2

Cholinergic Cells and Pathways

A. History of Methodological Development Needed to Define the Cholinergic Neuron, Explain Acetylcholine Release, and Establish Central Cholinergic Pathways

1. What Led to Establishing Cholinergic Pathways?

a. Sir Henry Dale and Sir William Feldberg and the Existence of Cholinergic Pathways

The central presence of cholinergic transmission was first hypothesized by Sir Henry Dale (1937). The Stedmans, Paul Mann, John Quastel, and Maurice Tennenbaum, Dale's associate William (now Sir William) Feldberg, and his coworkers, including Martha Vogt and Catherine Hebb, and Josiah Burn and Edith Bulbring provided additional evidence for Dale's hypothesis; this evidence included the demonstration of the central nervous system (CNS) presence of acetylcholine (ACh) and choline acetyl transferase (CAT), ACh synthesis and ACh release in the CNS, and the central and peripheral effects of muscarinics and anticholinesterases (antiChEs) (Stedman and Stedman, 1937; Feldberg and Vogt, 1948; Feldberg, 1945, 1950; see Eccles, 1964; Karczmar, 1967; and Barker et al., 1972; Mann et al., 1938a, 1938b; see also Chapter 8 A). Also, Henry Dale surmised early the presence of a ChE from his demonstration of the evanescence of the action of ACh (Dale, 1914, 1937; see also Chapters 7A, 8A, and 9A).

Feldberg was struck with the uneven distribution of ACh, sites of ACh release and synthesis, and activities of ChEs in the CNS; these findings led him to postulate that "the central nervous system is built of cholinergic and noncholinergic neurones," distributed in an alternative fashion (Feldberg, 1945). This was the first step toward the notion of a transmitter, including ACh CNS pathways; in fact, Feldberg (1945) was perhaps the first investigator to employ the term "central pathway" to denote "transmission . . . through the mediation of acetylcholine across a number of . . . central . . . synapses." The evidence in question was obtained via the use of several extraction methods and bioassays for extracted ACh, although occasionally chemical identification was attempted (Stedman and Stedman, 1937). Also, collecting released ACh whether from the cerebrospinal fluid or via perfusion of appropriate spinal or brain sites was helpful with formulating Feldberg's notion (Feldberg, 1945; Bulbring and Burn, 1941).

A digression is warranted. Although Zenon Bacq had already employed chemical identification of endogenous ACh in 1935, his method was complex and impracticable. Much earlier ACh bioassays were employed (see, for example, Fuhner, 1918); they were used by Loewi (1921), to identify the "Vagustoff" released by the vagus nerve, and their use continued for decades. The bioassays included Venus heart, frog rectus abdominis, and several other tissues, and generally they were sensitive to ACh concentrations of 10^{-8} to 10^{-10} molar. But one particular bioassay was sensitive to ACh concentrations of 10^{-21} molar (Nishi et al., 1967). It involved the toad lung, but only the Japanese team of Kyozo Koketsu, Syogoro Nishi, and Hiroshi Soeda is capable of

employing it successfully (see below, section C). Then an immunocytochemical method was developed for detection of ACh (Geffard et al., 1985); of course, this technique would be most useful in definitive identification of cholinergic neurons and pathways, but there was no follow-up with regard to its employment. Subsequently a number of chemical methods were worked out, including radioisotopic, gas chromatographic–mass spectrometric, fluorometric, and polarographic. The gas chromatography–mass spectrometry (GCMS) method, discovered by Israel Hanin, Don Jenden, and Bo Holmstedt (see Hanin and Goldberg, 1976) is commonly used today; it is sensitive at a nanogram level.[1]

Finally, when Maurice Israel and his associates wished to prove an unorthodox concept of ACh release, they needed an ultrasensitive ACh measurement method to prove their point and developed chemiluminescense to meet this need (Israel and Lesbats, 1981; Israel et al., 1990; see next section). Today, this method is widely used in industry as it allows researchers to deal with a large number of samples.

b. Cholinergic Ascending Reticular Alerting System

The evidence concerning several markers of cholinergic neurons and their CNS locus did not yield a specific description of cholinergic pathways; it suggested only that there may be many such pathways (Eccles, 1964) and that Feldberg's notion of alternative cholinergic-noncholinergic areas or sites may be not quite tenable.

The lucky thought of studying pharmacological effects of cholinergic drugs on the EEG and relating these effects to cholinoceptive sites brought about the first descriptions of specific cholinergic pathways. The alerting EEG effects (fast, low-voltage activity and the appearance of the theta rhythms; see Chapter 9 BIV-3) of ACh and cholinergic muscarinic agonists were noticed early by Frederic Bremer and Jean Chatonnet (1949). Actually, Bremer and Chatonnet ascribed these effects to the direct action of the cholinergic agents on a central cholinergic system, while Darrow and his associates (1944) stated that these phenomena are due the vasodilator actions of the muscarinics on the brain vascularization. But Joel Elkes, Phillip Bradley, and their associates (Bradley and Elkes, 1953), Franco Rinaldi and Harold Himwich (1955a, 1955b), and Vincenzo (Enzo) Longo and Bernardo Silvestrini (1957) obtained similar effects with either ACh or diisopropylfluorophosphonate (DFP) in the rabbit and in the cat, and they blocked these effects by means of atropine (see also Jasper, 1966; Karczmar, 1967). Also, they eliminated the possibilities of the peripheral or vasodilator origin of these effects, or of diffuse actions of the drugs in question on the cortex, as they obtained these effects of ACh or DFP via their carotid injection in the *cerveau isole* preparation but not in the isolated hemisphere preparation. Altogether, these investigators proposed that cholinergic alerting effects are dependent on a cholinergic alerting mesodiencephalic system or ascending reticular activating system (ARAS; Figure 2-1). A similar proposal was made by Kris Krnjevic and J.W. Phillis (1963): they proposed the existence of a cholinergic thalamocortical pathway concerned with projection and augmenting activity as they pointed out that ACh-sensitive cortical cells respond to thalamic or peripheral sensory stimulation with repetitive after-discharges and changes in the EEG. These notions were supported by the finding of Frank (Hank) MacIntosh and Paul Oborin (1953) of the increased release of ACh from the cortex during EEG arousal evoked by brainstem stimulation. In addition, McLennan (1963) proposed, on the basis of ACh release data and on the dependence of this release on functional states of the brain that there is a cholinergic pathway to the basal nuclei that originates in the nucleus ventralis lateralis.

It must be stressed that the very concept of the ARAS is based on the important, early discovery of Giuseppe Moruzzi and Horace Magoun that the stimulation of the reticular formation (within the midbrain tegmentum) induces the general activation, via the thalamus, of the whole forebrain including all cortices; they emphasized that this stimulation causes cortical arousal accompanied by behavioral arousal or awakening (Moruzzi, 1934; Moruzzi and Magoun, 1949). As valid as this discovery is, Moruzzi's and Magoun's identification of behavioral and EEG arousal is not quite correct (see Chapter 9 BIV-3).

Finally, the anatomical description of ascending cholinergic pathways based on cytochemical and immunochemical methods was first provided

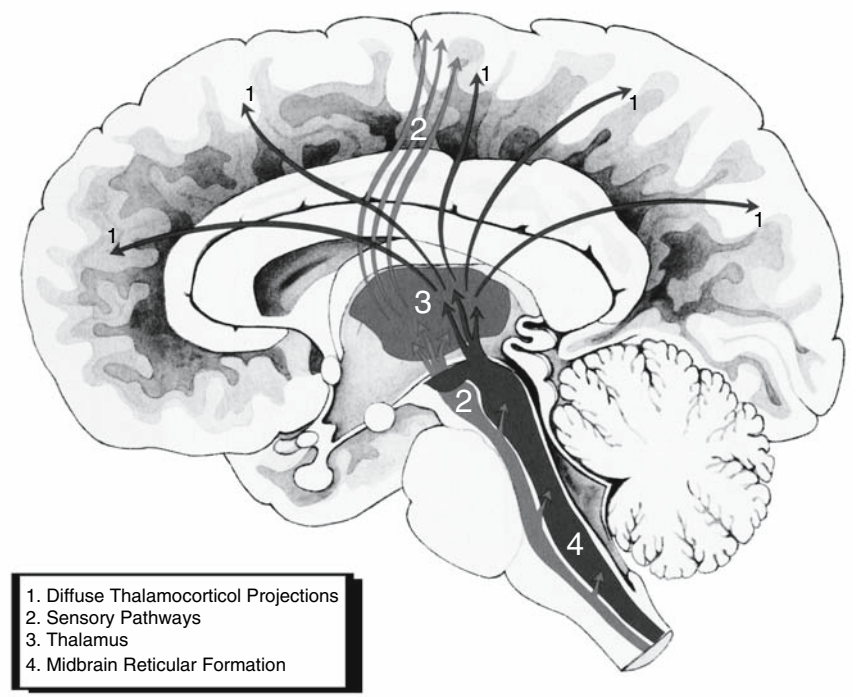

Figure 2-1. Reticular activating system according to Harold Himwich and Franco Rinaldi. (From Himwich, 1963).

by Charles Shute and Peter Lewis and Michel Gerebtzoff, and then by George Koelle; recent studies of the McGeers, Larry Butcher, and Marsel Mesulam are consistent with these findings (see section DI in this chapter). Since the investigations of Bradley, Krnjevic, Phillips, Himwich, Elkes, Bremer, Chatonnet, and Rinaldi, as well as those of Shute and Lewis, the notions of cholinergic alerting actions and their mesodiencephalic origin are generally accepted; it should be pointed out that this concept of a linearly extended cholinergic system is not in accordance with Feldberg's original postulate of alternative cholinergic and noncholinergic pathways.

c. Finally, a Definitive Description of Central Cholinergic Pathways

Histochemical and immunocytochemical means to identify AChE and CAT were the most effective and successful methods to delineate cholinergic pathways. During the 1940s and 1950s, several histochemical methods were developed by Giorgio Gomori, David Glick, and ultimately George Koelle to localize BuChE and AChE (see Koelle, 1963; Karczmar, 1963a, 1963b). All these methods are based on use of tissue slices and application of a substrate (such as a fatty acid ester, for example), hopefully specific for either AChE or BuChE; the substrate, when hydrolyzed by the enzyme, yields a colored or black precipitate, or still another reagent is added to produce the precipitate with the hydrolysate; specific inhibitors of AChE and BuChE are also applied to help, jointly with the use of enzyme specific substrates in identifying the enzyme that is being localized. Koelle and Friedenwald's famous microscopic histochemical method (1949) utilizes acetyl thiocholine or butyryl thiocholine as substrates as well as appropriate inhibitors. This method employs fresh frozen tissues or slices rather than fixed materials (making the method histochemical rather than histological) and produces remarkable resolution of the morphological location of the enzymes. Using his method in rats, rabbits, and cats, Koelle (1954) listed a number of central sites and nuclei that

exhibited "intense," "moderate," or "light" staining, including several components of the limbic system, several midbrain and medullary sites, several hypothalamic sites, basal ganglia, and reticular formation, but, surprisingly, he did not refer to ventral horn, although he found some staining in the dorsal horn.

While George Koelle stressed that his findings identified several brain areas that exhibit intense presence of cholinergic synapses, he did not describe, on the basis of these findings, the existence of specific cholinergic pathways. On the other hand, Koelle (1961, 1963) established the important concept of functional (membrane) versus storage or reserve AChE (he employed quaternary antiChEs to differentiate between the two); he also stressed that AChE is present both at the membrane of the soma (the postsynaptic enzyme) and at the nerve terminals (the presynaptic enzyme); also, he modified his original microscopic method so that it could serve for electronmicroscopic investigations.

Koelle's findings were confirmed and expanded by Gerebtzoff (1959) and Shute and Lewis (1967a, 1967b); Gerebtzoff's data may be less dependable than Koelle's, as Gerebtzoff applied Koelle's staining to formalin-fixed rather than fresh-frozen tissues. Shute and Lewis (1967a, 1967b) and Gerebtzoff (1959) also employed lesion techniques to identify the brain site origin of AChE. Similar to Koelle, Gerebtzoff did not use his data to describe specific cholinergic pathways. His sites of intense staining of AChE corresponded to those described by Koelle (i.e., thalamus and hypothalamus, basal ganglia, medullary and pontine sites, including pontine tegmentum, which was later identified as an important source of cholinergic radiation, etc.). Gerebtzoff also stressed a convergence of "cholinergic and noncholinergic fibres on the Purkinje cell," and, in contradistinction to Koelle, he described the presence of heavy AChE staining in the spinal ventral horn and its motoneurons, as well as in cranial motoneurons.

Charles Shute and Peter Lewis (1963, 1967a, 1967b) also reemployed Koelle's methods to advance significantly the understanding of the cholinergic pathways. They realized that "AChE-containing tracts . . . cannot be unequivocally traced back to their nuclei of origin," and they adopted a novel paradigm to resolve this difficulty: they discovered that "after involvement of AChE-containing tracts in surgical lesion, enzyme accumulated on the cell body side of the cut and disappeared from the opposite side, and that this phenomenon would provide a useful method of determining the polarity of cholinergic pathways"; also, this method allowed tracing a given pathway from the neurons of origin to their terminations. Finally, Shute and Lewis employed special micromethods to be sure that the lesions are applied to appropriate sites.

Their studies led them to define two pathways. The first is "the ascending cholinergic reticular system . . . arising . . . from reticular and tegmental nuclei of the brainstem, and from comparable groups of cells in the fore-brain" and extending to thalamus, subcortical, and cortical areas, hypothalamus, and limbic nuclei; they identified this system with the ARAS (which therefore corresponds to the Rinaldi-Himwich and Krnjevic pathways) and with the alerting EEG phenomena. The second pathway, the cholinergic limbic system, originates from the medial septum and diagonal band and projects to the hippocampal formation and the dentate gyrus, thence to the medial cortex, nuclei of the ascending system (ARAS), and the cerebellum. Again, Lewis and Shute proposed the involvement of this limbic system in such EEG phenomena as the hippocampal theta waves (see Chapter 9 BIV-3). It must be stressed how modern—that is, comparable to the work, 20 years later, of the McGeers, Mesulam, and others—these studies appear (Figures 2-2 and 2-3).

Subsequently, Peter Lewis (with Henderson, 1980) was the first to employ a dual cytochemical technique combining AChE histochemistry with horseradish peroxidase (HRP) procedures. When HRP is injected into the brain it is taken up and transported retrogradely to the neurons, which supply the area of HRP injection (Kristenson et al., 1971); this dual method confidently identifies the sites of origin of cholinergic pathways, and, in the hands of Lewis and Henderson it amply confirmed the Lewis-Shute conclusions. Since the studies of Lewis, Kristenson, and their associates, other agents became available to trace back the origin of axons and neuronal pathways, including certain neurotoxins, fluorescent and radio-autographic tracers such as Fluoro-Gold and Fluoro-Red (see, for example Li and Sakagachi, 1997) and [3H] choline (Jones and Beaudet, 1987).

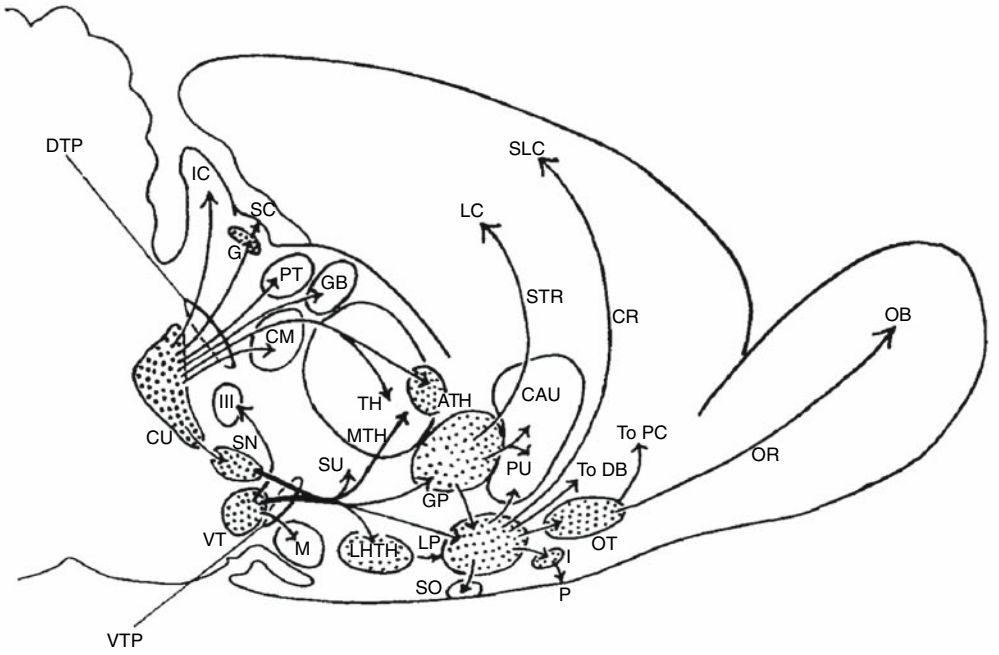

Figure 2-2. Diagram showing the constituent nuclei (stippled) of the ascending cholinergic reticular system in the mid-forebrain, with projections to the cerebellum, tectum, thalamus, hypothalamus, striatum, lateral cortex, and olfactory bulb. ATH, antero-ventral and antero-dorsal thalamic nuclei; CAU, caudate; CM, cetromedian (parafascicular) nucleus; CR, cingulate radiation; CU, nucleus cuneiformis; DB, diagonal band; DTP, dorsal tegmental pathway; G, stratum griseum intermediale of superior colliculus; GB, medial and lateral geniculate bodies; GP, globus pallidus and entopeduncular nucleus; LC, lateral cortex; LHTH, lateral hypothalamic area; LP, lateral preoptic area; M, mammilary body; MTH, mammillo-thalamic tract; OB, olfactory bulb; OR, olfactory radiation; OT, olfactory tubercle; P, plexiform layer of olfactory tubercle; PC, precallosal cells; PT, pretectal nuclei; PU, putamen; SC, superior colliculus; SLC, supero-lateral cortex; SN, substantia nigra pars compacta; SO, supraoptic nucleus; STR, striatal radiation; SU, subthalamus; TH, thalamus; TP, nucleus reticularis tegmenti pontis (of Bechterew); VT, ventral tegmental area and nucleus of basal optic root; VTP, ventral tegmental pathway. (Reprinted from Brain vol. 90, 497–517, 1967, "The Ascending Cholinergic Reticular System: Neocortical, Olfactory and Subcortical Projection" by C.C.D. Shute and P.R. Lewis by permission of Oxford University Press.)

Essentially, the early studies of Shute and Lewis stood the test of time, and the subsequent investigators, while expanding on their data via using different methodology (such as CAT immunocytochemistry; see below), confirmed their conclusions (Kasa, 1971a, 1971b; McGeer et al., 1987a, 1987b). In fact, this confirmation was also obtained by investigators using techniques similar to those employed by Shute and Lewis (see, for example, Krnjevic and Silver, 1965). In addition, in her 1985 study Paula Wilson, who used improved three-dimensional photography and the dual cytochemical technique of Lewis and Henderson (1980) to analyze histochemically the pathways in question, "endorsed . . . the concept . . . of dorsal tegmental projections to most of the nuclei first postulated by Shute and Lewis," although she described additional projections to the thalamus and the cortex, and stressed the significance of the nucleus basalis magnocellularis of Meynert (NBM) and parabrachial nucleus as sources of important cholinergic radiations; these notions were supported by the data obtained via CAT immunohistochemistry method (Fibiger, 1982; Woolf and Butcher, 1986; Bigl et al., 1982; see also next section). Altogether, the Shute-Lewis pathways as modified subsequently are a good approximation of the pathways established some

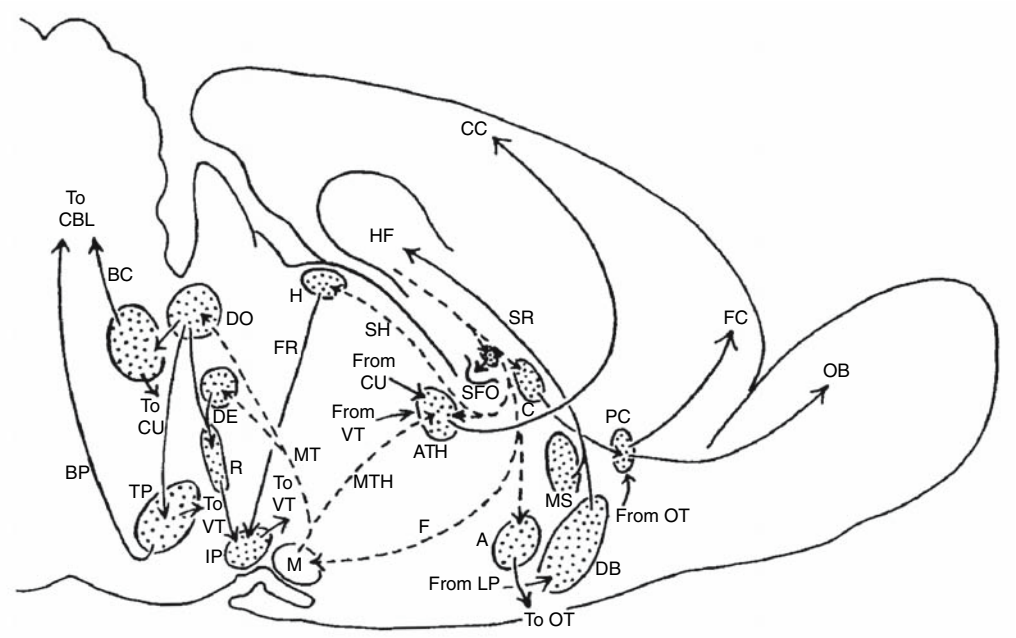

Figure 2-3. Diagram showing cholinesterase-containing nuclei of the midbrain and forebrain (indicated by stipple) connected with the hippocampus, their projections to the medial cortex, and their connections with the ascending cholinergic reticular system. Abbreviations: A, nucleus accumbens; ATH, antero-ventral and antero-dorsal thalamic nuclei; BC, brachium conjunctivum; BP, brachium pontis; C, interstitial nucleus of the ventral hippocampal commissure; CBL, cerebellum; CC, cingulated cortex (cingular and retrosplenial areas); CU, nucleus cuneiformis; DB, diagonal band; DE, deep tegmental nucleus (ventral tegmental nucleus of Gudden); DO, dorsal tegmental nucleus; F, formix; FC, frontal cortex (area infralimbica and anterior limbic area); FR, fasciculus retroflexus (habenula-interpeduncular tract); H, habenular nuclei; HF, hippocampal formation; IP, interpeduncular nucleus; LP, lateral preoptic area; M, mammilary body; MS, medial septal nucleus; MT, mammillo-tegmental tract; MTH, mammillo-thalamic tract; OB, olfactory bulb; OT, olfactory tubercle; PC, precallosal cells; R, dorsal and median nuclei of raphe (nucleus centralis superior); SFO, subfornical organ; SH, stria habenularis; SR, septal radiation; TP, nucleus reticularis tegmenti pontis (of Bechterew); VT, ventral tegmental area. (Reprinted from Brain vol. 90, 521–540, 1967, "The Ascending Cholinergic Reticular System: Neocortical, Olfactory and Subcortical Projection" by C.C.D. Shute and P.R. Lewis by permission of Oxford University Press.)

20 years later by employing CAT immunohistochemistry methodology. It should be stressed that the pathways in question overlap with the central sites, already discussed in this section, of cholinergic receptors, and ACh presence, release, and synthesis.

Nevertheless, is AChE histochemistry a dependable way to identify the cholinergic pathways? The McGeers and Henry Kimura, the pioneers of CAT immunohistochemistry, stressed that AChE "also occurs in non-cholinergic cells" and they adduced as examples "dopaminergic neurons of the substantia nigra, noradrenergic neurons of the locus ceruleus and serotonergic neurons of the raphe, which all stain intensely for AChE" . . . and . . . "are not cholinergic" (Mizukawa et al., 1986); similar arguments were raised by Nancy Woolf and Larry Butcher (1986). In agreement with this notion, several investigators who identified simultaneously AChE and CAT in brain neurons found that a percentage of neurons (usually quite small) exhibited only AChE but not CAT (Eckenstein and Sofroniew, 1981). The matter of the presence of AChE in noncholinergic cells relates to the presence of cholinergic receptors postsynaptically on noncholinergic neurons; this occurrence is characteristic for the synapses between cholinergic radiations and other transmitter systems and is relevant for the McGeers' reference to transmitter interaction. In addition, AChE is present presynaptically on axonal

terminals of cholinergic neurons, and this presence may be taken for marker of a cholinergic neuronal soma.

Do these reservations obviate the dependability of AChE histochemistry for the tracing of cholinergic pathways, as implied in the study of Mizukawa et al. (1986)? When AChE histochemistry is carried out without the lesions paradigm, then the point in question is well taken; however, appropriate lesions allow identifying the cholinergic neurons, as explained above, and this safety factor is reinforced by the use of one of the techniques for retrograde identification of the cholinergic neurons. These arguments are supported by the overlap of the pathways based on AChE histochemistry not only with such markers of the cholinergic system as cholinergic receptors and ACh release, but also with the pathways based on the CAT immunohistochemistry; in fact, in the 1980s, several investigators employed AChE histochemistry in conjunction with CAT immunohistochemistry and discovered that the findings obtained by two methods coincided to a great extent (Mizukawa et al., 1986; Woolf and Butcher, 1986;see also below, section IID).

Yet, it is apparent that either ACh or CAT with coenzyme A (CoA), the specific enzyme and coenzyme, which are, respectively, involved in ACh synthesis (see Chapter 3 B) constitute potentially better markers of a cholinergic neuron than AChE. Some attempts at histochemical or immunohistochemical visualization of ACh were made (Geffard et al., 1985; see Kasa, 1986); the histochemical technique employs heteropolyanions that precipitate and visualize ACh and choline, while the immunohistochemical technique uses anti-ACh antibodies. However, these attempts did not lead to any generally accepted methodology. A more successful approach concerned histochemical visualization of the CoA-SH group. This approach was first suggested by Barnett (1968); the actual technique was developed by Catherine Hebb and her associates (1970; see also Hebb and Whittaker, 1958) and Paul Kasa (1971a, 1971b), and Paul Kasa adapted the method for electron microscopy analysis. Using this method, Kasa (1971a, 1971b, 1978; see particularly his detailed and useful review of 1986) identified ascending cholinergic radiations to the several cortical areas, the limbic system, and the spinal cord, as well as intrinsic cholinergic systems in the cortex. Kasa opined that, whenever available, the histochemical CAT and AChE data agree with the more extensive mapping based on CAT immunohistochemistry, and with data concerning other components of the cholinergic system. Generally, this is true; however, his mappings, whether based on histochemistry or immunohistochemistry, differ in several respects from those described by Lewis and Shute (1967) on the basis of AChE histochemistry or by the McGeers, Mesulam, Butcher, Woolf, and others on the basis of CAT immunochemistry. Altogether, as AChE histochemistry is relatively limited in its specificity and discriminatory powers, as recognized by Kasa himself (1986), it is to the immunohistochemical identification of the distribution of CAT that we owe the definitive progress in this area.

The immunohistochemical tracing of CAT was first described by Eng et al. (1974) and Pat and Edith McGeer and Henry Kimura in the 1970s (McGeer et al., 1974); the McGeers and Kimura further developed this method and applied it extensively to the mapping of cholinergic pathways (Kimura et al., 1980, 1981); it is a dependable and most direct procedure for establishing cholinergic pathways, as CAT, per definition, identifies cholinergic neurons. The method is based on producing antibodies to the purified CAT protein, preparation of appropriate antisera, and applying histochemical staining techniques; it became successful only when the purification of the CAT protein became adequate and the specificity of the antibodies achieved. Additionally, the McGeers and the subsequent investigators (see section IIC, below) used lesions, retrograde marking and its visualization (Mesulam, 1978), and antiChEs to achieve precise mapping of the cholinergic pathways. The early, and yet quite advanced mapping was first presented by the McGeers and Henry Kimura (1980, 1981). They stressed the importance of brainstem systems, including cranial motor nuclei, parabrachial complex and tegmental nuclei, as well as forebrain systems including gigantocellular complex and several reticular nuclei; they described thalamic, limbic, and cortical radiations of the brainstem system and the presence of cholinergic cortical interneurons. The subsequent work of particularly Nancy Woolf, Marcel Mesulam, Larry Butcher, Bruce Wainer, and Hans Fibiger described in much detail the sources of origin and the radiations of both descending and ascending branches of the system (see below, section DII) and proposed novel,

specific nomenclature for the cholinergic sites of origin (see Chapters 1 through 6; Mesulam et al., 1983a, 1983b).

d. Conclusions

The work of Mesulam, Woolf, and others expanded the vision of Shute, Lewis, Kimura, the McGeers, and their associates and brought about something rare in the cholinergic field: an almost definitive statement concerning at least one area of that field, namely, cholinergic pathways (see below, section IIC). This accomplishment helped link the cholinergic system with central functions and behaviors (see Chapter 9). Furthermore, the early work concerning the identification within the cholinergic neurons of several components of the cholinergic system besides CAT, AChE, and CoA-SH, namely, synaptic vesicles and their dynamics, choline uptake into the cholinergic terminals, and ACh uptake into the vesicles, and so forth, served as a basis for establishing the functional and molecular characteristics of a cholinergic neuron (see section B, below).

2. The Story of Cholinergic Markers and of the Processes Leading to the Release of Acetylcholine

a. What Makes a Cholinergic Neuron Tick?

Besides the early evidence concerning CNS presence of ACh and CAT, additional lines of evidence were established in the 1950s and 1960s; these lines helped identify cholinergic synapses and cholinergic neurons, define their central sites, and explain what makes a cholinergic neuron tick. Of course, the primary line of evidence was initiated by Sir John Eccles as he demonstrated the presence of cholinergic synapses in the CNS; this demonstration included also the findings, important for the definition of the "ticking" of cholinergic neurons, of the cholinoceptivity of a central cholinergic neuron and of its release of ACh (Eccles et al., 1954; Eccles, 1964; see also Chapter 9 A and Karczmar, 2001a, 2001b). Canberra's team of David Curtis, Casmir Krnjevic, John Phillis, and John Crawford continued this line of research, and that of Philip Bradley and others in United Kingdom; it is described in more detail in Chapter 9 A (see also Eccles, 1964; Karczmar, 1967).

Another, related line of evidence concerned cholinoceptivity of brain sites, that is, the presence of cholinergic receptors, whether muscarinic or nicotinic (only monotypes of muscarinic and nicotinic receptors were recognized at the time), and evoked cholinergic potential; this research dealt also with the pharmacology of the receptor responses. This research, carried out initially by the Canberra team, employed the method of electrophoretic application of ACh and drugs such as atropine, beta-erythroidine and d-tubocurarine, muscarine, and nicotine. Their studies in the 1950s and 1960s showed that the central cholinoceptivity is represented mainly and ubiquitously by muscarinic receptors and responses to muscarinic agonists that are potentiated by antiChEs. Indeed, this early research demonstrated that muscarinic receptors are present in the hypothalamus, brainstem and medulla, striate, limbic sites, geniculate, thalamus, and cerebral cortex (see Eccles, 1964; Karczmar, 1967). While today (see Chapter 6 B) many nicotinic sites are distinguished, during the early post-Ecclesian era, besides the Renshaw cells only certain geniculate and cortical sites were recognized as nicotinic (Krnjevic, 1963, 1974; Tebecis, 1970a, 1970b). In addition it became recognized that cholinoceptive responses at cholinergic neurons evoke a "down-the-line" activity that results in the release of ACh from their axon terminals, this released ACh being responsible for the subsequent actions. Subsequently it became apparent that cholinoceptive receptors are present on noncholinergic cells, activation of these receptors resulting in release of other than ACh transmitters, and that cholinergic receptors are located at nerve terminals of cholinergic and noncholinergic neurons (see Chapter 9 BI and BIII). It should be added that, as shown recently (particularly with regard to the neuromyal junction), several proteins (neuregulins such as ARIA) and proteoglycans (such as agrin) mediate the expression, synthesis, and distribution of the cholinergic receptors, and these proteins also serve as markers for cholinergic and cholinoceptine neurons (see Fishbach and Ropsen, 1997).

It was already mentioned that ACh synthesis and its catabolism constituted important items in Dale's and Feldberg's reasoning concerning the

role of ACh as a central neurotransmitter. David Nachmansohn provided the crucial evidence in support of this notion, as he and Machado (1943) discovered the synthetic enzyme choline acetyltranferase (CAT; originally termed choline acetylase). Further studies of CAT, its coenzyme A, its central presence and distribution, its role, and its kinetic characteristics were carried out by William Feldberg, Martha Vogt and their associates (see above), Catherine Hebb, David Nachmansohn, Bernard Minz, John Quastel, and others (see Augustinsson, 1948; Quastel et al., 1936; Nachmansohn, 1963; Hebb, 1963).

Then the central (and peripheral) existence and role of a ChE, which was prophesied, as already mentioned, by Dale in 1914, were established by a number of distinguished investigators. The demonstrator of the peripheral cholinergic transmission, Otto Loewi himself, proved Dale's notion. He and Emil Navratil (1924) observed that aqueous extracts of the frog heart destroyed the Vagustoff, that is, ACh; the characteristics of the active extract were those of an enzyme, and Loewi termed it "acetylcholine esterase." Loewi and Navratil (1926) demonstrated subsequently that the potentiation of the vagal effect by physostigmine (they referred to physostigmine as "eserine") is due to physostigmine's antiChE action. Then Stedman et al. (1932) showed that ChEs have as their specific substrates choline esters, and David Glick was probably the first investigator to demonstrate in 1939 the presence of a ChE in the brain (see Glick, 1941). For further information on ChE-focused investigations, in particular on the significance of AChE as contrasted with that of ChEs, see Chapter 3 DI (see also Koelle, 1963; Augustinsson, 1948, 1963) These investigations determined that indeed AChEs are markers of cholinergic neurons and are involved in the question of "what makes the cholinergic neurons tick"; these studies established, however, that this particular marker is not completely reliable, as AChE is present in noncholinergic neurons as well (see Chapter 3 DI).

It should be mentioned that at least some of these findings were made long before Eccles' demonstration of the presence of cholinergic transmission in the central nervous system. As in the case of cholinoceptivity, these sites or markers were ubiquitous; whenever pertinent studies were carried out, the various markers coincided with one another as well as with cholinoceptivity (see, for example, Hebb and Whittaker, 1958; Hebb, 1963).

Additional markers were established subsequently, and their discovery was an important constituent of the evidence for the existence of cholinergic transmission and cholinergic pathways. Palay and Palade (1955) described the presence of synaptic vesicles in the brain, and Victor Whittaker (see Whittaker et al., 1964; Whittaker, 1990) and Eduardo De Robertis (De Robertis and Bennett, 1955) simultaneously expanded on this discovery as well as described definitive and elegant centrifugation methods for obtaining synaptosomes, that is, nerve terminal preparations containing synaptic vesicles.

The neurochemical and cytological analysis of synaptosomes, particularly in Whittaker's laboratory, first in Cambridge and then at Goettingen's Max-Planck-Institut, yielded remarkable results concerning the composition and dynamics of the cholinergic synaptic vesicles, synaptic neurolemmas, and plasma membranes. For example, Whittaker and his associates early purified and isolated cholinergic synaptic vesicles and subsequently developed antisera recognizing the presynaptic plasma membrane (PSPM) to isolate in pure form synaptosomes derived specifically from cholinergic terminals of the Torpedo (see Whittaker and Borroni, 1987; Whittaker, 1990). Note that the PSPM antigens involved in these processes are two gangliosides, Chol-1 alpha and beta, that are present in the mammalian brain (Ferretti and Borroni, 1986). Whittaker and his associates demonstrated also the presence of adenosine triphosphate and other entities subserving the nerve terminal of the cholinergic vesicles (Dowdall et al., 1974; Whttaker, 1992).

This analysis constitutes the basis for the subsequent studies of processes related to the release of ACh from cholinergic nerve terminals. In the modern era, it was shown that these processes require specialized protein systems, which are concerned with generation of ACh, nerve terminal uptake of choline, and vesicular uptake of ACh (see several sections of Chapter 3), as well as with vesicular transport phenomena and recycling of the vesicles; these systems are described in detail in sections B and C of this chapter. Furthermore, besides defining a number of cholinergic markers of the cholinergic neurons, the studies in question

led also to description of possibly several modes of ACh release, which I characterize below as classical and unorthodox hypotheses of ACh release (section C, below).

B. Morphology, Cytoanatomy, and Markers of Central Cholinergic Neurons

Is there a specific morphology and cytoanatomy of cholinergic cells that would distinguish them from noncholinergic neurons? Are there any cytoanatomical characteristics of a cholinergic synapse that would provide the basis for such a distinction? Or are the cytoanatomy and/or morphology of either the cholinergic neurons or their synapses not sufficiently specific for such a differentiation? Are additional cholinergic markers, such as the presence of CAT or a choline uptake system needed for the identification of a cholinergic cell? Answering these questions is important not only for the understanding of the characteristics and the function of cholinergic neurons but also for the definition of cholinergic pathways; these matters will be discussed in the two following sections.

1. Morphology and Cytoanatomy of Cholinergic Neurons

Few studies specifically focus on the morphology and cytoanatomy of cholinergic neurons (see Ruggiero et al., 1990; Famiglietti, 1983; Rodieck and Marshak, 1992; Martinez-Rodriquez and Martinez-Murillo 1994); among these investigations, the studies of Famiglietti (1983) and Rodieck with Marshak (1992) concern only 1 cholinergic cell type, namely, the amacrine cells of the retina. In some cases (see, for example, Butcher et al., 1976) the morphology of cholinergic cells is referred to only parenthetically; in other cases (see, for example, Woolf and Butcher, 1986, 1989) this morphology may be deduced from the photomicrographs included with the studies in question. In what follows, the cytoanatomy and morphology are described either on the basis of specific description of the neurons or on the basis of the pertinent photomicrographs. Finally, axons releasing various transmitters (or axons emanating from different nuclei that contain neurons synthesizing the same transmitter) may vary anatomically, but at this time it does not seem possible to differentiate anatomically cholinergic axons from noncholinergic axons. It must be stressed that the neurons are referred to in this section as cholinergic because they were identified as such by means of CAT immunohistochemistry or by means of additional markers (see below).

Altogether, cholinergic cells come in many shapes and sizes. Thus, the alpha motoneurons that supply the striated muscle endplates, whether located in the ventral horns of the spinal cord or in the brainstem, which is the origin of cranial nerves, are among the largest neurons of the nervous system: these polygonal, multipolar neurons may be up to 500 Å in diameter. On the other hand, the gamma motoneurons that supply the spindles are among the smallest neurons, as they range from 18 to 38 μm (Szentagothai and Rajkovits, 1955; see also Brodal, 1981). Other cholinergic neurons are frequently ovoid, round, or oval and elongated; they may be bipolar or multipolar, as in the case of the neurons of the mesencephalic interstitial nucleus of Cajal and nucleus basalis magnocellularis (NBM), respectively. Multipolar ovoid neurons are also present in the nucleus reticularis and in the nucleus ambiguus (Ruggiero et al., 1990). These multipolar neurons are 25 to 40 Å in diameter, although some of the multipolar neurons of NBM, other basal forebrain sites, and the pedunculopontine tegmental nuclei are considerably larger; these larger neurons are usually hyperchromatic (Butcher et al., 1977; Woolf and Butcher, 1989; Mesulam et al., 1983a, 1983b; Martinez-Murillo et al., 1989; Bigl and Arendt, 1992). Smaller (18 to 25 μm) ovoid or fusiform mutipolar neurons were found in the parabrachial complex, basal forebrain substantia nigra, raphe nuclei, periventricular gray, and hypothalamus (Ruggiero et al., 1990; Martinez-Murillo, 1989); also, ovoid or fusiform neurons are the cholinmergic neurons classified by Marsel Mesulam and his associates (Mesulam et al., 1983a, 1983b) as belonging to basal forebrain sectors Ch1 to 3. Martinez-Murillo and his associates (1989) were among the few investigators who described the cholinergic cells of cholinergic complexes of the forebrain, including NBM, in more detail ("cell nucleus showed one or more indentations . . . occupied central position and was surrounded by abundant cytoplasm rich in

organelles . . . large lipofuscin granules were also observed . . . the dendrites were thick").

And then there are the amacrine cells of the retina. While the amacrine cells of the innermost nuclear layer are peptidergic, the starburst amacrine cells of the inner plexiform layer are either cholinergic or gabaergic. These large neurons exhibit no obvious polarity and unique morphology (Famiglietti, 1983; Rodieck and Marshak, 1992); they were identified in the retinas of the rabbit, human, and primates (see Giolli et al., 2005).

As can be seen, it is difficult to decide purely on the basis of morphology that a given cell is cholinergic. In fact, neurons subserving noncholinergic transmitters (i.e., catecholaminergic, serotonergic, or peptidergic neurons; perhaps the easily distinguished central histaminergic mast cells may be an exception) may be similar in shape and size to one or another "type" of cholinergic neurons.

May we then look to synaptic morphology for the differentiation in question? In the 1960s, Gray (1969; see also Hutchins, 1987 and Shepherd and Harris, 1998) distinguished morphologically between synapses subserving excitatory and inhibitory transmission; they are referred to as Type 1 and Type 2 synapses, respectively. The excitatory Type 1 synapses have a wider synaptic cleft than Type 2 inhibitory synapses. The postsynaptic membrane of the Type 1 synapses is thick and dense, and occupies a great part of the postsynaptic area, while the Type 2 synapses exhibit dense material both pre- and postsynaptically; thus, Type 2 synapses are symmetrical while Type 1 synapses are asymmetrical. Finally, the synaptic region (synaptic or active zone) of Type 1 synapses is longer than that of Type 2 synapses. Cholinergic synapses are generally Type 1 synapses (see Kimura et al., 1981 and Eccles, 1964; there occasionally may be exceptions to this rule, see Smiley, 1996), but the Type 1 morphology cannot serve for reliable morphological identification of cholinergic transmission, as Type 1 synapses can be activated by noncholinergic excitatory transmitters such as glutamate. Finally, the axons and nerve terminals are characterized by varicosities and boutons (see, for example, Shepherd and Harris, 1998), but it does not appear that these may be used as dependable markers of cholinergic axons and terminals.

Other synaptic markers, the synaptic vesicles, may serve well to identify cholinergic transmission. Following Victor Whittaker's and Eduardo de Robertis' discoveries (see section IIA, above), dynamics of cholinergic vesicles were studied in detail; much of the pertinent research took place first at the Station Biologique of Arcachon, France, and then in Victor Whittaker's laboratory at Max-Planck-Institut in Goettingen, Germany. While this research was conducted with the Torpedo electric organ, the results are consistent with those obtained in mammals, including mammalian brain. Seen via electron microscopy of peripheral or central cholinergic nerve terminals, the ACh-containing cholinergic vesicles were round or oval and had a clear core that exhibited variable degrees of density and variable size (Zimmerman and Whittaker, 1977; see also Prior and Tian, 1955). In mammals, they vary in size from 45 to 50 Å in diameter; they are by far larger in the case of the vesicles of the Torpedo electric organ (Whittaker, 1992; Martinez-Murillo, 1989). Vesicles of similar form and size are seen in Whittaker's synaptosomal preparations (Whittaker, 1992). Occasionally large or fused vesicles are also present; they may be the source of the giant excitatory cholinergic postsynaptic potentials (Eccles, 1964). They usually form clusters throughout the terminals, as well as at the neurolemma of the terminal. Several other modes of cholinergic vesicles are also present: during the process of exocytosis some vesicles fuse with the terminal neurolemma and accordingly change in form from ovoid to flattened; empty vesicles or vesicular ghosts also appear as they are formed in neuronal perikarya as well as at the nerve terminal after the release of their ACh content in the course of vesicular recycling (see next section and Whittaker, 1992). There are also two or more density modes among the vesicles, that is, vesicles may vary in their "molecular acetylcholine content" (MAC; Whittaker, 1990) during the process of cycling (see below); the recycling vesicles that contain freshly synthesized ACh are denser, while the less dense vesicles are present in the axons of the electric fish (Whittaker, 1992).

On the whole, there is a clear distinction among cholinergic vesicles and vesicles containing other transmitters. For example, peptidergic and serotonergic vesicles have a dense core and are larger than cholinergic vesicles, while catecholaminergic vesicles are granular; yet, sometimes clear core vesicles of a size comparable to that of cholinergic vesicles are present in serotonergic or

catecholaminergic terminals (Van Bockstaele and Pickel, 1993; Horie et al., 1993; Doyle and Maxwell, 1993). It must be also remembered that peptides, such as the vasoactive intestinal peptide (VIP), are copresent with ACh in cholinergic vesicles (Agoston and Lisiewicz, 1989; see Whittaker, 1990). Altogether, the total picture of cholinergic terminals, cholinergic neurons, and cholinergic vesicles as described in this section is quite diagnostic for cholinergic nerves and synapses.

2. Neurochemical Systems as Markers of Cholinergic Neurons and Intraterminal ACh Motions

Processes of synthesis of ACh include several components such as nerve terminal choline uptake, CAT, acetyl coenzyme A and its synthetase, and synaptic vesicles and their dynamics; these components serve as dependable markers of cholinergic neurons and cholinergic nerve terminals. In addition, ChEs (particularly AChE) help identifying both cholinergic perikarya and nerve terminals of cholinergic neurons. Choline acetyltransferase and AChE characterization of cholinergic cells are discussed in detail in sections DI–DIII, below, and in Chapter 3 B1-3. In this section, the systems linked with the vesicular cycling and storage and release of ACh are specifically considered.

As illustrated by certain aspects of the cytomorphology of cholinergic neurons, described above, synaptic vesicles undergo a cycle that must be subserved by appropriate neurochemical systems. As ACh, CAT, and AChE, these systems define what it is to be a functional cholinergic cell (Weihe et al., 1998); in fact, the systems concerned with vesicular cycling, transport of ACh into the vesicles, and ACh synthesis are regulated by a single cholinergic locus gene (see Eiden, 1998; Mallet et al., 1998).

3. "Cycling" and "Recycling" Processes

A complicated process concerns formation and movement of synaptic vesicles, loading of the vesicles with ACh, the fusion of the vesicles with an endosomal component and the terminal plasmalemma, and the vesicular release of ACh. This process is referred to as cycling—or recycling, if one starts with the empty synaptic vesicles that have released their ACh.

a. Formation and Movement

The process begins with the formation of the empty vesicles and with their movement. Similar to CAT, empty synaptic vesicles are formed within the Golgi organelles and are transported anterograde-fashion at a fast rate (Kiene and Stadler, 1987). This fast transport involves microtubules, actin filaments, and neurofilaments; the vesicles are bound to these organelles by a family of proteins called synapsins (see Whittaker, 1992); mRNAs coded for these and other proteins are contained in the Golgi bodies of the perikaryon, and Whittaker (1992) suggests that these proteins subserve generally vesicular transport and related processes with regard to both cholinergic and noncholinergic vesicles. Phosphorylations and dephosphorylations serve to link with and liberate the vesicles from the elements of cytoskeleton and to mobilize the vesicles for exocytosis.

b. Acetylcholine Loading into the Vesicles

At the nerve terminal, a specific protein facilitates loading ACh into empty vesicles (this process was called "concentrative uptake" by Whittaker, 1992). The protein is referred to as vesicular ACh transporter (VAChT; Bahr and Parsons, 1986). Phenyl piperidines inhibit this process, (–) 2-(4-phenylpiperidino) cyclohexanol (vesamicol) being the most powerful and specific inhibitor of VAChT action; it is interesting and teleological that vesamicol shows a much higher affinity for empty vesicles (vesicle ghosts) than for loaded vesicles (Noremberg and Parsons, 1989; see also Whittaker, 1992). Sophisticated molecular studies of VAChT by Varoqui and Erickson (1998) indicated that the vesicular transport of ACh requires cholinergic-specific amino acids within the N-terminal portion of VAChT, and that this is the site of action of vesamicol. The important aspect of the transporter mechanism is that the genes for VAChT and CAT are colocalized: "the gene encoding the vesicular acetylcholine transporter has been localized within the first intron of the gene encoding acetycholinetransferase and is in

the same transcriptional orientation" (Mallet et al., 1998). In fact, certain polypeptide factors involved in cholinergic ontogeny such as cholinergic differentiation factor/leukemia inhibitory factor concomitantly increase VAChT and CAT mRNA levels. Furthermore, the regulation of expression of CAT and VAChT is coregulated by the cholinergic gene locus that contains genes both for CAT and for VAChT (Mallet et al., 1998; Wu and Hersh, 2004; Lim et al., 2000; for further details of this coregulation, see Chapter 3 B-1); thus, CAT and VAChT gene transcriptions share common promoters (Mallet et al., 1998) . It should be stressed that the transporter in question is not necessarily specific for cholinergic neurons and vesicles (Cervini et al., 1995; Mallet et al., 1998) and that different transporters subserve other transmitter systems such as monoaminergic and serotonergic systems (see, for example, Zucker et al., 2001).

Whittaker and his associates discovered early that ATP is copackaged with ACh into the cholinergic vesicles (as it is with catecholamines in granular catecholaminergic vesicles; Whittaker et al., 1964). The specific carrier for this uptake is saturable and of the high-affinity type (Whittaker, 1992); it was identified as the vesicle component 11 or vesicular ATP translocase, which is a protein-binding active factor (Lee and Witzemann, 1983). The ATPase, which is present in the vesicular wall, assists the ATP translocase, which maintains the proton gradient stimulating the translocation; this gradient also facilitates the VAChT-activated vesicular uptake of ACh (Whittaker, 1992, 1998). According to Whittaker (1992), similar to ACh, ATP is taken up preferentially into the pool of recycling vesicles, that is, into the pool of vesicles released empty from the nerve terminal plasma membrane after their fusion with the membrane and after the release of ACh; this is consistent with the notion of a "readily releasable pool" of vesicles (vesicle pool immediately available for release; see below). Acetylcholine and ATP do not course freely inside the vesicles but are adsorbed to an intravesicular proteoglycan matrix (Reigada et al., 2003); in fact, when the vesicular membrane is treated with distilled water, ACh and ATP remain attached to the matrix as long as the cations are not added; this explains why vesicular ACh is so stable upon stimulation of the terminal (at least until enzymically hydrolyzed) or upon purification (Yves Dunant, personal communication).

c. Docking and Fusion

Docking of the vesicles and the fusion of synaptic vesicle plasma membranes directly lead to ACh release (see Whittaker, 1992). This is a most important step, as the demonstration that the fusion of vesicles and the release of vesicular contents of ACh—or quanta—are linked is a part of the proof of the quantal nature of ACh release; subsequently, this demonstration was further helped by development of novel, refined techniques (see below, this section). The fusion factors include presynaptic membrane proteins, syntaxin, synaptotagmins and attachment proteins, SNAPs (particularly SNAP 25 and synaptobrevin), and synaptic vesicle proteins (VAMP1 and 2; Hou and Dahlstrom, 2000; Morel at al., 1998; Chapman et al., 1955; Robinson et al., 2004); these proteins interact with Ca^{2+} during processes of fusion and ACh release. Some or all of these proteins form fusion attachment protein receptor complexes called SNAREs; additional proteins of the vesicles such as synaptophysin (P38), Spring (a finger protein), and VMG also play a role in docking and fusion processes (Whittaker, 1992; Li et al., 2005; Fasshauer et al., 2003). SNAREs are cleaved by clostridial neurotoxins, "most potent inhibitors of neurotransmitter release known" (El Far et al., 1998; see Figure 2-4). Importantly, cholinergic stimulation—such as nicotinic, via alpha7 recptors—may activate attachment proteins (Liu et al., 2005).

It was proposed that SNAREs act as synapse-specific membrane recognition molecules, acceptors for docking and fusion catalysts (Morel et al., 1998; El Far et al., 1998; Rothman, 1994). In addition, other vesicular proteins, the RABs, as well as internal vesicular matrix (Reigada et al., 2003), subserve the vesicular plasmalemma fusion and ACh release; however, some RABs are concerned with an entirely different function, the endoplasmic fusion (Sudhof, 2000, 2005). The fusion and release processes produce empty synaptic vesicles, ready for recycling; this recycling—that is, reformation of ACh-loaded vesicles—may occur "directly" or via an endosomal intermediary, activated by a RAB protein (the role of several synaptic vesicle RAB proteins, such as rabphilin, is

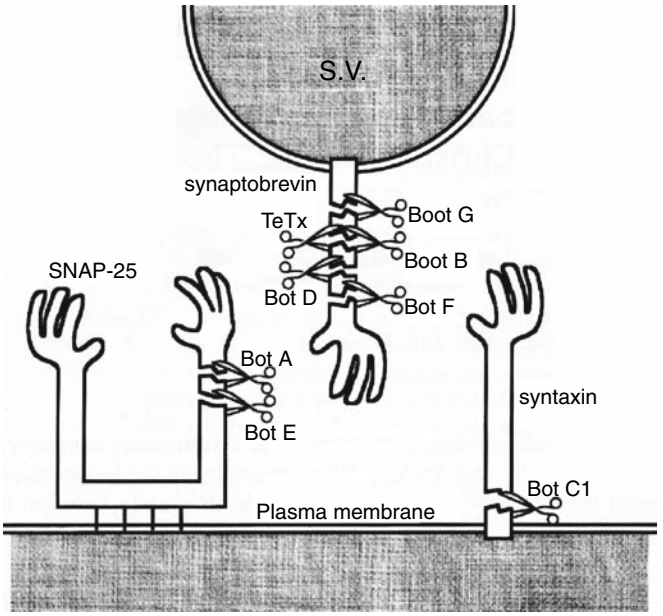

Figure 2-4. Clostridial toxin cleavage sites. The proteins cleaved by the clostridial neurotoxins are shown to highlight their juxtaposed membrane orientations in the synaptic vesicles (synaptobrevin) and plasma (syntaxin and SNAP-25) membranes. This oversimplifies the *in vivo* situation, as fractions of cellular syntaxin and SNAP-25 are found on synaptic vesicles. Transmembrane domains insert syntaxin and synaptobrevin into the membrane, whereas palmitoylation of cysteine residues is responsible for the membrane localization of SNAP-25. Note that Bot C1 may cleave both syntaxin and SNAP-25. (From El Far et al., 1988; reprinted by permission from Elsevier Press.)

at this time not clear; Sudhof, 2000; Lonart and Sudhof, 2001).

It must be added that fusion, whether between vesicles, between vesicles and presynaptic membranes, or between vesicles and the endosomal intermediate, was demonstrated *in vitro* (Whittaker, 1992; Sudhof, 2000). Several technical innovations contributed to this demonstration. Thus, the quick-freezing technique employed in the Bruno Ceccarelli Center of the University of Milan (see, for example, Torri-Tarelli et al., 1985, 1990) stabilizes the pertinent events within 1 ms. Then, precise monitoring of exo- and endocytosis of the vesicles was made possible by the use of lipophilic fluorescent probes such as FM dyes and the optic recording of their location within the cell and vesicular membranes (Cochilla et al., 1999; Sudhof, 2000, 2005). The employment of these techniques led to the description of exocytosis as consisting of the fusion of vesicles with the plasma membrane (plasmalemma) which creates small indentations in the latter and coated vesicles; after the emptying of the vesicles and the release of Ach, the empty, uncoated vesicle is ready for recycling and ACh uptake. The use of this technique jointly with appropriate immunocytochemistry methods linked time-wise the protein-activated fusion and the emptying of vesicles and added support for the theorem of the quantal hypothesis of the release of ACh. It should be stressed that, unlike the transporter proteins, fusion and exocytosis proteins (and other release proteins such as the mediatophore, which is discussed below, in section B2) might not be specific for the cholinergic vesicles (see, for example, Bacci et al., 2001; Israel and Dunant, 1998).

d. Postsynaptic Membranes and Cholinergic Receptors

A number of active proteins related to neurotrophins are engaged in the regulation and formation of cholinergic receptors and membrane ion channels. Neuroregulins, including ARIA, and laminin chains are among these proteins. While

ARIA is predominantly displayed at the neuromyal junctions, it may be also present centrally, as are the laminins (Fischbach and Rosen, 1997; Yin et al., 2003). Also, electrotactins and related proteins are involved in synaptogenesis; they were identified by Israel Silman, Joel Sussman, and their associates as adhesion proteins, which are members of the AChE family and which form a pattern of electrostatic potential that participates in the formation of active synapses (Botti et al., 1998; Rydberg et al., 2004).

While not listed generally as markers of the cholinergic neuron, it appears that these proteins could serve in this capacity (Sanes and Truccolo, 2003).

e. Cycling and Recycling of Synaptic Vesicles

The course of exocytosis involves several kinds of synaptic vesicles; Victor Whittaker remarked early on the heterogeneity of the vesicles as well as proposed the notion of vesicle cycling, if the total life cycle of the vesicles is considered, or recycling, if exocytosis is taken as the starting point of the phenomenon (Whittaker, 1992, 1998). This heterogeneity and cycling of vesicles were originally established in terms of the differences, before and after cholinergic nerve terminal stimulation, in the density, size, and specific radioactivity of ACh of the vesicles, following their loading with radioactive choline and their release of ACh; other means, such as fluorescent dyes, laser scanning, and so on, are also used for the analysis of vesicle heterogeneity and their cycling (Ryan, 2001). Cultured hippocampal neurons yielded much evidence with respect to this matter (see, for example, Murthy and Stevens, 1999).

As already referred to, essentially, there exist two kinds of cholinergic vesicles: empty and full (or ACh loaded). The empty vesicles are formed either in the neuronal perikarya or at the nerve terminal; they are generated at the terminal following ACh release and the emptying of the ACh-loaded vesicles (exocytosis). The empty vesicles formed in the perikarya of the cholinergic neurons migrate down the axon, carried by the axoplasmic flow. They become loaded in or near the nerve terminal as they incorporate ACh via the action of VAChT proteins (see above, this section) and the processes of ACh synthesis (see Chapter 3 B); the vesicles that become empty via ACh release are also subject to ACh loading. Following Whittaker's original description of the processes of recycling or cycling, he and others (see, for example, Prior and Tian, 1995; Pyle et al., 2000; Sudhof, 2000) included several pools of synaptic vesicles in the cycle.

Originally, Whittaker (1992) recognized four pools and defined their different roles during recycling and exocytosis. The empty vesicles arriving from the cell body represent the first pool. Then there is the reserve pool of ACh-loaded vesicles. The next pool is the pool of docked vesicles; Sudhof (2000) and others refer to this pool as "the readily releasable pool" that contains only relatively few vesicles docked in the active presynaptic zone. Finally, there is the stimulus-induced recycling pool of empty vesicles that preferentially takes up ACh that is newly synthesized in the cytoplasm (Barker et al., 1972). Pyle et al. (2000) determined that the latter pool of vesicles might be rapidly converted into ACh-loaded vesicles ("immediately after exocytosis"). Pyle used FM dyes in this instance (see also Sudhof, 2000; Ryan, 2001).

Some investigators recognize additional pools and processes. Thus, Thomas Sudhof (2000) writes of a "recycling pool," which he defines as labeled, by the FM-143 dye, under conditions of intensive synaptic stimulation; according to Sudhof, this pool consists of the "readily releasable pool" and a "reserve pool"; both include ACh-loaded vesicles. Then several proteins, in addition to those that regulate vesicular cycling via their role in docking in fusion (see above, this section), such as dynamine, are directly important in the organization of cycling (see Weible et al., 2004).

4. False Transmitters

The issue of false transmitters is of interest in the present context. The false transmitters are ACh analogues that are taken up by the vesicles and released in lieu of ACh. However, in general, false transmitters cannot be taken up by the nerve terminal choline uptake system; therefore, appropriate precursors, that is, choline analogues capable of being taken up by the terminal and then acetylated in the terminal by CAT, are used to form the false transmitters. Such precursors are, for example, pyrrolidinecholine, homocholine, and

triethylcholine (Whittaker, 1992); the generation of false transmitters by these precursors was established for the Torpedo electric organ and for the mammalian brain (von Schwarzenfeld, 1979; von Schwarzenfeld et al., 1979). Depending on the false transmitter, they can render the cholinergic transmission either ineffective (Jenden, 1990; Jenden et al., 1989) or partially effective, forming small endplate potentials (see next section).

5. Release Processes

The process of release is activated by the endogenous or electric presynaptic stimulation and ensuing depolarization of the membrane; this process is Ca^{2+} dependent. As is well known, Bernard Katz, Rodolfo Miledi, and Juan Del Castillo (Del Castillo and Katz, 1954; Katz, 1966; Katz and Miledi, 1965) demonstrated this dependence; they could even relate in situ the concentration of Ca^{2+} to the size of the endplate potentials. The phenomena of depolarization and Ca^{2+}-dependent ACh release may be duplicated with brain synaptosomes (see Whittaker, 1992). The saturable Ca^{2+} uptake is catalyzed by ATP and calmodulin. Calmodulin is a cytosolic protein regulating the formation of the cytoskeleton (Kretsinger, 1987; see also Whittaker, 1992), but it also facilitates, in the presence of ATP, Ca^{2+} uptake, presumably via activation of many protein phosphokinases. As calcium uptake and calmodulin dynamics can be visualized in situ or in the synaptosomes (see Whittaker, 1992), these components of ACh release may also serve as markers of a cholinergic nerve terminal (for additional, modern insights into the processes of ACh release, see below, section C).

It is important to remember that, since the days of Thomas Hokfelt and Victor Whittaker, it has been well known that ACh is coreleased with other transmitters, including catechol and indoleamines, and particularly peptides (see Chapter 9 BIII-2).

6. Cholinergic Markers in Noncholinergic Cells and Tissues

The markers of cholinergic cells appear in noncholinergic cells and tissues of the adult vertebrates, in ephemeral organs such as placenta (see Sastry, 1997), during preneurogenetic stages of ontogenesis (Karczmar, 1963a, 1963b; Chapter 8 BI and BII), as well as in monocellular and metazoan species devoid of nervous system (or of contractile systems, present in flagellates, which exhibit cholinergic correlates (see below, this section, and Chapter 8 BV). These phenomena pose a problem with regard to the identification of cholinergic neurons. The related question is that of the place of BuChE in this identification process.

The question of the presence of cholinergic markers such as SNAREs or RABs in noncholinergic or nonneuronal cells is moot, as this matter was not investigated to any extent in such cells. Yet, cholinoceptive receptors appear at the nerve terminals of noncholinergic neurons such as glutaminergic cells and/or the postsynaptic sites of noncholinergic neurons, including inhibitory interneurons; are other cholinergic markers present in these instances? (See section C, this chapter, and Chapter 9 BI; see also Atkinson et al., 2004).

Then, may other cholinergic components such as ChEs, CAT, VAChT, and ACh serve as cholinergic markers? Some of these components, particularly ChEs, appear in noncholinergic neurons and in nonnervous cells and tissues (for example, in blood), in preneurogenesis stages of development, in nonnervous tissues of invertebrates including bacteria, and in plants. Their presence and role in these cases is, to say the least, mysterious and piquant: What is the role of ChEs in bacteria and plants? Why do certain mammalian species exhibit AChE in the red blood cells and BuChE in the plasma while a reverse situation exists in other species? Why do platelets of some species contain AChE, while those of other species do not? Why are both AChE and BuChE as well as ACh present in the cerebrospinal fluid, as established early by Sir William Feldberg (Feldberg and Schriever, 1936; see also Koelle, 1963; Karczmar, 1967; Augustinsson, 1948; Fischer, 1971; Goedde et al., 1967)? These matters are discussed at length in section DIII of Chapter 3.

Of particular interest is the presence of BuChE in cholinergic neurons (Koelle, 1963; Giacobini, 2000, 2002; it is also present in the glia and in the plaques of Alzheimer's disease; see Chapter 10). Should BuChE be considered a cholinergic

marker? Ezio Giacobini seems to think so, as he opines that BuChEs of the central neurons protect against excess ACh that may occur in some "physiological" conditions. He bases this notion on his finding (Giacobini, 2002; see Chapter 3 DIII) that a specific antiBuChE augmented markedly rat cortical levels of ACh; does it follow from this evidence that BuChE may serve as a protector? What would be the physiological conditions under which BuChE could play this role? This is not to deny the possibility that both BuChE and AChE may play this scavenger role in the blood, where it would rapidly hydrolyze ACh that may be released into the blood from various tissues (Karczmar and Koppanyi, 1956; Chapter 3 DIII).

It appears altogether that a neuron may be deemed dependably as cholinergic when it exhibits the preponderance of cholinergic markers, while the presence of cholinergic components in nonnervous cells such as the erythrocytes, preneurogenetic embryos, and uninnervated organisms such as bacteria is both a mystery and a problem.

Certain speculations were already raised with regard to the role of BuChE (see above and Chapter 3 DIII). Furthermore, the ontogenetic presence of cholinergic components prior to neurogenesis and in species devoid of nerve cells may relate to the demonstrated function of cholinergic components as morphogens (see Chapter 8 BV and CIII and Chapter 11 A) or, perhaps, as metabotropes.

7. Conclusions

There are several problems with respect to the processes described in this section. For instance, what determines the ratio between empty vesicles generated in the cholinergic neuron perikaryon and the empty vesicles recovered at the nerve terminal plasma membrane after the release of ACh? Then, which mechanism moves the empty vesicles away from the terminal (see section C5)? It must also be stressed that while very many markers of the cholinergic neurons were identified, many more will be discovered in the future.

It seems clear that we cannot describe at this time a parsimonious mechanism that would link sequentially the proteins in question during the processes that involve the vesicles, ACh synthesis and vesicular uptake, and the preparation to release and the release of ACh. Are all the present and future SNAREs, SNAPs, RABs, and other paladins of the phenomena described necessary (Ybe et al., 2001)? Do myelin protein expressions of genes such as Nogo, which are inhibitory during neuronal growth and regeneration, assume a regulatory role in adulthood, as proposed by Roger Nitsch and his associates with respect to the hippocampus (Meier et al., 2003; see also Chapter 8 BII)? Is there some redundancy to this system? Are all these proteins and phospholipids specific for ACh release and for the cholinergic system, or are they needed for the release of other transmitters? These unsolved problems and unanswered questions will multiply when we consider the process of ACh release; indeed, a number of speculations do not fit within the picture presented in this section (see next section).

C. Classical and Unorthodox Hypotheses of Acetylcholine Release

There are several models of the release of ACh from a presynaptic cholinergic terminal: the classical model established in the 1950s by Victor Whittaker, Eduardo De Robertis, Bernard Katz, Ricardo Miledi, Paul Fatt, John Hubbard, Steve Thesleff, and Jose del Castillo (see Karczmar, 1967; Katz, 1966; Eccles, 1964; McLennan, 1963; Whittaker, 1992; see also section B5, above), and three current, neoclassical, or unorthodox pictures of the release of ACh. ACh release is regulated by cholinergic presynaptic receptors, and it is linked with postsynaptic responses. Electro- and neurophysiological details of the postsynaptic and presynaptic cholinergic receptor responses and detailed knowledge of synaptic potentials and currents and their ionic mechanisms are out of the scope of this section (see, however, Chapters 3, 6, and 9 BI). Nevertheless, certain aspects of postsynaptic responses are relevant to the quantal phenomena and hence are pertinent for this section.

1. The Classical ACh Release Model and Postsynaptic Responses

While much of the pertinent information concerning the classical model of ACh release deals

with the skeletal neuromyal junction and its endplate, vertebrate autonomic neurons, the Torpedo electric organ, and invertebrate neurons such as the giant synapses of the squid (Eccles, 1964; Karczmar et al., 1986; Whittaker, 1992), ample evidence confirms the validity of the model for the central cholinergic synapses.

The release occurs when the nerve terminal is depolarized by endogenous or electric presynaptic stimulation and ensuing depolarization of the terminal in the presence of Ca^{2+} and, presumably, calmodulin, as well as a number of fusion and related proteins (see above, sections B3 and B5). Then the presynaptic cholinoceptive receptors modulate ACh release when they abut on cholinergic terminals, and regulate the release of other transmitters when they are present at noncholinergic terminals; in fact, either the same or different proteins are involved in these two types of release regulation (see, for example, Atkinson et al., 2004).

As is well known, Bernard Katz, Ricardo Miledi, and Jose Del Castillo (Del Castillo and Katz, 1954; Katz, 1966; Katz and Miledi, 1965) demonstrated this dependence for the neuromyal junction; they could relate in situ the concentration of Ca^{2+} to the size of the endplate potentials. Subsequent work demonstrated this relationship with respect to excitatory postsynaptic potentials of the central or peripheral neurons. Further details of this phenomenon are presented in Chapter 9 BI.

The release is generally multiquantal; that is, it involves a number of synaptic vesicles, particularly when it elicits a productive response, such as a spike. A productive response may be a postsynaptic response originating at either the soma or dendrites of a cholinoceptive neuron, or it may originate at a presynaptic site of a cholinergic or, for that matter, a noncholinergic neuron. There is a delay of 1 to 2 ms before the postsynaptic response appears; in fact, this delay is a characteristic phenomenon of a chemically, transmitter-operated synapse, and its presence is a part of the proof of the existence of chemical transmission, since it does not occur at the sites of electric transmission (see Eccles, 1963; McGeer et al., 1987b).

The postsynaptic cholinergic response may be excitatory or inhibitory in nature; the excitatory and inhibitory responses are depolarizing and hyperpolarizing, respectively. The productive postsynaptic excitatory response is initiated, whether at the dendritic or somatic membranes, by an excitatory postsynaptic slow or fast potential (sEPSP and fEPSP; an endplate potential [EPP] is evoked at the striated muscle endplate). The sEPSP and fEPSP are generated at muscarinic and nicotinic neuronal sites, respectively. Ultimately, as it reaches the critical threshold (at about—40 mv), the fEPSP generates the postsynaptic spike, which is the definitive signal underlying interneuronal communication. The sEPSPs do not generate spikes, unless in combination with a facilitatory transmitter or compound, or with an antiChE; therefore, the sEPSP is a modulatory rather than a transmittive potential (see Krnjevic, 1969, 1974). The inhibitory and excitatory potentials represent the movement of appropriate ions and, therefore, specific currents (Eccles, 1964).

The nicotinic fEPSP may appear in the absence of a spike, if the receptive postsynaptic membrane is partially obtunded by postsynaptic blockers such as d-tubocurarine, curarimimetics, and certain toxins; if the axonal conduction is partially blocked by inhibitors of presynaptic, axonal conduction such as blockers of Na channels (e.g., tetradotoxin); if ACh synthesis is attenuated by blockers of choline uptake and/or ACh synthesis; and if the release of ACh is partially inhibited by blockers of presynaptic Ca^{2+} channels, including high concentrations of Mg^{2+}, and of calmodulin-Ca^{2+} interaction (McGeer et al., 1987b). It may be also generated by electrophoretic application of ACh at a concentration insufficient to generate a spike. Also, it is possible to visualize the fEPSP by applying a cellular microelectrode or a patch clamp electrode at an angstrom-small distance from the receptor site; in this case, following effective presynaptic stimulation the fEPSP appears as a shoulder of the rising spike (Kuffler, 1949; Katz, 1966; Kuffler et al., 1984; Thesleff, 1960).

During the 1950s and 1970s, Paul Fatt, Bernard Katz, and Ricardo Miledi proceeded to "miniaturize" the postsynaptic responses. First, Fatt and Katz (1952) demonstrated the presence at the quiescent neuromyal junction of spontaneously and randomly occurring "subthreshold" excitatory responses, which they referred to as miniature endplate potentials (mEPPs) or miniature endplate currents (mEPCs). After appropriate analysis it

was posited that these responses are due to spontaneous release of single "packets" of ACh that "might" correspond to the contents of single synaptic vesicles (Katz, 1966; notice the conditional mode of Sir Bernard's statement; Sir Bernard was careful to distinguish proven fact from hypothesis; see below, next section). Ultimately, this phenomenon, originally described for the neuromyal junction, was shown to extend to all cholinergic, peripheral, and central synapses.

Is there a relation between the "miniatures" and the fEPSPs? Such a relation, if demonstrated, would cement the notion of the "quantal" nature of postsynaptic responses. Bernard Katz and Ricardo Miledi provided the necessary proof. While of similar size, the EPSPs fluctuate randomly in size, and this fluctuation can be augmented *in vitro* by diminishing Ca^{2+} concentration or increasing Mg^{2+} concentration. These fluctuations may be analyzed statistically by the use of Poisson's theorem; using this analysis in his striated-muscle experiments Katz (1958) established that fEPSPs are composed of a certain number of quanta, that is, synaptic vesicles. Also, other methods may be used to evaluate quantal fEPSP content, including comparing the miniature fEPSPs or miniature EPPs size to fEPSPs, establishing the relationships among the elementary events, the "miniatures," and the fEPSPs (see below, this section), considering the size of the vesicles and the concentration of the vesicular ACh, and so on (McGeer et al., 1987b; Eccles, 1964; Thesleff et al., 1984). Investigators employing these various methods of analysis reported relatively similar values for the quantal contents of fEPSPs recorded in central or peripheral neurons; these values range from 100 to 200.

Syogoro Nishi, Hideho Soeda, and Kyozo Koketsu (1967) carried out a pertinent study on the toad sympathetic ganglion. These investigators employed Corsten's (1940) frog lung bioassay for ACh—which they rendered more sensitive by adapting it for the toad lung—for the measurement of ACh; ultimately, they related ACh release via a single volley (from a single synaptic knob), the fEPSP, and the "miniatures"; they estimated the quantal content of the fEPSPs at 100 to 200 and the ACh contents of the fEPSPs at 6,000 to 8,000 molecules. It should be pointed out that the bioassay employed by the Japanese investigators is most sensitive, as it responds to 10^{-21} M concentrations of ACh and as it is logarithmically linear over the range of 10^{-6} to 10^{-21} M concentrations. Yet, this bioassay is used very infrequently as it may be employed by only the most precise workers endowed with the most patient and delicate hands; Syogoro Nishi and his associates spent entire, long, and exhausting days to combine, for the purpose of a single experiment, electrophysiology, ACh collection, measuring the transmitter, and fixing the ganglion for the necessary microanatomy evaluation. These values are in agreement with those obtained at the central synapses, and even with those obtained recently by means of the ultrasensitive chemiluminescent method for the measurement of ACh release (see below, next section).

There is a great difference between synapses with regard to quantal size. In the case of neuromuscular and electroplaque junctions, the quantum size is 2 to 3 nÅ; this size is due to the release of 6,000 to 10,000 ACh molecules. This constitutes a large quantal size, and the quanta in question are composed of about 10 subunits of about 1,000 molecules each. The ganglionic and probably central synapses exhibit smaller quanta; in the case of the former, the quantum size comprises only about 1,000 ACh molecules (Yves Dunant, personal communication; see also Nishi et al., 1967; Bennet, 1995).

Then Katz and Miledi performed the second phase of "miniaturization"; it concerned the synaptic "noise." Many investigators noticed the microvolt "noise" in their neuromyal preparations following ACh electrophoresis or in quiescent preparations and attributed it to instrumental imperfections. Only Katz and Miledi (1970, 1973) had the serendipity to perceive that the "noise" is a physiological phenomenon; they proposed that there is a spontaneous leakage of ACh into the synaptic cleft (amounting to a 10^{-8} M concentration and inducing a low-level—a few microvolts—depolarization) and that a few molecules of this ACh generate single-channel responses. Katz and Miledi (1977) opined that this phenomenon obtains not only pharmacologically via ACh electrophoresis but also naturally; they referred to it as an "elementary" event, that is, the current generated by opening a single nicotinic receptor (a direct measurement of individual receptor openings was achieved by Neher and Sakmann [1992] via the patch clamp technique). Again, the

phenomenon in question obtains not only at the neuromyal junction but also at the central synapses (see also Masukawa and Albuquerque, 1978).

Still another miniaturization may be possible. Rene Couteaux identified at the frog myoneural junctions "active zones," which he described as thickenings of the junctional membranes and zones of concentrations of the synaptic vesicles; there may be from 100 to 300 active zones at the frog and mammalian myoneural junctions, and one quantum may react with each "active zone" (Couteaux and Pecot-Dechavassine, 1970; see also Kuno et al., 1971).

2. Nonclassical Notions on Release of Acetycholine

The notion of ACh leakage, proposed by Fatt and his associates, should be related to the unorthodox hypotheses concerning nonquantal release of ACh. There are three such hypotheses. The earliest one was posited in the 1970s as the "kiss and run" model of exocytosis by the late Bruno Ceccarelli; it was presented in a definitive form by Jacopo Meldolesi (1998). Meldolesi actually contributed earlier (Torri-Tarelli et al., 1985, 1990; Meldolesi and Ceccarelli, 1981) to the classical lore of quantal release of ACh by analyzing the role of fusion and transport proteins in the quantal phenomena (see above, section BI). More recently, Meldolesi (Valtorta et al., 1990, 2001) assumed a prudent position: he described Ceccarelli's concept as "fascinating" and proposed that the "kiss and run" process "operates in parallel with the classical . . . vesicle recycling." Meldolesi and Ceccarelli used the quick-frozen technique to demonstrate that the processes of plasmalemmal invagination, fusion, and vesicle recycling are not the rule for exocytosis: "many of the vesicles more intimately continuous with the plasmalemma . . . seem . . . to appear not as invaginations, open to extracellular space, but still as vesicles, sealed . . . by thin diaphragms . . . in direct continuity with the cell surface . . . [therefore,] there is incomplete fusion" (Meldolesi, 1998); this leads to effective and indeed rapid exocytosis and recycling, or to "kiss and run" (Stevens and Williams, 2000). Additional, supportive evidence indicates that exocytosis—perhaps a portion of the total process at any time—may be dissociated from recycling of membrane invaginations and vesicles (Henkel and Betz, 1995).

It should be noted that Meldolesi refers to "many," not all, vesicles as participating in the "kiss and run" process. Also, Henkel and Betz (1995) have only indirect evidence as to the amount of ACh that may be released independently of classical recycling (they consider the cycling of the membrane-ligated fluorescent dye FM1-43 as a monitor of exocytosis). Altogether, Meldolesi and his associates (Meldolesi, 1998; Valtorta et al., 2001) suggest that the "regulated" exocytosis with its complicated assembly of regulatory proteins and organelles is a phenotypic phenomenon, and that not all its components—"secretion competence factors"—may be expressed molecularly; therefore, he speculates that the two processes may run in parallel.

The second of these drastic hypotheses was posited in the 1970s and 1980s by Maurice Israel, Nicolas Morel, Bernard Lesbats, and Yves Dunant (see, for example, Israel et al., 1983; Israel, 2004; Israel and Dunant, 1998, 2004; Dunant, 2000).[2]

They isolated from cholinergic nerve terminals—but not from postsynaptic membranes—a protein, which they associated with Ca^{2+}-dependent release of ACh and which they called the mediatophore. Then they showed that among a number of cell lines, including glioma, fibroblast, and several neuroblastoma lines that were loaded with Ach, only those rich in the mediatophore could release the transmitter; furthermore, the release capacity could be given to cell lines incapable of release by transfecting them with plasmids encoded with mediatophore. Furthermore, antisense probes hybridizing the mediatophore messenger blocked the release of ACh from these preparations as well as from synaptosomes, "naturally" capable to release ACh.

All this could simply signify that the mediatophore belongs to the proteins, such as soluble N-ethylmaleimide attachment proteins (SNAPs), that are involved in docking of synaptic vesicles and in the exocytosis (see above, sections B-2 and B-3), and that it does not mediate any vesicle-independent release processes. In fact, mediatophore is a homo-oligomer of a 16-kDa subunit which is associated in a sector of the nerve terminal membrane which includes other proteins linked with release mechanisms such as vesicular ATPase (V-ATPase; Israel and Dunant, 1998); then the difference between mediatophore and

other cholinergic release proteins such as SNAPs would be only that the mediatophore plays a more general role than the latter, as it subserves translocations and release mechanisms in noncholinergic cells such as glia.

But there is another angle to the story that supports the notion of the mediatophore being essential for ACh release which is independent of the vesicles, and, in fact, the mediatophore subserves the main process of ACh release. Indeed, Israel's team presents data that suggest that this is indeed so in the case of the cholinergic cells. In addition, other investigators (see Prior and Tian, 1995) showed that, following labeling of cholinergic nerve terminals with radioactive choline, isotopic composition of released ACh matches closely the cytoplasmic (free) rather than vesicular (bound) transmitter. Also, this release relates to the decrease in the concentration of cytosolic ACh (Dunant and Israel, 1985). The French-Montreal-Geneva team suggested also that during the release process a constant number of mediatophore molecules may be activated "close to a calcium channel" (Israel and Morel, 1990), this phenomenon resulting in numerically quantal release, although the release is not originating from synaptic vesicles; thus, Maurice Israel and his colleagues can have their cake and eat it too. Ultimately, Israel and his associates (see Israel and Dunant, 1998) suggested that mediatophore molecules represent "elementary pores that translocate ACh from . . . either . . . the cytosol, or synaptic vesicles." It may be added that for the measurement of ACh, Israel, Dunant, Morel, and their associates used the elegant, ultrarapid, and ultrasensitive choline oxidase chemiluminescent method; the technique is sensitive at picomolar concentrations of ACh (see section A).

As pointed out by Maurice Israel and Yves Dunant (Israel and Dunant, 2004; see also Peters et al., 2001), mediatophore has a more general role than that of transmitter release. It appears to be needed (with Ca^{2+} and SNAREs) for membrane fusion (such as the fusion between vesicular and postsynaptic membranes) and proton translocation in V-ATPase processes.

Laurent Descarries and Daniel Umbriaco presented the third and final "radical" hypothesis (or speculation) of nonsynaptic, diffuse release of ACh in 1995. Descarries, Mircea Steriade, and associates expanded the hypothesis subsequently (Descarries, 1998; Descarries and Mechawar, 2000). These investigators employed CAT immunostaining and advanced electronmicroscopy to study cholinergic, monoaminergic, and serotonergic nerve terminals and their axonal varicosities in several rat brain areas. They claimed that in rat cerebral cortex, nestriatum and hippocampus 70% to 80% [*sic*] of varicosities showed "no hint" of synaptic differentiation" or "junctional complex." Altogether, Descarries and associates (Descarries et al., 1997, 2004; Descarries, 1998) proposed that these nonsynaptic varicosities subserve an evoked or spontaneous (diffuse) release of ACh (and other monoamines) into the synaptic cleft. This ACh release is nonsynaptic and results in ambient, low ACh concentrations in the synaptic cleft (or "extracellular space"; Descarries, 1998).

Descarries and associates felt that several findings supported their notion. For example, muscarinic and nicotinic receptors are present at non- or extrasynaptic sites, whether at somatic locations, dendritic spines, or dendritic branches (Mrzlyak et al., 1993). Also, many investigators (e.g. Newton and Justice, 1993, and Israel and Morel, 1990) apparently demonstrated that low ACh levels in the nanomolar range continually exist in the synaptic cleft. Finally, Victor Gisiger, Laurent Descarries, and others provided evidence suggesting that some AChE forms (i.e., G4) may preserve (not eliminate) ambient ACh levels in the synaptic cleft (see Descarries et al., 1997 and Chapter 3 D, III). A related hypothesis—or shall we call it a speculation?—posited by Coggan and his associates (2005), is that, at least at the parasympathetic ganglia transmission occurs not only synaptically but also ectopically, i.e., outside opf synaptic specializations; this mode would involve alpha7 nicotinic postsynaptic receptors.

3. Conclusions

Intense discussions are held at various meetings among adherents to the various hypotheses of nonvesicular release of ACh, such as Maurice Israel and his associates on the one hand, and Victor Whittaker on the other. Sometimes, positions taken by the adversaries seem to soften. Thus, Maurice Israel proposed a mechanism via which their mediatophore process yields a quantal (although not vesicular) release of ACh. Victor Whittaker is more intransigent and retains his position. Actually, in the 1970s he suggested that the since the recycling vesicles (Vesicles Peak

2, or VP2 vesicles) obtained in his experiments with radioactive choline were newly created following the intense stimulation, they must necessarily exhibit the isotopic ACh composition that matches that of the cytosol; this would explain Israel's process as effective only under special conditions.

Altogether, it is not easy to relate the three unorthodox ideas on the mode of ACh release to the classical, quantal hypothesis of release. There is no denying that much evidence supports one or more of the novel ideas, such as the presence of ambient levels of ACh in the synaptic cleft, the absence of synaptic specializations at certain nerve terminal sites where ACh release does occur, and the presence of mediatophore at cholinergic nerve terminals and its capacity for ACh release. Yet the evidence for the vesicular release of ACh is very strong (see Victor Whittaker's comment on this matter in his Foreword to this book). Thus, the relation between synaptic vesicles, mEPPs, and EPPs is well established, for both the central and the peripheral nervous system. Also well demonstrated are the relationships among recycling of the vesicles, the proteins involved in this process, and the release of ACh. Even certain minutiae of the release and vesicular processes are demonstrably connected; thus, the fast vesicle recycling supports and accompanies intense stimulation (Sara et al., 2002). Perhaps then we should try to combine the various release models. Do they all operate under physiological conditions? Do they represent alternative release modes? Do they operate under special circumstances only? Do the pertinent mechanisms and entities—and particularly the mediatophore—belong to the markers of the cholinergic neuron, such as VAChT, SNAPs, and SNAREs?

The classical model alone is immensely complex. It contains multiple components and processes of synthesis of ACh: concentrative—to use Whittaker's term—vesicular uptake of acetylcholine and the activating proteins involved in this uptake and regulated by the cholinergic gene locus; vesicular docking and plasmalemma fusion processes, again including activating proteins; and actual, Ca^{2+}-mediated release of acetylcholine. Processes of vesicular cycling and recycling accompany these phenomena, as the vesicles move from the empty to the loaded form, constituting several pools of vesicles. In part at least, the so-called motor proteins that move along the cytoskeleton filaments propagate this movement; they contribute to muscle contraction and to vesicular movement (see also Whittaker, 1992, 1998; Sudhof, 2000; Howard, 2001). The conceptualization involved in formulating these processes and their components is flabbergasting; it still leaves unanswered questions. How do the various proteins link (we have at this time just barely a notion of the formation of protein complexes such as SNAREs that activate vesicular fusion)? What promotes and organizes temporally the cycling motion of the vesicles? What is the precise molecular and genetic control of the expression of these multiple processes? What determines the ratio between empty vesicles and the vesicles generated in the cholinergic neuron perikaryon? Which is the mechanism that moves the empty vesicles away from the terminal?

ACh release would become even more complex if, aside from the classical, the unorthodox processes also participated in the release. Only a most advanced computer program could describe this phenomenon (or could it?). I once suggested that only a very advanced computer program might be capable of describing another phenomenon, the causative transit from synaptic transmission via the multiple cholinergic and noncholinergic pathways to specific functional or behavioral events (Karczmar, 1993, 2004; see Chapter 9 BVI-BIV and V). Can a superprogram be devised to describe the combined phenomena?

It must be added that the cholinergic markers of the cholinergic neuron are not pertinent only for the transmittive function of this neuron, and this chapter's discussion does not necessarily imply that transmission constitutes the only role of the cholinergic neuron. Several nontransmittive roles were proposed, beginning in the late twentieth century. For example, it is well established that the cholinergic system and its components have a trophic role and a regenerative role, with regard to both the adult nervous system and development; when enacting this role, they are referred to as "morphogens." Then John Eccles and the McGeers proposed that the cholinergic system and its components exert a metabotropic role, as ACh (as well as certain other transmitters) triggers the neuronal membrane to precipitate second messenger–generated effects leading to metabolic changes in the neuron (McGeer et al., 1987b). These and related matters are discussed in detail in Chapters 8 BIV, 3 DIII, and 11.

DI. Central Cholinergic Pathways—An Introduction

The work of Kimura, the McGeers, Mesulam, Woolf, Kasa, Butcher,Wainer, Woolf, Wenk, and others expanded the vision of Shute and Lewis, Koelle and Gerebtzoff, and their associates and brought about something rarely heard of in the cholinergic field: an almost definitive statement concerning at least one area of that field, namely cholinergic pathways, even though there is a need to clean up discrepancies between the various investigators' pathway maps and to clarify the inconsistencies in nomenclature they used (see section A, above, and sections DI, DII, and DIII, below). The main consequence of this accomplishment is that, combined with lesion studies and pharmacological investigations of central functions and behaviors, this work established the cholinergic correlates of functions and behaviors, as well of certain disease states (see Chapters 9 BIV, BV, and BVI and 10 A; details concerning distribution of ChEs that are discussed in Chapter 3 DIII are also pertinent in the present context).

The cholinergic pathways were studied within the last 30 years by modern methods—which include CAT immunocytochemistry and histochemistry, various methods of measurements of AChE and of nicotinic and muscarinic receptors, and lesion and retrograde staining techniques (see section A, above, and Chapter 9 BIV–BVI)—in monkeys, apes, marmosets, raccoons, chickens, humans, rabbits, rodents, and cats (the rat brain was most particularly investigated). Generally, there is a remarkable similitude among the cholinergic neuronal groups and pathways of these species; thus, cholinergic neuronal clusters of the raccoon's forebrain are remarkably similar to those described for the rat and monkey by Mesulam and his team (Mesulam et al., 1983a, 1983b; Mesulam, 1976, 1990, 2003, 2004). Yet, species differences exist and will be pointed out.

It was already mentioned (see section A) that maps obtained in the past via Koelle's histochemical method for staining AChE are not as dependable for the identification of cholinergic neurons as the CAT staining method (for example, the Koelle method identifies both cholinergic and cholinoceptive cells; see section IIA). It was also mentioned that, nevertheless, maps provided decades ago by Shute and Lewis (1966, 1967a, 1967b) and Krnjevic and Silver (1966; see section IIA) essentially agree with the mapping obtained by means of the CAT stain provided more recently (Wilson, 1985; Kasa, 1986; Mesulam, 2000). For example, Pamela Wilson's (1985) maps of an AChE-containing tegmental pathway correspond closely to the McGeers' maps obtained by means of CAT immunocytochemistry visualizing the parabrachial system. Furthermore, some investigators who employed jointly in single studies methods for detection of both CAT and AChE obtained parallel results (Kasa and Silver, 1969; Kasa, 1971a, 1971b; Eckenstein and Sofroniew, 1983; Satoh et al., 1983).

The McGeers, Mesulam, Fibiger, Wenk, Butcher, Woolf, Wainer, and their associates described CAT immunocytochemistry–based maps of cholinergic pathways for many species. There is considerable overlap among these maps, but there are also many differences—in substance, in terminology, and in the mode of subdividing the cholinergic system, as will appear clearly in this section.[3] The CAT immunohistochemistry maps of Kimura and the McGeers were prepared first (see section A). These maps, together with amplifications and improvements provided by Wainer, Woolf, Butcher, and Fibiger, and comparisons with the maps prepared later by Mesulam and his associates, are presented first; the Mesulam maps are described subsequently.

Chronology is not the only reason for this two-punch mode of presentation. In view of the manifold differences among the various maps, it is well-nigh impossible to present a single pathway description that would smoothly amalgamate the work of all the pertinent teams; separate presentation of two main systems and their juxtaposition should present a clear picture of the cholinergic system as a whole.

DII. The Cholinergic Pathways Presented by the McGeers, Kimura, and Kasa and Expanded by Butcher, Woolf, and Wainer

Upon developing the immunohistochemical staining method for CAT, the McGeers and Kimura stressed that the cholinergic marking obtained by this method is reliable and leads to a clear-cut identification of cholinergic neurons, their pathways, and radiations (McGeer and

McGeer, 1989, 1993; McGeer et al., 1983, 1984a, 1984b, 1987a, 1987b; Kimura et al., 1980, 1981); this is particularly true when the CAT marking is combined with retrograde staining techniques and other means of cholinergic identification. On the basis of these methods, the McGeers identified a number of cholinergic pathways. They distinguished, for several species including humans, 5 major and several minor cholinergic pathways or systems (McGeer et al., 1984b, 1987a). Their major systems include the medial forebrain system, parabrachial complex, reticular formation and its giganto- and magnocellular fields, motor nuclei subserving the peripheral nerves, and striatal interneurons (Figures 2-5 and 2-6; see below).

The inconvenience here is that the nomenclatures used by the McGeers and the subsequent investigators in the description of the pathways differ.[4] It also complicates the matter that nomenclature for the pertinent nuclei—such as those of the brainstem—is frequently revised (see, for example, Reiner et al., 2004). The major systems recognized by Nancy Woolf, Larry Butcher, Hans Fibiger, and Bruce Wainer include the motor nuclei and the striatal interneurons, but they refer to McGeers' medial forebrain and its nucleus basalis of Meynert (NBM) as either magnocellular forebrain or basal forebrain, and to the McGeers' parabrachial complex and its pediculopontine and lateral tegmental nuclei as components of the brainstem and the spinal cord, namely pontomesencephalic tegmentum (Woolf, 1991; Butcher, 1995; Woolf and Butcher, 1986 and 1989; Butcher et al., 1993; Butcher and Woolf, 2003; Wainer et al., 1993; to include the tegmentum in the brainstem jointly with the spinal cord may not be felicitous). Also, Woolf and others distinguish, in addition to the McGeers' systems, the diencephalic complex, while the McGeers distinguish, outside of the Woolf-Butcher systems, the

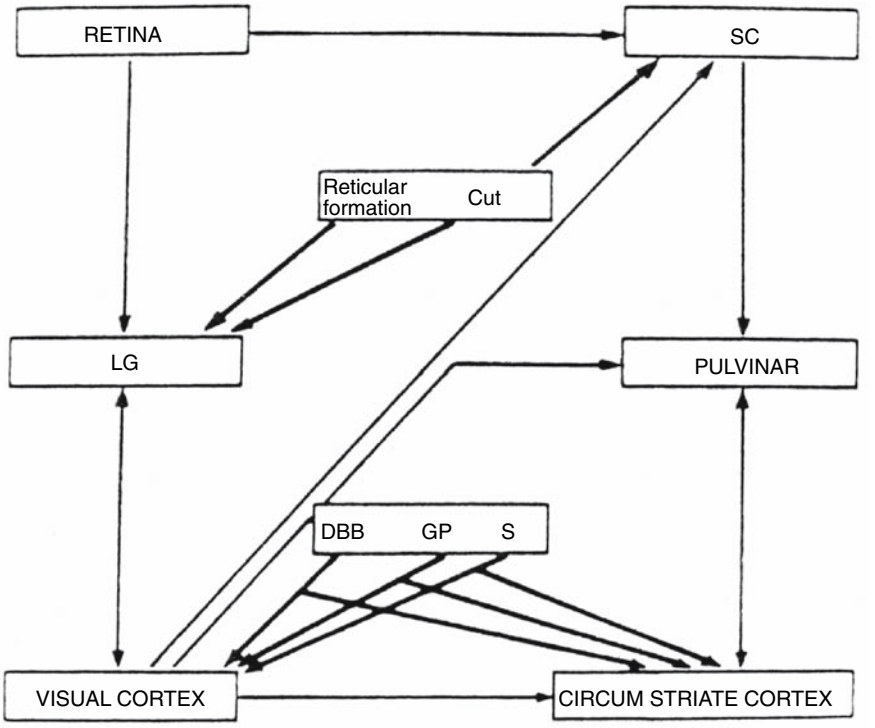

Figure 2-5. Diagram summarizing some of the connections between various parts of the visual pathways and visual cortical areas. The cholinergic routes are indicated by thick arrows. DBB, nucleus of the diagonal band (Broca); GP, golbus pallidus; LG, lateral geniculate nucles; S, stria terminalis; SC, superior colliculus. (From Kasa, 1986.)

reticular formation in their classification. Then Paul Kasa embraces still another approach (see Kasa, 1986 and Figure 2-5): he lists the major brain divisions and subdivisions (i.e., telencephalon, diencephalons, mesencephalon, etc., and their main subdivisions) and then describes the cholinergic projections to these locations. However, as described below, there are many similarities among the radiations described by the McGeers, Nancy Woolf, and Larry Butcher for the forebrain (or basal forebrain) and NBM.

1. The McGeers' Medial Forebrain System

The McGeers (see, for example, McGeer et al., 1987a) described the medial forebrain complex or system and its magnocellular component as consisting of a sheet of "giant" cholinergic cells; it "starts just anterior to the anterior commisure and extends in a caudolateral direction . . . to terminate . . . at . . . the caudal aspect of the lentiform nucleus." Divac described these projections in 1975, but not as cholinergic in nature. The system includes several nuclei; in a rostral to caudal order, they are: medial septum nucleus, nuclei of the vertical and horizontal limbs of the band of Broca, and the NBM. Woolf (1991) also includes substantia innominata and nucleus ansae lenticularis in this series. The system is essentially afferent in nature, and its descending radiation to the brainstem is mostly not cholinergic (Semba et al., 1989).

Nucleus basalis of Meynert is of primary importance as it projects to the frontal, occipital, parietal, and temporal cortical lobes, or the entire cortical mantle, as well as to the amygdala, the thalamus, and its reticular nuclei; the presence of the NBM radiations was stressed not only by the McGeers but also by Mesulam, Kasa, and the Woolf-Butcher team (Mesulam and Geula, 1988; Mesulam et al., 1983a, 1983b; Kasa, 1986; Wainer et al., 1993; see also Wenk, 1997; Woolf, 1991). The NBM is one of the earliest recognized brain structures, as it was identified (as *ganglion basale*) in 1872 by Meynert; it was described in detail by the Swiss biologist and neurologist Rudolf A. von Kolliker and named by him after Meynert. While it is well formed and extensive in several mammals including the dolphin and the human (Mesulam and Geula, 1988), it is frequently not listed specifically in neuroanatomy texts, except as a part of the ventral basic ganglia or substantia innominata (e.g., Brodal, 1981). It is absent in the rabbit, muskrat, and several other mammalian species; Gorry (1964) did not describe this site as cholinergic; he defined it morphologically as poorly organized and sparsely distributed within substantia innominata in the cat, dog, and rat. This notion was confirmed for the rat by investigators employing the CAT stain (see Wenk, 1997; Mesulam et al., 1983a; Mesulam and Geula, 1988); accordingly, Wenk (1997) referred to this structure in the rat as "homologous" to that of the nucleus basalis of, say, human; actually it is referred to frequently in the case of the rat as nucleus basalis magnocellularis (NBMC); other complications of the matter are discussed in section D2, below. Finally, in the zebra finch, the nucleus basalis has as its analog the ventral paleostriatum, which radiates in the bird to the forebrain and its song control nuclei; presumably, other bird species exhibit similar cholinergic systems (Li and Sakaguchi, 1997).

The cholinergic cells of the medial septum and vertical and horizontal limb of the forebrain's diagonal band are intensely projected to the limbic system and several cortical areas, including cingulate, pyriform, and entorhinal cortices. The McGeers, the Butcher-Woolf-Fibiger team, and other investigators (e.g., Wainer et al., 1993) emphasized the morphologic preponderance and behavioral significance of the diagonal band and projections from other regions of the forebrain to the limbic system, including the septum, amygdale, and hippocampus. The McGeers and others also described cholinergic diagonal band neuron projections to the olfactory bulb, the nucleus interpeduncularis, and the entorhinal and perirhinal cortex (Kasa, 1986; Dohanich and McEwen, 1986; Woolf and Butcher, 1989; Woolf, 1991; McGeer et al., 1987a, 1987b). The horizontal band extends, according to the McGeers (1987a, 1987b; see also Dohanich and McEwen, 1986), to the parietal, occipital, temporal, and frontal lobes, habenula, and amygdala; according to Woolf (1991), the horizontal band and magnocellular preoptic area extend efferently to, besides the nuclei and areas referred to by the McGeers (1987a), the olfactory bulb and pyriform nuclei, hypothalamus, tegmentum (peduculopontine and pediculopontine tegmental areas), raphe, and locus ceruleus. Some of

this radiation is frequently referred to as septohippocampal pathway (see Woolf, 1991, 1997, 1998; Butcher et al., 1993). Also, Woolf and Butcher used Mesulam's nomenclature when they referred to the neurons of the medial septum and the vertical limb of the diagonal band (which also contribute to the septohippocampal radiation) as Ch1 and Ch2 neurons (see the next section). While the preponderance—or at least a good proportion—of the cholinergic cells of the septum and the diagonal band, as well as of the cholinergic cells of NMBC, send ascending projections to the cortex and the limbic system, only a small proportion of the cells of these systems that send descending projection to the brainstem are cholinergic (Semba et al., 1989).

As referred to above, the McGeers and the Woolf-Butcher team have described cholinergic forebrain radiations to the olfactory bulb and to the entorhinal cortex (see also Halasz and Shepherd, 1983). While such radiations imply the existence of a cholinergic olfactory system, the two teams do not explicitly propose the presence of such a system; it is, however, proposed by Kasa (1986). Kasa stresses that CAT and AChE activity is present in several layers of the olfactory bulb; CAT levels are particularly high in the glomerular and internal plexiform layers. Besides the forebrain, this afferent activity originates in the olfactory tubercle and, perhaps, in the islands of Calleja (Shute and Lewis, 1967a, 1967b). On the other hand, it is not clear from the evidence presented by the McGeers, the Woolf-Butcher team, and Kasa whether or not cholinergic neurons contribute to the efferent outflow from the olfactory tubercle to the olfactory cortex (including enthorhinal and pyriform cortices); a nucleus basalis–olfactory pathway is, however, defined by Mesulam and his team (see Selden et al., 1998, and below, section D2).

It was already mentioned that the McGeers and their associates and the Woolf-Butcher team use the terms "medial forebrain complex" and "basal forebrain" (the "magnocellular forebrain"), respectively, for systems that are relatively similar but differ somewhat with respect to the nuclei or groups of origin and their radiations' targets. The nuclei and the radiations of the Woolf-Butcher system are somewhat richer than those proposed by the McGeers, and in her 1991 review Nancy Woolf describes in detail both efferents and afferents of the 7 nuclei that she recognizes in the case of her "basal forebrain" (see also Butcher and Woolf, 2003). Similarly, the Woolf-Butcher description of the functional significance of their system ranges more widely than the description offered by the McGeers for their complex. However, the McGeers, just as other investigators, assign major importance for loss of cognition and Alzheimer's disease to the loss of neurons and of the cholinergic systems in the "medial basal forebrain" and the nucleus basalis (see, e.g., McGeer et al., 1987a, 1987b). Indeed, Nancy Woolf (1997, 1998) assigns a major role for cognition and consciousness and self-awareness (see Chapter 9 BVI) to the basal forebrain and the reticular formation and its radiation to the cerebral cortex modules.

2. The McGeers' Parabrachial Complex

The McGeers and associates recognized the parabrachial complex as the second major system and the "most intense and concentrated cholinergic cell group in the brain stem." It surrounds conjunctivum commencing in the most rostral aspects of the pons and radiates along the brachium conjunctivum and superior cerebellar peduncles in the caudodorsal direction. The pathway contains several nuclei such as the pedunculopontine tegmental nucleus, medial and lateral parabrachial nuclei, and Kolliker-Fuse nuclei (occasionally there are disagreements between the McGeers and Mesulam's team [Mesulam et al., 1984] regarding the presence of CAT in some of the nuclei of the McGeers' parabrachial complex; see below, next section). According to the McGeers and other 1980s investigators, including Bruce Wainer and his associates (Saper and Loewy, 1982; Lee et al., 1988), the pedunculotegmental-parabrachial system ascends to all thalamic nuclei (including anterior nuclear, reticular nuclear, and posterior nuclear areas), the substantia nigra, and the cortex; Saper and Loewy (1982) also describe projections of this system to the hypothalamus and amygdala (see also Kasa, 1986). In 1987 (McGeer et al., 1987a), the McGeers opined, "this region is a major supplier of afferents to all parts of the diencephalon . . . including hypothalamus . . . and the limbic system and possibly to the cortex." It should be noted that the McGeers' term "parabra-

chial system" corresponds to an extent—but not entirely—to Nancy Woolf's term "pontomesencephalic formation"; this matter is discussed further below. Subsequently, Edith McGeer (Semba et al., 1989) stressed the importance of brainstem (pedunculopontine tegmental and dorsal raphe nuclei) in projecting to the cortex and receiving afferents from the magnocellular regions of the basal forebrain. Nancy Woolf and her associates (see, e.g., Woolf, 1991) add tectum and basal forebrain as destinations of the tegmental nuclei described by the McGeers, and this is an important addition to the McGeers' complex.

Nancy Woolf and Larry Butcher (see, e.g., Woolf, 1991; Woolf and Butcher, 1986; Butcher and Woolf, 2003; and Woolf et al., 1986) depart from the McGeers in not employing the term "parabrachial complex"; they instead localize a somewhat similar complex in the brainstem and refer to it as "pontomesencephalic tegmentum"; they emphasize the presence within this site of laterodorsal tegmental and pedunculopontine nuclei. Palkovits and Jacobowitz (1974) and Hoover and Jacobowitz (1979) include these neurons within the cuneiform nucleus. Butcher, Woolf, and others (Woolf and Butcher, 1986, 1989; Woolf, 1991) describe its efferent (ascending) radiations, which include, in addition to those enumerated by the McGeers, subthalamus, habenula, striatal areas and basal ganglia, reticular formation, medium septum, lateral geniculate, and stria terminalis. Woolf and other investigators opine that the descending projections of the pontomesencephalic tegmentum also reach the cranial nuclei, including the trigeminal, facial, and hypoglossal motor nuclei, vestibular nuclei, raphe, locus ceruleus, and pontine and medullary and pontine reticular nuclei (Jones and Yang, 1985; for further references, see Kasa, 1986; Woolf, 1991; Butcher and Woolf, 1986; 1989). Nancy Woolf (1991, 1996, 1997, 1998) emphasizes the role of the radiation from the pontomesencephalic tegmental system (the McGeers' parabrachial complex) to the basal forebrain, relaying thence to the cerebral cortex for processes of memory and consciousness; it is interesting that she states that this notion "replaces the older notion of a nonstop pathway originating in the cholinergic reticular formation" or system (Shute and Lewis, 1967a, 1967b). This matter is addressed again in the next section.

3. The McGeers' Reticular System

The McGeers' third major system, the reticular system, is "a scattered collection of very large cells," the giganto- and magnocellular neurons (McGeer et al., 1987). These cells aggregate medially with respect to the raphe and ventrally with respect to the inferior olive; longitudinally, they extend from the rostral pons to the caudal medulla. They contain nuclei reticularis pontis oralis and caudalis, reticularis tegementis pontis, reticularis gigantocelluralis and reticularis lateralis, as well as the formatio reticularis centralis (or medularis) and cuneiform nucleus. Particularly the hypoglossal and gigantocelluralis nuclei contain high levels of CAT and of ACh located in the soma and nerve terminals (Kimura et al., 1981), perhaps indicating the presence of cholinergic-cholinergic relays (see Woolf and Butcher, 1986). The system's neurons radiate to the thalamus and other rostral nuclei, cerebellum, superior colliculus, and spinal cord (see Kasa, 1986). Again, there is a substantial difference between this system as described by the McGeers and the same system described by others. While Woolf (1991) refers to the medicular reticular nuclei and the medullary tegmentum as the source of the medullary reticular radiation, she ascribes only afferent projections to this source and defines them as coursing to the cerebellar cortex; she adds as the medullary origin of this reticular outflow the prepositus hypoglossal nucleus. Mesulam and Jacobowitz include nucleus cuneiformis with the pedunculopontine nuclei, and they refer to these nuclei as the pontomesencephalic reticular formation, or the Sector Ch5 cluster (see next section), which the McGeers list under their parabrachial system (Mesulam et al., 1983a, 1983b; Mesulam, 1990; Palkovits and Jacobowitz, 1974; Jacobowitz and Palkovits, 1974).

Apparently, the McGeers' account of the reticular system and pontine tegmentum does not jibe with the original description of the reticular system and its identification with the ARAS by Shute and Lewis, Himwich and Rinaldi, and Krnjevic (see section A, above). But Woolf, Butcher, and Wainer emphasize the cortical radiations of the components of their parabrachial complex (tegmental nuclei) and assign to them a role in arousal, memory, and the sleep-wakefulness cycle; that is,

they synonymize this system with the classical ARAS (Woolf and Butcher, 1986, 1989; Woolf, 1991).

4. The McGeers' Striatal Interneurons

Striatal cholinergic interneurons constitute the fourth system of the McGeers; they identified it very early (McGeer et al., 1971). These neurons are present in the caudate, putamen, and accumbens; following their cholinergic identification by McGeer et al. (1971), they were recognized as such ("intrinsically-organized local circuits") by Woolf (1991), Kasa (1986), and Mesulam (1984). But, efferents also radiate from the striate and the basal ganglia, including substantia innominata and globus pallidum, to superior colliculus, cortex, and, possibly, substantia nigra (for references, see Kasa, 1986). The basal ganglia constitute a problem. As the rest of the striate, some components (e.g., substantia nigra) may or may not contain cholinergic interneurons (see Kasa, 1986). However, the literature agrees on the presence of CAT and AChE in the substantia nigra, but it is not clear whether the CAT stain represents afferents to this site, such as the postulated striatonigral path, or innervation radiating from peduculopontine tegmentum and nucleus accumbens (Kasa, 1986; McGeer et al., 1987a).

5. The McGeers' Motor Nuclei

The motor nuclei of the peripheral nerves are the McGeers' fifth system. These include cranial nerve nuclei, which are the source of the efferent nerves to the autonomic system, motor nuclei innervating the facial muscle, and motor counterparts in the anterior and lateral horns of the spinal cord. This is in agreement with the work of Kasa (1986), Woolf (1991), and others; similar to the McGeers, Woolf, Butcher, and their associates distinguish the cholinergic motor neurons as a separate component, which is a part of their brainstem and spinal cord system. The spinal cord should be mentioned in this context; it was not reviewed by the McGeers or the Butcher-Woolf team. AChE and CAT stain is obtained in the motor neurons, as referred to above, as well as in other parts of the ventral horn (see also Kasa, 1986; Dun et al., 2001); according to Kasa (1986) and Barber et al. (1984), cholinergic neurons may be also present in the dorsal and intermediate spinal cord, and the muscarinic nature of the dorsal horn receptors (of the M2, M3, and M4 type) was demonstrated by Zhang et al. (2005; see, however, Dun et al., 2001). The cholinergic innervation of these sites may originate in the nucleus ruber and from the reticular system of the McGeers, particularly the hypoglossal and magnocellular nuclei. Other descending cholinergic pathways originating in the hypothalamus and the brainstem (parabrachial system of the McGeers) may contribute to the spinal innervation (Loewy, 1990).

6. The Other (Sometimes Minor) Systems

The McGeers and others also recognize several additional cholinergic systems. For example, cholinergic interneurons are present in the cortex, striate, hypothalamus, interpeduncular nuclei, and hippocampus (Tago et al., 1987); however, the McGeers did not describe any cholinergic radiations emanating from the cholinergic diencephalic (hypothalamic) neurons. The intrinsic nature of some of the cortical and striatal cholinergic neurons was also evidenced in the studies of Butcher and Woolf (e.g. Butcher et al., 1975; Butcher and Woolf, 2004), Bolam and Wainer (e.g. Bolam et al., 1984), and Kasa (1971 and 1986); a variety of techniques including CAT and AChE staining were employed. Interpeduncular nucleus may contain the highest content of CAT, even higher that that of putamen and caudate (Kasa, 1986); this may be mostly derived from habenular and other cholinergic afferents abutting on the nucleus interpeduncularis. There also may be intrinsic cholinergic cells in the nucleus ruber, as well as cholinergic efferents descending spinally from this nucleus (rubrospinal cholinergic tract (see Kasa, 1986; Woolf, 1991). The axons and the dendrites of the intrinsic cortical cholinergic interneurons "elaborately intertwine and interlap" (Kasa, 1986), which is in agreement with the notions of Woolf and her functional interpretations of the role of the cholinergic system in cognition (1991, 1997; see Chapter 9 BV and BVI).

The cerebellum is another, probably minor locus of the cholinergic system that is rarely men-

tioned in the Woolf-Butcher or McGeers studies (see, however, Kasa, 1986). But CAT and AChE are present in the Golgi cells, intracerebral nuclei, and granular and molecular layers; they are also present in the mossy fibers and in the terminals of mossy and parallel fibers, as well as other cerebellar terminals (for literature, see Kasa, 1986). The source of this CAT and AChE is not known. Shute and Lewis (1967a, 1967b) opined on the basis of their AChE stain that cholinergic afferents reach the cerebellum via the 3 cerebellar peduncles. Furthermore, cholinergic receptors, notably of several nicotinic types, are present in all lobules of the cerebellar cortex, although their density may be small (De Filippi et al., 2005). Cholinergic origins of the afferents are not clear, although the red nucleus and the spinal afferent pathways may serve as such. Furthermore, cerebellar cortex neurons seem to exhibit cholinoceptivity (for references, see Kasa, 1986). Altogether, the cerebellum seems to have a cholinergic system that is ready to be defined.

Cholinergic cells are also found in the retina (amacrine cells) and other components of the visual system (Domino, 1973; Domino, et al., 1973). CAT activity is high in amacrine cells and the geniculate, lateral reticular nucleus, superior olivary complex, magnocellular nucleus, vestibular nuclei, inner ear spiral ganglion, and several auditory system components (see McGeer et al., 1987; Kasa, 1986a, 1986b; Woolf, 1991.)

Cholinergic contributions to visual and auditory systems require additional comments. Several components of the visual system (i.e., retina, optic nerve, lateral geniculate, superior colliculus, and visual cortex) contain CAT and AChE (Rasmusson, 1993; see also Chapter 9 BV and Kasa, 1986). Though there is ample evidence that retinal neurons send cholinergic projections to the geniculate, there seems to be no direct radiation from the geniculate or colliculus to the visual cortex (see Figure 19 in Woolf, 1991). Furthermore, forebrain areas, including the substantia innominata, corpus pallidus, and diagonal band, innervate several laminae of the visual cortex and also exhibit high AChE concentrations (Bear et al., 1985; see Kasa, 1986, and Woolf, 1991, for further references). The geniculate and superior colliculus are also innervated by the dorsal tegmentum and the reticular formation or the parabigeminal nucleus (see above, and Woolf, 1991; Kasa, 1986; Mesulam, 1990, McGeer et al., 1987a, 1987b). However, constructing a full diagram of the cholinergic visual system, though partial and speculative constructions have been offered, is impossible (Figure 2-5).

The auditory cholinergic system is infrequently described, yet cholinergic contribution to this system is undeniable (see Kasa, 1986). The denervation method and using CAT and AChE markers demonstrate a cholinergic presence in the superior olive, organ of Corti hair cells (or their base), spiral ganglion, and cochlea. Altogether, the generally accepted auditory pathway is the olivocochlear bundle (see also Chapter 9 BV). However, CAT and/or AChE are present in several auditory system components (i.e., the geniculate, inferior colliculus, and nucleus cuneiformis), and Kasa (1986) diagrammatized a cholinergic auditory pathway that unites the auditory apparatus with the auditory cortex via the nuclei in question.

Similar to the McGeers' "major" systems or complexes, the "minor" systems exhibit afferent terminals originating in many brain parts. For example, the interpeduncular nucleus is probably innervated by the basal forebrain, parabrachial (pontomesencephalic), and septum. All these findings agree with the demonstration of ACh release and the presence of cholinergic (muscarinic or nicotinic) receptors in these loci (for references, see Kasa, 1986; McGeer et al., 1987a, 1987b; Woolf, 1991.)

7. Comparing and Commenting on the McGeer and Woolf-Butcher Pathway Maps

The McGeer and the Woolf-Butcher pathway maps may be compared in several ways (see Figure 2-6). Butcher, Woolf, and their associates and the McGeers use the same terms and replicate to a great extent the McGeers' descriptions of certain pathways described by the McGeers. This is the case with the motor nuclei and the striatum, except that Butcher and Woolf (see Woolf, 1991; Butcher, 1995) add to the McGeers' striatum the islands of Calleja (of the olfactory tubercle) and the olfactory tubercle (anterior perforated substance). The cholinergic neurons of the islands may supply the olfactory band (Kasa, 1986). Also, Butcher and Woolf classify their striatum,

somewhat in the developmental sense, under telencephalon. Then they employ different nomenclature but define similar—but not identical—morphology as they describe the pontomesencephalic tegmentum of the brainstem and the spinal cord in lieu of the McGeers' parabrachial complex, and they refer to the basal forebrain rather than to the medial forebrain systems of the McGeers.

Finally, Butcher and Woolf adduce additional systems to those of the McGeers, such as the diencephalon complex with its hypothalamic nuclei and medial habenula (similar to Kasa, 1986). According to Woolf, the hypothalamic nuclei radiate to the cortex, while according to Kasa (1986), the habenula, a thalamic component, may radiate to the interpeduncular nucleus. Actually, several investigators assert that cholinergic cells are present in all parts of the hypothalamic nuclei (for references, see Woolf, 1991). These cells are frequently interneurons and form intrinsically organized local circuits. They form also the tuberoinfundibular pathway involved in the anterior pituitary hormone release; this classical pathway was discovered on pharmacological grounds by Mary Pickford and defined as cholinergic by George Koelle on the basis of his histochemical AChE stain (see Chapter 9 BIV-2); this notion was confirmed on the basis of CAT immunocytochemistry by Pat McGeer and his team (Tago et al., 1987).

Several general comments are appropriate. Comparing the pertinent figures and tables presented by the McGeers (McGeer et al., 1987a, 1987b; Semba et al., 1989), on the one hand, and Woolf with Butcher and Wainer, on the other (Woolf and Butcher, 1986, 1989; Wainer et al., 1993; see Figures 2-6 and 2-7), it is apparent that the cholinergic radiations depicted by Woolf and their associates are more ubiquitous and farreaching than those described by the McGeers; on the other hand, they correspond rather closely to those included in Mesulam's system (see next section).

Then there is the matter of targets and their afferents and efferents. Described by the McGeers or Woolf and Butcher, the afferent pathways supply cholinergic radiation to, and release ACh at, cholinergic or noncholinergic but cholinoceptive targets. Such pathways constitute cholinergic-noncholinergic or cholinergic-cholinergic relays similar to those existing in the autonomic sympathetic and parasympathetic system (see also Chapter 9 BI; see Figure 9-8). Moreover, the neuronal groups described by either the McGeers or the Woolf-Butcher team as sending afferents to cholinergic or noncholinergic targets generally also receive afferents from these targets. Altogether, the McGeers (Semba et al., 1989) and the Woolf-Butcher team (see Woolf, 1991; see also Kasa, 1986) describe mutual, multiple, and ubiquitous connections among all the systems or pathways that they describe, including the cholinergic hypothalamic and habenular (thalamic) neurons, pontomesencephalic complex (McGeers' parabrachial system), forebrain (or basal forebrain, including diagonal band nucleus), reticular system, and cortex. It is somewhat misleading that these and other investigators also mention efferent pathways. This is the case of the "efferents" of the motoneurons and autonomic preganglionic neurons, which radiate to the skeletal muscle and the ganglia, respectively, while the McGeers state that the brainstem parabrachial system (pedunculopontine tegmental nuclei) receives afferents from the magnocellular regions of their basal forebrain complex, which projects efferents to the parabrachial system (Semba et al., 1989); in either case, these "efferents" are, at the same time, afferents of the neurons in question.

Nancy Woolf (1991, 1997, 1998) adduces important generalizations to her and Larry Butcher's work. She emphasizes that the cholinergic system exhibits rich "interdigitation, interconnection" via the cholinergic dendrites and axon collaterals "and re-entrant circuits." Indeed, in her 1991 review she describes in detail not only radiations of her cholinergic systems of origins (including the basal brain and the brainstem) to ubiquitous target nuclei and cell groups, but also the afferents from noncholinergic and cholinergic complexes to these groups of origin. These hallmarks—interdigitations, reentrant circuits, and interconnections—of the cholinergic system contribute to its global character, and Nancy Woolf contrasts this global arrangement of the cholinergic system with the modular arrangement of the sensory and cortical circuits. She also attaches special importance to the innervation by individual cholinergic forebrain cells of the functional cortical units termed "macrocolumns" as she relates this feature to the phenomena of memory, learning, and consciousness (see Chapter 9 BV

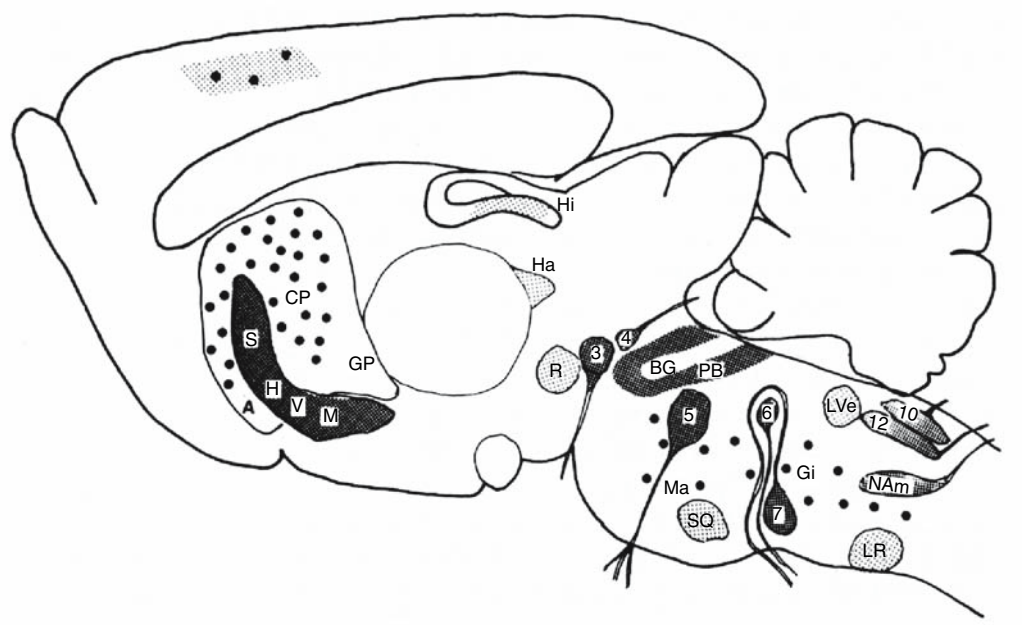

Figure 2-6. Sagittal view of rat brain illustrating ChAT-containing neurons. Major systems are indicated by black dots or heavy stippling, minor ones by light stippling. A, nucleus accumbens; Am, amygdala; BC, brachium conjectivum; CP, caudate-putamen; Gi, gigantocellular division of the reticular formation; GP, globus pallidus; H, Horizontal limb of diagonal band; Ha, habenula; Hi, hippocampus; IC, inferior colliculus; IP, interpenduncular nucleus; LR, lateral reticular nucleus; LVe, lateral vestibular nucleus; M, nucleus basalis of Maynert; Ma, magnocellular division of the reticular formation; NAm, nucleus ambiguous; PB, parabrachial complex; R, red nucleus; S, medial septum; SN, substantia nigra; SO, superior olive; V, vertical limb of diagonal band. (From McGeer et al., 1985. Reprinted by permission from Kluwer Academic/Plenum Publishers.)

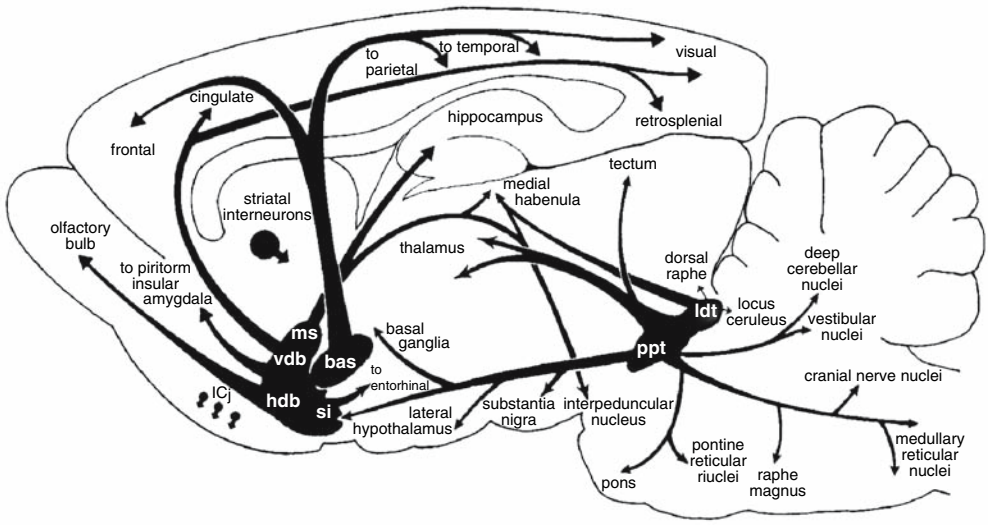

Figure 2-7. Cholinergic neurons in the basal forebrain and pontomesencephalon have widespread projections. Cholinergic neurons in the basal forebrain, including those in the medial septal nucleus (ms), vertical diagonal band nucleus (vdb), horizontal diagonal band nucleus (hdb), substantia innominata (si), and nucleus basalis (bas), project to the entire cerebral cortex, hippocampus, and amygdala. Cholinergic neurons in the pontomesencephalon include those in the pedunculopontine nucleus (ppt) and laterodorsal tegmental nucleus (ldt) and have ascending projections to the basal forebrain and thalamus. (From Woolf, 1997.)

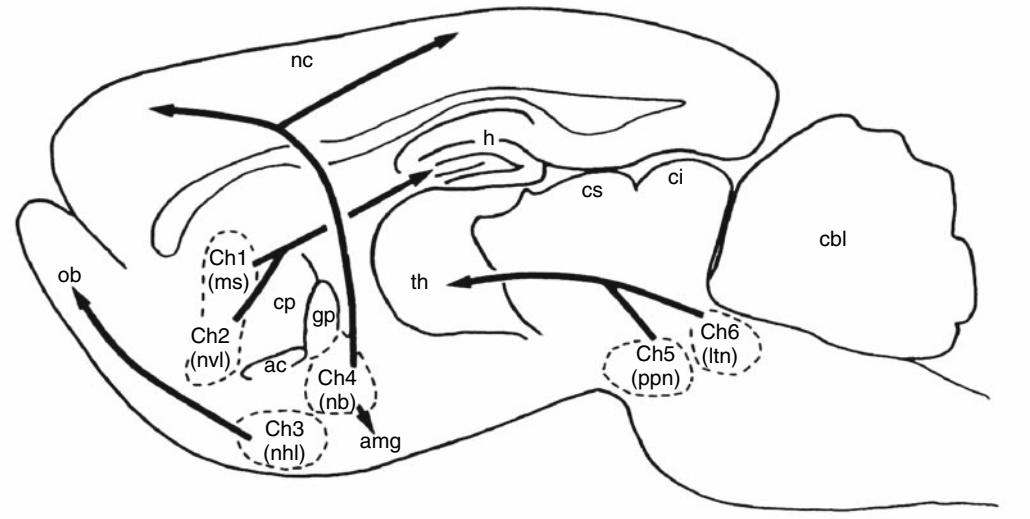

Figure 2-8. Schematic representation of some ascending cholinergic pathways. The traditional nuclear groups, which most closely correspond to the Ch subdivisions, are indicated in parentheses. However, the correspondence is not absolute. ac, anterior commissure; amg, amygdala; cbl, cerebellum; ci, inferior colliculus; cp, caudate-putamen complex; cs, superior colliculus; gp, globus pallidus; h, hippocampus; ltn, lateral dorsal tegmental nucleus; ms, medial septum; nb, nucleus basalis; nc, neocortex; nhl, horizontal limb nucleus; nvl, vertical limb nucleus; ob, olfactory bulb; ppn, pedunculopontine nucleus; th, thalamus. (From Mesulam et al., 1983a.)

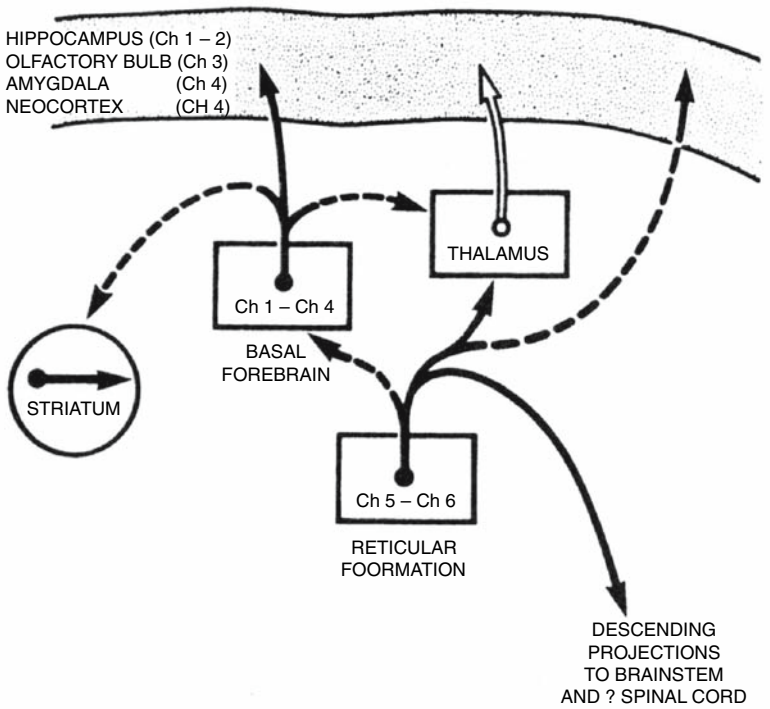

Figure 2-9. Diagrammatic representation of some cholinergic pathways. The solid arrows indicate major pathways and the broken arrows minor pathways. The open circle and arrow indicate that the thalamocortical pathway is noncholinergic. (From Mesulam, 1990. Reprinted by courtesy of Marsel Mesulam.)

and BVI and Figure 9-35 and 9-36). Also, the cholinergic neurons receive complete sets of somatosensory and proprioceptive information, and this juxtaposition prevents the neurons "ordinarily from being intensely driven by any individual input." Interestingly and perhaps inconsistently, Woolf also opines that "the bombardment of multiple weak inputs and the re-entrant circulation of activation . . . makes the cholinergic systems unstable . . . and . . . chaotic," but she feels also that this characteristic makes the system "exquisitely sensitive . . . to variations in initial input."

DIII. Cholinergic Pathways as Defined by Marsel Mesulam, His Associates, and Other Recent Investigators

Marsel Mesulam became involved in cholinergic pathways early, as in 1976 he had already embarked on the marking of central cholinergic afferents by the retrograde horseradish peroxidase method that he developed, combined with AChE cytochemistry. Subsequently, he begun to employ CAT immunocytochemistry as well, and also the classical lesion method, to accentuate somatic CAT visualization; moreover, he used DFP pretreatment to cause a hyperexpression of AChE in the perikarya (Butcher and Bilezikjian, 1975). Altogether, for more than 25 years he has been engaged in defining maps of the cholinergic system in rodents and primates, including humans.

Mesulam contributed new findings and much detail to the definition of cholinergic pathways; he also provided the cholinergikers with a useful subdivision of the cholinergic system into 8 "major sectors" (or "constellations," as sometimes referred to by Mesulam), which he labeled Ch1 to Ch8 (Mesulam et al., 1983a, 1983b; Mesulam, 1990). Mesulam claims that "the nomenclature for the nuclei that contain . . . cholinergic cells has engendered considerable inconsistency and confusion" (a sentiment expressed here by this author as well), and that his terminology eliminates the inconsistency (Mesulam et al., 1983a). The additional advantage of Mesulam's categorizing is that, as the sectors refer to circumscribed sites rather than brain parts (e.g., medial septum versus the McGeers' forebrain) their projections are also relatively limited and easy to define. Actually, only a few investigators appear to use Mesulam's terminology, although many refer to the nuclei listed within Mesulam's sectors without using his nomenclature (e.g., Jones and Cuello, 1989).

It is important that the Ch1 to Ch8 sectors are present as relatively homologous entities in rodents, cats, primates, and humans (Mesulam et al., 1983a, 1983b, 1984; Mesulam, 1990; Selden et al., 1998; Mesulam, 2003, 2004). There are, of course, species differences with respect to Mesulam's maps; some investigators opine that Mesulam's system applies more to primates than to rodents (for example, raccoon; Bruckner et al., 1992; see also Butcher and Semba, 1989), and Mesulam himself states that there are "potential difficulties for the Ch nomenclature," particularly for the Ch1 to Ch4 sectors, and he refers to species differences between the sectors. However, according to many investigators, the discrepancies appear small (see Vincent and Reiner, 1987, for references).

It is notable that the neurons of the various sectors differ in size, shape, or chromicity (coloring). It is also important that the sectors are, without exception, heterogeneous; that is, both cholinergic and noncholinergic neurons are present in the sectors; the ratio of cholinergic versus noncholinergic neurons differs among the sectors. Finally, the perikarya of the Ch1 to Ch8 neurons as well as the nerve terminals of cholinergic or noncholinergic radiations to these neurons contain AChE.

Four important sectors, Ch1 to Ch4, are present in the basal forebrain (Mesulam et al., 1983a, 1983b). Ch1 neurons are contained in the medial septal nucleus, particularly along the midline raphe and the outer edge of the septum. Mesulam et al. (1983a, 1983b) state that about 80% of Ch1 neurons are noncholinergic; the opinion of Mesulam and his team that the cholinergic neurons constitute a small minority of the Ch1's neurons is not shared by other teams (see, for example, Senut et al., 1989). Ch1's main outflow is the hippocampus, and this description agrees with that of the McGeers, Kasa, the Butcher-Woolf team, and Senut et al. (1989).

The boundaries between the Ch1 and Ch2 sectors are not well defined and, in Mesulam's diagrams, the Ch1 and Ch2 sectors form a

bean-shaped continuum (see Table 2-1 and Figures 2-8 and 2-9). The Ch2 sector consists of neurons located within the vertical limb of the diagonal band; 70% or more of Ch2's neurons are cholinergic. The cholinergic and noncholinergic neurons of the diagonal band form distinct clusters. According to Mesulam and his associates (1983a, 1983b), Ch2 neurons project to the hippocampus—similar to the Ch1 neurons—as well as to the hypothalamus and occipital cortex. The McGeer and Woolf-Butcher teams add to these projections of the vertical diagonal band projections to several cortices and to the interpeduncular nucleus, and their description agrees with that of Senut et al. (1989).

The Ch3 sector comprises neurons of the horizontal limb of the diagonal band. Mesulam states that he does not differentiate the horizontal limb from the preoptic magnocellular nucleus or area; he also opines, jointly with the Bigl-Butcher-Woolf team, that there may be an overlap between the Ch3 and Ch4 sectors (see Bigl et al., 1982). Depending on the site within this nucleus, the frequency of cholinergic cells varies between 25% and 75%. The main outflow of the Ch3 neurons is, according to Mesulam, the olfactory bulb; there is a general consensus between Mesulam and the Woolf-Butcher team as to this generalization. As the Ch3 and Ch4 sectors appear to overlap, the radiations from the overlap area extend to several neocortical areas. It may be added that the Woolf-Butcher team identifies the preoptic magnocellular nucleus as a separate area, and they describe the radiations from this nucleus as extending, similar to the Ch3 to Ch4 overlap area, to several neocortices.

Marsel Mesulam described the Ch4 sector of the monkey as "providing the major source of cholinergic projections to the cortical mantle" (Mesulam et al., 1983a, 1983b). Subsequently, Mesulam defined it as nucleus basalis magnocellularis and considered it homologous with the nucleus basalis of the rat (e.g., Mesulam and Geula, 1988). However, Shute and Lewis (1967a, 1967b), Jacobowitz and Palkovits (1974), and Emson et al. (1979) considered the nucleus basalis to be a part of the entopeduncular nucleus and globus pallidus, and Mesulam et al. (1983a, 1983b) suggest that neurons listed within substantia innominata and the preoptic magnocellular nucleus "should probably" also be considered parts of the nucleus basalis and of the Ch4 sector.

In monkeys and rats, about 90% of the neurons of nucleus basalis are cholinergic. However, Shute and Lewis (1967a, 1967b), Jacobowitz and

Table 2-1. Nomenclature for Cholinergic Projections of the Basal Forebrain and Upper Brainstem in the Rat

Cholinergic Cell Groups	Traditional Nomenclature for the Nuclei That Contain the Cholinergic Neurons	Major Source of Cholinergic Innervation for
Ch1	Medial septal nucleus	Hippocampus
Ch2	Vertical limb nucleus of the diagonal band	Hippocampus
Ch3	Lateral part of the horizontal limb nucleus of the diagonal band	
Ch4	Nucleus basalis of Meynert, globus pallidus	
Ch5	Substantia innominata	
	Nucleus of the ansa lenticulars, neurons lateral to the vertical limb nucleus and those on the medial parts of the horizontal limb nucleus of the diagonal band	
Ch6	(including parts of the preoptic magnocellular nucleus)	
	Nucleus pedunculopontinus, neurons within the parabrachial area	
	Laterodorsal tegmental nucleus	

See Woolf, 1997, for further details.

Palkovits (1974) and Emson et al. (1979) considered the nucleus basalis as being a part of the entopeduncular nucleus and globus pallidus (as defined by Mesulam and his team). Mesulam and his team opine also that Ch4 sector is the principal source of cholinergic projections to the neocortex and that it extends to the amygdala (Mesulam et al., 1983a, 1983b; Mesulam and Geula, 1988; Mesulam, 1990; Selden et al., 1998).

Mesulam and his team (e.g., Selden et al., 1998) distinguish two "discrete, organized" pathways, medial and lateral, that originate in the Ch4 sector (the lateral pathway is further subdivided by Mesulam into two "divisions"). Mesulam emphasizes that these pathways contain AChE, CAT, and nerve growth factor, but little NAPDH activity, while NAPDH is present in the Ch5 and Ch6 neurons (Selden et al., 1998).

The medial Ch4 pathway supplies the cingulate, parolfactory, percingulate, and retrospinal cortices, and it merges with the lateral pathway within the occipital lobe. The lateral pathway projects to frontal, parietal, temporal, and occipital cortices. The Ch4 sector (or nucleus basalis) increases in size and differentiation in the course of vertebrate evolution, culminating with its status in the human (Gorry, 1964); in fact, Mesulam (1990, 1998) distinguishes 4 "divisions" of the human Ch4, rather than the 2 "divisions" he differentiates in the rat and monkey. The notion that Ch4 (i.e., the nucleus basalis, including substantia innominata) is a major neocortex supplier is shared by the McGeers, the Woolf-Butcher team, Kasa, and Mesulam. These and other investigators also agree that Ch4 is important for memory, learning, and, more generally, cognitive function, as well as for cognitive disorders, aging, and Alzheimer's disease.

Sectors Ch5 and Ch6 are present in the human and animal reticular formation (Mesulam et al., 1989). Contrary to the forebrain sectors, these constellations exhibit high NAPDD activity levels. On the other hand, Ch4 neurons and other forebrain sectors are rich in nerve growth factor (NGF) protein, while the Ch5 and Ch6 sectors are not (Mesulam et al., 1989). How does this finding jibe with the information that Ch4 neurons are much more affected in Alzheimer's disease than the Ch5 and Ch6 neurons (Zweig et al., 1987)?

The Ch5 sector is localized in the pontomesencephalic reticular formation that comprises neurons within the pedunculopontine nucleus; some of the Ch5 neurons also extend into the cuneiform and parabrachial nuclei, as well central tegmental tract and other adjacent sites. As are other sectors, Ch5 is heterogeneous, as it contains noncholinergic neurons as well. The Ch5 sector radiates to the thalamus and its several nuclei, including anterior, lateral, and reticular nuclei, and it sends a minor projection to the neocortex. Mesulam emphasizes that the Ch5 (and Ch6) radiations correspond to the reticulothalamic pathway of Shute and Lewis (1967a, 1967b) and to the ascending reticular activating system of Moruzzi and Magoun (1949; see above, section A1–A3, and Chapter 9 BIV-3). Mesulam and his team (see Mesulam et al., 1983a, 1983b; Mesulam, 1990) do not exclude the possibility that the Ch5 sector also sends weak projections to the habenula and hypothalamus, to the olfactory bulb, and to the spinal cord and the brainstem.

Radiations from Ch5 and particularly the pedunculopontine nucleus are similar to the radiations of this nucleus described by the McGeers and the Woolf-Butcher team, although additional projections are described for the Mesualm system, or complex. However, there are divergences between the systems proposed by the McGeers and Mesulam. Thus, Mesulam's sector Ch5 corresponds to the parabrachial radiation of the McGeers rather than to their reticular system, and his adjudication to the Ch5 sector of the parabrachial and cuneiform nuclei of the pontomesencephalic reticular formation (with its pedunculopontine nuclei) relates the Ch 5 sector the Woolf-Butcher team's pontomesencephalic tegmentum (see above, section DII).

The Ch6 sector neurons are localized in the laterodorsal tegmental nucleus, which is confined within the periventricular gray. According to Mesulam, the Ch6 projections are similar to the Ch5 projections, as both Ch5 and Ch6 sectors innervate various thalamic neurons; the concern here is still with a reticulothalamic system (Mesulam et al., 1989). Moreover, Mesulam opined that, similar to the Ch5 neurons, the Ch6 neurons might project to the habenula, hypothalamus, neocortices, hippocampus, and olfactory bulb. The Ch6 sector seems to correspond more closely to the Woolf-Butcher pontomesencephalic tegmentum system with its laterodorsal tegmental nuclei than to the McGeers'

parabrachial complex. The Woolf-Butcher pontomesencephalic tegmental complex radiates, similar to Mesulam's sector Ch6, to the thalamic nuclei, parts of the limbic system, the cortices, and the habenula; however, Woolf and Butcher propose that their pontomesencephalic tegmental complex radiates also to additional brain parts, including basal ganglia and striate, lateral geniculate, and pontine reticular nuclei—a major difference between their complex and Mesulam's Ch6 sector (see section DII, above).

Marsel Mesulam is quite brief when describing sectors Ch7 and Ch8. He opined that the cholinergic neurons of sector Ch7 were located in the medial habenula and projected to the interpeduncular nucleus. This is in accordance with the Woolf-Butcher team's and Kasa's classifications. In fact, Kasa (1986) suggested that the lateral habenular nuclei also project to the interpeduncular nucleus and may serve as a relay for the diagonal band cholinergic cell communication with the nucleus. On the other hand, the McGeers did include the habenula in their projection system.

Finally, the Ch8 sector is localized in the parabigeminal nucleus of the pontomesencephalic region, and its neurons project to the superior colliculus and the lateral geniculate (Mesulam et al., 1989). The innervation of the colliculus constitutes a bewildering situation; Kasa (1986) refers to a number of origins of cholinergic innervation of the superior colliculus, including reticular nuclei, striate, and reticular, peduculopontine tegmental, or cuneiform nuclei. This cholinergic innervation of the superior colliculus and geniculate is important because these two constellations are a part of what may be a cholinergic visual system (see above, section DII).

DIV. Conclusions

When localized, CAT and AChE act as cholinergic markers, adding to the identification of cholinergic neurons and cholinergic pathways (see sections 2 B, above). Moreover, descriptions of these pathways establish the cholinergic system's ubiquity and its presence in brain parts crucial for sensory processing, functions, and behaviors (Karczmar, 2004). This role of the cholinergic system in sensory function needs stressing: while the importance of the cholinergic system in function and behavior has been recognized since the work of William Feldberg, it was denied with regard to the sensorium by Michail Michelson (1974; Michelson and Zeymal, 1970) and others.

Certain special features characterize the cholinergic networks and pathways. Nancy Woolf (1991) emphasized that they form complex interconnections, as they consist of afferents to both cholinergic and noncholinergic neurons and as they receive noncholinergic and cholinergic relays. Altogether, cholinergic pathways are not modular or linear in pattern, but global, and this ensures the subtle, point-to-point control of transmission across the networks in question.

Following the early work of Shute and Lewis, Gerebtzoff, and Koelle, assiduous investigations by the McGeers, Larry Butcher, Nancy Woolf, Marsel Mesulam, Paul Kasa, and Bruce Wainer almost definitely established the cholinergic pathways and networks. That is not to say that there are no differences in their views, and these differences were outlines in this section. On the whole, these divergences are small and do not interfere with our understanding of the major cholinergic pathways such as the forebrain (Mesulam's Ch1 to Ch4 sectors) and its nucleus basalis of Meynert, and the pontomesencephalic formation of the Woolf-Butcher team (Mesulam's Ch5 and Ch6 sectors). Also, this general understanding of the cholinergic pathways, nuclei, and networks helps in associating these entities with functions and behaviors that are endowed with cholinergic correlates (see Chapter 9). Yet, certain areas require more analysis, as in the case of descending pathways connecting supraspinal and spinal sites such as spinal motor and autonomic nuclei.

An important point must be raised. This section focuses on cholinergic sites of origin of cholinergic pathways and networks, yet the cholinergic pathways connect everywhere with pathways manned by other transmitters—in fact, peptides, monoamines, indoleamines, and amino acids interact throughout the brain. These multitransmitter interconnections are not clear, as studies that would simultaneously involve connections among several transmitters are difficult and, therefore, rare (see, however, Senut et al., 1989; Lee et al., 1988; Luppi et al., 1988; and Dun et al.,

1993). Yet, culling from studies concerning different single transmitters as well as those concerning several transmitters simultaneously, it became obvious that interconnections among all the existing transmitter systems are present throughout the brain. This notion is supported by the demonstration that all central functions and behaviors are regulated by multiple transmitters, even though some of them may exhibit preponderant cholinergic correlates (see Chapter 9 BIV–BVI).

Notes

1. This digression leads to a story. As a part of his PhD dissertation as a University of California, Los Angeles, graduate student in Don Jenden's laboratory, Israel Hanin developed the gas chromatography method in the 1960s and went with Jenden as a postdoc to develop, in Bo Holmstedt's laboratory at the Karolinska Institutet, the complete gas chromatography–mass spectrometry method using the equipment available in Holmstedt's laboratory. Thereupon, Jenden, Holmstedt, and Hanin proceeded to use the method for the first chemical identification of ACh in the brain. As they succeeded, Jenden and Holmstedt sent their results, by wireless, to Henry Dale: they felt that he would be happy to learn that his belief in the presence of ACh could be vindicated via a chemical brain method for identifying and measuring ACh. This was a few months before Dale's death in 1968 but, with his usual courtesy, Dale managed to tell Holmstedt and Jenden, via a letter, how much he enjoyed getting this news. Apparently, Hanin's name does not appear in that exchange (see Holmstedt, 1975).
2. This part of the story is quite piquant. The distinguished French neuroscientist, the late Rene Couteaux, who with David Nachmansohn was an early student of neuromyal AChE (see Couteaux, 1953, 1998), sent a promising young French scientist, Maurice Israel, to Whittaker's Cambridge laboratory to help Whittaker in the purification of synaptic vesicles of the Torpedo. When back in France, Maurice Israel continued the work on the purification of the vesicles and, subsequently, he became one of the exponents of the hypothesis of the nonvesicular release of ACh, and a vigorous opponent of Whittaker's classical image of the vesicular release of ACh (see below, this section, and section IIB5).
3. Frequently, the various teams do not refer to one another in their reviews; for example, the reviews of Butcher et al. (1993), Wainer et al. (1993), Woolf (1991), and Mesulam et al. (1983) do not refer to the work of the McGeers, and the 1988 paper of Lee and Wainer (Lee et al., 1988) manages not to quote either the Mesulam team or the Woolf-Butcher team. The review of Semba et al. (1989; with Edith McGeer as the coauthor) and of Butcher (1995) may be exceptional as they quote Hans Fibiger's and Mesulam's work, and Fibiger's, Mesulam's and the McGeers and Kimura's work, respectively.
4. This statement reminded Yves Dunant of Sir William Feldberg's dictum that "there is a type of scientist who, if given the choice, would rather use his colleague's toothbrush than his [or her] terminology" (cited by Katz, 1969).

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