nucleus basalis as visualized by ChATi is shown on page 75.



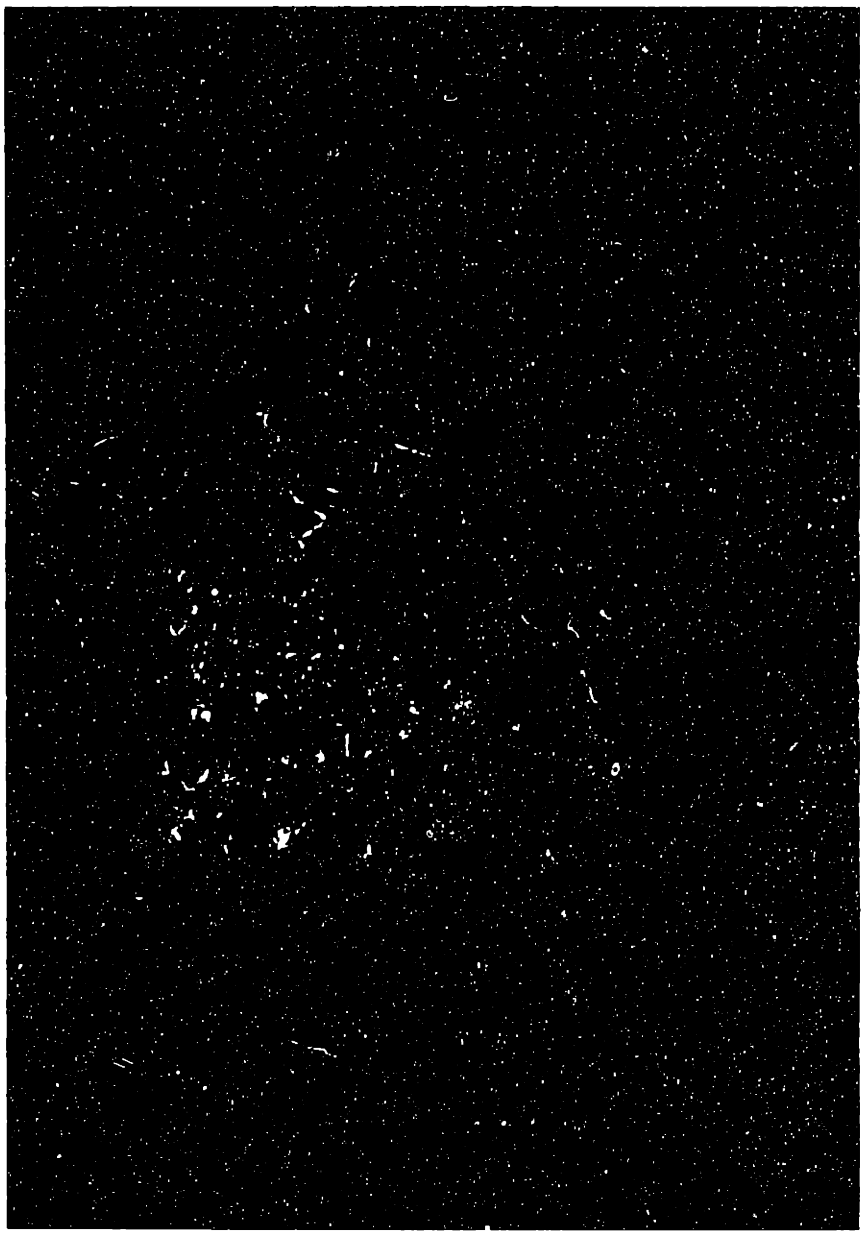
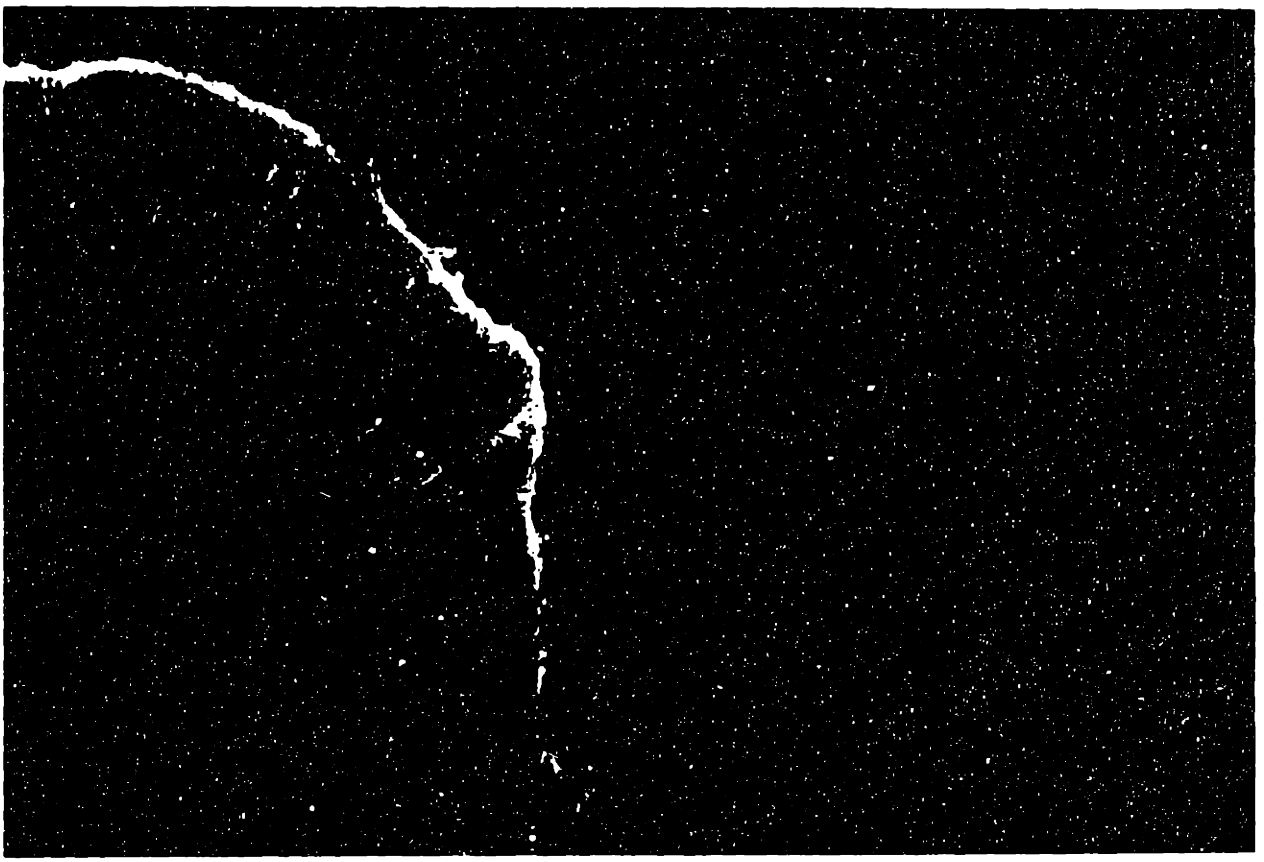


Figure 8

Choline Acetyltransferase Immunohistochemistry in Frontal Cortex Following Lesion of the Nucleus Basalis

Fig. 8) Photomicrograph shows control- and lesion-side cortex following nucleus basalis lesion as visualized by ChATi on the following page.



The stereotaxic coordinates used for the unilateral injections of ibotenic acid into the ventrobasal nucleus of the thalamus were based on the atlas of Paxinos and Watson. Two injections were employed as the nucleus extends over 2 mm in the anterior-posterior plane. The rats used for histological verification of the lesion were perfused 7 days after the ibotenic acid injection to allow for the formation of gliosis, which can be detected in Nissl stains by the appearance of small intensely stain granules and the loss of cell bodies. Adjacent sections to those used for Nissl stain from unilateral ventrobasal lesioned rats were processed using the Fink-Heimer staining method to detect degenerating axons.

A large gliotic lesion is clearly seen in sections processed for Nissl staining which correspond to the expected position of the ventrobasal nucleus (Fig 9,10).

Figure 9

The Ventrobasal Nucleus of the Thalamus in the Rat

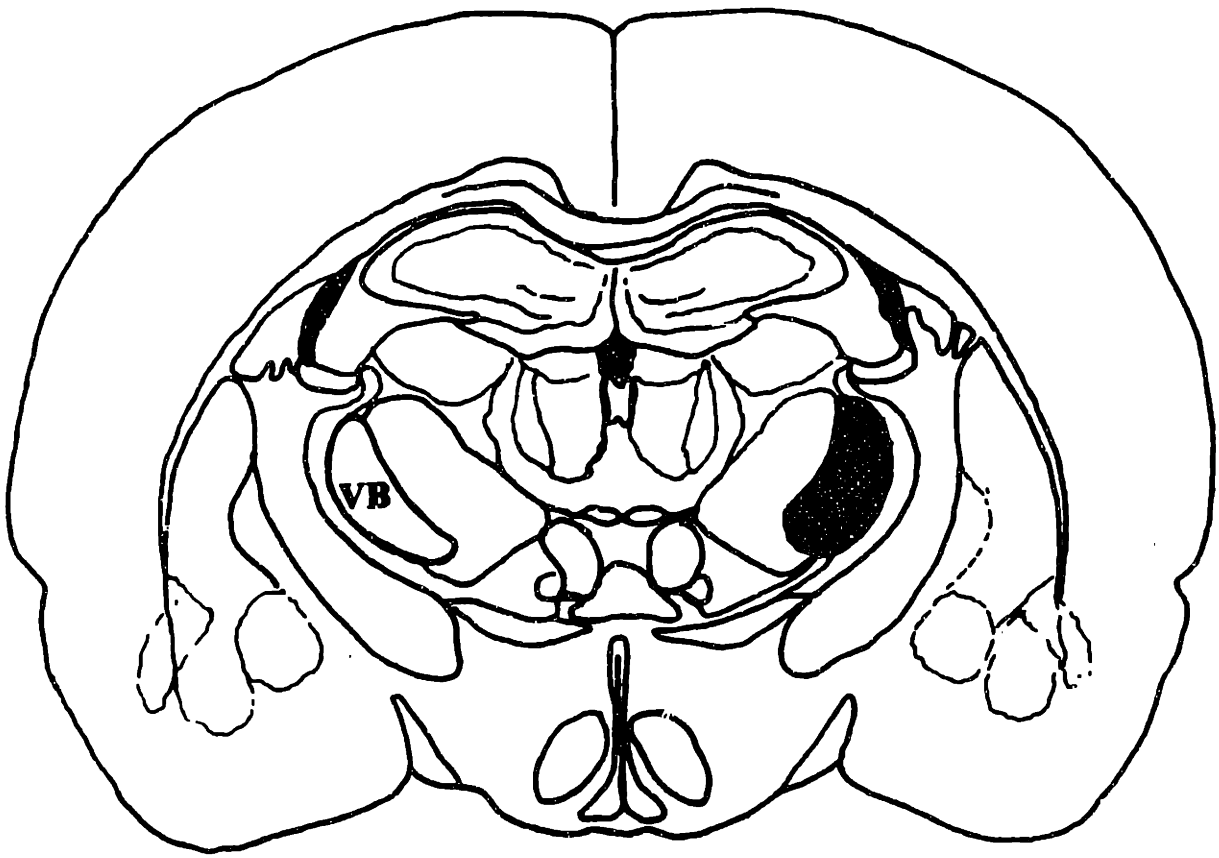


Fig. 9) The ventrobasal nucleus of the thalamus of the rat and its relation to the cortex and hippocampus are shown above (Adapted from Paxinos and Watson, The rat brain in stereotaxic coordinates).

Figure 10

Verification of Ibotenic Acid-Induced Lesion of the Ventrobasal Nucleus as shown by Gliosis

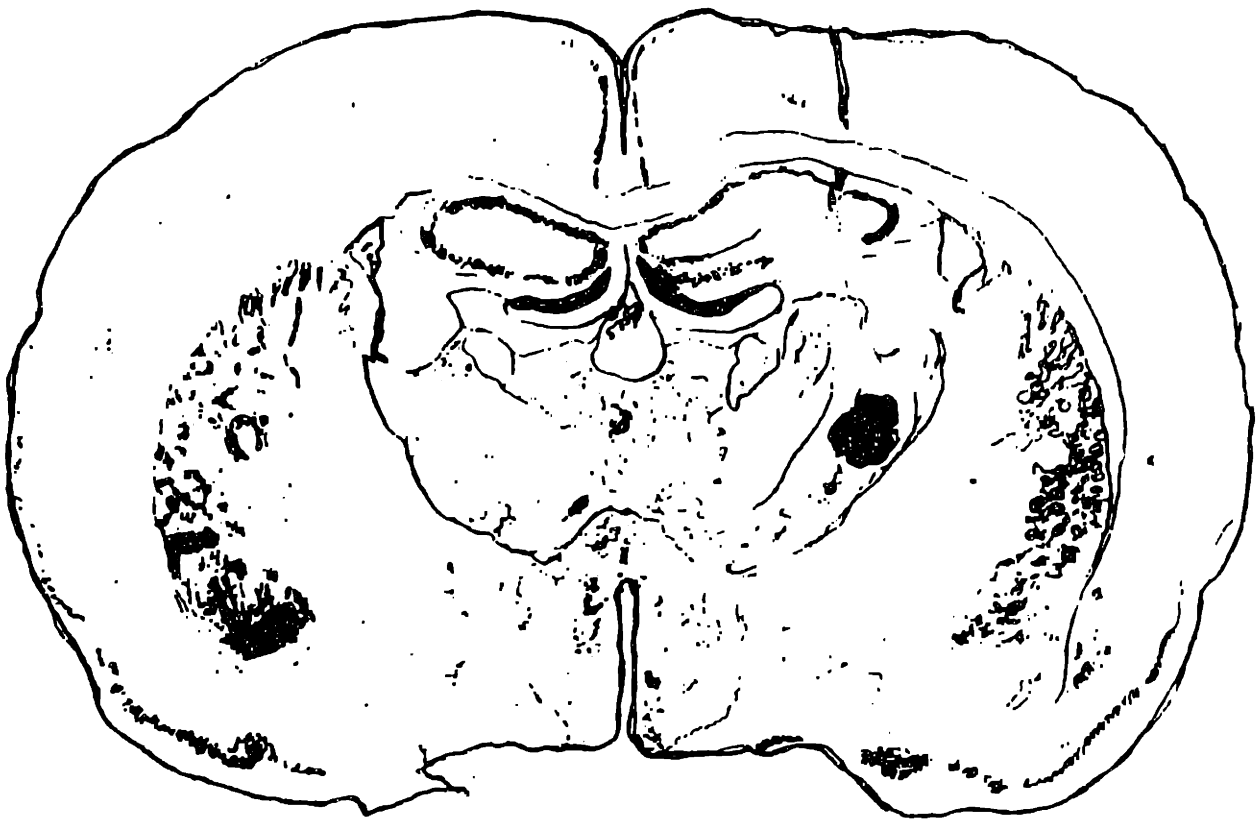


Fig. 10) Ibotenic acid injection into the ventrobasal nucleus results in lesion of the structure. The extent of the lesion is shown by gliosis.

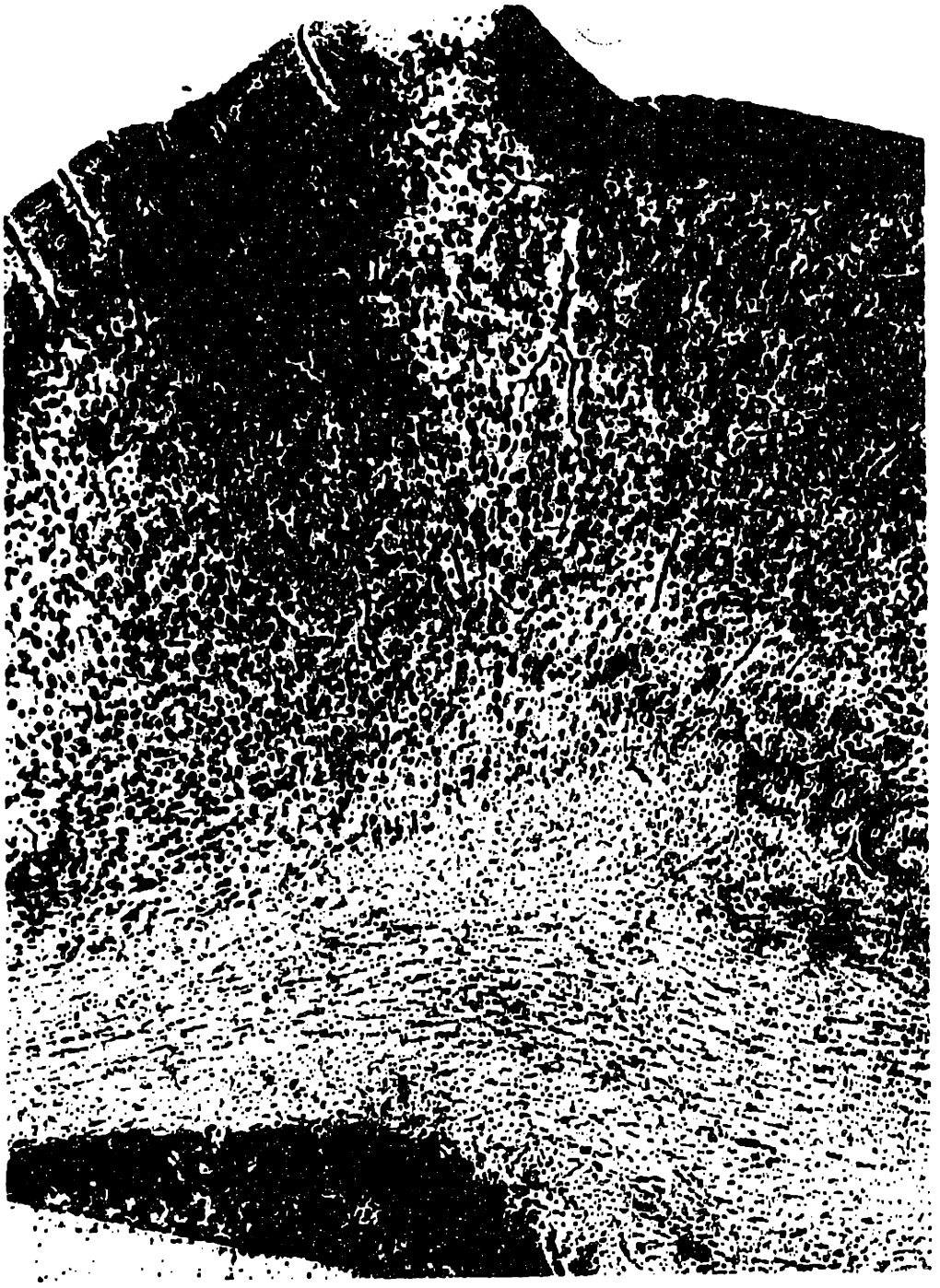
Cell loss appears to be restricted to the ventrobasal nucleus, the other thalamic nuclei do not exhibit signs of gliosis. Sections processed using the Fink-Heimer

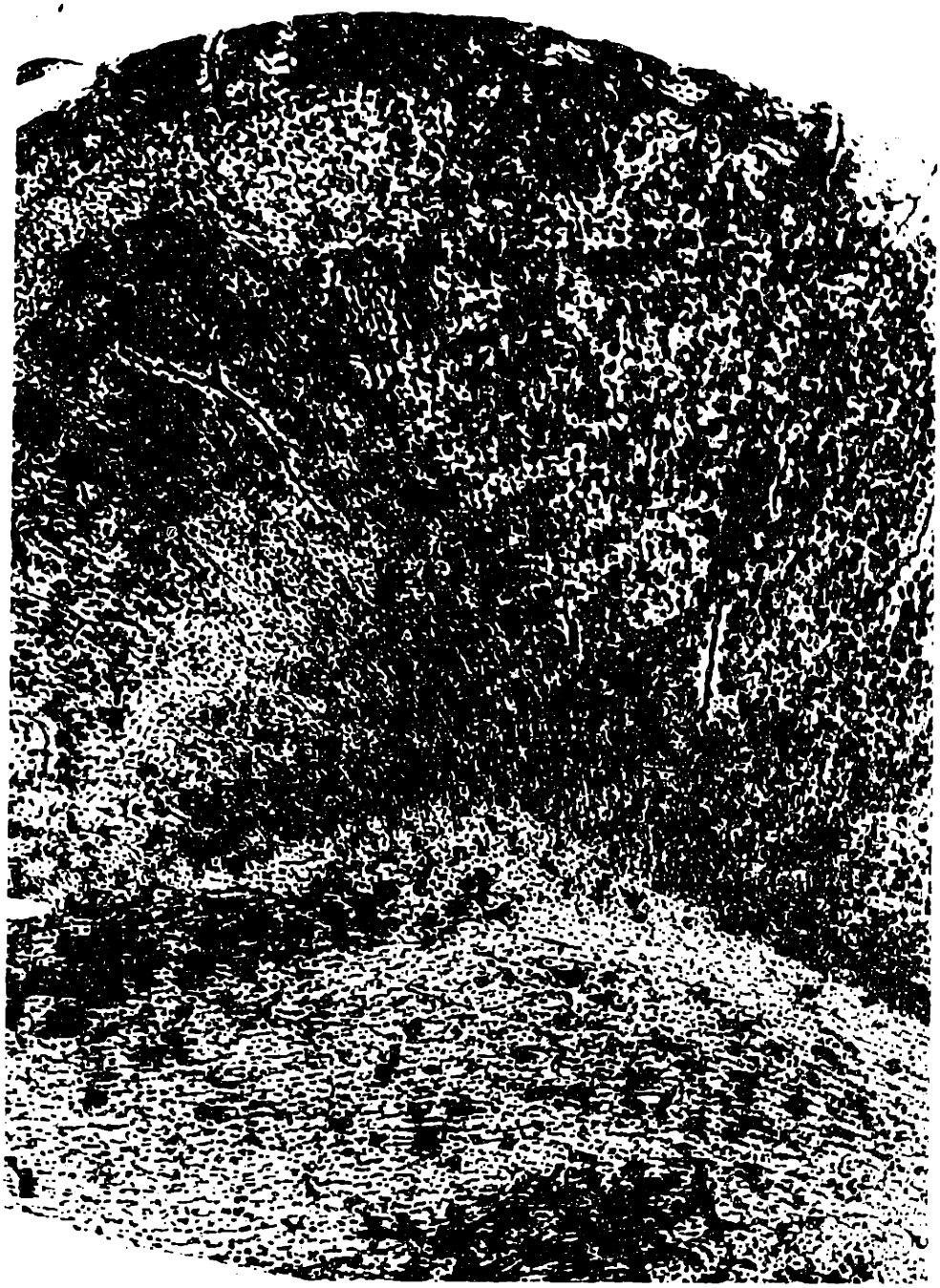
stain for the detection of fiber degeneration indicate the presence of the lesion. Degenerating axons entering the cortex are seen on the ventrobasal lesion side relative to the control side (Fig. 11). The cortex on the lesion side relative to the control side also shows degenerating fibers in superficial layers, but not in the deeper layers due to the high background staining in these areas.

Figure 11

The Degeneration of Thalamocortical Fibers Following Ventrobasal Lesions

Fig.11) Photomicrograph of control-side section of the fiber projection to cortex (page 82) is compared to a lesion-side section (page 83), showing degenerating fibers on the ventrobasal nucleus lesion-side.





Biochemical verification of ibotenic acid-induced and transection lesions

Ibotenic acid induced lesions of the NBM were verified by the comparison of ChAT activity in lesioned side vs. non-lesioned side cortical tissue. The effects of ibotenic acid-induced NBM lesions were also compared to a set of animals which received sham lesions. Unilateral lesion of the NBM was accomplished by the injection of 0.5 ul of ibotenic acid over 5 minutes with a fine glass cannula at the coordinates given above. The rats were sacrificed one week later for tissue harvest.

Another set of animals received sham lesions, where a glass infusion cannula containing phosphate buffered saline was stereotaxically placed in the brain at 0.5 mm ventral to the co-ordinates used for the ibotenic lesioned animals to avoid mechanical damage to the large cells of the NBM. Sham lesion rats were sacrificed one week after the operation.

Frontal cortical tissues were compared between the lesioned and unlesioned sides in both ibotenate and sham groups. Choline acetyltransferase (ChAT) activity was significantly lower in cortical tissue on the ibotenic NBM lesion side relative to control side cortex in the same animals (Table 1). There was no difference in ChAT activity in sham lesioned cortex relative to contralateral control cortex (Table 1).

Table 1

Effects of Nucleus Basalis Lesions on Frontoparietal Cortical ChAT Activity

Experimental Group	ChAT activity (nmol ACh/ug prot/hr)	% decrease relative to control
IBO control	395±14	
IBO lesion	291±15***	-26%
SHAM control	485±28	
SHAM lesion	464±25	-3%

Tissues were collected and assayed for choline acetyltransferase (ChAT) activity one week after rats were treated with ibotenic acid (IBO) injected in the nucleus basalis magnocellularis (nBM); or cannula placement without injection (SHAM). Data are expressed as means ± SEM, (n = 14 for IBO or 8 for SHAM). ***$p \leq 0.005$ differs from the control group.

The lesion side decrease in ChAT activity following ibotenic acid injection into the NBM indicates the loss of ChAT containing terminals in cortex which project from the nBM. ChAT is a pre-synaptic marker specific to cholinergic neurons. Anatomical studies combining ChAT immunohistochemistry combined with tracing and lesion methods have been utilized to determine the origin and distribution of cholinergic arbors in cortex (Eckenstein et al., 1988). It has been estimated that app. 40% of cortical cholinergic arbors are of intrinsic origin. Extrinsic projections account for the remainder, of which app. 50 % of the total cholinergic arbors project from the basal forebrain nucleus basalis and 10 % project from midbrain cholinergic cell groups associated with the reticular activating system. From this distribution of ChAT positive arbors, it follows that

a 25 % decrease in cortical ChAT from a NBM lesion reflects an approximate 50% loss of cells in the NBM. Thus, the observed lesion-induced decrease represents a partial lesion model of cholinergic deafferentation. The absence of change in the sham-lesion ChAT activity indicates this procedure does not damage the NBM projection to cortex. The magnitude of decrease in ChAT activity in the ibotenate-lesioned group is comparable to other studies using single injection lesions of the NBM.

The time course of cortical ChAT depletion following ibotenic acid induced NBM lesion was examined by comparing lesion side to non-lesioned cortical ChAT activity at 3, 7, 15, and 90 days after unilateral lesion. The greatest depletion of ChAT activity was observed at 7 days post-lesion (Fig 12).

Figure 12

Choline Acetyltransferase Activity in Frontoparietal Cortex Decreases, Then Recovers Following Nucleus Basalis Lesions

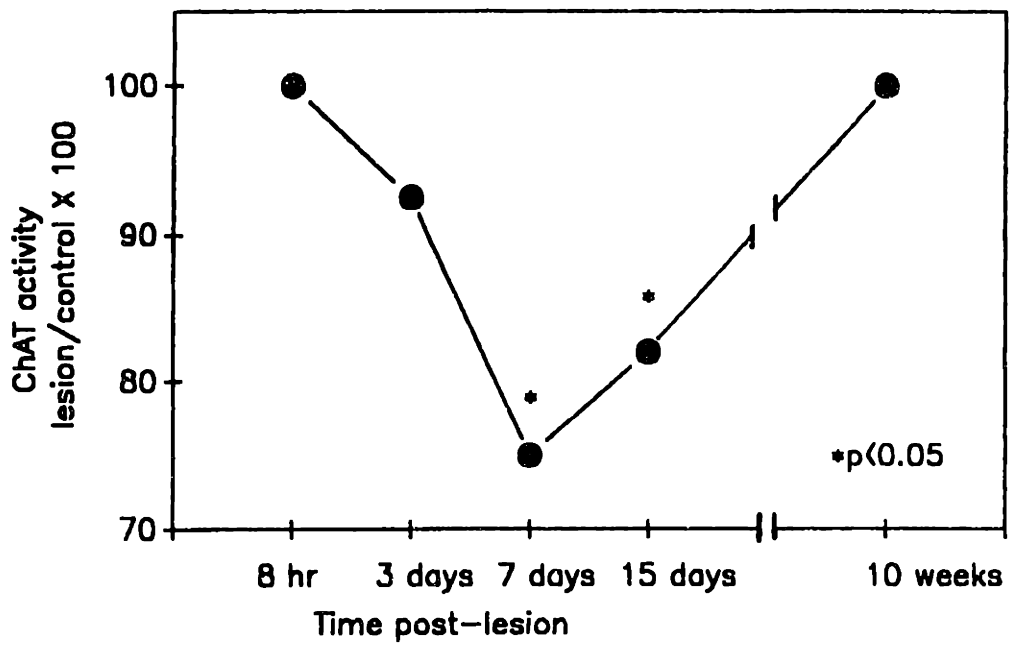


Fig. 12) The ratio of choline acetyltransferase (ChAT) activity in lesion- to control-side cortex was determined at post-lesion intervals ranging from 8 hours to 10 weeks. No significant change in cortical ChAT activity was observed at 8 hrs or 3 days following the unilateral injection of ibotenic acid. Significant decreases in cortical ChAT activity were observed at 7 days following the lesion, this deficit persisted at 15 days post-lesion. Cortical ChAT activity recovered to control-side levels by 10 weeks following the lesion. Data are expressed as the ratio of lesion to control side levels, * $p<0.05$, n=5-6/group.

ChAT activity was significantly lower at 15 days, but showed recovery to normal values at 90 days following the lesion (Fig 12).

The peak ChAT depletion in cortex at 7 days following NBM lesion is in agreement with most published reports (Wenk et al., 1984; Belleroche et al.,

1987). Recovery of ChAT activity following NBM is controversial as some groups observe full recovery within 90-180 days, while other groups do not see recovery at all (Wenk et al., 1984; Bartus et al., 1985; Belleroche et al., 1987). This discrepancy appears to be due to variations in the magnitude of the lesion. Partial lesions of the NBM using well controlled single injections of ibotenic acid leave some remaining cells and intact projections to the cortex. Larger lesions may completely destroy the nucleus, such lesions are induced by using higher concentrations of ibotenic acid, larger injection volumes, and/or multiple injections at different sites. The ChAT-positive cells of the nBM are diffusely distributed over 2 mm in the anterior-posterior plane and approximately 0.4 mm in the medial-lateral plane, so a procedure which destroyed the all cells of the NBM would also cause a great deal of non-specific damage to the globus pallidus and other adjacent structures. This procedure obviates the possibility of recovery, as there are no remaining cells or terminals. Such large non-specific lesions also disrupt the local basal forebrain circuitry and inputs, thereby impeding the function and recovery of remaining cholinergic cells (Lindefors et al., 1992).

The recovery of ChAT in cortical tissue following the lesion could reflect: 1) no change in the distribution of remaining ChAT positive arbors and an upregulation of ChAT per remaining terminal; 2) increased lateral compensatory sprouting of ChAT positive arbors into adjacent denervated areas; 3) compensatory sprouting from surviving cells of the ipsilateral and/or contralateral NBM (Fig 13).

Figure 13

Models for Choline Acetyltransferase Recovery

in Cortex Following Nucleus Basalis Lesions

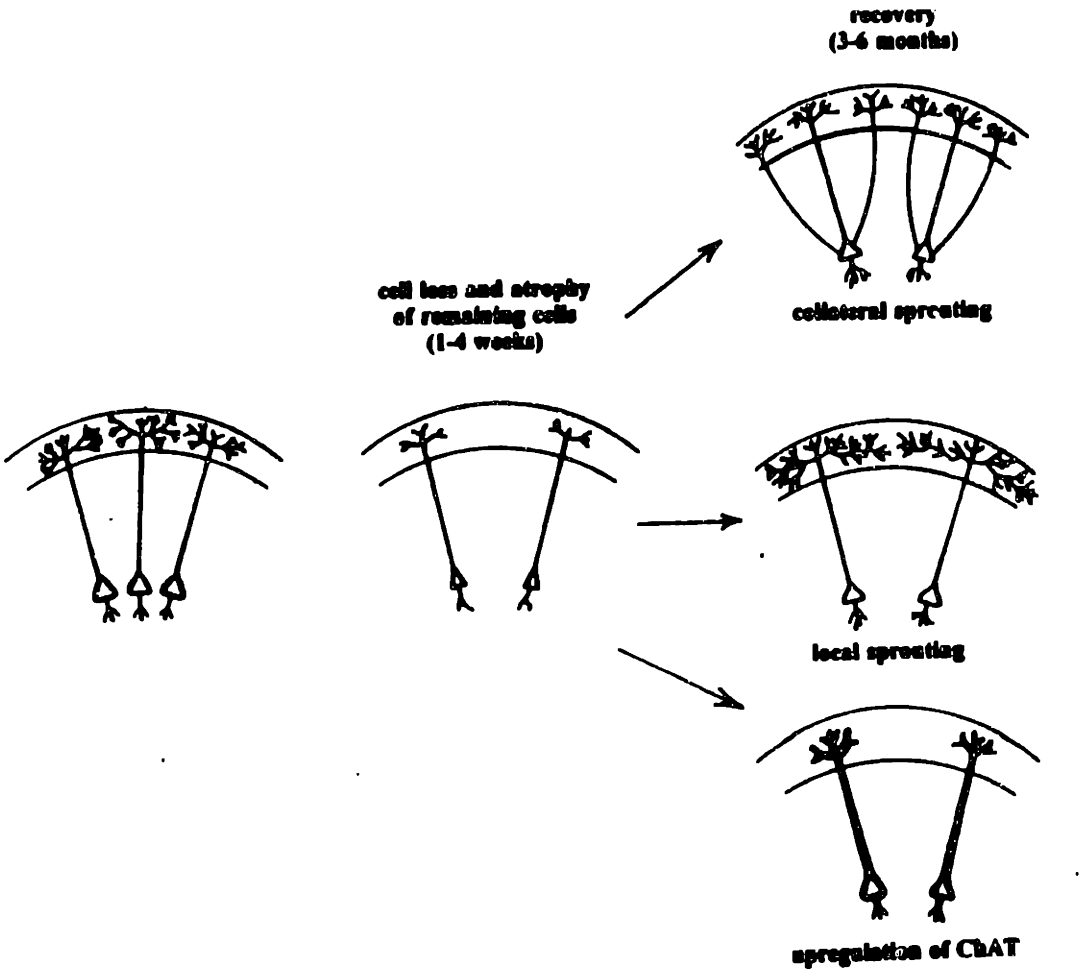


Fig. 13) The recovery of cortical choline acetyltransferase (ChAT) activity could occur by one or more mechanisms: sprouting from surviving cells of the nucleus basalis (A), local lateral sprouting in the cortex by remaining cholinergic axons (B), or upregulation of ChAT in remaining terminals without morphological changes in distribution (C).

Cortical acetylcholinesterase staining (AChase) and nerve growth factor receptor (NGFr) immunohistochemistry indicates that severe atrophy of putative cholinergic cells and disorganization of local fiber networks in the basal forebrain occurs between 1 and 4 weeks occurs following quisqualate lesions of the NBM (Unger and Schmidt, 1992). The density of AChase-positive fibers in cortex is severely reduced by 1 week post-lesion. Sprouting of AChase-positive fibers from surviving NBM cells is seen at 3 months but these projections are restricted to the basal forebrain and do not reach the cortex, additionally sprouting of intrinsic AChase-positive cells in cortex was not observed. AChase density does not recover in the cortex at 6 months following this type of excitotoxic lesion. The authors conclude that denervated regions of cortex do not receive compensatory cholinergic inputs following NBM lesions. In contrast to this finding, sprouting of AChase positive fibers from severed axons has been observed following cortical transection of medial cholinergic fibers (Farris et al., 1993). These fibers traverse the gliotic scar and apparently reinnervate the cortex. These authors report that the lamina specific pattern of AChase is re-established and interpret that their findings represent long distance sprouting of central cholinergic axons. This would be remarkable, as their results indicate that these axons also terminate in their correct target regions, as shown by the lamina-specific recovery. These results indicate that basal forebrain cholinergic axons have the capacity to regenerate in adult animals and are capable of migrating to their correct cortical targets in the absence of signals which presumably disappear following normal development. Unfortunately, AChase

staining is not specific to cholinergic neurons and is not a reliable marker for the assessment of cholinergic recovery. The staining pattern observed may be due to sympathetic fiber ingrowth, which is well characterized following surgical deafferentation of the hippocampus (Gage et al., 1984).

Cortical/fimbria fornix transections were performed to surgically sectioning the cholinergic fiber bundles entering these structures. These animals were sacrificed at either one week or three weeks after surgery, tissues anterior and posterior to the surgical section were collected.

Table 2

Effects of Surgical Transection of Cholinergic Afferent Pathways on Choline Acetyltransferase Activity in Frontoparietal Cortex and Hippocampus

Experimental Group	1 week post-lesion ChATactivity (dpm/mg prot)
Control cortex	222±32
Lesion cortex	97±17* (-56%)
Control hippocampus	305±14
Lesion hippocampus	127±9* (-58%)

Tissues were collected and assayed for ChAT activity one week after rats were treated with surgical transection of cholinergic afferent fibers. Data are expressed as means ± SEM, (n = 6). *$p \leq 0.05$ differs from the control group.

The ChAT depletion in the surgical section animals observed in lesion side

cortex is the result of sectioning the consolidated fibers of the NBM which project en route to posterior cortex. This procedure may also damage projections from midbrain cholinergic nuclei which preferentially target midline cortices. This manipulation appears to result in the maximal depletion of cortical ChAT in the rat because of the presence of cholinergic interneurons in this species. Sections from anterior cortex relative to the section showed no differences in ChAT activity between lesion and non-lesioned side. The expected result was an increase in ChAT in cortical tissues anterior to the section by the accumulation of axonally transported ChAT, although such an increase may have occurred before 7 days and subsequently normalized.

ChAT activity was also examined in the cortex of rats following ibotenic acid lesions of the ventrobasal thalamus in order to determine whether this procedure damaged cholinergic projections to the cortex, no significant difference was observed between lesioned side and non-lesioned side cortex.

Biochemical verification of the ventrobasal thalamic lesion procedure is problematic as the putative transmitter(s) for this projection are the excitatory amino acids glutamate/aspartate. ChAT activity is found exclusively in cells which synthesize and release acetylcholine, in contrast, all cells contain the enzymatic pathways for amino acid metabolism. Thus, enzymatic comparisons are not suitable for assaying the effects of lesioning. Almost all neurons appear to have glutamate receptors and there is no evidence for specific receptor subtype localized to presynaptic glutaminergic terminals. It has been suggested that high-affinity

glutamate uptake is a marker for glutamate neurotransmission and is restricted to nerve terminals which release glutamate. This suggestion has not been verified with pure preparations of glutaminergic nerve terminals (Levi and Raiteri, 1973,). High- and low-affinity glutamate uptake was examined in synaptosomes prepared from ventrobasal lesion and non-lesion cortex. Reductions were observed in both uptake systems in lesioned side relative to control side cortical synaptosomes; where low-affinity glutamate uptake was reduced by 46% and high-affinity uptake was reduced by 11%. This reduction, while suggestive, does not provide direct evidence for the loss of glutaminergic terminals.

Acetylcholine release from cortical slices was examined as a functional index of cholinergic function following NBM lesions. Acetylcholine release from cortical slices is significantly reduced at 7 days following NBM lesions (Fig 14b). Lesion-side cortical acetylcholine release recovers to control levels at 15 days and recovery persists 3 months after the lesion (Fig. 14cd).

Figure 14

Cortical Acetylcholine Release Following Nucleus Basalis Lesions

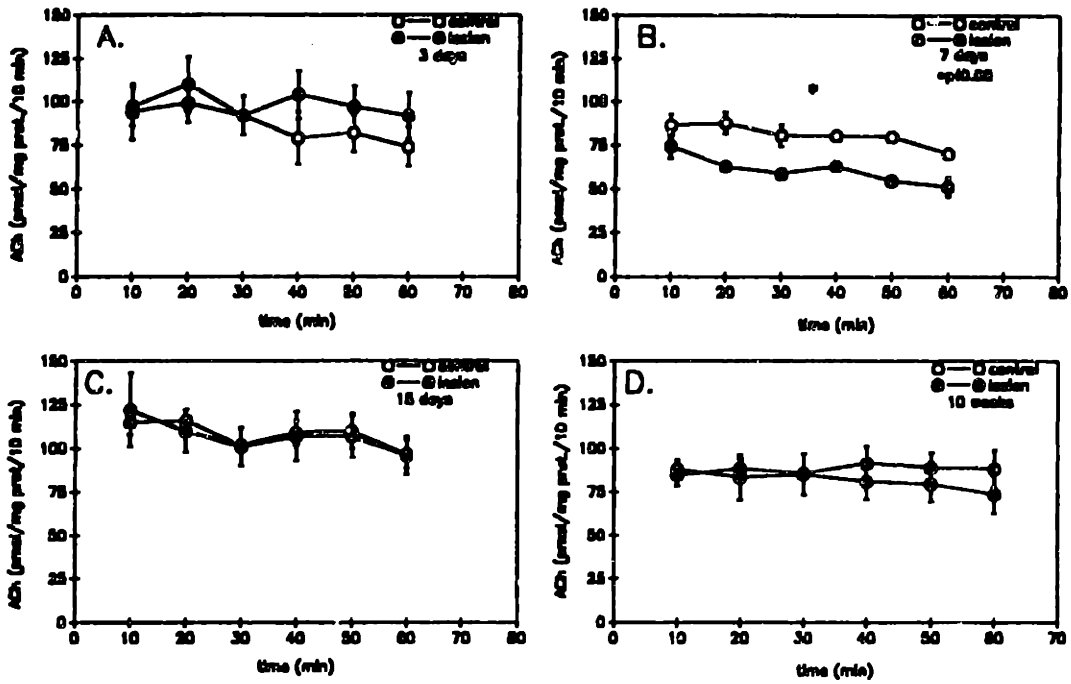


Fig. 14) Electrical evoked acetylcholine release was measured from control- and lesion-side superfused cortical slices at post-lesion intervals ranging from 3 days to 10 weeks following ibotenic acid-induced lesions of the nucleus basalis. No significant differences in evoked acetylcholine release from the lesion-side cortical slices were observed at 3 days following the ibotenic acid injection (A). Evoked acetylcholine release from lesion-side relative to control cortical slices was significantly lower at 7 days post-lesion (B, *$p \leq 0.01$, n=5). The lesion-induced deficit in acetylcholine release recovered to control-side levels at 15 days post-lesion (C), the recovery persisted at 10 weeks post-lesion (D).

The magnitude of the peak reduction of acetylcholine release at 1 week is consistent with other measures which indicate a partial cholinergic deafferentation of cortex following single site NBM lesions. This reduction co-incides with the

maximal reduction in ChAT activity. This is of interest as ChAT levels should not determine the synthesis and the release of acetylcholine. ChAT is not saturated under physiological conditions. The reduction in acetylcholine release may reflect transient metabolic factors which occur in parallel to ChAT depletions following the lesion. The release of acetylcholine is a more functional index of cholinergic neuronal activity than ChAT activity. The dissociation between these two cholinergic indices has been also observed in aging, were reductions in acetylcholine release are observed in the absence of changes in ChAT activity. The ChAT enzyme is apparently unsaturated under physiological conditions, precursor availability determines the synthesis rate and available pool for release of acetylcholine. Partial decreases observed in ChAT activity would not necessarily result in decreased acetylcholine release.

Choline efflux from cortical slices was measured in order to determine whether increased hydrolysis of choline containing lipids in lesioned side cortex, however there were no differences between lesioned and non-lesioned side slices (Fig 15).

Figure 15

Cortical Choline Efflux Following Nucleus Basalis Lesions

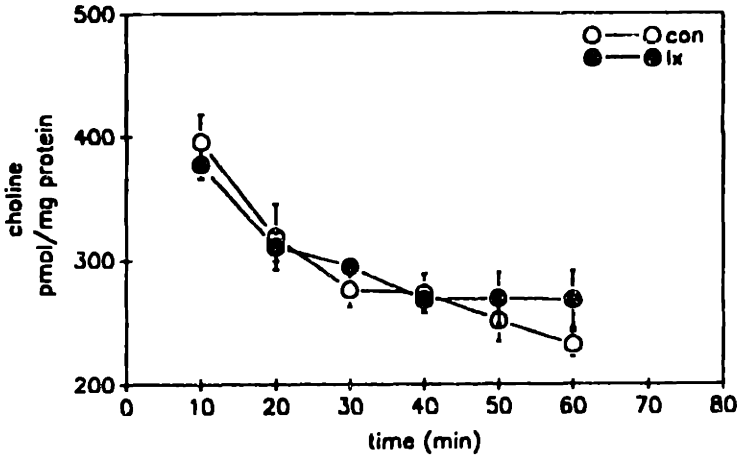


Fig 15) Stimulation evoked choline efflux from control- and lesion-side cortical slices did not differ at 7 days post-lesion.

Amino acid release was measured from slices of somatosensory cortex at 1 week following unilateral ventrobasal thalamic nucleus lesions to determine whether there were lesion-induced specific reductions in glutamate and aspartate release relative to other amino acids. Glutamate release from VB lesion-side cortex was significantly lower as compared to control-side cortex (21% decrease, $p \leq 0.05$), but the magnitude of this lesion-side decrease was similar to other non-transmitter amino acids (Table 3).

Table 3

Cortical Amino Acid Release Following Ventrobasal Lesions

Amino acid release from cortical slices following unilateral ventrobasal thalamic lesions: control- vs. lesion-side

Amino acid	control side	lesion side	% decrease on lesion side
Aspartate	34.6 + 4.4	27.1 + 1.5	22%
Glutamate	50.4 + 4.3	40.0 + 5.7*	21%
Asparagine	27.6 + 2.3	22.5 + 1.0	18%
Serine	132.7 + 13.6	117.0 + 12.8	12%
Glutamine	145.8 + 8.0	127.4 + 8.2	13%
Glycine	88.5 + 20.1	70.7 + 7.6	20%
Threonine	54.1 + 5.1	44.0 + 3.0	19%
Taurine	83.8 + 5.2	74.0 + 5.3	12%
Alanine	83.2 + 11.	63.4 + 6.6	24%
Tyrosine	38.3 + 4.6	27.2 + 3.7	29%
GABA	9.1 + 2.0	8.6 + 1.5	5%

All values are expressed as means $\pm$ SEM pg amino acid/10 ul superfusate/10 min/mg tissue protein, n=6, *$p \leq 0.05$, Newman-Keuls test.

All lesion-side amino acids measured were decreased relative to the control-side released, the magnitude of this decrease ranged from 5% for GABA to 29% for tyrosine. The average decrease for lesion-side amino acid release was 18%, which is similar to the observed decrease in glutamate, therefore, the decrease in glutamate is non-specific and is not useful for the identification of the

thalamocortical projection as glutaminergic. While it is clear that this pathway does not utilize acetylcholine or the catecholamines as transmitters, the identity of this pathway as glutaminergic is largely inferential (Jones, 1984). The complex metabolism and compartmentalization of amino acids do not allow for direct measurements of amino acid transmitter release.

Tissue protein following NBM lesion.

In order to determine whether tissue protein was an appropriate normalization factor for other biochemical measurements, the ratio of tissue total protein to tissue wet weight was compared with NBM lesion-side and control-side tissues. No differences were observed between for total protein ratios with lesion- and control-side cortex.

Individual tissue proteins were separated from NBM lesion- and control-side cortex in order to determine whether significant alterations in abundant proteins occurred 8 hr, 3 and 8 days after the lesion. There was no difference in the most abundant proteins at any of these times following the lesion. Several less abundant proteins were decreased in lesion-side cortex (Fig.16). These measurements indicate that tissue protein is an appropriate normalization factor for other biochemical measurements.

Figure 16

Cortical Proteins Following Nucleus Basalis Lesions

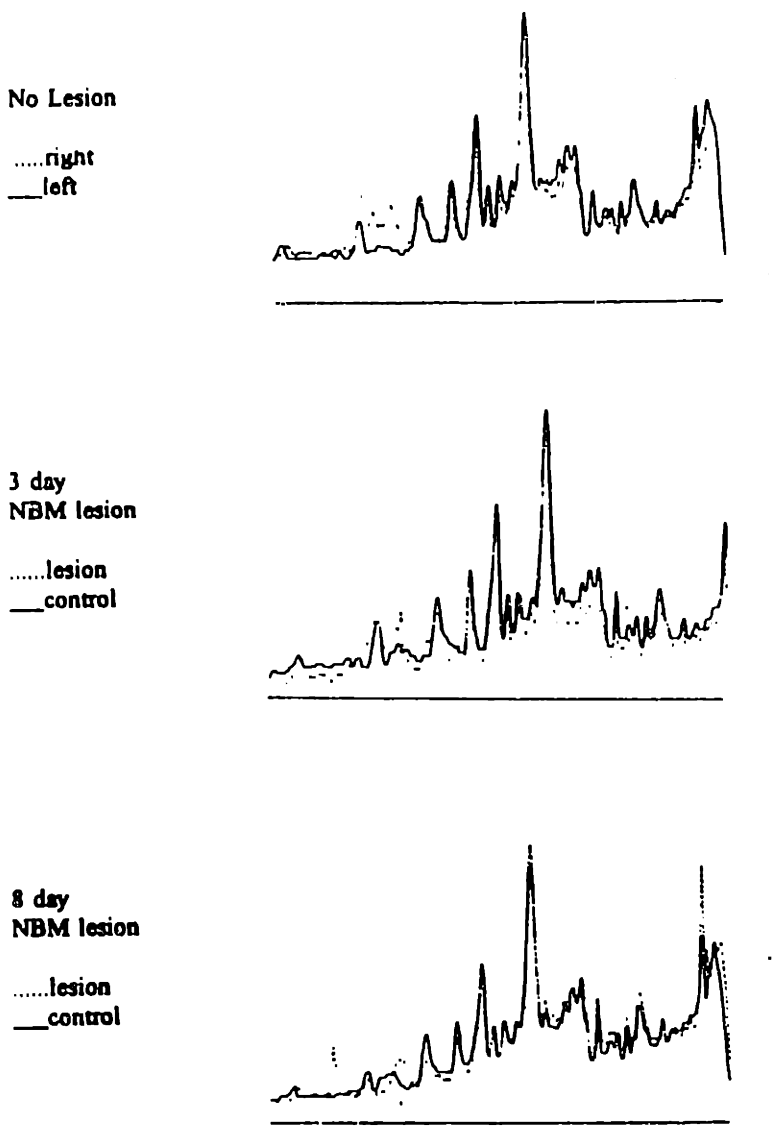


Fig. 16) Cortical protein contents was used as a normalization actor for other biochemical indices following nucleus basalis lesions. Individual proteins from control- and lesion-side cortex following ibotenic acid-lesions of the nucleus basalis were separated by gel electrophoresis in order to determine whether the levels of major cortical proteins were changed following the lesion. No major differences are seen between the control- and lesion-side, indicating that cortical protein is an appropriate normalization factor.

Phospholipid and phospholipid metabolites in cortex following lesions of ascending inputs: changes in mass.

In order to test the hypothesis that partial cholinergic deafferentation alters phospholipid metabolism in terminal cortical regions, rats received unilateral ibotenic acid-induced lesions of the nucleus basalis.

Tissue sections adjacent to those used for ChAT determination were homogenized, aliquots for phospholipid and water soluble compounds were separated by the method of Folch. Total phospholipid fractions and individual phospholipids were assayed for phosphatide content. Choline (Ch), and glycerophosphocholine (GPC) were separated by HPLC. All tissue measurements were normalized to tissue protein content (Lowry et al., 1951).

Phospholipid levels showed small, but significant decreases on the lesioned side (Table 4). GPC and Ch, water-soluble metabolites of phosphatidylcholine, also were significantly lower in lesion side cortex (Table 4). Tissue phospholipids and choline did not show differences between sham lesion and control cortex of the same animals, indicating that the changes seen following the NBM lesion are not due to non-specific cortical transneuronal degeneration following cannula placement.

Table 4

Phospholipids and Water-Soluble Phospholipid Metabolites Following Nucleus Basalis Lesions

Experimental Group	Phospholipid (nmol/mg prot)	Choline (nmol/mg prot)	Glycerophosphocholine (nmol/mg prot)
IBO control	480±20	1.16±0.10	5.50±0.40
IBO lesion	420±10* (-13%)	0.92±0.80*(-21%)	4.90±0.4*(-10%)
SHAM control	480±20	1.38±0.20	nd
SHAM lesion	480±20	1.28±0.16	nd.

Tissues were collected and assayed for phospholipid and water-soluble phospholipid metabolite levels one week after rats were treated with ibotenic acid (IBO) injected in the nucleus basalis magnocellularis (nBM); or cannula placement without injection (SHAM). Data are expressed as means ± SEM, (n = 14 for IBO, or 8 for SHAM). *$p \leq 0.05$ differs from the control group.

The plot of values of phospholipid vs. ChAT, lesion/control ChAT vs. lesion/control phospholipid, and phospholipid vs. choline are shown in figs. 17 and 18, the distribution of points indicates that these indices are not linearly related. In contrast, phospholipid vs. GPC do indicate a relation between these measures (Fig. 19).

Figure 17

Cortical Choline Acetyltransferase Activity vs. Phospholipid Levels Following Nucleus Basalis Lesions

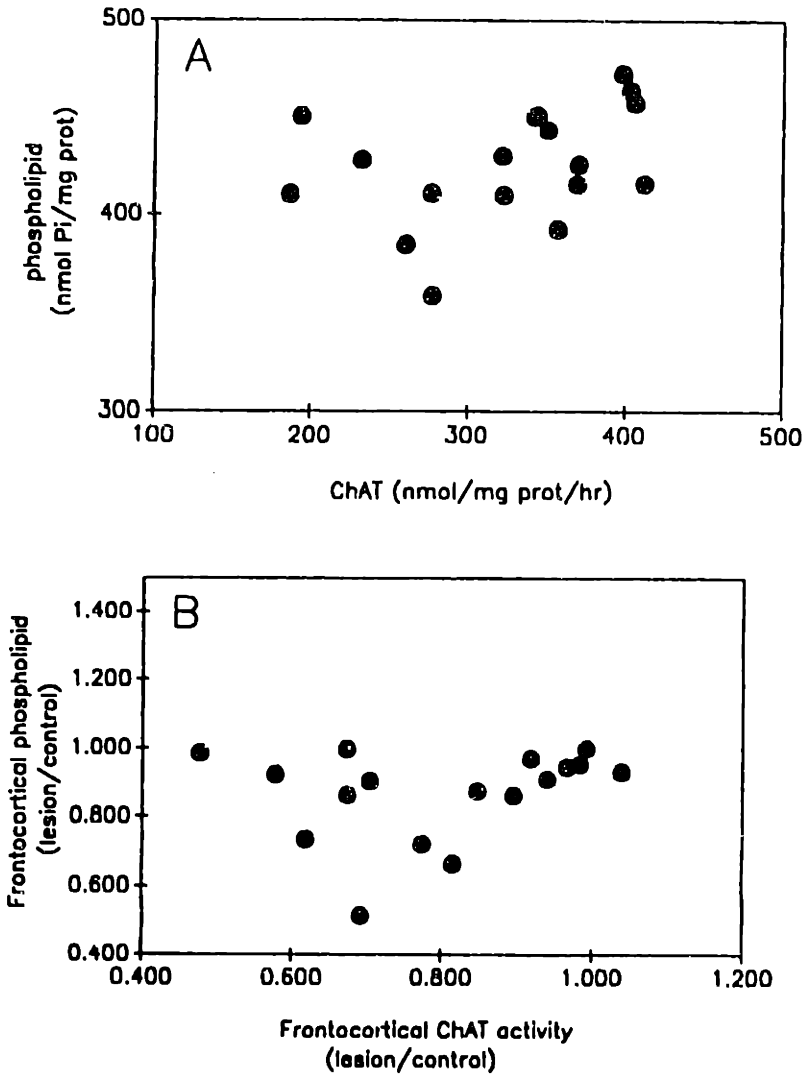


Fig 17) Cortical phospholipid levels vs. choline acetyltransferase activity (ChAT) from adjacent cortical sections were plotted in order to determine whether these indices are linearly related. There is no significant relation between phospholipid levels and ChAT activity (A). The ratio of lesion- to control-side phospholipid vs. ChAT was plotted in order to determine whether the magnitude of lesion-induced phospholipid depletion was related to the magnitude of ChAT depletion. There was no significant relationship between these ratios (B).

Figure 18

Cortical Phospholipid vs. Choline Following Nucleus Basalis Lesions

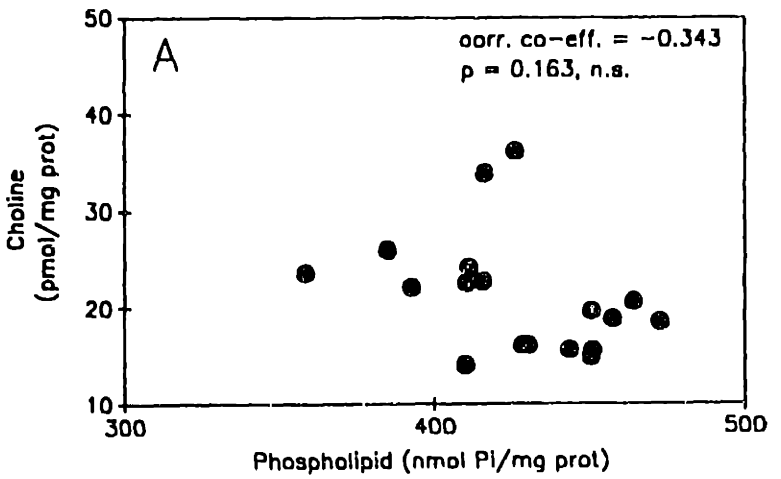


Fig. 18) Cortical phospholipid levels vs. choline levels were plotted in order to determine whether phospholipid was linearly related to the level of its precursor, choline. There was no significant relation between these indices.

Figure 19

Cortical Phospholipid vs. Glycerophosphocholine Following

Nucleus Basalis Lesions

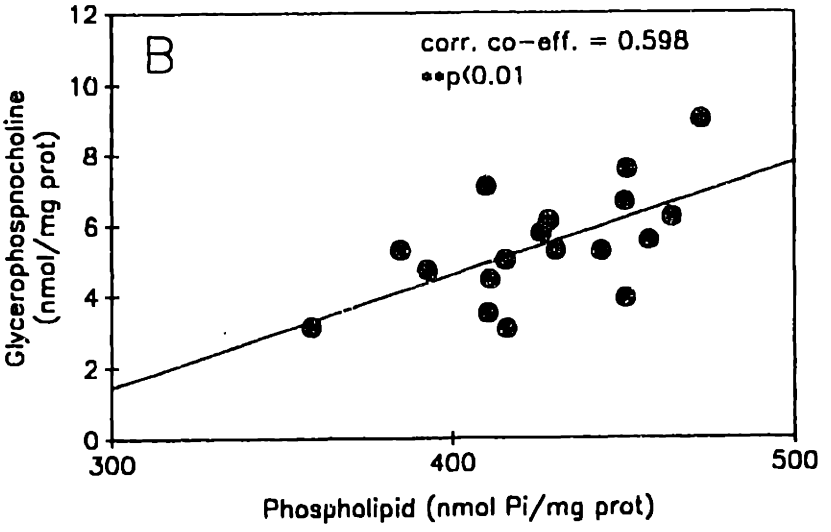


Fig. 19) Cortical phospholipid levels and glycerophosphocholine levels are linearly related (corr. co-eff.=0.598, $p \leq 0.01$, linear regression test) indicating that levels of the phospholipid catabolite glycerophosphocholine are related to phospholipid levels.

In order to determine if the NBM lesion-induced depletion in cortical phospholipids is a consequence of the initial hyperexcitation of NBM neurons by ibotenic acid, cortical tissues were collected at 8 hr after the lesion. No differences

were observed at this time (Fig 20). The maximal excitation of cortical neurons as indexed by c-fos mRNA induction occurs at this time. Thus, the cortical phospholipid depletion observed at 7 days after the lesion does not occur immediately after the injection of ibotenic acid.

Lesion- and control-side were examined at 3 days following the lesion as this time coincides with maximal decreases in metabolic indices such as SDHACU and glucose utilization. Lesion-side phospholipids were lower than the control side, but these differences are not significant at this time (Fig 20). The depletion is significant at 7 days after the lesion and recovers by 15 days to control values. The time course of NBM lesion-induced phospholipid depletion in cortex showed that the depletion is transient and differs from the time course of cortical ChAT depletion (Figs 12 & 20). The recovery of cortical phospholipid levels following nBM lesion is similar to the recovery of other metabolic indices such as SDHACU, ACh release (Fig. 14), and cortical glucose metabolism.

Tissue choline is decreased in lesion- relative to control-cortex at 7 days following the NBM lesion, but increases at 15 days post-lesion. The rapid recovery of cortical phospholipid is in contrast to the persistent depletion of ChAT at this time (Fig 12),

Figure 20

Cortical Phospholipids Levels Decrease, Then Recover Following Nucleus Basalis Lesions

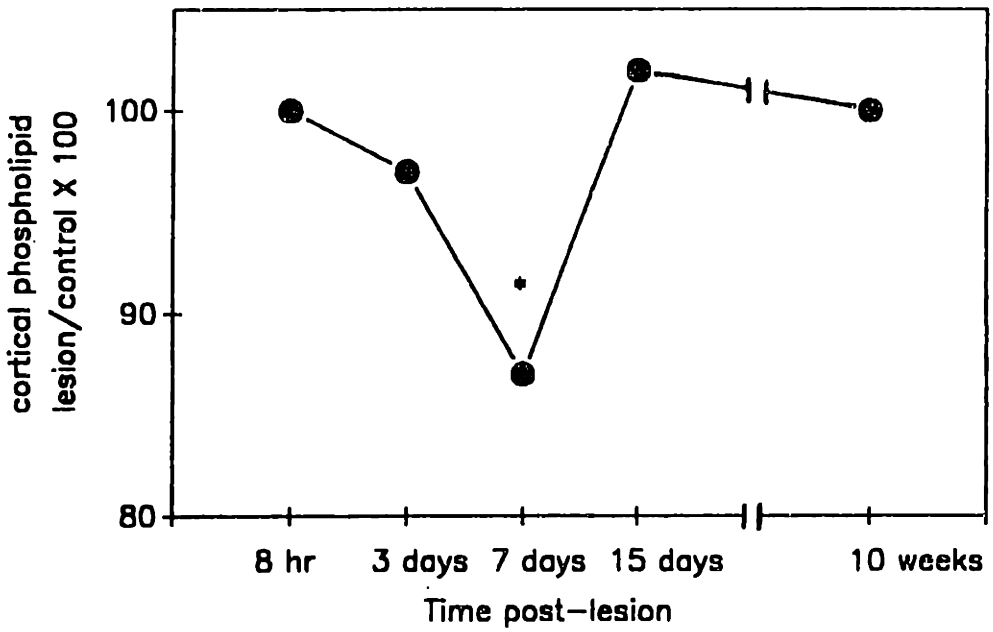


Fig. 20) The time course of the nucleus basalis lesion-induced depletion of cortical phospholipid contents is shown by plotting the ratio of tissue phospholipids in lesion- to control-side cortex at post-lesion intervals ranging from 8 hours to 10 weeks. No significant change in cortical phospholipid was observed at 8 hrs or 3 days following the unilateral injection of ibotenic acid. Significant decreases in cortical phospholipid contents were observed at 7 days following the lesion ($p \leq 0.05$), this deficit recovered at 15 days post-lesion. The post-lesion recovery of cortical lesion-side phospholipid persisted to 10 weeks following the lesion.

indicating a dissociation between the recovery rates for these two indices. The increase in tissue choline may contribute to the recovery of phospholipid levels seen at this time.

Surgical sectioning of the cortex results in a greater cholinergic deafferentation

in tissues posterior to the lesion (45% decrease) as compared to ibotenic acid lesions of the NBM (26% decrease). Phospholipid levels in cortex posterior to the surgical section are significantly decreased at 1 and 3 weeks following the lesion (Table 5). In contrast to the effects seen in cortex, cholinergic deafferentation of the hippocampus does not induce depletions of phospholipid (Table 5).

Table 5

Effects of Surgical Transection of Cholinergic Afferent Pathways on Phospholipid Levels in Frontoparietal Cortex and Hippocampus

Experimental Group	1 week post-lesion phospholipid (nmol/mg prot)	3 week post-lesion phospholipid (nmol/mg prot)
Control cortex	555±21	572±13
Lesion cortex	509±14* (-9%)	543±13* (-5%)
Control hippocampus	702±39	420±9
Lesion hippocampus	756±8	426±14

Tissues were collected and assayed for phospholipid levels one week or three weeks after rats were treated with surgical transection of cholinergic afferent fibers. Data are expressed as means ± SEM, (n = 7 for 1 week group, or 6 for three weeks). *$p \leq 0.05$ differs from the control group.

Cortical tissues in the posterior deafferented cortex show similar levels of phospholipid depletion to those seen following ibotenic acid NBM lesions at 1 week post-lesion in spite of the much larger decrease loss of ChAT activity (45% vs. 26% depletion), indicating that the absolute decrease in cortical phospholipids reaches a maximum decrease of approximately 10% (Table 4). The fiber section

causes a longer lasting depletion of cortical phospholipid as seen in Table 5 in contrast to the ibotenic acid induced-lesion of the NBM, where recovery was observed at 15 days post-lesion (Fig. 20). This may be due to the larger cholinergic depletion and/or greater non-specific damage caused by the transection.

Hippocampal tissues which showed a significant depletion of ChAT activity (mean 58% decrease on the lesion side, 1 week post-lesion) did not show phospholipid depletions at 1 and 3 weeks following the lesion (Table 5). This shows that the cholinergic deafferentation induced phospholipid is tissue specific to cortex. There are several differences in the physiological properties of the septohippocampal and basalocortical projections which may be account for the tissue specificity of the depletion: 1) activation of the NBM causes low voltage desynchronous activity in the cortex, while activation of the septal nucleus induces high voltage synchronous activity in the hippocampus, 2) the basalocortical projection is approximately 95% cholinergic, while the septohippocampal projection is less than 50% cholinergic, with GABAergic cells accounting for the majority of the projection. In addition to these well characterized differences, chemical trophic factors which influence phospholipid metabolism could be released in terminal regions by one projection, but not the other.

The transmitter specificity of the effect of deafferentation on terminal field phospholipid metabolism was examined following unilateral lesion of the ventrobasal nucleus of the thalamus (VB). The extent of mechanical non-specific damage of ibotenic acid injections into VB resemble NBM injections with respect

to cortical damage caused by the cannula, diffusion of the toxin. The transmitter identity of the VB projection is putatively glutaminergic based on indirect evidence and numerous studies indicate that it is not cholinergic or catecholaminergic (Jones, 1984). This thalamic nucleus has a topographically well defined unilateral major input into primary somatosensory cortex in the rat and consists entirely of projection neurons (Jones, 1984). The boundary of the cortical terminal region of this projection is discernable by the branch of the middle cerebral artery which allows for accurate microdissection (Welker and Sinha, 1972). Two ibotenic acid unilateral injections were made into VB to insure the maximal loss of thalamocortical axons and verification of the lesion was established by histological indices. A set of rats received unilateral ibotenic acid lesions in parallel as a positive control. The VB lesion did not effect phospholipid levels in the terminal cortical area (Table 6).

Table 6

The effects of unilateral ibotenic acid lesions of the thalamic ventrobasal nucleus (VB) and the nucleus basalis (NB) on phospholipid levels in frontoparietal cortex, one week post-lesion.

experimental group	VB lesion phospholipid (nmol/mg prot)	NB lesion phospholipid (nmol/mg prot)
control	652 + 50	445 + 13
lesion	627 + 35	394 + 10*
% lesion relative to control	-3%	-11%*

Results are expressed as means + SEM, *$p \leq 0.05$, n=7, Newman-Keuls test.

The lesion procedures were verified by histological and biochemical methods (see Figs. 10 and 11 for VB post-lesion histology). NBM lesions resulted in significant ChAT losses in frontoparietal cortex (23% depletion of ChAT on lesion side cortex, $p \leq 0.005$, n=7), while the VB lesion did not significantly alter cortical ChAT (n=7).

The unilateral NBM lesion resulted in the loss of phospholipids in frontoparietal cortex as measured one week after the lesion (11% decrease on lesion side, $p \leq 0.05$, n=7), while a similar lesion treatment of the VB nucleus did not effect phospholipid levels in the terminal cortical areas (Table 6). This indicates that the lesion-induced deficit is transmitter specific.

The depletion of cortical phospholipid levels seen after cholinergic deafferentation could reflect: 1) the loss of cholinergic terminals alone, with no changes in intrinsic cortical tissue, or 2) changes in phospholipid metabolism in cortical tissue by decreased phospholipid synthesis and/or increased phospholipid degradation. The first hypothesis is not congruent with several observations. ChAT depletion persists at 15 days after the lesion (18% decrease on lesion side), while cortical phospholipids have recovered to control levels at this time. Following cortical transection, the magnitude of ChAT depletion is nearly double that seen following ibotenic acid induced lesions of the NBM, but the magnitude of the depletion of cortical phospholipid is approximately the same following these procedures. If phospholipid depletion reflects the loss of cholinergic axons and their terminals, a much greater depletion following cortical transection would be

expected. The cortical transection procedure causes much greater non-specific damage than the ibotenic acid lesion, medial cortico-cortical projections are also transected. Non-specific axonal degeneration does not appear to contribute to the depletion of cortical phospholipid. The phospholipid depletion is tissue specific. Cholinergic deafferentation of the cortex results in depletion, while a similar deafferentation of hippocampus does not cause phospholipid depletion. If the depletion of phospholipids in terminal fields were simply due to the degeneration of cholinergic axons and terminals, one would expect similar depletions in the hippocampus following deafferentation. The deafferentation of the primary thalamic input to sensory cortex does not result in phospholipid depletion, in contrast to nBM lesions. The ventrobasal thalamic projection has between 1 and 2 orders of magnitude more axons terminating in sensory cortex compared to nBM projection in the rat, where the nucleus basalis consists of about 15,000 cells/side and the ventrobasal nucleus consists of about 250,000 cells/side. It would be difficult to explain the magnitude of the decreases seen in phospholipid, choline, and GPC without post-synaptic changes taking place. Anatomical and biochemical indices show that cholinergic synapses accounts for a small percentage of transmission in the cortex, where glutaminergic and gamma-aminobutyric acid (GABAergic) transmission predominates (Eckenstein et al., 1988). ChAT is a pre-synaptic marker specific to cholinergic neurons. Anatomical studies combining ChAT immunohistochemistry combined with tracing and lesion methods have been utilized to determine the origin and distribution of cholinergic arbors in cortex. It

has been estimated that app. 40% of cortical cholinergic arbors are of intrinsic origin. Extrinsic projections account for the remainder, of which app. 50 % of the total cholinergic arbors project from the basal forebrain nucleus basalis and 10 % project from midbrain cholinergic cell groups associated with the reticular activating system. From this distribution of ChAT positive arbors, it follows that a 25 % decrease in cortical ChAT from a NBM lesion reflects an approximate 50% loss of cells in the NBM. Based on this distribution of cholinergic inputs to cortex, it appears unlikely that the 13 % observed decrease in cortical phospholipid following a NBM lesion occurred entirely because of the loss of presynaptic elements (i.e. projections of NBM). This would require that a very large fraction of phospholipid in the cortex is in located in the cholinergic arbors of the 15,000 cells of the NBM. Morphological studies of NBM projections indicate that these cells have restricted arbors and are estimated to innervate a 1.0-1.5 mm sq area of cortex. Most cholinergic terminals are in layer V (Bigl et al., 1982; Lamour et al., 1982; Price and Stern, 1983). In comparison, estimates of cholinergic innervation of the rat striatum indicate that approximately 1% of the cells in this structure are ChAT positive (Ragsdale and Graybiel, 1985). The striatum contains five to ten times more cholinergic arbors than the cortex, based on tissue levels of ACh, ChAT activity, and ACh release. The changes in phospholipids and phospholipid metabolites in cortex following NBM lesion must reflect local post-synaptic changes in phospholipid metabolism. Gliosis, an index for local gliotic proliferation, could account for post-synaptic changes in

phospholipid if glial cells differed in their phospholipid composition from neurons. The measurement of gliosis in brains following ibotenic acid induced-NBM lesions using nissl or GFAP staining show no evidence of gliosis outside of the injection site, GFAP levels do not change in frontoparietal cortex following NBM lesions (Wallace et al., 1991).

Phospholipid biosynthetic and degradative enzyme activities in cortex following cholinergic deafferentation.

The alterations in phospholipid mass could reflect decreased biosynthesis and/or increased degradation. Enzyme activities were measured in tissue preparations from lesion-side and control-side frontoparietal cortex following ibotenic acid induced-NBM lesions.

Choline Kinase in cortex following nBM lesions.

Phosphorylation of choline by the cytosolic enzyme choline kinase directs choline into the de novo pathway for phosphatidylcholine biosynthesis. The assay conditions for choline kinase activity in rat brain homogenates was optimized for homogenate protein concentration and reaction incubation time (Fig. 21). The rate of formation of phosphocholine is linear from 0 to 30 ug, then decreases as protein concentration increases. Subsequent assays utilized protein concentrations within the linear range. The effects of reaction time indicate the formation of phosphocholine is linear in the range of 0 to 20 minutes (linear correlation co-

efficient = 0.994). The kinetic parameters of choline kinase activity in rat cortex homogenate was determined by varying the concentration of cold choline and measurement of the formation of labelled phosphocholine (Fig 21).

Figure 21

Choline Kinase Activity in Cortical Homogenates: Protein Concentration, Incubation Time, and Choline Concentration

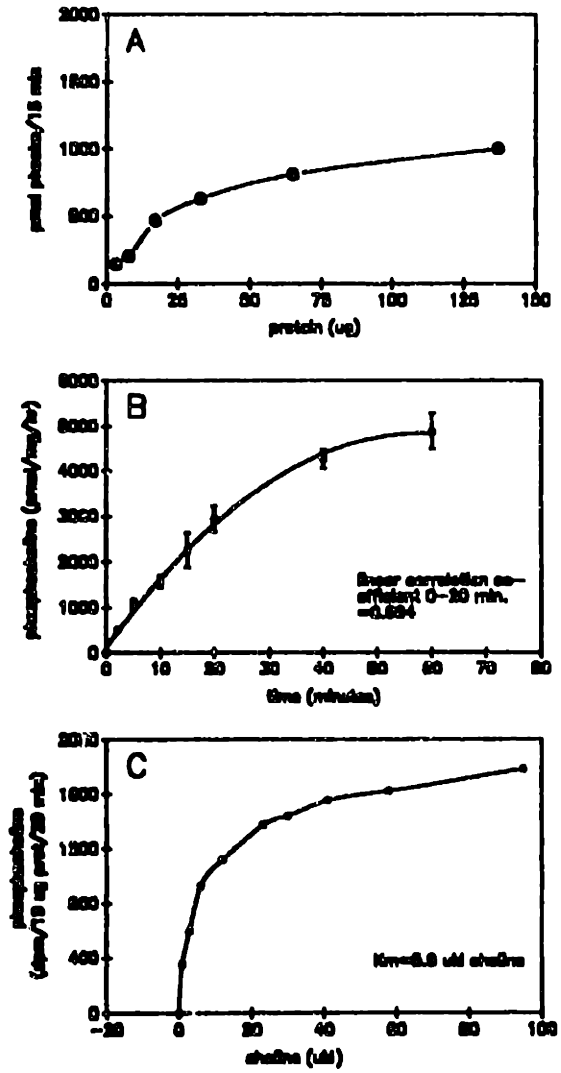


Fig. 21) Choline kinase activity in cortical homogenates was determined from 2-130 ug of protein in the reaction (A). The linearity of the reaction was determined over time, indicating that the reaction is linear from 0 to 20 minutes (B). The kinetic profile of the reaction was determined by varying the reaction choline concentration, indicating the K_m of choline is 5.9 uM and the reaction saturates near 40 uM of choline (C).

There was no detectable synthesis of phosphatidylcholine using these reaction conditions.

The effects of nucleus basalis lesion on cortical choline kinase was examined 1 week post-lesion. The lesion was verified by choline acetyltransferase activity, lesion-side activity in cortex was 28% lower as compared to the control-side. Homogenates from NBM lesion- and control side cortex incubated using reaction conditions with choline concentrations close to the K_m (10uM) and in the saturated range (80 uM). The results are shown in table 7.

Table 7

Effects of Nucleus Basalis Lesions on Choline Kinase Activity in Cortical Homogenates

Experimental Group	10 uM choline (dpm/mg prot)	80 uM Choline (dpm/mg prot)
IBO control	1207±75	1974±100
IBO lesion	1245±17	1605±50** (-19%)

Tissues were collected and assayed for choline kinase activity one week after rats were treated with ibotenic acid (IBO) injected in the nucleus basalis magnocellularis (NBM). Data are expressed as means ± SEM, (n = 5 for IBO). **$p \leq 0.01$ differs from the control group.

The lower choline concentration is in the K_m range, while the higher choline concentration is in the saturating range (Fig. 21C). There was no difference in

choline kinase activity between lesion- and control-side samples at low choline concentrations, but at saturating choline concentrations, there was a significant decrease in choline kinase activity in lesion-side samples (19% decrease, $p \leq 0.01$, n=5, Student's paired t-test). As choline kinase is the committed step for phosphatidylcholine synthesis, decreases in the formation of phosphocholine could lead to decreased synthesis of phospholipid and subsequent alterations in phospholipid mass.

The observed decrease in choline kinase activity in cortex following NBM lesions is coincident with decreased tissue levels of choline. Choline has been shown to regulate the formation of phosphocholine (Millington and Wurtman, 1982). Decreases in choline kinase activity have been demonstrated under conditions of low choline (Ando et al., 1987). Choline kinase activity is directly related to conditions of growth (Warden and Friedkin, 1985; Macara, 1988; Teegarden et al., 1990), thus the decreases in choline kinase activity following NBM deafferentation may reflect regressive events in the cortical terminal fields. It is also noteworthy that alterations in phospholipid mass in cortical terminal fields are specific to cholinergic deafferentation. The dual utilization of choline as a precursor for phosphatidylcholine and acetylcholine biosynthesis raises the interesting possibility of regulatory mechanisms between these two pathways that support acetylcholine synthesis in the cerebral cortex.

Base exchange activity in rat cortex following NBM lesions

Phospholipid remodelling can occur by the exchange one water soluble head group (choline, ethanolamine, or serine) for another. Alterations in base exchange activity could alter the ratios of phospholipids, but would not be expected to change their total mass.

Serine base exchange activity was examined in lesion- and control-side cortical homogenates in order to determine if this remodelling reaction is increased in cortex following NBM lesions. Cortical homogenates used for this experiment were prepared from tissue harvested one week following NBM lesions. The reaction was run in the presence of excess serine in order to calculate specific activity. There was no difference in serine base exchange activity between control (496 $\pm$ 25 pmol serine incorporated/ug protein/20 min, n=7) and lesion (485 $\pm$ 26 pmol serine incorporated/ug protein/20 min, n=7).

Radiolabelled Choline Incubation of Rat Cortical Slices Following NBM Lesion: Uptake and Incorporation into Phosphocholine and Phosphatidylcholine.

The uptake and metabolism of ^{14}C-choline was examined in cortical slices prepared from NBM lesioned rats in order to address the following questions. Does the uptake of labelled choline differ between lesion- and control-side cortex? Does phosphocholine formation and phosphatidylcholine formation decrease in lesion side cortex relative to control side cortex?

Slices were prepared from frontoparietal cortex, equilibrated for 1 hour in

superfusion chamber, and incubated for 1 hour in the presence of 14C-choline. Choline release from slices into the incubation medium was measured in initial experiments, which showed that the choline concentration at the end of 1 hour was 8 uM. Labelled slices were washed 4 times (10 ml/wash) with ice-cold fresh medium to remove excess label. The medium was counted after the final wash, indicating the absence of radioactivity in the medium. Choline containing compounds were separated using a Folch extraction. The labelling efficiency following 1 hr of incubation was 1% (dpm total label in slices/dpm total label in incubation medium) and approximately 15% of the label taken up was found in the organic fraction. In order to determine whether a significant fraction of choline uptake in cortical slices occurred by high-affinity choline uptake, slices were treated with 1 uM hemicholinium-3. This concentration specifically blocks the high-affinity carrier. Hemicholinium-3 treatment did not effect choline labelling or labelled phospholipid formation in slices, indicating that most choline uptake in cortical slices occurred through low-affinity carriers. This result reflects the paucity of high-affinity sites in cortex (Happe and Murrin, 1993).

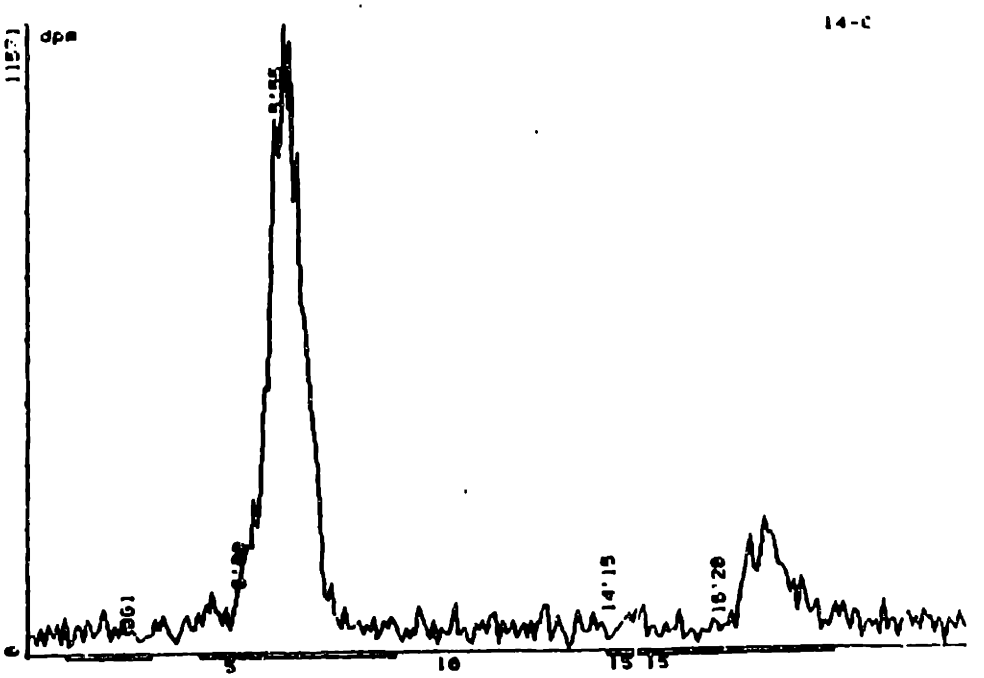
Total radioactivity in aqueous and organic fractions were examined following Folch extraction of labelled slices prepared from NBM lesion- and control-side cortex. Total radioactivity did not differ between lesion- and control-side cortical slices, indicating that total uptake did not differ (control: 63884 $\pm$ 5555 dpm, lesion: 59068 $\pm$ 5455 dpm).

Water-soluble labelled choline metabolites were separated and quantified.

Labelled choline and phosphocholine were clearly detected, but CDP-choline was not measurable in most samples (peak not distinguished from background) and labelled glycerophosphocholine was not apparent, indicating the absence of measurable breakdown products of phosphatidylcholine after the 1 hour label incubation (Fig 22).

Figure 22

Water-Soluble Choline Metabolites in Cortex Following Incubation with Radiolabelled Choline



Water-soluble choline metabolites were extracted and separated by normal phase high performance liquid chromatography from cortical slices following 1 hour of label incubation with ^{14}C-choline. Radiolabelled choline and phosphocholine were clearly detected. Glycerophosphocholine could not be detected. Refer to Fig. 3 for comparison to standards.

Distribution of ^{14}C label in cortical slices was determined following 1 hr incubation with 2 uCi ^{14}C-choline/tube. The labelling efficiency was 1% (aqueous + organic label extracted from slices/total label in incubation medium), the distribution of the label in slices is shown below (Table 8).

Table 8

Distribution of ^{14}C-Labeled Compounds in Cortical Slices Prepared from Nucleus Basalis Lesioned Rats

A) Non-Specific Distribution

Experimental Group	Total ^{14}C	Aqueous ^{14}C	Organic ^{14}C
IBO Control	63900±5600	55000±4300	9000±1400
IBO Lesion	59000±5500	51000±4200	8300±1400

B) Specific Distribution

Experimental Group	^{14}C-Choline	^{14}C-Phosphocholine	^{14}C-Phosphatidylcholine
IBO Control	7300±500	1700±500	2500±300
IBO Lesion	6600±500	1200±300	1900±200*

Cortical slices were prepared one week after rats were treated with ibotenic acid (IBO) injected in the NBM, the slices were incubated with 2 uCi of 14C-choline for 1 hour. Results are expressed as means ± SEM, (n=10). *$p \leq 0.05$ differs from the control group.

85% of the total label was found in the water-soluble fraction, while 15% of the total label in found in the organic fraction Table 8A). Individual labelled water-soluble choline metabolites were separated by normal phase HPLC. The recovery of these metabolites, based on the recovery of known standards is 80%. Individual labelled organic choline metabolites were separated by TLC. The recovery of these metabolites, based on recovery standards is 50%. Data above are normalized for quench and recovery standards (Table 8B). Choline accounts for 13% of the total aqueous radioactivity and 11% of the total radioactivity/sample (aqueous + organic). Phosphocholine accounts for 3% of the total aqueous radioactivity and 2.5% of the total radioactivity/sample. Phosphatidylcholine accounts for 28% of the total organic label and 10% of the total radioactivity/sample. The sum of the identified radioactivity/sample is 24% (Table 8B). Nearly all of the organic fraction was PC, although autoradiography showed that a very small fraction of radioactivity was in lyso-PC (these levels were counted and accounted for about 3% of the organic label). No evidence of label was seen in phosphatidylethanolamine or phosphatidylserine. Chromatographs of the water-soluble metabolites did not show peaks other than those for choline and phosphocholine (Fig. 22).

Labelled phosphatidylcholine was significantly lower in lesion-side slices relative to control (Table 8B, $p \leq 0.05$, $n=10$). A similar magnitude of decrease in lesion-side phosphatidylcholine was observed when these values were normalized for specific activity, although this difference did not reach significance (Table 8B).

These data were normalized to the total label for each sample (Table 9), in contrast to the protein normalization used for table 8

Table 9

Distribution of ^{14}C-Labeled Compounds in Cortical Slices Prepared from Nucleus Basalis Lesioned Rats

Specific Distribution: normalization to total label

Experimental Group	^{14}C-Choline	^{14}C-Phosphocholine	^{14}C-Phosphatidylcholine
IBO Control	0.844±0.035	0.194±0.014	0.148±0.013
IBO Lesion	0.831±0.026	0.129±0.024*	0.119±0.010

Cortical slices were prepared one week after rats were treated with ibotenic acid (IBO) injected in the nBM, the slices were incubated with 2 uCi of 14C-choline for 1 hour. Results are expressed as means ± SEM, (n=10). *$p \leq 0.05$ differs from the control group.

These results indicate that phosphatidylcholine biosynthesis is decreased in cortical slices following NBM lesion following the decrease in choline kinase activity. This decrease is the most likely mechanism for the decrease in phospholipid mass observed following NBM lesions. This decrease does not appear to be restricted to cholinergic neurons, as the label uptake occurred by low affinity uptake, which is found in all cortical cells.

Phospholipid catabolism in cortex following NBM lesions

Muscarinic receptor-coupled hydrolysis of inositol lipids was examined in cortex following NBM lesions in order to determine whether receptor sensitivity to agonist stimulation increases in response to cholinergic deafferentation. Muscarinic stimulation by carbachol activates all 3 major phospholipases (PL-A_2, PL-C, and PL-D). Phosphatidylinositol bisphosphate hydrolysis by PL-C was chosen as an assay system for muscarinic and serotonergic receptor responsiveness because of the stability of the reaction product inositol phosphate-1 (IP1). Serotonergic agonist sensitivity was examined in order to determine whether heterologous supersensitivity occurs following cholinergic deafferentation.

Receptor responsiveness to carbachol and serotonin with tranylcypromine was measured in cortical slices 1 week following unilateral NBM lesions. The ratio of ^{3}H-IP1:total sample ^{3}H was determined for each sample. This normalization factor was utilized in order to avoid error due to potential differences in inositol uptake, lipid incorporation, and lipid phosphorylation in the NBM lesion and control tissues. Such differences have been observed in aging. Response to agonist was expressed as the ratio of agonist stimulated to basal 3H-IP1 accumulation. Receptor sensitivity was expressed by the ratio of lesion to control side agonist response. The data were analyzed by linear regression comparing receptor sensitivity to percentage ChAT depletion as an index of the lesion.

NBM lesion significantly increased lesion-side receptor sensitivity to muscarinic

stimulation (Fig. 23, $p \leq 0.01$, n=12).

Figure 23

Muscarinic Receptor Sensitivity vs. Choline Acetyltransferase Activity in Cortex Following Nucleus Basalis Lesions

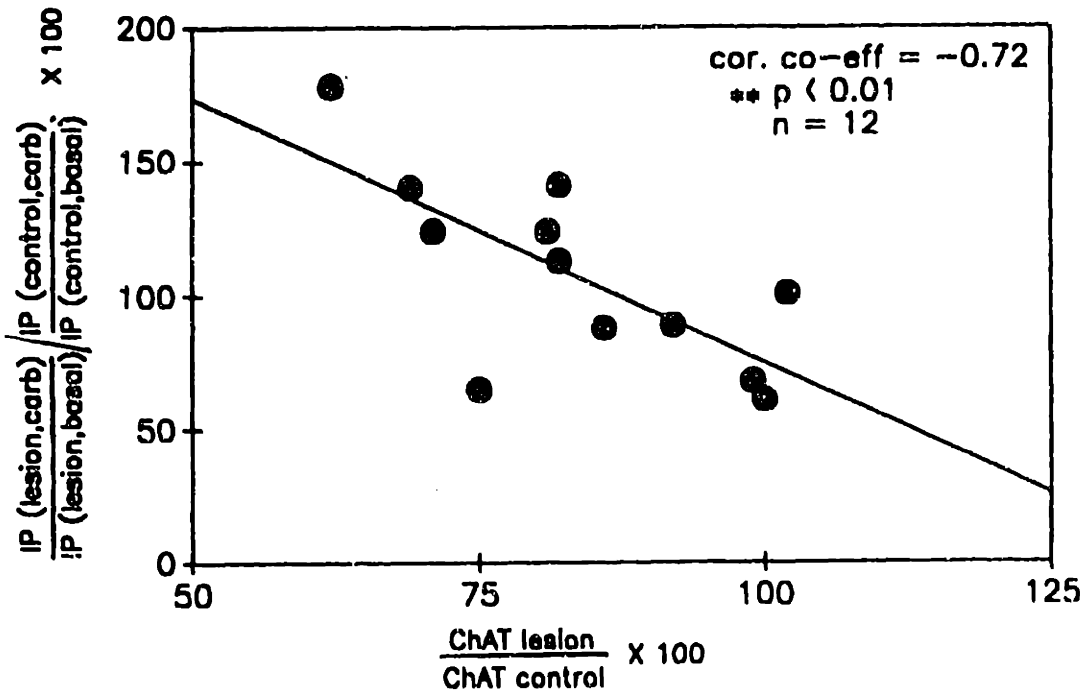


Fig. 23) The ratio of lesion- to control-side muscarinic receptor responsiveness to carbachol significantly increases relative to the severity of choline acetyltransferase depletion (correlation co-efficient=-0.72, **$p \leq 0.01$, n=12, linear regression test).

A similar increase in serotonergic receptor sensitivity to stimulation ($p \leq 0.05$, n=7) was observed following NBM lesions. Basal ^{3}H-IP1 and ^{3}H-lipid

accumulation did not significantly differ in lesion- and control-side cortex. The increase in lesion-side muscarinic receptor responsiveness is due to increased receptor-effector coupling of post-synaptic muscarinic receptors, as total muscarinic receptors and postsynaptic m1 receptors are acutely decreased following NBM lesions (McKinney and Coyle, 1982; Bellerache et al., 1985; Watson et al., 1985; Dawson et al., 1991).

The results presented above are in agreement with several reports increased muscarinic receptor responsiveness in cortex following NBM lesions by excitotoxin (Reed and Bellerache, 1988) and colchicine injection (Mundy et al., 1991). In contrast, several groups report no change in carbachol-induced inositol lipid hydrolysis following NBM lesions by excitotoxin injection (Raulli et al., 1989; Scarth et al., 1989) and electrolysis (Shoham et al., 1990). Several errors in experimental design and discrepancies were found in the papers which report negative findings: basal IP1 accumulation is not reported following NBM lesions (Raulli et al., 1989; Scarth et al., 1989; Shoham et al., 1991); biochemical verification of the lesion in cortex was not confirmed for the samples that were used for IP1 accumulation (Raulli et al., 1989; Shoham et al., 1989); the post-lesion time for tissue harvest was varied (2-3 weeks), but data were statistically treated as 1 group (Shoham et al., 1991). The increase in lesion-side carbachol response in cortex following NBM lesion is supported by electrophysiological and behavioral studies (Lamour et al., 1982; Mastropaulo and Crawley, 1988)

Similar experimental lesion systems indicate agonist-induced inositol lipid

hydrolysis is increased following septohippocampal lesions (Connor and Harrell, 1989) and lesion of putative glutaminergic pathways (Nicoletti et al., 1987). Changes in receptor sensitivity have been shown in partial lesion studies of the dopaminergic nigrostriatal pathway (Melamed et al., 1982). Other compensatory events including increases in the firing rate of remaining nigrostriatal cells in the dopaminergic partial lesion model raise the interesting possibility that the decreases in cortical phospholipids seen following NBM lesion may reflect increased membrane turnover in response to increased firing of remaining cholinergic cells. If this occurs, this could facilitate the demand for choline at these synapses.

These in vitro results indicate that compensatory receptor supersensitivity in terminal cortical fields occurs following cholinergic deafferentation, but does not address the issue of whether the magnitude of these events could contribute to increases in phospholipid turnover in vivo. One study has examined cortical inositol phospholipid turnover in vivo following NBM lesions and concluded that while agonist administration increased phospholipid turnover, no difference was observed between lesion- and control-side in the absence of pharmacological stimulation, thus basal inositol phospholipid turnover does not appear to be altered following cholinergic deafferentation (Nariai et al., 1991). Such receptor changes would contribute to compensatory increases in lipid turnover, but this increase does not appear to be large enough to account for the decreases seen in cortical phospholipid mass following NBM lesions.

Summary and conclusions

The results presented in this thesis show that basal forebrain cholinergic inputs exert control over cortical phospholipid metabolism as demonstrated by deafferentation by lesioning of cholinergic cell bodies or transection of cholinergic axons. Lesion procedures were verified by choline acetyltransferase immunohistochemistry, the presence of gliosis, and choline acetyltransferase activity in the lesion-side cortex. Ibotenic acid injection into the nucleus basalis results in decreases in cortical phospholipids at 7 days following the lesion, this depletion was not observed at 8 hours or 3 days following the injection. The lesion-induced depletion of cortical phospholipids recovered to control levels at 15 days. This post-lesion recovery co-incided with the recovery of evoked acetylcholine release from cortical slices. Transection of cholinergic axons caused similar depletions of cortical phospholipid levels at 7 days following the lesion. This depletion persisted to 21 days following the lesio The decreases in cortical choline acetyltransferase activity observed following axon transection lesion were greater than those seen following ibotenic acid injections into the nucleus basalis. Sham lesions did not change cortical phospholipid levels, indicating that non-specific damage to the cortex did not lead to the phospholipid depletion.

Choline kinase activity in nucleus basalis lesion-side cortical homogenates was decreased relative to control-side cortex under saturating levels of choline. Metabolic labelling of lesion- and non-lesion cortex with choline showed

decreased labelling of phosphatidylcholine. Serine base exchange activity in cortex did not change following nucleus basalis lesions. These results indicate that decreased cortical phospholipid synthesis occurs following nucleus basalis lesions.

Receptor-mediated inositol lipid hydrolysis in cortical tissues increased following nucleus basalis lesions. Responsivity to carbachol and serotonin increased, indicating that homologous and heterologous compensatory supersensitivity occured in response to the lesion.

Deafferentation of non-cholinergic ascending inputs (putative glutaminergic) did not alter cortical phospholipid levels, indicating that the cortical phospholipid depletion is transmitter-specific. Cholinergic deafferentation of the hippocampus did not result in phospholipid depletion.

The mechanism(s) of lesion-induced alterations in cortical phospholipid metabolism are of interest in comparison to complex human neurodegenerative disorders and allow for testing specific hypotheses at the cellular and systems level.

Suggestions for further research

To determine whether changes in cortical phospholipid metabolism are specific to decreased cholinergic input, lesion studies of other ascending neurotransmitters could be examined. The brainstem nucleus locus coeruleus (LC) provides the primary noradrenergic input to cerebral cortex. The effects of lesion of the LC on cortical phospholipid metabolism could be used to examine whether changes

described above are specific to decreases in cholinergic input to cortex.

Neurotransmitter precursor administration may be useful for ameliorating some of the these lesion effects. This strategy could be effective by augmenting neurotransmission by remaining intact cholinergic projections in a partial lesion or by altering phospholipid metabolism that occurs during degeneration (see appendix below). Such effects have been shown in an *in vitro* brain slice model (Ulus et al., 1989, Buyukuysal et al., 1991). Choline and aminopyridine administration have membrane protecting effects as well, both agents protect against electrical stimulation phosphatide depletion (Buyukuysal et al., 1991).

Aminopyridines have been shown to augment acetylcholine release in striatal slices from aged rats, another cholinergically compromised system (see appendix below). Acetylcholine release and tissue phospholipids following nucleus basalis lesions may be significantly increased by aminopyridine treatment.

Choline has been shown to counteract the depletion of tissue acetylcholine evoked by atropine (Wecker et al., 1978). Experiments on the effects of atropine administration on tissue phospholipid levels could provide additional evidence as to whether the NBM lesion induced changes in terminal cortical fields are transmitter specific. If such effects of atropine induced depletion of phospholipids are seen, it would be of obvious benefit to examine the effects of choline administration.

Nucleus basalis lesion-induced depletions of cortical phsopholipids may underlie compensatory mechanisms for promoting acetylcholine synthesis and release at the

Nucleus basalis lesion-induced depletions of cortical phsopholipids may underlie compensatory mechanisms for promoting acetylcholine synthesis and release at the expense of phospholipid synthesis. It would be difficult to examine this problem with choline labelling, as choline is the biosynthetic precursor and degradative product of phosphatidylcholine and acetylcholine metabolism. In order to determine whether choline liberated by phosphatidylcholine breakdown is preferentially shunted to acetylcholine biosynthesis, the choline phospholipid must be indirectly labelled to avoid choline pool artifacts. An alternate method has been developed to address such questions, this involves the step-wise incorporation of a labelled methyl group starting with phosphatidylethanolamine to form labelled phosphatidylcholine, using labelled methionine as the methyl donor (Blusztajn and Wurtman, 1983). After incorporation into phospholipid, label appears in free choline and acetylcholine from phospholipid hydrolysis. This unfortunately requires large quantities of labelled methionine as the vast majority (>95%) is incorporated into protein. To address these problems, a third phospholipid labelling strategy could be developed using ^{14}C-lyso-1-alkyl-phosphatidylcholine (^{14}C-lysoPAF). This compound has been shown to be incorporated in cells and re-acylated to form phosphatidylcholine, pulse-chase studies show the later appearance of label in choline, phosphocholine and acetylcholine (J.K. Blusztajn, personal communication). Initial brain slice labelling experiments with this compound were not successful, as shown by the absence of label in the lipid fraction. The successful application of this labelling method in brain tissue by

The distribution of mRNA expression for choline kinase could be examined by in situ hybridrization. This may reveal site specific alterations in the expression of this enzyme following NBM lesions. Electron microscopy of cortex following cholinergic deafferentation could reveal changes in cortical morphology.

In summary, the transmitter-specific lesion approach shows promise for the further study of phospholipid and transmitter interactions in vivo and in vitro and may be useful elucidating compensatory mechanisms for supporting neurotransmission following brain lesions.

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APPENDIX

Cholinergic hypofunction in the aged rat brain

There is evidence that brain cholinergic hypofunction occurs during senescence (Bartus et al., 1982). The locus of the hypothesized deficit is unclear as both pre- and post-synaptic changes have been reported. Decreases in radiolabelled acetylcholine release (Freeman and Gibson, 1987) and decreases in muscarinic receptors (Surichamorn et al., 1988; Beigon et al., 1989) have been reported in aged rat brain In order to further characterize this deficit, basal and evoked endogenous acetylcholine release was examined from striatal slices prepared from 3 and 24 month old rats as a pre-synaptic index of cholinergic activity. Potassium evoked acetylcholine release was examined using striatal tissues from young rats in other experiments (Fig. 1).

Figure 1

Potassium-Evoked Acetylcholine Release from Striatal Slices

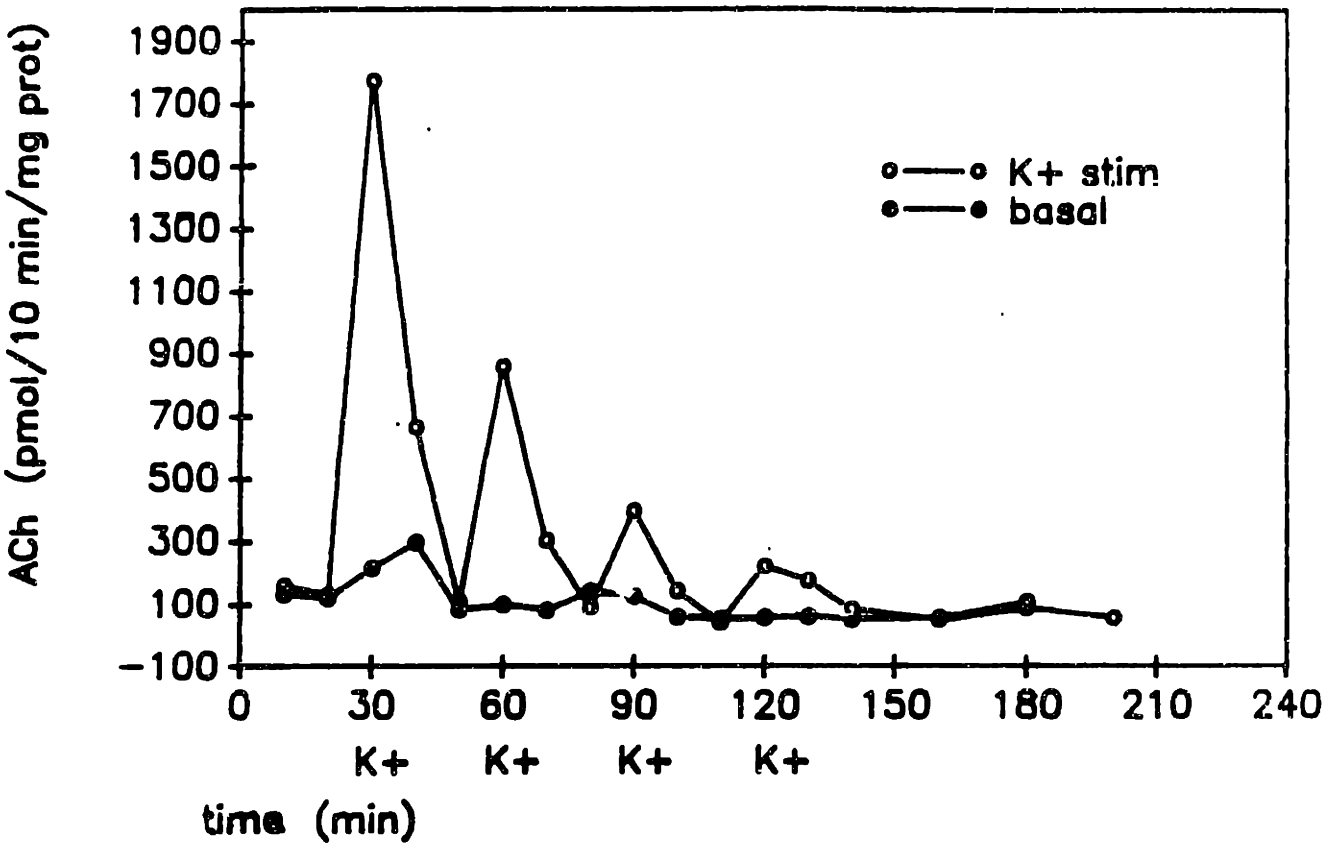


Fig. 1) Potassium-evoked acetylcholine release from striatal slices was robust for the first stimulation period, then markedly decreased during subsequent stimulation period. The inconstancy of this release may compared to the relative stability of electrical-evoked acetylcholine release shown in figures 14 in the thesis above.

The release profile showed that this release was not stable after periodic 5 minute pulses of high potassium medium, thus, this method was not used for further experiments. Carbachol stimulated inositol lipid hydrolysis in cortical slices was examined as a functional post-synaptic measure.

Basal endogenous acetylcholine release from striatal slices did differ between young and aged rats (Fig. 2a). The release profile was stable for 1 hour, the sum of the basal acetylcholine release (pmol/mg protein/hour) was 328 for young rats and 236 for aged rats. The addition of the potassium channel blocker 3,4-diaminopyridine (3,4-DAP, 10uM) to the superfusion medium significantly increased endogenous acetylcholine release from striatal slices prepared from both young ($p<0.0001$, $n=5$) and aged rats ($p<0.0005$, $n=5$) as shown in figure 3. The magnitude of the 3,4-DAP evoked increase over basal release was similar between the two age groups (young:193%, aged:231%). The potentiation of endogenous acetylcholine release by 3,4-DAP is in agreement with the finding that this treatment increased the release of radiolabelled acetylcholine (Gibson and Peterson, 1983).

Electrical field potential evoked acetylcholine release from striatal slices was significantly lower in aged rat slices (Fig. 2, $p<0.01$, $n=4$, 29% decrease), in contrast to basal release. Electrically evoked acetylcholine release was stable for the first 30 minutes of the stimulation period, then tended to decline for the subsequent 30 minutes.

Figure 2

Basal and Stimulated Acetylcholine Release from Striatal Slices Prepared from Young and Aged Rats

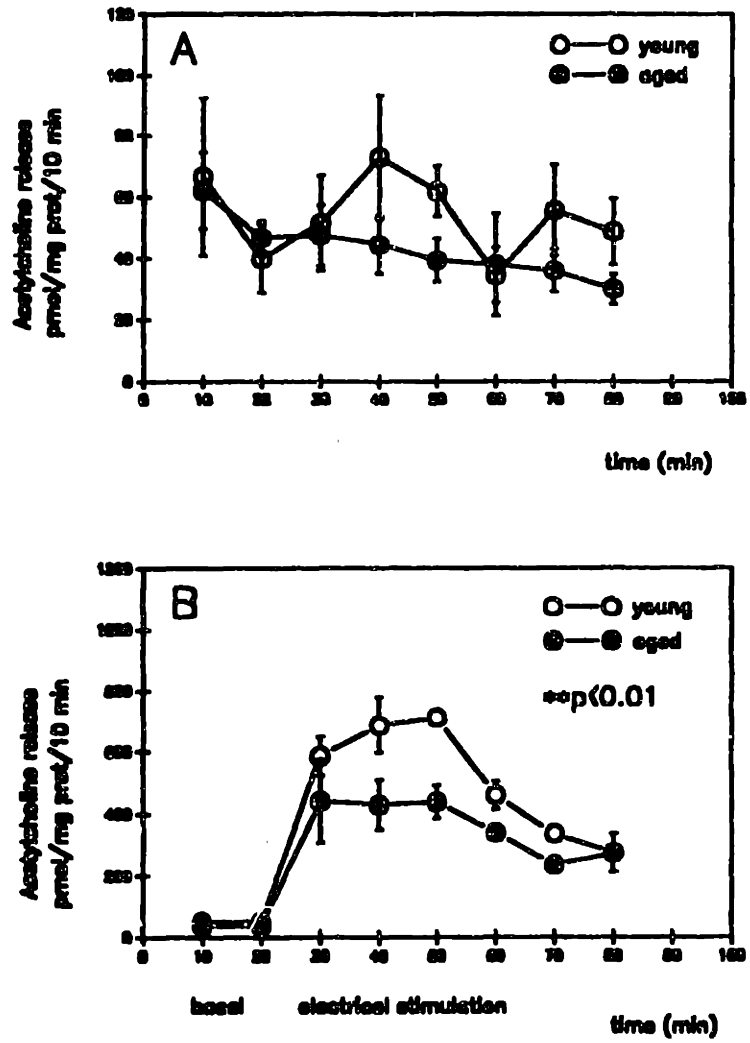
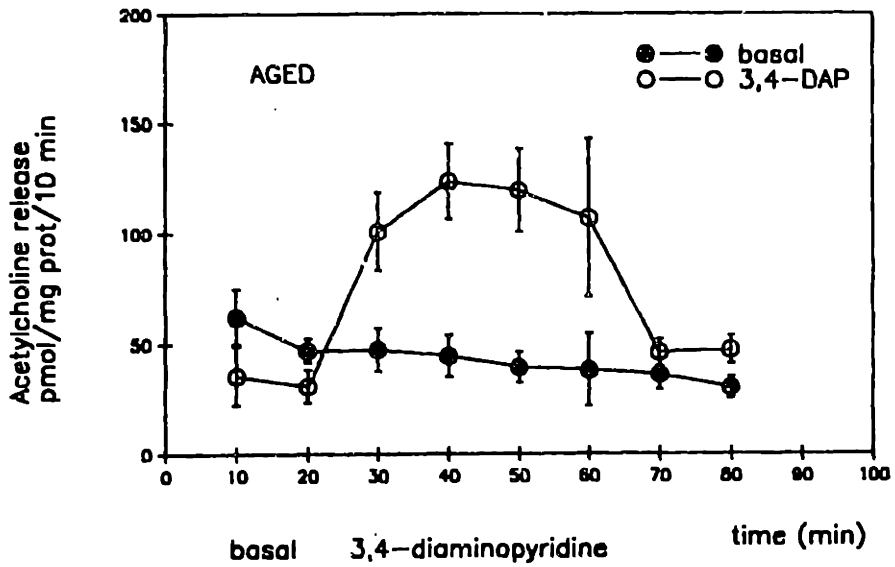


Fig 2) Basal acetylcholine release from striatal slices prepared from young rats (3 months) and aged rats (24 months) showed no significant differences (n=5) and was stable for both groups throughout the superfusion period. Electrically-stimulated acetylcholine release was significantly lower from slices prepared from aged rats relative to young rats ($p<0.01$, n=4).

Figure 3

3,4-Diaminopyridine Augments Basal Acetylcholine Release from Striatal Slices Prepared from Aged and Young Rats



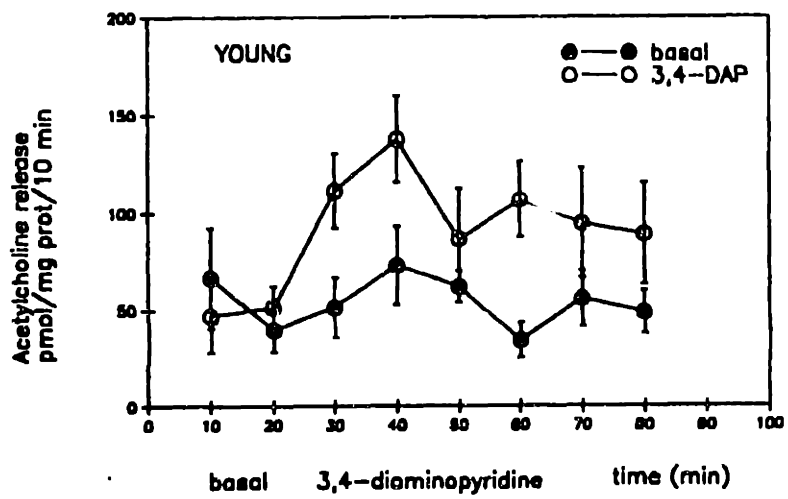


Fig 3) The potassium channel blocker 3,4-diaminopyridine markedly increased acetylcholine release from striatal slices prepared from aged ($p<0.0005$, $n=5$) and young rats ($p<0.0001$, $n=5$).

Figure 4

Age-Related Differences in 3,4-Diaminopyridine Effects on Stimulated Acetylcholine Release from Striatal Slices Prepared from Aged and Young Rats

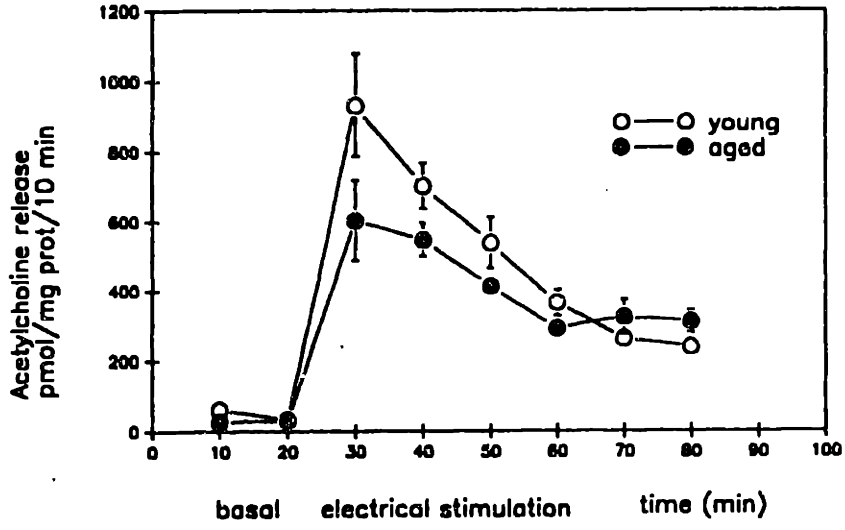


Fig 4) Striatal slices superfused with 3,4-diaminopyridine (10uM) containing medium were stimulated for 1 hour with electrical field potentials. Acetylcholine release was lower in aged rats ($p<0.05$, $n=5$) compared to young rats.

The age-related deficit in electrically evoked acetylcholine release from striatal slices is in agreement with decreases in radiolabelled acetylcholine release (Gibson and Peterson, 1987). The addition of 3,4-DAP (10 uM) to the superfusion medium did not abolish the age-related difference in electrically evoked acetylcholine release (Fig. 4, $p<0.05$, $n=5$, 18% decrease).

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Biographical Note

Todd Clayton Holmes was born to Barbara and Randall Holmes on April 18, 1961. He graduated from San Dieguito High School in 1979. In September, 1983, he entered the University of California, San Diego. During his undergraduate studies, the author worked in the laboratory of Dr. J. Anthony Deutsch for one year. He subsequently worked for three years in the laboratory of Dr. Steven Foote. The author received the Bachelor of Arts degree in biology, cum laude, and psychology, with honors, from the University of California, San Diego in 1988. In September, 1988, he entered the Massachusetts Institute of Technology as a graduate student in the Department of Brain and Cognitive Sciences. The author is a member of the Society for Neuroscience. During his graduate training, he was a recipient of an AASERT fellowship award.

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