Biology of Cholinergic Function

Edited by
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Preface

The goal of this book is to provide an integrated overview of cholinergic biology. To this end, topics have been selected to encompass all aspects of study relating to an understanding of the cholinergic system. The material is presented in an order conducive to a thorough study of cholinergic function. Each chapter introduces new concepts, which are further elaborated in subsequent chapters. We have not limited our approach to the many well-studied systems but have attempted to cover a very large and sometimes inconsistent literature. Classic areas—for example, anatomy, physiology, biochemistry, enzymology, and pharmacology—of the cholinergic system are reviewed. These chapters are followed by discussions of recent advances, which hitherto have been confined to isolated symposium-based reviews. In this category are the chapters on invertebrate and "simple" neuronal systems, behavioral and environmental aspects of cholinergic function, the biology of cholinergic receptors, and tissue culture systems. The chapters on cholinergically linked diseases represent the first such attempt to incorporate under specific headings the available knowledge on various disease states in which a malfunction in cholinergic mechanisms is known or suspected. Four comprehensive and extensively documented appendices provide a compendium of factual information on cholinergic parameters.

We requested from the authors not only that they provide information from their own investigations, but also that they integrate their results with those published by others. Furthermore, we urged them to be speculative in their interpretations in order to encourage exploration of additional directions and areas of investigation. To this end, the authors have presented sufficient detail that the information not only educates but encourages and challenges the reader. It is the concept of total integration—from *in vitro* to *in vivo*, from invertebrate to mammal, from animal to man—that hopefully will advance our understanding of cholinergic biology.

Several important areas related to cholinergic biology have intentionally not been included in this book. Some of these (e.g., cholinergic receptor isolation techniques, cyclic nucleotide and hormonal interactions with cholinergic systems) have been reviewed in detail in other books. Still other areas are the subject of relatively recent investigation, and the data are being generated but are still fragmentary—e.g., platelets as a possible model of cholinergic function, the role of dimethylaminoethanol (Deanol) in the process of ACh formation. Most of these topics, however, are referred to in the appropriate chapters, and their potential importance is clearly evident in each case.

This book will be useful to the basic research scientist, the physician, and all other individuals involved in the neurobiological sciences.

We are very grateful to all the authors who participated in this project. Their understanding, cooperation, and willingness to adhere to the ground rules made our job as editors so much easier. We would also like to express our sincere appreciation to Cathy A. Rupp, Joanne Kolego, and Patricia Orefice for providing excellent secretarial support throughout this endeavor.

The idea for this book was conceived in August 1973. It has taken almost 3 years for it to come to fruition. Our efforts in making this book complete and as comprehensive as possible have involved a true labor of love, some disagreements, and hours of stimulating and fruitful discussion. It is our sincere hope that the *Biology of Cholinergic Function* will meet a long overdue need and will provide a lasting and significant contribution to the biological sciences.

Alan M. Goldberg
Israel Hanin
(*March, 1976*)

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Introduction

Israel Hanin and Alan M. Goldberg

Origins of the study of cholinergic biology can be traced back to 1906 when Hunt and Taveau initially examined the physiological actions of "cholin" and its analogues, and found that the acetyl derivative of choline is the most potent of the analogues in eliciting a blood pressure reduction in rabbits. These observations were followed by a series of important experiments in the laboratories of Professor Otto Loewi and of Sir Henry Dale. Loewi developed the concept of neurotransmitter function, while Dale and co-workers provided specific indication for the role of acetylcholine as a neurotransmitter in the mammalian system. The principles outlined by these two scientific giants have withstood the test of time and were recognized in 1936 by the awarding of the Nobel Prize, to be shared jointly by Loewi and Dale.

The original isolation of acetylcholine from mammalian tissue was achieved by Dale and Dudley in 1929 using horse and ox spleens. It was not until 1939, however, that Mann, Tennenbaum, and Quastel demonstrated the synthesis of acetylcholine in brain tissue. It is of interest to note that almost 50 years were to elapse between the time of the original simple chemical analysis of acetylcholine used by Dale and co-workers and the confirmation and definitive chemical identification of this compound in rat brain extracts in 1968 using gas chromatography–mass spectrometry.

During the next decade Feldberg, as well as Chang and Gaddum, joined efforts with Dale in the pursuit of active isolation and identification of choline esters in a multitude of tissues and species. These findings were instrumental in prompting further research into the functional significance of acetylcholine in various tissues and species.

There have been major advances in understanding the functional role of acetylcholine in both the central and peripheral nervous systems. The studies of Eccles and co-workers during the early 1950s provided evidence to support the concept that acetylcholine is a neurotransmitter in the central nervous system – at least in the Renshaw cells at the level of the spinal cord. At about the same time, Katz and co-workers were instrumental in identifying and describing the quantal nature of acetylcholine release at the neuromuscular junction. The latter findings implied that a highly organized and quantitative system is involved in the regulation of neurotransmitter release.

The development of subcellular fractionation techniques by DeRobertis, Whittaker, and their associates provided the anatomical basis for future biochemical mechanistic studies. Moreover, histological analysis of these

fractions provided a lucid demonstration of the existence of distinct vesicles in the cholinergic nerve ending. These developments were instrumental in the advent within the 1960s of investigations into mechanisms regulating acetylcholine metabolism at the subcellular level, and to much of our present understanding of these mechanisms.

Throughout these studies, since the early investigations of Loewi, pharmacological agents affecting cholinergic function have helped in furthering the elucidation of biochemical and physiological events at the cholinergic nerve ending.

During the past decade several chemical techniques for the determination of choline and acetylcholine have been developed. Several of these are also applicable to the analysis of labeled tracer incorporation from the precursor choline to the product acetylcholine. This added dimension has enabled systematic study of the dynamic aspects of cholinergic function.

The various developments mentioned in this introductory chapter represent highlights of the experimental observations that have provided us with a better understanding of cholinergic biology. They have been detailed within the appropriate sections throughout this book.

A scientific discipline advances only when the questions asked of it become specific, integrative, and probing. The study of cholinergic biology is presently at the threshold of such integrative considerations. The use of "simple" neuronal models, tissue culture systems, and combined behavioral and neurochemical studies will ultimately serve to integrate basic phenomenology with cholinergically linked disease states. It is hoped that such studies will provide a better understanding of the defects in such diseases and thus lead to a rational approach to their therapy.

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The Anatomy of Cholinergic Neurons

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I. INTRODUCTION

The objective of this chapter is to point out the anatomical location of "cholinergic" neurons, that is, those neurons that release acetylcholine (ACh) from their nerve terminals onto postsynaptic receptors in the process of neurotransmission. There have been many presentations of the requirements for establishing that a given compound is a neurotransmitter (McLennan, 1963; Werman, 1966; Phillis, 1970). In a recent review, these requirements were restated simply and rigorously—the compound in question should be shown to have precisely the same postsynaptic actions as those elicited by presynaptic activation; also, the compound in question should be released from the presynaptic terminals in an amount sufficient to cause postsynaptic activation (Krnjević, 1974). Although this most rigorous proof is not available for all the neurotransmitter candidates, ACh at the neuromuscular junction comes closest to fulfilling these requirements. Thus, ACh is unique in this respect; many experiments that establish the neuro-

transmitter function of ACh have become "classic" and are important as models for establishing other compounds as neurotransmitters.

When speaking of cholinergic transmission, one generally envisions the process occurring at the nerve terminals, where ACh is released onto the postsynaptic receptors (*vide supra*). However, in the past there have been proposals suggesting some variations on the role of ACh in the process of neurotransmission. Although these alternative proposals have not been popular and are not well accepted, they have generated considerable interest.

One proposal is that ACh is involved in maintaining the propagation of the action potential along the length of the axon (Nachmansohn, 1959, 1963). This proposal states that ACh is released at specific anatomical locations along the axon causing subsequent depolarization and further release of the transmitter. Another role has been proposed for ACh in sympathetic neurons, which are usually thought to contain norepinephrine as their neurotransmitter. It has been proposed that postganglionic sympathetic neurons contain ACh as well as norepinephrine, and that the primary event at sympathetic nerve terminals is a release of ACh, which in turn causes a release of norepinephrine (Burn, 1963; Burn and Rand, 1965). Related to these latter issues is the question of whether a single neuron can release more than one neurotransmitter, i.e., whether there are postsynaptic receptors for two or more compounds that are released upon nerve stimulation. It is generally assumed that a cholinergic neuron contains only one neurotransmitter, namely ACh.

As it is impractical to summarize all available evidence for ACh as a neurotransmitter at all of the various neurons in the body, only a few critical experiments are summarized in this chapter, examples of which are presented where possible. We do not give detailed anatomical descriptions of the neuronal pathways that are thought to be cholinergic. Rather, these pathways are presented in simplified schematic diagrams, and reference made to neuroanatomy texts for additional details. There are many excellent reviews on the ultrastructure of synapses, a topic discussed in part in other chapters in this book (De Robertis, 1964; Peters, Palay, and Webster, 1970; Pappas and Purpura, 1972). Furthermore, only data from mammals are included here; wherever applicable, we have cited reviews and papers that contain appropriate summaries.

II. METHODS FOR LOCALIZING CHOLINERGIC NEURONS

A. Biochemical "Markers"

In the absence of a routine, workable, and specific histochemical method for cholinergic neurons, investigators have relied on a variety of methods for

identifying and localizing cholinergic tracts. Among the most useful of these methods is the measurement of various biochemical parameters that are intimately associated with cholinergic structures. These biochemical "markers" include ACh levels, choline acetyltransferase (ChAT), and acetylcholinesterase (AChE) activity, high-affinity choline uptake, and the presence of cholinergic receptors.

Although the presence of ACh and ChAT alone is inadequate for establishing a neurotransmitter role from ACh, in practice it has been the most convenient starting point for localizing cholinergic tracts. Over recent years, great advances have been made in measuring small quantities of ACh (Appendix I). However, a problem that still exists in measuring ACh levels is the post-mortem loss of ACh, presumably attributable to the widespread presence of AChE. Again, methodologies have recently evolved to overcome this problem, which include instant freezing of brain tissue (Veech, Harris, Veloso, and Veech, 1973) and the utilization of focused microwave radiation for purposes of killing animals (Stavinoha, Weintraub and Modak, 1973).

Although there are simple and sensitive methods for measuring both ChAT and AChE, there is little doubt that ChAT is a far more relevant marker for cholinergic structures than is AChE. ChAT—the enzyme responsible for the synthesis of ACh—might be a perfect marker for cholinergic neurons. Although in some cases AChE may also be a marker for cholinergic neurons, it cannot be specific because it is localized postsynaptically. AChE has a more widespread distribution than does ChAT, but it does not necessarily parallel the levels of ChAT and ACh, whereas the latter two compounds usually show remarkably similar distributions (Koelle, 1969).

The recent discovery that cholinergic nerve terminals contain a unique high-affinity uptake mechanism for choline, which appears to be rate-limiting and regulatory in the synthesis of ACh (Simon and Kuhar, 1975), provides a tool for localizing cholinergic terminals (Kuhar, 1973). Thus, one could obtain a relative measure of the cholinergic innervation of various tissues or brain regions by the relative levels of the observed high-affinity choline uptake. These various biochemical markers are especially powerful when used in combination with microdissection techniques and techniques involving placement of electrolytic lesions for localizing cholinergic tracts.

Yet another useful biochemical marker is the presence of what appear to be cholinergic receptors. Methods for determining these receptors by biochemical means are mainly of two types—one involving the generation of cyclic GMP by cholinergic muscarinic agonists (George, Polson, O'Toole, and Goldberg, 1970; Lee, Kuo, and Greengard, 1972) and the other involving the direct specific binding of cholinergic compounds to membranes,

presumably receptors (Beld and Ariens, 1974; Hiley and Burgen, 1974; Schleifer and Eldefrawi, 1974; Yamamura and Snyder, 1974). Although this is a new area of research, it will undoubtedly contribute to our understanding of the anatomy of cholinergic systems.

B. Histochemical Procedures

An essential tool for localizing cholinergic tracts would be a routine, workable histochemical method for either ACh or ChAT. One has only to observe the great volume of literature and knowledge that followed from the development of histochemical methods for the catecholamines and serotonin to visualize parallel potential areas of discovery in the cholinergic systems.

There are currently well accepted, workable procedures for identifying structures containing AChE at the light and electron microscopic levels. Initially, it was hoped that AChE would be a selective marker for cholinergic structures. However, it now appears fairly conclusive that one cannot generally state that AChE is such a marker, and the same remarks apply here as in the above section with regard to AChE. Whereas there may be some cases in which AChE is an adequate marker for cholinergic neurons (Koelle, 1969), such as in the hippocampus (Fonnum, 1970), an inherent difficulty with the specificity of AChE is the fact that it is localized to postsynaptic areas at cholinergic synapses and may be even more widespread. Therefore, the presence of AChE could not by itself distinguish between cholinergic neurons or "cholinoceptive" neurons.

The presumably unique localization of ChAT to cholinergic neurons would therefore provide one with the impetus for developing a histochemical method for this enzyme. There have been attempts to do this, utilizing the principle of trapping CoA with lead after hydrolysis of acetyl-CoA at the site of ChAT (Burt, 1970; Kasa, Mann, and Hebb, 1970). Although this method is not yet routine for mapping cholinergic cell bodies and their projections, it may become extremely valuable once it is perfected.

An alternative approach for localizing ChAT would be the development of immunohistochemical techniques (Goldman, 1968). This appears to be a feasible goal, especially because of the recent reports of complete purification and isolation of ChAT (Chao and Wolfgram, 1973; Husain and Mautner, 1973; Singh and McGeer, 1974). This procedure involves preparing an antibody to ChAT and utilizing this antibody in conjunction with a histochemical marker, such as the highly fluorescent compound fluorescein isothiocyanate, to localize the enzyme in tissue slices. Although this procedure has many problems and requires extensive controls, laboratories are working intensively on this problem, and within the next few years these methods may provide an answer to the problem of the localization of ChAT-containing neurons.

C. Stimulation and Electrophysiology

Once the localization of either ChAT- or ACh-containing neurons is known, those neurons may be investigated in more detail. It would become necessary to see if ACh is released upon stimulation and if there are cholinergic receptors in areas showing suspected cholinergic terminals.

One difficulty in performing stimulation–release experiments is in measuring release of a transmitter in inaccessible structures, such as brain tissues. However, various techniques, such as the "push–pull" cannula (Gaddum, 1961), are available to overcome these difficulties. Furthermore, electrophysiological methods are powerful tools for testing various neurons for sensitivity to iontophoretically applied ACh, and thus to characterize pharmacologically the receptors on these neurons.

D. Other Methods

Other methods that have been very useful for localizing cholinergic neurons include the placement of lesions and microdissection techniques. The placement of lesions is often combined with biochemical methods to show depletion of cholinergic "markers" following destruction of a suspected cholinergic input to a given region. Histochemical studies determining the size and location of lesions are always an important adjunct to these procedures. Microdissection techniques are sufficiently refined to permit analysis of cholinergic markers in single cell bodies or in small brain regions (see Appendix IV, *this volume*).

III. PERIPHERAL NERVOUS SYSTEM

A. Motor Neurons to Striated Muscle

1. Neuromuscular Junctions

The neuromuscular junction assumes a position of perhaps unique importance in neurobiology in that it is a model system that has been explored in many aspects. The cholinergic nature of neuromuscular transmission is firmly established and has been reviewed by many authors (Hebb and Krnjević, 1962; Katz, 1966; Krnjević, 1974). A few of the more critical and interesting historical experiments may be briefly summarized as follows.

In very early experiments, it was found that ACh was present in, and released from stimulated neuromuscular preparations (Hess, 1923; Brinkman and Ruiter, 1924). With this finding in mind, Sir Henry Dale and his collaborators devised experiments to test if ACh was released from the presynaptic nerve, the muscle, or both. Like others before them, they found that stimulation of motor nerve fibers to perfused voluntary muscles caused

the appearance of ACh in the eserinized perfusate. Even when the transmission of excitation from nerve to muscle was blocked by the presence of curarine, stimulation of the nerve fibers still resulted in the release of ACh, indicating that transmission was not necessary for this release. Whereas direct stimulation of muscle showed some release of ACh, there was no release in denervated muscle. These results suggested that the source of ACh release was probably a presynaptic site.

These classic experiments of Dale, Feldberg, and Vogt (1936) were very thorough. They used several neuromuscular preparations including the tongue of the cat, the gastrocnemius muscle of the cat and dog, and the hindleg muscles of the frog. They were careful to use nerve trunks with only motor nerves, and they assayed ACh on more than one bioassay system. As a result of these and a multitude of later experiments, it is now well accepted that ACh is liberated from presynaptic sites.

The next relevant question was whether ACh can cause depolarization of muscle membranes. This question has been answered by electrophysiological methods. Iontophoretic application of ACh to the muscle results in a depolarization of the muscle membrane, similar to that which one sees on stimulation of the presynaptic nerve. Because this effect is also observed on denervated muscle, it is apparently a direct postsynaptic action (Katz, 1966). Moreover, in recent years the ACh receptor itself has become intensely studied at the molecular level (De Robertis and Schacht, 1974).

One further important question regarding neuromuscular transmission is: "Is enough ACh released to stimulate the muscle fiber?" This is a very difficult question to answer for technical reasons in that it is difficult to know such things as the precise amount of ACh released onto the neuromuscular junction receptors, and to devise experiments whereby one can accurately measure the amount of ACh necessary to stimulate the muscle membrane. Nevertheless, careful experiments have been performed, and the estimates indicate that the amount of ACh released could very well be enough to cause this depolarization (Krnjević and Miledi, 1958; Krnjević and Mitchell, 1961).

Mammalian neuromuscular junctions of every type have not been extensively examined as to their cholinergic nature. Rather, we tend to assume this nature by analogy and on the basis of partially fulfilled criteria and lack of striking contradictory evidence. Such assumptions must be made with considerable caution. For example, at crustacean (Takeuchi and Takeuchi, 1964) and insect (Beranek and Miller, 1968) neuromuscular junctions, glutamate, rather than ACh, may be the neurotransmitter.

2. Cholinergic Spinal and Cranial Motor Nerves

Attached to the spinal cord are 31 pairs of segmentally arranged spinal nerves. Each spinal nerve arises from the cord by two roots—a dorsal root,

which contains afferent projections, and a ventral root, which contains efferents. When discussing spinal motor neurons, we are concerned with the efferent neurons in the ventral roots, the cell bodies of which are localized at the ventral horn in the gray matter of the spinal cord. Their fibers project uninterrupted throughout the body to all skeletal muscles. A schematic of these neuronal pathways is presented on the left side of Fig. 1.

In addition to these spinal motor neurons, there are 12 pairs of cranial nerves which emerge from the central nervous system (CNS) at supraspinal

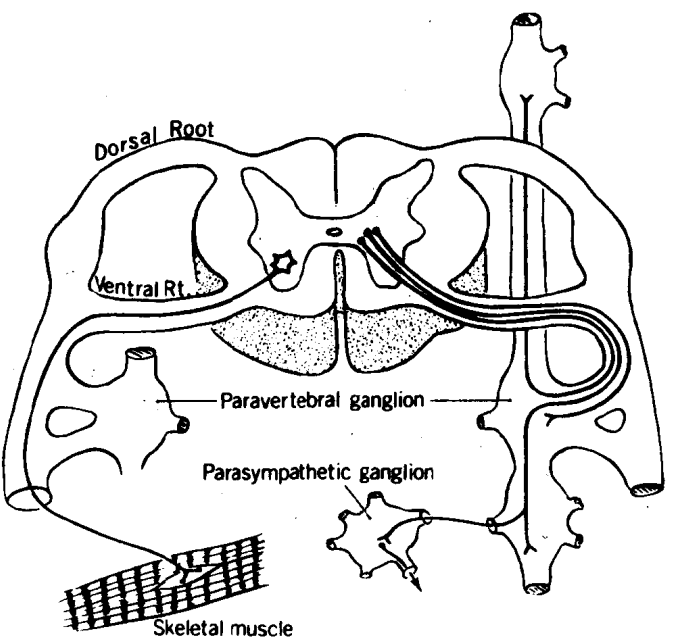

FIG. 1. Schematic diagram of a spinal segment and its associated cholinergic neurons. g, ganglion. Reproduced in modified form with permission (Koelle, 1970).

levels. These nerve trunks contain different types of functionally distinguishable nerves. Again, in this particular case we are concerned with motor nerves that are presumed to be cholinergic. The cranial nerves that contain motor nerves are the oculomotor (III), trochlear (IV), trigeminal (V), abducens (VI), facial (VII), glossopharyngeal (IX), vagus (X), accessory (XI), and hypoglossal (XII). These neurons project to muscles of the eye, jaw, face, neck, larynx, pharynx, and tongue. Their cell bodies are localized at nuclei in the brainstem, and their precise location and paths of projection are presented in detail in anatomy texts. The IIIrd, VIIth, IXth, and Xth nerves also contain cholinergic fibers which innervate the parasympathetic ganglia, but these fibers are part of the autonomic nervous system and are discussed in the following section.