Molecular Biology and Neurobiology of Choline Acetyltransferase

Paul M. Salvaterra

Division of Neurosciences
Beckman Research Institute of the City of Hope
1450 E. Duarte Road
Duarte, CA 91010

Contents

Abstract

In the 45 years since the first description of choline acetyltransferase (ChAT; EC 2.3.1.6.), significant progress has been made in characterizing the molecular properties of this important neurotransmitter synthetic en-

zyme. We are now on the verge of understanding its genetic regulation and biological function(s). The *Drosophila* cDNA has been cloned, sequenced, and expressed in both a eucaryotic and a procaryotic system. The levels of ChAT specific mRNA have been determined during *Drosophila* development. Monoclonal and polyclonal antibodies have been produced to the enzyme from a variety of sources and used for biochemical and immunocytochemical studies. Two well characterized genetic systems have identified the ChAT gene and described a series of useful alleles. As a nervous system specific protein expressed only in the subset of neurons using acetylcholine as a neurotransmitter, ChAT is a good model for uncovering the processes and factors responsible for regulating genes involved in neurotransmitter phenotype selection and maintenance. Recent studies have described the purification of a cholinergic factor from muscle conditioned medium and indicated the potential importance of nerve growth factor (NGF) for regulating ChAT expression in the central nervous system. These factors, or ones remaining to be discovered, may be involved in the etiology or disease process of neurodegenerative nervous system disorders such as Alzheimer's disease.

Index Entries: Choline acetyltransferase; biochemistry; molecular biology; immunology; gene regulation; neurotransmitter phenotype; Alzheimer's disease; NGF; review.

Introduction

Overview and Scope

Chemical neurotransmission plays a major role in controlling the behavior and health of all animals. A large effort is thus underway in the field of neurobiology to understand neurotransmission at many levels. The enzymes that control the synthesis of the so-called classical small molecule neurotransmitters are likely to be important regulatory points in the pathway from neural input to neural output. The purpose of this article is to briefly review the biochemical and biological information about the enzyme responsible for acetylcholine (ACh) production: acetyl CoA-choline-*O*-acetyltransferase (ChAT, EC 2.3.1.6).

Neurotransmitter biosynthetic enzymes were the subjects of numerous biochemical studies during the last several decades. In recent times the rate of these studies is increasing because neurotransmitters and their enzymes are reduced in several neurological diseases, reemphasizing the health consequences of altered neurotransmitters; and technological advances have allowed us to prepare new reagents, including specific antibodies and nucleic acid probes, for studying neurotransmitters and their enzymes not only with new precision but in entirely new ways. It is my intention to highlight only briefly the early work on ChAT and more thoroughly discuss current issues and newer studies in this area. While many significant biochemical studies of ChAT have been done, the exciting direction for ChAT research now lies in integrating this information into the context of the cell biology of the nervous system. Acetylcholine was the first neurotransmitter discovered (Loewi, 1921), and as a result significant historical importance is often associated with studies of the cholinergic system. Physiological studies of cholinergic neurotransmission, particularly at the experimentally accessible vertebrate neuromuscular junction, have served as the paradigm for later studies of other neurotransmitter systems (Fatt and Katz, 1951). Application of acetylcholine to electrically excitable tissue can result in a variety of agonist induced re-

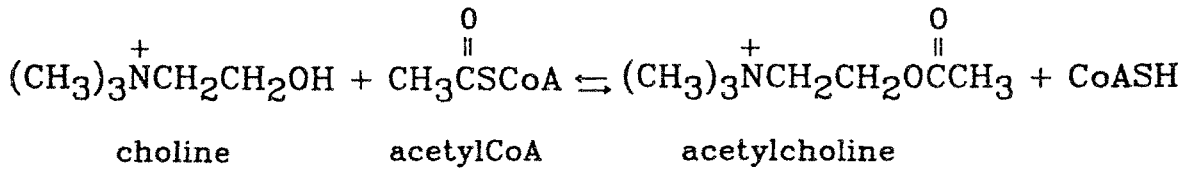

Fig. 1. Biochemical reaction catalyzed by ChAT.

sponses in the target cells. The response repertoire of the neurotransmitter can be the well known cation conductance increase observed at vertebrate neuromuscular junctions resulting in muscle contraction, or any one of a number of excitatory or inhibitory changes in central nervous system neurons from a variety of species (*see* Phillis, 1976; McCaman and Ono, 1982). Most of the physiological changes observed in cholinergically innervated neurons can be explained by the ligand gated opening of either cation or anion selective channels. Recent studies have also indicated the importance of various second messenger systems, such as cyclic nucleotides or phosphatidyl inositol derivatives (Nathanson, 1987) in translating acetylcholine action into an appropriate response. A great deal is known about the molecular properties of the two major acetylcholine receptor types in vertebrates. Recent cloning of the cDNAs for both the nicotinic and muscarinic receptors have been reported (Kubo et al., 1986; Mishina et al., 1985). Site specific in vitro mutagenesis is now being applied to these molecules in order to establish structure–function relationships (Mishina et al., 1985; Sakmann et al., 1985).

ChAT was also one of the first enzymes of neurobiological interest to be discovered and described (Nachmansohn and Machado, 1943). The enzyme catalyzes the biosynthesis of ACh according to the reaction scheme shown in Fig. 1. As a consequence of the extensive studies of ACh and ChAT, people who continue to work in this field are often viewed as "old-fashioned." It is my opinion that such a view is only partly correct. Enzymology is an old field. In a more modern frame of reference ChAT can be viewed as an example of a nervous system specific gene expressed in only a subset of neurons. The factors that regulate the initial decision of a neuron to express ChAT rather than a number of other neurotransmitter phenotypic choices are of interest to a variety of developmental neurobiologists. Understanding the biochemistry of ChAT is sure to help unravel the mysteries of neurotransmitter phenotype selection phenomena. Once a neuron decides to express ChAT it often keeps expressing it for the lifetime of the cell (a hundred years or more in some humans). The factor(s) that help maintain this expression should be of interest to both neurobiologists interested in aging as well as clinicians who deal with the neurodegenerative diseases especially prevalent in old age. These neurodegenerative diseases seem to have a certain amount of neurotransmitter specificity (i.e., Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease). The factors that can maintain and/or change the neurotransmitter phenotype are also of interest to both basic and clinical scientists in view of the potential for implanting cells (including nonneuronal cells) into the brain to alleviate a deficit in a particular neurotransmitter system. The eventual resolution of all of these interesting questions will be aided greatly by understanding more about the enzymes responsible for neurotransmitter synthesis. Even though we know a great deal about cholinergic neurobiology, the best is yet to come. Continued refinement of our knowledge about ChAT is sure to contribute not only to advances in basic neurobiological understanding of such important areas as development, but also to future therapeutic approaches to human dis-

ease such as senile dementia of the Alzheimer's type.

General Background

ChAT is distributed universally in the nervous systems of all species of animals (*see* Mautner, 1977; McGeer et al., 1984; Rossier, 1977). Although the specific types of neurons that use ACh as a neurotransmitter vary among species, it is likely that the distribution of ChAT in the nervous system indicates that it functions primarily by producing acetylcholine for use as a neurotransmitter. The highest tissue levels of ChAT have been reported for *Drosophila* heads (Driskell et al., 1978; Dewhurst et al., 1970) and the electromotor nucleus of *Torpedo* brain (Brandon and Wu, 1978; Salvaterra and Foders, 1979). These observations are not surprising, since insects are thought to use ACh as their primary sensory neurotransmitter (i.e., *see* Gerschenfeld, 1973; Pitman, 1971) and the major function of the electromotor nucleus is to provide the electric organ with a pure cholinergic innervation. ChAT has also been described in the nervous systems of other invertebrates (Emson et al., 1974; Husain and Mautner, 1973; Hildebrand et al., 1974).

Within vertebrate nervous systems there appears to be a close correlation between the levels of ChAT and the number of neurons thought to use ACh as a transmitter. Regions of mammalian CNS that have high levels of ChAT thus include the ventral horn of the spinal cord, the caudate-putamen, and the basal forebrain nuclei while cerebellum is low. Biochemical assay methods have so far provided the only quantitative measure of the levels of enzymatically active ChAT. Unfortunately, these methods are limited in their spatial resolution to the size of the tissue sample that can be dissected (i.e., *see* Cheney et al., 1976.) Many recent studies of ChAT distribution have refined the spatial resolution to the cellular and subcellular level by using immunocytochemical techniques (*see below*). Unfortunately, it is difficult to interpret these immunocytochemical studies in a quantitative way. In favorably organized nervous tissue where the neuronal cell bodies are spatially removed from their synaptic targets (i.e., *Torpedo* electromotor nucleus vs electric organ), there appears to be a surprising amount of active enzyme present in the cell bodies where it has no known functional significance (Rossier, 1977; Fonnum, 1970).

ChAT activity has also been observed in several nonnervous system cells and tissues including plants (Barlow and Dixon, 1973), bacteria (White and Cavallito, 1970; Alpert et al., 1966), primate placenta (Morris, 1966; Hersh et al., 1978; Hersh et al., 1978a), and spermatozoa (Bishop et al., 1976), although the latter has been questioned (Goodman et al., 1984). The functional significance of these observations is unknown. Several interesting proposals have been advanced, however, suggesting a role for ACh in nonsynaptic intercellular communication and/or regulation of the immune system (Mautner, 1977; Rossier, 1977).

Although ChAT seems in general to be distributed within the nervous system in a pattern consistent with its role in producing neurotransmitter, the importance of ChAT activity for regulating ACh levels is often not emphasized (i.e., *see* Tûcek, 1985). Other rate limiting steps for the production of ACh have been favored, including choline uptake, electrical activity, diet, and acetyl-Coenzyme A levels. Whatever the physiologically important rate controlling step for ACh production, it must involve ChAT in an intimate way. The enzyme is poised at a metabolic branch point for common cellular components (acetyl-Coenzyme A and choline), which could be used in a number of other biochemical pathways (i.e., general metabolic reactions or phospholipid biosynthesis).

Once these components encounter ChAT they are almost surely converted into ACh.

Biochemistry

Assay Methods

Much of the early biochemical controversy surrounding studies of ChAT can be traced to the difficulties in unambiguously assaying the enzyme. A thorough discussion of the various assay procedures for ACh can be found in the recent review of Mautner (Mautner, 1986) as well as his earlier summary of this field (Mautner, 1977). Most current biochemical studies of ChAT use a modification of the method originally described by Fonnum (Fonnum, 1969). The method involves transfer of a radioactively labeled acetyl group on acetyl-Coenzyme A to unlabeled choline. After acetylation, the newly synthesized radioactive ACh is separated from the unreacted acetyl-CoA by first forming a complex with tetraphenyl boron followed by solvent extraction into a hydrophobic phase. The advantages of this assay method include its simplicity, speed, and sensitivity. The major disadvantages include a lack of complete specificity for ChAT (unless the pH is carefully controlled, carnitine acetyltransferase will also give positive results) and the inability to resolve kinetic parameters for the enzyme reaction on a short time scale. Several alternative assay schemes have been used to circumvent these shortcomings with respect to kinetic measurements (Hersh et al., 1978; Hersh and Peet, 1977) with varying degrees of success. A significant amount of work has been devoted to identifying specific inhibitors of ChAT (*see* Mautner, 1986; Mauter, 1977), which should allow unequivocal identification of the enzyme. Unfortunately, many of the compounds that best inhibit ChAT activity (such as sulphydryl reagents) lack the requisite specificity to be used effectively in biological studies. Microassays for determining ChAT activity in single cells have also been developed (McCaman and Hunt, 1965).

Purification

The biochemical purification of an enzyme is often one of the earliest undertakings in understanding its function. With a pure protein it is usually possible to clarify the reaction mechanism, determine the amino acid composition and sequence, and use the pure protein as an antigen to generate monospecific antisera. When attempting these types of studies with mixture of unknown proteins, unambiguously interpretable results are difficult to obtain. In addition, it is only possible to evaluate the success of a protein purification scheme relative to a number of criteria, which generally improve with each advance in analytical biochemical methodology. A significant amount of the early literature on ChAT purification must therefore be interpreted with caution.

A key factor in successfully purifying any protein is often its relative abundance in the starting material. With ChAT very few good choices are available. The abundance of the protein can be estimated to vary from only one part in 40,000 for *Drosophila* heads (Slemmon et al., 1982) to less than one part in several million for mammalian brain (Eckenstein et al., 1981). Faced with such extreme purification factors, several groups have made progress in ChAT purification. The enzyme has been purified extensively from nematodes (Rand and Russell, 1985), *Drosophila* heads (Slemmon et al., 1982), squid head ganglia (Husain and Mautner, 1973), chicken brain (Johnson and Epstein, 1986), bovine (Cozzari and Hartman, 1983; Ryan and McClure, 1979) and porcine (Eckenstein et al., 1981) caudate nuclei, rat brain (Ryan and McClure, 1979; Malthe-Sørenssen et al., 1978; Dietz and Salvaterra, 1980), *Torpedo* electric organ (Brandon and Wu, 1978), human placenta (Hersh et al., 1978) and brain (Peng et al., 1980; Roskoski et al., 1975).

Successful purification protocols have combined a number of traditional biochemical techniques, including differential solubility in solutions of salt or polyethylene glycol, ion-exchange chromatography using either cation or anion exchangers, affinity columns made from inhibitors or substrate analogs, and often gel filtration or hydrophobic interaction chromatography (Mautner, 1986). As purification proceeds many investigators have noted the tendency of ChAT to become unstable.

An especially useful purification step that has been incorporated into several protocols involves group specific affinity chromatography using a column derivatized with a textile dye such as Cibacron blue or green-agarose (Roskoski et al., 1975; Hersh et al., 1978; Dietz and Salvaterra, 1980; Eckenstein et al., 1981; Slemmon et al., 1982). Several recent reports have also described the use of immunoaffinity columns derivatized with either monoclonal or polyclonal antibodies prepared against ChAT (Bruce et al., 1985; Levey et al., 1982; Peng et al., 1983; Braun et al., 1987). Immunoaffinity columns should be ideal for achieving large purification factors in a single step, however, their widespread use is limited by several factors. In many cases, the antibodies bind the protein so tightly that it must be denatured before being released from the column. This makes identification of the resulting polypeptide as ChAT problematic since enzyme activity is often essential for positive identification. It is also difficult to adequately characterize even partially denatured protein with respect to any catalytically related properties such as final specific activity that may be useful in quantitative assessment of the purification procedure. When polyclonal antibodies are used as an affinity ligand, their monospecificity must first be established (*see below*). Even monoclonal antibodies are known to react with functionally unrelated proteins that share a common epitope by chance.

With the exception of several earlier reports describing ChAT purification, most current work has resulted in a physical picture of this enzyme that is consistent not only between laboratories but also with respect to the enzyme obtained from different species. This convergence of results from most laboratories in the field is reassuring in the sense that information gained about the biochemistry of ChAT from one source, with a given set of techniques, may be confidently extended to the enzyme isolated from other sources by other procedures. It may, however, be purely coincidental since seemingly easily determined physical constants for ChAT, such as molecular mass and final specific activity, vary outside the range usually expected for purely experimental error (*see below*).

Physical Properties

One of the most fundamental physical properties of an enzyme is its molecular mass. The size of ChAT has been determined using a number of techniques, different sources for the enzyme, and in many different laboratories. Most recent determinations have produced values in the range of 67–69 kdalton using gel filtration or sucrose density gradient centrifugation (Ryan and McClure, 1979; Slemmon et al., 1982; Eckenstein et al., 1981; Peng et al., 1980; Rand and Russell, 1985), and detecting enzyme activity relative to other globular proteins of known molecular mass. The resolution of these techniques is usually not sufficient to determine molecular weights with a precision greater than ±10%. In general, where the enzyme has been sufficiently purified to examine the ChAT-related polypeptide(s) by SDS gel electrophoresis, molecular weights have been reported to be in the same range as the native enzyme activity (Slemmon et al., 1982; Bruce et al., 1985; Braun et al., 1987; Dietz and Salvaterra, 1980; Levey et al., 1982). Molecular weights determined by SDS gel electrophoresis usually have greater precision than gel filtration or sucrose density gradient techniques.

The agreement between molecular weights of the native enzyme and the denatured polypeptides indicates that ChAT is most likely a simple globular protein with no complicated subunit structure. The agreement between molecular weight determinations of *Drosophila* ChAT by sucrose gradient centrifugation and gel filtration reinforce the picture of a simple globular enzyme with little axial asymmetry (Slemmon et al., 1982a).

Some reports have argued for a more complicated subunit structure of ChAT (Chao, 1975; Chao and Wolfgram, 1973) or a simpler peptide component (Chao, 1978). Often relatively impure preparations were used for these studies with the attendant risk of ChAT interacting with one or more impurities (which could generate larger apparent molecular weights) and/or proteases (which could generate smaller apparent molecular weights) (i.e., *see* Hersh and Peet, 1978; Fonnum, 1968; Fonnum and Malthe-Sørenssen, 1973; Hersh et al., 1984).

In several cases, however, a molecular weight significantly greater than 69 kdalton has been reported for purified ChAT polypeptides (Cozzari and Hartman, 1983; Hersh et al., 1984; Badamchian and Carroll, 1985) and in addition multiple ChAT related polypeptides have been observed on SDS gels (Cozzari and Hartman, 1983; Hersh et al., 1984; Dietz and Salvaterra, 1980; Slemmon et al., 1982). Molecular weights larger than 69 kdalton could indicate that ChAT is synthesized initially as a larger protein and normally processed by cellular proteases to the size of the major activity species. It remains possible that in spite of efforts to control proteolysis during enzyme purification, the importance of this mechanism for generating smaller polypeptides during biochemical isolation procedures has been underestimated (Hersh et al., 1984; Mautner, 1986). Other mechanisms which could also result in multiple ChAT polypeptides include the possibility of multiple genes, post-translational modification, differential splicing of RNA, and/or even multiple translation initiation sites in a single mRNA. The relative contributions of any of these processes in generating a more complicated structural picture of ChAT are unknown, but we are now accumulating evidence for one or more of them operating in *Drosophila (see below).*

We have recently reinvestigated the molecular size of *Drosophila* ChAT using high titer polyclonal antibodies prepared against a fusion protein made from a ChAT cDNA clone (Muñoz-Maines et al., 1987). These antibodies allow us to directly examine the ChAT related polypeptides by immunostaining Western blots of fresh homogenates without any prepurification. Our results indicate that an immunologically detectable ChAT protein is present in fresh *Drosophila* head homogenates primarily as a single polypeptide with a molecular weight of about 75 kdalton (although smaller M_r immunoreactive polypeptides can also be detected). In contrast to this value, we have previously demonstrated that completely purified *Drosophila* ChAT shows a mixture of 3 polypeptides with molecular weights of 67, 54, and 13 kdalton (Slemmon et al., 1982). All of these peptides have a shared primary structure as determined by peptide maps (Slemmon et al., 1982a). The simplest interpretation of these results would be that in spite of our best efforts, we have been unable to prevent limited proteolysis of a higher molecular weight protein into smaller components during enzyme purification. This higher molecular weight form of ChAT may not be unique to *Drosophila* since a similar higher molecular weight protein has been observed in vertebrate ChAT preparations using rapid immunoaffinity purification procedures or eliminating proteolysis (Hersh et al., 1984; Cozzari and Hartman, 1983). The size of ChAT in cholinergic cells is thus still subject to some uncertainty.

The existence of various isoelectric forms of ChAT is also still subject to a certain amount of

uncertainty (Malthe-Sørenssen and Fonnum, 1972; Malthe-Sørenssen, 1976). It should be mentioned that limited proteolysis could give rise to ChAT activities with multiple isoelectric points (Hersh et al., 1984; Salvaterra and McCaman, 1985). The exact molecular nature of different charge forms of ChAT in vertebrates is thus unknown. In *Drosophila* there seems to be only a single gene for ChAT (Greenspan, 1980) and the two major isoelectric forms of the enzyme activity seem to be generated by protease action on a single protein (Salvaterra and McCaman, 1985) although other potential mechanisms have not yet been ruled out experimentally.

Subcellular Distribution

Like most other neuron specific proteins, ChAT is most likely synthesized in the cell body of the neuron and transported to the nerve endings where it catalyzes the synthesis of ACh. Several studies have indicated that ChAT is in the slowly transported pool of proteins (Frizell et al., 1970; Jablecki and Brimijoin, 1974; Kasa et al., 1973; Dziegielewska et al., 1976; Jablecki and Brimijoin, 1975; Tûcek, 1975; Tûcek, 1974; Osborne, 1979; Ohshiro et al., 1978; Dahlstrom et al., 1978; O'Brien, 1978; Cannas and Giacobini, 1978; Wooten and Cheng, 1980; Giacobini et al., 1979). It should be mentioned, however, that all studies dealing with the axoplasmic transport of ChAT have relied on detection of enzyme activity. Until we know more about the initial gene products transcribed from ChAT mRNA we should perhaps view the assignment of ChAT to the slow transport pool as tentative. In fact, it is rather surprising that immunocytochemically demonstrable ChAT protein in vertebrate nervous system appears to be more easily detected in neuronal soma rather than in nerve endings (Houser et al., 1983). This curious result may be caused by some technical limitation of the immunocytochemical technique (i.e., poor antibody penetration into nerve ending compartments) or it may reflect the distribution of ChAT polypeptides rather than activity (i.e., the possibility of a non-enzymatically active precursor or product of ChAT).

Even within nerve endings ChAT appears to exist in an aqueous soluble form as well as a membrane associated form (Fonnum, 1968; Fonnum and Malthe-Sørenssen, 1973; Benishin and Carroll, 1984; Benishin and Carroll, 1982; Badamchian and Carroll, 1985). Several interesting biochemical distinctions including catalytic properties and molecular size distinctions have been described for ChAT found in these two different subcellular compartments (Benishin and Carroll, 1984; Benishin and Carroll, 1982; Smith and Carroll, 1980; Badamchian and Carroll, 1985). ChAT has been known for some time to interact with other cellular proteins under a variety of conditions. It has even been demonstrated that some of these interactions may have potentially important consequences for regulating enzyme activity (i.e., *see* Rossier, 1977). So far, however, there appears to be no physiologically functional distinction for differentially distributed subcellular forms of ChAT.

Molecular Biology

The techniques of molecular biology, with their inherent sensitivity and ability to amplify molecular structures, are particularly important for studies of relatively rare nervous system specific proteins such as ChAT. The cDNA for *Drosophila* ChAT has recently been cloned using the lambda gt 11 expression vector and screening a cDNA library with a mixture of anti-ChAT monoclonal antibodies (Itoh et al., 1986). The nucleotide and deduced amino acid sequence of the single clone isolated in this initial screening is shown in Fig. 2. The identification of this cDNA clone as ChAT rests on sev-

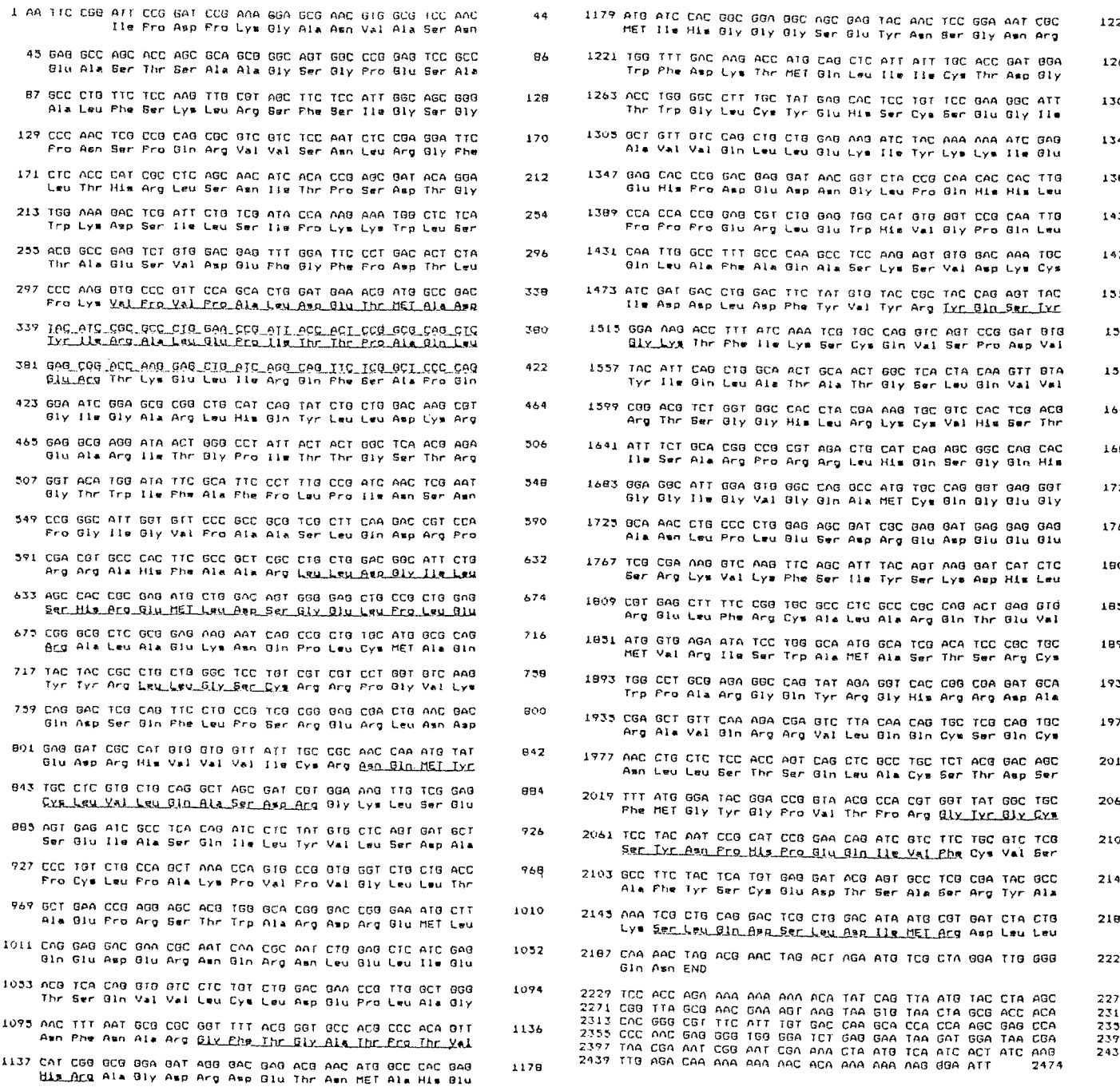

Fig. 2. Nucleotide and deduced amino sequence of *Drosophila* ChAT cDNA. The nucleotides are numbered in the 5' to 3' direction and the deduced amino acid sequence is shown below the nucleotide sequence. The amino acids confirmed by *N*-terminal sequencing of tryptic peptides are underlined. Data is adapted from Itoh et al. (1986).

eral independent criteria. Most importantly, 10 tryptic peptides determined by microsequencing purified enzyme are all represented in the deduced amino acid sequence of the cDNA clone. In addition, this clone maps to the same cytogenetic position for ChAT determined independently by segmental anueploid analysis of *Drosophila* (Greenspan, 1980). We have also recently demonstrated that cRNA synthesized in vitro by SP6 polymerase transcription of this cDNA clone can direct the synthesis of enzymatically active ChAT when injected into *Xenopus* oocytes or other in vitro translation systems (*see below*).

A curious finding during the antibody screening of the expression vector library was the identification of an alternative clone that produced an immunologically positive protein. This alternative clone, even though positive to ChAT monoclonal antibodies, was ruled out as an authentic ChAT cDNA based on its deduced amino acid sequence as well as its cytogenetic position (Itoh et al., 1986, unpublished results).

Several interesting structural findings are apparent from an examination of the ChAT cDNA sequence. There appears to be a high concentration of dibasic amino acid residues (13 pairs) (*see* Fig. 2). This structural feature is most often observed in peptide hormone precursors where these amino acids indicate protease processing sites used during the generation of active hormones. The biological significance of the dibasic amino acid residues in ChAT is unknown, but this structural feature may have important consequences for biochemists interested in purifying an intact protein. The ChAT sequence also contains a consensus glycosylation site even though there is no indication that enzymatically active ChAT contains sugar residues. The enzyme from a number of sources fails to bind to any lectin columns (Mautner, 1986, unpublished results).

The ChAT cDNA clone we have isolated is not a full length cDNA clone. Our clone is only 2.4 kbases in length, while the size of the ChAT mRNA in *Drosophila* is close to 5 kbases in length (Itoh et al., 1986). We have observed a qualitatively indistinguishable size for the ChAT mRNA at all developmental stages of *Drosophila*. It is therefore unlikely that different forms of ChAT mRNA are produced during different stages of the life cycle of flies. In addition, the ChAT cDNA sequence contains no methionine initiation codon or poly-A+ addition site. In spite of the fact that we have isolated only a partial ChAT cDNA, the size of the protein which could be produced by this 2.4 kbase cDNA is about 81 kdalton. This size protein is thus greater than that observed for purified *Drosophila* ChAT (i.e., 67 kdalton, Slemmon et al., 1982). In fact, the amino acid coding sequence represented in our clone could even code for a protein larger than the 75 kdalton immunoreactive band we have recently observed in homogenates of fresh fly heads (Muñoz-Maines et al., 1987). Perhaps *Drosophila* ChAT is synthesized initially as a larger protein that is modified by proteolytic processing to produce smaller molecular weight forms. Alternatively, multiple forms of ChAT protein could be synthesized from a single mRNA by using multiple protein translation initiation sites.

In order to test the ability of our partial cDNA to direct the synthesis of enzymatically active ChAT, we have recently completed two studies. The original pCha-2 cDNA (Itoh et al., 1986) was subcloned into pGEM-2 (Promega Biotec) and two independent plasmids were isolated with the cDNA insert in opposite orientations relative to the SP6 RNA polymerase initiation site in this vector. Injection of in vitro synthesized ChAT cRNA into *Xenopus* oocytes resulted in the production of enzymatically active ChAT detected in extracts of oocytes (*see* Fig. 3, McCaman et al., 1987).

Only RNA in the sense orientation produced active enzyme. Uninjected oocytes, oocytes injected only with water, or with an equivalent

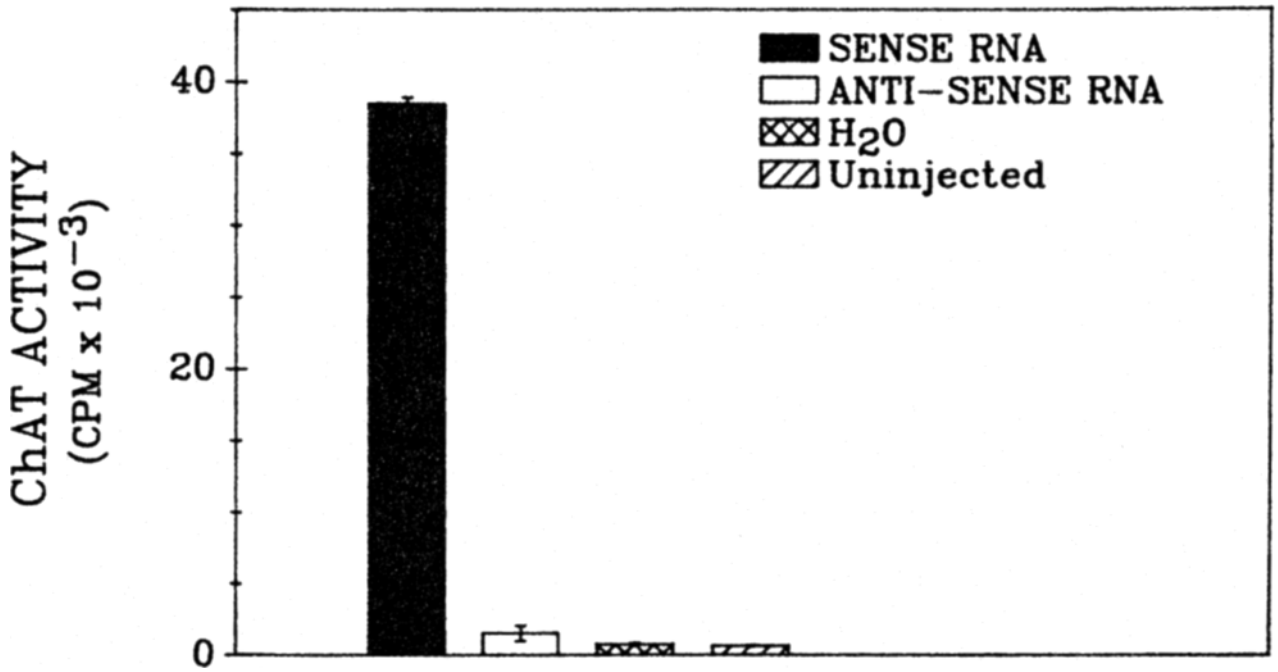

Fig. 3. In vitro synthesized ChAT cRNA in the sense orientation injected into *Xenopus* oocytes produces active ChAT. ChAT activity was measured in extracts of *Xenopus* oocytes which had been injected with 30 ng of SP6 transcribed cRNA in either the sense or anti-sense orientation, 50 nL of water, or uninjected. Two days after injection 3–10 oocytes were pooled, homogenized and assayed for ChAT activity. Values represent the means of triplicate determinations ± the standard error of the mean. Data adapted from McCaman et al. (1987).

amount of anti-sense-oriented cRNA, produced no detectable ChAT activity (*see* Fig. 3). The size of the immunoreactive protein produced by the sense cRNA-injected oocytes and detected with our anti-ChAT fusion protein antibodies is 75 kdalton, as shown in Fig. 4. Interestingly, this is also the size of the major form of ChAT protein recognized by these polyclonal antibodies in fresh *Drosophila* head homogenates. We do not know if an identical protein is being produced in *Drosophila* and the *Xenopus* extracts, since the pGEM vector could conceivably provide a necessary methionine initiation codon (a single inframe start codon is contained in the vector). It has also recently been demonstrated that a complex mixture of total mRNA prepared from either *Torpedo* brain electric lobe or rat CNS can direct the synthesis of enzymatically active ChAT when injected into *Xenopus* oocytes (Gundersen et al., 1985; Berrard et al., 1986).

We have also subcloned the pCha-2 insert into the pKK233-2 expression vector (Pharmacia) and tested the ability of this construct to produce active ChAT in transfected *E. coli*. This vector contains a tryptophan-lactose (TAC) promoter sequence and no possible methionine initiation codon. As shown in Fig. 5, active ChAT can easily be detected in extracts of *E. coli* that have been transfected with this plasmid in the proper orientation to produce sense RNA. In addition, the expression of the ChAT activity is clearly under the control of the TAC promoter since it can be induced by inclusion of IPTG in the medium. Unfortunately, it has so far been technically impossible to determine the size of the protein produced under these conditions since the anti-ChAT antisera we have available for immunostaining are contaminated with anti-*E. coli* antibodies (i.e., antigens present in the fusion protein immunogen). Our cDNA clone

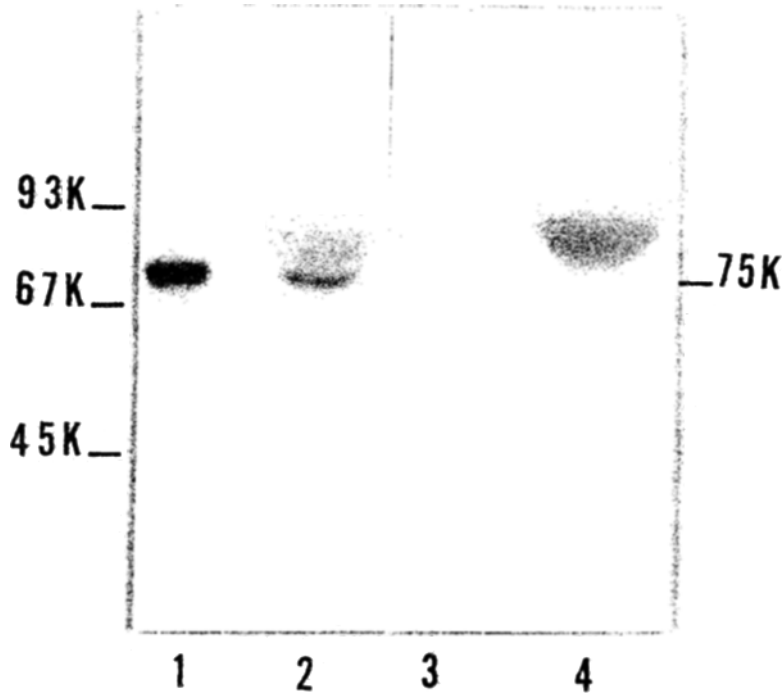

Fig. 4. Western blots of fresh *Drosophila* head homogenate and the protein produced after injection of SP6 transcribed ChAT cRNA into *Xenopus* oocytes. The samples in lanes 1 and 2 were stained with a rabbit polyclonal antiserum that recognizes *Drosophila* ChAT (Muñoz-Maines et al., 1987), whereas lanes 3 and 4 were stained with pre-immune serum from the same rabbit. Lanes 1 and 3 contained a sample of fresh *Drosophila* head homogenate boiled directly in SDS dissociation buffer and estimated to contain approximately 1.4×10^{-4} µmol/min of ChAT activity, whereas lanes 2 and 4 contained an aliquot of *Xenopus* oocyte extract 4 d after injection with SP6 transcribed ChAT cRNA in the sense orientation and estimated to contain 1.4×10^{-5} µmol/min of ChAT activity. Samples were electrophoresed and transferred to nitrocellulose and bands reacting with antibodies were visualized by autoradiography following incubation of the blots in ^{125}I-labeled Protein-A. Molecular weight markers are indicated on the left side of the figure in kdalton. Oocytes injected with ChAT cRNA in the antisense orientation, water, or uninjected, showed immunostaining patterns essentially identical to lane 4. Both the *Drosophila* head sample (lane 1) and the *Xenopus* oocyte extract (lane 2) show a major band specifically staining with antibodies at a M_r of 75 kdalton. Data adapted from McCaman et al. (1987).

can thus code for an enzymatically active protein in both a eucaryotic and procaryotic expression system.

An interesting feature of the *Drosophila* ChAT protein sequence is its homology with *Torpedo* acetylcholinesterase (AChE, Mori et al., 1987). Six homologous peptides can be identified and ordered along the sequence when comparing these two cholinergic macromolecules (*see* Fig. 6).

This type of homology may indicate that ChAT and AChE have evolved from a common ancestral gene. Several short homologous peptides have also been noted when comparing ChAT with a neuronal alpha-type nicotinic acetylcholine receptor subunit from rat (Mori et al., 1987). Although these homologous segments are not extensive enough to indicate a common origin for these two genes, it is interesting that the homology is strongest in the part of the receptor that is thought to be involved in ACh binding (Mishina et al., 1985). In addition to providing important evolutionary insight into the origins of cholinergic

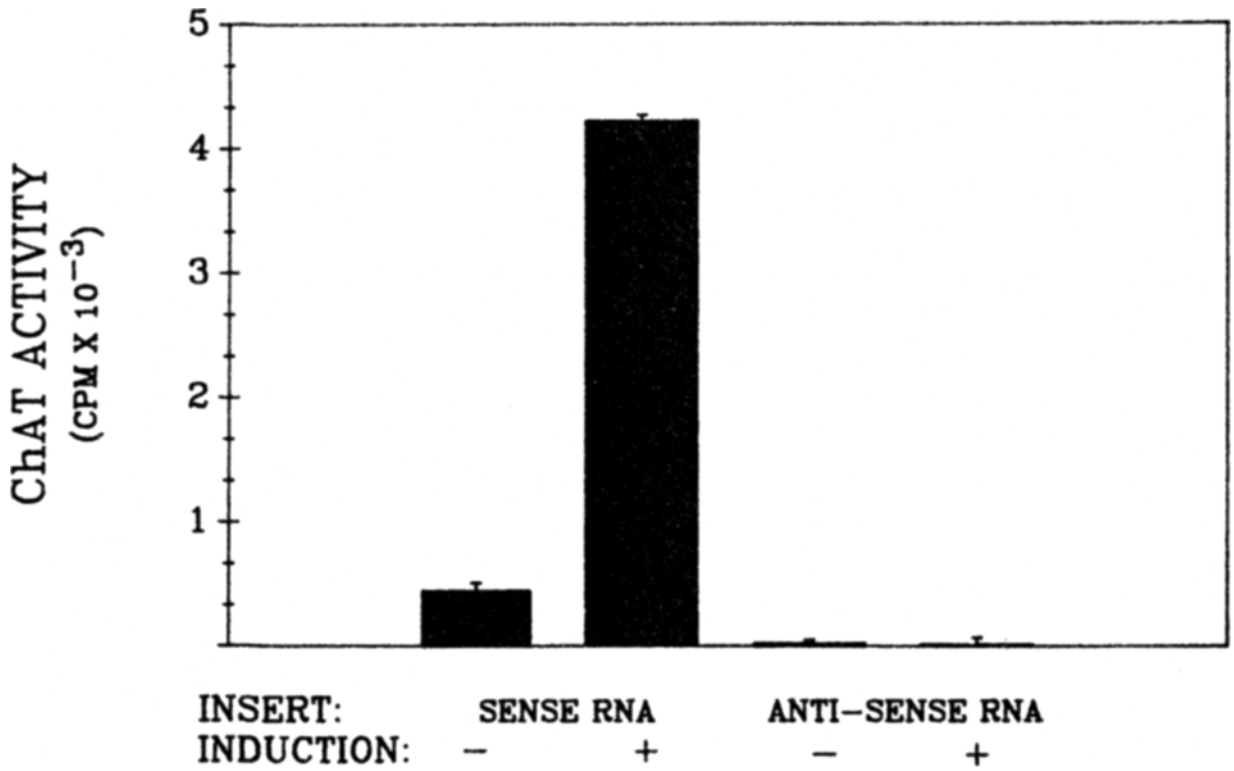

Fig. 5. *Drosophila* ChAT cDNA produces active ChAT under control of the TAC promoter in transfected *E. coli*. The *Drosophila* ChAT cDNA insert (Itoh et al. 1986) was subcloned into the EcoR1 site of pKK223-3 (Pharmacia) in both orientations. ChAT activity was determined in homogenates of transfected *E. coli* JM105 (overnight culture). Transfected cells were induced (+) by addition of 10 m*M* IPTG 1 h before assay. Insert refers to the orientation of the cDNA which produces either sense or antisense RNA. (Data from H. Sugihara and P. M. Salvaterra, unpublished).

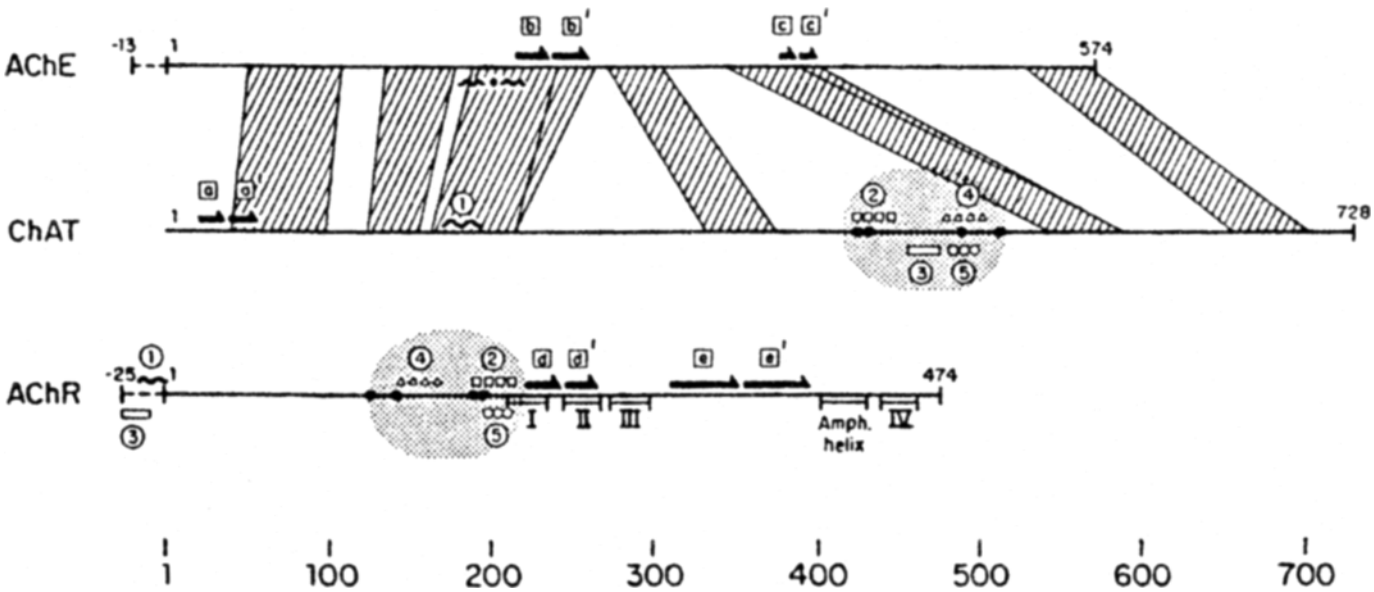

Fig. 6. ChAT, acetylcholine esterase (AChE), and acetylcholine receptor (AChR) share limited sequence homology. Schematic representation of structural homologies within and among *Drosophila* ChAT, *Torpedo* AChE, and rat neuronal nicotinic AChR alpha subunit. Each sequence is represented as a line, while a dashed line indicates the signal sequence or the sequence only included in precursors. The *N*-terminal position of the *Drosophila* ChAT has not yet been identified, and therefore position 1 only indicates the furthest upstream residue so far sequenced. Homologous regions between ChAT and AChE are indicated by shading between the two sequences. The active site residue (Ser 200) for AChE is marked by an asterisk between two thick wavy lines indicating the active site residues homologous to the other esterases and pancreatic proteases. The circled numbers denote the five homologous segments between ChAT and AChR alpha subunit, which are distinguished by different symbols located at approximate positions along each sequence. Thick arrows indicate internally duplicated sequences, which are named a, a', b, b', and so on. The four extracellular cysteine residues characteristic of AChR alpha subunit are marked along the sequence by closed circles. Also shown by closed circles are the four cysteine residues in ChAT. These residues are located in the vicinity of the homologous clustered segments (2–5) when comparing ChAT with the AChR alpha subunit. The two regions enclosed by an oval include all four cysteine residues and the homologous segments of ChAT and the AChR alpha subunit. The transmembrane regions of AChR (I, II, III, and IV) and the amphipathic helix are indicated. The amino acid residue positions are numbered below the lines. Data taken from Mori et al. (1987).

macromolecules, these homologous sequences found in ChAT may serve as useful guides for identifying structural domains that are important for the function of the enzyme. In vitro mutagenesis coupled with ChAT expression systems will now make it possible to design and test various amino acid changes in the ChAT sequence for their effect on enzyme activity.

Many of the unanswered questions regarding the size and detailed amino acid sequence of the primary ChAT translation product can be resolved by isolating a full length cDNA clone for the enzyme. We have made some recent progress in obtaining several additional clones both from our original *Drosophila* head cDNA library as well as alternative cDNA libraries. One clone we have obtained extends several hundred bases in the 5' direction whereas another contains 3.8 kbase pairs of cDNA extending mostly in the 3' direction from our current pCha cDNA. We are now sequencing these extended clone, as well as attempting to isolate additional clones to obtain a complete cDNA sequence.

An additional effort in our laboratory is underway to obtain genomic clones for ChAT. Several candidates have already been isolated and we are now determining the nucleotide sequences, restriction maps, and intron–exon structure of the *Drosophila* ChAT gene.

Immunology

Monoclonal Antibodies

A great deal of recent work on ChAT has gone into the production and use of both monoclonal and polyclonal antibodies. Monoclonal antibodies are excellent reagents for a variety of immunochemical and immunocytochemical studies. Their generation does not depend on the availability of rigorously purified antigen, an especially important feature in light of the controversy surrounding earlier attempts at producing monospecific antisera against ChAT (Rossier, 1975, Rossier, 1981). Several groups have succeeded in producing monoclonal antibodies to ChAT from a variety of species including *Drosophila* (Crawford et al., 1982), bovine (Levey et al., 1981), rat (Crawford et al., 1982a; Ishida et al., 1983; Levey et al., 1983; Ichikawa et al., 1983; Park et al., 1982; Strauss and Nirenberg, 1985), porcine (Eckenstein and Thoenen, 1982), and chicken (Johnson and Epstein, 1986). These antibodies have been shown in several cases to crossreact with ChAT from different species, indicating conserved immunological structural domains in the enzyme (Levey and Wainer, 1982; Salvaterra et al., 1986; Ichikawa et al., 1983; Johnson and Epstein, 1986). Some antibodies are thought to be directed at sequence specific domains since they can recognize ChAT related polypeptides after denaturation and SDS gel electrophorsis (Salvaterra and McCaman, 1985; Levey et al., 1983; Levey et al., 1981; Levey et al., 1982). Other monoclonal antibodies seem to recognize conformationally dependent epitopes since they can interact with only native enzyme (Ichikawa et al., 1983; Crawford et al., 1982a). Antibodies which directly inhibit enzyme activity (i.e., potentially active site directed) and non-inhibiting antibodies have been described (Crawford et al., 1982; Crawford et al., 1982a; Ishida et al., 1983). Several monoclonal antibodies have proven to be important ligands for ChAT purification (Bruce et al., 1985a; Bruce et al., 1984; Eckenstein and Thoenen, 1982) when attached directly to a gel matrix or as part of an immunoaffinity purification step. We have successfully used our anti-*Drosophila* ChAT monoclonal antibodies as screening reagents for isolating a cDNA clone (Itoh et al., 1986).

One of the major uses for anti-ChAT monoclonal antibodies has been the immunocytochemical mapping of cholinergic neurons in the brains of different species. The demonstration of immunocytochemical reaction product

related to a neurotransmitter biosynthetic enzyme is taken as good evidence that the stained cell makes and uses acetylcholine as a neurotransmitter. In other words, anti-ChAT staining can be used to mark the neurotransmitter phenotype of cholinergic neurons. Published work in this area has formed the basis of separate reviews and will not be detailed here (Cuello and Sofroniew, 1984; Wainer et al., McGeer et al., 1984).

Historically, AChE histochemical staining has provided the best picture of the cellular distribution of cholinergic neurons in the brain (i.e., *see* Bigl et al., 1982; Buther and Woolf, 1984; Fibiger, 1982). The application of anti-ChAT antibodies not only confirms many of these early studies, but also provides new information. In some regions of the nervous system AChE staining has not been entirely reliable for marking cholinergic cells (Mesulam et al., 1984; Armstrong et al., 1983; Matthews et al., 1987). ChAT immunocytochemistry has thus been directly combined or compared with AChE histochemistry to demonstrate the close but not absolute correlation of the distribution of these two cholinergic markers (Levey et al., 1984; Matthews et al., 1987; Eckenstein and Sofroniew, 1983; Satoh et al., 1983).

Many recent studies have revealed potentially significant relationships between ChAT containing neurons and other neurotransmitter systems (Chan-Palay and Palay, 1983). Several interesting studies have even demonstrated the colocalization of ChAT along with peptides thought to function as neuromodulators or neurotransmitters such as gallin, substance P, enkephalin, and vasoactive intestinal polypeptide (VIP) (Furness et al., 1984; Melander et al., 1986; Eckenstein and Baughman, 1984). Although these colocalization studies are usually interpreted in terms of separate actions for acetylcholine and the peptide, it is interesting to note that VIP has also been shown to activate ChAT activity (Luine et al., 1984). Some of the more interesting new findings provided by ChAT immunohistochemical studies involve the discovery of new cholinergic cells. For example, small populations of lightly stained intrinsic neurons have been identified in rodent cerebral cortex (Houser et al., 1983) and hippocampus (Matthews et al., 1987). Homologous ChAT positive intrinsic cells have not yet been described in primate cortex or hippocampus, so the generality of their occurrence in different species remains to be determined. It should be mentioned, however, that negative immunocytochemical results, like all negative results, are difficult to interpret. These intrinsic cortical and hippocampal neurons are only lightly stained with most of the available antibodies when compared to more "traditional" cholinergic neurons (*see* Fig. 7).

Even within an area of vertebrate CNS as well studied as the spinal cord, ChAT immunocytochemistry has provided a few surprises (*see* Fig. 7). In addition to intense positive staining of motor neurons, there appears to be an additional population of early developing cholinergic cells in embryonic spinal cord that are also stained. These cells reside primarily between the dorsal and ventral horn and have been named partition cells (Phelps et al., 1984).

Some of the most intensively studied brain regions using ChAT immunocytochemistry are the basal forebrain nuclei of animals. ChAT immunocytochemistry has helped construct a three-dimensional model for this rather widely dispersed collection of cells (Mesulam et al., 1984; Mesulam et al., 1983). The cells with a cholinergic phenotype have been emphasized primarily because of their potential relationship to the Nucleus basalis of Meynert in humans. Large cholinergic neurons in the Nucleus basalis have been shown to be substantially decreased in number in postmortem tissue taken from the brains of Alzheimer's disease patients (for review, *see* Terry and Davies, 1980). The reduction of ChAT positive neurons correlates well with the striking decrease in

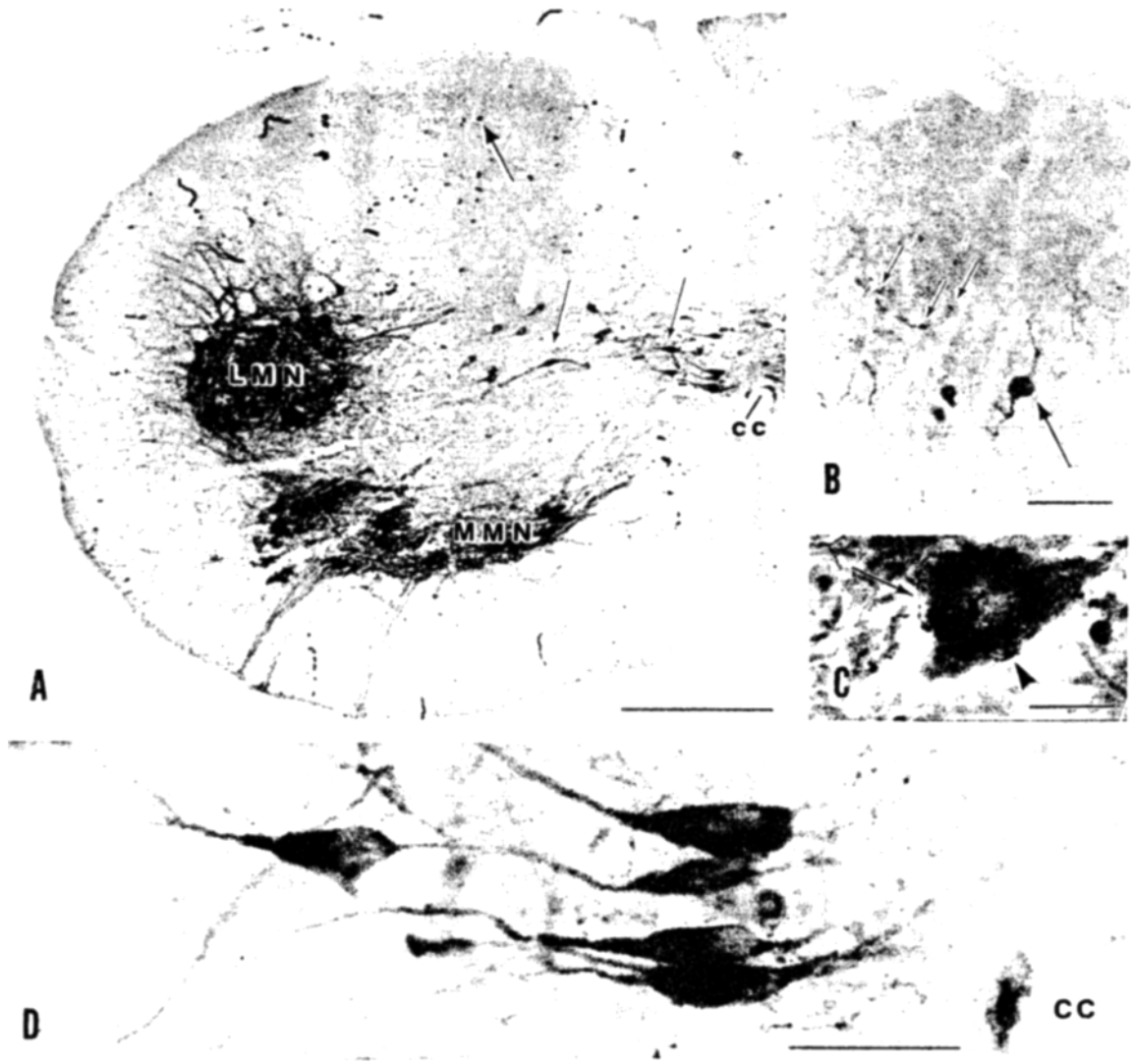

Fig. 7. Immunocytochemistry of rat spinal cord using a monoclonal anti-ChAT antibody and the PAP technique. Panel A shows a section of rat cervical spinal cord containing ChAT-positive medial (MMN) and lateral (LMN) motorneurons, as well as immunoreactive neurons (small arrows) in the intermediate gray matter and around the central canal (CC). A band of ChAT-positive punctate staining is seen in the dorsal horn along with a positively stained ChAT-positive neuron (large arrow: shown at higher magnification in panel B). Scale: 300 μm. Panel B is an enlargement of the small, ChAT-positive dorsal horn neuron (large arrow) shown in A. Additionally, ChAT-positive puncta (small arrows) interconnected by thin fibers are visible. Scale: 40 μm. Panel C illustrates a thin ChAT-positive axon (arrow) which courses toward a densely stained lateral motorneuron and terminates on it in a synaptic-like swelling. A second punctate structure (arrowhead) is also adjacent to the motorneuron. Electron microscopy of similar structures has shown them to be presynaptic elements of synaptic junctions. Scale: 20 μm. Panel D shows the intensely stained, ChAT-positive neurons located near the central canal in A at higher magnification to illustrate the extent to which immunoreaction product fills their processes. Scale: 40 μm. Photomicrographs provided by P. E. Phelps, R. Barber, and J. E. Vaughn.

ChAT activity seen in neurochemical analysis of cortical tissue samples. These large basal forebrain neurons are thought to project primarily to the cortex (*see below*).

In the insect nervous system, ACh is thought to function as the primary sensory neurotransmitter (for reviews, *see* Hildebrand, 1982; Pitman, 1971). ChAT immunocytochemistry of *Drosophila* supports this view where the enzyme appears to be localized primarily in the neuropil region of sensory ganglia (Gorczyca and Hall, 1987; Buchner et al., 1986). In a recent study it has also been demonstrated that immunohistochemical staining with monoclonal anti-ChAT antibodies reflect the levels of ChAT activity in *Drosophila* mutants containing temperature-sensitive alleles for the enzyme (Gorczyca and Hall, 1987). One of the interesting contrasts apparent when comparing the distribution of ChAT in *Drosophila* and the vertebrate nervous system concerns the subcellular distribution of the reaction product. In most vertebrate studies, the immunocytochemical reaction product appears more intensely in cell bodies and only lightly in neuropil regions, whereas in insects the major staining is in the neuropil region. The significance of this observation is not known. It is worth mentioning, however, that if ChAT functions exclusively to provide neurotransmitter, its appearance in cell bodies is curious since the major functional role of ACh in nervous system is for synaptic transmission. It is possible that only a small amount of active ChAT is necessary at nerve endings to maintain synaptic transmission. It is also possible that the enzyme is synthesized as an enzymatically inactive form in cell bodies that is nevertheless recognized by antibody probes.

It is likely that future studies of ChAT distribution using immunocytochemistry will provide not only more confirmatory information about the chemical anatomy of the brain but also exciting new findings that will have to be integrated into our overall picture of neurotransmitter phenotype specific neurons in the brain. These future studies may rely on the production of new ChAT monoclonal antibodies with specificity for individual forms of ChAT and may help to clarify the subcellular distribution of these different forms.

Antisera

Whereas monoclonal antibodies have been extremely useful in circumventing the difficulties encountered in ChAT purification and providing reagents with epitope specificity, polyclonal antibodies are useful for many other reasons. In general, a polyclonal antiserum contains a mixture of antibodies, many of which have higher affinity for the antigen than most monoclonal antibodies. This becomes an important consideration when antibodies are used to detect a rare protein species such as ChAT against a background of many other more abundant cellular proteins. It can also be important in providing more latitude with respect to procedures used during immunocytochemistry where certain fixation protocols may destroy many of the potential epitopes for a complex antigen such as ChAT. In addition, polyclonal antibodies should provide both conformationally dependent and sequence specific antibodies. Several laboratories have recently produced polyclonal anti-ChAT antibodies in response to these important considerations (Bruce et al., 1985; Bruce et al., 1984; Johnson and Epstein, 1986a; Cozzari and Hartman, 1980; Eckenstein and Thoenen, 1982; Lutz and Tyrer, 1987).

Earlier studies have also reported production of anti-ChAT antisera. The use of these preparations for immunocytochemical studies (Kan et al., 1980; McGeer et al., 1979; Kan et al., 1978; Chao et al., 1982; Kan and Chao, 1981; Kimura et al., 1981; Peng et al., 1981; Hattori et al., 1976; McGeer et al., 1974) has been ques-

tioned, however (*see* Rossier, 1975; Rossier, 1981). Even though an antiserum is not monospecific it can be used for a number of purposes, such as investigating the species cross-reactivity of ChAT or in constructing immunoaffinity columns for ChAT purification (Rossier et al., 1973; Peng et al., 1982; Malthe-Sørenssen et al., 1978; Peng et al., 1983; Rossier, 1976; Peng et al., 1983; Peng et al., 1980; Singh and McGeer, 1974; Polsky and Shuster, 1976; Wooten et al., 1978). In evaluating these efforts it is important to consider the purity of the antigen used for immunization as well as the purpose for which the antibodies will be used. In one study, antibodies produced to an impure preparation of ChAT were used to help construct an affinity column to remove the antigenic impurities (Rossier et al., 1973a).

In our laboratory we have recently produced three rabbit antisera to an enriched fusion protein isolated from *E. coli* lysates that have been transfected with lambda gt 11 phage containing a *Drosophila* ChAT cDNA insert (Muñoz-Maines et al., 1987). All three antisera can recognize native *Drosophila* ChAT using double immunoprecipitation assays. The titers against the native enzyme are low since the antigen should not contain a significant amount of ChAT secondary or tertiary structure. When these antisera are used to detect ChAT-related polypeptides by Western blotting, however, all three antisera have shown titers in excess of several thousand. With these high titer antibodies we have been able to detect immunostained polypeptides on Western blots of fresh homogenates of *Drosophila* without any purification. All three antisera recognize the 67 and 54 kdalton polypeptides previously identified as ChAT in completely purified preparations of *Drosophila* ChAT (Slemmon et al., 1982). Surprisingly, the major immunostained polypeptide recognized by all three antisera has a molecular size of 75 kdalton when no prepurification of ChAT was attempted (i.e., less risk of proteolysis). Rapid HPLC gel filtration of fresh *Drosophila* homogenates can partially separate the 67 and 75 kdalton polypeptides and both appear to be enzymatically active (Muñoz-Maines et al., 1987).

Biology

Genetics

An especially important advantage in working with *Drosophila* ChAT has been the availability of genetic information about the enzyme. The particular advantages in using this organism for genetic studies of neurobiology in general and cholinergic enzymes in particular has been discussed in an excellent review (Hall and Greenspan, 1979). The single *Drosophila* ChAT gene (*cha*) has been mapped using segmental aneuploids to the right arm of the third chromosome (Greenspan, 1980). This information led to the isolation of several mutant alleles, including presumptive nulls and conditional temperature sensitives (Greenspan, 1980; Hall et al., 1979). Using these temperature sensitive alleles it is possible to specifically perturb ChAT activity in a variety of experimental situations. It has thus been shown that decreased ChAT activity is associated with several important phenotypes, including altered electroretinogram traces, motor behavior, and even subtle alterations in courtship (Greenspan, 1980). The absence of the off transient in the electroretinogram of conditional *cha* mutants at restrictive temperature reinforces the long held notion that acetylcholine is involved in visual neurotransmission in insects. The precise physiologic mechanism(s) responsible for the other phenotypes are unknown. ChAT temperature-sensitive mutants have even been used to identify a specific *Drosophila* synapse which uses acetylcholine as the neurotransmitter

(Gorczyca and Hall, 1984). We have used these mutants to show a strong and direct correlation between in vivo ChAT activity and acetylcholine levels in *Drosophila* (Salvaterra and McCaman, 1985). Animals that have a complete homozygous deficiency for the *cha* locus can apparently develop normally throughout early embryogenesis, but fail to survive into the larval stages (Greenspan, 1980; Gorczyca and Hall, 1987).

ChAT has also been studied genetically in the soil nematode *Caenorhabditis elegans* (Rand and Russell, 1984). Mutant alleles have been identified that lead to a substantial decrease in enzyme activity in homozygous animals and result in uncoordinated movement, slow growth, and resistance to acetylcholinesterase inhibition. The *cha* gene maps to linkage group IV very close to the position of the *unc-17* allele, which also leads to uncoordinated movement. Interestingly, evidence has been presented to argue for a complex loci for *cha* and *unc-17* based on an unusual pattern of complementation between these two otherwise independent loci.

The genetic studies of ChAT have provided us with elegant tools to make a number of conclusions about cholinergic neurotransmission. It is surprising that it is possible to substantially reduce ChAT activity (often to levels less than 10% of normal) without observing any overt phenotypic changes. This may indicate a built-in redundancy in the neural circuits driving the eventually affected behaviors, or perhaps a substantial reservoir of excess neurotransmitter. It is not surprising that a complete absence of ChAT is lethal, but lethality is observed only after substantial early development (including much of neurogenesis).

Developmental Aspects

ChAT as well as other neurotransmitter biosynthetic enzymes belong to the class of proteins that are expressed in a tissue- and cell-type specific manner during development. As neurogenesis and nervous system development proceed, it has been possible to trace ChAT through the expression of enzymatic activity and immunocytochemically detectable protein. A number of studies have focused on the ontogenic appearance of the enzyme in a variety of tissues (either whole brain or microdissected brain regions) from several different species (Dewhurst et al., 1970; Nadler et al., 1974; Sorimachi and Kataoka, 1974; Giacobini, 1972; Loh, 1976; Burt and Narayanan, 1976). Sexual dimorphism has also been described for the developmental appearance of ChAT activity in rats (Brown and Brooksbank, 1979; Loy and Sheldon, 1987). Immunocytochemical studies have provided even more spatial resolution to the developmental appearance of the enzyme protein (i.e., *see* Phelps et al., 1984; Gorczyca and Hall, 1987).

We have recently investigated some aspects of the developmental expression of ChAT activity and steady state mRNA levels in *Drosophila*. *Drosophila* are holometabolus insects that pass through three larval stages, a pupal stage, and metamorphose into an adult. The body plan of the organism changes radically during this developmental history. Many of the neurons remain from the original larval nervous system throughout metamorphosis and innervate new structures in the adult. In addition, the adult nervous system adds new neurons as the sensory imaginal disks develop and differentiate during metamorphosis. This complicated life history presents the fly with significant challenges with respect to regulating neurotransmitter gene expression. As shown in Table 1, there are two developmental phases of increasing ChAT expression in *Drosophila*. Also, we have noted expression of both ChAT mRNA and enzyme activity very early in development (Between six and seven hours after oviposition) at a time when very few neurons are present and neurogenesis is still proceeding. The first phase of increasing

Table 1
ChAT Specific mRNA and Activity Measured at Different Developmental Stages in *Drosophila*

Developmental stage	ChAT mRNA/fly[a] fg ± SE; $N = 3$	ChAT activity/fly[b] pmol/min/fly ± SE; $N = 2$
1h	0	0
4h	0	0
7h	.24 ± .03	.17 ± .06
10h	.35 ± .02	.14 ± .01
13h	6.79 ± .78	.25 ± .07
1d (1st instar)	14.00 ± 1.15	19.80 ± 9.50
3d (3rd instar)	6.30 ± .45	42.70 ± 29.70
5d (pupal)	7.17 ± .89	30.20 ± 3.65
7d (pupal)	12.90 ± 1.15	19.60 ± 4.40
9d (imago)	307.00 ± 10.70	712.00 ± 106.00

[a]Samples were collected at the indicated times. Total RNA was isolated and the amount of ChAT specific mRNA was determined by dot blot hybridization and quantitative densitometry of the resulting autoradiograms. Values are the averages for three experiments.

[b]Values represent the average of two separate egg collections and are expressed as the amount of ^{14}C-acetylcholine formed in triplicate assays. Samples were collected at the same time as those used for the mRNA analysis. (Data from L. Carbini and P. M. Salvaterra, unpublished.)

steady state ChAT mRNA levels proceeds to the first larval instar stage and is temporally followed by increasing enzyme activity throughout the larval stages. In third instar larva and early pupa the steady state levels of ChAT mRNA decrease dramatically and are again followed temporally by a decrease in enzyme activity. At the end of pupation the levels of mRNA begin to rise again and in turn are followed by increasing levels of enzyme activity. These results indicate that the developmental expression of ChAT is consistent with the regulation of its transcript. Interestingly, the two phases of rapidly increasing ChAT-specific mRNA production seem to correlate best with the times when *Drosophila* neurons are making physical contact with each other, whereas the decreasing phase of mRNA expression, which reaches a minimum during early pupation, correlates with the degeneration of neural processes, many of which later grow back to innervate new adult body structures.

An equally interesting question with regard to the developmental expression of ChAT in specific subsets of neurons concerns the mechanism(s) responsible for maintaining expression throughout life. Apparently, once a neuron decides to become cholinergic by expressing ChAT the decision can be enforced throughout the life of the neuron. In other cases, however, only a transient plastic expression of ChAT has been argued (*see below*).

Regulation of Expression

One of the central areas of current biological research involves the investigation of the mechanisms responsible for the tissue and cell type specific expression of genes. A variety of both cis and trans regulatory elements have been identified as important features in a variety of eucaryotic gene expression systems (i.e., *see* Maniatis et al., 1987). So far, no one has

identified a promoter sequence for ChAT, but trans regulatory factors have been identified and studied in a number of biological and biochemical systems. This effort has been aided by studies relating environmental factors to the phenotypic expression of various neurotransmitters.

Vertebrate sympathetic neurons ordinarily express genes that allow them to produce and use adrenergic neurotransmitter. When neurons ordinarily fated to be adrenergic are exposed to certain environmental stimuli they can apparently switch their phenotypes and become cholinergic. This phenomena has been observed both in vivo (Landis and Keefe, 1983; LeDouarin, 1980; Cochard et al., 1979), and in vitro (Patterson, 1978) in a variety of experimental systems, and is thought to be a normal developmental event allowing for the plastic expression of neurotransmitter phenotype (Black, 1982; Patterson, 1978). Certain target tissue can apparently produce a soluble factor, which when presented to adrenergic neurons, causes them to switch their neurotransmitter phenotype to acetylcholine. The early work on neurotransmitter phenotype specification and cholinergic switching has been the subject of several excellent review articles (Patterson, 1978; Black, 1982; LeDouarin, 1980).

Recent work has resulted in the purification and preliminary biochemical characterization of the responsible glycoprotein that converts normally adrenergic sympathetic rat neurons to cholinergic neurons (Fukada, 1985). This factor apparently mediates a decision by the neuron to produce ChAT, but the mechanism responsible for this conversion has not been described. With the availability of adequate amounts of the purified cholinergic factor and molecular probes for the ChAT gene and mRNA, it should soon be possible to determine the molecular logic of neurotransmitter phenotype expression.

A great deal of excitement in the field of neurobiology has been generated recently by the finding that nerve growth factor (NGF) may function as a CNS neuronotrophic agent for certain types of mammalian neurons, including cholinergic neurons. NGF has long been known to influence the survival and differentiation of sensory and sympathetic neurons both in culture and in vivo (i.e., *see* Levi-Montalcini, 1968; Levi-Montalcini, 1982; Thoenen and Barde, 1980). It has now been demonstrated that NGF may also regulate survival and/or ChAT production in mammalian CNS neurons (Gnahn et al., 1983; Hefti et al., 1984; Mobley et al., 1985). In particular, there seems to be an emerging relationship between CNS neurons responsive to NGF in animals and the large cholinergic neurons that seem to degenerate in the brains of Alzheimer's patients. Although the specificity and selectivity of the NGF effect on CNS neuronal ChAT is difficult to establish directly, recent in vitro experiments seem to confirm and extend the original in vivo observations (Hefti et al., 1985; Shelton and Reichardt, 1986; Martinez et al., 1987). It is especially relevant in light of the central nervous system actions of NGF that a number of recent studies have reinvestigated the questions of NGF and NGF receptor localization in the brain. Using more sensitive assays than previously possible, it has now been unequivocally established the NGF, NGF mRNA, and NGF receptors are not only present in the brain but are also distributed in a manner that makes their action on the large cholinergic basal forebrain neurons even more likely (Shelton and Reichardt, 1986; Seiler and Schwab, 1984; Korsching et al., 1985). It will thus be important to establish the details of NGF action on cholinergic CNS neurons not only with respect to normal CNS development, function, and maintenance but also with regard to its role in neurodegenerative diseases.

Relationship to Alzheimer's Disease

Ever since the important observations documenting low levels of acetylcholine, ChAT, and cholinergic neurons in the brains of pa-

tients suffering from Alzheimer's disease (Bowen et al., 1976; Perry et al., 1977; Whitehouse et al., 1981; Whitehouse et al., 1982), a significant amount of work has been done both extending these observations and documenting new relationships between the cholinergic systems of normal human brain and the neurons that are lost in Alzheimer's disease (for reviews, *see* Davies, 1979; Marchbanks, 1982; Bird et al., 1983; Terry and Davies, 1980). Despite these intense efforts we still know very little about the intriguing relationship between cholinergic neurons and Alzheimer's disease (Davies and Maloney, 1976; McGeer et al., 1984a).

Initially, there appeared to be well justified optimism that new insights into the disease process would be established by linking cholinergic biochemical and neuropathological deficits with the clinical memory deficits so characteristic of Alzheimer's disease. A central role for the cholinergic systems of the brain had long been proposed and supported by numerous lines of investigation and is often termed the "cholinergic hypothesis of memory" (*see*, for example, Bartus et al., 1982). When cholinergic markers were shown to be deficient in Alzheimer's brains an understandable transformation of this older, more established theory was advanced into the "cholinergic hypothesis of Alzheimer's disease" (Bartus et al., 1982; Perry et al., 1977a; Collerton, 1986; Coyle et al., 1983). Unfortunately, the situation seems to be more complex than a simple relationship of degenerating cholinergic neurons which would explain the memory dysfunction. Other neurotransmitter systems or neuroactive peptides are also decreased in Alzheimer's tissue, calling the original cholinergic specificity into question (Whitehouse et al., 1985; Whitehouse et al., 1987). Other brain regions where acetylcholine is not thought to be a major neurotransmitter have also been implicated in the neurodegenerative cell groups affected in the disease (Hyman et al., 1984; Bondareff et al., 1982). It should also be mentioned that the decrease of any brain component in a neurodegenerative disease may only reflect the loss of the cells that ordinarily produce the decreased component and have little direct relationship to the specific etiology of the disease. An intriguing argument against the possible causal relationship between the degeneration of cholinergic neurons in Alzheimer's brain and reduced cortical ChAT has been advanced by showing that the reduction in ChAT activity seems to temporally precede the loss of basal forebrain cholinergic cells (Perry et al., 1982). There also seems to be a strong correlation with the pathological indices of Alzheimer's disease and the reduction in ChAT activity (Wilcock et al., 1982; Etienne et al., 1986).

Although neurotransmitter related hypotheses of the nature of Alzheimer's disease are still popular, the etiology remains unknown. Many possibilities have been considered, including viruses or prions, environmental toxins, and genetic predisposition. Recent evidence has provided a possible link between the neuropathological changes seen in Alzheimer's tissue and a gene derived from the amyloid protein (found in the characteristic plaques and tangles of Alzheimer's tissue), which maps to chromosome 21 (St. George-Hyslop et al., 1987). An especially attractive hypothesis has been advanced which emphasizes the importance of growth and differentiation factors to neuronal survival (Appel, 1984). None of these newer hypotheses, as well as the older "cholinergic" hypothesis have been entirely ruled out or established as the cause or even the effect of Alzheimer's.

It should be mentioned that the cholinergic hypothesis of Alzheimer's disease has attained the special status of being used as a rationale for the treatment of the disorder (i.e., *see* Kurz et al., 1986; Gottfreis, 1985; Greenwald and Davis, 1983). So called cholinergic drugs (i.e., either mimicking acetylcholine action, prevent-

ing acetylcholine hydrolysis or serving as a precursor for acetylcholine synthesis by ChAT) have been administered to patients either orally, intravenously, or infused directly into the brain (Harbaugh et al., 1984; Davis and Mohs, 1982, Levy et al., 1983). Unfortunately, the results of these approaches have not proven to be unequivocally successful in alleviating the symptoms of the disease. It is not clear whether this lack of success is because of complicated technical features of the attempted treatments (i.e., administering the wrong drugs or using the wrong application method, time course, or dose), a failure of the criteria used to evaluate the treatments (i.e., the evaluation of Alzheimer's symptoms in living patients remains somewhat subjective), or a failure of the "cholinergic hypothesis" itself. Obviously, much additional work on the fundamental biology of ChAT regulation remains to be done before we can even hope to evaluate the "cholinergic hypothesis" in relation to not only Alzheimer's disease but also to normal memory phenomena.

Similar to Alzheimer's disease other diseases also have been associated with deficits in subpopulations of cholinergic neurons. Amyotrophic lateral sclerosis, for example, is distinguished by a degeneration of normally cholinergic motor neurons. Also, similar to Alzheimer's the significance of the degenerative phenomena to the particular neurotransmitter phenotype remains unknown. In addition a variety of clinical features are observed in patients receiving "cholinergic" drugs. Usually these symptoms are not thought to be directly related to ChAT, but are more likely mediated through one or more cholinergic receptors.

Significant progress has been made in recent years describing the chemical anatomy of the brain, including definition of cellular neurotransmitter phenotypes using a variety of antibody and nucleic acid probes. Although impressive, this information has not yet proceeded to new insights establishing the causes of any neurodegenerative disease. Part of the difficulty in evaluating the relationship of neurotransmitter deficits to specific neurological diseases is directly related to our current ignorance about the regulatory features of neurotransmitter biosynthetic enzymes. What, if any, is the dependence of neuronal survival on continued expression of a neuron's chosen neurotransmitter? How does correct maintenance of neurotransmitter phenotype affect the survival and function of the postsynaptic targets of neurons undergoing reduced neurotransmitter production or switching of neurotransmitter phenotype? What is the chemical nature and biological function of factors necessary for the correct expression and maintenance of neurotransmitter phenotype in the degenerating cells?

Future Directions

It is not difficult to predict the direction of near term studies related to ChAT. The techniques of molecular biology will most certainly play a central role in helping us to understand the important features of this enzyme. With the ready availability of monoclonal and polyclonal antibodies, amino acid sequence information, and *Drosophila* cDNA and genomic clones, it is likely that one or more approaches will soon succeed in isolating clones for the cDNA and gene from other species including humans. When complete nucleic acid and protein sequence information is available for ChAT from a variety of species, we should be able to deduce the important structural features of the enzyme and gene that have been conserved during evolution. The techniques of in vitro mutagenesis will then be applied to test hypotheses about the structure and function of the enzyme.

Genomic sequences should lead to an identification of the promoter and other regulatory regions essential for ChAT expression. It is also likely that molecular biological studies

will resolve much of the current controversy about the physical and structural features of the protein.

This wealth of molecular information about ChAT, which is sure to accumulate in the next several years, is not guaranteed to increase our understanding of cholinergic biology. In order to answer important questions about the biology of neurotransmission in general and ChAT in *Drosophila* and C. *elegans*, as well as available and new mutant alleles, are sure to be important tools in achieving any new understanding. The construction of transgenic animals and cells will allow many hypotheses to be formulated and tested in satisfying ways. It should become possible to construct altered regulatory sequences that will allow for experimental manipulation of ChAT expression. Perhaps, new cholinergic "neuronal-like" cells will be constructed and used as therapeutically useful implants to provide a source of acetylcholine to a cholinergically deficient brain region.

Understanding the normal developmental regulation of ChAT expression will be an important goal approached from a variety of disciplines using a variety of techniques. The mechanism of maintenance of correct expression throughout the life of the committed differentiated cholinergically neuron will also be addressed. The plasticity of neurotransmitter phenotype selection should be either established or ruled out in other neuronal groups. We should also begin to appreciate the underlying molecular logic involved in the initial decision of the cholinergic phenotype as well as subsequent decisions to use an alternative neurotransmitter. The molecular aspects of cotransmitters should also be defined.

It is impossible to predict where ChAT research will lead us in the long term, but all the advances in this field should help provide a more complete picture of how the normal brain works through chemical neurotransmission as well as what doesn't work in diseased brain.

Acknowledgments

I wish to express thanks to my friends and colleagues J. R. Slemmon, G. D. Crawford, N. Itoh, V. Muñoz-Maines, H. Sugihara, L. Carbini, and other members of my laboratory whose contributions to studies of ChAT were essential. I also thank J. E. Vaughn, C. Houser, P. Phelps, R. Barber, K. Ikeda, E. Roberts, and N. Mori for their valuable contributions and collaborations. I am also grateful to I. Hayashi for encouragement and helpful discussions. The assistance of S. Webb in preparing the manuscript is gratefully acknowledged. Work in the author's laboratory has been generously supported by NIH-NINCDS.

References

Alpert A., Kisliuk R. L., and Shuster, L. (1966) A simple radioisotopic assay for choline acetyltransferase and its application in the *Lactobacillus plantarum* system. *Biochem. Pharmacol.* **15,** 465–473.

Appel S. H. (1984) Neuronotrophic factors and diseases of aging: An approach to ALS, Parkinsonism and Alzheimer's disease, in *Comparative Pathobiology of Major Age-Related Diseases: Current Status and Research Frontiers,* Scarpelli D. G. and Migaki G., eds., Alan R. Liss, NY, pp. 411–424.

Armstrong D. M., Saper C. B., Levey A. I., Wainer B. H., and Terry R. D. (1983) Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. *J. Comp. Neurol.* **216,** 53–68.

Badamchian M. and Carrol P. T. (1985) Molecular weight determinations of soluble and membrane-bound fractions of choline O-acetyltransferase in rat brain. *J. Neurosci.* **5,** 1955–1964.

Barlow R. B. and Dixon R. O (1973) Choline acetyltranferase in the nettle Urtica dioica L. *Biochem. J.* **132,** 15–18.

Bartus R. T., Dean R. I., Beer B. and Lippa A. S (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217,** 408–417.

Benishin C. G. and Carroll P. T. (1982) A comparison of soluble and membrane-bound forms of

choline-*O*-acetyltransferase in mouse brain nerve endings. *Proc. West. Pharmacol. Soc.* **25**, 343–345.

Benishin C. G. and Carrol P. T. (1984) Developmental differences between soluble and membrane-bound fractions of choline-*O*-acetyltransferase in neontal mouse brain. *J. Neurochem.* **43**, 885-887

Berrard S., Biguet N. F., Gregoire D., Blanot F., Smith J., and Mallet J. (1986) Synthesis of catalytically active choline acetyltransferase in *Xenopus* ooytes injected with messenger RNA from rat central nervous system. *Neurosci. Lett.* **72**, 93-98.

Bigl V., Woolf N. H., and Butcher L. L. (1982) Cholinergic projections from the basal forebrain to frontal, parietal, temporal, occipital and cingulate cortices: a combined fluorescent tracer and acetylcholinesterase analysis. *Brain Res. Bull.* **8**, 727–749.

Bird T. D., Stranahan S., Sumi S. M., and Raskind M. (1983) Alzheimer's disease; choline acetyltransferase activity in brain tissue from clinical and pathological subgroups. *Ann. Neurol.* **14**, 284-293.

Bishop, M. R., Sastry B. V., Schmidt D. E., and Harbison R. D. (1976) Occurrence of choline acetyltransferase and acetylcholine and other quaternary ammonium compounds in mammalian spermatozoa. *Biochem Pharmacol.* **25**, 1617-1622.

Black I. B. (1982) Stages of neurotransmitter development in autonomic neurons. *Science* **215**, 1198-1204.

Bondareff W., Mountjoy C. Q., and Roth M. (1982) Loss of neurons of origin of the adrenergic projection to cerebral cortex (nucleus locus coeruleus) in senile dementia. *Neurology* **32**, 164-168.

Bowen D. M., Smith C. B., White P., and Davison A. N. (1976) Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain* **99**, 459-496

Brandon C. and Wu J. Y. (1978) Purification and properties of choline acetyltransferase from *Torpedo* californica. *J. Neurochem.* **30**, 791-797.

Braun A., Barde Y. -A., Lottspeich F., Mewes W., and Thoenen H. (1987) N-terminal sequence of pig brain choline acetyltransferase purified by a rapid procedure. *J. Neurochem*, **48**, 16-21.

Brown, R. and Brooksbank B. W. (1979) Developmental changes in choline acetyltransferase and glutamate decarboxylase activity in various regions of the brain of the male, female, and neonatally androgenized female rat. *Neurochem. Res.* **4**, 127–136.

Bruce G., Wainer B. H., and Hersh L. B. (1984) Immuno-affinity-purification of human placental choline acetyltransferase. *Fed. Proc.* **43**, 2007.

Bruce G., Wainer B. H., and Hersh L. B. (1985) Immunoaffinity purification of human choline acetyltransferase: Comparison of the brain and placental enzymes. *J. Neurochem.* **45**, 611–620.

Buchner E., Buchner S., Crawford G., Mason W. T., Salvaterra P. M., and Sattelle, D. B. (1986) Choline acetyltransferase-like immunoreactivity in the brain of *Drosophila melanogaster*. *Cell Tissue Res.* **246**, 57–62.

Burt A. M. and Narayanan C. H. (1976) Choline acetyltransferase, choline kinase, and acetylcholinesterase activities during the development of the chick ciliary ganglion. *Exp. Neurol.*. **53**, 703–713.

Butcher L. L. and Woolf N. J. (1984) Histochemical distribution of acetylcholinesterase in the central nervous system: clues to the localization of cholinergic neurons, in *Handbook of Chemical Neuroanatomy*, Bjorklund A. and Kihar M. J., eds., Elsevier, Amsterdam, pp. 1–45.

Cannas M. and Giacobini G. (1978) Synthesis and transport of choline acetyltransferase in the somatic motoneuron of the chick. *Boll. Soc. Ital. Biol. Sper.* **54**, 863-866.

Chan-Palay V. and Palay S. L. (1983) *Coexistence of Neuroactive Substances*. Wiley, New York.

Chao L. (1975) Subunits of choline acetyltransferase (EC 2.3.1.6.). *J. Neurochem.* **25**, 261–266.

Chao L. P. (1978) Choline acetyltransferase. The absence of multiple forms and purification from mouse brain. *Neurochem. Res.* **3**, 549–561.

Chao L. P., Kan K. S., and Hung F. M (1982) Immunohistochemical localization of choline acetyltransferase in rabbit forebrain. *Brain Res.* **235**, 65–82.

Chao L. P. and Wolfgram F. (1973) Purification and some properties of choline acetyltransferase (EC 2.3.1.6) from bovine brain. *J. Neurochem.* **20**, 1075–1081.

Cheney D. L., Racagni G. and Costa E. (1976) Distribution of acetylcholine and choline acetyltransferase in specific nuclei and tracts of rat brain, in *Biology of Cholinergic Function*, Goldberg A. M. and Hanin I., eds., Raven Press, NY, pp. 655–659.

Cochard P., Goldstein M., and Black I. B. (1979) Initial development of the noradrenergic phenotype in autonomic neuroblasts of the rat embryo in vivo. *Dev. Biol.* **71**, 100–114.

Collerton D. (1986) Cholinergic function and intellectual decline in Alzheimer's disease. *Neurosci.* **19,** 1–28.

Coyle J. T., Price D. L., and DeLong M. R. (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* **219,** 1184–1190.

Cozzari C. and Hartman B. K. (1980) Preparation of antibodies specific to choline acetyltransferase from bovine caudate nucleus and immunohistochemical localization of the enzyme. *Proc. Natl. Acad. Sci. USA* **77,** 7453–7457.

Cozzari C. and Hartman B. K. (1983) Choline acetyltransferase. Purification procedure and factors affecting chromatographic properties and enzyme stability. *J. Biol. Chem.* **258,** 10013–10019.

Crawford G., Slemmon J. R., and Salvaterra P. M. (1982) Monoclonal antibodies selective for *Drosophila melanogaster* choline acetyltransferase. *J. Biol. Chem.* **257,** 3853–3856.

Crawford G. D., Correa L., and Salvaterra P. M. (1982a) Interaction of monoclonal antibodies with mammalian choline acetyltransferase. Proc. *Natl. Acad. Sci. USA* **79,** 7031–7035.

Cuello A. C. and Sofroniew M. V. (1984) The anatomy of the CNS cholinergic neurons. *TINS* **7,** 74–78.

Dahlstrom A., Heiwall P. O., Booj S., and Dahllof, A. G. (1978) The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons. *Acta. hysiol. Scand.* **103,** 308–319.

Davies P. and Maloney A. J. F. (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *The Lancet* **2,** 1403.

Davies P. (1979) Neurotransmitter related enzymes in senile dementia of the Alzheimer type. *Brain Res.* **171,** 319–327.

Davis K. L. and Mohs R. C. (1982) Enhancement of memory processes in Alzheimer's disease with multiple-dose intravenous physostigmine. *Am. J. Psychiatry* **139,** 1421–1424.

Dewhurst S. A., McCaman R. E., and Kaplan W. D. (1970) The time course of development of acetylcholinesterase and choline acetyltransferase in *Drosophila melanogaster. Biochem. Genet.* **4,** 499–508.

Dietz G. W. Jr., and Salvaterra P. M. (1980) Purification and peptide mapping of rat brain choline acetyltransferase. *J. Biol. Chem.* **255,** 10612-10617.

Driskell W. J., Weber B. H., and Roberts, E. (1978) Purification of choline acetyltransferase from *Drosophila melanogaster. J. Neurochem.* **30,** 1135–1141.

Dziegielewska K. M., Saunders N. R., Evans C. A., Skacel P. O., Haggendal C. J., Heiwall P. O., and Dahalstrom A. B. (1976) Effects of colchicine and vinblastine on axonal transport of choline acetyltransferase in rat sciatic nerve. *Acta. Physiol. Scand.*. **96,** 486–494.

Eckenstein F., Barde Y. A., and Thoenen H. (1981) Production of specific antibodies to choline acetyltransferase purified from pig brain. *Neuroscience* **6,** 993–1000.

Eckenstein F. and Baughman R. W., (1984) Two types of cholinergic innervation in cortex, one colocalized with vasoactive intestinal polypeptide. *Nature* **309,** 153–155.

Eckenstein F. and Sofroniew M. V. (1983) Identification of central cholinergic neurons containing both choline acetyltransferase and acetylcholinesterase and of central neurons containing only acetylcholinesterase. *J. Neurosci.* **3,** 2286–2291.

Eckenstein F. and Thoenen H. (1982) Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. *EMBO J.*; 363–368.

Emson P. C., Malthe-Sørenssen D., and Fonnum F. (1974) Purification and properties of choline acetyltransferase from the nervous system of different invertebrates. *J. Neurochem.* **22,** 1089–1098.

Etienne P., Robitaille Y., Wood P., Gauthier S., Nair N. P. V., and Quirion R. (1986) Nucleus basalis neuronal loss, neuritic placques and choline acetyltransferase activity in advanced Alzheimer's disease. *Neurosci.* **19,** 1279–1291.

Fatt P. and Katz B. (1951) An analysis of the endplate potential recorded with an intracellular electrode. *J. Physiol. (Lond)* **115,** 320–370.

Fibiger H. C. (1982) The organization and some projections of cholinergic neurons of the mammalian forebrain. *Brain Res. Rev.* **4,** 327-388.

Fonnum F. (1968) Choline acetyltransferase binding to and release from membranes. *Biochem. J.* **109,** 389–398.

Fonnum F. (1969) Radiochemical micro assays for the determination of choline acetyltransferase and acetylcholinesterase activities. *Biochem. J.* **115,** 465–472.

Fonnum F. (1970) Topographical and subcellular localization of choline acetyltransferase in rat hippocampal region. *J. Neurochem.* **17,** 1029-1037.

Fonnum F. and Malthe-Sørenssen D. (1973) Membrane affinities and subcellular distribution of the different molecular forms of choline acetyltransferase from rat. *J. Neurochem.* **20,** 1351–1359.

Frizell M., Hasselgren P. O., and Sjostrand J. (1970) Axoplasmic transport of acetylcholinesterase and choline acetyltransferase in the vagus and hypoglossal nerve of the rabbit. *Exp. Brain Res.* **10;** 526–531.

Fukada K. (1985) Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proc. Natl. Acad. Sci. USA* **82,** 8795–8799.

Furness J. B., Costa M., and Keast J. R. (1984) Choline acetyltransferase and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. *Cell Tissue Res.* **237,** 329–336.

Gerschenfeld H. M. (1973) Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiol. Rev.* **53,** 1–119.

Giacobini G., Cannas M., Panattoni G. L., and Filogamo G. (1979) A developmental model for studying choline acetyltransferase transport in chick motoneurons. *Dev. Neurosci.* **2,** 225–234.

Giacobini G. (1972) Embryonic and postnatal development of choline acetyltransferase activity in muscles and sciatic nerve of the chick. *J. Neurochem.* **19,** 1401–1403.

Gnahn H., Hefti F., Heumman R., Schwab M. E., and Thoenen H. (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Brain Res.* **285,** 45-52.

Goodman D. R., Adatsi F. K., and Harbison R. D. (1984) Evidence for the extreme overestimation of choline acetyltransferase in human sperm, human seminal plasma and rat heart: a case of mistaking carnitine acetyltransferase for choline acetyltransferase. *Chem. Biol. Interact.* **49,** 39–53.

Gorczyca M. and Hall J. C. (1984) Identification of a cholinergic synapse in the giant fiber pathway of *Drosophila* using conditional mutations of acetylcholine synthesis. *J. Neurogenet.* **1;** 289-313.

Gorczyca M. G. and Hall J. C. (1987) Immunohistochemical localization of choline acetyltransferase during development in Cha^{ts} mutuants of *Drosophila* melanogaster. *J. Neurosci.* **7;** 1361–1369.

Gottfries C. G. (1985) Alzheimer's disease and senile dementia: biochemical characteristics and aspects of treatment. Psychopharmacology (Berlin) **86,** 245–252.

Greenspan R. J. (1980) Mutations of choline acetyltransferase and associated neural defects in *Drosophila melanogaster. J. Comp. Physiol.* **137,** 83–92.

Greenwald B. S. and Davis K. L. (1983) Experimental pharmacology of Alzheimer disease. *Adv. Neurol.* **38,** 87–102.

Gundersen C. B., Jenden D. J., and Miledi R. (1985) Choline acetyltransferase and acetylcholine in *Xenopus* oocytes injected with mRNA from the electric lobe of *Torpedo. Proc. Natl. Acad. Sci. USA* **82,** 608.

Hall J. C. and Greenspan R. J. (1979) Genetic analysis of *Drosophila* neurobiology. *Ann. Rev. Genet.* **13,** 127-195.

Hall J. C., Greenspan R. J., and Kankel D. R. (1979) Neural defects induced by genetic manipulation of acetylcholine metabolism in *Drosophila. Soc. Neurosci. Symp.* **4,** 1-42.

Harbaugh R. E., Roberts D. W., Coombs D. W., Saunders R. L., and Reeder T. M. (1984) Preliminary report: Intracranial cholinergic drug infusion in patients with Alzheimer's disease. *Neurosurgery,* 514-518:

Hattori T., Singh V. K., McGeer E. G., and McGeer P. L. (1976) Immunohistochemical localization of choline acetyltransferase containing neostriatal neurons and their relationship with dopaminergic synapses. *Brain Res.* **102,** 164–173.

Hefti F., Dravid A., and Hartikka J. (1984) Chronic intraventricular injection of nerve growth factor elevates hippocampal choline acetyltransferase activity in adult rats with partial septolippocampal lesions. *Brain Res..* **293,** 305–311.

Hefti F., Hartikka J., Eckenstein F., Gnahn H., Heumann R., and Schwab M. (1985) Nerve Growth factor increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. *Neurosci.* **14,** 55–68.

Hersh L. B., Barker L. A., and Rush B. (1978) Effect of sodium chloride on changing the rate-limiting step in the human placental choline acetyltransferase reaction. *J. Biol. Chem.* **253,** 4966–4970.

Hersh L. B., Coe B., and Casey L. (1978a) A fluorometric assay for choline acetyltransferase and its use in the purification of the enzyme from human placenta. *J. Neurochem.* **30,** 1077–1085.

Hersh L. B. and Peet M. (1977) Re-evaluation of the kinetic mechanism of the choline acetyltransferase reaction. *J. Biol. Chem.* **252,** 4796–4802.

Hersh L. B. and Peet M. (1978) Effect of salts on the physical and kinetic properties of human placental choline acetyltransferase. *J. Neurochem.* **30,** 1087–1093.

Hersh L. B., Wainer B. H., and Andrews L. P. (1984) Multiple isoelectric and molecular weight variants of choline acetyltransferase. Artifact or real? *J. Biol. Chem.* **59,** 1253–1258.

Hildebrand J. G. (1982) Chemical signalling in the insect nervous system, in *Neuropharmacology of Insects*, Pitman, London, pp. 5-11.

Hildebrand J. G., Townsel J. G., and Kravitz E. A. (1974) Distribution of acetylcholine, choline, choline acetyltransferase and acetylcholinesterase in regions and single identified axons of the lobster nervous system. *J. Neurochem.* **23,** 951–961.

Houser C. R., Crawford G. D., Barber R. P., Salvaterra P. M., and Vaughn J. E. (1983) Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* **266,** 97–119.

Husain S. S. and Mautner H. G. (1973) The purification of choline acetyltransferase of squid head ganglia. *Proc. Natl. Acad. Sci. USA* **70,** 3749–3753.

Hyman B. I., Van Hoesen G. W., Damasio A. R., and Barnes C. L. (1984) Alzheimer's disease: Cell-specific pathology isolates the hippocampal formation. *Science* **225,** 1168–1170.

Ichikawa T., Ishida I., and Deguchi T. (1983) Monoclonal antibodies to choline acetyltransferase of rat brain. *FEBS Lett.* **155,** 306–310.

Ishida I., Ichikawa T., and Deguchi T. (1983) Immunochemical and immunohistochemical studies on the specificity of a monoclonal antibody to choline acetyltransferase of rat brain. *Neurosci. Lett.* **42;** 267-271.

Itoh N., Slemmon J. R., Hawke D. H., Williamson R., Morita E., Itakura K., Roberts E., Shively J. E., Crawford G. D., and Salvaterra P. M. (1986) Cloning of *Drosophila* choline acetyltransferase cDNA. *Proc. Natl. Acad. Sci. USA* **83,** 4081–4085.

Jablecki C. and Brimijoin S. (1974) Reduced axoplasmic transport of choline acetyltransferase activity in dystrophic mice. *Nature* **250,** 151–154.

Jablecki C. and Brimijoin S. (1975) Axoplasmic transport of choline acetyltransferase activity in mice: effect of age and neurotomy. *J. Neurochem.* **25,** 583–593.

Johnson C. D., and Epstein M. L. (1986) Monoclonal antibodies and polyvalent antiserum to chicken choline acetyltransferase. *J. Neurochem.* **46,** 968–976.

Kan K. S., Chao L. P., and Eng L. F. (1978) Immunohistochemical localization of choline acetyltransferase in rabbit spinal cord and cerebellum. *Brain Res.* **146,** 221–229.

Kan K. S., and Chao L. P., (1981) Localization of choline acetyltransferase at neuromuscular junctions. *Muscle Nerve* **4,** 91–93.

Kan K. S., Chao L. P., and Forno L. S. (1980) Immunohistochemical localization of choline acetyltransferase in the human cerebellum. *Brain Res.* **193;** 165-171.

Kasa P., Mann S. P., Karcsu S., Toth L., and Jordan S. (1973) Transport of choline acetyltransferase and acetylcholinesterase in the rat sciatic nerve: a biochemical and electron histochemical study. *J. Neurochem.* **21,** 431–436.

Kimura H., McGeer P. L., Peng J. H., and McGeer E. G. (1981) The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat. *J. Comp. Neurol.* **200,** 151–201.

Korsching S., Auburger G., Heumann R., Scott J., and Thoenen H. (1985) Levels of nerve growth factor and its mRNA in the central nervous systme of the rat correlate with cholinergic innervation. *EMBO J.* **4,** 1389–1393.

Kubo T., Fudada K., Mikami A., Maeda A., Takahashi H., Mishina M., Haga T., Haga K., Ichiyama A., Kanzawa K., Kojima M., Matsuo H., Herose T. and Numa S. (1986) Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature.* **323,** 411–416.

Kurz A., Ruster P., Romero B., and Zimmer R. (1986) Cholinergic treatment strategies in Alzheimer's disease. *Nervenarzt.* **57,** 558–569.

Landis S. C. and Keefe D. (1983) Evidence for neurotransmitter plasticity in vivo: developmental changes in properties of cholinergic sympathetic neurons. *Dev. Biol.* **98,** 349–372.

LeDourain N. M. (1980) The ontogeny of the neural crest in avian embryo chimaeras. *Nature* . **286,** 663–669.

Levey A. I., Aoki M., Fitch F. W. and Wainer B. H. (1981) The production of monoclonal antibodies reactive with bovine choline acetyltransferase. *Brain Res.* **218,** 383–387.

Levey A. I., Armstrong D. M., Atweh S. F., Terry R. D., and Wainer B. H. (1983) Monoclonal antibodies to choline acetyltransferase: production, specificity, and immunohistochemistry. *J. Neurosci.* **3,** 1–9.

Levey A. I., Rye D. B. and Wainer B. H. (1982) Immunochemical studies of bovine and human choline-O-acetyltransferase using monoclonal antibodies. *J. Neurochem.* **39,** 1652–1659.

Levey A. I. and Wainer B. H. (1982) Cross-species and intraspecies reactivities of monoclonal antibodies against choline acetyltransferase. *Brain Res.* **234,** 469–473.

Levey A. I., Wainer B. H., Rye D. B., Mufson E. J., and Mesulam M. M. (1984) Choline acetyltransferase-immunoreactive neurons intrinsic to rodent cortex and distinction from acetylcholinesterase-positive neurons. *Neuroscience* **13,** 341–353.

Levi-Montalcini R. (1968) Nerve growth factor. *Physiol. Rev.* **48,** 534–569.

Levi-Montalcini R., (1982) Developmental neurobiology and the natural history of nerve growth factor. *Annu. Rev. Neurosci.* **5,** 341–362.

Levy R., Little A., Chuaqui P., and Reith M. (1983) Early results from double-blind, placebo-controlled trial of high dose phosphatidyl choline in Alzheimer's disease. *Lancet* **1,** 987–988.

Loewi O. (1921) Uber humorale ubertragbarkeit der herzenwickung. *Pflugers Arch. ges Physiol.* **189,** 239–242.

Loh Y. P. (1976) Developmental changes in activity of choline acetyltransferase, acetyl-cholinesterase and glutamic acid decarboxylase in the central nervous system of the toad, *Xenopus* laevis. *Neurochem. 26;* 1303-1305.

Loy R. and Sheldon R. A. (1987) Sexually dimorphic development of cholinergic enzymes in the rat septohippocampal system. *Dev. Brain Res.* **34,** 156–160.

Luine V. N., Rostene W., Rhodes J., and McEwen B. S. (1984) Activation of choline acetyltransferase by vasoactive intestinal peptide. *J. Neurochem.* **42,** 1131–1134.

Lutz E. M. and Tyrer N. M. (1987) Immunohistochemical localization of choline acetyltransferase in the central nervous system of the locust. *Brain Res.* **407,** 173–179.

Malthe-Sørenssen D. (1976) Molecular properties of choline acetyltransferase from different species investigated by isoelectric focusing and ion exchange adsorption. *J. Neurochem.* **26,** 861–865.

Malthe-Sørenssen D. and Fonnum F. (1972) Multiple forms of choline acetyltransferase in several species demonstrated by isoelectric focusing. *Biochem J.* **127,** 229–236.

Malthe-Sørenssen D., Lea T., Fonnum F., and Eskeland T. (1978) Molecular characterization of choline acetyltransferase from bovine brain caudate nucleus and some immunological properties of the highly purified enzyme. *J. Neurochem. 30,* 35–46.

Maniatis T., Goodbourn S., and Fischer J. A. (1987) Regulation of inducible and tissue-specific gene expression. *Science* **236,** 1237–1244.

Marchbanks R. M. (1982) Biochemistry of Alzheimer's dementia. *J. Neurochem.* **39,** 9–15.

Martinez H. J., Dreyfus C. F., Jonakait G. M., and Black I. B (1987) Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. *Brain Res.* **412,** 295–301.

Matthews D. A., Salvaterra P. M., Crawford G. D., Houser C. R., and Vaughn J. E (1987) An immunocytochemical study of choline acetyltransferase-containing neurons and axon terminals in normal and partially deafferented hippocampal formation. *Brain Res.* **402,** 30–43.

Mautner H. G (1977) Choline acetyltransferase. *CRC Crit. Rev. Biochem.* **4,** 341–370.

Mautner H. G. (1986) Choline acetyltransferase, in *Neurotransmitter Enzymes,* Boulton A. A., Baker G. B., and Yu P. H., eds, Humana Press, Clifton, NJ, pp. 273-317.

McCaman R. E., Carbini L., Maines V., and Salvaterra P. M. (1987) Single RNA species injected in *Xenopus* oocyte directs the synthesis of active choline acetyltransferase. *Mol. Brain Res.* (in press).

McCaman R. E. and Hunt J. M. (1965) Microdetermination of choline acetylase in nervous tissue. *J. Neurochem.* **12,** 253–259.

McCaman R. E. and Ono J. K. (1982) Aplysia cholinergic synapses: a model for central cholinergic function, *Progress in Cholinergic Biology: Model*

Cholinergic Synapses, Hanin I. and Goldberg A. M., eds., Raven Press, New York, pp. 23–43.

McGeer P. L., McGeer E. G. and Peng J. H. (1984) Choline acetyltransferase: purification and immunohistochemical localization. *Life Sci.* **34**, 2319–2338.

McGeer P. L., McGeer E. G., Singh V. K., and Chase W. H. (1974) Choline acetyltransferase localization in the central nervous system by immunohistochemistry. *Brain Res.* **81**, 373–379.

McGeer P. L., McGeer E. G., Suzuki J., Dolman E. E., and Nagai T. (1984a) Aging, Alzheimer's disease, and the cholinergic system of the basal forebrain. *Neurol.* **34**, 741–745.

Melander T., Staines W. A., Hokfelt T., Rokaeus A., Eckenstein F., Salvatera P. M., and Wainer B. H. (1986) Galanin-like immunoreactivity in cholinergic neurons of the speptum-basal forebrain complex projecting to the hippocampus of the rat. *Brain Res.*. **30**, 130–138.

Mesulam M. M., Mufson E. J., Wainer B. H., and Levey A. I. (1983) Central cholinergic pathways in the rat: An overview based on an alternative nomenclature (Ch1-Ch6). *Neurosci.* **10**, 1185–1201.

Mesulam M. M., Mufson E. J., Levey A. I., and Wainer B. H. (1984) Atlas of cholinergic neurons in the forebrain and upper brainstem of the macaque based on monoclonal choline acetyltransferase immunohistochemistry and acetylcholinesterase histochemistry. *Neuroscience* **12**, 669–686.

Mishina M., Tobimatsu T., Imoto K., Tanaka K- I., Fujita Y., Fukuda K., Kurasaki M., Takahaski T., Morimoto Y., Hirose T., Inayama S., Takahashi T., Kuno K., and Numa S. (1985) Location of functional regions of acetylcholine receptor alpha-subunit by site-directed mutagenesis. *Nature* **313**, 364–369.

Mobley W. C., Rutkowski J. L., Tenekkoon G. I., Buchanan K., and Johnson M. V. (1985) Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science* **229**, 284–287.

Mori N., Itoh N., and Salvaterra P. M. (1987) Evolutionary origin of cholinergic macromolecules and thyroglobulin. *Proc. Natl. Acad. Sci. USA* **84**, 2813–2817.

Morris D. (1966) The choline acetyltransferase of human placenta. *Biochem. J.* **98**, 754–762.

Muñoz-Maines V. J., Slemmon J. R., Panicker M. M., Neighbor N., and Salvaterra P. M. (1987) Production of polyclonal antisera to choline acetyltransferase using a fusion protein produced by a cDNA clone. *J. Neurochem.* (in press).

Nachmansohn D. and Machado A. L. (1943) The formation of acetylcholine, a new enzyme. *J. Neurophysiol.* **6**, 397–403.

Nadler J. V., Matthews D. A., Cotman C. W., and Lynch G. S. (1974) Development of cholinergic innervation in the hippocampal formation of the rat. II. Quantitative changes in choline acetyltransferase and acetylcholinesterase activities. *Dev. Biol.* **36**, 142–154.

Nathanson N. M. (1987) Molecular properties of the muscarinic acetylcholine receptor. *Ann. Rev. Neurosci.* **10**, 195–236.

O'Brien R. A. (1978) Axonal transport of acetylcholine, choline acetyltransferase and cholinesterase in regenerating peripheral nerve. *J. Physiol. (Lond)* **282**, 91–103.

Ohshiro S., Fujiwara M., and Osumi Y. (1978) Axonal transport of norepinephrine and choline acetyltransferase in regenerating sciatic nerve of the rat. *Exp. Neurol.* **62**, 159–172.

Osborne N. N. (1979) Some properties and the axonal transport of choline acetyltransferase in nervous tissue of the snail *Helix* pomatia. *Biochem. Pharmacol.* **28**, 1257–1259.

Park D. H., Ross M. E., Pickel V. M., Reis D. J., and Joh T. H. (1982) Antibodies to rat choline acetyltransferase for immunochemistry and immunocytochemistry. *Neurosci. Lett.* **34**, 129–135.

Patterson P. H. (1978) Environmental determination of autonomic neurotransmitter functions. *Annu. Rev. Neurosci.* **1**, 1–17.

Pearson R. C. A., Sofroniew M. V., Cuello A., Powell T. P. S., Eckenstein F., Esiri M. M., and Wilcock G. K. (1983) Persistence of cholinergic neurons in the basal nucleus in a brain with senile dementia of the Alzheimer's type demonstrated by immunohistochemical staining for choline acetyltransferase. *Brain Res.* **289**, 375–379.

Peng J. H., Kimura H., McGeer P. L., and McGeer E. G. (1981) Anticholine acetyltransferase fragments antigen binding (Fab) for immunohistochemistry. *Neurosci. Lett.* **21**, 281–285.

Peng J. H., McGeer P. L., Kimura H., Sung S. C., and McGeer E. G. (1980) Purification and immuno-

chemical properties of choline acetyltransferase from human brain. *Neurochem. Res.* **5**, 943–962.

Peng J. H., McGeer P. L., and McGeer E. G. (1982) Cross-reactivity of antibodies to chicken brain choline acetyltransferase with mammalian enzyme. *J. Neuroimmunol.* **3**, 113–121.

Peng J. H., McGeer P. L., and McGeer E. G. (1983) Anti-human choline acetyltransferase fragments antigen binding (FAB)-sepharose chromatography for enzyme purification. *Neurochem. Res.* **8**, 1481–1486.

Perry E. K., Perry R. H., Blessed G., and Tomlinson B. E. (1977) Necropsy evidence of central cholinergic deficits in senile dementia. *Lancet* **1**; 189.

Perry E. K., Perry R. H., Gibson P. H., Blessed G., and Tomlinson B. E. (1977a) A cholinergic connection between normal aging and senile dementia in human hippocampus. *Neurosci. Lett.* **6**, 85–89.

Perry R. H., Candy J. M., Perry E. K., Irving D., Blessed G., Fairbairn A. F., and Tomlinson B. E. (1982) Extensive loss of choline acetyltransferase activity is not reflected by neuronal loss in the nucleus of Meynert in Alzheimer's disease. *Neurosci. Lett.* **33**, 311–315.

Phelps P. E., Barber R. P., Houser C. R. Crawford G. D., Salvaterra P. M., and Vaughn J. E. (1984) Postnatal development of neurons containing choline acetyltransferase in rat spinal cord: an immunocytochemical study. *J. Comp. Neurol.* **229**, 347–361.

Phillis J. W. (1976) Acetylcholine and synaptic transmission in the central nervous system, in *Chemical Transmission in the Mammalian Central Nervous System*, Hockman C. H. and Bieger D., eds., University Park Press, pp. 159–213.

Pitman R. M. (1971) Transmitter substances in insects: A review. *Comp. Gen. Pharmac.* **2**, 347–361.

Polsky R. and Shuster L. (1976) Preparation and characterization of two isozymes of choline acetyltransferase from squid head ganglia. II. Self-association, molecular weight determinations, and studies with inactivating antisera. *Biochim. Biophys. Acta.* **445**, 43–66.

Rand J. B. and Russell R. L. (1984) Choline acetyltransferase-deficient mutants of the nematode *Caenorhabditis elegans*. *Genetics* **106**, 227–248.

Rand J. B. and Russell R. L (1985) Properties and partial purification of choline acetyltransferase from the nematode *Caenorhabditis elegans*. *J. Neurochem.* **44**, 189–200.

Roskoski R., Lim C. T., and Roskoski L. M. (1975) Human brain and placental choline acetyltransferase: purification and properties. *Biochemistry* **14**, 5105–5110.

Rossier J. (1975) Immunohistochemical localization of choline acetyltransferase: real or artifact? *Brain Res.* **98**, 619–622.

Rossier J. (1976) Immunological properties of rat brain choline acetyltransferase. *J. Neurochem.*. **26**, 549–553.

Rossier J. (1977) Choline acetyltransferase: a review with special reference to its cellular and subcellular localization. *Int. Rev. Neurobiol.* **20**, 283–337.

Rossier J. (1981) Serum monospecificity: a prerequisite for reliable immunohistochemical localization of neuronal markers including choline acetyltransferase. *Neuroscience* **6**, 989–991.

Rossier J., Bauman A., and Benda P. (1973) Antibodies to rat brain choline acetyltransferase: species and organ specificity. *FEBS Lett.* **36**, 43–48.

Rossier J., Bauman A., and Benda P. (1973a) Improved purification of rat brain choline acetyltransferase by using an immunoabsorbent. *FEBS Lett.* **32**, 231–234.

Ryan R. L. and McClure W. O. (1979) Purification of choline acetyltransferase from rat and cow brain. *Biochemistry* **18**, 5357-5365.

Rye D. B., Wainer B. H., Mesulam M.-M., Mufson E. J., and Saper C. B. (1984) Cortical projections arising from the basal forebrain: a study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase. *Neuroscience* **13**, 627-643.

Sakmann B., Methfessel C., Mishina M., Takahashi T., Takai T., Kurasaki M., Fukuda K., and Numa S. (1985) Role of acetylcholine receptor subunits in gating of the channel. *Nature* **318**, 538–543.

Salvaterra P. M., Crawford G. D., Houser C. R., Matthews D. A., Barber R. P., and Vaugh J. E. (1986) Biochemistry and immunocytochemistry of choline acetyltransferase, *Dynamics of Cholinergic Function*, Hanin E., ed., Plenum, NY, pp. 51–63.

Salvaterra P. M. and Foders R. M. (1979) [^{125}I]-α-bungarotoxin and [^{3}H]quinuclidinylbenzilate binding in central nervous systems of different species. *J. Neurochem.* **32**, 1509–1517.

Salvaterra P. M. and McCaman R. E. (1985) Choline acetyltransferase and acetycholine levels in *Drosophila melanogaster*: A study using two temperature-sensitive mutants. *J. Neurosci.* **5**, 903–910.

Satoh K., Armstrong D. M., and Fibiger H. C. (1983) A comparison of the distribution of central cholinergic neurons as demonstrated by acetylcholinesterase pharmacohistochemistry and choline acetyltransferase immunohistochemistry. *Brain Res. Bull.* **11**, 693–720.

Seiler M. and Schwab M. E. (1984) Specific transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. *Brain Res.* **300**, 33–39.

Shelton D. L. and Reichardt L. F. (1986) Studies on the expression of the nerve growth factor (NGF) gene in the central nervous system: levels and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. *Proc. Natl. Acad. Sci. USA* **83**, 2714–2718.

Singh V. K. and McGeer P. L. (1974) Cross-immunity of antibodies to human choline acetyltransferase in various vertebrate species. *Brain Res.* **82**, 356–359.

Slemmon J. R., Salvaterra P. M., Crawford G. D., and Roberts E. (1982) Purification of choline acetyltransferase from *Drosophila melanogaster*. *J. Biol. Chem.* **257**, 3847–3852.

Slemmon J. R., Salvaterra P. M., and Roberts E. (1982a) Molecular characterization of choline acetyltransferase from *Drosophila melanogaster*. *Neurochem. Int.* **6**, 519–525.

Smith C. P. and Carroll P. T. (1980) A comparison of solubilized and membrane bound forms of choline-O-acetyltransferase (EC 2.3.1.6) in mouse brain nerve endings. *Brain Res.* **185**, 363–371.

Sorimachi M. and Kataoka K. (1974) Developmental change of choline acetyltransferase and acetylcholinesterase in the ciliary and the superior cervical ganglion of the chick. *Brain Res.*. **70**, 123–130.

St. George-Hyslop P. H., Tanzi R. E., Polinsky R. J., Haines J. L., Nee L., Watkins P. C., Myers R. H., Feldman R. G., Pollen D., Drachman D., Growdon J., Bruni A., Foncin J. -F., Salmon D., Frommelt P., Amadcci L., Sorbi S., Placentini S., Stewart G. D., Hobbs W. J., Conneally P. M., and Gusella J. F. (1987) The genetic defect causing familial Alzheimer's disease maps on chromosome 21. *Science* **235**, 885–890.

Strauss W. L. and Nirenberg M. (1985) Inhibition of choline acetyltransferase by monoclonal antibodies. *J. Neurosci.* **1**, 175-180.

Terry R. D. and Davies P. (1980) Dementia of the Alzheimer type. *Ann. Rev. Neurosci.* **3**, 77–95.

Thoenen H. and Barde Y. -A. (1980) Physiology of nerve growth factor. *Physiol. Rev.* **60**, 1284–1335.

Tûcek S. (1974) Transport and changes of activity of choline acetyltransferase in the peripheral stump of an interrupted nerve. *Brain Res.* **82**, 249–261.

Tûcek S. (1975) Transport of choline acetyltransferase and acetylcholinesterase in the central stump and isolated segments of a peripheral nerve. *Brain Res.* **82**, 259–270.

Tûcek S. (1985) Regulation of acetylcholine synthesis in the brain. *J. Neurochem.* **44**, 11–24.

Wainer B. H., Levey A. I., Mufson E. J., and Mesulam M.-M. (1984) Cholinergic systems in mamalian brain identified with antibodies against choline acetyltransferase. *Neurochem. Int.* **6**, 163–182.

White H. L. and Cavallito C. J. (1970) Inhibition of bacterial and mammalian choline acetyltransferase by styrylpyridine analogs. *J. Neurochem.* **17**, 1579–1589.

Whitehouse P. F., Price D. L., Clark A. W., Coyle J. T., and DeLong M. R. (1981) Alzheimer's disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann. Neurol.* **10**, 122–126.

Whitehouse P. J., Price D. L., Struble R. G., Clark A. W., Coyle J. T., and DeLong M. R. (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* **215**, 1237–1239.

Whitehouse P. J., Struble R. G., Hedreen J. C., Clark A. W., and Price D. L. (1985) Alzheimer's disease and related dementias: Selective involvement of specific neuronal systems. *CRC Crit. Rev. Neurobiol.* **1**, 319–339.

Whitehouse P. J., Vale W. W., Zweig R. M., Singer H. S., Mayeux R., Kuhar M. J., Price D. L., and DeSouza B. (1987) Reductions in corticotropin releasing factor-like immunoreactivity in cerebral cortex in Alzheimer's disease, Parkinson's disease, and progressive supranuclear palsy. *Neurology* **37**, 905–909.

Wilcock G. K., Esiri M. M., Bowen D. M. and Smith C. C. (1982) Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J. Neurol. Sci.* **57**, 407–417.

Wooten G. F. and Cheng C. H. (1980) Transport and turnover of acetylcholinesterase and choline acetyltransferase in rat sciatic nerve and skeletal muscle. *J. Neurochem.* **34,** 359–366.

Wooten G. F., Park D. H., Joh T. H., and Reis D. J. (1978) Immunochemical demonstration of reversible reduction in choline acetyltransferase concentration in rat hypoglossal nucleus after hypoglossal nerve transection. *Nature* **275,** 324–325.