ACETYLCHOLINESTERASE AND THE BASAL GANGLIA -
FROM CYTOLOGY TO FUNCTION

by

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ABSTRACT

Biochemical, anatomical, and histochemical studies were performed in the basal ganglia with an emphasis on the localization of the enzyme acetylcholinesterase (AChE). The existence of the enzyme in dopaminergic nigro-striatal neurons was demonstrated. Descending striato-nigral and pallido-nigral axons did not contain detectable amounts of AChE. A cell group called the nucleus basalis magnocellularis, intimately associated with the globus pallidus, was found to contain high levels of AChE; furthermore, these neurons were shown to be the source of a cholinergic projection to the neocortex.

In the striatum, large neurons containing high levels of AChE were found to be likely candidates as the cholinergic neuron of the striatum. Cholinergic perikarya were found to be absent in the neocortex; nor were perikarya synthesizing large amounts of AChE found in the neocortex. An empirical hypothesis was formulated on the basis of these and other findings regarding cholinergic neurons: High levels of AChE are a necessary but not sufficient criterion for identifying cholinergic perikarya.
# TABLE OF CONTENTS

Abstract .......................................................... ii  
Table of Contents ............................................... iii  
List of figures .................................................. iv  
Acknowledgements ................................................ v  
GENERAL INTRODUCTION .......................... 1  
  a) Historical overview of AChE ............. 1  
  b) The basal ganglia and cholinergic function 8  
  c) Brief description of research and rationale 10  
GENERAL DISCUSSION ........................................ 13  
  a) Review of new contributions reported in this thesis 13  
  b) The role of the striatum in motor and psychological function 15  
  c) Psychopharmacology of the striatum .......... 17  
      1) Psychopharmacology of dopamine in the striatum 17  
      2) Psychopharmacology of acetylcholine in the striatum 18  
  d) Interactions between dopamine and acetylcholine .......... 19  
      1) Psychopharmacological studies .......... 19  
      2) Measurement of biochemical parameters following pharmacological manipulations 20  
  e) The cytological relationship between the dopaminergic and the cholinergic neuron 21  
REFERENCES ......................................................... 26  
APPENDIX ............................................................. 35
LIST OF FIGURES

Figure 1. Traditional cytological model explaining dopamine - acetylcholine interactions 24

Figure 2. Alternative cytological model proposed to explain the same dopamine - acetylcholine interactions 25
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GENERAL INTRODUCTION

The general strategy adopted in this thesis was to investigate cholinergic systems of the extrapyramidal system, especially by studying the enzyme acetylcholinesterase (AChE). There is a special advantage to studying AChE - it may be assayed biochemically and also localized histochemically, with excellent resolution and contrast. Moreover, by pretreating animals with an irreversible AChE inhibitor, commonly diisopropylphosphorofluoridate (DFP), some time preceding sacrifice (Lynch et al., 1972), further advantages may be realized: Neurons which synthesize the enzyme at different rates may be distinguished. By the use of DFP, the subcellular distribution of the enzyme may be resolved with the light microscope, within limitations. The transport of AChE along axonal projections may be determined. For these reasons and others to be elaborated below, the study of AChE has been an integral part of each of the investigations described in this thesis.

a) Historical overview of AChE

Since much of the work comprising this thesis is concerned with AChE, what is known and what has been speculated about AChE over the past decades will be reviewed in some detail. The major early advances in understanding potential functional roles of AChE were made by Koelle (1962). He noted three major generalizations concerning AChE: First, its highest concentrations appeared to be on presynaptic cholinergic terminals, with the possible exception of the neuromuscular junction. Second, the enzyme was found in many neurons not thought to be cholinergic, although in dramatically lower
levels than in neurons thought to be cholinergic. Finally, there did appear to be nicotinic receptors on the terminals of cholinergic neurons, specifically motoneuron terminals, which could give rise to antidromic action potentials recorded in the alpha-motoneuron. This last observation would of course give AChE a raison d'être for being present presynaptically. Subsequent research has borne out the first two of these hypotheses. With regard to the third, today the existence of the nicotinic receptors on motoneuron terminals appears to be valid, although it is dubious that they are of physiological significance (Miyamoto, 1978). The first hypothesis, that high AChE levels appear to be a universal characteristic of cholinergic neurons, appears to be true at least as far as the perikaryon is concerned. Unfortunately, this hypothesis has been received poorly by the neuro-science community, since Shute and Lewis (1961, 1965) modified the hypothesis to state that all neurons which contained any level of AChE in their axons were cholinergic. This modification was accepted without further examination by a number of other prolific investigators (e.g., Krnjević and Silver, 1965, 1966), despite earlier data published by Koelle (1954) which clearly invalidated such a hypothesis.

The postulate that the post-synaptic occurrence of AChE implies cholinception remains unproven. It is certainly plausible in the peripheral nervous system (Koelle, 1962), but there exists at least one major exception in the central nervous system: The dentate gyrus of the hippocampal formation receives a massive cholinergic input (Lewis, Shute, and Silver, 1967), but none of the neurons of the entire hippocampal formation contain appreciable amounts of AChE (Lewis, Shute, and Silver, 1967; Lehmann, unpublished observations).
Some attempts have been made to support the hypothesis that low to intermediate levels of AChE in certain neurons of the central nervous system are indicative of cholinception (Butcher and Talbot, 1978a,b), but the majority of the evidence for these special cases is negative (Lehmann and Fibiger, 1979).

Two hypotheses were proposed decades ago in an attempt to explain completely AChE in all its locations. Nachmansohn (1959) believed that acetylcholine was released and hydrolyzed along the entire extent of axons in general, and that this process was essential to nerve impulse conduction. Burn and Rand (1959) believed that acetylcholine was contained in sympathetic (noradrenergic) terminals and that the release of acetylcholine, elicited by the action potential, mediated the subsequent release of noradrenaline, by stimulating a pre-junctional, extracellular cholinergic receptor. While there were perhaps data to support these hypotheses at one time, abundant data garnered over the decades have refuted both hypotheses (Silver, 1974). Champions of the Nachmansohn (1959) and Burn and Rand (1959) hypotheses are still to be found, however (Butcher et al., 1975).

It remains likely, however, that cholinergic receptors exist on noradrenergic neurons in the periphery (Sharma and Banerjee, 1978), and they may exist on catecholamine terminals in the central nervous system. The latter case is of course much more difficult to resolve experimentally (for review of literature, see Butcher and Talbot, 1978a,b). Thus exogenous acetylcholine may have a direct action on catecholamine terminals.

Recently, it has been proposed that butyrylcholinesterase serves as a precursor for AChE, on the basis of some rather equivocal
pharmacological data (Koelle et al., 1976). In studies from this laboratory to be reported below, a selective lesion of dopaminergic neurons in the substantia nigra resulted in a substantial depletion of AChE with no depletion in butyrylcholinesterase, suggesting that at least in these neurons butyrylcholinesterase cannot serve as the precursor for AChE. It has since been shown that AChE and butyrylcholinesterase show large differences in binding properties, in immunological cross-reactivity, and, in human plasma, have separate gene loci (Silman et al., 1979), making it difficult indeed to support this latest hypothesis of Koelle et al. (1976).

A great deal of research has been done on the different molecular forms of AChE, resolved by a number of biochemical techniques. Early studies were at disagreement with respect to the molecular weights and numbers of isoenzymes of AChE. This was apparently due to differing degrees of aggregation and solubilization occurring due to the different procedures used in the different laboratories. Vigny et al. (1976) succeeded in solubilizing the enzyme thoroughly and reproducibly by using the very high sodium chloride concentration of 1 M. They found three characteristic forms of AChE in muscle: 4s, 10s, and 16s, following ultracentrifugation. It appeared that the 16s form was specifically associated with muscle end-plate. Subsequently, the same group found that rat brain contained only the 4s and 10s forms (Rieger and Vigny, 1976). Furthermore, as rat brain developed, a predominance of the 4s form gave way to an almost exclusive occurrence of the 10s form. These data were very suggestive of a synaptic function of an oligomeric form of AChE. In superior cervical ganglion, 4s, 6.5s, 10s, and 16s forms
were found, although all forms but the 16s were found in preganglionic and postganglionic nerves (Gisiger et al., 1978). The 16s form disappeared from the superior cervical ganglion following denervation. However, other workers found small amounts of 16s AChE in peripheral nerves which apparently was transported with unusually high velocity (DiGiamberardino and Courard, 1978; Fernandez et al., 1979). The functional implications of these findings for AChE in synaptic function await further study. It is also noteworthy that AChE which is bound to the external surface of axons (representing about 84% of the total in the nerve section) apparently is not transported at appreciable velocities in either the anterograde nor retrograde direction. Instead, the minority of AChE contained within the axon undergoes rapid axoplasmic transport (Brimijoin et al., 1978).

In the nerve terminal, AChE appears to have an exclusively external, membrane-bound localization, although sometimes electron microscopic studies report reaction product within mitochondria or the Golgi apparatus, which was not eliminated by the appropriate histochemical controls, i.e., it does not represent true AChE (Eränko et al., 1967; Bridges et al., 1973; Koelle et al., 1974; Kuhar and Rommelspacher, 1974). This is in agreement with subcellular fractionation studies (Toschi, 1959; DeRobertis et al., 1963; Whittaker et al., 1964). All these observations emphasize the importance of presynaptic AChE, particularly that which is bound on the external surface of the cholinergic terminal, in the central nervous system. Obviously, AChE has a different role in regulating acetylcholine transmission than monoamine oxidase has in regulating catecholamine transmission (Geffen and Livett, 1971), monoamine oxidase having an intratermal,
primarily mitochondrial localization.

A very intriguing possible mechanism for regulating cholinergic transmission via AChE has been raised in recent years, namely the secretion of AChE either by cholinergic neurons or by the neurons or organs which receive cholinergic innervation. The hypothesis that AChE was secreted was first suggested by electron microscopic histochemical studies (Flumerfelt et al., 1973; Kreutzberg and Tóth, 1974; Kreutzberg and Schubert, 1975). In a rigorous electron microscopic histochemical study in the motoneuron, the possible cytological mechanism for secretion was proposed (Kreutzberg et al., 1975): Following synthesis in the rough endoplasmic reticulum, AChE apparently passes through the Golgi apparatus and becomes incorporated into the smooth endoplasmic reticulum. Since there is direct continuity between the smooth endoplasmic reticulum and the plasma membrane, this suggested a general cytological mechanism for AChE secretion. The observation that, rarely, synaptic vesicles appear to contain AChE (Bodian 1970), suggested that secreted AChE may not only be restricted to extracellular locations, but may play some role in intercellular communication as well. It is important to note that most indications suggest that if AChE indeed enters synaptic vesicles, it is by endocytosis.

AChE has subsequently been detected in cerebrospinal fluid (Chubb et al., 1976; Bareggi and Giacobini, 1978; Scarsella et al., 1979; Greenfield et al., 1979). Non-denaturing gel electrophoresis studies suggested that one isoenzyme was secreted (Chubb et al., 1976; Scarsella et al., 1979). Administration of chlorpromazine increases the amount of AChE secreted (Bareggi and Giacobini, 1978;
The release is much more pronounced in cisternal than in ventricular cerebrospinal fluid (Bareggi and Giacobini, 1978; Greenfield et al., 1979). The source of the released AChE is unknown; however, it is higher in activity than plasma AChE, and there is no concomitant increase in lactate dehydrogenase, a conventional marker for cell disruption (Greenfield et al., 1979). This phenomenon is of obvious importance in understanding control mechanisms governing cholinergic tone in the central nervous system. In the cases of the superior cervical ganglion (Gisiger and Vigny, 1977) and cat geniohyoid muscle (Inestrosa et al., 1977), afferent cholinergic neurons are not necessary to support the release of AChE. In the isolated rat hemidiaphragm, however, the ratio of 10s and 4s AChE released by electrical depolarization match the ratio of these forms in the phrenic nerve, and not muscle (Skau and Brimijoin, 1978). The latter authors suggested synaptic, exocytotic release of AChE as the source of extracellular AChE and as a means of regulating or perhaps supplying postsynaptic AChE. While it remains questionable whether cholinergic axons can secrete AChE, it certainly is true that organs which normally receive cholinergic innervation can secrete AChE, following chronic denervation and in non-depolarizing medium (Gisiger and Vigny, 1977; Inestrosa et al., 1977).

AChE release from whole brain synaptosomal fraction in non-depolarizing medium has been demonstrated (Burgun et al., 1977). What role exactly AChE release plays in the central nervous system is more difficult to answer than it is in the peripheral nervous system. It is not known, for instance, whether such released AChE reaches
cholinergic synapses, but its presence has not been reported in extracellular space in the brain except for cerebrospinal fluid.

b) The basal ganglia and cholinergic function

The postulate that AChE is concerned in some way with the function of acetylcholine remains such an attractive concept that the study of this enzyme was the major emphasis of the projects comprising this thesis. That the striatum is among the highest in biochemical markers for cholinergic transmission in the entire brain (Hoover et al., 1978; Kobayashi et al., 1978) makes this an attractive nucleus in which to study the cholinergic neuron. Many of the psychopharmacological effects produced by drugs acting on cholinergic receptors in the central nervous system, such as tremor and catalepsy (Karczmar, 1975) may be mediated in the striatum.

In the early part of the 1970's it became clear that the markers for cholinergic neurons, namely choline acetyltransferase and acetylcholine, were completely intrinsic to the striatum (McGeer et al., 1971; Butcher and Butcher, 1974). Lesions of the brain surrounding the striatum had no effect on either of these biochemical markers for cholinergic neurons. This led to the suggestion that the cholinergic neuron was an interneuron (McGeer et al., 1971). At about the same time, six different morphological types of neurons were identified in the striatum (Kemp and Powell, 1971). One goal of the neuroscientist is to construct a wiring diagram of the brain, as if it were a computer, and then go on to explain its function, and in cases of disease dysfunction, in terms of that wiring diagram. The first step, of course, is to identify the components.

In the case of the basal ganglia, the primary specific goal as
far as acetylcholine is concerned, was to identify the cholinergic neuron morphologically. This was first accomplished by the technique of immunohistochemistry, employing antibodies raised against purified choline acetyltransferase (Hattori et al., 1976). The most common type of neuron in the striatum, the medium spiny neurons, were identified as the putative cholinergic neurons. Biochemico-pharmacological data were used to support the identification of this as the cholinergic neuron, which received a direct dopaminergic input (Hattori et al., 1976).

When the experiments reported in this thesis were begun, the study of AChE in the striatum appeared to be as complex an approach as any other for studying cholinergic function. As noted above, AChE has complex forms and cellular localizations, not only within and on the plasma membranes of neurons in varying levels of activity, but also quite possibly in intercellular space. Histochemically, AChE activity in the striatum appears dense and uniform (Koelle, 1954; McGeer et al., 1971). Fortunately, a technique was developed which allowed the restriction of histochemical product formed by AChE to the neurons which synthesize the enzyme most rapidly (Lynch et al., 1972; Butcher et al., 1975): Administration of the irreversible inhibitor of AChE, diisopropylphosphorofluoridate (DFP), inhibits the AChE (as well as other enzymes) everywhere in the animal. If the animal is then sacrificed four to twelve hours after administration of DFP, only the enzyme which has been newly synthesized during that time is visualized histochemically. Thus, it became possible to study the intrinsic neurons of the striatum which synthesize AChE, a study which is still being pursued. Preliminary studies (Butcher
et al., 1975) showed that only a minority of neurons (less than 5%) synthesized the enzyme at appreciable levels, and of these still fewer synthesized very large amounts of the enzyme.

The papers which follow are entirely of an anatomical and biochemical nature. The functional implications, whereby some speculations concerning the role of the striatal cholinergic neurons in psychomotor function may be entertained, will be dealt with in the discussion. In the publications which follow, as the main body of this thesis, a broad data base was sought to substantiate hypotheses which were, and perhaps still are, considered unorthodox. The candidate is aware that in science, as in all endeavors, today's conclusions have a way of becoming tomorrow's fallacies.

c) Brief description of research and rationale

Butcher et al. (1975) reported that the dopaminergic neurons of the substantia nigra contained AChE. This conclusion was based mainly on the similar topographic distribution of AChE-containing neurons and dopamine-containing neurons, the latter having been localized by catecholamine histofluorescence studies (Dahlström and Fuxe, 1964; Ungerstedt, 1971). This was not, however, a compelling argument to support the conclusion. For this reason, the first investigation of this thesis was to test rigorously the hypothesis that dopamine-containing neurons of the substantia nigra contained AChE.

Selective, if not specific, destruction of dopamine-containing neurons can be achieved by the administration of the neurotoxin 6-hydroxydopamine by either of two routes. The toxin may be injected directly into the ascending axons of the dopaminergic nigrostriatal
Retrograde degeneration of the dopaminergic neurons then ensues. Alternatively, the toxin may be injected into the lateral ventricle. Desmethylimipramine, which blocks uptake of the toxin into noradrenergic neurons, must be administered also if noradrenergic fibres and perikarya are to be spared.

Both of these routes of administration were employed in the first investigation (A) to obtain a complete and specific lesion of the nigrostriatal dopaminergic projection. The extent of the lesions was determined by measuring the enzyme tyrosine hydroxylase, which is thought to be a specific marker for catecholamine neurons. Choline acetyltransferase was measured to assess the specificity of the lesion. Finally, AChE was both measured by radioenzymatic assay and examined histochemically. The biochemical characteristics of AChE, an enzyme which does not follow Michaelis-Menten kinetics, were also studied.

The histochemical demonstration of an AChE-containing striatono nigral projection had been proposed earlier (Olivier et al., 1970). The second investigation of this thesis set out to support this hypothesis also. The original investigation (Olivier et al., 1970) had employed electrolytic lesions, which destroy axons passing through the lesioned area, in addition to neurons. Since that investigation, a neurotoxin, kainic acid, has been found which spares axons of passage but destroys intrinsic neurons of the striatum. This second investigation (B) utilized kainic acid in order to obtain the more selective striatal lesion. Information on the localization and sources of AChE in the striatum were also obtained. Histochemical experiments were performed to determine if blockade of transport
along the striatonigral axons caused an accumulation of AChE within them.

A group of neurons, the nucleus basalis magnocellularis, which may belong to the extrapyramidal system (Divac, 1975) or to the reticular group of neurons (Das and Kreutzberg, 1968), became the focus of the next investigation (C). These neurons contain very high levels of AChE, and it had been speculated that they were the source of a cholinergic projection to the neocortex (Divac, 1975). All the most rigorous means of testing this hypothesis, by utilizing different sorts of lesions, neuroanatomical tracing techniques, and AChE histochemistry, were employed to test this hypothesis.

There has been a great deal of confusion and numerous hypotheses concerning the interpretations which may be drawn from the presence of AChE activity in different neurons. The investigations reported in this thesis have some bearing on these interpretations. A short review on the topic was therefore written (D). This review contains some original data, the most striking of which relate to the morphological identification of the striatal cholinergic neuron. Campochiara and Coyle (1978) had developed a selective lesion of the cholinergic neuron. This turned out to be the same morphologically described neuron previously speculated to be the cholinergic neuron on the basis of anatomical and histochemical arguments set forth in publication B.
GENERAL DISCUSSION

a) Review of new contributions reported in this thesis

From the preceding papers, three major findings have emerged:
1) A hypothesis for identifying potential cholinergic neurons on the basis of their high AChE activity has been proposed. 2) The cholinergic neuron of the striatum has been morphologically identified. 3) The origin of a major cholinergic projection to the cortex has been identified.

In the first investigation (A), the hypothesis proposed by Butcher et al. (1975) was supported: Selective lesion of the dopaminergic neurons resulted in a 30-40% depletion of AChE in the substantia nigra, and about a 12% depletion of AChE in the striatum. These results suggest that both the perikarya and axons of dopaminergic nigrostriatal neurons contain AChE. In this investigation, the source of a cholinergic input to the substantia nigra could not be found.

In the second investigation (B), the hypothesis of Olivier et al. (1970) was refuted. A massive kainic acid lesion of striatal neurons did not result in a detectable depletion of AChE in the substantia nigra. Furthermore, colchicine injections into the axons of the striatonigral pathway, which in general will cause proximal accumulation of AChE in axons which transport the enzyme, did not cause such an accumulation. While a major portion, if not all, of the choline acetyltransferase activity of the striatum originates from neurons whose perikarya reside within the striatum, about 50% of the AChE activity is derived from an external source.

The third investigation provided compelling evidence that the
nucleus basalis magnocellularis is the source of a cholinergic projection to the cortex (C). The efferent neurons were mapped (C, Figs. 1 and 2). It was not possible to assign this group of neurons to either the reticular formation or the extrapyramidal system, on the basis of currently available evidence.

The review (D), which evaluates the interpretations which can be made from the localization of AChE in various neurons, arrived at two basic general and empirical conclusions: 1) Very high levels of AChE four to twelve hours following DFP administration can be taken as a necessary but not sufficient criterion for a neuron to be cholinergic. This is a valuable rule, since it would eliminate 99% of the neurons in the brain as candidates for cholinergic neurons, since they contain but low to intermediate levels of AChE. For instance, the cerebral cortex is devoid of intensely AChE-reactive neurons, and thus, one would predict, does not contain cholinergic perikarya. This example was experimentally verified (D). 2) At this point in time, AChE activity contained in a neuron cannot be taken as evidence that such a neuron is cholinceptive.

In this investigation, evidence was also gathered to support the hypothesis that the intensely AChE-reactive neuron was the cholinergic interneuron of the striatum. When rat pups ten days of age received kainic acid injections, choline acetyltransferase activity and AChE activity were selectively depleted. While histologically no other neuron loss was detectable, the intensely AChE-reactive neuron was destroyed. The putative cholinergic neuron makes up only about 1% of the total striatal neuron population.

These fundamental studies of the putative cholinergic neuron
of the striatum are essential as a basis for a more comprehensive understanding of that neuron's more complex functions. It would appear to play an important part in the function of the striatum. For instance, of all striatal neurons, the large neurons (including the putative cholinergic neuron) undergo their final mitosis earlier than the rest of the striatal neurons (Brand and Rakic, 1979). They appear to develop sensitivity to kainic acid's neurotoxic effects earlier than the rest of the striatal neurons. These results raise the possibility that they form the primary neural foundation upon which subsequent striatal neural networks are built. A postnatal developmental study of the putative cholinergic neuron is currently in progress.

b) The role of the striatum in motor and psychological function

The title of the thesis promises some consideration of the function of the striatal cholinergic neuron. This is, after all, the ultimate intrigue of brain research. The current concepts concerning the general psychological function of the striatum will now be reviewed very briefly.

First, it must be considered that the striatum does not play an essential role in the control of basic metabolic function, elementary sensorimotor functions, or elementary cognitive functions (Villablanca et al., 1976). However, deficits in response initiation to a conditioned avoidance response occur following lesion of the dopaminergic nigrostriatal projection (Fibiger et al., 1974). Such deficits can be reversed by anticholinergic drugs, such as scopolamine and atropine (Fibiger et al., 1975). This is one example of an antagonism which appears very frequently between dopaminergic
and cholinergic transmission in the striatum.

The striatum appears to play its major role as one of the higher feedback systems controlling movement. Although traditionally it has been considered a more primitive motor output system which operates independently of the pyramidal (cortico-spinal) motor system, direct motor outputs have been found to be at most a minor portion of efferents from the basal ganglia. On the contrary, circuitry which links the striatum with thalamic pathways which feed back onto the cerebral cortex is the predominant neuroanatomical characteristic of the system (Carpenter, 1975). There appear to be rather direct routes for visual input to the striatum via the cortex, demonstrable both anatomically (Künzle and Akert, 1977) and by recording responses to visual stimuli (Pouderoux and Freton, 1979). Cortical afferents, not only from motor areas (Künzle, 1975) but also from all sensory and associational areas, innervate the striatum (Goldman and Nauta, 1977; Jones et.al., 1977). Somatic and visual sensory neglect can be induced by lesions of the extrapyramidal motor system (Feeney and Wier, 1979). In normal animals, single-cell recording in the striatum during visually and somatically guided hand movements indicate that the typically "quiet" striatum fires during correction of these movements (Anderson et al., 1979). A direct role of the cholinergic system of the striatum in modulating cortical processing of sensory information has been demonstrated by Roemer et al. (1978): Intracaudate injection of carbachol has direct effects on somatosensory-evoked cortical potentials. Cryogenic lesions of the striatum produce pursuit-tracking deficits (Bowen, 1969).
c) Psychopharmacology of the striatum

Anthropomorphism is considered to be a pitfall in science generally and in neuroscience in particular. However, since some drugs used in the studies to be discussed can induce a syndrome that closely resembles paranoid schizophrenia (e.g., amphetamine), and others are used therapeutically in schizophrenics (Snyder, 1974), some of the animal behaviors elicited by these drugs may be considered analogous to human psychological disorders (Mattyse, 1974). Thus the reader is encouraged to consider the following psychopharmacological discussion in terms of what roles in thought processes the transmitters dopamine and acetylcholine may play. It should be noted that while there is considerable evidence to indicate that site of actions of drugs described below is the striatum, this point is actually rarely adequately demonstrated.

1) Psychopharmacology of dopamine in the striatum

6-Hydroxydopamine lesions of the nigrostriatal projection result in decreased spontaneous locomotor activity (Creese and Iversen, 1973; Roberts et al., 1975), and an attenuation in the normal locomotor stimulation induced by amphetamine. Apparently the nucleus accumbens is more involved in the locomotor response to amphetamine, while the stereotypy response to higher doses of amphetamine is localized to the caudate-putamen (Creese and Iversen, 1975). Neuroleptics, which are thought to block dopamine receptors, cause catalepsy in high doses (Asper et al., 1973).

Electrophysiologically, it is thought by some that dopamine is mono-synaptically excitatory, although at longer latencies its overall effect is inhibitory, an effect probably mediated by at least one interneuron (Kitai et al., 1976; Richardson et al., 1977). Dopamine stimulates adenylate cyclase in the striatum (McGeer et al., 1976; Kebabian, 1978),
making it a candidate as a slower acting, humoral agent which may not
function exactly like a classical synaptic transmitter.

2) Psychopharmacology of acetylcholine in the striatum

Because cholinergic systems are found in so many parts of the
central and peripheral nervous systems, the only direct assessment
of the behavioral role of acetylcholine in the striatum is obtained
by injection of acetylcholine agonists and antagonists directly
into the striatum. Injection of muscarinic agonists or AChE
inhibitors into the striatum results in tremor, limb dystonia,
rigidity, and akinesia - all characteristics of Parkinson's disease
(Connor et al., 1966; Goldman and Lehr, 1976; Matthews and Chiou,
1978, 1979). All these effects are blocked by muscarinic antagonists.
Intrastriatal injection of atropine, on the other hand, elicits stereo­
typy - an effect which may also be elicited by increasing dopaminergic
tone (Zámbó et al., 1979).

Electrophysiologically, there is less agreement about the
effects of acetylcholine in the striatum. Some investigators found
predominantly inhibition of iontophoresed acetylcholine (Bloom et al.,
1965; McLennan and York, 1966) which was blocked by muscarinic anta­
gonists (McLennan and York, 1966). Systemically administered
cholinergic agonists and antagonists led others to believe that
cholinergic receptors in the caudate nucleus were inhibitory and
muscarinic (Koller and Berry, 1976). However, using striatal slices,
local stimulation evokes excitation which can be blocked by high
concentrations of curare or enhanced by physostigmine, while
muscarinic antagonists were without effect (Misgeld and Bak, 1979).
In contrast, another study reports both short and long latency
excitation by local stimulation of striatal slices, the first being blocked by nicotinic antagonists and the latter being blocked by muscarinic antagonists (Weiler et al., 1979).

d) **Interactions between dopamine and acetylcholine**

On the basis of pharmacological studies alone, it is not surprising to find that a large number of antagonistic effects exist between dopaminergic and cholinergic agonists, and between dopaminergic and cholinergic antagonists. While generally Barbeau (1962) is credited with creating the concept of a dopamine-acetylcholine balance in the striatum, for historical accuracy he credits the concept's inception to McGeer et al. (1961). Both groups of workers drew their conclusions from the effects of drugs on victims of Parkinson's disease, in whom dopaminergic agonists or cholinergic antagonists ameliorated, but dopamine antagonists or cholinergic agonists exacerbated the symptoms. The cholinolytic drugs which are successful in ameliorating the symptoms of Parkinson's disease are muscarinic (Duvoisin, 1967). Huntington's disease is essentially the inverse of Parkinson's disease - in these patients dopaminergic afferents to the striatum are intact, but the neurons of the striatum, including the cholinergic neurons, have undergone massive atrophy. The dopamine-acetylcholine balance model gained further support when the inverse pharmacological relationship held true once more - dopamine antagonists and cholinergic agonists ameliorate the symptoms of the disease (Aquilonius and Sjöström, 1971).

1) **Psychopharmacological studies**

In psychopharmacological studies in experimental animals, examples of the inverse actions of cholinergic and dopaminergic
drugs are innumerable. Just a few pertinent examples will be cited here. Methylphenidate induces stereotypy by increasing dopaminergic transmission; its effects are reversed by administration of the muscarinic agonist oxotermorine (Davis et al., 1978). The muscarinic agonist, pilocarpine, decreases amphetamine-induced increases in locomotor activity, while scopolamine, a muscarinic antagonist, potentiates the locomotor excitation produced by amphetamine (Fibiger et al., 1970). In mice with unilateral 6-hydroxydopamine lesions of the nigrostriatal projection, scopolamine produces circling towards the side of the lesion, indicating potentiation of dopaminergic tone on the intact side (Pycock et al., 1978). This effect is blocked by administration of tyrosine hydroxylase inhibitors (which prevent the synthesis of dopamine). Significantly, direct or indirect cholinergic agonists depressed the rates of circling caused by amphetamine or apomorphine, suggesting antagonism of dopaminergic transmission on the intact side.

2) Measurement of biochemical parameters following pharmacological manipulation

When instead of behavioral measurements, biochemical parameters indicating rate of transmitter turnover are measured in response to pharmacological manipulation, data consistent with the dopamine-acetylcholine balance are obtained. Cholinergic agonists increase dopamine turnover in the striatum, while muscarinic antagonists decrease dopamine turnover in the striatum, presumably as some sort of compensatory mechanism, as reviewed by Bartholini et al. (1975).

Systemic administration of neuroleptics (dopamine antagonists) causes a marked increase in the amount of acetylcholine which can
be collected from cat caudate nucleus by push-pull cannula (Stadler et al., 1973; Bartholini et al., 1975). More commonly, the level of a transmitter is measured following the pharmacological manipulation; the level is considered to be inversely related to the turnover rate of the transmitter. Thus, electrolytic lesion of the substantia nigra causes a transient decrease in striatal levels of acetylcholine, while amphetamine increases striatal acetylcholine levels, but requires an intact nigrostriatal projection in order to do so (Agid et. al., 1975). Neuroleptics such as haloperidol, spiroperidol, chlorpromazine, and pimozide, and the depleter of biogenic amines, reserpine, cause decreases in striatal acetylcholine levels (Consolo et al., 1975; Glick et al., 1976; Marco et al., 1976; Consolo et al., 1977; Bianchi et al., 1979). It may be noted that such results have not consistently been obtained in "olfacto-striatal" regions, namely the nucleus accumbens and olfactory tubercle (Consolo et al., 1977).

e) The cytological relationship between the dopaminergic and cholinergic neuron

The very orderly way in which the pharmacological data have suggested a constant and consistent antagonistic effect of dopaminergic inhibitory influence on the cholinergic neuron, together with immunohistochemical evidence, make the model of a direct synaptic contact from the dopamine neuron onto the cholinergic neuron very appealing (Hattori et al., 1976), and this model has been popular for a number of years (see Fig. 1). However, some of the points raised by the research contained in this thesis and to be found in the literature make this model appear less attractive.

First, electron microscopic data suggest that dopaminergic neurons
account for approximately 15% of the terminals of the striatum (Hattori et al., 1973; Hökfelt and Ungerstedt, 1973; Arluison et al., 1978a,b), and these terminals synapse almost exclusively on spines, or according to others, the dendritic shafts of spiny neurons (for review, see Pasik et al., 1979). It is quite difficult to argue for the existence of dendritic spines on the putative cholinergic neuron. Spines are not apparent in DFP-pretreated material stained for AChE; if they do exist one must postulate that they for some reason do not contain AChE, as does the rest of the dendrite at the same post-DFP survival time. Furthermore, since the putative cholinergic neuron only constitutes about 1% of the total neuron population, it must have very extensive dendritic arborization in order to receive a large proportion of 15% of the total striatal terminals. The possibility is thus raised that, psychopharmacologically, a neuron other than the cholinergic neuron receives the major portion of dopaminergic input.

In the final analysis, it is the psychopharmacological experiment which will make or break a hypothesis of neuronal connections. In an early study (Fibiger et al., 1970), it was shown that rats older than 25 days modulated their locomotor stimulation by amphetamine in response to pilocarpine (decreasing the stimulation) and scopolamine (increasing the stimulation). However, at 15 days of age, when the amphetamine stimulation could be demonstrated quite easily, the cholinergic agents were without effect. If the dopamine neuron were to transmit its information exclusively through the cholinergic neuron, as diagrammed in Figure 1, then these results would be impossible to obtain: With no cholinergic receptor to affect the amphetamine response, the cholinergic neuron is, at day 15, mute. The dopaminergic neuron must transmit its information through some other neuron, whose
post-synaptic receptor has matured by this age.

The model of the dopamine neuron synapsing directly onto the cholinergic neuron was the appropriate model for the data available at the time, since it was the simplest model. Instead of a "series" circuit, in which the cholinergic neuron acts as the essential link through which dopamine must act, we must now consider other models. The next simplest model may involve a dopaminergic and cholinergic neuron terminating in parallel on the same neuron, where they may exert their opposite effects, as diagrammed in Figure 2. With six types of neurons (at least) and at least four types of afferents to the striatum, the model is bound to become more complex as studies progress.

Experiments are currently in progress to test the hypothesis proposed above, namely that the cholinergic neuron does not receive a large proportion of the dopaminergic input to the striatum. More rigorous biochemical and histochemical studies are being performed to see if the selective cholinergic neuron lesion produced by striatal injects of kainic acid in neonatal rats is as selective as it appears. Binding studies, employing neuroleptics as ligands as well as dopamine agonists, should indicate no decrease in dopamine receptors following lesion of the cholinergic neurons if the hypothesis proposed here is correct. Likewise, dopamine-stimulated adenylate cyclase should not be altered by the selective cholinergic neuron lesion. Rotation (i.e., psychopharmacological) studies are planned to see if there is a functional asymmetry in receptors in the animal. Studies such as these will eventually yield the necessary information to construct a "road map" of the striatum. In time, we may be able to comprehend the "traffic patterns" that constitute nothing less than thoughts.
Figure 1. Pharmacological and immunohistochemical data were all neatly explained by this simple model, in which the cholinergic neuron is part of a "series" circuit. The dopamine neuron from the substantia nigra synapses directly onto the striatal cholinergic neuron, which then contacts other neurons of the striatum. In this model, the cholinergic neuron is an essential link in dopaminergic neurotransmission. The expression of dopaminergic function thus requires: 1) A dopamine receptor on the cholinergic neuron; 2) Functional acetylcholine release; 3) A functional cholinergic receptor on other striatal neurons.
Figure 2. Some psychopharmacological and anatomical considerations suggest that the cholinergic neuron does not receive direct dopaminergic input. Nonetheless, the "dopamine-acetylcholine" hypothesis has an inscrutable and large data base to support it. If this dopamine-acetylcholine interaction occurs in the striatum, a "parallel" circuit model may explain the interaction. A very simple model to explain the interaction is diagrammed above. The dopamine neuron and cholinergic neuron both synapse onto another striatal, each exerting opposite effects on that neuron.
REFERENCES


APPENDIX

List of Publications


ACETYLCHOLINESTERASE IN THE SUBSTANTIA NIGRA AND CAUDATE-PUTAMEN OF THE RAT: PROPERTIES AND LOCALIZATION IN DOPAMINERGIC NEURONS

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Abstract—In order to examine the hypothesis that acetylcholinesterase (AChE) is contained within dopaminergic neurons of the nigro-striatal projection, the effects of selective destruction of these neurons by 6-hydroxydopamine (6-OHDA) on cholinesterase, tyrosine hydroxylase, and choline acetyltransferase in substantia nigra (SN) and caudate-putamen (CP) were studied in the rat. Four to five weeks after intraventricular or intracerebral 6-OHDA injections tyrosine hydroxylase in these structures was reduced by 90% or more. Choline acetyltransferase was not affected in the SN or CP, but cholinesterase was reduced by about 40% in the SN and by 12% in the CP. To determine that the observed decreases in cholinesterase activity reflected true AChE and not butyrylcholinesterase (BChE), further experiments were conducted on tissues from animals with intracerebral 6-OHDA lesions. (1) Substrate specificity. Acetylcholine (ACh) was replaced by either acetyl-ß-methyl-choline (AcßMeCh) or butyrylcholine (BCh) in the cholinesterase assay. SN and CP from 6-OHDA lesioned rats showed 54% and 92% of control tissue cholinesterase activity respectively with AcßMeCh as substrate, in good agreement with values found using ACh. No decrease in activity toward BCh was observed. (2) Kinetics. The decrease in cholinesterase activities at different concentrations of ACh was determined. Analysis of the data revealed that cholinesterase in dopaminergic neurons was inhibited by high ACh concentrations, a characteristic property of AChE but not BChE. (3) Selective inhibitors. In the SN, cholinesterase in dopaminergic neurons was inhibited by the selective AChE inhibitors BW284C51 and ambenonium with a dose-response curve similar to erythrocyte AChE but different from serum BChE. The selective BChE inhibitor, tetraisopropylpyrophosphoramide, inhibited the enzyme in dopaminergic neurons only at concentrations which inhibited erythrocyte AChE, concentrations somewhat higher than those which inhibited serum BChE. These results support recent histochemical observations indicating that AChE is contained in dopaminergic neurons of the SN. Moreover, these experiments represent the first characterization of AChE from a homogeneous population of non-cholinergic neurons in mammalian CNS.

Although it has been known for some years that the zona compacta of the substantia nigra contains high levels of AChE (EC 3.1.1.7), only recently has evidence been provided for the existence of this enzyme within the dopaminergic neurons of this nucleus (Butcher et al., 1975). At present, however, there is no information regarding the biochemical properties of the cholinesterase in these neurons, and it is to this question that the present experiments were addressed. Two techniques were viewed as providing the means for studying AChE in DA cells of the SN as well as their terminals in the caudate-putamen. When used appropriately, 6-OHDA is a selective neurotoxin for catecholaminergic neurons (Javoy et al., 1976). For example, intraventricular injection of 6-OHDA can produce widespread destruction of catecholaminergic neurons without having significant effects upon neurons which contain other neurotransmitters (Uretsky & Iversen, 1970; McGeer et al., 1973). Furthermore, it has recently been demonstrated that stereotoxic injection of 6-OHDA into the axons of the nigrostriatal bundle results in both anterograde degeneration of DA terminals in the CP and retrograde degeneration of DA perikarya in the pars compacta of the SN (Clavier & Fibiger, 1977). Thus, by comparing the properties of AChE in the SN and CP of control and 6-OHDA lesioned rats, it is possible to deduce some of the characteristics of this enzyme in the DA neurons.

Cholinesterase was characterized by three criteria used to distinguish true AChE from BChE (EC 3.1.1.8). These criteria were: (1) Kinetics. Under certain conditions, AChE demonstrates inhibition by high concentrations of substrate, while BChE shows a monotonically increasing velocity with increasing

Abbreviations used: 6-OHDA, 6-hydroxydopamine; AcßMeCh, acetyl-ß-methylcholine; BCh, butyrylcholine; BChE, butyrylcholinesterase (EC 3.1.1.8); CAT, choline acetyltransferase (EC 2.3.1.6); CP, caudate-putamen; DA, dopamine; DMPA, 6,7-dimethyl-5,6,7,8-tetrahydropri­dine; isoOMPA, tetraisopropylpyrophosphoramide; NSB, nigrostriatal bundle; SN, substantia nigra; TH, tyrosine hydroxylase (EC 1.14.16.2).
acetylcholine concentrations. (2) Substrate selectivity. AChE and BChE hydrolyze different substrates with different velocities. For AChE, the hydrolysis rates for different substrates are ACh > AcβMeCh > BCh while for BChE, BCh > ACh > AcβMeCh (ADAMS, 1949). (3) Selective inhibitors. Several selective inhibitors of AChE and BChE exist (AUSTIN & BERRY, 1953; DUBOIS et al., 1950; ALDRIDGE, 1953; LANDS et al., 1955), but these inhibitors have not been tested in the brain using radioenzymatic assay techniques or on specific nuclei within the extrapyramidal system.

METHODS

Male Wistar rats were obtained from Woodlyn Laboratories, Guelph, Ontario. 6-OHDA (250 μg, dissolved in 25 μl of 0.9% saline, 0.1% ascorbic acid) was injected into the left lateral ventricle under light ether anesthesia, 1 h following pretreatment with pargyline (50 mg/kg). This procedure has been shown to produce extensive damage to both dopaminergic and noradrenergic neurons (BREESE & TRAYLOR, 1971). Controls received vehicle injections. Experimental animals were aphagic for an average of 3 days following injection, and were fed intragastrically to maintain body weight. Control and experimental animals were killed 4-5 weeks following injection.

Another group of rats received 4 μg of 6-OHDA dissolved in 2 μl of the same vehicle, stereotaxically injected into the left NSB at a rate of 0.2 μl/min under pentobarbital anesthesia. These animals received intraperitoneal injections of desipramine HCl (25 mg/kg) 30 min before the 6-OHDA injection to prevent concomitant damage to noradrenergic neurons (ROBERTS et al., 1975). These animals were also allowed to survive 4-5 weeks following surgery. Animals were killed by cervical fracture. Brains were removed rapidly and the CP was dissected on ice. These tissues included globus pallidus and nucleus accumbens, and averaged 45 mg wet tissue weight. The mesencephalon was sectioned on a freezing microtome and the SN care­
tiously co­me Co., North Carolina; and ambenonium chloride. Enzyme activity was cor­
tected for recovery from alumina (60%) and expressed as Vmax. Homogenates were frozen and aliquots were taken as needed for subsequent assays.

CAT was assayed in the incubation mixture described by McCAMAN & DEWHERST (1970) in a final volume of 50 μl. Incubations were for 30 min at 37°C. The product was extracted into 200 μl of 1.5% sodium tetraphenylboron in 3-heptanone, as described by FONNUM (1969). Since most batches of 3-heptanone acidify the aqueous phase, the solven­
t was previously washed in a separator funnel with 0.5 m-NaOH, followed by six washes with distilled water. This procedure reduces the blank and results in consistent 100% efficiency for ACh extraction. Following vigorous agitation and centrifugation, 100 μl of the supernatant was added to scintillation vials. Omission of choline or physostigmine virtually eliminated measured CAT activity.

Cholinesterase activity, using ACh, BCh, or AcβMeCh, was assayed in a final incubation volume of 50 μl, with final concentrations of 15 mM-sodium phosphate buffer (pH 7.00) and 5 mM-substrate, except where noted. Tissues were diluted in water by at least a factor of 500, so that endogenous substrates and ions known to affect AChE (e.g. K+, Ca2+, ACh) had insignificant final concentrations. Incubations were at 37°C for 30 min, and in no case was more than 10% of the substrate consumed. The reaction was linear with time. Extraction with tetraphenylboron (FONNUM, 1969) was used in this assay to remove the labeled substrate: 200 μl of 1.5% tetraphenylboron in washed 3-heptanone was added to stop the incubation, and agitated vigorously. Following centrifugation, the superna­
tant was aspirated, and the extraction procedure repeated. The pH of the aqueous phase remained at 7.0. Following the second aspiration, 25 μl of the aqueous phase was added to scintillation vials containing 0.5 ml of 0.1 m-NaOH.

Ten millilitres of Bray's solution was added to scintilla­
tions were for 20 min at 37°C. The product solutions were used as substrate in control sections.

The following chemicals were obtained from the sources listed: Catalase, Boehringer-Mannheim; ACh bromide, BDH chemicals; tyrosine, BCh, AcβMeCh, sodium tetra­
phenylboron, bovine erythrocyte AChE (Type I), and horse serum BChE (Type X), Sigma; acetylcholine A, DMPH4, Calbiochem; 3-heptanone. Eastman; [acetyl-1-3H]choline. [acetyl-1-14C]choline, [butyryl-1-14C]choline, [acetyl-1-14C]βMeCh, [U-14C]tyrosine. New England Nuclear; tetraisopropyl pyrophosphoramide (isoOMPA) K & K Laboratories; 1.5-bis-(4 allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51). Burroughs Wellcome Co., North Carolina; and ambenonium chloride, Sterling-Winthrop, Rensselaer, New York.

RESULTS

Four to five weeks following intraventricular injection of 6-OHDA, TH in the CP and SN were reduced by 90%, while CAT activity in these areas showed no significant change (Table 1). Cholinesterase activity decreased by 31% in the SN and by 12% in the CP, both of which results were statistically significant (P < 0.001, Student's two-tailed t test).

Four to five weeks following unilateral 6-OHDA injection into the NSB, similar decreases in TH were obtained in the ipsilateral CP and SN without altering CAT activity in these structures (Table 2). Cholinesterase activity was decreased in the SN by 43% (P < 0.001) and by 12% in the CP (P < 0.01). AChE histochemistry by the method of KARNOVSKY & ROOTS (1954) in a NSB-6-OHDA lesioned animal is shown in Fig. 1. The lesioned side (left) shows reduction in staining in the pars compacta and the ventral tegmental area [the A10 group of dopa­
mimetic cells according to UNGERSTEEDT (1971)]. This
FIG. 1. Section of mesencephalon has been stained for AChE. The pars compacta of the substantia nigra (SNC) and the A10 area stain quite densely for AChE. This staining is decreased on the left half of the section, where dopaminergic cells have been selectively lesioned with an injection of 6-OHDA placed in their ascending axons (the NSB).
TABLE 1. THE ACTIVITIES OF THREE ENZYMES FROM SN AND CP OF RATS INJECTED INTRAVENTRICULARLY WITH 6-OHDT EXPRESSED AS PER CENT OF CONTROL ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>TH</th>
<th>CAT</th>
<th>AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td>10.3% ± 1.7%*</td>
<td>88.0% ± 7.1%</td>
<td>69.0% ± 1.4%*</td>
</tr>
<tr>
<td>CP</td>
<td>9.3% ± 1.7%*</td>
<td>93.6% ± 2.6%</td>
<td>87.6% ± 1.8%*</td>
</tr>
</tbody>
</table>

Control values for TH were: TH. 1.14 nmol/mg tissue/h; CAT. 2.54 nmol/mg tissue/h; AChE. 659 nmol/mg tissue/h. Control values for CP were: TH. 1.36 nmol/mg tissue/h; CAT. 18.8 nmol/mg tissue/h; AChE. 2.12 µmol/mg tissue/h. n = 6.
* P < 0.001.

TABLE 2. THE ACTIVITIES OF ENZYMES FROM SN AND CP OF RATS INJECTED WITH 6-OHDA IN THE NSB EXPRESSED AS PER CENT OF CONTROL ± S.E.M.

<table>
<thead>
<tr>
<th>Cholinesterase with different substrates</th>
<th>TH</th>
<th>CAT</th>
<th>ACh</th>
<th>AcβMeCh</th>
<th>BCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td>9.9% ± 14%***</td>
<td>99.6% ± 6.0%</td>
<td>56.7% ± 1.6%***</td>
<td>53.7% ± 3.1%***</td>
<td>96.4% ± 6.0%</td>
</tr>
<tr>
<td>CP</td>
<td>3.4% ± 0.9%***</td>
<td>106.0% ± 4.3%</td>
<td>87.9% ± 1.7%***</td>
<td>92.0% ± 3.5%***</td>
<td>110.7% ± 6.5%*</td>
</tr>
</tbody>
</table>

Control values for SN were: TH. 1.21 nmol/mg tissue/h; CAT. 2.37 nmol/mg tissue/h. Control values for CP were: TH. 1.33 nmol/mg tissue/h; CAT. 18.8 nmol/mg tissue/h. Cholinesterase values for control tissues are listed in Table 3. n = 6.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

The first criterion employed to characterize these enzymes was the use of selective substrates (Table 2 and 3). Erythrocyte AChE hydrolyzed BCh at 1.6% of the rate that it hydrolyzed ACh, while BChE hydrolyzed AcβMeCh at only 0.4% of the rate that it hydrolyzed ACh. Activities reported here were not corrected for the racemic nature of AcβMeCh, only one enantiomer of which is reportedly hydrolyzable (Hoskin, 1963).

As shown in Table 3, the 6-OHDA lesions did not affect the already low rate of BCh hydrolysis in the SN, while there was a slight increase in BCh hydrolysis in the CP, which proved statistically significant (P < 0.05). In contrast, there was a marked decrease in the rate of AcβMeCh hydrolysis in the SN (46.3%, P < 0.001) and an 8% decrease in the CP (P < 0.001). This decrease in AcβMeCh hydrolysis correlates quite well with the decrease in ACh hydrolysis in both SN and CP after 6-OHDA lesions. Also, the relative rates of AcβMeCh hydrolysis to ACh hydrolysis in SN and CP compared closely to the ratio observed for erythrocyte AChE (12-14%).

For the comparison of the kinetic properties of the enzymes each point was graphed as per cent of activity at 1 mM-ACh in order to superimpose the

TABLE 3. SPECIFIC ACTIVITIES OF CHOLINESTERASES FROM FOUR DIFFERENT SOURCES USING THE THREE SUBSTRATES LISTED

<table>
<thead>
<tr>
<th></th>
<th>Specific activities of cholinesterases (nmol/mg/h)</th>
<th>ACh</th>
<th>AcβMeCh</th>
<th>BCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td>684.3</td>
<td>82.8</td>
<td></td>
<td>70.1</td>
</tr>
<tr>
<td>CP</td>
<td>1992</td>
<td>278</td>
<td></td>
<td>70.5</td>
</tr>
<tr>
<td>Erythrocyte AChE</td>
<td>44,300</td>
<td>4940</td>
<td></td>
<td>725</td>
</tr>
<tr>
<td>Serum BChE</td>
<td>583,000</td>
<td>2500</td>
<td></td>
<td>1,480,000</td>
</tr>
</tbody>
</table>

All substrates are 5 mM. n = 6 for brain tissues, n = 2 for commercially supplied erythrocyte AChE and serum BChE.
curves (Fig. 2) SN AChE and CP AChE apparently differ slightly in the substrate concentration giving highest activity, and erythrocyte AChE demonstrated a higher and sharper peak activity; all three enzymes, however, are inhibited by high concentrations of ACh, a property characteristic of AChE, but not BChE (inset, Fig. 2).

In order to quantitate the cholinesterase which is found in dopaminergic neurons, the difference between control and 6-OHDA animals at a single concentration of ACh was measured. That difference (ΔAChE) can be taken as a measure of the AChE activity found in the dopaminergic neuron, assuming that there was negligible plasticity in the other cholinesterase-containing neurons in the region. In order to characterize the kinetics of the dopaminergic cholinesterase, velocities at different concentrations of ACh were measured in SN from control and unilateral NSB 6-OHDA lesioned animals, and ΔAChE values were calculated and plotted in Fig. 3. ΔAChE showed the same typical kinetics as the control SN AChE, indicating that it is true AChE. Unfortunately, the low magnitude of AChE decrease in the CP (as well as the slight increase in BChE) prevented the same analysis from being performed reliably in that region.

Figure 4 shows the effect of increasing concentrations of isoOMPA on cholinesterase activity from the two brain areas, the two standard enzymes, and ΔAChE, calculated as described. isoOMPA selectively inhibited BChE only at concentrations around $10^{-5}$ M. Figures 5 and 6 show that BW284C51 and ambenonium were potent inhibitors of AChE, although there was some inhibition of BChE at higher concentrations. In Figs. 4–6 it is evident that SN AChE and ΔAChE resemble erythrocyte AChE more closely than they resemble BChE on these graphs, but CP AChE resembles erythrocyte AChE more faithfully than the enzymes from SN. The only case where ΔAChE differs markedly from SN AChE is in its dose–response to ambenonium: the $K_i$ for ΔAChE is an order of magnitude lower than for SN AChE, and ΔAChE is more completely inhibited by $10^{-4}$ M ambenonium as well (Fig. 6).

**DISCUSSION**

The decreases in AChE activity in SN and CP that were observed following 6-OHDA lesions of the dopaminergic nigrostriatal projection by two different methods confirm recent histochemical studies which have suggested that AChE is synthesized by the dopa-
AChE in dopaminergic neurons

Fig. 4. Per cent inhibition of cholinesterase by isoOMPA, $1 \times 10^{-6}$ M to $1 \times 10^{-4}$ M. ○ SN (control); △ ΔSN (SN control-SN lesioned); □ CP (control); ○ Erythrocyte AChE; ◆ Serum BChE.

Fig. 5. Per cent inhibition of cholinesterases by BW 284C51, $1 \times 10^{-9}$ M to $1 \times 10^{-4}$ M. ○ SN (control); △ ΔSN (SN control-SN lesioned); □ CP (control); ○ Erythrocyte AChE; ◆ Serum BChE.

Fig. 6. Per cent inhibition of cholinesterases by ambenonium, $1 \times 10^{-6}$ M to $1 \times 10^{-4}$ M. ○ SN (control); △ ΔSN (SN control-SN lesioned) □ CP (control); ○ Erythrocyte AChE; ◆ Serum BChE.
minergic neurons of the SN (Butcher et al., 1975; Butcher & Bilezikian, 1975; Butcher & Hodge, 1976). Both AChE levels measured by radioenzymatic assay and AChE histochemical staining were observed to decrease following treatment with 6-OHDA (Fig. 1 and Tables 1 and 2). Some transsynaptic plasticity of AChE in nondopaminergic cells in the SN and CP following lesion of DA neurons cannot be ruled out. However, in a histochemical study Butcher et al. (1975) examined the synthesis of AChE in the SN following irreversible inhibition of cholinesterase with disopropylfluorophosphate and traced the de novo synthesis of AChE within the DA cell bodies. Thus, transsynaptic plasticity can be ruled out as the sole factor mediating the decrease in AChE in the SN following 6-OHDA. This is not a surprising result, since it has been established that all known catecholamine neurons stain for AChE (Jacobowitz & Palkovits, 1974; Palkovits & Jacobowitz, 1974) and that total brain AChE decreases by 13% following intraventricular 6-OHDA injection in mice (Benton et al., 1975).

The histochemical regimen may not be as reliable as biochemical methods with respect to differentiating between BChE and AChE (Contestabile, 1976; Bridges et al., 1973). In the case of the dopaminergic neurons of the nigrostriatal system, however, the use of several substrates, selective inhibitors, and different concentrations of ACh have confirmed the suggestion that dopaminergic neurons contain true AChE (Butcher et al., 1975). ∆AChE, the AChE which is depleted from the SN by 6-OHDA lesions, was characterized by kinetic properties, substrate specificity, and response to selective inhibitors as true AChE. It is likely that ∆AChE is AChE contained in dopaminergic neurons of the SN. The failure to observe a decrease in BCh hydrolysis after 6-OHDA treatments indicates that BChE is not contained in these neurons.

The difference between AChE and BChE, using the three biochemical criteria, were confirmed with commercial preparations from erythrocytes and serum, respectively. The cholinesterases from the CP and SN fulfill all the criteria for true AChE: (1) SN AChE and CP AChE are inhibited by high concentrations of ACh. (2) The relative rates of ACh to AcβMeCh hydrolysis by SN AChE and CP AChE compared quite closely to the rates observed in this study for erythrocyte AChE. (3) The responses of AChE from these three sources to three selective inhibitors were essentially similar and were distinct from the response of BChE. Some qualitative differences between erythrocyte, SN, and CP AChE were observed. Specifically, SN AChE showed some differences from CP AChE with respect to kinetics (Fig. 2) and effects of selective inhibitors (Figs. 4–6). Second, the effects of selective inhibitors on CP AChE paralleled the effects of these inhibitors on erythrocyte AChE more closely than did SN AChE. Previous workers have also found differences in a kinetic parameter of AChE, the Michaelis constant, in tissue obtained from different regions of the nervous system (Tunnicliff et al., 1976). The qualitative differences of AChE with respect to kinetic properties and response to inhibitors may be related to the heterogeneous molecular properties of this enzyme (Chan et al., 1972; Vijayan & Bowson, 1974; McIntosh & Plummer, 1976; Rieger & Vigny, 1976; Somogyi & Chubb, 1976; Gurd, 1976). There are hazards, of course, with imputing a different molecular structure of AChEs from different sources on the basis of assays performed on a crude homogenate. Although the tissues were diluted at least 500 fold, some very potent component in the tissue may have exerted a modulatory effect on the enzyme’s properties. Likewise, the properties of the purified erythrocyte AChE may be due to an artifact of purification, e.g. a partial proteolysis at some stage of the procedure.

The presence of AChE on noncholinergic neurons has been suggested by some to indicate that those neurons receive a cholinergic input (Parent & Butcher, 1976). The present data and other considerations suggest however that AChE cannot be considered a reliable marker for cholinception. Primarily, this is because there is a dramatic lack of correlation between CAT and AChE in some regions of the nervous system. For example, the cerebellum of various species contains high levels of AChE, but low levels of CAT, and there is no correlation between the two enzyme levels in the different strata of cerebellar cortex (Goldberg & McCaman, 1967). In addition, rabbit dorsal root ganglion contains high levels of AChE, but insignificant levels of CAT (McCaman & Hunt, 1965). AChE appears therefore to be playing an unknown role in these areas where ACh is not being synthesized in significant amounts.

It is generally agreed that compared to the striatum the SN also contains a relatively low CAT to AChE ratio (McGeer et al., 1973; Fonnum et al., 1974) and this raises the question as to the function, if any, of AChE in the dopaminergic neurons of the nigro-striatal projection. At present a cholinergic afferent to the SN has not been demonstrated. Hemitranssections anterior or posterior to this nucleus have no effects upon nigral CAT activities (McGeer et al., 1973; unpublished observations). Furthermore, the failure of kainic acid injections into the SN to affect CAT activity in this nucleus suggests that CAT is not contained within perikarya in the SN which might synapse with DA neurons (Nagy, Vincent, Lehmann, Fibiger & McGeer, in preparation). At present therefore the localization of CAT within the SN is unknown and there is no firm evidence to indicate that the dopaminergic perikarya or dendrites receive cholinergic innervation. If AChE in the DA perikarya does not serve to hydrolyze ACh released upon them, this raises the possibility that the enzyme is synthesized in the SN but that its function with respect to cholinergic transmission occurs in the axon terminals of the NSB in the striatum. Specifically in view of
the growing evidence supporting dendro-axonic transmission (LLINAS & Hess, 1976), it is possible that dopaminergic neurons synthesize ACh to inactivate ACh released by cholinergic dendrites upon which the dopaminergic terminals are thought to synapse (HATTORI et al., 1976; GIORGUIEFF et al., 1976). Alternatively, cholinergic axons in the striatum may synapse on dopaminergic axons or boutons. According to conventional criteria, however, there is no ultrastructural evidence for the presence of axo-axonic synapses in the striatum (KEMP & POWELL, 1971; HATTORI, personal communication).

As a rule, cholinceptive cells may be incapable of localizing AChE to the area of cholinception. In addition to the above considerations, further support for this hypothesis is found in the peripheral sympathetic system, where AChE has been identified in noradrenergic terminals in the pineal gland (ERANKO et al., 1970; RODRIGUEZ DE LORES ARNAIZ & PELLIGRINO DE Iraldi, 1972), and where CAT is virtually negligible (Lehmann, unpublished observations). In view of the well-established cholinergic input to the noradrenergic cell bodies in the superior cervical ganglion which give rise to the noradrenergic terminals in the pineal gland, the presence of AChE in those terminals may be due to a transport process incapable of specifically localizing AChE to the area of cholinception. Therefore, although AChE may prove useful as a marker for cells which are cholinceptive at some locus of the cell, it clearly cannot be utilized to identify the point of cholinergic contact.

To the extent that the decrease in AChE in the CP can be attributed entirely to AChE present in dopaminergic axons and terminals, the cell bodies in the SN appear to transport a greater amount of the enzyme to their terminals than resides in the cell bodies: in terms of absolute enzyme activities, a decrease in the CP of 10,000 nmol/h/CP (i.e. 240 nmol/mg tissue/h × 45 mg tissue/CP) correlates with a decrease in the SN of 2400 nmol/h/SN (i.e. 300 nmol/mg tissue/h × 8 mg tissue/SN). Approximately four times as much AChE is exported to axons and terminals as is retained in the dopaminergic cell bodies and dendrites of the SN—roughly the same ratio as is observed for TH in this system.

The presence of AChE in dopaminergic neurons of the SN has been confirmed biochemically, and the transport of AChE by nigrostriatal axons is suggested. The results presented here shed no light on the question of cholinception by these dopaminergic neurons. Qualitative differences between AChE from CP, SN and bovine erythrocytes has been shown, with respect to kinetics and response to selective inhibitors. The nigrostriatal system may offer a good system for studying molecular isoenzymes of AChE from a homogeneous population of neurons in the CNS. The specific role of AChE on dopaminergic neurons in the SN is not understood. When the origin of CAT in this nucleus is elucidated, and when the question of cholinception by dopaminergic terminals is resolved, the function of the enzyme may be better understood.

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THE LOCALIZATION OF ACETYLCHOLINESTERASE IN THE CORPUS STRIATUM AND SUBSTANTIA NIGRA OF THE RAT FOLLOWING KAINIC ACID LESION OF THE CORPUS STRIATUM: A BIOCHEMICAL AND HISTOCHEMICAL STUDY

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Abstract—The distribution of acetylcholinesterase in the corpus striatum and substantia nigra was examined with the use of kainic acid lesions of the corpus striatum and pharmacohistochemical experiments. Histochemically identified acetylcholinesterase-containing neurons in the striatum were among those which were destroyed by kainic acid. Complementary biochemical studies demonstrated that approximately 50% of the total acetylcholinesterase activity in the striatum was localized in these acetylcholinesterase-containing neurons. Intrastriatal injections of kainic acid produced a substantial decrease in the activity of the glutamic acid decarboxylase in the substantia nigra, thus demonstrating that neurons contributing to the striato- and/or pallidonigral pathways had been lesioned. However, nigral acetylcholinesterase activity was not significantly reduced by the striatal kainic acid injections. Furthermore, stereotaxic injections of colchicine along the course of the striatonigral projection failed to produce an accumulation of acetylcholinesterase in these fibers proximal to the injection. In contrast, injections of colchicine into the nigrostriatal projection led to a proximal accumulation of acetylcholinesterase in the fibers of this system, thus confirming the presence of acetylcholinesterase in the ascending dopaminergic neurons.

It is concluded that the striato- and pallidonigral projections in the rat do not contain significant levels of acetylcholinesterase. Furthermore, acetylcholinesterase-containing neurons in the striatum appear to be interneurons rather than the source of striatal efferents. It is suggested that some of these acetylcholinesterase-containing neurons may be striatal cholinergic interneurons.

Certain nuclei within the extrapyramidal system such as the caudate, putamen and substantia nigra, pars compacta, contain among the highest levels of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) in the central nervous system (Silver, 1974). Recent studies from this laboratory suggest that approximately 40% of the AChE activity in the substantia nigra of the rat is contained within axons and terminals afferent to this nucleus (Nagy, Vincent, Lehmann, Fibiger & McGeer, 1978). Consistent with this finding are the results of earlier lesion studies which led Poirier & co-workers (Olivier, Parent, Simard & Poirier, 1970) to propose that in the cat the projection from the striatum to the substantia nigra contains AChE. Furthermore, it has been suggested that this projection may arise from aspiny neurons in the striatum (Poirier, Parent, Mar-

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; DFP, diisopropylphosphorofluoridate; GAD, glutamic acid decarboxylase.

CHAND & BUTCHER, 1977), which have been shown in pharmacohistochemical experiments to stain intensely for AChE (BUTCHER, Talbot & Bilezikjian, 1975a). In the present experiments, we sought to evaluate further the existence and source of AChE in the striatonigral and pallidonigral projections. In order to destroy these systems selectively without producing concomitant damage to the dopaminergic nigrostriatal projection, which is also known to contain AChE (BUTCHER et al., 1975a; BUTCHER, Talbot & Bilezikjian, 1975b; BUTCHER, 1977; Lehmann & Fibiger, 1978), kainic acid, a neurotoxin which is thought to destroy perikarya selectively, leaving afferent axons and terminals intact (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976), was injected intrastriatally and the effects on AChE and other neuronal enzyme markers in the corpus striatum were determined. Furthermore, the brains of rats lesioned with kainic acid were examined by AChE histochemistry to determine if cells which stained for AChE were destroyed by kainic acid.

217
EXPERIMENTAL PROCEDURES

Male Wistar rats (300–325 g) were obtained from Woodlyn Laboratories, Guelph, Ontario. Ten nmol kainic acid in 1 μl 10 mm sodium phosphate pH 6.5, 0.9% in NaCl was injected over 5 min unilaterally into the caudate-putamen of rats anesthetized with pentobarbital. From stereotaxic zeros, coordinates were: anterior 9.6 mm, lateral 2.8 mm; dorsal 4.5 mm. Two weeks later the rats were killed by cervical fracture and the corpora striata were dissected on ice. These tissues included the caudate-putamen, the globus pallidus, and the nucleus accumbens, and averaged 45 mg wet tissue weight. The mesencephalon was sectioned on a freezing microtome and the substantia nigra was carefully dissected from these sections on ice. Tissues included A9 and A10 areas and pars reticulata, averaging 8 mg wet tissue weight. Homogenization and assay of tyrosine hydroxylase (tyrosine, tetrahydropteridine: oxygen oxidoreductase 3-hydroxylating) EC 1.14.6.2), choline acetyltransferase (Acetyl-CoA: choline-O-acetyltransferase EC 2.3.1.6; ChAT) and AChE were as previously described (LEHMANN & FIBIGER, 1978). Glutamic acid decarboxylase (L-glutamate L-carboxylase EC 4.1.1.15; GAD) was assayed by the method of MCGEER & WADA (1971a). Protein was assayed by the method of LOWRY, ROSEBROUGH, FARR & RANDALL (1951).

Histochemical staining for AChE was performed by the method of KARNOFSKY & ROOTS (1964) at 24–48 h following pretreatment with diisopropylphosphorofluoridate (DFP). The irreversible inhibition of AChE by the non-specific phosphorylating agent DFP allows AChE to be visualized at various stages of regeneration, i.e. following de novo synthesis and subsequent transport to distal cell processes. Combined with other experimental manipulations as outlined in the figure captions, this technique permits the identification of AChE-positive neurons simultaneously with anatomical characterization.

RESULTS

Biochemistry

As seen in Table 1, the marked fall in the activities of GAD and ChAT, enzymes which are contained in neurons whose perikarya are located in the corpus striatum (MCGEER, MCGEER, FIBIGER & WICKSON, 1971b; MCGEER & MCGEER, 1975; HATTORI, SINGH, MCGEER & MCGEER, 1976; RIBAK, 1978), indicated that the kainic acid injections destroyed more than 70% of the γ-aminobutyrate-containing and cholinergic cell bodies. The activity of AChE was reduced by 40% in the same tissues whereas there was some increase in striatal tyrosine hydroxylase. In contrast, nigral tyrosine hydroxylase and ChAT activities were not significantly affected. Most important in terms of the possible presence of AChE in the striatonigral projection was the lack of a significant decrease in the activity of nigral AChE despite the extensive damage to the corpus striatum (Table 1 and Fig. 1) and the extensive damage to the striatonigral and/or pallidonigral projections as shown by the decrease in nigral GAD activity. It should be noted that histological examination of the extent of the kainic acid lesion indicated that it included the globus pallidus as well as the striatum. No significant cell destruction was observed outside the corpus striatum.

Histochemistry

DFP pretreatment suppresses ‘background’ AChE staining (which is presumably contained in axons, terminals and dendritic processes of neurons) and allows the visualization of discrete perikarya. Axonally transported AChE is also seen more clearly at longer survival times. For these reasons, DFP pretreatment was employed for all AChE histochemical experiments.

Intrastratial injections of kainic acid greatly reduced the amount of striatal AChE revealed by histochemistry (Fig. 1A). Furthermore, these injections eliminated the AChE-positive neuronal perikarya visualized by means of DFP pretreatment, which were seen in the unlesioned striatum of the same rat (compare Fig. 1B and C). In the uninjected caudate-putamen, stained perikarya appeared to have aspiny processes, at least at the light microscopic level (Fig. 1B). Injection of colchicine at various rostro-caudal points between the striatum and substantia nigra

| Table 1. Neurotransmitter-related enzymes in corpus striatum and substantia nigra 2 weeks after injection of kainic acid (10 nmol) in the caudate-putamen |
|---------------------------------|------------------|------------------|
|                                | % Control        | Control value ± S.E.M. |
| Corpus striatum                |                  |                  |
| acetylcholinesterase           | 62.5% ± 6.0%*    | 43.4 ± 2.07 μmol/mg protein/h |
| choline acetyltransferase      | 27.5% ± 7.3%*    | 109.9 ± 4.01 nmol/mg protein/h |
| glutamic acid decarboxylase    | 23.3% ± 3.4%*    | 103.7 ± 3.71 nmol/mg protein/h |
| tyrosine hydroxylase           | 126.8% ± 5.5%*   | 7.88 ± 0.319 nmol/mg protein/h |
| Substantia nigra               |                  |                  |
| acetylcholinesterase           | 92.0% ± 4.8%     | 10.9 ± 0.132 μmol/mg protein/h |
| choline acetyltransferase      | 104.0% ± 9.8%    | 16.7 ± 0.96 nmol/mg protein/h |
| glutamic acid decarboxylase    | 51.1% ± 3.8%*    | 265.0 ± 8.75 nmol/mg protein/h |
| tyrosine hydroxylase           | 93.9% ± 4.9%     | 5.59 ± 0.404 nmol/mg protein/h |

n = 12. *P < 0.001, Student’s two-tailed test.
Fig. 1. Loss of acetylcholinesterase-containing neuronal somata after infusion of 10 nmol/μl kainic acid into the right striatum (A). Non-infused side is shown on left side of A and in B; arrows point to individual cell bodies. Dashed lines in A delimit the area displaying loss of acetylcholinesterase activity, shown in detail in C. Acetylcholinesterase method as described previously (Butcher et al., 1975a). 1.5 mg/kg DFP was injected intramuscularly 24 h prior to death. cx, cerebral cortex; fb, fiber bundle perforating the striatum. Scale in A is 4 mm; scale in C is 400 μm and this magnification applies also to B.
FIG. 2. Partial trajectories of the nigrostriatal pathways (arrows, frames A and B) visualized by acetylcholinesterase histochemistry following unilateral intracerebral infusion of colchicine (0.5 μg in 1 μl 0.9% saline; rate = 0.25 μl/min) into the left globus pallidus and adjacent regions (frame A) or into the left medial forebrain bundle regions and contiguous areas (frame B). Rats were killed 48 h after treatment with 1.5 mg/kg DFP and 72 h after infusion of colchicine. In frame C is depicted the striatoni-gral pathway visualized according to the horseradish peroxidase procedure of DE OLMOs (1977); (see also BUTCHER & GISLER, 1977); 0.5 μl of a 40%, horseradish peroxidase solution was unilaterally infused into the caudate-putamen over a 5-min period; rats were killed 48 h after the injection. Anterograde transport of the enzyme reveals the partial trajectory of the striatoni-gral projection (frame C), which bears considerable resemblance to the striatoni-gral pathway as demonstrated by protein-incorporation autoradiography with [3H]proline (cf. BUTCHER, 1978). Horizontal sections are shown. Dashed lines in frame A bracket the area in which portions of the striatoni-gral pathway are contained. Scale = 4 mm. PC, substantia nigra, pars compacta; PR, substantia nigra, pars reticulata; IC, internal capsule; ct, cannula tract. The PELLEGRINO & CUSHMAN (1967) coordinates were: striatum: AP = 2.0, Lat = 3.0, vertical from cortical surface = 5.0; globus pallidus: AP = 0.8, Lat, 3.5, vertical = 6.5. Medial forebrain bundle and adjacent regions: AP = 1.8, Lat = 1.5, vertical = 8.2.
Fig. 3. Accumulation of acetylcholinesterase in fibers of ascending dopaminergic pathways from the substantia nigra and probably also the ventromedial mesencephalic tegmentum (bracketed by arrows in F and H) following unilateral infusion of 0.5 μg 1 μl colchicine into the ventral thalamus (coordinates according to Pellegrino & Cushman (1967): AP = 0.0, Lat = 1.5, vertical from cortical surface = 8.0). The non-infused side of the brain is shown at various levels in A, C, E and G corresponding to the same levels from the same brain sections on the infused side (B, D, F and H). Observe the absence of accumulation of acetylcholinesterase in processes of neuronal somata in the caudate-putamen complex on the infused side of the brain (B, D; compare with A and C, respectively). C and D are higher power depictions of A and B, respectively. E and F show the ventral thalamus and adjacent dorsolateral hypothalamus. G and H depict the substantia nigra and adjacent ventromedial mesencephalic tegmentum. Rats were killed 24 h after intramuscular injection of 1.5 mg/kg DFP and 72 h after intracerebral colchicine infusion. Scale in B is 500 μm and applies to A–B and E–H; scale in D is 300 μm and applies also to C. fb, fiber bundle perforating CP; PC, substantia nigra, pars compacta; PR, substantia nigra, pars reticulata.
reliably resulted in accumulation of AChE in axons of the nigrostriatal projection proximal to the injection site (Fig. 2A and B, Fig. 3E–H). In contrast, accumulation of AChE was never observed in the proximal segments of axons of the striatonigral or pallidonoigral systems after colchicine injections in the vicinity of these projections (Fig. 2A and B, Fig. 3A–D). Finally, unlike the accumulation and increased staining for AChE in the processes of the cells of the zona compacta of the substantia nigra after these colchicine injections, such a phenomenon was never seen in the striatum (compare Fig. 3E–H with 3A–D).

**DISCUSSION**

The present results failed to provide evidence for the presence of AChE in the striatonigral or pallidonoigral projections in the rat. Thus, although the kainic acid lesions extensively destroyed perikarya in the striatum and globus pallidus, including those which stain heavily for AChE, no statistically significant loss of this enzyme could be detected biochemically in the substantia nigra ipsilateral to the lesion. Furthermore, in agreement with conclusions drawn by Shute & Lewis (1967), colchicine injections in the vicinity of nigrostriatal and striatonigral fibers produced AChE accumulation caudal but not rostral to the injection. The factors underlying the apparent discrepancy between these results and those of previous investigators (Olivier et al., 1970) are presently not clear but could conceivably be related to species differences. However, inasmuch as Olivier et al. (1970) utilized electrolytic lesions and long survival times it is also possible that retrograde degeneration of the AChE-containing nigrostriatal projection may have contributed to their findings. This possibility has also recently been put forward by Poirier et al. (1977).

Nigral GAD activity was significantly decreased by these lesions, thus confirming previous suggestions that some of the striatonigral and/or pallidonoigral fibers contain GAD (Hattori, McGeer, Fibriger & McGeer, 1973; Fonnum, Grofova, Rivik, Storm-Mathisen & Walberg, 1974; Gale, Hong & Guidotti, 1977). The decrease in nigral GAD activity following striatal kainic acid lesions is complementary to the decrease in nigral GAD activity following intranigral kainic acid lesions (Nagy et al., 1978), suggesting that nigral GAD is partially derived from intrinsic nigral neurons and partially derived from striatonoigral and/or pallidonoigral afferents.

The modest decrease in striatal AChE observed after kainic acid lesions indicates that approximately 50% of the AChE activity in this structure is contained within neurons which are intrinsic to the corpus striatum. The figure of 50% is obtained by extrapolating to a 100% lesion of the ChAT and GAD markers. We have recently shown that approximately 12% of the total AChE activity in the striatum is contained within the axons and terminals of the dopaminerig no-striatal projection (Lehmann & Fibriger, 1978). Conceivably, cortical, thalamic and raphe afferents could make up the balance. However, two other possibilities must also be considered. First, some AChE may be contained within non-neuronal elements of the striatum (but cf. Butcher et al., 1975a). Second, denervation plasticity of the sort observed with AChE in the superior cervical ganglion (Somogyi & Chubb, 1976; Gisger, Vigny, Gaquare & Rieger, 1978) must be considered as a contributing factor to biochemical changes which result from lesions.

Our observation that kainic acid lesions, which produced extensive damage to the AChE-containing neurons in the striatum, did not result in a biochemically detectable change in AChE activity in the substantia nigra suggests that these AChE-positive cells do not project to the substantia nigra. AChE reactive neurons are few in number and appear aspiny at the light-microscopic level (Butcher et al., 1975a; Poirier et al., 1977). Furthermore, in the monkey (Poirier et al., 1977) AChE reactive neurons are larger (>25 μm) and fewer in number than the medium spiny neuron (12–18 μm, Kemp & Powell, 1971: 13–20 μm, Grofova, 1975). These observations suggest that neurons with high AChE activity are not medium spiny neurons, and that medium spiny neurons have low, if any, AChE activity. Although earlier work suggested that the large aspiny neurons were the source of striatal efferents to the globus pallidus and substantia nigra (Fox, Rafols & Cowan, 1975), more recent studies have demonstrated that the striatal efferents originate predominantly, if not exclusively, from the medium-sized, spiny neurons of Kemp & Powell (1971) (Grofova, 1975; Bunney & Agahianian, 1976; Kocsis, Preston & Kital, 1976; S. T. Kital, personal communication). Lack of AChE staining in these numerous medium-sized, spiny efferent cells is consistent with the absence of a detectable change in AChE activity in the substantia nigra after the kainic acid lesions.

Inasmuch as the aspiny AChE-containing neurons appear not to project to the substantia nigra, this raises the speculation that some of the AChE-reactive cells may be the cholinergic neurons which are also thought to be intrinsic to the corpus striatum (McGeer et al., 1971b). Arguing against this speculation is the immunohistochemical observation by Hattori et al. (1976) that some dendritic spines in the striatum contain ChAT, and that medium spiny neurons, as characterized by an unindented nucleus (Kemp & Powell, 1971), contain ChAT (T. Hattori, personal communication). If this is the case, then on the basis of presently available evidence it would have to be concluded that these cholinergic, spiny neurons do not contain AChE. Such a situation would be unprecedented for cholinergic neurons which typically contain very high levels of AChE activity. Clearly, additional work is required to identify the nature of AChE-containing neurons in the corpus striatum.
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REFERENCES


THE NUCLEUS BASALIS MAGNOCELLULARIS: THE ORIGIN OF A CHOLINERGIC PROJECTION TO THE NEOCORTEX OF THE RAT

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Abbreviations

AChE, acetylcholinesterase; CAT, choline acetyltransferase; DFP, diisopropylphosphorofluoridate; GAD, glutamic acid decarboxylase; HRP, horseradish peroxidase.
The cells of origin of a neocortical cholinergic afferent projection have been identified by anterograde and retrograde methods in the rat. Horseradish peroxidase injected into neocortex labeled large, acetylcholinesterase (AChE)-intense neurons in the ventromedial extremity of the globus pallidus. This same group of neurons underwent retrograde degeneration following cortical ablations. The region in which cell depletion occurred also showed significant decreases in the activities of choline acetyltransferase and AChE. Discrete electrolytic and kainic acid lesions restricted to the medial part of the globus pallidus each resulted in significant depletions of neocortical choline acetyltransferase and AChE. Hemitransections caudal to this cell group did not result in such depletions. Taken together these observations suggest that the AChE-intense neurons lying in the ventromedial extremity of the globus pallidus, as mapped in this study, constitute the origin of a major subcortical cholinergic projection to the neocortex. The utility of AChE histochemistry in DFP-pretreated animals in identifying cholinergic neurons is discussed in the light of this example. Specifically, it is proposed that high AChE activity 4–8 hrs after DFP pretreatment is a necessary, but not sufficient, criterion for the identification of cholinergic perikarya.

The neurons in question appear to be homologous to the nucleus basalis of the substantia innominata of primates, and are thus termed "nucleus basalis magnocellularis" (nBM) in the rat. No evidence was obtained to support the hypothesis that nucleus of the diagonal band projects to neocortex. However, striking similarities in size and AChE activity were observed among the putative cholinergic perikarya of the nBM, the nucleus of the diagonal band, and the medial septal nucleus.

Kainic acid lesions of the neocortex produced uniform and complete perikaryal destruction. These lesions decreased neocortical glutamic acid decarboxylase...
activity, suggesting that there are GABAergic perikarya in the neocortex. However, the same lesions did not affect neocortical choline acetyltransferase. This observation suggests that there are no cholinergic perikarya in the neocortex, a conclusion that is consistent with the absence of intensely AChE-reactive neurons in neocortex.
INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) per se has not proven to be a reliable marker for cholinergic neurons and their projections in the central nervous system (PHILLIS, 1976). For example, it is now known that high levels of AChE are contained in certain dopaminergic and noradrenergic neurons (SILVER, 1974; JACOBOWITZ & PALKOVITS, 1974; BUTCHER, TALBOT & BILEZIKJIAN, 1975; LEHMANN & FIBIGER, 1978).

Despite this lack of specificity for cholinergic neurons, histochemical studies of AChE have proven to be of value in suggesting potential cholinergic projections because all neurons that have been unequivocally characterized as cholinergic appear to contain very high levels of AChE (e.g., the septo-hippocampal projection: LEWIS, SHUTE, & SILVER, 1967; MESULAM, VAN HOESEN & ROSENE, 1977; LYNCH, ROSE & GALL, 1978; the motoneuron, KREUTZBERG, TÓTH & KAIYA, 1975; visceral efferent neurons of the intermediolateral spinal cord, BUTCHER, MARCHAND, PARENT & POIRIER, 1977). On the basis of currently available information it can therefore be postulated that a strong histochemical reaction for AChE is a necessary (but not sufficient) criterion for the identification of cholinergic neurons.

KODAMA (1929) first provided evidence for a projection from the magnocellular neurons of the basal forebrain to the neocortex and this observation has been confirmed and extended by others (DAS, 1971; KIEVET & KUYPERS, 1975; DIVAC, 1975; JONES, BURTON, SAPER & SWANSON, 1976). MESULAM & VAN HOESEN (1976) reported that neurons in the basal forebrain of the rhesus monkey that stain intensely for AChE are also labeled with HRP after injections of the latter enzyme into the neocortex. On the basis of this evidence these authors supported earlier suggestions (SHUTE & LEWIS, 1967; DIVAC, 1975) that there is a cholinergic projection from the basal forebrain, mostly from the nucleus basalis of the substantia innominata, which innervates the precentral neocortex. Re-
recently, KELLY & MOORE (1978) have published results that appear to be consistent with this hypothesis. They found that electrolytic lesions in the vicinity of the globus pallidus of the rat produced significant decreases in cortical choline acetyltransferase activity. EMSON & LINDVALL (1979) have also recently commented on various magnocellular forebrain nuclei as possible origins of the cholinergic innervation of the neocortex. The purpose of the present experiments was to provide further information concerning the origin and localization of cholinergic neuronal elements in the neocortex.

METHODS

Male Wistar rats weighing approximately 300 gm at the time of surgery were used in all experiments. All surgery was conducted while animals were under pentobarbital anesthesia. Horseradish peroxidase (HRP; Sigma type VI) was injected into various cortical areas in 0.1 µl volumes at a concentration of 30% in 0.9% saline. The histological protocol of MESULAM & VAN HOESEN (1976); MESULAM (1976a, b); MESULAM (personal communication) was followed for visualization of HRP with benzidine dihydrochloride: alternate sections were stained for both HRP and AChE, according to the same procedure. Twenty-four to thirty hours following HRP injections, the animals were perfused under deep pentobarbital anesthesia with 50 ml 0.9% saline at room temperature; this was followed by a perfusion of 400 ml cold fixative (1% paraformaldehyde and 1.25% glutaraldehyde, 10% sucrose, 0.1 M sodium phosphate buffer, pH 7.4). The brain was stored in fixative for 2-3 hrs, then transferred into 5% sucrose- 0.1 M sodium phosphate buffer (pH 7.4) and kept overnight at 4°C.

The HRP reaction was carried out essentially as previously described (MESULAM 1976a; MESULAM, personal communication). Free floating sections were rinsed in distilled water for 1 min. They were then incubated for one hour in incubation medium containing 4 mM acetylthiocholine, 10 mM glycine, 2 mM copper sulfate, and 50 mM sodium acetate. The pH of this incubation medium was main-
Sections were then rinsed in six changes of distilled water, 30 sec each. Then they were transferred to 50% benzidine dihydrochloride (Sigma), 0.1% nitroferricyanide, 0.01 M sodium acetate buffer pH 5.0, 10% in ethanol, for 10 min. The tray was lifted out of this last medium briefly, while 5 ml freshly prepared 0.3% H₂O₂ was added to an incubation volume of 100 ml. The tissues were reinserted into this incubation mixture and shaken gently for 4 to 4.5 min.

The HRP-reaction was stopped by transferring the sections into 9% sodium nitroferricyanide, 50% ethanol in 40 mM sodium acetate buffer (pH 5.0), which was freshly prepared and kept at 0-4°C. Following 20 minutes in this medium, sections were rinsed in six changes of distilled water. In order to visualize the AChE reaction product, the sections were immersed in 10% potassium ferri-cyanide for 15 min. This was followed by extensive rinsing in six changes of distilled water. Subsequently, sections were mounted from water onto glass slides, and air-dried. In some cases the sections were counterstained with cresyl violet.

Five types of brain lesions were utilized: (1) Extensive unilateral cortical lesions were made in seven animals by suction. Care was taken not to damage any subcortical structures such as the hippocampus, septum and striatum. The lesions included frontal, dorsal and lateral cortex anterior to the level of bregma. Gelfoam (T) was applied to fill the void. (2) A second group of nine rats received unilateral electrolytic lesions of the nucleus basalis magnocellularis (nBM). The lesions were made by applying a current of 2 mA for 20 sec at the coordinates AP + 8.1 mm, ML + 2.6 mm and DV + 3.6 mm from stereotaxic zero. The incisor bar was set at -4.2 mm. (3) Eleven animals were injected with two nmoles of kainic acid dissolved in NaPO₄ buffered (7.0) sterile saline in a volume of 0.2 μl over a period of seven minutes at the same coordinates used for the electrolytic nBM lesions. (4) Another nine animals received unilateral injections of kainic acid (10 nmol in 2 μl of the same vehicle...
over five minutes) into the frontal cortex at the coordinates AP + 10.8 mm, 
ML + 2.0 mm, and DV -2.2 mm from stereotaxic zero (incisor bar set at + 5.0 mm).

(5) Hemitransections of the brain at a level just posterior to the entopeduncular 
nucleus (AP + 5.9 mm from stereotaxic zero, incisor bar at - 4.2 mm) were made in 
seven animals according to the method of McGEER, FIBIGER, McGEER & BROOKE (1973).

All animals that had received unilateral cortical lesions by suction were 
used after a survival time of six months. Animals that had received electrolytic 
lesions, kainic acid lesions of nBM, and hemitransections were sacrificed two 
weeks postoperatively. Animals which received kainic acid lesions of the cortex 
were sacrificed one week postoperatively. Some brains were sectioned coronally 
on a freezing microtome. The sections were kept cold on ice and from these 
sections the nBM, which included the medial and central globus pallidus, was dis- 
sected from each side of the brain (see Fig. 1). The frontal cortical pole was 
obtained from unfrozen brains by making a coronal cut just anterior to the head 
of the striatum. Care was taken to remove the olfactory bulbs from the ventral 
surface of the frontal cortex.

The activities of choline acetyltransferase (Acetyl-CoA: choline-O-acetyl- 
transferase, EC 2.3.1.6; CAT) and AChE were measured according to modifications 
(LEHMANN & FIBIGER, 1978) of the method of FONNUM (1969). Glutamic acid decar- 
boxylase (L-glutamate 1-carboxylase, EC 4.1.1.15; GAD) was assayed by the method 
of CHALMERS, McGEER, WICKSON & McGEER (1970). Protein was measured according to 
LOWRY, ROSENBRUGH, FARR & RANDALL, (1951).

For histological verification of the kainic acid-induced lesions of the 
frontal cortex and nBM, and electrolytic lesions of the nBM, the animals were 
deeply anesthetized and then perfused with 10% Formalin - 0.9% saline. The brains 
were removed and placed in 10% Formalin - 0.9% saline for two weeks, sectioned 
at 50 μm at the lesion sites on a freezing microtome and stained with cresyl 
violet. Where possible these histological verifications were conducted on the
same brains that were analyzed for the various enzymes. For histochemical studies of AChE, animals were injected intramuscularly with diisopropylphosphorofluoridate (DFP, 1.5 mg/kg, Sigma, dissolved in peanut oil) and sacrificed at various times later. Histochemical staining for AChE was performed according to KARNOVSKY & ROOTS (1964) on 25 μm, free-floating sections. Perikaryal dimensions were estimated both photographically and with the aid of a measuring eyepiece, both of which were calibrated with a stage micrometer.
RESULTS

TOPOGRAPHY OF INTENSELY AChE-REACTIVE NEURONS IN THE BASAL FOREBRAIN

Nucleus of the diagonal band and medial septal nucleus

The organization of intensely AChE-reactive neurons is given in Figs. 1 and 2. At the most rostral level (Fig. 1A-B), numerous intensely AChE-reactive neurons in the medial septal nucleus and vertical limb of the nucleus of the diagonal band (nDB) can be identified. Moving caudally (Fig. 1C-D), the nDB becomes more laterally and ventrally located, and is called the horizontal limb of the nDB (PRICE & POWELL, 1970). At more caudal levels (Fig. 2A-E), this cell group becomes much less compact; now scattered, magnocellular intensely AChE-reactive neurons occupy the regions termed magnocellular preoptic nucleus and lateral hypothalamic area (WYSS, SWANSON & COWAN, 1979). All of these intensely AChE-reactive neurons are more or less contiguous at some point with one another, although as noted there are marked differences in the population densities of the neurons.

Ventral pallidum

A few scattered, magnocellular, intensely AChE-reactive neurons can be seen in the region termed "ventral pallidum" by HEIMER & WILSON (1975) (Fig. 1B-E). It is important to note that the term ventral pallidum refers to the rat's putative homologue of substantia innominata of primates, and does not refer to the ventral part of the globus pallidus (HEIMER & WILSON, 1975). Along the medial border of the globus pallidus is a group of intensely AChE-reactive neurons which may be continuous with the nDB at the more dorsal and caudal aspects of the nDB. However, these neurons, which are believed to be the rat's homologue of the primate nucleus basalis of the substantia innominata (see discussion), have a characteristic morphology and topographical distribution.

Distribution of the nucleus basalis magnocellularis

The rostral pole of the nucleus basalis magnocellularis (nBM) is located
approximately just caudal to the decussation of the anterior commissure. At this point it is situated along the ventral and medial boundaries of the globus pallidus (Fig. 1E) until the tail of the globus pallidus is reached (Fig. 2D). It should be noted, however, that a few scattered characteristically large, intensely AChE-reactive neurons typical of nBM are found deep within the core of the globus pallidus at all rostrocaudal levels. Furthermore, at its most caudal extent, the nBM extends ventrolaterally into the medial half of the tail of the globus pallidus (Fig. 2E). At this point and further caudally (Fig. 2F), many of the intensely AChE-reactive neurons are located interstitially (DAS & KREUTZBERG, 1968), that is, their perikarya and dendrites are situated between the fibre bundles of the internal capsule. At the most caudal levels investigated in this study (Fig. 2F), these large intensely AChE-reactive neurons are located in the ventral edge of the internal capsule, predominantly lateral to the entopeduncular nucleus, which would appear in sections slightly more caudal than Fig. 2F.

Injections of HRP into the frontal and antero-dorsal neocortex resulted in numerous HRP-labeled neurons in nBM (Fig. 3A). With the rostrally located injections used in these experiments, the most anteriorly labeled cells were observed just ventral to the GP in nBM. In those cases in which there was diffusion of HRP from the injection site in the neocortex to subcortical regions, a few labeled cells were occasionally observed in the nDB. However, when the HRP diffusion remained within the confines of the neocortex, no labeling of nDB was observed. The majority of neurons labeled by HRP in the present experiments were found in the group of large, multipolar neurons located in the ventral and medial regions of the posterior half of the GP. These corresponded precisely to the location of the AChE-intense neurons seen in Figs. 1F and 2A. With the rostral cortical HRP injections employed in this study, the caudal extent of
labeled cells in nBM corresponded to the AChE-intense neurons in Fig. 2B. In subsequent preliminary experiments it has been found that more posterior cortical HRP injections (e.g., occipital cortex) label magnocellular neurons at more posterior regions of the nBM, i.e., at the level of the entopeduncular nucleus. In order to determine if the cortical HRP injections did in fact label these intensely AChE-reactive neurons of the nBM, the method of MESULAM & VAN HOESEN (1976) was utilized to demonstrate HRP and AChE in the same section. Although AChE staining was somewhat reduced by this procedure, it nevertheless revealed that some cells in the nBM with a high AChE content also contained HRP reaction product.

MORPHOLOGICAL OBSERVATIONS OF THE INTENSELY AChE-REACTIVE NEURONS IN THE BASAL FOREBRAIN

Nucleus of the diagonal band, medial septal nucleus, and caudate-putamen

The AChE-intense neurons of the medial septal nucleus and nDB have similar morphological features, with the size of the major axis of the perikarya ranging from 19-42 μm, and averaging 29 μm. AChE-intense aspiny neurons of the caudate-putamen have similar dimensions (Fig. 3C, range, 23-47 μm; average, 34 μm).

Since intensely AChE-reactive neurons in the striatum are less densely packed than the intensely AChE-reactive neurons of the medial septal nucleus and nDB, their dendrites are more easily visualized, although for all three of these nuclei, the appreciable "background" staining for AChE limits the distance from the soma at which the dendrite may be seen clearly.

Nucleus basalis magnocellularis

While the perikarya of the nBM have similar dimensions to those neurons described above (major axis ranging from 25 to 45 μm, averaging 35 μm), their dendrites are easily seen against the background of white matter in which they are usually found. Thus the neuropil that these neurons occupy distinguishes them anatomically from the other intensely AChE-reactive neurons of the basal
forebrain. The most striking morphological feature of the neurons of nBM is their "isodendritic" nature (RAMON-MOLINER & NAUTA, 1966; DAS & KREUTZBERG, 1968): their dendrites taper off very gradually from the perikaryon, indeed making it difficult to delineate exactly where the soma ends and the dendrite begins (Fig. 3B). This morphological feature in all probability accounts for the discrepancy between the absolute dimensions reported here and those reported by PARENT, GRAVEL & OLIVIER (1979). The dendrites project directly somatofugally, with minimal branching or deviation in course. Often these cells are arranged in tightly packed clusters, so that the morphology of individual neurons cannot easily be ascertained. These clusters are quite often so densely packed that at low magnification they appear to form a giant neuron. Further, the morphology of these large, interstitial neurons is variable and irregular compared to those in the medial septal nucleus and nDB.

LESION STUDIES

Retrograde degeneration of nucleus basalis magnocellularis

Extensive lesions of the neocortex anterior to bregma resulted in a marked loss of large, intensely AChE-reactive neurons that were located in the medial and ventral aspects of the GP (Fig. 2A-F), in agreement with data presented by DAS (1971) for the rabbit. Of the few AChE-intense neurons that remained in this region on the lesioned side of the brain, the majority appeared to be somewhat shrunken and pyknotic (Fig. 4). In sections that were counterstained with cresyl violet, there was no apparent decrease in the population of the smaller diameter neurons that did not stain intensely for AChE and that were located in the region of the ventral and medial GP. The intensely AChE-reactive neurons of the medial septal nucleus and nDB did not appear to undergo retrograde changes or degeneration after the cortical lesions. On the cortically-lesioned side choline acetyltransferase (CAT) and AChE activity were decreased in the region of nBM, while glutamic acid decarboxylase (GAD) activity was normal (Table I).
Anterograde degeneration following lesions of nucleus basalis

Electrolytic and kainic acid lesions of the nBM resulted in similar and parallel depletions of CAT and AChE activities in the ipsilateral frontal cortex (Table II). Fig. 5 shows, diagrammatically, the extent of electrolytic and kainic acid lesions. Comparison of the areas encompassed by the lesions with the distribution of the large, intensely AChE-reactive, interstitial neurons in the medial and ventral globus pallidus indicated that the lesions damaged a significant number of these neurons. Hemitransections just caudal to the entopeduncular nucleus did not result in significant depletions of either CAT or AChE (Table II).

Cortical kainic acid lesions

Kainic acid injections in the frontal cortex of the rat resulted in uniform neuronal destruction in all layers of cortex as assessed by cresyl violet histology. The affected areas composed roughly 50% of the cortical tissue sample dissected and assayed for CAT, AChE and GAD. These tissues showed no depletion in CAT, a minor depletion in AChE, and a major depletion in GAD (Table III). The kainic acid lesioned tissues did not shrink, since there was no difference between weights of lesioned, contralateral, and control tissues. Histological examination also did not indicate that any shrinkage had occurred one week after the kainic acid lesion.

DISCUSSION

The nucleus basalis - substantia innominata complex

In primates, the delineation of the substantia innominata is relatively straightforward, and likewise the clusters of magnocellular, AChE-intense neurons found within the substantia innominata is clear; hence this cell group is appropriately termed "nucleus basalis of the substantia innominata" in primates (KIEVET & KUYPERS, 1975; JONES et al., 1976; MESULAM & VAN HOESEN, 1976). However, the topography of these nuclei is not as clear in cat and rat (present observations and PARENT, personal communication). HEIMER & WILSON (1975) have
attempted to clarify the current understanding of the topography of the rat's homologue to the substantia innominata called the "ventral pallidum" in the rat, describing it as an area which 1) receives an input from nucleus accumbens and/or olfactory tubercle, 2) lies adjacent to striatal structures, and 3) has neuropil identical to the globus pallidus.

According to descriptions of the nucleus basalis in various species, these neurons are characteristically intensely AChE-reactive, large (25-45 µm), and project to the neocortex (DAS & KREUTZBERG, 1968; DAS, 1971; DIVAC, 1975; JONES et al., 1976; MESULAM & VAN HOESEN, 1976; PARENT et al., 1979). These characteristics have been used as operational criteria for mapping nBM in Figs. 1 & 2. It is clear that in the rat, the neuropil surrounding these neurons does not always resemble that of the globus pallidus; neither does the distribution of these neurons always follow the region of the ventral pallidum outlined by REIMER & WILSON (1975) and NAUTA, SMITH, FAULL & DOMESICK (1978), especially in the more caudal sections (Figs. 1E-2F). This leads us to question the applicability of the term "nucleus basalis of the substantia innominata" for the rat. Hence, we have adopted the more parsimonious nomenclature, "nucleus basalis magnocellularis," and it is suggested that in the rat this cell group is homologous to the nucleus basalis of the substantia innominata in primates. The homology of the nBM in rat and nucleus basalis of the substantia innominata in primates is supported by their common characteristic cortical projections, morphology, and intense AChE activity.

Organization of intensely AChE-reactive neurons in the basal forebrain

The similarities and apparent continuity of large, intensely AChE-reactive neurons of the rat forebrain has already been noted (DIVAC, 1975). On the basis of differences in projection areas of these neurons (EMSON & LINDVALL, 1979), the intensely AChE-reactive neurons in the basal forebrain of DFP-pretreated rat can be divided into at least three main groups: (a) the medial septal nucleus,
(b) the nucleus of the diagonal band of Broca, and (c) the nucleus basalis magnocellularis (nBM). The intensely AChE-reactive neurons of the medial septum are probably the origin of the well known cholinergic septo-hippocampal projection (see LYNCH et al., 1978). The intensely AChE-reactive neurons of the medial septum are continuous with those of the rostral portion of the nDB (Fig. 1A, B). Furthermore, like the medial septal nucleus, some neurons in the nDB also project to the hippocampus (CONRAD & PFAFF, 1976; MEIBACH & SIEGEL, 1977). This also suggests that at least part of the nDB can be viewed as a caudal extension of the medial septal nucleus. The other areas to which the nDB projects include the habenula, the anteromedial nucleus of the thalamus, the interpeduncular nucleus and the mamillary nucleus (CONRAD & PFAFF, 1976; MEIBACH & SIEGEL, 1977; HERKENHAM & NAUTA, 1977). It may be noted that the caudal border of nDB is indistinct from that of the magnocellular nucleus of the preoptic area. Earlier studies (JACOBOWITZ & PALKOVITS, 1974) have suggested that the tightly packed, intensely AChE-reactive cell group of nDB continues into a region not recognized as nDB but generally termed lateral preoptic area (EMSON, PAXINOS, LE GAL LA SALLE, BEN-ARI & SILVER, 1979). It is apparent from Figs. 1 & 2 that the population density of the magnocellular, intensely AChE-reactive neurons decreases in the lateral preoptic area (Fig. 2A-C) and lateral hypothalamic area (Fig. 2D). Despite the present difficulties in defining the boundary, biochemical evidence supports the concept that distinct differences between nDB and lateral preoptic area do exist. Specifically, nDB has much higher choline acetyltransferase activity than the lateral preoptic area (HOOVER et al., 1978). Further studies, based on retrograde neuroanatomical techniques combined with histochemical and morphological identification are required to clarify the distinction between the intensely AChE-reactive neurons in the nDB and those in the magnocellular nucleus of the preoptic area (WYSS et al., 1979). In a similar vein, the projections of the intensely AChE-reactive neurons in the magnocellular nucleus of
the preoptic area and in the lateral hypothalamic area are not presently known and require investigation.

The present results suggest that the nDB does not project to neocortex. Thus, HRP injections confined to the neocortex did not result in labeled cells either in the medial septum or the nDB, in agreement with JONES et al. (1976). However, it remains possible, of course, that the nDB may project to neocortical areas that were not investigated in the present experiments.

DIVAC (1975) found some labeled cells in the medial septum and nDB after cortical HRP injections but according to his Fig. 3, it appears possible that this was due to diffusion of the injected HRP to the hippocampus. Consistent with our failure to label cells in medial septal nucleus and nDB after cortical HRP injections is the finding that the magnocellular, intensely AChE-reactive neurons that characterize these nuclei did not appear to undergo retrograde degeneration or loss after the extensive cortical ablations.

Characterization of the nBM

In contrast to the lack of labeling of the medial septum and nDB, cortical injections of HRP labeled many neurons in nBM (Fig. 3A). These results confirm the findings of DIVAC (1975). The location of these labeled neurons correspond to the distribution of the magnocellular, intensely AChE-reactive neurons which were found to undergo extensive retrograde atrophy or loss after cortical ablation. Furthermore, in one series of animals in which the tissues were processed for both HRP and AChE histochemistry, it was found that all the cells in the nBM that contained HRP reaction product also stained intensely for AChE. These results are in substantial agreement with MESULAM & VAN HOESEN'S (1976) observations in the monkey. It should be noted, however, that these latter authors found a few HRP-labeled, AChE-reactive neurons in nDB after cortical HRP injections. Since nDB labeling was not observed in the present experiments, it is not known whether this discrepancy is due to species differences or to other
factors such as those discussed above. In any event, on the basis of the present observations and in agreement with previous findings (DIVAC, 1975), it can be concluded that the nBM projects widely upon the neocortex of the rat and that the origin of this projection is the group of magnocellular, largely interstitial, intensely AChE-reactive neurons identified in Figs. 1 & 2.

The lesion experiments indicate that the nucleus basalis-neocortical projections is cholinergic. Thus, electrolytic lesions of this region resulted in significant reductions in the neocortical activity of a reliable enzyme marker for cholinergic neurons, choline acetyltransferase. Furthermore, lesions with kainic acid, a neurotoxin which destroys neuronal perikarya but generally leaves fibres of passage intact (MASON & FIBIGER, 1979) yielded the same decreases in neocortical choline acetyltransferase activity. This suggests that the destruction of neuronal perikarya in the region of the electrolytic lesions, and not damage to fibres of passage, was responsible for the decrease in cortical choline acetyltransferase caused by the electrolytic lesions. The observations that hemitranssections just caudal to the entopeduncular nucleus did not affect choline acetyltransferase activity in the frontal cortex supports this conclusion and indicates that neurons caudal to this level do not contribute significantly to the cholinergic innervation of this part of the neocortex. The hypothesis that nBM is a source of a cortical cholinergic projection is further supported by the selective decrease in choline acetyltransferase in nBM caused by retrograde degeneration following cortical lesions (Table I).

Whither the balance of cortical choline acetyltransferase?

While lesions of the nBM resulted in significant decreases in neocortical choline acetyltransferase activity, in no instance was this loss complete. The present lesions were smaller than those employed by KELLY & MOORE (1978); thus it is not surprising that smaller choline acetyltransferase depletions resulted. Cortical isolation also produces much larger depletions in cortical choline
acetyltransferase (GREEN, HALPERN & VAN NIEL,1970). These results have been reproduced in this laboratory (LEHMANN, ATMADJA & FIBIGER, in preparation). The failure of the present lesions to produce a complete depletion of choline acetyltransferase in the cortex could therefore be explained by 1) incomplete lesion of the nBM; 2) the existence of other subcortical neurons which are cholinergic and project to the neocortex, or 3) the existence of cholinergic perikarya in the neocortex itself. Several lines of evidence argue against this last possibility. First, kainic acid injections into the frontal cortex did not significantly affect choline acetyltransferase activity in the cortical region damaged by the kainic acid. These cortical injections did, however, produce histological evidence of massive neuronal loss in the frontal cortex and this was corroborated by the significant decrease in cortical glutamic acid decarboxylase activity. This latter observation suggests that there are GABAergic perikarya in the neocortex. This is consistent with the identification of GABAergic perikarya by autoradiography of labeled GABA uptake in parietal cortex (HÖRFELT & LJUNGDAHL, 1972) and immunohistochemical identification of GABAergic perikarya in visual cortex (RIBAK,1978). Second, there are no intensely AChE-reactive perikarya in the neocortex of control or DFP-pretreated rats. Inasmuch as all identified cholinergic perikarya stain strongly for AChE, this absence in the neocortex is consistent with a completely extrinsic source of cholinergic innervation of the neocortex of the rat. This conclusion is at variance with initial immunohistochemical observations (McGEER, McGEER, SINGH & CHASE, 1974). The possible basis of this discrepancy has been discussed recently by EMSON & LINDVALL (1979). The small depletion of AChE that occurred in the kainic acid lesioned cortical tissues is consistent with both our and previous (KRNJEVIC & SILVER, 1965) observations that weakly AChE-reactive neurons exist in the cortex. These may emit commissural fibres which contain AChE (KRNJEVIC & SILVER, 1965).
Functional considerations

The relationship between nBM and the ventral pallidum, the latter apparently being the rat's homologue for the primate substantia innominata, has important implications for a functional understanding of these neurons. The substantia innominata appears to be a part of the system which is intermediate between extrapyramidal and limbic, termed "olfacto-striatal," and elegantly discussed by HEIMER & WILSON (1975). Since the nucleus basalis is found within the substantia innominata in primates, it may be functionally integrated with the olfacto-striatal system. Whether this association occurs in cat and rat is less clear. On the other hand, some evidence has been presented by DAS & KREUTZBERG (1968) that these AChE-rich neurons may be a rostral extension of the reticular formation. Additional observations relating to this question are found in a recent comparative study of nucleus basalis in rat, cat, and monkey (PARENT et al., 1979). Because of the very different functions these systems are thought to subserve, assignment of nBM to either the descending olfacto-striatal system or the reticular system will be of considerable value in assigning functions to this cortical cholinergic projection. The present results raise the possibility that the dramatic depletion in cortical choline acetyltransferase and AChE in cerebral cortex of victims of Alzheimer's disease (DAVIES, 1979), may be due to a lesion of these subcortical neurons.
ACKNOWLEDGEMENTS

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Figure 1: Distribution of AChE-reactive perikarya in rat forebrain

AChE stain according to KARNOVSKY & ROOTS (1964) in animals pretreated with 1.5 mg/kg DFP i.m. 5 hours preceding sacrifice. A few scattered intensely AChE-reactive neurons can be seen in the regions described by HEIMER & WILSON (1975) and NAUTA et al. (1978) as ventral pallidum (Fig. 1B-E). The distribution of the intensely AChE-reactive neurons operationally defined as nBM (see discussion) differs from the topography of the ventral pallidum, which is thought to represent the rat's homologue to primate substantia innominata (HEIMER & WILSON, 1975; WALAAS & FONNUM, 1979). Although many groups of intensely AChE-reactive neurons are contiguous, if not continuous, the histochemical regimen employed here is not sufficient by itself to clarify the topographical boundaries, for instance, of the nDB which merges with medial septal nucleus along its vertical limb (Fig. 1B) and with the magnocellular preoptic nucleus at caudal levels (Fig. 2A and further caudal). The organization of AChE-reactive neurons in the thalamus and hypothalamus of the rat has been previously described (PARENT & BUTCHER, 1976).

Abbreviations: AC, anterior commissure; CP, caudate-putamen; F, fornix; GP, globus pallidus; IC, internal capsule; LHA, lateral hypothalamic area; LPO, lateral preoptic area; MaPO, magnocellular preoptic nucleus; MS, medial septal nucleus; nBM, nucleus basalis magnocellularis; nDB, nucleus of the diagonal band; OT, olfactory tubercle; SM, stria medullaris; TAD, antero-dorsal thalamic nucleus; TAV, anteroventral thalamic nucleus; TR, reticular thalamic nucleus; TV, ventral thalamus; VP, ventral pallidum, diagrammed according to HEIMER & WILSON (1975).

Calibration bar: 1mm.
Figure 2: Distribution of AChE-reactive perikarya in rat forebrain (cont.)

At more caudal levels, perikarya of nBM become more numerous. In each section, neurons morphologically typical of nBM are occasionally found within the core of the GP (Fig. 2A, B) while the population of neurons comprising the bulk of nBM increases greatly between GP and the internal capsule, forming a network of perikarya and processes as described by PARENT et al. (1979) (Fig. 2B-D). The interstitial character of nBM is seen most dramatically near the tail of the GP (Fig. 2E). The population of nBM perikarya diminishes as the level of the entopeduncular nucleus is approached (Fig. 2F). Note the very low "background" AChE staining in the region of the nBM compared to all other regions described which contain intensely AChE-reactive neurons. Experimental conditions as in Fig. 1. Abbreviations are found in legend to Fig. 1. MaPO, LPO, and LHA have been located according to the description of WYSS, SWANSON & COWAN (1979).

Calibration bar: 1 mm
Figure 3: HRP-labeled (A) and AChE-stained (B) neurons of nucleus basalis magnocellularis

HRP injected into frontal cortex labeled neurons in nBM with morphology identical to those of neurons in nBM when stained for AChE (B) 5 hours following DFP pretreatment. The-HRP labeled section was counter-stained with cresyl violet (A), while the AChE-stained section was not counterstained (B). In some experiments, sections were stained for both AChE and HRP following DFP pretreatment. Neurons which contained the blue HRP product always stained with the characteristic Hatchett's brown for AChE. No other neurons in the areas lesioned (see Fig. 5) contained HRP product. These micrographs were taken at the level of Fig. 1F. The topography of HRP-labeled neurons following cortical injections followed the topography of intensely AChE-reactive neurons labeled nBM in Figs. 1 and 2.

Calibration bar: 50 μm
Figure 4: **Retrograde degeneration of nucleus basalis magnocellularis**

produced by cortical lesions

A,C - lesioned side; B,D - control side.

In low power micrographs (A,B), depletion and shrinkage of nBM perikarya are evident (A). The smaller, weakly staining neurons do not appear to be adversely affected. The micrograph is taken from a section at the level of Fig. 2B. The animal was sacrificed 6 months after the cortical lesions and 12 hours after 1.5 mg/kg DFP. AChE staining was performed according to KARNOVSKY & ROOTS (1964).

Calibration bar: 200 μm

Under higher power (C,D), these micrographs reveal the somal shrinkage and apparent dendritic atrophy sustained by surviving nBM neurons (C).

Calibration bar: 50 μm
Figure 5: The extent of damage caused by electrolytic (A) and kainic acid (B) lesion, as assessed by cresyl violet histology, is indicated diagramatically.

Abbreviations: AC, anterior commissure; EP, entopeduncular nucleus; F, fornix; GP, globus pallidus; IC, internal capsule; SM, stria medullaris; St, caudate-putamen.
Table I. Neurotransmitter-related enzymes in the region of the nucleus basalis magnocellularis six months after extensive unilateral cortical lesions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Control</th>
<th>Control activity ± S.E.M.</th>
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<tbody>
<tr>
<td>choline acetyltransferase</td>
<td>64.1 ± 6.0%**</td>
<td>63.9 ± 2.9 nmol/mg protein/h</td>
</tr>
<tr>
<td>acetylcholinesterase</td>
<td>80.6 ± 3.4%*</td>
<td>19.5 ± 1.6 umol/mg protein/h</td>
</tr>
<tr>
<td>glutamic acid decarboxylase</td>
<td>107.0 ± 9.3%</td>
<td>296 ± 31 nmol/mg protein/h</td>
</tr>
</tbody>
</table>

n=4
*P < .02; **P < .001, Student's two-tailed test.
Table II. Choline acetyltransferase and acetylcholinesterase activities in the frontal cortex after lesions in the region of nucleus basalis magnocellularis

<table>
<thead>
<tr>
<th>Lesion Type</th>
<th>% Control</th>
<th>Control activity ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kainic acid lesions (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>78.0% ± 2.2%***</td>
<td>30.5 ± 0.70 nmol/mg protein/h</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>79.3% ± 4.0%***</td>
<td>5.51 ± 0.10 umol/mg protein/h</td>
</tr>
<tr>
<td>Electrolytic lesions (n=9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>76.2% ± 4.9%*</td>
<td>27.8 ± 1.94 nmol/mg protein/h</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>74.2% ± 3.9%**</td>
<td>5.85 ± 0.36 umol/mg protein/h</td>
</tr>
<tr>
<td>Hemitransections - caudal to nBM (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>95.7% ± 5.3%</td>
<td>26.9 ± 1.63 nmol/mg protein/h</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>96.1% ± 7.5%</td>
<td>5.20 ± 0.45 umol/mg protein/h</td>
</tr>
</tbody>
</table>

*P < .02; **P < .01; ***P < .001, Student's two-tailed test.
Table III. Neurotransmitter-related enzymes in the frontal cortex after local kainic acid injections

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Lesioned side, % Control</th>
<th>Contralateral, % Control</th>
<th>Control (unoperated) activity, ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>choline acetyltransferase</td>
<td>92.2% ± 4.9%</td>
<td>96.4% ± 5.5%</td>
<td>29.1 ± 2.1 nmol/mg protein/h</td>
</tr>
<tr>
<td>acetylcholinesterase</td>
<td>83.4% ± 5.3%*</td>
<td>86.6% ± 4.6%</td>
<td>3.59 ± 0.22umol/mg protein/h</td>
</tr>
<tr>
<td>glutamic acid decarboxylase</td>
<td>59.3% ± 5.0%**</td>
<td>100.4% ± 4.1%</td>
<td>190 ± 7.3 nmol/mg protein/h</td>
</tr>
</tbody>
</table>

n=6
*P < .05; **P < .001, Student's two-tailed test.
Fig. 1
Fig. 2
Fig. 3
Fig. 5A
The following two figures are appended to the manuscript for the copies included in the thesis only. Fig. 6 depicts the area of cortical ablation which resulted in retrograde degeneration of the nBM. Fig. 7 depicts the HRP injection site at its greatest two-dimensional size.
Fig. 7
MINIREVIEW

ACETYLCHOLINESTERASE AND THE CHOLINERGIC NEURON

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Division of Neurological Sciences
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Vancouver, British Columbia, V6T 1W5
Canada

The study of acetylcholinesterase (AChE) dates as far back as the discovery of acetylcholine (1,2). It has widespread distribution and very high activity, generally two orders of magnitude higher than choline acetyltransferase (3). It is stable, and there are many simple assay and histochemical techniques for measuring its activity. For these reasons, AChE has been the subject of a vast amount of research. Yet today no teleological model has been found to explain the distribution of AChE in the central nervous system: The existence of AChE on a given neuron is not sufficient information to predict that neuron's relationship with acetylcholine.

In general there is an excellent correlation between AChE and choline acetyltransferase activity in the rat forebrain on a regional basis (3). It has long been recognized, however, that AChE is radically disproportionate with acetylcholine and choline acetyltransferase in some brain regions (4). For instance, the cerebellum is high in AChE compared to its content of choline acetyltransferase (5), while the inverse holds true for the medial habenula (3) and median eminence (6).

On a cellular level, AChE is found in fairly high activity on some neurons which are known not to be cholinergic and furthermore are not thought to be cholinceptive. Two salient examples are the dopaminergic neurons of the substantia nigra (7-9) and the noradrenergic neurons of the locus coeruleus (10). It is clear from just these two examples, as Koelle pointed out in 1955 (11), that the presence of AChE in a given neuron is not sufficient evidence to indicate that such a neuron is cholinergic.

Nonetheless, on occasion AChE has proven worthy of study in the pursuit which may be called "biochemical neuroanatomy", i.e., the identification of neurons 1) morphologically, 2) by afferent and efferent connections, and 3) by the transmitter(s) used. The study of AChE is a useful adjunct to more specific enzyme markers and conventional neuroanatomical techniques as an arbitrary and characteristic marker of certain classes of neurons. For example, AChE histochemistry formed the link between the biochemical and anatomical characterization of the non-homogeneous organization of the striatum (12,13). It has also proven useful in the identification of similar classes of neurons within the CNS (14) and across species (15). Furthermore, when an irreversible inhibitor such as diisopropylphosphorofluoridate (DFP) is administered in vivo some time preceding sacrifice, the morphological features of neurons that contain AChE are revealed with detail exceeded only by the Golgi method. This powerful modi-

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fication of the AChE histochemical technique was introduced by Lynch and coworkers (16) and has been applied extensively with technical improvements by Butcher and collaborators, particularly in the striatum (7).

Beyond these pragmatic applications, we have lately re-examined the validity of an hypothesis conceived as long ago as 1954 by Koelle (17). Having divided nuclei of the brain into four categories according to the intensity of AChE staining, Koelle generalized from the single example of the motoneuron "... it may be postulated that neurons in the intensely and moderately stained categories are likewise cholinergic." In the light of our understanding 25 years later, it is striking to note how many correct examples of cholinergic neurons are listed in the "intensely stained" category and how few correct examples are listed in the "moderately stained" category. Today, comparison of the density of AChE-staining in various perikarya, although still qualitative, is facilitated by advances in histochemical technique. Thus, well-characterized cholinergic projections originating in the central nervous system have been shown to arise from somata which synthesize large amounts of AChE following DFP-pretreatment. Examples of such cholinergic neurons include the septo-hippocampal projection (18,19) and the motoneuron (20,21).

Most importantly, there is no known example of a cholinergic neuron that does not have high levels of AChE. We may therefore form an empirical generalization based on cases in the central and peripheral nervous systems which adhere to the rule: high AChE activity is a necessary but not sufficient characteristic for identifying cholinergic neurons. If indeed this rule holds true, the easily determined distribution of AChE will greatly accelerate the elucidation of cholinergic neuroanatomy.

The value of this rule depends upon its validity in each circumstance; the discovery of one cholinergic neuron without high levels of AChE will destroy the rule's utility. This laboratory has made use of the rule in three cases in which it seemed most likely to fail. Here we summarize the course of those investigations in three areas of the brain: the striatum, the cerebral cortex, and the globus pallidus.

The Striatum

There are six types of neurons described in the striatum of the cat by Kemp & Powell (22): the large, so-called "aspy" neuron (22-30 μm, mean of major and minor axes), comprising less than 1% of the total neuron population; the medium spiny (12-18 μm), comprising 96% of the population; three other medium-sized neurons (16-18 μm, 16-18 μm, and 12-14 μm), together comprising 3% of the population; and the small neuron (5-9 μm) filling out the last 1%. Traditionally, the large aspiny neuron was considered to be the sole source of the descending projections from the striatum (23,24). This concept has been revised in the light of data gathered in the last decade; now at least 50% of the medium spiny neurons are known to have descending projections from the striatum (23,24). This concept has been revised in the light of data gathered in the last decade; now at least 50% of the medium spiny neurons are known to have descending projections (25,26), while the large aspiny neuron is thought to be an interneuron (27).

In the striatum from DFP-pretreated animals, the large aspiny neuron is unique in that it stains intensely for AChE; the small neuron stains lightly, and the medium cells are generally judged not to stain at all (7). However, immunohistochemical evidence, obtained with antibodies directed against purified choline acetyltransferase, previously suggested that medium spiny neurons were cholinergic (28,29). Similar results were recently obtained by Kiyaga et al. (30) employing a conventional histochemical reaction for localizing choline acetyltransferase ultrastructurally. These observations are
in disagreement with the hypothesis that high levels of AChE are necessarily contained in cholinergic neurons. This empirical rule led us to suspect that the large aspiny neurons, rather than the medium-sized neurons, were the elusive cholinergic interneurons of the striatum (27), the existence of which was originally proposed by McGeer et al. (31).

Data reported by Campochiara & Coyle (32) has indicated that kainic acid injected into the striatum of 10-21 day old rats preferentially depletes choline acetyltransferase, compared to the GABAergic marker glutamic acid decarboxylase. Kainic acid is a neurotoxin which in general destroys neuronal perikarya while leaving afferent axons and terminals intact (33,34). However, striatal neurons appear to require a functional glutamatergic innervation in order to be susceptible to kainic acid's neurotoxic action, as first demonstrated by McGeer et al. (35,36). The simplest hypothesis to explain the preferential depletion of choline acetyltransferase at early postnatal times is that the glutamatergic corticostriatal projection (37,38) establishes a functional synaptic contact with cholinergic neurons slightly earlier than it does with the other neurons of the striatum. The data reported by Campochiara & Coyle (32) thus presented an opportunity to test a prediction of the hypothesis that the cholinergic neuron of the striatum was the AChE-intense large aspiny neuron.

### TABLE I

<table>
<thead>
<tr>
<th></th>
<th>% of Control</th>
<th>Velocity, contralateral striatum ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>74.7 ± 2.0**</td>
<td>153 ± 4.1 nmol/mg protein/h</td>
</tr>
<tr>
<td>AChE</td>
<td>78.4 ± 2.8*</td>
<td>48.5 ± 2.8 umol/mg protein/h</td>
</tr>
<tr>
<td>GAD</td>
<td>96.5 ± 4.6</td>
<td>102 ± 5.3 nmol/mg protein/h</td>
</tr>
</tbody>
</table>

n = 5
*P < .002; **p < .001, Student's two tailed test.

10 nmol kainic acid in 0.5 µl sodium phosphate buffered (pH 7.4) isosmolar Ringer solution was injected unilaterally into the corpus striatum of rats 10 days post partum. 14 days after surgery, choline acetyltransferase (CAT) and acetylcholinesterase (AChE) were significantly reduced, while glutamic acid decarboxylase (GAD) activity was unaffected.

This laboratory reproduced the biochemical data of Campochiara & Coyle (32) in the ten-day old neonate (see Table I) and performed histochemical studies in parallel. The kainic acid injections resulted in a striatum with large, irregularly shaped areas entirely void of the AChE-intense, large aspiny neuron, when visualized by AChE histochemistry following DFP-pretreatment. In contrast, cresyl-violet stained neurons (being composed 96% of medium spiny neurons) and small, weakly AChE-reactive neuron populations were unaltered (Table II). These data argue strongly that neither the medium spiny nor the small neuron of the striatum is cholinergic. It is possible that one of the three minority medium-sized neurons identified by Kemp & Powell (22) may be cholinergic, but by far the favored candidate is the large, AChE-intense aspiny neuron. Furthermore, in recent developmental studies of the striatum pursued in this laboratory, the latero-medial progression of the postnatal development of large, AChE-intense neurons is
paralleled exactly by the regional development of choline acetyltransferase activity (Lehmann & Fibiger, in preparation). Finally, it should be noted that in more recent immunohistochemical experiments aimed at the neuronal localization of choline acetyltransferase, preliminary evidence has been obtained that implicates the large aspiny neuron of the striatum as a cholinergic neuron (39). These observations demonstrate that the criterion requiring high AChE activity as a necessary but not sufficient characteristic of cholinergic neurons was capable of predicting the morphology of the cholinergic neuron in the striatum.

### TABLE II
Quantitation of morphologically identifiable neurons in regions made void of putative cholinergic neuron by neonatal kainic acid lesions

<table>
<thead>
<tr>
<th>Type of neuron</th>
<th>% of Control</th>
<th>Density, contralateral striatal ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresyl-violet stained</td>
<td>106.5 ± 8.1</td>
<td>1574 ± 57.4 neurons/mm^2</td>
</tr>
<tr>
<td>Sml weakly AChE-reactive</td>
<td>124.4 ± 10.6</td>
<td>17.2 ± 1.39 neurons/mm^2</td>
</tr>
<tr>
<td>Large AChE-intense</td>
<td>Zero</td>
<td>12.2 ± 0.60 neurons/mm^2</td>
</tr>
</tbody>
</table>

Identically lesioned rats from the same group on which biochemical assays were performed (Table I) were injected intramuscularly with 2.0 mg/kg DFP. 6 hr later, they were perfused and the brains processed for AChE histochemical staining. Alternate sections were cresyl-violet counterstained. At least 22 sample areas entirely void of large AChE-intense neurons were counted from 3 rats; the corresponding striatal region contralaterally served as control. There were no significant changes in the density of either of the other morphologically identifiable neurons. Note that each of the AChE-reactive neurons represents roughly 1% of the total neuronal population as estimated by cresyl-violet staining.

### The Cerebral Cortex

There are three major lines of evidence that have argued for the existence of cholinergic perikarya in the neocortex: 1) Chronic isolation of cortical slabs results in a large (65% - 80%) but not complete depletion of choline acetyltransferase (40-42). Other workers have reported no decreases in cortical choline acetyltransferase activity following similar operations (38,43). 2) Local electrical stimulation of a chronically-isolated cortical slab results in a long-lasting inhibition of glutamate-induced firing which is blocked by atropine and mimicked by acetylcholine (44,45). 3) Antibodies directed against choline acetyltransferase stain large numbers of neurons in the cortex (46). Further support for the existence of cholinergic perikarya in the cortex has derived from the arguments that the decrease in choline acetyltransferase following cortical isolation may be due to retrograde degeneration of cholinergic perikarya (46) or a "secondary effect of denervation" (38), and the observation that the cortical inhibition evoked by surface stimulation of the cortex in the slab is identical to that found in intact cortex (44).

Arguing against this hypothesis is the observation that there are no intensely AChE-reactive perikarya in the cerebral cortex (17,47,48). This observation becomes more striking in rat cerebral neocortex 5 hours follow-
ing DFP-pretreatment (Lehmann, Atmadja & Fibiger, in preparation), a condition which causes known cholinergic neurons to stain intensely for AChE. The "necessary but not sufficient" rule therefore predicts that there are no cholinergic perikarya in the neocortex. Again, the opportunity to test the validity of the "necessary but not sufficient" criterion presented itself.

Kainic acid was employed by this laboratory to effect a complete and uniform neuronal lesion in frontal cortex of rat, as assessed by cresyl-violet histology. The volume of complete perikaryal depletion comprised approximately 50% of the assayed tissue. In this experiment it was found that glutamic acid decarboxylase activity and high affinity glutamate uptake were decreased in the lesioned tissue by approximately 50 percent. However, choline acetyltransferase activity did not change (Table III). In order to escape the conclusion that there are no cholinergic neurons in the cortex, it would be necessary to invoke the condition that putative cholinergic interneurons of the cortex project heavily for distances exceeding one centimeter, rather than terminating locally. This condition is in disagreement with Arguments 1) and 2) cited above as support for the existence of cholinergic neurons in the cortex. Again, pending agreement of future data, the necessary but not sufficient rule for AChE activity in cholinergic neurons predicted successfully the absence of cholinergic perikarya in the cerebral cortex suggested by the results of the kainic acid lesion experiment (Table III). It should be noted that an explanation for both residual choline acetyltransferase activity and stimulus-evoked release of acetylcholine following cortical isolation is still lacking. Similar large-ly unexplained failures to observe complete depletions of either choline acetyltransferase or acetylcholine release have been observed distal to peripheral cholinergic nerves following positively complete transection in several species (49-56). It is difficult of course to extrapolate across species and from peripheral to central nervous system in order to suggest that an analogous phenomenon does or does not occur in the neocortex of the rat. To perform such a comparison on a percentage basis (57) is all the more hazardous in view of the very low specific activity of choline acetyltransferase in the neocortex compared to the peripheral nerves studied.

Globus Pallidus

On the basis that AChE-rich axons projecting to the neocortex appeared to originate from the pallidum, Shute & Lewis (58) originally proposed the existence of a cholinergic pallido-neocortical projection. This suggestion was subject to question, however, since it was well-known at that time that AChE content was not sufficient to characterize a projection as cholinergic (11). Furthermore, there was no known projection from the globus pallidus to the cortex; and the globus pallidus was known to have extremely low levels of AChE and choline acetyltransferase (59). Yet in 1976, Kelly & Moore (60) found that pallidal lesions did indeed result in substantial decreases of choline acetyltransferase in large areas of neocortex.

Reporting retrograde transport of HRP injected into the cortex, Divac (61) speculated that what Shute & Lewis (58) had identified as neurons in the globus pallidus were actually the rat's homologue of the primate nucleus basalis of the substantia innominata, which also projects to neocortex (62-64). Mesulam & van Hoesen (63) demonstrated that in the primate, HRP was transported to AChE-rich neurons of the nucleus basalis of the substantia innominata, and joined Divac (61) in speculating that these were the source of a cholinergic projection to the neocortex. Ensuing experiments designed to prove that these AChE-rich neurons were the source of the cholinergic projection from the pallidal region in the rat became obvious.
TABLE III

Neurotransmitter-related enzymes in the frontal cortex after local kainic acid injections

<table>
<thead>
<tr>
<th>Lesioned side, % of Control</th>
<th>Control (unoperated) velocity, ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT 92.2 ± 4.9</td>
<td>29.1 ± 2.1 nmol/mg protein/h</td>
</tr>
<tr>
<td>AChE 83.4 ± 5.3*</td>
<td>3.59 ± 0.22 umol/mg protein/h</td>
</tr>
<tr>
<td>GAD 59.3 ± 5.0**</td>
<td>190 ± 7.3 nmol/mg protein/h</td>
</tr>
<tr>
<td>Glu-up 56.6 ± 4.5**</td>
<td>1.23 ± 0.04 umol/mg protein/h</td>
</tr>
</tbody>
</table>

n = 6
*P < .05; **P < .001, Student's two-tailed test.

One week following injection of 10 nmol kainic acid in 2 µl sodium phosphate buffered (pH 7.4) saline into frontal cortex of rat, choline acetyltransferase (CAT) was not significantly decreased. Acetylcholinesterase (AChE) activity was slightly decreased, while major decreases in glutamic acid decarboxylase (GAD) and high-affinity glutamate uptake (Glu-up) were observed.

Introducing the minor refinement of suppressing non-perikaryal AChE staining by DFP-pretreatment to Mesulam & van Hoesen's protocol for simultaneously visualizing HRP and AChE, this laboratory replicated Mesulam & van Hoesen's (63) findings in the rat; HRP injected in the neocortex labelled only neurons of nucleus basalis magnocellularis (nBM) which stained heavily for AChE. Discrete lesions of nBM produced either electrolytically or with kainic acid produced identical and parallel depletions in choline acetyltransferase and AChE in frontal cortex, while hemitranssections slightly caudal to nBM did not affect these enzymes (Lehmann, Nagy, Atmadja & Fibiger, submitted). Six months following cortical ablations, retrograde degeneration in nBM visualized by AChE-histochemistry following DFP pre-treatment was paralleled by choline acetyltransferase and AChE depletions in nBM (Table IV). What had been speculation in 1967 became an obvious conclusion by the close of the seventies: the source of the cholinergic innervation of the neocortex arising from the pallidal region is the group of intensely AChE-reactive neurons, the nBM.

Looking Forward

The above have been but three examples where the predictive utility of high AChE levels in identified neurons can be demonstrated. Several other examples have been cited above where the necessary but not sufficient rule also applies, and there is no clear exception. It must be conceded, however, that relatively few cholinergic neurons in the CNS have been unequivocally characterized. In searching for an exception to the rule that all cholinergic neurons contain high levels of AChE, the habenular complex particularly presents a challenge. The tightly-packed cluster of neurons comprising the medial habenula stains very weakly for AChE following DFP pretreatment, while the lateral habenula contains only moderately-staining neurons (65; Lehmann & Fibiger, unpublished observations). Although at one time the medial habenula was thought to be the sole source of a massive cholinergic projection to the interpeduncular nucleus (66-71), it is now believed that...
the cholinergic input to the interpeduncular nucleus derives at least 50% from the nucleus of the diagonal band (72), which does contain large, intensely AChE-reactive neurons (73) and probably projects along stria medullaris (72,74), although others believe that the source is entirely from cholinergic perikarya residing in the lateral habenula (75,76). Kainic acid injections in the habenular region led to approximately 50% depletion of choline acetyltransferase in the interpeduncular nucleus (77), supporting the notion that the habenular complex was not the sole source of choline acetyltransferase in the interpeduncular nucleus. However, the necessary but not sufficient criterion of high AChE levels indicates that no choline acetyltransferase may originate from perikarya in the habenular complex. It may be possible to account for the data obtained with kainic acid by McGeer et al. (77) on the basis of partial lesion of fibers of passage, which has been demonstrated in a similar dense fiber bundle, namely the dorsal noradrenergic bundle (78). Apart from the nucleus of the diagonal band, at present we are not able to suggest another candidate as an origin for the cholinergic innervation of the AChE-rich interpeduncular nucleus. However, it should be noted that the "simple" neuroanatomy of this complex remains to be clarified, and that the habenular complex represents the most striking anomaly in the central nervous system with regard to disproportionate AChE and choline acetyltransferase activities (3).

### TABLE IV

<p>| Neurotransmitter-related enzymes in the region of nBM six months after decortication |
|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>% of Control</th>
<th>Control velocity ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>64.1 ± 6.0**</td>
</tr>
<tr>
<td>AChE</td>
<td>80.6 ± 3.4*</td>
</tr>
<tr>
<td>GAD</td>
<td>107.0 ± 9.3</td>
</tr>
</tbody>
</table>

n = 4

*P < .02; **P < .001, Student's two-tailed test.

The retrograde degeneration of AChE-intense neurons identified as nucleus basalis magnocellularis (nBM) was paralleled by decreases in choline acetyltransferase (CAT) and acetylcholinesterase (AChE), but not glutamic acid decarboxylase (GAD). Data from Lehmann, Nagy, Atmadja and Fibiger, submitted.

### Summary

While the distribution of AChE in the central nervous system remains largely unexplained, neurons with very high levels of AChE are frequently identified as cholinergic, and cholinergic neurons always have high levels of AChE. This supports the utility of the empirical rule, that identification of cholinergic neurons requires demonstration of high levels of AChE. The application of this rule in conjunction with neuroanatomical and biochemical data has already provided substantial new insights into the organization of central cholinergic systems. It is evident that AChE histochemistry continues to be a valuable neurobiological tool, particularly in characterizing neurons that may be cholinergic, and also in identifying those neurons that cannot be cholinergic.
Acknowledgements

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55. S. Tucek, Brain Res. 82 289-286 (1974).